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Identification, by a Monoclonal Antibody, of a 53-kD Protein Associated with a Tubulo-Vesicular Compartment at the *cis*-side of the Golgi Apparatus

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Abstract. Purified Golgi membranes of the human intestinal adenocarcinoma cell line Caco-2 were used as an antigen to produce a monoclonal antibody, G1/93, which specifically labels a tubulovesicular compartment near the *cis* side of the Golgi apparatus, including the first *cis*-cisterna itself, as visualized by single and double immunoelectron microscopy with antibodies against galactosyltransferase. The antigen recognized by G1/93 was identified as a protein with a subunit size of 53 kD. Pulse-chase experiments revealed that the 53-kD protein dimerizes immediately after synthesis followed by formation of oligomers of ~310 kD, probably homohexamers. The protein has a transmembrane topology with only a short cytoplasmic segment as assessed by protease protection experiments. Glycosidase digestion studies indicated that the protein is probably not glycosylated. The unique subcellular distribution of the G1/93 antigen in close vicinity to the *cis*-Golgi is in line with the notion that this protein may delineate the biosynthetic transport pathway from the endoplasmic reticulum to the Golgi apparatus. Moreover, G1/93 is a useful marker to identify the *cis* side of the Golgi apparatus in a variety of human cells.

RADIOAUTOGRAPHIC and cell fractionation studies performed with acinar cells of the exocrine pancreas have led to the concept that newly synthesized secretory and plasma membrane proteins leave the rough endoplasmic reticulum rough (ER)[1] in small vesicles which pinch off at specific sites, the so-called transitional elements of the rough ER, near the *cis*-side of the Golgi apparatus (Jamieson and Palade, 1967*a*, *b*; Palade, 1975). Transport of proteins from the ER to the Golgi region requires energy (Jamieson and Palade, 1968) as assessed by the use of respiratory inhibitors or inhibitors of oxidative phosphorylation, is asynchronous for many proteins (Lodish et al., 1983), and can be blocked by low temperatures (Matlin and Simons, 1983; Saraste and Kuismanen, 1984; Tartakoff, 1986).

Despite recent progress in reconstituting the ER to Golgi transport in a cell-free system (Balch et al., 1986) or in semi-intact cells (Beckers et al., 1987) the mechanisms underlying this pathway remain unknown. In particular, it has not been unequivocally established whether protein exit from the ER can occur at the transitional elements only or whether all of the ER can generate transport vesicles. Furthermore, it is not known whether export from the ER is mediated by transport receptors as proposed by Lodish et al. (1983) or occurs by default; i.e., resident proteins of the ER would carry a retention signal while proteins destined for export would lack such a signal (see Pfeffer and Rothman, 1987). Yet another hypothesis postulates that resident proteins of the ER recycle via a salvage compartment interposed between transitional elements and the *cis*-Golgi apparatus (Pelham, 1988; Warren, 1987). The lack of marker proteins for transitional elements, transport vesicles and *cis*-Golgi apparatus has prevented a direct assessment of these questions experimentally.

In the present communication an integral membrane protein is described which might qualify as a marker protein for the ER to Golgi biosynthetic pathway. This protein was identified by a monoclonal antibody, which was produced in mice by using purified Golgi membranes of the human intestinal cell line Caco-2 as an antigen.

1. *Abbreviations used in this paper*: DSP, dithio-bis (succinimidylpropionate); DSS, disuccinimidyl suberate; ER, endoplasmic reticulum.

Materials and Methods

Materials and Chemicals

Reagents and supplies were obtained from the following sources: [^{35}S]methionine (translation grade) and EN^{3}HANCE from New England Nuclear (Boston, MA), Na^{125}I (carrier-free) from the Eidgenössisches Institut für Reaktorforschung (Würenlingen, Switzerland). DME (4.5 g/liter of glucose), penicillin, streptomycin, fungizone, nonessential amino acids, minimum essential medium selectamine kit, and dialyzed FCS from Gibco (Paisley, Scotland), FCS from Biological Industries (Israel), 0-glycanase from Genzyme (Boston, MA), endoglycosidase H from Boehringer Mannheim (Federal Republic of Germany), disuccinimidyl suberate (DSS), di-

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thio-bis (succinimidylpropionate; DSP), PMSF, antipain, benzamidine, pepstatin aprotinin, and type III-O trypsin inhibitor from Sigma Chemical Co. (St. Louis, MO), proteinase K and SDS-PAGE reagents from Serva (Heidelberg, FRG), subclass-specific secondary antibodies, TRITC-goat-anti-mouse IgG, TRITC-goat anti-rabbit Ig, FITC-goat-anti-mouse IgG, FITC-rabbit-anti-mouse IgG and FITC-goat-anti-rabbit Ig from Cappel (West Chester, PA) or Nordic (Tilburg, Netherlands), sulfosuccinimidyl (hydroxyphenyl) propionate from Pierce (Rockford, IL), cell culture dishes from Falcon (Becton, Dickinson, Cockeysville, MD), multichamber slides from Miles Laboratories (Naperville, IL), and trypsin from Worthington Biochemical Corporation (Freehold, NJ).

Purification of a Fraction Enriched in Golgi Membranes from Caco-2 Cells. Golgi membranes were isolated essentially according to Stieger et al. (1988) from four 100-mm plates of Caco-2 cells of the 70th passage. The FII fraction containing the Golgi membranes was used as an antigen to produce monoclonal antibodies.

Isolation of a "Total Membrane Fraction" of Caco-2 Cells. Caco-2 cells grown to confluency in a Mini-Marbrook chamber (2-cm diameter) were scraped from the nitrocellulose filter with a rubber policeman into 1 ml PBS, pH 7.2, and homogenized by passing 10 times through a 25 G needle connected to a 1 ml tuberculine syringe. The homogenate was centrifuged at 2,800 *g* for 15 min and the resulting supernatant was spun at 100,000 *g* for 45 min. The pellet was designated "total membrane fraction".

Monoclonal Antibody Technique. Mice were injected intracutaneously with 425 μg Golgi membrane protein in 200 μl PBS emulsified with 300 μl complete Freund's adjuvant. A booster injection with 610 μg antigen in PBS, mixed with incomplete Freund's adjuvant, was administered subcutaneously 7 wk later. 11 wk after the first immunization a final booster of membrane protein without adjuvant was injected intravenously (342 μg) and intraperitoneally (342 μg) and 3 d later cell fusion was carried out according to established techniques (Fazekas de St. Groth and Scheidegger, 1980; Galfré et al., 1977) as described (Hauri et al., 1985) using PA1 myeloma cells. Screening of antibodies was carried out by means of a dot blot procedure using Golgi membranes as an antigen (Hawkes et al., 1982). Positive cultures were expanded and the cells were frozen in liquid nitrogen. 10× concentrated (by ammonium sulfate precipitation) culture supernatants were further screened by immunofluorescence using Caco-2 cells. A hybridoma cell line secreting an antibody exhibiting a Golgi-like pattern was subcloned by limiting dilution. The resulting antibody was designated G1/93. Another monoclonal antibody (G1/139) obtained from the same fusion was specific for a lysosomal 100-kD membrane glycoprotein. Furthermore, a monoclonal antibody was prepared against purified human liver epoxide hydrolase (Skoda et al., 1988) and used as a smooth ER-specific probe (Galteau et al., 1985). All the monoclonal antibodies were of the IgG1 subclass.

Immunofluorescence. Caco-2 cells were grown on 8-well multichamber slides to 50% confluency. The cells were washed twice with PBS and fixed with 3% formaldehyde in PBS, pH 8.3, for 30 min. After fixation the cells were washed with PBS, pH 7.2, and free aldehyde groups were quenched with 20 mM glycine in PBS twice for 5 min. The cells were then permeabilized with 0.1% saponine in glycine-PBS for 20 min and incubated for 30 min with 10 μl 10-fold concentrated culture supernatant (containing monoclonal antibodies) in 250 μl PBS–0.1% saponine. A rabbit antiserum against human milk galactosyltransferase (kindly provided by Dr. E. Berger, Zürich) was used at a 1:250 dilution. After rinsing the cells four times (10 min in total) with PBS–0.1% saponine, primary antibody binding was visualized with rabbit-anti-mouse-FITC, goat-anti-mouse-FITC, goat-anti-rabbit-FITC or goat-anti-rabbit-TRITC (incubation time 30 min). After four rinses with PBS–0.1% saponine the chambers were disassembled and the cells were embedded in 90% glycerol-10% PBS–0.1% phenylendiamine. The specimens were examined with a Reichert Polyvar microscope equipped with epifluorescence and Nomarski DIC in conjunction with methods of video microscopy as previously described (Bächi et al., 1988). For double immunofluorescence the formaldehyde-fixed and saponine-permeabilized cells were sequentially incubated with rabbit anti-galactosyltransferase serum, goat anti-rabbit Ig-TRITC, monoclonal antibody G1/93, and goat anti-mouse IgG-FITC.

Immunoelectron Microscopy. Filter-grown Caco-2 cells were fixed 7 d after confluency with a mixture of 1% formaldehyde, freshly prepared from paraformaldehyde, and 0.1% glutaraldehyde in 0.15 M sodium-bicarbonate pH 7.4 for 1 h. The cells were then scraped from the filter and pelleted in 10% gelatin. The pellet was fixed and stored in 1% formaldehyde in 0.1 M phosphate buffer, pH 7.4, until use. HepG2 cells were fixed in a mixture of 1% formaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 for 1 h. The cells were scraped and pelleted as above. Human intestinal biopsies were obtained from subjects being examined for gastrointestinal disorders. Only freshly obtained material showing no pathological alterations by light and electron microscopy and having normal levels of brush border enzyme activities was used in this study. Biopsies were fixed in a mixture of 2% formaldehyde, freshly prepared from paraformaldehyde, and 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h, and stored in 2% formaldehyde until use. Ultrathin cryosectioning was done essentially as described previously (Fransen et al., 1985). Sections were incubated with the monoclonal antibody, a rabbit-anti-mouse antibody, and finally with protein A complexed to colloidal gold (Slot and Geuze, 1985). For double-labeling experiments, the sections were sequentially incubated with the monoclonal antibody, rabbit-anti-mouse, and protein A–10 nm colloidal gold, followed by a blocking step with protein A alone (2 mg/ml, 5 min), and finally with the polyclonal antibody against galactosyltransferase, followed by protein A–5 nm colloidal gold. The sections were examined in a Philipps EM 410 electron microscope operating at 80 KV.

Cell Culture and Metabolic Labeling with [^{35}S]Methionine. Caco-2 cells (kindly provided by Dr. A. Zweibaum) were grown in Optilux petri dishes according to Pinto et al. (1983) as described (Hauri et al., 1985). All the experiments were performed with cells of passage 136 to 150. Labeling of Caco-2 cells with [^{35}S]methionine was carried out with cells grown on filters 5 to 11 d after reaching confluency. Before labeling, the cells were rinsed with PBS and preincubated upside-down in PBS–1% nonessential amino acids–20% dialyzed FCS at 37°C. After a 5- or 15-min pulse with 100 μCi [^{35}S]methionine in 250 μl PBS–20% dialyzed FCS at 37°C, which was added to the basolateral side, the cells were washed with and chased in normal culture medium in the presence of 10 mM unlabeled methionine. HepG2 cells (kindly provided by Dr. M. Spiess) were grown in 30-mm dishes in DME supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 μg/ml fungizone. Confluent cells were metabolically labeled with 100 μCi [^{35}S]methionine (in 1 ml methionine-free medium containing 1% nonessential amino acids and 20% dialyzed FCS) per dish overnight.

Immunoprecipitation, SDS-PAGE, and Fluorography. Antigens were immunoprecipitated from Triton X-100-solubilized cells essentially as described (Hauri et al., 1985) by means of monoclonal antibodies adsorbed to protein A–Sepharose at pH 8.0. After four wash steps with 100 mM sodium phosphate containing 0.2% BSA, 1% Triton X-100, 0.1% sodium azide and 40 μg/ml PMSF (pH 8.0), one wash step with 100 mM sodium phosphate, pH 8.0, and one step with 10 mM sodium phosphate, pH 8.0, the immunocomplexes were released from the beads by boiling in electrophoresis sample buffer. Proteins were separated on 10% SDS–polyacrylamide slab gels or 4 to 10% SDS–polyacrylamide gradient slab gels using the Laemmli system (1970) and visualized by fluorography using EN^{3}HANCE and preflashed Kodak X-omat AR films.

Chemical Crosslinking. Intact total membranes or Triton X-100-solubilized 100,000 *g* 1 h supernatants of Caco-2 cells were incubated with the crosslinking reagent (0.857 mM final concentration) at room temperature. The noncleavable crosslinker DSS or the cleavable crosslinker DSP were used. After 20 min the reaction was stopped with a 45 mM final concentration of ethanolamine.

Protein Labeling with Na^{125}I. A total membrane fraction of Caco-2 cells originating from one filter culture was either labeled with Na^{125}I using the lactoperoxidase-glucoseoxidase method as described (Hauri et al., 1985) or the ^{125}I-sulfo-SHPP procedure (Thompson et al., 1987). 20 μl sulfo-SHPP and 1 mCi Na^{125}I were used to prepare ^{125}I-sulfo-SHPP which was immediately added to a volume of 500 μl PBS containing the membrane vesicles. After 30 min at room temperature the reaction was stopped with 1 ml PBS containing 1 mg lysine. The tube was filled with 4 ml PBS and the sample was spun at 100,000 *g* for 1 h. The supernatant was discarded and the pellet was used for the protease protection experiment.

Phase-Partitioning in Triton X-114 and Sodium Carbonate Extraction. A total membrane fraction of [^{35}S]methionine-labeled Caco-2 cells was solubilized in preclouded 2% Triton X-114 and the 100,000 *g* 1 h supernatant was subjected to phase partitioning according to Bordier (1981). The detergent phase and the aqueous phase were separated and subjected to immunoprecipitation experiments. Membrane association was also tested by the sodium carbonate procedure (Fujiki et al., 1982) as described (Gorr et al., 1988).

Digestion with Glycosidases. These experiments were performed with the immunoprecipitated ^{35}S-labeled antigen as described previously (Gorr et al., 1988).

Protease Protection Experiments. A total membrane fraction (see above) was prepared from filter-grown ^{35}S-labeled or unlabeled Caco-2 cells. Aliquots of the membrane pellet corresponding to cells of one filter (in 500 μl PBS) were incubated for 1 h on ice with either trypsin (250 μg/ml final concentration) or proteinase K (20 μg/ml final concentration) in the presence or absence of 0.1% (wt/vol) Triton X-100. After 1 h the reaction

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was stopped by the addition of chicken trypsin inhibitor (500 μg/ml) and PMSF (40 μg/ml) followed by a 1 h solubilization step with 1% Triton X-100 and centrifugation (100,000 *g*, 1 h). The supernatants were analyzed by immunoprecipitation and SDS-PAGE.

Results

A Tubulo-Vesicular Compartment Near the cis-Golgi Apparatus Defined by Monoclonal Antibody G1/93

A fraction enriched in Golgi membranes of intestinal adenocarcinoma cells, Caco-2, was used as an antigen to produce organelle-specific monoclonal antibodies. When screened by immunofluorescence on saponine-permeabilized Caco-2 cells, one of the antibodies exhibited a punctated intracellular staining pattern which was reminiscent of the Golgi apparatus. Its relation to the Golgi apparatus was studied by double immunofluorescence microscopy using the Golgi marker galactosyltransferase which in intestinal absorptive cells in vivo is known to be localized in medial and *trans* but not *cis* cisternae of the Golgi apparatus (Roth et al., 1986). In Caco-2 cells the Golgi apparatus is dispersed in many small stacks and hence antibodies against galactosyltransferase in many instances yield a dot- or dash-like staining pattern (Fig. 1 *b*) rather than a single bright complex in a juxtanuclear position as in many other cells. Fig. 1 *a* shows the immunofluorescence pattern obtained with antibody G1/93. This compartment seems to overlap with the galactosyltransferase-positive area but it was always somewhat larger and more diffuse. In the hepatoma cell line HepG2 where the Golgi apparatus displays the classical compact structure (Fig. 1 *e*) the G1/93-positive compartment was also more defined and showed partial identity with the galactosyltransferase positive area (Fig. 1 *d*). This indicated to us that the G1/93 antigen might be structurally or functionally related to the Golgi apparatus. The pattern obtained with G1/93 was markedly different from that obtained with mAb G1/139 against lysosomal membranes (Fig. 1, *c* and *f*) and with EH 1/8 against the smooth ER marker epoxide hydrolase (not shown).

Immunoelectron microscopy carried out with ultrathin frozen sections of Caco-2 cells (Fig. 2, *a* and *b*) or normal human small intestinal mucosa (Fig. 2, *c* and *d*) in conjunction with the colloidal gold–protein A technique revealed that antibody G1/93 specifically labeled clusters of vesicles and tubules on one side of the Golgi apparatus including one fenestrated cisterna of the Golgi itself (Fig. 2 *c*). A larger magnification (Fig. 2 *e*) shows more clearly the labeling in one Golgi cisternae. No other regions of the cells showed any specific labeling. These vesicles exhibit an electron dense interior and very often a coat on their outside which is less developed than with clathrin-coated vesicles (Fig. 2 *d*). In most instances the immunolabel was observed within the vesicles and tubules suggesting that the epitope recognized by G1/93 is present on the extracytoplasmic side of the membrane. Immuno double-labeling experiments with antibody G1/93 and anti–galactosyltransferase performed on the same section demonstrated that the tubulovesicular compartment is topographically related to the *cis* side of the Golgi apparatus. In the normal intestine, galactosyltransferase, an accepted marker of the *trans*-Golgi apparatus (Roth and Berger, 1982), labeled preferentially the cisternae of the medial and the *trans* side of the Golgi apparatus, while the tubulovesicular compartment was localized at the *cis* side (Fig. 3 *b*). In Caco-2 (Fig. 3 *a*) and HepG2 (Fig. 4) cells galactosyltransferase labeled predominantly the cisternae at the *trans* side of the Golgi apparatus, while the G1/93 positive compartment was again localized on the *cis* side of the Golgi apparatus. In these cells the medial part of the Golgi apparatus was free of label.

G1/93 Recognizes a 53-kD Protein

The antigen recognized by monoclonal antibody G1/93 was identified in Caco-2 and HepG2 cells by metabolic labeling with [^{35}S]methionine followed by immunoprecipitation and SDS-PAGE. In both cell types the antibody precipitated a single protein with a relative molecular mass of 53 kD (Fig. 5, lanes *2* and *3*). The antigen synthesized in Caco-2 cells was further characterized and found to have no oligosaccharide side chains by two independent criteria. First, it did not undergo an apparent upward shift in molecular mass on SDS-gels with longer labeling periods (Fig. 5, lane *8* vs. lane *4*), and second, neither digestion with endoglycosidase H, which cleaves N-linked high-mannose side chains, nor with O-glycanase which cleaves O-linked side chains, lead to a decrease of its apparent relative molecular mass on SDS-gels (Fig. 5). The results suggest that the 53-kD protein is probably not or only minimally glycosylated.

Membrane Association and Topology of the 53-kD Protein

A possible membrane association of the 53-kD protein was tested by the sodium carbonate extraction procedure (Fujiki et al., 1982) and by phase partitioning using Triton X-114 (Bordier, 1981). Caco-2 cells were labeled overnight with [^{35}S]methionine and a total membrane fraction was prepared which was subjected to the carbonate extraction procedure. Fig. 6 *A* shows that the 53-kD protein remained membrane associated after this procedure. For the phase-partitioning experiment Caco-2 cells were labeled with [^{35}S]methionine and phase partitioning was performed in 2% Triton X-114. Fig. 6 *B* demonstrates that most of the 53-kD protein molecules partitioned into the detergent phase as did the two integral brush border membrane enzymes aminopeptidase N and dipeptidylpeptidase IV which were used as a control. We conclude therefore that the 53-kD antigen is an integral membrane protein.

To study the topology and potential transmembrane disposition protease protection experiments were carried out. In a first series of experiments a total membrane fraction was prepared by differential centrifugation from [^{35}S]methionine-labeled Caco-2 cells. The membrane pellet was resuspended by means of a syringe connected to a 25-gauge needle to generate sealed vesicles. When intact vesicles were incubated with trypsin or proteinase K, the 53-kD protein was largely protected from digestion (Fig. 7, lanes *3* and *5*). However, the mobility of the protein slightly but consistently increased by ~0.5 to 1.0 kD due to protease treatment while the protein was completely digested when the vesicles were permeabilized with Triton X-100 before protease treatment (Fig. 7, lanes *4* and *6*). Assuming a right-side-out orientation of intracellular membranes these results suggest that a short segment of the 53-kD protein is exposed at the cytoplasmic side

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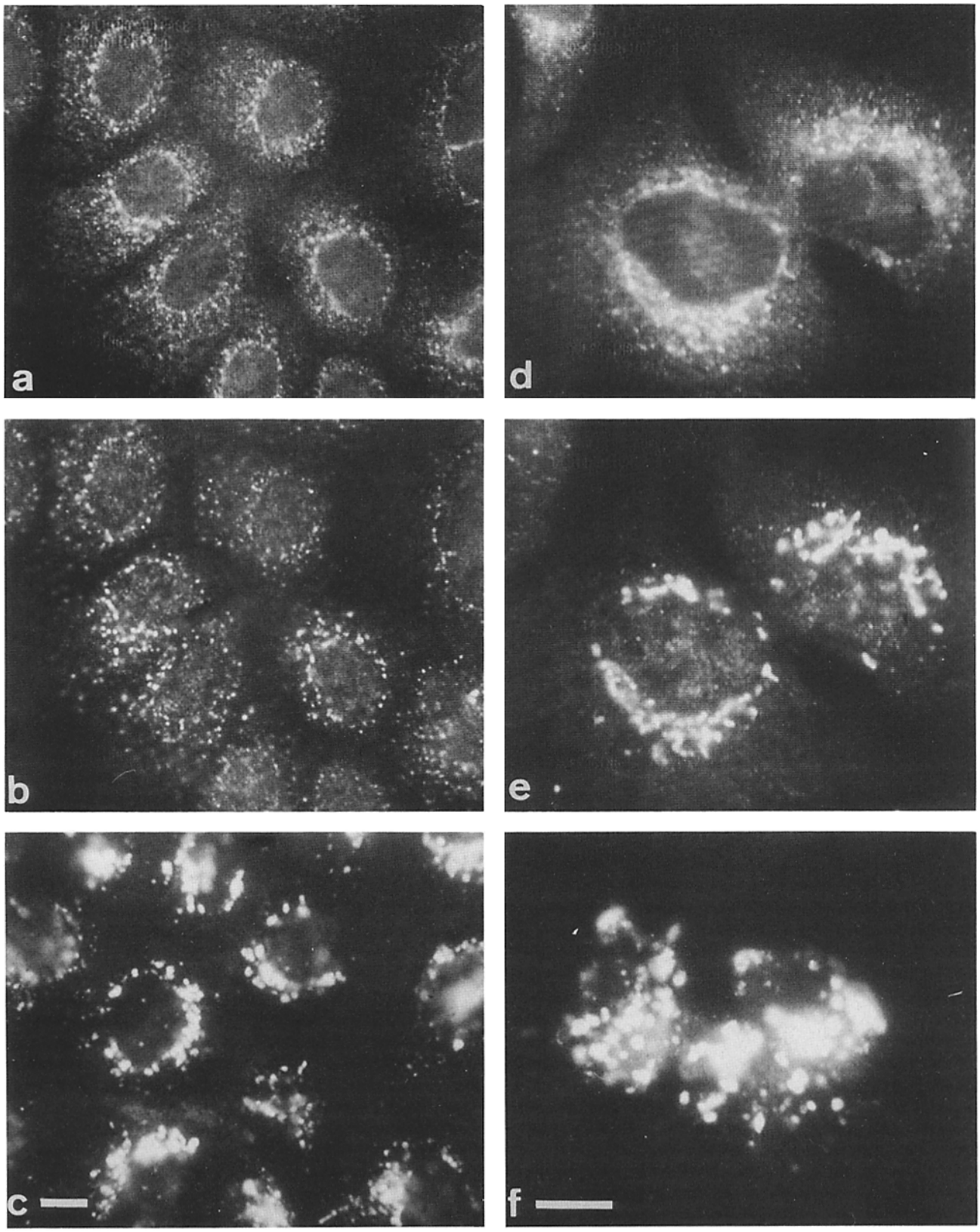

Figure 1. Double immunofluorescence localization of the G1/93 antigen and of galactosyltransferase and single immunofluorescence localization of lysosomes in Caco-2 cells (*a–c*) and HepG2 cells (*d–f*). For the double immunofluorescence Caco-2 cells were grown in uncoated and HepG2 cells in polylysine-coated multichamber slides. The cells were fixed with 3% formaldehyde, permeabilized with 0.1% saponine and sequentially incubated with rabbit anti-galactosyltransferase, rhodamine-labeled goat anti-rabbit IgG, monoclonal antibody G1/93, and fluorescein-labeled goat anti-mouse IgG. For single immunofluorescence localization of lysosomes the cells were fixed and permeabilized as above. They were then incubated with mab G1/139 against a lysosomal membrane protein followed by rhodamine-labeled goat anti-mouse

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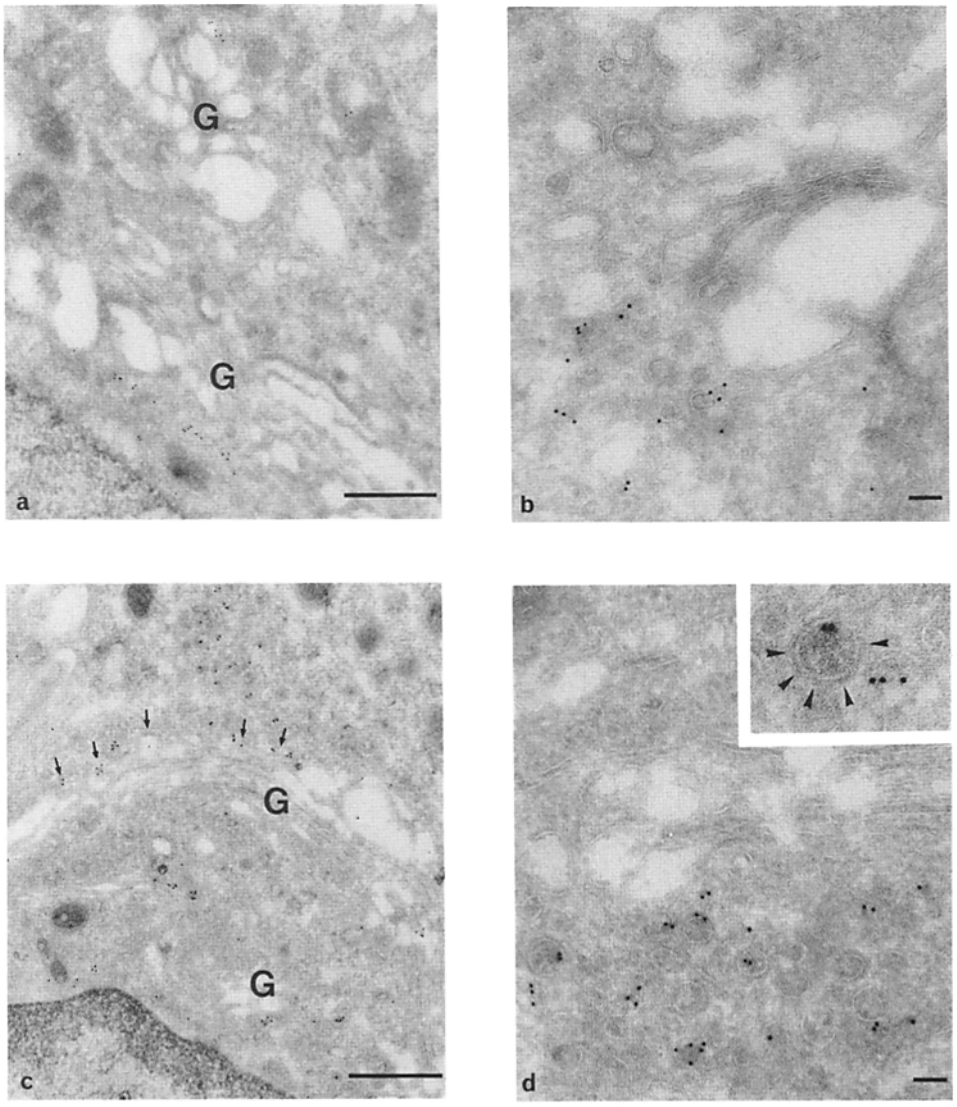

Figure 2. Immunogold labeling of a tubulo-vesicular compartment in Caco-2 cells (*a* and *b*) and in normal human enterocytes (*c*, *d*, and *e*) with G1/93 antibody (ultrathin cryosections). In both cell types the vesicles often exhibit an electron dense interior and a coat on their outside (*arrowheads* in inset of *d*). In addition, a single fenestrated cisterna of the Golgi apparatus is occasionally labeled (*arrows* in *c* and *e*). Bar, 0.25 μm.

IgG. Images were taken with a SIT camera connected to a Reichert Polyvar fluorescence microscope. (*a* and *d*) Localization of the G1/93 antigen; (*b* and *e*) localization of galactosyltransferase; (*c* and *f*) localization of a lysosomal 100-kD protein defined by mAb G1/139. Bars, 10 μm.

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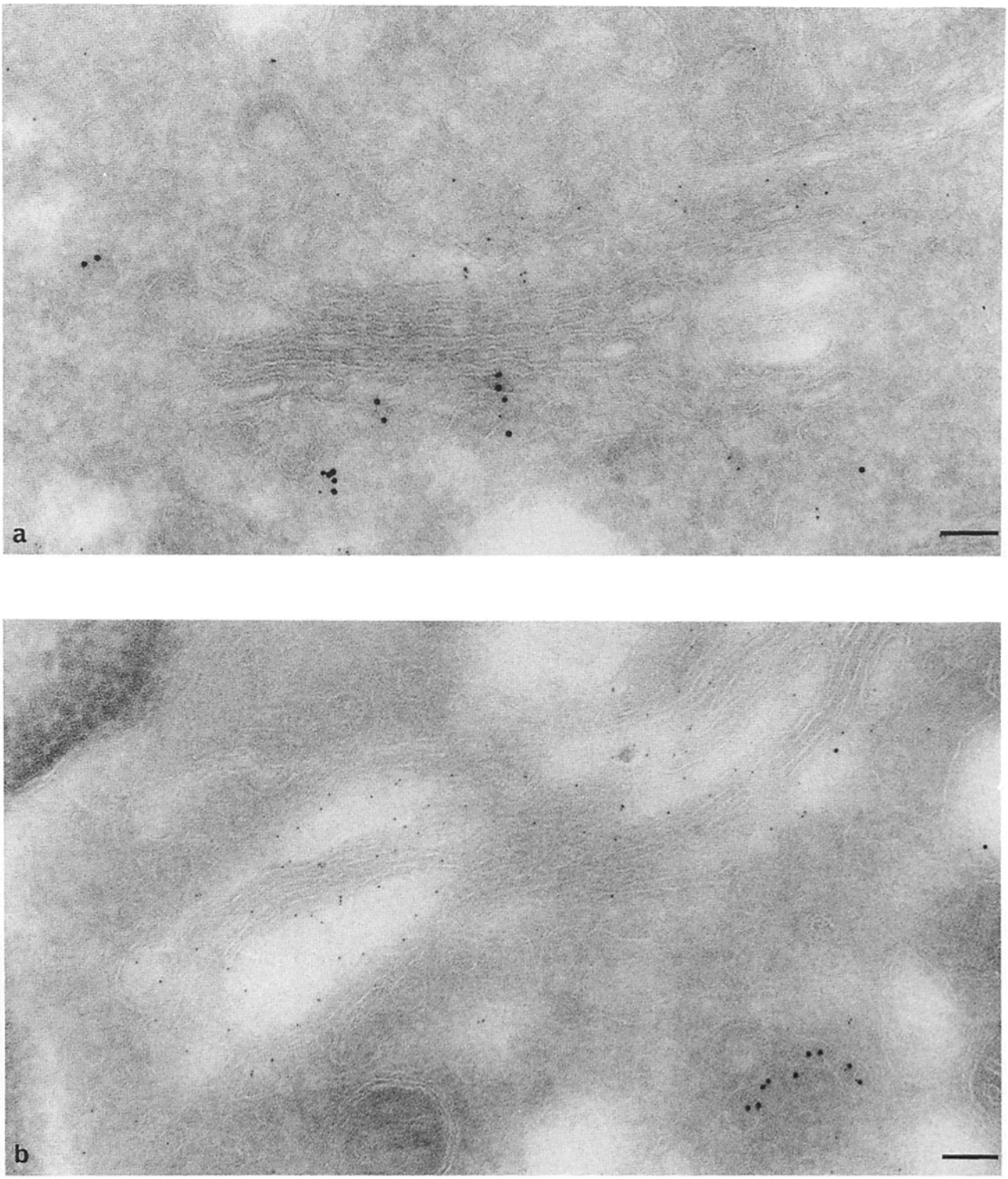

Figure 3. Double labeling of Caco-2 cells (*a*) and normal human enterocytes (*b*) with G1/93 (large gold) and anti-galactosyltransferase (small gold). In Caco-2 cells (*a*) galactosyltransferase is localized on one side (i.e., the *trans* side) of the Golgi apparatus, while G1/93 stains vesicles and tubules on the opposite side. The cisternae of the medial part are free of label. In the normal enterocytes (*b*) the cisternae of the medial part of the Golgi apparatus are also stained for galactosyltransferase. Bars, 0.1 μm.

while the bulk of the protein is either buried in the lipid bilayer or protruding into the extracytoplasmic space.

To further evaluate if the 53-kD protein is indeed exposed at the cytoplasmic side intact vesicles were radiolabeled with ^{125}I by two different nonpermeant labeling procedures (Fig. 7), i.e., the lactoperoxidase-glucoseoxidase method or the ^{125}I-sulfo-SHPP procedure (Thompson et al., 1987). These procedures can be expected to radiolabel sealed right-side-

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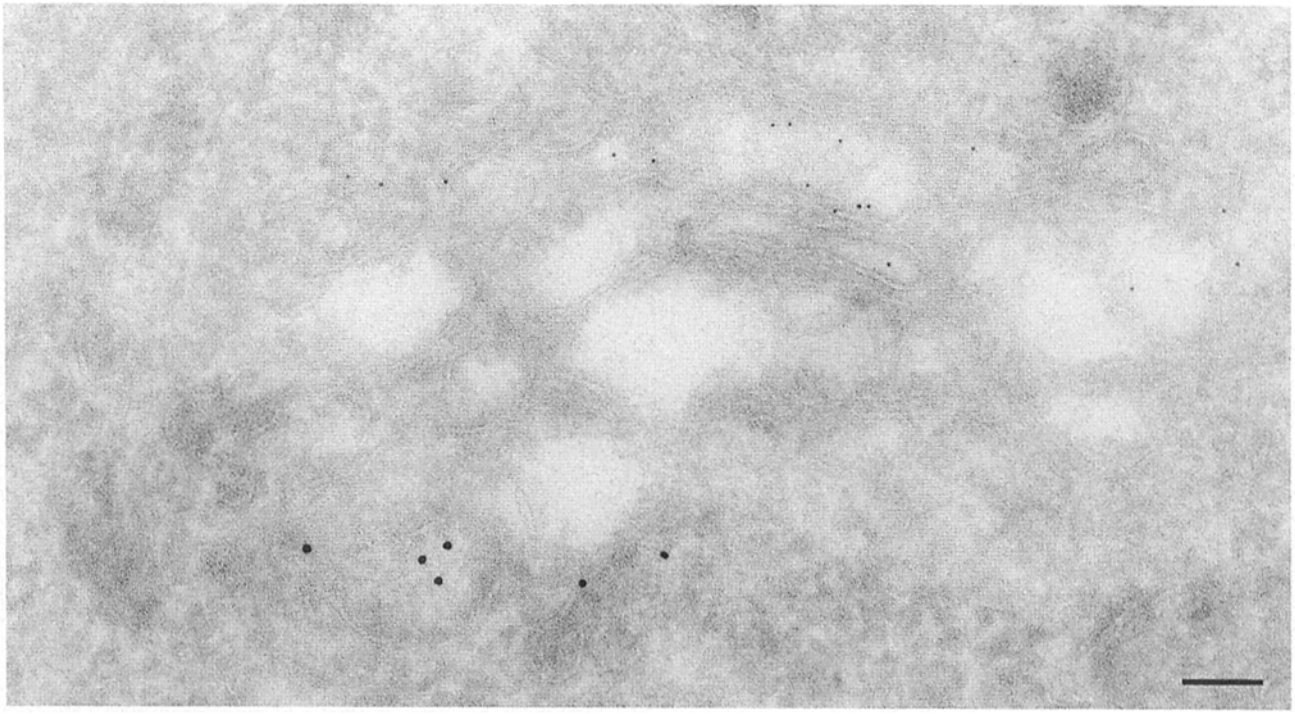

Figure 4. Double immunogold labeling of the G1/93 antigen (large gold) and galactosyltransferase (small gold) on ultrathin cryosections of HepG2 cells. As in Caco-2 cells galactosyltransferase is localized on one side of the Golgi apparatus while G1/93 labeled vesicles and tubules on the opposite side of the Golgi apparatus. Bar, 0.1 μm.

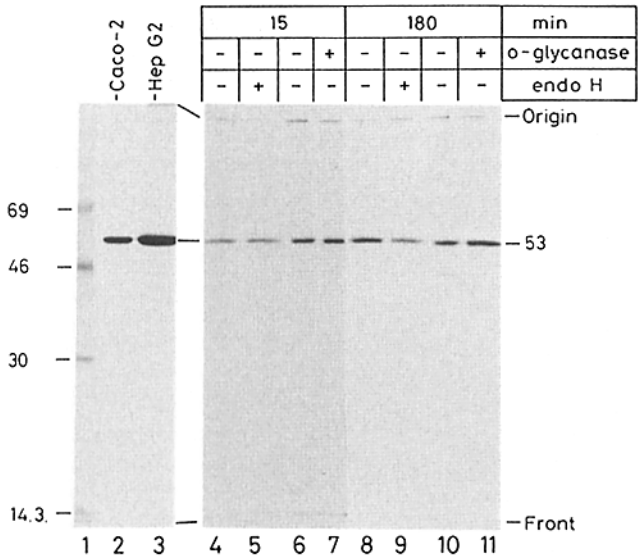

Figure 5. Identification of 53-kD protein in Caco-2 and HepG2 cells by immunoprecipitation with monoclonal antibody G1/93 (fluorogram). Caco-2 (lane *2*) or HepG2 (lane *3*) cells were labeled over night with [^{35}S]methionine and subjected to immunoprecipitation as described under Materials and Methods. To test for the presence of oligosaccharide side chains Caco-2 cells were labeled for 15 min (lanes *4–7*) or 180 min (lanes *8–11*) with [^{35}S]methionine and the immunoisolated G1/93 antigen was subjected to endo-H or O-glycanase treatment as indicated. Mock-treated controls without glycosidase are shown in lanes *4* (for lane *5*), *6* (for *7*), *8* (for *9*), and *10* (for *11*). Lanes *4–7* and *8–11* were assembled from two fluorograms of the same 10% SDS-gel which had to be exposed for different lengths of time. The numbers at the left margin of the gel (lane *1*) indicate ^{35}S-marker proteins in kilodaltons.

out vesicles of intracellular origin from only the cytoplasmic side and the label should therefore largely disappear after protease digestion. This was indeed so. However, mock-treated ^{35}S-labeled vesicles included as a control were found to be somewhat less sealed than under the optimal conditions used for the biosynthetic-labeling experiment (Fig. 7,

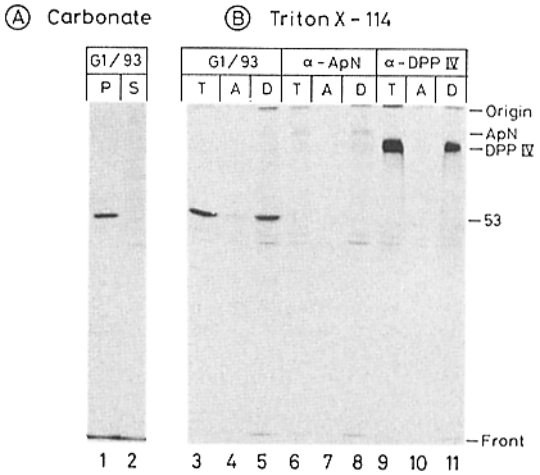

Figure 6. Membrane association of the ^{35}S-labeled 53-kD protein defined by antibody G1/93 in Caco-2 cells (fluorogram). Confluent cells were metabolically labeled with [^{35}S]methionine and subjected to the carbonate extraction procedure (*A*) or phase partitioning in Triton X-114 (*B*) as described under Materials and Methods. (*P*) Pellet; (*S*) supernatant; (*T*) total membrane fraction; (*A*) aqueous phase; (*D*) detergent phase; (*APN*) aminopeptidase N; (*DPPIV*) dipeptidylpeptidase IV.

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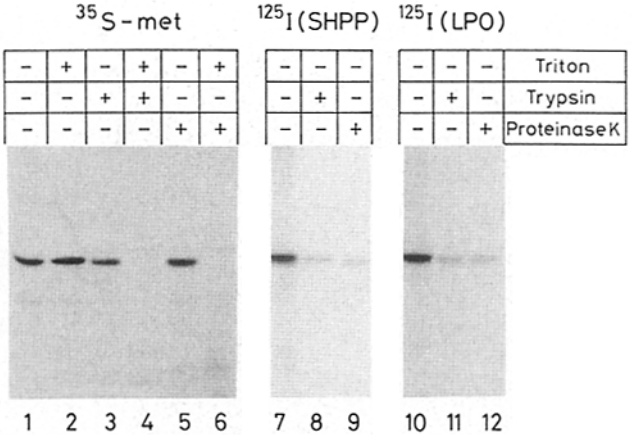

Figure 7. Protease protection experiments with the 53-kD protein in a total membrane vesicle fraction of Caco-2 cells. Confluent cells were metabolically labeled with [^{35}S]methionine (*^{35}S-met*) for 75 min and a total membrane fraction was prepared and subjected to protease treatment. Alternatively total membrane fractions of unlabeled cells were radioiodinated with ^{125}I-sulfosuccinimidyl (hydroxyphenyl) propionate (*^{125}I-SHPP*) or by the lactoperoxidase-glucose-oxidase method (*^{125}I-LPO*) before protease treatment. See Materials and Methods for details. *Triton*, pretreatment of the membranes with 0.1% Triton X-100.

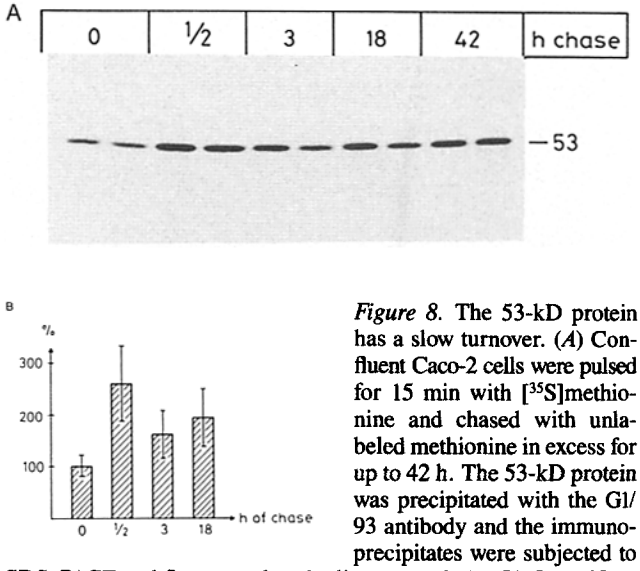

Figure 8. The 53-kD protein has a slow turnover. (*A*) Confluent Caco-2 cells were pulsed for 15 min with [^{35}S]methionine and chased with unlabeled methionine in excess for up to 42 h. The 53-kD protein was precipitated with the G1/93 antibody and the immunoprecipitates were subjected to SDS–PAGE and fluorography (duplicate samples). (*B*) Quantification by densitometric scanning of pulse-chase experiments as illustrated in *A*. The bars indicate the mean ± 1 SD (n = 8).

lanes *1* to *6*). Nevertheless, trypsin removed 74% and proteinase K removed 84% of the radioactivity from the ^{125}I 53-kD protein after labeling with the lactoperoxidase-glucoseoxidase procedure while the corresponding values were 48 and 46%, respectively, for the mock-treated ^{35}S-labeled protein. Similar results were obtained with the ^{125}I-sulfo-SHPP procedure. Trypsin was found to remove 75% and proteinase K 77% of the radioactivity while in the mock-treated control samples the values were 52 and 45%, respectively. Collectively these results suggest a transmembrane disposition of the 53-kD protein.

Life Cycle and Oligomeric State of the 53-kD Protein

Pulse-chase experiments with Caco-2 cells suggested that the once synthesized 53-kD protein is long-lived as little degradation was apparent by SDS–PAGE after long chase periods (Fig. 8 *A*). This impression was confirmed by quantification of the fluorograms (Fig. 8 *B*).

The oligomeric state of the 53-kD protein was investigated by SDS–PAGE under nonreducing conditions and after covalent chemical cross-linking. Under nonreducing conditions little 53-kD subunit protein was observed on the gel (Fig. 9, lane *10*). Instead a number of high molecular mass proteins were precipitated with the G1/93 antibody the most prominents of which had an apparent molecular mass of ~110 kD and ~310 kD. When such gel lanes were cut out and subjected to a second slab gel electrophoresis under reducing conditions all of these bands displayed a molecular mass of 53-kD suggesting that all the high molecular mass bands resulted from oligomerization of the 53-kD protein probably by disulfide bonds (Fig. 9 *C*). Thus the 110-kD band may be a homodimer and the 310-kD a homohexamer of the 53-kD protein. Fig. 9, lane *10* shows at least two additional strong bands. At present we do not know what these bands are, but their intensity was variable in different experiments.

To probe for next neighbor relationships covalent crosslinking experiments were carried out with a Triton X-100 detergent extract of ^{35}S-labeled Caco-2 cells (Fig. 9 *B*) or with a total membrane fraction of these cells (Fig. 9 *A*) by using either the noncleavable crosslinker DSS or the very similar cleavable crosslinker DSP. With Triton X-100 extracts both crosslinkers gave identical results when the samples were treated and separated under nonreducing conditions, i.e., in the absence of dithiothreitol (DTT) (Fig. 9, lanes *11* and *12*). After immunoprecipitation with the G1/93 antibody most of the radioactivity was found in the 300-kD range of the gel. As expected, with DSS a similar pattern was also observed under reducing conditions, i.e., in the presence of DTT (Fig. 9, lane *8*). Most important, however, is the finding that after DSS treatment almost no free subunits were present on the gel indicating a strong subunit interaction. Most of the crosslinked radioactivity was due to the 53-kD protein since the reduction of the DSP-crosslinked material predominantly yielded the expected subunits (Fig. 9, lane *9*). However, under these conditions additional bands appeared on the gel and it was of interest therefore to assess if the 53-kD protein indeed interacts with other proteins or whether these additional proteins were artefactually crosslinked in a Triton X-100 environment. For this purpose the crosslinking experiments were repeated with intact membrane vesicles before detergent solubilization. Fig. 9 *A* shows that DSP more efficiently crosslinked the protein than DSS (Fig. 9, lane *6* vs. *5*). When the DSP-crosslinked material was reduced virtually all of it was recovered as separate subunits (Fig. 9 *A*, lane *3*) with slightly higher relative molecular mass which is most likely due to attached crosslinker molecules. Moreover, it is evident that no additional protein are specifically crosslinked with the 53-kD protein under these more native conditions. Collectively the crosslinking data support the notion that the 53-kD protein may form dimers and hexamers under native conditions.

The biogenesis of oligomers was studied by pulse-chase experiments followed by SDS–PAGE under nonreducing

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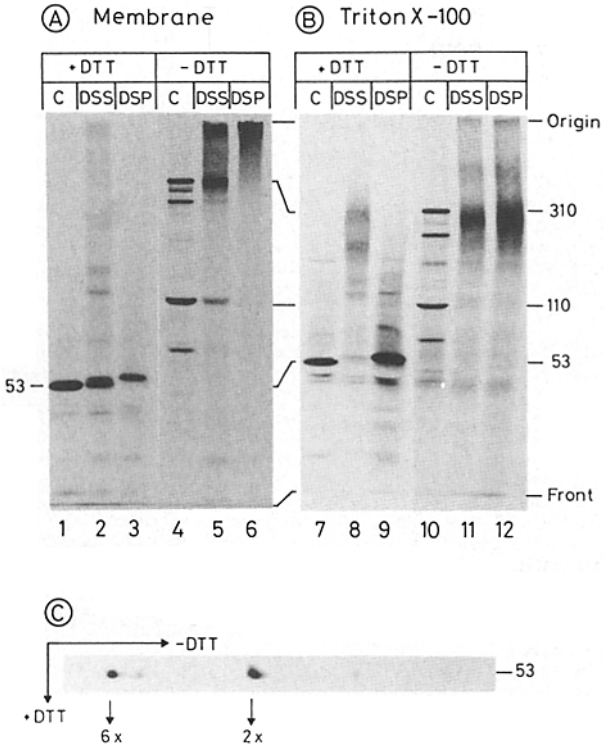

Figure 9. The oligomeric state of the 53-kD protein. (*A*) Caco-2 cells were labeled overnight with [^{35}S]methionine and a total membrane fraction was subjected to chemical crosslinking using DSS or DSP and compared to mock-treated controls (lanes *1* and *4*) after immunoprecipitation with G1/93. (*B*) Chemical crosslinking was performed in a Triton X-100 cell extract of [^{35}S]methionine-labeled Caco-2 cells. (*C*) Two-dimensional SDS–PAGE of an immunoprecipitate obtained with G1/93. The precipitate was separated under nonreducing conditions (*−DTT*) in the first dimension and under reducing conditions (*+DTT*) in the second dimension. The fluorogram shows that the high molecular mass bands apparent in lane *4* are composed of 53-kD subunits. (*2x*) Position of dimer; (*6x*) position of hexamer in the first dimension.

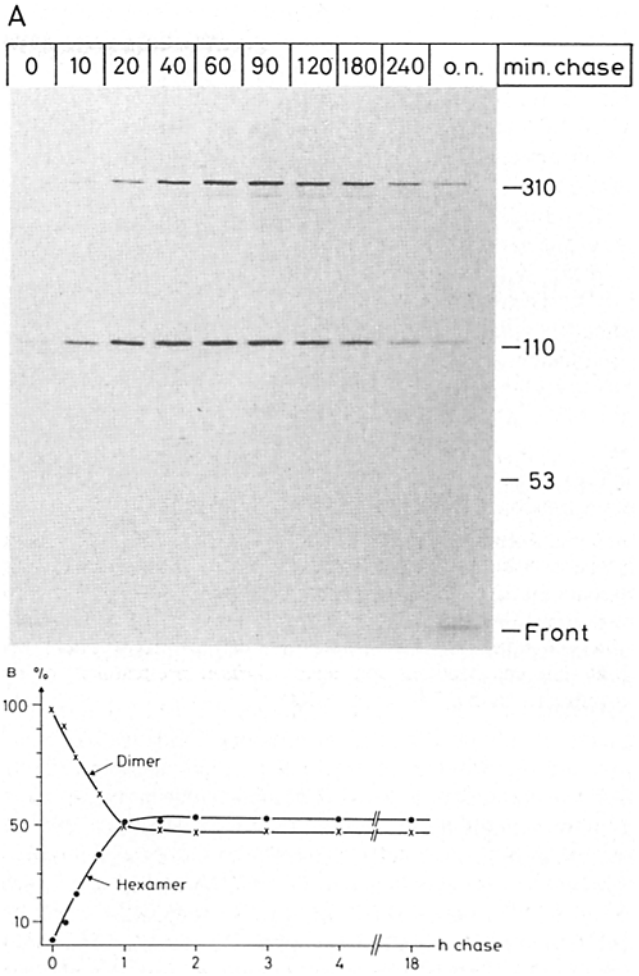

Figure 10. Biogenesis of dimers and hexamers of the 53-kD protein. (*A*) Caco-2 cells were pulsed with [^{35}S]methionine for 5 min and chased for the indicated time intervals (*o.n.*, overnight). The immunoprecipitates obtained with the G1/93 antibody were separated on a 4–10% gradient SDS-polyacrylamide gel under nonreducing condition (i.e., in the absence of DTT). (*B*) Quantification by densitometric scanning of dimer and hexamer formation during the chase period. 100% is the sum of dimers and hexamers at a given chase time. Monomers were only visible at 0 min chase and were therefore not included in the quantification. Each time point is the mean of three experiments (variations ≤7%).

conditions (Fig. 10). After a 5-min pulse a sizable fraction of the 53-kD protein had already undergone dimerization. During the chase period the monomer rapidly disappeared with concomitant appearance of the dimer and slightly delayed appearance of the ~310-kD hexamer. After ~120 min a steady state is reached in which ~50% of the molecules are in dimers and 50% in hexamers.

Discussion

In this study we have identified and characterized a novel 53-kD protein by means of the monoclonal antibody G1/93 which specifically labels a tubulovesicular compartment in close proximity to the Golgi apparatus. A number of observations suggest to us that this protein may delineate the biosynthetic protein pathway from the ER to the Golgi apparatus in intestinal epithelial cells. Firstly, immunoelectron microscopy showed that in addition to the vesicular tubular compartment the first fenestrated *cis*-cisterna (Rambourg et al., 1987) of the Golgi apparatus occasionally comprised the 53-kD protein in intestinal absorptive epithelial cells, Caco-2 cells, and HepG2 cells. It is currently unknown if part of the tubules decorated with antibody G1/93 represents transitional elements of the rough ER, i.e., the presumed exit site of secretory and membrane proteins (Palade, 1975), since transitional elements cannot be identified with certainty in ultrathin cryosections of intestinal cells. It is clear though that G1/93 does not label the whole rough ER. Secondly, the immunofluorescence double-labeling experiments demonstrated that the morphology of the compartment stained with antibody G1/93 paralleled that of the Golgi apparatus as revealed by an antibody to galactosyltransferase. These observations are consistent with a current model of biosynthetic protein traffic which predicts that transport vesicles shuttle back and forth between ER and the Golgi apparatus (Farquhar, 1985). In intestinal epithelial cells it has not been possible to visualize major membrane proteins as brush border hydrolases in transport vesicles near the *cis*-Golgi due to the

small amount of protein in transit to the Golgi apparatus (Fransen et al., 1985). Detection of a protein in transport vesicles by the protein A-gold technique may require its accumulation by means of a recycling mechanism operating between ER and *cis*-Golgi. The subcellular distribution of the 53-kD protein and its long half-life are in line with such a mechanism.

What might be the function of the 53-kD protein? A number of functions related to carbohydrate processing are thought to reside in *cis*-Golgi elements (Kornfeld and Kornfeld, 1985). These include α-mannosidase I, *N*-acetylglucosaminyltransferase I as well as *N*-acetylglucosaminylphosphotransferase and *N*-acetylglucosamine-1-phosphodiester α-*N*-acetylglucosaminidase whose sequential action generates the mannose-6-phosphate signal on lysosomal hydrolases. These proteins have not yet been purified and it is therefore of interest to determine whether the 53-kD protein is responsible for one of these enzyme activities.

A striking feature of the 53-kD protein relates to its oligomeric structure. Under nonreducing conditions the protein appears on SDS-gels as a homooligomer, probably partly as a hexamer and partly as a dimer, suggesting the presence of intermolecular disulfide bonds. A hexameric structure may point to a channel function (Lear et al., 1988).

Alternatively the 53-kD protein may be involved in the recycling of soluble ER resident proteins. Evidence has been provided recently that luminal ER proteins are sorted from secreted proteins in a post-ER compartment by means of a common COOH-terminal peptide, KDEL (Pelham, 1988). Recycling of these proteins to the ER would require a transport receptor. Our conclusion that the 53-kD protein most likely recycles between ER and *cis*-Golgi would be in line with a function as a sorting receptor. Our inability to cross-link the 53-kD protein to any other cellular component does not necessarily rule out such a function.

Finally the 53-kD protein may be part of the machinery mediating the formation, transport or recycling of the shuttle vesicles themselves.

Very recently Saraste et al. (1987) have described a novel antigen with similar location to the 53-kD protein. The antigen, defined by polyclonal antibodies, was identified as a 58-kD protein which was localized predominantly in the *cis*-Golgi apparatus and in some of the tubules and vesicles located along the *cis* face of the Golgi apparatus in myeloma cells. It was reported, however, that the 58-kD protein is a glycoprotein while we have not found any evidence for glycans in the 53 kD protein. It remains to be shown if the two proteins are related.

In conclusion, we have identified a 53-kD protein that is associated with tubulovesicular elements at the *cis* side of the Golgi apparatus in a variety of human cells. This protein might serve to elucidate molecular mechanisms underlying the ER to Golgi biosynthetic pathway of secretory and membrane proteins.

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References

Bächi, T. 1988. Direct observation of the budding and fusion of an enveloped virus by video microscopy of viable cells. *J. Cell Biol.* In press.

Balch, W. E., and D. S. Keller. 1986. ATP-coupled transport of vesicular stomatitis virus G protein. *J. Biol. Chem.* 261:14690–14696.

Beckers, C. J. M., D. S. Keller, and W. E. Balch. 1987. Semi-intact cells permeable to macromolecules: use in reconstitution of protein transport from the endoplasmic reticulum to the Golgi complex. *Cell.* 50:523–534.

Bordier, C. 1981. Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* 256:1604–1607.

Farquhar, M. G. 1985. Progress in unraveling pathways of Golgi traffic. *Annu. Rev. Cell Biol.* 1:447–488.

Fazekas de St. Groth, S., and D. Scheidegger. 1980. Production of monoclonal antibodies: strategy and tactics. *J. Immunol. Methods.* 35:1–21.

Fransen, J. A. M., L. A. Ginsel, H. P. Hauri, and J. Blok. 1985. Immunoelectronmicroscopical localization of a microvillus membrane disaccharidase in the human small-intestinal epithelium with monoclonal antibodies. *Eur. J. Cell Biol.* 38:6–15.

Fujiki, Y., A. Hubbard, S. Fowler, and P. B. Lazarow. 1982. Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. *J. Cell Biol.* 93:97–102.

Galfré, G. S., C. Howe, C. Milstein, G. W. Butcher, and J. D. Howard. 1977. Antibodies to major histocompatibility antigens produced by hybrid cell lines. *Nature (Lond.).* 266:550–552.

Galteau, M.-M., A. Antoine, and H. Reggio. 1985. Epoxide hydrolase is a marker for the smooth endoplasmic reticulum in rat liver. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:2793–2800.

Gorr, S.-V., B. Stieger, J. A. Fransen, M. Kedinger, A. Marxer, and H. P. Hauri. 1988. A novel marker glycoprotein for the microvillus membrane of surface colonocytes of rat large intestine and its presence in small-intestinal crypt cells. *J. Cell Biol.* 106:1937–1946.

Hauri, H. P., E. E. Sterchi, D. Bienz, J. A. M. Fransen, and A. Marxer. 1985. Expression and intracellular transport of microvillus membrane hydrolases in human intestinal epithelial cells. *J. Cell Biol.* 101:838–851.

Hawkes, R., E. Niday, and J. Gordon. 1982. A dot-immunobinding assay for monoclonal and other antibodies. *Anal. Biochem.* 119:142–147.

Jamieson, J. D., and G. E. Palade. 1967*a*. Intracellular transport of secretory proteins in the pancreatic exocrine cell. I. Role of the peripheral elements of the Golgi complex. *J. Cell Biol.* 34:577–596.

Jamieson, J. D., and G. E. Palade. 1967*b*. Intracellular transport of secretory proteins in the pancreatic exocrine cell. II. Transport to condensing vacuoles and zymogen granules. *J. Cell Biol.* 34:597–615.

Jamieson, J. D., and G. E. Palade. 1968. Intracellular transport of secretory proteins in the pancreatic exocrine cell. IV. metabolic requirements. *J. Cell Biol.* 39:589–603.

Kornfeld, R., and S. Kornfeld. 1985. Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* 54:631–664.

Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Lear, J. D., Z. R. Wassermann, and W. F. DeGrano. 1988. Synthetic amphiphilic peptide models for protein ion channels. *Science (Wash. DC).* 240: 1177–1181.

Lodish, H., N. Kong, M. Snider, and G. J. A. M. Strous. 1983. Hepatoma secretory proteins migrate from rough endoplasmic reticulum to Golgi at characteristic rates. *Nature (Lond.).* 304:80–83.

Matlin, K., and K. Simons. 1983. Reduced temperature prevents transfer of a membrane glycoprotein to the cell surface but does not prevent terminal glycosylation. *Cell.* 34:233–243.

Palade, G. E. 1975. Intracellular aspects of the process of protein transport. *Science (Wash. DC).* 189:347–358.

Pelham, H. R. B. 1988. Evidence that luminal ER proteins are sorted from secreted proteins in a post-ER compartment. *EMBO (Eur. Mol. Biol. Organ) J.* 7:913–918.

Pfeffer, S. R., and J. E. Rothman. 1987. Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi. *Annu. Rev. Biochem.* 56:829–852.

Pinto, C., S. Robine-Leon, M. D. Appay, M. Kedinger, N. Triadou, E. Dassaulx, B. Lacroix, P. Simon-Assmann, K. Haffen, J. Fogh, and A. Zweibaum. Enbesocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biol. Cell.* 47:323–330.

Rambourg, A., Y. Clermont, L. Hermo, and D. Segretain. 1987. Tridimensional structure of the Golgi apparatus of nonciliated epithelial cells of the ductuli efferentes in rat: an electron microscope stereoscopic study. *Biol. Cell.* 60:103–116.

Roth, J., and E. G. Berger. 1982. Immunocytochemical localization of galactosyltransferase in Hela cells: codistribution with thiamine pyrophosphatase in trans-Golgi cisternae. *J. Cell Biol.* 92:223–229.

Roth, J., D. J. Taatjes, J. Weinstein, J. C. Paulson, P. Greenwell, and W. M. Watkins. 1986. Differential subcompartmentation of terminal glycosylation in the Golgi apparatus of intestinal absorptive and goblet cells. *J. Biol. Chem.* 261:14307–14312.

Saraste, J., and E. Kuismanen. 1984. Pre- and post-Golgi vacuoles operate in the transport of Semliki Forest virus membrane glycoproteins to the cell surface. *Cell.* 38:535–549.

Saraste, J., G. E. Palade, and M. G. Farquhar. 1987. Antibodies to rat pancreas Golgi subfractions: identification of a 58-kD cis-Golgi protein. *J. Cell Biol.* 105:2021–2030.

Skoda, R. C., A. Demierre, O. W. McBridge, F. J. Gonzales, and U. A. Meyer. 1988. Human microsomal xenobiotic epoxide hydrolase. Complementary DNA sequence, complementary DNA-directed expression in Cos-1 cells, and chromosomal localization. *J. Biol. Chem.* 263:1549–1554.

Slot, J. W., and H. J. Geuze. 1985. A new method of preparing gold probes for multiple labeling cytochemistry. *Eur. J. Cell Biol.* 38:87–93.

Stieger, B., K. Matter, B. Baur, K. Bucher, M. Höchli, and H. P. Hauri. 1988. Dissection of the asynchronous transport of intestinal microvillar hydrolases to the cell surface. *J. Cell Biol.* 106:1853–1861.

Tartakoff, A. 1986. Temperature and energy dependence of secretory protein transport in the exocrine pancreas. *EMBO (Eur. Mol. Biol. Organ.) J.* 5: 1477–1482.

Thompson, J. A., A. L. Lau, and D. D. Cunningham. 1987. Selective radiolabeling of cell surface proteins to a high specific activity. *Biochemistry.* 26: 743–750.

Warren, G. 1987. Signals and salvage sequences. *Nature (Lond.).* 327:17–18.