

Giantin, a Novel Conserved Golgi Membrane Protein Containing a Cytoplasmic Domain of at Least 350 kDa

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The Golgi complex consists of a series of stacked cisternae in most eukaryotes. Morphological studies indicate the existence of intercisternal cross-bridge structures that may mediate stacking, but their identity is unknown. We have identified a 400-kDa protein, giantin, that is localized to the Golgi complex because its staining in double immunofluorescence experiments was coincident with that of galactosyltransferase, both in untreated cells and in cells treated with agents that disrupt Golgi structure. A monoclonal antibody against giantin yielded Golgi staining in one avian and all mammalian cell types tested, indicating that giantin is a conserved protein. Giantin exhibited reduced mobility on nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis, was recovered in membrane fractions after differential centrifugation or sucrose flotation, and was not released from membranes by carbonate extraction. Thus, giantin appears to be an integral component of the Golgi membrane with a disulfide-linked luminal domain. Strikingly, the majority of the polypeptide chain is cytoplasmically disposed, because large (up to 350 kDa) proteolytic fragments of giantin could be released from intact Golgi vesicles. This feature, a large contiguous cytoplasmic domain, is present in the calcium-release channel of muscle that cross-bridges the sarcoplasmic reticulum and transverse tubule membranes. Therefore, giantin's localization, conservation, and physical properties suggest that it may participate in forming the intercisternal cross-bridges of the Golgi complex.

INTRODUCTION

The Golgi apparatus participates in the glycosylation and transport of proteins and lipids in the secretory pathway (Mellman and Simons, 1992). Although our knowledge of the proteins carrying out these functions is far from complete, a substantial number of glycosylation enzymes and components involved in vesicular transport have been characterized (Rothman and Orci, 1992; Shaper and Shaper, 1992). Lagging considerably behind is the identification of another class of proteins, those that determine the structure and spatial organization of the Golgi complex itself.

A striking and highly conserved feature of the Golgi apparatus is its cisternal organization. Stacks of flattened Golgi membrane saccules can be identified in almost all eukaryotic cells (Morré *et al.*, 1971; Farquhar and Palade, 1981). Although regions of a cisterna can be highly fenestrated (Rambourg and Clermont, 1990), the inter- and intracisternal membrane distances, usually <15 nm (Mollenhauer and Morré, 1991), appear to be relatively constant over the surface area of the saccules. The cis-

ternal morphology is somehow maintained despite tremendous membrane flow through the Golgi complex. It is known that the cisternae are polarized with respect to this flow and also with respect to the location of the carbohydrate trimming and transferase enzymes that reside in the Golgi complex (Dunphy and Rothman, 1985; Kornfeld and Kornfeld, 1985; Rothman and Orci, 1992). Thus, the Golgi consists of a series of subcompartments, although the polarity in enzyme distribution appears to reflect different mixtures in each cisterna rather than cisterna-specific localization for each enzyme (Nilsson *et al.*, 1993). It is likely that the stacking of cisternae plays a role in establishing and maintaining this polarity. In addition, stacking probably serves to increase intra-Golgi transport efficiency by minimizing the distance between successive subcompartments. The flattened shape of Golgi saccules may increase glycosylation efficiency by reducing the luminal volume (Mellman and Simons, 1992). Flattening may also promote vesicle and tubule formation, thereby facilitating transport.

Because of the near ubiquity of Golgi stacks in eukaryotes, it is likely that both stacking and flattening involve highly conserved mechanisms. Little is known about either process. Protease treatment of isolated Golgi complexes leads to unstacking but not necessarily rounding of cisternae, suggesting that flattening involves the interaction of luminal components, whereas stacking depends on cytoplasmically disposed components (Cluett and Brown, 1992). It has been hypothesized that the electron densities that can be visualized between cisternae in electron micrographs of plant and invertebrate cells (Mollenhauer, 1965; Turner and Whaley, 1965) or animal cells (Franke *et al.*, 1971; Cluett and Brown, 1992) represent proteinaceous filaments or bridges holding the Golgi cisternae together. Indeed, these structures are no longer apparent on cisternae after protease treatments that result in unstacking (Morré *et al.*, 1973; Cluett and Brown, 1992). Obviously, it will be of great importance to identify the components that give rise to this structure and to characterize their role in the cisternal organization of the Golgi.

Additional aspects of Golgi structure are its spatial disposition and continuity (degree of fragmentation), which can vary extensively between different cell types and during different stages of the cell cycle. Certain organisms (e.g., higher plants, fungi, and invertebrates) exhibit a number of discrete stacks (Mollenhauer and Morré, 1991), whereas in many animal cells the Golgi has a "ribbon-like" appearance because of the fact that the flattened cisternae, which form a mass of loops and turns readily visualized by light microscopy (see Figure 1), are actually part of one long continuous membrane network (Rambourg and Clermont, 1990). During interphase this structure is localized in a juxtannuclear region in close proximity to the microtubule organizing center (Kreis, 1990). At mitosis the Golgi fragments and redistributes to the cell periphery, and at telophase it reforms at the cell center (Zeligs and Wollman, 1979; Lucocq *et al.*, 1987, 1989). It is likely that interactions between the Golgi and microtubules are important in this reorganization, because the microtubule network is depolymerized at mitosis, and agents that induce disruption of the microtubule network cause Golgi dispersal (Kreis, 1990). Cytoplasmic dynein, a microtubule minus-end-directed motor protein, has been implicated in the postmitotic localization of the Golgi to the centrosome, where the minus ends of microtubules are located (Ho *et al.*, 1989; Cortesy-Theulaz *et al.*, 1992). It therefore seems likely that there is a Golgi protein with a cytoplasmic domain that mediates the linkage of the organelle to motor components. In addition, there may exist a more direct linkage of Golgi membranes to microtubules because the *in vitro* interaction of Golgi membranes and microtubules can take place in the absence of cytoplasmic dynein (Karecla and Kreis, 1992; Murata *et al.*, 1992).

Consideration of the structural aspects of the Golgi apparatus thus indicates that unidentified proteins must exist whose function is the establishment and regulation of Golgi structure. Features of such proteins are likely to include specific Golgi localization, conservation in evolution, expression in all tissue types, membrane association, relatively large domain facing the cytoplasm (in contrast to the glycosylation enzymes, which have short cytoplasmic tails [Paulson and Colley, 1989]), and the ability to interact with cytoskeletal components. Here we describe our initial experiments with an antibody that recognizes a novel Golgi protein, giantin, that has many of these features. In particular the finding that giantin has a cytoplasmic domain of ≥ 350 kDa suggests that it is likely to be part of the structure that cross-bridges the cisternae of the Golgi complex.

MATERIALS AND METHODS

Materials

Protease inhibitors (used at the following final concentrations: 1 $\mu\text{g}/\text{ml}$ pepstatin, 1 $\mu\text{g}/\text{ml}$ leupeptin, 1 $\mu\text{g}/\text{ml}$ antipain, and 0.2 mM phenylmethylsulfonyl fluoride) were from Sigma (St. Louis, MO). Proteinase K and Triton X-100 were from Serva (Heidelberg, Germany). Trypsin was from Worthington (Freehold, NJ). Antibodies used included: a polyclonal antiserum against galactosyltransferase (Oscar, gift of E. Berger, University of Zürich) and a purified monoclonal antibody (mAb) against tubulin (1A2, gift of T. Kreis, University of Geneva). In addition the following mAb hybridoma supernatants (~ 10 -fold concentrated by ammonium sulphate precipitation) were used: G1/133 (anti-giantin), G1/221 (anti-human transferrin receptor) (Sander and Hauri, unpublished data), G1/93 (anti-ERGIC 53) (Schweizer *et al.*, 1988), G1/296 (anti-p63) (Schweizer *et al.*, 1993a), GT2/36/118 (anti-galactosyltransferase) (Berger *et al.*, 1986), and A1/118 (anti-118a) (Linstedt, Schweizer and Hauri, unpublished data). ^{35}S -methionine was isolated from *Escherichia coli* grown in the presence of ^{35}S -sulfate under sulfur-limited conditions.

Preparation of Anti-Golgi mAbs

The mouse mAb G1/133 was generated in a fusion described previously (Schweizer *et al.*, 1988). Briefly, Golgi membranes were isolated from Caco-2 cells (Stieger *et al.*, 1988), mixed with Freund's adjuvant (DIFCO Labs, Detroit, MI), and injected into mice. After fusion of spleen and PA1 myeloma cells, the culture supernatants were screened for reactivity to the Golgi membranes by dot blot (Hawkes *et al.*, 1982). The cultures positive by dot blot were expanded and tested for reactivity in Caco-2 cells by immunofluorescence. G1/133 was positive in both tests and therefore was subcloned by limiting dilution and found to be of the IgG1 subtype.

Immunofluorescence

All experiments were carried out on eight chamber glass slides (Miles Labs, Naperville, IL). Vero cells (African green monkey kidney cells) were plated on uncoated slides. All other cell types were plated on slides coated with either poly-L-lysine or Cell-Tak (Beckton Dickinson, Bradford, MA). The cell types included: chick hind limb primary culture (provided by M. Ruegg, Biocenter of University of Basel), insect SF9 (*Spodoptera frugiperda*, provided by A. Kempf, Biocenter of University of Basel), MRC-5 (provided by T. Bächli, University of Zürich), and *Xenopus* A6 (provided by F. Verrey, University of Zürich). Treatments with 10 $\mu\text{g}/\text{ml}$ brefeldin A (provided by D. Römer and E. Rissi, Sandoz AG, Basel), 1 μM okadaic acid (Boehringer Mannheim, Mannheim, Germany), or 10 $\mu\text{g}/\text{ml}$ nocodazole (Aldrich, Milwaukee, WI) were

in normal growth media for the times indicated in the figure legends. In experiments where the cells were extracted before fixation, the cells were rinsed with phosphate-buffered saline (PBS) and then extracted by two consecutive 5-min incubations in 250 μ l of extraction buffer (1% Triton X-100, 80 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) [PIPES] pH 6.8, 1 mM MgCl₂, 1 mM EDTA, and protease inhibitors) at 37°C. After any of the above treatments (drug, extraction, or none), the cells were fixed with 3% paraformaldehyde for 30 min, washed twice with PBS and twice with PBS containing 20 mM glycine, and then permeabilized for 20 min with PBS-glycine containing 0.1% saponin. A 200- μ l solution of PBS-saponin containing either none, one, or two primary antibodies was added for 30 min. The dilutions used were as follows: anti-giantin (G1/133) at 1:25, anti-galactosyltransferase (Oscar) at 1:200, anti-tubulin (1A2) at 1:2000, phalloidin at 1 μ g/ml, anti-ERGIC 53 (G1/93) at 1:100. After five washes with PBS-saponin, 200 μ l PBS-saponin containing one or both of the following antibodies was added: fluorescein isothiocyanate-labeled goat anti-rabbit (dilution 1:50; Cappel, West Chester, PA), rhodamine isothiocyanate-labeled goat anti-rabbit (dilution 1:50; Cappel). After five final washes with PBS-saponin, the wells were rinsed with PBS, and a coverslip was attached over mounting media (glycerol containing 0.1 mg/ml phenylenediamine). Analysis was with either a Reichert Polyvar microscope (Vienna, Austria) or a Bio-Rad MRC-500 confocal laser scanning microscope system (Richmond, CA).

Metabolic Labeling and Immunoprecipitation

Metabolic labeling with ³⁵S-methionine was as described (Stieger *et al.*, 1988). Cell lysates were prepared by addition of IP buffer (1% Triton X-100, 0.2% sodium dodecyl sulfate [SDS], 100 mM NaCl, 5 mM EDTA, 30 mM triethanolamine, pH 8.1, and protease inhibitors) and passage through a 25-gauge needle. After incubation for 30 min on ice, the lysate was centrifuged at 100 000 \times g for 60 min, and the resulting supernatant (1 ml for each 2-cm filter of Caco-2 or 10-cm plate of Vero cells) was rotated for 90 min at 4°C with 12 μ l G1/133-coated protein A-Sepharose (Pharmacia, Piscataway, NJ). The Sepharose beads were collected and washed four times with 1 ml IP buffer, rinsed twice to remove excess salt and detergent, boiled in sample buffer, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (7% gels) and fluorography. Each 12 μ l of protein A-Sepharose was coated by sequential incubations with 2 μ l rabbit anti-mouse IgG (Cappel) and 30 μ l G1/133.

Immunoblotting

Where indicated, samples were first adjusted to 10% trichloroacetic acid, incubated on ice for 15 min, and centrifuged for 15 min in a microcentrifuge. The resulting protein pellet was washed with acetone and dissolved in sample buffer containing 3 M urea. The proteins were then subjected to SDS-PAGE on either 7 (Laemmli, 1970) or 3% (Weber and Osborn, 1969) gels and transferred to nitrocellulose at 32 V for 2 h in buffer containing 25 mM tris(hydroxymethyl)aminomethane (Tris), 192 mM glycine, and 0.1% SDS. The nitrocellulose was rinsed with 50% methanol to remove SDS and was stained with Ponceau S (Serva). All subsequent incubations were in PBS containing 5% nonfat dry milk and 0.05% Tween-20 (Serva). The nitrocellulose was blocked for 60 min followed by incubation with G1/133 (1:100) for 60 min, three 5-min washes, incubation with peroxidase coupled goat anti-mouse IgG (1:1000) (Cappel) for 60 min, and three final washes. The nitrocellulose was processed using enhanced chemiluminescence (Amersham, Arlington Heights, IL). In cases where the immunoblots were reprobed with additional antibodies, the procedures and antibody dilutions were identical.

Cell Fractionation

Caco-2 cells grown to confluency were rinsed twice with ice-cold PBS, scraped into PBS, and collected by centrifugation. The pellet was resuspended and centrifuged in homogenization buffer (250 mM

sucrose, 1 mM EDTA, 10 mM tetraethylammonium, pH 7.4, and protease inhibitors). The washed pellet was resuspended and homogenized in homogenization buffer (1 ml/10 cm plate) by 20 passages through a 25-gauge needle. The postnuclear supernatant was obtained by centrifugation of the homogenate at 1000 \times g for 5 min. To obtain the P2 and S2 fractions, the postnuclear supernatant was centrifuged at 27 000 \times g for 10 min. The S2 was then separated into P3 and S3 fractions by centrifugation at 200 000 \times g for 30 min. To obtain the F1 fraction, the postnuclear supernatant was adjusted to 50% sucrose (6-ml volume) and placed in the bottom of a centrifuge tube. Then 45% (4 ml) and 10% (1.5 ml) sucrose steps were carefully overlaid. The resulting step gradient was centrifuged for 3 h at 30 000 rpm in a Kontron 41.14 swinging bucket rotor (Kontron, Zürich, Switzerland). The F1 fraction was collected from the 45%/10% interface with a pipette. Alternatively, the gradient was collected (1 ml/fraction) from the bottom.

Triton X-114 Phase Partitioning

Triton X-114 (Fluka, Buchs, Switzerland) was prepared as described (Bordier, 1981). The lysis buffer was 1% Triton X-114, 0.5 M NaCl, 10 mM Tris-Cl (pH 7.4) containing the protease inhibitor cocktail, and 10 mM bromophenol blue (Serva). The Caco-2 F1 fraction was solubilized with the lysis buffer (1 ml/200 μ g protein) and centrifuged for 15 min in a microfuge. The lysate was incubated for 3 min at 37°C and then centrifuged at 37°C for 3 min. The detergent pellet was saved on ice. The supernatant was extracted two additional times by addition of Triton X-114 to 1% followed by the 37°C incubation and centrifugation. The combined detergent pellets, the final aqueous phase supernatant, and an untreated aliquot of the original lysate were all adjusted to equal salt and detergent concentrations and then precipitated with trichloroacetic acid and were analyzed by immunoblotting.

Glycosylation Assay

Immunoprecipitations were scaled up as follows. Caco-2 cell lysates (4 15-cm plates in 16 ml IP buffer) were incubated with 130 μ l of G1/133 for 60 min on ice, 13 μ l rabbit anti-mouse (Cappel) was added for another 30 min on ice, 130 μ l protein A-Sepharose was added, and the sample was rotated at 4°C for 60 min. The beads were collected and washed three times by rotation for 5 min in 20 ml IP buffer and rinsed twice and boiled in 200 μ l sample buffer. The depleted extract was then subjected to a second immunoprecipitation using the A1/118 antibody. Each sample was loaded onto two lanes (100 μ l/lane), separated by SDS-PAGE, transferred to nitrocellulose, and stained as described above. The nitrocellulose was then cut in two (leaving identical samples on each half) and processed for carbohydrate detection using Glycotrack as described by the distributor (Oxford Glycosystems, Abingdon, UK). The procedure involved sequential incubations in periodate, biotin-hydrazide, and blocking buffer as specified. To control for nonspecific biotinylation, the periodate incubation was omitted for one-half of the nitrocellulose. The nitrocellulose was then probed with 1 μ g/ml streptavidin-peroxidase (Pierce, Rockford, IL) and processed using enhanced chemiluminescence.

Velocity Sedimentation

Caco-2 cells were extracted by scraping and passing through a 25-gauge needle in buffer containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, and protease inhibitors. Aliquots (100 μ l) of the extract were layered on 4-ml linear 10–25% glycerol gradients containing the extraction buffer and centrifuged for 12.5 h at 40 000 rpm in an SW56 rotor (Beckman, Palo Alto, CA). Fractions (300 μ l each) were collected from the top with a pipette. The protein in each fraction was precipitated with trichloroacetic acid and assayed for giantin by immunoblotting. The migration, in parallel gradients, of sedimentation markers (bovine thyroglobulin and sweet potato beta-amylase, Sigma) was determined by protein assay (Pierce). The minimum axial ratio of giantin was estimated from the Perrin parameter

(Cantor and Schimmel, 1980). The Perrin parameter was calculated using equations and assumed values as previously described (Bloom *et al.*, 1988), except that the Svedberg equation was solved for the diffusion coefficient by assuming a minimum native molecular weight of 400 000 for giantin.

RESULTS

Monoclonal Antibody G1/133 Recognizes an Epitope Localized to the Golgi Apparatus

A membrane fraction enriched in Golgi markers was prepared from the human intestinal cell line Caco-2 and used to generate mAbs (Schweizer *et al.*, 1988). One antibody, G1/133, was of the IgG1 subclass and appeared to recognize an epitope restricted to the Golgi apparatus. In Figure 1A is the pattern generated by indirect immunofluorescence staining of the human fibroblast cell line MRC-5 with G1/133, as analyzed by confocal laser scanning microscopy. The striking juxtanuclear staining pattern with a ribbon or worm-like pattern is typical for Golgi proteins. Indeed, the specific staining pattern of galactosyltransferase in double immunofluorescence experiments was nearly indistinguishable from that of G1/133 (Figure 1B). Background staining of other structures with the G1/133 antibody was extremely low. Unfortunately, this was not always the case for the polyclonal antibody against galactosyltransferase. Nevertheless, we noted a consistent minor difference between the two patterns. Wherever a loop or ring was clearly outlined, the G1/133 pattern was restricted to the outermost part of the loop, whereas the galactosyltransferase staining was restricted to the

inner part of the loop (compare arrows in Figure 1, A and B). As a control, we compared the patterns, in double-stained Vero cells, of two antibodies both against galactosyltransferase (one monoclonal and one polyclonal) and did not observe any difference.

Our attempts to use the G1/133 antibody for immunoelectron microscopic experiments have been unsuccessful. As an alternative method to confirm that the G1/133 epitope is specific to the Golgi apparatus, we have performed immunofluorescent experiments on cells treated with agents that are known to affect Golgi structure. Again, the cells were simultaneously stained with the anti-galactosyltransferase antibody to provide a Golgi marker.

First, Vero cells were treated for various times with the fungal metabolite brefeldin A, which causes a redistribution of Golgi proteins via membrane tubules to sites in the cell periphery (Doms *et al.*, 1989; Lippincott-Schwartz *et al.*, 1989; Sandvig *et al.*, 1991). The results are shown in Figure 2 for 0 (A and B), 2 (C and D), 10 (E and F), and 30 (G and H) min of treatment. The G1/133 and galactosyltransferase patterns underwent similar changes, and at early time points both epitopes were localized in the same tubules emanating from the Golgi region toward the cell periphery. The second compound used was the microtubule depolymerizing agent nocodazole, which causes the fragmentation of the Golgi into "mini" Golgi stacks (Pavelka and Ellinger, 1983; Rogalski and Singer, 1984; Ho *et al.*, 1989). We confirmed that microtubules were disrupted by using an anti-tubulin antibody. After nocodazole treatment the galactosyltransferase and G1/133 patterns were coincident

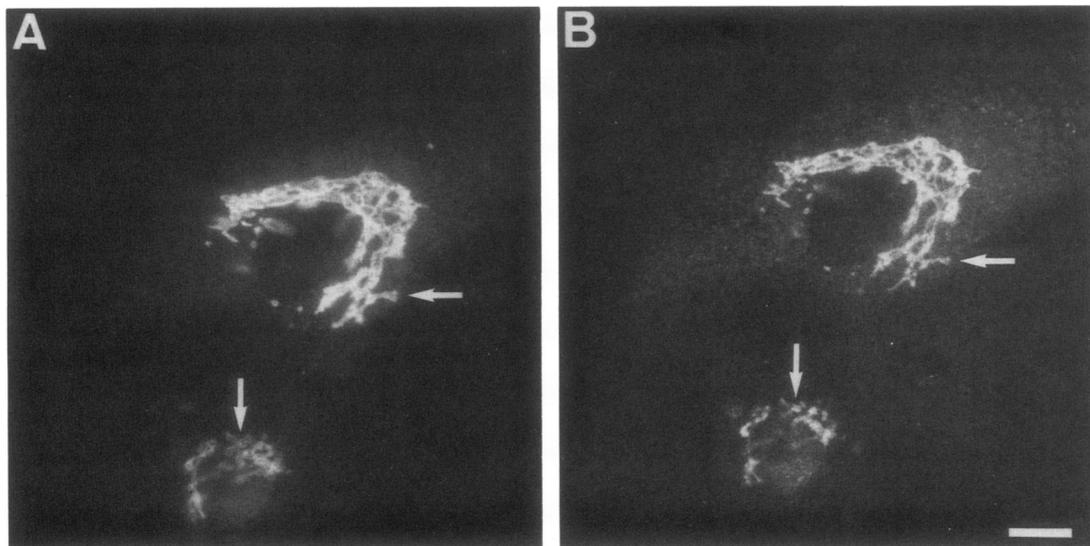


Figure 1. Comparison of G1/133 (A) and anti-galactosyltransferase (B) staining patterns in MRC-5 cells by confocal immunofluorescence microscopy. Note that the stained structures occupying a perinuclear position were nearly identical except where the galactosyltransferase appeared to be localized inside loops outlined by G1/133 staining (arrows). Bar, 8 μ m.

and included numerous ring-shaped structures dispersed throughout the cytoplasm. Finally, we used the phosphatase inhibitor okadaic acid, which causes a vesiculation of the Golgi much like that observed during mitosis (Lucocq *et al.*, 1991). Okadaic acid treatment resulted in rounding of the cells and dispersal of both the galactosyltransferase and G1/133 staining patterns. Reversal of the drug treatment led to accumulation of both galactosyltransferase and G1/133 in patches before final recovery of the Golgi pattern.

In summary, the G1/133 pattern was nearly indistinguishable from that of galactosyltransferase in cells where the Golgi was left intact (untreated), redistributed into the cell periphery (brefeldin A), fragmented into dispersed small stacks (nocodazole), or completely vesicularized (okadaic acid). When cells were labeled with the two antibodies, neither the time course of disruption nor the intermediates visualized were significantly different. We conclude that the G1/133 monoclonal recognizes an epitope restricted to the Golgi apparatus.

The Epitope Recognized by G1/133 Is Conserved in Evolution

Because of the characteristic morphology of the Golgi apparatus in most cell types, we used an immunofluorescence assay to test the tissue and species distribution of the Golgi epitope recognized by G1/133. This assay gave a strong specifically localized signal with a characteristic Golgi pattern; there was virtually no staining in control incubations with no antibody or with a different monoclonal as primary antibody. The G1/133 antibody labeled the Golgi in cell lines from human intestine (Caco-2), human lung fibroblast (MRC-5) (Figure 1A), human liver (HepG2), monkey fibroblast (Vero) (Figure 2A), rat pheochromocytoma (PC12) (Figure 3D), rat kidney fibroblast (normal rat kidney), hamster ovary (Chinese hamster ovary [CHO]) (Figure 3C), hamster kidney (baby hamster kidney [BHK]) (Figure 3B), mouse fibroblast (3T3) (Figure 3A), and an explant from chick hind limb (Figure 3E). The antibody did not stain a *Xenopus* cell line (A6) or an insect cell line (SF9) (Figure 3F). The widespread crossreactivity indicates that the epitope is expressed in a variety of tissues and has been conserved during vertebrate evolution.

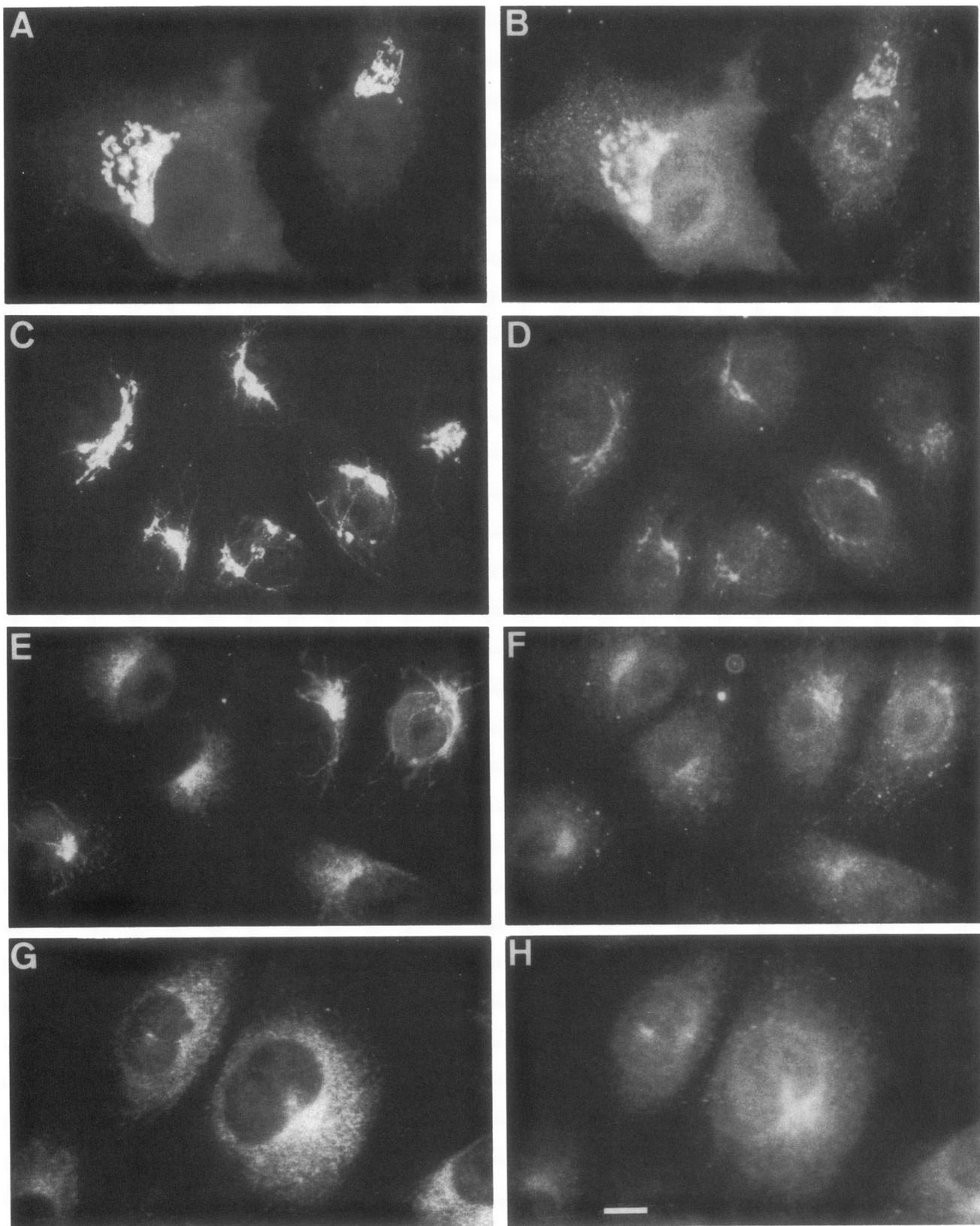
The G1/133 Epitope Is Contained in Giantin, a Novel Golgi Protein of Apparent Molecular Weight 400 kDa

Immunoblotting and immunoprecipitation experiments were carried out on extracts from either the human Caco-2 cell line or the monkey Vero cell line using the G1/133 monoclonal antibody. Additional cell lines have not yet been examined. Both techniques revealed that the antibody reacts specifically with a protein present in both cell types of extremely low mobility on SDS-

PAGE (Figure 4, A and B). The apparent molecular weight of the protein, 400 kDa, was calculated using the migration of cross-linked phosphorylase B as a reference (Figure 8, lane 1). The use of this set of molecular weight markers required an alternative SDS-PAGE system (Weber and Osborn, 1969). To our knowledge very few proteins, and no Golgi-localized proteins, with a monomeric molecular weight of 400 kDa have been characterized. Therefore, we conclude that the G1/133 antibody recognizes a previously unidentified protein that we have named giantin. There was a variable amount of specific reactivity found at, or just beyond, the interface between the stacking and resolving gels (Figure 4, A and B). It has not been determined whether or not this material represents nonspecific aggregates of giantin.

The specificity of the antibody reactions and the fact that a protein of the same apparent size was recognized in two different cell types indicated that giantin was probably the Golgi protein identified in our immunofluorescence experiments. Additional biochemical experiments suggested that giantin is a membrane protein. Giantin was recovered in a sedimentable form after cell fractionation by differential centrifugation and was not present in the cytosol (Figure 4B). To ensure that the sedimentation properties of giantin were due to membrane association, postnuclear supernatants were subjected to flotation gradient analysis. Giantin was recovered from the 10%/45% sucrose interface (Figure 4C, fraction 10), confirming its designation as a membrane protein. Figure 4B demonstrates that giantin was enriched in the floated fraction, termed F1, because all lanes were loaded with equal amounts of protein and processed in parallel.

The membranes in the F1 fraction were subjected to various treatments, including pH 11.5 extraction in carbonate buffer, and were collected by centrifugation. Pellet and supernatant fractions were then assayed for the presence of giantin by immunoblotting. The extent of extraction of proteins into the supernatant was monitored by Ponceau S staining of the nitrocellulose filter used for the immunoblot. After carbonate extraction the majority of the stainable bands were extracted. In contrast, giantin remained with the membrane pellet under these conditions (Figure 5A, lanes 7 and 8). Eight separate experiments using vesicles derived from either Vero or Caco-2 cells yielded the same result. Giantin was also recovered in the pellet fraction after incubation in low salt, high salt, or urea-containing buffer (Figure 5A, lanes 1-6). The minor amount of giantin recovered in the supernatant after urea treatment (Figure 5A, lane 6) was not present in a second experiment in which a higher urea concentration (7 M) was used. Treatment with detergent was required to fully extract giantin into the supernatant (Figure 5A, lanes 9 and 10). These results suggest that giantin is likely to be an integral component of the Golgi membrane.



Surprisingly, giantin was recovered entirely in the aqueous phase after samples were subjected to phase partitioning with Triton X-114 (Figure 5B). However, some integral membrane proteins have been observed to partition into the aqueous phase of Triton X-114, presumably because this partitioning correlates with the amount of hydrophilic surface that a given protein displays (Alvarez *et al.*, 1984). As controls in our experiments, the same blots were reprobed with antibodies against two known integral membrane proteins. One, the transferrin receptor, was recovered in the detergent phase as expected, but the other, p63 (Schweizer *et al.*, 1993a,b), was recovered in the aqueous phase (Figure 5B). Taken together, the extraction and Triton X-114 phase partitioning experiments suggest that giantin is an integral membrane protein with a large amount of hydrophilic surface area (see below).

Giantin exhibited a slow turnover rate when cells were metabolically labeled for 15 min and chased for up to 18 h (Figure 6A). In these experiments giantin migrated as a relatively sharp band on SDS-PAGE and showed no change in mobility during the chase period. No increase in molecular weight because of glycosylation was apparent. Because it is uncertain whether a shift in the mobility of a 400-kDa protein would be detected, an independent test for glycosylation was also used. In this experiment giantin was immunoprecipitated and transferred to nitrocellulose where it was visualized by Ponceau S staining. The nitrocellulose was then either left untreated or treated with periodate. The periodate treatment results in oxidation of carbohydrates, which can then be labeled with biotin-hydrazide (Bayer *et al.*, 1990). Periodate-specific biotinylation was found for several control proteins (the 118 antigen [Linstedt and Hauri, unpublished data], antibody heavy chains, and ovalbumin) but not for giantin (Figure 6B). Giantin showed a weak and apparently nonspecific labeling both with and without periodate treatment. Taken together, the lack of a mobility shift and the lack of specific labeling with biotin-hydrazide suggest that giantin is not significantly glycosylated.

Giantin Has a Cytoplasmic Domain of ≥ 350 kDa

We used protease treatment of vesicles isolated from Caco-2 cells to determine whether the G1/133 epitope is in a cytoplasmic or luminal domain of giantin. Addition of either 50 $\mu\text{g}/\text{ml}$ trypsin or 20 $\mu\text{g}/\text{ml}$ proteinase K to the F1 fraction (1 mg/ml protein) resulted in complete loss of immunoreactivity on immunoblots (Figure 7A). A control protein also localized to the Golgi com-

plex, the 118 antigen (Linstedt and Hauri, unpublished data), was protected under these conditions but digested if detergent was added (Figure 7A). This result suggests that the G1/133 epitope is protease sensitive and in a cytoplasmic domain of giantin.

When protease digestion was carried out in the presence of 25 times higher protein concentration (25 mg/ml postnuclear supernatant), both trypsin and proteinase K generated large fragments of giantin, presumably the result of incomplete digestion. Interestingly, unlike intact giantin these protease fragments did not sediment with membranes (Figure 7B). The vesicles in these experiments were shown to be intact by reprobing the blot with antibody against the 118 antigen that, as expected, was completely protected (Figure 7B). The apparent molecular weights of the proteinase K fragment and the largest trypsin fragment (calculated from their migration on the Weber and Osborn gel system) were 200 and 350 kDa, respectively (Figure 8, lanes 2 and 3). Because protease digestion releases these fragments (containing the G1/133 epitope) into a freely soluble form and because the membrane vesicles were intact, it can be concluded that giantin has a cytoplasmic domain with the minimum size of 350 kDa.

Giantin Is a Highly Elongated Disulfide-bonded Particle

Giantin was recovered at a significantly retarded position after SDS-PAGE under nonreducing conditions (Figure 8, lane 4), suggesting that it may exist in a disulfide-linked complex. The extracts in these experiments were prepared in the presence of iodoacetamide to prevent disulfide bond formation after lysis. As a further control, the trypsin and proteinase K fragments of giantin were analyzed on the same gels. Because these fragments are exclusively from a cytoplasmic domain of giantin, they should not show a change in mobility under nonreducing conditions unless disulfide bond formation took place after lysis. As expected, the migration of the cytoplasmic domain fragments was not affected by nonreducing conditions (Figure 8). The finding that giantin has a slower mobility on SDS-PAGE under nonreducing conditions is further evidence that giantin spans the Golgi membrane, allowing a luminal domain to be modified by disulfide cross-bridging. The apparent molecular weight of the disulfide-bonded species was about 600 kDa, but this calculation required extrapolation beyond the largest marker protein and is therefore only approximate. Because specific coprecipitating proteins were not recovered after immunopre-

Figure 2. Comparison of G1/133 (A, C, E, and G) and anti-galactosyltransferase (B, D, F, and H) immunofluorescence patterns after brefeldin A treatment. Vero cells treated with 10 $\mu\text{g}/\text{ml}$ brefeldin A for 0 (A and B), 2 (C and D), 10 (E and F), or 30 (G and H) min were double stained and processed for normal immunofluorescence microscopy. The disruption of the stained structures was simultaneous, and frequently single tubules were stained by both antibodies. Bar, 8 μm .

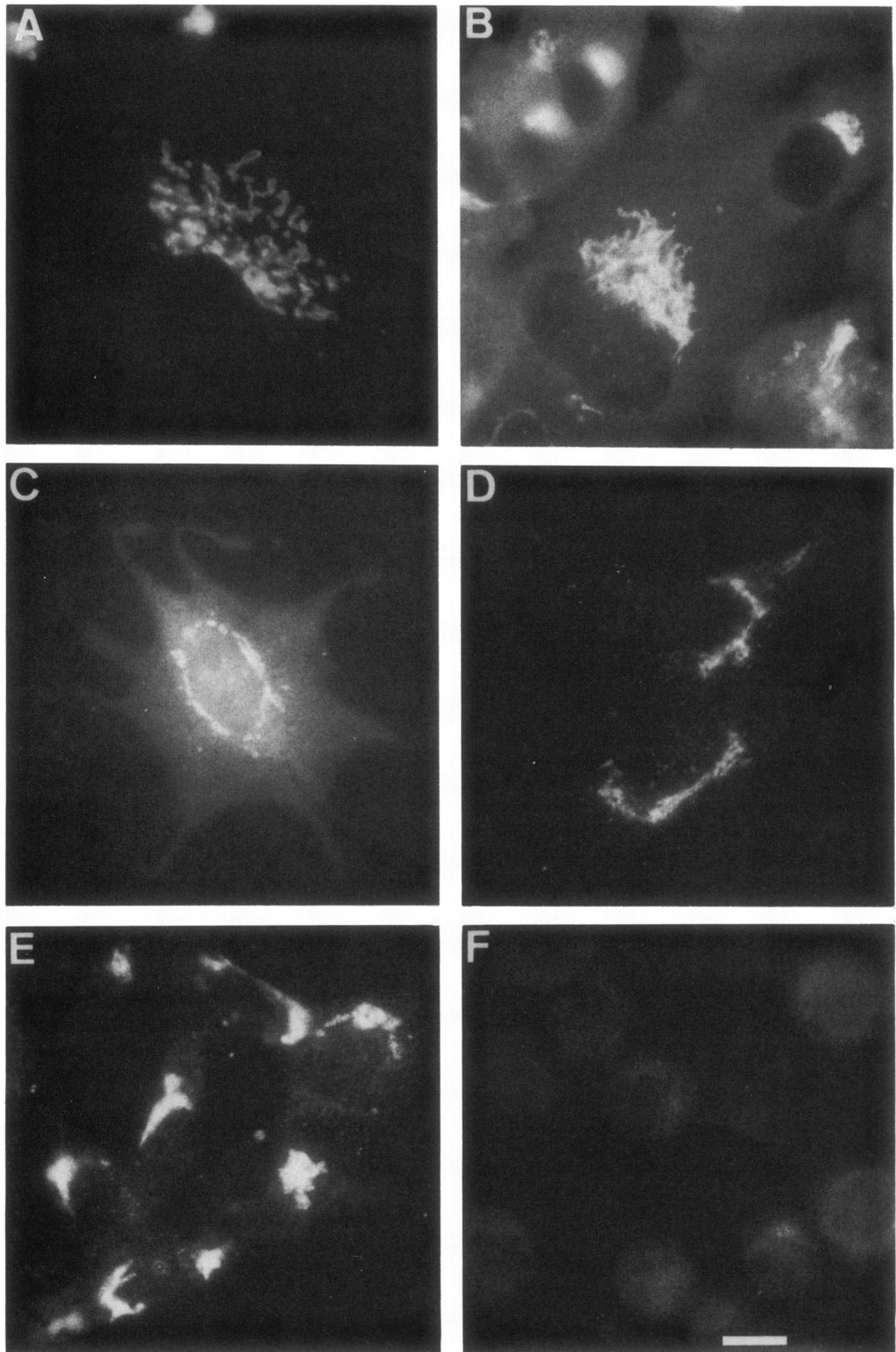


Figure 3. G1/133 fluorescence pattern in various cell types. The cell types are 3T3 (A), BHK (B), CHO (C), PC12 (D), chick hind limb explant (E), and SF9 (F). Bar, 10 μ m.

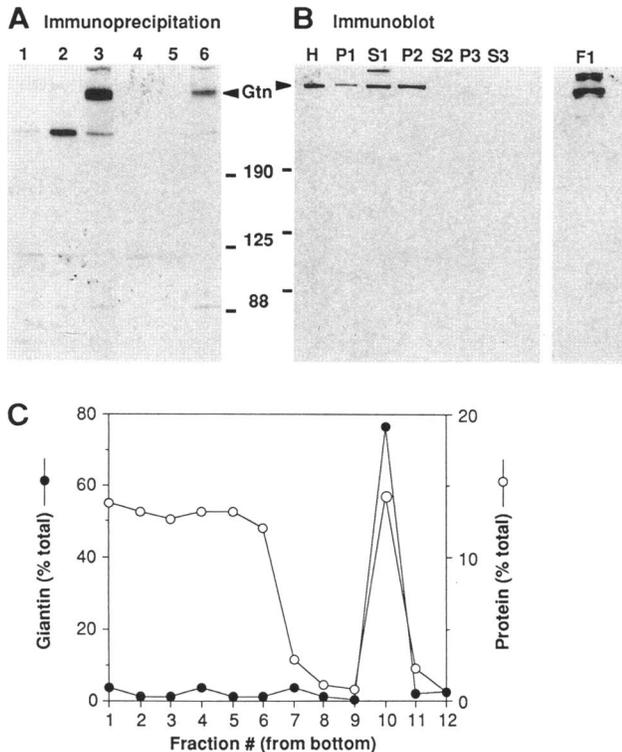


Figure 4. (A) Immunoprecipitation with G1/133. Metabolically labeled extracts of Vero cells (lanes 1–3) or Caco-2 cells (lanes 4–6) were subjected to immunoprecipitation with no primary antibody (lanes 1 and 4), a nonspecific primary antibody (lanes 2 and 5), or G1/133 (lanes 3 and 6) followed by SDS-PAGE and fluorography. A specific reaction resulted in precipitation of a very large protein designated giantin (Gtn). Note that there was also material at the stacking/resolving gel interface. Recovery of material at this position was variable in our experiments. (B) Immunoblotting after differential centrifugation. Caco-2 cells were separated by differential centrifugation and equal amounts (150 μ g protein) of each fraction were analyzed by immunoblotting with the G1/133 antibody. Shown are the homogenate (H), the pellets (P), and the supernatants (S) from successive spins at $1000 \times g$ (P1, S1), $27\,000 \times g$ (P2, S2), and $200\,000 \times g$ (P3, S3). Giantin was recovered in the P2 fraction and was not detectable in the S3 (cytosol) fraction. An equal amount of the F1 fraction (see below) was applied to the same gel; in the photograph this lane was separated for presentation purposes. (C) Flotation gradient. A Caco-2 postnuclear supernatant was adjusted to 50% sucrose and overlaid with 45% sucrose and 10% sucrose steps. After centrifugation the gradient was fractionated from the bottom, and each fraction was subjected to an immunoblotting and densitometric scanning to assay for giantin and a protein assay. Fractions 1–6 represent the material that remained in the 50% sucrose layer, which contained most of the cellular protein but no detectable giantin. Giantin was recovered in fraction 10 (designated F1) that encompassed the 45%/10% sucrose interface.

precipitation of giantin (Figures 4A and 6A), it is unlikely that giantin is part of a disulfide-linked hetero-oligomer. It also seems unlikely that intrachain disulfides could account for such a large retardation in the mobility of giantin. For these reasons we speculate that the disulfide-linked species is a dimer of two 400-kDa giantin subunits.

Detergent extracts of Caco-2 cells were analyzed on glycerol velocity gradients to determine the sedimentation properties of giantin. In each of five experiments, giantin comigrated with beta-amylase, which has a sedimentation coefficient of 9S (Figure 9). Given that the giantin particle in the lysate was ≥ 400 kDa (note that these experiments were performed under nonreducing conditions), this rate of sedimentation is surprisingly low, because the molecular weight of beta-amylase is 200 kDa. Using the sedimentation coefficient and the minimum molecular weight of the giantin particle, a minimum estimate of the axial ratio of the particle can be calculated (see MATERIALS AND METHODS). For a 400-kDa prolate ellipsoid with a 9S sedimentation coefficient, the calculated axial ratio is 25 and the dimensions are 2.5 nm by 62.5 nm. If the actual native molecular weight of giantin were greater, this would imply an even greater axial ratio. Although these are at best rough estimates, it seems likely that giantin is a large elongated structure.

Giantin Is Resistant to Extraction by Triton X-100

The large dimensions and predominantly cytoplasmic disposition of giantin support the hypothesis that it

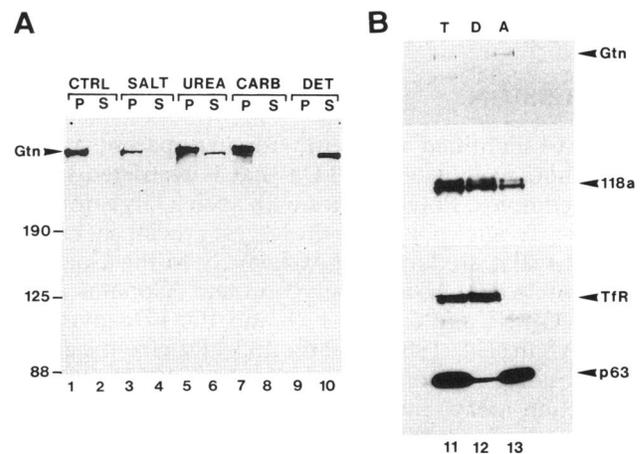


Figure 5. (A) Extraction of giantin from Caco-2 membrane vesicles. Aliquots of the Caco-2 F1 fraction (200 μ g/ml) were incubated on ice for 30 min in various extraction buffers and then collected by centrifugation ($200\,000 \times g$ for 30 min). The recovery of giantin (Gtn) in the pellet fractions (P) and in trichloroacetic acid-precipitated supernatant fractions (S) was assayed by immunoblotting. The extraction buffers were 250 mM sucrose buffer (CTRL), 1 M NaCl buffer (SALT), 5 M urea buffer (UREA), 100 mM carbonate, pH 11.5 (CARB), and a detergent buffer (DET) containing 1% NP40, 0.5% deoxycholate, 0.1% SDS. (B) Triton X-114 phase partitioning of giantin. The Caco-2 F1 fraction (400 μ g) was solubilized with Triton X-114 lysis buffer and divided into two equal aliquots. One aliquot was subjected to phase partitioning, whereas the other aliquot was left untreated. The recovery of giantin in trichloroacetic acid pellets of the detergent (D) and aqueous (A) phases was assayed by immunoblotting. The untreated sample was assayed similarly to determine the total (T) amount of giantin (Gtn) present in the experiment. The immunoblot was reprobed with antibodies against transferrin receptor (TfR), p63, and the 118 antigen (118a) (Linstedt and Hauri, unpublished data) to determine the partitioning of other membrane proteins.

performs a structural role in the Golgi apparatus. To begin to address this question, we took advantage of the observation that cytoskeletal and cytoskeleton-associated proteins are relatively stable to extraction by 1% Triton X-100 (Brown *et al.*, 1976). To test whether giantin has similar properties, Vero cells were extracted with 1% Triton X-100 in a microtubule stabilizing buffer (Navone *et al.*, 1992) for 10 min at 37°C before fixation. The cells were then processed for immunofluorescence microscopy using the antibody against giantin as well as several control antibodies. In these experiments there was no detectable extraction of giantin (compare Figure 10, A and B). In fact, except for a slight loss in the resolution of individual Golgi loops, the basic structure of the Golgi as visualized by staining with the antibody against giantin was unperturbed. In contrast, three integral membrane proteins, one localized exclusively to the Golgi apparatus (galactosyltransferase; Figure 10, C and D) and two that are found both in the Golgi and in other compartments (ERGIC-53, Figure 10, E and F, and mannose-6-phosphate receptor), were completely extracted. As expected, both the microtubule network (Figure 10G) and the actin network (Figure 10H) were left intact under these conditions. The inextractability of giantin may mean that it is associated with the cytoskeleton.

DISCUSSION

We have identified a protein with an apparent subunit molecular weight of 400 kDa that is restricted to the Golgi complex. Polypeptides with such a large unit size are rare. To our knowledge the largest previously known proteins that are localized exclusively to the Golgi are MG-160, a 160-kDa sialoglycoprotein (Gonatas *et al.*, 1989); GIMP_c and GIMP_i, 130- and 100-kDa glycoproteins (Yuan *et al.*, 1987); and the 133-kDa enzyme alpha-mannosidase II (Moremon and Robbins, 1991). Protease protection assays indicate that these proteins are predominantly luminal with small cytoplasmic domains. Large proteins with partial Golgi localization include the 215-kDa cation-independent mannose 6-phosphate receptor, which recycles between the Golgi and a prelysosomal compartment (Griffiths *et al.*, 1988); two peripheral membrane proteins of ~200 kDa that dissociate from the Golgi membrane after brefeldin A treatment (Kooy *et al.*, 1992; Narula *et al.*, 1992); and Sec7p, a 227-kDa peripheral membrane protein required for protein transit through the yeast Golgi apparatus (Franzoso *et al.*, 1991).

We believe giantin to be an integral component of the Golgi membrane, because it was not extracted from the membrane by high salt, urea, or alkaline carbonate (Figure 5A). The tight association of giantin with the Golgi membrane was also apparent in fractionation experiments where intact giantin was recovered exclusively in membrane-containing fractions (Figure 4, B

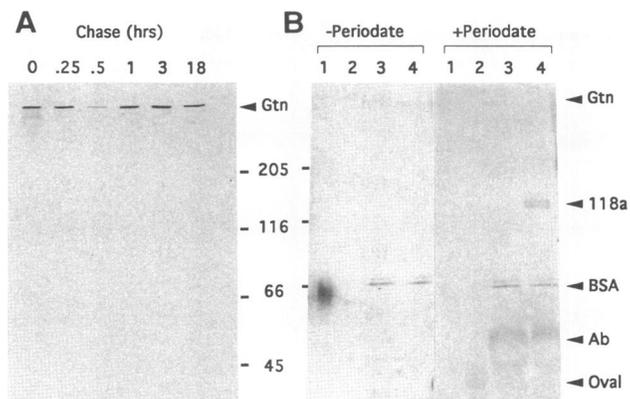


Figure 6. (A) Recovery of giantin after various times of chase. Caco-2 cells were pulse labeled for 15 min with [³⁵S]methionine and subsequently chased for the indicated times. Giantin was recovered by immunoprecipitation and the immunoprecipitates were analyzed by SDS-PAGE and fluorography. Note that there was neither a change in mobility nor an apparent loss of giantin with increasing chase times. (B) Carbohydrate detection assay. Identical sets of samples were separated by SDS-PAGE and transferred to nitrocellulose. As indicated, one set was treated with periodate and the other left untreated. Both were then incubated with biotin-hydrazide, and the biotinylated proteins were detected with streptavidin-peroxidase. The samples were 5 μg bovine serum albumin (lane 1), 5 μg ovalbumin (lane 2), immunoprecipitated giantin (lane 3), and immunoprecipitated 118 antigen (lane 4). Note that a periodate-specific signal is evident for the 118 antigen (118a), the antibody heavy chains (Ab), and ovalbumin (Oval) but not for giantin (Gtn) or bovine serum albumin (BSA).

and C). Moreover, giantin remained associated with Golgi membranes, as judged by coincident staining with galactosyltransferase, during major perturbations of Golgi structure with various pharmacological agents (Figure 2). Under nonreducing conditions giantin had a decreased mobility, suggesting that the protein contains a disulfide-linked luminal domain (Figure 8), and protease experiments indicated that the protein also contains a significant cytoplasmic domain (Figure 7). The finding that giantin partitions into the aqueous phase after Triton X-114 extraction does not contradict the conclusion that giantin is an integral membrane protein, because the distribution of a protein between the detergent and aqueous phases reflects the overall balance between the hydrophobic and hydrophilic properties of the protein's surface (Alcaraz *et al.*, 1984). That the majority of the surface area of giantin is hydrophilic was indicated by limited protease digestion, which liberated a number of freely soluble fragments, including one with an apparent molecular weight of 350 kDa (Figures 7 and 8).

Two findings support the conclusion that this 350-kDa fragment is part of a contiguous cytoplasmic domain of giantin. First, the digestion was carried out under conditions where intracellular membranes, including Golgi-derived vesicles, were shown to be intact and sealed (Figure 7B). Second, Golgi vesicles could be im-

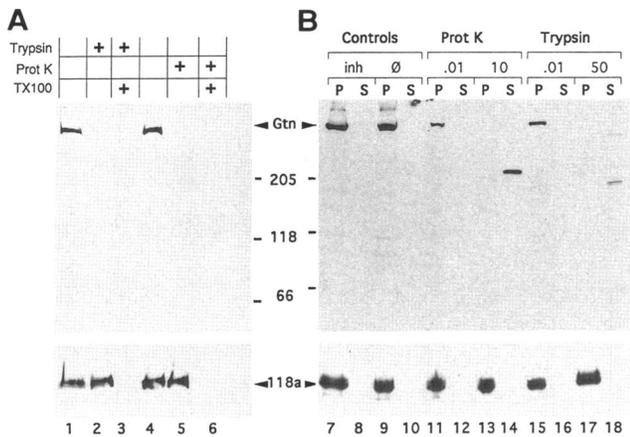


Figure 7. (A) Protease protection of giantin. Membrane vesicles (1 mg/ml Caco F1 fraction) were incubated on ice for 60 min with additions of trypsin (50 μ g/ml), proteinase K (20 μ g/ml), and Triton X-100 (1%) where indicated. Each sample was adjusted to equal amounts of protease inhibitors, protease, and detergent before precipitation with trichloroacetic acid. Giantin (Gtn) recovery was assayed by immunoblotting. To control for vesicle lysis the immunoblot was reprobed with an antibody that recognizes a lumenally-oriented Golgi antigen (118a). (B) Release of soluble fragments of giantin. Aliquots of Caco-2 postnuclear supernatants (25 mg/ml) were incubated on ice for 30 min with a protease inhibitor cocktail (inh), no addition (\emptyset), proteinase K (0.01 or 10 μ g/ml), or trypsin (0.05 or 50 μ g/ml) as indicated. The fractions were separated into membrane pellets (P) and supernatants (S) by centrifugation at 200 000 \times g for 30 min, and the recovery of giantin fragments was assayed by immunoblotting. As in A, the blot was reprobed with an antibody against 118a to demonstrate vesicle intactness.

munoisolated with beads coated with the G1/133 monoclonal antibody (Linstedt and Hauri, unpublished data). Velocity gradient analysis indicated that giantin has a highly elongated shape; therefore, it seems likely that giantin's large cytoplasmic domain projects away from the Golgi membrane.

The conservation of the G1/133 epitope among avian and mammalian species indicates that the antibody binding site itself may be an important domain in the function of giantin. Because the antibody recognizes this site after denaturation of the protein with SDS, it is likely that the antibody binds a contiguous stretch of amino acids. We will need additional antibodies against giantin, or gene sequence information, to determine whether other stretches within the protein are similarly conserved. Several antibodies have been described that recognize conserved peripheral components of the Golgi membrane (Willison *et al.*, 1989; Goud *et al.*, 1990; Stearns *et al.*, 1990; Kooy *et al.*, 1992), but the degree of conservation we have found for the G1/133 epitope is unique among the known integral components of the Golgi complex. Although the glycosylation of secretory proteins exists in all cell types with a secretory pathway, most mAbs against glycosylation enzymes show species and even tissue specificity in their cross-reactivity.

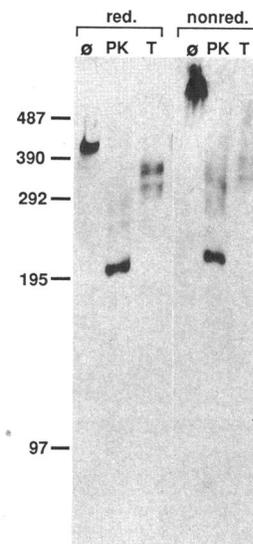


Figure 8. Migration of giantin and proteolytic fragments of giantin on nonreducing SDS-PAGE. Caco-2 cell lysates (\emptyset) were prepared in a buffer containing iodoacetamide (2% SDS, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 5.4 mM iodoacetamide, and the protease inhibitor cocktail). Reducing (red.; 100 mM dithiothreitol final concentration) or nonreducing sample buffer (nonred.) was added, and the proteins were separated as described (Weber and Osborn, 1969) and transferred to nitrocellulose. The position of giantin was determined by immunoblotting. Also analyzed were the high-speed supernatants from proteinase K (PK)- and trypsin (T)-treated postnuclear supernatants (see legend to Figure 7B).

The conservation of the G1/133 epitope on giantin allows us to study the distribution of a single Golgi protein in a variety of tissues and species. From such studies it is likely that our knowledge of Golgi structure and

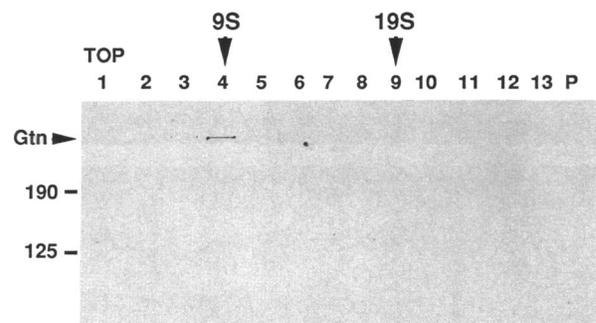
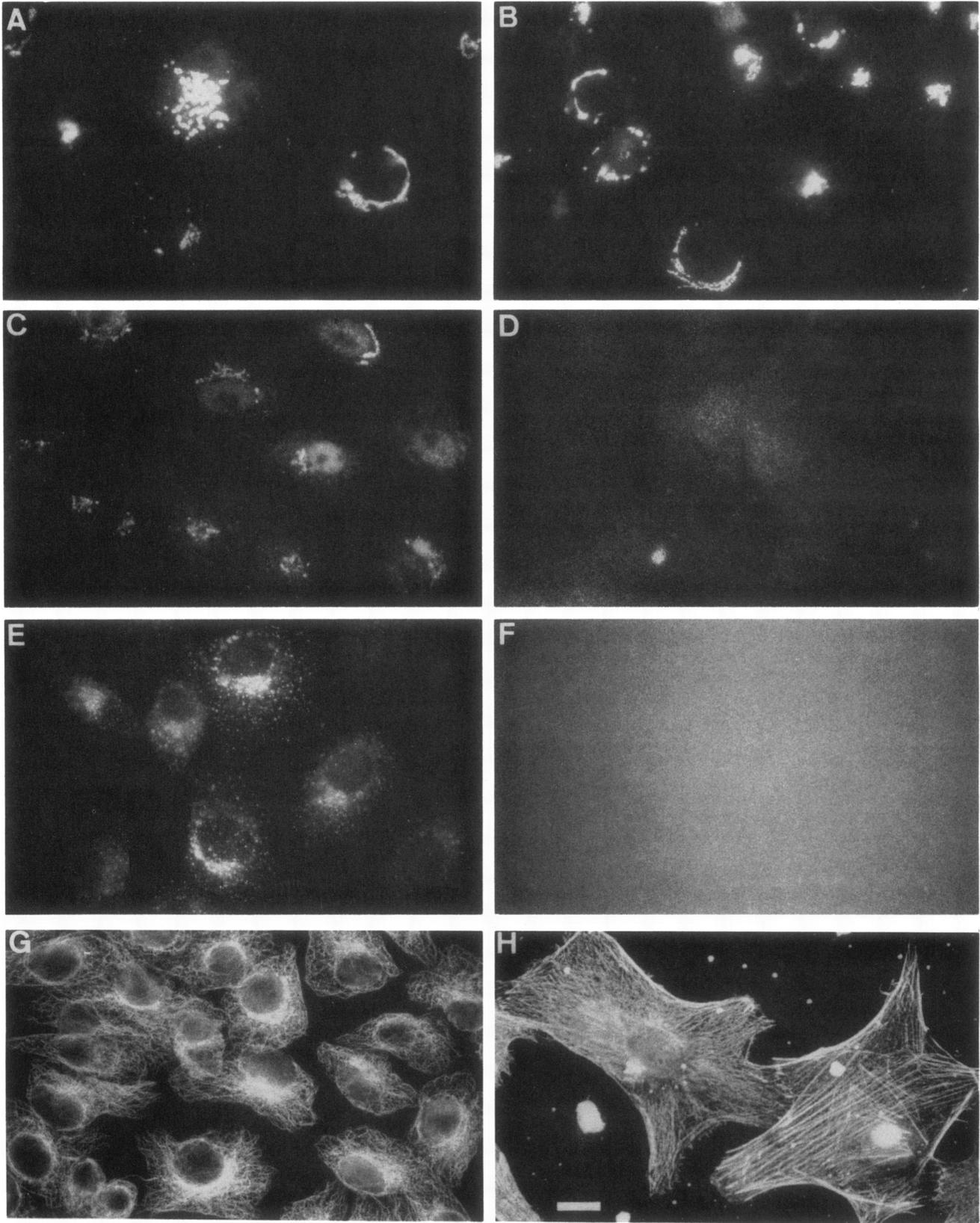


Figure 9. Velocity sedimentation of giantin. After sedimentation through glycerol gradients, the recovery of giantin in each fraction was determined by immunoblotting. Fraction numbers start from the top of the gradient. The peak positions of two reference proteins, thyroglobulin (19S) and beta-amylase (9S), were determined from parallel gradients. Giantin was not detectable in the material recovered as a pellet (P) at the bottom of the gradient. For this experiment Caco-2 cells were extracted by scraping and passage through a 25-gauge needle in ice-cold buffer containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, and the protease inhibitor cocktail.



subcompartmentalization can be extended. Unfortunately, giantin has not yet been localized by immunoelectron microscopy. Until this is accomplished, the precise location of giantin within the Golgi will not be known. However, two observations suggest that the protein is not uniformly distributed throughout the Golgi apparatus in Vero cells. First, the immunofluorescence pattern shares a characteristic of markers such as alpha-mannosidase II and galactosyltransferase, which are thought to be restricted to subcompartments of the Golgi. These markers, like giantin, yield a precise pattern in which the loops and extensions of the Golgi ribbon are often resolved from one another (Figure 1). In contrast, if the Golgi complex is uniformly labeled; for example, with certain lectins or with antibodies against secretory proteins transiting the Golgi complex, a significantly more diffuse pattern is usually obtained (Lewis and Pelham, 1992; Ridgway *et al.*, 1992). Second, we consistently observed areas where the galactosyltransferase and giantin patterns were segregated from one another, albeit by extremely short distances (Figure 1). There is precedence for resolution of Golgi subcompartments using fluorescence microscopy (Antony *et al.*, 1992; Nilsson *et al.*, 1993). Nevertheless, we feel that firm conclusions cannot be drawn without an electron microscopic analysis.

The biochemical features of giantin are sufficiently distinct from those of known Golgi proteins to warrant the speculation that giantin has a novel function. As described in the INTRODUCTION, proteins localized to the Golgi complex would be expected to fall into at least three functional categories: those involved in post-translational modifications such as glycosylation, those involved in transport and sorting, and those that establish and regulate the Golgi structure. Although there are no known members of this latter group, we hypothesize that giantin serves a structural role in the Golgi as its physical characteristics resemble those that would be predicted for such proteins.

Thus, the intercisternal filaments or cross-bridges that are proposed to mediate Golgi stacking (Mollenhauer and Morré, 1991; Cluett and Brown, 1992) are likely to be conserved proteins with an exclusive Golgi localization, tight membrane binding, and large cytoplasmic domains. Particularly striking is the similarity of the size and apparent membrane topology of giantin with the calcium-release channel (also known as ryanodine receptor) localized to the sarcoplasmic reticulum of skeletal muscle and the inositol 1,4,5-trisphosphate receptor (InsP₃ receptor, also known as P₄₀₀) localized to

subcompartments of the endoplasmic reticulum. The calcium-release channel is an integral membrane protein with a molecular mass of 565 223 (≈ 400 kDa on SDS-PAGE) of which 90% comprises a contiguous cytoplasmic domain (Takeshima *et al.*, 1989). This domain forms a foot structure that links the membranes of the sarcoplasmic reticulum and the transverse tubule, a specialization of the muscle cell plasma membrane (Chadwick *et al.*, 1988; Lai *et al.*, 1988). Indeed, Cluett and Brown (1992) were struck by the morphological similarity between the calcium-release channel foot structure and the intercisternal cross-bridges they visualized in rat liver Golgi. The InsP₃ receptor has sequence similarity with the calcium-release channel and is also a large (313 kDa) integral membrane protein containing a large contiguous cytoplasmic domain (Furuichi *et al.*, 1989). In Purkinje cells this receptor is highly enriched in a subcompartment of the endoplasmic reticulum identified as stacked cisternae separated by regularly spaced cross-bridges (Satoh *et al.*, 1990). The identity of the cross-bridges is unknown; however, their size and shape correspond to that of the isolated InsP₃ receptor (Maeda *et al.*, 1990). It will be interesting to see whether the InsP₃ receptor, the calcium-release channel, and giantin are part of a class of large multifunctional integral membrane proteins that contain large cytoplasmic domains capable of forming intermembrane cross-bridges.

Resistance to extraction with nonionic detergent is a common feature of the cytoskeleton and its associated proteins (Brown *et al.*, 1976). Further experiments are necessary to determine the basis of the inextractability of giantin. The fact that Triton X-100 extraction had very little effect on the giantin pattern, whereas other Golgi membrane proteins were completely extracted (Figure 10), suggests that giantin may be part of a Golgi skeleton. The existence of such a structure would have implications for mechanisms of Golgi organization and the retention of endogenous Golgi proteins.

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Figure 10. Immunofluorescent staining of Vero cells extracted before fixation. Shown are the fluorescence patterns of giantin in unextracted (A) and extracted (B) cells, galactosyltransferase in unextracted (C) and extracted (D) cells, ERGIC-53 in unextracted (E) and extracted (F) cells, tubulin in extracted cells (G), and actin in extracted cells (H). Extraction was performed by treatment at 37°C for 10 min with 1% Triton X-100 in 80 mM PIPES-KOH pH 6.8, 1 mM EGTA, 1 mM MgCl₂, and a protease inhibitor cocktail (see MATERIALS AND METHODS). These cells were then fixed and processed in parallel with the unextracted cells. The giantin photographs (A and B) were taken with identical exposure times. There was no apparent extraction of giantin. Bar, 10 μ m.

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