Proteins Containing an Uncleaved Signal for Glycophosphatidylinositol Membrane Anchor Attachment are Retained in a Post-ER Compartment

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Abstract. Glycophosphatidylinositol (GPI)-anchored membrane proteins are initially synthesized with a cleavable COOH-terminal extension that signals anchor attachment. Overexpression in COS cells of hGH-DAF fusion proteins containing the GPI signal of decay accelerating factor (DAF) fused to the COOH-terminus of human growth hormone (hGH), produces both GPI-anchored hGH-DAF and uncleaved precursors that retain the GPI signal. Using hGH-DAF fusion proteins containing a mutated, noncleavable GPI signal, we show that uncleaved polypeptides are retained inside the cell and accumulate in a brefeldin A-sensitive, Golgi-like juxtanuclear structure. Retention requires the presence of either a functional or a noncleavable GPI signal; hGH-DAF fusion proteins containing only the COOH-terminal hydrophobic domain (a component of the GPI signal) are secreted.
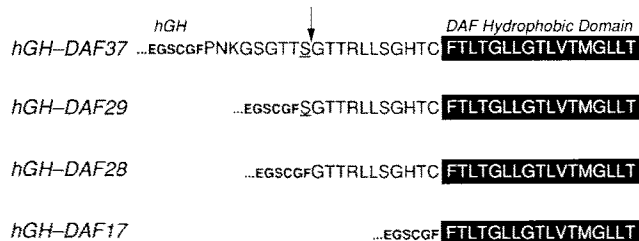
Immunofluorescence analysis shows colocalization of the retained, uncleaved fusion proteins with both a Golgi marker and with p53, a marker of the ER-Golgi intermediate compartment. Since N-linked glycosylation is postulated to facilitate the transport of proteins to the cell surface, we engineered a glycosylation site into hGH-DAF. Glycosylation failed to completely override the transport block, but allowed some uncleaved hGH-DAF to pass through the secretory pathway and acquire endoglycosidase H resistance. The retained molecules remained endoglycosidase H sensitive. We suggest that the uncleaved fusion protein is retained in a sorting compartment between the ER and the medial Golgi complex. We speculate that a mechanism exists to retain proteins containing an uncleaved GPI signal as part of a system for quality control.

A small but diverse class of cell surface proteins is now known to be anchored to the plasma membrane by a glycophosphatidylinositol (GPI)[1] structure covalently attached to the COOH terminus of the protein (Cross, 1990; Low, 1989; Ferguson and Williams, 1988). The GPI membrane anchor contains ethanolamine, carbohydrate, and phosphatidylinositol (Ferguson et al., 1988), and is apparently preassembled in the ER by sequential glycosylation of phosphatidylinositol (Masterson et al., 1989). GPI attachment to protein requires translocation of the protein across the membrane of the RER; proteins that lack a signal peptide fail to become GPI-anchored (Caras, 1991). Attachment is presumed to occur at the luminal side of the ER, following which the GPI-anchored protein is transported to the cell surface via the Golgi apparatus. GPI-anchored proteins are initially synthesized with a cleavable signal at the COOH terminus (Boothroyd et al., 1981; Tse et al., 1985; Caras et al., 1987). GPI-attachment, directed by this signal, occurs in a coordinated processing event in which 17-31 COOH-terminal residues are proteolytically removed, and the GPI anchor is then attached to the new COOH terminus. The processing enzyme(s), possibly a transamidase, has not yet been identified.

We have used the GPI-anchored protein, decay accelerating factor (DAF), as a model system to study the signal for anchor attachment. The last 29 to 37 amino acids of DAF, when fused to the COOH terminus of a normally secreted protein, human growth hormone (hGH), will target the resulting hGH-DAF fusion protein to the plasma membrane by means of a GPI anchor (Caras et al., 1987; Moran et al., 1991). Further analysis of this COOH-terminal DAF sequence indicates that the GPI signal contains two critical features: a COOH-terminal hydrophobic domain (17 residues) and a cleavage/attachment site for anchor addition. The latter is comprised of a pair of small residues, optimally positioned 10 to 12 residues NH_2-terminal to the hydrophobic domain (Caras et al., 1989; Moran and Caras, 1991). The DAF GPI signal is cleaved at a Ser-Gly sequence (Fig. 1) (Moran et al., 1991) releasing a 28-residue COOH-terminal peptide that includes the hydrophobic domain. Small residues that function well at the attachment site include Ser, Gly, Ala, Asp, Asn, and possibly Cys (Moran et al., 1991; Micanovic et al., 1990). Substitution of one or both residues surrounding the cleavage/attachment site with a large or bulky residue, abolishes both cleavage and anchor attach-

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1. *Abbreviations used in this paper:* BFA, brefeldin A; CGN, *cis*-Golgi network; COS, African green monkey CV-1 cells constitutively expressing SV40 T-antigen; DAF, decay accelerating factor; GPI, glycophosphatidylinositol; hGH, human growth hormone; PDI, protein disulfide isomerase; rGH, rat growth hormone.

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                hGH           ↓        DAF Hydrophobic Domain
hGH–DAF37  ...EGSCGFPNKGSGTTSGTTRLLSGHTC FTLTGLLGTLVTMGLLT

hGH–DAF29                   ...EGSCGFSGTTRLLSGHTC FTLTGLLGTLVTMGLLT

hGH–DAF28                      ...EGSCGFGTTRLLSGHTC FTLTGLLGTLVTMGLLT

hGH–DAF17                              ...EGSCGF FTLTGLLGTLVTMGLLT
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Figure 1. Schematic diagram showing the COOH-terminal sequences of the hGH-DAF fusion proteins. The fusion proteins shown contain the COOH-terminal 37-, 29-, 28-, or 17-residues of DAF as indicated (*large type*), fused to the COOH-terminus of hGH (*small type*) (Moran et al., 1991; Caras et al., 1989). The 17-residue COOH-terminal hydrophobic domain, a critical component of the GPI signal (Caras et al., 1989), is indicated by a black box. The cleavage site is denoted by an arrow and the anchor addition site, Ser319 of DAF (Moran et al., 1991), is underlined.

ment (Moran et al., 1991; Moran and Caras, 1991). The uncleaved fusion proteins are not transported to the cell surface, but inside accumulate inside the cell. In addition, when hGH-DAF fusion proteins containing a functional GPI signal are overexpressed in COS cells, processing is incomplete, leading to significant accumulation of both uncleaved as well as GPI-linked forms of the protein (Moran et al., 1991). Whereas GPI-linked hGH is transported to the plasma membrane via the secretory pathway, the uncleaved fusion protein remains trapped inside the cell.

In this report we studied the fate of hGH-DAF fusion proteins bearing an uncleaved GPI signal. We describe experiments designed to ask where in the secretory pathway the transport block occurs. We also studied the effect of introducing a glycosylation site into the normally unglycosylated hGH backbone. The rationale for this was twofold: first, analysis of *N*-linked carbohydrate can determine whether a protein has traversed the Golgi apparatus; and second, because *N*-linked glycosylation has been shown to facilitate the transport of some proteins to the cell surface (Guan et al., 1985; Machamer et al., 1985), we asked whether glycosylation could reverse the retention of hGH-DAF proteins containing an uncleaved GPI signal. We speculate that the uncleaved GPI signal acts as a retention signal that prevents transport to the cell surface, possibly as a form of quality control.

Materials and Methods

Antibodies

Mouse monoclonal antibody G1/93 against the intermediate compartment marker, p53 (Schweizer et al., 1988, 1991), was generously provided by Dr. H.-P. Hauri (University of Basel, Basel, Switzerland); mouse monoclonal antibody, 5D3, against the 72-kD KDEL receptor (Vaux et al., 1990) was from Dr. D. Vaux (European Molecular Biology Laboratory, Heidelberg, Germany); a rabbit antibody against protein disulfide isomerase was provided by R. Freedman (University of Canterbury, UK); the anti-Golgi complex mouse monoclonal antibody, recognizing a 52-kD protein purified from an isolated Golgi fraction from a human breast carcinoma, was from Upstate Biotechnology (Lake Placid, NY; catalogue no. 05-137); Affinity-purified rabbit antibody against hGH was supplied by the Medicinal and Analytical Chemistry Department at Genentech, Inc. (South San Francisco, CA); fluorescent anti-mouse or anti-rabbit IgG was from Cappel Laboratories (Cochranville, PA). Rhodamine-conjugated wheat germ agglutinin was from Molecular Probes, Inc. (Eugene, OR).

Recombinant Plasmids and Fusion Proteins

Plasmids encoding hGH-DAF37, hGH-DAF29 and hGH-DAF28 have been previously described (Moran et al., 1991). To produce glycosylated forms of these fusion proteins, oligonucleotide-directed mutagenesis (McClary et al., 1989) was used to introduce a consensus sequence for *N*-linked glycosylation (Asn-X-Ser/Thr). To produce fusion proteins glycosylated at Asn-63 in the hGH sequence (proteins designated gly63), Glu-65 was mutated to Ser-65; a second mutation introduced a glycosylation site at position 98 in the hGH sequence (proteins designated gly98) by the conversion of Ala-98 to Asn-98. DNA manipulation was then used to transfer these mutations to hGH-DAF37, hGH-DAF29 and hGH-DAF28, to produce a gly63- and a gly98-form of each fusion protein. The recombinant DNAs were cloned into a mammalian expression vector containing a cytomegalovirus enhancer/promoter as previously described (Caras et al., 1989).

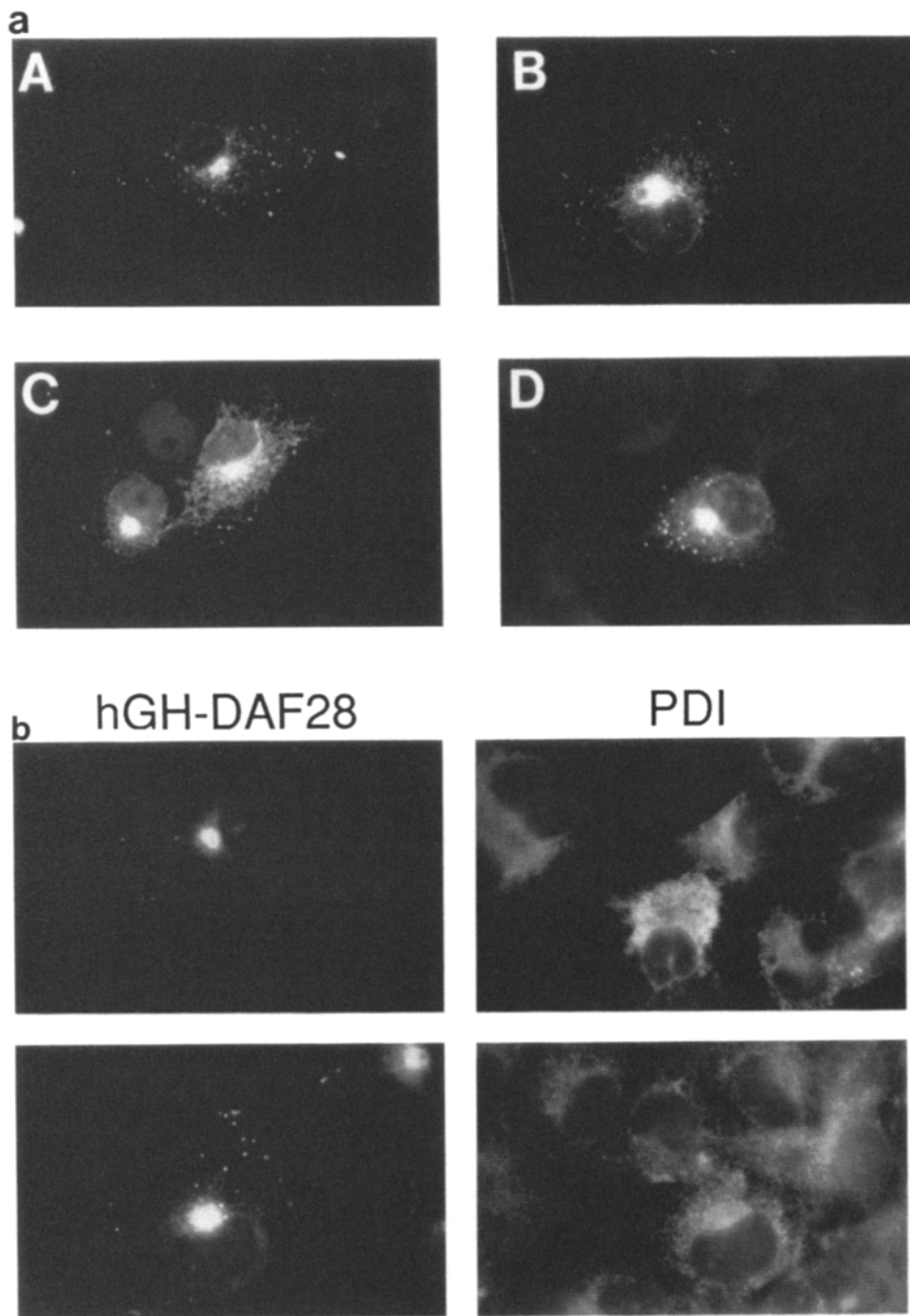## Transfections, Metabolic Labeling, and Immunoprecipitation

COS cells were transfected using the DEAE dextran method as described by Selden (1987) using 2 μg of plasmid DNA per 35-mm dish and DEAE-dextran at 400 μg/ml. Metabolic labeling of cells with [35S]methionine was as previously described (Caras et al., 1989). The cells were harvested by scraping and lysed with 1% NP-40 in the presence of a protease inhibitor (1 mm PMSF). Immunoprecipitations were carried out as described by Anderson and Blobel (1983).

Pulse-Chase Experiments

Transfected cells in 6-well dishes were incubated in methionine-free Dul-
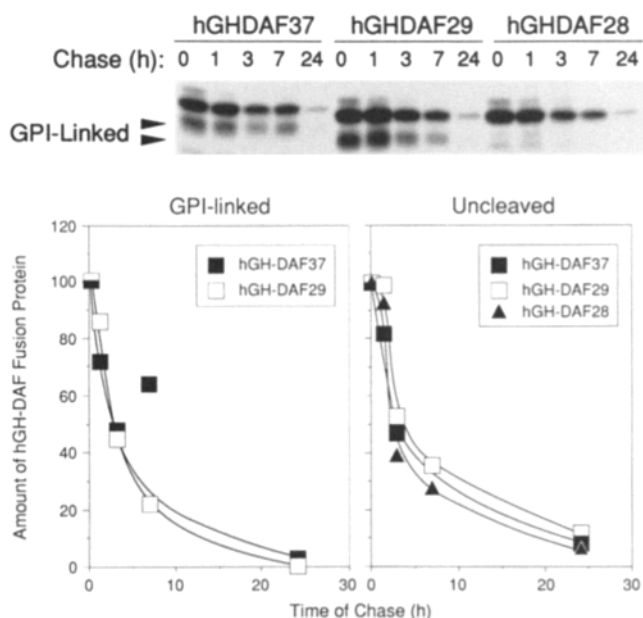
Figure 2. (*a*) Lack of a precursor-product relationship between uncleaved hGH-DAF polypeptides and GPI-linked molecules. Transfected COS cells were pulse labeled with [35S]methionine for 25 min, washed, and then chased from 1 to 24 h as described in Materials and Methods. Cell lysates were immunoprecipitated with a purified goat anti-hGH antibody and subjected to SDS-PAGE on a 15% acrylamide gel. The radioactive bands were visualized by autoradiography. The processed GPI-linked forms of hGH-DAF37 and hGH-DAF29 migrate faster than the corresponding uncleaved fusion proteins (Moran et al., 1991). Additional faint lower molecular weight bands might represent degradation products of the fusion proteins. (*b*) Degradation of GPI-linked and uncleaved hGH-DAF fusion proteins. The radioactive bands shown in *a* were quantitated directly from the dried gel using a Fuji BAS2000 Bio-Imaging analyzer. The amount of each fusion protein remaining during the chase was normalized to the amount present directly after the pulse.

becco's minimal essential medium (DMEM) for 15 min and then pulsed for 20–25 min with [^{35}S]methionine (125 μCi per well; >1,000 Ci/mmol) in methionine-free DMEM. The cells were then washed and chased with complete medium for up to 24 h and analyzed by immunoprecipitation.
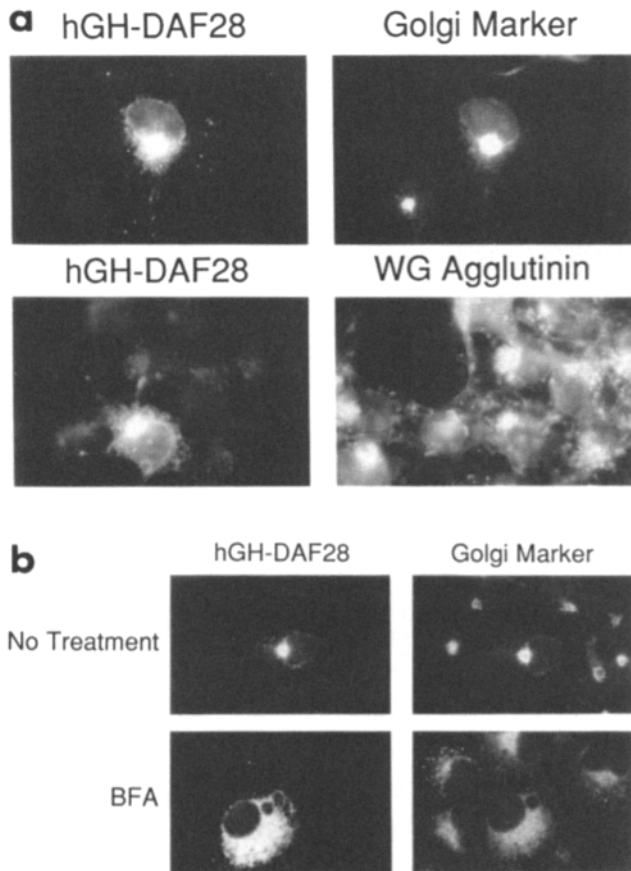
Endoglycosidase H and Neuraminidase Digestions

Immunoprecipitated proteins bound to protein A, were resuspended in 40 μl of 0.05 M sodium phosphate buffer, pH 6, containing either 0.02 units of endo H (Genzyme or Genencor, S. San Francisco, CA) or 0.01 units of neuraminidase (Genzyme Corp., Cambridge, MA) and incubated at 37°C overnight. The digested samples were then washed once with 0.05 M sodium phosphate buffer, pH 6, and subjected to SDS-PAGE. The radioactive bands were visualized by autoradiography at −70°C using an intensifying screen. The radioactive bands were quantitated directly from the dried gel using a Fuji BAS2000 Bio-Imaging analyzer (Fuji, Stamford, CT).

Immunofluorescent Labeling

Immunofluorescent labeling of intact cells (cell surface labeling) or permeabilized cells (internal labeling) was carried out essentially as described (Caras et al., 1989) except that 0.5% Triton X-100/PBS was used to permeabilize the cells. Cells were incubated with a purified rabbit antibody against hGH, followed by fluorescein-conjugated goat anti-rabbit antiserum (Cappel Laboratories).

Results

We have studied the expression in COS cells of chimeric proteins containing a GPI signal from the COOH-terminus of DAF, fused to the COOH-terminus of hGH, a normally

a hGH-DAF28   Golgi Marker

hGH-DAF28   WG Agglutinin

b   hGH-DAF28   Golgi Marker

No Treatment

BFA

Figure 4. (*a*) Double label immunolocalization of hGH-DAF28 and a Golgi marker (*upper*); and hGH-DAF28 and wheat germ agglutinin (*lower*). Transfected COS cells were treated as follows: upper panels, cells were fixed and permeabilized and incubated with rabbit anti-hGH IgG and a mouse monoclonal antibody against the Golgi apparatus, followed by fluorescein-conjugated anti-rabbit and rhodamine-conjugated anti-mouse IgG; (*lower*) cells were fixed and incubated with wheat germ agglutinin (2 mg/ml in PBS) for 60 min at room temperature to saturate cell surface binding sites. The cells were then permeabilized and incubated with rhodamine-conjugated wheat germ agglutinin, followed by rabbit anti-hGH IgG, followed by fluorescein-conjugated anti-rabbit IgG. (*b*) Double label immunolocalization of hGH-DAF28 and a Golgi marker before and after treatment with brefeldin A (BFA). 24 h after transfection, COS cells were treated with 10 µg/ml BFA for 2 h as indicated. Cells were fixed and permeabilized and incubated with rabbit anti-hGH IgG and a mouse monoclonal antibody against the Golgi apparatus, followed by fluorescein-conjugated anti-rabbit and rhodamine-conjugated anti-mouse IgG. The Golgi marker recognizes a 52-kD protein purified from an isolated Golgi fraction from a human breast carcinoma (Upstate Biotechnology). Both punctate and diffuse staining was observed after treatment with BFA, often within the same cell.

secreted protein. As previously reported (Moran et al., 1991), cells expressing the fusion proteins hGH-DAF37 and hGH-DAF29 (Fig. 1), both of which contain a functional GPI-signal, produce two products precipitable with an anti-hGH antibody: a lower molecular weight GPI-linked form that is transported to the cell surface, and a larger uncleaved form that is not GPI-linked, but retains the GPI-signal. To determine whether the uncleaved protein is a newly synthesized precursor to the GPI-linked form, we performed a series of pulse-chase experiments. 24 h after transfection, the

cells were pulsed with [^{35}S]methionine for 25 min and then chased for various times. Cell lysates were analyzed by immunoprecipitation (Fig. 2 *a*). Both the GPI-linked species and the uncleaved protein were present in the cell lysates immediately after the pulse, and there was no evidence of any precursor-product relationship between the two species. Both proteins disappeared from the cells with similar kinetics (Fig. 2 *b*), and the uncleaved protein could be detected in the cell lysates for up to 24 h after the chase, indicating that this species is a terminal product of the cells rather than a newly synthesized intermediate.

We have previously shown that substitutions at the cleavage-attachment site of the GPI-signal abolish both cleavage and anchor attachment (Moran et al., 1991). For example, cells expressing the noncleavable fusion protein hGH-DAF-28 (containing Phe in place of Ser at the cleavage-attachment site of the GPI-signal [Fig. 1]), produce only the larger uncleaved protein product (Fig. 2 *a*), which disappeared from the cells with the same kinetics as the uncleaved forms of hGH-DAF37 and hGH-DAF29 (Fig. 2 *b*). The uncleaved hGH-DAF28 fusion protein was not present on the cell surface (Moran et al., 1991) and only trace amounts could be detected in the cell media, suggesting that the protein does not traverse the secretory pathway, but instead is degraded inside the cells. The half-life was 3 to 4 h for both hGH-DAF28 and the uncleaved forms of hGH-DAF37 and hGH-DAF29, and ∼3 h for the GPI-anchored forms of hGH-DAF-37 and hGH-DAF29.

Immunofluorescence Analysis

Because cells expressing an hGH-DAF fusion protein with a noncleavable GPI-signal produce exclusively the uncleaved product which remains inside the cell, the retained protein can be localized without the complication of co-expression of a GPI-anchored, cell-surface form. Immunofluorescence analysis of such cells suggests that noncleavable hGH-DAF fusion proteins accumulate in a number of distinct intracellular structures (Fig. 3 *a*). In addition to staining of the reticular network of the endoplasmic reticulum, we observed intense staining of a Golgi-like juxtanuclear structure, as well as of vesicular structures scattered throughout the cytoplasm. Double label immunofluorescence microscopy with an ER marker, protein disulfide isomerase, indicated that this staining pattern is distinct from the diffuse reticular staining seen with protein disulfide isomerase (Fig. 3 *b*). These data suggest that the uncleaved fusion proteins are retained in a post-ER compartment. Colocalization of the uncleaved hGH-DAF28 fusion protein with a Golgi marker as well as with wheat germ agglutinin (Fig. 4 *a*) points to a Golgi localization.

Treatment of cells with the drug brefeldin A has been shown to induce rapid redistribution of Golgi proteins into the ER (Lippincott-Schwartz et al., 1989). We therefore tested whether the juxtanuclear accumulation of hGH-DAF-28 is affected by exposure of the cells to brefeldin A. In the presence of brefeldin A both the hGH-DAF28 fusion protein and the Golgi marker redistributed to a diffuse staining pattern (Fig. 4 *b*). Upon removal of the drug, both markers resumed their original juxtanuclear position (not shown). We concluded that the noncleavable hGH-DAF28 fusion protein accumulates in a brefeldin A-sensitive intracellular compartment, possibly the Golgi apparatus.
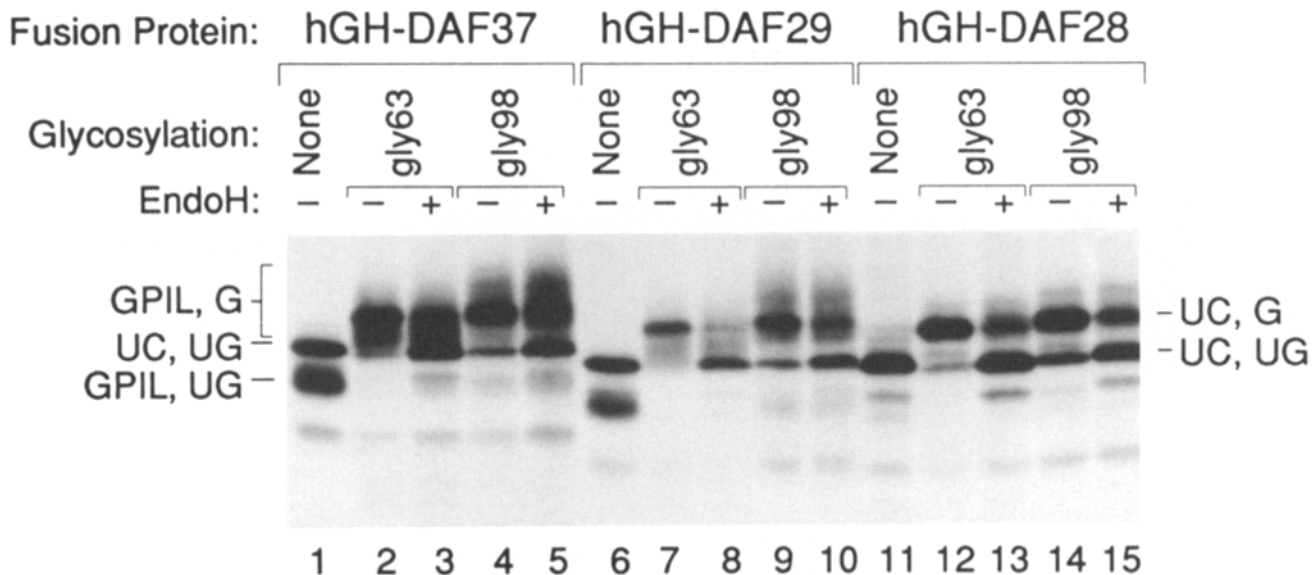
Figure 5. N-glycosylation and acquisition of Endo H resistance, of hGH-DAF fusion proteins containing a consensus sequence for N-glycosylation. COS cells were transfected with DNAs encoding hGH-DAF fusion proteins as indicated, containing a potential glycosylation site at position 63 (gly63) or 98 (gly98) as indicated. 24 h after transfection the cells were labeled with [^{35}S]methionine (166 μCi per 35 mm well) for 6 h. Immunoprecipitates of the cell lysates were divided in two equal portions and incubated overnight in the presence (+) or absence (−) of endo H as described in Materials and Methods, before being subjected to SDS-PAGE. The unglycosylated form of each fusion protein is included for reference (lanes 1, 6, and 11). Band assignments are as follows: GPIL, GPI-linked; UC, uncleaved; G, glycosylated; UG, unglycosylated. The positions of the glycosylated forms of uncleaved hGH-DAF37 and hGH-DAF29 (UC, G) can be determined by reference to the glycosylated form of hGH-DAF28. The glycosylated GPI-linked species (GPIL, G) are presumably represented by the broad smeared bands visible in lanes 2 to 5 and 7 to 10.

Analysis of Glycosylated Forms of GPI-Linked and Uncleaved hGH-DAF Fusion Proteins

Localization of a protein within the Golgi complex can in some instances be confirmed by biochemical analysis of the N-linked carbohydrate. Conversion from endoglycosidase H (endo H) sensitivity to resistance occurs in the medial Golgi complex (Kornfeld and Kornfeld, 1985) as a result of trimming of the high-mannose type oligosaccharides added in the ER, followed by the addition of other sugars. Because hGH is normally unglycosylated, we used site directed mutagenesis to introduce a novel glycosylation site, either at position 63 or 98 in the amino acid sequence. In both cases, a single amino acid was mutated to produce a consensus sequence (Asn-X-Ser/Thr) for N-linked glycosylation, and DNA manipulation was then used to create potentially glycosylated forms of the fusion proteins, hGH-DAF37, hGH-DAF29 and hGH-DAF28.

To determine that these mutations indeed resulted in glycosylated products, transiently transfected COS cells were labeled with [^{35}S]methionine, and the cell lysates were then analyzed by immunoprecipitation. After the introduc-

Table I. Endo H Resistance of Glycosylated hGH-DAF28, Determined by Quantitation of the Bands in Fig. 5

	hGH-DAF28/Gly63		hGH-DAF28/Gly98	
	−Endo H	+Endo H	−Endo H	+Endo H
		%		
Glycosylated	87	16	70	25
Unglycosylated	13	84	29	75

tion of a glycosylation site at position 63 or 98, both the GPI-linked (broad lower band) and the uncleaved forms (upper band) of hGH-DAF37 and hGH-DAF29 migrated more slowly relative to the unglycosylated species (Fig. 5, compare lane 1 with lanes 2 and 4; and lane 6 with lanes 7 and 9). Although the two unglycosylated species (GPI-linked versus uncleaved) were well separated on a 15% polyacrylamide gel, the corresponding glycosylated forms appeared to overlap, indicating a larger mobility shift upon glycosylation for the GPI-linked species. This observation suggests that the GPI-linked and uncleaved species may undergo differential processing of the N-linked carbohydrate. The noncleavable hGH-DAF28 protein showed a similar shift in electrophoretic mobility (compare lane 11 with lanes 12 and 14), indicating the addition of carbohydrate.
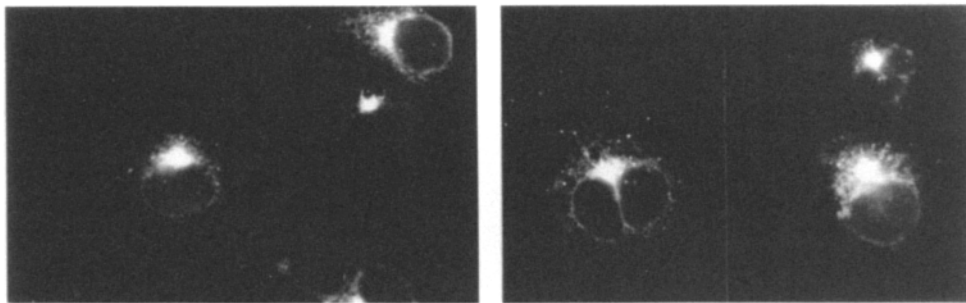
To determine whether any or all of these glycosylated proteins had entered the medial Golgi apparatus, we analyzed their sensitivity to cleavage by endo H. The uncleaved forms of hGH-DAF37 and hGH-DAF29 were largely endo H-sensitive; a prominent band that comigrates with the unglycosylated species reappeared after exposure to endo H (Fig. 5, lanes 3, 5, 8, and 10). In contrast, the GPI-linked forms of hGH-DAF37 and hGH-DAF29 appeared to be largely endo H-resistant; only trace amounts of a species corresponding to the unglycosylated form reappeared after treatment with endo H. The noncleavable fusion protein, hGH-DAF28, remained largely sensitive to endo H. Quantitation of the bands (possible for hGH-DAF28 but not hGH-DAF37 and hGH-DAF29 due to overlap of the GPI-linked and unprocessed species) indicated that only 16–25% of the molecule were endo H-resistant (Table I). These data suggest that whereas the GPI-linked molecules are transported through

hGH-DAF28/gly63   hGH-DAF28/gly98

Figure 6. Immunolocalization of glycosylated hGH-DAF28. Transfected COS cells were fixed, permeabilized and stained with an anti-hGH antibody as described in Materials and Methods.

the medial Golgi apparatus on their way to the cell surface, acquiring endo H-resistance, only a small fraction of the uncleaved molecules reach the medial cisternae, where modification of high mannose oligosaccharides is believed to take place. We conclude from these results that the bulk of the uncleaved fusion protein accumulates in a compartment proximal to the medial Golgi compartment. Staining of cells expressing glycosylated hGH-DAF28 (Fig. 6) revealed a pattern indistinguishable from that seen with the unglycosylated fusion protein (Fig. 3). Both species accumulate in a juxtanuclear organelle, suggesting a post-ER localization.

The Presence of an Uncleaved GPI-Signal Blocks Secretion

Since it has been proposed that N-linked glycosylation may facilitate transport of some proteins to the cell surface (Guan et al., 1985; Machamer et al., 1985), we also examined the ability of the glycosylated versus unglycosylated hGH-DAF fusion proteins to be secreted relative to authentic hGH. Transfected COS cells were labeled with [^{35}S]methionine

for 6 h, and the cell lysates and culture media were then analyzed by immunoprecipitation. During the 6 h labeling period, 66.5% of the authentic hGH synthesized was secreted into the medium (Fig. 7 and Table II). Fusion of the 17-residue COOH-terminal hydrophobic domain of DAF to the COOH-terminus of hGH (as in hGH-DAF17, Fig. 1), slowed but did not prevent secretion; 26.5% of the fusion protein appeared in the medium. In contrast, the uncleaved form of hGH-DAF29 and the noncleavable protein hGH-DAF28, were almost totally retained inside the cells. As expected, the GPI-anchored form of hGH-DAF29, which is known to be on the cell surface (Moran et al., 1991), remained cell associated. These results suggest that whereas the addition of a hydrophobic domain alone slows but does not block secretion of hGH, the presence of an uncleaved GPI signal provides a strong signal for retention. The addition of N-linked carbohydrate resulted in secretion of a fraction (15–22%) of the hGH-DAF28 molecules synthesized, but failed to completely override the block to secretion. Glycosylation appeared not to facilitate the secretion of unprocessed hGH-DAF29 molecules.

To further analyze the fate of hGH-DAF28 molecules and more precisely pinpoint the block in the secretory pathway, we carried out a pulse-chase experiment in which transfected COS cells were pulsed with [^{35}S]methionine for 20 min and chased for 7 h. The cell lysates and culture media were then analyzed by immunoprecipitation following treatment with or without endo-H or neuraminidase (an enzyme that removes sialic acid residues added in the *trans*-Golgi cisternae [Kornfeld and Kornfeld, 1985]). This protocol favored the
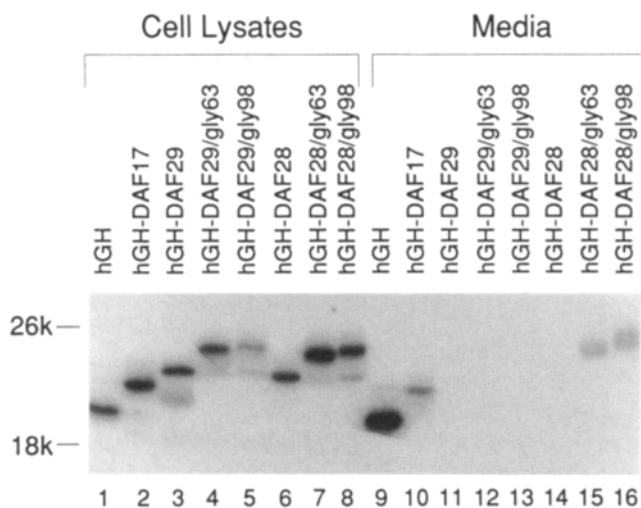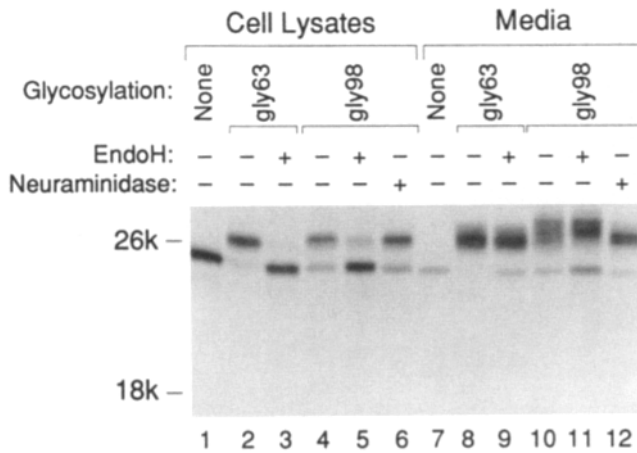

Figure 7. Secretion of hGH and hGH-DAF fusion proteins. COS cells were transfected with DNAs encoding hGH or hGH-DAF fusion proteins as indicated. After 24 h, the cells were labeled with [^{35}S]methionine (166 μCi per 35 mm well) for 7 h and the cell lysates and culture media were analyzed by immunoprecipitation and SDS-PAGE. (The relative amounts of GPI-linked versus uncleaved hGH-DAF29 varied in different experiments [compare Figs. 2 *a*, 5, and 7]. The reason for this is unclear).

Table II. Relative Distribution of hGH-DAF Fusion Proteins in Cell Lysates and Culture Medium During a 6 h Labeling Period, Determined by Quantitation of the Bands in Fig. 7

Protein	Cells	Medium
	(%)	(%)
Authentic hGH	33.5	66.5
hGH-DAF17	73.5	26.5
*hGH-DAF29	100.0	0
*hGH-DAF29/Gly63	99.7	0.3
*hGH-DAF29/Gly98	99.5	0.5
hGH-DAF28	99.7	0.3
hGH-DAF28/Gly63	84.3	18.7
hGH-DAF28/Gly98	78.0	22.0

* GPI-linked protein plus uncleaved polypeptide.

Cell Lysates | **Media**

Glycosylation: None gly63 gly98 | None gly63 gly98

EndoH: − − + − + − − − + − + −
Neuraminidase: − − − − − + − − − − − +

26k −

18k −

1 2 3 4 5 6 7 8 9 10 11 12

Figure 8. Endo H- and neuraminidase-resistance of secreted and retained fractions of glycosylated hGH-DAF28. COS cells expressing either glycosylated or unglycosylated hGH-DAF28 (shown for reference) as indicated, were pulsed with [^{35}S]methionine for 20 min and then chased for 7 h. Immunoprecipitates of the cell lysates and culture media were divided in equal portions and incubated overnight in the presence (+) or absence (−) of endo H or neuraminidase, and then subjected to SDS-PAGE. We previously determined that the gly98, but not gly63, forms of hGH or hGHDAF acquired sialic acid and became neuraminidase-resistant. Neuraminidase digestions are therefore included for the gly98 species only.

detection of molecules that escaped the block to secretion and appeared in the medium, allowing us to compare this secreted fraction with the fraction that was retained inside the cell. The secreted hGH-DAF28 protein was endo H-resistant and neuraminidase-sensitive (at position 98), indicating passage through the Golgi Apparatus (Fig. 8). The protein that remained inside the cell was endo H-sensitive and neuraminidase-resistant, indicating that these molecules had failed to reach the medial and *trans*-Golgi cisternae where terminal modification of the carbohydrate takes place. This result suggests that the rate-limiting step in the secretion of the hGH-DAF28 fusion protein is passage into the medial Golgi complex.

Colocalization of the hGH-DAF28 Fusion Protein with a Marker of the ER-Golgi Intermediate Compartment

Newly synthesized proteins upon exiting the ER, are believed to pass through a recently identified intermediate compartment on their way to the Golgi apparatus (Saraste and Kuismanen, 1984; Lodish et al., 1987; Schweizer et al., 1988). This ER-Golgi intermediate compartment is defined by p53, a 53-kD nonglycosylated transmembrane protein present in a tubulo-vesicular membrane system close to the cis-side of the Golgi apparatus (Schweizer et al., 1988, 1991), and possibly includes the *cis*-most stakes of the Golgi Apparatus (Mellman and Simons, 1992). It has recently been suggested that this compartment, which functions in receiving and sorting proteins delivered from the ER, be known as the *cis*-Golgi network (CGN) (Hsu et al., 1991; Mellman and Simons, 1992). To determine whether this might be the structure in which the hGH-DAF28 molecules accumulate, we analyzed transfected cells by double label immunofluorescence, using an anti-p53 monoclonal antibody together with rabbit anti-hGH IgG. Colocalization of

both markers to the same juxtanuclear structure (Fig. 9), together with the observation that the retained molecules remain endo H-sensitive, is consistent with a conclusion that the uncleaved hGH-DAF28 fusion protein is retained in the CGN.

Because the salvage of resident ER proteins by the KDEL receptor (Munro and Pelham, 1987) is thought to occur in the CGN (Pelham, 1988; Warren, 1987; Mellman and Simons, 1992), we asked whether the 72-kD glycoprotein, one of two proteins postulated to be the KDEL receptor (Vaux et al., 1990), colocalizes with hGH-DAF28 in the same juxtanuclear structure. Double label immunofluorescence indicated that this is not the case (Fig. 9). An antibody to the 72-kD KDEL receptor labeled a series of vesicles that occasionally showed partial overlap with vesicles containing hGH-DAF28. However, the juxtanuclear structure that was intensely stained with anti-hGH IgG, was not labeled, suggesting that for the most part the two proteins are contained in different compartments or subcompartments.

Discussion

The Process of GPI Attachment

Our observations indicate that the processing of hGH-DAF fusion proteins bearing a GPI signal is incomplete after overexpression in COS cells (Moran et al., 1991). A similar observation has been made using alkaline phosphatase (Micanovic et al., 1990). This suggests that the machinery for anchor attachment, or the availability of the preassembled anchor, is limiting. Our pulse-chase analysis indicates that hGH-DAF polypeptides that initially escape COOH-terminal processing and anchor attachment, never become GPI-anchored proteins. As a possible explanation we suggest that GPI attachment occurs in the membrane during translocation, and that uncleaved polypeptides become separated from the processing machinery after extrusion into the lumen of the ER. It is tempting to speculate that the machinery for GPI attachment might be part of a translocation complex that includes signal peptidase, oligosaccharyltransferase, glucosidases, and possibly proteins involved in folding. Assuming that the machinery for GPI attachment is limiting such that not all translocation sites are capable of GPI attachment, the function of the hydrophobic component of the GPI signal might be to briefly retard exit from the membrane, thereby increasing the chance of an encounter with the processing activity. Our observation that the COOH-terminal hydrophobic domain of DAF slows secretion of hGH is consistent with this hypothesis.

An Uncleaved GPI Signal Causes Retention

hGH-DAF fusion proteins containing substitutions at the cleavage site that prevent processing of the GPI signal, fail to be transported to the cell surface as judged by an absence of fusion protein in the culture medium or on the plasma membrane. Similarly, incompletely processed hGH-DAF37 or hGH-DAF29 polypeptides, bearing a functional but uncleaved GPI signal, are not transported to the cell surface. Intracellular accumulation of these uncleaved fusion proteins suggests the presence of a retention signal that prevents passage through the secretory pathway. These observations

hGH-DAF28   p53
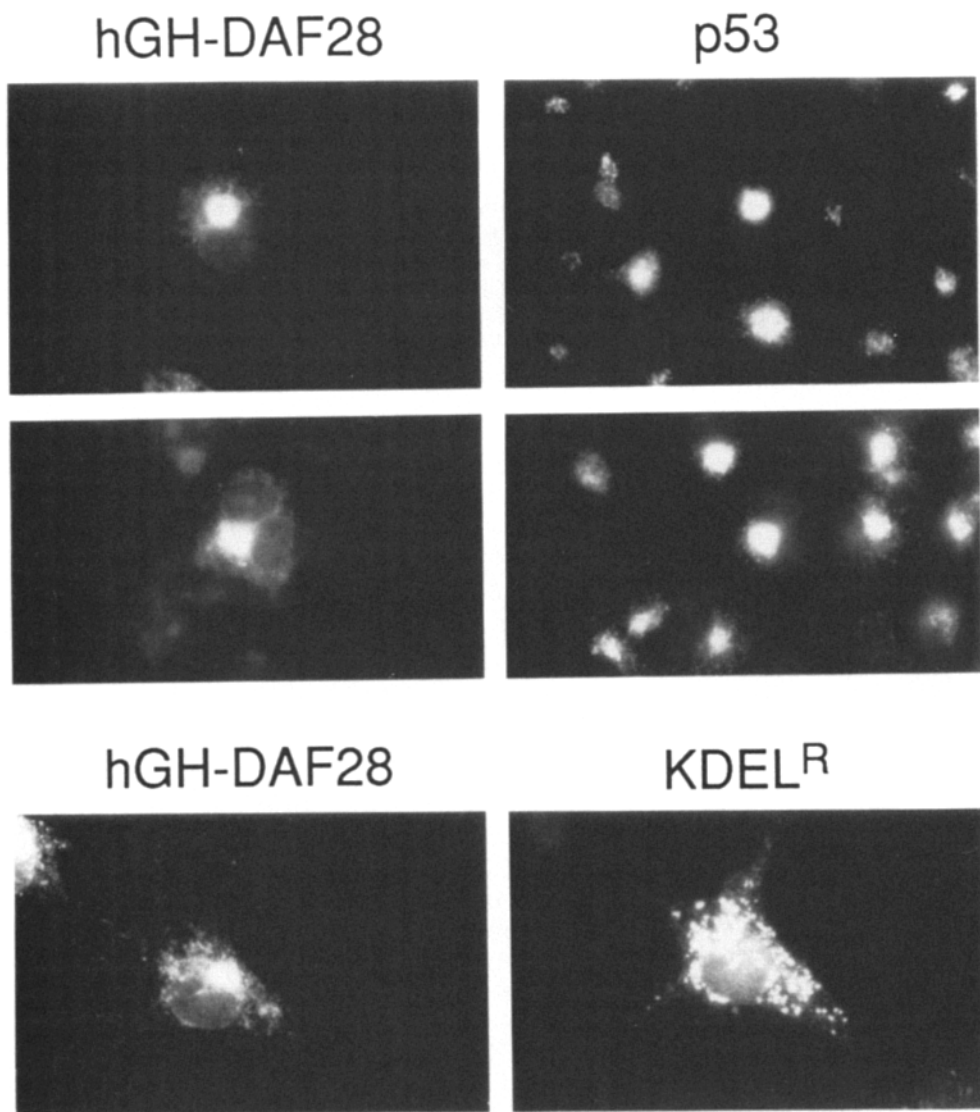
hGH-DAF28   KDEL^R

Figure 9. Double label immuno-localization of hGH-DAF28 with either p53 or the 72-kD KDEL receptor. Transfected COS cells grown on coverslips were fixed and permeabilized and treated with rabbit anti-hGH IgG and a mouse monoclonal antibody against either p53 or the 72-kD KDEL receptor, followed by fluorescein-conjugated anti-mouse and rhodamine-conjugated anti-rabbit IgG.

are consistent with at least two possibilities: (*a*) the un-cleaved COOH-terminal extension induces misfolding and/or aggregation of the attached protein, resulting in retention; or (*b*) there is a retention signal associated with the uncleaved COOH-terminal extension itself. Although we cannot at present distinguish between these two models, our results suggest that the structural requirements of a COOH-terminal extension causing retention, are somewhat similar to the re-quirements for GPI attachment itself. The last 29 amino acids of DAF constitute the minimal DAF sequence that will signal anchor attachment (Moran et al., 1991). The 17-residue COOH-terminal hydrophobic domain alone neither triggers anchor attachment (Caras et al., 1989) nor causes retention; hGH-DAF17 (containing only the 17-residue hy-drophobic domain of DAF fused to the COOH terminus of hGH) is secreted, albeit more slowly than authentic hGH. A COOH-terminal extension comprised of the COOH-terminal hydrophobic domain combined with 10 or more of the adjacent DAF residues, containing either a partial or complete cleavage/attachment site, results in retention; un-cleaved hGH-DAF27 (Caras et al., 1989), hGH-DAF28, and hGH-DAF29 are all retained. In contrast, a fusion protein containing the COOH-terminal hydrophobic domain of DAF

plus an irrelevant 8-residue hydrophilic spacer, fused to the COOH-terminus of hGH, fails to become GPI-anchored and is secreted (hGH-Syn17, Caras et al., 1989). Consistent with our observations, mutations that block cleavage of the alka-line phosphatase GPI signal similarly block transport to the cell surface (Micanovic et al., 1990), indicating that this phenomenon is not specific to the DAF GPI-signal.

N-Linked Glycosylation and Secretion

In experiments using rat growth hormone (rGH), Guan and Rose (1984) showed that conversion of rGH to a membrane protein by the addition of the transmembrane-cytoplasmic domains of the vesicular stomatitis virus glycoprotein, prevented transport to the cell surface. The introduction of *N*-linked glycosylation reversed the secretion block and al-lowed membrane-attached rGH to reach the plasma mem-brane (Guan et al., 1985). The authors suggested that a sort-ing signal required for the secretion of rGH was not functional when the molecule was bound to the membrane, and that *N*-linked glycosylation provided the missing signal for transport to the cell surface. Our data using hGH indicate that GPI-anchored hGH is efficiently transported to the cell surface (Caras et al., 1989; Moran et al., 1991), suggesting

either that attachment to the membrane per se does not prevent secretion of hGH, or that the GPI-signal facilitates transport to the plasma membrane. The latter possibility is not unlikely in view of recent evidence that whereas truncated T-cell receptor chains fail to be secreted, GPI-linked chains are efficiently transported to the cell surface (Lin et al., 1990). Our present results indicate that the addition of N-linked glycosylation partially alleviates the intracellular retention of uncleaved hGH-DAF28, but not hGH-DAF29, allowing a fraction of the unprocessed fusion protein to be secreted. If retention is due to misfolding and/or aggregation, induced by the uncleaved COOH-terminal extension, we would have to assume that randomly introduced carbohydrate facilitates correct folding, or prevents aggregation, of hGH-DAF28 but not hGH-DAF29 (these proteins differ only by the presence or absence of a single Ser residue). Alternatively, if the uncleaved COOH-terminal extension is recognized directly as a retention signal, N-linked glycosylation might in some way provide an opposing signal, resulting in partition. The presence of a complete GPI signal with a functional cleavage/attachment site (as in hGH-DAF29) might provide a stronger retention signal than a defective GPI signal with a nonfunctional cleavage site (hGH-DAF28), resulting in the failure of glycosylation to override the secretion block in the former case.

Intracellular Localization of the Retained hGH-DAF Fusion Proteins

Immunofluorescent staining suggested that the uncleaved hGH-DAF fusion proteins are transported out of the ER and accumulate in a BFA sensitive structure that colocalizes with both a Golgi marker and with p53, a marker of the ER-Golgi intermediate compartment. The retained molecules remained endo H-sensitive whereas molecules that escaped the transport block acquired resistance. This suggests that the retained molecules do not reach the medial Golgi complex. Taken together, these observations argue that the uncleaved fusion protein accumulates in a compartment intermediate between the ER and the medial Golgi complex. Using the three compartment model of the Golgi complex proposed by Hsu et al. (1991), Pelham (1991) and Mellman and Simons (1992) we propose that the uncleaved fusion proteins accumulate in the CGN, which is comprised of the cis-most cisternae of the Golgi Apparatus and the adjacent tubulovesicular structures known as the intermediate compartment (Mellman and Simons, 1992; Schweizer et al., 1988; Lewis et al., 1990). The CGN is postulated to be a salvage compartment in which proteins are selected for return to the ER (Warren, 1987; Pelham, 1991; Mellman and Simons, 1992). There is evidence suggesting that unassembled major histocompatibility complex class I molecules that fail to be transported to the cell surface, recycle between the ER and the CGN (Hsu et al., 1991). In view of the postulated role of this compartment as a sorting compartment, we speculate that incompletely processed precursors to GPI-linked proteins progress out of the ER and enter the CGN, where further transit into the medial Golgi complex is prevented by a quality control mechanism. As stated above, the uncleaved COOH-terminal extension might function as a retention signal by driving aggregation or misfolding of the attached protein. (Quality control mechanisms preventing the secretion of incompletely assembled multisubunit proteins or misfolded

proteins have been reviewed by Hurtley and Helenius, 1989; Klausner, 1989; Rose and Doms, 1988). Alternatively, it is possible that the uncleaved GPI-signal is recognized directly as a retention signal, in which case an additional editing function of the CGN might be the sorting and retention of improperly processed precursors to GPI-linked proteins.

The KDEL receptor is believed to function in the retrieval of ER proteins from a post-ER salvage compartment, presumably the CGN (Pelham, 1988, 1991; Mellman and Simons, 1992). Two different KDEL receptors have been described (Vaux et al., 1990; Lewis and Pelham, 1990). In transfected COS cells, the 26-kD KDEL receptor (Lewis and Pelham, 1990) was localized to a Golgi-like structure similar to that containing hGH-DAF28. However, our data indicate that in COS cells, the 72-kD KDEL receptor (Vaux et al., 1990) is localized in a vesicular compartment distinct from the juxtanuclear structure containing uncleaved hGH-DAF-28. These observations may reflect the heterogeneous or dynamic nature of the salvage compartment.

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