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# Characterization of an Endoplasmic Reticulum Retention Signal in the Rubella Virus E1 Glycoprotein

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**Rubella virus contains three structural proteins, capsid, E2, and E1. E2 and E1 are type I membrane glycoproteins that form a heterodimer in the endoplasmic reticulum (ER) before they are transported to and retained in the Golgi complex, where virus assembly occurs. The bulk of unassembled E2 and E1 subunits are not transported to the Golgi complex. We have recently shown that E2 contains a Golgi-targeting signal that mediates retention of the E2-E1 complex (T. C. Hobman, L. Woodward, and M. G. Farquhar, Mol. Biol. Cell 6:7–20, 1995). The focus of this study was to determine if E1 glycoprotein also contains intracellular targeting information. We constructed a series of chimeric reporter proteins by fusing domains from E1 to the ectodomains of two other type I membrane proteins which are normally transported to the cell surface, vesicular stomatitis virus G protein (G) and CD8. Fusion of the E1 transmembrane and cytoplasmic regions, but not analogous domains from two control membrane proteins, to the ectodomains of G and CD8 proteins caused the resulting chimeras to be retained in the ER. Association of the ER-retained chimeras with known ER chaperone proteins was not detected. ER localization required both the transmembrane and cytoplasmic regions of E1, since neither of these domains alone was sufficient to retain the reporter proteins. Increasing the length of the E1 cytoplasmic domain by 10 amino acids completely abrogated ER retention. This finding also indicated that the chimeras were not retained as a result of misfolding. In summary, we have identified a new type of ER retention signal that may function to prevent unassembled E1 subunits and/or immature E2-E1 dimers from reaching the Golgi complex, where they could interfere with viral assembly. Accordingly, assembly of E2 and E1 would mask the signal, thereby allowing transport of the heterodimer from the ER.**

Rubella virus (RV) is a small enveloped RNA virus in the togavirus family, whose members also include the well-studied prototype alphaviruses, Sindbis virus and Semliki Forest virus (SFV). RV is the causative agent of German measles, and infection during the first trimester of pregnancy poses drastic medical problems for human fetuses (for reviews see references 13 and 57). Although RV is structurally similar to alphaviruses, its replication and maturation differ significantly from those of its togavirus cousins. For example, in infected cells, the time course of viral macromolecular synthesis is comparatively delayed, and the levels of protein and RNA produced are much lower than for prototype alphaviruses (13). In addition, alphaviruses typically bud from the plasma membrane of infected cells, whereas RV matures primarily on intracellular membranes (56).

Virions contain three structural proteins which are derived from a common polyprotein precursor (44): an RNA-binding capsid protein which is located in the virus particle interior; and two membrane-spanning glycoproteins, E2 and E1 (45). The glycoproteins are targeted to the endoplasmic reticulum (ER) by two independently functioning amino-terminal signal peptides (19, 22), whereas capsid, a phosphoprotein, remains in the cytoplasm (40). Dimerization of E2 and E1 in the ER is required for efficient transport of both proteins to the Golgi complex (3, 24). Whether E2-E1 dimers are further oligomerize into $(E2\text{-}E1)_3$ trimers as in alphaviruses has not been resolved (14). The transmembrane (TM) domain of E2 acts as a Golgi-targeting signal which mediates retention of the E2-E1 heterodimer at this site (25). As for alphaviruses (15, 54), RV budding is thought to result from the interaction between the nucleocapsid (capsid plus RNA) and the cytoplasmic (CT) domains of E2 and/or E1 (21). During an earlier study (25), we obtained preliminary evidence indicating that E1 may also contain intracellular targeting information, specifically, an ER retention domain. In this investigation, we have characterized the retention signal in RV E1 glycoprotein in greater detail by studying the effects of fusing E1 domains to the ectodomains of two different cell surface glycoproteins, vesicular stomatitis virus G protein (VSV G) and CD8, which have the same membrane topology as E1. We report that the TM and CT domains of E1 glycoprotein confer retention in the ER by a mechanism which does not involve misfolding or aggregation. As far as we are aware, this is the first ER retention signal that requires both a TM and a CT domain. We hypothesize that these E1 domains play a critical role early in the viral assembly pathway by modulating the transport of newly assembled E2-E1 heterodimers from the ER. The time required for transport of E2-E1 heterodimers from the ER to the Golgi appears to be correlated with the folding rate of E1, which is ~10 times slower than that of E2 (24). Since E2-E1 dimerization occurs while folding of E1 is under way (24), the E1 ER retention signal may serve to prevent immature glycoprotein spike complexes from leaving the ER before maturation of E1 is completed.

## MATERIALS AND METHODS

**Reagents.** Reagents and supplies were from the following sources. Protein A- and G-Sepharose were purchased from Pharmacia (Alameda, Calif.). Fibronectin, sodium dodecyl sulfate (SDS), brefeldin A (BFA), nocodazole, dialyzed fetal bovine serum (FBS), and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, Mo.). Promix ([$^{35}$S]methionine-cysteine; 1,000 Ci/mmol) and $^{14}$C-labeled protein standards were purchased from Amersham Corp. (Arlington Heights, Ill.). Texas red-conjugated goat anti-mouse Immunoglobulin

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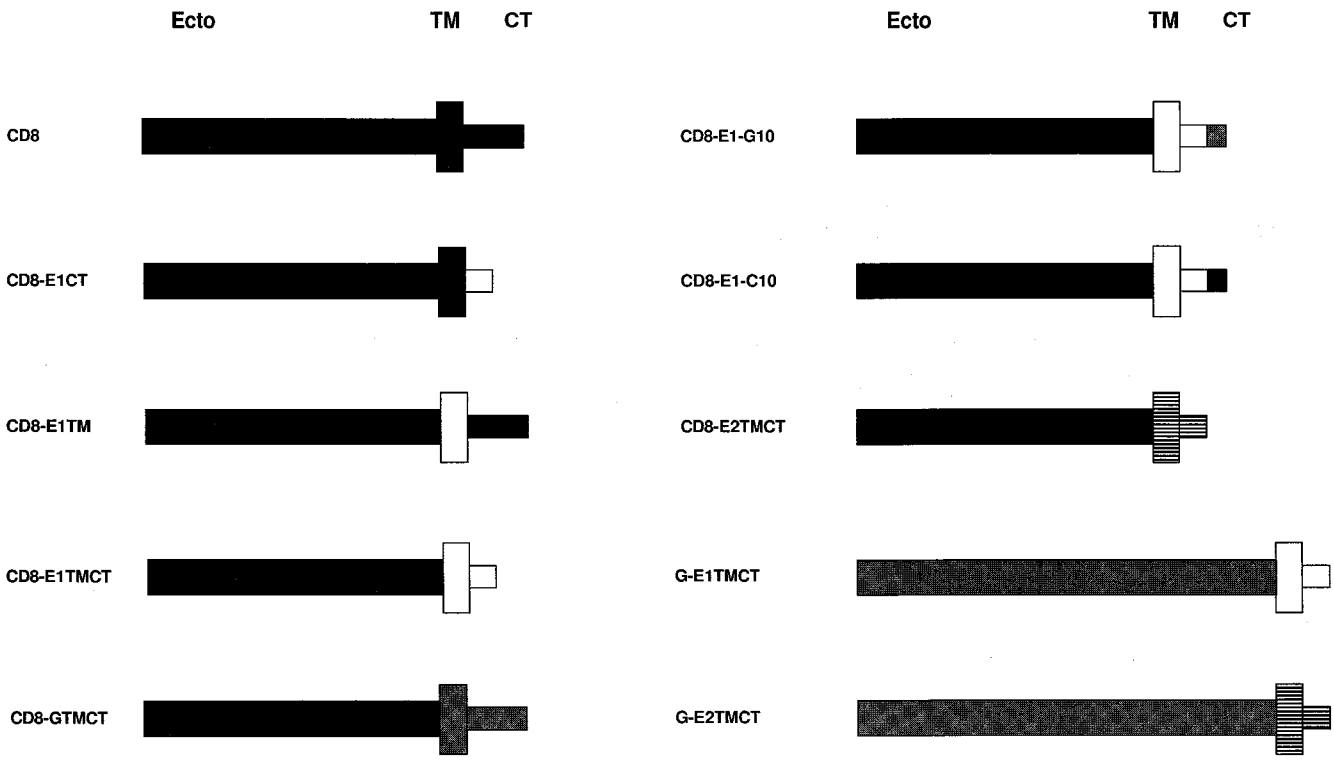


FIG. 1. Expression constructs. The ectodomain (Ecto) and the TM and CT domains of each cDNA are indicated and are used in naming the constructs accordingly. Each domain is shaded to signify its origin: CD8, black; E1, white; VSV G, grey; and E2, horizontal lines. For example, CD8-E1$_{CT}$ encodes the CD8 ectodomain and TM domain (black) fused to the E1 CT domain (white). CD8-E1-G10 and CD8-E1-C10 are exactly the same as CD8-E1$_{TMCT}$ except that the E1 CT domain has been lengthened by fusing the last 10 amino acids from VSV G and CD8, respectively. Each cDNA construct was subcloned into a mammalian cell expression vector and stably transfected into CHO cells. The chimeric proteins were analyzed by indirect immunofluorescence and biosynthetic labeling using monoclonal antibodies against the ectodomains of VSV G and CD8.

G (IgG) and fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG (each double-labeling grade) were purchased from Jackson ImmunoResearch Laboratories (West Grove, Pa.). The SFV RNA expression system, Lipofectin, Optimem serum-free medium, FBS, alpha minimal essential medium (MEM) without nucleosides, and reagents for in vitro transcription were obtained from Life Technologies Inc. (Gaithersburg, Md.). MEM lacking cysteine and methionine was purchased from ICN Biomedicals (Irvine, Calif.). DOSPER transfection reagent, Pefabloc, and *Pwo* polymerase were purchased from Boehringer Mannheim Corporation (Laval, Quebec, Canada). Rabbit antiserum to α-mannosidase II (Man II) and rough ER (RER) membrane proteins were obtained from Marilyn Farquhar (University of California, San Diego) and William Dunn (University of Florida, Gainesville), respectively. The mouse hybridomas OKT8 and BW8G65, which secrete antibodies to the ectodomains of CD8 and VSV G, respectively, were obtained from the American Type Culture Collection (Rockville, Md.) and Bill Balch (Scripps Research Institute, La Jolla, Calif.), respectively. The CD8 cDNA (34) was obtained from Richard Wozniak (Department of Cell Biology and Anatomy, University of Alberta).

Rabbit antiserum to the CT domain of RV E1 was produced by immunizing rabbits with a synthetic peptide, $NH_2$-KGLYYLRGAIAPR-COOH, coupled to rabbit serum albumin with gluteraldehyde. All injections and bleeds were done by Chemicon (Temecula, Calif.).

**Reporter proteins.** G-E1$_{TMCT}$ is a cDNA that encodes the ectodomain of VSV G fused in frame to the TM and CT domains of E1 (Fig. 1). Its construction has been described previously (25).

G-E2$_{TMCT}$ is a cDNA that encodes the ectodomain of VSV G fused in frame to the TM and CT domains of E2 (Fig. 1). Its construction has been described previously (25).

CD8-E1$_{TMCT}$ is a cDNA that encodes the ectodomain of CD8 fused to the TM and CT domains of RV E1 glycoprotein (Fig. 1). The cDNA was constructed by replacing an *Eco*RV-*Hin*dII fragment from pCMV5-CD8 which encodes the CD8 TM and CT domains with an *Eco*RV-*Hin*dIII fragment from pCMV5-E1 (20) which encodes the analogous domains from E1.

CD8-G$_{TMCT}$ is a cDNA that encodes the ectodomain of CD8 fused to the TM and CT domains of VSV G (Fig. 1). The cDNA was constructed by replacing an *Eco*RV-*Hin*dII fragment from pCMV5-CD8 which encodes the CD8 TM and CT domains with an *Eco*RV-*Hin*dIII fragment from pCMV5-G$_{EcoRV}$ (25) which encodes the analogous domains from G protein.

CD8-E1$_{CT}$ is a cDNA that encodes the ectodomain and TM domain of CD8 fused to the CT domain of E1 glycoprotein (Fig. 1). An antisense oligonucleotide (5′-G CAT GGA TCC CTA GCG CGG TGC TAT AGC GCC GCG CAA GTA GTA CAA GCA TTT GTG GTT GCA GTA AAG GGT G-3′) encoding the entire CT region of E1 and a portion of the CD8 TM domain was used in a PCR to introduce the E1 CT domain in place of the CD8 CT domain.

CD8-E1$_{TM}$ is a CD8 derivative that contains the E1 TM domain in place of the CD8 TM domain (Fig. 1). A sense oligonucleotide (5′-CGC GGA TAT CCA TTG GTG GCA GCT CAC TCT GGG CGC CAT TTG CGC CCT CCC ACT CGC TGG CTT ACT CGC TTG CTG TGCACAG GAA CCG AAG ACG TGT T-3′) containing an *Eco*RV site fused to the coding region for the E1 TM domain was used in a PCR to fuse the E1 TM domain to the CD8 CT domain coding region. The E1-TM-CD8-CT PCR product was ligated to ectodomain of CD8 via the *Eco*RV site.

CD8-E1-G10 is identical to CD8-E1$_{TMCT}$ except that the C-terminal 10 amino acids from VSV G are fused to the end of the E1 CT domain. An antisense oligonucleotide (5′-CGC AAG CTT ACT TTC CAA GTC GGT TCA TCT CTA TGT CTG TGC GCG GTG CTA TAG CGC C-3′) encoding the 10 amino acids from VSV G and a portion of the E1 CT domain was used in a PCR to make this construct.

CD8-E1-C10 is identical to CD8-E1$_{TMCT}$ except that the C-terminal 10 amino acids from CD8 are fused to the end of the E1 CT domain. An antisense oligonucleotide (5′-GCG AAG CTT TTA GAC GTA TCT CGC CGA AAG GCT GGG CTT GTC GCG CGG TGC TAT AGC G-3′) encoding the 10 amino acids from CD8 and a portion of the E1 CT domain was used in a PCR to make this construct.

All cDNAs were subcloned into the mammalian cell expression vector pCMV5 (2).

**PCRs.** *Pwo* polymerase was used in PCRs according to the manufacturer's instructions to introduce coding regions for the E1 TM and CT domains separately into the CD8 cDNA. Generally, 20 to 30 cycles were used for each reaction to minimize the chances of introducing second-site mutations. All products were verified by DNA sequencing.

**Cell culture and transfection.** CHODG44 cells were cultured and stably transfected exactly as described previously (23). BHK-21 cells were cultured in Glasgow MEM-tryptose phosphate containing 5% FBS. COS cells cultured in high-

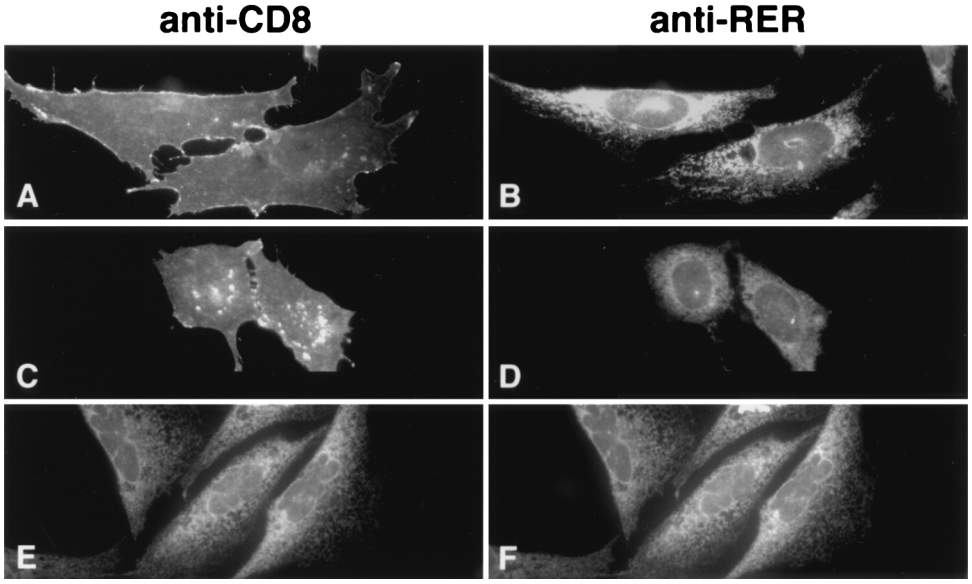


FIG. 2. Retention of the CD8 reporter protein in the ER is specific to the RV E1 TM and CT domains. CHO cells stably expressing native CD8 (A and B), CD8-$G_{TMCT}$ (C and D), or CD8-$E1_{TMCT}$ (E and F) were fixed with methanol and incubated with mouse anti-CD8 (A, C, and E) and rabbit anti-RER (B, D, and F), followed by anti-mouse–Texas red and anti-rabbit–fluorescein isothiocyanate. Similar to CD8, CD8-$G_{TMCT}$ is transported to the cell surface and does not colocalize with the RER marker. In contrast, CD8-$E1_{TMCT}$ (E) is restricted to the RER, as indicated by coincidental staining with anti-RER (F).

glucose Dulbecco modified Eagle medium containing 5% FBS were transfected by using Lipofectin according to manufacturer's instructions.

**Metabolic labeling and radioimmunoprecipitation.** Confluent 35-mm-diameter dishes of cells were washed once with phosphate-buffered saline (PBS) and incubated in MEM without cysteine and methionine and containing 5% dialyzed FBS for 15 min at 37°C. Cells were labeled for 10 to 15 min with 150 μCi of Promix ($^{35}$S label) in 250 μl of the same medium followed by chase periods in growth medium containing 25× excess methionine and cysteine. Radiolabeled cells were washed three times with ice-cold PBS and lysed on ice in 500 μl of 1% Nonidet P-40–150 mM NaCl–50 mM Tris-HCl (pH 8.0) containing 100 μg of the protease inhibitor Pefabloc per ml. Lysates were centrifuged at 14,000 × *g* for 5 min at 4°C before immunoprecipitation with 10 to 20 μg of OKT8 IgG and protein A-Sepharose. Immune complexes were washed twice with 1% Triton X-100–500 mM NaCl–50 mM Tris-HCl (pH 7.4), once with 0.2% Triton X-100–1.0 M NaCl–50 mM Tris-HCl (pH 7.4), and once with water. Samples were boiled in 2× SDS-gel sample buffer for 5 min before loading onto gels. Radioimmunoprecipitation of VSV G chimeras with antibody BW8G65 to the ectodomain of VSV G and protein G-Sepharose and endoglycosidase (endo H) H digestions were performed exactly as described previously (25).

**RNA-mediated transfection.** BHK-21 cells grown in 35-mm-diameter dishes ($3 \times 10^5$/dish) were transfected with in vitro-synthesized capped SFV-G-$E1_{TMCT}$ and SFV-G-$E2_{TMCT}$ RNAs as described previously (25).

**SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography.** VSV G and CD8 chimeric proteins were separated on 10 and 12.5% polyacrylamide gels, respectively (30). Gels were fixed in isopropanol-water-acetic acid (25:65:10) for 30 min before treatment with 1.0 M sodium salicylate–0.01% 2-mercaptoethanol for 20 min. After drying, gels were exposed to Kodak XAR film at −80°C. Protein bands were quantitated with a Bio-Rad densitometer.

**Immunofluorescence microscopy.** Cells were grown on fibronectin (10 μg/ml)-coated 12-mm-diameter glass coverslips, fixed with methanol at −20°C, and processed for indirect immunofluorescence as described previously (23). To trap proteins in the ER-Golgi intermediate compartment (ERGIC), cells were first treated with BFA (5 μg/ml from methanol stock) for 60 min, followed by addition of nocodazole (10 μg/ml from dimethyl sulfoxide stock) for an additional 120 min at 37°C prior to processing for indirect immunofluorescence.

## RESULTS

**The carboxy terminus of E1 functions as an ER retention signal.** In an earlier study, we noticed that replacement of the TM and CT domains of VSV G with those from RV E1 glycoprotein resulted in a chimeric protein, G-$E1_{TMCT}$, that was not transported from the ER (25). We reasoned that there could be at least two explanations to account for this observation: (i) G-$E1_{TMCT}$ was nonspecifically retained in the ER as a result of misfolding or (ii) G-$E1_{TMCT}$ is not misfolded but is specifically retained in the ER by a retention signal in the C-terminal region of E1. Before attempting to distinguish between these two possibilities, it was important to determine if these E1 domains could mediate the retention of another type I membrane protein, CD8, which like VSV G is normally transported to the cell surface. CD8 is a human T-cell surface glycoprotein (34) which has been used extensively as a reporter protein to study intracellular targeting signals (27, 41). The CD8 gene contains an *Eco*RV site immediately 5′ to the coding region for the TM and CT domains (34), which enabled us to easily append different TM and CT domains onto the ectodomain of CD8 (Fig. 1). The coding region for the E1 TM and CT domains was fused to the ectodomain of CD8, and the resulting cDNA, CD8-$E1_{TMCT}$, was expressed in stably transfected CHO cells. Double indirect immunofluorescence with OKT8, a monoclonal antibody to the ectodomain of CD8, and a polyclonal antibody to RER membrane proteins (11) was used to determine the intracellular localizations of the different CD8 chimeras. In contrast to normal CD8, which at steady state was detected primarily on the cell surface (Fig. 2A), CD8-$E1_{TMCT}$ was found to overlap exclusively with the RER marker (Fig. 2E and F). Retention of CD8-$E1_{TMCT}$ was stable, as treatment of cells with 1 mM cycloheximide for 3 h did not result in movement of the glycoprotein to the Golgi complex or plasma membrane (not shown). As a control to show that replacement of the CD8 TM and CT domains did not result in nonspecific ER retention, the TM and CT domains from VSV G were fused to the ectodomain of CD8 (Fig. 1). The resulting chimera, CD8-$G_{TMCT}$, was expressed in stably transfected CHO cells, and its steady-state localization was determined by indirect immunofluorescence. Similar to wild-type CD8, CD8-$G_{TMCT}$ was found primarily on the cell surface and in the Golgi complex and did not overlap with the RER marker in double indirect immunofluorescence (Fig. 2C and D).

The intracellular transport properties of the CD8 chimeras were also investigated by metabolic labeling and radioimmunoprecipitation. The biosynthesis and maturation of CD8 have

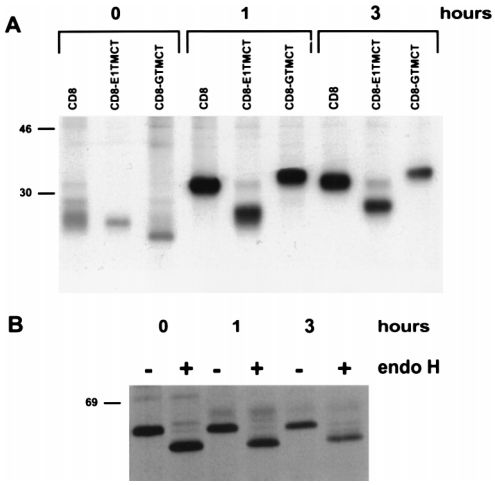


FIG. 3. The bulk of CD8-E1$_{TMCT}$ and G-E1$_{TMCT}$ do not undergo complete glycosylation. Transfected CHO cells grown in 35-mm-diameter dishes were pulse-labeled with 150 μCi of [$^{35}$S]methionine-cysteine for 10 min and chased in the absence of radioactivity for periods of 0, 1, and 3 h before lysis. Immunoprecipitates were prepared by using mouse anti-CD8 or anti-VSV G and protein A or G-Sepharose, respectively, separated by SDS-PAGE, and fluorographed. Positions of protein standards (in kilodaltons) are indicated at the left. In panel A, the mature forms (32 to 34 kDa) of CD8 and CD8-G$_{TMCT}$ which have traversed the Golgi complex are evident at 1 and 3 h of chase. In contrast, most of CD8-E1$_{TMCT}$ does not reach the Golgi complex and therefore does not receive its full complement of O-linked sugars. The anti-CD8 monoclonal antibody used in this study is conformation dependent and therefore does not bind efficiently to the CD8 ectodomain immediately after the pulse (0 h). (B) Immunoprecipitates prepared from CHO cells stably expressing G-E1$_{TMCT}$ cells were incubated with or without endo H before separation on SDS-PAGE (10% gel) and fluorography. After 3 h, >90% of G-E1$_{TMCT}$ remains endo H sensitive, indicating that the glycoprotein is largely confined to the ER.

been extensively studied in transiently and stably transfected cells (27, 41, 46). The mature glycoprotein is a disulfide-bonded homodimer which contains only O-linked sugars and is transported to the cell surface rapidly (half-time of <30 min). Stably transfected CHO cells were labeled for 10 min with [$^{35}$S]methionine-cysteine followed by chases for various time periods with medium containing excess methionine and cysteine. Radiolabeled CD8 proteins were immunoprecipitated from cell lysates by using OKT8 and subjected to SDS-PAGE and fluorography. Immediately after the 10-min pulse, the immature forms of CD8 and CD8-G$_{TMCT}$ migrated with apparent molecular masses of ~25 to 29 kDa (Fig. 3A, 0-h lanes), in close agreement with previous studies on the biosynthesis of CD8 (46). Newly synthesized CD8-E1$_{TMCT}$ migrated at approximately 27 kDa and displayed less electrophorectic heterogeneity at this time point (Fig. 3A, 0-h lane). The epitope recognized by OKT8 is conformation dependent, and consequently the antibody binds efficiently only to completely folded CD8 proteins. This is why very little radiolabeled material was precipitated immediately after the 10-min pulse. After 1 and 3 h, radiolabeled CD8 migrated at 32 to 34 kDa, indicating that the glycoprotein had become terminally glycosylated in the Golgi complex (Fig. 3A, 1- and 3-h lanes). For unknown reasons, the mature size of CD8-G$_{TMCT}$ was slightly larger than that of CD8 even though the predicted molecular masses of these proteins excluding glycosylation are virtually identical. Both VSV G and CD8 are palmitylated in their TM or CT domains (46, 48), and we did not notice any significant differences in the amount of charged residues in these domains which could potentially alter the binding of SDS and subsequent migration of the proteins on SDS-PAGE (Fig. 4). Therefore, at this point, we can only speculate that a posttranslational modification other than palmitylation, which is specific to the VSV G CT domain, accounts for the apparent difference in molecular mass. In contrast to CD8 and CD8-G$_{TMCT}$, the bulk of CD8-E1$_{TMCT}$ remained in an immature form (~28 kDa) during the 3-h chase period, with less than 10% conversion to the Golgi-processed form (Fig. 3A, 1- and 3-h lanes). Between 1 and 3 h of chase, there was a slight increase in the size of CD8-E1$_{TMCT}$, from 27 to 28 kDa, which may have been due to the addition of *N*-acetylgalactosamine (46) or some other unknown posttranslational modification as has been described for other ER-retained CD8 chimeras (27, 41). We favor the latter hypothesis since addition of *N*-acetylgalactosamine is thought to occur in a post-ER compartment (46, 51), and as documented below, we did not find any convincing evidence that much CD8-E1$_{TMCT}$ leaves the ER.

In a parallel experiment to verify our previous results, stably transfected CHO cells expressing G-E1$_{TMCT}$ were biosynthetically labeled and immunoprecipitated with a monoclonal antibody to the VSV G ectodomain (Fig. 3B). After a 3-h chase period, >90% of G-E1$_{TMCT}$ remains sensitive to endo H, indicating that it is not transported to the medial Golgi complex during this time period (Fig. 3B).

Transmembrane/Cytoplasmic domains which do mediate ER retention

| | |
|---|---|
| Rubella E1 | WWNLTLGAICALPLVGLLACCAKCLYYLRGAIAPR |
| Ad2 E19 | _____KYKSRRSFIDEKKMP |
| CD3ε | _____EPIRKGQRDLYSGLNQRRI |
| TCRα | NLSVMGLRILLLKVAGFNLLMTL---------- |

Transmembrane/Cytoplasmic domains which do not mediate ER retention

| | |
|---|---|
| VSV G | SSIASFFFIIGLIIGLFLVLRVGIHLCIKLKHTKKRQIYTDIEMNRLGK |
| CD8 | DIYIWAPLAGTCGVLLLSLVITLYCNHRNRRRRVCKCPRPVVKSGDKPSLSARYW |
| Rubella E2 | AFAAFVLLVPWVLIFMVCRRACRRR |

FIG. 4. Comparison of E1 TM and CT domains to those of other type I membrane proteins. The predicted membrane-spanning region of each protein is underlined. The sequence of the human T-cell receptor subunit α-subunit (TCRα) CT domain is not shown since it is not required for retention and degradation. Notice the two charged residues (R and K) within the TM sequence of this protein. The sequence of the adenovirus E19 protein (Ad2 E19) and human T-cell receptor subunit CD3ε TM domains are not shown since they are not required for ER retention. Amino acids that are known to be important for retention are shown in a larger shadowed font.

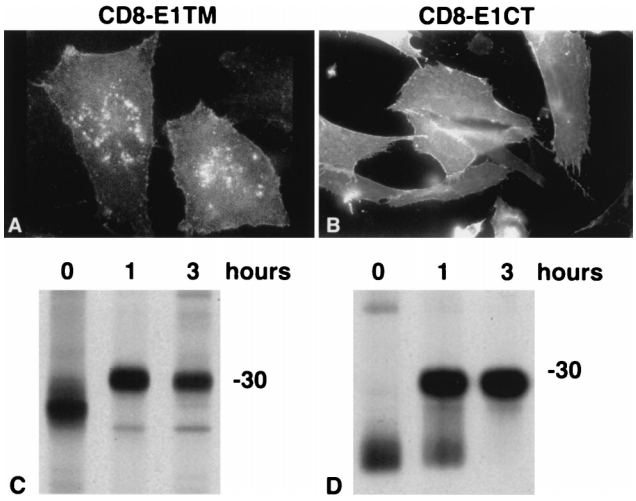


FIG. 5. Both the TM and CT domains of RV E1 are required for retention in the ER. CD8 reporter constructs containing the E1 TM or CT domain in place of the CD8 TM (CD8-$E1_{TM}$) or CD8 CT (CD8-$E1_{CT}$) domain were expressed in stably transfected CHO cells and processed for indirect immunofluorescence, biosynthetic labeling, and radioimmunoprecipitation using anti-CD8 as described for Fig. 3. Both CD8 chimeras are transported to the cell surface (A and B) and become fully glycosylated after 1 h (C and D), indicating that neither glycoprotein is retained in the ER. CD8-$E1_{CT}$ (D) is slightly smaller than CD8-$E1_{TM}$ (C) because the E1 CT domain is shorter than that of CD8. Sizes are indicated in kilodaltons.

We monitored the fate of newly synthesized CD8-$E1_{TMCT}$ for longer chase periods to see if more of the protein exited the ER during extended chase periods. After 7 h, the vast majority of CD8-$E1_{TMCT}$ was still in an immature form (not shown). Even though CD8-$E1_{TMCT}$ was not exported from the ER, it was not subject to rapid degradation, as less than 30% of CD8-$E1_{TMCT}$ was degraded after 7 h, compared with >35% for CD8 (not shown). Separation of CD8-$E1_{TMCT}$ immunoprecipitates on nonreducing gels revealed that it migrates as a disulfide-linked homodimer identical to CD8 and does not form cross-linked aggregates (not shown). Formation of disulfide-linked aggregates is a common fate of misfolded proteins which are retained in the ER (38). To determine if CD8-$E1_{TMCT}$ and/or G-$E1_{TMCT}$ were stably associated with ER chaperones, we used lysis and coimmunoprecipitation conditions known to preserve interactions between nascent and/or misfolded proteins with ER chaperones (17, 37). From these experiments, we were not able to detect any stable interactions between the two ER-retained proteins and chaperones such as BiP, calnexin, GRP94, or calreticulin (data not shown). These results suggest that CD8-$E1_{TMCT}$ and G-$E1_{TMCT}$ are retained in the ER by a mechanism that does not involve the ER quality control system or aggregation due to misfolding.

**Both the TM and CT domains of E1 are required for retention in the ER.** Generally, retention signals within resident ER membrane proteins have been localized within TM or CT domains (16, 27, 50, 55, 58). In addition, some ER membrane proteins contain two independently functioning retention signals, one in the TM domain and the other in the CT domain (49). E1 does not contain any of the previously characterized motifs which are known to cause ER retention of type I membrane proteins within its TM or CT domain. Specifically, there are no charged amino acids in the TM domain or any recognizable versions of dilysine or YXXLXXR in the CT region (Fig. 4). Therefore, it was not possible to predict, based on homology, whether the E1 TM or CT domain was responsible for ER localization. We next sought to map more precisely the E1 ER retention signal by constructing CD8 chimeras containing either the E1 TM or CT domain alone to see if either of these domains could independently mediate retention. CD8-$E1_{TM}$ and CD8-$E1_{CT}$, which contain the E1 TM and CT domains in place of the CD8 domain, respectively (Fig. 1), were stably expressed in CHO cells as described above. The localization of these proteins was investigated by indirect immunofluorescence and biosynthetic labeling. Under steady-state conditions, neither of these CD8 chimeras was confined to the ER; instead both were localized to the cell surface and the Golgi (Fig. 5A and B). Similarly, both glycoproteins were rapidly converted into their mature 30- to 32-kDa forms after 1 h of chase (Fig. 5C and D). The CT domain of E1 consists of 13 amino acids, compared to 27 for CD8, and consequently the mature size of CD8-$E1_{CT}$ is ~30 kDa, 2 kDa less than that of CD8-$E1_{TM}$. These results indicate that both chimeras were rapidly transported from the ER and that neither the E1 TM nor E1 CT domain alone is sufficient for retention.

**Expression of the E2 TM and CT domains does not mediate release of ER retention caused by E1 TM and CT domains.** RV glycoproteins E2 and E1 form a heterodimer in the ER before efficient transport to the Golgi complex occurs (3, 24). We therefore reasoned that the retention signal on E1 might be masked during assembly with E2 in order to allow transport of the E2-E1 heterodimer from the ER. This could potentially be effected by direct interaction of the E2 and E1 TM and/or CT domains as has been described for other oligomeric complexes (4, 7, 31). We decided to test whether coexpression of CD8-$E1_{TMCT}$ with a CD8 chimera containing E2 TM and CT do-

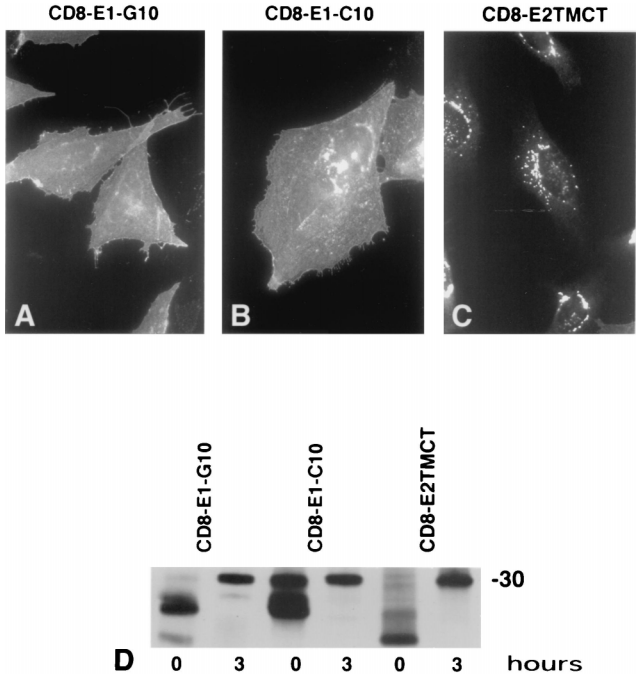


FIG. 6. Extending the length of the E1 CT domain by 10 amino acids abolishes ER retention. CHO cells stably transfected with CD8-E1-G10 (A), CD8-E1-C10 (B), and CD8-$E2_{TMCT}$ (C) were grown on fibronectin-coated coverslips and processed for indirect immunofluorescence using an anti-CD8 monoclonal antibody as described for Fig. 5. CD8-E1-G10 (A) and CD8-E1-C10 (B) were efficiently transported to the cell surface, in contrast to CD8-$E2_{TMCT}$ (C), which is retained in the Golgi complex. Cells were also labeled with [$^{35}$S]methionine-cysteine for 15 min and chased for 0 and 3 h before lysis and radioimmunoprecipitation. All three CD8 chimeras are rapidly transported through the Golgi complex and become fully glycosylated in less than 3 h.

mains would result in transport of the former from the ER. CD8-$E2_{TMCT}$ was constructed by ligating the coding regions for the E2 TM and CT domains to the ectodomain of CD8 as described above (Fig. 1). CD8-$E2_{TMCT}$ was stably expressed in CHO cells, and as expected, this glycoprotein was efficiently transported from the ER to the Golgi complex (Fig. 6C and D), where it was retained by the Golgi retention signal in the E2 TM domain (25). CD8-$E2_{TMCT}$ and CD8-$E1_{TMCT}$ are predicted to be similar in size; therefore, we engineered a 10-amino-acid epitope tag from VSV G onto the end of CD8-$E1_{TMCT}$ so that it could be distinguished from CD8-$E2_{TMCT}$. The resulting construct, CD8-E1-G10 (Fig. 1), could then be differentiated from CD8-$E2_{TMCT}$ by indirect immunofluorescence in cotransfected cells by using monoclonal antibody P5D4 directed against the carboxyl-terminal 10 amino acids of the VSV G CT domain (29).

Stably transfected CHO cell lines were constructed to ensure that CD8-E1-G10 was targeted to the ER similarly to CD8-$E1_{TMCT}$. Unexpectedly however, indirect immunofluorescence and radioimmunoprecipitation experiments revealed that the epitope-tagged chimera was efficiently transported to the plasma membrane (Fig. 6A and D). Results from a previous study are consistent with the possibility that the CT of VSV G may in fact facilitate transport from the ER (10). In light of this finding, it was important to determine if it was the VSV G epitope per se that was causing the CD8 chimera to be transported from the ER or whether simply lengthening the E1 CT domain was abrogating retention. We constructed CD8-E1-C10 (Fig. 1), which is identical to CD8-E1-G10 except that the 10-amino-acid P5D4 epitope (G10) was replaced with the carboxy-terminal 10 amino acids from the CD8 (C10). This glycoprotein was also transported from the ER to the Golgi complex and cell surface (Fig. 6B and D) very rapidly, indicating that loss of ER retention was not specific to the P5D4 epitope but rather resulted from increasing the length of the E1 CT domain.

Since it was not possible to use a carboxy-terminal epitope-tagged version of CD8-$E1_{TMCT}$ for coexpression experiments, a polyclonal antibody was generated to the CT domain of E1. This antibody worked well for immunoprecipitation but not for immunoblotting or indirect immunofluorescence (not shown). CHO-CD8-$E1_{TMCT}$ cells were transiently transfected by using DOSPER with an expression plasmid containing CD8-$E2_{TMCT}$. Forty-eight hours posttransfection, the cells were lysed in PBS–1% Triton X-100 after biosynthetic labeling with [$^{35}$S]methionine-cysteine for 15 min and a chase period of 3 h. Rabbit anti-E1 CT serum was used to immunoprecipitate CD8-$E1_{TMCT}$ from cells transfected with or without CD8-$E2_{TMCT}$, and radiolabeled proteins were visualized by SDS-PAGE and fluorography. We did not observe an increase in Golgi-specific processing of CD8-$E1_{TMCT}$ in cells that were transfected with CD8-$E2_{TMCT}$ (data not shown). This may have been due to the fact that retransfection of CHO-CD8-$E1_{TMCT}$ cells with the CD8-$E2_{TMCT}$ plasmid was not very efficient. We obtained better coexpression efficiency (>30%) following cotransfection of wild-type CHO cells with plasmids encoding CD8-$E1_{TMCT}$ and CD8-$E2_{TMCT}$; however, we still did not observe increased processing of CD8-$E1_{TMCT}$ when coexpressed with CD8-$E2_{TMCT}$ (data not shown). Thus, it did not appear that coexpression of the E2 TM and CT domains can mediate release of CD8-$E1_{TMCT}$ from the ER.

The quaternary structure of the RV glycoprotein spike complex is unknown. Specifically, it has not been determined if the E2-E1 heterodimers oligomerize further to $(E2\text{-}E1)_3$ structures as in alphaviruses (14). Should this be the case, it may be that more than one copy of the E2 TM domain is required for

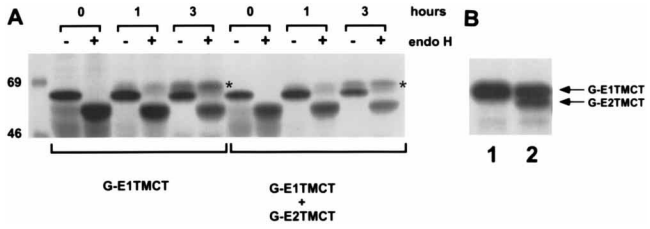


FIG. 7. Coexpression with G-$E2_{TMCT}$ is not sufficient to rescue G-$E1_{TMCT}$ from the ER. BHK-21 cells were transfected with in vitro-synthesized capped RNAs encoding G-$E1_{TMCT}$ alone or along with G-$E2_{TMCT}$ as indicated at the bottom of panel A. Four hours posttransfection, cells were labeled for 10 min with [$^{35}$S]methionine-cysteine and chased for the indicated time periods in the absence of radioactivity. Radioimmunoprecipitates were prepared from cell lysates by using rabbit anti-E1 CT serum (A) or a monoclonal antibody to the ectodomain of VSV G (B) and incubated with or without endo H as indicated. Samples were then subjected to SDS-PAGE on 10% acrylamide gels before fluorography. The fraction of endo H-resistant G-$E1_{TMCT}$ present after 1 and 3 h (asterisk) is not increased when it is coexpressed with G-$E2_{TMCT}$. The leftmost lane in panel A contains $^{14}$C-methylated protein standards (positions indicated in kilodaltons). (B) To show that both glycoproteins were expressed at similar levels in the transfected cells, G-$E1_{TMCT}$ and G-$E2_{TMCT}$ were immunoprecipitated from the aliquots of the same cell lysates used for panel A immediately after the 10-min pulse, using a monoclonal antibody to the ectodomain of VSV G. Samples were then subjected to SDS-PAGE and fluorography.

neutralizing the E1 retention domain during assembly. The quaternary structure of CD8 is a dimer (34), which could potentially explain why coexpression of CD8-$E2_{TMCT}$ did not result in increased transport of CD8-$E1_{TMCT}$ from the ER. Indeed, the oligomeric status of proteins is thought to affect their trafficking between the ER and Golgi complex (10, 26, 27). We therefore decided to determine whether coexpression of G-$E2_{TMCT}$ could rescue G-$E1_{TMCT}$ from the ER, the rationale being that VSV G normally forms trimers. We reasoned that two G-$E2_{TMCT}$ molecules could potentially oligomerize with one G-$E1_{TMCT}$ since trimerization of VSV G is effected by the ectodomain (9, 10). To increase the efficiency of coexpression of G-$E1_{TMCT}$ and G-$E2_{TMCT}$, we used an RNA-mediated transfection procedure based on the SFV replicon (25, 32). BHK-21 cells were transfected with self-replicating in vitro-transcribed RNA encoding G-$E1_{TMCT}$ with or without G-$E2_{TMCT}$ RNA. Four hours later, cells were biosynthetically labeled with [$^{35}$S]methionine-cysteine for 10 min and chased for up to 3 h before lysis and radioimmunoprecipitation with anti-VSV G antibodies or anti-E1 CT serum. In Fig. 7A, it can be seen that coexpression of G-$E2_{TMCT}$ has no measurable effect on the processing of G-$E1_{TMCT}$. After 1 and 3 h of chase, the bulk of G-$E1_{TMCT}$ remained sensitive to endo H whether or not G-$E2_{TMCT}$ was present (Fig. 7A). Figure 7B shows that with this transfection method, G-$E1_{TMCT}$ and G-$E2_{TMCT}$ are expressed at similar levels in BHK cells. A small amount of endo H-resistant material was evident after 3 h, indicating that some G-$E1_{TMCT}$ leaves the ER both in the presence and in the absence of G-$E2_{TMCT}$ (Fig. 7A, asterisk). In contrast, more than 50% of G-$E2_{TMCT}$ had become resistant to digestion with endo H after a 60-min chase (not shown). These results suggest that coexpression and juxtaposition of the E2 TM and CT domains are not sufficient to overcome $E1_{TMCT}$-mediated retention.

**The E1 ER retention signal functions by static retention.** RNA-mediated transfection of G-$E1_{TMCT}$ into BHK-21 cells caused higher levels of expression than in stably transfected CHO cells, which consequently resulted in significantly more endo H-resistant G-$E1_{TMCT}$ accumulating after 3 h of chase (compare Fig. 7A and 3B). Similarly, overexpression of G-$E1_{TMCT}$ and CD8-$E1_{TMCT}$ in COS cells (Fig. 8) led to signif-

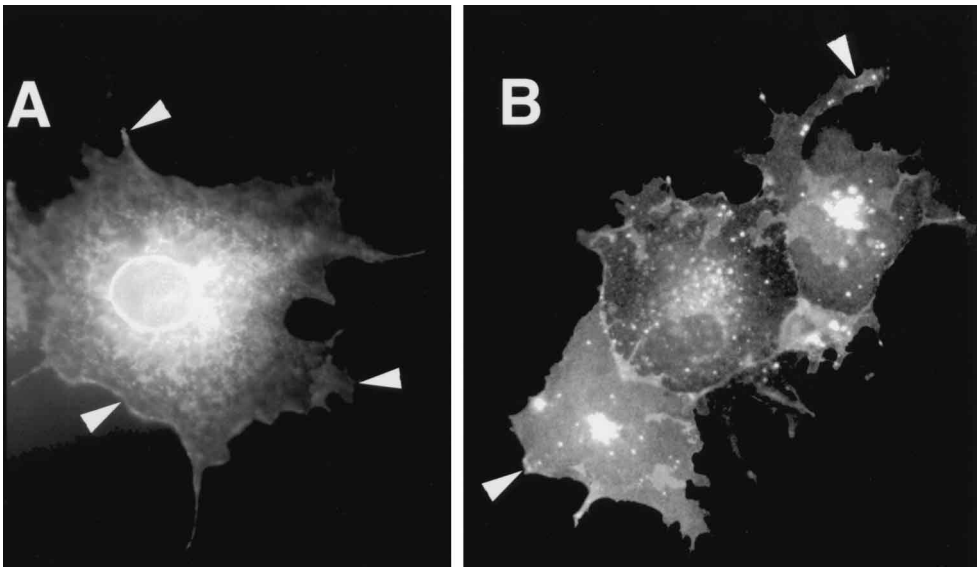


FIG. 8. Hi-level expression of G-E1$_{TMCT}$ and CD8-E1$_{TMCT}$ in COS cells results in transport of a fraction of the glycoproteins to the cell surface. COS-7 cells were transfected with plasmids encoding CD8-E1$_{TMCT}$ (A) and G-E1$_{TMCT}$ (B) and at 48 h posttransfection were fixed in methanol. They were subsequently processed for indirect immunofluorescence by using monoclonal antibodies to the ectodomain of CD8 (A) or VSV G (B) followed by Texas red-conjugated goat anti-mouse IgG. In both cases, high-level transient expression results in a significant proportion of the glycoproteins being transported to the cell surface. Cell surface staining is indicated by the arrowheads at the edges of the cell borders. Significantly more G-E1$_{TMCT}$ than CD8-E1$_{TMCT}$ was present on the plasma membrane, suggesting that this chimera is not retained as efficiently in COS cells.

icant levels of cell surface expression (compare to Fig. 2E and 9A). In such instances where extremely high levels of transient protein expression are achieved, it is not uncommon to observe mislocalization of proteins due to saturation of the protein retention systems (28, 43). In contrast, transient expression of the two glycoproteins in BHK-21 and CHO cells by using plasmid-based transfection did not lead to significant levels of cell surface expression (data not shown). From these data, we reasoned that it was possible that transport of the E1 chimeras out of the ER was due to saturation of a static or retrieval-based retention system in the transfected COS and RNA-transfected BHK-21 cells.

To address whether the E1 ER retention signal operated by retrieval, we used conditions which have been shown to trap proteins which cycle between the ER and Golgi complex through the ERGIC (18, 27, 33). CHO-G-E1$_{TMCT}$ cells were used for these experiments because in some cells, the G-E1 chimera was detected in vesicular elements which did not correspond to ER-associated structures, indicating that a fraction of G-E1$_{TMCT}$ is able to leave the ER in CHO cells (data not shown). CD8-E1$_{TMCT}$ was never detected in vesicular structures in CHO cells, suggesting that retention of this chimera was more efficient than retention of G-E1$_{TMCT}$. Jackson et al. also noticed that retention efficiency of the dilysine ER targeting motif varied according to the reporter protein used (27). CHO-G-E1$_{TMCT}$ cells were treated with the fungal metabolite BFA for 60 min, followed by addition of nocodazole for 120 min at 37°C. Under these conditions, Golgi membrane proteins first redistribute to the ER due to the action of BFA. In the presence of both BFA and nocodazole, proteins that normally leave the ER do so and become trapped in ERGIC, since BFA blocks movement from ERGIC to the Golgi complex, and nocodazole prevents microtubule-dependent redistribution of proteins from ERGIC to the ER (33). As expected, Man II, a Golgi membrane protein, redistributes to the ER and perinuclear vesicular structures under these conditions (compare Fig. 9B and D). In a small fraction of cells, G-E1$_{TMCT}$ was found to colocalize with Man II in the vesicular structures (Fig. 9C and D, arrowheads). However, most of the cells in Fig. 9C contain G-E1$_{TMCT}$ in the ER only, and thus the two cells with vesicular staining are the exception rather than the rule. Analysis of more than 20 microscopic fields confirmed that >85% of cells contained G-E1$_{TMCT}$ in the ER only (not shown).

We also used temperature shift experiments in an effort to detect cycling of G-E1$_{TMCT}$ between the ER and Golgi complex. CHO-G-E1$_{TMCT}$ cells were incubated at 15°C for 2 h followed by incubation at 37°C for 5 and 30 min. Under these conditions, ER membrane proteins that cycle between the ER and Golgi complex will accumulate in ERGIC at 15°C and synchronously move toward the Golgi complex upon warming to 37°C (27, 33, 39, 52). We did not detect any significant change in the distribution of G-E1$_{TMCT}$ or CD8-E1$_{TMCT}$ in the temperature shift experiments (not shown). If ER localization of these two proteins did involve retrieval from post-ER compartments, we would have detected their presence in post-ER structures in all cells under these conditions, and this was clearly not the case. Thus, from the BFA-nocodazole and temperature shift experiments, we conclude that the majority of G-E1$_{TMCT}$ and CD8-E1$_{TMCT}$ molecules do not leave the ER. Accordingly, we believe that the E1$_{TMCT}$ which was localized to non-ER vesicles, represents the small fraction of protein which escapes the ER and proceeds to the cell surface rather than being recycled. Similarly, we believe that the G-E1$_{TMCT}$ and CD8-E1$_{TMCT}$ found on the cell surface of transfected COS cells represents the fraction of protein that leaks out of the ER and is not recycled.

## DISCUSSION

In this study, we have characterized an ER retention signal present in the RV E1 glycoprotein. An emerging theme is that membrane and soluble proteins are localized to the ER by multiple mechanisms which can involve one or more recognizable retention signals. Similarly, with regard to E1, there

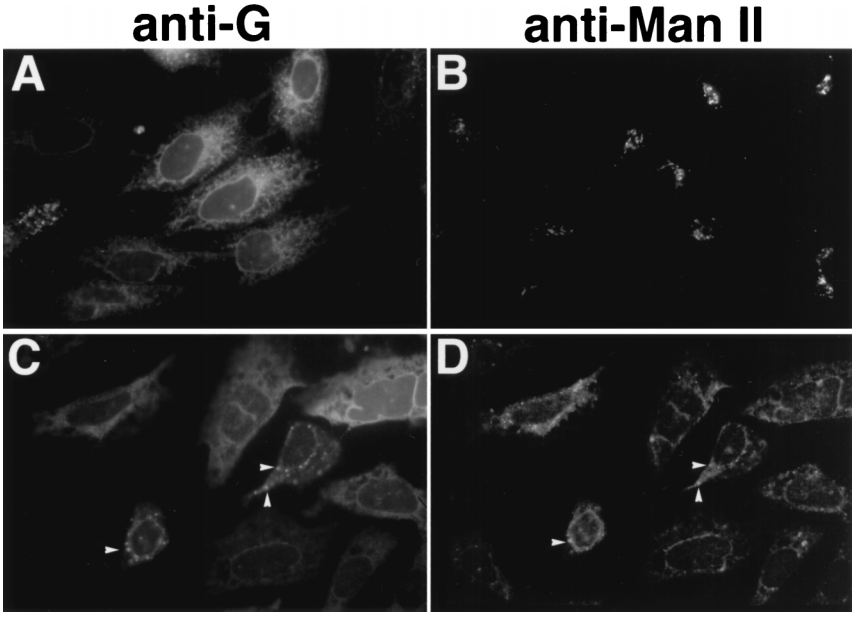


FIG. 9. The bulk of G-E1$_{TMCT}$ does not leave the ER. CHO cells stably expressing G-E1$_{TMCT}$ were processed for indirect immunofluorescence after growth at 37°C with no further treatment (A and B) or after treatment with 5 μg of BFA per ml for 60 min followed by BFA and nocodazole (10 μg/ml) for 120 min (C and D). G-E1$_{TMCT}$ (A and C) and the Golgi marker Man II (B and D) were stained with a monoclonal antibody to the VSV G ectodomain and rabbit anti-Man II, respectively. In the presence of BFA and nocodazole, the distribution of Man II changes from a compact juxtanuclear pattern (B) to punctate perinuclear (D), indicating its entrapment in the ER and ERGIC. The field of cells in panels C and D was chosen to show that in a minority of cells, overlap between G-E1$_{TMCT}$ and Man II in vesicular structures (arrowheads) was observed after treatment. However, in the vast majority of cells, the distribution of G-E1$_{TMCT}$ did not change in response to BFA and nocodazole (panel C and data not shown).

appear to be at least two distinct means of preventing unassembled subunits from reaching the Golgi complex and budding. Unassembled E1 subunits accumulate in a tubular pre-Golgi compartment when synthesized in the absence of E2 (23). Sorting of E1 into this compartment is contingent upon the lumenal domain only and is therefore independent of the carboxy-terminal retention domain described in this study (25a). Our current view holds that E1 plays a central role at two key points in the RV maturation pathway by (i) modulating the assembly and transport of the E2-E1 dimer from the ER to the Golgi complex (24) and (ii) driving the budding reaction through binding of nucleocapsids to its CT domain. Direct binding of the E1 CT domain to nucleocapsids has not been demonstrated as yet, but we have provided indirect evidence for this previously (21). If the E1 CT domain does interact directly with nucleocapsids during the budding reaction, it would be advantageous for RV to have evolved one or more mechanisms to prevent unassembled and presumably nonfunctional E1 subunits from reaching the budding site, where they could interfere with viral assembly by competing for nucleocapsid binding.

**Mechanism of ER retention.** A growing number of ER retention signals have been identified recently. The best characterized of these motifs are KDEL-COOH, KKXX-COOH, YXXLXXR-COOH (where X is any amino acid), and $NH_2$-RR, all of which seem to function by retrieval from post-ER compartments (27, 39, 47, 50). For KDEL-bearing proteins at least, the primary means of ER retention is not retrieval since deletion of this sequence does not necessarily affect ER localization (53). This finding suggests that the KDEL signal may actually function as a fail-safe mechanism to retrieve small amounts of lumenal proteins that do escape the ER. As mentioned above, a given protein may have two types of retention signals, one that functions by static retention and one that functions by retrieval. For instance, the localization of the ER membrane protein Sec12p is facilitated by both static retention (CT domain mediated) and retrieval (TM domain mediated) from post-ER compartments (49). Yet another means of ER localization utilized by some cytosolic proteins is via short hydrophobic sequences at or near their carboxy termini (1, 12, 36, 42, 58). Finally, in the case of certain multiprotein complexes, unassembled subunits often have exposed motifs that mediate their ER retention and rapid proteolysis (6). For the T-cell antigen receptor complex subunits, ER retention and degradation information is contained in the TM and/or CT domains of α and β subunits (6). In addition, a third component of this complex, CD3ε, has been shown to contain a tyrosine-based ER retention motif (Fig. 4) near the end of its CT domain (39). Subunit assembly results in masking of the degradation motifs, thereby ensuring that only properly assembled and therefore functional complexes leave the ER.

The RV ER retention signal (Fig. 4) does not possess any obvious homologies to any of the aforementioned motifs and may therefore represent a new class of targeting motif. As far as we are aware, it is the only ER retention signal whose function requires both a TM and a CT domain. Clearly, these E1 domains do not act as a degradation signal which targets proteins for rapid ER degradation, since CD8-E1$_{TMCT}$ was even more stable than native CD8. With regard to whether the E1 retention signal mediates ER localization by static retention or retrieval, our results are consistent with the former. Under steady-state conditions, in a very small fraction of cells, G-E1$_{TMCT}$ but not CD8-E1$_{TMCT}$ was localized to vesicular structures that did not correspond to ER and was occasionally detected on the cell surface (data not shown). In addition, transfected COS cells contained significantly more G-E1$_{TMCT}$ on the cell surface than CD8-E1$_{TMCT}$ (Fig. 8). Taken together, these data suggest that CD8-E1$_{TMCT}$ is retained more effi-

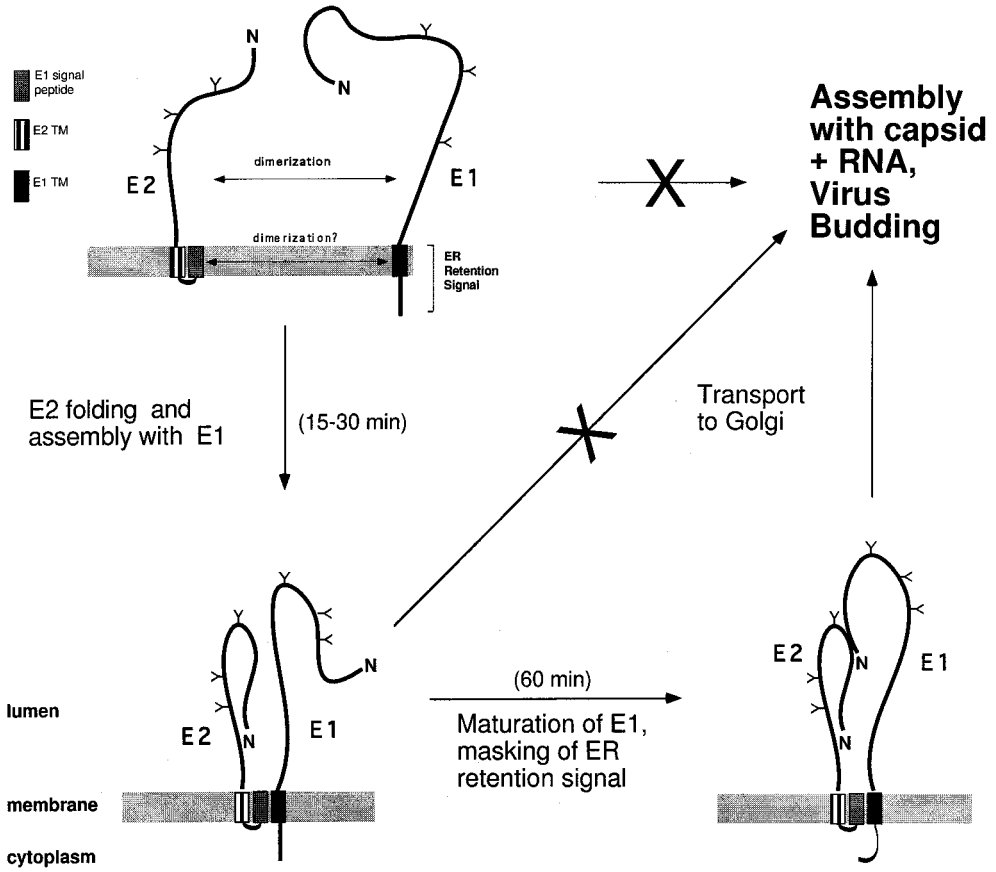


FIG. 10. Model for assembly of RV glycoproteins E2 and E1. Following translocation into the ER, E2 and E1 are tethered in the membrane by TM domains (striped and black rectangles) located near their carboxy termini. E2 also contains the E1 signal peptide at its extreme carboxy terminus (grey rectangle). Between 5 and 10 min postsynthesis, E2 protein folding is completed and dimerization of E2 and E1 occurs through ectodomain and possibly endodomain interactions. Transport to the Golgi complex does not occur until folding of E1 is completed. This process can take more than 60 min in CHO cells. The ER retention signal on E1 functions to retain unassembled E1 subunits and immature E2-E1 dimers in the ER until folding and heterodimer formation and maturation are completed. In the presence of E2, maturation of E1 results in a conformational change that masks the ER retention domain in the C terminus of E1, thereby allowing transport to the Golgi complex. E2-E1 dimers are retained in the Golgi complex by the retention signal in the E2 TM domain, and the E1 CT domain is available for binding to nucleocapsids. Amino termini (N and N-linked glycans [Y]) are indicated. The approximate times required for completion of E2 folding, assembly with E1, and E1 folding are shown in parentheses.

ciently than G-E1$_{TMCT}$ and that retention efficiency is dependent on the reporter protein used. Indeed, for the dilysine ER targeting sequence, CD8-KKXX-COOH chimeras were found to be retained more efficiently than CD4-KKXX-COOH chimeras (27). Experimental conditions designed to detect recycling between the ER and Golgi complex (33) did reveal that some G-E1$_{TMCT}$ was able to leave the ER and colocalize with Man II in the ERGIC; however, the vast majority of the protein remained in the ER. Similarly, temperature block experiments revealed that very little G-E1$_{TMCT}$ or CD8-E1$_{TMCT}$ left the ER of stably transfected CHO cells. Clearly, when G-E1$_{TMCT}$ or CD8-E1$_{TMCT}$ was expressed at very high levels in COS cells or RNA-transfected BHK-21 cells, significant amounts of the chimeras, particularly G-E1$_{TMCT}$, were detected beyond the ER. This was not at all surprising, as other investigators have reported that high level expression of heterologous proteins in transfected COS cells can result in aberrant localization of the expressed proteins (28, 43). The presence of G-E1$_{TMCT}$ in vesicular structures in stably transfected CHO cells likely represents the small population of protein that escapes from the ER and is eventually transported to the cell surface rather than recycling to the ER. From these observations, we conclude that the E1 ER retention signal works primarily by static retention and that the small amount of protein that escapes the ER is not retrieved.

Of critical importance is how the E1 retention signal is masked or otherwise rendered nonfunctional to allow transport of E2-E1 dimers to the Golgi complex. In the case of other oligomeric complexes such as the T-cell antigen receptor complex, ER retention and degradation motifs which are present in the TM domains of unassembled subunits are masked by juxtaposition of the motifs during subunit assembly (4–6). Likewise, assembly of the IgE receptor complex and subsequent transport from the ER involves steric masking of a dilysine retention motif present in one of the subunits (31). The E2-E1 heterodimer is efficiently transported from the ER to the Golgi complex, indicating that the E1 retention signal is no longer functional after a certain point in the E2-E1 maturation pathway. In the present study, coexpression of two different reporter proteins, CD8 and VSV G, containing the E1 and E2 TM and CT domains did not result in significantly increased transport of chimeras containing the E1 domains from the ER. This finding suggests that simple juxtaposition or assembly of the E2 and E1 TM and/or CT domains is not the mechanism by which the E1 retention signal is masked.

In CHO cells, the half-time for transport of E2 and E1 to the

Golgi is 60 to 90 min; however, assembly of E2-E1 heterodimers can be detected in <5 min postsynthesis and is completed between 15 and 30 min (24). There is a considerable lag time (~30 min) from the start of when heterodimer formation occurs to when Golgi-specific modification of E2 and E1 is first detectable. This delay is most likely due to the slow rate of E1 folding, which requires >10 times as long as that of E2 for maturation (24). Thus, it appears that E1 undergoes the majority of its maturation while bound to E2. Furthermore, this finding suggests that the E1 ER retention signal may also function to retain immature E2-E1 dimers until the folding of E1 is completed. Therefore, inactivation or masking of the retention motif in E1 would be the result of a change in the tertiary structure of E1 during maturation, rather than a direct consequence of dimerization with E2 (Fig. 10). Other togavirus envelope glycoprotein dimers are known to undergo drastic changes in their tertiary or quaternary structures during maturation. For example, a late step in the maturation of alphavirus E2-E1 dimers results in changes to the membrane topology of the E2 CT domain (35). In addition to masking of the retention signal, such a conformational change in E1 could also position its CT domain in the proper context for nucleocapsid binding. Presumably, this occurs during or after leaving the ER, since capsid protein does not become concentrated in the ER region to the same extent as it does in the Golgi complex (20, 21). Currently, we are investigating whether regions of E2 other than the TM and/or CT domains can trigger maturation of E1 by coexpressing segments of E2 that lack the TM and CT domains with E1 and assaying whether transport from the ER occurs. Data obtained so far indicate that the E2 TM domain is required for export of E1 from the ER (reference 25 and unpublished observations).

In addition to its potential importance in the virus assembly process, the E1 ER retention motif may represent a new type of targeting signal which, like other ER retention signals, operates by interaction with cellular proteins (8, 49). To this end, we now have evidence that the E1 TM and CT domains function as an intracellular retention signal in yeast (data not shown), and work is currently in progress to determine which if any endogenous cellular proteins are required for these domains to mediate ER retention.

## ACKNOWLEDGMENTS

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