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A Golgi Retention Signal in a Membrane-spanning Domain of Coronavirus E1 Protein

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Abstract. The E1 glycoprotein from an avian coronavirus is a model protein for studying retention in the Golgi complex. In animal cells expressing the protein from cDNA, the E1 protein is targeted to cis Golgi cisternae (Machamer, C. E., S. A. Mentone, J. K. Rose, and M. G. Farquhar. 1990. *Proc. Natl. Acad. Sci. USA.* 87:6944–6948). We show that the first of the three membrane-spanning domains of the E1 protein can retain two different plasma membrane proteins in the Golgi region of transfected cells. Both the vesicular stomatitis virus G protein and the alpha-subunit of human chorionic gonadotropin (anchored to the membrane by fusion with the G protein membrane-spanning domain and cytoplasmic tail) were retained in the Golgi region of transfected cells when their single membrane-spanning domains were replaced with the first membrane-spanning domain from E1. Single amino acid substitutions in this sequence released retention of the chimeric G protein, as well as a mutant E1 protein which lacks the second and third membrane-spanning domains. The important feature of the retention sequence appears to be the uncharged polar residues which line one face of a predicted alpha helix. This is the first retention signal to be defined for a resident Golgi protein. The fact that it is present in a membrane-spanning domain suggests a novel mechanism of retention in which the membrane composition of the Golgi complex plays an instrumental role in retaining its resident proteins.

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SORTING of newly synthesized proteins in the exocytic pathway is a fundamental problem in cell biology which has received a great deal of attention in recent years. Secreted and plasma membrane proteins follow a common pathway through the cell: from the ER, through the Golgi complex, to the cell surface (32). Resident proteins of the ER and the Golgi complex are specifically retained. Although much is known regarding the signal sequence–mediated translocation of proteins across the membrane of the ER (46), less is known regarding the trafficking of proteins once they have entered this pathway.

One current hypothesis involves the idea that proteins destined for constitutive secretion or insertion at the plasma membrane are transported by default with the bulk flow of lipid (35). Proteins destined for lysosomes or secretory granules (in cells which perform regulated secretion) are directed by specific signals once they have traversed the Golgi complex. This hypothesis requires that resident proteins of the ER and Golgi complex have specific signals that cause their retention in the appropriate compartment. Evidence is accumulating to support this idea. A tripeptide which is presumed to lack any signals for transport is secreted rapidly from cells and defines the rate of "bulk flow" (47). Retention signals for both soluble and membrane-bound ER proteins have been identified (14,30,31). The mannose-6-phosphate modification on lysosomal hydrolases is recognized by a receptor in the Golgi complex which targets these proteins to lysosomes (20).

The Golgi complex plays a central role in the processing and sorting of newly synthesized proteins (reviewed in reference 9). Its characteristic morphology (stacks of flattened saccular membranes) and central location (peri- or juxta nuclear) in the cell may be important for these functions. Four Golgi subcompartments have been defined functionally: *cis*-, *medial*-, *trans*-, and *trans*-Golgi network. Newly synthesized proteins are thought to move vectorially through the Golgi complex subcompartments via vesicular transport, from the *cis*- to the *trans*-side of the stack. Endogenous Golgi proteins such as the glycosyltransferases and glycosidases that are involved in the processing of asparagine-linked oligosaccharides are each enriched in a specific subcompartment (8). It has been suggested that this arrangement allows sequential and orderly processing of glycoproteins as they are vectorially transported through the Golgi complex.

In addition to its role in protein processing, the Golgi complex is instrumental in correct sorting of protein traffic. Lysosomal hydrolases, regulated secretory proteins, and proteins destined for the apical or basolateral plasma membrane domains in polarized kidney epithelia are sorted in the *trans*-most cisternae of the Golgi, the *trans*-Golgi network (15,43). The *cis*-side of the Golgi complex may also be involved in sorting, since escaped ER resident proteins must be separated from those that are transported forward (34).

We have been studying the sorting of newly synthesized proteins in the exocytic pathway using a model Golgi protein, the E1 glycoprotein of the avian coronavirus infectious bron-

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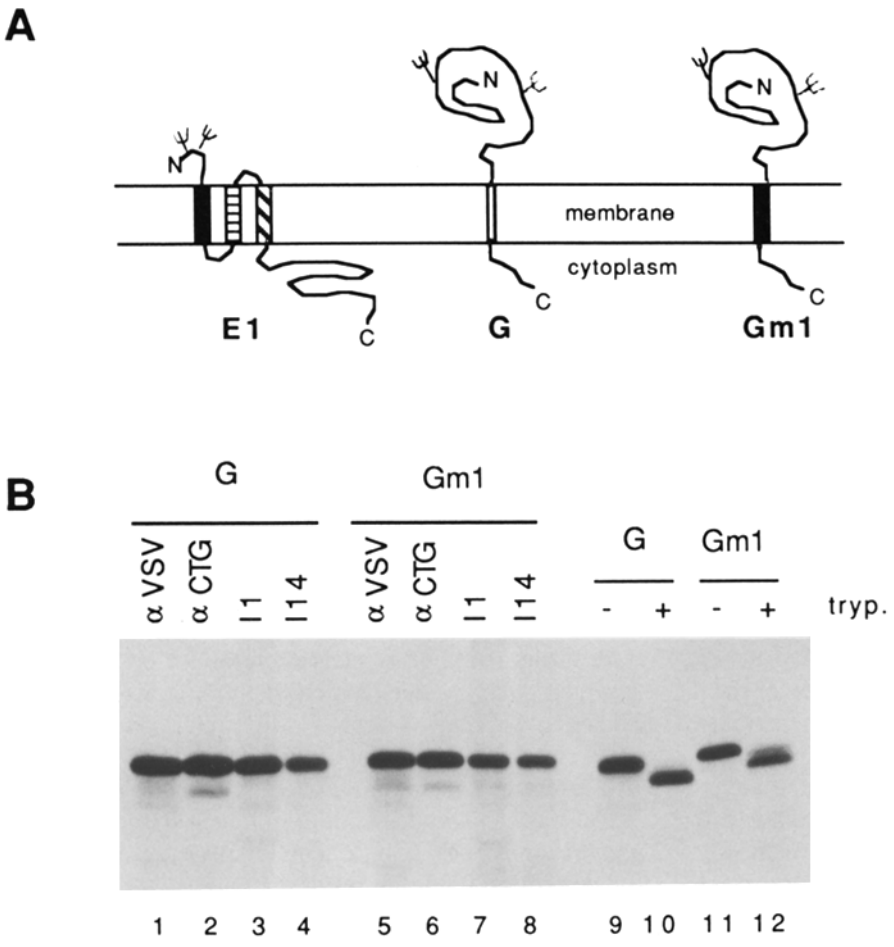

Figure 1. The m1 sequence functions as a normal membrane-spanning domain in the VSV G protein. (*A*) Schematic representation of the E1 protein, VSV G, and the chimeric protein Gm1. Locations of *N*-linked oligosaccharides are marked. (*B*) HeLa cells expressing either G (lanes 1–4) or Gm1 (lanes 5–8) were labeled with [^{35}S]cysteine for 30 min and an aliquot of each cell lysate was immunoprecipitated with antibody to the ectodomain of G protein (αVSV), the cytoplasmic tail (αCTG), or one of two conformation-specific mAbs (I1 and I14). Microsomal membranes from transfected HeLa cells labeled for 10 min were incubated with (lanes *10* and *12*) or without (lanes *9* and *11*) trypsin, solubilized, and immunoprecipitated with polyclonal anti-VSV serum. Samples were electrophoresed and the gel was fluorographed.

chitis virus (IBV)[1]. The E1 protein consists of a short, glycosylated amino-terminal domain, three membrane-spanning domains, and a long carboxy-terminal cytoplasmic domain. The restricted intracellular localization of the E1 protein in coronavirus-infected cells is believed to direct virus assembly at intracellular membranes (45). When cDNA is expressed in animal cells in the absence of the other viral proteins, the IBV E1 protein is targeted to *cis*-Golgi membranes (29). Deletion of the first and second, or the second and third of the three E1 membrane-spanning domains showed that the first membrane-spanning domain was apparently required for intracellular retention (26). We show here that the first membrane-spanning domain is a Golgi retention signal, since it is both necessary and sufficient for Golgi retention. Unlike ER retention signals that have been identified at the carboxy-termini of proteins (on either the lumenal or cytoplasmic side of the ER membrane), this Golgi retention signal is buried in the membrane. Our results suggest the novel possibility that the membrane composition of Golgi subcompartments may play an important role in retaining resident proteins in this organelle.

Materials and Methods

Cells and Transfection

COS-7 and HeLa cells were maintained in DME with 5% FBS. COS-7 cells plated in 35-mm dishes (70% confluent) were transfected with an SV-40-based expression vector using DEAE-dextran as described (28). E1 expression was analyzed 44 h posttransfection. For expression using the vaccinia-T7 system, HeLa cells (70% confluent) were infected with the recombinant vaccinia virus vTF7-3 encoding T7 RNA polymerase (10) at a multiplicity of infection of 20. After adsorption for 30 min at 37°C, the inoculum was replaced with 0.75 ml of serum free medium containing 4 μg of a vector (pAR2529) encoding the appropriate gene behind the T7 promoter and 10 μl of the cationic lipid "TransfectACE" (Bethesda Research Laboratories, Gaithersburg, MD; and reference 37). Expression was analyzed by metabolic labeling starting at 4 h postinfection.

Mutagenesis and Production of Chimeric Proteins

For most of the mutations, the Kunkel method of oligonucleotide-directed mutagenesis (21) was used. The E1, Δm2,3, and Gm1 genes were cloned into the M13 vector mp8, and single strands produced in *Escherichia coli* RZ1032 (*dut*, *ung*). The exception was production of the chimera Gm1, which was produced by domain replacement using the oligonucleotide

5′-CAGTAGTTGGAAAAGCTATAATTTATTTATAACTG-
CATTCTTGTTGTTCT
TAACCATAATACTTCAGTATGGCTATGCAACCCGG-
GTTGGTATCCATC-3′

using single stranded G template from *E. coli* JM103 and screening plaques by differential hybridization. The following oligonucleotides were used for mutation of E1 (with mutated nucleotides underlined):

NI_{22}: 5′-AAAGAGTATATCTTATTTATAACTG-3′;
TI_{33}: 5′-GTTGTTCTTAATTATAAATACTTCAG-3′;
QI_{37}: 5′-CATAATACTTATATATGGCTATGC-3′;
mIins: 5′-ACTGCATTCTTGATAATATTGTTCTTAACC-3′; and
LQ_{30}: 5′-CTGCATTCTTGCAGTTCTTAACCA-3′.

$NI_{22}TI_{33}$ was produced using both NI_{22} and TI_{33} oligonucleotides as

1. *Abbreviations used in this paper*: IBV, infectious bronchitis virus; VSV, vesicular stomatitis virus.

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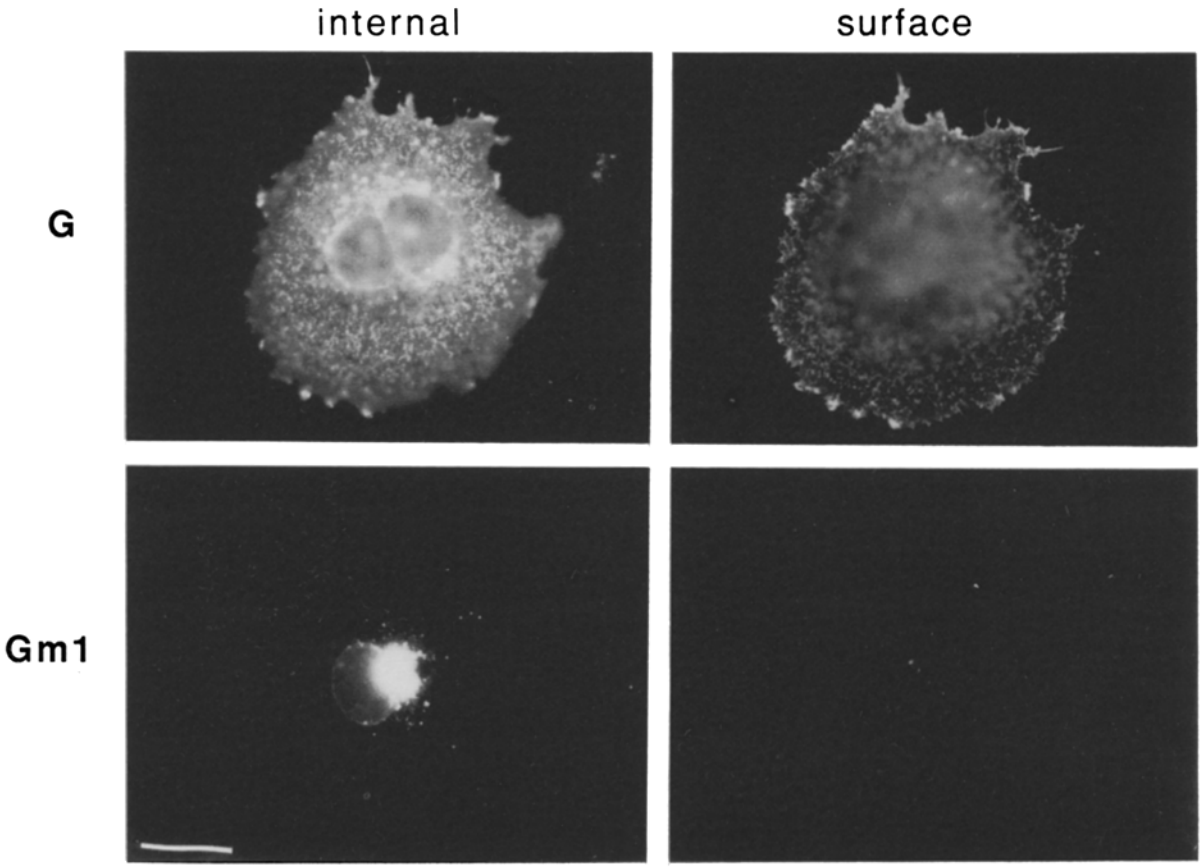

Figure 2. Gm1 is retained in the Golgi region of transfected cells. COS cells expressing either G or Gm1 were fixed and stained by double-label indirect immunofluorescence microscopy. Surface G protein was detected by staining with rabbit anti-VSV serum followed by a Texas red–conjugated second antibody. Internal G protein was detected after permeabilization with a monoclonal anti–G antibody and a fluorescein-conjugated second antibody. Left panels were photographed with the fluorescein filter, and those on the right are the same field photographed with the rhodamine filter. Bar, 10 μm.

primers for second strand synthesis. These same oligonucleotides were used to create the mutations in the E1 deletion mutant, Δm2,3. Gmlins was produced with the mlins oligonucleotide, but GmlQI was obtained only after a longer oligonucleotide,

5′-CTTAACCATAATACTTATCTATGGCTATGCAACCC-3′

was used.

T4 DNA polymerase (Biolabs) was used for second strand synthesis, and the double-stranded molecules were transfected into *E. coli* NM522. Single-stranded DNA from three to six plaques was sequenced using the dideoxy procedure (Sequenase, USB) to select the desired mutations. The mutated genes were excised from the double-stranded replicative form DNA and subcloned into both the SV-40 expression vector pJC119 (44) and the T7 expression vector pAR2529 (10). All general recombinant DNA techniques were as described (41).

The membrane-spanning domain of αm was replaced with either the IBV E1 m1 or m3 domain using restriction sites in the coding sequence. To create αm1G, a BamHI to RsaI fragment (encoding the α subunit) was filled in with the Klenow fragment of DNA polymerase I, digested with XhoI, and ligated with a HpaII to BamHI fragment (encoding the m1 domain and G tail) from Gm1 which was prepared similarly. To create αm3G, the same α-encoding fragment described above was ligated with a DraI to BamHI fragment from Δm1,2 (encoding the m3 domain) and a BamHI to XhoI fragment (encoding the G tail) from the G mutant TMB (which has a BamHI site introduced at nucleotide 1483; reference 36).

Indirect Immunofluorescence Microscopy

COS-7 cells grown on coverslips were fixed, permeabilized, and stained 44 h posttransfection essentially as described (26, 27). For detection of E1 and mutant E1 proteins, an affinity-purified rabbit anti–peptide antiserum recognizing the COOH-terminus of E1 was the primary antibody (1:40, ~5 μg/ml), followed by Texas red-conjugated, affinity-purified goat anti–rabbit IgG (1:500; Jackson Immuno Research Laboratories Inc., Avondale, PA). For detection of G protein and mutant G proteins by double labeling, non-permeabilized fixed cells were first stained with a rabbit anti-VSV serum (1:200) followed by Texas red–conjugated, affinity-purified goat anti–rabbit IgG. After permeabilization with 0.5% Triton X-100, internal G protein was detected by staining with a monoclonal anti–G antibody (I1, 4 μg/ml; reference 23), followed by fluorescein-conjugated affinity-purified goat anti–mouse IgG (1:200, Jackson Immuno Research Laboratories Inc.). Cells expressing the chimeric αm proteins were stained with an affinity-purified rabbit anti–peptide antibody which recognizes the G cytoplasmic tail (1:20; reference 29) followed by the Texas red–conjugated second antibody described above. Cells were visualized with a Nikon Microphot microscope (Nikon Inc., Garden City, NJ) equipped with epifluorescence illumination and a Nikon 60× oil immersion plan apochromat objective. Photographs were taken with Tri-X Pan film (Eastman Kodak Co., Rochester, NY) and processed with Diafine developer (Accufine, Inc., Chicago, IL).

Radiolabeling and Immunoprecipitation

COS-7 cells expressing E1 and mutant E1 proteins, or G and mutant G proteins were labeled ~44 h posttransfection. E1 proteins were labeled for 1 or 2 h in 0.5 ml cysteine-free DME with 100 μCi [^{35}S]cysteine (1,300 Ci/mmol; Amersham Corp., Arlington Heights, IL). Cells were harvested immediately, or after a 3 h chase in regular growth medium containing a threefold excess of unlabeled cysteine. Cells were lysed in detergent solution (50 mM Tris, pH 8.0, 1% NP-40, 0.4% deoxycholate, 62.5 mM EDTA, and 0.13 TIU/ml aprotinin) and E1 proteins immunoprecipitated using the anti-

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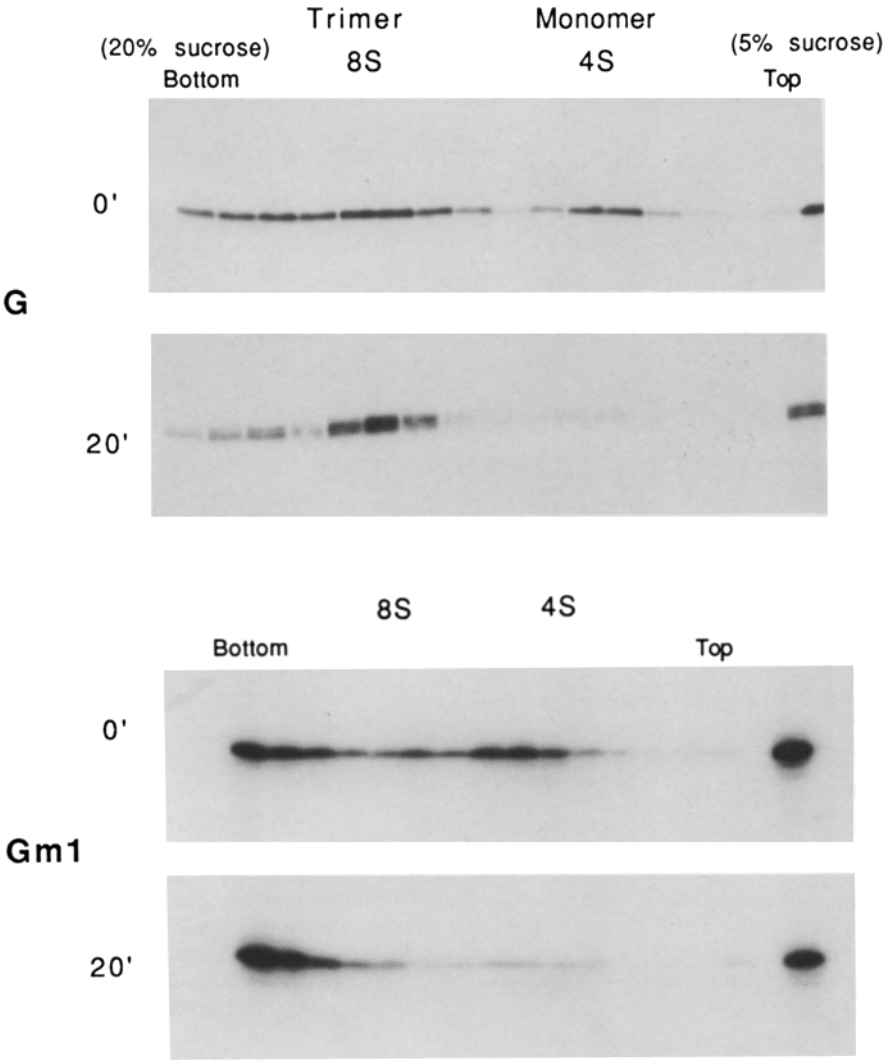

Figure 3. Gm1 forms an oligomer larger than a trimer. HeLa cells expressing either G or Gm1 were labeled for 10 min, and lysed immediately or after a 20 min chase in unlabeled cysteine. Lysates were centrifuged in 5 to 20% continuous sucrose gradients, and gradient fractions were immunoprecipitated with anti-VSV serum (see Materials and Methods). A portion (20%) of each cell lysate was immunoprecipitated directly, and run in the far right-hand lane of each gel. Although apparently a monomer after synthesis, Gm1 formed a large (>15S) aggregate during the chase.

peptide serum and fixed *Staphylococcus aureus* (Calbiochem-Behring Corp., San Diego, CA) as described previously (26).

For analysis of G proteins, HeLa cells (4 h postinfection) or COS-7 cells (44 h posttransfection) were incubated in cysteine-free medium for 10 min and then labeled for 30 min in 0.5 ml cysteine-free medium containing 50 μCi [^{35}S]cysteine. Cells were harvested immediately, or after various times of chase as above. Cells were lysed as above, and G proteins immunoprecipitated with either 3 μl of a polyclonal rabbit anti-VSV serum, 3 μl of a rabbit anti-peptide serum which recognizes the G cytoplasmic tail (27), or with 2 μl of mAbs I1 or I14 (23). To show that Gm1 spanned the membrane, HeLa cells were labeled for 10 min, scraped from the dish, dounced 50 times with a tight-fitting pestle, and treated with or without 100 μg/ml TPCK-trypsin (Boehringer-Mannheim Biochemicals, Indianapolis, IN) for 60 min at 0°C. PMSF was added to 50 mM, microsomes were solubilized in detergent solution as above, and G proteins immunoprecipitated with the polyclonal anti-VSV serum.

E1 proteins were electrophoresed in 15% polyacrylamide gels containing SDS, and G proteins were electrophoresed in 10% gels (22). Marker proteins were [^{14}C]methylated standard molecular weight markers (Amersham Corp.). Labeled proteins were detected by fluorography (2).

Analysis of Oligosaccharides

E1 oligosaccharides were analyzed after E1 proteins in transfected COS-7 cells were labeled for 2 h and chased for 3 h. *S. aureus* pellets were eluted, and aliquots were treated with endo H (0.1 mU; ICN Radiochemicals, Irvine, CA), *N*-glycanase (peptide:*N*-glycosidase F, 0.05 mU; Genzyme Corp., Boston, MA), or buffer alone using the protocol described previously (29).

For G proteins, the kinetics of oligosaccharide processing were determined in cells labeled for 10 min followed by various chase times. Immunoprecipitates were treated with endo H (0.1 mU) as described (28). Fluorograms were quantitated by densitometry.

Trimer Assay

Oligomerization of the Gm1 protein was analyzed by velocity gradient centrifugation in sucrose essentially as described (7). Continuous 5 to 20% sucrose gradients were poured over a 0.25 ml 60% sucrose cushion in SW50.1 tubes. All solutions were in 20 mM Tris, 30 mM MES, pH 5.8, 1% Triton X-100, 100 mM NaCl. HeLa cells expressing either G or Gm1 were labeled with [^{35}S]cysteine for 10 min and harvested immediately or after 20 min of chase in unlabeled cysteine. Lysates were loaded on top of the gradients and spun at 47,000 rpm for 16 h. Fractions (0.33 ml) were collected, immunoprecipitated with anti-VSV antibody, and electrophoresed to determine the location of G protein in the gradient.

Results

Retention of a Plasma Membrane Protein

In an earlier study (26), we found that deletion of the first and second membrane-spanning domains of E1 resulted in a mutant protein (Δm1,2) which was efficiently transported to the plasma membrane. However, when the second and third membrane-spanning domains were deleted (Δm2,3),

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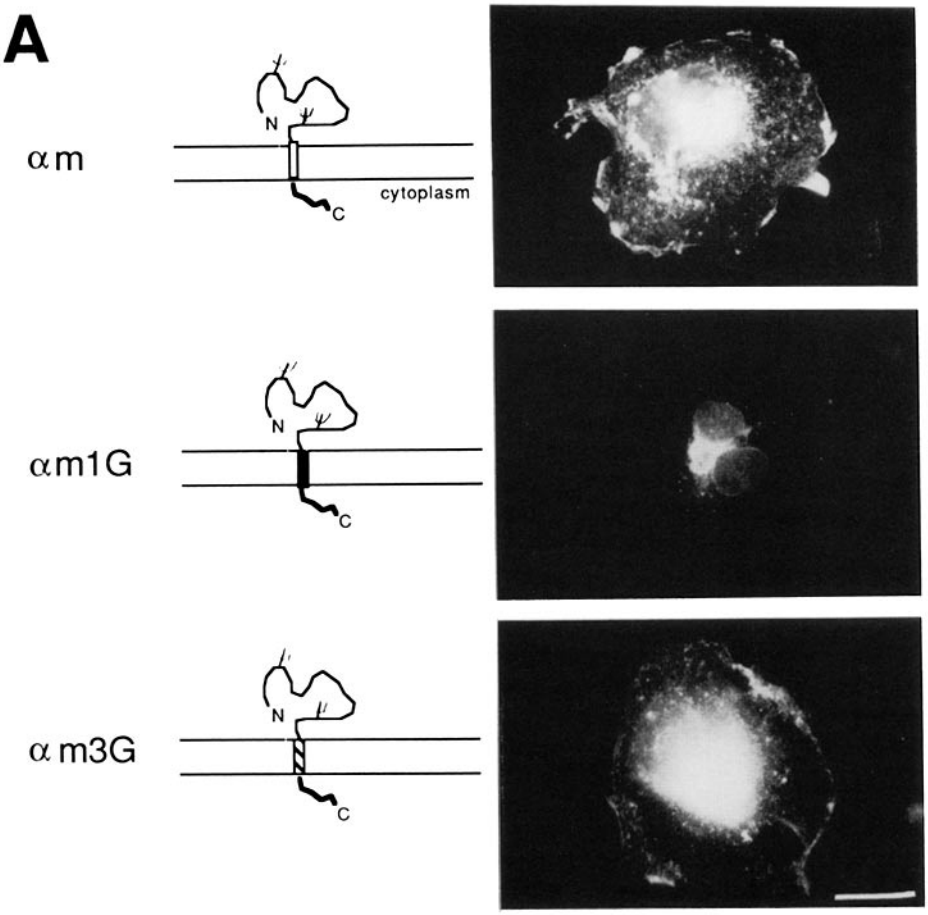

Figure 4. The m1 domain, but not the m3 domain, retains another plasma membrane protein in the Golgi region. (*A*) Transfected COS cells expressing αm, αm1G, or αm3G were fixed, permeabilized, and stained for indirect immunofluorescence microscopy with an anti-peptide which recognizes the G cytoplasmic tail, and a Texas red–conjugated second antibody. (*B*) The amino acid sequences (single letter code) are shown for the transmembrane domains of VSV G protein, and both the m1 and m3 domains of the IBV E1 protein. Bar, 10 μm.

the mutant protein was retained in the Golgi region of transfected cells. Both mutant proteins were inserted into and spanned the membrane properly. These results suggested that either the Golgi retention signal was in the first membrane spanning domain (m1), or that the deletion creating Δm1,2 disrupted a retention signal elsewhere in the molecule. To distinguish between these two possibilities, we asked whether m1 could retain a protein normally transported to the plasma membrane.

The G protein of vesicular stomatitis virus (VSV) is transported rapidly and efficiently to the plasma membrane in transfected cells, and much is known about its folding and oligomerization (7). The single membrane-spanning domain of the G protein was replaced with that of m1 from IBV E1 (Fig. 1 *A*). The domain replacement was performed precisely using oligonucleotide-directed mutagenesis. The chimeric G protein, called Gm1, was expressed transiently in COS cells using a SV-40–based vector (28), or in HeLa cells, using a vaccinia virus-T7 RNA polymerase expression system (10). Gm1 was recognized by polyclonal antibodies to both the G ectodomain and the cytoplasmic tail, and by two mAbs which recognize conformation-sensitive epitopes (Fig. 1 *B*, lanes *5–8*). In addition, Gm1 spanned the membrane since the cytoplasmic tail was susceptible to trypsin digestion in microsomal membranes (Fig. 1 *B*, lane *12*). These results indicated that m1 functioned as a proper membrane-spanning domain in Gm1, and that the chimeric protein was not grossly misfolded.

Gm1 was not transported to the plasma membrane, however. Indirect immunofluorescence microscopy demonstrated that Gm1 was absent from the cell surface but present in a juxtanuclear region consistent with Golgi localization (Fig. 2). In addition, the two *N*-linked oligosaccharides added to Gm1 were not processed to an endo H–resistant form as they

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were on wild-type G protein. After a 10 min pulse label, wild-type G protein became endo H resistant with a half-time of about 20 min, whereas Gm1 was endo H sensitive even after 4 h of chase (see Fig. 10). This suggested that the Gm1 protein was retained in a pre-medial Golgi compartment, like the wild-type E1 protein.

G protein has been shown to form a noncovalently associated homotrimer before its exit from the ER (6). We tested the oligomeric structure of Gm1 on sucrose gradients after a pulse-chase label (Fig. 3). After a 10 min label, wild-type G protein was ~50% trimer (8S) and 50% monomer (4S), consistent with the results of Doms et al. (6, 7). After 20 min of chase, all the G protein was found in the 8S trimer peak. Although apparently a monomer after the 10 min label, Gm1 pelleted after the 20 min chase. Other centrifugation conditions suggested this oligomer was between 15 and 20S (data not shown). Several mutant G proteins that are grossly misfolded were also shown to pellet under the standard gradient conditions (7; and unpublished results), but unlike Gm1, they pelleted immediately after the pulse label. The simplest interpretation of our results is that Gm1 was retained specifically by the m1 sequence. However, we cannot distinguish whether inability to trimerize resulted in retention of Gm1 in a subcompartment of the ER (near the Golgi region) or if the large oligomers (with or without other proteins) were the result of specific retention in the Golgi complex. These points will be discussed below.

Retention of Another Plasma Membrane Protein

In addition to the VSV G protein, the m1 sequence was also able to retain another plasma membrane protein called αm. The αm protein consists of the alpha subunit of human chorionic gonadotropin fused to the membrane-spanning domain and cytoplasmic tail of VSV G (16), and appears to be transported to the cell surface as a monomer (17). In this case, we were able to replace the single membrane-spanning domain of αm with either m1 or the third membrane-spanning domain (m3) from the E1 protein using restriction sites in the coding sequence. These chimeric proteins are termed αm1G and αm3G, respectively. Whereas αm3G was transported to the plasma membrane like the parent molecule, αm1G was retained in the Golgi region of transfected COS cells (Fig. 4). The two *N*-linked oligosaccharides on αm1G remained endo H sensitive, whereas those on αm and αm3G were processed to an endo H–resistant form (data not shown).

Point Mutations in m1 Release Retention of Δm2,3, but not the Full-length E1 Protein

We attempted to define the sequence requirements for retention of E1. The amino acid sequence of m1 is not unusual for a membrane-spanning domain (Fig. 4 *B*). When comparing the sequences of four E1 proteins from different coronaviruses however (18), we noticed that the polar uncharged residues spaced throughout the m1 domain were conserved (Fig. 5 *A*). These polar residues line up on one side of a predicted alpha helix when the sequence is modeled. We asked if three of these polar residues (Asn22, Thr33, and Gln37) were required for proper targeting of E1 by changing them individually or in combination to hydrophobic isoleucines. In addition, we inserted two isoleucines in the middle of m1 to disrupt the potential amphipathicity of the helix. We also changed one of the conserved hydrophobic residues (Leu30) to a polar Gln (see Fig. 5 *A* for a summary of mutations). The mutations were introduced into both the wild-type E1 protein and the mutant protein Δm2,3, which has only the first of the three membrane-spanning domains and is retained in the Golgi region like the wild-type protein.

The mutant proteins were all inserted into the membrane and glycosylated as shown by immunoprecipitation from [^{35}S]cysteine-labeled transfected COS cells (Fig. 5 *B*). Mutation of Asn22 (NI_{22}) reduced the amount of fully glycosylated protein (most had one *N*-linked oligosaccharide instead of two), perhaps by conformationally altering the amino-terminal domain. Localization of the mutant proteins was determined by indirect immunofluorescence microscopy. With the exception of the Leu30 to Gln change (LQ_{30}), all of the mutations introduced into the full-length E1 protein appeared to hinder transport out of the ER (Fig. 6). This is seen by the reticular staining pattern which includes nuclear envelope. This suggested that the Ile mutations might be disrupting proper folding of the E1 protein, perhaps by interfering with association of m1 with the other two membrane-spanning domains, or with insertion into the membrane. In contrast, the LQ_{30} mutation had no apparent effect on targeting of E1. Since mutating the polar residues to Ile prevented the exit of the mutant proteins from the ER, we were not able to assess their effects on retention in the Golgi complex.

The same mutations were tested in the Δm2,3 background, where m1 is the only membrane-spanning domain. In this case, all of the Ile mutations, including the two amino acid insertion, resulted in transport of the proteins to the plasma membrane with varying efficiency (Fig. 7). The Leu to Gln mutation ($\Delta m2,3/LQ_{30}$) again had no effect on targeting of Δm2,3.

These results were confirmed and quantitated by analyzing the processing of the *N*-linked oligosaccharides. The two oligosaccharides of the nonretained E1 mutant protein Δm1,2 are processed to an endo H-resistant, polylactosamine-containing form as the protein is transported through the Golgi complex to the plasma membrane (27). Since the processed form is heterogeneous, it is difficult to quantitate on SDS gels. To determine the fraction of each protein with processed oligosaccharides, we subtracted the amount of unprocessed material (endo H sensitive) from the total (*N*-glycanase sensitive; Fig. 8). As shown in the quantitation below the lanes, $\Delta m2,3/QI_{37}$, and Δm2,3/ins were the most efficiently transported, with 90 and 85% endo H–resistant oligosaccharides after a 3-h chase, respectively.

Point Mutations in m1 Release Retention of Gm1

Our results suggested that m1 was indeed a retention signal when it was the only membrane-spanning domain in the protein. To confirm that the chimeric VSV G protein Gm1 was retained specifically by the m1 sequence, we introduced the two mutations found to release retention of Δm2,3 most efficiently (QI_{37} and mlins). Both Gm1QI and Gmlins were transported to the plasma membrane, as shown by indirect immunofluorescence (Fig. 9). Both of these proteins were transported efficiently, but less rapidly than wild-type G protein, as shown by the half times of oligosaccharide processing (Fig. 10). Gm1QI and Gmlins were processed with half times of 25 and 35 min, respectively, as compared to 18 min for wild-type G protein. When assayed for oligomerization

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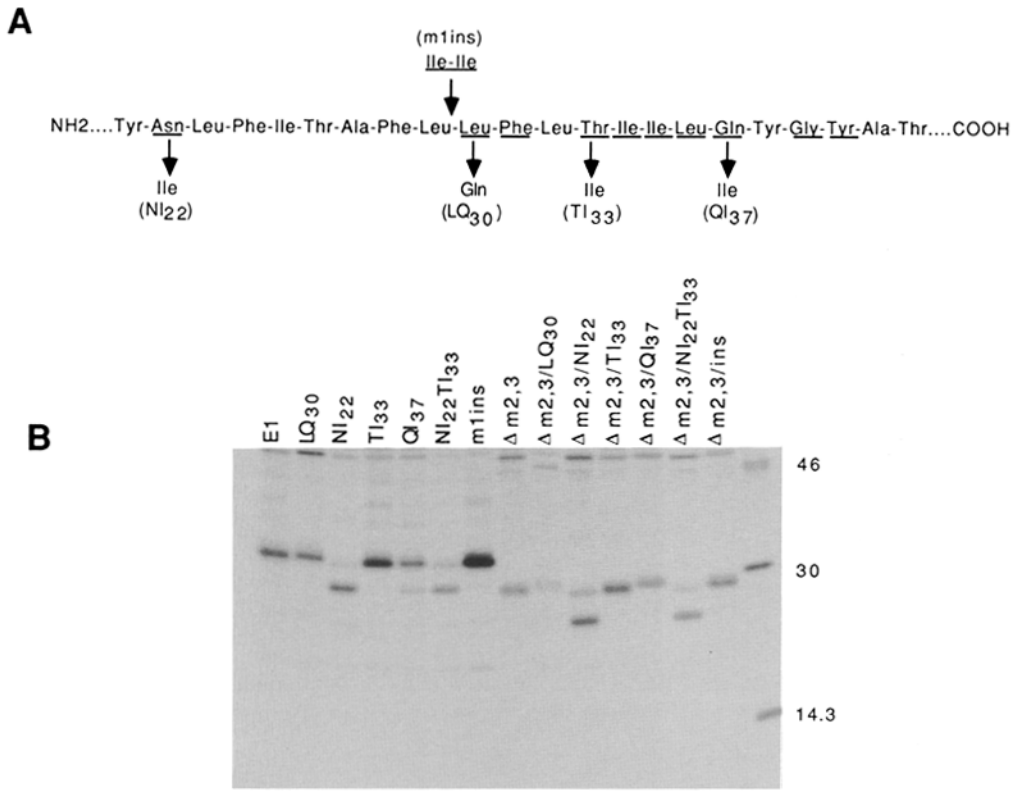

Figure 5. Point mutations in the m1 domain. (*A*) The amino acid sequence of the m1 domain of IBV E1 is shown with residues conserved in at least three of four coronavirus E1 proteins underlined. The mutations introduced into this sequence by oligonucleotide-directed mutagenesis are also shown (with mutant protein name in parentheses). (*B*) Transfected COS cells expressing each of the mutant E1 proteins were labeled for 1 h with [^{35}S]cysteine, and lysates were immunoprecipitated with anti-E1 antibody. Molecular weight standards ($\times 10^{-3}$) are shown on the right. Only one of the two *N*-linked oligosaccharide addition sites was efficiently used when Asn22 was changed to Ile (NI_{22}).

on sucrose gradients, both GmlQI and Gmlins were found to form normal trimers (not shown). These results suggest that Gml is retained specifically via the m1 sequence and not nonspecifically because of misfolding.

Discussion

A Membrane-spanning Domain Contains a Golgi Retention Signal

In this paper, we have shown that the first of the three membrane-spanning domains of the model Golgi protein IBV E1 contains a signal for retention. When the single membrane-spanning domain of a model plasma membrane protein (VSV G) was replaced with the m1 sequence from E1, the chimeric Gml was retained in the Golgi region of transfected cells. Another plasma membrane protein (αm), was also retained in the Golgi region when m1 replaced the normal membrane-spanning domain, but not when m3 was inserted. The uncharged polar residues that line one face of the alpha helix predicted for m1 seem to be the important feature of this sequence. Changing any of three polar residues to an Ile, or insertion of two Ile residues into the middle of m1 in the E1 protein Δm2,3 (with the second and third membrane-spanning domains deleted) resulted in transport to the plasma membrane. Thus, m1 was shown to be necessary and sufficient for Golgi retention when it was the only membrane-spanning domain in the protein. With further mutagenesis, we are in the process of determining whether the exact sequence of polar residues is required for Golgi retention, or if the polar nature of one side of the helix is the important feature.

Several possibilities could explain our inability to release retention of the full-length E1 protein with the mutations we introduced. First, certain mutations in m1 might affect assembly of the protein in the membrane because of the proximity of the m2 and m3 domains (we are unable to predict these interactions). Second, there could be a sequence in addition to m1 that is involved in retention of full-length E1 (perhaps in m2). Third, retention of E1 could occur via a different mechanism than retention of Δm2,3 and Gml. Replacing the polar residues in m1 with a hydrophobic residue containing a smaller side chain than isoleucine might be less disruptive to folding and assembly of E1, and allow us to assess the contribution of m1 to retention of E1. We have recently replaced Gln37 in the full-length E1 protein with alanine, and this replacement appears to release retention and allow transport to the plasma membrane (unpublished results). We therefore favor the interpretation that m1 is involved in retention of full-length E1 as well as Δm2,3 and Gml. However, more mutations need to be analyzed before concluding that the m1 sequence in the full-length E1 protein is fully responsible for retention.

The Gml chimeric protein provides strong evidence that m1 contains retention information. The finding that two of the mutations in m1 that released retention of Δm2,3 (Δm2,3/QI_{37} and Δm2,3/ins) also allowed efficient transport of Gml to the cell surface supports the idea that Gml is retained specifically. It is unlikely that the single glutamine to isoleucine change in the middle of the membrane-spanning domain of GmlQI would prevent misfolding of Gml. It is more likely that this glutamine residue is a key component of the retention signal, and its replacement eliminates retention.

It should be noted that the only protein whose localization we have determined at the electron microscope level is the wild-type E1 protein. Although both the immunofluores-

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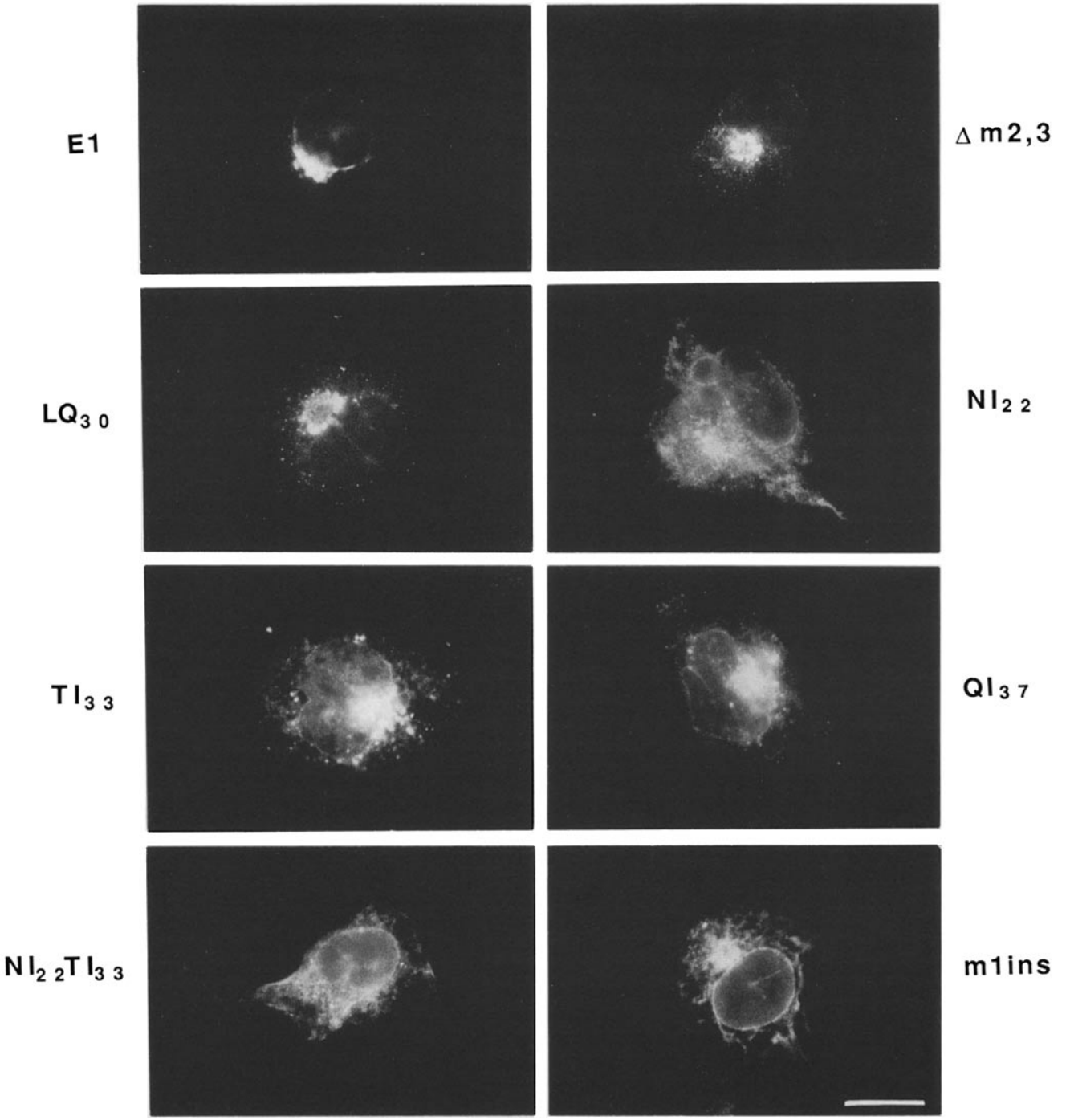

Figure 6. Indirect immunofluorescence microscopy of mutant E1 proteins. Transfected COS cells were fixed, permeabilized, and stained for E1 using the anti-peptide antibody which recognizes the E1 cytoplasmic tail, followed by a Texas red–conjugated second antibody. With the exception of LQ_{30}, all of the mutation appeared to hinder exit of the mutant proteins from the ER. Bar, 10 μm.

cence data and the endo H–sensitive oligosaccharides on Δm2,3 and Gm1 are consistent with *cis*-Golgi localization, absolute verification awaits immunoelectron microscopy.

The polar residues that were mutated are conserved in the E1 proteins from four different coronaviruses (18). Although there is little amino acid conservation overall in these proteins, the membrane topology is predicted to be the same. The first and second membrane-spanning domains show the highest overall region of identity (18). This suggests an important function for this region, and our results indicate this might be intracellular retention.

Contrasting results with an E1 protein from a different coronavirus, mouse hepatitis virus, have recently been reported by Armstrong et al. (1). They found that an E1 protein lacking the carboxy-terminal 40 amino acids, as well as a deletion mutant comparable to our Δm1,2 was not retained in the Golgi region. (Although these proteins were not retained in the Golgi region, they were detected in lysosomes,

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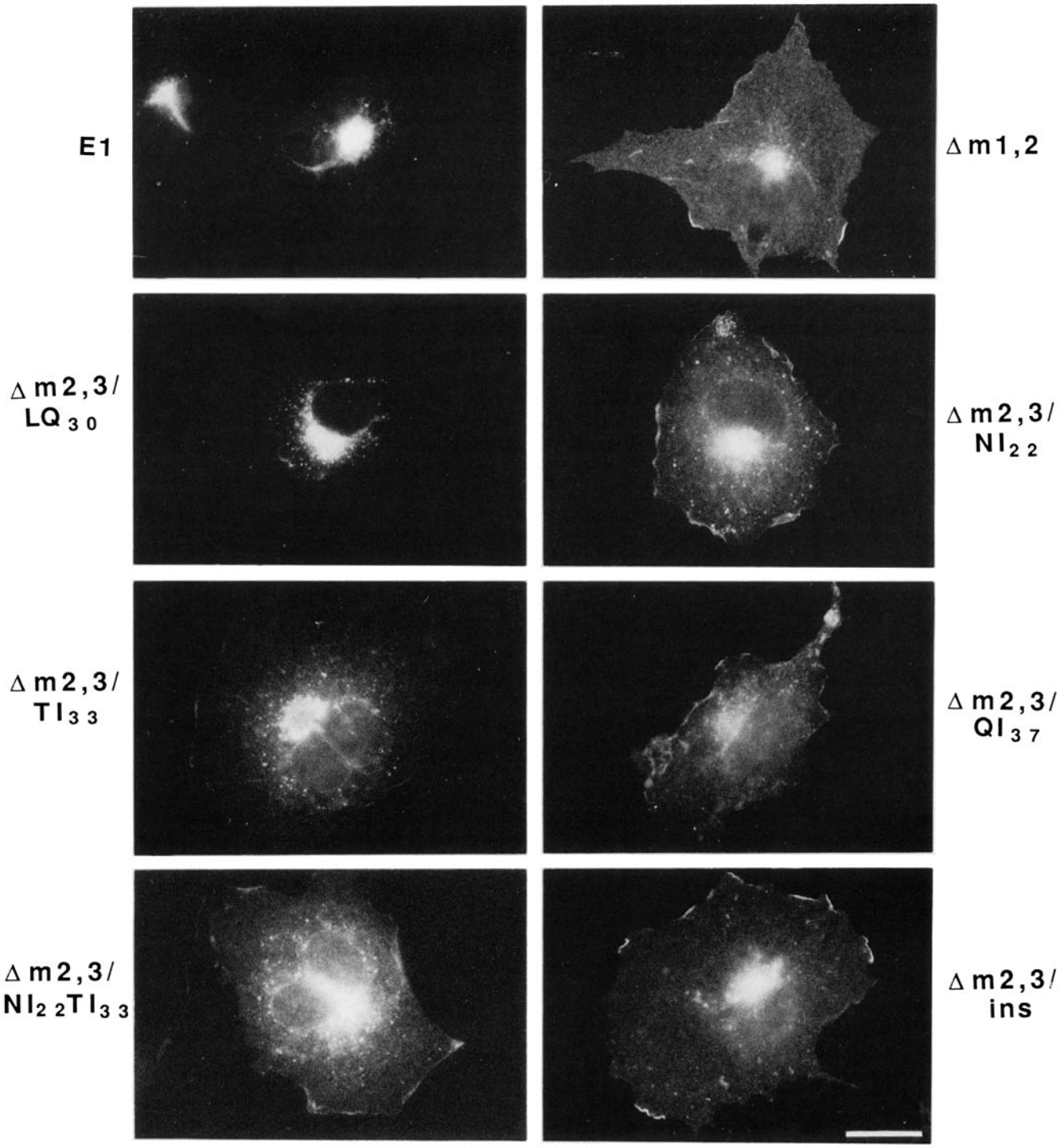

Figure 7. Indirect immunofluorescence microscopy of Δm2,3 with mutations in the m1 domain. Transfected COS cells were stained as in Fig. 6. When m1 was the only membrane-spanning domain in the protein, all of the Ile mutations resulted in transport of the mutant proteins to the plasma membrane. Mutation of Leu30 to Gln (*Δm2,3/LQ30*) had no effect on Golgi localization. Bar, 10 μm.

not at the plasma membrane). We have produced a number of deletions in the cytoplasmic tail of IBV E1, and have seen no effect on Golgi localization (unpublished results). Although localization of the mouse hepatitis virus E1 protein in transfected cells at the electron microscopic level has not been reported, the protein appears to acquire carbohydrate modifications characteristic of the late Golgi region when expressed in COS cells (39). Thus, it is possible that the mouse hepatitis virus E1 protein reaches a later Golgi subcompartment than the IBV E1 protein, and that the retention mechanism could be different. Interestingly, both TGN38 (a *trans*-Golgi network protein; reference 25) and Kex2p (believed to be a late Golgi protease in *S. cerevisiae*; reference 11) are not retained in the Golgi complex when their cyto-

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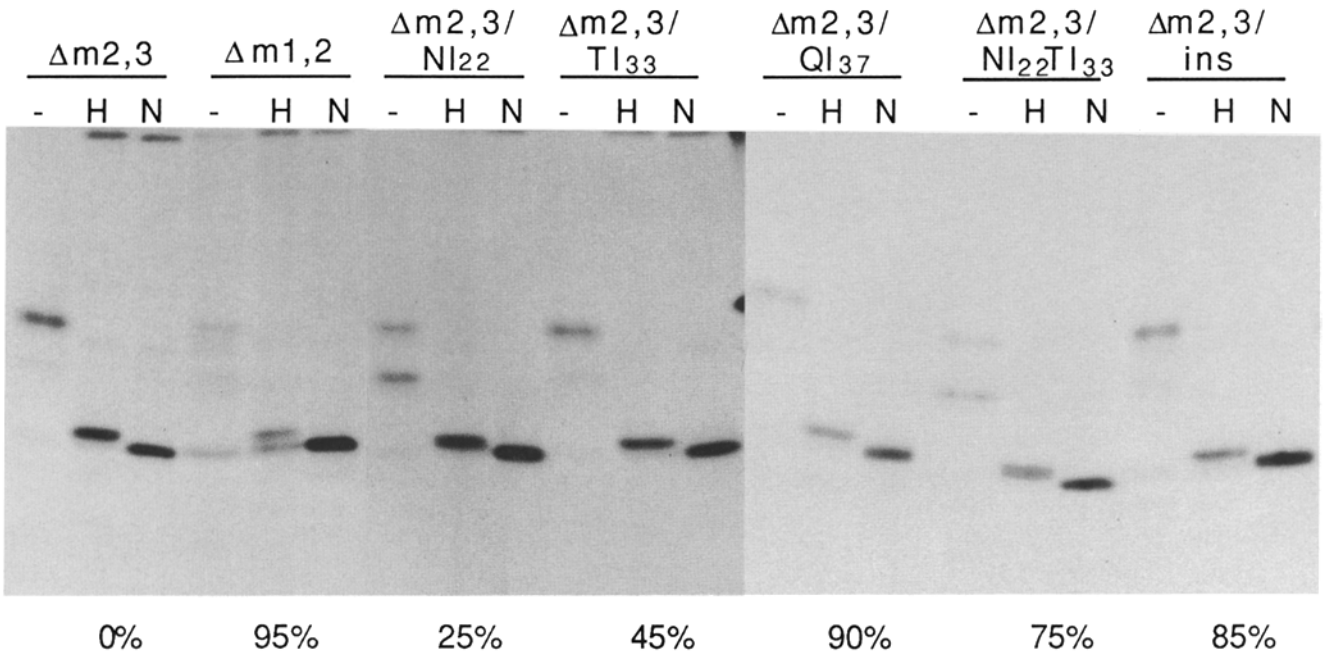

Figure 8. Analysis of the *N*-linked oligosaccharides on mutant E1 proteins. Transfected COS cells were labeled with [^{35}S]systeine for 2 h, and chased in unlabeled cysteine for 3 h. Lysates were immunoprecipitated with anti-E1 antibody, and precipitates were eluted and split into three aliquots. These were untreated (−), digested with endo H (*H*), or digested with *N*-glycanase (*N*) to remove all of the carbohydrate. Fluorograms were quantitated by densitometry, and the amount of processed (polylactosaminoglycan containing) oligosaccharides was determined by subtracting the amount of unprocessed material (endo H sensitive) from the total (*N*-glycanase sensitive). The percentage of E1 with processed oligosaccharides is shown below each set of three lanes.

plasmic tails are deleted. This suggests that retention of membrane proteins in the *trans*-Golgi network may involve sequences on the cytoplasmic side of the membrane.

Mechanism of Retention

How might a sequence buried in the lipid bilayer function in retention? The Lys-Asp-Glu-Leu (KDEL) sequence found at the carboxy-terminus of a group of soluble resident ER proteins (HDEL in *S. cerevisiae*) has been shown to be necessary for their retention in the ER (30, 34). Recently, putative receptors which recognize these sequences have been identified (24, 42, 48). It has been suggested that these receptors retrieve escaped ER residents from a pre-Golgi compartment termed the salvage compartment (19, 34). If the m1 sequence is recognized by a receptor, it would have to be a transmembrane receptor whose own membrane-spanning domain recognized m1. As predicted from the structure of the photosynthetic reaction center of *Rsp. viridis* (5), polar residues in membrane-spanning domains might be expected to interact with other polar amino acids, and hydrophobic residues with membrane lipids. Although the oligomeric structure of Gm1 suggests that it could be associated with one or more different proteins, to date we have been unable to detect other proteins by co-immunoprecipitation or cross-linking.

Another, nonreceptor-mediated mechanism of retention is also conceivable. A structural change such as aggregation could occur when a protein arrives in a new compartment, preventing movement into transport vesicles. This type of retention has been demonstrated for many mutant proteins that fail to fold correctly after synthesis, resulting in retention in the ER (37). The *cis*-Golgi is thought to differ from the ER in several ways, most notably in lipid composition and divalent cation concentration. The early Golgi is the first compartment where a newly synthesized protein comes into contact with glucosylceramide and sphingomyelin, which are synthesized there (12, 13). In addition, the Ca^{2+} concentration is presumed to be significantly lower than in the ER (3). We are currently analyzing the oligomeric structure of the E1 protein and the mutant protein Δm2,3. Since Gm1 is found in an oligomer greater than 15S, the possibility exists that retention of these proteins occurs indirectly via aggregation. We are using both the E1 protein mutants and the Gm1 mutants to address the possible direct (receptor mediated) or indirect mechanisms of retention.

Implications for Retention of Endogenous Golgi Proteins

Since E1 is a viral protein, our results need to be confirmed for endogenous Golgi proteins. Unfortunately, cDNAs are not yet available for endogenous *cis*-Golgi proteins, although cDNAs encoding *trans*-Golgi glycosyltransferases have been isolated (reviewed in 33). All Golgi glycosyltransferases that have been sequenced have a "type II" membrane topology, with the amino terminus in the cytoplasm, the carboxy terminus in the lumen, and an uncleaved signal sequence which also serves as the membrane-spanning domain (33). There is no obvious sequence homology between the m1 domain of E1 and the membrane-spanning domains of these proteins. However, there are several observations which support the idea that sequences associated with the lipid bilayer might be involved in retention of proteins in the *trans*-Golgi complex. Colley et al. (4) have shown that α2,6 sialyl transferase is efficiently secreted from transfected cells if a cleavable signal sequence is engineered in place of the normal signal an-

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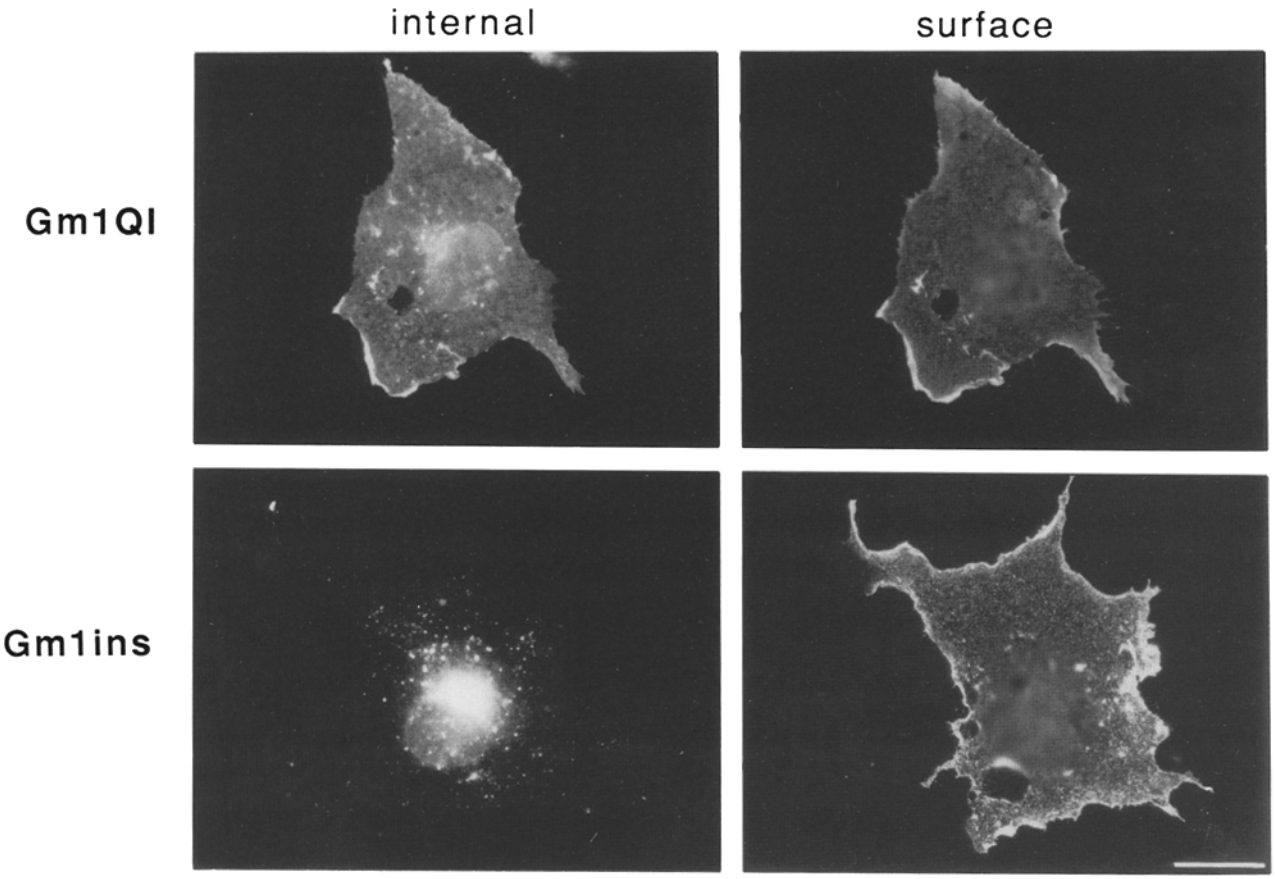

Figure 9. Two different mutations in the membrane-spanning domain of Gm1 release retention from the Golgi region. The conserved Gln (Gln 37 in E1) was changed to Ile (*Gm1QI*), and the 2 Ile residue insert was introduced into Gm1 (*Gm1ins*). Double-label indirect immunofluorescence microscopy was performed as described in Fig. 2. Both Gm1QI and Gm1ins were readily detected on the plasma membrane. Bar, 10 μM.

chor. Russo et al. (40) have found that the amino terminus of β1,4 galactosyl transferase (including the cytoplasmic tail and membrane-spanning domain) is sufficient to target a marker protein, pyruvate kinase, to Golgi membranes (40; Russo, R. N., N. L. Shaper, and J. H. Shaper, manuscript in preparation). In addition, no lumenal, nonmembrane-bound Golgi proteins have yet been identified. All of these observations are consistent with the idea that sequences bu-

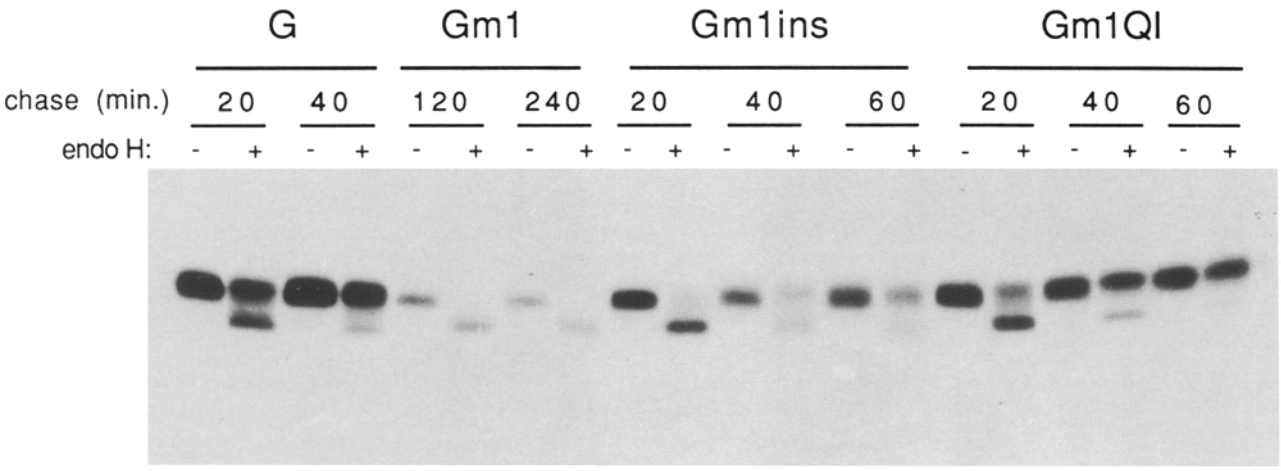

Figure 10. Rate of transport of Gm1QI and Gm1ins through the Golgi. Transfected COS cells expressing G, Gm1, Gm1QI, or Gm1ins were labeled for 10 min with [^{35}S]cysteine, and chased in unlabeled cysteine for the times shown. Lysates were immunoprecipitated, and left untreated (−) or digested with endo H (+). The oligosaccharides on both Gm1QI and Gm1ins are processed to an endo H–resistant form efficiently, but somewhat more slowly than those on the wild-type G protein.

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ried in the lipid bilayer of the Golgi are important for retention in this organelle. Further experiments using the IBV E1 protein as a model for Golgi retention should provide additional insight into the fundamental problem of protein sorting in the exocytic pathway.

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References

1. Armstrong, J., S. Patel, and P. Biddle.1990. Lysosomal sorting mutants of coronavirus E1 protein, a Golgi membrane protein. *J. Cell Sci.* 95:191–197.
2. Bonner, W. M., and R. A. Laskey. 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* 46:83–88.
3. Carafoli, E. 1987. Intracellular calcium homeostasis. *Annu. Rev. Biochem.* 56:395–433.
4. Colley, K. J., E. U. Lee, B. Adler, J. K. Browne, and J. C. Paulson. 1989. Conversion of a Golgi apparatus sialyltransferase to a secretory protein by replacement of the NH_2-terminal signal anchor with a signal peptide. *J. Biol. Chem.* 264:17619–17622.
5. Deisenhofer, J., and H. Michel. 1989. The photosynthetic reaction center from the purple bacterium *rhodopseudomonas viridis*. *Science (Wash. DC).* 245:1463–1473.
6. Doms, R. W., D. S. Keller, A. Helenius, and W. E. Balch. 1987. Role for ATP in regulating the assembly and transport of vesicular stomatitis virus G protein trimers. *J. Cell Biol.* 105:1957–1969.
7. Doms, R. W., A. Ruusala, C. Machamer, J. Helenius, A. Helenius, and J. K. Rose. 1988. Differential effects of mutations in three domains on folding, quaternary structure, and intracellular transport of vesicular stomatitis virus G protein. *J. Cell Biol.* 107:89–99.
8. Dunphy, W. G., and J. E. Rothman. 1983. Compartmentation of asparagine-linked oligosaccharide processing in the Golgi apparatus. *J. Cell Biol.* 97:270–275.
9. Farquhar, M. G. 1985. Progress in unraveling pathways of Golgi traffic. *Annu. Rev. Cell Biol.* 1:447–488.
10. Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss. 1986. Eukaryotic transient expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc. Natl. Acad. Sci. USA.* 83:8122–8126.
11. Fuller, R. S., A. J. Brake, and J. Thorner. 1989. Intracellular targeting and structural conservation of a prohormone-processing endoprotease. *Science (Wash. DC).* 246:482–486.
12. Futerman, A. H., and R. E. Pagano. 1991. Determination of the intracellular sites and topology of glucosylceramide synthesis in rat liver. *Biochem. J.* In press.
13. Futerman, A. H., B. Stieger, A. L. Hubbard, and R. E. Pagano. 1990. Sphingomyelin synthesis in rat liver occurs predominantly at the cis and medial cisternae of the Golgi apparatus. *J. Biol. Chem.* 265:8650–8657.
14. Gabathuler, R., and S. Kvist. 1990. The endoplasmic reticulum retention signal of the E3/19K protein of adenovirus type 2 consists of three separate amino acid segments at the carboxy terminus. *J. Cell Biol.* 111:1803–1810.
15. Griffiths, G., and K. Simons. 1986. The trans Golgi network: sorting at the exit site of the Golgi complex. *Science (Wash. DC).* 234:438–443.
16. Guan, J.-L., H. Cao, and J. K. Rose. 1988. Cell-surface expression of a membrane-anchored form of the human chorionic gonadotropin alpha subunit. *J. Biol. Chem.* 263:5306–5313.
17. Guan, J.-L., A. Ruusala, H. Cao, and J. K. Rose. 1988. Effects of altered cytoplasmic domains on transport of the vesicular stomatitis virus glycoprotein are transferable to other proteins. *Mol. Cell Biol.* 8:2869–2874.
18. Kapke, P. A., F. Y. T. Tung, B. G. Hogue, D. A. Brian, R. D. Woods, and R. Wesley. 1988. The amino-terminal signal peptide on the porcine transmissible gastroenteritis coronavirus matrix protein is not an absolute requirement for membrane translocation and glycosylation. *Virology.* 165:367–375.
19. Kelly, R. B. 1990. Tracking an elusive receptor. *Nature (Lond.).* 345:480–481.
20. Kornfeld, S. 1990. Lysosomal enzyme targeting. *Biochem. Soc. Trans.* 18:367–374.
21. Kunkel, T. A., J. D. Roberts, and R. A. Zakour. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* 154:367–382.
22. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
23. Lefrancois, L., and D. S. Lyles. 1982. The interaction of antibody with the major surface glycoprotein of vesicular stomatitis virus. *Virology.* 121: 168–174.
24. Lewis, M. J., and H. R. B. Pelham. 1990. A human homologue of the yeast HDEL receptor. *Nature (Lond.).* 348:162–163.
25. Luzio, J. P., B. Brake, G. Banting, K. E. Howell, P. Braghetta, and K. K. Stanley. 1990. Identification, sequencing and expression of an integral membrane protein of the trans Golgi network (TGN38). *Biochem. J.* 270: 97–102.
26. Machamer, C. E., and J. K. Rose. 1987. A specific transmembrane domain of a coronavirus E1 glycoprotein is required for its retention in the Golgi region. *J. Cell Biol.* 105:1205–1214.
27. Machamer, C. E., and J. K. Rose. 1988. Influence of new glycosylation sites on expression of the vesicular stomatitis virus G protein at the plasma membrane. *J. Biol. Chem.* 263:5948–5954.
28. Machamer, C. E., R. Z. Florkiewicz, and J. K. Rose. 1985. A single N-linked oligosaccharide at either of the two normal sites is sufficient for transport of vesicular stomatitis virus G protein to the cell surface. *Mol. Cell Biol.* 5:3074–3083.
29. Machamer, C. E., S. A. Mentone, J. K. Rose, and M. G. Farquhar. 1990. The E1 glycoprotein of an avian coronavirus is targeted to the cis Golgi complex. *Proc. Natl. Acad. Sci. USA.* 87:6944–6948.
30. Munro, S., and H. R. B. Pelham. 1987. A C-terminal signal prevents secretion of luminal ER proteins. *Cell.* 48:899–907.
31. Nilsson, T., M. Jackson, and P. Peterson. 1989. Short cytoplasmic sequences serve as retention signals for transmembrane proteins in the endoplasmic reticulum. *Cell.* 58:707–718.
32. Palade, G. E. 1975. Intracellular aspects of the processing of protein synthesis. *Science (Wash. DC).* 189:347–358.
33. Paulson, J. C., and K. J. Colley. 1989. Glycosyltransferases. Structure, localization, and control of cell type-specific glycosylation. *J. Biol. Chem.* 264:17615–17618.
34. Pelham, H. R. B. 1989. Control of protein exit from the endoplasmic reticulum. *Annu. Rev. Cell Biol.* 5:1–23.
35. Pfeffer, S. R., and J. E. Rothman. 1987. Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi. *Annu. Rev. Biochem.* 56:829–852.
36. Puddington, L., C. E. Machamer, and J. K. Rose. 1986. Cytoplasmic domains of cellular and viral integral membrane proteins substitute for the cytoplasmic domain of the vesicular stomatitis virus glycoprotein in transport to the plasma membrane. *J. Cell Biol.* 102:2147–2157.
37. Rose, J. K., and R. W. Doms. 1988. Regulation of protein export from the endoplasmic reticulum. *Annu. Rev. Cell Biol.* 4:257–288.
38. Rose, J. K., L. Buonocore, and M. Whitt. 1991. A new cationic liposome reagent mediating nearly quantitative transfection of animal cells. *Biotechniques.* 10:520–525.
39. Rottier, P. J. M., and J. K. Rose. 1987. Coronavirus E1 glycoprotein expressed from cloned cDNA localizes in the Golgi region. *J. Virol.* 61:2042–2045.
40. Russo, R. N. 1990. Two forms of beta-1,4-galactosyltransferase: analysis of membrane orientation and Golgi retention. Ph.D. thesis. The Johns Hopkins University.
41. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning. A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 545 pp.
42. Semenza, J. C., K. G. Hardwick, N. Dean, and H. R. B. Pelham. 1990. ERD2, a yeast gene required for the receptor-mediated retrieval of luminal ER proteins from the secretory pathway. *Cell.* 61:1349–1357.
43. Simons, K., and A. Wandinger-Ness. 1990. Polarized sorting in epithelia. *Cell.* 62:207–210.
44. Sprague, J., H. Condra, H. Arnheiter, and R. A. Lazzarini. 1983. Expression of a recombinant DNA gene coding for the vesicular stomatitis virus nucleocapsid protein. *J. Virol.* 45:773–781.
45. Tooze, J., S. A. Tooze, and G. Warren. 1984. Replication of coronavirus MHV-A59 in sac$^-$ cells: Determination of the first site of budding of progeny virions. *Eur. J. Cell Biol.* 33:281–293.
46. Walter, P., and V. R. Lingappa. 1986. Mechanism of protein translocation across the endoplasmic reticulum membrane. *Annu. Rev. Cell Biol.* 2:499–516.
47. Wieland, F. T., M. L. Gleason, T. O. Serafini, and J. E. Rothman. 1987. The rate of bulk flow from the endoplasmic reticulum to the cell surface. *Cell.* 50:289–300.
48. Vaux, D., J. Tooze, and S. Fuller. 1990. Identification by anti-idiotype antibodies of an intracellular membrane protein that recognizes a mammalian endoplasmic reticulum retention signal. *Nature (Lond.).* 345:495–502.

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