E-selectin as anti-inflammatory drug target:

- **Expression, purification and charactrization for structural studies**
- **Assay development for antaginists evaluation**

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Auf Antrag von

Professor Beat Ernst
Doktor Mauro Zurini

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Professor Dr. Hans-Jakob Wirz, Dekan

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To my parents and my sister

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Table of content

Summary 10
1 Introduction 12
1.1 Physiology of inflammation and leukocyte differentiation 12
1.1.1 Leukocyte differentiation 14
1.1.2 Molecular mechanism of the inflammatory cascade regulation 17
1.1.3 Adhesion molecules involved in leukocyte migration from blood vessels during the inflammatory response 19
1.1.3.1 Integrins 19
1.1.3.2 Immunoglobulin superfamily of adhesion molecules 20
1.1.4 Excessive inflammatory reaction as a basis for pathological processes 22
1.2 Selectins 24
1.2.1 Structural specificities of P-selectin 29
1.2.2 P-selectin ligands 30
1.2.3 Structural specificities of L-selectin 32
1.2.4 L-selectin ligands 34
1.2.5 Structural specificities of E-selectin 35
1.2.6 E-selectin ligands 37
1.2.7 Molecular mechanisms of selectin-ligand interaction 39
1.2.8 Development of selectin antagonists 40
1.3 Recombinant protein expression systems 42
1.3.1 Mammalian expression system 42
1.3.2 Bacterial expression system 43
1.3.3 Insect cells expression system 43
1.4 Elucidation of protein-ligand interactions 44
2 The Aim 47
3 Materials and methods 49
3.1 E-, P-, L-selectin/IgG expression in CHO cells, purification and characterization 49
3.1.1 E-, P-, L-selectin/IgG purification on protein A affinity chromatography 49
3.1.2 E-, P-, L-selectin/IgG purification by gel filtration 50
3.1.3 SDS-PAGE electrophoresis and gel visualization 50
3.1.3.1 Silver staining 51
3.1.3.2 Coomassie staining 51
3.1.4 Protein concentration determination by Bradford assay 52
3.1.5 Mass spectrometry sequence analysis 52
3.1.6 Western blot analysis (Immunoblot analysis) 52
3.1.7 *N*- and *O*-deglycosylation 53

3.1.8 Molecular weight determination 56
3.2 Expression of LecEGF domain of human E-selectin in *E.coli* 56
3.2.1 General proceedings 56
3.2.1.1 Preparation of $CaCl_2$ competent cells [248] 57
3.2.1.2 Preparation of competent *E.coli* cells for electroporation 57
3.2.1.3 Bacterial glycerol stock preparation 58
3.2.2 Cloning into pEZZ18 58
3.2.2.1 Genomic DNA isolation from CHO cells 58
3.2.2.2 PCR generation of Lectin and EGF-like domains (LecEGF) of human E-selectin 58
3.2.2.3 DNA electrophoresis analysis 60
3.2.2.4 Restriction enzyme digestion 60
3.2.2.5 Dephosphorylation of the vector 61
3.2.2.6 DNA-purification 61
3.2.2.7 Ligation 61
3.2.2.8 Chemical transformation of competent *E.coli* cells 62
3.2.2.9 Plasmid isolation and clone analysis 62
3.2.2.10 Protein expression analysis by affinity chromatography on IgG sepharose 63
3.2.3 Cloning into vector pET-15b 63
3.2.3.1 PCR generation of LecEGF domain of human E-selectin 63
3.2.3.2 Restriction enzyme digestion of insert and vector 63
3.2.3.3 Vector dephosphorylation 63
3.2.3.4 DNA-purification with GenEluteTM Gel Purification Kit 64
3.2.3.5 Ligation into pET-15b 64
3.2.3.6 Chemical transformation into *E.coli* 64
3.2.3.7 DNA isolation and clone analysis 64
3.2.3.8 Protein expression optimization 64
3.2.3.9 HisLecEGF purification in native conditions 65
3.2.3.10 Isolation of inclusion bodies 66
3.2.3.11 HisLecEGF purification under denaturing conditions 66
3.2.3.12 Mass spectrometry analysis of proteins 67
3.2.3.13 Protein refolding by dialysis 67
3.2.3.14 Protein refolding by fast dilution and thrombin cleavage 68
3.2.3.15 ELISA based screening on protein refolding and activity 72
3.2.3.16 HisTag cleavage with thrombin 73
3.2.3.17 Screening for the refolding conditions on the asymmetrical field-flow fractionator (Wyeth) 73
3.2.3.18 HisLecEGF expression in M9 minimal medium 76
3.2.4 Cloning into pINompA I 77
3.2.4.1 Restriction enzyme digestion of insert and vector 77
3.2.4.2 Vector dephosphorylation 77
3.2.4.3 DNA-purification with GenEluteTM Gel Purification Kit 77
3.2.4.4 Ligation into pINompA I 77
3.2.4.5 Chemical transformation into *E.coli* 78

3.2.4.6 Electro transformation into *E.coli* 78
3.2.4.7 DNA isolation and clone analysis 78
3.2.5 Cloning into pET-11c 78
3.2.5.1 DNA cloning and manipulation 79
3.2.5.2 Optimization of protein expression 79
3.2.5.3 Protein preparation and analysis on MS 79
3.2.5.4 Protein purification by ion-exchange chromatography 79
3.2.5.5 Protein purification by RP-HPLC 79
3.2.5.6 Optimization of RP-HPLC for LecEGF separation 80
3.2.5.7 Protein refolding by fast dilution 81
3.3 Expression of LecEGF domain of human E-selectin in Baculovirus-infected insect cells 82
3.3.1 Intracellular expression of LecEGF 82
3.3.1.1 Cloning into pBacPack (BD Pharmingen) 82
3.3.1.2 PCR generation of LecEGF 82
3.3.1.3 PCR integration of His-Precision-LecEGF (HPLE) into pBakPack [251] 83
3.3.1.4 Purification of PCR-integration product 84
3.3.1.5 Electro transformation into *E.coli* 84
3.3.1.6 Colony PCR for colony analysis 84
3.3.1.7 Cloning into pFastBac (Invitrogen) 85
3.3.1.8 PCR generation of HPLE compatible with pFastBac 85
3.3.1.9 PCR integration of HPLE in pFastBac 85
3.3.1.10 Purification of PCR product 85
3.3.1.11 Transformation into *E.coli* 86
3.3.1.12 Colony PCR for colony analysis 86
3.3.1.13 Preparation of transposition agar plates for *E.coli* DH10Bac (Invitrogen) 86
3.3.1.14 Transformation into *E.coli* DH10Bac and transposition reaction 86
3.3.1.15 Clone analysis and bacmid DNA isolation 86
3.3.1.16 PCR transposition control 87
3.3.1.17 Transfection into *Sf 9* insect cells 88
3.3.1.18 Viral amplification and plaque assay 88
3.3.1.19 Protein production analysis 89
3.3.1.20 Ni-NTA purification under native conditions 90
3.3.2 LecEGF secretion into the medium 90
3.3.2.1 PCR generation of LecEGF 90
3.3.2.2 PCR integration of SSLecEGF+Flag (SSLecEGFFlag), and SSLecEGF into pFastBac 92
3.3.2.3 Purification of PCR-integration product 92
3.3.2.4 Transformation into *E.coli* 92
3.3.2.5 Colony PCR for colony analysis 92
3.3.2.6 Cloning into pFastBac 92
3.3.2.7 PCR generation of SSLecEGFFlag and SSLecEGF compatible with pFastBac 92
3.3.2.8 PCR integration of SSLecEGFFlag and SSLecEGF in pFastBac 92

3.3.2.9 Purification of PCR product 92
3.3.2.10 Transformation into *E.coli* 93
3.3.2.11 Colony PCR for colony analysis 93
3.3.2.12 Transformation into *E.coli* DH10Bac and transposition reaction 93
3.3.2.13 Clone analysis and Bacmid DNA isolation 93
3.3.2.14 PCR analysis of recombinant bacmid DNA 93
3.3.2.15 Transfection into *Sf 9* insect cells 93
3.3.2.16 Viral amplification and plaque assay 93
3.3.2.17 Protein production analysis and optimization 93
3.3.2.18 Viral amplification for scale-up 94
3.3.2.19 Production scale-up 94
3.3.2.20 Anti-Flag affinity chromatography 94
3.3.2.21 Western blot with the anti-Flag antibodies 94
3.3.2.22 NMR analysis of ligand binding 95
3.3.2.23 Protein deglycosylation 95
3.3.2.24 Size exclusion chromatography 96
3.3.2.25 Ion exchange chromatography 96
3.3.2.26 MS analysis 96
3.4 Bioassay development 96
3.4.1 Cell-free assay 97
3.4.1.1 Sialyl Lewis^a-polymer preparation 97
3.4.1.2 Molecule-molecule assay development, optimization and IC_{50} determination for selectin antagonists with E-, P-, L-selectin/IgG 97
3.4.1.3 Molecule-molecule assay development, optimization and IC_{50} determination for selectin antagonists with LecEGFFlag 98
3.4.1.4 Acute human promyelocytic leukemia cell line cultivation 98
3.4.1.5 Cell-based assay development, optimization and IC_{50} determination for selectin antagonists 99
4 Results 101
4.1 E-, P-, L-selectin/IgG expression in CHO cells and characterization 101
4.1.1 E- and P-selectin/IgG expression in CHO cells 101
4.1.1.1 Protein purification by affinity chromatography and size exclusion chromatography 101
4.1.2 E-selectin/IgG characterization 103
4.1.2.1 Immunoblot in native conditions 103
4.1.2.2 N-deglycosylation of E-selectin/IgG 103
4.1.2.3 Molecular weight determination of E-selectin/IgG 105
4.1.2.4 E-selectin/IgG binding to the monoclonal blocking antibody 7A9 105
4.1.2.5 MS analysis for identity confirmation of E-selectin/IgG 106
4.1.2.6 NMR analysis of E-selectin/ligand binding 106
4.1.3 P-selectin/IgG expression and characterization 107
4.1.4 L-selectin/IgG 108
4.2 Bioassay development with E-, P-, and L-selectin/IgG 109
4.2.1 Cell-free assay 109

4.2.2 Cell-based assay 109
4.3 Expression of lectin and EGF-like domain of human E-selectin in *E.coli* 111
4.3.1 LecEGF cloning into pEZZ18 111
4.3.1.1 Protein expression analysis 114
4.3.2 LecEGF cloning into pINompA I 114
4.3.3 LecEGF cloning into pET15b 115
4.3.3.1 HisLecEGF expression, purification and refolding 116
4.3.3.2 Screening of refolding conditions 121
4.3.4 LecEGF cloning into pET11c 122
4.3.4.1 Protein expression analysis, purification and refolding 123
4.4 Expression of lectin and EGF-like domains of human E-selectin in baculovirus-infected insect cells 125
4.4.1 LecEGF cloning and expression into pFastBac: intracellular expression of a His-tagged protein 125
4.4.1.1 LecEGF purification and analysis 126
4.4.2 LecEF cloning and expression in baculovirus infected insect cells: protein secretion into the medium 127
4.4.2.1 LecEGFFlag cloning and expression 127
4.4.2.2 LecEGFFlag purification and characterization 129
4.4.2.3 Mass spectrometry analysis and NMR analysis of LecEGFFlag 131
4.4.3 Bioassay development 131
5 Discussion 134
5.1 E-, P-, L-selectin/IgG expression, purification and characterization in CHO cells 134
5.2 Expression of lectin and EGF-like domain of human E-selectin in *E.coli* 141
5.2.1 Lectin and EGF-like domain (LecEGF) secretion into the medium 142
5.2.2 LecEGF secretion to the periplasm 144
5.2.3 LecEGF expression in the cytoplasma of *E.coli* 146
5.3 Expression of LecEGF domain of human E-selectin in baculovirus-infected insect cells 152
5.3.1 Intracellular expression of LecEGF 153
5.3.2 Extracellular expression of LecEGF 155
5.4 Bioassay development 159
5.4.1 E-selectin/IgG-sLe^a polyacrylamide assays 159
5.4.2 E-selectin/IgG- HL-60 assays 160
5.4.3 LecEGFFlag-sLe^a-polyacrylamide assay 161
6 Conclusions and Outlook 163
7 References 166
8 Abbreviations 182
9 Curriculum vitae 185

Summary

E-, P- and L-selectin belong to the C-type lectin family of cell adhesion molecules that initiate inflammatory response. Infammation *per se* is a physiologocal defense mechanism, but excessive leukcyte extravasation leads to numerous pathological and disease states, as well as metastatic cancer spread. Leukocyte tethering and rolling toward inflammatory site start with the interaction of selectins and the carbohydrate epitope of their glycoprotein ligands, sialyl Lewis^x. Therefore inhibitors of selectin-ligand interaction are of high pharmaceutical interest as potent anti-inflammatory agents. Tetrasaccharide sialyl Lewis^x serves as a lead strucure in chemical and computational search for selectin antagonists. Structural NMR and X-ray studies indicated binding mode of sialyl Lewis^x with E-, and P-selectin, but improved structural studies with the second and third generation antagonists is missing.

We expressed recombinant human E-, P- and L-selectin/IgG as secreted proteins in mammalian expression system and purified them to homogniety. Acitivity of the proteins was confirmed with blocking monoclonal antibodies and ligand binding confirmed by NMR.

Bioassays were developed in cell-free and cell-based formats with E-selectin/IgG to evaluate inhibitory potencies of in-house synthesized selectin antagonists. Due to variation and instabilities on day-to-day and batch-to-batch basis, assays were used only for preliminary antagonists screen.

To enhance further structural studies, we developed a new system for the expression of truncated form of human E-selectin (lectin and EGF-like domains). Initialy we tried to express these two domains in *E.coli*, but refolding of expressed inclusion bodies was inefficient. Therefore lectin and EGF-like domains of human E-selectin were expressed as secreted form in baculovirus-infeced insect cells with a flag-epitope on its C-terminus. Expressed protein (LecEGFFlag) was monomeric in solution, correctly folded and active, as confirmed in the reaction with monoclonal blocking antibodies, and NMR studies. Protein was expressed in two distinct glycosylation forms, with apparent molecular weigts of 19.96 kDa and 21.15 kDa.

In addition, we developed for the first time a cell-free assay with truncated form of E-selectin (aforementioned LecEGFFlag) for the evaluation of of E-selectin inhibitors. In a proof-of-concept manner, three different E-selectin antagonists were tested and obtained

IC_{50} values were in close agreement with published results. Reproducibility and stability of the assay on day-to-day and batch-to batch basis make it suitable not only for the preliminary screening, but also to quantify inhibitory potencies of E-selectin antagonists. Developed system is suitable for expression and similar characterization of P- and L-selectin as well.

1 Introduction

1.1 Physiology of inflammation and leukocyte differentiation

Humans have to defend themselves against various different pathogens including viruses, bacteria, fungi, protozoan and metazoan parasites. As a consequence, numerous body-defending mechanisms agents have been developed. Inflammation is a complex stereotypical reaction of the body in the response to damage of its cells and vascularized tissues. The discovery of the detailed processes of inflammation has revealed a close relationship between inflammation and the immune response.

Five basic symptoms of inflammation - redness (*rubor*), swelling (*tumour*), heat (*calor*), pain (*dolor*) and deranged function (*functio laesa*) have been known since the ancient Greek and Roman era. These signs are due to extravasation of plasma and infiltration of leukocytes into the site of inflammation [1]. Early investigators considered inflammation a primary host defence system, since inflammation is the key reaction of the innate immune response. However, over-inflammatory responses lead to death, as in anaphylactic shock, or debilitating diseases, as in arthritis and gout. According to different criteria, inflammatory responses can be divided into several categories [2]. The criteria include:

1. **Time** - hyperacute (peracute), acute, subacute, and chronic inflammation
2. **Main inflammatory manifestation** - alteration, exudation, proliferation
3. **Degree of tissue damage** - superficial, profound (bordered, not bordered)
4. **Characteristic picture** - nonspecific, specific
5. **Immunopathological mechanisms**
    - Allergic (reaginic) inflammation
    - Inflammation mediated by cytotoxic antibodies
    - Inflammation mediated by immune complexes
    - Delayed type hypersensitivity reactions

The inflammation brings the fluid, proteins, and cells from the blood into the damaged tissues. Thus, mechanisms exist that allow cells and proteins to gain access to extravascular sites where and when they are needed in case damage and infection have

occured. The main features of the inflammatory response are: **vasodilation**, i.e. widening of the blood vessels to increase the blood flow to the infected area; **increased vascular permeability**, which allows diffusible components to enter the site; **cellular infiltration** by chemotaxis, or the directed movement of inflammatory cells through the walls of blood vessels into the site of injury; **changes** in biosynthetic, metabolic, and catabolic **profiles** of many organs; and **activation** of cells of the immune system as well as of complex enzymatic systems of blood plasma.

Inflammation can be divided into several phases. The earliest event of an inflammatory response is temporary vasoconstriction. This is followed by several phases that occur over minutes, hours and days later, as outlined below.

The **acute vascular response** follows within seconds of the tissue injury and last for some minutes. This results from vasodilatation and increased capillary permeability due to alterations in the vascular endothelium, which leads to increased blood flow (*hyperaemia*) that causes redness (*erythema*) and the entry of fluid into the tissues (*oedema*). If there has been sufficient damage to the tissues, or if infection has occured, the **acute cellular response** takes place over the next few hours. The hallmark of this phase is the appearance of granulocytes, particularly neutrophils, in the tissues. These cells first attach themselves to the endothelial cells within the blood vessels (*margination*) and then cross into the surrounding tissue (*diapedesis*). During this phase erythrocytes may also leak into the tissues and a haemorrhage can occur. If the vessel is damaged, fibrinogen and fibronectin are deposited at the site of injury, platelets aggregate and become activated, and the red cells stack together in what are called "rouleau" to help stop bleeding and aid clot formation. The dead and dying cells contribute to pus formation.

If the damage is severe, a **chronic cellular response** may follow over the next few days as an appearance of a cell infiltrate composed of macrophages and lymphocytes. The macrophages are involved in microbial killing, in clearing up cellular and tissue debris, and they also seem to be very important in re-modeling the tissues.

Over the next few weeks, **resolution** may occur, meaning that the normal tissue architecture is restored. Blood clots are removed by fibrinolysis. If it is not possible to return the tissue to its original form, *scarring* results from in-filling with fibroblasts, collagen, and new endothelial cells. Generally, by this time, any infection will have been overcome. However, if it has not been possible to destroy the infectious agents or to

remove all of the products that have accumulated at the site completely, it is walled off from the surrounding tissue in *granulomatous tissue*. A **granuloma** is formed when macrophages and lymphocytes accumulate around material that has not been eliminated, together with epitheloid cells and gigant cells (perhaps derived from macrophages) that appear later, to form a ball of cell.

Inflammation is often considered in terms of **acute inflammation** that includes all the events of the acute vascular and acute cellular response (described above), and **chronic inflammation** that includes the events during the chronic cellular response and resolution or scarring. A large number of additional effects occur during inflammation. These include: the production of **acute phase proteins**, including complement components, by the liver; **fever**, caused by pyrogens acting on the hypotalamus in the brain; and systemic immunity, resulting in part from lymphocyte activation in peripheral lymphoid tissues.

1.1.1 Leukocyte differentiation

The cells of the immune system originate in the bone marrow, from where they migrate to the peripheral tissues, circulating in the blood and lymphatic system. All the cellular elements of blood, red blood cells, platelets, and the white blood cells of the immune system, derive ultimately from the same progenitor or precursor cells-the pluripotent hematopoietic stem cells in the bone marrow. The different types of blood cells and their lineage relationships are summarized in *figure* 1 [2].

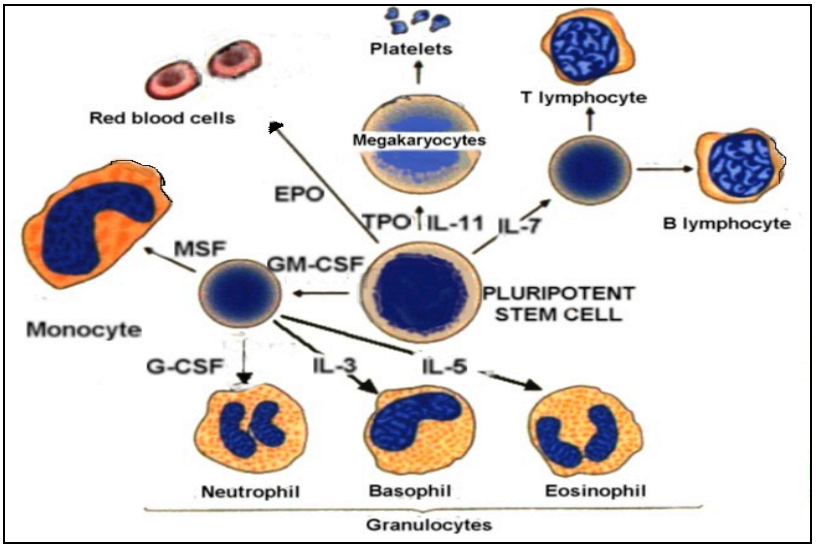


Figure 1.1: Hematopoiesis and leukocyte differentiation under the controlled by different cytokines.

Interleukin-7 (IL-7) is the major cytokine that stimulates bone marrow stem cells to start lymphoid progenitor, which is through activity of interleukin 6 (IL-6) transferred to B and T lymphocyte [2,3]. Thrombopoietin (TPO) enhances production of megakaryocyte, which will differentiate further to platelets under the activity of TPO and interleukin-11 (IL-11). Erythropoietin (EPO) stimulates pluripotent stem cells to ertythroid progenitor production, and its maturation to red blood cells. On the other hand, stem cells stimulated with granulocyte-monocyte colony-stimulating factor (GM-CSF) lead to myeloid progenitor formation and sends cells down the path leading to granulocytes and monocytes formation. Stimulated by macrophage colony-stimulating factor (M-CSF), the myeloid progenitor cells differentiate into monocytes, the precursors of macrophages. Under the influence of granulocyte colony-stimulating factor (G-CSF), myeloid progenitors differentiate into neutrophils. Further stimulation by interleukin 3 (IL-3) differentiates cells into basophils, and stimulation with interleukin 5 (IL-5) leads to eosinophil formation. The myeloid progenitor is the precursor of the granulocytes, macrophages, dendritic cells, and mast cells of the immune system.

- **Granlocytes**, also called polymorphonuclear leukocytes, have densely staining granules in their cytoplasm. There are three types of granulocyte, all of which are relatively short lived and are produced in increased numbers during immune responses, when they leave the blood to migrate to sites of infection or inflammation.

Neutrophils, which are the third phagocytic cell of the immune system, are the most numerous and most important cellular component of the innate immune response: hereditary deficiencies in neutrophil function lead to overwhelming bacterial infection which is fatal if untreated.

Eosinophils are thought to be important mostly in defense against parasitic infection, because their increased number during this type of infection.

The function of basophils is similar and complementary to that of eosinophils and mast cells.

- **Macrophages** are one of the three types of phagocyte in the immune system and are distributed widely in the body tissues, where they play a critical part in innate immunity. They are the mature form of monocytes, which circulate in the blood and differentiate continuously into macrophages upon migration into the tissues.

- **Dendritic cells** are specialized to take up an antigen and display it for recognition by

lymphocytes. Immature dendritic cells migrate from the blood to reside in the tissues and are both phagocytic and macropinocytic, ingesting large amounts of the surrounding extracellular fluid. Upon encountering a pathogen, they rapidly mature and migrate to lymph nodes.

- **Mast cells**, whose blood-borne precursors are not well defined, also differentiate in the tissues. They mainly reside near small blood vessels and, when activated, release substances that affect vascular permeability. Although best known for their role in orchestrating allergic responses, they are believed to play a part in protecting mucosal surfaces against pathogens.

The common lymphoid progenitor gives rise to the **lymphocytes**. There are two major types of lymphocyte: **B lymphocytes** or B cells, which when activated differentiate into plasma cells that secrete antibodies; and **T lymphocytes** or T cells, of which there are two main classes. One class differentiates on activation into cytotoxic T cells, which kill cells infected with viruses, whereas the second class of T cells differentiates into cells that activate other cells such as B cells and macrophages [4-6]. Most lymphocytes are small, featureless cells with few cytoplasmic organelles and much of the nuclear chromatin inactive. These cells have no functional activity until they encounter antigens, which is necessary to trigger their proliferation and the differentiation of their specialized functional characteristics.

Lymphocytes induce a specific immune response against any foreign antigen, because each individual lymphocyte matures bearing a unique variant of a prototype antigen receptor. In this way, the population of T and B-lymphocytes bear receptors of a high diversity in their antigen-binding sites. The B-cell antigen receptor (BCR) is a membrane-bound form of the antibody that the B cell will secrete after activation and differentiation to plasma cells. The T-cell antigen receptor (TCR) is related to immunoglobulin, and is specially adapted to detect antigens or pathogens derived from foreign proteins that have entered into host cells.

A third lineage of lymphoid cells, called **natural killer cells**, lack antigen-specific receptors and are part of the innate immune system. These cells circulate in the blood as large lymphocytes with distinctive cytotoxic granules. They are able to recognize and kill some abnormal cells, like tumor cells and virus-infected cells, and are important in the innate immune defense against intracellular pathogens.

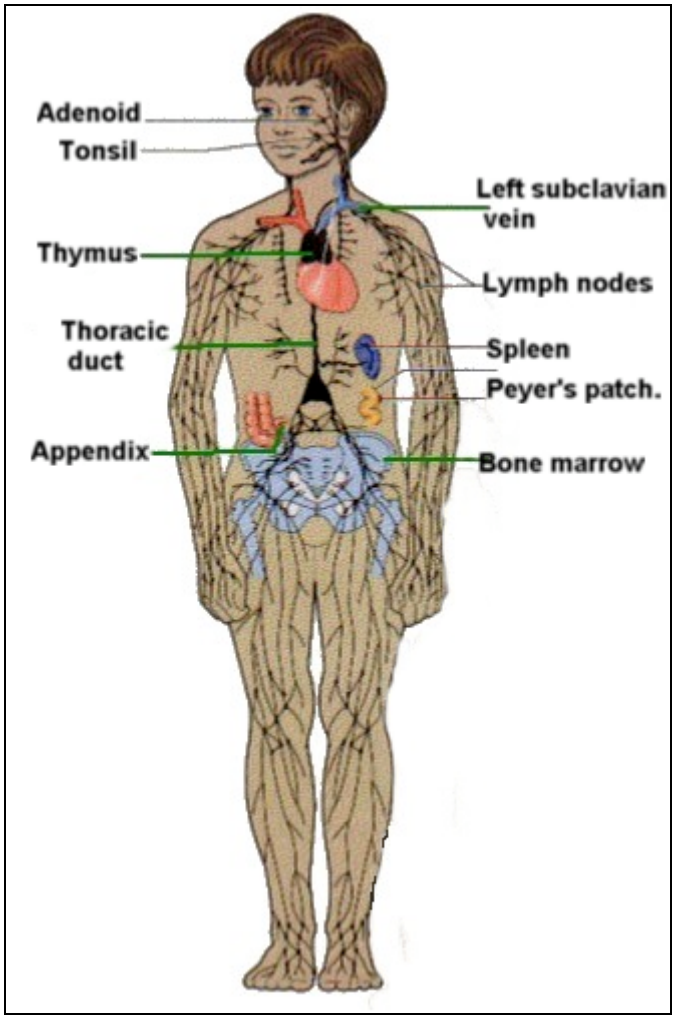


Figure 1.2: The lymphoid organs and tissue distribution. Adopted from [2].

Both B and T lymphocytes originate in the bone marrow; B lymphocytes mature there and T lymphocytes migrate to the thymus to undergo their maturation. Once they have completed their maturation, both types of lymphocyte enter the bloodstream, from which they migrate to the peripheral lymphoid organs: the lymph nodes, the spleen, and the mucosal lymphoid tissues. Each of these tissues traps the antigen from sites of infection and presents it to migratory small lymphocytes, thus inducing adaptive immune responses.

1.1.2 Molecular mechanism of the inflammatory cascade regulation

The migration of leukocytes from the vasculature to the sites of pathogenic exposure is one of the most important mechanisms of the innate immune system. In response to

inflammatory signals produced by cytokines (TNF-α, IL-1, IL-6 and others), leukocytes migrate through the endothelium toward the inflamed tissue [3], thus leave the blood circulation. These events predominantly take place in the postcapilary venuls, which are lined with specialized endothelial cells, and termed high endothelial venules (HEV). Receptor-mediated adhesion to the endothelial cell wall is a pre-requisite for leukocyte extravasation. Since this process includes several overlapping steps mediated through different cell adhesion molecules, it is called inflammatory adhesion cascade [4], (*figure* 1.3).

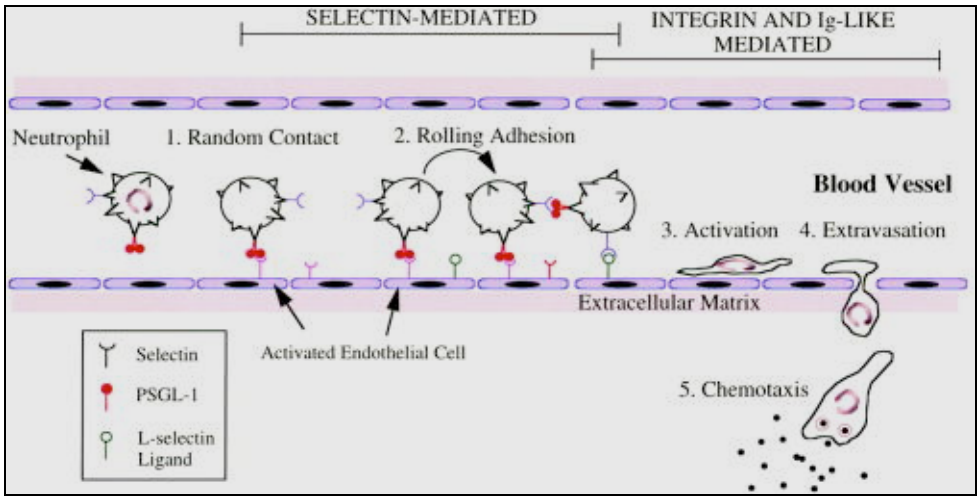


Figure 1.3: Shematic representation of leukocyte adhesion cascade in inflammation. Adopted from [7].

Neutrophils are the predominant leukocyte subset that mediates the acute inflammation. When an injury occurs, inflammatory mediators like histamine, thrombin and lipopolisaccharide will induce cytokine release from the damaged tissue. Cytokine stimuli direct neutrophil migration toward the inflamed tissues and stimulate endothelium to transiently express P- and E-selectin [5,6] whereas L-selectin is constitutively expressed on rolling neutrophils [8]. The interactions of selectin family of cell adhesion molecules with their ligands expressed on either endothelium, or leukocytes, are of low affinity and present a molecular base for transient leukocytes-endothelium contacts. At velocity of 2 mm/s, flowing leukocytes are directed from the blood flow toward the endothelial cell wall and present the first events in the inflammatory cascade. The initial contacts are still reversible and alternate with phases of free-moving cell. They are therefore termed “leukocyte capture”, or “tethering” [9].

Based on this mechanism, the number of fast associating and dissociating ligand-

receptor interactions increases, leading to leukocytes rolling across the affected endothelium. The leukocytes are slowed down in this phase, rolling at 20 μm/s, concentrating them locally on the vessel wall and enabling transmission of secreted cytokines from endothelial cells [10]. The cytokine stimulus trigger expression and activate other families of adhesion molecule: integrins on leukocytes interact with their ligands from the immunoglobulin (IgG)-superfamily (ICAM-1, VCAM-1), that are expressed on the activated endothelium [11]. This interaction is much stronger then one mediated by selectins and leads to the firm leukocyte adhesion and subsequent extravasation into the tissue. The endothelial cell monolayer in its resting state is associated through homophilic interaction between the IgG superfamily molecule PECAM-1. Homodimers are then formed with molecules on the opposing endothelial cells [9,12]. Extravasation finally takes place when leukocytes integrins bind to PECAM-1, interrupting the aforementioned homophilic interactions, allowing the leukocytes to transmigrate to sub-endothelial matrix.

1.1.3 Adhesion molecules involved in leukocyte migration from blood vessels during the inflammatory response

Endothelial cells lining the vascular spaces of postcapillary venules in peripheral tissues are among the first regulators of inflammation. The upregulation of adhesion molecules by endothelial cells, as well as the ability of endothelial cells to present chemo-attractant cytokines, leads to the adhesion of leukocytes to the endothelium, a prerequisite step before their transmigration into inflamed tissue [12-15]. The overlapping extracellular adhesive properties of cell adhesion molecules important for leukocyte recruitment during inflammation, namely integrins, the IgG-superfamily of adhesion molecules and selectins are discussed in the following text.

1.1.3.1 Integrins

Integrins are a group of heterodimeric transmembrane glycoproteins found on different cells that mediate cell-cell and cell-extracellular matrix adhesions [13]. All integrins comprise one α- and one β-subunit, which together form an extracellular ligand-binding site. Cytoplasmic tails of integrins provide phosphorylation sites and are related to the integrin-mediated assembly of cytoskeletal linkages involved in signal transduction [16]. These serve to modulate many aspects of cell behavior, including proliferation, survival/apoptosis, shape, polarity, motility, gene expression and differentiation. There

are 8 different β- subunits (β_1-β_8) that associate with one of 18 α-subunits to form at least 24 known receptors in a variety of cells, including lymphocytes, leukocytes, and platelets. Integrins are capable of mediating cell-cell binding but also are involved in cell interactions with extracellular proteins such as laminin, fibronectin, vitronectin, and fibrinogen. Integrins are mostly expressed in an inactive state. In case of leukocytes, integrins are inactive in the "resting" state, but are rapidly activated upon cytokine stimulation. The mechanism of integrin activation relays on a conformational switch between the closed form of low affinity or "inactive" form, and the open form that is active. This switch is coupled with ligand binding or with known activation stimuli, such as metal ions. The leukocyte firm adhesion to the activated endothelium is mediated primarily by integrins that consist of β_2-subunits (CD18). These are macrophage antigen-1 (Mac-1; $\alpha_M\beta_2$; CD11b/CD18), lymphocyte-associated function antigen-1 (LFA-1; $\alpha_L\beta_2$; CD11a/CD18), and a third CD18 integrin (CD11c/CD18; $\alpha_x\beta_2$). LFA-1 is the predominate integrin used for lymphocyte migration, whereas Mac-1 has emerged as the more critical integrin in most models of neutrophil-dependent inflammatory responses [16-18]. Pre-formed Mac-1 is stored in three separate PMN compartments: secretory vesicles, specific-granules, and gelatinase granules and can be rapidly mobilized to the PMN surface. This occurs after exposure to degranulation stimuli such as the bacterial peptide formyl-methionyl-leucyl-phenylalanine (fMLP), as well as to weaker stimuli as LPS and TNF-α. Inflammatory stimuli can also promote transcription and translation of Mac-1 genes, thus prolonging the integrin involvement during inflammation. Very late antigen (VLA-4, $\alpha_4\beta_1$ integrin) has been identified on both activated human lymphocytes and neutrophils, and mediates tethering, rolling and firm adhesion [19,20]. Leukocyte integrins mediate firm adhesion to the activated endothelium through the interaction of their counter receptors of the immunoglobulin superfamily [21].

1.1.3.2 Immunoglobulin superfamily of adhesion molecules

The immunoglobulin superfamily of cell adhesion molecules comprises a very diverse group of adhesive receptors. Members of this family are defined by the presence of one or more copies of the IgG fold, a compact structure with two cysteine residues separated by 55-75 amino acids, arranged as two antiparalel β sheets [20]. Typically IgG CAMs have a large amino-terminal extracellular domain containing IgG folds, a single transmembrane helical segment, and a cytoplasmic tail [22]. Members of the IgG CAM function in a wide variety of cell types and mediate many different functions, including

acting as receptors for growth factors and mediating cell-cell adhesion rather than cell-extracellular matrix interactions [23,24]. Predominantly members of this family function as adhesion receptors, including intercellular adhesion molecule-1 (ICAM-1; CD54), intercellular adhesion molecule-2 (ICAM-2), vascular cell adhesion molecule-1 (VCAM-1; CD106), platelet-endothelial cell adhesion molecule-1 (PECAM-1; CD31) and the mucosal addressin cell adhesion molecule-1 (MAdCAM-1). ICAM-1, ICAM-2 and VCAM-1 are involved in the adhesion of T cells to endothelial cells by serving as surface ligands for the integrins LFA-1 ($\alpha_L\beta_2$) and $\alpha_4\beta_1$ [12].

ICAM-1 and ICAM-2 (CD102) are expressed on a variety of haematopoietic and nonhaematopoietic cells, including B and T cells, fibroblasts, keratinocytes and endothelial cells. The expression level of ICAM-1 can be up regulated by various cytokines such as TNF-α and IL-1β. ICAM-2, however, is expressed constitutively and its expression is not cytokine-inducible. These molecules are counter-receptors for LFA-1 (CD11a/CD18). A portion of the cytoplasmic region of ICAM-1 has been shown to bind to the cytoskeleton of COS cells transfected with the cDNA of human ICAM-1, indicating homophilic interaction [12,24-26]. Linkage with the cytoskeleton may localize ICAM-1 within regions of the endothelial cell membrane in order to facilitate leukocyte adherence and transmigration. The expression of ICAM-1 in primary melanoma is related to the presence of distant metastases, although precise involvement of ICAM-1 in the process of metastasis has not been clear yet. Elevated levels of soluble ICAM-1, which still retain the binding site for LFA-1 in its extracellular domain, have been found in the serum of melanoma and ovarian cancer patients. It has been suggested that this active form of ICAM-1 can inhibit binding sites on cytotoxic T-cell and natural killer cells and that in the presence of ICAM-1 tumor cells might escape immune destruction. Since human melanoma cells have been shown to release ICAM-1, and its increased level in serum results in a non-specific inflammatory response, it has been proposed that circulating levels of ICAM-1 reflect the formation of metastases, possibly as a consequence of facilitating disseminating tumor cell binding [27].

VCAM-1, a 90-110 kDa glycoprotein is expressed on the surface of activated endothelium and variety of other cell types, including dendritic cells, tissue macrophages and bone marrow fibroblasts. VCAM-1 expression on endothelial cells can be up-regulated by several cytokines, such as IL-1β, IL-4, TNF-α and interferon-γ (IFN-γ). VCAM-1 interacts with the leukocyte integrin $\alpha_4\beta_1$ on many different cells including

eosinophils, monocytes, and also certain tumor cells, and with $\alpha_4\beta_7$ on activated peripheral T cells [19,20]. Thus, $\alpha_4\beta_1$/VCAM-1 interactions, like LFA-1/ICAM-1 interactions regulates the movement of lymphocytes out of blood vessels to the inflammatory sites. Interaction of VCAM-1 with $\alpha_4\beta_1$ integrin expressed on certain tumor types presents an important mechanism for the development of metastases in cancers such as melanomas, osteosarcomas and neuroblastomas [14,21].

The platelet-endothelial cell adhesion molecule PECAM-1, also knows as CD31 or endoCAM, is a 130 kDa glycoprotein found on endothelial cells, platelets, monocytes and neutrophils [23,24]. Several observations suggest that PECAM-1 is involved in leukocyte adhesion, transmigration, and particularly in the preferential migration of naive and CD8+ T cells across HEV [28]. PECAM-1 is homologous to both CEA and ICAM-1 proteins and has been shown to contribute to both homotypic and heterotypic cell adhesion by interaction with either itself or with the integrin $\alpha_v\beta_3$. Unlike ICAM-1, which is expressed over the entire surface of resting endothelial cells, PECAM-1 is expressed at intercellular junctions of endothelial cells and its surface expression is not increased by treatment with cytokines, such as TNF-α and IL-1 or by combinations of TNF-α and IFN-γ. Binding of mAbs to PECAM-1 enhanced CD8+ T-cell adhesion to fibronectin or VCAM-1, but not to fibrinogen or collagen, suggesting that it may cause activation of integrins containing α_4 subunits such as $\alpha_4\beta_1$ or $\alpha_4\beta_7$. Inhibition of PECAM-1 expression on leukocytes or endothelial cells by treatment with soluble PECAM-1 or blocking PECAM-1 mAbs inhibits monocyte or neutrophil transmigration in vitro. Treatment of either the endothelium or the leukocytes separately is equally effective, suggesting that homophilic adhesive interaction is involved [12].

1.1.4 Excessive inflammatory reaction as a basis for pathological processes

Excessive leukocyte accumulation in the inflamed tissue is a basis for pathological inflammatory reaction, angiogenesis and tumor metastasis. In case of atherosclerosis, myocardial and cerebral ischemia, hemorrhagic shock, thrombosis, diabetes-caused microangiopathies, or rheumatoid arthritis, one has observed strong deregulation of selectin expression and function in disease etiopathology. Atherosclerotic changes are caused by accumulation of LDL (“low density lipoprotein”) in the blood vessel wall and consequent inflammatory fibroid reaction. Monocytes are invading the inflammatory site

to oxidize and remove materials from lipids and cholesterol, endothelial lumen constraints, and arteries loose elasticity. The apparent monocyte's invasion and pathogenesis of atherosclerosis is mediated by E-, P-, and L-selectin and induced by LDL. Higher expression of selectins, ICAM-1 and VCAM-1 and their correlation with the atherosclerotic plaques was also proven in the animal models [26,29-31]. Nevertheless, in E-selectin molecule, there are mutations typically found in young patients with hard coronary disease that influence its functionality in vitro [31-33], whereas L-selectin mediates "secondary capture" of leukocytes to the atherosclerotic liaisons. The level of soluble selectins in serum was also thought to correlate with the expression of atherosclerotic plaques, however, these observation are still contradictory [26,34-37].

In the case of apoplexy, or cerebral ischemia, there is an acute disruption of cerebral oxygen supply, caused by the massive hemorrhage, or the closure of cerebral atherosclerotic blood vessels. In myocardial infarction, however, a dysfunction of blood circulation results in deficiency of myocardial oxygen supply, and irreversible damage of the affected tissues. As a consequence of cerebral and myocardial ischemia, sometimes reperfusion results in pathological acute inflammation, where leukocytes extensively migrate in the tissue [38,39]. P- and E-selectine have been up-regulated in these cases, and blockade of their function has been shown to significantly decrease excessive neutrophile migration [40-42].

Hemorrhagic shock, a disparity between heart volume and actual systemic circulation needs, is first manifested on the macrocirculatory level, and then as a microcirculatory dysfunction. Together with subsequent recirculation, it is also considered as an ischemia/reperfusion injury, and lead to the excessive neutrophile infiltration and increased adhesion to the affected tissue. This may cause severe pathological changes in organ that finally lead to the systemic organ failure. Blocking selectin function turned out to be an effective and protective approach in many *in vitro* studies, as well as in animal models [8,43-49].

One of the most prominent autoimmune diseases, rheumatoid arthritis is caused by pathological reaction of the immune system. Pathophysiological incident is an inflammation of the synovial membrane of the joints, characterized with a strong infiltration of the phagocytic leukocytes. Synovial membrane is enlarged and presses on the surrounding bones. This way synovial membrane liberates permanently inflammatory mediators that support chronicle inflammation. Although the exact molecular

mechanisms of rheumatoid arthritis have not been known yet, E-selectin accumulation in rheumatoid tissue, and its active role in pathogenesis of rheumatoid arthritis is very well documented. Serum levels of soluble E- and P-selectin have been used as the molecular markers for the active inflammation in rheumatoid arthritis [50-53]. Antirheumatoid therapeutics probably exert partial effect on the adhesion of polymorhonuclear neutrophils to the endothelial cells: they reduce cytokine mediated adhesion and expression of E-selectine on activated HUVECs. In case of many malignant diseases, primary tumor cells migrate through vascular system to different sites in organisms in a complex process:

1. Tumor cells ablation and migration in surrounding area by adhesion on the components of the exracellular matrix.
2. Hydrolytic activity of tumor cells, matrix destruction and consequent cell migration
3. Tumor cells proliferation through the blood and lymph, avoiding immune system, and adhesion to appropriate part of blood vessels wall.
4. Tumor cells invasion through the endothelium and the extra cellular matrix in the parenchyma beneath.

It is known that tumor cells can adhere to the endothelium, at least in some cases, following the mechanism of leukocyte adhesion to the endothelium in the inflammatory reaction. In addition, selectins play an important role in metastasis as well [54-58]. There is a huge body of evidence that in case of colon carcinoma, tumor cells expression of E-selectin ligands with the sLe^x and sLe^a epitopes correlates with the metastatic behavior of the tumor [59-62]. For the breast tumor, aforementioned correlation is also worth, whereas in other tumors it is still controversial. Cancer studies with animals have been conducted mostly on colon and lung carcinomas, focusing predominantly on metastasis-dependent induction and up-regulation of selectins [63-69]. Tumor tissues and serum of patients have also been examined in order to determine and quantify changes in selectin levels [5,69-84].

1.2 Selectins

The selectins mediate the initiation of the cell contact between leukocytes and endothelial cell. This selectin-mediated docking of lekocytes to the blood vessel wall in combination with the rapidly flowing blood stream leads to a rolling movement of leukocytes on the endothelial cell surface. Capturing leukocytes from the rapidly flowing

blood stream to the blood vessel wall is a very special example of cell contact formation, which has to overcome considerable shear forces. Thus, in most cases, these interactions are characterized by relatively rapid on/off kinetics of binding. The selectin family of cell adhesion molecules is the most recently identified gene family of adhesion molecules [3,5,46-49] that consists of only three member proteins. The members of this family, termed L-selectin, E-selectin, and P-selectin, have cluster designations CD62L, CD62E, and CD62P, whereas nomenclature corresponds to the expression site [85]. Selectins share an overall structure and primary sequences and exhibit similarities in the cognate glycans that contribute to their adhesion function. Genomic organization of selectins has been examined for human and mouse [86,87]. *In situ* hybridization indicated that all three selectins are members of a gene complex on human chromosome 1, bands q21-24. Human E-, P-, and L-selectin share a common structural motif including a N-terminal C-type lectin domain, an epidermal growth factor-like domain, a variable number of consensus repeats homologous to those in complement binding proteins (CR domains), a membrane spanning segment, and a short cytoplasmic region (*figure 1.4*).

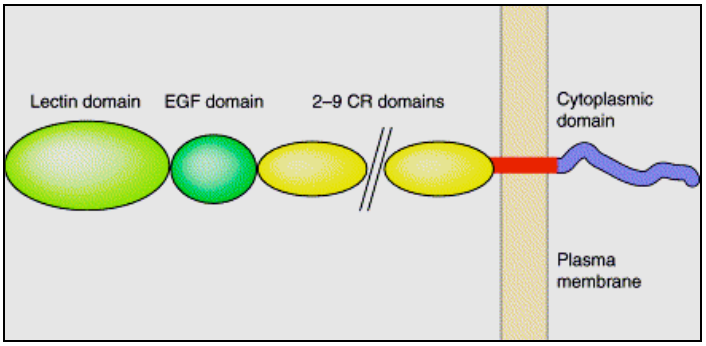


Figure 1.4: Domain organization of selectins. Adopted from [58].

Amino acid sequence identity within the lectin domain among different selectins is 52%, EGF-like domain 47%, and consensus repeats 35%. The high degree of conservation supports the central role of lectin and EGF domains in interaction with carbohydrate determinants of the ligands, but raises questions as to how the specificity of each selectin for its ligand is accomplished.

The C-terminal part of the proteins is located in the cytoplasma and it is connected to the cytoskeleton. The functional role of this part has not been thoroughly elucidated yet, but it plays an important role in signal transduction. In comparison with the other parts of molecule, the cytoplasmic tail is rather small, 17 amino acids in L-selectin, 32 amino

acids in E-selectin, and 35 in P-selectin, followed by the single transmembrane domain. Further molecular extension into extracellular part is accomplished through consensus repeats. Each consensus repeat is 60 amino acids long, contains 3 disulfide bridges and differs throughout the selectin family: human L-selectin contains 2, E-selectin 6, and P-selectin 9 consensus repeats. The evidences for the role of consensus repeats are partially contradictory. Majority indicates that, although the CRs are not required for ligand recognition, they do enhance ligand binding affinity. It has been shown that the reduction in the number of consensus repeats decreases the ability of P-selectin to support leukocyte rolling [88]. This indicates the importance of sufficient distance between plasma membrane and carbohydrate binding domain of selectins, which is provided and mediated by consensus repeats. The L-selectin adhesion-blocking antibody Mel 14 recognizes an epitope in the lectin domain. It showed very weak binding to a construct lacking the CR domains, and a decrease in lectin-specific interaction with the peripheral lymph node endothelium, suggesting again that the CR domains may be involved in induction of lectin domain conformation and enhancement of its activity [89].

Similar data were obtained in case of E-selectin, where soluble constructs containing the lectin and EGF-like domain with different number of consensus repeats have been generated by several groups [90-92]. Experimental data showed decreased selectin binding activity toward leukocytes or engineered cells, in case of reduced number of consensus repeats. However, the studies with recombinant chimeric selectins generated by domain switch between E- and L-selectin, and with not less then two CRs, implied that only lectin and EGF-like domains were the minimal structural unit required for specific ligand binding [92]. On the other hand, the functional importance of the CR domains was also supported by the isolation of the mAb EL-246 with an epitope in a common region of the CR domains of both E- and L-selectin. Since this antibody was blocking the function of both selectins, these results demonstrated the presence of a conserved epitope in the CR domains of L-and E-selectin crucial for leukocyte-endothelial interaction [89].

Domain-deletion studies of E-selectin and domain-exchange studies between L-, and P-selectin suggested that EGF-like domain is also involved in cell adhesion, either in stabilizing the conformation of the lectin domain, or in direct interaction with the ligand [90,93-99]. Studies with the blocking antibodies against EGF-like domain of L-selectin showed the inhibition of L-selectin mediated cell adhesion [100,101], consistent with the

studies done with a peptide derived from the EGF-like domain residues 127-139, which effectively inhibited monocyte adhesion to activated endothelial cells [102]. However, isolated EGF-like domain of P-selectin failed to inhibit HL-60 cell adhesion to CHO cells expressing P-selectin, which may argue against the direct involvement of the EGF-like domain in the adhesion. The experiments with the panel of monoclonal blocking antibodies for all three selectins have determined the functional epitopes in the lectin domains. Identification of the amino acids critical for the ligand binding in mutational studies, and predictions based on comparison with the crystal structure of mannose binding protein (MBP) was consistent [96,97,103]. These data were confirmed with the X-ray structure of the lectin and EGF-like domains of E-selectin and aforementioned co-crystallized complexes of E-, and P-selectin [99,104]. In case of E-selectin, lectin domain possesses a globular structure with the very flat and shallow binding pocket for the sugar ligand, whereas P-selectin LecEGF construct has a very similar conformation. The movement of different loops in the EGF domain of P-selectin and slightly changed surface amino-acids exposures causes minor differences in binding pockets, with different charges present.

The regulation of selectins presence on the cell surface is important for the control of leukocyte extravasation. In case of E- and P-selectin, this ensures that these selectins are only present on the endothelium in inflamed tissues. L-selectin, in contrast, is constitutively expressed on leukocytes, in agreement with its special function in the continuous process of lymphocyte homing. E-selectin is induced by cytokines such as TNF-_ or IL-1β and by lipopolysaccharide (LPS) as was first found for HUVECs [100,105]. Induction occurred on the transcriptional level, with the expression profile desand within 3–4 h after stimulation, maximal levels of E-selectin protein are expressed at the cell surface and basal levels are reached again after 16–24 h [5]. The 5' flanking region of human E-selectin were sequenced and regulatory elements for the activation of E-selectin promoter are NF_-B binding sites [106-110] and one is an ATF-binding element [111-113]. Several other inducers, like interleukin-10 interleukin-3, oncostatin M have been studied as well, which, however, differ in the overexpressed loci and overexpression intensity [114-116]. LPS from gram-negative bacteria, and lipoteichoic acid from gram-positive bacteria were also found to induce E-selectin expression [117]. Apart from soluble factors, leukocytes-contacting endothelial cell surface can also modulate E-selectin expression [118,119].

Inhibition of E-selectin mediated adhesion can be achieved with IL-4 through STAT6 signaling, glucocorticoids, transforming growth factor-β, and elevated cAMP can counteract cytokine-induced expression of E-selectin [120-124].

P-selectin is inducible by two different mechanisms. It is stored in the _-granules inside of platelets, and in Weibel-Palade bodies, wherefrom can be rapidly mobilized (minutes, even seconds for platelets) to the cell surface of endothelial cells upon the stimulation with thrombin, histamine, phorbol esters or Ca^{2+} ionophores [125]. Although both E- and P-selectin are rapidly internalized, only P-selectin molecules can be recycled from endosomes into the *trans*-Golgi network, where they are targeted to Weibel-Palade bodies, or are delivered, as in case of E-selectin, into the lysosomes [126,127]. A second regulation mechanism for P-selectin is similar to those observed for E-selectin, whereas TNF-α stimulates the transcript and the protein level of P-selectin [128-130]. On the contrary, in HUVECs P-selectin expression could neither be stimulated by LPS nor by TNF-α or IL-1β but with oncostatin M and interleukin-4. L-selectin, which is constitutively expressed on myeloid cells and large sets of the lymphocytes can be down-regulated on the transcriptional level during lymphocyte differentiation from a naive to memory cell type [131-133].

Induction of L-selectin mediated adhesion in the inflammatory process is achieved by the up-regulation of L-selectin ligands upon cytokine activation, mostly PSGL-1 [105]. On lymphocytes as well as on neutrophils, cell activation causes rapid down-regulation of L-selectin within minutes by proteolitic cleavage of L-selectin at an extracellular part proximal to the cell membrane. Proteolytic shedding occurs on neutrophils within 1-5 min, and can be induced by a variety of chemoatractants and activating factors such as fMLP, leukotriene B_4, IL-8, TNF, GM CSF, calcium ionophores but not by granulocyte CSF, macrophage CSF, IL-1, or interferon-γ [134,135].

In addition to a direct role in leukocyte capturing, selectins and their ligands are involved in signal transduction, and the up-regulation of endothelial selectins has also been modified through complex signaling effects [136]. Different studies had implicated nonreceptor tyrosine kinases such as c-Src, and the MAP kinase cascade in selectin signaling, as well as c-JUN N-terminal kinase and p38 kinase regulation pathways [137-139]. Selectins also play a role in triggering the activation of β2 integrins. The E-selectin led to enhanced β2 integrin-mediated binding to co-expressed ICAM-1 in the tissue culture monolayer. The trigger was blocked by the inhibitors of Erk/MAP kinase cascade,

indicating that the glycoprotein counter receptor for E-selectin on neutrophils actes in these cells by MAPK-dependent pathway. PSGL-1 on leukocytes also led to the activation of β2 integrins, in process blocked with tyrosine kinase inhibitors, whereas L-selectin expressed on neutrophils and cross-linked by antibodies, activates β2 integrins through p38 MAP kinase [94,140-142].

1.2.1 Structural specificities of P-selectin

P-selectin is found within the Weibel-Palade bodies of endothelial cells and in α-granules of platelets. Sequences within the cytoplasmic domain of P-selectin mediate sorting to the granule through the interactions with molecules that direct this process. Splice variants of P-selectin transcripts yield forms of P-selectin that are without a transmembrane domain, consistent with the fact that some P-selectin is released as a soluble form [143-148]. The cytosolic tail of P-selectin undergoes phosphorylation at specific threonine, tyrosine, and histidine residues, and myristoylation at a single cysteine in this domain. However, post-translational modifications are still not thoroughly desribed in terms of functionality.

P-selectin expression is under transcriptional control, with induction by TNF-α, IL-1β, or LPS. The role of P-selectin in leukocyte recruitment and inflammation is a function of both acute and chronic expression of P-selectin by endothelium and by platelet activation-dependent P-selectin expression. P-selectin $^{-/-}$ mice exhibit a substantial delay in neutrophil recruitment in the context of acute inflammation relative to wild-type mice, indicating that P-selectin plays an important part in the early phases of leukocyte recruitment. Endothelial P-selectin contributes to chronic inflammation, whereas platelet-derived P-selectin also contributes to leukocyte trafficking, as well as to wound healing and blood clotting mediating the adhesion of neutrophils, monocytes and natural killer cells. This adhesion represents a mechanism to augment the recruitment of leukocytes and platelets to sites of vascular compromise. Platelet-derived P-selectin contributes to hemostatic processes by its ability to stimulate monocytes to express tissue factor and by facilitating fibrin deposition during clot formation, and can also mediate platelets function in tumor angiogenesis [84,149,150].

Crystal structure of lectin and EGF domain (LE) of P-selectin has been solved as well as the structure of P-selectin co-crystalized with sLe^x and *N*-terminus of the PSGL-1 [99]. The P-LE crystal structure has an overall conformation similar to that of E-LE, consistent

with the 60% sequence identity between selectins in these domains. The sLe^x binding site has a common feature, and the basis for the metal-dependency of selectin binding is the coordination of a calcium ion by the side chains of Glu80, Asn82, Asn105, Asp106, and the backbone carbonyl of Asp106. Differences in the binding site are defined by the Ser97-Pro98-Ser99-Ala100. Structural reasons are also responsible for P-selectin lower affinity toward sLe^x compared to E-selectin (7.8 mM *vs*.1 Mm, respectively). In the P-selectin molecule there is a small number of contacts between lectin and EGF-like domain [104,151,152] within the residue 135-139, which argues that this interface is inherently flexible. P-selectin may be the most flexible in this region, because of substitution of Gly138 for Asn138, which can not form stabilizing hydrogen bonds to the lectin domain, and the flexibility of the molecule may be essential for maximizing contacts between P-selectin and PSGL-1 on free flowing leukocytes.

1.2.2 P-selectin ligands

Carbohydrate binding epitope for P-selectin is fucosylated lactosaminoglycan from blood group determinants, Le^x, and of the higher affinity, its sialylated form, sialyl $lewis^x$ (Neu5Acα2,3Galβ1,4[Fucα1,3]GlcNAc-*O*-R) or its stereoisomer sialyl $Lews^a$ (Neu5Acα2,3Galβ1,3[Fucα1,4]GlcNAc-*O*-R) [7,148,153-155]. The affinity for sLe^x is in a low millimolar range, but there are other glycoprotein ligands with the nanomolar binding affinity for P-selectin. The higher affinity for these ligands is a consequence of either carbohydrates composition presented, or multivalent interactions because of oligomerization of many epitopes

The binding of P-selectin to neutrophils is abolished upon sialidase treatment, suggesting that sialic acid might be a critical determinant required for P-selectin recognition. However, the binding of P-selectin to neutrophils is of much higher affinity than to nonmyeloid cells, originally suggesting that myeloid cells possess a unique ligand(s) for P-selectin. P-selectin glycoprotein-1 (PSGL-1) is so far the best characterized ligand for P-selectin, isolated using 125I-labeled P-selectin blotting and affinity chromatography on immobilized human P-selectin. As a functional ligand, PSGL-1 is expressed on essentially all blood leukocytes, myeloid, lymphoid and dendritic cells wherefrom the purified ligand PSGL-1 is isolated, as a disulfide-bonded glycoprotein of approximately 250-kDA [156,157]. In contrast to other selectin ligands, PSGL-1 binding to P-selectin is characterized by high-affinity binding (Kd in the range of 100 nm) and very fast association and dissociation rates [98].

The cDNA encoding PSGL-1 was expression-cloned in COS cells transfected to coexpress an _1–3(4)-fucosyltransferase (human FucT-III), which allows both sialyl Lex and sialyl Lea synthesis in these cells. Each subunit of human PSGL-1 contains 70 serine and threonine residues in the extracellular domain that are potential sites for *O*-glycosylation and three potential sites for *N*-glycosylation. The murine PSGL-1 also contains numerous extracellular serine and threonine residues and two potential sites for *N*-glycosylation.

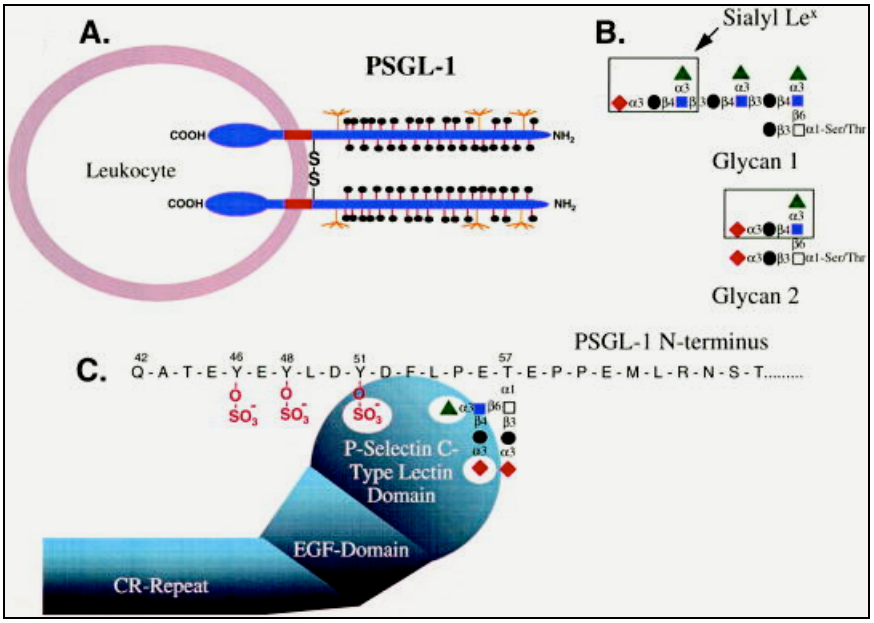


Figure 1.5: PSGL-1 structural requirements. Adopted from [7].

Treatment of purified PSGL-1 with sialidase abolishes its binding to P-selectin, but treatment of neutrophil-derived PSGL-1 with peptide *N*-glycosidase F, which removes most, if not all, *N*-glycans on the molecule, does not affect its recognition by P-selectin [154,158]. The involvement of *O*-glycans was supported by the observation that treatment of either neutrophils or purified PSGL-1 with the *O*-sialoglycoprotease from Pasteurella hemolytica, an enzyme that degrades sialylated mucins, blocks binding of the cells or ligand, respectively, to P-selectin. In addition, when HL-60 cells are treated with benzyl-_-Gal*N*Ac, which inhibits extension of *O*-glycans, the cells bind less to P-selectin. Other studies demonstrated that treatment of isolated PSGL-1 with endo-_-galactosidase, significantly reduces binding to P-selectin, thus indicating that polylactosamine, presumably on *O*-glycans, may also be important for the binding. The

structures of the *O*-glycans of native PSGL-1 purified from human HL-60 cells reveal that most contain a simple Core 2 structure with one or two sialic acid residues and generally lack fucose residues. Further binding epitope is determined by first twenty amino acids on N-terminus of PSGL-1[155,159,160]. Sulfation as requirement for P-selectin binding was confirmed by treatment of purified PSGL-1 with a bacterial aryl sulfatase that abrogates binding of the molecule to P-selectin. The critical binding domain is in the extreme amino terminus of PSGL-1. Furthermore, recombinant forms of PSGL-1 in which the three tyrosine residues have been changed to phenylalanine also fail to bind P-selectin. Such data point to a model in which the combination of tyrosine sulfate residues and oligosaccharides on the protein are required for high-affinity binding to P-selectin. Replacement of all three amino-terminal tyrosine residues with phenylalanine abolishes binding of the recombinant PSGL-1 to P-selectin, but not E-selectin. Interestingly, only one of the three potential tyrosine sulfate residues is absolutely necessary for binding to P-selectin. Together with the threonine mutation to alanine, these data support the model where the C-type lectin domain of PSGL-1 may contact tyrosine sulfate residues and the sialyl Le^x structure on a core-2 *O*-glycan at Thr-57.

The specific in vivo functions of PSGL-1 have been explored using blocking antibodies to the protein and recombinant forms. Monoclonal antibody PL1and its F(ab) fragments dramatically reduced rolling of human polymorphonuclear neutrophils and HL60 cells in venules of acutely exteriorized rat mesentery, indicating that PSGL-1 is important *in vivo* for rolling of myeloic cells in mesenteric venules at physiologic shear stress. In a rat model of ischemia/reperfusion injury, treatment of animals with recombinant PSGL-1 significantly enhanced rat survival and liver function and recovery. PSGL- blocking antibodies to mouse PSGL-1 blocks entry of T-helper-1 cells into inflamed areas of the skin during a cutaneous delayed-type hypersensitivity reaction model [157].

1.2.3 Structural specificities of L-selectin

L-selectin is a glycoprotein that varies in size according to the type of leukocyte in which it is produced. The protein is termed L-selectin and has been previously referred to as LEC-CAM, LAM-1, CD62L. The polypeptide sequence derived from the cloned cDNAs predicted a molecule corresponding to a type I transmembrane glycoprotein with a major amino-terminal extracellular segment [46,161]. The amino-terminal segment of the mature protein shows primary sequence similarity to other members of the C-type lectin family. The amino-terminal 116 residues of mature L-selectin protein share

approximately 25–30% amino acid sequence identity with other C-type lectins, especially mannose-binding proteins. The functional contributions of each domain within L-selectin have been examined using antibody blocking approaches [97] and recombinant L-selectin chimeras. In general, these studies confirm that the CRD in L-selectin is directly involved in adhesion interactions with glycan-based ligands, including especially amino acids within the carboxy-terminal portion of the CRD. Such studies also indicated that the EGF-like domain plays an important part in helping to maintain the CRD in its proper configuration for ligand recognition, and the two CR repeat segments within L-selectin are required to maintain it optimal [89]. Apart from ligand recognition, the cytoplasmic domain of the receptor is essential for L-selectin to support leukocyte rolling and binding of lymphocytes to HEV [162].

Unlike in case of endothelial selectins, crystal structure of L-selectin has not been solved yet. Pioneer models [97,163] have been made based predominantly on the structure of the rat manose binding protein, until the crystal structure of E- and P-selectin was solved. Since the lectin domains of selectins are highly homologous, and hydrophobic cores are absolutely conserved, it is ti expect that the structures of E-, P- and L-selectin will share extensive similarity as well. L-selectin is expressed on blood monocytes, neutrophils, subsets of natural killer cells, and T and B lymphocytes, including virtually all lymphocytes of the “naive” phenotype, but not by lymphocytes that exhibit the “memory” phenotype. L-selectin is also expressed in both early and mature hematopoietic cells in the bone marrow, except that cells of the B-lymphocyte lineage seem to express this molecule only near the latter stages of the B-lineage developmental program.

One unique feature that distinguishes L-selectin from other known adhesion molecules is that it is cleaved from the cell surface within minutes after cellular activation in process catalyzed by one or more metalloproteinases (secretases) [135,164]. L-selectin cleavage generates a functionally active receptor that is present at relatively high levels (1-2 μg/ml) in serum therefore is proposed to moderate leukocyte/endothelial interaction and leukocyte entry into tissue [165,166]. There is evidence that calmodulin regulates the protease-dependent shedding process. It has been proposed that L-selectin shedding serves to facilitate release of adhesion of cells from the endothelium during the transmigration process [167].

1.2.4 L-selectin ligands

Adhesion mediated by L-selectin is characterized by interactions of apparently low affinity (0.1 mM range), exhibiting relatively rapid “on-rate” and “off-rate” characteristics. These adhesive interactions operate predominantly under conditions of vascular shear flow. Four glycoprotein ligands for L-selectin have been identified, which posses all structural requirements for the successful binding: sulfation, sialylation and fucosylation are required [168-170]. Initially, ligands were found on mouse lymph node HEV using MECA-79. Glycosylation dependent cell adhesion molecule-1 (GlyCAM-1) is so far the best characterized L-selectin ligand expressed by HEV of lymph nodes. This is a secreted sialomucin [171,172] of 132 amino acids and 50 kDa, which contains numerous serine and threonine residues with the high content of *O*-linked sugars. The sequence of GlyCAM-1 does not predict a transmembrane segment, suggesting that any association between GlyCAM-1 and the HEV cell surface must be mediated by virtue of a 21-residue amphipathic helix at its carboxyl terminus, or more likely by interactions with one or more other membrane-tethered molecules, as GlyCAM-1 binding to lymphocytes can stimulate the activation of β_1 and β_2 integrins [173,174]. Protein is detectable in serum and has been proposed to regulate the adhesive and/or activation state of L-selectin-bearing leukocytes through interactions with L-selectin on such cells [175].

A second L-selectin ligand expressed by HEV is CD34, which is expressed throughout the endothelial cells of the vasculature, on hematopoietic precursor cells, on a number of embryonic fibroblast cell lines and in the brain [176,177]. Correctly glycosylated for L-selectin recognition was expressed only in HEV of lymph nodes. CD34 is a type I transmembrane sialomucin containing several mucin-like domains with numerous serine and threonine residues predicted to be heavily *O*-glycosylated. Since CD34 is a transmembrane glycoprotein, this L-selectin ligand is positioned to contribute substantially to the process of tethering and adhesion of circulating lymphocytes to lymph node HEVs. However, CD34 is clearly not the sole contributor of L-selectin ligand activity in HEVs, since CD34-null mice maintain virtually normal L-selectin-dependent lymphocyte homing activity [177].

A third molecule identified as an L-selectin ligand is termed MAdCAM-1, for mucosal vascular addressin cell adhesion molecule [178]. MAdCAM-1 had been studied previously as the ligand for the integrin-type lymphocyte homing receptor $\alpha_4\beta_7$, a molecule responsible for the bulk of lymphocyte homing to Peyer's patches. Biochemical

experiments indicate that MAdCAM-1 expressed in mesenteric lymph nodes is decorated with O-glycans that can support L-selectin-dependent leukocyte adhesion. MAdCAM-1 is therefore predicted to maintain ligand activity for both L-selectin and the $\alpha 4\beta 7$ integrin [179,180] A fourth HEV-expressed L-selectin ligand is the transmembrane sialomucin podocalyxin-like protein or PCLP. Like CD34, PCLP can be secreted, or expressed on some vascular endothelia, but it is also expressed on the foot processes of glomerular podocytes Levels of GlyCAM-1and secreted PCLP are downregulated 3-4 days after the induction of an immune response, whereas the levels of the cell-associated ligands, CD34 and PCLP remain largely unaltered throughout the immune response [181,182].

1.2.5 Structural specificities of E-selectin

The sequence of the human E-selectin cDNA predicts a type-I transmembrane glycoprotein that belongs to the group of C-type lectins [5,95,183,184]. Structural predictions and domain organization first were done in mutational studies [97]. To increase the understanding of E-selectin structure and its interactions with the ligand, a recombinant fragment of CRD and EGF-like domain of E-selectin was crystallized and solved [104], as well as the structure of LecEGF domain co-crystalized with sLe^x [99]. E-selectin posses 21 amino acids long signal sequence, cleaved in mature protein. An extracellular, amino-terminal C-type lectin domain (carbohydrate recognition domain) has 118 amino acids and forms a ligand binding site. This domain has 4 cystein residues (Cys 19, 90, 109 and 117) that form 2 disulfide bridges in 1-4 and 2-3 pathways. Lectin domain contains one potential N-linked glycosylation sites, the asparagine that is closest to the N-terminus and is buried in the protein core. Overall fold of lectin domain is very similar to the lectin domain of the rat mannose binding protein [185].

Epidermal growth factor-like domain shares the same general fold and arrangements of disulfide bonds as other EGF-like domains [186]. It is a 36 amino acids long domain that contains the 6 Cys residues forming 3 disulfide bridges (Cys122 and Cys133, Cys127 and Cys142, Cys144 and 153). Inner EGF-like domain, there are 2 *N*-glycosylation sites, which are more surface-accessible. Interestingly, the interaction between lectin and EGF domains is based only on the residues 135-139 of the EGF domain that make contact with the lectin domain. These contacts include several hydrogen bonds that involve main-chain atoms, whereas side chains of Glu135 and Gln30 is the only exception. Although the contacts are limited, relative orientation of two domains is not prone to

dramatic changes. The calcium binding site has been coordinated by the amino acids as described on *figure* x and table x. It has been determined that E-selectin contains a high affinity Ca^{2+} site with a K_D of ≅ 3.5 μM [187]. However, at high Ca^{2+} concentration, more then one calcium was able to bind to E-selectin, consistent with compared crystallographic data that differed in crystallographic conditions. When limited proteolysis was used as a probe to monitor E-selectin conformation, Ca^{2+} binding protected the protein from Glu-C endopeptidase [151,187,188]. This protective effect was further augmented upon sLe^x binding, indicating that Ca^{2+} binding induces conformational changes that facilitate ligand binding, which in turn, stabilizes Ca^{2+} binding. Loop adjacent to the calcium binding region (residues 94-103), although does not directly interact with it, adopts a conformation that tilts the loop toward Ca^{2+}. Since Tyr94 and Arg97 are critical for mediating cell adhesion, it is possible that unusual conformation of this loop enhances proteins interaction with carbohydrate ligand. In case of Glu-C digestion, major cleavage site was Glu98, within mentioned loop. Although proteolytic fragments were still associated with each other through disulfide bonds, they did not retain Ca^{2+}, or ligand binding activity. Thus, maintaining the rigid conformation of this loop is critical for E-selectin function. Residues Glu92 and Glu107, minor cleavage sites are close to each other and adjacent to the Ca^{2+} site, and probably the removal of Ca^{2+} in E-selectin induces conformational changes in this region.

Consensus repeat domains in human E-selectin are 62 amino acids long and further contribute to the *N*-linked glycosylation of the native protein, that can be up to 50% of predicted molecular weight [90]. Whether this part is indirectly involved in binding or not, is still not clearly understood. Short membrane spanning region of 21 amino aicds is interesting in elucidating the mechanisms of E-selectin proteolytic cleavage, and structural requirements for protease recognition. Cytoplasmic, intracellular domain is involved in signal transduction events that are mediated through E- selectin, although these mechanisms are still under investigation.

E-selectin expression during inflammation requires de novo gene transcription for expression, and that is why it does not play significant role in the earliest phases (<2 h). In E-selectin knockout mice, no defect of leukocyte recruitment to the inflamed peritoneum is evident, unless P-selectin function is simultaneously blocked with monoclonal antibodies [189]. The E-selectin serves an important role as a tissue-specific homing receptor for leukocyte recruitment specifically to the skin, particularly for memory

T cells. Leukocyte recruitment into human skin grafted onto severe combined immunodeficient mice depends on E-selectin, as well as T-cell infiltration into sites of cutaneous DTH, dermatologic disorders, and malignancy [190,191]. Since chronic expression is frequently seen in the skin, it has been hypothesized that E-selectin serves as a tissue specific “homing receptor” for leukocyte recruitment to the skin. Molecular dynamics of adhesion receptors at the neutrophil membrane upon E-selectin binding, show that E-selectin is unique among selectins in its capacity for clustering sialylated ligands and transducing signals leading to neutrophil arrest in shear flow [94].

1.2.6 E-selectin ligands

E-selectin ligands are expressed by neutrophils, monocytes, eosinophils, memory-effector T-like lymphocytes, and natural killer cells [184,190,191]. Each of these cell types is found in acute and chronic inflammatory sites in association with expression of E-selectin, thus implicating E-selectin in the recruitment of these cells to such inflammatory sites [184,192]. E-selectin interaction with its ligands could be described as a receptor-ligand-epitope interaction. Structural analysis of glycans present on the cells that interact with E-selectin revealed glycoprotein and glycolipid components of the myeloid cell membranes with the epitope defined as sialyl Le^x tetrasaccharide as a typical representative of a family of α2-3-sialylated and α1-3-fucosylated glycans that exist on *N*-glycans, on lipid-linked glycoconjugates, and on *O*-glycans [193], *figure* 1.6. Sialyl Lewis^a is structural isomer that has also been recognized as E-selectin ligand [194]. The latter set includes core-2-based lactosamine and polylactosamine chains. PSGL-1, fucosylated variably, at one or more GlcNAc residues in the polymer has been proposed as ligand for E-selectin [154] on neutrophils, HL-60 cells and T cells, without necessity for the sulfation of exposed sugars. This binding is of lower affinity compared to P-selectin and L-selectin binding to PSGL-1. Binding to E-selectin is also unaffected by OSGP treatment, in contrast to P-selectin binding, which is abolished.

A second candidate E-selectin ligand is a glycoprotein purified from bovine γ/δ T lymphocytes, as a bovine homolog of PSGL-1 [195,196]. This protein has an unreduced molecular mass of 250 kDa, and may also function as a ligand for P-selectin.

A third E-selectin ligand candidate, termed ESL-1, is a 150 kDa glycoprotein that has been purified and characterized from mouse neutrophils and from murine myeloid lineage cells Because of its location along the surface of microvilli, ESL-1 may function

in a step after capturing. Although ESL-1 is a strongly preferred target for generation of E-selectin –binding carbohydrates [196]. The human neutrophils had been found to interact with E- selectin through L-selectin [197], in a binding that was dependent on the sialic acid expressed on L-selectin. In recent studies has been demonstrated that that E-slectin binding to neutrophils in suspension, or as they role on a monolayer cell substrate in shear flow results in clustering of PSGL-1 and L-selectin, which was facilitated by fluid shear and dependant on MAPK-regulated membrane transport processes [94]. Other suggested E-selectin ligands include members of the non specific cross-reactive antigen (NCA) family present on the human neutrophils [198], subpopulation of the β_2 integrins that carry sLe^x and the heavily sLe^x modified lysosomal membrane protein lamp-1 [199]. As already described, E-selectin affinity for the ligand epitope defined by sLe^x is 8-15 fold higher compared to P-selectin and L-selectin (0.8-1 mM) [200]. The insight into molecular mechanism of this interaction was gained in co-crystalization of E-selectin lectin and EGF-like domain with the sLe^x. These data revealed that the interactions were almost entirely electrostatic in nature and that total buried surface area is relatively small (549 $Å^2$). The 3- and 4-hydroxyl groups of Fuc residue within sLe^x ligate the selectin-bound calcium ion. These two groups make additional interactions with the protein side chains that also bind the calcium ion. The 4-hydroxyl-group of galactose binds to the side chain of Tyr94, while 6- hydroxyl group binds to the side chain of Glu92. The sialic acid makes two interactions in the E-selectin, one to the side chain of Arg97, and the other to the Tyr48. In P-selectin Arg97 is replaced by serine, thus eliminating an ion-ion interaction and could partially rationalize higher affinity of sLe^x in E-selectin over P-selectin [201].

Table 1.1: Interactions in binding site of E-selectin and sialyl $Lewis^x$ (bb: backbone, sc: side chain) [103].

Interacting moieties	
Ca^{2+}	-Fuc O-3
Ca^{2+}	-Fuc O-4
Glu80 sc	-Fuc O-4
Asn82 sc	-Fuc O-4
Tyr94 sc	-Gal O-4
Glu92 sc	-Gal O-6
Arg97 sc	-Gal O-1
Tyr48 sc	-*N*euAc COOH
Arg97 sc	-*N*euAc COOH
Ca^{2+}	-Glu80 sc

Ca^{2+}	-Asn82 sc
Ca^{2+}	-Asn82 sc
Ca^{2+}	-Asn105 sc
Ca^{2+}	-Asp106 bb
Ca^{2+}	-Asp 106 sc

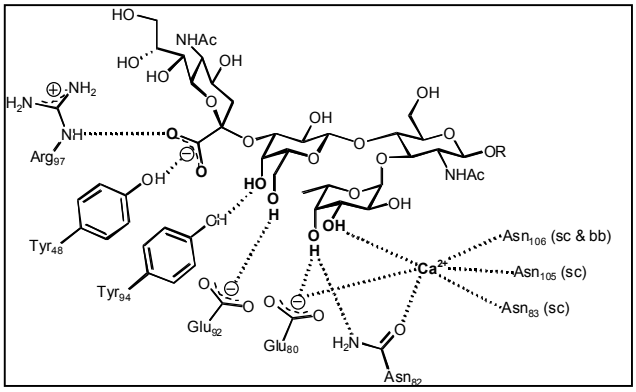


Figure 1.6: Model of E-selectin binding to sLe^x

1.2.7 Molecular mechanisms of selectin-ligand interaction

The principles that have emerged about the lectin-ligand interactions are following: the binding sites are of relatively low affinity and are found in shallow grooves on the surface of the proteins. Second, selectivity is mostly achieved via a combination of hydrogen bonds (involving the hydroxyl groups of the sugars) and via van der Waals' packing of the hydrophobic faces of monosaccharide rings against aromatic amino acid side chains. Third, further selectivity can be achieved by additional contacts between the saccharide and the protein, sometimes involving bridging water molecules or divalent cations. Finally, the actual region of contact between the saccharide and the polypeptide typically involves only one to three monosaccharide residues. As a consequence of all of the above, these lectin-binding sites tend to be of relatively low affinity, but of high specificity. The ability of such low-affinity sites to mediate biologically relevant interactions in the intact system thus appears to require multivalency. Special molecular mechanisms and contact between the cells are required for the recruitment of leukocytes from the rapidly flowing blood stream.

To support leukocyte rolling, selectins have been proposed to have rapid k_{on} (bond association constant) and rapid k_{off} (bond dissociation constant), as well as special mechanical properties linking tensile forces and bond dissociation [202,203]. The ability

to initiate rolling (capture or tethering) is most closely related to *on* rate, consistent with the L-selectin being more efficient, because its intrinsic cellular *on* rate is higher then those of vascular selectins. Actual rolling velocity, however, is determined predominantly by the *off* rate; therefore, higher *off* rate caused higher rolling velocity. L-selectin indeed has higher off rate, which might be due in part to shedding of the L-selectin, whereas E-selectin has the slowest velocity. The differences in *off* rate between selectins are reflected in bond life-times: the shorter the lifetime of the selectin-ligand bond, the higher the *off* rate, and faster the velocity. Next important property of selectins to mediate the rolling is the resistance to an increase in the *off* rate with increasing force applied to the bond, phenomena termed "tensile strength". This resistance to increased *off* rate is important for the rolling, otherwise, selectin bonds would have very short lifetime, lower then the minimum required to maintain rolling bonds on the trailing edge are broken, whereas there would be no time for the new ones to form. Selectin ligand bond also shows paradoxal lost of adhesion at very low shear, indicating requirements for a threshold hydrodynamic shear, especially for L-selectin [204]. This is likely related to the shorter bond lifetime of L-selectin, because very low shear may be insufficient to promote new bond formation on the leading edge of the cell, before existing bonds break. A threshold shear requirement may serve to inhibit leukocyte accumulation in situations of abnormally low shear in the vasculature.

1.2.8 Development of selectin antagonists

Selectins are required for the earliest leukocyte recognition and rolling event and therefore present an attractive therapeutic target to treat inflammatory diseases. Several animal studies have successfully validated the use of selectin inhibitors for the treatment of inflammation in applications such as asthma, arthritis, ischemia/reperfusion, and transplantation [205-208]. SLe^x is a key structure mediating selectin-ligand interaction and pharmacophores of sLe^x-selectin interaction have been described [209,210], *figure* 1.7.

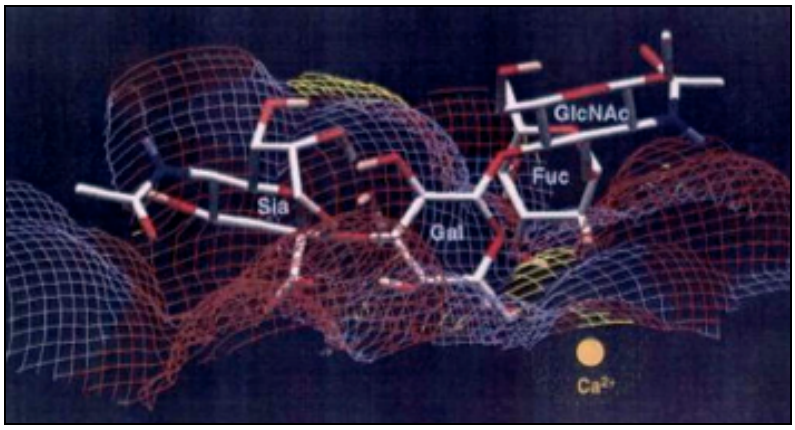


Figure 1.7: The bioactive conformation of sialyl Le^x is docked into the lectin domain on the surface of E-selectin.

However, according to the Lipinski's "rule of 5" that defines optimal requirements for potent new chemical entity, sLe^x is only a good carbohydrate scaffold that has to be optimized toward lead and further toward new chemical entity that can reach the market [211]. Therefore, based on the rational design of the bioactive conformation of the native ligand, different approaches have been taken to improve its drugability, i.e. to simplify sLex structure and improve stability, cost-effectiveness, and synthesis. Mimics of sLe^x could be sorted on the basis of number of sugars in the molecule to three, two, and one sugar-containing molecules, however, maintaining the key interactions with the protein [212]. Several different glycommimetics have been developed on this carbohydrate scaffold and E-selectin inhibitors with low micromolar range IC_{50} have been synthesized [213-216]. Since the selectin-ligand interactions are multivalent, inhibitors presented in a multivalent form should posses increased potency. Polymers, liposomes and protein conjugates containing sLe^x or similar carbohydrates have shown increased binding to E- and P-selectins relative to their monomeric derivatives [217].

Several other groups have produced libraries of compounds, fucopeptides or β-C mannoside-based peptides [218-220]. Some of the earliest inhibitors of the selectins were monoclonal antibodies directed against specific selectin molecules. These antibodies have the advantage of a high degree of a specificity, but they cross reacted with a few species, and were effective only at doses of 1-2 mg [221]. The most advanced selectin inhibitor TBC-1269, a low molecular weight dimeric glycomimetic is currently in the phase II clinical trial for childhood psoriasis whereas it failed to show any

benefit in phase II clinical trial for asthma [222,223]. The anti-inflammatory effects of this mimetic are still contradictory [224].

1.3 Recombinant protein expression systems

With the rapid progress in biotechnology, optimal protein expression is of great importance, since it has to meet challenging requirements of structural biology, chemistry and drug discovery. Parameters to be considered, when choosing an expression system, are the nature of the protein to be expressed, amount of recombinant protein needed in combination with the intended use, the design of a suitable expression vector for an expression system (including a potential tagging) and the availability or establishment of appropriate assay systems and a tentative purification protocol for the recombinant protein. For each individual product, the most suitable expression system has to be identified and optimized individually, by taking into account the properties of product and organism. The overall decision criteria for the choice of an expression system are the pharmacological activity profile of the yielded protein in context with posttranslational modifications and rentability.

1.3.1 Mammalian expression system

Mammalian system for recombinant protein expression is particularly useful for the expression of proteins with complex post-translational modifications, predominantly disulfide bond formation and complex glycan pathways. Although expression of many functional proteins can be achieved in *E.coli,* sometimes it is possible to express extracellular domains of mammalian proteins exclusively in mammalian system. Compared to the other expression systems, the yield, time and costs are more consuming in mammalian systems, although significant progress has been done especially in system optimization in preparative scale protein production [225,226]. The key factors for success in recombinant protein expression are transfer techniques, optimal cell culture maintenance and careful selection of expression plasmid [227,228]. Gene transfer via transfection plasmid leads either to stable integrated copies of the transgene into the host genome (stable expression), or to an episomally replicating plasmid which are gradually lost (transient expression). Preferable are mammalian systems like CHO, BHK cells and myeloma cells for stable protein expression, and COS cells for transient expression [229]. These systems have the further advantage of being

recognized as safe regarding infectious and pathogenic agents and therefore have a higher acceptance by regulatory bodies.

1.3.2 Bacterial expression system

The most widely used recombinant protein expression systems are those bacterial systems using *Escherichia coli* as host cell. *E.coli* can be easily genetically manipulated, has a short doubling time and can be cultivated on inexpensive media. Strategies for gene expression in *E.coli* include intracellular expression, secretion of the protein into the periplasmic space and very rarely used protein secretion into the medium. However, for functional analysis the expressed proteins have to be in their native state. Due to the membrane structure, the low chaperone and foldase level and the high periplasmic protease concentration, major problems arise that include cell toxicity, inefficient translation, improper processing or post-translational modifications [230]. Some of these problems can often be solved by testing differently tagged/non-tagged proteins and *E.coli* strains, optimizing expression conditions by lower growth temperature and varying the induction levels. Furthermore, it may be reasonable to test different *E.coli* strains with different genetic background, because certain strains tolerate some proteins better than others and allow higher levels of expression before forming inclusion bodies. Modulation of the protein primary structure can also condition *E.coli* strains to exert a strong influence on productivity and efficiency, mostly in the case of secreted proteins [231,232]. The intracellular protein content is often a balance between the amount of soluble protein in the cells, the formation of inclusion bodies and protein degradation. In case when protein is only expressed as inclusion body, protein solubilization under denaturing conditions and subsequent protein refolding [233] can lead to the correct folded, native protein. The best refolding conditions have to be determined empirically and are very specific for each protein, what can be time- and cost consuming, sometimes even impossible.

1.3.3 Insect cells expression system

As an expression system, baculovirus infected insect cells characterize gene transfer via viral infection on rather transient, then stable basis [228,234,235]. Large DNA portions can be transfected by incorporation into baculovirus genome, and subsequently into insect cells. Recombinant DNA transfer can be done in transfection (DNA of interest incorporated into the viral genome prior to the transfection into insect cells), or co-

transfection, where DNA of interest and linearized viral DNA are simultaneously transfected into insect cells. Based on the chosen method, wide variety of vectors is available. For the recombinant protein production, *Autographa californica* nuclear polyhedrosis virus (AcNPV), and *Bombyx mori* nuclear polyhedrosis virus (BmPV) are mainly employed. Insect cells mostly used are of the species *Spodoptera frugiperda* (*Sf9* and *Sf21* insect cells), and for the secreted proteins *Trychlopusia ni (High five*™ insect cells*).* Baculovirus expression of recombinant proteins permits folding, post-translational modification and oligomerization in manners that are often identical to those that occur in mammalian cells. These enable proper proteolysis, *N*- and *O*-glycosylation, acylation, amidation, carboxymethylation, phosphorylation, and prenylation. Proteins may be secreted from cells or targeted to different subcellular locations. Protein expression can be placed under the control of different promoters, dependent of the protein characteristics. For secreted, glycosylated proteins an early promoter can improve the functionality of the protein, but in lower yields, whereas stronger polyhedrin promoter enhances the protein yield, and is mostly used, allowing levels of expression of up to 30% of the total cell protein. For commercial applicator, scale-up techniques are under optimization, as well as construction of new innovative vectors and coexpression of chaperons, foldases and folding factors [234, 236].

1.4 Elucidation of protein-ligand interactions

The analysis of molecular interactions is a key part of the drug discovery process. In the case of structure-based drug design, major analyses are done using X-ray crystallography, nuclear magnetic resonance, surface plasmon resonance and classical competitive binding assays. X-ray crystallography and NMR are mostly used for structural assessments of ligand binding site on the target, as well as the binding epitope on the ligand, whereas surface plasmon resonance and binding assays quantify binding constants in high throughput fashion [237,238]. Mutational studies and structure prediction based on a similarity with already solved protein structures can generate very good models [97,151]. However, X-ray crystallography of the target protein, or protein co-crystallized with its physiological ligand or first generation compounds, is an absolute must and necessity for the *in silico* generations of ligands and proof of concept. Major drawbacks of X-ray crystallography are milligrams of pure, homogeneous protein that are required, what can be very expensive if protein cannot be produced in *E.coli.* In addition, it is not trivial to find the optimal crystallographic conditions and the procedure

is not straightforward. Once solved, crystal structure reveals a static complex of protein and ligand, whereas NMR gives a dynamic characterization of protein-ligand complexes, on a time scale from pico/nano seconds to milliseconds. Small proteins often do not get crystallized, whereas NMR has no such size limitations. Structure determination of weakly bound ligands and theirs bioactive conformation, even if receptor is large, can be easily assessed by NMR using NOEs, STDs, and T1rho relaxation time [209,210,215]. For structure determination, protein size limit is around 30-40 kDa, although large deuterated proteins can be solved with TROSY experiments. Isotope labeling with 15N for proteins 10-20 kDa, 13C and 15N for proteins > 20 kDa, and 13C, 15N and 2H for larger proteins is highly recommended [237,239]. The choice between uniform or selective labeling depends on recombinant protein source and prior knowledge about the structure. Protein in milimolar range, 90% purity is required for structural studies, whereas for an NMR screening few milligrams of non-labeled protein suffice. Surface plasmon resonance exploit evanescent wave phenomenon, where binding of molecules in solution to surface-immobilized receptors alters the refractive index of the medium. This change is monitored in real time and can measure accurately the amount of bound analyte, the kinetics of interactions with the k_{off} and k_{on} constants, or the amount of the active protein in the sample [238]. Applicable through the whole drug discovery process, SPR requires microgram amounts of proteins and ligands. Low-molecular mass drugs, as well as multiprotein complexes can be used with the interaction affinities from milimolar to picomolar range. Major drawbacks are sometimes complicated receptor-immobilization procedure and purity of the sample.

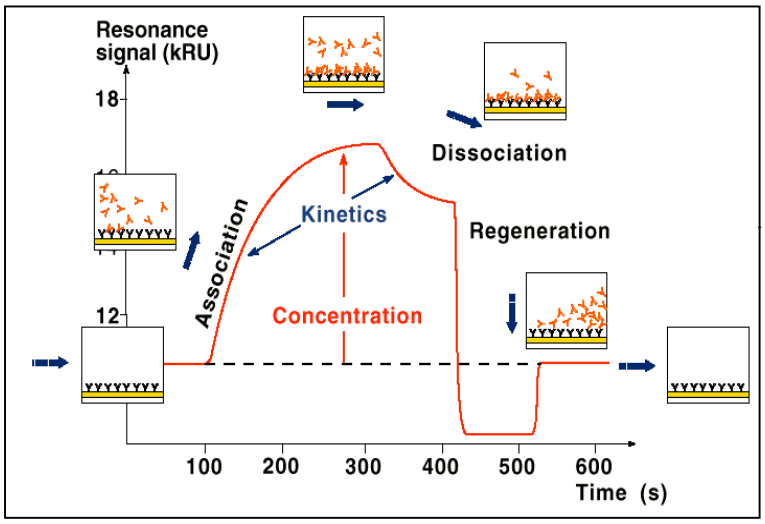


Figure 1.8: Surface plasmon resonance sensogram

Conventional bioassay is mostly used for screening and quantification of protein-ligand interactions. Originally created for 96-wells performance, nowadays it is adopted to 384- and even 2080-format, applicable in HTS and ultra-HTS screening. Bioassay is performed as cell-based assay appropriate for target classes as ion channels, receptors, transcription factors, or biochemical assay that utilizes isolated protein [217,240,241], *figure* 1.9. Typical target classes here are kinases, proteases and binding proteins. Classical readout technologies are colored enzymatic reactions, fluorescence polarization, fluorescence intensity or time-resolved fluorescence. Major drawbacks of the assay are occasional instability or too high sensitivity that might lead to false positive or negative results, as well as compound interference with the readout technology.

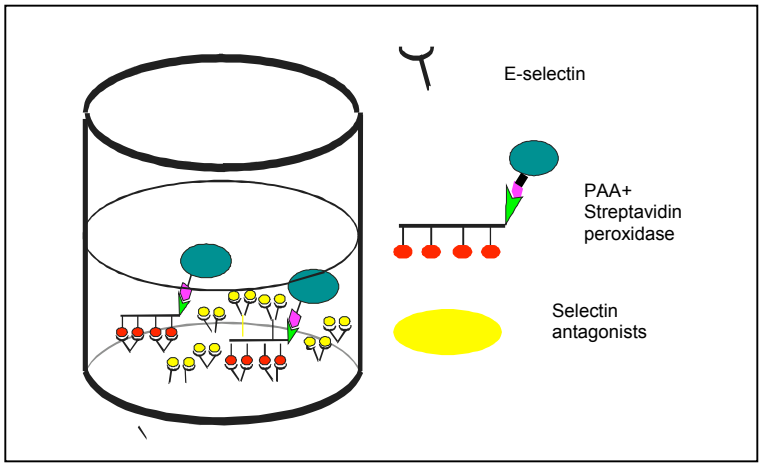


Figure 1.9: Molecule-molecule type of biochemical assay for evaluation of E-selectin antagonists

2 The Aim

The selectins family of adhesion molecules mediates the inflammatory process, i.e the extravasation of leukocytes from blood vessels into the neighboring tissue. Although this represents an essential defense mechanism, excessive or pathological leukocyte accumulation results in numerous disease states and promotes metastatic cancer spread. E- and P-selectin are expressed only as a consequence of an inflammatory stimulus; time-limited and localized to the inflammatory area. Since their inhibition could prevent local pathological inflammation at an early time-point, without undisered effects on other tissues, selectins became a promising target for the pharmaceutical industry. Detailed characterization of ligand-receptor interactions is a prerequisite for successful drug discovery and development. In NMR and X-ray crystal structure studies interaction of the lectin and EGF-like domains of E- and P-selectin co-crystalized with their natural ligand sLe^x have been solved [188,209]. Based on these findings, molecular modeling studies were developed in search for potent selectin antagonists [201]. To develop second generation of E-selectin antagonists, improved structural studies have to be performed with the more precise analysis of protein-carbohydrate interactions that define selectin binding mode. These include crystal structure analysis with the first generation of selectin antagonists that would support further modeling studies, NMR analysis with $^{15}N/^{13}C$ labelled protein, real-time kinetic studies using surface plasmon resonance technology and microcalorimetric studies. In addition, synthesized antagonists have to be rapidly screened in a reproducible and accurate bioassay. Therefore in the first step our aim was to obtain and characterize active, recombinant E-, P- and L-selectin/IgG that consist of lectin, EGF-like and consensus repeat domains of each of the selectins, fused to the Fc part of human IgG. In the second step, we aimed to develop a cost- and time-effective system to express and purify lectin and EGF-like domain of E-selectin in preparative yields sufficient to meet requirements for structural studies. In addition, expressed protein should be thoroughly characterized in terms of in-solution status (monomer, dimer, oligomer) and activity. Therefore our aim is to express lectin and EGF-like domain of human E-selectin in baculovirus-infected insect cells. We hypothesize that lectin and EGF-like domains of E-selectin expressed in this system could be used in a bioassay for evaluation of selectin antagonists. We aim to develop a cell-free assay that will be more accurate and reproducible compared to exisisting E-selectin assays. This

we will test in the proof-of-concept manner evaluating three known E-selectin antagonists and comparing the results to the published [216].

3 Materials and methods

3.1 E-, P-, L-selectin/IgG expression in CHO cells, purification and characterization

For expression of E-, P- and L-selectin / IgG, stably transfected chinese hamster ovary (CHO) cells were grown in the MEM α^- medium (Invitrogen), supplemented with 5% FCS (GIBCO) and 0.5 mg/ml gentamicin (GIBCO). Cells at 10^6 were seeded in 20 ml conditioned medium in 75 cm^2 flask for back-up cultures. Cells were confluent in 3 days and then split and seeded for either a new back-up cultures or for production. Protein production was carried out in the 160 cm^2 culture flasks and in roller bottles, 420 cm^2. 10^6 cells were seeded in 60 ml medium in 160 cm^2 culture flasks and let to grow until confluence for 5-6 days. Medium was harvested and new medium added to the cells in the production phase, which lasted up to another two weeks. For production in roller bottles $2x10^7$ cells were seeded in 200 ml medium and grown as adherent cultures in 5% CO_2 atmosphere, at 37°C and 90 rpm speed. Cells were grown for 1 week before starting the production phase, when 100 ml fresh medium was added and protein production continued for up to total of three weeks. To establish the optimal conditions for cell harvesting, small-scale analysis was done. Every day an aliquot of the conditioned medium was collected and purified over protein A chromatography. Also, pH value of the medium was daily controlled and cells examined under microscope. After production, medium was harvested by centrifugation at 1000xg, 4°C and 20 min. pH value of the supernatant with the secreted protein was adjusted to 7.6 and the salt concentration was set up to 0.15 M NaCl. Medium was filtrated through 0.22 μm sterile filter and prepared for protein A chromatography.

3.1.1 E-, P-, L-selectin/IgG purification on protein A affinity chromatography

All purification steps were carried out at 4°C. For purification purposes, 5 ml protein-A agarose column (BIO-RAD) was used on Fast Protein Liquid Chromatography (FPLC) system BioLogic Duo-Flow (BIO-RAD) with the software BioLogic Duo-Flow 3.0 (BIO-RAD). The column was equilibrated with 10 column volumes of binding buffer; TTBS (0.15 M NaCl, 0.05 M Tris, 0.05% Tween 20, pH 7.6), until stable baseline. Conditioned

medium was applied in 200 ml portions to avoid overloading of column. In the next step unbound material was washed with 10 column volumes of binding buffer, followed by 50 ml washing buffer (0.005 M $CH_3CH_2COONH_4$, pH 5.0) to remove all unspecific-bound proteins. Proteins specifically bound to the column were eluted with 3 column volumes of elution buffer (0.5 M CH_3CH_2COOH, pH 3.4). Usually 3.5-4 ml fractions were eluted as a single sharp peak. Immediately upon elution pH was adjust with 1 M Tris buffer to pH 7.0. Overall chromatography flow-rate was 1ml/min.

3.1.2 E-, P-, L-selectin/IgG purification by gel filtration

As a second purification step, gel filtration was performed to separate proteins bound with Fc part of IgG to the protein-A agarose. Sephadex column 200 ml, (Amersham Pharmacia) was equilibrated in TBS buffer (0.15 M NaCl, 0.05 M Tris, pH 7.6) until stable baseline. Protein-A-eluted fractions were pooled together, concentrated up to 4 ml and applied to the gel filtration column with a flow rate of 1 ml/min. Proteins were separated in the total volume of 120 ml, in the 4 ml fractions. After SDS PAGE analysis, E-sel/IgG-containing fractions were pooled together and concentrated in the Amicon concentration device, 30 kDa cut-off. Concentration was done at 4°C, 4000 rpm in the Sorvall centrifuge with H8-rotor.

3.1.3 SDS-PAGE electrophoresis and gel visualization

The whole purification procedure and final protein products, as well as all protein characterizations were analyzed by SDS-PAGE electrophoresis. Denaturing gel, 8% and protein samples were prepared according to the standard procedure [242] using buffers described in the *table* 3.1, *table* 3.2, and *table* 3.3. For visualization, separated proteins were predominantly Coomassie stained, and in cases when higher staining sensitivity was required, proteins were stained with silver. During electrophoresis, the applied current was 30 mA/gel in stacking, and 40 mA/gel in separating gel.

Table 3.1: Protocols for buffers used for SDS-PAGE

Buffer	(10x) Running buffer	(1x) Separating gel buffer	(1x) Stacking gel buffer	(3x) Reducing sample buffer
Components:	0.25 M Tris-HCl 2 M Glycin 1% (w/v) SDS pH 8.3	1.875 M Tris-HC pH 8.8	1.25 M Tris-HCl pH 6.8	0.065 M Tris-HCl 20% (v/v)Glycerin 10% (v/v) β-Mercaptoethanol 4% (w/v) SDS Spatule tipp of Bromphenolblau, pH 6.75

Table 3.2: Protocol for 5 ml 8% stacking gel preparation

Substance	Volume [ml]
H_2O	2.3
30% Acrylamide	1.3
1.5 M Tris (pH 8.8)	1.3
10% SDS	0.05
40% APS	0.05
TEMED	0.003

Table 3.3: Protocol for 2 ml 5% separating gel preparation

Substance:	Volume [ml]
H_2O	1.4
30% Acrylamid	0.33
1.5 M Tris (pH 6.8)	0.25
10% SDS	0.02
40% APS	0.02
TEMED	0.002

3.1.3.1 Silver staining

Gels were stained with the prepared kit from Amersham, Pharmacia, according to the supplier's protocol [243].

3.1.3.2 Coomassie staining

Gels were fixed in the fixation solution for up to two hours, and incubated in the staining solution for 2 hours. Destaining was done with several destaining solution exchanges. *Table* 3.4 describes solutions used for Coomassie staining.

Table 3.4: Protocol for 1L Coomassie staining solutions

Fixation solution	Volume [ml]	Staining/solution Destaining	Volume [ml]
CH_3OH	75	CH_3OH	75
C_2H_5COOH	100	C_2H_5COOH	100
H_2O	825	H_2O	825
-	-	Coomassie Brilliant Blue	1 g

3.1.4 Protein concentration determination by Bradford assay

Protein concentration was determined by Bradford assay [244]. The standard curve was prepared using bovine serum albumine in a concentration range from 0-200 μg/ml. For the preparation of Coomassie reagent, 10 mg Coomassie Brilliant Blue G-250 (BIO-RAD) were dissolved in 10 ml 85% phosphoric acid and 5 ml 95% ethanol, and filled up to 100 ml with water. Solution was filtrated prior to use.

20 μl protein samples were mixed with 50 μl 1M NaOH solution in an eppendorf tube. 1 ml reagent solution was added, and the reaction incubated at RT for 15 min. The absorption, which is directly proportional to the protein concentration, was measured on BIO-RAD Smart Spec spectrophotometer at 595 nm.

3.1.5 Mass spectrometry sequence analysis

Protein identity was confirmed by MS analysis. Single band representing purified protein was excised from the Coomassie stained 8% gel and digested with trypsin overnight at 37°C. Cleaved fragments were analyzed by LC/MS on Mass Spectrometer Finnigan TSQ 7000 and compared with predicted amino acid sequences.

3.1.6 Western blot analysis (Immunoblot analysis)

Western blot analysis performed on Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell BIO-RAD. Proteins were separated on 4% native polyacrylamide gel under native PAGE (all buffer components as described in the *tables* 3.1, 3.2, and 3.3, without SDS), at constant current of 20 mA/gel, and at 4°C. For the protein transfer to the nitrocellulose membrane, the following buffers were prepared:

Anode buffer I: 0.3 M Tris, 20% methanol (v/v)

Anode buffer II: 0.025 M Tris, 20% methanol (v/v)

Cathode buffer: 0.4 M 6-aminocapronic acid, 20% methanol (v/v)

Nitrocellulose membranes and 12 pieces of Whatman-paper were prepared in dimension 6.5x8.5 cm. After proteins were separated, the gel was incubated in cathode buffer for 5 min. 4 filters were incubated in anode buffer I and 2 filters in anode buffer II. The nitrocellulose membrane was first incubated for 2 min. in water, and then for 5 min. in anode buffer II. Filters incubated in anode buffer I were first prepared and then filters in anode buffer II, followed by membrane and the gel. The gel was covered with 6 filters

soaked in cathode buffer and finally with graphic cahtode. Protein transfer was done at 14 V for 1 hour. Successful membrane transfer was verified by staining with Ponceau S solution (SIGMA), and the membrane was immediately blocked for 1 h at RT in 3% BSA in 2 mM Ca^{2+} TTBS-buffer. After blocking, the membrane was washed 3 times for 5 min. with 2 mM Ca^{2+} TTBS-buffer and incubated with primary antibody at 10 μg/ml in 2 mM Ca^{2+} TTBS, 0.1% NaN_3 and 1% BSA. Incubation with primary antibody was done overnight, at 4°C, followed by membrane washing 3 times for 5 min. The membrane was shaked with the secondary antibody, the alkaline-phosphatase conjugated goat, anti-mouse IgG for 1.5 hours at RT, and washed twice as described above, while last time in 2 mM Ca^{2+} TBS, instead of 2 mM Ca^{2+} TTBS-buffer.

For the membrane visualization, developing buffer was prepared with 0.1 M Tris, pH 8.8, 0.1 M NaCl and 0.005 M $MgCl_2$.

NBT-BCIP substrate solution (40 μL) was added to 10 ml buffer, and alkaline phosphatase catalyzed reaction was developed until violet protein bands appeared. The membrane was washed in water to stop the reaction, dried and kept in the dark.

3.1.7 *N*- and *O*-deglycosylation

Deglycosylation was performed on the analytical scale with *N*-Glycosidase F (Roche) under reduced and native conditions [245]. Reaction set-up (*table* 3.5) was optimized according to the supplier's prescription:

Table 3.5: Reaction set-up for deglycosylation under reduced and native conditions

Reducing conditions	Native conditions
0.1 M NaPi	0.1 M NaPi
0.025 M EDTA	0.025 M EDTA
0.1% SDS	0.1% SDS
2% Tween-20	2% Tween-20
2 U *N*-Glycosidase F	2 U *N*-Glycosidase F
3 μg protein	3 μg protein
1 % 2-mercaptoethanol	–
H_2O up to 100 μl	H_2O up to 100 μl

Deglycosylation in denaturing conditions required protein with all reaction components to be boiled at 99°C for 5 min., prior to enzyme's addition. PNGase F was added only when sample were cooled down to RT. Components were mixed together and incubated at 37°C for up to 48 hours. For time course optimization, aliquots were taken at different time points and extent of deglycosylation was analyzed by SDS-PAGE.

N-deglycosylation under native conditions was done as described for 6 hours and the activity of deglycosylated protein was examined by native Western blot.

Combined deglycosylation

O-Glycosidase (Roche) was used in combination with *N*-Glycosidase in both reducing and native conditions [246], as described in *table* 3.6. Aliquots were taken at different time points and analyzed on SDS-PAGE.

Table 3.6: Reaction set-up for combined *N*-and *O*-deglycosylation

Reducing conditions	Native conditions
0.02 M NaPi	0.1 M NaPi
0.025 M EDTA	0.025 M EDTA
0.1% SDS	0.1% SDS
2% Tween-20	2% Tween-20
2 U *N*-Glycosidase F	2 U *N*-Glycosidase F
3 μg protein	3 μg protein
1 % 2-mercaptoethanol	_
2.5 mU *O*-Glycosidase	2.5 mU *O*-Glycosidase
H_2O up to 100 μl	H_2O up to 100 μl

Separated deglycosylation

N-deglycosylation was performed first, under reducing conditions. After 6 hours, aliquot was taken and the incubation buffer components adjusted to the ones described in the *table 3.7*.

Table 3.7: Buffer exchange and reaction set-up for subsequent *N*- and *O*-deglycosylation

Reducing conditions Buffer exchange
0.0175 M NaPi

0.025 M EDTA
0.1% SDS
2% Tween-20
1.65 mU *O*-Glycosidase
57.45% H_2O

After 18 h incubation with *O*-Glycosidase, deglycosylation extent was analyzed on 8% reducing gel. To address the protein activity, reaction set-up in native conditions was done and deglycosylated protein tested in native Western blot.

O-deglycosylation

Reaction set-up for *O*-deglycosylation and the incubation buffer were prepared as described in the *table 3.8*, [246]. *O*-deglycosylation was performed for up to 24 hours. Aliquots were taken after 3 and 24 hours and controlled by 8% SDS-PAGE.

Table 3.8: Reaction set-up for *O*-deglycosylation

Reaction conditions
10 μg protein
0.25 mU *O*-Glycosidase
Phosphate-citrate buffer up to 50 μl

N-Deglycosylation with *N*-Glycosidase A

Further *N*-deglycosylation was performed with *N*-Glycosydase A (Roche), according to the manufacturer prescription and in conditions described in the *table 3.9*. Samples were analyzed after 3 and 24 hours on reducing 8% SDS-PAGE.

Table 3.9: Reaction set-up for *N*-deglycosylation with *N*-Glycosidase A

Reaction conditions
10 μg protein
0.2 mU *N*-Glycosidase A
0.1% SDS
Phosphate-citrate buffer up to 50 μl pH 5.5

3.1.8 Molecular weight determination

Protein's molecular weight was determined in native conditions using non-denaturing protein molecular weight kit (SIGMA). Native PAGE was performed on the gels with different acryl amide percentage, using buffers described in the *table 3.10*. The molecular weights of selectins were determined by indirect calculation as described [247].

Table 3.10: Buffers for non-denaturing molecular weight determination:

Electrode buffer pH 8.3	Tris Buffer pH 6.7	Sample buffer pH 6.7
1.2 g Tris	5.98 g Tris	1 ml Tris buffer
5.76 g Glycine	0.46 ml TEMED	1 ml glycerol
ad 2 L H_2O	Ad 100 ml H_2O	1 ml H_2O + 0.25 mg Bromphenol Blue

Protein samples were mixed in equal volumes with the sample buffer and loaded on the gel. Front determination was done with sample buffer alone. The gels were run at 4°C and 10 mA/gel. Rf-value and relative electrophoretic mobility on different gels were determined as a function of molecular weight for each protein.

3.2 Expression of LecEGF domain of human E-selectin in *E.coli*

3.2.1 General proceedings

All work with bacterial cultures was done in sterile conditions under laminar flow. Commonly used medium and procedures are listed below and decribed in the *tables* 3.11-3.13. pH of the medium was set to 7.5 with 2N NaOH and autoclaved. Sterile filtrated antibiotics solutions were added just prior to use. Autoclaved medium was cooled down to 50°C and ampicilin stock solution at 100 mg/ml was added to the final concentration of 150 μg/ml. Agar plates were prepared by adding 15 g agar/liter LB medium. 25 ml medium was poored per plate, cooled down to RT and kept in dark at 4°C for 1 month.

Table 3.11: Components for 1 liter LB medium

Bacto-Trypton	10.0 g
Yeast Extract	5.0 g
NaCl	10.0 g
Aqua dest.	ad 1 liter

Table 3.12: Components for 1 liter TB medium

Bacto-Trypton	12.0 g
Yeast Extract	24.0 g
Glycerol	4.0 ml
Aqua dest	ad 1liter
TB phosphate (10X)	100 ml (2.31 g KH_2PO_4, 12.54 g K_2HPO_4)

3.2.1.1 Preparation of $CaCl_2$ competent cells [248]

Competent cells are used to take foreign DNA by transformation. Over-night culture of DHα5 *E.coli* was done in 10 ml LB medium at 37°C, shaking. On the next day 50 ml LB medium was inoculated with 4 ml over-night culture and was grown at the 37°C until the $O.D._{600}$ of 0.5-0.7. All subsequent steps followed at 0-4°C. Cells were centrifuged at 3000xg for 30 min. and the pellet was resuspended in 10 ml of 50 mM $CaCl_2$ solution. Suspension was incubated on ice for 30 min. and the cells finally centrifuged at 2000xg for 10 min. Final pellet was re-suspended in 1 ml 50 mM $CaCl_2$ solution with 20% glycerol and aliquot at 50 μl. Glycerol aliquots were freezed in liquid nitrogen, and stored at -80°C.

3.2.1.2 Preparation of competent *E.coli* cells for electroporation

Table 3.13: Components for 1 Liter SOB-Medium

Bacto-Tryptone	20 g
Bacto-Yeast extract	5 g
NaCl	0.5 g

250 mM KCl	10 ml
Aqua dest.	ad 1liter

Desired *E.coli* strain was streaked on agar plates w/o antibiotics and incubated overnight at 37°C. Single colony was picked and inoculated in 5 ml SOB medium without $MgCl_2$ and cultures were grown overnight at 37°C, shaking.

Pre-cultures (250 ml) were inoculated in SOB medium supplemented with 10 mM $MgCl_2$ at 1: 50 ratio and grown at 37°C up to $O.D._{600}$ of 0.5-0.7. Cells were harvested by centrifugation in a GS3 rotor at 5000 rpm and 4°C for 10 min. Pellet was re-suspended in 250 ml of 10% cold, sterile glycerol and centrifuged as described. Cells were resuspended in 82.5 ml cold 10% glycerol and centrifuged again. Finally, the pellet was resuspended in 1 ml cold 10% glycerol and cells aliquoted in 50 µl in pre-chilled eppendorf tubes. Cells were frozen in liquid nitrogen and stored at -80°C.

3.2.1.3 Bacterial glycerol stock preparation

A single colony was picked from the agar plate and grown in LB medium with ampicilin at 37°C, until mid-logarithmic phase. 0.85 ml culture was mixed with 0.15 ml sterile glycerol in an eppendorf tube, freezed in liquid nitrogen and stored at -80°C.

3.2.2 Cloning into pEZZ18

Plasmid vector pEZZ18 (Amersham, Pharmacia) contains ZZ domain of protein A upstream from the multiple cloning site and enables recombinant protein's secretion into the medium.

3.2.2.1 Genomic DNA isolation from CHO cells

CHO cells expressing human E-selectin/IgG were trypsinized, resuspended and centrifuged 5 min. at 13 000xg, followed by the washing 4 times with PBS buffer (Invitrogen). Genomic DNA was isolated from the cells with WIZARD Genomic Purification Kit (Promega). Genomic DNA was stored at 4°C.

3.2.2.2 PCR generation of Lectin and EGF-like domains (LecEGF) of human E-selectin

The DNA sequence coding for the LecEGF of human E-selectin was amplified from genomic DNA isolated from CHO cells in a Hot Start PCR. Restriction enzyme

recognition sequences were introduced in the sequence: *EcoR*I restriction site at the 5' end, followed by thrombin cleavage site, and *BamH*I site at 3' end, as well as stop codon.

Primer sequences:

LecEGFpEZZ18 (5'-Primer):

5'-ATG C**GA ATT C**GC TGG TGC CGC GCG GCA GCT GGT CTT ACA ACA CCT CCA CG-3'

GAA TTC: *EcoR*I restriction site

CTG GTG CCG CGC GGC AGC: Thrombin cleavage site

LEZZ3 (3'-Primer):

5'-GCT C**GG ATC C**TT ACA GGG CTG TAC AGT TCA C-3'

GGA TCC: *BamH*I restriction site

TAA: Stop codon

Lyophilized primers were dissolved in sterile H_2O at 100 pmol/µl and incubated at 65°C for 5 min., followed by 3 min. shaking at 650 rpm. For the PCR reaction, primers were diluted to 10 pmol/µl. Mastermix I was prepared as described in the *table* 3.14, and 25 µl were pipetted in the special PCR-tubes (GeNunc 0.2ml, PP, RNAse- and DNAse-free). Finally, mastermix II (*table* 3.15) was added without polymerase, which was added directly to the reaction mix in the PCR machine at 94°C.

Table 3.14: Mastermix I

Nucleotide mix 10 mM	200 µM each dNTP	1 µl	1 µl
5'primer 10 pmol/µl	0.1-0.6 µM	2 µl	2 µl
3'primer 10 pmol/µl	0.1-0.6 µM	2 µl	2 µl
Template DNA	0.1-0.25 µg	1 µl	3 µl
Autoclaved H_2O	ad 25µl	ad 25µl	ad 25µl

Table 3.15: Mastermix II

Pfu-PCR-Puffer 10x	1x	5 µl
Pfu-DNA-Poly	1.25 U/reaction	1 µl
Autoclaved H_2O.	ad 25µl	19 µl

PCR was performed using thermocycler BIO-RAD with the program described in the *table* 3.16.

Table 3.16: PCR reaction parameters for LecEGF amplification

Initial denaturation	94°C	4’	
Denaturation	94°C	60’’	30 cycles
Annealing	58°C	60’’	30 cycles
Elongation	72°C	30’’	30 cycles
Finale elongation	72°C	7’	
Cooling	4°C	-	

Obtained PCR product was purified with GenPCR purification kit (SIGMA) according to the manufacturer instruction.

3.2.2.3 DNA electrophoresis analysis

All DNA analyses were done by agarose gel electrophoresis in TBE buffer (*table* 3.17) DNA fragments migrate in the agarose gel from cathode toward anode dependent on their charge.

Table 3.17: 1 L TBE buffer

Tris	44.6 mM
Boric acid	44.6 mM
EDTA*2H_2O	10 mM

3.2.2.4 Restriction enzyme digestion

Prior to the ligation, PCR generated insert, as well as plasmid vector were digested with restriction enzymes *EcoR*I (New England Biolabs) and *BamH*I (New England Biolabs). Both enzymes have 100% activity in the digestion buffer NEB-buffer 2. Reaction components desribed in the *table* 3.18 were mixed and incubated at 37°C for 3.5 h.

Table 3.18: Restriction enzyme digestion set up

	Insert	Vector
Insert/Vector	60 µl	45 µl
Sterile H_2O	3.5 µl	0.5 µl
NEB-buffer 2 (10x)	7.5 µl	5.5 µl
*EcoR*I	2 µl ≅ 4 U	2 µl ≅ 4 U
*BamH*I	2 µl ≅ 4 U	2 µl ≅ 4 U

3.2.2.5 Dephosphorylation of the vector

After restriction enzyme digestion, vector was dephosphorylated with calf intestine alkaline phosphatase (CIP) (Roche) in a reaction mix described in the *table* 3.19.

Table 3.19: Dephosphorylation reaction set up

Vector	55 µl
Sterile H_2O	2 µl
CIP-buffer 10x	6.5 µl
Alkaline Phosphatase (1U/µl)	1.5 µl

Reaction components were mixed and incubated at 37°C for 1h, followed by enzyme inactivation at 65°C for 10 min. Digested and dephosphorylated vector and insert were further purified by agarose gel electrophoresis. Samples of total reaction mixtures were prepared as described under 3.2.1.6. After the separation, bands corresponding to the insert and vector were cut out from the gel under the UV light.

3.2.2.6 DNA-purification

DNA fragments from agarose gel were purified with the GenElute™ Gel Purification kit (SIGMA) according to the supplier's prescription. DNA was eluted in 40 µl elution buffer and stored at -20°C.

3.2.2.7 Ligation

Ligation is an ATP-dependent reaction, where recombinant ligase effectively joins blunt or cohesive DNA ends. Ligase catalyzes formation of a phosphodiester bond between OH- and phosphate- group of DNA strand. Different vector to insert ratio and reaction set up was tested and successful reaction set up is given in the *table* 3.20. Ligation was conducted over-night at 4°C.

Table 3.20: Ligation reaction set up

Vector	2 µl (0.1-5 µg)
Insert	5 µl (0.1-5 µg)
Ligation buffer 10x	1 x
ATP 10 mM	1 mM
Ligase	20-500 U
Sterile H_2O	Ad 10 µl

3.2.2.8 Chemical transformation of competent *E.coli* cells

Transformation is a process of plasmid-DNA up-take in the competente bacterial cells. The addition of $CaCl_2$ to the cells makes the membrane porous, and the haet schock enables DNA uptake.

Bacterial cells were thawed on ice and 100 μl cells were mixed gently with 5 μl ligation mixture. Cells were incubated on ice for 30 min. and then at 42°C for 80 sec. Cells were cooled down on ice for 3 min, 500 μl SOC medium (described in the *table* 3.21) was added and phenotypic expression started for 1 h at 37°C and 550 rpm shaking. Finally, cell suspension (100-400 μl) was plated on pre-warmed agar plates, containing 150 μg/ml Amp. When dried, plates were incubated at 37°C over-night.

Table 3.21: Components of 1 L SOC medium

D-Glucose	1M
$MgCl_2$	2M
in LB-Medium	

3.2.2.9 Plasmid isolation and clone analysis

For ligation and transformation controle, single colony was picked, inocculated in 3 ml LB medium with the appropriate antibiotics and grown to stationary phase at 37°C. Plasmid DNA was isolated from the cells with GFX^{TM}Micro Plasmid Prep Kit (Amersham Pharmacia) by alkaline lyses [248]. Final DNA was eluted in 70 μl TE buffer (*table* 3.22}

Table 3.22: TE buffer preparation

Tris	10 mM
EDTA	1 mM
pH set to 8 with HCl	

Isolated plasmid DNA was first controled for the presence of correct fragment by restriction enzyme digestion with *EcoR*I and *BamH*I, or EcoRI und PstI restriction enzymes, and by PCR.

For the sequencing, single-clone culture was re-streaked on the agar plate in a dilution maner. Plate with the grown colonies was sent to Microsynth GmbH Sequencing Group, Balgach.

3.2.2.10 Protein expression analysis by affinity chromatography on IgG sepharose

50 ml LB medium with 100 μg/ml ampycilin was inoculated with 0.5 ml culture and grown over-night at 37°C. Medium was clarified by centrifugation at 4°C and 3000 g, sterile filtrated and applied to IgG sepharose column. Affinity purification was done on FPLC system (BIO-RAD). 2 ml IgG sepharose (Amersham, Pharmacia) was equilibrated with 10 column volumes of TTBS buffer, pH 7.6. After achieving stable base-line, clarified medium was applied at the flow-rate of 1 ml/min. Column was extensively washed with TTBS buffer to get stable base-line, and unspecificaly bound contaminants were washed away with 10 mM acetate buffer, pH 5.0. Bound proteins were eluted in 5 column volumes of 0.2 M acetic acid, pH 3.4. 1 M Tris was used to adjust pH to 7.6. Chromatography fractions were analyzed on 15% SDS-PAGE.

3.2.3 Cloning into vector pET-15b

Cloning vector pET-15b (Novagen) enables inducible expression of recombinant proteins with N-terminal His tag and possibilty of tag removal by thrombin.

3.2.3.1 PCR generation of LecEGF domain of human E-selectin

DNA sequence coding for LecEGF was PCR amplified as described in3.2.2.2 with primers:

FW pET15LE (5'-Primer):

5'-AGA TCG A**CA TAT G**TG GTC TTA CAA CAC CTC CA-3'

CAT ATG: *Nde*I restriction site

LEZZ3 (3'-Primer):

5'-GCT C**GG ATC C**TT ACA GGG CTG TAC AGT TCA C-3'

GGA TCC: *BamH*I restriction site

TAA: Stop codon

3.2.3.2 Restriction enzyme digestion of insert and vector

Restriction enzyme digestion was performed as described under 3.2.2.3, with enzymes *Nde*I and *BamH*I (New England Biolabs).

3.2.3.3 Vector dephosphorylation

Vector pET-15b was dephosphorylated as described under 3.2.2.4

3.2.3.4 DNA-purification with GenEluteTM Gel Purification Kit

Insert and vector DNA were purified and prepared for ligation as described under 3.2.2.5

3.2.3.5 Ligation into pET-15b

The ligation reaction was prepared as described under 3.2.2.6, and conducted over-night at 4°C.

Table 3.23: Ligation reaction set-up for ligation of LecEGF into pET-15b

Vector	4 μl (0.1-5μg)
Insert	6 μl (0.1-5μg)
Ligation buffer 10x	1.5 μl, 1 x
ATP 10 mM	1 mM
Ligase	20-500 U
Sterile H_2O	Ad 15 μl

3.2.3.6 Chemical transformation into *E.coli*

Transformation with ligation mix into *E.coli* DHα5 was done as described under 3.2.2.10. For protein production, 1 μl plasmid DNA HisLecEGFpET-15b (0.1 μg) was transformed into *E.coli* strains AD 494, Rosetta Gami and BL 21 (DE3), according to the same procedure.

3.2.3.7 DNA isolation and clone analysis

DNA isolation was done as described under 3.2.13. Isolated plasmid DNA was controlled for the presence of correct fragment by restriction enzyme digestion with *Nde*I and *BamH*I, or *Nde*I und *Pst*I restriction enzymes, and by PCR. For the sequencing, DNA was isolated as described, eluted in sterile H_2O and sent for sequencing to MWG genomic company, Ebersberg, Germany, (www.THE-MWG.com), and Syngene AG, Zürich, Switzerland.

3.2.3.8 Protein expression optimization

To establish the best conditions for HisLecEGF protein expression, LB medium and TB medium were tested, as well as 3 *E.coli* strains: AD 494, Rosetta gami and BL 21(DE3). 3 ml overnight culture was prepared from all three strains in 2 different mediums. 50 ml either LB, or TB medium with 100 μg/ml ampycilin was inoculated as described under 3.2.13, and cells were grown at 37°C until $O.D._{600}$ reached 0.6-0.8. The culture was

inoculated with 1 mM IPTG. 1 ml aliquots were taken at different time points, for up to 24 h post-induction and protein expression analyzed on SDS-PAGE. Non-induced cells were taken as negative control.

3.2.3.9 HisLecEGF purification in native conditions

Purification of HisLecEGF was done in native conditions using Ni-NTA (Ni^{2+}-Nitrilotriacetic acid) agarose (Qiagen), according to the manufacturer prescription. Collected bacterial culture was centrifuged at 4°C and 5000xg for 20 min. Pelleted cells were re-suspended in cell lyses buffer at 5 ml per gram wet weight. Lysozyme was added at 1 mg/ml and incubated on ice for 30 min. Cells were sonicated on ice with 70% output, 6x10 sec with 10 sec break. Cell lysate was centrifuged at 4°C and 10 000xg for 30 min. to pellet the cellular debris, and supernatant was filtrated through 0.22 μm sterile filter. Collected soluble cytoplasmic fraction was mixed 1:1 with the binding buffer prior to the immobilized-metal affinity chromatography. The affinity purification was performed on the FPLC system Biologics (BIO-RAD). For purification of His-tagged protein, 5 ml BIO-RAD column was assembled and packed according to the manufacturer prescription. Column was equilibrated with 5 column volumes of lysis buffer with a flow rate of 1 ml/min, until stable base-line. Cell lysate was applied, and column washed with 10 column volumes of lysis buffer until stable base line. With 10 column volumes of wash buffer, all non-specifically bound impurities were removed, and after achieving stable base-line, his-tagged proteins were eluted in 3 column volumes of elution buffer. After the use, column was kept in 30% sterile filtrated ethanol. All the buffers used (*table* 3.24) were sterile filtrated through 0.22 μm filter.

Table 3.24: Buffers for Ni-NTA IMAC under native conditions

Lysis Buffer	50 mM NaH_2PO_4 300 mM NaCl 10 mM imidazole, pH 8.0
Wash Buffer	50 mM NaH_2PO_4 300 mM NaCl 20 mM imidazole, pH 8.0
Elution Buffer	50 mM NaH_2PO_4 300 mM NaCl 250 mM imidazole, pH 8.0

All chromatography steps were analyzed on SDS-PAGE.

3.2.3.10 Isolation of inclusion bodies

250 ml collected bacterial culture was centrifuged at 4°C and 5000xg for 20 min. Cell pellet was thoroughly resuspended in 12.5 ml ice cold wash buffer (ratio 1:20 to the starting culture), and protease inhibitor cocktail (Sigma) was added. Resuspended culture was sonicated on ice with 70% output, in the intervals of 6x20 sec and 10 sec break between. Cell resuspension was centrifuged at 4°C and 10 000g for 20 min., and supernatants were preserved for the control purposes. Pellet was washed 5x in the same manner, until extracted supernatant was cleared. In the last step insoluble material was solubilized in denaturing binding buffer (*table* 3.25). Pellet was washed, sonicated and centrifuged as described above, filtrated through 0.22 μm sterile filter and prepare for the affinity purification.

3.2.3.11 HisLecEGF purification under denaturing conditions

Ni-NTA affinity column was prepared as described under 3.2.21. For the purification under denaturing conditions, either 8M Urea, or 6M Guanidine were used as strong chaotrops and denaturing agents. Column was equilibrated with 5 column volumes of denaturing binding buffer until stabilized base-line.Isolated protein fraction was applied at the flow rate of 1ml/min, and column was thoroughly washed with the binding buffer. For the removal of non-specifically bound proteins, column was washed with 10 column volumes of wash buffer. His-tagged proteins were eluted with 5 column volumes of low pH elution buffer, and the column was immediately re-equilibrated with the loading buffer of neutral pH. pH value of the eluted fraction was set up to 7.0 with 1 M Tris buffer. All chromatography fractions were analyzed by SDS-PAGE.

Table 3.25: Inclusion bodies wash buffer and buffers for the Ni-NTA IMAC under denaturing conditions

Inclusion bodies wash buffer	100 mM Tris pH 8.0
Lysis (Binding) Buffer	100 mM NaH_2PO_4 10 mM Tris 8 M Urea or 6 M Gu-HCl, pH 8.0

Wash Buffer	100 mM NaH_2PO_4 10 mM Tris 8 M Urea or 6 M Gu-HCl, pH 6.3
Elution Buffer	100 mM NaH_2PO_4 10 mM Tris 8 M Urea or 6 M Gu-HCl, pH 4.5

3.2.3.12 Mass spectrometry analysis of proteins

Protein identity was confirmed by analysis of protease-digested fragments, and by mass determination. Protein was eluted in either 8 M Urea, or 6 M GuHCl under denaturing conditions, reduced with 10 mM DTT for 30 min. at RT, and alkylated when necessary (to prevent re-oxidation of reduced disulfides with 50 mM iodoacetamide (final conc.). Alkylation was done at RT for 15 min. in the dark, and reaction was stopped by decreasing pH bellow pH 4.5. Protein was desalted on reverse-phase HPLC (Amersham, Pharmacia) using C_4 Vydac column for hydrophobic interactions. Protein was eluted in 10-80% gradient of 0.1% TFA in methanol and 80% acetonitrile in H_2O. Protein presence in eluted fractions was confirmed by SDS-PAGE, and molecular weight of the protein in volatile buffer was either directly analyzed or proteins were digested with proteases. Proteins were digested with different proteases as follows: for the trypsin digestion, 5 μg protein was digested in 0.1M Tris buffer, pH 8.0, overnight at 37°C, with 0.5 μg trypsin (SIGMA). For digestion of 5 μg protein, 1 μg V8 protease (SIGMA) was used, and digestion was done in 0.1 M phosphate buffer, pH 6.8, overnight at 37°C. Lys-C-protease (SIGMA) digestion was also performed in 0.1M Tris buffer, pH 8.0, for 45 min. at 37°C.

3.2.3.13 Protein refolding by dialysis

Protein was eluted as described in *table* 3.25, and 11 ml protein solution at 0.8 mg/ml was reduced for 2h at 37°C with 0.1 M DTT. Reduced protein was diluted to 2M urea, 100 mM NaH_2PO_4, 10 mM Tris, pH 8.0, keeping protein concentration at 220 μg/ml, and was stirred overnight at 4°C. Dialysis was done in buffers described in the *table* 3.26 in tubes with 10 kDa cut off.

Table 3.26: Protein refolding by dialysis, conditions

Tris-HCl, pH 8.0	EDTA	GSH	GSSG
100 mM	1 mM	10 mM	1 mM
100 mM	1 mM	1 mM	1 mM
100 mM	1 mM	1 mM	10 mM

First dialysis step was done at the ratio 1:40 of protein solution:dialysis buffer for 24 h at 4°C with buffer exchanges every 8-10h. After 24 hours protein solution was centrifuged at 4°C and 10 000xg for 30 min. and dialyzed for another 48 h at 4°C against TBS supplemented with 10 mM Ca^{2+}, pH 7.6 at the same protein to buffer ratio, and buffer was exchanged every 12 h. Aliquots were taken throughout complete procedure in intervals of 4-12 hours and analyzed on SDS-PAGE and in an activity test. Second dialysis set up was done with 5 ml protein solution at 1.55 mg/ml, which was reduced with 100 mM DTT for 2h at RT. Protein was fast diluted with 45 ml cold H_2O and dialyzed in ratio 1:40 against 2 L of 25 mM Tris, pH 7.6 at 4°C. Since precipitates appear after 20 min., pH was raised to pH 8 with 1M NaOH, material was dialyzed overnight and was ready for subsequent thrombin cleavage. Aliquots were also taken for the activity analysis to monitor refolding.

3.2.3.14 Protein refolding by fast dilution and thrombin cleavage

Protein refolding by fast dilution was performed either as direct refolding, or with first His tag removal and subsequent refolding, as described in *tables* 3.27-3.31. For the first set up, protein that was eluted as described in *table* 3.25 at 1.55 mg/ml and reduced as described. By fast dilution protein concentration was set at 100 μg/ml in cold buffer, and refolding attempted as described in table. The quality of the material was checked on the analytical RP-HPLC (Agilent Technologies).

Table 3.27: Protein refolding by fast dilution, set-up 1

Direct refolding (small scale, 10 ml total): effect of proteins ligands
• -IB isolation • -Ni-NTA ⇒ 1.55 mg/ml, 120 mg/l total • -Reduction with 100 mM DTT • -HPLC check, soluble material

• [HisLecEGF] decreased to 100 μg/ml • Fast dilution in cold buffers		
• 1M L-Arg • 1:10 GSSG:GSH • 1 mM EDTA • pH 8.0	• 1M L-Arg • 1:10 GSSG:GSH • 1 mM EDTA • 1:10 Fucose • pH 8.0	• 1M L-Arg • 1:10 GSSG:GSH • 1 mM EDTA • 1:10 NeuNAc • pH 8.0
• 60 h stir at 4°C • HPLC control • SDS-PAGE, 4-20% Tris-Glycine gel, reducing and non-reducing conditions		
• Scale up and gel filtration		
• Scale up to 250 ml set-up for fast dilution ⇒ 25 mg protein • Gel filtration: 4 peaks, SDS-PAGE, 4-20% Tris-Glycine gel ⇒ not visible ⇒ concentrating • ELISA based screening with blocking monoclonal antibodies		

After HPLC control of each reaction, first reaction set up was scaled up to 250 ml to get enough material for further activity investigation. After 60 h fast dilution, protein was concentrated prior to the gel filtration in an Amicon 500 ml cell concentration device with the cut off membrane of 10 kDa at 4°C for 3.5 h, and the volume was decreased to 0.5 ml (500x concentration factor). Material was filtrated through 0.22 μm sterile filter prior to the application onto size exclusion chromatography column Sephadex (Amersham, Pharmacia). Proteins were chromatographied in TBS buffer with 2 mM Ca^{2+} at 4°C and the flow rate of 1 ml/min. Collected fractions were analyzed on SDS-PAGE and by activity test.

Table 3.28: Protein refolding by fast dilution; set-up 2

Tag removal prior to the refolding (small scale, 5 ml total): Dilution and dialysis
• IB isolation • Ni-NTA in 6 M GuHCl⇒ 1.55 mg/ml • Reduction with 100 mM DTT • HPLC check, soluble material

• [HisLecEGF] decreased to 155 μg/ml • Fast dilution 1:10 in H_20, pH 7.4⇒ removal of 6 M GuHCl, 100 mM DTT and 1 M L-Arg	
• Dialysis against 2 L H_20 ⇒ precipitation in 20 min • pH increase to 10 ⇒ soluble, stir for 24 hours	
• **Thrombin cleavage**	
• Human thrombin: cleavage ratio(w/w) thrombin:protein = 1:100 • Buffer adjusted to 50 mM Tris, 150 mM NaCl, 2.5 mM $CaCl_2$ ⇒ • Precipitation upon $CaCl_2$ addition, removed by EDTA	
• Cleavage at pH 8.0 for 12 h at RT	• Cleavage overnight at 20°C -37°C
• Precipitation, SDS-PAGE analysis, activity test	

Table 3.29: Protein refolding by fast dilution; set-up 3

Tag removal prior to the refolding (small scale, 5 ml total): Dialysis and dilution
• IB isolation • Ni-NTA in 6 M GuHCl⇒ 1.55 mg/ml • Reduction with 100 mM DTT • HPLC check, soluble material
• Dialysis 1:40, 2X against 6 M GuHCl, pH 8.0, 18 h at 4°C • Dilution 1:10 in 50 mM Tris, 150 mM NaCl, pH 8.0, 2.5 mM $CaCl_2$ ⇒ • Precipitation upon $CaCl_2$ addition, removed by EDTA
• **Thrombin cleavage**
• Human thrombin: cleavage ratio(w/w) thrombin:protein = 1:100 • Cleavage in 50 mM Tris, 150 mM NaCl, w/o $CaCl_2$, 18 h at 20°C
• After 18 h precipitation • SDS-PAGE analysis, activity test, Bradford: 10 μg/ml HisLecEGF in supernatant

Table 3.30: Protein refolding by fast dilution; set-up 3

Folding by dilution and dialysis, tag removal, oxidation		
• IB isolation • Ni-NTA ⇒ 1.55 mg/ml • Reduction with 100 mM DTT • HPLC check, soluble material		
• Fast dilution: [HisLecEGF] decreased to 100 μg/ml ⇒ 37.2 mg in 372 ml in • 0.1 M Tris-HCl, pH 8.0, overnight at 4 °C		
• Concentration and dialysis: • Amicon cell (cut off 10 kDa) 3h at 4°C conc. factor 7.44, 50 ml final • Dialysis 1:40 against 50 mM Tris, 2 mM EDTA, pH 8.0		
• **Thrombin cleavage**		
• Human thrombin: cleavage ratio(w/w) thrombin:protein = 1:100 • Cleavage in 50 mM Tris, 150 mM NaCl overnight at 37°C • Thrombin removal with benzimidine sepharose (Amersham, Pharmacia)		
• **Oxidation and folding**		
• GSSG:GSH • 1 mM : 0.1 mM	• GSSG:GSH • 1 mM :1 mM	• GSSG:GSH • 10 mM :1 mM
• Dialysis against TBS+2 mM Ca^{2+} • Activity test		

Table 3.31: Protein refolding by fast dilution; set-up 4

Folding by dilution, dialysis, tag removal
• IB isolation • Ni-NTA ⇒ 1.2 mg/ml • Reduction with 100 mM DTT • HPLC check, soluble material

• Fast dilution: [HisLecEGF] decreased to 68 μg/ml in 1.06 L cold buffer 0.1 M Tris-HCl, pH 8.0, 1M L-Arg, 1 mM EDTA, 48 h at 4°C • Concentration: 5 h at 4°C to 50 ml • Dialysis 1:40 against 50 mM Tris-HCl, pH 8.0, 2X in 48 h total
• **Thrombin cleavage**
• Human thrombin: cleavage ratio(w/w) thrombin:protein = 1:100 • Cleavage in 50 mM Tris, 150 mM NaCl overnight at 37°C • Thrombin removal with benzimidine sepharose (Amersham, Pharmacia)
• HPLC check: no correct peaks (aggregation w/o precipitation) • SDS-PAGEanalysis: reducing, non-reducing conditions cleavage succesful, aggregates in the sample • Activity test

3.2.3.15 ELISA based screening on protein refolding and activity

ELISAs with monoclonal blocking antibodies against human E-selectin were performed to test the correct folding and the activity of expressed LecEGF domain of E-selectin. As postive control and standard molecule, E-selectin/IgG, produced as secreted protein in CHO cells was used. NuncMaxiSorp plates (Invitrogen) were coated with 100 μl protein in standard TBS buffer with 2 mM calcium, pH 7.6, (TAB) or appropriate folding buffer. For the standard curve, E-sel/IgG was plated at 1 μg/ml - 0.05 μg/ml, and LecEGF in various concentrations. Plate was incubated overnight at 4°C and washed 3 times with TAB. Wells were blocked with 3% BSA in TAB at RT for 2h, and washed as described above. 1 μg mouse, anti-human E-selectin mAb 7A9 [249] in TAB was added to each well and the plate was incubated for 4 h at 37°C or at 4°C overnight. After the plate was washed, a secondary antibody (SIGMA A-5278), peroxidase-conjugated goat anti-mouse IgG diluted 1:6000 was added. The plate was incubated overnight at 4°C to allow complex formation between the antibodies, and washed as usual. *Ortho*

phenylen-diamine (OPD) tablets (SIGMA) were used as peroxidase substrate. Tablets were dissolved in 0.05 M phosphate-citrate buffer, pH 5.0 at the final concentration of 0.4 mg/ml, and 10 μl 35% H_2O_2 solution was added to 10 ml OPD-solution. 100 μl substrate solution was added to each well and the color was developed for 5-10 min. at RT before reaction was stopped by adding 1 M H_3PO_4. Absorbance was measured at 490 nm in SoftMaxPro plate reader (Molecular Probes), and protein calculated from the standard plot for E-selectin/IgG.

3.2.3.16 HisTag cleavage with thrombin

The thrombin cleavage sequence in vector pET-15b is placed between HisTag and LecEGF sequence. To generate native protein for further analysis, tag was cleaved with thrombin. The optimization was done with the thrombin (Novagen), following supplier prescriptions. Reaction set up was done at 1:25 and 1:100 dilution of the protease stock solution (1U/µl) at 16°C for 22h. Cleavage was performed in both native and denaturing conditions, as described in the *table* 3.32. Denaturing conditions were done in 6M GuHCl, and for native conditions proteins were dialyzed against PBS.

Table 3.32:. Reaction set up for thrombin cleavage

		1:25 in PBS	1:100 in PBS	1:25 in 0.6M GuHCl	1:100 in 0.6M GuHCl
Cleavage Buffer 10x	1x	8 µl	8 µl	8 µl	8 µl
Thrombin solution dilution	1 µl	1µl (1:25) = 0.04 U	1µl (1:100) = 0.01 U	1µl (1:25) = 0.04 U	1µl (1:100) = 0.01U
Protein solution	10 µg protein	64 µl	64 µl	32.3 µl	32.3 µl
H_2O dest.	Ad 80 µl	7 µl	7 µl	38.7 µl	38.7 µl

During 22h of protein digestion aliquots were taken at different time-points. Control reaction was performed with the control protein (48 kDa) supplied by Novagen and was cleaved in the two subunits of 35 and 13 kDa. Negative control was done with dilution buffer instead of protein solution.

3.2.3.17 Screening for the refolding conditions on the asymmetrical field-flow fractionator (Wyeth)

Proteins eluted from Ni-NTA IMAC in 6 M GuHCl, 50 Mm Tris, pH 8.0, were reduced in 100 mM DTT at RT and concentrated to 5 mg/ml. Sample status in solution and concentration were controlled on analytical RP-HPLC.

For the screening set up, 50 µl protein solution at 5.2 mg/ml were fast diluted in 2 ml of each of the buffers listed in *table* 3.34, in a 24-wells cell culture plate. Samples were incubated at 4°C for 12-48 hours, and inspected visually for possible precipitation.

Reaction conditions with no visible precipitation were further analyzed on AfFF matrix and HPLC for protein status in solution. The best reactions conditions (*table* 3.33) were scaled up to 50 ml, in which protein concentration was kept under 100 μg/ml.

Table 3.33: Reaction set up for optimal refolding conditions

Buffer 1b	Buffer 1c	Buffer 3c	Buffer 13c	Buffer 20b
0.5 M CHES, pH 9.5	0.5 M CHES, pH 9.5	0.5 M CHES, pH 9.5	0.5 M CHES, pH 9.5	0.5 M Tris, pH 8.0
1,2-propanediol, 25%	1,2-propanediol, 25%	2-etoxyethanol, 20%	Hexa-decyltrimethylam monium bromide, 10 mM	Octyl glucoside, 0.05%
83.2 μg/ml protein	83.2 μg/ml protein	83.2 μg/ml protein	83.2 μg/ml protein	83.2 μg/ml
3.4 mM GSH 0.5 mM GSSG	3.4 mM GSH 0.5 mM GSSG	3.4 mM GSH 0.5 mM GSSG	3.4 mM GSH 0.5 mM GSSG	3.4 mM GSH 0.5 mM GSSG

Table 3.34: Buffer composition for the screening on the Asymmetrical Field-Flow Fractionator

Buffer Components	**A: 0.5 M citrate, pH 8.0**	**B: 0.5 M Tris, pH 8.0,**	**C: 0.5 M CHES, pH 9.5**
1. 1,2-propanediol, 25%		17 Lithium sulphate, 0.2 M.	
2. 1,6-hexanediol, 10%		18. Magnesium formate, 0.2 M	
3. 2-etoxyethanol, 20%		19. Na/K tartarate ,0.5M	
4. 2-methyl-2,4-pentanediol (MPD), 30%		20. Octyl glucoside, 0.05%	
5. Ammonium acetate, 0.2 M		21. Pentaerythritol propoxylate, 30%	
6. Ammonium sulphate, 0.2 M		22. Polyacrylic acid, 20%	
7. Bicine, 0.5 M		23. Polyethyleneglycol monomethyl ether 550, 10%	
8. Di-ammonium tartrate, 0.2 M		24. Polyviniylpyrrolidone, 20%	
9. Dioxane, 10%		25. Potassium thiocyanate, 0.1 M	
10. Ethanol, 20%		26. Sodium chloride, 1 M	
11. Ethylene imine polymer, 2%		27. Sodium citrate, 0.2 M	
12. Heparin sulphate, 5 mM		28. Sodium malonate, 0.2 M	
13. Hexadecyltrimethylammonium bromide, 10 mM		29. Sodium succinate, 0.2 M	
14. Immidazole-HCl, 0.2 M		30. Tacsimate, 25% (v/v)	
15. Isopropanol, 15%		31. Tert-butanol, 25%	
16. Jeffamine M-600, 30%		32. Trimethylamine N-oxide, 0.2 M	

After 48 h of protein refolding in described buffer, the redox system (0.5 mM GSSG and 5 mM GSH, respectively) was added to each reaction set up to get 1:10 final ratio of oxidized to reduced agent. Because of the presence of 1.6 mM DTT in the buffer, 3.4 mM GSH and 0.5 mM GSSG were added. To maintain the appropriate environment for redox reaction, solution pH was controlled, and for CHES containing buffers adjusted to pH 8.0-8.5 with sulphonic acid. Samples were incubated at 4°C for 48-72 hours, filtrated through 0.22 μm sterile filter and concentrated in a Vivaspin 20 ml concentrator (7 kDa cut off) in an Eppendorf centrifuge 5804 R, at 3000 rpm and 4°C for the concentration factor 30. Refolding reaction was further analyzed on gel filtration and controlled for the "in-solution" status on analytical RP-HPLC.

3.2.3.18 HisLecEGF expression in M9 minimal medium

Protein expression was optimized in M9 minimal medium with clone pET-15bHisLecEGF in *E. coli* strains AD 494, BL 212 (DE3) and Rosetta gami. Medium was prepared as described in the *table* 3.35 and supplemented according to the *table* 3.36. pH was set to 7.4 with 2M NaOH, and the solution autoclaved.

Table 3.35: Components of 1 L M9 minimal medium

Na_2HPO_4 anhydr.	10.4 g
KH_2PO_4	3 g
NaCl	0.5 g
NH_4Cl	1 g

Table 3.36: Supplements to M9 minimal medium

1M $MgSO_4$	2 ml
1M $CaCl_2$	0.1 ml
20% Glucose	10 ml
The solutions were sterile filtrated. Prior to use, 12.1 ml were added per 1 L medium.	

Biotin solution at 1 mg/ml and sterile filtrated thiamin solution at 10 mg/ml were added to the medium at 1:100 and 1:1000 ratio, respectively. 3 ml culture set up was done from all 3 strains in LB-medium with 50 μg/ml Ampicilin and was grown overnigt at 37°C. 240 μl cell suspension was centrifuged at 4°C and 4000xg for 5 min., and pellets were resuspended in 4 ml M9 minimal medium. The cultures were further cultivated at 37°C, until they reached OD_{600} of 0.6-0.8. 1 mM

IPTG was added for induction. 500 µl aliquots were taken at different time points and analyzed for protein expression on SDS-PAGE.

3.2.4 Cloning into pINompA I

Cloning vector pINompA I possesses secretion signal sequence of the bacterial protease, outer membrane protein A [250]. DNA sequence coding for LecEGF domain of human E-selectin was PCR amplified from genomic DNA as described under 3.2.2.2.
Primers' sequences:
FW pINompALE (5'-Primer):
5'-ATC G**GA ATT C**CT GGT CTT ACA ACA CCT CCA
GAA TTC: *EcoR*I restriction site

LEZZ3 (3'-Primer):
5'-GCT C**GG ATC C**TT ACA GGG CTG TAC AGT TCA C
GGA TCC: *BamH*I restriction site
TAA: Stop codon

3.2.4.1 Restriction enzyme digestion of insert and vector

Restriction enzyme digestion was performed as described under 3.2.2.3, with the enzymes *EcoR*I and *BamH*I.

3.2.4.2 Vector dephosphorylation

Vector pINompA I was dephosphorylated as described under 3.2.2.4.

3.2.4.3 DNA-purification with GenEluteTM Gel Purification Kit

Insert and vector DNA were purified and prepared for ligation as described under 3.2.2.5

3.2.4.4 Ligation into pINompA I

Ligation reaction was prepared as described under 3.2.2.6 and *table* 3.37 and was conducted overnight at 4°C using DNA ligase (New England Biolabs).

Table 3.37: Ligation reaction set up for ligation of LecEGF into pINompA I

Vector	4 μl (0.1-5μg)
Insert	6 μl (0.1-5μg)
Ligation buffer 10x	1.5 μl, 1 x
ATP 10 mM	1 mM
Ligase	20-500 U
Sterile H_2O	Ad 15 μl

3.2.4.5 Chemical transformation into *E.coli*

Transformation with ligation mix into *E.coli* DHα5 was done as described under 3.2.2.7.

3.2.4.6 Electro transformation into *E.coli*

Prior to the electroporation, the ligation mix was dialysed to remove salts present in ligation buffer. 10 μl ligation mix was dialyzed against autoclaved H_2O for 1 h at RT with the filter VSWP 02500 Typ VS, 0.025 μm (Milipore). Dialyzed mixture was added to competent cells and carefully mixed. Cells were pipetted in the pre-cold electroporation cuvette. Electroporation was done at 400 Ω, 1.75 kV and 25 μF with the time constant between 6-8 ms. Just after the electro-pulse, 1ml SOC-medium was added to the cells which were subsequently incubated at 37°C, and 550 rpm for 1 h. After phenotypic expression, 100-500 μl transformed cells were plated on the agar plate and colonies grown overnight at 37°C.

Positive control was plasmid w/o insert, and for the negative control dephosphorylated vector.

3.2.4.7 DNA isolation and clone analysis

DNA isolation was done as described under 3.2.2.8

Isolated plasmid DNA was controlled for the presence of correct fragment by restriction enzyme digestion with *EcoR*I and *BamH*I, or *Nde*I and *Pst*I and by PCR.

For the sequencing, DNA was isolated as described, eluted in sterile H_2O and sent for sequencing to Firma Microsynth GmbH Sequencing Group, Balgach.

3.2.5 Cloning into pET-11c

pET-11c vector enables cloning of recombinant proteins without any tag, so that expressed recombinant protein corresponds, is as native as possible.

3.2.5.1 DNA cloning and manipulation

All DNA manipulations were done as described in 3.2.2.2 to 3.2.2.9.

3.2.5.2 Optimization of protein expression

To find the optimal conditions for protein expression in terms of bacterial strain, time, and medium composition, 50 ml culture set up was prepared as described under 3.2.3.8. 100 μl culture was taken at different times and centrifuged for 5 min at 16 000g, RT and pellet was resuspended in an appropriate volume of SDS-PAGE reducing buffer. Protein expression was analyzed on SDS-PAGE.

3.2.5.3 Protein preparation and analysis on MS

Protein was isolated from the total cell protein and prepared for MS as follows: protein-prep from 250 ml culture was solubilized as described under inclusion bodies isolation. Vydac C_{18} column was used for RP-HPLC. Buffer A for column equilibration and protein binding was 0.1% TFA in H_2O, and proteins were eluted in gradient of 60-75% B (80% acetonitrile in 0.1% TFA) at 1 ml/min B in 10 min. Collected fractions were analyzed by SDS-PAGE and protein-containing fractions were pooled together and lyophilized. Lyophilization was done in dry ice, overnight, resulting in 13.4 mg lyopihilized protein. Lyophilizate was dissolved at 2 mg/ml in 6M GuHCl, and reduced further as described under 3.2.2.9. MS analysis of intact protein and protein digests were prepared as described under 3.2.2.10 respectively.

3.2.5.4 Protein purification by ion-exchange chromatography

Inclusion bodies isolation from 750 ml culture was done as previously described, with extensive wash in 0.1M Tris, pH 8.0, 5 mM DTT and in the presence of protease inhibitor. Final solubilization step was done in 8M urea buffer, which was purified over Amberlite, an ion exchanger resin. Anion exchange chromatography was done on Mono Q column, Amersham, Pharmacia, first equilibrated with 10-column volume of buffer A (6M urea, pH 8.0, 1 mM DTT, 20 mM Tris). 30 ml protein solution, at 2.8 mg/ml were applied, and the ion exchange was done in gradient 0-80% buffer B (6M urea, pH 8.0, 1 mM DTT, 20 mM Tris, 1M NaCl) in 60 min. with the flow rate 1.5 ml/min. 4 ml fractions were collected and analyzed on SDS-PAGE.

3.2.5.5 Protein purification by RP-HPLC

Reverse phase HPLC was done on preparative scale on Source 5 RP column ST 4.6/150 with the polystirene/divinyl benzene beads. Protein-containing fractions from ion-exchange

chromatography were pooled together and starting material for RP-HPLC was 15 ml protein solution at 2.3 mg/ml. Prior to the application, material was controlled on analytical RP-HPLC and reduced with 100 mM DTT at RT for 30 min. Buffer A was 0.1% TFA, and buffer B 80% acetonitrile in 0.1% TFA. Gradient was programmed at 0-50% B with the flow rate of 1% B/min, followed by 50-95% B in 5 min. All eluted peaks were collected and analyzed on SDS-PAGE. Fractions were pooled together and lyophilized overnight as described.

3.2.5.6 Optimization of RP-HPLC for LecEGF separation

RP-HPLC on analytical scale was done on Amersham RP-HPLC device on C_4 column in order to separate two expressed forms of LecEGF. 0.1% TFA in H_2O was used as equilibration buffer A, whereas elution buffer B was 0.1% TFA in acetonitrile. 50μl protein in 6M GuHCl, 5 mM DTT, pH 8.0 at 100 μg/ml was injected, and different separations conditions were tested, as described in the *table* 3.38. Eluted fractions were analyzed on SDS-PAGE.

Table 3.38: RP-HPLC conditions for LecEGF separation

Time (min.)	(%A)	(%B)	Flow (ml/min)	Time (min.)	(%A)	(%B)	Flow (ml/min)
0.00	100	0	1.00	0.00	100	0.00	1.0
10.00	62.00	38.00	1.00	5.00	50.00	50.00	1.0
20.00	62.00	38.00	1.00	10.00	50.00	50.00	1.0
45.00	0	100.00	1.00	30.00	30.00	70.00	1.0
55.00	100	0.00	1.00	35.00	100	0.00	1.0
60.00	100	0.00	1.00				
60.10	89.00	11.00	1.00				
Time	(%A)	(%B)	Flow	Time	(%A)	(%B)	Flow
0.00	100	0.00	1.0	0.00	100	0.00	0.5
5.00	50.00	50.00	1.0	5.00	50.00	50.00	0.5
20.00	45.00	55.00	1.0	10.00	50.00	50.00	0.5
30.00	30.00	70.00	1.0	30.00	30.00	70.00	0.5
35.00	100	0.00	1.0	35.00	100	0.00	0.5

3.2.5.7 Protein refolding by fast dilution

Material lyophilized after RP-HPLC was dissolved in 6 M GuHCl, 10 mM Tris, pH 8.0, at 1 mg/ml and was reduced for 30 min. at RT with 100 mM DTT. Fast dilution started at 4°C (*Table* 3.39) in folding buffer with final protein concentration of 50 μg/ml.

Table 3.39: Procedure for fast dilution of LecEGF

• Lyophilizate dissolved at 1 mg/ml in in 6 M GuHCl, 10 mM Tris, pH 8.0, at 1 mg/ml and reduced for 30 min. at RT with 100 mM DTT.
• **Fast dilution and oxidation**
• [LecEGF] decreased to 50 μg/ml in 16 and 20 ml, resp. in 0.5 M Tris-HCl, pH 8.0 0.05% dodecyl maltoside, 10 mM $CaCl_2$, for 1 h at 4°C • Added 0.5 mM GSSG for 5 days at 4°C • **HPLC control and protein concentration** • **Dialysis**
• Dialysis 1:30 against 150 mM NaCl, 50 mM Tris, pH 8.0, 0.05% dodecyl maltoside, 10 mM $CaCl_2$ 3 times in 72 hours total at 4°C
• **HPLC control, protein concentration, SDS-PAGE and activity test**
• HPLC check: correct, smaller peaks (still soluble material, no aggregation) • Protein concentration: lower • SDS-PAGE reducing, non-reducing: different species present • ELISA –based activity test: inactive
• **Dialysis to remove detergent and prolonged oxidation**
. Dialysis 1:30 against 150 mM NaCl, 50 mM Tris, pH 8.0, 10 mM $CaCl_2$ • 3 times in 72 hours total at 4°C • Prolonged oxidation with 1 mM GSH and 0.1 mM GSSG
• **HPLC control, protein concentration**

3.3 Expression of LecEGF domain of human E-selectin in Baculovirus-infected insect cells

Baculovirus infected insect cells have been chosen as an expression system to facilitate proper protein folding and disulfide bond formation in LecEGF domain of human E-selectin. Recombinant protein was constructed with an N-terminal His tag, followed by the Precision protease restriction site and N-terminus of native protein.

3.3.1 Intracellular expression of LecEGF

3.3.1.1 Cloning into pBacPack (BD Pharmingen)

Cloning into pBacPack generated recombinant LecEGF with an N-terminal His tag and Prescission™ protease (Amersham, Pharmaca) restriction site. DNA sequence coding for LecEGF domain of human E-selectin was PCR integrated into pBacPack cloning vector as described bellow (*tables* 3.40 and 3.41).

3.3.1.2 PCR generation of LecEGF

DNA sequence coding for LecEGF domain of human E-selectin was PCR amplified from template plasmid pET-11cLE, in which sequence of LecEGF was cloned into vector pET-11c

Primers' sequences:
ESE1 (5'- primer):
5'-GAA GTT CTG TTC CAG GGG CCC TGG TCT TAC AAC ACC TCC ACG
Underlined: Precision cleavage site: GAA GTT CTG TTC CAG GGG CCC
ESE2 (3'-primer):
5'-CTC GGT ACC AGA TCT TCT AGA TTC G**TT A**CA CAA TTT GCT TCA CAC TT
In bold: STOP codon: TAA

Table 3.40: PCR reaction set up for LecEGF amplification

dNTPs (10 μM)	0.6μl
Oligo 1(100 μM)	0.5 μl
Oligo 2 (100 μM)	0.5 μl
Template DNA (plasmid)	0.3 μl = 0.05 μg
10 X Turbo Pfu buffer	5.0 μl

Turbo Pfu	1.0 μ
Sterile H_2O	Add 50 μl

PCR reaction was done under following conditions (*Table* 3.41) with the hot start.

Table 3.41: PCR conditions for LecEGF amplification

Initial denaturation	95°C	5 min.
Denaturation	95°C	30 sec 45 cycles
Annealing	55°C	30 sec 45 cycles
Elongation	72°C	1 min 45 cycles
Final elongation	72°C	10 min
Pause	4°C	∞

After the amplification, PCR fragment was purified with the PCR purification kit from SIGMA, according to the manufacturer instruction.

3.3.1.3 PCR integration of His-Precision-LecEGF (HPLE) into pBakPack [251]

For the integration of HPLE in vector pBacPack, following reaction set-up was used:

Table 3.42: PCR set-up for HPLE integration

dNTPs (10 μM):	0.6 μl
PCR fragment	2.0 μl (200-300 ng)
Recipient plasmid	0.5 μl (50-100 ng)
Turbo Pfu (Stratagene)	1.0 μl
10 X Turbo Pfu buffer	5.0 μl
Sterile H_2O	Ad 50 μl

PCR reaction was performed as for the usual amplification, except that elongation step was run at 68°C for 2 min/kb of generated plasmid.

Table 3.43: PCR conditions for HPLE integration into pBakPack

Initial denaturation	95°C	30 sec.
Denaturation	95°C	30 sec 18 cycles
Annealing	55°C	30 sec 18 cycles
Elongation	68°C	13 min 18 cycles
Final elongation	68°C	10 min
Pause	4°C	∞
*Dpn*I digestion (10 U)	37°C	3 h

At the end of PCR reaction, restriction enzyme digestion was performed with *Dpn*I to digest methylated DNA originating from recipient plasmid.

3.3.1.4 Purification of PCR-integration product

PCR integration mixture was purified. using the DNA gel extraction kit (Qiagen) according to the manufacturer prescription. Finally, purified DNA was eluted in 40 μl sterile H_2O.

3.3.1.5 Electro transformation into *E.coli*

E.coli cloning strain XL1-Blue was transformed with DNA by electroporation.

50 μl electrocompetent cells were thawed on the ice, 4 μl DNA was added and thoroughly mixed. Cells with DNA were pipetted into pre-chilled electroporation cuvette (BIO-RAD), and were pulsed at 1.5 kV, as described under 3.2.4.6. 1 ml SOC medium was added and phenotypic expression conducted for 1 h at 37°C, 550 rpm shaking. 100 μl and 300 μl cell suspension were plated on agar plates and incubated overnight at 37°C.

3.3.1.6 Colony PCR for colony analysis

Grown colonies were analyzed in PCR reaction with the specific LecEGF primers. PCR was done in the set-up described in the *table* 3.36. Instead of template plasmid DNA, grown colonies were touched with the sterile toothpick that was inoculated in the PCR mix and then in the wells with LB medium.

Table 3.44: PCR reaction set up for analysis of grown colonies

dNTPs (10 μM)	0.16 μl
Oligo 1(100 μM)	0.2 μl
Oligo 2 (100 μM)	0.2 μl
Template DNA (plasmid)	–
10 X Taq Poly buffer	2.0 μl
Taq Poly	0.5 μl
Sterile H_2O	Ad 20 μl

Reaction was prepared as master mix to avoid pipetting imprecision and was done as a hot start PCR. 150 μl LB medium/well with the appropriate antibiotic was plated into 96-wells plate. Single colony from the plates that were analyzed by PCR, were also inoculated in LB medium, and the plate was incubated at 37°C. For colonies which were found to be positive by PCR, 3 ml culture was started at 37°C in the 96-well plate. From 3 ml overnight culture in the LB medium with the appropriate antibiotic. DNA was then isolated as described previously, with the Mini Prep extraction kit (Amersham Pharmacia) and was sequenced with either standard primers, or with the insert-specific primers.

3.3.1.7 Cloning into pFastBac (Invitrogen)

HPLE was subcloned into pFastBac to enable transfection of recombinant viral DNA into insect cells.

3.3.1.8 PCR generation of HPLE compatible with pFastBac

PCR reaction was performed as described under 3.3.1.2

3.3.1.9 PCR integration of HPLE in pFastBac

PCR integration was done as described under 3.3.1.3.

3.3.1.10 Purification of PCR product

Purification of PCR-integration product was done as described under 3.3.1.4:

3.3.1.11 Transformation into *E.coli*

Transformation into *E.coli* XL Blue-1 was done as described under 3.3.1.5

3.3.1.12 Colony PCR for colony analysis

Colony PCR for colony analysis was done as described under 3.3.1.6.

3.3.1.13 Preparation of transposition agar plates for *E.coli* DH10Bac (Invitrogen)

Agar solution was prepared as described previously and autoclaved. When cooled to 55°C, antibiotics stock solutions were added and mixed with the agar solution before pouring plates under sterile conditions. Plates were stored in the dark at 4°C, stable for up to four weeks. Aqueous solutions of compounds were sterilized by filtration through a 0.22 µm filter and stored in small aliquots in light tight containers at -20°C.

Table 3.45: Antibiotics used for the preparation of transposition plates

Stock Solution	Concentration	Final concentration in agar
Kanamycin	50 mg/ml in water	50 µg/ml
Tetracycline	10 mg/ml in ethanol	10 µg/ml
Gentamycin	7 mg/ml in water	7 µg/ml
X-gal	100 mg/ml in methanol	100 µg/ml
IPTG	40 mg/ml in water	40 µg/ml

3.3.1.14 Transformation into *E.coli* DH10Bac and transposition reaction

DH10Bac chemically competent cells have viral DNA and transposases in the genome, which enables transposition reaction between recombinant plasmid and viral DNA. DH10Bac competent cells were thawed on ice and 100 µl aliquots were dispensed into 1.5 ml microfuge tubes. 1 µg/5 µl recombinant donor plasmid was added and DNA was gently mixed with the cells. The mixture was incubated on ice for 30 min. and was heat-shocked by transferring to 42°C for 45 sec. The mixture was chilled on ice for 2 min., 900 µl SOC medium was added and suspension was incubated at 37°C with medium agitation (225) for 4-6 hours. Cells were serially diluted using SOC medium to 10^{-1}, 10^{-2} and 10^{-3}. 100 µl of each dilution was spread evenly over the agar plates with the appropriate antibiotics and plates were incubated at 37°C for 48 hours.

3.3.1.15 Clone analysis and bacmid DNA isolation

48 h after plating, white colonies containing the recombinant bacmid DNA were selected for isolation of recombinant bacmid DNA. Before isolating DNA, candidate colonies were picked and streaked to fresh plates containing X-gal and IPTG, to verify the phenotype. Plates were incubated again for 48 h at 37°C.

From a single colony that was confirmed to have a white phenotype, 4 ml LB medium supplemented with 50 µg/ml kanamycin, 7 µg/ml gentamicin, and 10 µg/ml tetracycline was inoculated and grown to stationary phase (up to 24 hours) shaking at 300 rpm. 1.5 ml of culture was centrifuged at 14000xg for 1 min. Supernatant was removed and each pellet resuspended in 0.3 ml of solution I (15 mM Tris, pH 8.0, 10 mM EDTA, 100 µg/ml RNase A). 0.3 ml of solution II (0.2 N NaOH, 1% SDS) was added and gently mixed. Mixture was incubated at room temperature for 5 min., when 0.3 ml 3 M potassium acetate pH 5.5 was added slowly and mixed gently during addition. A thick white precipitate of protein and *E.coli* genomic DNA was formed. The sample was incubated on ice for 10 min. and centrifuged for 10 min. at 14000 g. The supernatant was transferred to the tube containing 0.8 ml isopropanol, mixed by gently inverting tube a few times and incubated on ice for 10 min. The mixture was centrifuged for 15 min. at 14000xg, supernatant was carefully removed and 0.5 ml 70% ethanol was added to microcentrifuge tube. After another centrifugation at 14000xg for 5 min., supernatant was removed and the pellet was air-dried for 10 min. at RT. DNA was dissolved in 40 µl sterile water and kept at 4°C. 0.9 ml recombinant bacmid culture was combined with 0.1 ml sterile glycerol, and stored at -80°C as a glycerol stock.

3.3.1.16 PCR transposition control

PCR analysis was performed to verify the presence of transposed HPLE in DNA isolated from *E.coli* DH10Bac. Reaction was performed according to the manufacturer instruction using M13 standard primers.

Primers' sequences:
M 13 FW (5' primer):
5'-GTT TTC CCA GTC ACG AC-3'
M 13 RW (3' primer):
5'-CAG GAA ACA GCT ATG AC-3'

Table 3.46: PCR reaction set-up for the transposition control

dNTPs (10 µM)	1 µl
Oligo 1(10 µM)	1.25 µl
Oligo 2 (10 µM)	1.25 µl
Template DNA (100 ng)	1 µl
10 X Taq Poly buffer	5.0 µl
Taq polymerase	1.0 µ
Sterile H_2O	Ad 50 µl

The PCR reaction was performed with the standard program recommended by supplier as described in the *Table* 3.47.

Table 3.47: PCR reaction parameters:

Initial denaturation	93°C	3 min.
Denaturation	94°C	45 sec 30 Cycles
Annealing	55°C	45 sec 30 Cycles
Elongation	72°C	5 min 30 Cycles
Final elongation	72°C	7 min
Pause	4°C	∞

The PCR products were analyzed on a standard (1%) agarose gel.

3.3.1.17 Transfection into *Sf 9* insect cells

Sf 9 insect cells used for transfection were grown as adherent cultures in TC100 medium (Invitrogen) supplemented with 10% FCS (Invitrogen) in T-flasks (25 or 75 cm^2, Nunc). Cells were regularly passaged twice per week in 1:3 dilution. 3 days after splitting healthy, confluent cells were used for transfection. After microscope inspection of the cells, medium was decanted and attached cells thoroughly re-suspended in 10 ml fresh medium with serum. Cells were seeded in a 6-well plate at $1x10^6$ in not less then 1.5 ml SFM. Cells were let to attach for 20 min. at RT.

2 μg of DNA were mixed with 100 μl of SFM in PST tube I and 5 μl Cellfectin reagent (Invitrogen) with 100 μl of SFM in PST tube II. Cellfectin solution was added to the DNA mixture and incubated at RT for 15 min., occasionally gently tapping the tube to mix the solution. 0.8 ml SFM was added and the solution was ready for the transfection. Attached cells (max. 50-60% confluent) were washed twice with 2 ml medium and finally 1 ml medium with prepared DNA was added. Plate was incubated at 28°C for 5 h, and medium was aspirated and exchanged. Plate was further incubated for 5 days, cells were inspected and medium was harvested for plaque assay and viral amplification.

3.3.1.18 Viral amplification and plaque assay

Plaque assay was performed for isolation of single virus and viral titer determination. For this purpose *Sf 21* cells were used. Cells were cultivated as described under 3.2.16 and *Sf 9* insect

cells, grown in a T25 culture flask and resuspended in 12 ml TC100 medium with 10% serum. Cells were plated at 10^6 cells/well in 12-wells plate. Cells were attached for 30 min at RT. Serial 10 folds virus dilutions were prepared (from 10^{-1} to 10^{-6}) in 1.5 ml TC100 serum free medium and mixed thoroughly in the plate. 0.5 ml virus suspension was given to the cells which were infected for 1 h at RT. The 0.5% agarose overlay was prepared in Excell400 (JRH Bioscience) medium+10% FCS and pre-warmed to 41.5°C. Viral suspension was removed from the cells and 2 ml agarose overlay gently poored over the cells. The overlay was solidified in 20 min. The plate was then sealed with parafilm and incubated 4-5 days at 28°C. To facilitate plate inspection and viral counting after 4-5 days, 250 µl of neutral red solution was poored over the agarose and incubate at RT for 1 h. Formed viral plaques were counted to calculate viral titar, and single plaque was isolated for further amplification. Virus was amplified in *Sf* 9 suspension cells at $1.2x10^6$ cells/ml in SF 900 medium for 3-4 days at 28°C. The state of the culture infection was determined by cell number, viability and cell diameter, using the CEDEX cell counter (Innovatis, GmBH). The culture supernatant was harvested by centrifugation at 400xg, for 10 min. at 10°C, when cell viability dropped below 90%. The titer of the viral stock was determined again by plaque assay prior to the protein production.

3.3.1.19 Protein production analysis

Protein production was initially followed on a small scale by analyzing pellet from the first viral amplification. 1 ml of insect cell culture supernatant was centrifuged as described above and pellet was washed in 0.5 ml PBS buffer. Centrifugation step was repeated, final pellet resuspended in 250 µl 5 X reducing SDS-PAGE buffer and analyzed on SDS-PAGE and reducing, denaturing Western blot with anti-His tag antibodies. Time-course of protein expression was also followed. Culture suspension of 10^6 *Sf 9* cells/ml in Sf900 II medium (GIBCO) was infected at M.O.I (multiplicity of infection) of 0.6, cells were grown in suspension at 28°C, 90 rpm and aliquots were taken at different time points, starting at 24 h p.i. (post infection), up to 96 h p.i. For standard protein production, suspension of 10^6 *Sf9* cells/ml was infected at M.O.I of 0.6, cells were grown in suspension at 28°C, 90 rpm and harvested 72 h post infection. Prior to the harvest, cells were inspected for cell number, viability and diameter, and were centrifuged at 10°C, 1000 rpm for 10 min. Supernatant was decanted, and cells lysed in a lysis buffer (0.05 M Tris, pH 8.0, 0.5 M NaCl, 1 mM PMSF, protease inhibitor cocktail, 0.5 ml). Cells were first resupenden and then sonicated on ice with burst at 70% output, 4x 20 sec with 10'' break in between. Finally, cell-lysates were centrifuged for 1 h at 4°C and 22000 rpm in ultracentrifuge Sorval 1000. Cell pellet was washed twice as described, and isolated soluble

cytoplasmic fracton was analyzed on SDS-PAGE, Western blot and by Ni-NTA affinity chromatography under native conditions.

3.3.1.20 Ni-NTA purification under native conditions

Ni-NTA affinity chromatography was used to purify HPLE from soluble cytoplasm under native conditions. The column was prepared and run as described under 3.2.3.9. Buffers used for purification are described in the *Table* 3.48. All chromatography steps were analyzed on the SDS-PAGE, and also in Western blot with mAb 7A9 and anti-His tag antibody.

Table 3.48: Buffers for Ni-NTA IMAC purification of HPLE under native conditions

Lysis Buffer	50 mM NaH_2PO_4 300 mM NaCl 10 mM imidazole, pH 8.0
Wash Buffer	50 mM NaH_2PO_4 300 mM NaCl 20 mM imidazole, pH 8.0
Elution Buffer	50 mM NaH_2PO_4 300 mM NaCl 250 mM imidazole, pH 8.0

3.3.2 LecEGF secretion into the medium

Cloning into pAcGP67a (Pharmingen) generated recombinant LecEGF with an N-terminal signal sequence from insect acidic glycoprotein GP67. Either Flag peptide (DYKDDDK) was introduced as a C-terminal tag, or recombinant protein was produced w/o any tag on the C-terminus. DNA sequence coding for LecEGF domain of human E-selectin was PCR integrated into pAcGP67a cloning vector, as described in the *Table* 3.49 and 3.50.

3.3.2.1 PCR generation of LecEGF

DNA sequence coding for LecEGF domain of human E-selectin was PCR amplified from template plasmid pFastBacHPLE, in which sequence of LecEGF was cloned into vector pFastBac.

Primers' sequences:
ESEL1 (5'- primer):
5'-GCG CAT TCT GCC TTT GCG TGG TCT TAC AAC ACC TCC ACG
ESEL2 (3'-primer):

5'-GGT CCC AGG AAA GGA TCA GAT TAC **TTA** TCG TCA TCG TCC TTG TAG TCC ACA ATT TGC TCA CAC TTG

Underlined: Flag peptide (GGT CCC AGG AAA GGA TCA GAT TAC)

Underlined in bold: stop codon **(TAA)**

ESEL3 (3'-primer): 5'-GGT CCC AGG AAA GGA TCA GA**T TA**C ACA ATT TGC TCA CAC TTG A-3'

Underlined in bold: stop codon **(TAA)**

Table 3.49: Cloning into pAcGP67a: PCR reaction set up for LecEGF+/-Flag

dNTPs (10 μM)	0.6 μl
Oligo 1(100 μM)	0.5 μl
Oligo 2 (100 μM)	0.5 μl
Template DNA (plasmid)	0.3 μl = 0.05 μg
10 X Turbo Pfu buffer	5.0 μl
Turbo Pfu	1.0 μ
Sterile H_2O	Ad 50 μl

PCR reaction was done under following conditions with hot start.

Table 3.50: PCR conditions for LecEGF amplification

Initial denaturation	95°C	5 min.
Denaturation	95°C	30sec 45 Cycles
Annealing	55°C	30sec 45 Cycles
Elongation	72°C	1min 45 Cycles
Final elongation	72°C	10 min
Pause	4°C	∞

After the amplification, PCR fragment was purified with the PCR purification kit (SIGMA) according to the manufacturer instruction.

3.3.2.2 PCR integration of SSLecEGF+Flag (SSLecEGFFlag), and SSLecEGF into pFastBac

For the integration of SSLecEGFFlag and SSLecEGF in vector pAcGP67a reaction set-up was used as described under the 3.3.1.2., and the PCR conditions were as described in the 3.3.1.3. At the end of the PCR reaction, restriction enzyme digestion was performed with *Dpn* I to digest methylated DNA originating from recipient plasmid.

3.3.2.3 Purification of PCR-integration product

Purification of PCR product was done as described in 3.3.1.4.

3.3.2.4 Transformation into *E.coli*

E.coli strain XL1-Blue was transformed with plasmid DNA as described in 3.3.1.5.

3.3.2.5 Colony PCR for colony analysis

Grown colonies were analyzed in PCR reaction as described in 3.3.1.6.

3.3.2.6 Cloning into pFastBac

SSLecEGFFlag and SSLecEGF were subcloned into pFastBac to enable transfection of recombinant viral DNA into insect cells.

3.3.2.7 PCR generation of SSLecEGFFlag and SSLecEGF compatible with pFastBac

PCR reaction was performed as described in 3.3.1.8.
ESEL4 (FW- primer):

5'-ATC CCG GTC CGA AGC GCG CGG ATG CTA CTA GTA AAT CAG TCA-3'

ESEL5 (RW-primer):
5'- CAG GCT CTA GAT TCG AAA GCG TTA CTT ATC GTC ATC GTC CTT GTA GTC-3'
ESEL6 (RW-primer):
5'-CAG GCT CTA GAT TCG AAA GCG TTA CAC AAT TTG CTC ACA CTT GAG-3'

3.3.2.8 PCR integration of SSLecEGFFlag and SSLecEGF in pFastBac

PCR integration was done as described in 3.3.1.9.

3.3.2.9 Purification of PCR product

Purification of PCR-integration product was done as described in 3.3.1.10.

3.3.2.10 Transformation into *E.coli*

Transformation into *E.coli* XL Blue-1 was done as described in 3.3.1.11.

3.3.2.11 Colony PCR for colony analysis

Colony PCR for colony analysis was done as described in 3.3.1.12.

3.3.2.12 Transformation into *E.coli* DH10Bac and transposition reaction

Transformation into *E.coli* DH10Bac and transposition reaction was done as described in 3.3.1.14.

3.3.2.13 Clone analysis and Bacmid DNA isolation

Clone analysis and Bacmid DNA isolation was done as described under 3.3.15

3.3.2.14 PCR analysis of recombinant bacmid DNA

PCR analysis was performed as described in 3.3.1.16.

3.3.2.15 Transfection into *Sf 9* insect cells

Transfection into *Sf 9* insect cells was done as described in 3.3.1.17. Attached cells (max. 50-60% confluent) were washed twice with 2 ml medium and finally 1 ml medium with prepared DNA was added. The plate was incubated at 28°C, 5 h, and medium was aspirated and exchanged. Plate was further incubated for 5 days, cells were inspected and medium was harvested for plaque assay and viral amplification.

3.3.2.16 Viral amplification and plaque assay

Plaque assay was performed as described in 3.3.2.16.

3.3.2.17 Protein production analysis and optimization

Preliminary analyses of protein production were performed with both LecEGFFlag and LecEGF. 50 ml insect cells culture were infected at 10^6 cells/ml, M.O.I of 10 and were grown in suspension at 28°C, 90 rpm shaking for 72 h p.i. Total culture was centrifuged at 10°C, 1000 rpm for 10 min., as well as 1 ml aliquot. Supernatant of 50 ml was filtrated through 0.22 μm filter and stored for further purification. 1 ml aliquot was analyzed by SDS-PAGE for protein expression in the supernatant and pellet. Optimization of protein production and subsequent scale-up was done with recombinant LecEGFFlag. Insect cell line, time-course of expression (as described under 3.3.14) and M.O.I. were optimized.

3.3.2.18 Viral amplification for scale-up

Scale up of protein production was done in a mid-scale with 1.5 L medium. 300 ml amplified viral stock was produced at $1.4x10^6$ *Sf 9* cells/ml in TC100 medium with 10% serum and at M.O.I. of 0.1. Culture was grown at 28°C for 72 h and harvested by centrifugation under standard conditions.

3.3.2.19 Production scale-up

LecEGFFlag was produced in 1.5 L culture of Hi 5 insect cells, Sf900 medium at $1.2x10^6$ cells/ml and M.O.I 10 for standard conditions. Medium with secreted protein was harvested 72 h p.i. and centrifuged at 5000 rpm, 20' at 10°C. Conditioned medium was purified on an anti-Flag chromatography.

3.3.2.20 Anti-Flag affinity chromatography

1 ml column (Amersham, Pharmacia) was packed with the mouse, anti-Flag M2 monoclonal antibodies agarose matrix (SIGMA) as described for column preparation under 3.2.3.11. Column was equilibrated at 1.0 ml/min. with 10 column volume of binding buffer (TBS) until stable base line. Protein solution was applied at the same flow rate for 30-100 ml, and was subsequently washed with 10 column volumes of binding buffer to remove unspecific bound proteins. When the base line was stable again, specifically bound protein was eluted with either 0.5 ml 100 μg/ml Flag peptide solution (SIGMA) in TBS, or with 10 column volume of 0.1 M glycine buffer, pH 3.5. After the run, column was equilibrated again in TBS buffer, and for longer period was stored at 4°C in TBS with 50% glycerol.

3.3.2.21 Western blot with the anti-Flag antibodies

Western blot analysis with the anti-Flag antibodies (SIGMA) was done under native and reducing conditions. In native conditions, reaction was done as described previously under 3.1.6, except that proteins were separated on 5-15% gradient native gel. Western blot in reducing conditions was done according to the same protocol, with following differences in performance. Proteins were separated on 15% reducing and denaturing SDS-PAGE, blotted to the membrane and stained with Ponceau solution to verify electrotransfer. Membrane was blocked for 1 h at RT in 5% skim milk solution in TBS buffer with 0.1% Tween 20. To the 10 ml of this solution, 2 μl antibody stock solution at 4.9 mg/ml was added and the membrane

incubated at RT for 1 h. All wash steps, incubation with the secondary antibody and developing reaction were done as described before.

3.3.2.22 NMR analysis of ligand binding

For NMR analysis, T1 rho relaxation time was measured, as well as STD to qualitatively verify the ligand binding to the protein. Protein was concentrated in Eppendorf centrifuge 5408R, at 3500 g and 4°C for 15 min in a 7 ml concentration device (Amicon, 10 kDa cut-off). 110 μl concentrated protein at 0.992 mg/ml were dialysed in a small dialysis device (Pierce) in 1:10 ratio of d_2O at 4°C, with the buffer exchange every 3-12 h for 2 days.

3.3.2.23 Protein deglycosylation

Protein was deglycosylated with *N*-glycanase (PNGaseF) in native and denaturing conditions. Optimization was done regarding the enzyme amount and used detergent. Reactions were performed in 0.02 M Na-phosphate-buffer, pH 6.8. Aliquots have been taken at different time points for reaction I (or it was performed over-night at 37°C). Finally, protein was deglycosylated for 48 h at 37°C, with an enzyme addition after 24 h.

Table 3.51: *N*-deglycosylation in denaturing conditions, reaction set-up

Reaction	I	II	III	IV	V
Protein	16 μg	4 μg	4 μg	4 μg	4 μg
EDTA	25 mM final	25 mM final	25 mM final	25 mM final	25 mM final
SDS	0.1% final	0.2% final	0.2% final	0.2% final	0.2% final
Tween 20	2% final	2% final	2% final	-	2% final
NP-40	-	0.75% final	0.75% final	-	0.75% final
2-mercaptoethanol	1% final	1% final	1% final	1% final	1% final
Enzyme	0.6 U/μg	1.2 U/μg	0.6 U/μg	3.0 U/μg	0.6 U/μg

Table 3.52: *N*-deglycosylation in native conditions

Reaction	I	II
Protein	16 μg	4 μg
EDTA	25 mM final	25 mM final
SDS	-	-
Tween 20	0.05% final	2% final
NP-40	-	-
2-mercaptoethanol	-	-
Enzyme	0.6 U/μg	1.2 U/μg

3.3.2.24 Size exclusion chromatography

Fractions eluted in anti-Flag chromatography, which showed presence of impurities were submitted to the size exclusion chromatography on Sephadex column (Amersham, Pharmacia), 100 kDa exclusion. 100 ml column was equilibrated with 5 volumes of TBS buffer and 2 ml protein solution at 0.927 mg/ml were applied at the flow rate of 0.5 ml/min. Chromatography was done overnight at 4°C, 5 ml fractions were collected and analyzed on SDS-PAGE. Fractions containing LecEGFFlag were pooled together and concentrated to 1.4 mg/ml.

3.3.2.25 Ion exchange chromatography

Anion exchange chromatography was performed for separation of two different glycosylation forms of LecEGFFlag, which were both eluted in anti-Flag chromatography. Mono Q anion exchange column (BIO-RAD) with 1.3 ml bed volume was equilibrated with binding buffer A (20 mM Tris, pH 8.0) until stable base line. 0.35 ml protein solution at 1.4 mg/ml were mixed with 0.7 ml binding buffer and applied to the column at the flow rate 0.3 ml/min. Column was washed again with 4 column volumes of buffer A. Gradient was formed to 50% B (20 mM Tris, 1 M NaCl, pH 8.0) in 15 ml, followed by steeper gradient up to 100% B in 6 ml. Buffer B was linear at 100% for next 6 ml, and then in next 10 ml it dropped to 0% B. Eluted fractions were analyzed on SDS-PAGE.

3.3.2.26 MS analysis

For molecular weight determination of LecEGFlag ESI/MS analysis was performed. Prior to the measurement, protein sample was desalted. Desalting was done on the RP-HPLC 1100 (Agilent Technologies) using C4 Vydac HPLC column. Prior to the protein load, column was equilibrated in 98% buffer A (0.1% TFA) and 2% buffer B (80% acetonitrile in 0.075% TFA). 100 μl protein solution at 1 mg/ml was applied to the column, and gradient was formed of 75% B in 60 min. Two separated peaks were eluted, and chromatography profile was analyzed on SDS-PAGE. Molecular weight of eluted proteins was determined by ESI/MS analysis.

3.4 Bioassay development

For the evaluation of selectin antagonists, bioassays have been developed in cell-free and cell-based format.

3.4.1 Cell-free assay

3.4.1.1 Sialyl Lewis^a-polymer preparation

Biotinylated sialyl Lewis^a-polyacrylamide polymer (Glycotech) was dissolved in Hepes buffer (HAB: 20 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM Ca^{2+}) at 1 mg/ml and stored at -20°C. Polymer complex with streptavidin-peroxidase (Roche) was prepared as described in the *Table* 3.53 and formed at 37°C for 2 h. Complex was kept at 4°C and it was stable over several weeks.

Table 3.53: sLe^a-polymer-streptavidin-POD complex preparation

sLe^a-polymer	20 μL
Steptavidin-POD conjugate	80 μL
Fetal calb serum	20 μL
HAB	ad 200 μL

3.4.1.2 Molecule-molecule assay development, optimization and IC_{50} determination for selectin antagonists with E-, P-, L-selectin/IgG

100 μl mouse IgG1 antibody against human Fc-IgG (SIGMA) or recombinant human E-, P-, and L-selectin/IgG were coated overnight at 4° C in flat bottom NuncMaxi Sorp 96-well plates. Solutions were discarded and plate dried by gentle tapping on several layers of tissue paper. Plate was washed three times with 200 μl HAB, blocked with 3% BSA in assay buffer at 4°C for 4 h and washed as described. Selectin antagonists were diluted in HAB, and 50 μl of 2x concentrated solution was added to each well, followed by the addition of 2x concentrated 50 μl sLe^a–polymer-streptavidin-POD complex. Plate was incubated at 37°C for 4 h in a humid chamber, and washed again as described above. In order to detect bound polymer, 100 μl/well ABTS substrate solution (BIO RAD) was added and the color was developed 5-10 min. Reaction was stopped by adding 100 μl/well 2% oxalic acid and the O.D. was measured at 415 nm with the spectrophotometer Softmax (Molecular Probes). IC_{50} values for tested compounds were calculated relative to the control polymer using software PrismPad. Optimization was done regarding the amount of sLe^a-polymer and protein in standard assay conditions, incubation time and temperature.

Table 3.54: Optimization of the cell-free assay for E-, P-, L-selectin/IgG

Optimization parameter	E-sel/IgG (μg/ml)	P-sel/IgG (μg/ml)	L-sel/IgG (μg/ml)	sLe^a –polymer (μg/ml)	Temperature (°C)
Conditions	0.3	3	3	0.03	37
Conditions	1.0	10	10	0.1	4

3.4.1.3 Molecule-molecule assay development, optimization and IC_{50} determination for selectin antagonists with LecEGFFlag

Anti-Flag M2 monoclonal antibody, or LecEGFFlag were diluted to 10 μg/ml and 3 μg/ml, respectively in either TBS or carbonate buffer for antibody, or HAB 20 for LecEGFFlag (HAB 20: 20 mM Hepes, pH 8.5, 150 mM NaCl, 20 mM Ca^{2+}). 100 μl protein solution were plated onto NuncMaxi Sorp 96-wells plate, overnight at 4°C. Unbound proteins were washed gently with 200 μl HAB 20, three times, and either LecEGFFlag was added to the coated antibodies, or plate was blocked 3 h at RT with 200 μl 3% BSA in HAB 20. Plate was washed again as described. Selectin antagonists were diluted in HAB 20, and 50 μl of 2x concentrated solution was added to each well, followed by the addition of 50 μl 2x concentrated sLe^a-polymer-streptavidin-POD complex. Plate was incubated at RT for 3 h, shaking at 57 rpm and washed again as described. In order to detect bound polymer, 100 μl/well ABTS substrate solution was added and the color was developed 5-10 min. Reaction was stopped by adding 100 μl/well 2% oxalic acid and the O.D. was measured at 415 nm. IC_{50} values for tested compounds were calculated relative to the control polymer using software GraphPrismPad.

Table 3.55: Optimization of the cell-free assay for LecEGFFlag

Optimization parameter	LecEGFFlag (μg/ml)	sLe^a –polymer (ng/ml)	Assay buffer.	Temperature (°C)
Conditions	0.3	30	TBS	37
Conditions	1.0	100	HAB 1 mM Ca^{2+}	RT
Conditions	10	300	HAB 20 mM Ca^{2+}	-
Conditions	-	1000	-	-

3.4.1.4 Acute human promyelocytic leukemia cell line cultivation

For cell.based assay, acute human promyelocytic leukemia cell line (HL-60 cells) were grown in suspension in RPMI 1640 medium (GIBCO), supplemented with 10% fetal calf serum and 1%

penicillin-streptomycin (GIBCO) at 37°C in humidified atmosphere with 5% CO_2. Cells suspension was centrifuged at RT and 1000 rpm for 10 min. in 5804 R centrifuge (Eppendorf). Pelleted cells were gently resuspended in PBS buffer, visually examined for viability and counted. $5x10^4$ cells/ml were seeded in 20 ml medium in T75 culture flask, and regularly passaged twice a week.

3.4.1.5 Cell-based assay development, optimization and IC_{50} determination for selectin antagonists

HL-60 cells were centrifuged as described and viability was examined (95-100%). Cells were seeded at $9x10^5$-$1.2x10^6$ cells/ml 24-36 h prior to the experiment. 100 μl/well selectins was coated on the Nunc MaxiSorb 96-well plate and incubated overnight at 4°C. Plate was washed 2x with 200 μl HAB and gently tapped on the tissue paper. 200 μl 3% BSA in HAB was added to each well and the plate was incubated for 3 h at RT. HL-60 cells, $2.2x10^7$ cells/plate were centrifuged as described and resuspended in 5 ml pre-warmed PBS with 5 μg/ml BCECF-AM (Roche) fluorescent dye. Cells were then incubated for 30 min at 37°C in the dark. After the incubation, cells were washed again in PBS and resuspended to the final of $2x10^5$ cells/well. Plate was washed as described and 50 μl of 2x concentrated tested compounds have been added to the well. 50 μl of cell suspension was added to each well and plate was incubated at 37°C for 1 h. Plate was washed carefully 3 times in HAB and fluorescence was measured in Fluoroskan II Perkin Elmer fluorometer at excitation wavelength of 485 nm, and emission wavelength of 538 nm. Inhibitory potencies for selectin antagonists have been expressed as a percentage of control, and calculated using software PrismPad. Optimization of cell-molecule assay was done regarding the number of the HL-60 cells used in the assay, protein concentration and the number of wash steps. The compounds that were tested in cell-molecule assay have been described in the *table* 4.6.

Table 3.56: Optimization of the cell-molecule assay

Optimization parameter	E-sel/IgG (μg/ml)	P-sel/IgG (μg/ml)	L-sel/IgG (μg/ml)	No.of HL-60 cells/well	Wash steps
Conditions	1	1	1	$2x10^5$	2x
Conditions	3	3	3	$1x10^5$	3x
Conditions	10	5	5		
Conditions		10	10		
Conditions		15	15		

4 Results

4.1 E-, P-, L-selectin/IgG expression in CHO cells and characterization

4.1.1 E- and P-selectin/IgG expression in CHO cells

Standard expression conditions have been established for culture maintenance, protein expression and production following published procedures [92]. Cells have been visually inspected and their outlook, as well as viability, was the parameter to follow prior to medium harvest. Cells reached 80% confluence after 3-4 days in 75 cm^2 flasks and were seeded in either 150 cm^2 flasks in 60 ml medium at 10^6 cells/flask, or in roller bottles at 1.5-2x10^6 cells/ml in 200 ml medium. After 5-7 days cells were confluent again, and medium with secreted protein was harvested after 2 weeks.

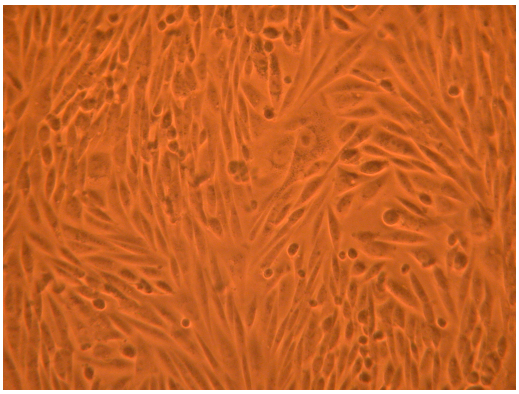


Figure 4.1: CHO-K1 cells in logarithmic growth phase

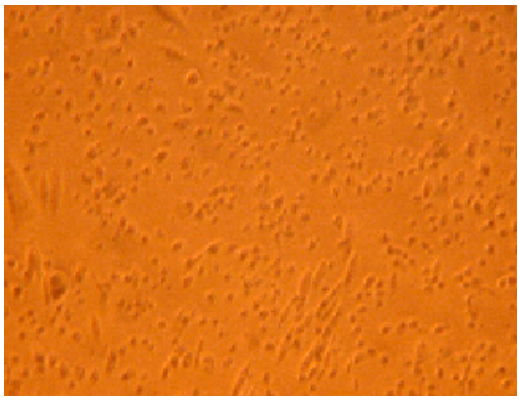


Figure 4.2: CHO-KI cells in production phase, 20% viability

4.1.1.1 Protein purification by affinity chromatography and size exclusion chromatography

IgG-fusion proteins are eluted from protein A as single peaks (*Figure* 4.3 and *figure* 4.4). Reducing SDS PAGE analysis of the eluted peaks has shown major protein bands at 148 kDa, and 110 kDa, for E- and P-selectin/IgG, respectively. Minor impurities consisting of serum albumin (66 kDa) and bovine IgGs (55 kDa and 25 kDa for heavy and light chains respectively), were also detected. These are standard impurities from serum-containing media, and size exclusion chromatography was used for their efficient removal. *Figure* 4.5 shows a typical size

exclusion elution pattern with superimposed calibration markers (dotted line). SDS-PAGE analysis of eluted fractions is shown in *figure* 4.6.

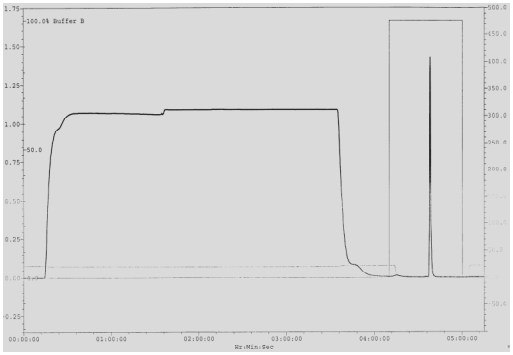
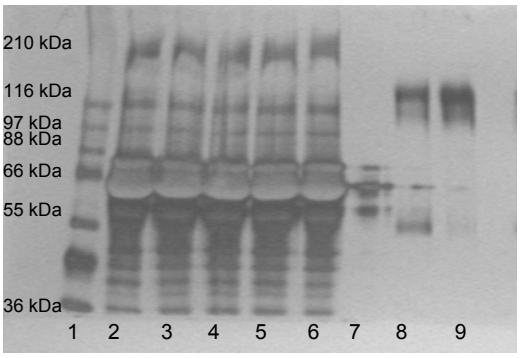


Figure 4.3: Protein A affinity chromatography of E-selectin/IgG. Protein was eluted at pH 3.4



Lane 1: HMW marker
Lane 2: Crude extract
Lane 3-6: Flow-through
Lane 7: Wash
Lane 8: Elution 1
Lane 9: Elution 2

Figure 4.4: 8% SDS-PAGE analysis of protein A chromatography

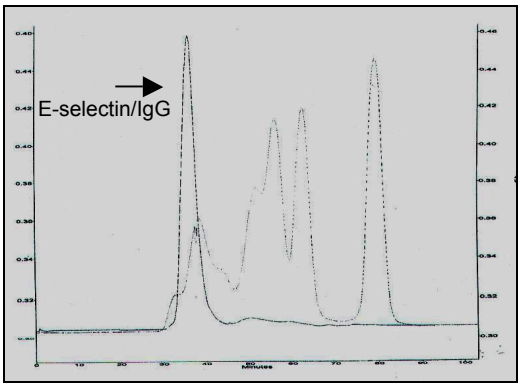
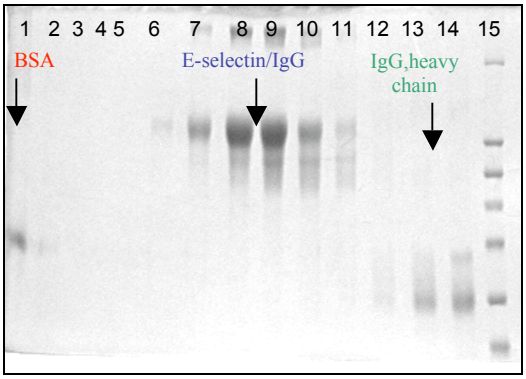


Figure 4.5: Size exclusion chromatography of E-selectin/IgG. The protein elutes between the peak of oligomers and thyroglobulin



Lanes 1,2: BSA aggregates
Lanes 3-5: -
Lanes 6-11: E-sel/IgG
Lanes 12-14: IgG
Lane 15: HMW standard

Figure 4.6: SDS-PAGE analysis of size exclusion chromatography. This method efficiently separates E-selectin/IgG from BSA aggregates and IgGs

A purification overview for E-selectin/IgG, starting with culture supernatant produced either in roller bottles, or tissue flasks, is given in the *table* 4.1. Protein concentration was determined by Bradford assay with BSA as standard.

Table 4.1: E-selectin/IgG purification overview with the protein concentration throughout the purification procedure. Conditioned medium considers total proteins of cell culture medium subtracted from cell culture medium that contains secreted proteins.

Device	Conditioned medium	Protein A pool	Size exclusion pool	Total yield/L (%)
Roller bottles (total 1.5L)	15 mg	8.5-12 mg	4.9-9.6 mg	7.3 mg (48.6%)

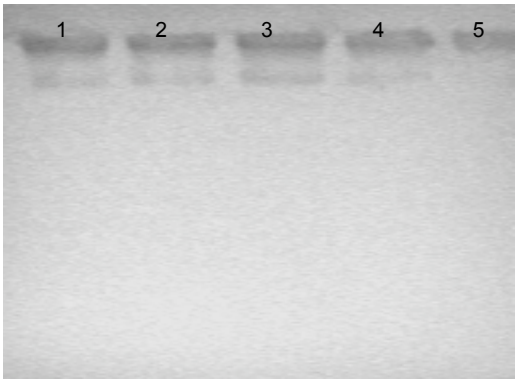
T 160 flask (total 1.5L)	1.45 mg total	0.695 mg	0.0625 mg	0.43 mg (29.65%)

4.1.2 E-selectin/IgG characterization

The activity of expressed and purified protein was addressed in native immunoblots and ELISA-based assays with a monoclonal blocking antibody, as described in materials and methods section. The glycosylation status was examined by *N*-deglycosylation. NMR analyses were used to confirm ligand binding to E-selectin/IgG (described in result section 4.1.2.6).

4.1.2.1 Immunoblot in native conditions

As previously described, immunoblots of E-selectin/IgG were performed using a monoclonal blocking antibody (mouse anti-human E-selectin antibody 7A9). In native conditions, the reaction was positive, whereas in reducing, or non-reducing, denaturing conditions no antibody binding was observed. A typical membrane with a positive immunoblot reaction is shown in *figure* 4.7.



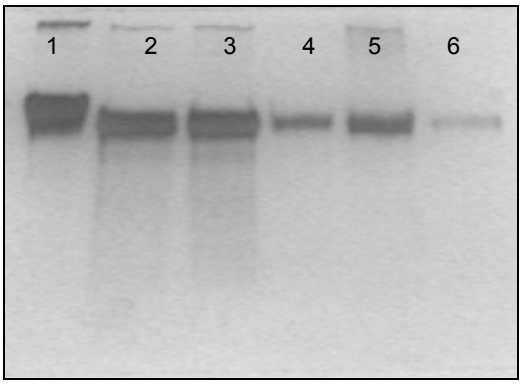
Lanes 1-5: E-selectin/IgG, samples after gel-filtration. Correspond to lanes 6-10 on *figure* 4.6.

Figure 4.7: Nitrocellulose membrane blotted with the native E-selectin/IgG, in reaction with the mAb 7A9 and 2nd antibody AP-conjugated.

4.1.2.2 N-deglycosylation of E-selectin/IgG

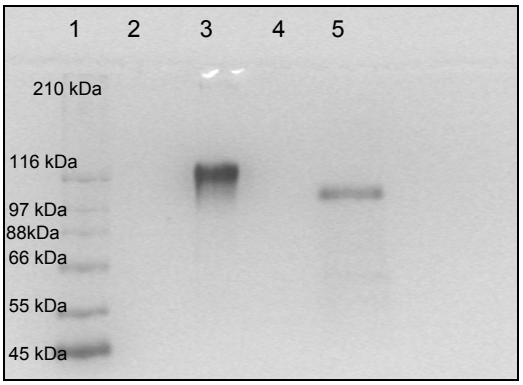
For N-deglycosylation with PNGaseF (*N*-glycanase), E-selectin/IgG was either denaturated or deglycosylated under native conditions. E-selectin/IgG deglycosylation in denaturing conditions after 48 hours is shown in *figures* 4.8, and 4.9. Results from a deglycosylation time-course have shown no significant difference in the deglycosylation extent after 1 hour and 48 hours, indicating that maximal deglycosylation takes place within an hour. The molecular weight of the protein was extrapolated from the SDS-PAGE migration distances, and was 148 kDa for the

fully glycosylated protein under reducing conditions, and 108.3 for deglycosylated, reduced E-sel/IgG.



Lane 1: E-selectin/IgG glycosylated
Lane 2: E-selectin/IgG 1 h deglycosylation
Lane 3: E-selectin/IgG 6 h deglycosylation
Lane 4: E-selectin/IgG 12 h deglycosylation
Lane 5: E-selectin/IgG 18 h deglycosylation
Lane 6: E-selectin/IgG 24 h deglycosylation

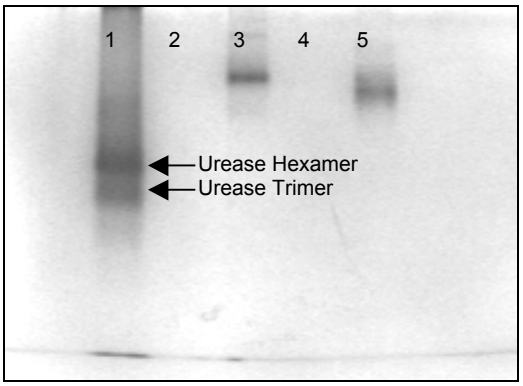
Figure 4.8: Time-course of N-deglycosylation of E-selectin/IgG in denaturing conditions, 8% silver stained gel.



Lane 1: HMW marker
Lane 2: -
Lane 3: E-selectin/IgG glycosylated
Lane 4: -
Lane 5: E-selectin/IgG 48 hours deglycosylated

Figure 4.9: 48 hours N-deglycosylation of E-selectin/IgG in denaturing conditions, 8% gel.

Deglycosylation under native conditions was performed for 48 hours and analyzed by native PAGE, as shown in *figure* 4.10. The difference in migration profile of deglycosylated and native E-selectin/IgG indicates removal of *N*-linked sugar residues. Partial deglycosylation did not affect binding activity toward monoclonal blocking antibodies, based on positive immunoblot reactions.



Lane 1: Molecular weight marker:
Urease, trimer: 272 kDa, hexamer: 544 kDa
Lane 2: -
Lane 3: E-selctin/IgG native
Lane 4: -
Lane 5: E-selctin/IgG deglycosylated for 48 hours

Figure 4.10: 48 hours *N*-deglycosylation of E-selectin/IgG in native conditions, 5% gel.

4.1.2.3 Molecular weight determination of E-selectin/IgG

The molecular weight of E-selectin/IgG in both reducing and native conditions was determined via indirect calculations, based on the protein's migration in PAA gels. In reduced and denatured conditions E-selectin/IgG has a molecular weight of 148 kDa. Molecular weight under native conditions was calculated based on the standard curve shown in *table* 4.2, and found to be 829.85 kDa, possibly a hexameric form.

Table 4.2: Proteins used as standards for molecular weight determination of E-selectin/IgG in the native conditions. As described in materials and methods, each protein was run on gels of different percentages. Migration distance plotted versus gel percentage generated a slope that describes the migration profile of each protein. The migration profile expressed as log (-slope) was plotted against the log of known molecular weights to generate a standard curve. Molecular weight of E-selectin/IgG was extrapolated from this curve.

Protein	-slope	log(-slope)	MW (kDa)/log MW	% of gels run
α-Lactalbumin	2.0	0.3	14.2 / 4.15	7, 8, 9, 10
Carbonic Anhydrase	3.7	0.568	29 / 4.46	6, 7, 8, 9
Albumin, ch.egg	3.84	0.584	45 / 4.65	7, 8, 9, 10
Albumin, bov. serum, mon.	5.34	0.728	66 / 4.82	7, 8, 9, 10
Albumin, bov. serum, dimer	7.9	0.897	132 / 5.12	7, 8, 9, 10
Urease, trimer	12.59	1.1	272 / 5.43	4.5, 5, 5.5, 6
Apoferitin, monomer	13.24	1.12	450 / 5.65	4.5, 5, 5.5, 6
Urease, hexamer	16.78	1.2	545 / 5.73	4.5, 5, 5.5, 6
Apoferitin, dimer	28.622	1.46	900 / 5.95	4.5, 5, 5.5, 6
E-selectin/IgG	23.1	1.36	829.85/5.919	4.5, 5, 5.5, 6

4.1.2.4 E-selectin/IgG binding to the monoclonal blocking antibody 7A9

Binding of E-selectin/IgG to the mouse anti-human E-selectin monoclonal blocking antibody 7A9 was performed to address the intactness of the active site of E-selectin/IgG. In an ELISA based assay, with a secondary enzyme-based colorimetric output, the binding was shown to be concentration dependent, with a K_d of 0.72 ±0.03 μg/ml E-sel/IgG, or 4.86 nM.

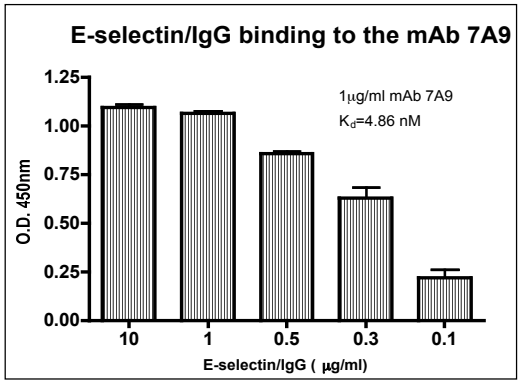


Figure 4.11: Concentration-dependent binding of E-selectin/IgG to the mAb 7A9.

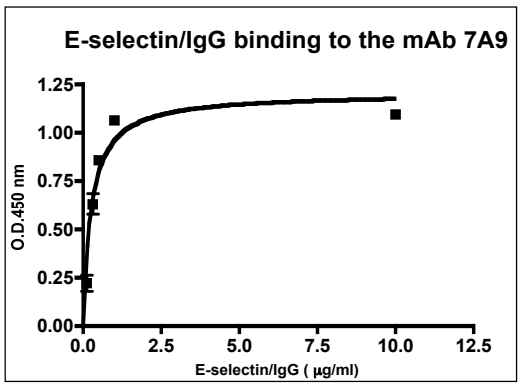


Figure 4.12: Saturation binding curve of 4.11

4.1.2.5 MS analysis for identity confirmation of E-selectin/IgG

MS analysis of a trypsin-digested E-selectin/IgG had covered the sequence to confirm the identity of E-selectin/IgG.[i]

4.1.2.6 NMR analysis of E-selectin/ligand binding

STD NMR was done to analyze ligand binding to E-selectin/IgG. In addition to the mAb 7A9 binding, the intactness of the binding site on E-selectin/IgG was examined using STD NMR of an E-selectin antagonist, **BW69669**. Spectra were recorded in presence and in the absence of protein. In the presence of protein, several peaks appear in the STD spectrum indicating binding of **BW69669** to E-selectin/IgG. Comparison of the experimental and control data are shown in the *figure* 4.13.

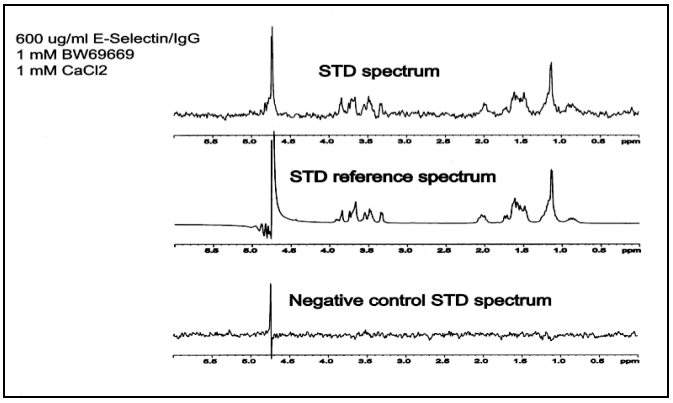


Figure 4.13: STD NMR of **BW69669** on E-selectin/IgG. Comparison with the control spectra indicates binding of **BW69669** to the protein.

4.1.3 P-selectin/IgG expression and characterization

P-selectin/IgG was expressed secretorily in CHO cells in adherent cultures, in 175 cm^2 tissue culture flasks under the conditions described for E-selectin/IgG. The production phase lasted for two weeks and 2.5 L medium were collected and purified.

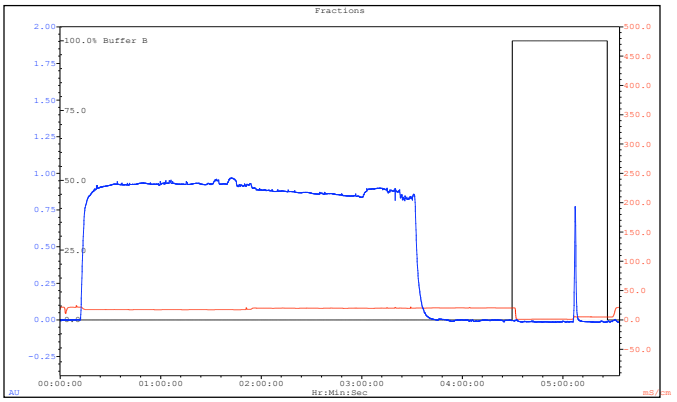
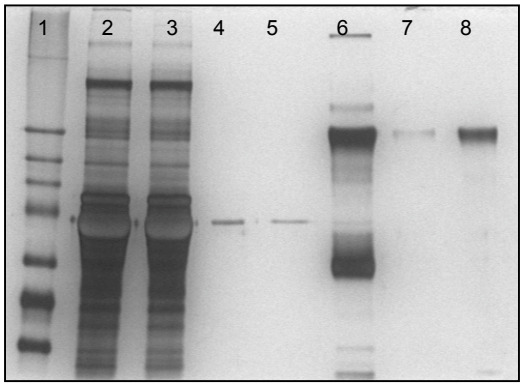


Figure 4.14: Protein A chromatography profile of P-selectin/IgG purification. P-selectin was eluted as a single peak and analyzed on 8% SDS-PAGE.



Lane 1: HMW marker, as described in *figure* 4.9
Lane 2: Crude extract
Lane 3: Flow through
Lanes 4,5: Wash
Lane 6: Elution
Lane 7: Size exclusion fraction, P-selectin/IgG at 110 kDa
Lane 8: Size exclusion, concentrated

Figure 4.15: SDS-PAGE analysis of the complete P-selectin/IgG purification.

An overview of the expression and production of P-selectin/IgG is presented in the *table* 4.3.

Table 4.3: P-selectin/IgG purification overview with the protein concentration throughout the purification procedure.

Fraction	Conditioned medium	Protein A pool	Size exclusion pool	Total yield
Concentration(μg/ml)	950	163	1000	0.7mg/l in 2.5 L
Total amount (mg)	9.45	2.15	2.0	⇒ 1.75 mg

Reaction with an antibody against human P-selectin, a mouse monoclonal blocking antibody (CD62 P) was used to address the activity of the protein. In native western blots good binding of the blocking antibody was observed. The final color reaction, catalyzed by a secondary

antibody, goat anti-mouse IgG coupled to alkaline phosphatase, was visible after less then 1 minute.

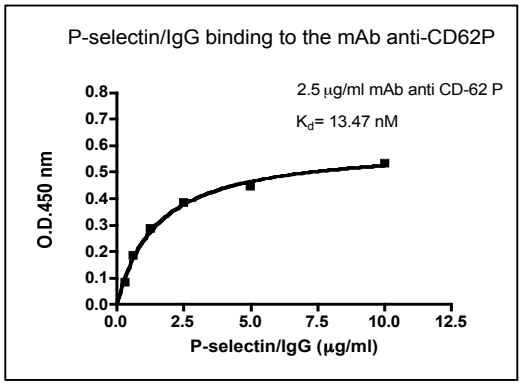


Figure 4.16: Concentration dependent binding of P-selectin/IgG to the mAb CD-62P.

For the quantification of the P-selectin binding to the blocking antibody, an ELISA-based assay was performed, where P-selectin/Ig was serially diluted and incubated with 2.5 μg/ml antibody. A secondary, HRP-conjugated antibody, with orto-phenilendiamine (OPD) as a substrate was used for the next step. Binding was quantified at 450 nm, and a binding constant of 13.47 nM could be calculated. The molecular weight of P-selectin/IgG was calculated in native conditions to be 233 kDa, indicating that protein is a dimer.

4.1.4 L-selectin/IgG

L-selectin/IgG was expressed and purified as described for E- and P-selectin/IgG and is available in purified form. Protein was assayed for the activity with a blocking antibody and was shown to be active under native western blots. The molecular weight of the L-selectin/IgG was calculated to be 87 kDa under reducing and denatured conditions, whereas native L-selectin/IgG, according to the migration profile in native PAGE, had a molecular weight of 209.7 kDa, corresponding to a dimer.

4.2 Bioassay development with E-, P-, and L-selectin/IgG

The availability of purified and active E-, P-, and L-selectin/IgG enabled the development of molecule-molecule (cell-free) and cell-molecule (cell-based) type of assays to evaluate potential selectin antagonists.

4.2.1 Cell-free assay

The cell-free assay used the reaction of protein coated to the wells and the ligand oligosaccharide (sialyl Lewis^a) presented by biotinylated polyacrylamide copolymer. Binding to the protein of this copolymer matrix in the presence or absence of candidate inhibitors is detected with the aid of streptavidin-peroxidase conjugate. The quantity of bound conjugate is estimated from the rate at which the chromogenic substrate, ABTS, is oxidized to colored products of λ_{max} 414 nm by horseradish peroxidase. For the evaluation of standard conditions in cell-free assay, several parameters were optimized. The amount of protein coated in the 96-well plate, the amount of sLe^a polymer used as control, incubation time and temperature, and wash steps. The optimal assay conditions are presented in *table* 4.4., with the screening results for the controls **BW69669** and sLe^x. E-selectin antagonists, with their structures and relative IC_{50} values, are presented in *table* 4.6 and 4.7. We had planned to measure selected antagonists in three independent experiments. However the IC_{50} values of the tested compounds and controls were fluctuating somewhat throughout the whole experimental set. As a consequence the IC_{50} determination has not been finalized.

Table 4.4: Optimized standard conditions in cell-free assay for studies of E-selectin-carbohydrate interactions.

Coating	E-selectIn/IgG	sLe^a polymer	Control
Direct E-selecin/IgG	3 μg/ml	0.1	**BW69669** (IC_{50}=42.5±12 μM) sLe^x (IC_{50}=800 μM)

4.2.2 Cell-based assay

HL60 cells that express physiological ligand of selectin were successfully cultivated and maintained according to the standard operating procedure given in the attachment. Standard conditions for cell-molecule assay and the evaluation of selectin antagonists were optimized as described in *table* 4.5. IC_{50} values in this assay are expressed relative to **BW69669**, known to have 10-12 times higher affinity for E-selectin/IgG as compared to sLe^x. Final results are given in *figure* 4.17 and *table* 4.6.

Table 4.5: Optimized standard conditions of a cell-based assay for studies of E-selectin-carbohydrate interactions.

Coating	HL60 cells/well	Incubation time/ temperature	Wash steps	Control
Direct E-selecin/IgG 1 μg/ml	100 000	1 hour 37°C	3	**BW69669** IC_{50}=200±80μM

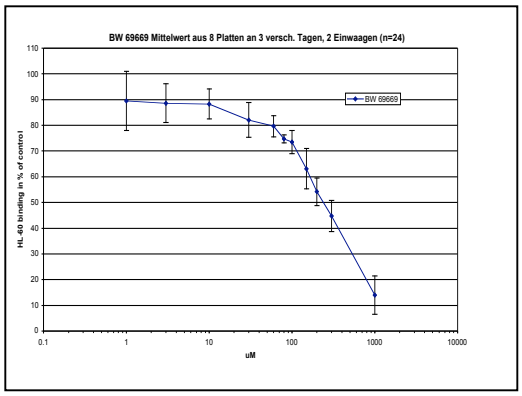
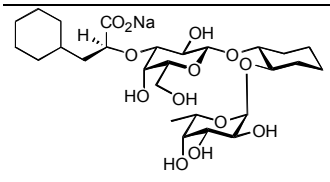
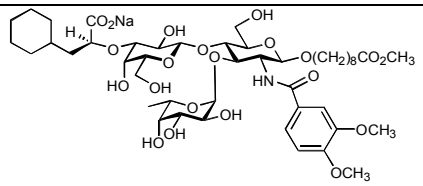
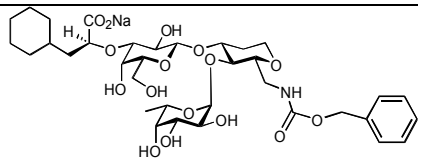


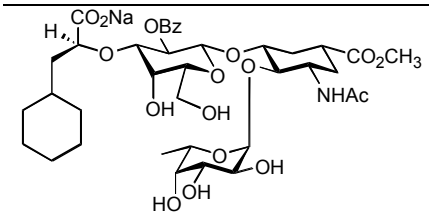
Figure 4.17: **BW69669**-mediated Inhibition of E-selectin/IgG binding to HL60 cells. Data represent the average of three independent experiments done in triplicates. Typically for this cell-based assay, **BW69669** has a higher IC_{50} value (200 μM), as compared to the cell-free assay, although in a good agreement.

Table 4.6: IC_{50} values for E-selectin antagonists obtained with a cell-based assay. Data represent an average of three independent experiments done in triplicates.

Antagonist	IC_{50} (μM)
BW75145	140±70
BW75757	146.7±73.3
BW186211	83.3±17
BW2091	50±10

Table 4.7: Structures of E-selectin antagonists synthesized in house and tested in cell-free and cell-based assays.

BW69669	**BW75757**	**BW75145**
		

BW186211	BW2091
	

In conclusion P- and L-selectin/IgG have been preliminary tested in cell-free and cell-based assay and standard conditions were established for both. The optimized assay conditions are listed in the *table* 4.8.

Table 4.8: Optimized standard conditions for cell-free and cell-based assay with P- and L-selectin/IgG. for studies of selectin-ligand interactions.

Protein in cell-free assay	sLe[a] polymer	Protein in HL60 assay	Incubation time/temp.
P-selectin/IgG 5 μg/ml	0.3 μg/ml	P-selectin/IgG 10 μg/ml	4 hours/4°C, 37°C
L-selectin/IgG 5 μg/ml	0.3 μg/ml	L-selectin/IgG 5 μg/ml	4 hours/4°C

4.3 Expression of lectin and EGF-like domain of human E-selectin in *E.coli*

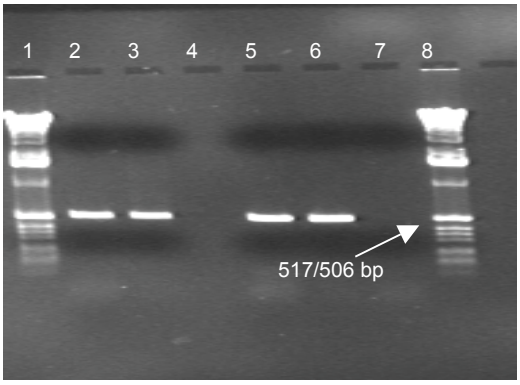
The milligram amounts of protein that would be necessary to conduct the desired structural studies, directed our strategy toward bacterial expression systems. Constructs comprising the lectin and EGF-like domains of human E-selectin (further in the text referred to as LecEGF) were cloned to yield recombinant fusion proteins with different tags to facilitate protein expression and purification. Different cloning strategies were carried out and are described in this chapter.

4.3.1 LecEGF cloning into pEZZ18

Cloning vector pEZZ18 has an export sequence that contains the so called ZZ domain of staphylococcal protein A. Fused to this sequence a recombinant protein of interest should be secreted in the culture medium. This should also improve protein folding and stability.

Genomic DNA was isolated from CHO-K1 cells expressing E-selectin/IgG, as described in the materials and methods. Plasmid pcDNA/Neo codes for E-selectin/IgG and it was used as a template for the amplification of the LecEGF domain of human E-selectin.

PCR amplification with the described primers (see materials and methods section) generated a fragment of 514 bps. The 5' primer had 4 bases prior to the *EcoR*I restriction site, followed by a thrombin cleavage site (24 bases). To maintain the open reading frame, one base (G) was inserted just prior to the sequence coding for LecEGF and annealing to the template DNA was done with the first 18 bases corresponding to the LecEGF sequence. The 3' primer introduced a stop codon after the LecEGF sequence and a *BamH*I restriction site followed by the 4 bases necessary for the optimal restriction digestion. Although the 5' primer, that was 51 bases long, had a melting temperature of 62°C, in PCR primers were successfully annealed at 58°C. The analysis of PCR products is presented in *figure* 4.18 and a molecular weight standard, DNA marker X (Roche), used for all DNA analyses, is schematically presented in *figure* 4.19.



Lane 1: DNA marker X:
Lane 2: PCR 1
Lane 3: PCR 2
Lane 4: PCR – ctrl.:
Lane 5: PCR 3
Lane 6: PCR 4
Lane 7: PCR – ctrl.
Lane 8: DNA marker X

Figure 4.18: Analysis of PCR amplified LecEGF fragment for cloning into pEZZ18.

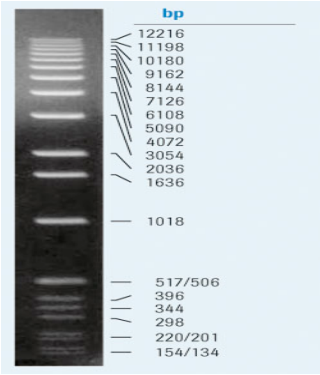
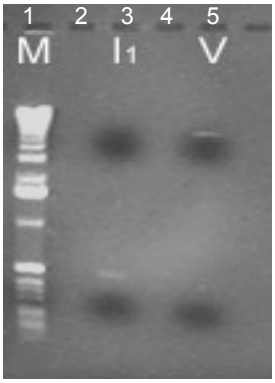


Figure 4.19: DNA marker X, with the designated bases.

A PCR fragment of the expected size was successfully amplified whereas negative controls without template DNA (lanes 4 and 7) did not give any product, indicating that the obtained product was a result of a specific reaction.

Plasmid pEZZ18 (4054 bp) was initially expressed in an *E.coli* expression strain (DH_5). pEZZ18 was isolated from *E.coli* using alkaline-lysis and analyzed by restriction enzymes (*Nde*I/*EcoR*I, *Nde*I/*BamH*I) digestion to verify plasmid identity. Multiple cloning sites of pEZZ18 contain recognition sequences of several restriction endonucleses, including EcoRI and BamHI. Insert and vector were therefore digested with these two endonucleases, electrophoresed on 1% agarose, and excised from the gel. After purification, both DNAs were controlled in another electrophoresis to verify purity and molar ratio between these two DNA fragments. *Figure* 4.19 shows control of the vector and insert, prior to the ligation.

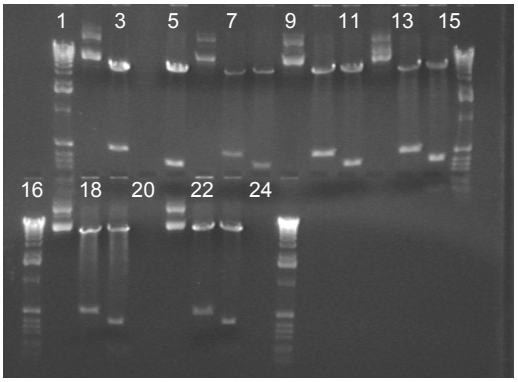


Lane 1: DNA marker X
Lane 2:-
Lane 3: LecEGF digested, purified,
Lane 4:-
Lane 5: Plasmid pEZZ18, digested, purified

Figure 4.20: 1% agarose gel analysis of purity and molar ratio between plasmid pEZZ18 DNA, and LecEGF DNA.

Intensity of the two DNA bands was similar, and since the molecular weight ratio of the insert and vector was 1:9, molar ratio was the same.

The ligation reaction was set up with these DNA fragments, predominantly using the ratios described under materials and methods. The afore mentioned *E.coli* strain DHα5, as well as another competent *E.coli* cloning strain, Top10, were either electroporated or chemically transformed with the ligation mixtures. In total, 31 clones were cultivated, DNA isolated and analyzed. The analysis included restriction enzyme digestion with *EcoR*I/*BamH*I endonucleases to generate a 496 bp insert and 4059 bp DNA that corresponds to the plasmid pEZZ18. *EcoR*I/*Pst*I digestion cuts the DNA sequence in the EGF domain and generates 3 fragments of 315, 150, and 4059 bps. Control PCR was done with the primers used for the starting LecEGF amplification or with an intern 5' primer (FWZZLE) that contains a 21 bp sequence of the EGF-part of the LecEGF. *Figure* 4.21 shows restriction enzyme digestion control of pEZZ18LecEGF.



Lanes 1, 15, 16, 25: DNA marker X
Lanes 2, 6, 9, 12, 17, 21: undigested DNA
Lanes 3, 7, 10, 13, 18, 22: pEZZ18LecEGF EcoRI/BamHI digestedl
Lanes 5, 8, 11, 14, 19, 23: pEZZ18LecEGF EcoRI/PstI digested

Figure 4.21: Restriction enzyme digestion of pEZZ18LecEGF with EcoRI/BamHI and EcoRI/PstI

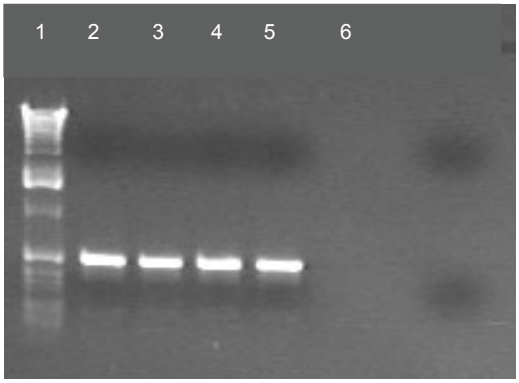
4.3.1.1 Protein expression analysis

Isolated pEZZ18LecEGF DNA that had an insert of the expected size after restriction enzyme digestion and PCR control, was transformed into the production *E.coli* strain AD 494, which should allow formation of disulfide bonds. Cells were grown overnight at 37°C in LB medium with 100 μg/ml Ampicillyn. Intracellular or secreted protein overexpression was analyzed by SDS-PAGE. Usual for non-inducible expression systems, there was no strong and clear overexpression. Culture medium and periplasmic fraction were subjected to IgG-sepharose affinity chromatography since proteins containing the ZZ domain of protein A should bind it. The protein eluted from this affinity column was a polypeptide of about 15 kDa, as revealed by SDS-PAGE in reducing conditions, corresponding to the sole ZZ domain of protein A (14 kDa). Identity of the band was confirmed also by dot blot analysis with a mouse IgG conjugated to the alkaline phosphatase. Sequence analysis of different clones has revealed that the sequence of the fusion protein was incorrect: either deletion or insertion has happened in the DNA sequence, always in the domain of the 5' primer. These had resulted in the shift of the open reading frame and it was actually only the ZZ domain with few (5) amino acids of LecEGF that could have been correctly translated before the appearance of the stop codon. After sequencing 31 clones, this strategy was abandoned and another mode of expression of LecEGF in *E.coli* was addressed.

4.3.2 LecEGF cloning into pINompA I

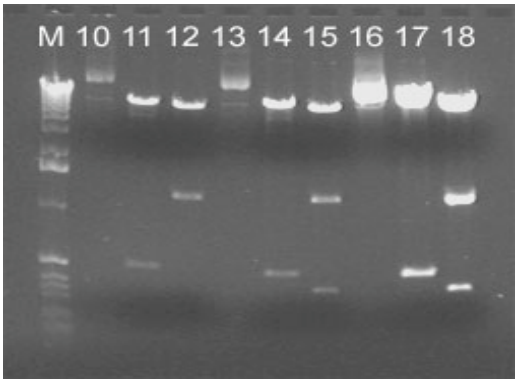
Plasmid vector pINomp A has a secretion sequence of the outer bacterial membrane protease A that should direct expressed protein to the periplasm. The oxidative environment of periplasm, in comparison to the cytoplasmic one, is more favorable for correct expression of proteins with disulfide bridges. The recombinant protein should be fused to the signal sequence and, upon correct processing would have an additional Met-Ser prior to the first Trp of E-selectin. Genomic DNA isolated from the CHO-K1 cells that expressed human E-selectin/IgG was used as a template to PCR amplifies the LecEGF domain. The forward primer introduced an EcoRI restriction site and one additional base prior to the LecEGF sequence in order to maintain the open reading frame. The 3' primer introduced a stop codon followed by a *BamH*I restriction site. PCR was done as described, and reaction products controlled on 1% agarose gels. The amplified fragment should be 514 bps in lenght, corresponding to the size of the fragment on *figure* 4.22. After restriction enzyme digestion of LecEGF and of the plasmid pINompA with *Eco*RI and *Bam*HI restriction enzymes, ligation reactions were started as described in materials and methods. Further cloning steps were conducted as described for pEZZ18. Restriction

enzyme digestion analysis of positive clones is shown in *figure* 4.23. 3 clones were sequenced but the sequence of each clone had point mutations, insertions or deletions at the very beginning of the sequence coding for LecEGF. This shifts the open reading frame and creates a random sequence with a stop codon within the LecEGF domain. Ultimatively, the attempt to export LecEGF over the bacterial periplasmic membrane failed and this cloning strategy was abandoned as well.



Lane 1: DNA marker X
Lanes 2-5: PCR product analysis
Lane 6: -ctrl.

Figure 4.22: PCR amplification of LecEGF for cloning into pINompA. PCR products analysis



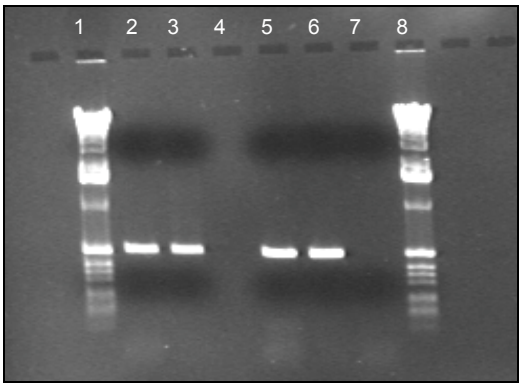
Lane 1: DNA marker X
Lanes 10, 13, 16: Non-digested pINompALecEGF
Lanes 11, 14, 17: *EcoR*I / *BamH*I digested pINompALecEGF
Lanes 12, 15, 18: *EcoR*I / *Pst*I digested pINompALecEGF

Figure 4.23: Restriction enzyme digestion of pINompALecEGF with *EcoR*I / *BamH*I and *EcoR*I / *Pst*I.

4.3.3 LecEGF cloning into pET15b

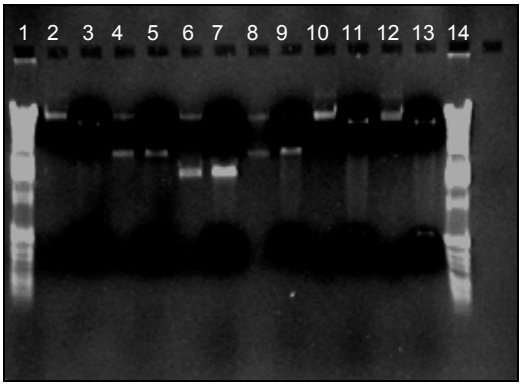
Plasmid vector pET15b enables expression of fusion proteins with an N-terminal 6XHistidine tag followed by a thrombin cleavage site for tag removal. This is an efficient system for protein expression that can be induced with IPTG. However protein expression is directed into the bacterial cytoplasm, disadvantageous for proteins with disulfide bridges. pET15b has a multiple cloning site with *NdeI* and *BamHI* restriction enzymes. The DNA sequence coding for LecEGF domain was amplified as described. The 5' primer introduced a *NdeI* restriction site, whereas the 3' primer introduced a stop codon and a *BamH*I restriction site. PCR products analysis is

shown in *figure* 4.24 and restriction digestion control of the analyzed clones with pET15b HisLecEGF in *figure* 4.25.



Lane 1, 8: DNA marker X
Lanes 2, 3, 5, 6: PCR product
Lanes 4, 7: negative control

Figure 4.24: Amplification of LecEGF for cloning into pET15b. 1% agarose gel analysis.



Lanes 1, 14: DNA marker X
Lanes 2, 4, 6, 8, 10, 12: undigested DNA pET15bHisLecEGF
Lanes 3, 5, 7, 9, 11, 13: DNA pET15bHisLecEGF digested with *NdeI/BamHI*

Figure 4.25: Restriction enzyme digestion of pET15b HisLecEGF. Three positive clones were sequenced.

The generated PCR product was of about the expected size of 512 bps. The expected size of the fragment after restriction enzyme digestion should be 496 bp in length and the obtained DNA band visible in lines 5 and 6, *figure* 4.25 were of about the expected size. PCR was also done for control purposes prior to the sequencing and in total 6 clones were sequenced, 5 of which were correct.

Further experiments were done with the clone named HLE2 (His LecEGF 2).

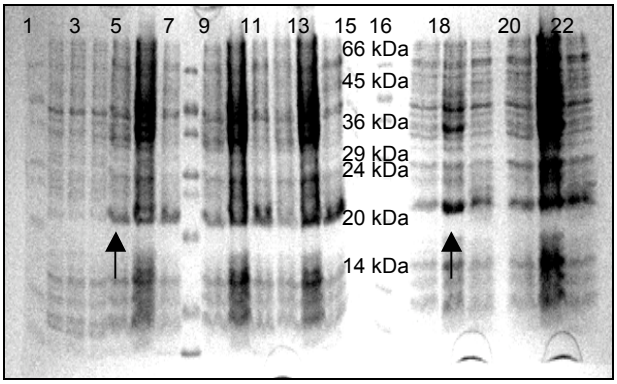
4.3.3.1 HisLecEGF expression, purification and refolding

E.coli strains AD 494 (DE3), BL 21 (DE3), and Rosetta gami (DE3) were transformed with HLE2 and, on SDS-PAGE, HisLecEGF was clearly overexpressed, giving a band between 20 and 24 kDa consistent with the expected molecular weight (21.85 kDa). Protein expression conditions were optimized based on a time-course, temperature and culture media using SDS-PAGE analysis and visual inspection of the expression level. Expression in the *E.coli* BL 21 (DE3) for 12 hours at 37°C in TB medium was the best condition. On a small scale these results were

confirmed after preliminary protein purification and concentration following Ni-NTA chromatography. The comparison of expression is given in table 4.3 and *figure* 4.24.

Table 4.9: Overview of the tested expression conditions during the optimization of HisLecEGF expression.

E.coli strain	Temperature	Medium	Total yield (Ni-NTA pool)
AD 494 (DE3)	30°C / 37°C	LB / TB	20 / 22 mg/L
BL 21 (DE3)	30°C / 37°C	LB / TB	38 / 120 mg/L
Rosetta gami (DE3)	30°C / 37°C	LB / TB	4.5 / 8 mg/L

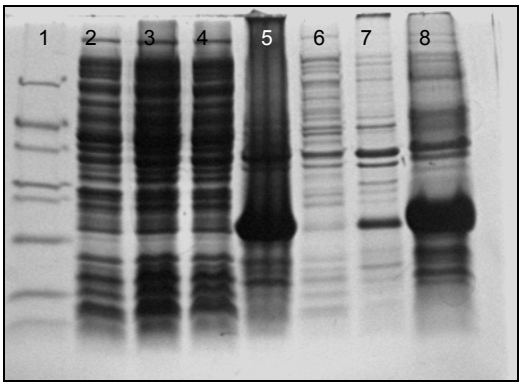


Lanes 1, 8, 16: LMW standard
Lanes 2-4: Expression, 0h post induction (p.i.)
Lanes 5-7: Expression, 3h p.i.
Lanes 9-11: Expression, 6h p.i.
Lanes 12-14: Expression, 9h p.i
Lanes 17-19: Expression, 12h p.i
Lanes 20-22: Expression, 18h p.i

Figure 4.26: Time-course expression of HisLecEGF in *E.coli* AD 494, BL 21, and Rosetta gami. Arrows point overexpressed HisLecEGF.

Initially protein purification on a Ni-NTA column was done under native conditions. A soluble bacterial cytoplasmic fraction was isolated and subjected to Ni-NTA chromatography. Ni-NTA chromatography under native conditions did not yield any pure protein. The variety of proteins that were present in the eluat is rather typical for Ni-NTA chromatography in case where there is no appreciable amount of overexpressed his-tagged protein, what indeed was the case. This indicated that the majority of the protein was produced as an insoluble inclusion bodies fraction.

Extensive inclusion bodies washing and solubilization in 8M urea or 6M GuHCl were therefore applied. Results are shown in *figure* 4.27. The final solubilized fraction contained much less contaminant proteins and the overall yield of purified HisLecEGF was 100-120 mg/L medium.



Lane 1: Low molecular weight standard
Lanes 2-4: Protein isolation, wash in 0.1 M Tris-HCl, pH 8.0, soluble fraction
Lane 5: Protein isolation, 5X wash in 0.1 M Tris-HCl, pH 8.0, insoluble fraction
Lane 6: Protein isolation, 5X wash in 0.1 M Tris-HCl, pH 8.0, soluble fraction
Lane 7: Protein isolation, 5X wash in 6 M GuHCl, pH 8.0, insoluble fraction
Lane 8: Protein isolation, 5X wash in 6 M GuHCl, pH 8.0, soluble fraction

Figure 4.27: HisLecEGF enrichment by inclusion bodies solubilization with 8M urea or 6M GuHCl. 15% SDS-PAGE, coomassie staining.

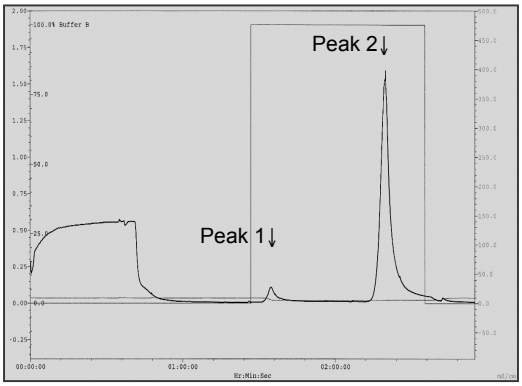
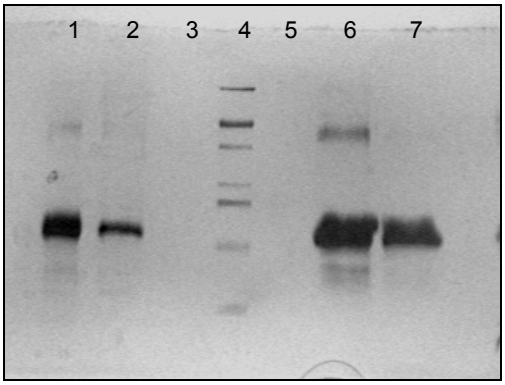


Figure 4.28: Chromatography profile on Ni-NTA column under denaturing conditions.



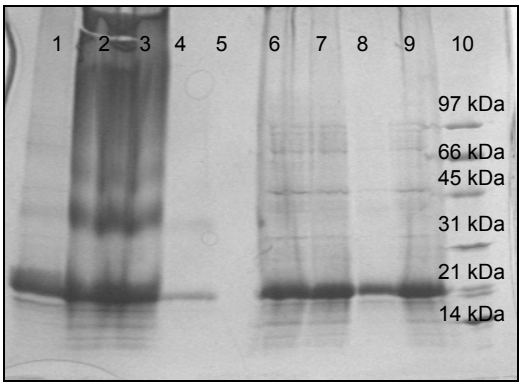
Lane 1: Crude extract
Lane 2: Flow through
Lane 3: -
Lane 4: LMW standard
Lane 5: -
Lane 6: Elution, peak 1
Lane 7: Elution, peak 2

Figure 4.29: SDS-PAGE analysis of chromatography profile on Ni-NTA column under denaturing conditions.

Protein purified on Ni-NTA under denaturing conditions is shown in *figure* 4.28 and its SDS-PAGE analysis on *figure* 4.29. The protein thus purified was analyzed by MS. The complete sequence was confirmed with fragments obtained by trypsin, V8, and C-Lys digestion and the molecular weight of the intact protein confirmed as 21 350 Da. The first methionine in the sequence was not processed, rather typical for bacterial protein expression. Since the protein

isolated in the presence of 8M urea showed an increase in molecular weight of 28 Da, all subsequent protein solubilizations were done exclusively in 6M Guanidine-HCl to avoid carbamoylation.

Refolding experiments were tried with purified, denatured HisLecEGF either by dialysis or fast dilution. Dialysis led to a quantitative protein precipitation with no active protein in the soluble fraction as tested with the blocking mAb 7A9 either in western blot or in ELISA based assays. Fast dilution was used as an alternative approach. Initial tests were monitored by analytical RP-HPLC. In reduced state the protein was easily eluted from the column, whereas oxidation caused fast formation of protein aggregates, which did not elute from the column. SDS-PAGE analysis, however, showed the presence of different protein forms in solution as shown in *figure* 4.30. By size exclusion chromatography it was possible to isolate separate forms, including the monomer fraction (*figure* 4.30 and *figure* 4.31). These fractions were subjected to the usual activity screen, but the observed activity was minor as compared to a positive control (*figure* 4.33).



Lane 1: Folding standard, non-reduced
Lane 2: Folding in 1M L-Arg, non-reduced
Lane 3: Folding in 1M L-Fuc, non-reduced
Lane 4: Folding in 1M Neu*N*Ac, non-reduced
Lane 5: -
Lane 6: Folding standard, reduced
Lane 7: Folding in 1M L-Arg, reduced
Lane 8: Folding in 1M L-Fuc, reduced
Lane 9: Folding in 1M Neu*N*Ac reduced

Figure 4.30: 4-20% gradient gel for the SDS-PAGE analysis of the refolding conditions.

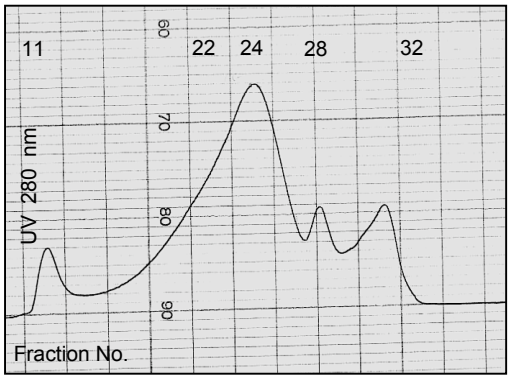
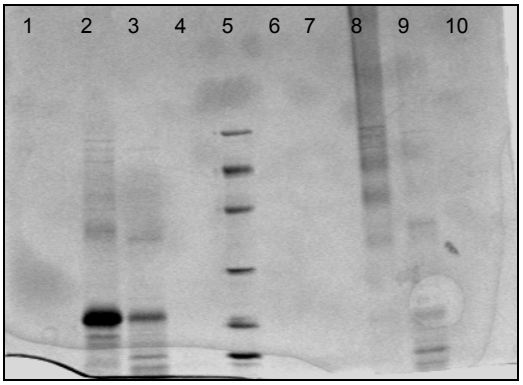


Figure 4.31: Size exclusion chromatography profile of HisLecEGF after initial folding screen.



Lane 1: Fraction 11
Lane 2: Fraction 24
Lane 3: Fraction 28
Lane 4: Fraction 32
Lane 5: Prot. Standard
Lane 6: -
Lane 7: Fraction 11 non-red.
Lane 8: Fraction 24 non-red.
Lane 9: Fraction 28 non-red.

Figure 4.32: SDS-PAGE analysis of size exclusion chromatography of HisLecEGF presented in *figure* 4.31.

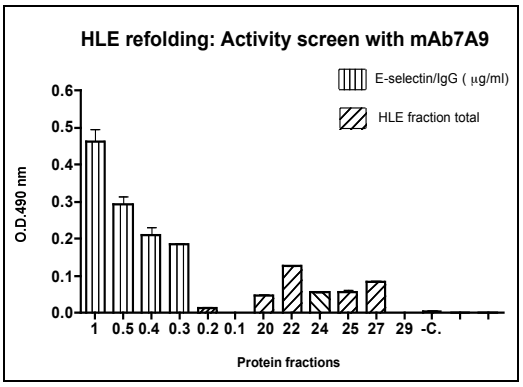


Figure 4.33: ELISA-based refolding screening with mAb 7A9.

Extensive optimization of refolding conditions was not successful and did not result in any sufficiently active protein. An overview of the screening results is presented in the *table* 4.10.

Table 4.10: Different approaches were tested in order to refold active HisLecEGF. Refolding set ups are described in material and methods section. Percentage of the active HisLecEGF is calculated as a fraction of total protein subjected to refolding.

Refolding method	Fast dilution	Tag removal / dialysis	Dialysis/ tag removal	Fast dilution/ concentration/ tag removal	Fast dilution/ concentration/ dialysis/ tag removal
% active protein	**1.08**	**Precipitation**	**0.27**	**3.68-0.17**	**0.01%**

4.3.3.2 Screening of refolding conditions

Asymmetrical Field-Flow Fractionation (AfFF) screening was used to find the best buffer composition preventing protein precipitation and promoting correct protein folding prior to oxidation. Light scattering and UV detection were combined to screen for, and distinguish between folded protein structures or protein aggregates. Among 95 buffer conditions examined, 5 were chosen for scale-up, and *figure* 4.34 shows the best screen result. In these conditions there was neither observable protein precipitation nor aggregation as confirmed by analytical RP-HPLC. After the addition of redox reagents (GSH, GSSG), however, quantitative precipitation occurred, except in solution with buffer 20b, which contained 0.05% n-octlyglucoside in 0.5M Tris, pH 8.0. Unfortunately, a gel filtration run performed on a concentrated sample after refolding and oxidation in this buffer did not yield any protein whatsoever. In a repeated experiment, the concentration step was monitored by RP-HPLC and revealed protein precipitation on the membrane of the concentrating device as soon as a concentration factor of 5 was achieved (volume reduced 5 times), with only about 7% of the protein remaining in solution. Activity of this fraction was tested with the blocking antibody but only 0.3% of the total protein was active.

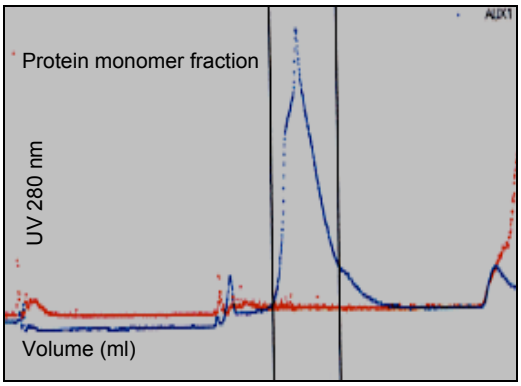
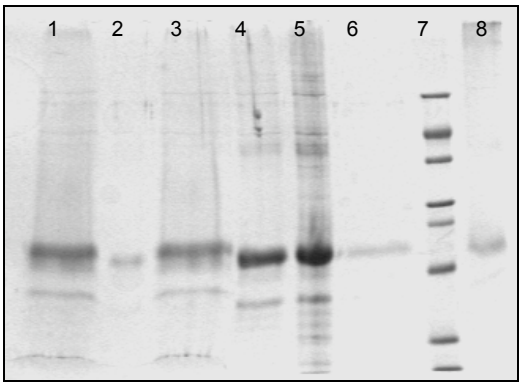


Figure 4.34: AfFF report in the screen for the optimal folding conditions. Light scattering and absorption at 280 nm showed protein behavior and the soluble fraction amount in each buffer.

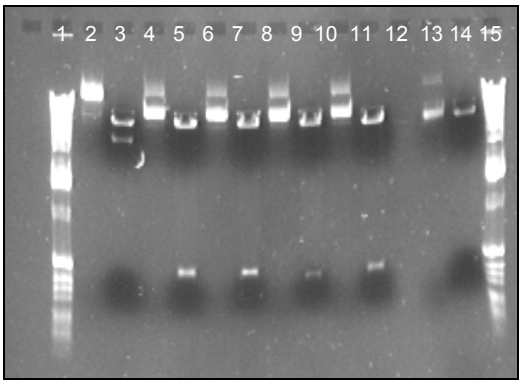


Lane 1: Set up 13c, suspension
Lane 2: Set up 13c, pellet
Lane 3: Set up 13c, supernatant
Lane 4: Set up 20b, suspension
Lane 5: Set up 20b, supernatant
Lane 6: Set up 20b, pellet
Lane 7: Low MW standard
Lane 8: Set up 20b, non-reduced

Figure 4.35: SDS-PAGE control of protein folding in different buffers as result of the AfFF screen. Different fractions were analyzed to address the status of the protein in different refolding solutions.

4.3.4 LecEGF cloning into pET11c

Plasmid vector pET11c directs expression of recombinant proteins in the bacterial cytoplasm w/o any tag. This is an advantage of the system, especially for complex protein folding, as folding of HisLecEGF was. Protein expression is IPTG-inducible and the vector is compatible with DE3 *E.coli* production strains. PCR amplification of LecEGF introduced *Nde*I and *BamH*I restriction sites on the 5' and 3' DNA end, respectively. Ligation and subsequent electroporation into *E.coli* DHα5 were also successful, as controlled by restriction enzyme digestion (*figure* 4.36). 4 clones have been sequenced, and 3 sequences were correct. A clone named LE2 was chosen for further experiments.

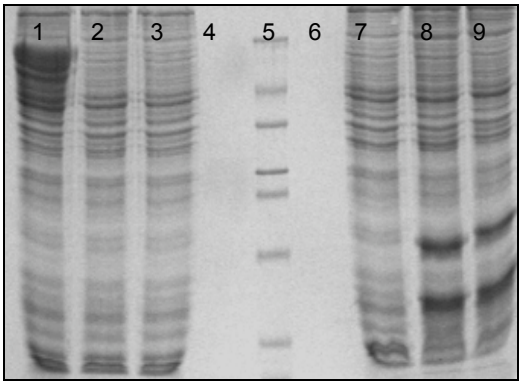


Lanes 1, 15: DNA marker X
Lanes 2, 4, 6, 8, 10, 13:
Undigested pET11cLecEGF
Lanes: 3, 5, 7, 9, 11, 14:
pET11cLecEGF digested with *Nde*I*, BamH*I

Figure 4.36: Restriction enzyme digestion of the pET11cLecEGF, with the undigested DNA of 6153 bps. and LecEGF of 478 bps.

4.3.4.1 Protein expression analysis, purification and refolding

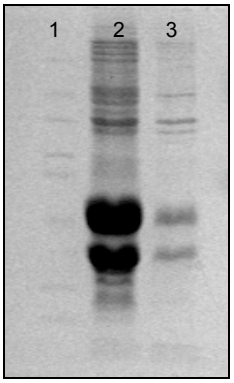
E.coli BL 21 (DE3), AD 494 and Rosetta gami were chemically transformed with the DNA LE2. A protein running at expected molecular weight of 18 kDa was overexpressed together with another shorter species, both however exclusively in inclusion bodies.



Lane 1: BL 21, pET11c induction 0
Lane 2: BL 21, pET11cLecEGF 2, induction 0
Lane 3: BL 21, pET11cLecEGF 8, induction 0
Lane 4: -
Lane 5: Low MW standard
Lane 6: -
Lane 7: BL 21, pET11c 10h post induction
Lane 8: BL 21, pET11cLecEGF 2, 10h post induction
Lane 9: BL 21, pET11cLecEGF 8, 10h post induction

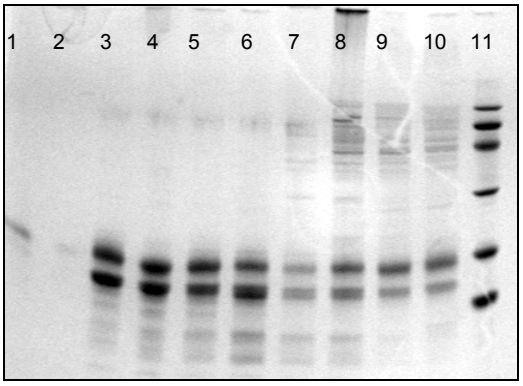
Figure 4.37: SDS-PAGE analysis of protein expression in BL 21 (DE3).

Further protein characterization was done by MS analysis of in-gel digests of both lower and higher molecular weight overexpressed proteins. This revealed that the shorter protein lacked the C-terminal last 30 amino acids, whereas the sequence of the higher molecular weight protein was correct. On analytical RP-HPLC, it was not possible to really separate these two protein species. A first purification attempt was performed by anion exchange chromatography in 8M urea. *Figure* 4.38 shows the isolated crude extract fraction, where full-length protein was the predominant species.



Lane 1: LMW standard
Lane 2: Fraction soluble in 8M urea
Lane 3: Insoluble pellet

Figure 4.38: Inclusion bodies fraction solubilized in 8M urea. 15% SDS-PAGE.



Lane 1: Fr. 1, RP-HPLC
Lanes 2-10: Fr. 2-10 RP-HPLC
Lane 11: MW standard

Figure 4.39: LecEGF purification on preparative RP-HPLC.

Since protein purification and separation of the two LecEGF species by anion exchange was not successful, preparative RP-HPLC was performed. As shown in *figure* 4.39, this method was partially successful but the intact species was present only in fraction 1, whereas fractions 3-6, containing both, were pooled together. Both pools were lyophilized.

Refolding was tried in parallel with both samples and there was no visible precipitation or aggregate formation as monitored by analytical RP-HPLC. Upon oxidation, a peak shift to shorter retention time was observed, a good indication of successful disulfide formation. However, the activity of the recovered protein was only 2% as compared to a positive control. The RP-HPLC conditions were optimized in an attempt to improve the separation of the two protein species prior to the any refolding trial, but this was not possible. Since the yields and the activity were very low, the strategy was abandoned.

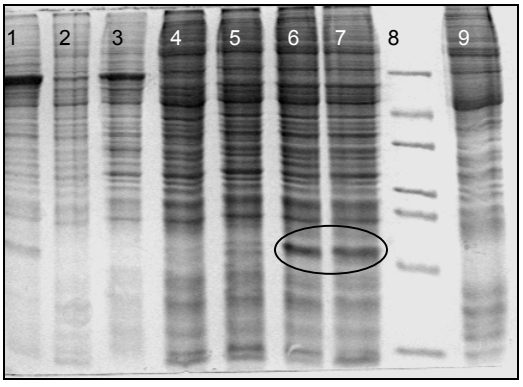
4.4 Expression of lectin and EGF-like domains of human E-selectin in baculovirus-infected insect cells

Baculovirus-infected insect cells have been chosen for LecEGF expression as a eukaryotic expression system that facilitates correct protein folding and disulfide bond formation. The system is potentially effective on time- and cost-scale for the preparation of the amounts of protein necessary for structural studies.

4.4.1 LecEGF cloning and expression into pFastBac: intracellular expression of a His-tagged protein

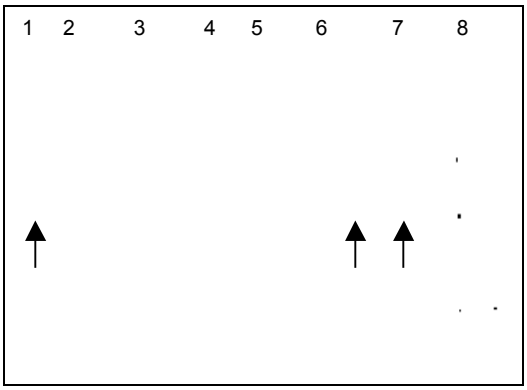
DNA coding for LecEGF (1-157) domain was successfully PCR amplified and PCR integrated in the plasmid pBacPak. DNA sequence was amplified with primers that enabled PCR intergration into the pFastBac cloning vector and a construct His-Prescission-LecEGF (pFastBacHPLE) was generated. Positive clones were screened by PCR and DNA sequenced. *E.coli* DH10Bac was transformed with the generated pFastBacHPLE and, after 48 h growth and selection, 10 colonies were further analyzed. A 2850 bps fragment was amplified by PCR with M13 standard primers. This corresponds to the size of bacmid DNA in between these two primers and the size of His-Prescission-LecEGF (HPLE).

Sf9 insect cells were successfully transfected with the transposed bacmid DNA DH10BacHPLE. A first preliminary screen for protein expression has revealed the presence of a 20.1 kDa protein expressed in the cells and reacting positive with anti-His antibodies, as shown in *figure* 4.41.



Lane 1: Transf. 1
Lane 2: P1 ampl.
Lane 3: +ctrl., His tagged protein
Lane 4: TL 42h p.i.
Lane 5: TL 54h p.i.
Lane 6: TL 66h p.i.
Lane 7: TL 72h p.i.
Lane 8.: LMW marker
Lane 9.: -ctrl., non-transf. *Sf*9 cells

Figure 4.40: HPLE expression kinetic analysis in *Sf9* insect cells. Overexpressed HPLE is designated.



Lane 1: Transfection1
Lane 2: P1 ampl.
Lane 3: +ctrl.
Lane 4: Total cell lysate 42h p.i.
Lane 5: Total cell lysate 54h p.i.
Lane 6: Total cell lysate 66h p.i.
Lane 7: Total cell lysate 72h p.i.
Lane 8: LMW marker

Figure 4.41: HPLE anti-his WB during expression kinetic analysis samples. Positive signals indicated by arrows appear only 66 and 72h p.i. at MW of 20 kDa.

In parallel to the protein expression optimization, a plaque assay was performed and a single viral clone isolated, purified and titer determined. The P2 viral titer that was used in further experiments was 0.6 pfu/ml.

4.4.1.1 LecEGF purification and analysis

Since LecEGF had an N-terminal His tag, and was expressed intracellularly, *Sf*9 insect cells were lysed under mild native conditions and that extract was purified over a Ni-NTA column. Fractions eluted with either an imidazole gradient or isocraticly at 500 mM imidazole in native conditions contained a variety of proteins. Under a variety of different conditions tested the elution profile seemed to be rather unspecific. Western blot analysis of cell lysate with anti-His antibodies was negative in both native and denaturing conditions, whereas it was positive for the rest of insoluble cell material tested under denaturing conditions. In agreement, western blot analysis with mAb 7A9 was also negative. To improve expression and production conditions different parameters were optimized, but the results were unchanged. *Table* 4.11 shows a summary of the optimization parameters tested to express and produce active LecEGF.[ii]

Table 4.11: Different parameters known to influence protein expression and production in insect cells were tested to produce functional and active LecEGF, i.e. His-Prescission-LecEGF (HPLE).

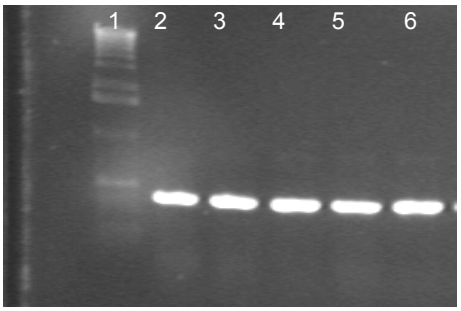
Viral Data	Cell line	Infection conditions:	M.O.I	Time course/ Temperature	Protein isolation methods:	Protein isolation methods:
$4.6x10^7$ pfu/ml	-*Sf9* suspension culture -*Sf21* -*High five*	-$1x10^6$ cells/ml -$1.2X10^6$ cells/ml	-1.0 -0.6 -0.06	-72h p.i -39-84h p.i. // -28°C -21°C	-Cell lysis -Sonication -Ultracentrifuge	Cell lysis+ Detergents -0.1%NP-40 -0.5%NP-40 -0.1% Triton x-100 -1% NP-40

4.4.2 LecEF cloning and expression in baculovirus infected insect cells: protein secretion into the medium

In order to express the LecEGF domain of E-selectin as a secreted form, the signal sequence of endogenous insect acidic glycoprotein 67 was fused to the N-terminus of LecEGF to direct its secretion into the medium. For analytical and purification purposes a Flag-tag was introduced on the C-terminal of the EGF-like domain.

4.4.2.1 LecEGFFlag cloning and expression

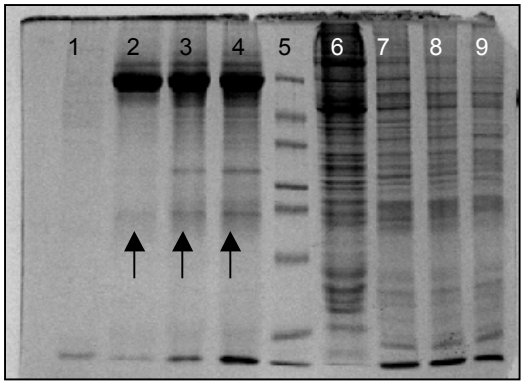
The LecEGF sequence was successfully PCR amplified with 5' and 3' overhangs enabling PCR integration into vector pAcGP67a. The purified mixture was electroporated into *E.coli* XL-1 Blue electro competent cells. Grown colonies were screened by PCR and positive ones have been sequenced. DNA coding for LecEGF with signal sequence and Flag epitope (SSLecEGFFlag) was subcloned into the vector pFastBac, as described above. The colony PCR screening procedure was as described, using primers that anneal to the vector sequence (3' MG 593) and FWZZLE that starts at bp 152 of the LecEGF sequence. 6 positive clones were isolated and sequenced, and *Figure* 4.42 shows a screen result. 5 colonies were sequenced, 4 were positive and DNA of clone 2 was taken for further cloning into *E.coli* DH10Bac and transposition reaction to generate bacmid DNA containing SSLecEGFFlag.



Lane 1: DNA marker X
Lanes 2-6: PCR product, screen of colonies1-5.

Figure 4.42: Colony PCR screens with the primers FWZZLE and MG 593 amplified a fragment of the expected 479 bps.

Bacmid DNA was isolated as described in materials and methods and those with the highest purity and concentration were chosen for the transfection experiments. After initial infection of *Sf*9 insect cells, the virus was purified by plaque assay and amplified. Titer of the virus to be used in further infections was determined. LecEGFFlag production and secretion in the medium was followed on small scale for 96 hours by SDS-PAGE and Western blot with anti-Flag antibodies. As shown in the *figure* 4.43, LecEGFFlag secretion in the medium started 48 h post-infection (p.i.), whereas maximal protein secretion was achieved at 72 and 96 hours p.i. There were still some amounts of unsecreted and unprocessed LecEGFFlag in the cells but this protein fraction was not further analyzed.



Lane 1: -ctrl: Medium of the non-infected *Sf*9 cells
Lane 2: Conditioned medium of infected Sf9 cells, 48h post-infection (p.i.)
Lane 3: Conditioned medium of infected Sf9 cells, 72h (p.i.)
Lane 4: Conditioned medium of infected Sf9 cells, 96h (p.i.)
Lane 5: LMW marker
Lane 6: -ctrl: Pellet of the non-infected *Sf*9 cells
Lane 7: Pellet of the infected *Sf*9 cells, 48 h p.i.
Lane 8: Pellet of the infected *Sf*9 cells, 72 h p.i.
Lane 9: Pellet of the infected *Sf*9 cells, 96 h p.i.

Figure 4.43: Time-course analysis of the LecEGFF expression. Arrows indicate LecEGFF secreted in the medium.

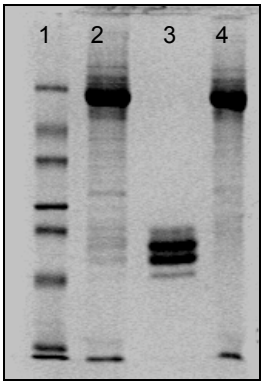
In order to improve yields of the secreted LecEGFFlag, apart from a time-course optimization, the influence of M.O.I. and an alternative cell line (*Trichopulsia Ni,* referred to as *Hi5*™) were optimized as well. *Table* 4.12 shows the best protein production conditions.

Table 4.12: Optimized conditions for LecEGFF protein production. Parameters that influence culture conditions and rotein production have been subjected to extensive optimization.

Cell line	Medium	Viral titer	No. cells	M.O.I.	Expression time	Temp.
Sf 9 *H5* √	Sf 900 No serum No antibiotic	9.6 x10^7	1x10^6	3-10 10 √	48-96h 72h √	28°C

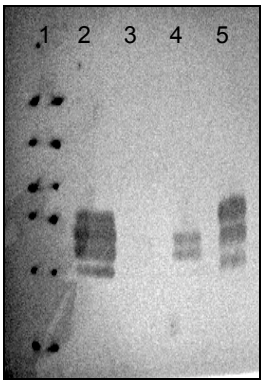
4.4.2.2 LecEGFFlag purification and characterization

LecEGFFlag was purified using a commercial M2 monoclonal anti-FLAG antibody resin, as described in materials and methods. Eluted under mild conditions with competing flag peptide, the protein was purified to homogeneity in a single step as judged by SDS-PAGE and shown in *figure* 4.44. A Western blot analysis of eluted protein, as well as fractions of flow through and conditioned medium with the anti-Flag monoclonal antibody M2 is shown in *figure* 4.45. According to the LecEGFF migration profile on reducing SDS-PAGE, the protein shows an apparent molecular weight of 20-24 kDa.



Lane 1: LMW marker
Lane 2: Crude extract, *Sf* 9 cells conditioned medium
Lane 3: Elution
Lane 4: Flow through

Figure 4.44: LecEGFF purification profile over the Anti-flag affinity chromatography.

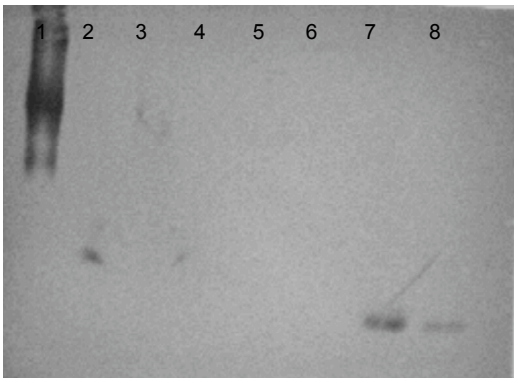


Lane 1: LMW marker
Lane 2: Elution
Lane 3: Flow through
Lane 4: Crude extract, *Sf* 9 cells conditioned medium
Lane 5: Cell pellet

Figure 4.45: Western blot analysis of Anti-flag chromatography fractions.

Activity of the LecEGFFlag secreted into the medium was addressed in a native western blot with the mAb 7A9. The recombinant protein gave positive signals in this reaction, indicating that

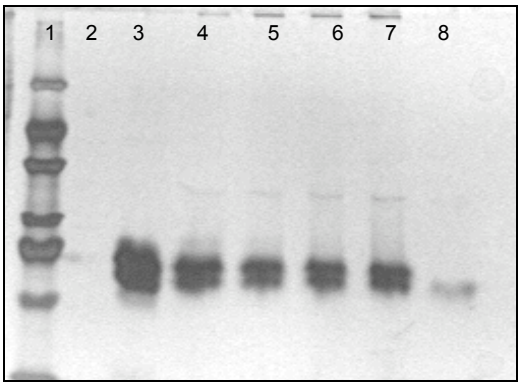
the LecEGF of E-selectin was correctly folded upon expression (*figure* 4.46). Western blot analysis with the M2 Anti-flag antibodies in native conditions was also positive; indicating that the C-terminal end of the EGF domain is free in the space, and accessible to the anti-flag antibodies.



Lane 1: E-selectin/IgG
Lane 2: -
Lane 3: P-selectin/IgG
Lane 4.: -
Lane 5.: L-selectin/IgG
Lane 6.: -
Lane 7.: LecEGFFlag
Lane 8.: LecEGFFlag deglycosylated

Figure 4.46: Western blot analysis under native conditions with anti-selectin blocking antibodies 7A9. Proteins were separated on 5-15% gradient native gel to analyze both E-selectin/IgG and LecEGFFlag.

Further protein characterization addressed the aggregation state of the expressed (exported) protein in solution. Since the migration profile on SDS-PAGE in non-reducing and native conditions was the same as in reducing conditions, it could be concluded that the protein is monomeric. The glycosylation status of LecEGFF was analyzed by treatment with PNGaseF under both native and denatured conditions. While deglycosylation of denatured LecEGFF completely removed *N*-glycans (as judged by SDS-PAGE), in native conditions there was a shift in migration profile but two bands were still present in the sample pointing to two predominant glycosylated forms. *Figure* 4.47 shows *N*-deglycosylation in denaturing conditions.



Lane 1: LMW standard
Lane 2: -
Lane 3: LecEGFFlag glycosylated
Lane 4: *N*-deglycosylation, 15 min.
Lane 5: *N*-deglycosylation, 30 min.
Lane 6: *N*-deglycosylation, 45 min.
Lane 7: *N*-deglycosylation, 60 min.
Lane 8: *N*-deglycosylation, 48 h.

Figure 4.47: Time-course of *N*-deglycosylation of LecEGFFlag with PNGaseF. Protein was denatured and reduced prior to the deglycosylation that was complete after 48 hours, when a single protein band was detected.

In order to control the deglycosylation extent, glycosylated and deglycosylated LecEGFFlag were stained with Schiff reagent since only proteins with bound glycans give a positive reaction. Since deglycosylated LecEGFFlag did not react with the Schiff reagent, we concluded that all glycans had been removed from the protein and that deglycosylation was complete.

4.4.2.3 Mass spectrometry analysis and NMR analysis of LecEGFFlag

Molecular weight of the expressed LecEGFFlag was determined by LC/MS. Prior to the MS analysis protein was desalted on an analytical RP-HPLC Vydac C_4 column, with the concomitant separation of the two differentially glycosylated protein species. The first eluted fraction was the one with higher molecular weight (with higher sugar content and therefore more hydrophilic). Molecular weight determination has shown heterogeneity of these isoforms, with a predominant mass at 21151.87 Da and 19958.57 Da, respectively.

NMR analysis of the binding activity of LecEGFFlag was done in the presence of an antagonistic sLe^x tetrasachharide mimetic **BW69669** that has 12 times higher affinity as compared to the carbohydrate epitope sLe^x. STD NMR and T1 rho relaxation time were measured in the presence of protein and significant change in STD NMR was observed, as shown in *figure* 4.48.

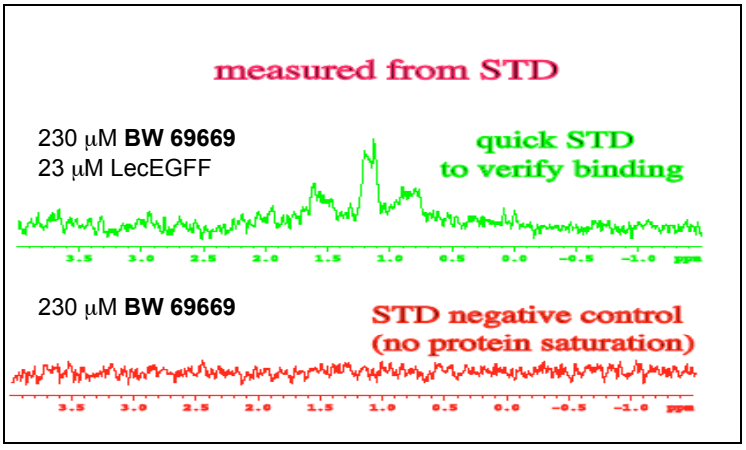


Figure 4.48: STD of **BW69669** in presence and absence of LecEGFFlag indicates ligand binding and confirms protein activity.

4.4.3 Bioassay development

In order to evaluate potential E-selectin antagonists, a bioassay was developed using the recombinant LecEGFFlag as a target molecule. Biotinylated polyacrylamide-sLe^a polymer (PAA-sLe^a) coupled to streptavidin peroxidase, should bind to LecEGFFlag. The intensity of the colored reaction using the chromogenic substrate ABTS, can quantitatively describe PAA-sLe^a

binding to the LecEGFFlag. A preliminary screen was done to establish the best assay conditions with different protein and polymer concentrations, as shown in *figure* 4.49. Having established the best protein concentrations, the affinity for sLe^a-polymer was assayed, as shown in *figure* 4.50.

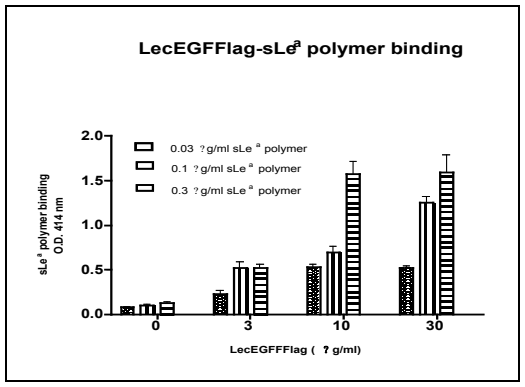


Figure 4.49: Optimization of LecEGFFlag and sLe^a-polymer concentration for bioassay. The standard optimal conditions were chosen in respect to the signal to background ratio.

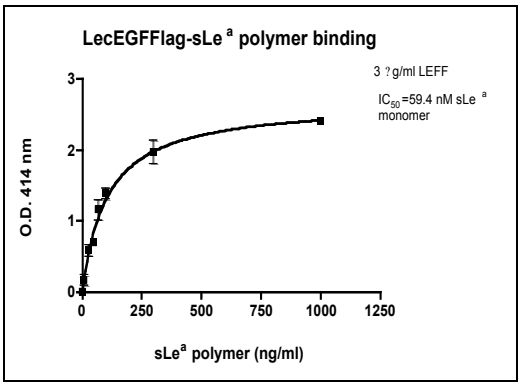


Figure 4.50: Determination of IC_{50} value for sLe^a polymer toward LecEGFFlag, as a measure of binding affinity.

The optimal protein and polymer concentration to give stable signal were chosen as 3 μg/ml LecEGFFlag and 100 ng/ml polymer, because these had at least 5x higher O.D. values as compared to background, what is considered as stable signals. All the subsequent tests were done under these conditions.

IC_{50} of LecEGFFlag toward the sLe^a polymer was determined as 274.5-356.5 ng/ml, or 54-62 nM. All IC_{50}, shown in *table* 4.8 were calculated as relative to this value. Measurements were done in triplicates or quadruplicates of three independent experiments and were overall reproducible. 3 different E-selectin inhibitors, as well as negative control (Fucα1-3, Glc*N*Ac) were tested in this assay, and the results obtained are presented on *figure* 4.52 and in *table* 4.13.

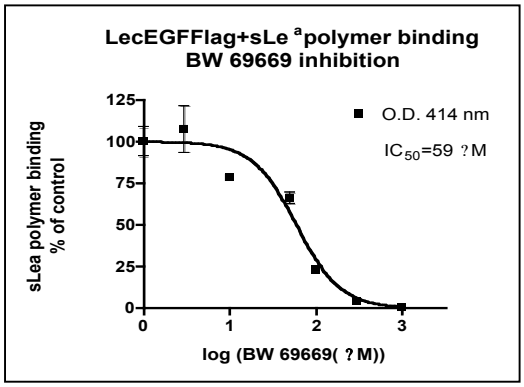


FIgure 4.51: Concentration dependence curve of BBW 69669. Data presented are means of three independent experiments done in triplicates.

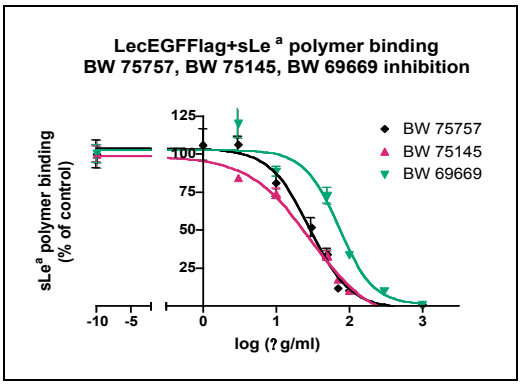


Figure 4.52: Superimposed concentration dependence curves of E-selectin antagonists tested in a bioassay. Data present three independent experiments done in triplicates.

Table 4.13: E-selectin antagonists, sLe^x mimetics analyzed in a molecule-molecule assay with LecEGFFlag and sLe^a polymer. Calculated IC_{50} values are relative to sLe^a polymer as control.

Antagonist	IC_{50} (μM)	Signal/ Bckg.	Signal/ Noise	Ctrl±SD	Bckg±SD
BW69669	63±6.4	10-13	11-20	1.06±0.19	0.06±0.028
BW75757	28±3	12-15	18-22	1.14±0.12	0078±0.03
BW75145	29.3±1	10-16	15-17	1.38±0.2	0.082±0.02
sLe^a-polymer	K_d=63.1±8.2 nM				0.075±0.01

In contrast to cell-free assay, cell-based assay with HL-60 cells showed very weak binding of LecEGFFlag compared to E-selectin/IgG, even as a control. Different incubation times and temperature were tested, but a stable control signal was not obtained.

5 Discussion

5.1 E-, P-, L-selectin/IgG expression, purification and characterization in CHO cells

For the efficient production of recombinant proteins, a variety of expression systems are available. Depending on the nature of the protein of interest and the amounts of protein necessary to conduct the desired studies, the most appropriate expression system is to be chosen. Limiting factors may be, however, the nature of the protein and its complex structure, folding pattern, or post-translational modifications. In this case the protein should be expressed in an environment as similar as possible to its natural counterpart. The expression conditions have additionally to be optimized to get optimal expression levels versus time- and cost-scale. Since their discovery in the late 80-es, recombinant human selectins were expressed on analytical and preparative scale for both functional and structural studies. A large body of evidence has defined chinese hamster ovary (nomenclatured in the text as CHO cells) and green African monkey cells as a mammalian systems optimal for expression of recombinant selectins [92,97,99,252-254]. Selectins are transmembrane proteins with extracellular domains (Lectin, EGF-like and consensus repeats) involved in ligand binding. Lectin and EGF-like domains contain 5 disulfide bridges and the whole molecule has eleven potential N-glycosylation sites. These data imply complex post-translational modifications and the necessity for the protein to pass through the Golgi and the oxidative environment of the endoplasmic reticulum for correct protein folding, disulfide-bond formation and N-glycosylation. Since glycosylation itself is not a pre-requisite for the activity of selectins [151], differences in glycosylation profiles of hamster, monkeys and humans do not influence protein activity. Other post translational modifications have not been thoroughly investigated for selectins so far, except for phosphorylation, which takes place only intracellularly and is implied in signal transduction.

Although one of the advantages of using cells such as CHO for expression is the possibility to closely mimic glycosylation patterns found in humans, over the production process duration, the degree of glycosylation can vary (several glycans, one glycan or no glycans bound to the peptide-backbone).

In different expression strategies, recombinant selectins have been mostly expressed as fused to the human/mouse Fc part of immunoglobulin [92,240,253,254]. There are many arguments

for this approach that works not only for selectins, but also for many different extracellular protein-domains expressed in this fashion. The production of receptor-immunoglobulin constant region chimera enables good secretion and easier purification using very simple protein A purification protocols. The ability of these molecules to dimerize is expected to add to the avidity of the interactions between the receptor and its ligands. First designed for CD4 immunoadhesins for AIDS therapy, the human heavy chain IgG1 constant region cassette was thoroughly characterized [255]. Subsequently, the murine homing receptor, a non-immunoglobulin superfamily member, was successfully produced exploiting this strategy [86]. It was demonstrated that the joining of molecules near the hinge region resulted in chimeric molecules that were both efficiently synthesized and dimerized in the absence of any light chain production. These molecules might find the utility as the modulators of inflammatory proceses and may be exploited as affinity reagent to isolate endothelial and cell ligands. A novel antibody-like form of human selectins was shown to be very suitable to bioassay development as well, since it can easily be coupled to any affinity or coating support [252]. Throughout a number of different selectin studies, the originally described expression strategy was modified in such a way that recombinant selectins containing different extracellular parts were not secreted into the medium, but expressed on the cell surface via fusion to CD16 (component of low affinity Fc receptor, FcgRIII), which contains a signal sequence for cell surface anchorage via glycosylphosphatidylinositol (GPI) linkeage [256]. In addition, recombinant proteins containing only selectin domains have been expressed in secreted form, [91], [151], as fused to the ZZ domain of protein A and either secreted or expressed on the cell surface [257]. Extracellular expression and secretion of selectins in the medium was usually governed by the native signal sequence, which, in the absence of a membrane anchor, led to production of soluble selectins. This has enabled the measurement of binding both in solution and on solid surfaces preventing any artifacts introduced by either low or high levels of expression on the cell surface.

E-, P- and L-selectin/IgG described in this study were constructed as IgG-fusion chimeras (*figure* 5.1), where the lectin (carbohydrate recognition domain-CRD), the EGF-like domain, 6 consensus repeat domains each for E-, and P-selectin and 2 for L-selectin were fused to the hinge region of human IgG1 immediately after the last consensus repeat domain. The endogenous signal sequence of each of the selectins directed protein expression into the medium. Amino acids 1-155 for E-, and L-selectin, and 1-157 for P-selectin, constitute the lectin and EGF-like domains, whereas boundaries of expressed consensus repeats were taken as amino acids 535 and 332 for E-and L-selectin respectively, and amino acids 540 for P-selectin. The resulting PCR products were spliced in a PCR-amplified product consisting of a few bases

from the 3' end of the CH1 domain and the complete hinge region and CH2 and CH3 domains of genomic human IgG1. The resulting chimeric selectin/IgG cDNAs were introduced at the *Not*I restriction site of the expression vector pcDNA/Neo. Chinese hamster ovary cells were transfected with the isolated pcDNA/neo and positive, producing transformants were selected in gentamicin (G-418) containing medium. Production conditions were set up based on the general rules for protein production in CHO cells [225,258] considering seeding density and cell life- and production-cycles. In order to enhance the expression of recombinant protein, selectins DNA sequences were placed behind the potent SV-40 promoter in frame with the neo gene for gentamicin resistance and cells were cultivated in medium with high G-418 content (0.5 mg/ml). Analysis done on a small scale compared protein production of adherent cultures grown in culture flasks and in roller bottles. While the growth phase was rather similar for both cultivating systems, the production in roller bottles resulted in higher protein yields, as is presented in *table* 4.1. The production time was prolonged in this system, which provides for better aeration and medium circulation. Yields of 7.3 mg/L medium, based on protein A chromatography, have far exceeded published results and represent a significant improvement over the initial 2 mg/L protein produced in culture flasks. All productions were done on a laboratory small scale and generated sufficient protein for analytical characterization, structural studies and bioassay development. Protein expression analysis revealed that expressed selectins were exported, soluble IgG recombinants. Purification of recombinant selectins was done on protein A resins, exploiting the affinity between the constant region of immunoglobulins and staphylococcal protein A. The dissociation constant for this interaction has been determined to be $2x10^{-8}$M [260]. Although the Fc part of human IgG1 preferentially binds to protein A, some bovine IgG could be detected in eluted fractions. These data are rather consistent with published results [92], because the binding specificity of protein A toward human Fc/IgG is not high enough to completely eliminate binding of other IgGs present in the medium. Although 5% fetal calf serum used in cell medium was found to be on optimal concentration, this rather high serum percentage led to contaminations of bovine IgGs in purified fractions. As a polishing step, size exclusion chromatography was done using either a small 15 ml Sephadex column for analytical, or a 200 ml Superose column for preparative applications. E- and P-selectin/IgG were both successfully separated from the rest of contaminating proteins by size exclusion. The chromatographic profile of the two columns has shown differences and has raised questions on the oligomerization status of E-selectin as will be discussed later. The molecular weight of the purified proteins was calculated in reduced and denatured conditions, based on the protein migration profile on 8% SDS-PAGE. As indicated in results, *figure* 4.4, E-selectin/IgG migrates

at about 148 kDa, whereas P-and L-selectin/IgG migrate at 110 and 87 kDa, respectively. Based on protein sequence the molecular weights of monomer E-. P-and L-selectin/IgG constructs were calculated to be 83.37 kDa, 85 kDa, and 56.84 kDa, respectively. The higher apparent molecular weight of the proteins in SDS-PAGE analysis indicates a high glycosylation extent of all of three selectins. Non-reducing SDS-PAGE indicated high molecular weight proteins exceeding the calibration range of the standards. Although the best method to address the molecular weight of the soluble proteins in native conditions would be mass spectrometry, the high molecular weight of recombinant selectins was well outside the range for MS analysis [261]. In native conditions, the molecular weight was examined by size exclusion chromatography and native PAGE. Based on the molecular organization of Fc-IgG chimeras, the theoretical molecular weight of each IgG chimera in native conditions is expected to be the double of what is found in reducing and denaturing conditions, namely 296 kDa, 220 kDa, and 174 kDa for E-, P-, and L-selectn/IgG respectively. When chromatographed on a 15 ml size exclusion Sephadex column and compared with standard proteins of known molecular weight, E-selectin/Ig was eluted between the peaks corresponding to protein aggregates and thyroglobulin (670 kDa), indicating a molecular weight of 840 kDa. These data would point to an oligomerization of E-selectin/IgG in solution in native conditions (or an oligomerization that takes place on the size exclusion matrix). Similar observations have been reported in some of the previous E-selectin characterization studies , however not for the E-selectin/IgG chimera but for a construct consisting of the lectin domain, EGF-like and 6 consensus repeat domains. In this study the apparent molecular weight of this soluble E-selectin was 310 kDa, whereas in reducing conditions on SDS-PAGE the protein migrated at 80 kDa. Further analyzes of this construct with equilibrium analytical ultracentrifugation have revealed that soluble E-selectin was an elongated monomer and that its abnormal behavior on size exclusion chromatography was an artifact, probably caused by the protein non-globular structure and its interaction with the column material. We tried to address the nature of this complex oligomerization that were formed by reproducing the same size exclusion conditions, except for the running buffers, which were either buffer with 8 M urea or phosphate buffer with and without calcium. The chromatographic pattern was unchanged in 8 M urea, pointing to covalent interactions that were calcium independent. The 200 ml size exclusion chromatography was done without a calibration with standard proteins. Here E- and P-selectin/IgG eluted in front of contaminating IgG molecules, consistent with the protein molecular weight. The size of this column yields to higher resolution and is suitable for preparative scale separations. However, for several reasons, size exclusion was abandon as an appropriate method to determine the molecular weight of the

proteins. IgG fusion proteins do not have globular shape and additionally protein interactions with the chromatographic matrix can cause retention and influence the elution profile. As already described [262], high protein glycosylation can also enhance the interaction with the chromatographic matrix. For these reasons we decided to address the question of the molecular weight of selectin/IgG chimeras in native state by native PAGE. The results obtained indicated a complex oligomerization state of E-selectin/IgG, whereas the molecular weights of P-, and L-selectin/IgG were in agreement with what had been observed for reduced and denatured proteins. In native PAGE proteins migrate not only depending on their molecular weight but also on their shape and overall charge. To avoid misinterpretation based on these factors, proteins were run on different percentage gels and a constant was generated, which described the migration profile of each of the tested proteins [247]. A similar approach for characterization of different E-selectin constructs was described recently [94], where the predominant part of the E-selectin/IgG fraction was reported to be 300 kDa and a 5% protein fraction a dimer thereof. In our case the protein migrates homogenously as an 840 kDa protein. However, this approach has not been really validated and the results are questionable. It is also possible that in equilibrium in solution, the protein exists in different forms: as a cluster but also as a dimer. For ligand binding studies, the characterization of the protein part that carries the ligand binding site was of higher importance. Based on the E-selectin/IgG construct used, the monomeric molecule has 1 lectin domain and therefore 1 ligand binding site. In case of P- and L-selectin/IgG there was no indication for protein oligomerization, which is consistent with published data [125,263]. Selectin dimerization or oligomerization happens *in vivo* and *in vitro* and enhances ligand binding, but this is mediated exclusively through the transmembrane and cytoplasmic part of the protein [264,265]. The above mentioned study done by Green *et al*. [94], has analyzed also E-selectin constructs made with lectin and EGF-like domain fused to the Fc part of IgG, as well as lectin and EGF-like domain alone. Only constructs that contained 6 consensus repeats fused to the Fc-IgG formed oligomers in solution. One could presume therefore that, in case of E-selectin/IgG, consensus repeats contribute to the oligomerization. However, partially contradictory are studies where LecEGF with 6 consensus repeats did not oligomerize but was found to have a rod-like shape of an asymmetric monomer. Taken together, in the case of E-selectin/IgG consisting of lectin, EGF-like domain and 6 consensus repeat domains, the consensus repeats and the Fc part of IgG could promote molecular clustering and oligomer formation. High increase in molecular weight as compared to what is expected for the protein sequence is consistent with the fact that E-, P-, and L-selectin have 11, 7 and 8 potential N-linked glycosylation sites in their extracellular domains, respectively. As already mentioned,

many different recombinant selectins have been produced in animal cells, predominantly CHO and COS cells, but the exact glycosylation profile of these proteins has not been described so far [266]. CHO cells can produce complex and hybrid *N*-linked structures. These can be incompletely processed and are termed high mannose carbohydrates, or partially processed and then substituted with Glc*N*Ac (± Gal ± Sia) and called hybrid moieties. Molecules that are completely processed to the core sugar sequence and subsequently elongated to contain Glc*N*Ac (±Gal ± Sia) at both the Man α (1,3) and the Man α (1,6) sugars are termed complex moieties. Different studies have shown that in animal cells the majority of glycoproteins posses high mannose- or complex-type asparagine-linked oligosaccharides [7,267]. To characterize the high molecular weight, we have addressed the glycosylation profile of recombinant E- and P-selectin/IgG by enzymatic cleavage with different glycanases, including PNGaseF, PNGaseA and *O*-glycanase [268]. Other lines of evidence showed that the treatment of the extracellular part of the protein with PNGaseF removed 78% of all posttranslational modifications, whereas in our experiments 62.5% of carbohydrates were removed in an extensive, denaturing (up to 48 hours) treatment with PNGaseF, section results, *figure* 4.5 [5]. Similar results were obtained in *N*-deglycosylation of E-selectin/IgG for NMR studies [269].

In case of P-selectin, and L-selectin/IgG, *N*-deglycosylation decreased the molecular weight by about 65%, and 45% respectively. *O*-linked glycosylation or ubiquitination were proposed as post-translational modifications on E-selectin in the early work of Bevilacqua. However, in our experiments protein treatment with *O*-glycanase did not noticeable affect the molecular weight. Interestingly, the crystal structure determination [151] of E- and P-selectins' lectin and EGF-like domain, in complex with sLe^x [188], has confirmed that complete *N*-deglycosylation does not affect ligand binding nor the protein capability to block neutrophile adhesion to the endothelium. These data would indicate that, at least in lectin und EGF-like domains, there are no other posttranslational modifications apart from *N*-glycosylation, which can be successfully removed. A brief overview of the glycosylation extent of E-selectin produced in animal cells has been given by Li *et al*. [90]. In this work it was demonstrated that glycosylation within the lectin and EGF-like domains increases the molecular weight predicted from protein sequence by 8 kDa, in the first two consensus repeats by another 26 kDa, and in the last four consensus repeats by 41 kDa, or 13 and 15 kDa per consensus repeat. Deglycosylation in native conditions was performed to address the contribution of sugars on protein activity. In native gel, there was a clear difference in the migration profile after extensive (48 h) deglycosylation. Consistent with published data, the protein binding site was still functional, as judged by positive binding of a blocking monoclonal antibody, described below.

Throughout the work with E-selectin, a monoclonal blocking antibody (7A9, mouse IgG1 subtype) was used to address protein activity. This antibody has been raised against IL-1 stimulated HUVEC, expressing E-selectin [270]. X-ray structure of the antibody has revealed a groove in the binding site, where probably E-selectin binds to the antibody. The lectin domain loop between β-strands β4 and β5 with exposed Tyr-94 and Arg-97 side chains, known to be involved in ligand binding, interact with the antibody, which is consistent with its blocking function [249]. This antibody shows blocking activity in vitro, blocking protein adhesion to the HL-60 cells, and inhibiting granulocytes adhesion to the stimulated HUVECs in a concentration dependent way. Different experimental set-ups were performed to investigate binding characteristics of E-selectin/IgG and mAb 7A9. This interaction is clearly calcium dependent, consistent with the general requirements of this ion for selectin activity. For binding and functional studies, native Western blot conditions were to be used, since reduced and denatured protein does not bind the antibody. Appearance of two bands on the western blot membrane (*figure* 4.5) is due to the heterogeneous glycosylation profile of the protein that, however, does not influence its activity. The binding constant of E-selectin/IgG toward the 7A9 antibody was characterized with a K_d of 5 nM, typical for an antibody-antigen interaction. There was no cross reactivity between mAb 7A9 and L- and P-selectin, as experimentally confirmed in parallel native Western Blots.

The functionality of expressed P-, and L-selectin/IgG were characterized in a reaction with different specific monoclonal blocking antibodies, anti-human CD62P, a mouse IgG1κ in case of P-selectin/IgG, and an anti-human CD62L, LAM1-116, a mouse IgG2aκ antibody in case of L-selectin [145,146,271]. Both glycosylated and non-glycosylated form of the proteins reacted with these antibodies, in a calcium-dependent manner and only under native conditions. Blocking antibodies against both P-, and L-selectin were highly specific without any cross reactivity with other proteins of this familly.

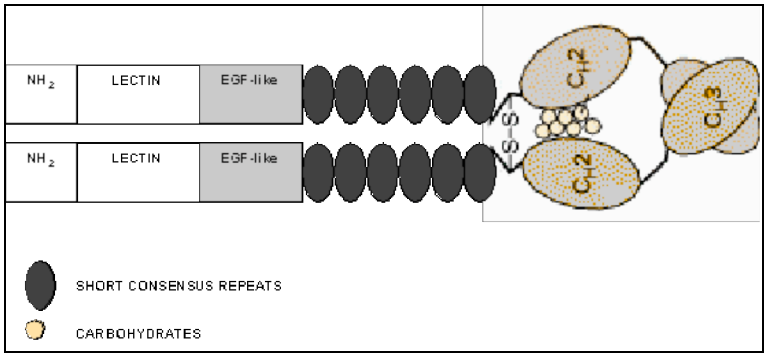


Figure 5.1: Shematic representation of E-selectin/IgG

5.2 Expression of lectin and EGF-like domain of human E-selectin in *E.coli*

The expression of cloned genes in *E. coli* for the production of recombinant proteins has provided a valuable system for studying protein structure and function. Production of recombinant proteins in *E.coli* is the basis of the multibillion dollars biotechnology industry. Bacterial or smaller eukaryotic proteins are usually easily over-produced in *E.coli*, whereas more complex, mammalian proteins are often more difficult to express in an active form. Proteins have been successfully produced in either the cytoplasmic or periplasmic space of *E.coli*. In general, proteins are more easily produced in the cytoplasm, but often aggregate into insoluble inclusion bodies, necessitating a refolding step. Proteins or protein domains that are normally secreted or extracellular part of the protein, have better chance to properly fold in *E.coli* when they are exported into the more oxidizing environment of the periplasmic space where disulfide bond formation can take place [272,273]. To obtain efficient translocation of a recombinant protein across the cytoplasmic membrane is often difficult and there is no guarantee of proper folding. In addition, proteins produced in the periplasm can also aggregate into insoluble inclusion bodies. Some fusion proteins are designed to cross both inner and outer bacterial membranes and are secreted into the media. This has drawbacks, however, and can be undesirable, due to the harsh agitation and oxidizing conditions of the culture during growth. Proteins secreted into the media can undergo unwanted modifications such as over-oxidation and deamidation.

The observation that deglycosylation of E-selectin does not affect protein activity [151] directed our expression strategy toward *E.coli*. On time and cost scale, this expression system would have been by far the most appropriate for the planned structural studies. For NMR studies, it is desirable to incorporate $^{15}N/^{13}C$ isotopes into the protein sequence. This has led our effort to express the lectin and EGF-like domains of human E-selectin in *E.coli* as a minimal active protein part of 18.5 kDa. However, lectin and EGF-like domains of E-selectin belong to the extracellular part of the protein. Consequently, these domains are post-translationaly modified on three *N*-linked glycosylation sites and have five disulfide bridges, two of which are in the lectin domain, and three in the EGF-like domain. Although these disulfide bridges are not directly part of the ligand binding site, their contribution to the overall protein structure and stability is significant. In the native protein, disulfide bonds are formed between Cys residues C19-C119, C92-C111, C124-C135, C129-C144 and C146-C155, following the 1-4, 2-3, 5-7, 6-8 and 9-10 pattern, *figure* 5.1. These challenging protein characteristics and the complex disulfide

pattern have directed our strategy to express LecEGF as a fusion protein secreting in the medium.

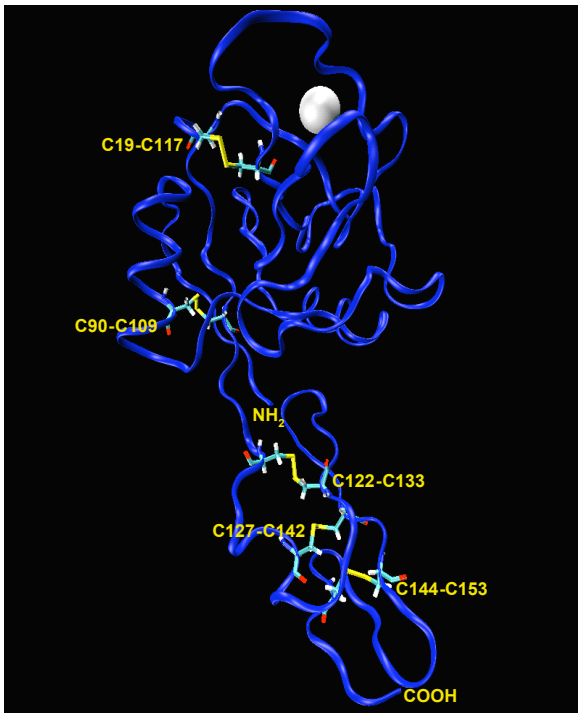


Figure 5.1: LecEGF of E-selectin with designated cysteins involved in formation of five disulfide bridges.

5.2.1 Lectin and EGF-like domain (LecEGF) secretion into the medium

The construction and use of fusion vectors based on the gene for staphylococcal protein A has been described for secretion of proteins into the culture medium, and for the rapid recovery of the fusion proteins from crude lysates by affinity chromatography (human insuline-like growth factor). It was demonstrated that fusion proteins containing only two (EE or ZZ) of the five (A, B, C, D, E, Z) IgG-binding domains of protein A were secreted into the growth medium of *E.coli* [274,275]. These findings led to the construction of the so called pEZZ18 plasmid vector with the described features of secretion- and IgG binding. pEZZ18 has both the lac UV5 and staphylococcal protein A promoters. The system is not inducible, but expression can be increased by heat shock i.e., by raising the culture temperature to 44°C for 2-6 hours during the stationary growth phase [274]. Promoters were fused to the export domains of protein A followed by a multiple cloning site.

Fusion protein with the 14 kDa long ZZ domain of protein A should have improved protein solubility and secretion and also facilitate single-step protein purification by IgG-sepharose

chromatography. The DNA sequence coding for human E-selectin was available from isolated genomic DNA of recombinant CHO cells. A fragment of 512 bps coding for the lectin and EGF-like domains was amplified as described in material and methods (3.2.2.2). In our case one base (G) had to be added to the cloned DNA sequence in order to maintain the open reading frame. The initial methionine at the transcription start was provided by the vector, whereas the stop codon for the transcription termination had to be provided by the insert. For further structural protein studies, it would have been necessary to remove the ZZ tag from the protein. Therefore, a thrombin cleavage site has been introduced into the sequence between the ZZ and the LecEGF domains. *EcoR*I and *BamH*I restriction sites were introduced to the 5' and 3' primers, respectively, and the stop codon after the amplified selectin sequence. These features have led to a 51 base pairs long 5' oligo, with a rather high melting temperature of 72°C as compared to 58°C for the 3'oligo. Although the 5' oligo was extraordinary long, analysis of its composition did not indicate any significant possibility for hairpin formation or primer-dimer formation.

Consistent with this observation, initial cloning reactions were performed with high efficiency and reproducibility using standard PCR conditions and did not require any significant optimization. Successfully transformed *E.coli* cloning strains Top10 and DHα5, generated sufficient number of colonies who's DNA obviously contained desired insert. *E.coli* strain AD 494 (DE3) was chosen for protein expression and production, since this strain is lacking the tyoredoxin gene and should facilitate disulfide bond formation even in the cytoplasm of *E.coli*. Once transformed into *E.coli* AD 494, it was not possible to detect recombinant protein in any of the bacterial expression compartments. Follow-up sequence analyses done on over thirty clones have revealed mutations, deletions or insertions of a single base, mostly within the sequence of the 5' primer, or just after it. Shortening the size of this oligo to 24 bps did not abolished mutations, although all the cloning and PCR steps were done with *Pfu* polymerase that has a proofreading activity. The fact that mutations have always been located either in the region covered by the 5' oligo or close to the beginning of the LecEGF protein sequence, and that were always on different position lead us to believe that this was a host protection mediated mechanism. Mutated DNA resulted in the shift of the open reading frame on the very beginning of the LecEGF sequence and only few amino acids of E-selectin were correctly translated before a stop codon. DNA was sequenced from all grown colonies and was always mutated. Therefore we concluded that the LecEGF domain of E-selectin which is fused on its N-terminal to the secretion sequence and ZZ domain of protein A is a toxic protein for a bacterial host, and

in case of its expression, the cell protection mechanism introduced DNA mutations to prevent protein synthesis.

5.2.2 LecEGF secretion to the periplasm

Translocation of proteins across the cytoplasmic membrane requires a “translocation competent” or unfolded state. Proteins that are exported by a cotranslational or translation-linked mechanism, have to be still unfolded, since folding into stable secondary and tertiary structures is thought to preclude translocation [276]. The cytoplasmic chaperons that are thought to bind newly synthesized secretory proteins, like SecB, keep them unfolded until translocation occurs. Exporting recombinant proteins into the periplasmic space of *E.coli* has several advantages. In case of LecEGF expression, the most important one is that in the more oxidizing environment of the periplasmic space the possibility for obtaining a properly folded and disulfide bridged proteins is enhanced. Periplasmic proteins are protected from degradation by cytoplasmic proteases and a simple treatment of the cells by osmotic shock releases them for easier purification. The translocation of foreign proteins however, is usually not readily accomplished. Proteins that are normally cytoplasmic in nature will not be translocated efficiently. Most mature exported proteins lack any positively charged residues in their *N*-terminal region. Even in case of success, translocation is often the rate-limiting step in the production, and numerous parameters such as promoter, signal peptide or sequence of the mature protein can all affect translocation.

Approximately 20% of *E.coli* proteins are efficiently translocated across the cytoplasmic membrane. Their signal peptides (STII, PhoA, OmpA, MalE, etc.) have therefore been used to export heterologous proteins. In the case of extracellular mammalian proteins the signal sequences are similar to the *E.coli* counterparts, but usually do not function as well in *E.coli*. Generally, in periplasmic protein production, there are two potential products to differentiate. Ideally, the mature protein with the signal peptide properly cleaved will be produced, but the unprocessed precursor protein containing the signal peptide may also accumulate.

Disulfide bond formation and isomerization in bacterial periplasma is catalyzed by enzymes similar to eukaryotic protein disulfide isomerase (PDI). A set of these bacterial enzymes with similar properties to those of PDI has been identified and named DsbA family. *DsbA* genes code for a 21 kDa periplasmic protein with a CXYC motive in the active site, where X and Y vary [277]. Within this consensus sequence, the first cysteine residue is characterized by an unexpectedly low pKa value, thus guaranteeing fast and efficient reaction with the thiol groups of nascent proteins, leading to enhanced disulfide bridge formation. DsbA coexpression has

been shown to increase protein solubility, stability and correct folding in several cases [278]. Several studies have defined the essential features of signal peptides: exclusively located at the amino terminal, they have a long hydrophobic region that is usually preceeded by one or more positively charged residues in a short, generally hydrophilic region [279]. In our efforts to express correctly folded and oxidized LecEGF in *E.coli*, secretion into the periplasmic space was a promising approach in view of correct formation of the disulfide bridge pattern.

For LecEGF expression in the periplasm we have used the pINompA cloning vector containing the signal sequence of an outer membrane protein A (ompA), a bacterial protease that is normally located in the periplasma [250]. The signal sequence is 22 amino acids long and is preceeding the LecEGF sequence. Multiple cloning sites of this vector enabled us cloning into *EcoR*I and *BamH*I restriction sites. The expressed fusion protein prior to the processing would contain a methionine from the signal sequence. Prior to the LecEGF sequence an amino acids triplet (ANS) would be introduced as a consequence of *EcoR*I restriction site. This vector is a low copy vector and utilizes a potent *lpp* promoter to efficiently initiate transcription of the cloned gene. Transcription via the lpp promoter is controlled by the lac UV5 promoter-operator, inserted downstream from the lpp promoter, and enables controlled induction of the cloned gene. The signal sequence is known to be cleaved of from the ompA precursor protein by the nonspecific signal peptidase I. This expression system has been used for the secretion of β-lactamase and mouse metallothionein, where the subcloning of a ZZ-fusion protein into the pINompA cloning vector finally enabled protein secretion in the medium [280]. The fusion of a signal and export sequences did not harm in this case expression of correctly processed protein.

Initial cloning into DHα5 cloning strain has shown some similarities as compared to our previous cloning into pEZZ18: low number of grown colonies and, although corrected by restriction enzyme digestion, all sequenced DNAs had a point mutations, either deletions or insertions. These mutations took place just downstream of the restriction site, or at the beginning of the sequence coding for LecEGF. Consequently, the open reading frame of the translated protein was shifted, resulting in a non-functional protein. In order to improve the basic cloning strategy, DHα5 cells were electroporated, as a more potent transformation method. In this case, however, we did not get a single colony to grow, although wide varieties of ligation and transformation conditions were tested. Control experiments, with plasmid w/o the insert were positive, therefore excluding *E.coli* cells as a source of negative results. The fact that electroporated, cells did not grow at all, and when chemically transformed only few of them grew however with the incorrect DNA sequence, led us to the conclusion, that fusion of LecEGF with

a signal transport sequence is toxic for the bacterial hosts. These negative attempts to clone LecEGF as a secretory protein in the bacterial periplasma were consistent with the negative results in pEZZ 18, and further strengthened the hypothesis of the toxicity of this fusion protein.

5.2.3 LecEGF expression in the cytoplasma of *E.coli*

The advantages of intracellular versus periplasmic protein production are generally that higher expression levels are achived (up to 70% of total cell proteins), that there is no need for protein translocation and that protein is protected from degradation by periplasmic proteases. In addition, expressed proteins very often form inclusion bodies, which can be easily isolated, providing a rapid purification step However, the solubilization has to be achieved in denaturing medium followed by refolding [233]. In general, translation initiation of foreign genes can be often inefficient and may require optimization. In addition, cytoplasmic proteases can quickly degrade a foreign protein, especially if it is small and soluble, and an extra amino terminal methionine residue that may be retained by the recombinant protein could disturb protein structure. In case of successful protein expression, preventing inclusion bodies formation is sometimes a challenge. To date, the precise physiochemical parameters that contribute to the formation of inclusion bodies remain unclear [281]. According to some evidence [282], the propensity to form insoluble aggregates does not really correlate with the protein characteristics such as its size, the use of fusion constructs, the subunit structure or its relative hydrophobicity Statistical analysis of the amino acid composition of proteins that do and do not form inclusion bodies in *E.coli* concluded that 6 parameters might be correlated with their formation: charge average, turn-forming residues, cysteine and proline amount, hydrophilicity and total number of residues [281]. Especially the first two parameters were found to strongly correlate with inclusion body formation. After the first pioneering studies [283] on protein folding, numerous examples of successful *in vitro* protein refolding demonstrate that all the information required for the formation of the native three-dimensional structure of a given protein is encoded in its amino acid sequence. This is however a multi-step process, since A number of proteins assisting in the folding of nascent polypeptide chains have been described [233].

Since the efforts to express correct LecEGF as secreted fusion protein have failed, cytoplasmic expression was tried. Cloning vector pET15b was used, with the strong T7 late promoter. The activity of this system depends on a transcription unit that supplies the T7 RNA polymerase, whose tight repression is essential to avoid leakiness of the T7 promoter [284]. Multiple cloning sites enable sequence insertions in *Nde*I and *BamH*I restriction sites, and the vector provides a 6-His tag to facilitate purification. A thrombin cleavage site follows the His-tag and enables its

removal after successful purification, as shown in *figure* 5.2A. In total, 16 amino acids are preceding the native terminal tryptophan of LecEGF. The DNA sequence coding for LecEGF was successfully cloned into pET15b and expressed in DHα5 cells in the first attempt. This was consistent with the hypothesis that protein fusion to a secretion signal, and forced translocation over the cell membrane was the reason for DNA mutations and unsuccessful cloning in prior attempts. Sequencing from two independent sources (MWG and Syngene) has confirmed the correct DNA sequence, which was subcloned in production *E.coli* strains. In preliminary screenings under different conditions, purification of expressed HisLecEGF was attempt in both isolated native and denaturing conditions by Ni-NTA chromatography.

As shown in section results, *figure* 4.27, the protein was exclusively expressed as inclusion bodies. There are several different approaches to minimize the formation of inclusion bodies when producing heterologous proteins intracellularly in *E.coli*. Overproduction itself, i.e., the increase in concentration of nascent polypeptide chain, is sometimes sufficient to induce the formation of inactive aggregates. Based on these findings, a kinetic model was proposed that shows that the yield of native protein increases (and that of inclusion body formation correspondingly decreases) with a decreasing rate of protein expression [285]. A reduction in the rate of protein synthesis can be achieved by using moderate or weak promoters or by only partial induction of a strong promoter [286]. Other means of reducing the protein-synthesis rate is by growing the culture at lower temperature or to add non-metabolizable carbon sources to the medium. Increased formation of inclusion bodies is also observed at slow folding rates of recombinant proteins. Slow folding may be expected upon cytosolic expression of heterologous proteins containing disulfide bonds in their native state. Although some *in vitro* folding studies have demonstrated clearly that native disulfide bonds can be formed even in the reducing environment found in the cytosol [287,288], disulfide bond formation may be extremely slow under these reducing conditions.

Considering all these general reasons for inclusion body formation, the aggregation and insolubility of expressed HisLecEGF was not surprising. Parameters that may influence protein production rate have been optimized. *E.coli* strain BL 21 (DE3) has been widely used for protein production. Strains AD 494 and Rosetta Gami could have been of interest, in the case of soluble protein production, or in case of incompatible codon usage for HisLecEGF expression in *E.coli*. These optimizations, however, did not improve HisLecEGF solubility.

Since preparative amounts of active protein would be needed for our studies, inclusion bodies were isolated, denaturated, and subsequent attempts to refold the protein have been

undertaken. Inclusion bodies have a high specific density, and are easily recovered and separated from the cell membranes by centrifugation and extensive washing in buffer with low reductant concentrations and protease inhibitors [289]. Although inclusion body proteins may contain relatively high secondary structure content, they do not readily dissolve in physiological buffers. Inclusion body solubilization requires rather strong denaturant, such as 6M guanidine hydrochloride (GuHCl) or 6-8M urea, where GuHCl is preferable to urea for two reasons. First, it is a stronger chaotrop, which may allow solubilization of extremely sturdy inclusion bodies that are resistent to urea. Second, urea solutions may contain isocyanate and formaldehyde, which, even if present in a very low concentration may lead to carbamoylation of free amino groups of the polypeptide, especially upon long-term incubation at alkaline pH [290]. In the case of proteins containing cysteines, inclusion bodies usually form interchain, scrambled disulfide bonds which reduce their solubility. The addition of low molecular weight thiol reagents in combination with a chaotrop allows complete reduction at mild alkaline pH and enhances solubilization.

HisLecEGF was initially solubilized in 8M urea, pH 8.0, and purified over a Ni-NTA column with an average yield of 120 mg/L. MS analysis of reduced and alkylated protein has shown an increase of the predicted molecular weight of 28-84 Da, which would correspond to different carbamoylation extents on ε-NH2 groups of lysine. Since HisLecEGF has 7 lysines, three of which influence ligand binding (K111, K112, and K113), this modification would irreversibly damage the protein. Therefore, all subsequent inclusion bodies solubilizations have been done in 6M GuHCl. Although protein folding processes are not strongly affected by other proteins present in the same renaturation system, better yields are obtained with pure protein. Ni-NTA chromatography under denaturing conditions allowed purification of HisLecEGF to homogeneity prior to the refolding. Purified protein (section results, *figure* 4.29) was digested with trypsin, Lys-C, and V8 protease, and analyzed by MS with 100% sequence coverage. The initial methionine was not processed, leaving a total of 10 additional amino acids on the N-terminus of HisLecEGF.

For the refolding process, several points had to be considered: the folding method, the fact that the native protein has 5 disulfide bridges, and the influence of small molecular weight additives as buffer components that can enhance refolding and stabilize correctly folded species. In a first refolding attempt, different dialysis protocols were applied [291,292]. Since HisLecEGF is a C-type lectin, calcium was added to all buffers that were used. Upon controlled removal of excess denaturant, the protein has quantitatively precipitated within the first hour, regardless of

temperature, pH, and buffer composition. During the slow removal of denaturant by dialysis, the protein was exposed to decreasing denaturant concentrations for an extended period of time. This might have a negative effect on the yield of *in vitro* refolding [285,289]. The major problem in refolding experiments is kinetic competition of folding and aggregation due to competing intra- and intermolecular interactions. In folding intermediates, which were formed at intermediate denaturant concentrations, residues that are normally buried inside the protein were exposed on the outer side, causing aggregation and precipitation upon slow denaturant removal. Once aggregates are formed, further aggregation is kinetically favored [293,294].

Unsuccessful dialysis led us to another approach, namely fast dilution. Dilution of the solubilized, reduced protein directly into a renaturation buffer is the most commonly used method in small-scale refolding studies [233]. However, protein concentration has to be carefully controlled to prevent aggregation. Unproductive aggregation processes may originate both from nonspecific (hydrophobic) interactions of predominantly unfolded, nascent polypeptide chains, as well as from incorrect interactions of partially structured folding intermediates. On the other hand, folding intermediates have been shown to shift equilibrium toward fully folded protein [233,289,295]. Aggregation reactions are second- (or higher) order processes, whereas correct folding is a first-order reaction. Thus, aggregation predominates upon renaturation above an optimal concentration of denatured protein [296,297], usually not higher than 100 μg/ml but not lower then 20 μg/ml. In addition, folding with concomitant disulfide bridge formation requires not only correct protein structure, but oxidation of the correct disulfide bonds. In case of a five disulfide bonds-containing proteins, there are 945 combinations for their formation [283]. However, correct cysteine pairing and disulfide bond formation should be favoured by the free energy gained upon formation of the correct native conformation. Once formed, correct native disulfide bridges are stable and should not be further shuffled.

Oxidation of cysteine residues can be performed with different reagents. The most common reagents used are low molecular weight thiols, particularly reduced and oxidized glutathione in different ratios. Because thiol-disulfide exchange reactions are rapidly reversible, “oxido-shuffling” reagents increase both the rate and the yield of correct disulfide bond formation by rapid reshuffling of improper disulfide bonds. Another method of oxidative protein folding relies on the formation of mixed disulfides on the protein simultaneously to dilution refolding [296,298]. The introduction of mixed disulfides to the denatured state increases the solubility of the unfolded protein by enhancing the hydrophilic character of the unfolded polypeptide chain. Considering low molecular weight additives, it is common knowledge that these can have a

tremendous effect on the folding efficacy. These low molecular weight additives can modulate protein solubility, stability, and interactions with other molecules and structures in solution [282,299].

Correct refolding of our construct can be monitored by analytical RP-HPLC, which gives information on the aggregation state of the protein, and by biological activity as addressed by binding of the blocking mAb 7A9, which binds only to native, correctly refolded protein. In light of all these parameters, different experimental set ups were designed and tested for the optimization of HisLecEGF refolding by fast dilution, but initially no appropriate conditions could be found. Adding redox reagents during the dilution process or after a presumed refolding did not make any difference. We tried, however, to separate different protein species present in solution after refolding by size exclusion chromatography [300], but the best fractions showed only minor activity. The possibility of a negative impact of the His tag on the refolding process was investigated by removing of the tag with thrombin under intermediate denaturant concentration. However, no positive effect could be observed. A broad screen of buffer compositions that would provide an adequate refolding environment was performed using screening system called “Affinity flow-field fractionation”. Some similarities have been observed between experimental conditions for protein crystallography and protein refolding. For this reason low molecular weight buffer additives usually used for crystallography have been screened as additives for protein refolding [301]. *Figure* 4.34, section results, shows the best screening result (high protein-monomer fraction after fast dilution). The protein precipitated in all buffers with pH 5.0, regardless of additives, indicating that buffers of higher pH (8-9.5) and ionic strength were more conducive to refolding. Addition of 0.005%-0.05% n-octylglucoside to the buffer resulted in the highest yield of monomeric protein fraction as detected by AfFF analysis and RP-HPLC. *N*-octylglucoside is a non-ionic detergent consisting of an aliphatic chain and a glucose moiety and is usually used for solubilization and stabilization of membrane proteins [302]. Since this detergent showed significant improvement on the monomeric fraction present after dilution, this might lead to the question whether glycosylation might be a prerequisite for the refolding of this particular protein. In a eukaryotic organism the addition of sugar chains, which happens simultaneously with the synthesis of the nascent polypeptide chain, might prevent exposure of hydrophobic protein parts. Once these are buried within the correctly folded protein, *N*-glycans can be removed without affecting protein stability and, in the case of the LecEGF domain of E-selectin, without affecting activity as well. However, protein stabilized in the presence of this detergent precipitated immediately after addition of redox reagents.

To avoid possible influence of a N-terminal tag on the rate of protein refolding, in the last attempt, amino acids 1-157 of LecEGF were cloned directly w/o any tag into pET11c plasmid vector (*figure* 5.2B), that has the same system characteristics as described for pET15b (Novagen). Cytoplasmic expression and inclusion body production was straightforward, consistent with the observation that DNA mutations occurred just in case of constructs that would lead to secretion of LecEGF. This expression strategy led to the co-expression of two bands: truncated LecEGF missing the last 40 amino acids on the C-terminus, and full-length LecEGF, as confirmed by MS. This protein truncation occured at translational level, since different *E.coli* strains which were transformed with different sequenced DNAs, lower IPTG concentration, temperature and expression time resulted in the same expression profile. As presented in *figure* 4.39, the charge, size, and hydrophobic differences of full-length and truncated forms were insufficient to separate the different species, even after extensive optimization. Only 26% of the total fraction was isolated as monomer on preparative RP-HPLC. Nevertheless, refolding was tried combining the screen results from AfFF, and with n-dodecylmaltoside, a non-ionic detergent for solubilization and stabilization of membrane proteins. Initial screens indicated that after refolding the protein was soluble in TBS buffer and readily eluted from an analytical RP-HPLC column, prior to the oxidation. Oxidation was also monitored by analytical RP-HPLC, using a decrease in retention time as diagnostics [303], since a correctly folded protein, with a correct disulfide pattern is more compact. However, when analyzed with mAb7A9, our presumably refolded protein was inactive. Prolonged oxidation did not improve its activity; on the contrary, the protein aggregated and could not be eluted from RP-HPLC any more. The formation of inter-molecular disulfide bridges was confirmed in a non-reducing SDS-PAGE gel, which has shown existence of monomers, dimers, and higher molecular weight oligomers in solution. Although the native disulfide pattern should have priority during *in vitro* formation, in case of LecEGF the oxidation conditions did not push native disulfide formation. The addition of oxidant rather enhanced protein aggregation without any significant active protein formation.

Further improvements of the folding strategy could have been achieved by co-expression of chaperons or co-secretion with the DsbA family of protein responsible for isomerization of disulfides, although in latter case yield of active protein was in range of hundred micrograms per liter [272]. However, it is questionable whether the latter strategy would work, since LecEGF could never be forced to secrete. Clearly in conclusion, *E.coli* turned out not to be the expression strategy for the production of lecEGF of human E-selectin.

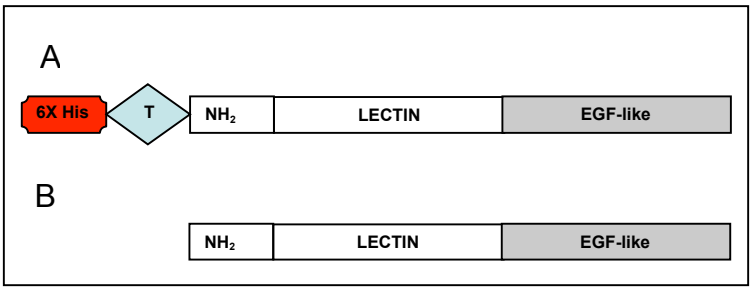


Figure 5.2 A and B: Shematic representation of HisLecEGF and LecEGF constructs of E-selectin expressed in the cytoplasma of *E.coli.* **A**: Construct in pET-15b, **B**: construct in pET-11C. **6xHis**: His tag, **T**:Thrombin cleavage site

5.3 Expression of LecEGF domain of human E-selectin in baculovirus-infected insect cells

Baculovirus infected insect cells are one of the most powerful expression systems. One of the major advantages over bacterial expression systems is its ability to produce functional complex heterologous proteins [137], [304,305]. The most relevant feature of this expression system is its eukaryotic-like environment for protein production, particularly important for the production of heterologous mammalian proteins [235]. Expression levels in baculovirus infected insect cells can vary from 25-100% of the total cell proteins, and can be as high as 1 g protein per 10^9 cells, or 1 liter culture. Protein expression and production is rather simple, and cultivation is performed at 24°C or 27°C. The rod shaped capsids of baculovirus can extend to accommodate an additional 100 kbp of DNA or larger and allows for the simultaneous expression of a number of genes. Baculovirus vectors are helper-virus independent and therefore simple to use and considerably faster and simpler then the construction of a cloned, high expressing recombinant eukaryotic cell line. Nevertheless, a baculovirus expression system takes a longer time to develop then a typical *E.coli* system and is usually based on transient transfection system. Important parameters in designing a baculovirus-infected insect cells system for recombinant protein production are the selection of an expression vector, providing the best expression and production results. Insect cell lines, growth media (serum supplemented, or serum-free), [306] and feeding strategies should be carefully chosen to allow for the optimal recombinant virus and protein expression.

Baculoviruses are the most prominent viruses known to affect insect population. More then 500 baculovirus isolates have been identified, most of which originate in arthropods, particularly insects of the order of Lepidoptera. The most common isolates used in foreign gene expression

are Autographa californica multiple nuclear polyhedrosis virus (AcMNPV) and *Bombyx mori* (silkworm) nuclear polyhedrosis virus (BmNPV) [307].

Naturally occurring infection and recombinant in vitro infection are similar with the corresponding three phases of viral replication, an early phase, a late phase and a very late phase [235]. The major difference is that the naturally occurring polyhedrin gene within the wild-type bacuovirus genome is replaced with a recombinant gene or cDNA., and correspondingly a recombinant product is expressed in place of the naturally occurring polyhedrin promoter.

5.3.1 Intracellular expression of LecEGF

For the above mentioned characteristics of heterologous protein expression, insect cells have been chosen for the expression of LecEGF. It was planned to express recombinant protein with an *N*-terminus His-tag, followed by a Prescission™-protease restriction site preceeding the LecEGF sequence (*figure* 5.3). A His-tag fused to the Prescission site was available in cloning vector pBacPak and therefore LecEGF was initially cloned into this vector to generate the above construct. To avoid any additional amino acids in the protein sequence as a consequence of restriction endonucleases sites, LecEGF was PCR integrated in pBacPak, according to the methodology described by Geiser *et al* [251]. This method utilizes extended protocols for site directed mutagenesis and exploits endogenous bacterial ligases for efficient gene insertion into the vector In the next step, HisPreLecEGF was PCR integrated into the pFastBac vector, which contains elements that enable transposition reactions with the bacmid DNA. In the pFastBac vector a strong polyhedrin promoter is governing protein expression. In contrast to some other promoters, (e.g. p10 promoter), this promoter belongs to the group of late viral promoters, leading to a start of protein expression only 48-72 hours after viral infection. The choice of promoter can play a significant role for the expression of functional proteins in insect cells, especially in cases of expression of secreted proteins [235]. The Prescission™ protease recognition site (LEVLFQ↓GP) was introduced between the *N*-terminal His-tag and LecEGF, because of a greater specificity as compared to other serin protease. Additionally, the enzyme is very stable under a wide variety of pH and temperature conditions. The generation of a correct DNA construct was confirmed by sequencing. *E.coli* DH10Bac was successfully transformed with pFastBacHPLecEGF. This E.coli strain possesses bacmid DNA and transposition helper elements that enable transposition reactions between pFastBac and bacmid DNA. Accordingly, the gene coding for HisPreLecEGF with a polyhedrin promoter and translation important elements has been incorporate into bacmid DNA. The successful incorporation was defined by blue-white colony selection, enabled by recombinant bacmid DNA. Transfection of *Sf9* insect

cells (*Spodoptera frugiperda*) was successful, as indicated by the first signs of viral propagation in the cells and cell monitoring by cytometry and microscopy. Viral infection takes three-four days and during this period insect cells undergo changes in their number, shape and viability.

The first transfection of *Sf9* insect cells was visually monitored and cells were analyzed on a cytometer for the signs of infection as extended cell diameter and decreased viability. Improved transfection protocols were used (S.Rieffel, personal communication), where infection at lower multiplicity of infection and higher cell number led to the faster generation of a virus with a stronger infection potency. For single virus isolation and subsequent amplification, as well as for determining the viral titer, the plaque assay was used. Viral data, as well as infection conditions were in the standard range of 10^6 plaque forming units (pfu) and a multiplicity of infection (M.O.I.) of 6. In order to keep production costs as low as possible and to enhance purification, cells were grown in Sf 900 medium without serum and without antibiotics. This did not influence cell growth. HisPreLecEGF expression was directed to the cytoplasma of insect cells, since it has been reported from different studies that intracellular protein expression usually has higher yields as compared to secretory constructs. Another advantage of intracellular production has to do with purification over Ni-NTA. In fact, components of the insect cells-medium and endogenous proteins secreted from insect cells interfere with the Ni-NTA matrix, necessitating medium dialysis prior to purification. It is known that the cytoplasma of insect cells is less reductive as compared to *E.coli*. In addition, there are few reports on the successful production of proteins with concomitant disulfide bonds formation in the cytoplasma of the insect cells, although this compartment would be more suitable for the expression of intracellular proteins or their domains [235]. HisPreLecEGF was produced intracellularly, as shown in *figure* 4.38 and confirmed in reducing and denaturing western blot with an anti-His antibody. However, protein was not isolated after mild, native lyses, indicating that it was insoluble and inactive. In native western blots and ELISA with mAb 7A9 it was not functional to any significant extent. Although insect cells possess processing machineries and chaperons in their cytoplasm that facilitate and improve protein refolding [68,308]. We have investigated and optimized infection rate, slower protein production and shorter production time, as factors that influence protein production. A lower rate of protein synthesis could facilitate the work of the folding machinery in the insect cells and result in correctly folded protein. However, extensive optimization did not result in any active protein production, pointing to problems in recombinant protein design, and choice of the expression compartment (i.e. protein with a tag on the N-terminal, and protein expression directed to the cytoplasm). In conclusion, lecEGF of human E-selectin expressed in insect cells intracellularly with an amino terminal His-tag, is not functional and active.

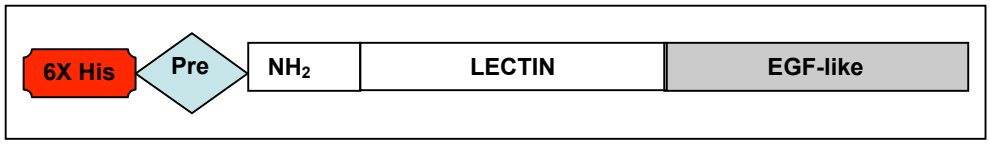


Figure 5.3: Shematic representation of HisPreLecEGF construct of E-selectin expressed in the cytoplasm of insect cells. **6xHis**: His tag, **Pre**: Prescission™ protease cleavage site.

5.3.2 Extracellular expression of LecEGF

Generally, regarding the ability to produce fully processed and biologically active recombinant proteins, insect cells with their secretion capabilities are second only to mammalian cells. The fundamental maturation step is protein folding, followed by quite diverse post-translational modifications, depending on the protein destination (intracellular or secreted). Although LecEGF has its native signal sequence and those of mammalian origin are well recognized in insect cells, an endogenous insect signal sequence, i.e., the signal for the acidic glycoprotein gp67 was used in order to improve the likelihood of correct protein processing. The acidic glycoprotein gp67 is the most abundant envelope surface glycoprotein of the *Autographa californica* nuclear polyhedrosis virus (AcNPV), and is essential for the entry of baculovirus particles into susceptible cells. Large amounts of this protein are secreted and anchored to the virus peplomer. Therefore its gene contains one of the most effective baculovirus-encoded signal sequences for protein secretion. During the transport across the cell membrane, theoretically, the signal peptide is cleaved after Ala38, leaving mature protein with its native N-terminal. At the C-terminus of the EGF-like domain a short tag has been placed, i.e. a FLAG™ tag [309], *figure* 5.4. This is a hydrophilic octapeptide (DYKDDDDK), and different anti-Flag antibodies are available against this epitope [310,311]. The Flag tag could be placed either at the amino-terminus, the carboxy-terminus or in association with other tags. It will usually not interfere with fusion protein expression, proteolytic maturation or activity. [312]. The most recent uses of this technique are in transfection of eukaryotic mammalian cells and insect cells and even transgenic systems. It can be used for a protein coupling to a chip, or for assays performed in 96-well plates [312,313]. Initially, the DNA construct containing lecEGF of E-selectin, and a flag-epitope (SSLecEGFFlag) was generated in plasmid pAcGP67, which contains the desired endogenous signal sequence of gp67, as described above. Using PCR integration methods described earlier, the LecEGFFlag sequence was integrated without restriction sites and the generation of overhangs [251]. In a second cloning step, the DNA fragment coding for SSLecEGFlag was PCR integrated in the final vector pFastBac,

downstream from the potent polyhedrin promoter, generating our final and pFastBacSSLecEGFFlag construct. Colony PCR screening and subsequent sequencing confirmed the correct DNA sequence and *E.coli* strain DH10Bac has been transformed with the pFastBacSSLecEGFFlag. *Sf*9 insect cells have been transfected with the recombinant bacmid DNA and 96 hours later, first signs of infection appeared. After viral purification by the plaque assay and a first amplification round, 96 hours post infection cells number and viability dropped and diameter increased indicating that infection had occurred. Based on the viral titer, the cells were infected at a M.O.I of 5, i.e., a stronger infection conditions then in the case of intracellular LecEGFFlag production. As shown in *figure* 4.43, protein secretion in the medium had started at 48 hours p.i., whereas between 72 and 96 hours p.i. there was not significant difference in the level of secreted protein. Therefore, protein was harvested 72 hours p.i. in order to avoid proteolytic cleavage. Baculovirus infected insect cells undergo a lytic cycle, and proteases can be expressed and released at every step of the limited time span (72-96h) of protein production [236,314]. Insect cells can produce proteases after baculovirus infection as a stress response; proteases can be produced from the baculovirus vector itself during the infection cycle, or can be released following cell lysis (e.g. lysosomal proteases).

On SDS-PAGE, overexpressed protein migrated between 20 kDa and 24 kDa as a smeary band, typical for glycosylated proteins. The predicted molecular weight of non-glycosylated LecEGFFlag is of 19.01 kDa. The overexpression of 24 kDa protein was also significant in the cells (approximately the same amount), as indicated by Western blot analysis under reducing conditions using the anti-flag M2 antibodies. When expressing secretory proteins in insect cells, post-translational processing is not completely efficient and partially unprocessed protein will be retained in the cells, probably in the endoplasmatic reticulum [314]. The higher molecular weight of this protein fraction is therefore consistent with unprocessed material.

Secreted flag-tagged protein was purified in a single step over a Sepharose affinity matrix coupled to the anti-flag monoclonal antibody M2. The flag-tagged proteins can bind to M2 antibody independently of the tag placement, in a calcium independent mode. The binding and elution requirements were very mild with 150 mM NaCl, and neutral pH. Bound LecEGFFlag was initially eluted by competing mode with excess of flag peptide and purified to homogeneity in a single step. However, at a later stage we opted for a lowering of the pH to 2.7 for elution (0.1 M glycine), because of the costs for commercial flag peptide. This pH decrease did not affect protein activity, since the pH was immediately corrected to neutral with 1 M Tris. However, some BSA unspecifically bound to the sepharose matrix was also eluted under these conditions,

but easily removed by a subsequent gel filtration. The activity of the eluted protein was further tested in Western blots with the 7A9 antibody under native conditions. Positive reaction with this blocking antibody has confirmed the prediction that once secreted, LecEGFFlag will be active. The secretion mechanism of the insect cells directs the protein into the ER and Golgi apparatus, where the environment is oxidative enough to catalyze disulfide bond formation of the nascent protein chain [314]. The protein migration profile on the SDS-PAGE and its apparent molecular weight indicated that LecEGFFlag is glycosylated, consistent with the fact that LecEGFlag posses three potential N-glycosylation sites, *figure* 5.5 The comparison with E-selectin/IgG in the reaction with mAb 7A9, under native Western blot conditions, shows that LecEGFFlag has a somewhat lower affinity toward this antibody. Since it has been reported that protein clustering increases affinity and avidity of this reactions, this might be a consequence of E-selectin/IgG dimerization. Therefore we have investigated the LecEGFFlag status in solution. Since glycosylation in insect cells did not increase significantly the molecular weight of the expressed protein, the LecEGFFlag is more appropriate for this analysis, as compared to the LecEGF secreted from CHO or COS cells.

Under different SDS-PAGE and native PAGE conditions, LecEGFFlag migrated as a double band, but as a monomer in solution, consistent with the previous molecule description of an asymmetric monomer [91]. STD NMR [208,216]. with the sialyl Lewis^x antagonist **BW69669** has further confirmed the activity of LecEGFFlag However, signals obtained in this measurement were less intense as compared to the signals obtained and published for E-selectin/IgG [210,269,315]. This can not be solely explained as a lower protein activity but also by the fact that the size of the protein unit that carries 1 binding site is seven times smaller in the LecEGFFlag then E-selectin/IgG (21 kDa, vs. 148 kDa resp.). In addition, the dimerization of E-selectin/IgG can contribute as well. It has been published that lower intensities of STD NMR signals are proportional to the molecular weight of the investigated protein and are less sensitive for the proteins of less then 30 kDa [316]. Since to the best of our knowledge these would be the first NMR experiments with a truncated version of E-selectin, differences in the NMR intensities and interpretation should be thoroughly examined The glycosylation status of LecEGFFlag has been examined by treatment with PNGaseF and *O*-glycosidase. Under reducing and denaturing conditions, the protein was fully deglycosylated with PNGaseF, confirming the typical glycosylation pattern of insect cells [307,317]. High mannose type of *N*-linked glycans could be completely removed by this enzyme (Glc*N*Ac$_2$Man$_{5-9}$). These results indicate the absence of any branching, or fucosylation in 3 position of mannose. In native, fully glycosylated conditions the molecular weight of two main glycosylated species was 21151.87

Da, and 19958.57 Da respectively. According to the predicted molecular weight of the naked protein, this would account for additional 2203.03 and 855.41 Da respectively, and pointing to 2 distinct *N*-linked sites that are in different stages of glycosylation. MS analysis has revealed protein heterogeneity even within the two separated species. In conclusion and after many unsuccessful attempts, active lectin and EGF-like domains the end we conclude that active lecEGF of human E-selectin could be successfully expressed in either insect cells *Sf9*, or *Trichoplusia Ni* (High five™), in sufficient amounts (18 mg/l) for structural studies and with an easy purification protocol by anti-flag affinity chromatography.

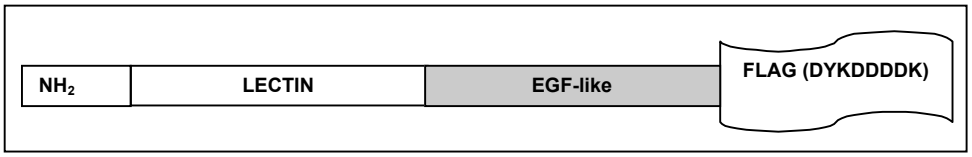


Figure 5.4: Shematic representation of LecEGFFlag secreted from insect cells

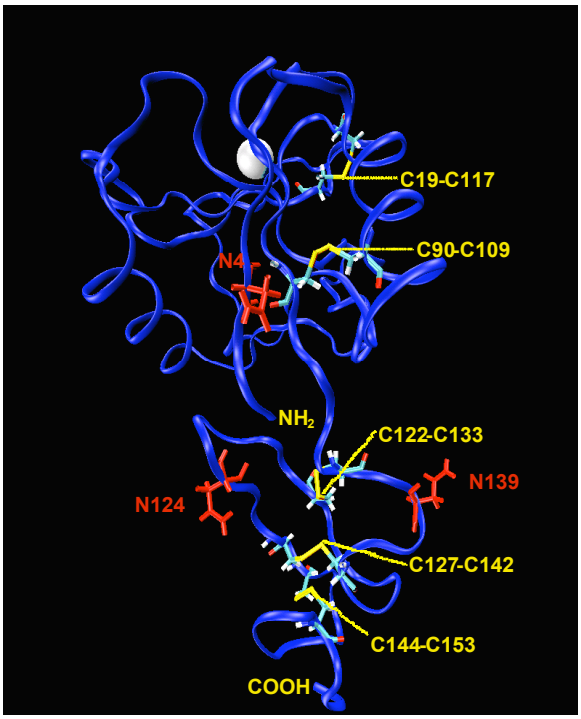


Figure 5.5: LecEGF of E-selectin with designated cysteins involved in formation of 5 disulfide bridges and potential *N*-glycosylation sites.

5.4 Bioassay development

In our efforts to discover potential selectin antagonists, it was necessary to develop, adapt and evaluate novel screening assays appropriate for molecular targets and lead validation. In a process of drug discovery, potential ligands are evaluated with different, independent experimental approaches, such as bioassays, NMR, or surface plasmon resonance. Moreover, bioassay for measuring the interactions of carbohydrates and lectins can be developed in a variety of solid or solution phase formats with sensitivity from nano- to the millimolar range. Availability of recombinant selectins and their thorough functional characterization allowed the development of a bioassay for the quantitative evaluation of selectin antagonists. Selectin-based bioassays were created using different approaches, leading to target-based and function-based formats. The latter were cellular assays based on inflammatory induction and subsequent selectin up-regulation or neutrophil reperfusions. There are, however, significant differences among data obtained with different assays and a high throughput robust screen is still lacking [241,257,318,319].

Selectin-expressing cells binding to a solid phase of ligand glycolipids [217,218]. In order to test our “in house” potential antagonists under different experimental conditions, but as close as possible to the physiological ones, molecule-molecule and cell-molecule assays were developed using a sLe^a-bearing polymer and a human promyelitic leukemia cell line (HL-60 cells) known to express selectin ligands, respectively [160].

5.4.1 E-selectin/IgG-sLe^a polyacrylamide assays

Molecule-molecule assays were based on binding of the sLe^a-polymer in the presence of selectin antagonists with an optical density-measurable read-out, as described under materials and methods. The assay was designed in a standard 96-wells format and optimized for standard conditions. When the plate was pre-coated with an anti-human Fc mouse IgG1, the control signal was not as stable and strong as when the protein was coated directly to the plate. Reasons for this are probably steric hindrance and/or inaccessibility of the ligand binding site on the protein. Further optimizations were directed toward the amount used in the assay. As a measure for standard conditions, a triple increase in the signal intensity as compared to the background (wells without proteins) was taken, according to Weitz-Schmidt [240]. The signal to noise and signal to background ratio could be one of the major reasons for inconsistencies in the assay performance among different groups. In the case of E-selectin/IgG, protein concentration in the wells was 3 μg/ml, whereas in the case of L-selectin and P-selectin/IgG, the standard protein amount was higher, up to 15 μg/ml. These differences point out the necessity

to characterize the protein preparation in term of the really active fraction. The amount of sLe^a-polymer used was the same in all three assays, i.e. 100 ng/ml, comparable with published results [92]. Since the physiological selectin-carbohydrate interaction is in the millimolar range, the sensitivity has to be higher. Assays were run under mild conditions, optimizing incubation time and number of the washing steps. Incubation at 37°C and for 4 hours gave the most stable and reproducible signals for standard conditions. The reliability of the assay for preliminary antagonist screening was based on the results obtained for sLe^x, and **BW69669**, an E-selectin antagonists thoroughly tested in similar assays and in animal models. The obtained data were in close agreement with the published one [201,208,216]. This consistency has enabled the interpretation of IC_{50} values of tested antagonists relative to the sLe^a polymer control. There were, however, several drawbacks in the assay in terms of stability and robustness. The optical density as an experimental read-out is rather sensitive to even small differences in the assay performance. This can have a great impact on the assay results and shift the IC_{50} values significantly. That is why an effort has to be made to utilize maybe fluorescence as a read-out and to find more robust assay conditions. The shifts in the IC_{50} values of some antagonists, and even of controls, were either due to this instability, or because of the quality of used protein. Selectin antagonists were tested in the polymer-based assay discussed above as a proof-of-concept with the aim of decreasing standard deviations, and improving assay reproducibility and accuracy. The use of a polymer presenting the natural carbohydrate epitope in a multivalent form has been discussed from several points of view [213,217,257]. Since the nature of selectin ligand interaction and physiological rolling conditions require fast and transient contacts, a polymer-presented ligand would form a more stable interaction and would promote the likelihood of interactions at other position on the polymer backbone. Although this is supposed to enhance the inhibitor effect in physiological conditions, the static assay stability should be improved in this way as well [217].

5.4.2 E-selectin/IgG- HL-60 assays

Cell-molecule assay with cells measure the potency of the inhibitor to block the interaction between naturally expressed selectin ligands on the cells and proteins coated to the plastic support of a 96-wells plate. Studies have been showing that the protein orientation, or more precisely, the orientation of the protein biding site, plays an important role in its ability to interact with the ligands expressed on the HL-60 cells [320]. The presence of the Fc-part of human IgG and consensus repeats on each of the selectins assayed seems to enable a correct protein orientation and protein-ligand interactions. As mentioned for the molecule-molecule assay,

standard conditions regarding protein amount coated, cell number used in the assay, and wash steps were also optimized. For standard conditions, proteins were used at 10-15 μg/ml, a higher concentration as compared to the molecule-molecule assay. The read-out in this cell-molecule bioassay is fluorescence, a more robust and preferable method, as compared to the optical density. Since fluorescence is expressed in arbitrary units, a direct comparison of the different data from assay to assay is not possible, rather, data have to be expressed relative to controls. In our case **BW69669** has been taken as a control, and the IC_{50} values of tested antagonists were expressed relative to this antagonist. The obtained IC_{50} values were higher as compared to the values from molecule-molecule assay. This inconsistency is rather typical for cell-based assay. One of the reasons is the uncontrolled cell machinery that synthesizes and expresses functional ligands and difficulties with the cell maintenance [160]. All tested compounds were run in triplicates of three independent experiments, but again the IC_{50} values were fluctuating on a day-to day basis. In case of L- and P-selectin/IgG only preliminary screens were done, aiming at defining standard assay conditions. The need to use higher protein concentration, as compared to the E-selectin/IgG assay could be the consequence of the protein quality, since P-selectin/IgG should bind to HL-60 cells with the highest affinity. PSGL-1 is the most abundant selectin ligand on these cells and binds P-selectin with a nanomolar binding constant. The incubation time and temperature in HL-60 assays were consistent with physiological conditions for all three selectins (at 37°C, and incubation time was reduced to only one hour). This shorter incubation was predominantly due to the toxicity of the fluorescent BCECF dye. The cell-molecule assay would need improvements in terms of reproducibility and accuracy but is very useful for preliminary antagonist screening.

5.4.3 LecEGFFlag-sLe^a-polyacrylamide assay

Up to date all experimental systems for studying selectin-carbohydrate interactions, have been exploiting recombinant E-selectin that contains complete extracellular domain. Truncated E-selectin version with LecEGF domains only was screened for the activity with the ligand bearing cells [90] but was never used for the antagonists screening assay. To further characterize the biological activity LecEGFFlag, a cell-free assay for a quantitative screen of E-selectin antagonists was developed in 96-wells format. Several previous assay formats that were established for E-selectin/IgG [240], were adopted and adjusted to LecEGFFlag. Li *et al.*, reported on unsuccessful coating with LecEGF expressed in COS cells and pointed out the necessity to pre-coat the wells with the non-blocking anti-E-selectin antibody 1D6 [97] to capture the protein. Accordingly, we have initially pre-coated the wells with an anti-flag M2 antibody, but

we did not get any signal that would indicate even very low affinity binding to the sLe^a polymer. Most probably sterical hindrance prevents the reaction between the sLe^a bearing polymer and the lectin domain of LecEGFFlag, although the polymer presents a scaffold with a high density of sLe^a molecules. Similar results were observed in the case of E-selectin/IgG when precoating with an anti-human Fc antibody resulted with lower signal intensity the direct E-selectin/IgG coating. When LecEGFFlag was directly coated to the wells, the result was a clear concentration-dependent sLe^a-polymer binding. Optimization of a protein and polymer concentration was performed in a similar way as for E-selectin/IgG. Apparent activity and the assay performance with LecEGFFlag was comparable to that of E-selectin/IgG. Apparent LecEGFFlag affinity for the sLe^a, based on the saturation binding to the polymer was slightly lower then reported for E-selectin/IgG [92,103], 72 nM vs. 49 nM, respectively, but well within the experimental error (range). Specificity of the assay was confirmed with the non-sialylated saccaride, which, in a wide concentration range did not block the polymer binding to any extent. The IC_{50} values for the tested antagonists [216] were expressed relative to sLe^a-polymer as control and were in close agreement with either published values, or those obtained in assay.. On the other hand, these data could also indicate that the presence of ith E-selectin/IgG as a target molecule. One of the major advantageous of a cell-free assay using LecEGFFlag is an excellent reproducibility, stability and accuracy, i.e., there were no fluctuations in the results on a day-to-day or batch-to-batch base, as described for E-selectin/IgG. The good reproducibility of the assay makes it very suitable for the quantitative determination of sLe^a-polymer interaction with LecEGFFlag and particularly for the rapid screen and determination of inhibitory potencies of selectin antagonists. The cell-based assay with HL-60 was not functional since very low fluorescence values were obtained in control experiments. This is in agreement with peviously described studies with the LecEGF of E-selectin [90]. Kinetic analysis of molecules and ligand orientations supports the important role of domain orientation and exposure for ligand binding in cell-based assay. The fact that LecEGFFlag does not have consensus repeats that could improve lectin domain orientation might explain the lack of functionality in this type of assay.

6 Conclusions and Outlook

Selectins, belonging to the C-type lectin family of cell adhesion molecules, initiate the inflammatory cascade by mediating leukocytes tethering and rolling prior to the extravasation into the inflammed tissue. According to their expression topology, selectins have been termed L-(leukocytes), P-(platelets and endothelium) and E-(endothelium) selectin [85]. Although inflammation represents a physiological defense mechanism, an excessive leukocyte extravasation leads to numerous pathological and disease states including angiogenetic and metastatic cancer spread. These findings have strengthened the pharmacological hypothesis that inhibitors of selectin-ligand interactions would block the subsequent cell adhesion events preceding inflammation and would thus be prospective anti-inflammatory agents [321]. The physiological ligands of all three selectins contain a common tetrasaccharide epitope, the so-called sialyl Lewis^x (sLe^x). It serves as a lead structure in our search for selectin antagonists. The bioactive conformation of sLe^x bound to the full length extracellular E-selectin (lectin, EGF-like and 6 consensus repeat domains) has been solved by NMR technologies and enabled rational design of E-selectin antagonists [209,322,323]. The structures of truncated E- and P-selectin (lectin and EGF-like domains) co-crystalized with sLe^x have been solved as well [188]. Despite all these efforts, there are still no potent selectin antagonists on the market. To get detailed insight into selectin/ligand interactions refined structural studies including selectin co-crystallization with the second and third generation antagonists and NMR studies of E-selectin/ligand complexes with $^{15}N/^{13}C$ labeled protein should be conducted. For aforementioned structural studies, sufficient amounts of expressed, active and characterized protein are needed.

Recombinant human E-, P- and L-selectin/IgG have been expressed as secreted proteins in mammalian expression system (CHO cells) and purified to homogeneity by protein A affinity- and size exclusion chromatography. Activity of the expressed proteins has been confirmed in reaction with blocking monoclonal antibodies and in NMR studies ligand binding was confirmed. Although expressed in a highly glycosylated state, protein activity was resistant to the treatment with PNGaseF, i.e., partial removal of *N*-linked sugars.

Bioassays were developed in cell-free and cell-based formats with E-selectin/IgG to quantify binding affinity of in-house synthesized E-selectin antagonists. In a cell-free assay we utilized polymer-presented sLe^a as a control and IC_{50} values obtained for tested antagonists have been expressed relative to this ligand. In a cell-based format, physiological ligands were presented by HL-60 cells. However, the reproducibility of the assays was unsatisfactory with variations on

day-to-day and batch-to-batch base to accurately quantify inhibitory potencies of tested antagonists.

To fullfil the requirements for structural studies, we decided to express truncated form of E-selectin that contains only the lectin and EGF-like domains. This protein of approximately 20 kDa would be suitable for X-ray crystal structure determination (co-crystallization), as well as NMR studies with labeled protein. In addition, for kinetic studies with surface plasmon resonance, the smaller protein would be more suitable as well. Initially, LecEGF domains have been cloned and expressed in *E.coli* as either secreted into the medium, periplasma or in form of inclusion bodies. Subsequent protein refolding was not successful and despite preparative amounts of expressed protein (80-120 mg/L), yields of active protein obtained under a variety of refolding conditions (0.8-2%) were unsuitable for any scale-up.

In search for an alternative expression system lectin and EGF-like domains have been cloned and expressed in baculovirus infected insect cells. This eukaryotic expression system enables post translational modifications and disulfide bonds formation. Initially expressed intracellularly protein was inactive. Finally, LecEGF was cloned in a secretable form as a fusion protein with the C-terminal flag epitope (DYKDDDK) as an affinity tag. Protein expressed in *Sf9* and *Hi5* insect cells was secreted in the medium and purified to homogeneity by an anti-flag M2 antibodies-affinity column. Activity was confirmed by the intereaction with the monoclonal blocking antibody against human E-selectin and in STD NMR studies. LecEGFFlag was monomeric in solution, as judged by its migration profile in reducing, non-reducing and native conditions. Two glycosylation isoforms were present in solution with molecular weights of 19.96 kDa and 21.15 kDa. In denaturing conditions, deglycosylation with *N*-glycanase (PNGase F) was complete. In native conditions, however, deglycosylation remained incomplete. The expression of LecEGFFlag as secreted protein in baculovirus infected insect cells with yields of 18 mg/L is efficient enough to envisage protein production for structural studies. Directed protein secretion into the medium enabled it to pass through the endoplasmatic reticulum and Golgi where it was posttranslationally modified (glycosylated and with correctly formed disulfide bonds).

For the first time, a cell-free assay was developed with a truncated form of E-selectin. LecEGFFlag was used for the characterization of E-selectin antagonists with the sLe^a polymer as a control ligand. In addition, similar to the assay developed for E-selectin/IgG, the same concentrations of polymer and protein (0.1 μg/ml polymer and 3 μg/ml protein) were used in standard assay conditions. The assay has shown an excellent stability, reproducibility and

accuracy on day-to-day and batch-to-batch basis. Three characterized selectin antagonists have been tested for proof-of-concept and the results obtained were in close agreement with published data [216]. Therefore, we conclude that cell-free polymer assay developed with LecEGFFlag as a target protein is indeed suitable for evaluation and precise quantification of inhibitory potencies of E-selectin antagonists.

In outlook, LecEGFFlag expressed in baculovirus infected insect cells will be used for X-ray crystal structure analysis of E-selectin co-crystallized with second and third generation antagonists. Prior to these studies, LecEGFFlag should be further purified over an anti E-selectin blocking monoclonal antibody column to isolate only functional protein.

Selective $^{15}N/^{13}C$ amino acids labeling of the protein expressed in insect cells and subsequent NMR studies will further refine the characterization of the bioactive conformation of the ligand and the pharmacophores/amino acids involved in binding.

The expression system that was developed for E-selectin is straightforward and applicable for P- and L- selectin. Therefore, the structural characterization of ligand binding to these two selectins can be solved or significantly refined as well. More precise data could further point to differences in binding modes of all three selectins and contribute to the development of highly selective antagonists. These experiments and novel information will rapidly improve the search for potent selectin antagonists as anti-inflammatory agents.

7 References

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8 Abbreviations

ABTS	(2'2-azino-di(3-ethylbenzthiazoline)-6-sulfonic acid)
AcNPV	Autographa californica nuclear polyhedrosis virus
Arg	Arginine
Asn	Asparagine
Asp	Aspartate
ATP	Adenosine triphosphate
BCECF-AM	2',7'-Bis(2-carboxyethyl)-5(6)-carboxy-fluorescein tetrakis(acetoxymethyl) ester
BHK	Baby hamster kidney
BmPV	Bombyx mori polyhedrosis virus
BSA	Bovine serum albumine
cAMP	Cyclic adenosine monophosphate
CD	Cluster of definition
cDNA	Complementary deoxyribonucleic acid
CEA	Carcinoembrionic antigen
CH_3CN	Acetonitrile
CHO	Chinese hamster ovary
CIP	Calf intestine phosphatase
COS	African green monkey's kidney cells
CRD	Consensus repeat domains
Cys	Cystein
DTH	Delayed type hypersensitivity
DTT	Ditrhiothreithol
E.coli	Escherichia coli
EDTA	Ethylendiaminotetraacetic acid
EGF	Epidermal growth factor
E-LE	E-selectin lectin and EGF-like domains
E-sel/IgG	E-selectin lectin, EGF-like and consensus repeat domains fused to the Fc part of human IgG
FBS	Fetal bovine serum
FCS	Fetal calb serum
fMLP	formyl-methionyl-leucyl-phenylalanine
FPLC	Fast protein liquid chromatography
Fuc	Fucose
FucT	Fucosyl transferase
Gal	Galactose
G-CSF	Granulocyte-monocyte colony-stimulating factor
GlcNAc	N-acetyl glucosamine
Glu	Glutamine
GlyCAM	Glycosylation dependent cell adhesion molecule
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GuHCl	Guanidium hydrochloride
HAB	Hepes assay buffer
HEV	High endothelial venules
His	Histidine

HisLecEGF, HLE	E-selectin lectin and EGF-like domains with N-terminal 6XHistidine
HisPreLecEGF, HPLE	E-selectin lectin and EGF-like domains with N-terminal 6XHistidine and prescission protease restriction site
HL-60	Human promyelocytic leukemia cells
HTS	High throughput screening
HUVECs	Human umbilical vein endothelial cells
ICAM	Intracellular adhesion molecule
IFN-γ	Interferon γ
IgG	Imminoglobulin G
IL	Interleukin
IMAC	Immobilized metal ion affinity chromatography
IPTG	Isopropyl-_-D-Thiogalactosid
JNK	c-Jun *N*-terminal kinase
K_d	Dissociation constant
LB	Lauria Bretani
LDL	Low density lipoprotein
LE	E-selectin lectin and EGF-like domains
LecEGF	E-selectin lectin and EGF-like domain
LecEGFFlag	E-selectin lectin and EGF-like domains with C-terminal Flag epitope
LFA	Leukocyte function associated antigen
LPS	Lipopolysaccharide
L-sel/IgG	L-selectin lectin, EGF-like and consensus repeat domains fused to the Fc part of human IgG
M.O.I.	Multiplicity of infection
mAb	Monoclonal antibody
Mac	Macrophage antigen
MadCAM	Mucosal addressin cell adhesion molecule
MAPK	Mitogen-activated protein kinase
M-CSF	Mavrophage colony-stimulating factor
MECA	multi-endocrine cellular antigen
MEM	Minimal essential medium
MS	Mass spectrometry
NBT/BCIP	Nitro-blue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate
NCA	Non-specific cross reactive antigen
NeuNAc	N-acetylneuraminic acid
NF-κB	Nuclear factor kappa B
Ni-NTA	Nickel-nitrilotriacetic acid
NMR	Nuclear magnetic resonance
NOE	Nuclear overhouser effect
OD	Optical density
PBS	Phosphate buffer saline
PCLP	Podocalyxin-like protein
PCR	Polymerase chain reaction
PECAM	Platelet endothelial cell adhesion molecule
Pfu	Plaque forming units
P-LE	P-selectin lectin and EGF-like domains
PMN	Polymorphonuclear leukocytes

PMSF	Polymethylsulfonile fluoride
PNGaseF	*N*-glycosidase F
POD	Peroxidase
P-sel/IgG	P-selectin lectin, EGF-like and consensus repeat domains fused to the Fc part of human IgG
PSGL-1	P-selectin glycoprotein ligand-1
PST	Polystirene
RP-HPLC	Reversed phase high pressure liquid chromatography
RT	Room temperature
SDS-PAGE	Sodium-dodecyl-sulfat polyacrylamide-gel-electrophoresis
Sf	Spodoptera frugiperda
SFM	Serum-free medium
Sia	Sialic acid
sLe^a	Sialyl Lewis^a
sLe^x	Sialyl Lewis^x
SN	Supernatant
SPR	Surface plasmon resonance
STAT	Signal transducer and activator of transcription
STD	Saturation transfer difference
TBS	Tris buffered saline
TCR	T-cell antigen receptor
TEMED	N,N,N',N'-Tetramethylethylendiamine
TFA	Trifluoroacetic acid
TNF-α	Tumor necrosis factor α
TPO	Thrombopoetin
Tris	(Hydroxymethyl)-Aminomethan
TTBS	TBS with 0.05% Tween 20
Tyr	Tyrosine
VCAM-1	Vascular cell adhesion molecule
VLA	Very late antigen

9 Curriculum vitae

PERSONAL

Name: Zorica Dragic

Birth Date: 24.02.1974.

E-mail: zorica.dragic@unibas.ch

EDUCATION

2001 – 2005 University of Basel, Institute of Molecular Pharmacy, Basel, Switzerland

PhD thesis with Prof. Beat Ernst:

E-selectin as anti-inflammatory drug target:

- **Expression, purification and characterization for structural studies**
- **Assay development for antagonist evaluation**

1993 – 1999 University of Zagreb, Croatia

Faculty of Pharmacy and Biochemistry: M.Sc. in Medical Biochemistry

Faculty of Pharmacy and Biochemistry: M.Sc. in Pharmacy

1989 – 1993 Gymnasium, Osijek, Croatia

PARTICIPATION AND POSTER PRESENTATION

2004 University of Basel, Switzerland: Key Issues in Drug Discovery

Graduate study program in Drug Discovery and Development

2003 Pfizer, Sandwich, UK

Drug Discovery 2003, Meeting young scientists in drug discovery

Poster presentation

PUBLICATIONS

- Maj M, V. Bruno, Z. Dragic, R. Yamamoto, G. Battaglia, W. Inderbitzin, N. Stoehr, T. Stein, F. Gasparini, I. Vranesic, R. Kuhn, F. Nicoletti, P.J. Flor.

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i

Attachment 1:

Human E-selectin bibliography:

Expasy address: http://au.expasy.org/cgi-bin/niceprot.pl?P16581

Name: LYAM2_HUMAN

Synonyms: Endothelial leukocyte adhesion molecule 1 (ELAM-1)

Leukocyte-endothelial cell adhesion molecule 2 (LECAM2)

CD62E antigen

Domain organization:

SIGNAL	1 21	21	
CHAIN	22 610	589	E-selectin.
DOMAIN	22 556	535	Extracellular *(Potential)*.
TRANSMEM	557 578	22	*Potential*.
DOMAIN	579 610	32	Cytoplasmic *(Potential)*.
DOMAIN	22 139	118	C-type lectin.
DOMAIN	140 175	36	EGF-like.
DOMAIN	178 239	62	Sushi 1.
DOMAIN	240 301	62	Sushi 2.
DOMAIN	303 364	62	Sushi 3.
DOMAIN	366 427	62	Sushi 4.
DOMAIN	429 490	62	Sushi 5.
DOMAIN	491 549	59	Sushi 6.

Attachment 2:

SsLecEGFFlag sequences:

DNA

SSLecEGF+Flag

```
1   ATGCTACTAG TAAATCAGTC ACACCAAGGC TTCAATAAGG AACACACAAG
60  CAAGATGGTA AGCGCTATTG TTTTATATGT GCTTTTGGCG GCGGCGGCGC
120 ATTCTGCCTT TGCGTGGTCT TACAACACCT CCACGGAAGC TATGACTTAT
180 GATGAGGCCA GTGCTTATTG TCAGCAAAGG TACACACACC TGGTTGCAAT
240 TCAAAACAAA GAAGAGATTG AGTACCTAAA CTCCATATTG AGCTATTCAC
320 CAAGTTATTA CTGGATTGGA ATCAGAAAAG TCAACAATGT GTGGGTCTGG
380 GTAGGAACCC AGAAACCTCT GACAGAAGAA GCCAAGAACT GGGCTCCAGG
440 TGAACCCAAC AATAGGCAAA AAGATGAGGA CTGCGTGGAG ATCTACATCA
500 AGAGAGAAAA AGATGTGGGC ATGTGGAATG ATGAGAGGTG CAGCAAGAAG
560 AAGCTTGCCC TATGCTACAC AGCTGCCTGT ACCAATACAT CCTGCAGTGG
620 CCACGGTGAA TGTGTAGAGA CCATCAATAA TTACACTTGC AAGTGTGACC
680 CTGGCTTCAG TGGACTCAAG TGTGAGCAAA TTGTGGACTA CAAGGACGAT
740 GACGATAAGT AA
```

Protein:

```
1   MLLVNQSHQG FNKEHTSKMV SAIVLYVLLA AAAHSAFAWS YNTSTEAMTY
50  DEASAYCQQR YTHLVAIQNK EEIEYLNSIL SYSPSYYWIG IRKVNNVWVW
100 VGTQKPLTEE AKNWAPGEPN NRQKDEDCVE IYIKREKDVG MWNDERCSKK
150 KLALCYTAAC TNTSCSGHGE CVETINNYTC KCDPGFSGLK CEQIVDYKDD
200 DDK
```

1-37: Signal sequence of gp67a insect acidic glycoprotein

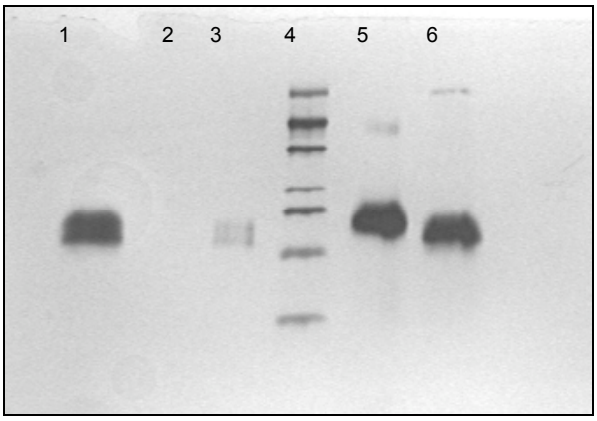
38-194: LecEGF of human E-selectin (1-157)

195-202: Flag tag: DYKDDDDK

Underlined: potential N-glycosylation sites

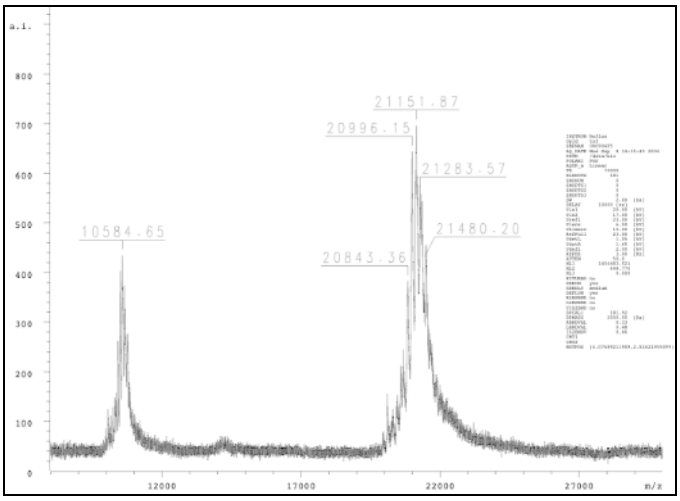
Attachment 3:

Molecular weight determination of LecEGFFlag by MS

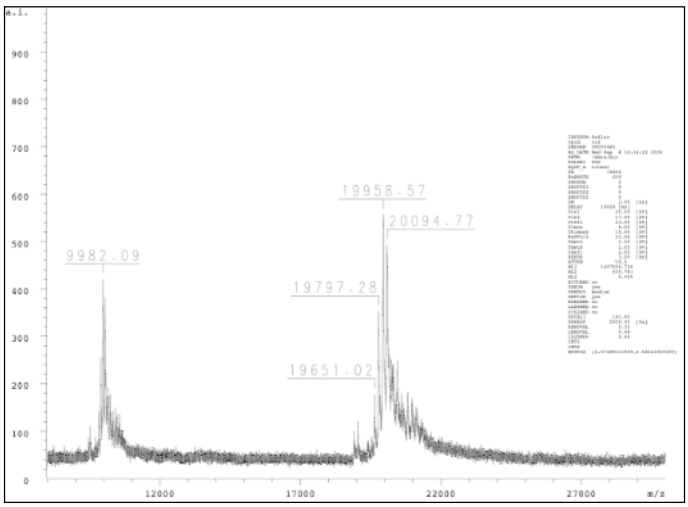


Lane 1.: Load, non-separated LecEGFFlag, 4 μg
Lane 2.: -
Lane 3.: Load, non separated LecEGFFlag, 0.5 μg
Lane 4.: LMW marker
Lane 5.: Peak 1
Lane 6.: Peak 2

RP-HPLC desalting and separation of two protein species of LecEGFFlag



MW determination of peak 1: Heterogenuos protein populati with prepredominant MW of 21.15 kDa.



MW determination of peak 2: Heterogenuos protein populati with prepredominant MW of 19.96 kDa.