Retardation of a Surface Protein Chimera at the Cis Golgi

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ABSTRACT: Dipeptidyl peptidase IV (D4) and the α subunit of human chorionic gonadotrophin (αhcg) are plasma membrane and secretory proteins, respectively. In the course of studies to understand mechanisms involved in transport along the exocytotic pathway, the ectoplasmic domain of D4 was replaced by the mature polypeptide of αhcg, resulting in the membrane anchored chimera, D4αhcg. Surprisingly, when transfected into Chinese hamster ovary (CHO) and Madin−Darby canine kidney (MDCK) cells, strong perinuclear Golgi staining was predominant, in addition to the expected surface staining. By following the biogenesis and transport of the molecule, it was established that newly synthesized D4αhdg is eventually transported to the cell surface but only after a significant retardation in the Golgi apparatus. The compartment of retardation was identified as the early or cis Golgi, before the medial Golgi, where resistance to endoglycosidase (endo) H is conferred. As a result of the transport retardation of the chimera, we were able to document the appearance of an endo D sensitive intermediate, which is usually too transient to be apparent in normal cells. The retardation of this chimera in the cis Golgi complements our previous report in which the D4 molecule with its transmembrane domain replaced by that of aminopeptidase N resulted in retardation in the trans Golgi/trans Golgi network in MDCK and CHO cells [Low, S. H., Tang, B. L., Wong, S. H., & Hong, W. (1994) *J. Biol. Chem. 269*, 1985−1994). Together, these reports indicate that transport along the exocytic pathway may not be simply by default but requires some sort of signal, the disruption of which results in inefficient intra−Golgi and/or Golgi to surface transport.

Proteins destined for the plasma membrane or secretion are transported to the cell surface via the exocytotic pathway, which consists of several physically distinct, membrane-bounded organelles. Newly synthesized proteins are cotranslationally translocated into the endoplasmic reticulum (ER),[1] transported by vesicles to the Golgi, where they traverse the various subcompartments of the Golgi, and are finally sorted into appropriate transport vesicles bound for the surface of the cell (Mellman & Simons 1992; Rothman & Orci, 1992). Each compartment contains its own unique set of proteins (including carbohydrate-modifying enzymes), and the progress of a protein as it is transported through the exocytotic pathway can be monitored by the extent of its carbohydrate modification (Brandli, 1991).

The Golgi apparatus has been divided into three subcompartments by virtue of the immunocytochemical and biochemical localization of enzymes involved in N-linked glycan modification. The mannosidase (Man) I enzyme has been localized to the cis Golgi, as it has been shown to act on proteins that have just exited the ER (Balch *et al.*, 1986), giving rise to the endoglycosidase (endo) D-sensitive moiety $Man_5GlcNAc_2$. Under normal circumstances, the transient nature of this molecule renders it extremely difficult to detect in wild-type cells (Kornfeld & Kornfeld, 1985). However, in the mutant CHO cell line CHO 15B (Gottlieb *et al.*, 1975), which is deficient in *N*-acetylglucosaminyltransferase (NT) I, the endo D-sensitive intermediate is stable as a result of the missing enzyme, which is crucial for the subsequent processing of the oligosaccharide. The second or medial Golgi subcompartment contains NT I and Man II, which together are responsible for producing the endo H-resistant $GlcNAcMan_3GlcNAc_2$ intermediate (Alberts *et al.*, 1989). The final stages of carbohydrate modification involve the addition of galactose and sialic acid residues carried out by galactosyltransferase (GT) and sialyltransferase (ST), respectively, localized to the trans face of the Golgi [for review see Kornfeld and Kornfeld (1985)]. Since transport from the ER to the medial Golgi is usually very rapid, no endo D-sensitive intermediate has ever been detected in normal cells. Thus, it is possible that the cis and medial Golgi may not be physically separated and represent the same compartment (Mellman & Simons, 1992).

Proteins are believed to be transported along the exocytotic pathway by default unless they possess retention or retrieval signals, which keeps them localized intracellularly despite the heavy, surface-destined protein traffic [for review, see Pfeffer and Rothman (1987) and Rothman (1987)]. Such signals have been identified for lumenal (Munro & Pelham, 1987) and transmembrane (Jackson *et al.*, 1990) ER resident proteins [for review see Hong and Tang (1993)]. Our laboratory and others have also shown that the transmembrane domains of NT I (Tang *et al.*, 1992b), GT (Nilsson *et*

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[1] Abbreviations: MDCK, Madin−Darby canine kidney; CHO, Chinese hamster ovary; D4, dipeptidylpeptidase IV; αhcg, α subunit of the human chorionic gonadotrophin; ER, endoplasmic reticulum; Ig, immunoglobulin; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TGN, trans-Golgi network; Man, mannosidase; ST, sialyltransferase; GT, β-1,4-galactosyltransferase; NT, *N*-acetylglucosaminyltransferase; endo, endoglycosidase; FITC, fluorescein isothiocyanate; BFA, brefeldin A; DME, Dulbecco's modified Eagle's medium, ARF, ADP-ribosylation factor.

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al., 1991) and α-2,6-ST (Munro, 1991; Wong *et al.*, 1992) play a major role in Golgi retention. While the mechanism of retention of these Golgi glycosyltransferases is not known, a recent report by Weisz *et al.* (1993) showed that the retention of a protein chimera, Gm1, in the Golgi was due to oligomerization or aggregation. In this construct, the transmembrane domain of the vesicular stomatitis virus G (VSV-G) protein was replaced by the first membrane-spanning domain of the M glycoprotein of avian coronavirus. Oligomerization was mediated by the membrane-spanning domain and coincided with the arrival of newly synthesized proteins at the Golgi complex.

Human chorionic gonadotropin (hcg) is a glycoprotein hormone which exists as a heterodimer made up of a noncovalently linked α subunit and a β subunit (Pierce & Parsons, 1981). The αhcg subunit is a secretory protein that contains two sites for N-linked oligosaccharide addition (Endo *et al.*, 1979) and can be secreted as a free subunit (Ruddon *et al.*, 1981). Dipeptidylpeptidase IV (D4) is a plasma membrane protein of type II orientation, with a 6-residue cytoplasmic domain, a 22 amino acid transmembrane domain, and a large catalytic ectodomain (Hong & Doyle, 1990). With the intention of examining the role played by various domains of D4 in its intracellular transport, we constructed several chimeras, in which various domains of D4 were replaced by other molecules. In this study, we have attached the cytoplasmic and transmembrane domains of D4 to the N-terminus of the αhcg molecule, generating the chimera D4αhcg. When transfected into CHO and MDCK cells, this chimera was retarded in its transport to the surface at the cis Golgi compartment. As a result of the retardation we were able to detect the customarily short-lived endo D-sensitive intermediate. In a previous paper (Low *et al.*, 1994), we showed that the transmembrane replacement of D4 with that of aminopeptidase N resulted in the retardation of the chimera in the trans Golgi/TGN of MDCK and CHO cells. In concert, studies on these two chimeras further enhance the veiw that traffic along the exocytotic pathway may be dependent on some sort of transport signal which, if disrupted or altered, could result in a retardation of transport.

MATERIALS AND METHODS

Materials. DNA modification and restriction enzymes were purchased from Gibco−Bethesda Research Labs (Gaithersburg, MD) or Amersham Corp. (Arlington Heights, IL). Cell culture media, fetal bovine serum (FBS), and dialyzed FBS and geneticin (G418) were ..om Gibco Labs (Grand Island, NY). Monoclonal antibodies against αhcg were obtained from Calbiochem-Behring Corp. (San Diego, CA). Brefeldin A (BFA) was from Epicentre Technologies (Madison, WI). [^{35}S]Met (>1000 Ci/mmol) was from Amersham Corp. FITC-conjugated goat anti-mouse IgG, endoglycosidases H and D, and β-galactosidase were from Boehringer Mannheim Corp. (Indianapolis, IN). Streptavidin−agarose and s-NHS-biotin were from Pierce Chemical Co. (Rockford, IL). All other reagents were from Sigma Chemical Co. (St. Louis, MO). Monoclonal antibodies against rat D4 have been described previously (Mendrick & Rennke, 1988; Hong *et al.*, 1989) and were a generous gift from Dr. D. L. Mendrick (Harvard Medical School, Cambridge, MA).

Construction of the D4αhcg Chimera. Standard procedures of DNA manipulation, polymerase chain reaction, and transfection were followed (Sambrook *et al.*, 1989). A DNA fragment corresponding to the cDNA sequence of αhcg immediately after the signal peptidase cleavage site was generated by polymerase chain reaction (PCR) using the sense oligonucleotide 5′-GCATGTTCTCGCGGCCGCTC-CTGATG-3′ and the antisense oligonucleotide 5′-CGGATC-CTCGAGTATATCCTTGAAGCGTG-3′. The sense oligonucleotide contains sequences which included a *Not*I site corresponding to the *Not*I site found immediately after the transmembrane domain of D4, and the antisense oligonucleotide contained a *Bam*HI site. Digestion of the PCR product with *Not*I and *Bam*HI therefore generated a fragment which could be ligated directionally to replace the entire ectodomain of D4 with the αhcg sequence without its signal peptide (Figure 1A). The sequence of the chimeric construct was confirmed by DNA sequencing and inserted into the mammalian expression vector pRSN (Low *et al.*, 1991a,c) under the control of a Rous sarcoma virus−SV 40 promoter−enhancer system.

Cell Culture and Transfection. CHO cells were from American Type Culture Collection (Rockville, MD). They were maintained in DME supplemented with 10% FBS. Stable transfection was achieved by the calcium phosphate precipitation method. Transfectants were selected and maintained in DME with 2500 μg/mL G418 for CHO cells (Low *et al.*, 1991a).

In Vitro Translation and Sodium Bicarbonate Treatment. This was performed exactly as previously described (Hong and Doyle, 1990).

Immunofluorescence Microscopy. This was performed as previously described (Wong *et al.*, 1992). Cells were cultured as a monolayer on glass cover slips and fixed with 2.7% paraformaldehyde and not permeabilized for surface staining or permeabilized with 0.1% saponin for total staining. Cells undergoing treatment were incubated with the appropriate drugs added to the culture medium for 1 h prior to processing for immunofluorescence. Cell labeling was visualized using an Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY) with epifluorescence optics and photographed with Kodak Tri-X 400 film.

Metabolic Labeling of Cells. Cells were washed twice with Hanks' buffered saline solution and then incubated for 45 min at 37 °C in methionine-free medium, supplemented with 10% dialyzed FBS (labeling buffer). The cells were then pulse-labeled with [^{35}S]Met (0.5 mCi/mL in labeling buffer) for 15 min, washed, and chased in medium containing excess cold methionine (100 mg/L) for various times as indicated in each figure.

Cell Surface Biotinylation and Immunoprecipitation. This was performed as previously described (Low *et al.*, 1991a).

SDS−PAGE and Analysis by Fluorography. This was performed as described (Low *et al.*, 1991c).

RESULTS

D4αhcg Is Translocated, Glycosylated, and Membrane-Anchored. Previous studies have shown that the *N*-terminal 34 amino acid sequence of D4 is the signal/anchor sequence (Hong & Doyle, 1990). In order to determine whether D4αhcg containing the cytoplasmic (6 residues) and transmembrane (22 residues) domains of D4 is membrane-anchored, its mRNA was translated in rabbit reticulocyte lysate. In the absence of microsomes, a polypeptide of 20

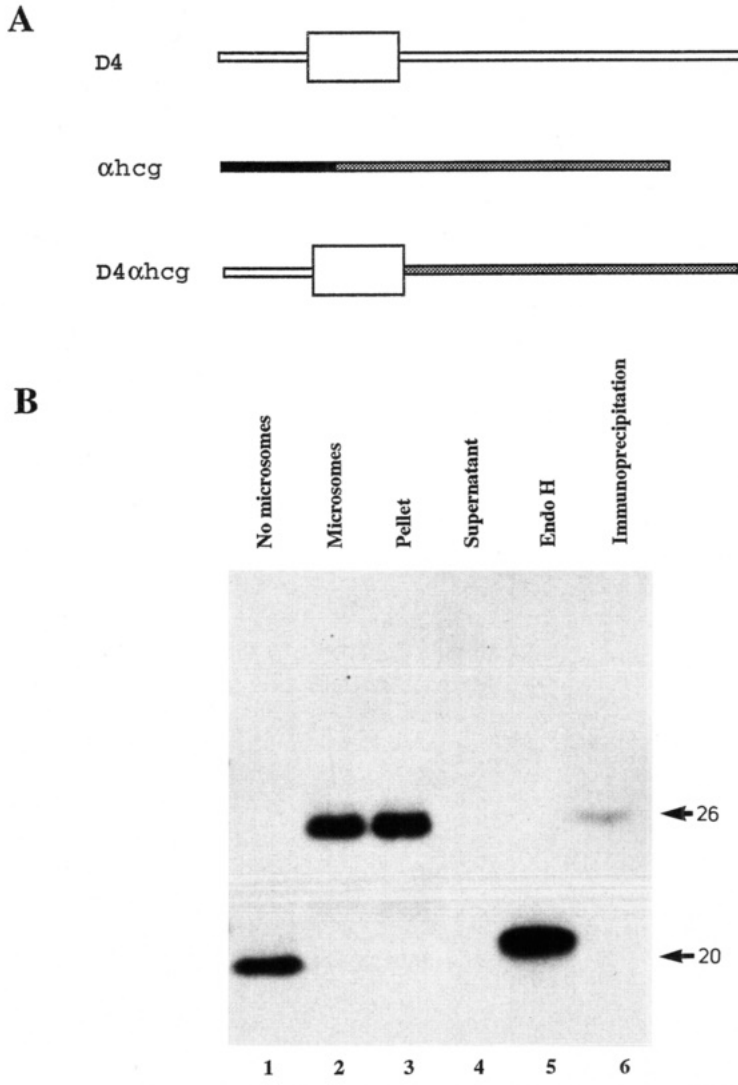

FIGURE 1: (A) Schematic diagram of the native D4 and αhcg and the chimeric construct D4αhcg. Portions of the chimera derived from different parent molecules are coded differently: D4, white; αhcg, shaded; signal peptide, black. (B) Membrane anchorage of the D4αhcg chimera. The mRNA of the chimeric construct was translated in rabbit reticulocyte lysate with (lanes 2−6) or without microsomes (lane 1). Ten microliters of the total translation reaction was diluted in 100 μL of sodium bicarbonate, pH 11.0, and the membrane-associated proteins (lane 3) separated from those extractable by this treatment (lane 4) by centrifugation. Aliquots (10 μL each) of the translation reaction were digested with endo H (lane 5) or subjected to immunoprecipitation with a monoclonal antibody against αhcg.

kDa was obtained (Figure 1B, lane 1). When dog pancreatic microsomes were included in the reaction, a slower migrating form of 26 kDa was obesrved (lane 2), which represents translocated polypeptides that have acquired two asparagine-(Asn-) linked oligosaccharide chains. The glycosylated product was selectively immunoprecipitated with antibody against αhcg (lane 6). Since the αhcg molecule has been previously shown to contain two sites for Asn-linked glycosylation (Endo *et al.*, 1979), the increase in size was as expected. This was confirmed by endo H digestion (lane 5), which converted the 26-kDa glycoprotein into a molecule almost identical in size to the unglycosylated product in lane 1.

To determine if the translocated protein was anchored in the membrane, the translation reaction was diluted in carbonate buffer, pH 11, and the membranes were collected by centrifugation. The glycosylated product was recovered only from the membrane pellet (lane 3), whereas none of the protein was detected in the supernatant (lane 4). Since the high pH failed to extract the molecule from the membrane fraction, this suggests that D4αhcg is an integral membrane protein with the glycosylated αhcg portion localized to the lumen. Altogether, the above experiments show that D4αhcg is very efficiently translocated and glycosylated and the resulting product remains firmly anchored in the membrane.

Accumulation of D4αhcg in the Golgi Apparatus. To investigate the effect of replacing the ectoplasmic domain of D4 with αhcg, the construct was stably transfected into CHO cells and the localization of the protein was determined by indirect immunofluorescence. Transfected cells were cultured on cover slips and either permeabilized or not for total and surface staining, respectively. Visualization of the proteins was achieved by sequential incubation with monoclonal antibodies and goat anti-mouse IgG conjugated with FITC. The cover slips were then processed for microscopy and photography.

CHO cells expressing D4 exhibited staining over the entire cell surface in both permeabilized and nonpermeabilized cells (Figure 2), typical of a plasma membrane protein. In nonpermeabilized cells, D4αhcg also showed surface staining, although of a lesser intensity compared to D4-expressing cells. In permeabilized cells, however, in addition to the

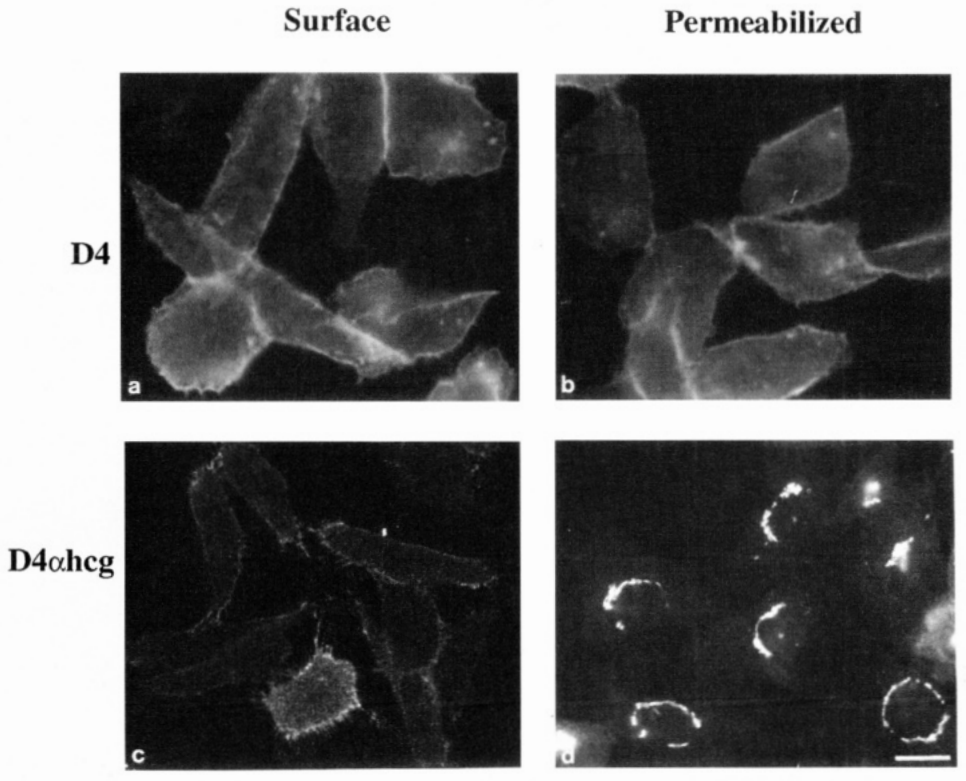

FIGURE 2: Indirect immunofluorescence microscopy of stably transfected CHO cells. Cells expressing native D4 (a and b) or D4αhcg (c and d) were processed for surface staining (a and c) or permeabilized with 0.1% saponin for total staining (b and d) by sequential incubation with a monoclonal antibody to αhcg and anti-mouse Ig-FITC. Bar, 10 μm.

slight surface staining, an intense perinuclear ring was observed, characteristic of the Golgi apparatus (Louvard *et al.*, 1982; Lipsky & Pagano, 1985). This was confirmed by its colocalization with mannosidase (Man) II as an endogenous Golgi marker (Velasco *et al.*, 1993). In contrast to D4αhcg (Figure 3a,c,e), staining for Man II yielded strictly Golgi staining, with no surface staining detectable (Figure 3b,d,f). Brefeldin A (BFA) is a fungal metabolite which causes the redistribution of Golgi-localized proteins into the endoplasmic reticulum (ER) (Lippincott-Schwartz *et al.*, 1990; Tang *et al.*, 1992c). BFA treatment at 10 μg/mL caused both D4αhcg and Man II to redistribute into diffuse reticular punctate staining, present throughout the cytoplasm, with a distinct nuclear envelope (Figure 3c,d). This staining pattern is typical of the ER (Louvard *et al.*, 1982; Terasaki *et al.*, 1984). The microtubule-disrupting agent nocodazole (25 μg/mL) fragmented the perinuclear staining of both proteins into bright spots distributed throughout the cell (Figure 3e,f), indicating the disruption of the Golgi apparatus (Tang *et al.*, 1992c).

In order to determine whether this phenomenon is specifically seen only in CHO cells, the chimera was also stably expressed in MDCK cells. As shown in Figure 4, in addition to its presence on the cell surface, immunofluorescence staining for D4αhcg in permeabilized cells also revealed intense Golgi localization, which was redistributed into the ER with BFA treatment and dispersed into vesicular structures throughout the cytoplasm in the presence of nocodazole. All these experiments confirm that D4αhcg is retained in the Golgi apparatus of CHO as well as MDCK cells.

ER to Medial Golgi Transport Is Delayed. In order to study the biogenesis of D4αhcg and its transport kinetics through the exocytic pathway, pulse−chase experiments were performed. Transfected CHO cells were pulse-labeled with [^{35}S]Met for 15 min and chased for varying lengths of time as indicated in Figure 5. Labeled D4αhcg molecules were recovered by immunoprecipitation from cell lysates and half the sample was treated with endo H, while the remaining half that was mock-treated served as a control. The products were analyzed by SDS−PAGE and fluorography.

D4αhcg is synthesized as a 26-kDa precursor (Figure 5, lane 1). This form represents the first step in N-linked glycosylation, where the oligosaccharide complex $Glc_3Man_9GlcNAc_2$ is transferred *en bloc* from the lipid carrier, dolichol, onto an asparagine residue. During the subsequent 1-h chase period, a 25-kDa intermediate was observed, before the molecule was finally converted into a high molecular weight smear of approximately 30−50 kDa (lanes 5−7). The high molecular weight smear was due to heterogenous polylactosamine glycosylation (Figure 6, lane 13). This phenomenon of polylactosamine addition to membrane-bound, but not secretory, αhcg molecules has been described before (Fukuda *et al.*, 1988). Even after 2 h of chase, the low molecular weight early precursors of D4αhcg were still sensitive to endo H digestion and reduced to a homogenous band of 20 kDa (lanes 8−13). This implies that they have not been processed by NT I and Man II, which reside in the medial Golgi compartment (Dunphy & Rothman, 1983; Farquhar, 1985; Kornfeld & Kornfeld, 1985). This represents a significant delay in ER to medial Golgi transport as compared to native D4 molecules, which became totally endo H resistant after only 45−60 min of chase (data not shown), whereas less than 10% of D4αhcg molecules had acquired endo H resistance in the equivalent time. In contrast, the high molecular weight smear was endo H-resistant (lanes 12−14) and must therefore have reached the medial Golgi and been modified by NT I and Man II prior to its acquiring large, heterogeneous polylactosamine moieties.

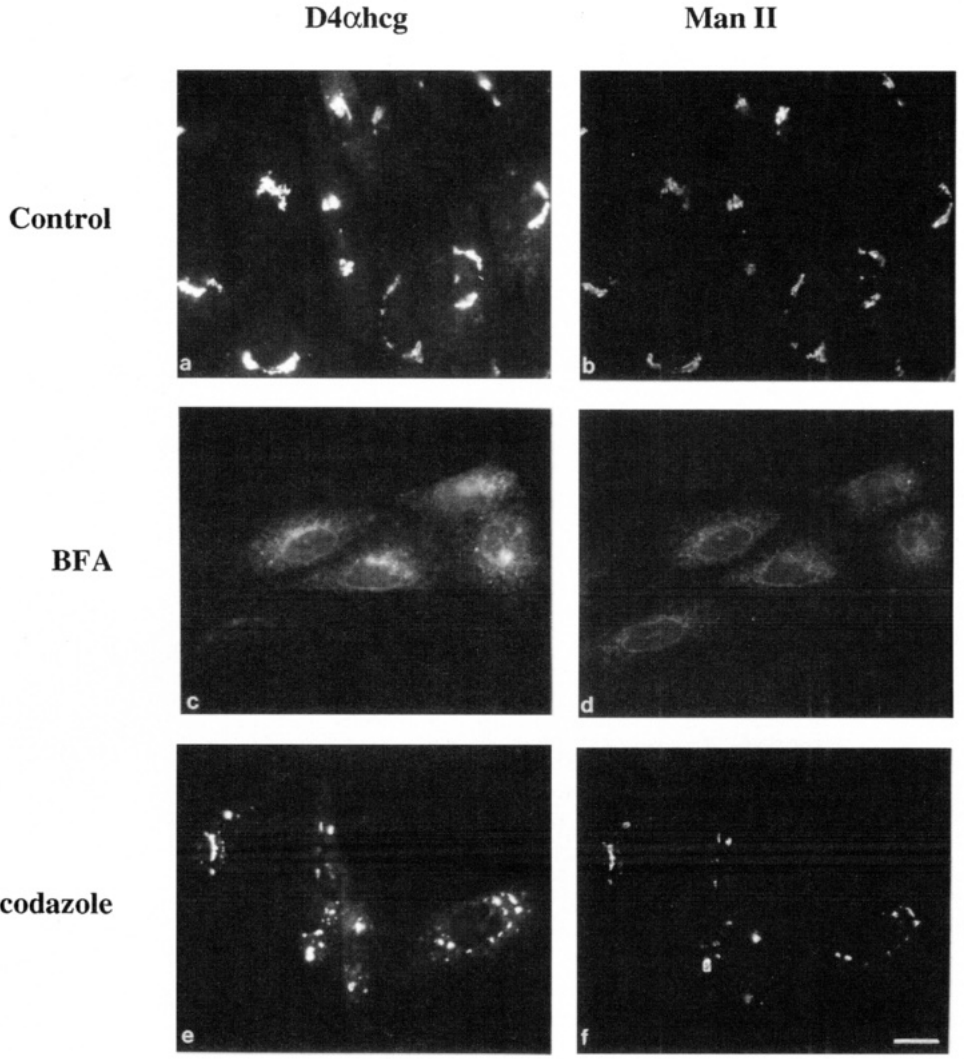

FIGURE 3: Colocalization of D4αhcg with the endogenous Golgi marker Man II. Cells stably expressing D4αhcg were either untreated (a and b) or treated with BFA at 10 μg/mL (c and d) or nocodazole at 25 μg/mL (e and f) for 1 h at 37 °C, followed by fixation on ice. The cells were permeabilized and incubated with a monoclonal antibody against αhcg and polyclonal antisera against Man II, followed by anti-mouse Ig-FITC and anti-rabbit Ig-rhodamine. The D4αhcg chimera was visualized and photographed using the FITC channel (a, c, and e) and Man II by the rhodamine channel (b, d, and f). Bar, 10 μm.

The High Molecular Weight Smear Is Transported to the Cell Surface. The transport of D4αhcg to the cell surface was studied using the technique of pulse−chase and selective cell-surface biotinylation (Low *et al.*, 1991a,c). The CHO cells were pulse-labeled for 15 min and chased for various lengths of time as indicated in Figure 6. Proteins on the surface of the cell were selectively biotinylated on ice, before cell lysis and immunoprecipitation. Proteins arriving on the cell surface were recovered by a subsequent step of incubation with streptavidin−agarose. One-tenth of the total D4αhcg recovered was loaded onto the SDS−polyacrylamide gel as a control (lanes 1−6).

Only the mature form of the molecule that has acquired polylactosamine modification was transported to the surface of the cell, as evidenced by the detection of the high molecular weight smear with streptavidin−agarose immunoprecipitation (lanes 9−12). The immature, ER and Golgi-retarded intermediates present intracellularly, and therefore not biotinylated, were recovered only from the total cell lysate (lanes 1−3) but not with streptavidin−agarose. The high molecular weight smear of D4αhcg was first detected after 2 h of chase (lanes 3 and 9), and the amount recovered from the cell surface was seen to increase up to 5 h of chase (lane 10), after which the amount remained constant up to 22 h of chase (as judged by the intensity of the smear in lanes 10−12), implying that the molecule was not degraded. To confirm that the smear was actually the D4αhcg molecule that had acquired heterogenous polylactosamine modification, a portion of the product recovered from streptavidin agarose after 5 h of chase was digested with endo-β-galactosidase (endo β), which hydrolyzes β-galactosidic linkages from polylactosamines (Fukuda, 1985). As a result of endo β digestion, the high molecular weight smear was converted to a band of 26 kDa (lane 13), the expected size of D4αhcg with N-linked core oligosaccharides. From the above results, it is confirmed that only D4αhcg molecules that have obtained polylactosamine glycosylation is transported to the cell surface.

The Compartment of Retardation Is Identified as the Cis Golgi. Transport from the ER to the Golgi can be measured by the trimming of the high-mannose ($Man_{8-9}GlcNAc_2$) asparagine-linked oligosaccharide intermediate to the $Man_5GlcNAc_2$ species by Man I, which resides in the cis Golgi (Balch *et al.*, 1986). This intermediate is uniquely sensitive to digestion by endo D and therefore can easily be distinguished by its reduced molecular weight during SDS−PAGE.

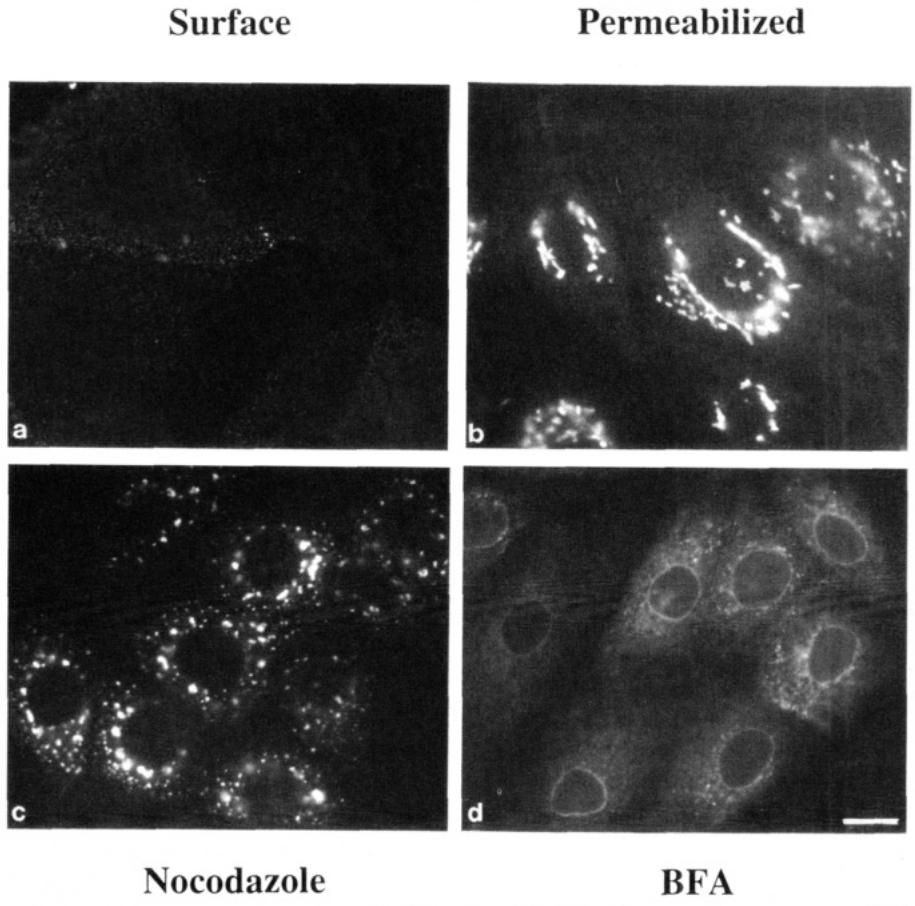

FIGURE 4: Localization of D4αhcg stably transfected into MDCK cells. MDCK cells were either not permeabilized (a) or permeabilized (b−d) with 0.1% saponin and processed for indirect immunofluorescence as in Figure 2. The cells were treated with 25 μg/mL nocodazole (c) or 50 μg/mL BFA for 1 h at 37 °C (d), prior to fixation on ice and antibody binding. Bar, 10 μm.

To determine whether we could document the appearence of this normally short-lived form, a pulse−chase experiment followed by endo D digestion was performed. As seen in Figure 7, the 25-kDa intermediate was sensitive to endo D digestion, resulting in the isolation of a 23-kDa band during the 30-min−2-h chase points (lanes 9−12). This reduction in size is consistent with the removal of one oligosaccharide chain. This suggests that one of the two endo H-sensitive glycans of this intermediate is sensitive to endo D digestion.

Under ordinary circumstances, where the ER to medial Golgi transport is not retarded, the endo D-sensitive intermediate is usually not observed, due to the extremely rapid rate of conversion of the endo D-sensitive intermediate $Man_5GlcNAc_2$ into the endo D-resistant intermediate $GlcNAcMan_5GlcNAc_2$ by NT I. In the case of D4αhcg, however, the molecule seems to be retarded in an early Golgi compartment, as evidenced by the Golgi localization and endo H sensitivity data. Coupled with the results of the endo D assay, it can be concluded that transport of the chimera is retarded in an early Golgi compartment, most likely the cis Golgi, which is distinct from the NT I and Man II-containing medial Golgi compartment.

DISCUSSION

Golgi Retardation of D4αhcg. It has been previously shown that the native D4 molecule is transported rapidly from the ER to the Golgi, and within 40 min, approximately 80% of the pulse-labeled molecule was converted into an endo H-resistant Golgi form (Low *et al.*, 1992). Replacement of its ectoplasmic domain with αhcg, as shown in this study, results in significant retardation in transport from the ER to the medial Golgi and only about 10% of the newly synthesized molecule was converted into a post medial Golgi form within 60 min of chase. Several lines of evidence suggest that this delay in transport to the medial Golgi is most likely due to a retardation in transport from the early or cis Golgi to the medial Golgi. First, the perinuclear steady-state staining pattern of the chimera strongly indicates that the chimera is localized to the Golgi apparatus. This steady-state accumulation in the Golgi would suggest that exit from the ER and ER-to-Golgi transport are not affected to any significant extent. This is in contrast to the case in which an ER retention signal was appended to D4 (Tang *et al.*, 1992a), with the resulting chimera having a steady-state staining pattern characteristic of the ER. Second, the significant delay of the chimera in receiving endo H resistance modifications as compared to the native molecule is highly indicative of a block proximal to the medial Golgi. This is further confirmed by the appearance of endo D-sensitive intermediates. Finally, kinetic correlation between the time of appearance of the endo H-resistant form and that required by the pulse-labeled protein to reach the cell surface suggests that post medial Golgi transport is not affected.

Most of the Golgi proteins cloned and studied so far have been found to mediate their Golgi localization by signals in and around their transmembrane domains (Munro, 1991; Nilsson *et al.*, 1991; Swift & Machamer, 1991; Russo *et al.*,

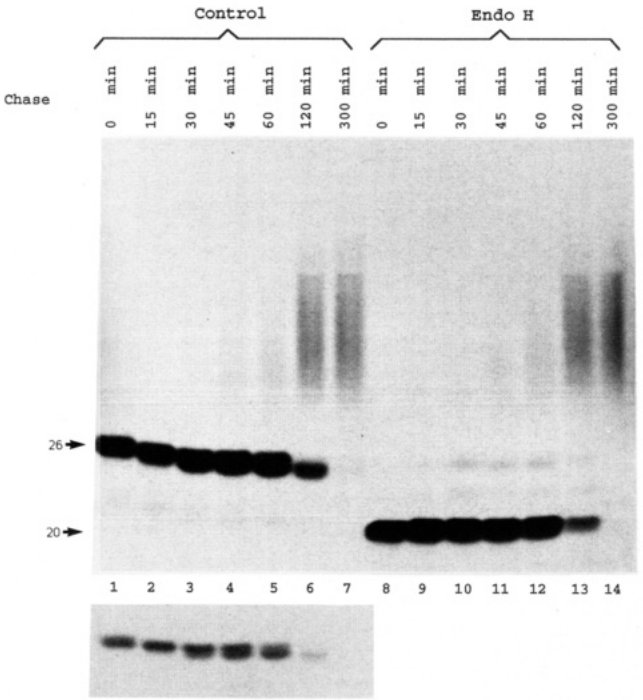

FIGURE 5: Kinetics of D4αhcg biogenesis. Stably transfected cells were pulsed with [^{35}S]Met for 15 min and chased for the various times as indicated. D4αhcg molecules were recovered by immunoprecipitation from the cell lysates with monoclonal antibodies bound to protein A−Sepharose beads. Half of the sample was treated with endo H (lanes 8−14), while the other half was mock-treated to serve as a control (lanes 1−7). The proteins were resolved on an SDS−13.5% polyacrylamide gel, and ^{35}S-labeled proteins were detected by fluorography. A shorter exposure of the gel from lanes 1 to 7 is shown below for a clearer resolution of the pre-endo H-resistant intermediates.

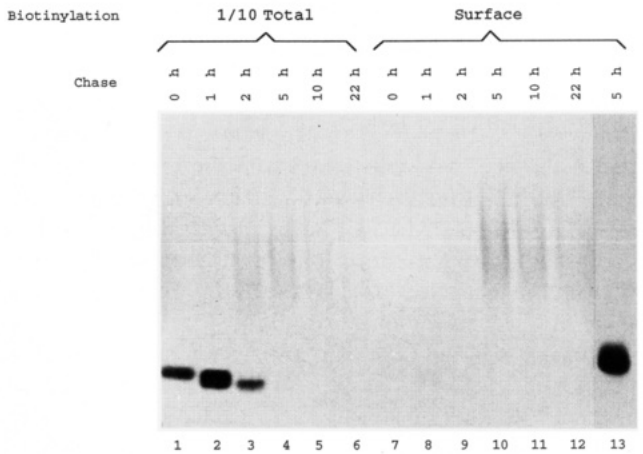

FIGURE 6: Surface biotinylation of cells expressing D4αhcg. Cells were pulsed for 15 min and chased for various times as indicated in the figure. The cell surface was biotinylated with NHS-biotin. After lysis, all the D4αhcg was recovered by immunoprecipitation. One-tenth of the immunoprecipitate (lanes 1−6) was loaded onto the gel and served as a measure of the amount of newly synthesized chimera. The biotinylated proteins on the surface of the cell were captured by absorption to streptavidin−agarose (lanes 7−13). Lane 13 contains D4αhcg molecules recovered from streptavidin−agarose beads after a 5-h chase and treated with endo β for 24 h at 37 °C. All the proteins were resolved by SDS−13.5% PAGE and detected by fluorography.

1992; Tang *et al.*, 1992b; Wong *et al.*, 1992). Despite this fact, no primary sequence homology has been observed among the membrane-spanning domains of these proteins and the mechanism of retention remains unknown, with one

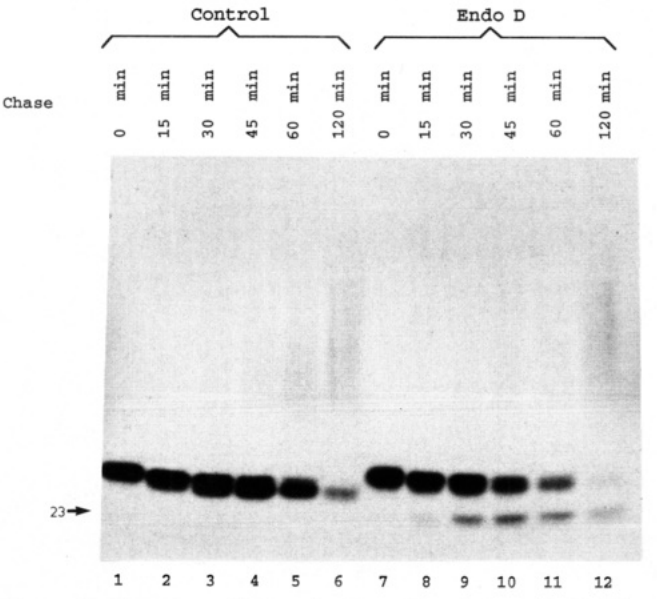

FIGURE 7: Detection of the endo D-sensitive intermediate. The cells were pulsed with [^{35}S]Met for 15 min and chased for different times as indicated in the figure. D4αhcg was recovered from the cell lysates by immunoprecipitation. Half of each sample was digested with endo D for 24 h at 37 °C, while the other half, processed identically without endo D, served as a control. The proteins were resolved by SDS−13.5% PAGE and detected by fluorography.

exception. The Golgi-localized protein chimera constructed by Weisz *et al.* (1993) consists of replacing the transmembrane domain of the VSV-G protein by the first membrane-spanning domain of the M glycoprotein of the avian coronavirus. Oligomerization of the molecule in the Golgi was discovered to be the cause of retention and mutants of the chimera which failed to form oligomers were able to be transported to the surface of the cell.

In view of this, efforts were also made to determine the oligomeric state of D4αhcg by sucrose gradient centrifugation and whether its retardation in the Golgi could also be the result of aggregation. It was discovered that the newly synthesized and endo D-sensitive intermediates, as well as the polylactosamine-modified mature species, were recovered from similar low molecular weight fractions of the gradient (data not shown), ruling out any possibility of aggregate formation.

The question of whether the Golgi consists of several distinct subcompartments connected together by vesicular or tubular transport or exists as one physically integrated unit remains unresolved (Mellman & Simons, 1992). The detection of the endo D-sensitive intermediate is interesting, as this would suggest that the activity of the Man I enzyme, responsible for endo D sensitivity, is distinct and separated from the NT I and Man II, endo H resistance-conferring enzymes. This is in contrast to two recent publications, which demonstrated that the distribution of NT I (Nilsson *et al.*, 1993) and Man II (Velasco *et al.*, 1993) was by no means rigid but was present over several Golgi subcompartments. Future experiments will be required to resolve this discrepency.

Mechanism of Golgi Retention. It is widely accepted that transport along the exocytotic pathway occurs by default in the absence of specific retention signals [for review see Pfeffer and Rothman (1987)]. However, the identification of retention/retrieval signals does not necessarily exclude the

existence of other types of signals required for efficient passage along the secretory pathway. The signal-mediated transport of proteins to the apical or basolateral domains of polarized epithelial cells has been well documented. Apical targeting has been shown to be mediated by a glycosylphosphatidylinositol (GPI) anchor (Lisanti *et al.*, 1989). In GPI-deficient cells, the uncleaved precursor of the GPI-anchored protein is retained in the ER (Delahunty *et al.*, 1993). Basolateral targeting signals of membrane proteins have also been identified within the cytoplasmic domain (Casanova *et al.*, 1991; Matter *et al.*, 1992; Mostov *et al.*, 1992).

BFA affects many steps of trafficking along the exocytotic pathway. It has been shown to inhibit ER-to-Golgi transport and cause the redistribution of Golgi proteins back to the ER (Doms *et al.*, 1989; Lippincott-Schwartz *et al.*, 1990), by causing a 110-kDa protein, β-COP, to dissociate from Golgi membranes (Donaldson *et al.*, 1990). It causes the fusion of the TGN components with the endosomal system (Lippincott-Schwartz *et al.*, 1991; Wood *et al.*, 1991). BFA has also been shown to interfere with sorting of basolaterally (Matter *et al.*, 1993) and apically (Low *et al.*, 1992) targeted proteins at the TGN and endosomes. This implies that there may be common components involved in each of these steps. That these components should be analogous, but by no means identical, is clearly illustrated by the different BFA concentrations needed to exert its effect at varied locations (Low *et al.*, 1991b, 1992). One of the actions of BFA is to prevent the binding of the ADP-ribosylation factor (ARF), and consequently coat proteins, to Golgi membranes. ARF binding is a prequisite for coat protein binding (Donaldson *et al.*, 1991), which precedes vesicle budding and transport. The process of vesicular budding and transport is thus a complicated, multistep reaction, tightly controlled by small GTP binding and heterotrimeric G proteins (Balch, 1992; Bomsel & Mostov, 1992). Proteins destined for transport by these vesicles must therefore also be under some form of regulation and it would not be surprising that some kind of signals are required for this incorporation. These signals may be disrupted or weakened in the chimeras, resulting in inefficient vesicle incorporation and transport.

Besides D4αhcg, DAD (Low *et al.*, 1994) and other mutant proteins retained in the Golgi [summarized in Table 1 of Low *et al.* (1994)], we have additional chimeras and deletion mutants of plasma membrane proteins which exhibit clear Golgi staining by immunofluorescence (Low *et al.*, unpublished results). D4Stu is a C-terminal truncation of the D4 molecule, and DSD is the result of a transmembrane domain substitution of D4 by that of sucrase isomaltase. In spite of the vastly differing structural alterations to these four molecules, their shared Golgi retardation lends credence to the notion that some sort of transport signal has been affected, resulting in their Golgi retardation.

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