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) 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, 1967a, 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.

Materials and Methods

Materials and Chemicals

Reagents and supplies were obtained from the following sources: [35S]methionine (translation grade) and EN3HANCE from New England Nuclear (Boston, MA); Na125I (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), diisuccinimidyl suberate (DSS), di-
disorders. Only freshly obtained material showing no pathological alterations was used. HepG2 cells were fixed in a mixture of 1% formaldehyde, 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 epithelial cells were fixed in 1% paraformaldehyde, and 0.1% glutaraldehyde in 0.15 M sodium-bicarbonate 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 epithelial cells were fixed in 1% paraformaldehyde, and 0.1% glutaraldehyde in 0.15 M sodium-bicarbonate buffer, pH 7.4, until use. HepG2 cells were fixed in a mixture of 1% formaldehyde, 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 (Franzen 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 Philips EM 410 electron microscope operating at 80 KV.

Cell Culture and Metabolic Labeling with [35S]Methionine. Caco-2 cells (kindly provided by Dr. A. Zweibaum) were grown in Opti-Lux 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 [35S]methylamine was carried out with cells grown on filters 5 to 11 d after reaching confluence. 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 [35S]methylamine 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 fresh medium in presence of 50 μg/ml PMSF (pH 8.0), 3 μg/ml trypsin inhibitor, 1 μg/ml streptomycin, and 1 μg/ml fungizone. Confluent cells were metabolically labeled with 100 μCi [35S]methylamine (in 1 ml methionine-free medium containing 1% nonessential amino acids and 20% dialyzed FCS) per dish overnight.

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 tuberculin 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 0.1 ml of GalTog membrane protein in Balb/c murine culture medium in incomplete Freund's adjuvant. A booster injection with 60 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., 1981). Hybridoma cells were selected after the antibody-cytotoxicity assay. Hybridoma-like patterns were subcloned by limiting dilution. The resulting antibody was designated GI/93. Another monoclonal antibody (GI/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 (Galfré et al., 1981) as described (Hauri et al., 1985) using PAl 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. 10X concentrated (by ammonium sulfate precipitation) culture supernatants were further screened by immunofluorescence using Caco-2 cells. A hybridoma cell line that bound antibody well was subcloned and antigen-like pattern was subcloned by limiting dilution. The resulting antibody was designated GI/93. Another monoclonal antibody (GI/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 (Galfré et al., 1981) as described (Hauri et al., 1985) using PAl myeloma cells.

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Protein Labeling with Na235S. A total membrane fraction of Caco-2 cells was labeled from one filter culture was either labeled with Na235S using the lactoperoxidase-glucose oxidase method as described (Hauri et al., 1985) or the 25I-sulfo-SHPP procedure (Thompson et al., 1987). 20 μg of protein was 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 protection.

Phase-Partitioning in Tritton X-I44 and Sodium Carbonate Extraction. A total membrane fraction of [35S]methylamine-labeled Caco-2 cells was solubilized in preclouded 2% Tritton X-144 and the 100,000 g 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 so-called "Total Membrane Fraction" method of video microscopy as previously described (Bachi et al., 1988). For double immunoaffinolysis the formaldehyde-fixed and saponin-permeabilized cells were sequentially incubated with rabbit anti-galactosyltransferase serum, goat anti-rabbit IgG, monoclonal antibody GI/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 buffer 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 biopsy specimens 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. Ultra thin cryosectioning was done essentially as described previously (Fazekas de St. Groth and Scheidegger, 1980). 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 nonlymphocyte-precipitated 35S-labeled antigen as described previously (Gorr et al., 1988).

Protease Protection Experiments. A total membrane fraction (see above) was prepared from filter-grown 35S-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
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 G1/39 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 exocytoplasmic 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 [35S]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 [35S]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 [35S]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 [35S]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
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.
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-glucose oxidase method or the $^{125}$I-sulfo-SHPP procedure (Thompson et al., 1987). These procedures can be expected to radiolabel sealed right-side-
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 [35S]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 [35S]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 35S-marker proteins in kilodaltons.

Figure 6. Membrane association of the 35S-labeled 53-kD protein defined by antibody G1/93 in Caco-2 cells (fluorogram). Confluent cells were metabolically labeled with [35S]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; (DPP IV) dipeptidylpeptidase IV.
tracted 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.

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 prominent 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 35S-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 pattern 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 conditions.
Figure 9. The oligomeric state of the 53-kD protein. (A) Caco-2 cells were labeled overnight with [35S]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 [35S]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 [35S]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%).

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-acetylgalactosamine-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 account for one of these enzyme activities.

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 glycoprotein while we have not found any evidence for glycosyltransferases in the pancreatic exocrine cell. 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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