THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 269, No. 14, Issue of April 8, pp. 10830-10837, 1994
Printed in U.S.A.
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Retention and Degradation of Proteins Containing an Uncleaved Glycosylphosphatidylinositol Signal*

(Received for publication, September 17, 1993, and in revised form, January 13, 1994)

Mark C. Field‡§, Paul Moran‡, Wenlu Li¶, Gilbert-André Keller¶, and Ingrid W. Caras‡‖

From the Departments of ‡Neurobiology and ¶Pharmacology, Genentech Inc., South San Francisco, California 94080

Glycosylphosphatidylinositol (GPI) membrane anchor attachment is directed by a COOH-terminal signal that is proteolytically removed and replaced with a preformed GPI anchor in a coupled reaction. Failure to complete proteolytic cleavage and anchor addition results in the retention of an uncleaved precursor in a post-endoplasmic reticulum (ER) compartment. In this report, we address three issues: (i) the exact position of the transport block, (ii) the subsequent fate of the retained molecules, *i.e.* where are they degraded, and (iii) the mechanism whereby these proteins are selected for retention. Using decay accelerating factor (DAF), we provide evidence that failure to cleave the GPI signal totally prevents *O*-glycosylation, suggesting that the uncleaved polypeptides are not transported into the *cis*-Golgi complex. This implies that transport is blocked at the boundary between the ER-Golgi intermediate compartment and the Golgi stacks. The degradation of an intracellularly retained human growth hormone (hGH)-DAF fusion protein containing a nonfunctional GPI signal shows some features of ER degradation, *i.e.* the degradation is insensitive to leupeptin, chloroquine, and ammonium chloride, and is inhibited at 16 °C or after ATP depletion. However, morphological evidence points to a pathway resembling autophagy. To reconcile these observations, we suggest either that hGHDAF is degraded by two distinct pathways (ER degradation and autophagy) or that ER degradation takes place in an ER-associated vesicular compartment in a process resembling autophagy. Using as probes a soluble hGH receptor and an antibody recognizing only native hGH, we show that a significant fraction of the retained protein is correctly folded, ruling out general misfolding as the basis for retention. We also show that hGHDAF fusion proteins are present in high molecular weight, disulfide-linked aggregates in COS cells. We suggest a model for retention in which the uncleaved GPI signal drives the formation of large micelle-like aggregates that cannot be secreted.

A number of diverse cell surface proteins are anchored to the cell membrane by a glycosylphosphatidylinositol (GPI)[1] glycolipid covalently attached to the COOH terminus of the protein (reviewed in Ferguson and Williams (1988), Low (1989), Cross (1990), and Field and Menon (1993)). GPI attachment to protein requires translocation of the polypeptide across the ER membrane and involves the cleavage of a COOH-terminal signal and its replacement, in a coupled process, by a preformed GPI lipid. The protein is then transported through the Golgi apparatus to the cell surface.

The GPI signal of human decay accelerating factor (DAF) has been localized to the COOH-terminal 29 amino acids (Moran *et al.*, 1991). The last 17 residues of this signal constitute a hydrophobic domain that is preceded by a cleavage/attachment site for anchor addition. Cleavage occurs between two small side chain amino acids (serine and glycine in the case of DAF), positioned 10–12 residues NH_2-terminal to the hydrophobic domain (Caras *et al.*, 1989; Moran *et al.*, 1991; Gerber *et al.*, 1992, see also Fig. 1). Fusion of this 29-residue signal to the COOH terminus of human growth hormone (hGH), a secreted protein, is sufficient to produce GPI-anchored hGH on the cell surface (Caras *et al.*, 1987; Moran *et al.*, 1991). The general features of the GPI signal of DAF are conserved among other GPI-anchored proteins (see Cross (1990) and Field and Menon (1993)).

Conditions that interfere with the GPI-attachment process, preventing both cleavage and anchor attachment, include: (i) substitution of one or both of the residues flanking the cleavage site with a bulky amino acid (Moran *et al.*, 1991; Moran and Caras, 1992); (ii) defects in the biosynthesis of the GPI lipid, *e.g.* in the human disease, paroxysmal nocturnal hemoglobinuria (Selvaraj *et al.*, 1987), or in certain thymoma mutants (Fatemi and Tartakoff, 1988); and (iii) treatment of cells with mannosamine (Lisanti *et al.*, 1991; Field *et al.*, 1993) or phenylmethanesulfonyl fluoride (Masterson and Ferguson, 1991). Also, when GPI-anchored proteins are overexpressed in COS cells, processing is incomplete, resulting in the production of uncleaved as well as GPI-linked products (Moran *et al.*, 1991). In all of these cases, the loss of GPI addition results in failure of the unprocessed proteins to reach the cell surface. Thus, an uncleaved GPI signal acts as a cellular retention signal.

Prior studies in COS cells have indicated that hGHDAF fusion proteins containing an uncleaved GPI signal are retained in a post-ER compartment (Moran and Caras, 1992). However, neither the exact identity of this compartment, nor the mechanism of the intracellular retention, nor the ultimate fate of these proteins was determined. These questions are addressed in this report.

MATERIALS AND METHODS

Reagents, Enzymes, and Antibodies—Brefeldin A and peroxidase-conjugated lectins were from Sigma. Endoglycosidase H (EC 3.2.1.96) was from Boehringer Mannheim. Phosphatidylinositol-specific phospholipase C (EC 3.1.4.10) from *Bacillus thuringiensis* was the gift of Dr. M. Low, Columbia University, New York. Concanavalin A-Sepharose was from Pharmacia LKB Biotechnology Inc. Antibodies to human growth hormone (hGH), gp120 from HIV-1, and DAF were supplied by the Medicinal and Analytical Chemistry Dept. at Genentech, Inc. An antibody to human cathepsin D was the gift of Dr. S. Kornfeld, Wash-

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§ Present address: Department of Biochemistry, Imperial College of Science, Technology, and Medicine, London, SW7 2AY, UK.

‖ To whom correspondence and reprint requests should be addressed.

[1] The abbreviations used are: GPI, glycosylphosphatidylinositol; COS, African green monkey CV-1 cells, constitutively expressing SV40 T-antigen; DAF, decay accelerating factor; hGH, human growth hormone; ER, endoplasmic reticulum; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.

ington University, St Louis, MO. An anti-Golgi complex antibody (against p52) was from Upstate Biotechnology, Lake Placid, NY. A plasmid expressing a soluble form of gp120 was obtained from Dr. P. Berman, Immunology Dept., Genentech, Inc. A GPI-anchored form of gp120 (gp120DAF) was produced by mutagenesis of this plasmid. Soluble hGH-receptor was obtained from the Protein Engineering Dept., Genentech, Inc.

Plasmids and Cell Lines—Construction of all recombinant DNAs has been previously described (Caras *et al.*, 1989; Moran and Caras, 1992), except the diglycosylated hGHDAF variants which were produced by gene manipulation of hGHDAFAsn^{63} and hGHDAFAsn^{98}. A stable CHO cell line, expressing hGHDAF28, was produced by co-transfection with plasmids encoding hGHDAF28 and dihydrofolate reductase using the calcium phosphate method (Sambrook *et al.*, 1989).

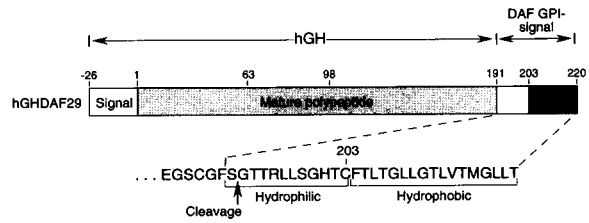
Transfection, Metabolic Labeling, and Immunoprecipitation—COS and L(tk$^-$) cells were transiently transfected by the DEAE-dextran method (Selden, 1987) using 4–8 μg of DNA/10-cm dish or 2 μg of DNA/35-mm dish and DEAE-dextran at 400 μg/ml. For some experiments (those involving treatment with lysosomotrophic reagents) cells were transfected in 10-cm dishes, allowed to recover overnight, and then plated out in 35-mm dishes for 48 h before radiolabeling. Cells were prestarved in methionine/cysteine-deficient Dulbecco's modified Eagle's medium, containing 10% diafiltered fetal calf serum, for at least 1 h, then labeled with Tran^{35}S-label (DuPont NEN) for variable times at 100–200 μCi/ml in the same medium. For pulse-chase experiments, cells were chased by replacing the labeling medium with complete medium containing methionine and cysteine. Cells were harvested by incubation in 7 mM EDTA/PBS, pelleted, and then extracted with 1% Triton X-100 containing a mixture of protease inhibitors (leupeptin, chymostatin, antipain, pepstatin (all at 2 μg/ml), 50 μg/ml L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone, and 1 mM phenylmethanesulfonyl fluoride). In some experiments, 1 mM *N*-ethylmaleimide was added to the extraction buffer to minimize artifactual oxidation of disulfide bonds (Braakman *et al.*, 1992). The Triton extracts were then precleared and immunoprecipitated with the relevant antibodies (Anderson and Blobel, 1983). Protein secreted into the medium was immunoprecipitated by harvesting the medium (1 ml), adding 6 μl of 20% SDS, 50 μl of 20% Nonidet P-40, 24 μl of 250 mM EDTA, pH 7.0, and protease inhibitors and treating exactly as the cell extracts. In experiments using the soluble hGH receptor or anti-hGH monoclonal antibodies, boiling of the Triton X-100 extracts in SDS was omitted. For some experiments, the Triton X-100 extract was treated with dithiothreitol and iodoacetamide before addition of antibody to effect complete denaturation.

Endoglycosidase H Digestion—Immunoprecipitates were solubilized by boiling in 20 μl of 0.5% SDS for 10 min. The beads were pelleted, and the supernatant was removed. Five-microliter aliquots were transferred to individual tubes, and 10 μg of bovine serum albumin/1 mM phenylmethanesulfonyl fluoride were added, followed by 40 milliunits of endoglycosidase H in 40 μl of 50 mM sodium phosphate, pH 7.0. The samples were incubated at 37 °C for 4 h, after which a second aliquot of endoglycosidase H (20 milliunits/20 μl) was added and the digestion continued overnight. The samples were then analyzed by SDS-PAGE. Mock samples were treated identically, except that the enzyme was omitted from the buffer.

To check the identity of some of the bands obtained by endoglycosidase H digestion, the samples were precipitated from sample buffer with 1 ml of acetone (−20 °C/4 h), washed once with 500 μl of acetone, and dried in a Speed Vac for 10 min. The pellet was solubilized by adding 20 μl of 0.05% Triton X-100 in PBS/1 mM $CaCl_2$. Twenty microliters of concanavalin A-Sepharose were then added, and the slurry was mixed on a rocker for 30 min at room temperature. After this time, the beads were pelleted and the supernatant was removed (unbound fraction). The beads were washed three times with PBS, and the bound material was released by boiling in a 20-μl sample buffer/250 mM α-methylmannoside. The resulting fractions were then analyzed by SDS-PAGE.

Pharmacological Treatments—The following compounds were used at the concentrations given and were added from 100× stocks 1–2 h prior to metabolic labeling; ammonium chloride (50 mM (aq)), chloroquine (40 μg/ml (aq)), leupeptin (0.1 mg/ml (in dimethyl sulfoxide)), brefeldin A (10 μg/ml (in 90% ethanol)), tunicamycin (2 μg/ml (in ethanol)), sodium azide (0.1%) plus sodium fluoride (20 mM (aq)).

Immunofluoresence and Confocal Microscopy—Immunofluorescent labeling of intact or permeabilized cells was carried out as described (Caras *et al.*, 1989). Cells were permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature. Images were obtained using a Zeiss fluorescent microscope. Confocal analysis was performed using a Molecular Devices confocal microscope (Molecular Devices). Image analysis was performed using the Sarastro software suite.



Construct	Alterations from hGHDAF29	Reference
hGHDAF29	–	Moran et al., 1991
hGHDAF29Asn63	Mutation to introduce AsnXSer at +63	Moran and Caras, 1992
hGHDAF29Asn63Asn98	Mutations to introduce AsnXSer at +63 and +98	This paper
hGHDAF28	Deletion of Ser192. GPI-signal nonfunctional	Moran et al., 1991
hGHDAF28Asn63	Mutation to introduce AsnXSer at +63	Moran and Caras, 1992
hGHDAF28Asn98	Mutation to introduce AsnXSer at +98	Moran and Caras, 1992
hGHDAF28Asn63Asn98	Mutations to introduce AsnXSer at +63 and +98	This paper
hGH	Authentic hGH, terminates at Phe191	Generic
hGHDAF17	Deletion of Ser192-Cys203	Moran and Caras, 1992
hGHDAFSyn17	Replacement of Ser192-Cys203 with synthetic spacer	Caras et al., 1989

FIG. 1. **Structures of hGHDAF fusion proteins used in this study.** Shown is the parent fusion protein, hGHDAF29, containing the 29-residue, fully functional GPI signal of DAF fused to the COOH terminus of hGH. The positions of important amino acid are indicated, and the COOH-terminal hydrophobic domain (17 amino acids) is represented in *black*. The sequence of the DAF GPI signal is given beneath, showing the position of the cleavage/attachment site. Additional constructs are tabulated below, together with their original citations. In hGHDAF28, the serine residue flanking the cleavage site on the NH_2-terminal side has been deleted, producing a noncleavable fusion protein.

Gel Electrophoresis and Western Blotting—Immunoprecipitates were separated on 15% discontinuous SDS polyacrylamide gels (Laemmli, 1970), and the gels were impregnated with En^{3}Hance, dried, and fluorographed as described (Field *et al.*, 1991). Gradient polyacrylamide gels (4–15%) were obtained from Bio-Rad Inc. Where necessary, the radioactive bands were quantitated from the dried gel using a Fujix BAS2000 Bio-Imaging system. Proteins were blotted onto nitrocellulose sheets, stained with lectins at 5 μg/ml, and visualized using peroxidase reagent from Bio-Rad.

Immunoperoxidase Electron Microscopy—Nontransfected parental cells and cells expressing either hGHDAF28 or a secreted protein, DNase, were fixed for 30 min in periodate-lysine-3% paraformaldehyde (McLean and Nakane, 1974). After washing with PBS, the cells were permeabilized by gentle agitation at room temperature in 0.5% saponin in PBS for 5 min and then incubated for 1 h with an affinity-purified rabbit antibody against hGH or DNase. After washing in 0.5% saponin-PBS, the cells were treated with peroxidase-conjugated goat anti-rabbit IgG (Cappel). The cells were then fixed in 1.5% glutaraldehyde in 0.1 M cacodylate, 5% sucrose for 15 min at room temperature and incubated in 0.2% diaminobenzidine for 2–5 min, after which 3% H_2O_2 was added to a final concentration of 0.01%. The reaction was stopped with 50 mM Tris, pH 7.4. The samples were washed, postfixed in 1% reduced osmium overnight (Karnovski, 1971), washed in water, dehydrated through graded ethanols and propylene oxide, and embedded in EPONETE 12 (Ted Pella, Inc., Redding, CA). Ultrathin sections were cut on a Reichert Ultracut E microtome, counterstained with ethanolic uranyl acetate and lead citrate, and examined in a Philips CM12 transmission electron microscope.

RESULTS

The various hGH derivatives used in this study are described in Fig. 1.

Retained Proteins Do Not Become O-Glycosylated—hGHDAF fusion proteins containing an uncleaved GPI signal are blocked in transport to the cell surface and are retained in a post-ER compartment, possibly the ER-Golgi intermediate compartment (Moran and Caras, 1992). To further define the position of the transport block, we analyzed the processing of the GPI-anchored glycoprotein, human DAF. Mature DAF is a 70-kDa glycoprotein containing one *N*-linked and several processed *O*-linked glycans. Inhibition of *O*-glycosylation does not affect transport of DAF to the cell surface (Reddy *et al.*, 1989), but

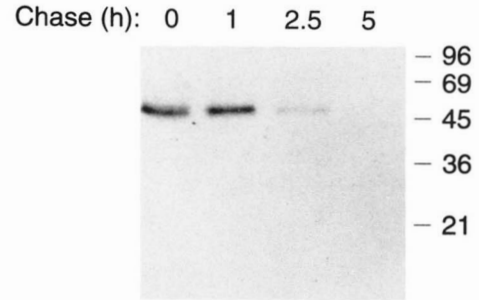


FIG. 2. **DAF fails to become *O*-glycosylated in murine L cells defective in GPI addition.** Murine L(tk⁻) cells, transiently expressing authentic DAF, were pulse-labeled for 1 h and then chased for 0, 1, 2.5, and 5 h as indicated. DAF was immunoprecipitated from cell extracts and analyzed by SDS-PAGE. The positions of molecular weight markers are shown on the *right*.

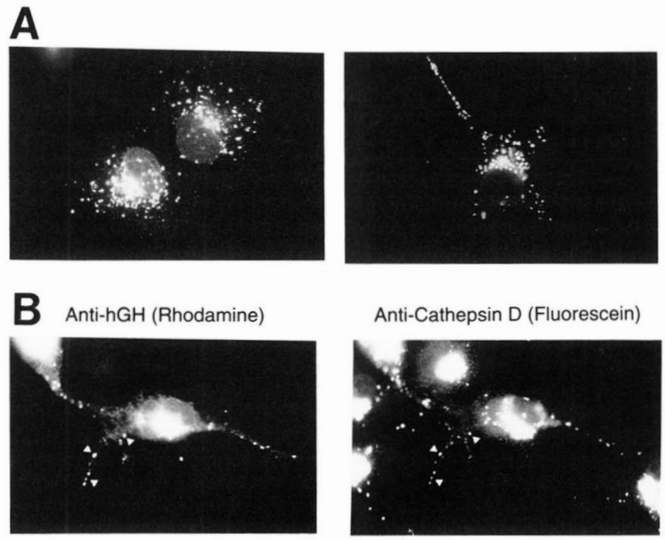


FIG. 3. **Localization of retained hGHDAF.** *A*, vesicular localization of retained hGHDAF. Shown are transiently transfected COS cells expressing two different hGHDAF fusion proteins containing an uncleavable GPI signal, stained with an anti-hGH antibody as described under "Materials and Methods." *B*, co-localization with cathepsin D. COS cells transiently expressing hGHDAF28 were fixed, permeabilized, and stained with mouse antibodies to hGH and a rabbit antibody to cathepsin D, followed by rhodamine-conjugated goat anti-mouse IgG and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG. *White arrowheads* indicate regions of overlap of the two markers.

prevents processing of the 46-kDa precursor to the 70-kDa mature form. DAF was transiently expressed in L(tk⁻) cells which cannot add GPI anchors due to a defect in synthesis of GPI lipids (Singh *et al.*, 1991). As expected, DAF was neither expressed on the cell surface as a GPI-anchored protein, nor secreted into the cell medium, indicating that the uncleaved protein is retained inside the cell (data not shown). Cell lysates contained only the 46-kDa DAF species (Fig. 2). This indicates that the failure to become GPI-anchored prevents *O*-glycosylation, implying that the retained DAF does not reach the Golgi stacks where *O*-glycosylation occurs (Dekker and Strous, 1990). To distinguish between a complete lack of *O*-glycosylation *versus* a lack of *O*-glycan elaboration, we analyzed DAF synthesized in L cells by immunoprecipitation followed by Western blotting using lectin-horseradish peroxidase conjugates. No reactivity was observed with the lectins from *Glycine max*, *Helix pometia*, or the B_4 isolectin from *Vicia villosa*, all of which bind terminal GalNAc (data not shown). We also saw no reactivity in the 46-kDa region of blots made from total extracts of L cells expressing DAF despite binding of all three lectins to ~10 glycoproteins. This suggests a complete lack of *O*-glycosylation of DAF in L cells, indicating that DAF fails to reach the *cis*-Golgi compartment where *O*-glycosylation is believed to initiate (Dekker and Strous, 1990). DAF was recognized by the lectin from *Canavila ensiformis*, indicating that it did contain an *N*-glycan (data not shown).

When DAF was overexpressed in COS cells, two species were produced: the 70 kDa, GPI-linked, cell-surface species and a 46-kDa intracellular species, presumably representing molecules that escaped GPI addition as has been observed following overexpression of hGHDAF in COS cells (Moran and Caras, 1992). Pulse-chase analysis indicated that this 46-kDa DAF species was degraded without conversion to a higher molecular weight form (data not shown), suggesting that, as in L cells, it never entered the Golgi apparatus. These data are consistent with our earlier observations using hGHDAF fusion proteins, suggesting that proteins containing an uncleaved GPI signal are blocked in transport at the boundary between the Intermediate compartment and the Golgi apparatus (Moran and Caras, 1992).

Co-localization of hGHDAF28 in Vesicles Containing Cathepsin D—Immunofluorescent localization of retained hGHDAF fusion proteins in transfected COS cells revealed that in addition to the ER and Intermediate compartment, hGHDAF is present in large vesicles in the peripheral cytoplasm (Fig. 3*A* and Moran and Caras (1992)). To test whether these vesicles might be lysosomes, we analyzed cells expressing hGHDAF28 (see Fig. 1) by double-label immunofluorescence microscopy, using antibodies to hGH and cathepsin D, a known lysosomal protease. Cathepsin D was detected in both small and large (1–2 μm) vesicles distributed throughout the cytoplasm and in vesicles close to the Golgi apparatus (Fig. 3*B*). A subpopulation of the large vesicles also contained hGH. Confocal microscopy confirmed the co-localization of cathepsin D and hGH in the same vesicular structures; the two fluorescent markers overlapped in three spacial dimensions when the image was rotated, ruling out apparent co-localization due to two separate structures lying one over the other (data submitted for review but not shown).

Nonlysosomal Degradation of hGHDAF28—Retained hGHDAF fusion proteins are degraded intracellularly. The morphological observations described above suggested that lysosomes might be involved. To test this, we treated COS cells expressing hGHDAF28 with ammonium chloride, leupeptin, and chloroquine, inhibitors of lysosomal degradation (Ascoli, 1979; Maxfield, 1982; Lippincott-Schwartz *et al.*, 1988). Leupeptin and chloroquine had essentially no effect on the degradation of hGHDAF28, while ammonium chloride slowed, but did not prevent, the degradation (Fig. 4). The effect of ammonium chloride was undramatic and highly variable. In repeated experiments, the amount of hGHDAF28 remaining in treated samples after 1–3 h of chase was 1.2- to 2-fold greater than that in untreated samples; after 5–6 h of chase, treated and control samples were indistinguishable. Taken together, these results suggest that hGHDAF28 is not degraded in lysosomes, despite the small effect of ammonium chloride, pointing instead to an ER degradation pathway (Bonifacino and Lippincott-Schwartz, 1991). We also found that brefeldin A had no significant protective effect (Fig. 4), supporting the conclusion that the Golgi complex is not involved (Klausner *et al.*, 1992). As reported for other proteins that undergo ER degradation, incubation at 16 °C (which inhibits membrane fission and fusion (Dunn, 1980a; Saraste *et al.*, 1986)) or depletion of cellular ATP levels with sodium azide and sodium fluoride efficiently prevented degradation (Fig. 4), indicating that the process is energy-dependent and suggesting that a transport step may be required (Lippincott-Schwartz *et al.*, 1988; Amara *et al.*, 1989).

Retention and Degradation of hGHDAF28 in CHO Cells—We

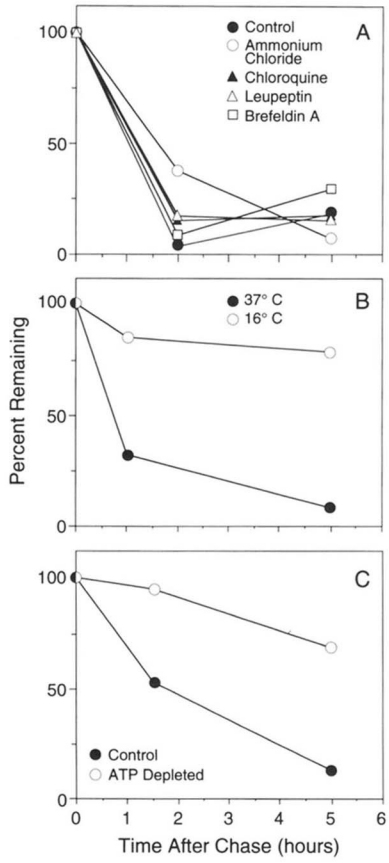


FIG. 4. **Degradation of hGHDAF28 in COS cells.** The degradation of hGHDAF28 was investigated under different conditions in transiently transfected COS cells. Cells were pulse-labeled for 30 min and chased for various times as indicated. hGHDAF28 was immunoprecipitated from cell lysates and analyzed by SDS-PAGE and fluorography. Radioactive bands were quantitated directly from the dried gel using a Fuji BAS2000 Bio-Imaging analyzer. Cells were pretreated with the indicated compound for 30 min before labeling, and the inhibitors were present during both the pulse and the chase. Concentrations of the inhibitors are given under "Materials and Methods." *Panel A*, effect of inhibitors of lysosomal function and of brefeldin A; *panel B*, effect of temperature; *panel C*, effect of ATP depletion by sodium fluoride and sodium azide, used in combination. The amount of hGHDAF28 remaining during the chase is shown as a percentage of the amount present immediately following the pulse.

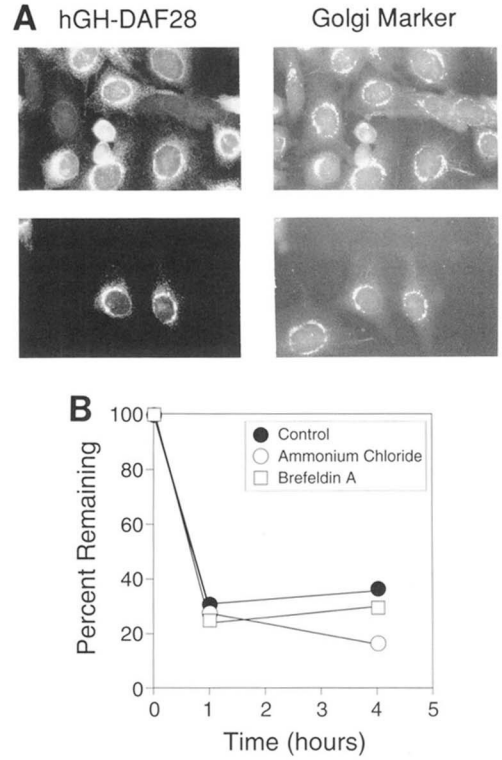


FIG. 5. **Retention and degradation of hGHDAF28 in CHO cells.** *A*, double-label immunolocalization of hGHDAF28 (*left panels*) and a Golgi marker (*right panels*) in CHO cells. A stable CHO cell transfectant expressing hGHDAF28 was fixed, permeabilized, and incubated with rabbit anti-hGH IgG and a mouse monoclonal antibody against the Golgi apparatus (Upstate Biotechnology), followed by fluorescein-conjugated anti-rabbit IgG and rhodamine-conjugated anti-mouse IgG; hGHDAF28 is observed in a post-ER compartment close to the Golgi apparatus as well as in the ER. *B*, degradation of hGHDAF28; CHO cells stably expressing hGHDAF28 were pulse-labeled for 30 min and then chased for various times as indicated. hGHDAF28 was immunoprecipitated from cell lysates and analyzed as described in the legend to Fig. 4. Cells were treated with ammonium chloride or brefeldin A (added 30 min before labeling) as described under "Materials and Methods." The amount of hGHDAF28 remaining during the chase is expressed as a percentage of the amount present immediately following the pulse.

also analyzed the fate of hGHDAF28 in a stable Chinese hamster ovary (CHO) cell transfectant. Essentially no hGHDAF was present on the cell surface or in the medium, nor did phosphatidylinositol-specific phospholipase C release any hGHDAF from these cells, confirming that, as in COS cells, hGHDAF28 fails to become GPI-anchored and is retained (data not shown). The immunofluorescent staining pattern of hGH-DAF28 in CHO cells was similar to that observed in COS cells (Fig. 5*A*), suggesting retention in a post-ER compartment close to the Golgi apparatus (presumably the ER-Golgi intermediate compartment). Ultrastructural analysis (see below) confirmed that hGHDAF28 fails to enter the Golgi stacks. The degradation of hGHDAF28 in CHO cells was completely insensitive to ammonium chloride and brefeldin A (Fig. 5*B*), suggesting that as in COS cells, hGHDAF28 is disposed of by a nonlysosomal, ER-type degradative pathway.

Retained hGHDAF28 Is Not Grossly Misfolded—Whereas secretory and GPI-linked forms of hGH or DAF are efficiently transported to the cell surface, the presence of an uncleaved GPI signal causes an essentially total transport block in both CHO and COS cells. We next asked what is the basis for this selection? We considered the possibility that the uncleaved GPI signal induces misfolding of the hGH domain in order to bury the hydrophobic COOH terminus, thereby leading to aggregation and retention (Tooze *et al.*, 1989; Hurtley and Helenius, 1989). To test for misfolding, COS and CHO cells expressing hGHDAF28 were stained either with a soluble form of the hGH receptor (shGHR), which makes extensive contact with the hGH surface (deVos *et al.*, 1992), or with a monoclonal anti-hGH antibody, Hybr72.3, that recognizes a native conformation-dependent epitope (Jin *et al.*, 1992) (Fig. 6). The staining patterns were essentially identical to those obtained using a polyclonal anti-hGH antibody (Moran and Caras (1992) and Fig. 3*A*), showing native hGHDAF28 present in the ER, a Golgi-like organelle, and (in COS cells) in large vesicles scattered throughout the cytoplasm. These observations suggest that there is correctly folded, but retained, hGH present in various compartments of the secretory pathway.

To quantitate the amount of correctly folded hGH, we immunoprecipitated hGHDAF28 or authentic hGH from radiolabeled COS cell lysates, using Hybr72.3. Residual material not precipitated by this antibody was recovered by reprecipitation with a polyclonal antibody to hGH. Hybr72.3 precipitated ~80% of both hGHDAF28 and hGH, but did not precipitate either protein following reduction and alkylation, confirming that the epitope recognizes only native hGH. These data indicate that a significant fraction of the hGHDAF28 molecules

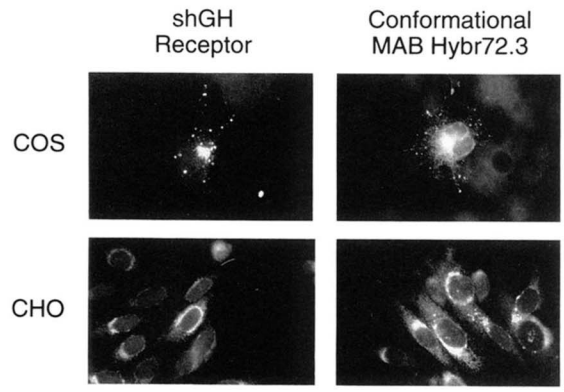


FIG. 6. **Correctly folded intracellular hGHDAF28 detected by conformation probes.** Transiently transfected COS cells, or CHO cells stably expressing hGHDAF28, were fixed, permeabilized, and incubated as follows, with one of two reagents that detect only correctly folded hGH: (i) recombinant soluble hGH (*shGH*) receptor, followed by a mouse antibody against the hGH receptor, followed by fluorescein-conjugated goat anti-mouse IgG or (ii) a monoclonal antibody against hGH (*MAB Hybr72.3*) that recognizes a conformation-dependent epitope, followed by fluorescein-conjugated goat anti-mouse IgG. The staining patterns observed were essentially identical with those obtained with a polyclonal antibody to hGH (see Fig. 5*A* for CHO cells and Fig. 3*A* and Moran and Caras (1992) for COS cells).

present in COS cells contain a correctly folded hGH domain. Since the block to secretion is essentially total, gross misfolding cannot explain the retention of hGHDAF28.

Aggregation of hGHDAF Fusion Proteins—We next considered the possibility that the hydrophobic GPI signal might drive aggregation (resulting in retention) by inducing the formation of micelle-like structures with the hydrophobic COOH termini forming the core. We therefore examined the aggregation states of hGHDAF28 and hGHDAF29 (see Fig. 1) in transfected COS cells. hGHDAF29 contains a functional GPI signal but is incompletely processed following overexpression in COS cells, giving rise to uncleaved, retained molecules as well as GPI-linked hGHDAF on the cell surface (Moran and Caras, 1992). Under nonreducing conditions, hGHDAF28 was recovered in both high molecular weight and monomeric forms (Fig. 7). Following reduction, only monomeric hGHDAF28 was observed. Quantitation of the monomeric species before and after reduction suggested that ~50% of the hGHDAF28 was present in a high molecular weight form. This is a minimum estimate as the immunoprecipitation procedure will disrupt some noncovalent interactions. Disulfide-linked aggregates of hGHDAF28 were also recovered after a 5-min pulse, indicating that aggregation occurs rapidly upon completion of the polypeptide (data not shown). Interestingly, the GPI-anchored (cell surface) species of hGHDAF29 was exclusively monomeric, whereas the uncleaved species (which is retained) behaved identically to hGHDAF28 (Fig. 7). Thus, the aggregation of hGHDAF28 is not due to the fact that the GPI-signal is nonfunctional. Fractionation on a 4–15% gradient polyacrylamide gel indicated that the hGHDAF28 aggregates are heterogenous in size and include dimers, trimers, and larger complexes (data not shown).

The aggregates appeared to contain only hGHDAF. To rule out that the lack of other bands is due to heterogeneity of associated proteins, hGHDAF28 was transiently co-expressed with gp120DAF (a GPI-linked fusion protein containing the DAF GPI signal fused to gp120 from HIV-1 (Schreier *et al.*, 1994)).[2] Lysates from doubly transfected COS cells were immunoprecipitated with antibodies to gp120 or hGH. Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) following reduction demonstrated no co-precipitation of gp120DAF with hGHDAF28 (Fig. 8), indicating that hGHDAF28 does not form mixed aggregates with other proteins in the secretory pathway.

[2] gp120DAF is correctly processed to gain a GPI anchor and is exported to the cell surface. In common with the parent glycoprotein from HIV IIIB, gp160, gp120DAF remains within the ER for ~1.0 h (M. C. Field and I. W. Caras, unpublished observations), and therefore provides a probe for nonspecific aggregation within the ER lumen.

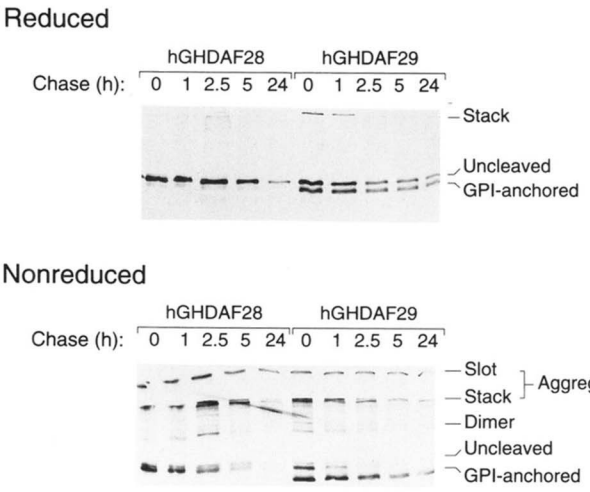


FIG. 7. **Uncleaved hGHDAF is present in disulfide-linked aggregates.** Immunoprecipitates of hGHDAF28 and hGHDAF29 from COS cell lysates were analyzed by SDS-PAGE under reducing conditions (*A*) or nonreducing conditions (*B*) on 15% gels. Cells were pulse-labeled for 1 h and chased for 0, 1, 2.5, 5, and 24 h as indicated. Note that the GPI-linked species of hGHDAF29 (*lower band*) is essentially unaltered by reduction, while the amounts of hGHDAF28 and uncleaved hGHDAF29 (*upper band*) present in monomeric form are significantly increased on reduction.

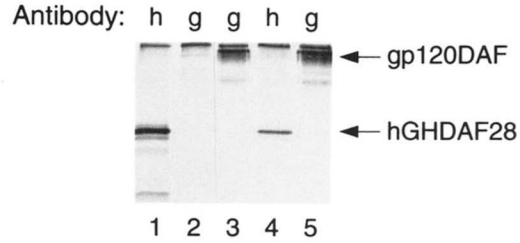


FIG. 8. **hGHDAF28 does not form mixed aggregates with other proteins in the secretory pathway.** COS cells were transiently transfected with hGHDAF28 alone (*lanes 1* and *2*), gp120DAF alone (*lane 3*), or both (*lanes 4* and *5*). The cells were metabolically labeled for 4 h and then analyzed by immunoprecipitation of cell lysates, using either anti-hGH (*h*) or anti-gp120 (*g*) antibodies as indicated. The positions of hGHDAF28 and gp120DAF are indicated at *right* by *arrows*. Co-expression of the two fusion proteins does not result in co-precipitation, indicating that mixed complexes are not formed.

Factors Affecting Retention/Secretion: N-Glycosylation and the Length of the Attached GPI Signal—Previous experiments using continuously labeled cells suggested that the addition of a single *N*-glycan (by DNA manipulation) to hGHDAF28 and hGHDAF29 allowed some molecules to escape the transport block and be secreted (Moran and Caras, 1992). We now added a second *N*-glycan and analyzed the fate of both the singly and doubly glycosylated fusion proteins in a pulse-chase experiment (Table I). Our results show that despite a minor level of secretion, the predominant fate of all of the uncleaved species remained intracellular degradation.

HGHDAF17, which contains only the 17-residue hydrophobic domain of the DAF GPI signal (Fig. 1), and hGHDAFSyn17, containing the hydrophobic domain plus an 8-residue synthetic spacer, are secreted from COS cells (Moran and Caras, 1992). Analysis by pulse-chase showed that these fusion proteins are

TABLE I

Secretion of hGH and hGHDAF fusion proteins

Pulse-chase analyses of the secretion of hGH and various hGHDAF fusion proteins from transiently expressing COS cells. Cells were pulse-labeled for 1 h and chased for the indicated times. hGH was immunoprecipitated from cell lysates or culture medium, fractionated by SDS-PAGE, and quantitated with a Fuji BAS2000 Bio-Imager.

Construct	Time of chase (h)					
	Cells			Medium		
	0	1.5	5.0	0	1.5	5.0
Influence of glycosylation						
hGHDAF28	100[a]	21.9	17.3	0.3	0.6	0.3
hGHDAF28Asn^{63}	100	21.0	20.1	0.3	2.8	6.5
hGHDAF28Asn^{63}-Asn^{98}	100	6.6	15.8	0.5	2.9	9.2
hGHDAF29	100	7.7	28.0	0	0	0
hGHDAF29Asn^{63}	100	45.8	43.2	0	5.3	10.1
hGHDAF29Asn^{63}-Asn^{98}	100	15.0	17.7	0	7.7	12.5
Influence of distance between hGH and hydrophobic domain						
hGH	100	11.3	7.2	0	35.8	87.9
hGHDAF17	100	43.6	26.6	0	50.5	54.8
hGHDAFSyn	100	29.6	8.5	0	58.3	58.8

[a] Numbers shown represent the amount of fusion protein recovered at the indicated time point expressed as a percentage of the amount present at the end of the pulse.

secreted almost as efficiently as hGH itself, with 55–60% of the fusion protein appearing in the medium after the 4-h chase (Table I). In contrast, less than 1% of hGHDAF28 is detected in the medium. Nonreducing SDS-PAGE indicated that, whereas hGH is essentially monomeric in both cell lysates and culture medium, ~20% of hGHDAF17 and hGHDAFSyn17 is present in large disulfide-linked aggregates in cell lysates, while only monomers and dimers are present in the medium (data not shown). These observations suggest that structures larger than dimers are not secreted and point to the size of the aggregate as a major factor determining whether these proteins are retained or secreted.

Ultrastructural Analysis of CHO Cells Expressing hGHDAF28—The distribution of retained hGHDAF28 in a stably transfected, clonal CHO cell line was analyzed by immunoperoxidase staining and transmission electron microscopy. In agreement with our biochemical analysis, the ER was heavily labeled (Fig. 9*A*) while the Golgi stacks were totally devoid of label (Fig. 9, *A* and *C*). Interestingly, hGHDAF28 was unevenly distributed in the lumen of the ER and appeared to be particulate, possibly reflecting the presence of micellar aggregates. To ensure that this unusual morphology is not a staining artifact, we similarly analyzed an amplified CHO cell line secreting DNase. In contrast to the staining of hGHDAF28 in Fig. 9*A*, the ER staining of DNase was uniform and nonparticulate (Fig. 9*B*). hGHDAF28 was also detected in large (1–3 μm) vesicles resembling autophagic vacuoles (Dunn, 1990a, 1990b), some of which appeared to contain partially degraded cellular organelles in addition to hGHDAF28 (Fig. 9, *D*, *E*, and *F*).

DISCUSSION

Retention and Degradation of Proteins Containing an Uncleaved GPI Signal—Based on the persistent endoglycosidase H sensitivity of glycosylated variants of hGHDAF28, we previously suggested that proteins containing an uncleaved GPI signal fail to enter the medial Golgi compartment (Moran and Caras, 1992). To further define the position of the transport block, we analyzed the glycosylation state of DAF in both L cells, which do not add GPI anchors to proteins (Singh *et al.*, 1991), and in COS cells, where overexpression leads to the production of uncleaved as well as GPI-linked molecules. In the absence of GPI addition, DAF completely lacked *O*-glycans (although it contained one *N*-glycan), indicating a failure to enter the *cis*-Golgi stacks where *O*-glycosylation is believed to initiate (Dekker and Strous, 1990). Together with previous immunofluorescence staining (Moran and Caras, 1992) co-localizing hGHDAF28 with p53, a marker of the ER-Golgi intermediate compartment (Schweizer *et al.*, 1988; Hauri and Schweizer; 1992), these observations strongly suggest that an uncleaved GPI signal causes a transport block at the boundary between the Intermediate compartment and the *cis*-Golgi stacks. In agreement with this conclusion, ultrastructural analysis of hGHDAF28 in CHO cells indicated that, despite intense labeling of the ER, the Golgi stacks were totally devoid of hGHDAF. Although there is no direct evidence that the ER and the Intermediate compartment are physically connected, these results are consistent with a notion that traffic between these two compartments is unrestricted; the requirement for vesicular transport to reach the Golgi apparatus makes this boundary a convenient sorting point at which to apply quality control.

Our pharmacological data suggest that the retained hGHDAF28 is degraded in a nonlysosomal compartment by an ER-based mechanism similar to that described by Klausner and co-workers (Lippincott-Schwartz *et al.*, 1988; Bonifacino *et al.*, 1989; see Bonifacino and Lippincott-Schwartz (1991) for review). This mechanism has been shown to operate for a number of multisubunit proteins, *e.g.* the asialoglycoprotein receptor (Amara *et al.*, 1989) and the T-cell receptor (Lippincott-Schwartz *et al.*, 1988), that fail to oligomerize correctly. The hallmark of this form of intracellular degradation is the insensitivity of the process to compounds that inhibit lysosomal function, *e.g.* chloroquine, ammonium chloride, and leupeptin, as was observed for hGHDAF28 in both COS and CHO cells. As ER degradation takes place in a pre-Golgi compartment, the process is unperturbed by brefeldin A, which inhibits ER to Golgi transport. We also found that the rate of degradation was significantly inhibited by decreased temperature, *i.e.* incubation at 16 °C, and by depletion of cellular ATP, both of which perturb ER degradation (Lippincott-Schwartz *et al.*, 1988; Bonifacino *et al.*, 1989; Amara *et al.*, 1989). The requirements for energy and physiological temperatures are both suggestive of a sequestration and/or transport step from the site of polypeptide synthesis to another (degradative) site.

Our morphological data show that in COS cells hGHDAF28 is present in numerous cytoplasmic vesicles that are clearly distinct from the ER. A subset of these also contain cathepsin D, an enzyme found in lysosomes and autophagosomes. Analysis of stably transfected CHO cells by electron microscopy showed the presence of hGHDAF28 in the ER and in densely stained vesicles that lie close to the ER. Some of these vesicles contained complex membrane whorls, similar to the structures reported for intermediate form autophagosomes (Dunn *et al.*, 1990a, 1990b; Noda and Farquhar, 1992). These observations suggest that hGHDAF28 might be degraded by an autophagic pathway. Autophagy generally occurs in metabolically stressed cells and has been described mainly on the basis of ultrastructural studies (Tooze *et al.*, 1990; Dunn, 1990a, 1990b; Seglen and Bohley, 1992). During autophagy, pieces of ER membrane and contents pinch off to form autophagosomes, which gradually acquire lysosomal markers, *e.g.* cathepsins and lamp glycoproteins (Dunn, 1990b; Tooze *et al.*, 1990; Rabouille *et al.*, 1993), possibly by fusing with endosomal vesicles (Tooze *et al.*, 1990). Several discrete stages, intermediate between ER and true lysosomes, have been described (Dunn, 1990b; Noda and Farquhar, 1992).

How can these morphological observations, which point to an autophagic pathway, be reconciled with our pharmacological data suggesting ER degradation? Possible explanations include the following. (i) There are two distinct pathways for disposing of the retained hGHDAF28, an ER degradation pathway and

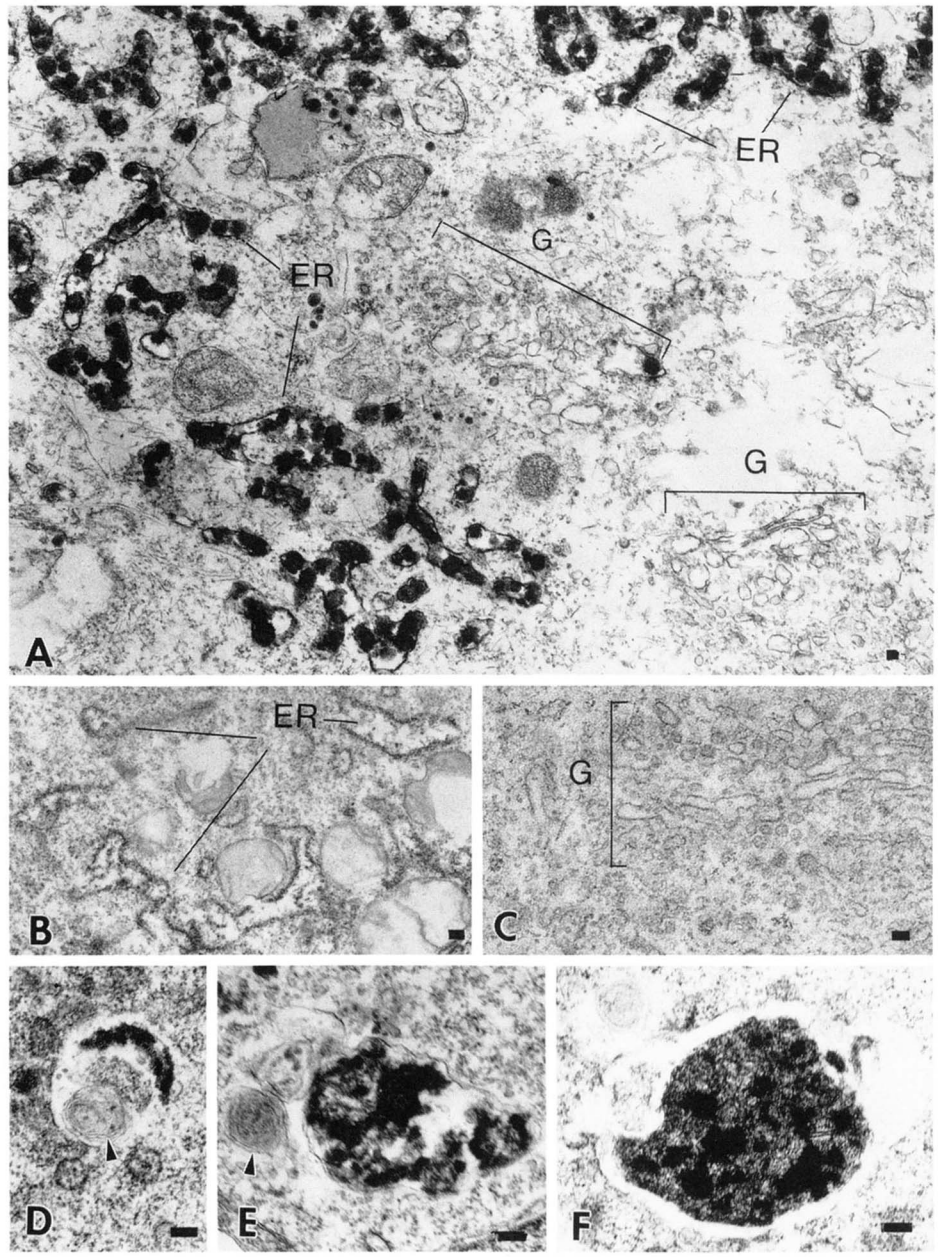


FIG. 9. **Ultrastructural localization of hGHDAF28 in a stable CHO cell line.** *A*, overview, showing intense staining of hGHDAF28 in the endoplasmic reticulum (*ER*) but not in the Golgi apparatus (*G*). Note that the staining is nonuniform and particulate (particle size: ~0.1 μm); *B*, control staining, showing DNase in an amplified stable CHO cell line. Note that the ER staining is uniform and nonparticulate; *C*, higher magnification view of another cell expressing hGHDAF28, showing that the Golgi apparatus is unlabeled; *D*, *E*, and *F*, autophagic vesicles containing hGHDAF28. Note the presence of partially degraded cellular organelle profiles in *D* and *E* (*arrowheads*). *Scale bars*: 0.1 μm.

an autophagic pathway. (ii) ER degradation, at least for some proteins, occurs in an ER-associated vesicular compartment by a process that resembles autophagy, *i.e.* ER degradation might be a specialized form of autophagy. Such a notion is consistent with the requirement for energy and the sensitivity to temperature. It also provides a mechanism for the sequestration of the degradative function of the ER away from synthetic functions. While the process of sequestering the protein into degradative vesicles might resemble autophagy, it is possible that these vesicles are distinct from classical autophagosomes. If the vesicles do indeed mature into late autophagosomes, most of the protein might be degraded before this occurs, resulting in resistance to lysosomal inhibitors. Further, if proteins are rapidly degraded once they enter this compartment, the amount of protein detected in vesicles at any point in time may represent only a small fraction of the total amount that passed into this compartment. Determining the role of autophagy or an autophagic-like process in the disposal of newly synthesized ER proteins clearly requires further investigation.

Sorting of Proteins Destined for Degradation—Intracellular hGHDAF28 was recognized by a soluble hGH receptor and by an anti-hGH monoclonal antibody that binds only native hGH, indicating that a significant fraction of the molecules contain a correctly folded hGH domain despite the complete block in export. These data rule out the possibility that the uncleaved GPI signal causes gross misfolding of the fusion protein, leading to nonspecific aggregation with consequent retention and degradation. Rather, we favor the interpretation that, like other amphipathic molecules, hGHDAF28 has a tendency to form micellar structures, with the GPI signal domains forming a hydrophobic core. Consistent with this, we found considerable levels of disulfide-linked homoaggregates of hGHDAF28, as well as staining of apparently aggregated hGHDAF28 within the ER by electron microscopy. This phenomenon provides a sorting mechanism, allowing the uncleaved hGHDAF28 to be partitioned away from other proteins in the ER. A similar sorting mechanism based on specific aggregate formation has been described by Tooze *et al.* (1989). The intimate association of molecules in the aggregate would allow some disulfide bond shuffling to occur and may account for the formation of covalently linked complexes. It should also be noted that the aggregation does not trap other proteins, as we were unable to

co-precipitate a correctly processed GPI-linked protein, gp120DAF, with hGHDAF28.

It has been suggested that *N*-linked carbohydrate facilitates the secretion of some proteins by increasing the solubility of folding intermediates in the ER, thereby inhibiting aggregation and promoting correct folding of the polypeptide (Machamer *et al.*, 1985). In this case, however, *N*-glycosylation did not lead to efficient secretion of hGHDAF, consistent with our interpretation that the transport block is not caused by general misfolding of the fusion protein.

hGHDAF17 and hGHDAFSyn17 are secreted despite the fact that they both contain a hydrophobic COOH-terminal extension (that based on Triton X-114 binding, appears to be exposed) and would therefore be expected to form micellar structures. Although we saw some aggregation of hGHDAF17 and hGHDAFSyn17 in the cell lysates, only monomers and a small amount of dimers were recovered in the medium. This suggests that the cell is only able to efficiently secrete monomeric hGHDAF, while dimeric hGHDAF is exported less efficiently and larger aggregates are retained. To explain why hGHDAF17 and hGHDAFSyn17 are not retained, we propose that the length of hydrophilic sequence between the hGH domain and the COOH-terminal hydrophobic domain limits the size of the aggregate, *i.e.* if the hydrophobic domain is too close to the globular hGH domain, steric hindrance might become an important factor, keeping the aggregate size small. In hGHDAF17, the hydrophobic domain is attached directly to the globular hGH domain, while hGHDAFSyn17 contains a truncated 8-residue spacer between hGH and the hydrophobic domain, compared to the slightly longer spacer of 11 residues in hGHDAF28. Smaller aggregates may be less stable than larger aggregates, making the probability of dissociation and hence secretion more likely.

We therefore propose that the retention of proteins containing an uncleaved GPI signal is due to the formation of high molecular weight homoaggregates. Such a model can account for the observation that many different GPI-anchored proteins (*e.g.* Thy-1 (Fatemi and Tartakoff, 1988), LFA-3 (Selvaraj *et al.*, 1987), alkaline phosphatase (Micanovic *et al.*, 1990), DAF, and hGHDAF) fail to be exported when processing of the COOH terminus is prevented in some manner, obviating the need to explain how different GPI signals, having different sequences, can cause retention of many different proteins. In agreement with our results, Delahunty *et al.* (1993) recently showed that an uncleaved GPI signal changes the aggregation state of $Q7^b$ and suggested that this might cause the observed retention in the ER. This model is further supported by the observation that deletion or disruption of the hydrophobic domain of several GPI-anchored proteins results in efficient secretion (Caras *et al.*, 1989; also see Cross (1990) for review).

Acknowledgments—We thank Dr. S. Kornfeld for antisera to cathepsin D, Dr. M. Low for PI-phospholipase C, and Dr. David Hanzel (Molecular Devices Inc., Sunnyvale, CA) for analysis of confocal data. M. C. F. thanks R. V. W. for inspiration.

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