Degradation of vasopressin precursor and pathogenic mutants in diabetes insipidus

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Abbreviations

ADH	Anti-diuretic hormone
ADNDI	Autosomal dominant neurohypophyseal diabetes insipidus
ALLN	N-Acetyl-leucyl-leucyl-norleucinal
AQP2	Aquaporin 2
ATF 6	Activating transcription factor 6
AVP	Arginine vasopressin
b-ZIP	Basic leucine zipper
CFTR	Cystic fibrosis transmembrane conductance regulator
COP I	Coat protein I
COP II	Coat protein II
CPY*	Mutated vacuolar enzyme carboxypetidase
DI	Diabetes insipidus
E1	Ubiquitin activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin-protein ligase
EDEM	ER degradation-enhancing 1,2-mannosidase-like protein
eIF2α	Eukaryotic translation initiation factor 2
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
ERGIC	ER-Golgi intermediate compartment
ERSD	ER storage disease
ERSE	ER stress response element
Glc	Glucose
GlcNAc	N-acetylglucosamine
GRP	Glucose-regulated protein
GT	UDP-glucose:glycoprotein glucosyltransferase
JIK	Jun N-terminal inhibitory kinase
Man	Mannose
MRI	Magnetic resonance imaging
NP II	Neurophysin II
OST	Oligosaccharyltransferase

PAI-2	Plasminogen activator inhibitor-2
PDI	Protein disulfide isomerase
PERK	Protein kinase-like ER kinase
PKA	Protein kinase A
PrP	Prion protein
PrP^{Sc}	Prion protein scrapie (infectious)
PTHrP	Parathyroid hormone-related peptide
RAP	Receptor-associated protein
RB	Russell bodies
S1P	Site-1 protease
S2P	Site-2 protease
SP	Signal peptidase
SRP	Signal recognition particle
TGN	Trans-Golgi network
TRAM	Translocating chain-associated membrane protein
TRAP	Translocon-associated protein
UBA	Ubiquitin-associated domain
UBL	Ubiquitin like domain
UPR	Unfolded protein response
UPRE	Unfolded protein response element
V2R	Vasopressin receptor V2
VP	Vasopressin

Summary

The nonapeptide hormone, arginine vasopressin, plays a decisive role in the regulation of fluid balance by reducing free water clearance through reabsorption of water in the renal collecting ducts. Mutations in the gene encoding arginine vasopressin cause autosomal dominant neurohypophyseal diabetes insipidus, a disease characterized by excessive urine production and strong thirst. Post mortem examination of affected individuals suggests a selective degeneration of vasopressinergic neurons in the hypothalamus. On a molecular level, the disease is linked to a trafficking defect. Mutant vasopressin precursor is retained in the endoplasmic reticulum, while the wild-type is transported to mature secretory granules at synaptic processes. How this trafficking defect of the vasopressin precursor is interrelated with the degeneration of neurons is unknown. A plausible hypothesis is that mutant proteins, or degradation products thereof, are toxic to neurons.

Accordingly, we analyzed the fate of mutant vasopressin precursor arrested in the endoplasmic reticulum of transfected cell lines. Proteasomal, but not lysosomal, inhibitors induced stabilization of mutant precursors and the accumulation of three distinct non-glycosylated cytosolic species: pre-pro-vasopressin, pro-vasopressin, and an N-terminally truncated form. These results provide evidence that mutant precursor, after translocation into the ER lumen, is retrotranslocated to the cytosol and degraded by the proteasome. Furthermore, a fraction of the newly synthesized precursor, even of wild-type, was found not to be translocated, but to be synthesized into the cytosol due to inefficiency of the vasopressin signal peptide.

In autosomal dominant neurohypophyseal diabetes insipidus, neurotoxicity may thus result from degradation intermediates and/or by ER retention directly. Both mistargeted and retrotranslocated proteins add to the cytosolic pool of these degradation products. Neurodegeneration might occur in heterozygous individuals once a critical concentration of toxic material is exceeded.

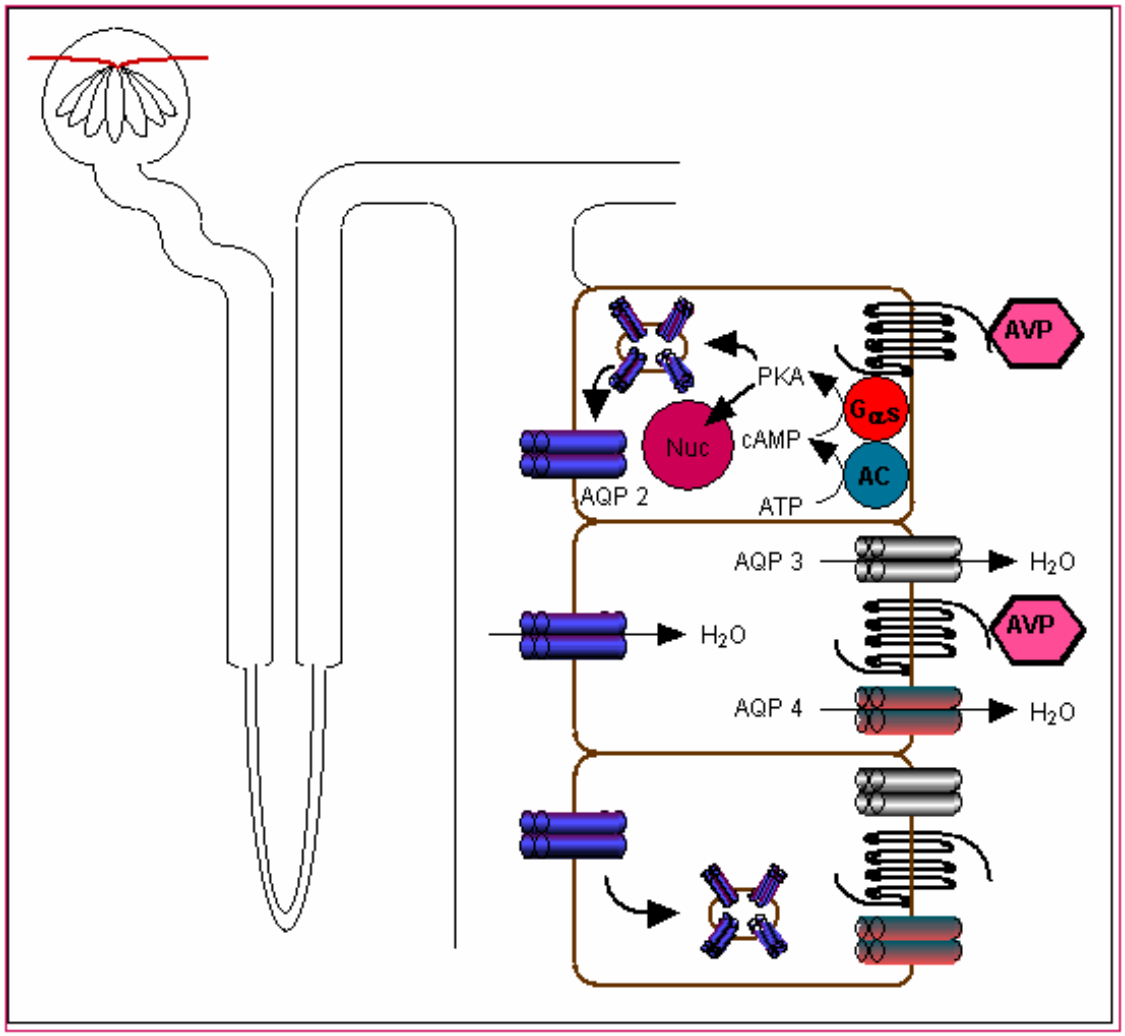
Introduction

1. Diabetes insipidus

1.1 Water regulation by the antidiuretic hormone

Water homeostasis is critical to survival of cells and organisms. Accordingly, the balance of water uptake and excretion needs to be carefully controlled. We ingest water as part of our daily diet. Loss of water occurs during the maintenance of body temperature by sweating, or when removing waste products by either urination or defecation. Additional moisture is lost during exhalation, but the largest loss of fluid will ordinarily occur through urination. The amount of urine an individual produces varies depending on kidney activity, while the quantity of water filtered from the blood passing through the kidney remains relatively constant under physiological conditions. An adult human produces about 180 litres of primary filtrate per day, of which about 90% is held back by the proximal compartments of the nephron. The remaining 10% reach the distal collecting tubules, where water reabsorption is controlled by anti-diuretic hormone (ADH), also known as vasopressin (VP). ADH-dependent water reabsorption reduces the volume we excrete each day to an approximate 1% of the original primary filtrate.

Vasopressin synthesis occurs in magno- and parvocellular neurons of the hypothalamus. Hypovolemia and/or hypernatremia stimulate release of vasopressin to the blood stream. Water reabsorption in the kidney is achieved via a G-protein coupled receptor cascade. Vasopressin from the bloodstream binds to the vasopressin receptor V2 (V2R) at the basolateral side of the cell in renal collecting ducts (Figure 1). This leads to an elevation of intracellular cAMP levels by the activation of adenylate cyclase and subsequent phosphorylation of the water channel aquaporin 2 (AQP2) at its cytoplasmic C-terminus by protein kinase A. Consequently, transport vesicles containing AQP2 fuse with the apical plasma membrane and permit increased reabsorption of water from the collecting ducts. In parallel, synthesis of AQP2 is induced. Water molecules leave the cell through aquaporin 3 and 4 channels at the basolateral membrane. Removal of AVP from the V2R results in the internalisation of AQP2, ending the cycle (Rutishauser and Kopp, 1999; Levin et al., 2001).



Rutishauser and Kopp (1999)

FIG. 1. Arginine vasopressin (AVP) binds to its receptor and activates protein kinase A (PKA), inducing fusion of aquaporin 2 containing vesicles with the plasma membrane. Water resorption persists as long as AVP remains bound to its receptor. Subsequently AQP2 is reinternalized.

1.2 Disorders of water homeostasis

Failure to efficiently concentrate urine will lead to polyuria and consequently polydipsia. This clinical condition, characterized by the production of large quantities of dilute urine, is known as diabetes insipidus (DI). Three mechanisms are known to cause DI. Deficiency of VP itself causes neurohypophyseal diabetes insipidus. Renal resistance to the antidiuretic action of VP, e.g. due to injury of the nephron or to mutations in the VP receptor or the aquaporins, results in nephrogenic diabetes insipidus. Finally, inappropriate and excessive drinking without a somatic cause leads to dipsogenic diabetes insipidus.

DI and its subtypes can be diagnosed by a water deprivation test. It compares plasma osmolality with urine osmolality during a defined time period of water deprivation, and assesses the response of these parameters to exogenous VP administration. Neurohypophyseal diabetes insipidus results from acquired pathologies affecting the vasopressinergic cells, such as trauma, infiltrating or inflammatory diseases, or tumors. Rarely, the disease is congenital due to mutations in the gene encoding pre-pro-vasopressin. This disease is called autosomal dominant neurohypophyseal diabetes insipidus (ADNDI). Although it is rather rare, the disorder shows a high penetrance with symptoms beginning weeks to months after birth. Presentation of neurohypophyseal diabetes insipidus implies destruction or loss-of-function of more than 80% of magnocellular neurons. Further clinical information may be obtained from magnetic resonance imaging (MRI). In normal subjects the posterior pituitary shows a high intensity signal on T1-weighted images. Absence of such a signal has been correlated with neurohypophyseal DI, although studies have failed to display a strict cosegregation of morphological abnormalities and clinical symptoms (Miyamoto et al., 1991; Rutishauser et al., 1996; Gagliardi et al., 1997).

1.3 Vasopressin: gene structure and mutational analysis in ADNDI

The vasopressin gene, located on chromosome 20p13, consists of three exons (Figure 2). The first exon encodes a signal peptide of 19 amino acids, followed by the 9 amino acid hormone, a 3 amino acid linker, and the 9 N-terminal amino acids of neurophysin II (NPII), vasopressin's carrier protein. Exon 2 encodes 67 amino acids, which form the central part of NPII. Exon 3 constitutes the C-terminal 17 amino acids of NPII, a single amino acid linker, and a C-terminal glycopeptide of 39 amino acids, also known as copeptide, bearing a single N-linked glycosylation site.

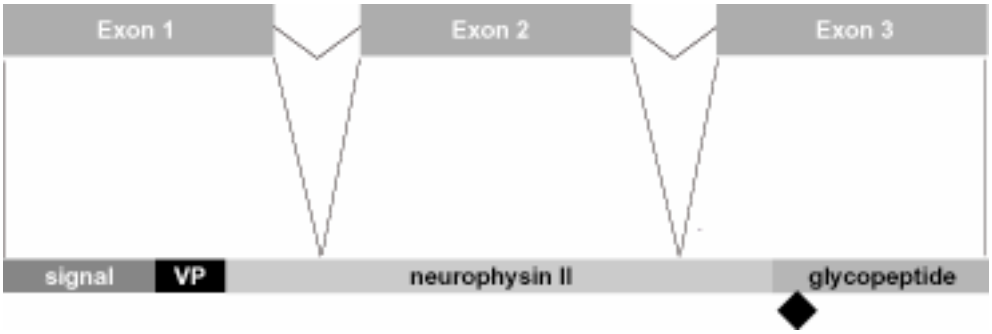


FIG. 2.Gene structure and the spliced precursor of vasopressin.

Mutations in the VP-NPII gene cause autosomal dominant neurohypophyseal diabetes insipidus. The phenotype is not linked to a specific mutation or set of mutations in the vasopressin gene, and no hot spots have been determined. More than 40 mutations spread over all three exons are known to cause a similar clinical phenotype (Figure 3 and Table I). The observed mutations are located mainly in neurophysin II, although some have been found in the signal peptide and the hormone itself, including the only mutation known to cause recessive inheritance. This exception likely results from the reduced binding affinity of the mutant vasopressin to its receptor in the collecting duct (Willcutts et al., 1999). No mutations have been found in the copeptide so far. Interestingly, severity of the phenotype appears largely independent of a particular mutation. Moreover, individuals carrying the same mutant may exhibit various degrees of polyuria, and symptoms may regress with age (Rutishauser et al., 1996).

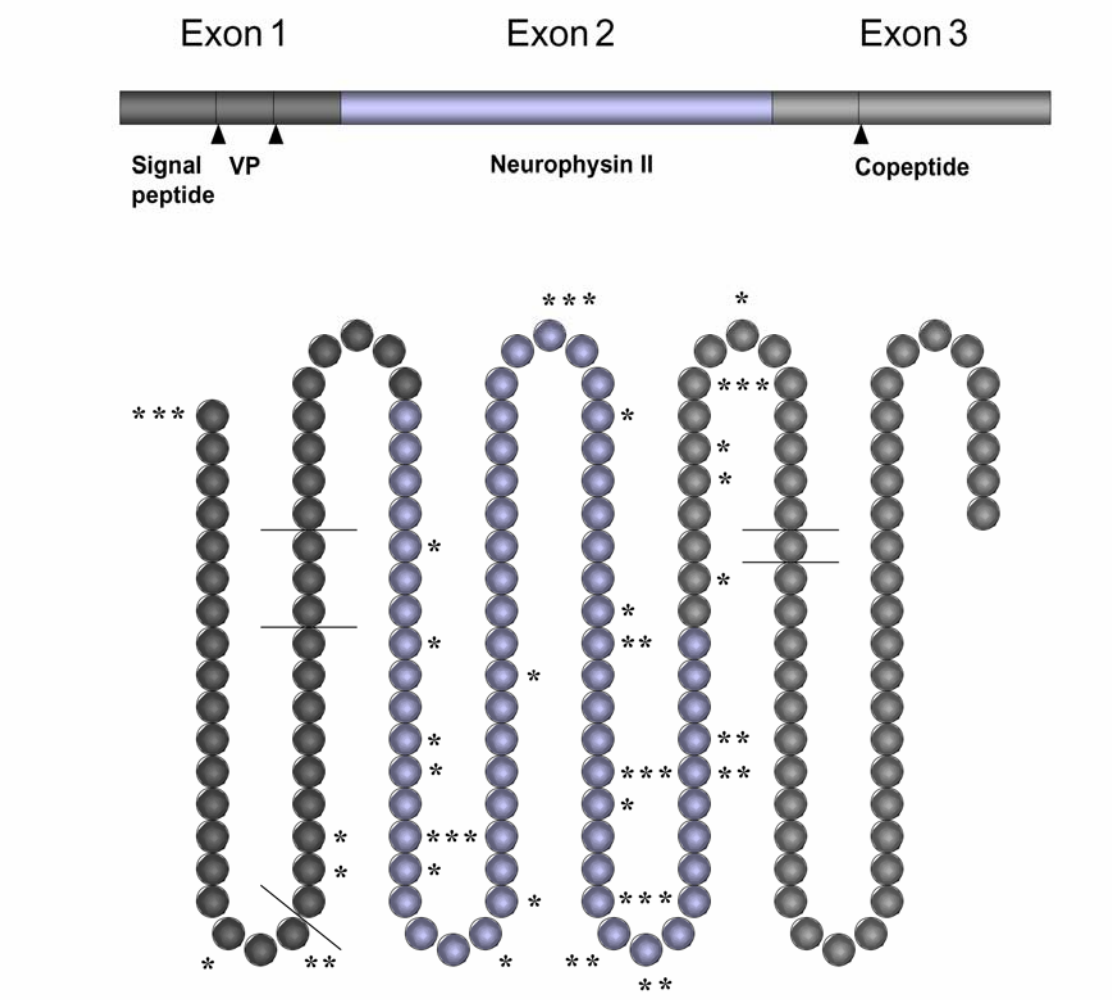


FIG. 3. Scheme depicting the domains of pre-pro-vasopressin (above) and the sites of known mutations (below). Processing sites are marked by triangles. Lines indicate domain borders. Asterisks mark sites of a known mutation. Note that more than one mutation has been found at some sites.

Table I

Exon	Nucleotide #	Base Mutation	Amino Acid #	Amino Acid Substitution	Kindred	Reference
1	225	A→G	SP -19 to -16	del MPDT	1	Christensen (2004)
	227	G→A	SP -19 to -16	del MPDT	1	Christensen (2004)
	227	del G	SP -19 to -16	del MPDT	1	Rutishauser (1996)
	274	C→T	SP -3	S→F	1	Rittig (1996)
	279	G→A	SP -1	A→V	8	Ito (1993) McLeod (1993) Rittig (1996) Calvo (1998) Siggaard (1999) Boson (2003) Christensen (2004)
	280	C→T	SP -1	A→T	3	Rittig (1996) Heppner (1998) Christensen (2004)
	285	T→C	VP 2	Y→H	2	Rittig (1996) Rittig (2002)
	287-289	del CTT	VP 3	del F	1	Wahlstrom (2004)
2	1730	G→C	NP 14	G→R	1	Rittig (1996)
	1740	G→T	NP 17	G→V	1	Bahnsen (1992)
	1748	C→T	NP 20	R→C	1	Rittig (1996)
	1751	T→C	NP 21	C→R	1	Gonking (1997)
	1757	G→C	NP 23	G→R	3	Heppner (1998) Rutishauser (2002) Christensen (2004)
	1757	G→A	NP 23	G→R	1	Calvo (1999)
	1758	C→T	NP 23	G→V	1	Gagliardi (1997)
	1761	C→T	NP 24	P→L	1	Repaske (1994)
	1772	T→C	NP 28	C→R	1	Hansen (1997)
	1773	G→A	NP 28	C→Y	1	Skordis (2000)
	1774-1776	del CGC	NP 28/29	del C/ A→W	1	Fluck (2001)
	1797	T→C	NP 36	V→A	1	Christensen (2004)
	1824-1829	del AGG	NP 47	del E	7	Yuasa (1993) Rittig (1996) Mahoney (2002) Christensen (2004) Ye (2005)
	1829	G→A	NP 47	E→K	1	Miyakoshi (2004)
	1830	A→G	NP 47	E→G	2	Rittig (1996) Christensen (2004)
	1839	T→C	NP 50	L→P	1	Rittig (1996)
	1857	C→T	NP 56	S→F	1	Grant (1998)
	1859	G→A	NP 57	G→S	2	Ito (1991) Rittig (1996)
	1859	G→C	NP 57	G→R	1	Rittig (1996)
	1872	G→C	NP 61	C→S	2	Rittig (1996) Bullmann (2002)
	1872	G→A	NP 61	C→Y	2	Grant (1998) Rutishauser (2002)

	1873	C→A	NP 61	C→X	3	Rittig (1996) Grant (1998) Christensen (2004)
	1874	G→T	NP 62	G→W	1	Nagasaki (1995)
	1883	G→T	NP 65	G→C	2	Rittig (1996) Christensen (2004)
	1884	G→A	NP 65	G→D	1	Christensen (2004)
	1884	G→T	NP 65	G→V	2	Ueta (1996) Rauch (1996)
	1886	C→T	NP 66	R→C	1	Rutishauser (1999)
	1887	G→C	NP 66	R→P	1	Mundschenk (2001)
	1889	T→G	NP 67	C→G	1	DiMeglio (2001)
	1891	C→A	NP 67	C→X	1	Nagasaki (1995)
	1907	T→G	NP 73	C→G	1	Christensen (2004)
	1908	G→T	NP 73	C→F	1	Santiprabhob (2002)
	1910	T→C	NP 74	C→R	1	Rutishauser (2002)
	1911	G→A	NP 74	C→Y	1	Fujii (2000)

3	2094	C→A	NP 79	C→X	1	Rittig (1996)
	2101	G→T	NP 82	E→X	2	Calvo (1998)
	2106-2107	CG→GT	NP 83	E→X	2	Rittig (1996) Bullmann (2002)
	2110	T→G	NP 85	C→G	2	Abbes (2000) Nijenhuis (2001)
	2110	T→C	NP 85	C→R	1	Abbes (2000)
	2112	C→G	NP 85	C→W	1	Christensen (2004)
	2116	G→T	NP 87	E→X	1	Rittig (1996)

X = stop codon

1.4 ER retention of mutant VP precursor

Like many secreted molecules, vasopressin is cotranslationally targeted to the endoplasmic reticulum (ER). Once the N-terminal pre-pro-hormone has entered the ER, signal peptidase removes the signal peptide. The folding of the pro-hormone is assisted by chaperones and the binding of vasopressin to the pocket of folded neurophysin II stabilizes dimerization of the pro-hormone (de Bree and Burbach, 1998). In addition, the copeptide is modified by attachment of a glycan to asparagine at position 5. If quality control is passed, the hormone travels via the ER-Golgi intermediate compartment (ERGIC) to the Golgi apparatus, where the glycan is further modified. At the trans-Golgi network (TGN), the pro-hormone is sorted to the regulated secretory pathway. Immature secretory granules containing higher oligomers of pro-vasopressin bud off from the TGN, and as they mature, prohormone convertase 1 cleaves the prohormone and frees the vasopressin peptide. Mature secretory granules travel along the axon to the cell periphery and are stored near the synapse for subsequent regulated release. Vasopressin circulates freely in the blood stream, although it does bind to specific receptors on platelets. Circulating vasopressin has a half-life of 5 to 15 minutes. Endothelial and circulating endo- and amino-peptidases are responsible for its eventual degradation, and plasma levels are low under basal conditions.

Several groups have studied the effect of the mutations on pre-pro-hormone trafficking using heterologous expression systems.

Stable expression of the wild-type and a mutant (G17V) in the pituitary cell line AtT-20 showed a distinct difference in processing and secretion of the molecules (Olias et al., 1996). The wild-type precursor was correctly glycosylated and processed, and NPII was detected in the culture medium. The mutant was core-glycosylated but remained endoglycosidase H-sensitive, indicating that the protein did not reach the trans-Golgi network. Secretion was drastically reduced. Immunofluorescence studies showed that NPII in cells expressing the wild-type was concentrated in the tips of the cell processes where secretory granules accumulate. In G17V cells, NPII staining was restricted to the ER, determined by colocalization with the ER-resident protein BiP.

Beuret et al. (1999) expressed the ΔG227 mutation, leading to a truncated signal peptide, in COS-7 cells. Glycosylation of the resulting precursor showed the truncated signal to be functional for ER-import. However, most of the precursor was observed with a higher apparent molecular weight, suggesting a failure in signal peptide cleavage. The mutant

precursor was almost completely retained in the ER, corroborated by costaining for the ER resident protein p63.

Nijenhuis et al. (1999) studied a variety of mutants by stably expressing them in the neuroendocrine cell line Neuro2a and the rat pheochromocytoma cell line PC12/PC2. When comparing G14R, E47R, ΔE47, G57S, and G65V to the wild-type, all mutants were found to be impaired in processing and secretion, albeit to different extents (in decreasing order of impairment: G65V≥G14R>ΔE47≥E47G>G57S). Sensitivity to endoglycosidase H indicated retention of the precursors in the ER. Immunofluorescence studies using transiently transfected Neuro2a cells demonstrated that the mutant prohormone was found in large accumulations in the ER, which colocalized with the ER marker protein disulfide isomerase (PDI).

Analogous studies with the E87X mutant in PC12/PC2 and the mouse pituitary cell line AtT-20 also showed reduced processing and secretion and colocalization with PDI (Nijenhuis et al., 2000).

Expression studies in PC12/PC2 cells using wild-type vasopressin and the C85G mutant confirmed previous observations. Processing and secretion was only observed for the wild-type (Nijenhuis et al., 2001). The mutant was retained in the ER, as indicated by endoglycosidase H sensitivity. Transient transfection of Neuro2a cells demonstrated that the mutant was not only confined to the normal reticular ER. Costaining with PDI showed these areas to represent enlarged ER subcompartments. Such large areas of altered ER morphology could be responsible not only for severe dysfunction but also for death of the host cells *in vivo* (Aridor and Hannan, 2000; Rutishauser and Spiess, 2002).

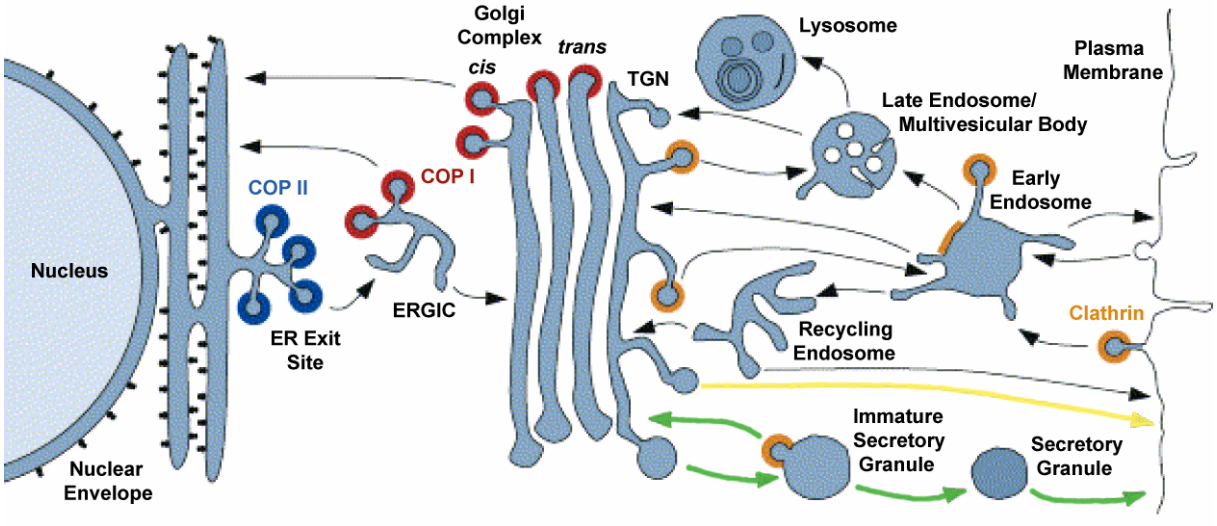
Siggaard et al. (1999) examined the signal peptide mutant A(-1)T in Neuro2a cells. They observed an 8-fold reduction in vasopressin secretion when compared to the wild-type, accompanied by the accumulation of improper signal cleavage. The precursor was found to colocalize with glucose-regulated protein 78, indicating an ER localization.

Although the degree of retention varies among different mutants, inefficient ER exit leading to reduced secretion of mutant hormone precursors apparently is a common denominator in the pathogenesis of ADNDI. In order to understand the processes leading to this trafficking defect, we need to briefly look at the mechanisms involved in secretion of proteins from the cell.

2. The secretory pathway

Hormones are molecules acting on target tissues which are typically remote from the site of production. In order for secretion to occur, the proteins must thus enter the secretory pathway. While all cells possess the means to constitutively release proteins form the cell, endocrine cells need to regulate hormone release in response to a specific stimulus. Therefore, they are endowed with an additional release mechanism, the regulated secretory pathway (Figure 4).

If a protein is to be released from the cell, it is targeted to the ER. In mammalian cells this is a cotranslational process. As the newly synthesized polypeptide emerges into the ER lumen, it may be modified by core glycosylation. In addition, chaperones promote proper folding. Before the secretory protein can leave the ER it needs to pass quality control, a mechanism which prevents further transport of incorrectly folded precursors, such as proteins which are either non-functional or thermo-labile because they have not attained their native conformation. The protein then travels via the ERGIC to the Golgi apparatus. Here further modifications such as complex glycosylation or sulfation may occur. The TGN is the compartment where proteins destined for regulated secretion are segregated from those taking other pathways. The hormone precursors are sorted to immature secretory granules, which mature as they travel to the cell periphery. During the maturation process, the pro-hormones are activated by pro-hormone convertases. The mature secretory granules are then stored near the cell periphery, from where they can be rapidly released in response to the proper stimuli.



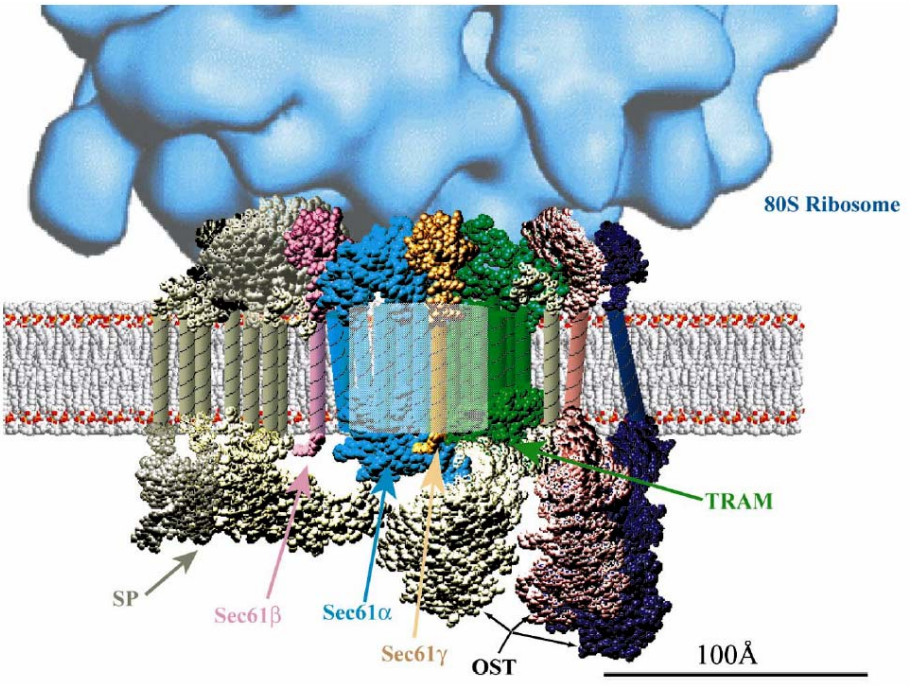
Bonifacino and Glick (2004)

FIG. 4. Schematic representation of the intracellular transport system. Some vesicle budding and cargo selection steps are mediated by known coat components (e.g. coat proteins I (COPI) or II (COPII), or clathrin). Others, such as sorting to immature secretory granules, remain to be determined. Constitutive secretory pathway (yellow) and regulated secretory pathway (green) are highlighted.

2.1 ER entry/translocation

Protein secretion is initiated by targeting the molecule to the ER. Proteins possess signal sequences, exhibiting a stretch of hydrophobic amino acids. As these amino acids emerge from the ribosome, they are recognized and bound by the signal recognition particle (SRP). Binding of SRP attenuates translation and by the binding of SRP to the SRP receptor, the nascent peptide chain is targeted to the ER. Entry into the ER lumen is conferred by the translocon, a gated channel formed by the heterotrimeric sec61 αβγ complex (Figure 5). Concurrent with entry, signal peptidase cleaves the signal peptide.

Entry into the ER, however, depends on additional components. Reconstitution experiments using lipid vesicles demonstrated the requirement of translocating chain-associated membrane protein (TRAM) beside the heterotrimeric sec61 complex and SRP receptor (Gorlich and Rapoport, 1993).



Johnson and van Waes (1999)

FIG. 5. Ribosome docked to the translocon complex. Only one of the Sec61 heterotrimers (Sec61αβγ) is depicted. SP: signal peptidase, OST: oligosaccharyltransferase, TRAM: translocating chain-associated membrane protein

While these are the essential components required for translocation, some substrates such as the prion protein are inefficiently or improperly translocated using this minimal system. Translocon-associated protein (TRAP) complex was found to alleviate this inefficiency (Fons et al., 2003). Other substrates were found to be only partially dependent on TRAP. Analysis of their respective signal sequences implied that this dependence was linked to a functional property of the signal, rather than a physical parameter such as hydrophobicity. The efficiency of the signal in targeting the nascent chain to the ER, specifically gating activity, correlates inversely with TRAP dependence. The gating step commits the substrate to initiate translocation of its N-terminus. Substrates whose signal sequence shows weaker gating activity exhibit a stronger requirement of TRAP during their translocation than those with stronger gating activity.

The signal sequence itself also carries information which determines the efficiency of ER import. Critical factors are the length of the hydrophobic region in the signal, the overall

distribution of charged amino acids with respect to the hydrophobic region, and the position of the signal in relation to the start codon, i.e. the size of the N-terminal region synthesized prior to the signal (Wahlberg and Spiess, 1997; Goder and Spiess, 2001).

2.2 Quality control in the ER

Transfer of nascent chains to the ER via the Sec61 translocon is accompanied by the binding of several ER resident proteins which stabilize and modify them. Quality control in the ER can be subdivided into different levels. Primary quality control applies to all proteins, working in a general fashion. Secondary quality control, on the other hand, is restricted to selected categories of proteins.

Shared structural and biophysical features distinguishing native from non-native conformation are the basis for primary quality control. These include exposed hydrophobic regions, unpaired cysteine residues, and the tendency to aggregate. The molecular chaperones involved in primary quality control include members of the heat shock protein family such as BiP/GRP78 and glucose-regulated protein 94 (GRP94), the lectins calnexin and calreticulin, and the thiol-disulfide oxidoreductases PDI and Erp57. Acting in concert, these molecules ensure the retention of incompletely folded precursors and unassembled oligomers. Correctly folded proteins are not detected by the system and are free to leave the ER.

The cues allowing the targeting of these chaperones to non-native peptide chains have only been partially determined. Some information is available for BiP as well as for the calnexin/calreticulin system. *In vitro* data suggests that BiP binds heptapeptides bearing aliphatic amino-acid side chains in alternating positions (Flynn et al., 1991). The binding of BiP is limited by the heptapetides accessibility. Only exposed areas bearing the sequence are used in protein folding.

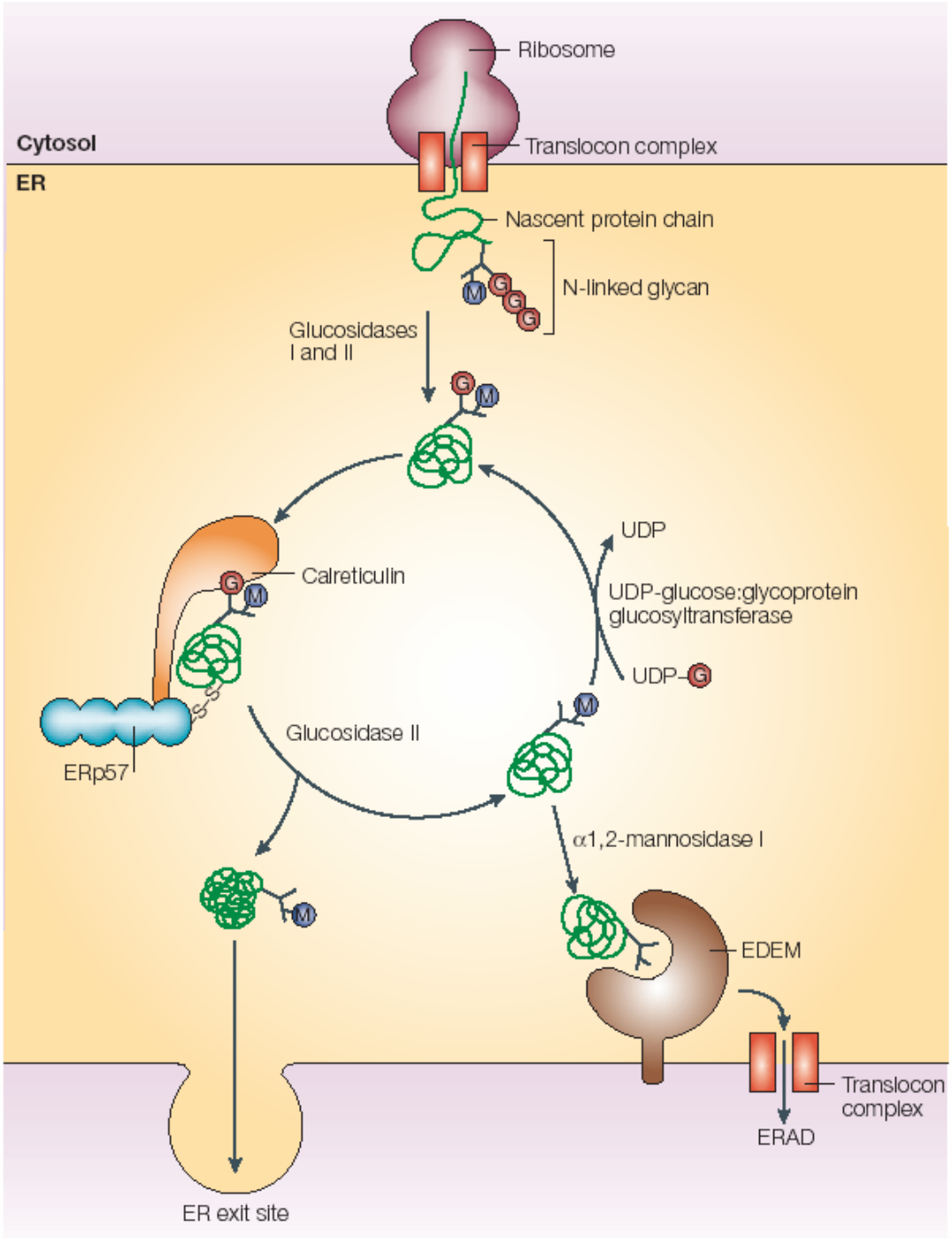
The calnexin/calreticulin cycle is involved in the quality control of glycoproteins (Figure 6). Improperly folded material is retained in the ER, and if failure to reach the native conformation persists, eventually targeted for degradation. The two lectins interact with trimmed intermediates of N-linked core glycans on nascent protein chains. They recognize the $Glc_1Man_7GlcNAc_2$ structure, where Glc is glucose, Man is mannose, and GlcNAc is N-acetylglucosamine. Either the soluble calreticulin, the transmembrane calnexin, or both lectins together are associated with most glycoproteins synthesized in the ER. Both lectins form

complexes with the thiol-disulfide oxidoreductase Erp57. Erp57 forms transient disulfide bonds with calnexin/calreticulin-bound glycoproteins.

Two independent enzymes mediate the timer for retention and release in the control cycle. Glucosidase II hydrolyses glucose from monoglucosylated core glycans and permits dissociation of the glycoprotein from the respective lectin. UDP-glucose:glycoprotein glucosyltransferase (GT) reglucosylates the substrate if the glycoprotein is improperly folded and thus allows for reassociation of the substrate with the lectin (Parodi, 2000). GT serves as a folding sensor which prevents exit of improperly folded protein by tagging chains with glucose. *In vitro* experiments suggest the presence of hydrophobic amino-acid clusters as the recognition motif for reglucosylation (Caramelo et al., 2003). The cycle of deglucosylation and reglucosylation continues until the protein has reached the proper conformation, or until the chain is targeted for degradation.

Trimming of a mannose from the middle branch of the glycan by ER α1,2-mannosidase I targets glycoproteins for degradation. Removal of the mannose leads to an association of the oligosaccharide with ER degradation-enhancing 1,2-mannosidase-like protein (EDEM). ER α1,2-mannosidase I acts more slowly than glucosidase II and GT. Only terminally misfolded proteins which have failed to attain proper conformation during multiple cycles of deglucosylation and reglucosylation are removed from the calnexin/calreticulin cycle and eliminated (Molinari et al., 2003).

Acting in concert with the primary quality control mechanisms, select subsets of proteins are subject to additional scrutiny. Secondary quality control relies on specific recognition mechanisms for individual proteins or protein families (Figure 7). The proteins involved in secondary quality control, which is often cell type specific, have been grouped into three classes: ‘outfitters’, ‘escorts’, and ‘guides’ (Herrmann et al., 1999).



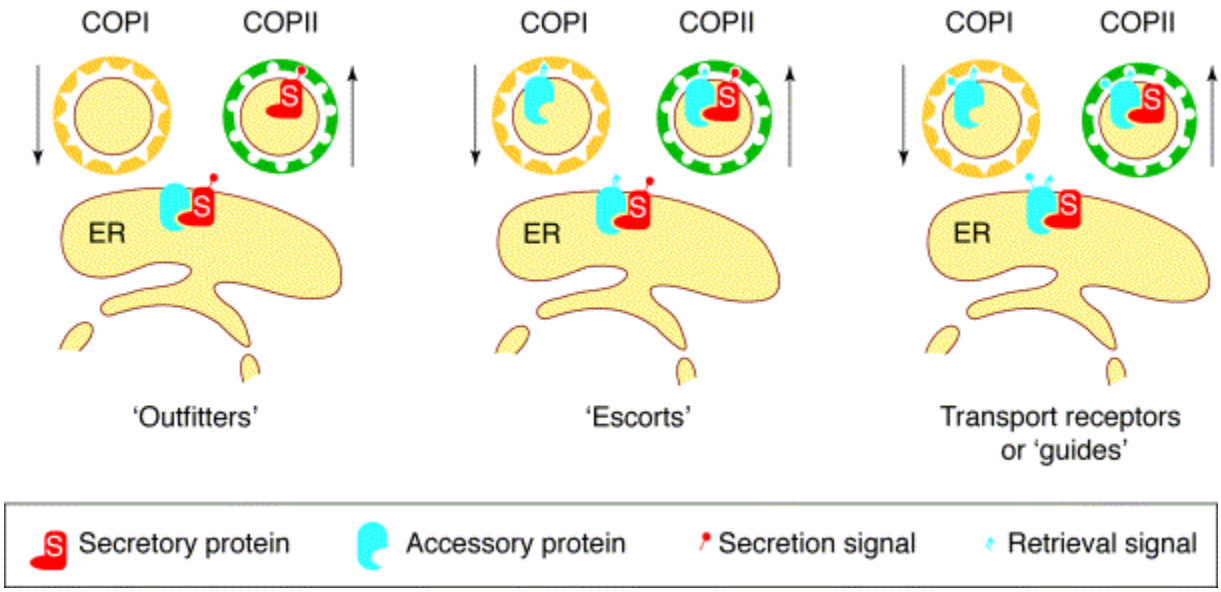
Ellgaard and Helenius (2003)

FIG. 6. Scheme depicting the steps of quality control undertaken by an N-linked glycoprotein. The processing of the core glycan tree helps ensure folding fidelity. Properly folded proteins will eventually leave the ER via exit sites, while terminally misfolded material is targeted for degradation via the cytosolic proteasome pathway. M (mannose), G (glucose)

Outfitters are ER resident proteins which help establish or maintain secretion competence of secretory proteins. They function as folding catalysts and chaperones. One example is a yeast integral ER membrane protein, Shr3p. While it is non-essential, it is necessary for proper trafficking of general amino acid permease to the plasma membrane. In its absence, the amino acid permeases are retained in the ER in an aggregated state (Kota and Ljungdahl, 2005).

Escorts act similarly to outfitters, but they leave the ER and cycle to the Golgi. They include regulatory molecules which prevent premature activation of their substrates. Receptor-associated protein (RAP) is a well-characterized example of this category. RAP binds and stabilizes newly synthesized receptors of the low-density lipoprotein receptor family. In the absence of RAP, premature binding of ligand to the receptor leads to receptor aggregation in the ER, followed by the degradation of the receptor-ligand complex. Ordinarily, the receptor-RAP complex dissociates in the Golgi due to the lower pH, from where RAP cycles back to the ER (Bu and Schwartz, 1998)

Guides or transport receptors, differ from the previous groups in that they provide information required for the selective uptake of cargo into transport vesicles. They bind to the secretory protein and interact directly or indirectly with the coat proteins, serving as adaptors which cycle between ER and Golgi. One well-known representative of the guides is the lectin ERGIC-53 which cycles between ER and Golgi. It serves as a transport receptor for certain proteins bearing high-mannose N-linked glycans (Appenzeller et al., 1999).



Hermann and Schekman (1999)

FIG. 7.Overview of the three different classes of accessory proteins involved in secondary quality control.

3. UPR and degradation of ER-retained proteins

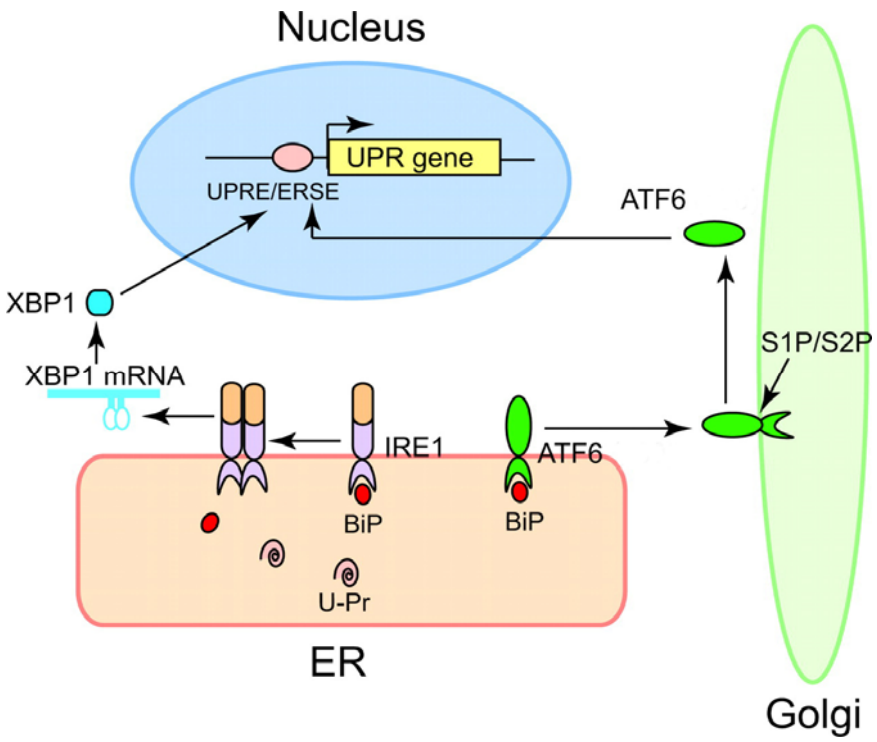
3.1 UPR

The presence of irrevocably misfolded proteins in the ER is detrimental to the cell over a prolonged time period. The material interferes with proper trafficking by detaining components of the secretory pathway. Two processes aid eukaryotic cells to reduce this bulk load: ER-associated degradation (ERAD) and the unfolded protein response (UPR). The two responses are interconnected. Induction of UPR leads to an increase in ERAD capacity, while efficient ERAD is dependent on an intact UPR. Loss of ERAD results in a constitutive UPR induction. Loss of both ERAD and UPR strongly diminishes cell viability.

One of the key classes of target genes of UPR is ER-resident chaperones. An increase in chaperone concentration helps to reduce the amount of unfolded proteins and precludes their aggregation.

One of the elementary players in the unfolded protein response (Figure 8) is IRE1, an ER transmembrane glycoprotein (Liu and Kaufman, 2003; Zhang and Kaufman, 2004). It encompasses kinase and RNase activities in the cytoplasmic domain and a BiP interacting domain in the lumenal domain. BiP regulates IRE1 activity in an inverse manner. If unfolded proteins accumulate, the reduction of available BiP will result in the dimerization and autophosphorylation of IRE1. This in turn activates its RNase activity to catalyse the splicing of the mRNA of its substrate, transcription factor XBP1. The spliced product activates transcription of UPR target genes in the nucleus, many of which bear the mammalian ER stress element in the promoter region.

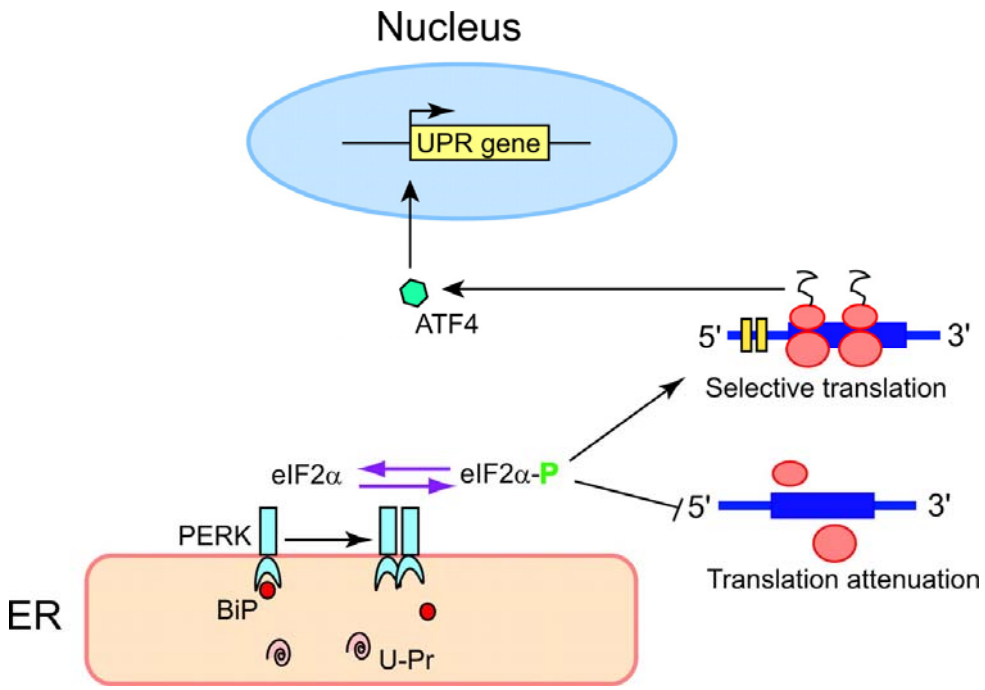
Higher eukaryotic cells possess two additional UPR transducers: activating transcription factor 6 (ATF6), and the double-stranded RNA-activated protein kinase-like ER kinase (PERK). ATF6 possesses an N-terminal basic leucine zipper (b-ZIP) domain in the cytosol and a C-terminal ER-stress sensing domain in the ER lumen. Upon ER-stress, ATFα and ATFβ transit to the Golgi compartment where they are cleaved by site-1 protease (S1P) and site-2 protease (S2P) yielding cytosolic fragments which activate transcription of their target genes in the nucleus. These include ER-resident molecular chaperones and folding enzymes.



Zhang and Kaufman (2004)

FIG. 8. Illustration of the interplay of IRE1 and ATF6 in promoting the expression of genes involved in both the unfolded protein response and the ER stress response via the respective response elements (UPRE and ERSE).

Similar to IRE, PERK contains a lumenal stress-sensing domain and a cytosolic domain which phosphorylates the α-subunit of eukaryotic translation initiation factor 2 (eIF2α) under ER stress conditions (Figure 9). This phosphorylation leads to a general translation initiation attenuation, by reducing the formation of translation initiation complexes. The cell is, therefore, confronted with less newly synthesized proteins to fold, freeing chaperones to alleviate the current burden. Some mRNAs have a lower requirement for eIF2α. Their initiation complex is enhanced. One such example is ATF4, which serves not only as a feedback loop to reverse the phosphorylation of eIF2α and release translational attenuation, but also induces expression of CHOP upon longer UPR induction



Zhang and Kaufman (2004)

FIG. 9. The effect of PERK on general translation, as well as its influence on induction of specific unfolded protein response genes.

Prolonged activation of UPR leads to apoptotic cell death through two of the key players. Activated IRE1 recruits Jun N-terminal inhibitory kinase (JIK) and TRAF2. JIK activates apoptosis-signaling kinase 1, which in turn activates JNK and mitochondria/Apaf1-dependent caspases. TRAF2 release from the ER-associated apoptosis effector procaspase-12, permits the clustering and activation of caspase 12. It acts on caspase 9 which in turn activates caspase 3, leading to apoptosis.

The b-ZIP transcription factor CHOP induced by prolonged UPR via the eIF2α and ATF4 pathway activates caspase 3 through unknown intermediates leading to cell death (Kaufman, 2002; Rutkowski and Kaufman, 2004).

In yeast, activation of UPR induces transcription of several genes encoding ERAD proteins. Among these are ubiquitin-conjugating enzymes and ubiquitin-ligases, as well as a player of the (retro-)translocon, Hrd3. Studies in mammalian cells have demonstrated the necessity of the IRE-XBP1 signaling in regulating ERAD. Notably, induction of EDEM depends solely on this pathway. The efficient targeting of defective glycoproteins for degradation is therefore preceded by the activation of the unfolded protein response.

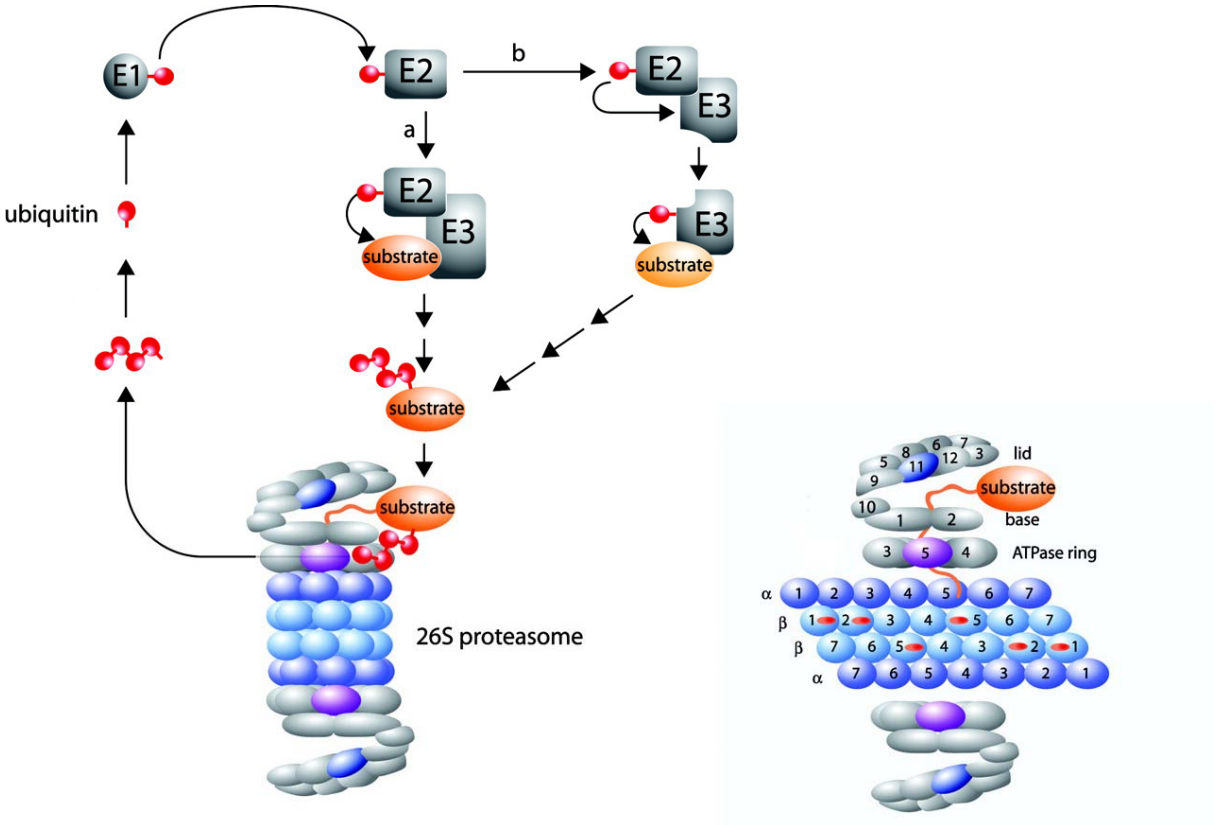
3.2 Retrotranslocation and degradation

It has been estimated that as much as 30% of all newly synthesized proteins fail to pass quality control and need to be degraded (Schubert et al., 2000). Degradation of such misfolded proteins occurs outside the ER, thus requiring retrotranslocation. The protein destined for degradation is unfolded and exits the ER via the Sec61 complex (Wiertz et al., 1996). Studies in yeast using mutated vacuolar enzyme carboxypetidase Y (CPY*) have elucidated many of the players involved in ER quality control and degradation (Kostova and Wolf, 2003). The transport from ER to the cytosol requires directionality. This might be mediated by the existence of two subsets of translocons with distinct compositions (Plemper et al., 1999). An important player for the removal of proteins from the ER to the cytosol is the Cdc48-Ufd1-Npl4 complex. Cdc48, p97 in mammals, belonging to the AAA ATPase family, is thought to pull the polypeptide chain through the pore in an ATP-dependent manner (Ye et al., 2001). Cdc48/p97 possesses a homo-hexameric ring structure. ATP hydrolysis promotes a strong conformational change. The emerging model suggests that the substrate emerges from the (retro-)translocon and becomes polyubiquitinated which allows binding of the Cdc48-Ufd1-Npl4 complex. The conformational change of ATP hydrolysis then serves as the racheting mechanism providing unidirectionality of retrotranslocation.
The tagging of proteins for degradation by ubiquitin is a multi-step process (Figure 10). Initially, ubiquitin is activated by a trans-esterification reaction requiring ATP which attaches it to a cysteine residue of ubiquitin activating enzyme (E1). The ubiquitin is subsequently transferred to the cysteine residue of a number of ubiquitin-conjugating enzymes (E2). These may transfer the ubiquitin directly to the substrate, although a third class of enzymes, ubiquitin-protein ligases (E3) are often involved as facilitators.
Efficient delivery of proteins destined for degradation by the proteasome requires the presence of at least four ubiquitin moieties. The initial tag is extended by the attachment of additional ubiquitin to the preceding individual, usually to Lys48. The factors driving chain elongation have not been determined, although it is thought that the presence of additional component such as ubiquitylation factor E4 may be required.

Delivery of ubiqitinated ER substrates occurs via Dsk2p and Rad23p (Medicherla et al., 2004). Both proteins contain an N-terminal ubiquitin like domain (UBL) which may interact with the regulatory particle of the proteasome, and a C-terminal ubiquitin-associated domain (UBA) permitting the binding of polyubiquitin chains. Furthermore, Rad23p is thought to

play a role in deglycosylation of retrotranslocated glycoproteins by recruiting N-glycanase (Suzuki et al., 2001).

The tagged substrate can bind to the 26S proteasome, the active degradation machinery, which deubiquitylates and unfolds it prior to degradation. The proteasome consists of the 20S core cylinder encompassing three catalytically active sites, and two 19S cap components responsible for recognition, binding and unfolding of ubiquitnated proteins. The cap consists of two functionally distinct parts, the base and the lid. The lid is necessary for deubiquitylation, freeing ubiquitin for another round of protein targeting. The base, consisting of a ring of six ATPases, both binds the tagged substrate and is thought to control access to the core in a similar manner to Cdc48 in retrotranslocation.



Kostova and Wolf (2003)

FIG. 10. The cycle undertaken by ubiquitin moieties used in tagging defective protein substrate for degradation (right). Schematic of the 26S proteasome with a linearized core segment. The active sites are marked in red (left).

4. Neurotoxicity hypothesis in ADNDI

Mutations in a secretory protein like pro-vasopressin are likely to disturb correct folding, leading to retention of the protein by quality control. Such material will typically be degraded via the ubiquitin-proteasome pathway. In a heterozygous situation, one might expect the products of the wild-type allele to generate functional hormone, possibly of a reduced amount. However, the ADNDI mutations listed in Table I are all dominant.

The mechanism explaining dominant inheritance has currently not been determined. Dimerization of pro-vasopressin, beginning in the ER, might explain retention of the product of the wild type allele only to some extent. The dimers consisting of one mutant and one wild type pro-hormone would likely fail to pass quality control, leading to their eventual degradation. Ito et al (1999) addressed this question when they epitope-tagged the wild-type and several mutants (A(-1)T, ΔE47, G57S, and C67X) and transfected them into human embryonic kidney cells, tsa 201. Crosslinking experiments revealed homo- and heterodimer formation between wild-type and mutant precursors. Furthermore, mutant precursor was found to inhibit trafficking of wild-type precursor from the ER to the Golgi apparatus. Ultimately, fewer vasopressin-containing secretory granules would be available for release at the cell periphery. However, the cell might adapt to the stronger demand for vasopressin by increasing expression of the gene. This would still result in a recessive phenotype. Notably, in similar experiments we were not able to confirm interaction of wild-type and mutant vasopressin precursor.

Clinical data from individuals affected by diabetes insipidus has led to the development of a hypothesis about the aetiology of the disease. A decrease in circulating vasopressin paralleled by gliosis and hypocellularity of vasopressinergic neurons in the hypothalamus supported the notion that vasopressin mutants exert a toxic effect on their host cells, resulting in neurodegeneration. Support for this has been obtained from a limited number of post mortem studies (Hanhart, 1940; Gaupp, 1941; Braverman et al., 1965; Nagai et al., 1984; Bergeron et al., 1991). The selective destruction of the vasopressinergic magnocellular cells expressing mutant hormone would explain the dominant inheritance. Progressive cell death could account for the gradual development of clinical symptoms. How the mutants exert this detrimental effect remains the major open question in understanding the disease.

Support for the toxic hypothesis came from other heterologous expression studies. Ito et al. (1997) used stably transfected Neuro2a cells to asses an effect of vasopressin mutants on cell viability. Expression of mutant precursors (A(-1)T, ΔE47, G57S, and C67X) as such was not found to considerably affect growth of cells. When the cells were differentiated to a neuronal phenotype with valproic acid, however, viability was significantly reduced (C67X>A(-1)T>G57S>ΔE47). Concurrent metabolic labeling and immunofluorescence studies confirmed the reduced secretion of mutant precursors and their accumulation within the ER observed by other groups

Beuret et al. (1999) investigated the molecular consequences of the signal peptide mutant ΔG227. The mutation leads to translation initiation at an alternative ATG site, and displays reduced cleavage of the signal peptide. Transient transfection of COS-7 cells showed that the mutant was retained in the ER, and led to the formation of disulfide-linked aggregates. This was not surprising since the uncleaved signal contains an unpaired cysteine at position -11. Abolishing this unpaired cysteine did not alter the properties of the precursor protein. The double mutant ΔG227/ C(-11)S showed a similar phenotype as ΔG227, ER-localized precursor with an uncleaved signal peptide. To determine if uncleaved signal peptide interferes with disulfide bond formation, a cysteine residue in the vasopressin region of a wild-type precursor was altered to serine. The mutant, C6S, mimicked the aggregation phenotype, while being largely confined to the ER, despite proper cleavage of the signal peptide. Evidently, two independent processes, unpaired cysteine residues leading to aberrant disulfide bond formation, and interference of uncleaved signal peptide with correct folding, contribute to ER retention and formation of disulfide-linked aggregates.

The accumulation of material in the ER is a phenomenon common to a group of disorders known as ER storage diseases (ERSD)(Rutishauser and Spiess, 2002). The material stems from nascent proteins failing quality control mechanisms in the ER. The disease phenotypes may result from the deficiency of a particular protein at its site of action, these are generally recessive diseases, or the mutant protein may exert a toxic effect. In the latter case, the mutant protein itself or one of its degradation products is detrimental to cell viability. Mutant proteins might aggregate and thus prevent further transport, detaining chaperones and clogging the ER until parts are rendered non-functional and need to be degraded.

The use of animal models to examine ADNDI has met with limited success. A mutation (del G in NP 65, aka Brattleboro mutation) causes a frameshift leading to a strongly altered C-

terminus. The precursor, bearing a poly Lys tail extending beyond the translational stop codon, causes a recessive disease phenotype in the rat (Schmale et al., 1984). Furthermore, the C67X mutation failed to reproduce a human disease phenotype in rats (Si-Hoe et al., 2000). Although the mutant was retained in the ER and water homeostasis was affected, no cell death or atrophy of magnocellular neurons was observed. Nevertheless, a recent paper supported the neurodegeneration hypothesis in ADNDI using a murine knock-in model (Russell et al., 2003). Mice expressing the C67X neurophysin II mutation showed polyuria and polydipsia which worsened with progressing age. This was paralleled by loss of neurons in the supraoptic and paraventricular nuclei, along with an induction of the chaperone BiP in these cells. Furthermore, VP gene products could not be found in the neuronal projections, indicating a trafficking defect. The observed neurodegenereation, however, did not appear in mice expressing the common A(-1)T mutation.

5. Aim of this thesis

Experiments have shown ADNDI to be linked to a defect in protein trafficking. Protein precursors of mutant vasopressin were found to accumulate in the ER. In addition, experiments in our lab have demonstrated the formation of disulfide-linked aggregates formed by mutant- but not wild-type vasopressin precursor (Beuret et al., 1999). Whether the appearance of aggregates plays a direct or indirect role in the progression of the disease will warrant further examination. The accumulation of proteins in the ER has been linked to various classes of human disorders. Generally, terminally misfolded secretory protein precursors are removed from the ER by shuttling them into the ERAD pathway. Apparently, mutant vasopressin leads to toxic proteins or degradation fragments thereof, resulting in cell death.

To investigate the basis of the neurotoxic effect, we decided to examine the mechanism of degradation of ADNDI mutant pro-vasopressin.

Results

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Degradation of Wild-type Vasopressin Precursor and Pathogenic Mutants by the Proteasome*

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Mutations in the gene encoding the antidiuretic hormone arginine vasopressin cause autosomal dominant neurogenic diabetes insipidus. Autoptic data in affected individuals suggest that the neurons expressing mutant vasopressin undergo selective degeneration. Expression studies have shown that the mutants are retained in the endoplasmic reticulum, but how this trafficking defect is linked to neurotoxicity is unknown. One possibility is that unsecreted mutant precursors, or degradation products thereof, are cytotoxic. We therefore investigated the fate of endoplasmic reticulum-retained pathogenic mutants. Our data show that the mutants are retrotranslocated to the cytosol and degraded by the proteasome. In the presence of proteasomal inhibitors, three distinct un- or deglycosylated cytosolic species of vasopressin precursors were stabilized: pre-pro-vasopressin, pro-vasopressin, and an N-terminally truncated form. In addition to the retrotranslocated forms, a fraction of the newly synthesized precursor was not translocated, but was synthesized into the cytosol due to inefficient function of the vasopressin signal peptide. As a result, cytosolic pre-pro-vasopressin and its degradation product were also recovered when wild-type vasopressin was expressed. Cytosolic forms of vasopressin might trigger cytotoxicity *in vivo*, as has been proposed in the case of prion protein, which also contains an inefficient N-terminal signal peptide.

The antidiuretic hormone, arginine vasopressin, is synthesized in vasopressinergic neurons of the hypothalamus as a precursor consisting of three moieties (Fig. 1): the 19-amino-acid signal sequence, the nonapeptide hormone, the vasopressin-associated carrier protein neurophysin II, and a 39-amino-acid glycopeptide (copeptide) with a single *N*-glycosylation site (1). The precursor is cotranslationally targeted to the ER,[1] where the signal is cleaved off by signal peptidase and the copeptide is core glycosylated. The prohormone contains a total of eight disulfide bonds. After complex glycosylation in the Golgi apparatus, the matured precursor is cleaved into its three moieties and targeted to secretory granules at the distal end of the axons. From there, the hormone is released into the circulation upon osmotic and non-osmotic stimuli. Vasopressin binds to its receptor on cells of the renal collecting duct, initiating a signaling cascade that leads to the mobilization of aquaporin-2 water channels, allowing regulated water reabsorption. Through this mechanism, vasopressin mediates the conservation of as much as ~20 liters of fluid/day, thereby playing an important role in water homeostasis.

Lack of circulating vasopressin causes diabetes insipidus. Affected individuals suffer from polyuria and polydipsia due to the inability to concentrate their urine. In rare cases, the condition is caused by mutations in the vasopressin gene and is inherited in an autosomal dominant manner (2, 3). Around 40 mutations have been reported that alter the signal peptide (4, 5), the hormone (6, 7), or the neurophysin II moieties, respectively (8–14). Autosomal dominant neurohypophyseal diabetes insipidus (ADNDI) appears to be a neurodegenerative disease. Postmortem histologic examinations revealed only few magnocellular neurons and scar tissue replacing much of the vasopressinergic nuclei (15–18). A degenerative process specific to cells expressing the mutant protein would also explain the complete penetrance in heterozygous individuals (19) and the delayed onset of the symptoms weeks to months after birth. The neurodegeneration hypothesis was further supported by a study that showed decreased viability of cultured cells stably expressing mutant vasopressin (20). A number of expression studies have shown that the mutant vasopressin precursors are retained in the ER (11, 20–24). Together, the data suggest a cytotoxic effect of retained mutant precursors or of their degradation products. We therefore studied the degradation of vasopressin mutants associated with ADNDI and found it to occur by the proteasomal machinery following retrotranslocation into the cytosol. Analysis of the degradation intermediates furthermore showed that a significant portion of the primary translation products fails to enter the ER lumen. Both pathways of degradation, via the ER lumen and directly from the cytosol, were also found to some extent for the wild-type protein. The cytotoxic effect of mutant vasopressin prohormone may result from processes that are quantitatively, but not fundamentally, different from those occurring in cells expressing the wild-type protein.

EXPERIMENTAL PROCEDURES

Plasmids and Constructs—cDNAs for the wild-type vasopressin precursor and the mutants A−1T, ΔE47, and G57S were a gift from M. Ito (Northwestern University, Chicago, IL). The signal peptide of enkephalin was fused to wild-type and ΔE47 pro-vasopressin and, to delete the vasopressin hormone sequence, to the wild-type neurophysin II-glycopeptide sequence (in the same manner as described in Ref.

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[1] The abbreviations are: ER, endoplasmic reticulum; ADNDI, autosomal dominant neurohypophyseal diabetes insipidus; DMEM, Dulbecco's modified Eagle's medium; ALLN, *N*-acetyl-leucyl-leucyl-norleucinal; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PrP, prion protein.

Degradation of wild-type vasopressin precursor and pathogenic mutants by the proteasome

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Summary

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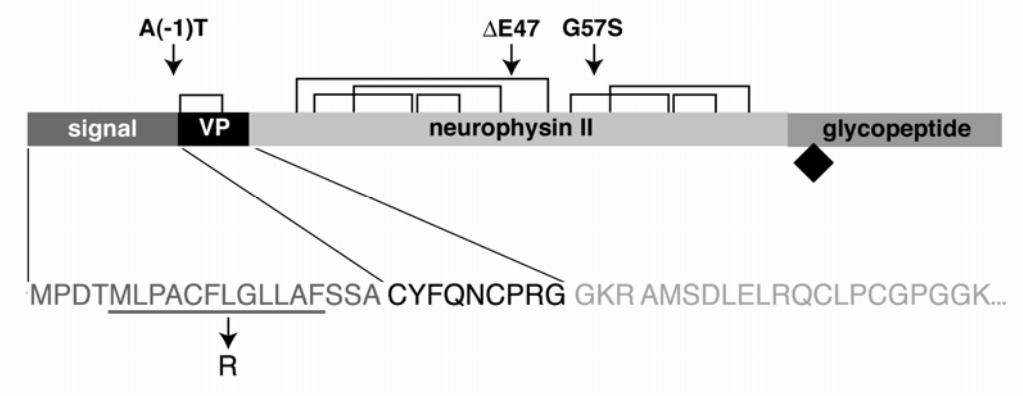

FIG. 11. **Wild-type and mutant vasopressin precursor and its signal sequence.** The domain organization of the vasopressin precursor is shown with disulfide bridges as line connections and the glycosylation site as a diamond. The positions of mutations in mutant vasopressin precursors used in this study are indicated by an arrow. Below, the sequence of the signal peptide and the N-terminal portion of pro-vasopressin is shown. To prevent ER targeting, L(-9) of the signal peptide was mutated to R as indicated.

Autosomal dominant neurohypophyseal diabetes insipidus (ADNDI) appears to be a neurodegenerative disease. Post-mortem histologic examinations revealed only few magnocellular neurons and scar tissue replacing much of the vasopressinergic nuclei (Hanhart, 1940; Gaupp, 1941; Braverman et al., 1965; Bergeron et al., 1991). A degenerative process specific to cells expressing the mutant protein would also explain the complete penetrance in heterozygous individuals (Mahoney et al., 2002) and the delayed onset of the symptoms weeks to months after birth. The neurodegeneration hypothesis was further supported by a study which showed decreased viability of cultured cells stably expressing mutant vasopressin (Ito and Jameson, 1997). A number of expression studies have shown that the mutant vasopressin precursors are retained in the ER (Olias et al., 1996; Beuret et al., 1999; Ito et al., 1999; Nijenhuis et al., 1999; Siggaard et al., 1999; Evans et al., 2000). Together, the data suggest a cytotoxic effect of retained mutant precursors or of their degradation products.

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Experimental Procedures

Plasmids and constructs

cDNAs for the wild-type vasopressin precursor and the mutants A(-1)T, ΔE47, and G57S were a gift from M. Ito (Northwestern University, Chicago, IL). The signal peptide of enkephalin was fused to wild-type and ΔE47 pro-vasopressin and, to delete the vasopressin hormone sequence, to the wild-type neurophysin II-glycopeptide sequence (in the same manner as described in (Cescato et al., 2000). Point mutations D(-17)R, L(-9)R, and R(8)E/K(11)E/R(12)E, were generated by polymerase chain reaction. All cDNAs were subcloned into the pRc/RSV expression plasmid (Invitrogen) and verified by DNA sequencing.

Cell culture and transient transfection

COS-1 and CV-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine at 37°C in 7.5% CO_2. Neuro2A cells COS-1 cells were transiently transfected in 6-well plates using Lipofectine (Life Technologies, Inc.) and used 2–3 days after transfection. Neuro2a were grown in DMEM containing 4500 mg/l glucose in 5% CO_2. They were transfected using Metafectene (Biontex Laboratories). To produce stably expressing cell lines, the cDNA of the vasopressin precursor was subcloned into the expression vector pCB6 and transfected into CV-1 cells using calcium phosphate precipitation. Clonal cell lines resistant to 0.5 mg/ml G418-sulfate were isolated and screened for pro-vasopressin expression by immunofluorescence.

Metabolic labeling and immunoprecipitation

For labeling experiments, transfected cells were starved for 30 min in DMEM without cysteine and methionine (Sigma) supplemented with 2 mM L-glutamine. Cells were labeled for the times indicated with 100 µCi/ml [^{35}S] protein labeling mix (DuPont-NEN) in starvation medium and chased in starvation medium supplemented with excess cysteine and methionine. Cells were transferred to 4°C, washed with phosphate-buffered saline (PBS), lysed in 500 µl of lysis buffer (PBS, 1% Triton X-100, 0.5% deoxycholate, 2 mM phenylmethylsulfonyl fluoride), and scraped. After 10 min centrifugation in a microfuge, the lysate was subjected to immunoprecipitation using rabbit polyclonal anti-neurophysin II or anti-vasopressin antibodies (ICN). The immune complexes were isolated with protein A-Sepharose (Zymed) and analyzed by electrophoresis on 10% polyacrylamide Tris/trycine SDS-gels and autoradiography. For deglycosylation, immunoprecipitates were either boiled for 2 min in 50 µl 50 mM Na-citrate, pH 6, 1% SDS and incubated with 1 mU endo-β-N-acetylglucosaminidase H (Roche Biochemicals) for 2 h at 37°C, or they were boiled in 100 µl 0.1 M Na-phosphate, pH 6.8, containing 50 mM EDTA, 1% β-mercaptoethanol, 0.1% SDS and incubated with 0.25 U endoglycosidase F/N-glycosidase F (Roche Biochemicals) for 3 h at 37°C

Protease inhibition

Stock solutions of 10 mM N-Acetyl-leucyl-leucyl-norleucinal (ALLN), 1 mM lactacystin, 1 mM pepstatin A (all from Sigma) in DMSO, and of 10 mM leupeptin (Roche Biochemicals) in water were prepared. For application to the cells, they were diluted into DMEM to final concentrations of 250 µM ALLN, 25 or 40 µM lactacystin, 100 µM leupeptin, and 5 µM pepstatin A. ALLN was added to the cells 90 min and lactacystin 10 min prior to the experiment and was freshly added to the starvation, labeling, and chase media. The lysosomal inhibitors leupeptin and pepstatin A were applied to the cells 16 h before the experiment and were present during all further incubations.

Cytosol extraction

To separate the cytosol from microsomes, labeled cells were incubated at 4°C in swelling buffer (15 mM HEPES/KOH, pH 7.2, 15 mM KCl) supplemented with 2 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (from a 500-fold concentrated stock of 1 mg/ml each of pepstatin A, leupeptin, chymostatin, antipain, and 5 mg/ml benzamidine, dissolved in 40% DMSO and 60% ethanol) for 15 min at 4°C, scraped, and centrifuged for 30 min at 136000×g. The supernatant containing the cytosol and the resuspended organelle pellet were subjected to immunoprecpitation and analyzed as above.

Results

Proteasome inhibitors stabilize mutant vasopressin precursors and degradation intermediates

To test the fate of wild-type and mutant pre-pro-vasopressin in COS-1 cells, transiently transfected cells were radiolabeled with [^{35}S]methionine/cysteine for 1 h and chased with excess unlabeled methionine/cysteine for 0 or 6 h. Cells and media were subjected to immunoprecipitation using an antibody directed against neurophysin II followed by SDS-gel electrophoresis and fluorography (Fig. 2A, lanes 1–8). Upon pulse-labeling, wild-type protein and the mutants ΔE47 and G57S were found as a major species of ~21 kDa corresponding to N-glycosylated pro-vasopressin. The mutant A(-1)T, in which mutation of the last residue of the signal sequence causes inefficient signal cleavage (Ito et al., 1993), appeared as two major products corresponding to glycosylated pre-pro-vasopressin and pro-vasopressin. In all cases, additional faint bands in the range of~17–19 kDa were produced. After 6 h of chase, wild-type pro-vasopressin and the signal-cleaved fraction of the A(-1)T mutant were secreted into the medium. Since COS cells lack prohormone processing enzymes, intact glycosylated pro-vasopressin of 21 kDa was recovered. Hardly any protein could be detected in the cells, indicating that the mutants ΔE47, G57S, and the uncleaved fraction of A(-1)T had been retained and degraded.

To test for degradation via the ER-associated degradation pathway, the proteasomal peptide inhibitor N-acetyl-leucyl-leucyl-norleucinal (ALLN) was added to the medium 90 min before and during the pulse and the chase periods (Fig. 12A, lanes 9–16). ALLN stabilized the putative degradation intermediates of ~17–19 kDa for wild-type and mutant precursors, and to variable extent also the full-size, glycosylated band of the mutant precursors, consistent with proteasomal degradation of retained protein. This was confirmed by experiments using lactacystin, a more specific proteasomal inhibitor. Addition of 25 μM lactacystin stabilized low molecular weight forms that were indistinguishable from those seen with ALLN treatment (Fig. 12B). In contrast, a mixture of leupeptin and pepstatin A, two inhibitors of lysosomal degradation, had no stabilizing effect on the mutant ΔE47 (Fig. 12C). These results indicate that mutant pro-vasopressin as well as a fraction of wild-type pro-vasopressin is degraded by the proteasome in a process that involves intermediates of 17–19 kDa.

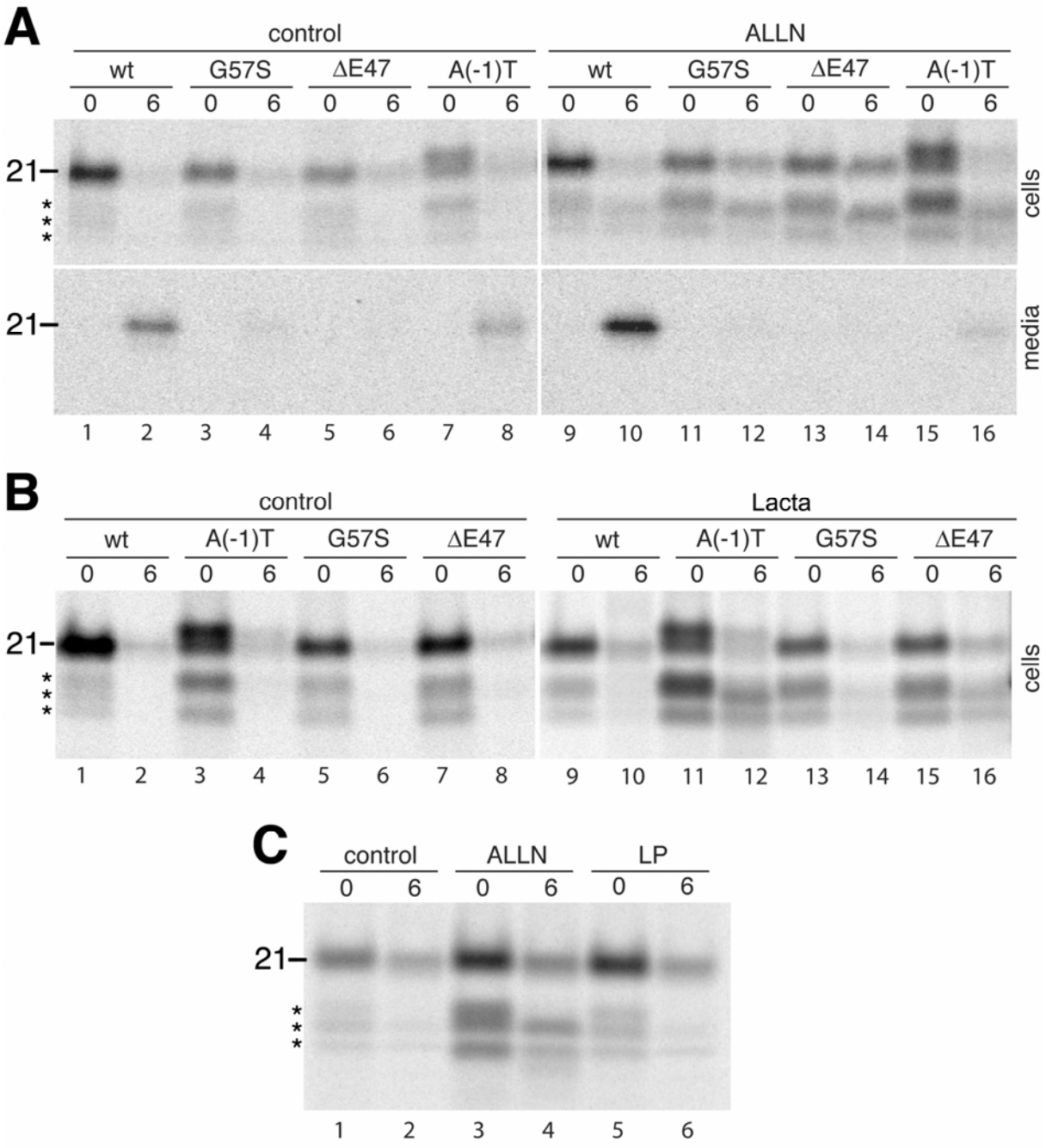

FIG. 12. **Effect of proteasomal inhibitors on the stability of wild-type and mutant vasopressin precursors.** Wild-type (wt) vasopressin precursor and the mutants G57S, ΔE47, and A(-1)T were expressed in COS-1 cells, labeled with [^{35}S]methionine/cysteine for 1 h, and chased for 0 or 6 h without inhibitors (control) or in the presence of 250 μM ALLN (panel A), 25 μM lactacystin (panel B), or of the lysosomal inhibitors leupeptin and pepstatin A (LP; panel C), as described in *Experimental procedures*. Cells and media were subjected to immunoprecipitation using an antibody directed against neurophysin II, and immunoprecipitates were analyzed by SDS-gel electrophoresis and autoradiography. The apparent molecular weight of glycosylated pro-vasopressin of 21 kDa is indicated. Products of lower apparent molecular weight of ~17–19 kDa are pointed out by asterisks.

Three cytosolic degradation intermediates are stabilized in the presence of proteasome inhibitors

In addition to the expected glycosylated pro-vasopressin and in the case of A(-1)T to glycosylated pre-pro-vasopressin, up to three different lower-molecular weight forms could be distinguished. To analyze potential precursor–product relationships, we performed a time-course of labeling of cells expressing either wild-type or mutant ΔE47 vasopressin precursor in the presence or absence of ALLN (Fig. 13A). In addition to an increasing signal of glycosylated pro-vasopressin (form 1), the three smaller species (forms 2–4) appeared with distinct kinetics. Form 2 appeared with highest relative intensity after the shortest pulse times of 5 min. Form 3, however, appeared and increased in intensity in parallel with glycosylated pro-vasopressin. Form 4 only accumulated after 30–60 min and in the presence of ALLN. The same bands were observed using the more specific proteasome inhibitor lactacystin (Fig. 13B). The patterns of products generated by wild-type and mutant precursors were qualitatively similar, indicating that they are not related to specific mutations.

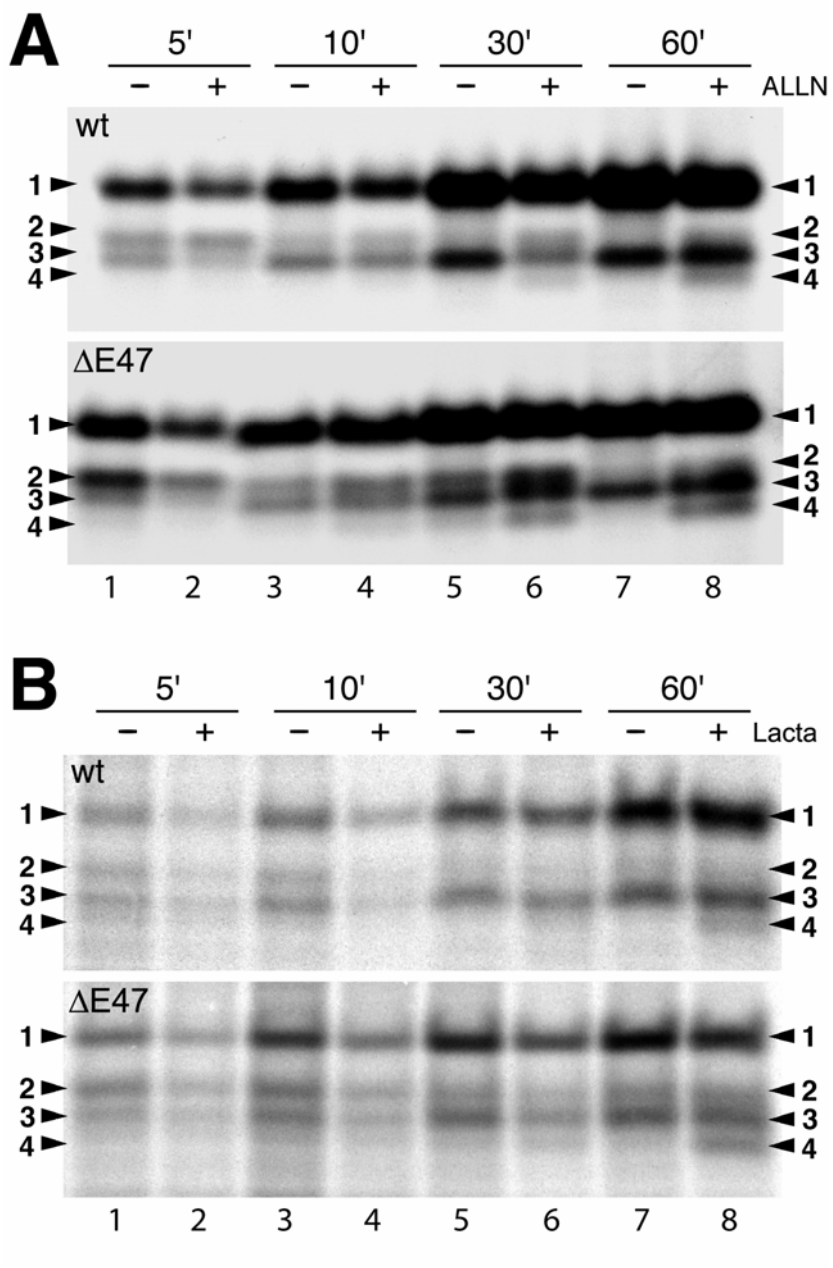

FIG. 13. **Time-course of appearance of different vasopressin precursor forms.** COS-1 cells expressing wild-type (wt) or ΔE47 mutant vasopressin precursor were labeled for 5–60 min with [^{35}S]methionine/cysteine. Cells were incubated with proteasome inhibitor (+) before and during labeling as described in *Experimental procedures* or were untreated (–). ALLN (250 μM) was used as inhibitor in panel A, and lactacystin (40 μM) in panel B. Vasopressin products were immunoprecipitated and analyzed by SDS-gel electrophoresis and autoradiography.

To characterize the different forms, immunoprecipitates of ALLN-treated labeled cells expressing ΔE47 vasopressin precursor were incubated with endoglycosidase H or F (Fig. 14A, lane 1–3). The 21-kDa form 1 was deglycosylated to an apparent molecular weight of ~18 kDa corresponding to form 3. In contrast, the lower bands were insensitive to deglycosylation. This suggested that product 3 corresponds to un- or deglycosylated pro-vasopressin and product 4 to a subsequent degradation intermediate lacking a short segment of the polypeptide at the N- or C-terminus. Upon immunoprecipitation using an antibody directed against the vasopressin hormone, form 4 was not recovered (Fig. 14A, lane 4), indicating that it lacks the hormone sequence at the N-terminus.

Based on its size, product 2 likely represents pre-pro-vasopressin, the primary translation product that had not been translocated to the ER lumen. For comparison, we expressed various mutant precursors to serve as size markers (Fig. 14B). A mutant with a nonfunctional signal sequence (L(-9)R; lane 2), in which an arginine disrupts the hydrophobic core, comigrated with form 2. Only a very small fraction was glycosylated but not processed by signal peptidase (arrowhead). A mutant lacking the signal peptide entirely (ΔSP), i.e. pro-vasopressin synthesized into the cytosol, migrated like band 3 (lane 3). In a further construct the hormone domain was deleted (ΔVP) by fusing NPII-glycopeptide to the signal sequence of pre-pro-enkephalin. In addition to a glycosylated product of ~21 kDa, this construct also produced a 17-kDa form comigrating with band 4 (lane 4). Interestingly, this product of 17 kDa was generated by all constructs, indicating that N-terminal clipping occurred independently of whether the protein was initially inserted into the ER or synthesized directly into the cytosol, and whether a signal sequence was still attached or not.

ER-associated degradation involves the retrotranslocation of unfolded or misfolded proteins from the ER lumen back to the cytosol where they are exposed to cytosolic N-glycanase (Hirsch et al., 2003; Kostova and Wolf, 2003). To determine the localization of the low-molecular weight forms, cells expressing wild-type or ΔE47 vasopressin precursor were labeled for 1 h in the presence of ALLN, broken by swelling and scraping, and subjected to ultracentrifugation. We then analyzed the immunoprecipitated products in the membrane pellet (M) and the cytosol fraction (C) in comparison to the unfractionated total cell lysate (L; Fig. 14C). The experiment was performed with cells labeled for 5 min (lanes 1–3), producing predominantly form 2, or for 60 min (lanes 4–9), generating forms 3 and 4 in addition to

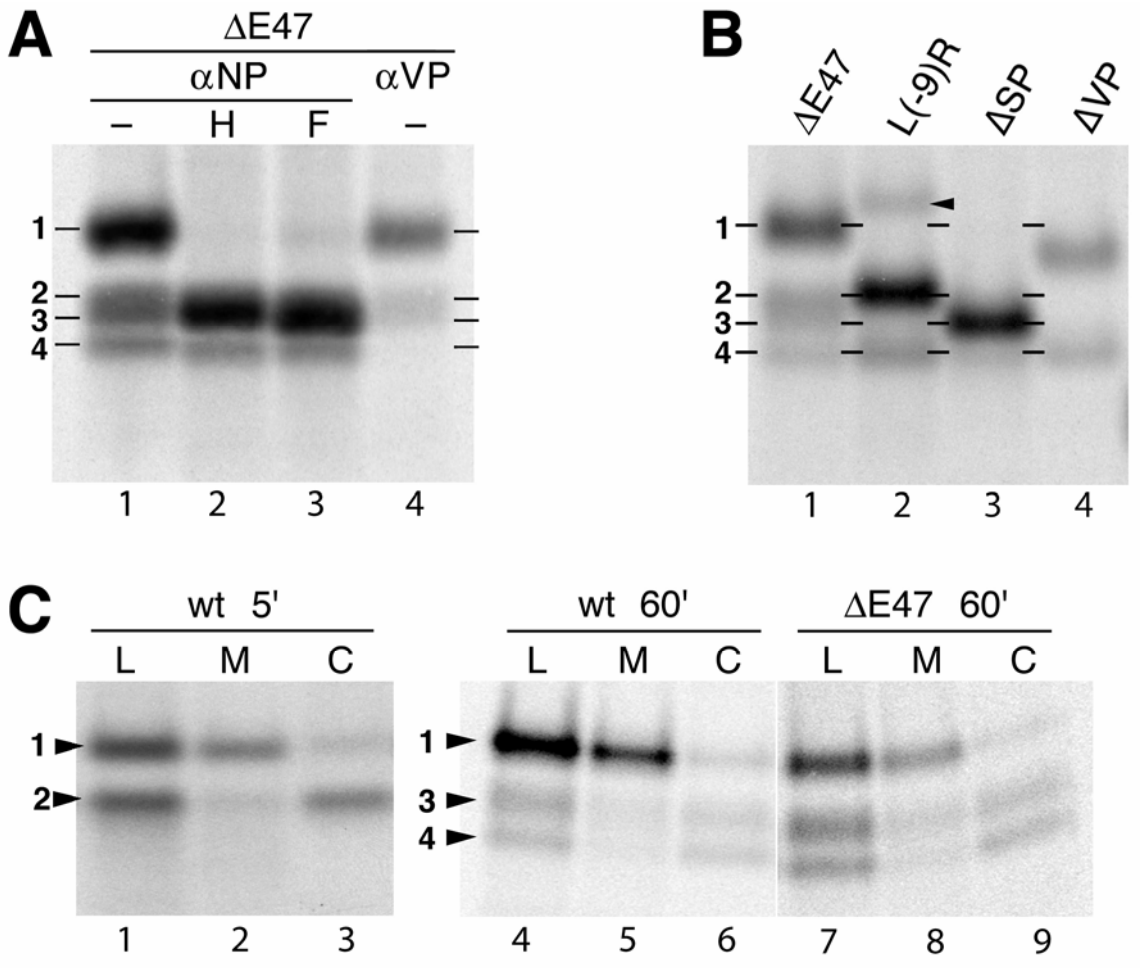

FIG. 14. **Characterization of low molecular weight vasopressin products.** Panel A: COS-1 cells expressing the vasopressin precursor mutant ΔE47 were incubated with ALLN, labeled with [^{35}S]methionine/cysteine for 1 h, subjected to immunoprecipitation using anti-neurophysin II (αNP) or anti-vasopressin (αVP) antibodies and analyzed either directly (–) or after deglycosylation using endoglycosidase H (H) or endoglycosidase F (F). Panel B: COS-1 cells expressing the following mutant precursors were labeled for 30 min in the presence of ALLN and analyzed as above: ΔE47, the signal peptide mutant L(-9)R, the signal peptide deletion mutant ΔSP, and the mutant ΔVP which lacks the vasopressin hormone sequence. Panel C: COS-1 cells expressing wild-type or ΔE47 mutant precursor as indicated were incubated with ALLN and labeled for 5 min to generate (besides form 1) predominantly form 2, or for 60 min to generate predominantly forms 3 and 4. The labeled cells were then broken by swelling and centrifuged at high speed. The supernatants containing cytosolic proteins (C) and the membrane pellets (M) were analyzed in parallel to unfractionated aliquots of total cell lysates by immunoprecipitation, gel electrophoresis and autoradiography.

glycosylated pro-vasopressin. The membrane fraction contained almost all of the glycosylated wild-type and mutant pro-vasopressin, whereas the smaller products were predominantly recovered in the cytosolic fraction. These products were therefore either retrotranslocated from the ER lumen or had never been targeted into the ER.

The native signal peptide of vasopressin precursor is inefficient in ER targeting

The occurrence of unglycosylated pre-pro-vasopressin indicates that the native signal sequence is inefficient in mediating translocation across the ER membrane. To test this, we expressed wild-type and ΔE47 mutant precursor with the native signal sequence in parallel with the same proteins containing the signal peptide of pre-pro-enkephalin. Upon labeling for 3–60 min, form 2 was only produced by the constructs with the native vasopressin signal sequence, but not with the enkephalin signal (Fig. 15A). In contrast, forms 3 and 4 were generated with either signal, indicating that they are derived from form 1, glycosylated pro-vasopressin in the ER lumen, after retrotranslocation. The fact that wild-type and ΔE47mutant proteins behaved identically indicates that the mutation is not responsible for the phenomenon and that the native signal of the vasopressin precursor is inherently inefficient.

The signal of the vasopressin precursor is unusual in that it contains a negative charge near the N-terminus (D(-17)) and is C-terminally followed by a cluster of positive charges (Fig. 15B). The enkephalin signal, in contrast, has a positive N-terminus and a longer hydrophobic core. To test whether the unusual charge distribution is responsible for inefficient translocation of the vasopressin precursor, D(-17) was mutated to R, or the residues R(8), K(11), and R(12) were mutated to E. However, upon expression of these charge mutants in COS-1 cells, the pre-pro-vasopressin form (form 2) was still detected (Fig. 15C). Inefficiency to translocate the protein is thus not, or not solely, due to the unusual charge distribution.

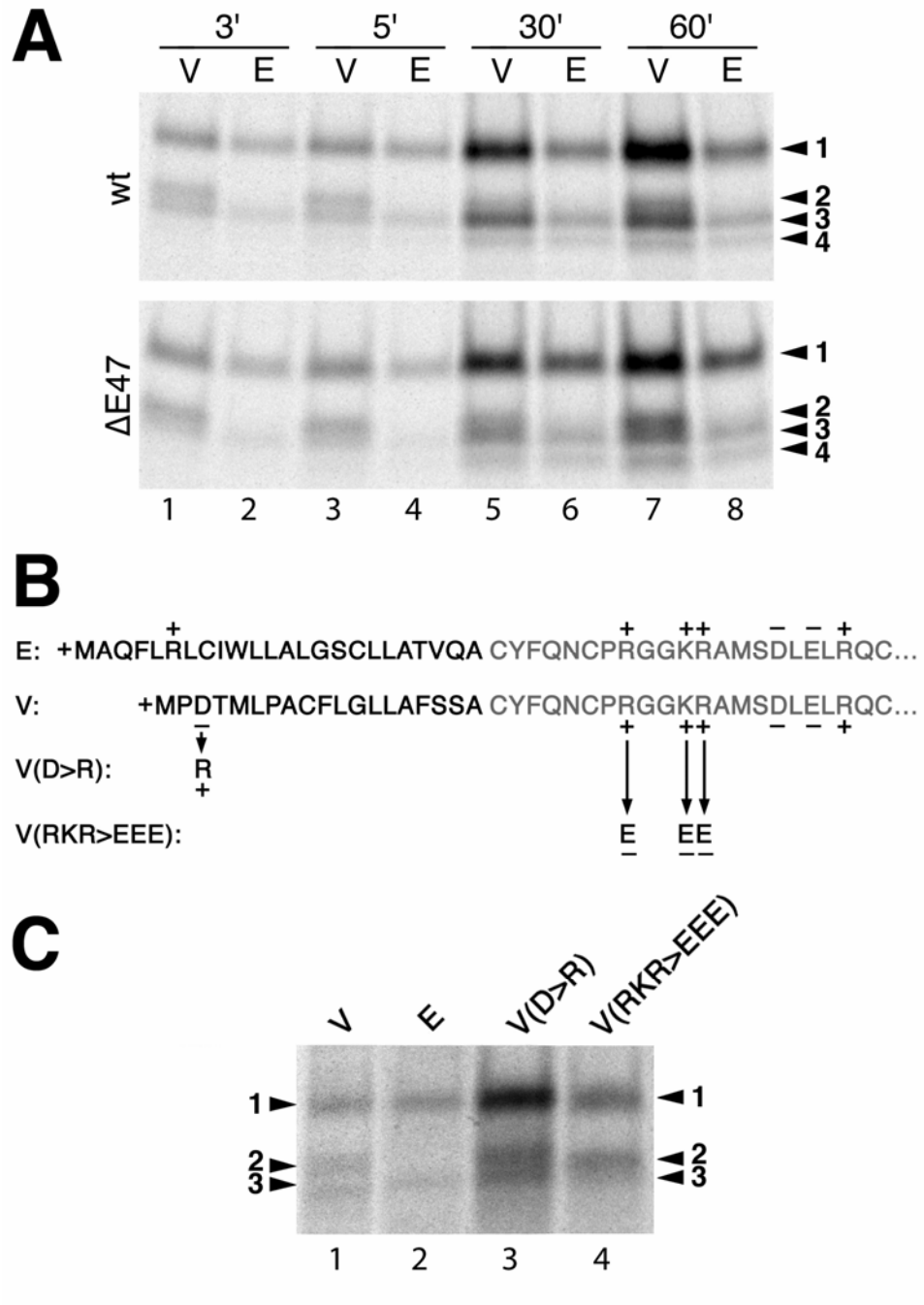

FIG. 15. **Signal dependence of form 2 generation.** Panel A: COS-1 cells expressing wild-type or mutant ΔE47 precursor with either the native vasopressin signal (V) or the signal sequence of pre-pro-enkephalin (E) were incubated with ALLN and labeled for 3–60 min with [^{35}S]methionine/cysteine before immunoprecipitation, gel electrophoresis and autoradiography. Panel B: The sequence of the enkephalin signal (E) fused to pro-vasopressin (gray) is shown in comparison to the native vasopressin signal (V). Mutation of the charges flanking the hydrophobic core of the signal in constructs V(D>R) and V(RKR>EEE) are indicated. Panel C: COS-1 cells expressing the vasopressin precursor with the native signal (V), with the enkephalin signal (E), or with the charge mutants V(D>R) and V(RKR>EEE) were labeled for 5 min and analyzed as in A.

Missorting of pre-pro-vasopressin is not due to overexpression and also occurs in neuronal cells

To exclude the possibility that mistargeting of pre-pro-vasopressin is simply a consequence of high-level expression in COS cells, we examined the polypeptides produced in stably transfected CV-1 cells, the parental cell line of COS-1 cells lacking the large T antigen driving the SV40 promoter/origin of replication present in our expression plasmids. In labeling and immunoprecipitation experiments, the stable CV-1 cell line expressing wild-type vasopressin precursor yielded a somewhat lower signal from the same number of cells than COS cells of which only 5–10% were transfected. The CV-1 cells are therefore producing at least 10–20 fold less of the protein per cell. Even in this situation, all three low molecular weight forms were made (Fig. 16A). In particular, form 2, pre-pro-vasopressin, was made as well. The inefficiency of the vasopressin signal is thus also apparent at moderate rates of synthesis.

In vivo, the vasopressin precursor is expressed by hypothalamic secretory neurons. We tested whether the products observed in fibroblasts were also generated in Neuro2a cells, a mouse neuroblastoma cell line that endogenously expresses secretogranin II and that had been shown to sort exogenous pro-opiomelanocortin into dense-core granules (Chevrier et al., 1991). In transfected Neuro2a cells expressing wild-type or ΔE47 mutant vasopressin precursor and labeled for 5–60 min, pre-pro-vasopressin (form 2) and unglycosylated pro-vasopressin (form 3) were detected and stabilized by ALLN as in COS-1 and CV-1 cells (Fig. 16B). However, form 4 corresponding to N-terminally truncated pro-vasopressin could not be detected. Whereas the inefficiency of the vasopressin signal is observed in all cell types tested, the cytosolic protease responsible for N-terminal truncation of vasopressin precursors in COS-1 and CV-1 cells, is missing in Neuro2a cells.

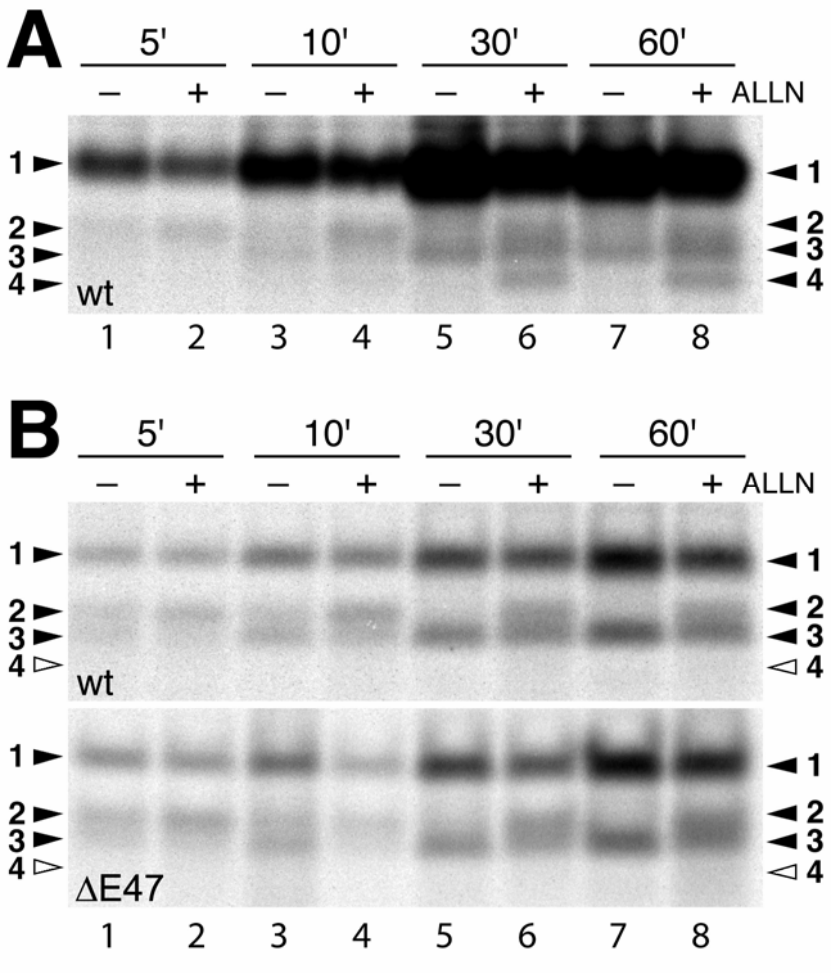

FIG. 16. **Generation of unglycosylated pre-pro-vasopressin (form 2) in a stable CV-1 cell line and transfected Neuro2a cells.** A CV-1 cell line stably expressing the wild-type vasopressin precursor (panel A) and transiently transfected Neuro2a cells expressing wild-type (wt) or ΔE47 mutant precursor (panel B) were labeled for 5–60 min with [^{35}S]methionine/cysteine with (+) or without (–) incubation with ALLN. Vasopressin products were immunoprecipitated and analyzed by SDS-gel electrophoresis and autoradiography. Open arrowheads indicate the absence of form 4 production in Neuro2a cells.

Discussion

Degradation of vasopressin precursor occurs via proteasomes

In cells expressing mutant vasopressin precursor, products of lower molecular weight than full-size glycosylated pro-vasopressin were stabilized by proteasomal inhibitors. No stabilization was observed with inhibitors of lysosomal proteases. This confirmed the expectation that the mutant proteins retained in the ER by the lumenal quality control system are subject to ER-associated degradation (ERAD), i.e. proteolysis by the cytosolic proteasome. Interestingly, significant stabilization of the same types of intermediates was also observed in cells expressing the wild-type protein. This may be due to a considerable number of polypeptides that never attained the native structure owing to errors in translation or post-translational processes necessary for proper protein folding. It has previously been estimated that about one third of newly synthesized total protein is rapidly degraded (Schubert et al., 2000).

Proteins targeted for proteasomal degradation are often, but not always, tagged by ubiquitin (Ciechanover, 1994; Hiller et al., 1996; Fisher et al., 1997; Zhou et al., 1998; Yewdell, 2001). We were unable to demonstrate ubiquitination of vasopressin polypeptides using multiple approaches, including immunoblotting of immunoprecipitated vasopressin precursor with anti-ubiquitin antibodies, increasing the cells' ubiquitin pool by overexpressing a ubiquitin cDNA, or coexpression of dominant-negative ubiquitin constructs (K48R and K48RG76A). It is unclear whether we did not reach sufficient amounts of ubiquitinated material, whether the ubiquitinated form might not be recognized by our antibodies, or whether the vasopressin mutants are targeted to the proteasome through alternative pathways, such as neddylation or sumoylation (Gong et al., 2000; Kamitani et al., 2001; Aguilar and Wendland, 2003). To detect ubiquitinylated proteins is notoriously difficult. In general only a small amount of the protein is detectable in ubiquitinated forms, which furthermore are heterogeneous in size. In addition, rapid deubiquitination may occur in cell lysates.

With proteasome inhibitors three unglycosylated forms accumulate in the cytosol

Upon incubation with proteasome inhibitors, three vasopressin precursor forms of molecular weights in the range of 17–19 kDa were stabilized (forms 2–4). All three of them were unglycosylated and cytosolic. Comparison of their electrophoretic mobility with that of different mutant vasopressin precursors, and immunoreactivity with antibodies directed against the hormone domain indicate that these forms correspond to unglycosylated pre-pro-vasopressin (form 2), deglycosylated pro-vasopressin (form 3), and N-terminally truncated pro-vasopressin (form 4). Small amounts of form 3 have previously been observed in untreated cells (Beuret et al., 1999), but have been interpreted to be the product of incomplete glycosylation in the ER lumen. That this form is largely released into the supernatant of broken cells indicates that it has been retrotranslocated and deglycosylated.

The data support a scenario (illustrated in Figure 17) in which pre-pro-vasopressin is inserted into the ER, cleaved by signal peptidase, and modified to glycosylated pro-vasopressin (form 1). Mutant proteins which are unable to fold into the native structure, but to some extent also wild-type polypeptides, are retrotranslocated to the cytosol and rapidly deglycosylated (form 3). In addition, a fraction of the translation products is not transported into the ER (form 2), based on the finding that the signal sequence was not cleaved and the protein remained unglycosylated. In the presence of proteasomal inhibitors, both forms 2 and 3 may be N-terminally clipped to produce form 4 before degradation. The protease responsible for this slow clipping is unknown and appears to be expressed in a cell type specific manner, since form 4 was not detectable in Neuro2a cells. Cytosolic nonproteasomal proteases are known to be involved in the processing of antigenic peptides to be presented by MHC class I molecules (e.g. ER aminopeptidase associated with antigen processing, (Serwold et al., 2002)).

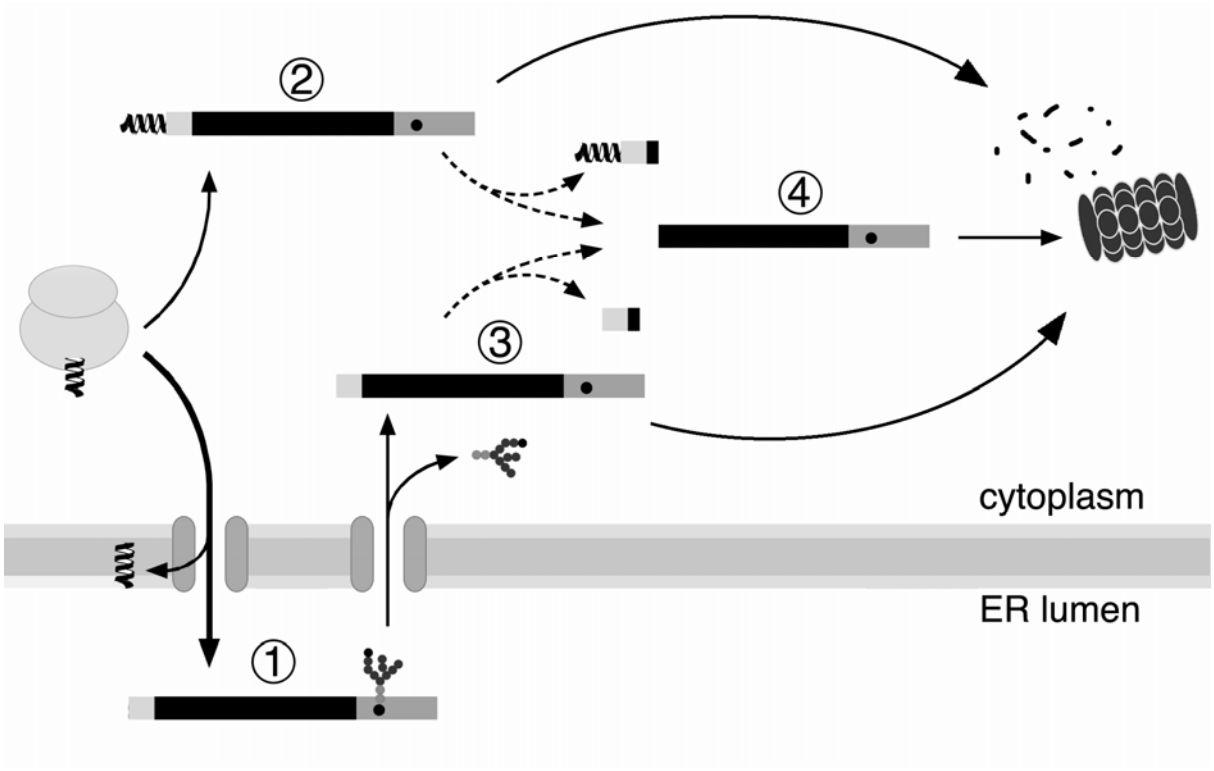

FIG. 17. **Schematic summary of the products and degradation intermediates of vasopressin precursor.** The majority of vasopressin precursor is translocated into the ER lumen, cleaved by signal peptidase and glycosylated to form 1 (glycosylated pro-vasopressin). Molecules unable to fold are retrotranslocated into the cytosol and deglycosylated to form 3 (deglycosylated pro-vasopressin). A fraction of precursor proteins (even of the wild-type) is not translocated and is found as cytosolic pre-pro-vasopressin (form 2). Forms 2 and 3 are stabilized by proteasomal inhibitors. In COS-1 and CV-1 cells, but not in Neuro2a cells, they give rise to an N-terminally processed form 4. Primarily forms 2, 3, and 4 accumulate upon addition of proteasome inhibitors indicating that they are substrates of the proteasome (shown on the right).

The vasopressin signal functions inefficiently

The production of pre-pro-vasopressin suggests that the native vasopressin signal is inefficient. This is not due to incomplete signal cleavage, since no glycosylated pre-pro-vasopressin could be detected. In ADNDI mutants affecting the cleavage efficiency of the signal, such as A(-1)T (mutation of the cleavage site (Ito et al., 1993)) and ΔG227 (truncation of the signal; (Beuret et al., 1999)), glycosylated pre-pro-vasopressin is easily detected. A(-1)T also revealed increased form 2 (Fig. 12A, lane 15, and Fig. 12B, lane 11), since retrotranslocated polypeptides add to those that were primarily synthesized into the cytosol. Therefore, the defect in the native vasopressin signal is due to inefficient targeting or translocation. The phenomenon is not an artefact of overexpression and potential saturation of the secretory route, since it is also observed in CV-1 cells expressing at least ten times less protein per cell. Moreover, it is detected in Neuro2a cells which have characteristics of neuroendocrine cells and is thus likely to occur also in vasopressinergic cells *in vivo*.

The charges flanking the hydrophobic core of signal sequences largely determine signal orientation in the ER translocation machinery (Goder and Spiess, 2001). Typically, the N-terminal portion of signal peptides is positively charged (the positive-inside rule; (von Heijne, 1990)), or at least more positive than the C-terminal flanking sequence (Hartmann et al., 1989). This is not the case for the vasopressin signal where the charge difference Δ(C–N) calculated according to the rules by Hartmann et al. (Hartmann et al., 1989) is +2. Surprisingly, however, the unusual charge distribution is not responsible for the translocation inefficiency: mutation of D(-17) to R or of R(8), K(11), and R(12) to E did not reduce the production of pre-pro-vasopressin despite an improved charge difference of 0 and -4, respectively. The efficiency of the enkephalin signal fused to pro-vasopressin is thus also not just due to the positive N-terminus. The hydrophobic core of the enkephalin signal is longer and more hydrophobic (in total and on average) than that of the vasopressin signal. This might account for more efficient recognition by signal recognition particle (Hatsuzawa et al., 1997).

Inefficient function of signal sequences is observed rarely. Plasminogen activator inhibitor-2 (PAI-2) is found as a secreted and a cytosolic form because of an internal, uncleaved signal that is inefficient both in binding signal recognition particle and in subsequent interaction with the translocation complex (Belin et al., 1996). Parathyroid hormone-related peptide (PTHrP) precursor is in part found as pre-pro-PTHrP in the cell and accumulates in nucleoli due to a nucleolar targeting signal (Amizuka et al., 2000). Although

this signal has a positive N-terminus, its C-terminal sequence is even more positively charged. In both cases, dual localization may reflect separate functions in different compartments.

Of special pathophysiological interest is the case of the prion protein (PrP). A particular misfolded conformation of PrP (PrP^{Sc}), which is favored by certain mutations in the protein, causes neurodegenerative disorders (prion diseases). The N-terminal signal of PrP is inefficient and membrane targeting of a fraction of the protein is rescued by a C-terminal hydrophobic sequence (Hölscher et al., 2001). PrP can adopt multiple membrane topologies, including a fully translocated form (^{Sec}PrP), two transmembrane forms with either the N- or the C-terminal portion of the polypeptide translocated into the ER lumen (^{Ntm}PrP and ^{Ctm}PrP, respectively), and a cytosolic form (Hegde et al., 1998; Hegde and Rane, 2003). Again, the charge difference of the signal is the opposite of that typical for secretory signal peptides. It has been shown that mutant PrP as well as a significant portion of wild-type PrP is degraded via the proteasome, since cytosolic, unglycosylated forms accumulate upon treatment with proteasomal inhibitors (Ma and Lindquist, 2001; Yedidia et al., 2001). It has recently been proposed that the cytosolic forms of PrP trigger at least some neurodegenerative prion diseases, because expression of a cytosolic form of PrP lacking the N-terminal signal and the C-terminal glycosylphosphatidylinositol anchor sequence was toxic in Neuro2a cells and rapidly caused neurodegeneration in transgenic mice (Ma et al., 2002). In addition, conversion to a PrP^{Sc}-like conformation was found to be increased for a mutant PrP causing heritable prion disease correlating with its increased transport into the cytosol (Ma and Lindquist, 2002). To which extent the situation of mutant vasopressin precursor parallels that of PrP remains to be determined.

It is not known at present whether ADNDI results from a toxic product in the cytosol or in the ER lumen. Several factors that are induced by ER stress have been shown to mediate cell damage. The transcription factor CHOP has been shown to induce an apoptotic response in renal tubular cells of mice injected intraperitoneally with tunicamycin (Zinszner et al., 1998), and downstream effectors of CHOP have been characterized (Wang et al., 1998). In a mouse model of non-autoimmune diabetes mellitus, hyperglycemia occurs as a consequence of apoptotic destruction of pancreatic β cells induced by ER stress due to ER retention of a mutant insulin precursor. Targeted deletion of CHOP delays the onset of diabetes in these mice (Oyadomari et al., 2002). Tunicamycin-induced apoptotic destruction of renal tubular cells is significantly alleviated in mice deficient in caspase-12, which is located at the ER and mediates ER-associated apoptosis in a specific manner (Nakagawa et al., 2000). Caspase-12 also mediates amyloid-β-induced toxicity in primary cortical neurons cultured *ex vivo*, but is

not activated by non-ER-associated apoptotic signals, such as cycloheximide, tumor necrosis factor, or anti-Fas, again illustrating its specific role in ER-associated cell damage.

In ADNDI, cytotoxic pathways triggered by ER-retention directly and/or by retrotranslocated degradation intermediates in the cytosol may be involved in the degeneration of vasopressinergic neurons. Our findings indicate that proteasomal degradation of mistargeted precursors occurs even when two wild-type alleles are expressed. In heterozygous individuals, a critical concentration of toxic molecules may be exceeded, leading to neuronal degeneration.

Acknowledgements

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General Discussion and Outlook

In a subset of patients with DI, dominant-negative mutations in the gene of vasopressin, the anti-diuretic hormone, are responsible for the disease. Diverse mutations (Table I) lead to a similar phenotype, clinically characterized by polyuria and polydipsia. Post mortem examination of affected individuals revealed a hypocellularity and gliosis of vasopressinergic magnocellular neurons. The processes linking vasopressin deficiency and/or mutant precursor retention to eventual cell death have not been established and remain to be clarified.

Misfolded vasopressin precursors are degraded by the proteasome

Our metabolic labeling experiments demonstrated that vasopressin mutants (ΔE47 and G57S completely, and A(-1)T partially) failed to exit the cell, as no protein was detected in the supernatant of transfected cells. Immunofluorescence staining showed the precursors to be localized to and retained in the ER. The material found in the ER remained there for only a short period of time, as pulse-chase experiments of mutant vasopressin failed to show a signal after a chase period of 6 hours. During this time, the bulk of the unreleased material must have been removed from the cell by a degradative mechanism.

Experiments using different protease inhibitors suggested that the retained material is shuttled into the ERAD pathway and degraded by the proteasome. Inhibitors of the 26S proteasome, ALLN and lactacystin, used in different cell types (COS-1 and N2a) provide evidence that the improperly folded material is cleared from the cell via the proteasome. The use of pepstatin and leupeptin, inhibitors of the lysosome, did not stabilize mutant vasopressin precursor. Interestingly, the use of proteasomal inhibitors revealed that a considerable fraction of the precursor material is unable to enter the ER lumen, indicated by uncleaved signal peptide remaining attached to the precursor molecule. This was true not only for the diverse mutants examined, but also for the wild-type. Replacing the native signal peptide of vasopressin with the signal peptide of enkephalin enhanced ER translocation, inasmuch as no precursor with signal was detectable. The findings suggest an inefficiency of the vasopressin signal peptide in conferring translocation into the ER lumen.

Potential explanations for signal peptide inefficiency

Poor transport of a nascent peptide chain into the ER can be due to different factors. Entry into the secretory pathway is initiated by the recognition of a hydrophobic stretch of amino acids in the signal peptide of a secretory protein by SRP. The hydrophobic stretch in the wild-type vasopressin signal is shorter than in the more efficient enkephalin signal sequence (Figure 15 B). Decreased binding of SRP to the hydrophobic stretch in the signal peptide would reduce ER delivery.

Another possibility has been brought up by Fons et al. (2003). Subsequent to targeting a nascent chain to the ER via SRP and its receptor, the protein needs to be translocated across the ER membrane. This gating step was re-enacted using purified components in a proteoliposome system (Gorlich and Rapoport, 1993). Fons et al. (2003) demonstrated that efficient targeting of nascent chains to the ER lumen may depend on TRAP. PrP exhibits poor targeting in proteoliposomes supplemented with the minimal translocation machinery. Import was improved with increasing amount of TRAP in the proteoliposomes. Different signal peptides fused to the PrP mature domain demonstrated an inverse relationship between TRAP dependence for ER import and the signal peptide's ability to induce translocation, the signal's gating strength (Kim et al., 2002). Only if the gating capability of the signal is very strong, does TRAP become dispensable. TRAP dependence could, however, not be linked to an obvious property of the signal peptide, such as overall length, length of the hydrophobic domain, or charge differential across the hydrophobic domain. Since the gating strength does not depend on a structural but rather on a functional parameter, comparison of signal sequences cannot provide a reliable prediction in this direction. A testing series analogous to Fons et al. (2003), using the vasopressin signal fused to PrP might help determine if this underlies the observed signal inefficiency. A construct bearing an enkephalin signal should thus exhibit a lower dependence on TRAP than one bearing the native vasopressin signal peptide. Low TRAP expression in magnocellular neurons could prevent efficient ER targeting of vasopressin precursor, predisposing the cells to an increase in mistargeted proteins if additional mutations occur.

Finally, failure of the signal to properly orient the precursor in the translocon could prevent translocation. The unusual charge distribution of the vasopressin signal is in disagreement with von Heijne's positive-inside rule. However, we have shown that this is not the main reason for inefficient targeting, since charge mutations in the signal did not abrogate the observed phenotype (Figure 15 C). Even a charge distribution favouring the translocation of

the C-terminal part of the precursor was not adequate to prevent the appearance of pre-pro-vasopressin. The relatively short hydrophobic region would also not be a likely candidate for hindering reorientation of the signal from a head-on insertion to translocation of its C-terminus. Extending the hydrophobic stretch by leucines was found to actually impede reorientation of pre-pro-vasopressin (Eusebio et al., 1998).

Dominance and mechanisms of cell death in ADNDI

Mutations in the vasopressin precursor leading to DI are dominant. The reason for this is most likely a cytotoxic effect generated by the mutant proteins. Successive loss of vasopressinergic magnocellular neurons leading to a declining quantity of secreted vasopressin corresponds to the progressive nature of the disease. The irreversible decline of hypothalamic cells would further account for the observed high penetrance.

Several mechanisms may be responsible for cytotoxicity. The large variety of mutations described for vasopressin all produce a similar phenotype. This makes it improbable that each mutation is directly involved in toxicity. It appears more likely that they all act in a similar way to prevent normal folding and to generate a common misfolded toxic conformation. A dramatic example for a toxic protein is the prion protein which can adopt an extremely stable folding conformation leading to scrapie in sheep or Creutzfeldt-Jacob disease in humans by promoting the misfolding of normal prion protein. This novel conformation is inherently toxic, poisoning the host cell by interfering with essential processes.
In general, misfolding of the protein due to mutations may cause toxicity in different ways. The protein may aggregate, induce autophagy, or overload the quality control and degradative mechanisms, all possibly cumulating in cell death. The cell uses chaperones to prevent the accumulation of misfolded proteins, and to keep unfolded proteins in solution. Terminally misfolded proteins are targeted for degradation to clear the cellular machinery of potentially toxic aggregates. Our experiments showed that expression of mutant vasopressin pro-hormone leads to formation of disulfide-linked aggregates (Beuret et al., 1999)(and unpublished). These aggregates were not observed when expressing wild type. Immunofluorescence studies showed no obvious ER deformation in transfected cells. Thus, aggregate formation may represent the beginning of neuronal toxicity. Eukaryotic cells use two major routes to remove undesired material. The ubiquitin-proteasome system removes short-lived nuclear and

cytosolic, as well as ER-synthesized proteins. The proteasome requires at least partial unfolding of the substrate for degradation to proceed and is, therefore, relatively inefficient at degrading aggregated proteins. Larger complexes and even whole organelles are degraded by the autophagy-lysosomal pathway. This involves the formation of double-membrane structures which eventually fuse with lysosomes.

ER-synthesized proteins which are destined for degradation need to clear two hurdles if they are to be efficiently eliminated. Initially, they need to be extracted from the ER lumen, and subsequently shuttled to the proteasome for degradation. Inefficiency of the former process will lead to the deposition of material in the ER lumen, which is the hallmark of ER storage diseases (ERSD) (Kim and Arvan, 1998; Aridor and Hannan, 2000; Rutishauser and Spiess, 2002). Russell bodies (RB) are dilated ER cisternae containing condensed immunoglobulins. Valetti et al (1991) demonstrated that synthesis of a mutated immunoglobulin which is neither secreted nor degraded is sufficient to induce formation of RB in different tissues among multiple species. RB membranes are separated from the ER lumen, and while the soluble BiP and PDI are excluded from these membranes, ribosomes and calnexin, both associated with membranes, are found on ER as well as on RB. Dilated cisternae are found in a number of secretory cells of different origin. This suggests that condensation of transport-incompetent proteins in the ER may be the common cause for these elements. One such example is the accumulation of mutated α1 anti-trypsin in hepatocytes in patients carrying the Z-variant allele (PiZ). Deposition of PiZ damages the hepatocytes, leading to liver disease (Carlson et al., 1989).

How RB are ultimately disposed of is not clearly understood. One model suggests fusion of cisternae with lysosomes, another speculates on the formation of autophagic vesicles. In ADNDI, there is no evidence that RB might be formed in neuronal cells transfected with vasopressin mutants (Beuret et al., 1999; Nijenhuis et al., 1999; Friberg et al., 2004). This is illustrated by the fact that the addition of lysosomal inhibitors will neither cause accumulation of vasopressin precursor nor the formation of RB.

Proteins which have been retrotranslocated but not degraded immediately run a high risk of aggregating in the cytosol. Integral membrane proteins endowed with highly hydrophobic regions are prime examples of such candidates. One well-studied paradigm is the Δ508 mutant form of the cystic fibrosis transmembrane conductance regulator (CFTR). Dislocation from the ER results in indigestible cytoplasmic aggregates which are ultimately sequestered to inclusion bodies termed aggresomes (Johnston et al., 1998). Aggresomes are found around the microtubule organizing center. Proteins are collected there by active minus end transport

along microtubules. Characteristic proteins found in aggresomes include the intermediate filament vimentin, molecular chaperones and proteasome subunits. The reason for sequestering aggregated proteins to a central cell site is not quite clear. It is thought that it might facilitate disposal of aggregates by an autophagic route, particularly in large mammalian cells such as neurons (Kopito, 2000). In post-mitotic cells effective clearance is very important because accumulation of material cannot be diluted by cell proliferation. Immunofluorescence and electron microscopy studies in stably transfected and differentiated neuronal cells, challenged by proteasomal inhibitors, might help determine if such a degradation pathway is followed by mutant vasopressin precursor as an alternate degradation route. Webb et al. (2003) found that α-synuclein, the major aggregate protein in Parkinson's disease, was indeed degraded by both the proteasome and autophagy.

Castino et al. (2005) introduced autophagy to the issue of cell debris degradation in ADNDI. Expression of the C67X mutant in rat and Neuro2a cells led to autophagic structures in the cell body, implicating the lysosome in the degradation process. The mutant caused the appearance of enlarged vesicles in the ER of transfected cells, confirmed by colocalization with calnexin and PDI. These markers set them apart from RB. A subset of C67X protein appeared to be found in the TGN, where it colocalized with the lysosomal marker cathepsin D. Inhibition of the autophagic-lysosomal pathway induced apoptosis of the mutant expressing cells, confirmed by the release of cytochrome c from mitochondria and the activation of caspases. Apparently, activation of autophagy serves as a survival mechanism to prevent cell death caused by the stress of mutant vasopressin precursor. In contrast, our findings did not demonstrate a role for the lysosome in vasopressin degradation. Their observation may thus indicate a step occurring at a later time point, perhaps as a result of the gradual build up of aggregated mutant vasopressin which obstructs the ER.

Overloading the cellular control systems can have other detrimental effects. If traffic to the proteasome is impeded or if the amount of misfolded protein surpasses the degradative capacity of the ubiquitin-proteasome pathway, material will agglomerate in the cytosol.

One interesting example is the cellular phenotype of mutations in the proteolipid protein gene which are responsible for a demyelination disease, Pelizeaeus-Merzbacher disease. These mutants lead to the accumulation of the gene products in the ER, causing ER stress. Eventually, the oligodendrocytes undergo apoptosis (Gow et al., 1998). In motor neuron disease, kinesin accumulates in sphere-like structures among massive accumulations of highly phosphorylated neurofilaments. These spheroids which impair anterograde transport in the axons of motor neurons are thought to result from disturbed transport of neurofilaments

(Toyoshima et al., 1998). One could speculate that mutation of the vasopressin wild-type which is already targeted inefficiently leads to an overload of the degradation pathway causing an accumulation of an ordinarily removed toxic intermediate form. Alternatively, overloading the degradation system may prevent the efficient removal of other proteins, allowing them to accumulate to toxic levels in the cell.

Ideally, one would like to observe the fate of vasopressinergic neurons *in vivo*. Specific loss of magnocellular neurons in the hypothalamus is difficult to study. Mouse models offer evidence that the destruction of neurons occurs only among this subspecies of vasopressinergic neurons. This loss which Russell et al. (2003) were able to mimic in their knock-in mice was only observable in the C67X mutant mice. Those carrying the A(-1)T mutation, the most predominant among human patients, did not exhibit death of the corresponding magnocellular neurons.
The mechanism of cell death itself is unclear in the animals. Accumulation of unfolded protein in the ER should invoke UPR. Failure to cope with the material present would then lead to ERAD and possibly to the induction of cell death via the expression of CHOP. Yet, C67X animals lacked an observable CHOP induction, perhaps due to the relatively small population of cells dying at any given time.

A suitable system which permits the study of cell death in response to its exposition to mutant vasopressin precursor has remained intangible. Ito et al. (1997) had most closely achieved this goal. His Neuro2a neuroblastoma cell line exhibited reduced cell viability in differentiated cells expressing various mutant vasopressins, but not wild-type over a time period of six weeks. Our future goal is to build on their results and try to define a minimal toxic element of vasopressin precursor by stably expressing wild-type, several mutants, and selected tagged deletion constructs of vasopressin precursor in Neuro2a and rat hypothalamic cells. The toxic fragment would have to lie N-terminal of the shortest known truncation mutant found to date, C61X. To ensure comparable expression levels, all constructs will be transfected into the same mother cell line bearing a Flp recombination site. In parallel, we will examine the possible contribution of the inefficient signal to toxicity. The mutant coagulation factor X, “Santo Domingo”, causes a severe bleeding disorder. It is imported into the ER but its signal remains uncleaved (Racchi et al., 1993). Its recessive inheritance establishes it as a non-cytotoxic control protein with failed trafficking properties. The enkephalin signal peptide-bearing vasopressin precursor could serve as a control for efficient ER import. It will be

interesting to determine whether the enkephalin signal rescues or ameliorates the survival rate of otherwise toxic mutants, and whether these fusion constructs still induce aggregate formation.

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Education

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1987 - 1989	Greenwood Lakes Middle School, Lake Mary, FL, USA
1989 - 1991	Lake Mary High School, Lake Mary, FL, USA (gifted class)
Aug. - Dec. 1991	Kantonsschule Trogen, Switzerland
Dec. 1991 - Dec. 1994	Gymnasium Muttenz, Typus C (mathematics/science)
Oct. 1995 - Apr. 2000	University of Basel, study area: Biologie II
Sept. 1998 - Feb. 2000	Diploma-Thesis under Prof. Dr. Markus A. Rüegg, Biozentrum Basel: "Cadherins: The effect of dominat negative constructs on post-synaptic differentiation at the neuromuscular junction"
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Practical Experience

1994	Tutorial, middle school level
July 1994	Assistant camp counselor in a sailing camp by the Sozialpädagogischer Dienst Basel in Le Prese, GR
Mai 1995	Practical training as an orderly during the military in Liestal, BL
Aug. - Oct. 1995	Worked in the PHP-Betrieb TTS, Ciba-Geigy Werke Stein, AG
Oct. 2001 - Feb.2002	Biology tutorial
Sept. 2002	School project supervision of Peter Blattmann at the Biozentrum
Dec. 2002	Assistant of the Biochemistry lab course
Jan. 2004	Lab head of the Biochemistry lab course
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Languages

German	native speaker
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Other interests and activities

	Reading, computer, sports: Basketball (player, referee, coach)
1992 - 1994	Coaching assistant of the Jugendriege Magden
1994	Committee member of the Turnverein Magden
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since 2001	Chairman of the technical committee of the Turnverein Magden
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