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A C-terminally-anchored Golgi protein is inserted into the endoplasmic reticulum and then transported to the Golgi apparatus

A. D. LINSTEDT*†, M. FOGUET*, M. RENZ‡, H. P. SEELIG‡, B. S. GLICK§, AND H.-P. HAURI*¶

*Department of Pharmacology, Biozentrum, University of Basel, CH-4056 Basel, Switzerland; ‡Institute of Immunology and Molecular Genetics, D-76133, Karlsruhe, Germany; and §Department of Molecular Genetics and Cell Biology, The University of Chicago, Chicago, IL 60637

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ABSTRACT **Unlike conventional membrane proteins of the secretory pathway, proteins anchored to the cytoplasmic surface of membranes by hydrophobic sequences near their C termini follow a posttranslational, signal recognition particle-independent insertion pathway. Many such C-terminally-anchored proteins have restricted intracellular locations, but it is not known whether these proteins are targeted directly to the membranes in which they will ultimately reside. Here we have analyzed the intracellular sorting of the Golgi protein giantin, which consists of a rod-shaped 376-kDa cytoplasmic domain followed by a hydrophobic C-terminal anchor sequence. Unexpectedly, we find that giantin behaves like a conventional secretory protein in that it inserts into the endoplasmic reticulum (ER) and then is transported to the Golgi. A deletion mutant lacking a portion of the cytoplasmic domain adjacent to the membrane anchor still inserts into the ER but fails to reach the Golgi, even though this mutant has a stable folded structure. These findings suggest that the localization of a C-terminally-anchored Golgi protein involves at least three steps: insertion into the ER membrane, controlled incorporation into transport vesicles, and retention within the Golgi.**

Conventional membrane proteins of the secretory pathway are inserted cotranslationally into the endoplasmic reticulum (ER) membrane and then carried to their final destination in transport vesicles. The initial targeting of these proteins to the ER is mediated by hydrophobic signal sequences, which are recognized during translation by the signal recognition particle (1). In contrast, proteins that become anchored to membranes by hydrophobic stretches near their C termini do not bind signal recognition particles and are inserted into membranes posttranslationally (2). Many C-terminally-anchored proteins have restricted intracellular locations (3), suggesting that a different mechanism targets the insertion of these proteins at specific subcellular sites. The simplest possibility would be that C-terminally-anchored proteins are inserted directly into their final target membrane. However, our results suggest that the actual sorting mechanism is more complex.

We analyzed the membrane insertion of giantin, a protein that is attached to Golgi membranes by a putative C-terminal membrane anchor. Several lines of evidence indicate that giantin's cytoplasmic domain forms a long coiled-coil rod: this domain is composed almost entirely of heptad repeats (4); giantin is recovered from detergent extracts as a highly elongated homodimer whose dimensions can be estimated to be 3.5 × 250 nm (5); and anti-giantin antibodies labeled Golgi-associated filaments, as detected by immunoelectron microscopy (T. Misteli, A.D.L., and H.-P.H., unpublished results). Here we report that this rod-shaped structure first becomes membrane-associated at the cytoplasmic surface of the ER and then is transported to the Golgi apparatus.

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METHODS

Subcellular Fractionation. The fractionation will be described in detail elsewhere (M.F. and H.-P.H., unpublished results). Briefly, HepG2 cells grown to confluence on three 15-cm plates were homogenized by 10 passages through a ball-bearing homogenizer (6) with a clearance of 20 μm in 3 ml of 120 mM NaCl/5 mM KCl/25 mM $NaHCO_3$, pH 7.4, containing protease inhibitors (0.2 mM phenylmethanesulfonyl fluoride and pepstatin, leupeptin, and antipain each at 1 μg/ml). After centrifugation at 750 rpm for 10 min in an SS-34 rotor (Sorvall), the resulting postnuclear supernatant was applied to the top of a 12-ml linear Nycodenz (Life Technologies, Basel) gradient (7) [13–29% (wt/vol) with a 1-ml 35% cushion; all solutions contained 10 mM triethanolamine (pH 7.4), 1 mM EDTA, and protease inhibitors]. After centrifugation at 25,500 rpm for 3 hr in a TST-28.17 rotor (Kontron, Zurich), the gradients were fractionated from the bottom, and each fraction was assayed for density, content of total protein (Bio-Rad), glucose-6-phosphatase (8), GalNAc transferase (9), and galactosyltransferase (10). The amounts of ERGIC-53 protein (data not shown) (11) and giantin were determined by densitometry after transfer from SDS/PAGE gels and immunoblotting. To determine the distribution of newly synthesized proteins, HepG2 cells (one 15-cm plate per experiment) were incubated with 2 mCi (1 Ci = 37 GBq) of [^{35}S]methionine for 5 min and chased as indicated prior to fractionation. Each fraction was diluted, the membranes were collected by centrifugation, and the content of labeled giantin and dipeptidylpeptidase IV (DPPIV) was determined by immunoprecipitation, SDS/PAGE, and fluorography.

Transfection. Full-length giantin was generated as follows using partial clones encoding different fragments of giantin (4). cDNA clones G1 and G14 were joined at a shared *Eco*RV site. A three-piece ligation joined R7 (a 5′ rapid amplification of cDNA ends product) to G15 (cDNA) at a *Pst* I site and G15 to G12 (cDNA) at a shared *Eco*RI site. A unique *Kpn* I site was used to join the two giantin halves (R7/G15/G12 to G1/G14). The entire giantin open reading frame was cloned into the expression vector pXMT3, which contains the human adenovirus promoter. The anchor-minus construct was generated by restriction at a unique *Bsu*36I site (nucleotide 9503) upstream of the predicted membrane-spanning domain. Filling in at this site created an in-frame stop codon. The two internal deletions

Abbreviations: ER, endoplasmic reticulum; ERGIC, ER–Golgi intermediate compartment; DPPIV, dipeptidylpeptidase IV.

†Present address: Department of Biology, University of California at San Diego, La Jolla, CA 92093.
¶To whom reprint requests should be sent at: Department of Pharmacology, Biocenter of the University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland.

(amino acids 2172–3138 and 1233–3141) were created by treating the *Bsu*36I-digested vector with mung bean nuclease. All mutations were confirmed by restriction and sequence analysis. DEAE-dextran was used for transfection of COS cells, and calcium phosphate precipitation was used for HeLa and CHO cells (12). All experiments were performed 2 days after transfection. Immunofluorescent analysis was performed as described (5), except that the anti-giantin monoclonal antibody was used at a 1:100 dilution. Velocity gradient sedimentation, extraction with alkaline carbonate, and determination of the turnover rate were as described (5), except that the experiments were performed on transfected and metabolically labeled COS cells, and the various forms of giantin were recovered by immunoprecipitation. Unless otherwise indicated, the labeling was for 3 hr in medium containing [^{35}S]methionine at 100 μCi/ml followed by a 2-hr chase in medium containing excess unlabeled methionine.

RESULTS AND DISCUSSION

To monitor the intracellular distribution of giantin, we fractionated postnuclear supernatants from HepG2 cells on Nycodenz gradients (M.F. and H.-P.H., unpublished results). Based on the sedimentation of marker proteins, this gradient system resolved ER, cis/medial-Golgi, and trans-Golgi membranes (Fig. 1*A*). Plasma membrane and membranes of the ER–Golgi intermediate compartment (ERGIC) cosedimented with ER and trans-Golgi, respectively. First, we determined the steady-state distribution of giantin. Giantin cosedimented with membranes containing the cis-Golgi marker GalNAc transferase (13) and was well separated from membranes containing galactosyltransferase, a trans-Golgi marker (14) (Fig. 1*B*). This result indicates that giantin resides primarily in cis- and/or medial-Golgi cisternae. We then determined the distribution of newly synthesized giantin. After a 5-min metabolic labeling period at 37°C, radioactive giantin was not present in the Golgi but was recovered exclusively in the ER-containing fractions at the bottom of the gradient (Fig. 2*A*). After 60 min of chase in the presence of excess unlabeled methionine, the labeled giantin had attained its steady-state Golgi distribution.

We tested whether the intracellular transport of giantin shares characteristics with the conventional secretory pathway by measuring the distribution of newly synthesized giantin after a 15°C chase, a treatment that blocks vesicle-mediated ER-to-Golgi transport (15). As a control, newly synthesized DPPIV, a conventional plasma membrane protein, was recovered in ER- and ERGIC-containing fractions after a 15°C chase (Fig. 2*B*). Giantin was also recovered in these fractions, with no accumulation detectable in the cis- and medial-Golgi-containing fractions (Fig. 2*B*). Therefore, giantin behaves like a conventional secretory protein with respect to its intracellular transport at 15°C. Although colocalization on a gradient is not a definitive test for the intracellular location of a protein, the results shown in Fig. 2 are consistent with the idea that giantin inserts into the ER before being transported to the Golgi. The immunofluorescence data described below support this conclusion.

To confirm that giantin is attached to Golgi membranes by its predicted C-terminal anchor, we analyzed a mutant form of giantin lacking this anchor sequence. First, a full-length clone of giantin was generated by combining five partial cDNA clones at shared restriction endonuclease sites. Transfection of this construct into COS cells led to overproduction of a protein whose mobility on SDS/PAGE was indistinguishable from that of endogenous giantin (see Fig. 4*A*). The transfected giantin appeared to undergo proper membrane integration, as it was not extracted by treatment with alkaline carbonate buffer

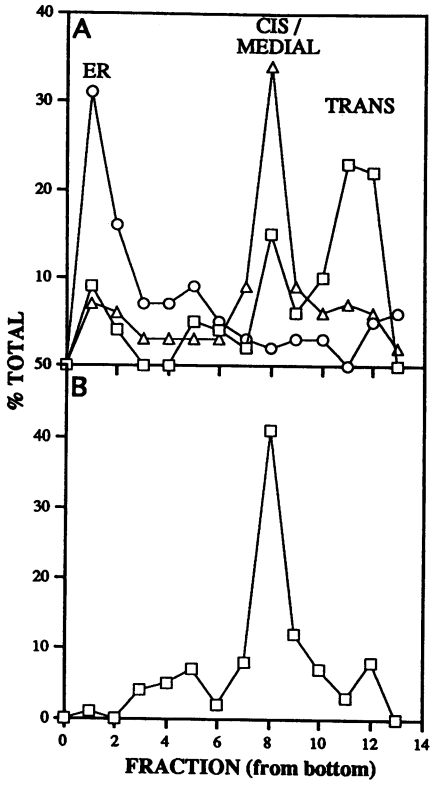

FIG. 1. Steady-state distribution of markers for several intracellular compartments (*A*) and of giantin (*B*) on Nycodenz gradients. The peak positions of glucose-6-phosphatase (○; ER), GalNAc transferase (△; cis-Golgi), and galactosyltransferase (□; trans-Golgi) are indicated.

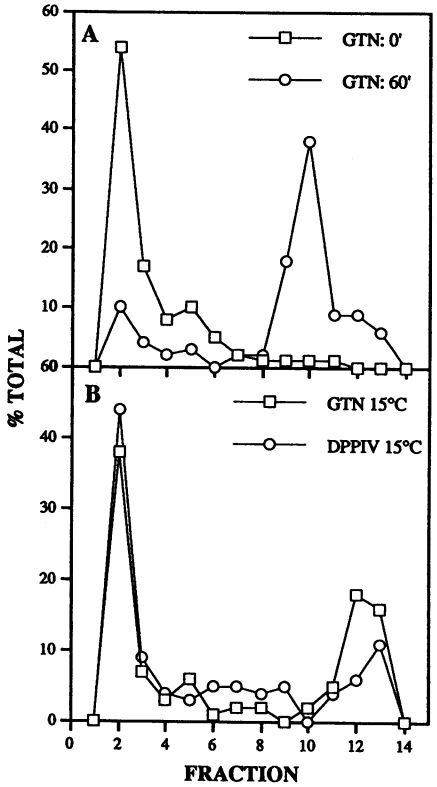

FIG. 2. Distribution of newly synthesized giantin (GTN) after a 0- or 60-min chase at 37°C (*A*) or after 3 hr at 15°C (*B*). The distribution of newly synthesized DPPIV after 3 hr at 15°C is also indicated (*B*). As expected, the radiolabeled DPPIV recovered after the 15°C chase contained only pre-Golgi carbohydrate modifications (not shown).

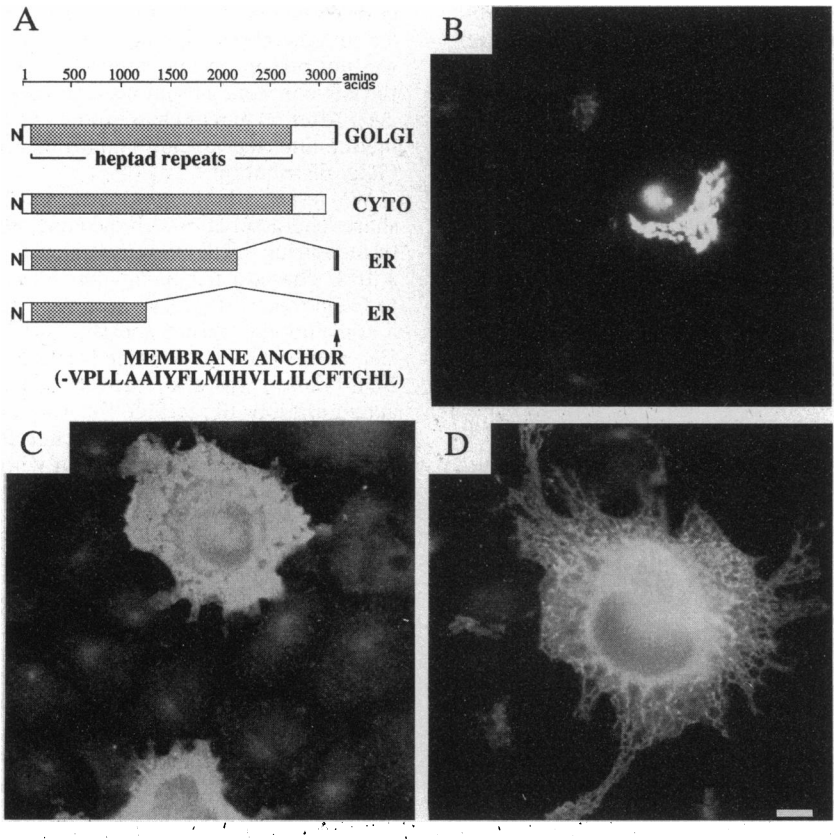

FIG. 3. (*A*) Schematic diagram of constructs for expressing giantin, anchor-minus giantin, and two internal deletion mutants. (*B–D*) Immunofluorescent patterns after transfection of COS cells with full-length giantin (*B*), anchor-minus giantin (*C*), or the mutant containing the smaller internal deletion (*D*). The last 25 amino acids comprising giantin's C terminus are also shown (in single-letter code). (Bar = 6μm.)

(data not shown). To generate the anchor-minus construct, a stop codon was introduced near the 3′ end of the giantin open reading frame (Fig. 3*A*). After expression in COS cells, this truncated version of giantin (361 kDa) did not sediment with membranes (data not shown) but was localized to the cytoplasm as judged by immunofluorescence (Fig. 3*C*), indicating that the C-terminal stretch of hydrophobic amino acids is indeed required for membrane association of giantin.

We also used immunofluorescence to determine the localization of two giantin mutants with substantial deletions adjacent to, but not including, the membrane anchor (Fig. 3*A*). As a control, overexpressed wild-type giantin was properly

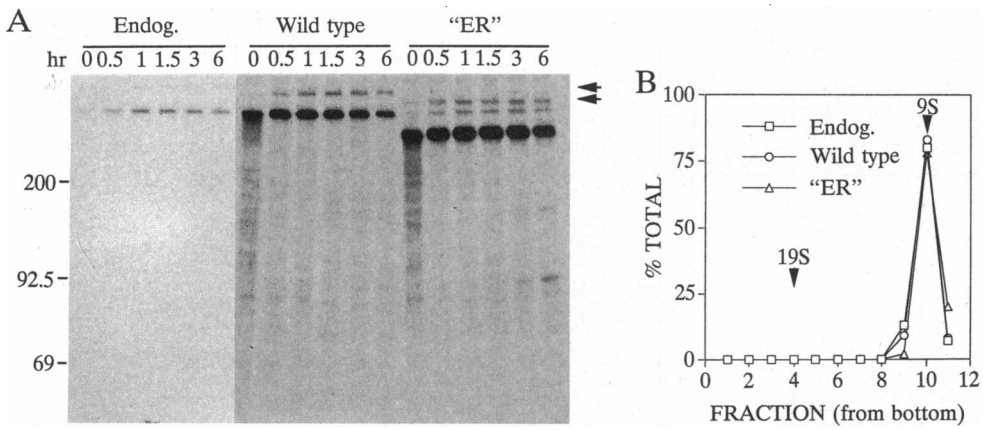

FIG. 4. Comparison of the stability (*A*) and sedimentation rate (*B*) of endogenous giantin, overexpressed wild-type giantin, and the mutant containing the smaller internal deletion (designated "ER"). To determine the stability of the proteins, COS cells that were either mock-transfected (Endog.) or transfected with DNA encoding wild type or the ER-localized deletion mutant ("ER") were pulsed labeled for 5 min and then chased for the indicated times. The wild-type and mutant forms of giantin were recovered by immunoprecipitation, and the immunoprecipitates were analyzed by SDS/PAGE and fluorography. Although we performed the SDS/PAGE under reducing conditions, a small amount of dimer formation was visible with both wild-type giantin and the deletion mutant (arrows). Monomeric endogenous giantin migrated between the monomeric and dimeric forms of the deletion mutant. Only the dimeric forms were recovered after analysis on nonreducing SDS/PAGE (not shown). To determine the sedimentation rate of the proteins, cell lysates from the transfected COS cells were sedimented on glycerol velocity gradients. The fractions were assayed for giantin by immunoprecipitation. The peak positions of two reference proteins, thyroglobulin (19S, 669 kDa) and β-amylase (9S, 200 kDa), are indicated. Note that the relatively slow sedimentation rate of the giantin dimers (molecular masses ≥ 722 kDa) is expected for highly elongated particles.

targeted to the Golgi (Fig. 3*B*). In contrast, the two deletion mutants gave staining patterns characteristic of ER accumulation (Fig. 3*D*). Identical results were obtained when the transfection experiments were performed with HeLa or CHO cells instead of COS cells (data not shown). The ER localization of these giantin mutants indicates that after insertion into the ER membrane, the altered forms of giantin cannot access the intracellular transport machinery. To test whether this defect was due to misfolding, aggregation, or rapid degradation of the mutant giantin proteins, we compared the biochemical properties of the ER-localized mutant that contained the smaller internal deletion with those of full-length giantin. Previously we demonstrated that giantin forms a highly elongated disulfide-linked homodimer (5). The ER-localized mutant was indistinguishable from overexpressed wild-type giantin in sedimentation rate, dimer formation, and turnover time (Fig. 4), suggesting that the mutant protein had a folded conformation similar to that of wild-type giantin. Therefore, it seems unlikely that the ER localization of this mutant protein is due to the action of a quality control mechanism on the cytoplasmic face of the ER.

The internally deleted giantin mutants appear to undergo only the initial ER insertion step of the giantin sorting pathway. Because the ER is the most abundant intracellular membrane, this insertion reaction could involve nonspecific partitioning into membranes. However, we did not detect any accumulation of newly synthesized giantin or the ER-localized giantin mutants in other membranes, suggesting that this C-terminally-anchored protein may be inserted into the ER by a signal-mediated pathway.

Many C-terminally-anchored proteins remain in the ER (3), whereas giantin is rapidly transported to the Golgi. Thus access to the transport machinery must be regulated. It is possible that some C-terminally-anchored proteins are actively retained in the ER. However, it seems unlikely that the internal deletions in the ER-localized giantin mutants could have generated an ER retention signal. Our observations imply that some C-terminally-anchored proteins are excluded from transport vesicles because they lack a sorting signal or because they cannot be incorporated into a forming vesicle for steric reasons. Interestingly, the sequences missing from the two ER-localized giantin mutants might represent a flexible hinge at the base of the giantin rod, as these sequences do not contain the heptad motif present throughout the remainder of the protein. This putative hinge region could be required to allow the 250-nm giantin rod to project through the 10-nm coat of transport vesicles that are only 50 nm in diameter (16).

After being transported, giantin is retained in the Golgi. The retention mechanism remains to be elucidated. Sed5, a C-terminally-anchored protein of the ERGIC, seems to contain localization information in both its membrane anchor and its cytoplasmic domain (17). Retention of Golgi glycosylation enzymes, which all have type II topologies and short cytoplasmic tails (18), depends upon signals within the membrane-spanning domain (19). Type II proteins may be retained in the Golgi by forming large heterooligomeric complexes that are excluded from transport vesicles (20, 21). This hypothesis explains why overexpression of certain Golgi enzymes causes them to accumulate in the ER (22–26): overexpression presumably leads to premature oligomerization. In contrast, overexpressed wild-type giantin did not accumulate in the ER (Fig. 3*B*) even with high levels of expression, suggesting that this protein does not form large oligomers.

The experiments reported here indicate that other mechanisms mediate the ER insertion, exit from the ER, and retention in the Golgi of the C-terminally-anchored protein giantin. Additional work needs to be directed at identifying the targeting signals in the giantin molecule and characterizing the machinery that mediates the intracellular sorting of C-terminally-anchored proteins.

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