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Targeting of a Heterodimeric Membrane Protein Complex to the Golgi: Rubella Virus E2 Glycoprotein Contains a Transmembrane Golgi Retention Signal

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Rubella virus (RV) envelope glycoproteins, E2 and E1, form a heterodimeric complex that is targeted to medial/trans-Golgi cisternae. To identify the Golgi targeting signal(s) for the E2/E1 spike complex, we constructed chimeric proteins consisting of domains from RV glycoproteins and vesicular stomatitis virus (VSV) G protein. The location of the chimeric proteins in stably transfected Chinese hamster ovary cells was determined by immunofluorescence, immunoelectron microscopy, and by the extent of processing of their N-linked glycans. A trans-dominant Golgi retention signal was identified within the C-terminal region of E2. When the transmembrane (TM) and cytoplasmic (CT) domains of VSV G were replaced with those of RV E2, the hybrid protein (G-E2$_{TMCT+}$) was retained in the Golgi. Transport of G-E2$_{TMCT+}$ to the Golgi was rapid ($t_{1/2}$=10–20 min). The G-E2$_{TMCT+}$ protein was determined to be distal to or within the medial Golgi based on acquisition of endo H resistance but proximal to the trans-Golgi network since it lacked sialic acid. Deletion analysis revealed that only the TM domain of E2 was required for Golgi targeting. Although the cytoplasmic domain of E2 was not necessary for Golgi retention, it was required for efficient transport of VSV G-RV chimeras out of the endoplasmic reticulum. When assayed in sucrose velocity sedimentations gradients, the Golgi-retained G-E2$_{TMCT+}$ protein behaved as a dimer. Unlike virtually all other Golgi targeting signals, the E2 TM domain does not contain any polar amino acids. The TM and CT domains of E1 were not required for targeting of E2 and E1 to the Golgi indicating that a heterodimer of two integral membrane proteins can be retained in the Golgi by a single retention signal.

INTRODUCTION

Targeting of proteins for retention in specific organelles of the exocytic pathway is signal-dependent. For example, some lumenal resident proteins are maintained in the endoplasmic reticulum (ER)[1] by a C-terminal tetrapeptide (Pelham, 1989), and several ER membrane proteins are retained by cytoplasmically disposed peptide sequences (Jackson *et al.*, 1993; Nilsson *et al.*, 1989; Schutze *et al.*, 1994), via receptor-mediated retrieval systems. Similarly, localization of membrane proteins such as TGN38 and furin to the trans-Golgi network (TGN) appears to be a dynamic process that is in part dependent upon a cytoplasmic retrieval motif (Bos *et al.*, 1993; Bosshart *et al.*, 1994; Humphrey *et al.*, 1993). Recently a Golgi localization signal that apears to operate via a different mechanism than the cytoplasmic retrieval motif was found in the transmembrane (TM) domain of TGN38 (Ponnam-

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[1] Abbreviations used: CHO, Chinese hamster ovary; CT, cytoplasmic; endo H, endoglycosidase H; ER, endoplasmic reticulum; Man II, α-mannosidase II; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RV, rubella virus; SDS, sodium dodecyl sulfate; SFV, Semliki Forest virus; TGN, trans-Golgi network; TM, transmembrane; VSV, vesicular stomatitis virus.

balam *et al.*, 1994). Considerably less is known about how proteins are retained in the stacks of the Golgi complex although transmembrane sequences appear to be involved (Machamer, 1991). The first of three membrane-spanning domains of infectious bronchitis virus M protein is necessary for its retention in the Golgi (Machamer *et al.*, 1990; Machamer and Rose, 1987); similarly, a number of glycosyltransferases, all type II membrane proteins that reside in the Golgi stack contain targeting signals in and around their signal/anchor, membrane-spanning domains (see Machamer [1993] for review). No homology has been found between the various TM domains of Golgi proteins.

Three different models have been proposed to account for retention of membrane proteins in the Golgi stacks. 1) Formation of large insoluble aggregates or lattices: according to this model, resident Golgi proteins form large insoluble complexes that are unable to enter transport vesicles. It is based on the finding that a Golgi-retained chimeric protein Gm1 (vesicular stomatitis virus [VSV] G protein in which the transmembrane domain is replaced with the first membrane-spanning domain from infectious bronchitis virus M protein), forms large, sodium dodecyl sulfate (SDS)-resistant oligomers (Swift and Machamer 1991; Weisz *et al.*, 1993). 2) Bilayer-mediated sorting: this model is based on the observation that the TM domains of endogenous Golgi enzymes are shorter (17 residues on average vs. 21 for plasma membrane proteins). It holds that Golgi membrane proteins are inherently less stable in thicker, cholesterol and sphingolipid-rich membranes (e.g., plasma membrane) and, as a result, partition into the thinner cholesterol/sphingolipid-poor bilayers of the Golgi (Bretscher and Munro, 1993). 3) Kin recognition: this model proposes that Golgi enzymes are homodimers (stabilized by lumenal domain interactions) which are retained via interactions between the TM domains of adjacent "kin" homodimers in the same cisterna, thus forming long heterooligomers (Nilsson *et al.*, 1993, 1994). Retention is believed to be enhanced by interaction of the cytoplasmic domains of Golgi proteins with an intercisternal matrix (Nilsson *et al.*, 1993; Slusarewicz *et al.*, 1994).

We have been using rubella virus (RV) envelope glycoproteins to study the requirements for Golgi targeting of heterodimeric membrane proteins. RV is one of several types of simple enveloped RNA viruses that bud into Golgi membranes. We have previously shown that RV glycoproteins, E2 and E1 (both of which are type I membrane proteins), are targeted to the Golgi when stably expressed in Chinese hamster ovary (CHO) cells (Hobman *et al.*, 1993). E2 and E1 are derived by endoproteolytic cleavage of a polyprotein precursor (Oker-Blom, 1984; Oker-Blom *et al.*, 1984), dimerize in the ER, and are transported to, and retained in, the Golgi as a heterodimeric complex (Hobman *et al.*, 1993). It is not yet known how heterodimeric protein complexes are retained in the Golgi. Specifically, are one or two Golgi retention signals involved? To determine how the RV E2/E1 complex is retained in the Golgi, we have constructed stably transfected cell lines expressing chimeric proteins consisting of RV and VSV G protein domains. We have identified a Golgi retention signal within a 21-amino acid peptide region near the C-terminus of E2 glycoprotein. When this E2 domain was attached to the ectodomain of VSV G protein, the chimeric protein was targeted to the Golgi and behaved as a dimer on sucrose gradients. Thus the oligomerization properties of the Golgi-retained G protein were affected, but insoluble aggregates were not seen. Here we demonstrate that the RV spike complex can be retained in the Golgi via a single retention signal (the E2 transmembrane domain) since replacement of the E1 transmembrane and cytoplasmic domains did not affect targeting of E2 or E1 to this organelle. The results are discussed with regard to current models of Golgi retention.

MATERIALS AND METHODS

Reagents

Reagents and supplies were from the following sources: protein A- and G-Sepharose were purchased from Pharmacia (Alameda, CA). Fibronectin, SDS, and bovine serum albumin were purchased from Sigma (St. Louis, MO). [^{35}S]Cysteine (800 Ci/mM) was from ICN Biomedicals (Irvine, CA) and ^{14}C-labeled protein standards from Amersham (Arlington Heights, IL). Endoglycosidase H (endo H) and neuraminidase (*Arthrobacter ureafaciens*) were obtained from Boehringer Mannheim (Indianapolis, IN). Rhodamine (tetramethylrhodamine isothiocyanate; TRITC)-conjugated goat anti-mouse IgG and fluorescein (fluorescein isothiocyanate; FITC)-conjugated donkey anti-rabbit IgG (each double-labeling grade) were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). FITC-conjugated goat anti-human IgG was purchased from Zymed (San Francisco, CA). The Semliki Forest virus (SFV)-based plasmid expression system, Lipofectin, Optimem serum-free media, and reagents for in vitro transcription were obtained from Life Technologies (Gaithersburg, MD). A plasmid encoding VSV G and rabbit anti-VSV serum were kindly provided by Dr. Carolyn Machamer (Johns Hopkins University, Baltimore, MD). Human anti-RV serum was obtained from Dr. Aubrey Tingle (University of British Columbia, Vancouver, Canada) or was purchased from Vital Blood Products (Calabasa, CA). Mouse monoclonal antibodies that recognize the ectodomains of E2 and E1 (ascites) were kindly provided by Dr. John Safford (Abbott Laboratories, North Chicago, IL). Rabbit antiserum to α-mannosidase II (Man II) was prepared as described (Velasco *et al.*, 1993). Rabbit polyclonal serum to rough ER membrane proteins was obtained from Dr. William Dunn (University of Florida, Gainesville, FL). Mouse monoclonal antibodies to the ectodomain (BW8G65) and cytoplasmic domain (P5D4) (Kreis, 1986) of VSV G protein were obtained via Dr. William Balch (Scripps Research Institute, La Jolla, CA).

Recombinant Plasmids

The construction of the cDNA encoding RV E2 and E1 and VSV G protein (Figure 1) have been described (Hobman *et al.*, 1990; Rose and Bergmann, 1982). To easily switch the TM domains of RV glycoproteins and G protein, in frame *Eco*RV sites were engineered into the coding sequences of the ectodomains immediately adjacent to the TM regions. A 600-bp *Bam*HI–*Hin*dIII fragment encoding the

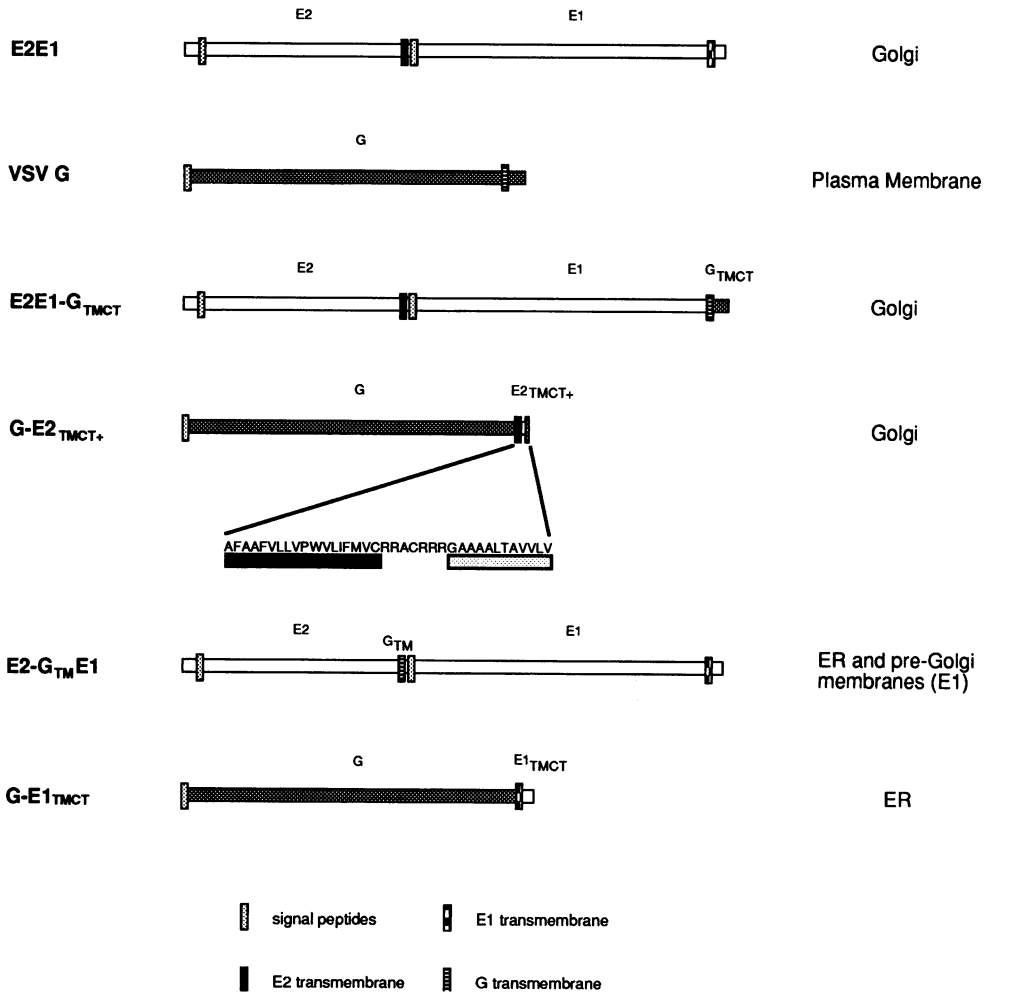


Figure 1. Schematic of RV, VSV G, and chimeric cDNA constructs indicating localization of encoded proteins in stably transfected CHO cells. E2E1, signal peptides for E2 and E1 are indicated by stippled rectangles. The TM regions of E2 and E1 are indicated by black and checkered rectangles, respectively. VSV G, the signal peptide is marked by a stippled rectangle and the TM region is represented by a striped rectangle. E2E1-G_{TMCT}, the E1 TM and CT domains were replaced by the TM and CT domains of VSV G. G-$E2_{TMCT+}$, the TM and CT domains of G were replaced by the TM and CT region of E2. The sequence of the C-terminus of G-$E2_{TMCT+}$ is also shown. In addition to the TM region of E2, this construct contains the short cytoplasmic domain of E2 and about half of the signal peptide for E1. E2-G_{TM}E1, the TM domain of E2 was replaced by the corresponding segment from VSV G. G-$E1_{TMCT}$, the TM and CT domains of G were replaced by the TM and CT regions of E1. All cDNAs were subcloned into the expression vector pCMV5.

3′ one-third of E1 was excised from pCMV5-E2E1 and ligated into the phagmid vector pT7T319u (Pharmacia Biotech, Piscataway, NJ). Similarly, a 627-bp *Kpn*I–*Bam*HI fragment from the 3′ end of VSV G was subcloned into phagmid vector pBKS(+) (Stratagene). Recombinant plasmids were used to transform *Escherichia coli* strain CJ236 and uracil-containing single-stranded DNA was prepared. Mutagenic oligonucleotides E1EcoRV (5′-GGAGTGGGCTGATATCCATTGGTGGCAG-3′) and VSVGEcoRV (5′-GCAATAGAGCTTTTGATATCACTGAACCAACC-3′) were used to introduce inframe *Eco*RV sites in the E1 and G genes, respectively, as described (Kunkel, 1985). The new *Eco*RV site in E1 resulted in changing Ala^{444}-ALA^{445} to Asp^{444}-Ile^{445} and in G protein, Ser^{460}-Trp^{461} was changed to Asp^{460}-Ile^{461}. The mutations were confirmed by restriction analysis and DNA sequencing. Fragments bearing the mutations were subcloned back into pCMV5-E2E1 and pCMV5-G which were then used to stably transfect CHODG44 cells as described (Hobman *et al.*, 1992). The mutations did not appear to have any effect on the rate or efficiency of transport of E2 and E1 to the Golgi complex or G protein to the cell surface.

E2E1-G_{TMCT} (E2 and E1 Ectodomain Fused to TM and Cytoplasmic [CT] Domains of VSV G Protein). The 200-bp *Eco*RV–*Bam*HI fragment (encoding the TM and CT domains of E1) from pCMV5-$E2E1_{EcoRV}$ was replaced with a 280-bp *Eco*RV–*Bam*HI fragment from pCMV5-G_{EcoRV} (encoding the TM and CT domains of G protein) using a three part ligation strategy. The resulting plasmid, pCMV5-E2E1-G_{TMCT} (Figure 1) encodes RV E2 and E1 ectodomain fused to the TM and CT domains of VSV G protein.

G-$E2_{TMCT+}$ (VSV G Ectodomain Fused to TM and CT Domains of E2 plus 12 N-Terminal Amino Acids of E1 Signal Peptide). Attempts to introduce an inframe *Eco*RV site in the ectodomain of E2 adjacent to the TM anchor using conventional oligonucleotide-directed mutagenesis were unsuccessful; therefore we used mutagenic primers in conjunction with a polymerase chain reaction (PCR) to accomplish this. A cDNA encoding RV E2 was excised from pCMV5-E2 (Hobman *et al.*, 1990) with *Eco*RI and *Bam*HI and ligated into pT7319u such that the *Bam*HI 3′ end of the E2 cDNA was adjacent to the T7 promoter. The recombinant plasmid was linearized with *Sca*I which cuts once within pT7T319u and subjected to PCR using the 5′ mutagenic primer E2EcoRVPCR (5′-CTGTCTGATATCCACGCGTTCGCGGCC-3′) and T7 primer (5′-TTAATACGACTCACTATAG-3′) as the 3′ primer. Reactions contained 50 ng template and were cycled as follows: 5 min at 95°C (1×), 1 min at

Table 1. Sequence of C-terminal regions of constructs

G-protein (WT)	DIKSSIASFFFIIGLIIGLFLVLRVGIHLCIKLKHTKKRQIYTDIEMNRLGK
G-E1$_{TMCT}$	DIHWWNLTLGAICALPLVGLLACCAKCLTTLRGAIAPR
G-E2$_{TMCT+}$	DIHAFAAFVLLVPWVLIFMVCRRACRRRGAAAALTAVVLV
G-E2$_{TMCT}$	DIHAFAAFVLLVPWVLIFMVCRRACRRR
G-E2$_{TM}$	DIHAFAAFVLLVPWVLIFMVCRR

Listed are the sequences of the carboxy-terminal regions of selected cDNA constructs. Sequences of the peptides are given in the one-letter code for amino acids. The predicted membrane-spanning region of each construct is underlined. WT, wild type.

95°C, 1 min at 40°C, 3 min at 75°C (3×), 1 min at 95°C, 1 min at 55°C, 1 min at 75°C (30×), and 10 min at 75°C. The 250-bp PCR product was digested with *Eco*RV and *Bam*HI, and the resulting 180-bp fragment encoding the E2 TM domain (18 amino acids), CT (7 amino acids) plus the N-terminal 12 residues of the E1 signal peptide (+domain) was gel-purified, cloned into pBKS(−) (Stratagene), and sequenced. The E2$_{TMCT+}$ fragment was then ligated to the ectodomain of VSV G by replacing the *Eco*RV–*Bam*HI (280 bp) 3′ fragment from pCMV5-G$_{EcoRV}$ with the 180 bp PCR generated product. The resulting plasmid, pCMV5-G-E2$_{TMCT+}$ (Figure 1) encodes VSV G luminal domain fused to the RV E2$_{TMCT+}$ domains.

G-E2$_{TMCT}$ (VSV G Ectodomain Fused to TM and CT Domains of E2). PCR was used to engineer a stop codon and *Hin*dIII site after the seventh Arg residue in the CT region of E2 so that translation stopped before the E1 signal peptide region (Figure 1 and Table 1). A 108-bp fragment encoding the E2$_{TMCT}$ domains was amplified using the 5′ primer, E2EcoRVPCR and 3′ primer E2CTR7 (5′-GAGGGCGGCAAGCTTCTAGCGGCGGCGACAGG-3′) as described above. The PCR product was blunt-end ligated into pCR-Script (Stratagene) and sequenced. The E2$_{TMCT}$ cDNA was excised from pCR-Script vector by digestion with *Eco*RV and *Hin*dIII, gel purified and in a three part reaction, ligated to the G ectodomain (*Eco*RI–*Eco*RV fragment) and pCMV5.

G-E2$_{TM}$ (VSV G Ectodomain Fused to TM Domain of E2). PCR was used to place a stop codon and *Hin*dIII site after the second Arg of the E2 CT domain (Fig. 1 and Table 1) using E2EcoRVPCR as a 5′ primer and E2TMHIN3 (5′-GCGCCGCGGAAGCTTACTCAGCG-GCGGCACACC-3′) as a 3′ primer. The PCR product was digested with *Eco*RV and *Hin*dIII, ligated into pBKS(−) and sequenced. pBKS(−)E2$_{TM}$ was digested with *Eco*RI and *Eco*RV and religated in the presence of the G ecto domain (*Eco*RI-*Eco*RV fragment). The resulting recombinant plasmid was digested with *Eco*RI and *Hin*dIII to release G-E2$_{TM}$ which was then subcloned into pCMV5.

E2-G$_{TM}$E1 (E2 TM Domain Replaced by TM Domain from VSV G Protein). A four step PCR strategy was used to replace the E2 TM domain with the corresponding region from VSV G protein (Figure 1). Using a cDNA encoding wild type VSV G protein as a template (250 ng), the primers E2-VSVGTM5′ (5′ GCGAACGCGCTGTCTCT-TGACAGCTCTATTGCCTCTTTTTTC-3′) and E2-VSVGTM3′ (5′ G-CGGCGGCGACAGGCGCGGCGGAGAACCAAGAATAGTCCAA-T3′) were used to amplify the TM domain of G protein such that it was now flanked by the nucleotides encoding part of the ecto and cytoplasmic domains of E2. Reactions contained 100 ng each primer, 20 mM Tris-HCl (pH 8.2), 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 mM each dNTP and 2.5 U of Pfu polymerase (Stratagene) and cycling conditions were as follows: 5 min at 95°C (1×), 1 min at 95°C, 1 min at 40°C, 3 min at 75°C (3×), 1 min at 95°C, 1 min at 48°C, 1 min at 75°C (30×), and 10 min at 75°C. The resulting PCR product was used as a primer in two separate amplification reactions in order to fuse the coding region for the VSV G TM domain to sequences encoding the ectodomain of E2 and the entire E1 gene, respectively. Oligonucleotides that flank ends of the multiple coding site of the expression vector pCMV5 (Andersson *et al.*, 1989) were used as the other primers for these reactions. The resulting E2- and E1-derived PCR products which overlap via sequences encoding the G TM domain were gel-purified and combined in the presence of Pfu polymerase and dNTPs for 5 cycles of 95°C/1 min; 55°C/1 min; 75°C/3 min to extend the complementary regions. Subsequently, primers that flank the pCMV5 multiple cloning site were added and the reaction was subjected to 30 cycles of PCR as above. The presence of the G TM domain was confirmed by sequencing the E2/E1 junction region. The PCR-derived E2-G$_{TM}$E1 cDNA was then reconstructed by restriction fragment replacement of the E2 ectodomain and E1 regions to ensure that no other PCR-induced mutations would be present in the final product.

G-E1$_{TMCT}$ (VSV G Ectodomain Fused to TM and CT Domains of E1). The 280-bp *Eco*RV-*Bam*HI fragment (G$_{TMCT}$) in pCMV5-GEcoRV was replaced with the 200-bp *Eco*RV-*Bam*HI (E1$_{TMCT}$) fragment from pCMV5-E2E1$_{EcoRV}$ in a two part ligation reaction. The resulting plasmid, pCMV5-G-E1$_{TMCT}$ encodes the ectodomain of VSV G protein fused to the TM and CT domains of RV E1 (Figure 1).

Transient Expression

VSV G cDNAs were blunt end-ligated into the *Sma*I site of pSFV1 which contains the four nonstructural proteins of SFV under the transcriptional control of the SP6 polymerase promoter (Liljestrom and Garoff, 1991). This vector was chosen for the reason that very high levels of heterologous protein expression can be obtained because the polycistronic mRNAs are self-replicating since they encode SFV replication enzymes. Recombinant plasmids were linearized with *Spe*I prior to in vitro transcription. Transcription reactions (50 μl) contained 2 μg linearized plasmid, 40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine-$(HCl)_3$, 1 mM dithiothreitol, 1 mM $m^7G(5')ppp(5')G$, 0.5 mM GTP, 1 mM each ATP, CTP and UTP, 1 U/μl placental RNAse inhibitor and 40 U of SP6 RNA polymerase. After 2 h at 37°C, transcription reactions were terminated by the addition of 50 μl of water, 10 μl of 3 M sodium acetate (pH 4.8–5.4) and 72 μl of isopropanol (−20°C). RNA was recovered by centrifugation in a microcentrifuge, washed with 70% ethanol, dried, and resuspended in 50 μl TE (pH 7.5). RNA (~2 μg) mixed with 10 μg of Lipofectin (in 1 ml of Optimem) was used to transfect 2.5×10^5 CHODG44 cells for 3–4 h at 37°C. The transfection mixture was removed, and the cells were incubated in complete medium for 2–4 h before use in biosynthetic labeling experiments. By immunofluorescence analysis, we observed that 50–90% of transfected cells expressed the transgenes by this method.

Infection with VSV

CHODG44 cells grown in 35-mm dishes were infected with VSV in serum-free medium at a multiplicity of infection of 10–25. After 40 min at 37°C, the virus-containing medium was removed and replaced with serum-containing medium for 2–3 h after which time the cells were metabolically labeled.

Metabolic Labeling, Radioimmunoprecipitation, and Endo H and Neuraminidase Digestion

Radiolabeling of cells with [^{35}S]cysteine, immunoprecipitation of RV glycoproteins, and digestion with endo H or neuraminidase was

performed as described previously (Hobman *et al.*, 1993). When using the BW8G65 monoclonal antibody to VSV G protein, NET buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.1% Nonidet P-40, 0.25% gelatin, and 0.02% sodium azide) was used for washing of immune complexes.

SDS-Polyacrylamide Gel Electrophoresis (PAGE) and Autoradiography

Proteins were separated on 10% polyacrylamide gels (Laemmli, 1970). Gels were fixed in isopropanol:water:acetic acid (25:65:10) and soaked in Amplify (Amersham) before drying and exposure to Kodak XAR film at −80°C. Protein bands were quantitated using a Bio-Rad (Richmond, CA) densitometer.

Sucrose Gradient Sedimentation

A velocity gradient sedimentation procedure (Doms *et al.*, 1987) was used to compare the oligomeric state of native G and $GE2_{TMCT+}$. Radiolabeled cell lysates were prepared in MNT buffer (100 mM NaCl, 20 mM Tris-HCl, 30 mM 2-(*N*-morpholino) ethanesulfonic acid (MES), pH 5.8) containing 1% Triton X-100, and were loaded onto 5–20% sucrose gradients prepared in MNT buffer containing 0.1% Triton X-100 over a 0.25-ml cushion of 60% sucrose. Gradients were centrifuged at 45,000 rpm (208,000 × *g*) using an SW60 rotor for 14–17 h at 4°C. Fifteen fractions (0.3 ml) were collected from the bottom of the tubes or by pumping out fractions from the top with 65% sucrose containing bromophenol blue. Each fraction was diluted with 0.5 ml of 50 mM Tris-HCl (pH 8.0), 12.5 mM EDTA, 1% Nonidet P-40 and 0.3% SDS before immunoprecipitation with 2.5 μl of rabbit anti-VSV serum and 5 μl of protein A-Sepharose.

Immunofluorescence Microscopy

Cells were plated onto fibronectin (10 μg/ml)-coated chamber slides or 12-mm glass coverslips at a density of 2×10^4 cells/cm^2 and processed for indirect immunofluorescence as described (Hobman *et al.*, 1992).

Electron Microscopy

CHOG-$E2_{TMCT+}$ cells grown to confluency were fixed by sequential incubation with 8% and then 4% paraformaldehyde in phosphate-buffered saline, each for 1 h at room temperature. The anti-VSV G monoclonal BW8G65 was used to detect G-$E2_{TMCT+}$ by immunoperoxidase of whole cells or immunogold labeling of ultrathin cryosections as previously described (Hendricks *et al.*, 1993, Hobman *et al.*, 1992, Lundstrom *et al.*, 1993).

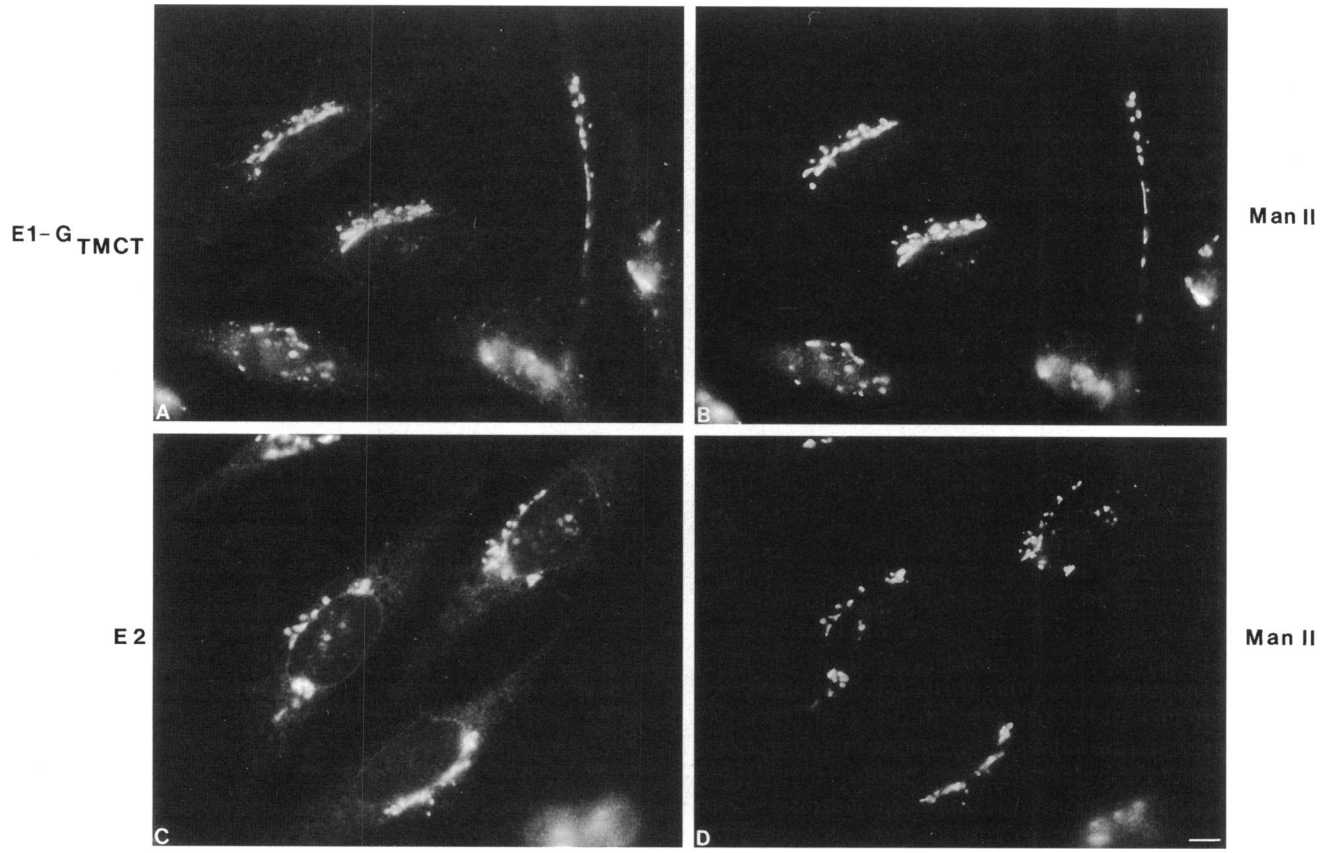
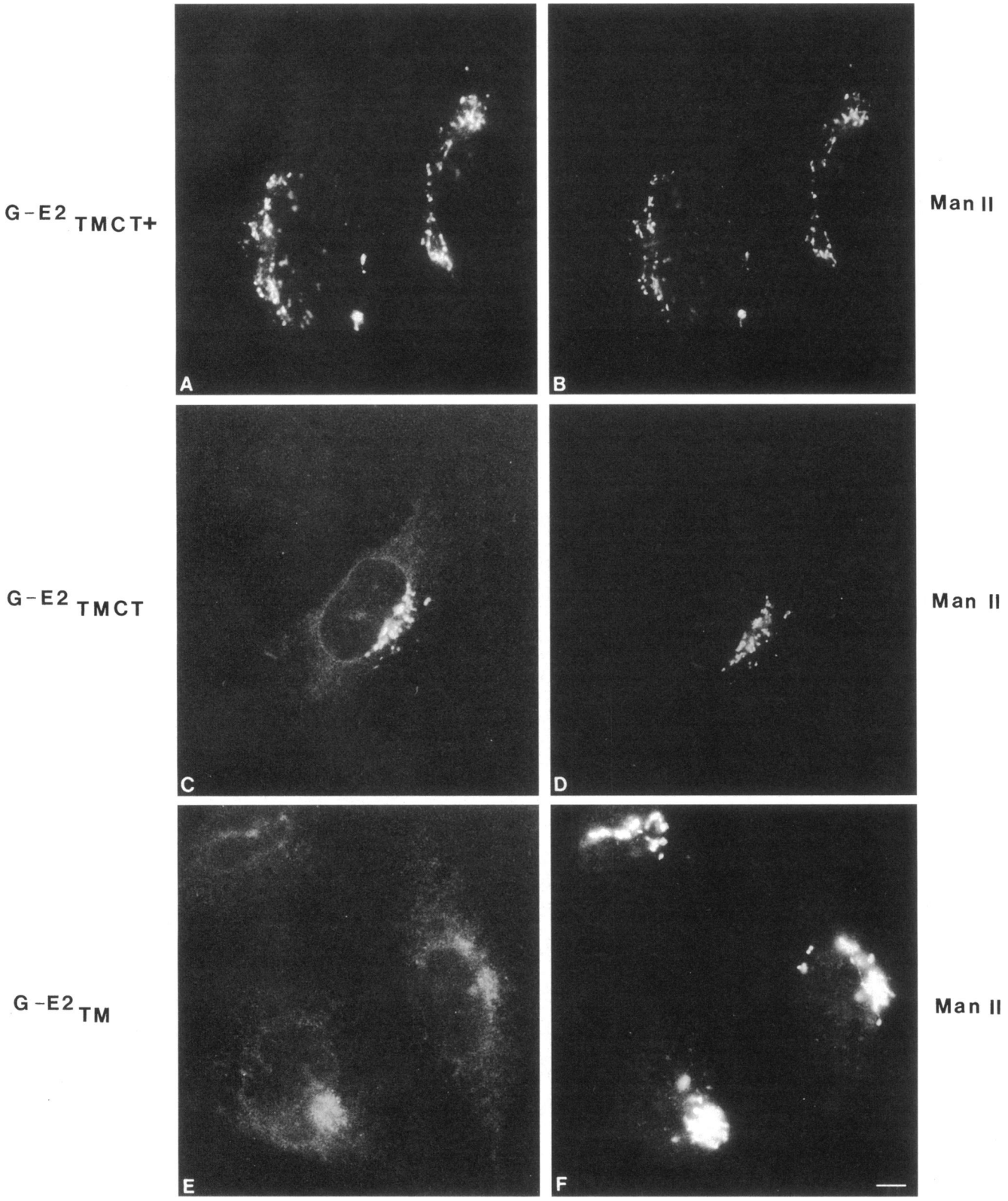


Figure 2. The TM and CT domains of E1 are not required for Golgi retention. CHO cells stably expressing E2 and E1 fused to the TM and CT domains of E1 (CHOE2E1-G_{TMCT} cells) were grown on fibronectin-coated coverslips, fixed and permeabilized with 100% methanol at −20°C, and doubly stained with monoclonal antibodies to the ectodomain of E1 (to visualize E1-G_{TMCT}) and E2, and with rabbit anti-Man II to visualize the Golgi complex. The localization of E1-G_{TMCT} (A) overlaps with that of Man II (B) indicating that this chimeric protein is targeted to the Golgi. Similarly, E2 (C) and Man II (D) colocalize in the Golgi complex. Bar, 5 μm.


G-E2 TMCT+
Man II
A
B
G-E2 TMCT
Man II
C
D
G-E2 TM
Man II
E
F

RESULTS

The E1 TM and CT Domains Are Not Required for Targeting of E2 and E1 to the Golgi Complex

Since Golgi retention signals reside within membrane-spanning domains of many resident Golgi proteins (Machamer, 1991), we concentrated our search for RV Golgi retention signals around the hydrophobic, presumptive membrane-spanning domains of E2 and E1. The strategy employed was to construct chimeric proteins between RV glycoproteins and VSV G protein, a type I membrane glycoprotein that is transported to the plasma membrane. The latter was chosen since cDNA clones and well characterized antibodies against VSV G are available. To conveniently exchange TM domains between RV glycoproteins and VSV G, *Eco*RV sites were engineered into the RV and VSV G cDNAs immediately adjacent (ectodomain side) to the coding regions for the membrane-spanning domains. This resulted in changing two amino acids in each of E2, E1, and G proteins to an Asp–Ile pair; these changes did not alter the targeting or rate of transport of the viral proteins in CHO cells in any detectable manner.

To determine if the TM and CT regions of E1 were necessary for retention of the E2/E1 heterodimer, we replaced them with the analogous segments from VSV G. The first cDNA construct, E2E1-G_{TMCT}, encodes the entire E2 protein and the E1 ectodomain fused to the TM and CT domains of G protein (Figure 1). CHO cell lines stably expressing this construct were made and analyzed by indirect immunofluorescence to determine their location. Both E2 and E1-G_{TMCT} were localized to the Golgi complex as determined by costaining with the Golgi marker protein Man II (Figure 2) (Velasco *et al.*, 1993). The glycoproteins were stably retained in the Golgi as there was no change in their distribution after incubation with cycloheximide for 3 h. E1-G_{TMCT} was also localized to the Golgi using a monoclonal antibody to the CT of G protein (Kreis, 1986). In biosynthetic labeling experiments, both E2 and E1-G_{TMCT} became endo H-resistant indicating transport as far as the medial Golgi. These results indicate that the TM and CT domains of E1 are not required for targeting of the RV spike complex to the Golgi.

E2 Contains a Golgi Retention Signal

We next investigated whether the C-terminal region of E2 contained an autonomous Golgi targeting signal that could affect transport of VSV G. PCR was used to generate a cDNA fragment encoding the C-terminus of E2 which was then fused to the ectodomain of G protein. This construct, G-$E2_{TMCT+}$, encodes the ectodomain of G protein fused to the TM (18 amino acids) and CT (7 amino acids) domains of E2 plus the N-terminal 12 amino acids of the E1 signal peptide (Figure 1 and Table 1). In stably transfected CHO cells, G-$E2_{TMCT+}$ was targeted to the Golgi complex as determined by co-localization with Man II (Figure 3, A and B), and it was retained there because incubation for 3 h with cycloheximide did not result in its transport to the cell surface.

After metabolic labeling and immunoprecipitation, G-$E2_{TMCT+}$ was first detected as three distinct species—57, 60, and 64 kDa (Figure 4). The 57- and 60-kDa bands represent endo H-sensitive precursors that diminish with time. By 20-min chase >50% of G-$E2_{TMCT+}$ was present as the 64-kDa, endo H-resistant form, indicating that the glycoprotein was rapidly transported to the medial Golgi. Immunoprecipitates were also treated with neuraminidase to determine if the *N*-linked glycans of the chimeric G protein had been exposed to sialyltransferases which reside in the trans-Golgi cisternae and TGN (Krinsje-Locker *et al.*, 1992, Roth *et al.*, 1985). At 120-min chase G-$E2_{TMCT+}$ was not neuraminidase-sensitive, even though the glycoprotein was completely endo H-resistant by this time (Figure 5). In contrast, wild-type G protein from CHO cells infected with VSV was sensitive to neuraminidase by 40 min (Figure 5). Based on the finding that G-$E2_{TMCT}$ does not become sialylated, we conclude that transport of G-$E2_{TMCT+}$ is blocked between the medial Golgi and the TGN.

When the distribution of G-$E2_{TMCT+}$ was determined at the EM level after immunoperoxidase and immunogold labeling, it was restricted to the Golgi stacks and associated vesicles (Figure 6, A and B) and was not found in the TGN or at the cell surface. We conclude that the C-terminus of E2 glycoprotein contains an autonomous signal that specifies localization to the medial/trans-Golgi.

Figure 3. Immunolocalization of G-E2 chimeric proteins (G-$E2_{TMCT+}$, G-$E2_{TMCT}$, and G-$E2_{TM}$). Cells were doubly stained with a mouse monoclonal antibody to the ectodomain of VSV G (BW8G65) and rabbit anti-Man II to visualize the Golgi complex. (A and B) G-$E2_{TMCT+}$ (VSV G ectodomain fused to E2 TM and CT domains of E2 plus 12 amino acids from E1 signal peptide) is located predominantly in the Golgi as demonstrated by overlap with Man II. (C and D) G-$E2_{TMCT}$ (VSV G ectodomain fused to E2 TM and cytoplasmic domains of E2) also largely colocalizes with Man II; however, a small amount of the protein is detected in the ER and perinuclear cisterna. (E and F) G-$E2_{TM}$ (VSV G ectodomain fused to TM domain of E2) also overlaps with Man II in the Golgi, but a larger fraction of the protein is found in the ER. Bar, 5 μm.

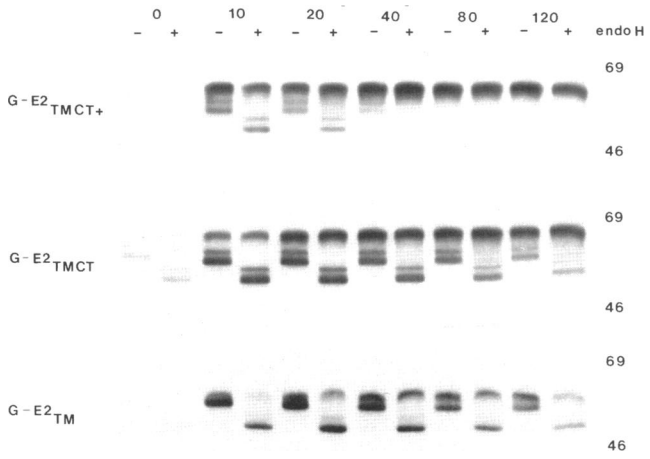


Figure 4. Truncation of the cytoplasmic domain of E2 retards transport from the ER. Cells were pulse labeled for 10 min with [^{35}S]cysteine, chased with excess cysteine (min), and immunoprecipitated using a monoclonal antibody to G protein. Where indicated (+), samples were treated with endo H. Immunoprecipitates from CHOG-E2$_{TMCT+}$, CHOG-E2$_{TMCT}$, and CHOG-E2$_{TM}$ cells are shown. Labeled proteins are under-represented at zero min chase because the anti-G monoclonal recognizes an epitope found only on folded G proteins. G-E2$_{TMCT+}$ is transported most rapidly to the Golgi, followed by G-E2$_{TMCT}$, then G-E2$_{TM}$, as evidenced by the rate at which the glycoproteins become resistant to endo H.

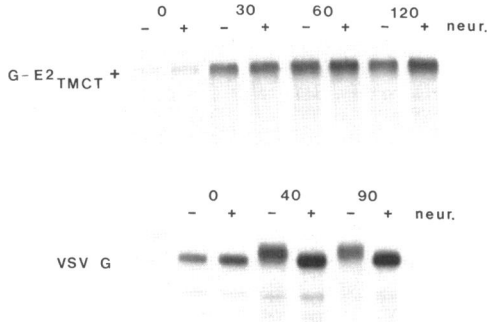


Figure 5. G-E2$_{TMCT+}$ is not sialylated. Upper panel: CHOG-E2$_{TMCT+}$ cells were pulse-labeled for 15 min and incubated with chase medium as above. Immunoprecipitates were prepared with a monoclonal antibody to G (BW8G65) and digested with neuraminidase (*A. ureafaciens*) before SDS-PAGE and fluorography. No sensitivity of G-E2$_{TMCT+}$ to neuraminidase was detected up to 120 min of chase even though the glycoprotein is completely resistant to endo H by this time (see Figure 4). Lower panel: As a positive control, CHODG44 cells were infected with VSV, and immunoprecipitates prepared after 0, 40, and 90 min of chase were digested with neuraminidase. By 40 min, VSV G is completely neuraminidase-sensitive, indicating sialylation of its *N*-linked glycans.

The Hydrophobic Signal Peptide of E1 and the Cytoplasmic Tail of E2 Are Not Required for Golgi Retention

Recently it was determined that the E1 signal peptide remains attached to mature E2 glycoprotein (Baron *et al.*, 1992). Since G-E2$_{TMCT+}$ contains 12 amino acids from the N-terminal region of the E1 signal sequence, it was important to determine whether these amino acids play any role in Golgi retention. Two additional cDNA constructs were made that do not include this region—G-E2$_{TMCT}$ which has a stop codon after the last residue (Arg-7) in the putative CT domain of E2, and G-E2$_{TM}$, in which a stop codon was placed after the second amino acid of the E2 CT domain (Arg-2) (Figure 1 and Table 1).

In CHO cells stably expressing G-E2$_{TMCT}$, this chimera (like G-E2$_{TMCT+}$) was localized primarily to the Golgi complex by immunofluorescence (Figure 3, C and D), and it was not chased out of this organelle after 3-h incubation in the presence of cycloheximide. One obvious difference between the two glycoproteins was that G-E2$_{TMCT}$ was transported to the Golgi at a slower rate than G-E2$_{TMCT+}$: deletion of the N-terminus of the E1 signal peptide resulted in a slight accumulation of the G protein-E2 chimera in the ER by immunofluorescence (compare Figure 3, A and C), and endo H-sensitive forms were still present after a 120-min chase (Figure 4). In CHO cells stably expressing G-E2$_{TM}$, the glycoprotein was localized throughout the ER and Golgi but not on the cell surface by immunofluorescence (Figure 3, E and F), and there was no transport of G-E2$_{TM}$ to the plasma membrane even after a 3-h cycloheximide chase. Processing of the N-linked glycans on G-E2$_{TM}$ to endo H-resistant forms took place, although it occurred at a slower rate ($t_{1/2}$ = 80–120 min) than G-E2$_{TMCT}$ (Figure 4). It appears that >50% of G-E2$_{TMCT}$ and G-E2$_{TM}$ were rapidly exported from the ER, but a significant fraction took considerably longer to reach the Golgi (Figure 4). By immunofluorescence, the chimeras were still detectable in the ER after prolonged chase in the presence of cycloheximide, suggesting that a minor fraction of these chimeric glycoproteins do not leave the ER. Taken together these results indicate that 1) the cytoplasmic domain of E2 is required for efficient export from the ER, and 2) only the E2 TM region is required for Golgi retention.

The TM domain of E2 Is Required for Transport of the RV Spike Complex to the Golgi

The TM domain of E2 was replaced with that of VSV G (Figure 1) to determine if the RV spike complex would be transported to the cell surface or retained in the Golgi. If the latter proved to be the case, it would infer that another Golgi retention signal is present in the TM and or CT domains of E1. Unexpectedly, the distributions of E2-G$_{TM}$ and E1 coincided mainly with that of ER membrane proteins in stably transfected cells (Figure 7, A–D). Incubation with cycloheximide for 3 h did not result in detectable transport of these glycoproteins to the Golgi or cell surface indicating

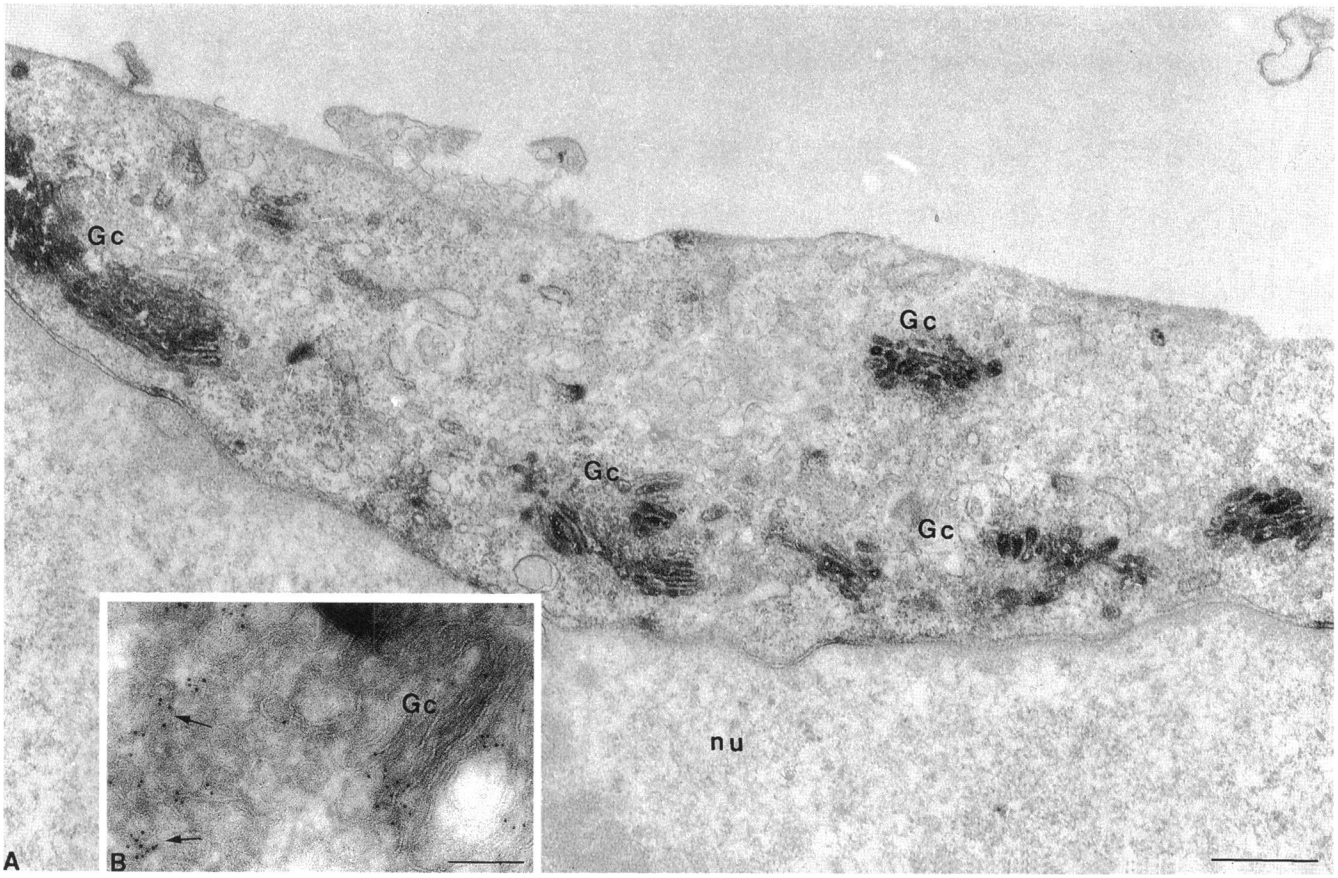


Figure 6. G-E2$_{TMCT+}$ is found in the Golgi stacks. (A) Immunoperoxidase labeling of CHOG-E2$_{TMCT+}$ cells using mouse anti-G (monoclonal antibody BW8G65). Staining is seen throughout the Golgi stacks (Gc). Bar, 0.5 μm. (B) Immunogold labeling of ultrathin cryosections of CHOG-E2$_{TMCT+}$ cells using an anti-G monoclonal antibody. Gold particles are concentrated on membranes of the Golgi stack (Gc) and associated vesicles (arrows). Bar, 0.1 μm.

that they are stably retained in the ER. Interestingly, some E1, but not E2-G$_{TM}$ was detected in structures (Figure 7C, arrows) that resemble the pre-Golgi elements composed of tubular networks of smooth membranes found in cells expressing E1 alone (Hobman *et al.*, 1992). Since E2-G$_{TM}$ and E1 are derived from the same polyprotein precursor and therefore synthesized in equimolar amounts, this finding indicates that at least a fraction of E1 never associates with E2-G$_{TM}$ or dissociates from this subunit after heterodimer assembly. Thus the E2 TM domain appears to be required for transport of the RV spike complex from the ER. Accordingly, it is not possible to ascertain whether the E1 TM and CT domains contain a Golgi retention signal by analysis of the E2-G$_{TM}$E1 construct.

The E1 TM and CT Domains Retain the Ectodomain of VSV G Protein in the ER

Because it was not possible to determine whether or not the E1 C-terminus could retain the RV spike complex in the Golgi in the absence of the E2 TM domain, a corollary strategy was used in which the E1 TM and CT domains were fused to the ectodomain of VSV G protein. By indirect immunofluorescence, G-E1$_{TMCT}$ was localized primarily to the ER (Figure 8A), whereas CHO cells expressing wild-type G protein showed strong cell surface staining (Figure 8B). After incubation with cycloheximide for 3 h, the bulk of G-E1$_{TMCT}$ remained in the ER although occasional weak cell surface staining was observed in some cells. This is consistent with the results from biosynthetic labeling experiments which show that only limited processing of *N*-linked glycans occurs after 3 h (Figure 8C). Since G-E1$_{TMCT}$ is largely retained in the ER, it was not possible to determine if the TM or CT domains of E1 contains a Golgi retention signal that functions autonomously. It remains to be determined if the E1 TM and/or CT domains specifically mediate ER retention as do the KK and RR motifs found on

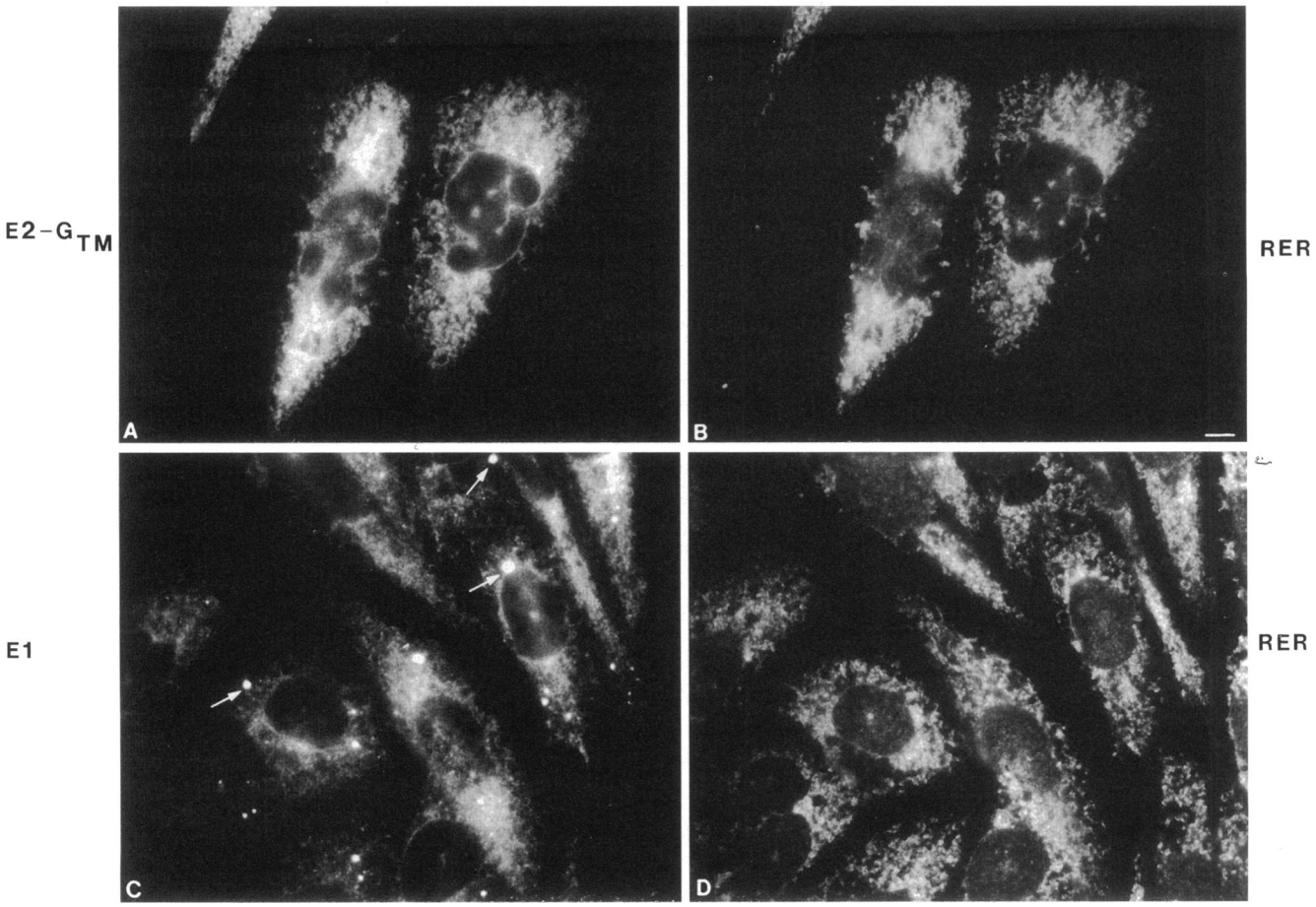


Figure 7. The TM domain of E2 is required for transport of the RV spike complex to the Golgi. CHO cells stably expressing E2-G_{TM} and E1 were processed for indirect immunofluorescence (as above) and were doubly stained with monoclonal antibodies to the ectodomains of E2 (to visualize E2-G_{TM}) and E1, and with a rabbit polyclonal antibody to rough ER membrane proteins. E2-G_{TM} (A) coincides perfectly with the rough ER marker (B). Similarly, most of E1 (C) is found in the rough ER except for the fraction that is localized to the cytoplasmic structures (arrows) that are devoid of ER membrane proteins (D). Bar, 5 μm.

certain type I and type II ER membrane proteins respectively (Jackson *et al.*, 1990, Schutze *et al.*, 1994).

Oligomerization of G-E2$_{TMCT+}$

Machamer and coworkers found that a VSV G/coronavirus M protein chimera which is retained in the Golgi region forms large, SDS-resistant oligomers (instead of the homotrimers formed by the wild type G protein) and suggested that oligomerization could serve as a mechanism for retention in the Golgi complex (Swift and Machamer, 1991; Weisz *et al.*, 1993). To determine if G-E2$_{TMCT+}$ assembly involves formation of large oligomers, we compared the size of native VSV G trimers to those formed by G-E2$_{TMCT+}$ on sucrose gradients. Since permanently transfected cell lines expressing native G protein were unstable and did not synthesize enough protein to facilitate biochemical analysis, native G and G-E2$_{TMCT+}$ were expressed transiently in CHO cells using a Semliki Forest virus (SFV) based plasmid system. In vitro synthesized mRNA consisting of SFV nonstructural proteins (which form an RNA-dependent RNA replication complex) fused to VSV G genes was transfected into CHO cells. Transfected cells were radiolabeled with [^{35}S]cysteine, solubilized in MNT buffer (pH 5.8) containing 1% Triton X-100, and lysates were subjected to centrifugation in 5–20% sucrose gradients (Doms *et al.*, 1987).

Following the pulse, native G protein was found in fractions 6 through 13 (Figure 9) which is the typical distribution of unassembled G subunits in this type of gradient (Doms *et al.*, 1987; Swift and Machamer, 1991). By 60 min virtually all of the G protein had trimerized and was located near the bottom of the gradient (fractions 11, 12, and 13)

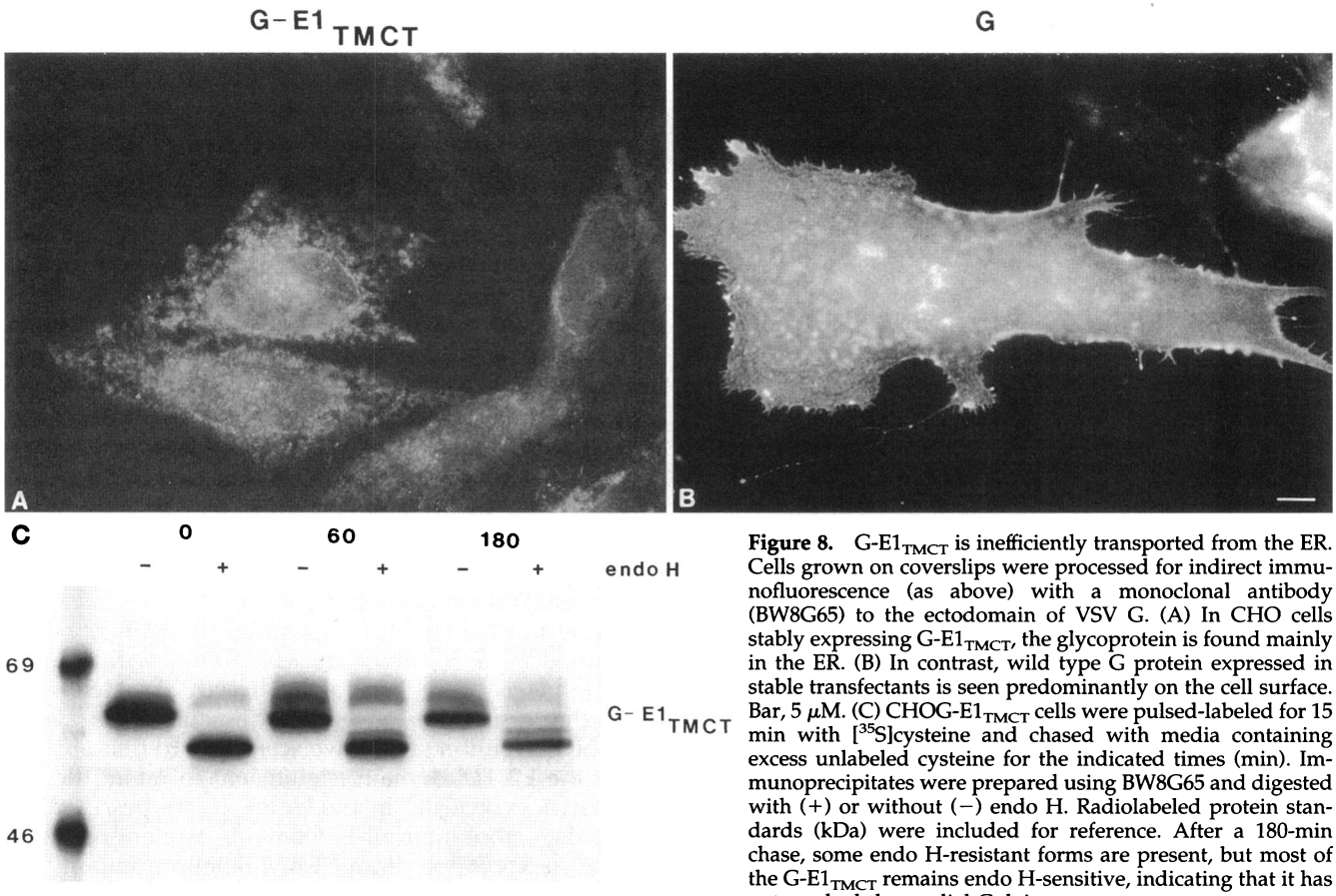


Figure 8. G-E1$_{TMCT}$ is inefficiently transported from the ER. Cells grown on coverslips were processed for indirect immunofluorescence (as above) with a monoclonal antibody (BW8G65) to the ectodomain of VSV G. (A) In CHO cells stably expressing G-E1$_{TMCT}$, the glycoprotein is found mainly in the ER. (B) In contrast, wild type G protein expressed in stable transfectants is seen predominantly on the cell surface. Bar, 5 μM. (C) CHOG-E1$_{TMCT}$ cells were pulsed-labeled for 15 min with [^{35}S]cysteine and chased with media containing excess unlabeled cysteine for the indicated times (min). Immunoprecipitates were prepared using BW8G65 and digested with (+) or without (−) endo H. Radiolabeled protein standards (kDa) were included for reference. After a 180-min chase, some endo H-resistant forms are present, but most of the G-E1$_{TMCT}$ remains endo H-sensitive, indicating that it has not reached the medial Golgi.

(Figure 9). Similar results were obtained using VSV-infected CHO cells.

When assayed on sucrose gradients, G-E2$_{TMCT+}$ behaved identically to wild-type G immediately following the pulse, but after 60 min its distribution had shifted and peak amounts of the protein were found in fractions 9, 10, and 11 (Figure 9). The fact that G-E2$_{TMCT+}$ has a lower sedimentation rate than that of wild-type G protein trimers suggests that it forms a smaller oligomer, presumably a dimer. G-E2$_{TMCT+}$ expressed in stably transfected CHO cells behaved similarly on sucrose gradients. When cell lysates from separately transfected cells expressing G and G-E2$_{TMCT+}$ were mixed and fractionated on the same gradient, the G trimers were also found closer to the bottom of the gradient than the G-E2$_{TMCT+}$ oligomers. Analysis of radiolabeled G-E2$_{TMCT+}$ by SDS-PAGE under non-reducing conditions indicated that, unlike the Golgi enzyme α-mannosidase II (Moremen *et al.*, 1991), the dimers were not disulfide-linked. From these data we conclude that G-E2$_{TMCT+}$ assembles into smaller oligomers (e.g., dimers) than native G protein.

DISCUSSION

In this study we have investigated how a heterodimeric protein complex composed of rubella E2 and E1 glycoproteins is retained in the Golgi complex. Fusion of the G ectodomain to the C-terminal region of E2 (which includes the 18-amino acid membrane-spanning domain) resulted in a chimera, G-E2$_{TMCT+}$, that was rapidly and efficiently transported to the Golgi. Although G-E2$_{TMCT+}$ was transported to the medial Golgi as evidenced by acquisition of endo H resistance ($t_{1/2}$ 10–20 min), it was not sialylated. Thus transport was arrested between the medial Golgi and the TGN where sialylation is presumed to occur. Immunoelectron microscopy confirmed that G-E2$_{TMCT+}$, like the RV glycoproteins, was confined to Golgi stacks and associated vesicles. E2 and E1, but not G-E2$_{TMCT+}$, became partially sialylated when expressed in CHO cells. This finding may be explained by fact that trace amounts (less than 5%) of RV glycoproteins are transported to the plasma membrane of CHOE2E1 cells (Hobman *et al.*, 1993). Although the E2 CT domain did not alter targeting to the Golgi, it was

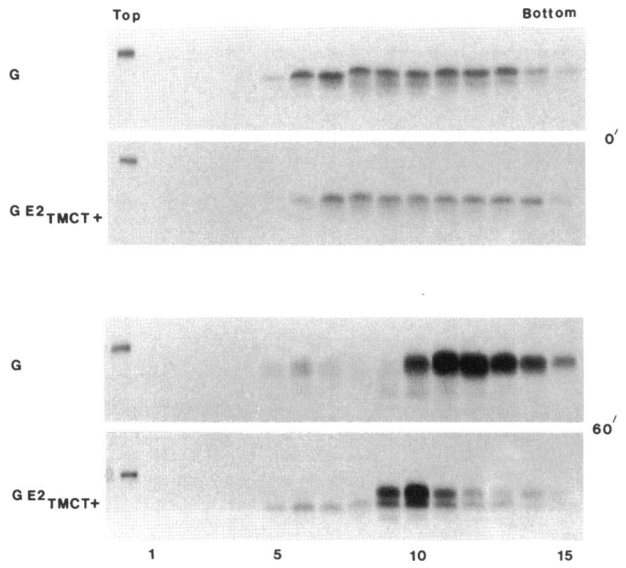


Figure 9. G-E2$_{TMCT+}$ forms small oligomers. CHO cells were transfected with RNA encoding VSV G or G-E2$_{TMCT+}$ and labeled with [^{35}S]cysteine for 5 min at 4 h post transfection. Cells were lysed in MNT buffer (pH 5.8) containing 1% Triton X-100 immediately after the pulse (0 min) or after a 60 min chase (60 min). Lysates were loaded onto a 5–20% sucrose gradient (in MNT buffer containing 0.1% Triton X-100), and centrifuged at 45,000 rpm (208,000 × *g*) for 14–17 h in a SW60 rotor at 4°C. Fractions were immunoprecipitated with rabbit anti-VSV serum and subjected to SDS-PAGE separation and fluorography. Following the pulse, unassembled G and G-E2$_{TMCT+}$ proteins were localized throughout fractions 6–14. After 60 min the highest levels of VSV G trimers were consistently found in fractions 11, 12, and 13 in the bottom half of the gradients, whereas G-E2$_{TMCT+}$ oligomers were always found in fractions 9, 10, and 11. The fraction numbers (1–15) are given at the bottom of the figure.

found to be necessary for efficient transport to the Golgi since deletion of the 12 C-terminal amino acids from G-E2$_{TMCT+}$ slowed export from the ER. Alterations in the length and sequence of the cytoplasmic domain of VSV G have also been shown to affect its rate and efficiency of transport from the ER (Doms *et al.*, 1988).

Replacement of the C-terminal TM and CT domains of E1 with the corresponding regions from VSV G protein did not alter targeting of the E1/E2 complex to the Golgi, indicating that these E1 domains are not required for Golgi retention. Whether or not these domains contain a second targeting signal which is sufficient to retain both E2 and E1 in the Golgi could not be resolved using our domain switching strategy since replacement of the E2 TM domain with that of VSV G (which presumably does not contain a targeting signal), resulted in transport arrest of E2-G$_{TM}$ and E1 proximal to the Golgi. Interestingly, a significant portion of E1 did not associate with E2-G$_{TM}$ and was sequestered in what appeared to be a pre-Golgi compartment distinct from the rough ER, comparable to the site where unassembled E1 subunits accumulate (Hobman *et al.*, 1992). Therefore one possible explanation for the finding that E2-G$_{TM}$ and E1 are not transported from the ER, is that the E2TM domain may be directly or indirectly required for oligomerization of the RV glycoproteins which precedes transport from the ER. This can be investigated in the future by comparing the folding and dimerization rates of E2-G$_{TM}$ and E1 with that of E2 and E1.

When the E1 TM and CT domains were appended to the VSV G ectodomain, the resulting chimera G-E1$_{TMCT}$ was also confined to the ER. Thus the E1 TM and CT domains are not sufficient to direct G protein to the pre-Golgi tubular network where unassembled E1 subunits accumulate (Hobman *et al.*, 1992). We are currently attempting to determine if the retention of VSV G protein in the ER by these domains is due to a specific ER retention signal in the E1 C-terminus or simply a result of misfolding of the G lumenal domain. If the former is true, such a signal would have to be masked during assembly with E2.

How is E1 retained in the Golgi? Most likely E1 is retained as a result of its association with E2. Although we were not able directly to determine whether or not the TM and CT domains of E1 possess a Golgi retention signal, we were able to demonstrate that the E2 TM domain is sufficient to retain the E2/E1-G$_{TMCT}$ construct in the Golgi. It can be ruled out that the ectodomain of E1 contains a retention signal since coexpression of an E1 TM deletion mutant with E2, results in secretion of soluble E1 into the media (Hobman *et al.*, 1994). Thus only a single Golgi targeting signal is required to retain a heterodimer of two membrane proteins in this organelle. Neither E2 or E1 is efficiently transported to the Golgi when expressed alone in CHO cells (Hobman *et al.*, 1993). The inability of individual glycoprotein subunits to be transported to the Golgi would ensure that only properly assembled spike heterodimers reach the virus budding site. Since the E1 TM and CT domains are not required for transport of the spike complex to the Golgi, formation of a transport-competent heterodimer must occur primarily by interactions between the ectodomains of E2 and E1. Similarly, intersubunit interactions between other viral membrane glycoproteins are largely confined to their ectodomains (Doms *et al.*, 1993). The C-terminus of E1 may stabilize interactions between E2 and E1, as coexpression of E2 with an anchorless form of E1 results in secretion of a considerable proportion of the truncated E1 into the media (Hobman *et al.*, 1994).

Relationship to Current Models of Golgi Retention

Do our results fit any of the recently proposed models of Golgi retention? Our data is most consistent with the bilayer-mediated sorting model (Bretscher and Munro,

1993), as the E2 TM domain is comparable to other Golgi retention signals (18 amino acids in length), and contains 3 phenylalanine residues. This model also predicts that longer TM domains (like E1 which is 22 amino acids) can coexist in the thinner, cholesterol/sphingolipid-rich membranes of the Golgi. Presumably, interactions between the ectodomains of E2 and E1 (which may be stabilized further by the TM and CT domain interactions), are strong enough to prevent E1 from partitioning into the thicker cholesterol/sphingolipid-rich membranes of budding vesicles. The finding that G-E2$_{TMCT+}$, like native E2 and E1, forms dimers instead of large oligomers is not consistent with the aggregation model of Golgi retention (Swift and Machamer, 1991). Moreover, the E2 TM domain does not contain any polar amino acids which have been proposed to be important for retention in this model (Machamer *et al.*, 1993, Weisz *et al.*, 1993). A serious flaw of the aggregation model is that unlike Gm1, the native Golgi-associated M protein itself does not form large oligomers (Swift and Machamer, 1991), nor has this been documented for other Golgi membrane proteins. The possibility that E2 and E1, G-E2$_{TMCT+}$ and M proteins form higher order structures which dissociate during centrifugation cannot be entirely ruled out at present.

Our finding that G-E2$_{TMCT+}$ forms a noncovalently linked dimer is partly consistent with the kin recognition model (Nilsson *et al.*, 1993, 1994) but the situation becomes more complex when considering the E2/E1 and E2/G$_{TMCT}$ heterodimers since the TM domain of VSV G can not independently confer Golgi retention, nor is it likely that the E1 TM domain does either. E2 and E1 form a heterodimer which is stabilized mostly through ectodomain interactions, and unless an E2 TM domain can interact with more than one adjacent TM domain (thus forming a branched oligomer or a trimer) or E1 or VSV G TM domains can interact with each other or E2 TM, the largest linear oligomer that could be formed would be E1-E2-E2-E1 (or E1G$_{TMCT}$-E2-E2-E1G$_{TMCT}$). It is important to emphasize that the structures of Golgi enzymes and virus glycoproteins and the functions performed by each is different (enzymatic vs. structural, respectively). Furthermore, it seems unlikely that the CT domains of RV E2 and/or E1 would be tightly associated with an intercisternal Golgi matrix (Slusarewicz *et al.*, 1994) since they must remain accessible for binding to nucleocapsids during viral assembly. Therefore it is likely that the intra-Golgi quaternary arrangement of viral glycoproteins differs from that of Golgi enzymes. The quaternary structure of the RV spike complex is at the simplest a single E2/E1 heterodimer, but it may be arranged as a hexamer (i.e., (E2/E1)$_3$) as with the structurally related alphaviruses (Fuller, 1987; Vogel *et al.*, 1986). If the latter is true, the RV glycoproteins would exist in a two dimensional array in Golgi membranes. It seems unlikely that a linear oligomer of viral spike heterodimers would directly support virus budding without some kind of rearrangement of the spikes into a two-dimensional array.

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