

The Rubella Virus E2 and E1 Spike Glycoproteins Are Targeted to the Golgi Complex

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Abstract. Rubella virus (RV) has been reported to bud from intracellular membranes in certain cell types. In this study the intracellular site of targeting of RV envelope E2 and E1 glycoproteins has been investigated in three different cell types (CHO, BHK-21 and Vero cells) transfected with a cDNA encoding the two glycoproteins. By indirect immunofluorescence, E2 and E1 were localized to the Golgi region of all three cell types, and their distribution was disrupted by treatment with BFA or nocodazole. Immunogold labeling demonstrated that E2 and E1 were localized to Golgi cisternae and indicated that the glycoproteins were distributed across the Golgi stack. Analysis of immunoprecipitates obtained from stably transfected

CHO cells revealed that E2 and E1 become endo H resistant and undergo sialylation without being transported to the cell surface. Transport of RV glycoproteins to the Golgi complex was relatively slow ($t_{1/2} = 60-90$ min). Coprecipitation experiments indicated that E2 and E1 form a heterodimer in the RER. E1 was found to fold much more slowly than E2, suggesting that the delay in transport of the heterodimer to the Golgi may be due to the slow maturation of E1 in the ER. These results indicate that RV glycoproteins behave as integral membrane proteins of the Golgi complex and thus provide a useful model to study targeting and turnover of type I membrane proteins in this organelle.

THE study of enveloped viruses has been highly instrumental in understanding the pathways and mechanisms of intracellular transport in eukaryotic cells. While most viruses mature at the plasma membrane, a limited number of viruses acquire their envelopes by budding into intracellular compartments. Viral assembly may take place on the inner nuclear membrane (herpesvirus), ER membranes (coronavirus, flavivirus, and rotavirus), intermediate compartment (coronavirus), and Golgi complex (coronavirus, bunyavirus, and pox virus) (43). The site of budding of a particular virus is likely determined by the targeting of one or more viral glycoproteins to the budding site. For this reason, viral glycoproteins have been useful tools for the study of protein targeting.

Rubella virus (RV)¹ is a Togavirus which has been reported to bud from intracellular membranes in some cell types (4, 51). RV contains two envelope glycoproteins, E2 and E1, both of which are type I membrane proteins, and a capsid protein which associates with the positive-strand genomic RNA in the virion interior (40). The subgenomic RNA which encodes these proteins (41) has been cloned and sequenced (10, 13, 14). Expression of the cDNAs encoding

E2 and E1 together or E1 alone in transfected COS cells resulted in their accumulation in a juxtannuclear location suggestive of the Golgi (18). We have recently demonstrated that E1 expressed in the absence of E2 accumulates in a post-ER, pre-Golgi compartment composed of a network of tubular smooth membranes (20). In this paper, we have studied the fate of E2 and E1 and have found that when expressed together they are targeted to the Golgi cisternae in several cell types. Results obtained with agents that perturb the organization of the Golgi, e.g., nocodazole and brefeldin A (BFA), indicate that E2 and E1 behave as integral membrane proteins of the Golgi. The results suggest that E2 and E1 are transported as a complex and retained in the Golgi apparatus. Thus, one or both of these membrane proteins must contain a Golgi retention signal.

Materials and Methods

Materials

Reagents and supplies were obtained from the following sources: BFA was from Epicentre Technologies (Madison, WI). Protein A- and G-Sepharose were purchased from Pharmacia (Alameda, CA). Geneticin (G418) was from Gibco Laboratories (Grand Island, NY). Fibronectin, SDS, nocodazole, BSA, and dialyzed fetal FBS (DFBS) were purchased from Sigma Chemical Co. (St. Louis, MO). [³⁵S]cysteine (1,000 Ci/mM) was from ICN Biomedicals Inc. (Irvine, CA). Amplify, ¹⁴C-labeled protein stan-

1. *Abbreviations used in this paper:* BFA, brefeldin A; DFBS, dialyzed FBS; NEM, N-ethylmaleimide; RV, rubella virus.

dards, and goat anti-rabbit IgG-gold conjugate (5 nm) were purchased from Amersham Corp. (Arlington Heights, IL). Endoglycosidases D (endo D) and H (endo H), and neuraminidase (*Arthrobacter ureafaciens*) were purchased from Boehringer Mannheim Biochemical (Indianapolis, IN). TRITC-conjugated goat anti-mouse IgG and FITC-conjugated donkey anti-rabbit IgG were purchased from Jackson Immuno Research Laboratories (West Grove, PA). FITC-conjugated goat anti-human IgG was purchased from Zymed Labs, Inc. (San Francisco, CA). Sulfo-NHS-biotin and streptavidin agarose were obtained from Pierce Chemical Co. (Rockford, IL). The expression vector Moloney leukemia virus (MLV)-neo was a gift from Dr. Allen Wells (University of Alabama, Birmingham, AL).

Antibodies

Human anti-rubella virus serum was obtained from Dr. Aubrey Tingle (University of British Columbia, Vancouver, B.C.). Mouse monoclonals against E2 and E1 (ascites) were kindly provided by Dr. John Safford (Abbott Laboratories, North Chicago, IL). Polyclonal rabbit anti-RV serum was a gift from Dr. Christian Oker-Blom (Abo Akademi, Turku, Finland). Rabbit anti-Ig μ 120 serum was provided by Dr. William Dunn (University of Florida, Gainesville, FL). Rabbit antibodies to the rat cation-independent mannose-6-phosphate receptor were characterized previously (9). Rabbit polyclonal antiserum to ER membrane proteins was from Dr. D. Louvard (Pasteur Institute, France) (31). mAb to a conserved epitope on the endodomain of human transferrin receptor was a gift from Dr. Ian Trowbridge (Salk Institute, La Jolla, CA). Rabbit antiserum to α -mannosidase II (man II) was prepared with antigen provided by Dr. Kelley Moremen (University of Georgia, Athens, GA) using antigen prepared as described (39). Mouse mAb BW8G5 to the luminal domain of VSV G protein was a gift from Dr. William Balch (Scripps Research Institute, La Jolla, CA). Mouse monoclonal antibody (X22) to clathrin heavy chain was obtained from Dr. Francis Brodsky (University of California, San Francisco, CA) and rabbit anti- β -COP (anti-EAGE) serum was a gift from Dr. Thomas Kries (European Molecular Biology Laboratory, Heidelberg, Germany) (12).

Cell Culture and Transfections

CHOE2E1 are stably transfected CHO cells that express both E2 and E1 glycoproteins (20). CHOE2 cells were established by cotransfecting CHODG44 cells with pCMV5E2 (18) and pFR400 as described (20). The RV genes are under the transcriptional control of the human cytomegalovirus immediate early promoter in the expression vector pCMV5 (1, 18). Cells were grown in MEM α supplemented with 10% DFBS, 100 U/ml penicillin, 100 μ g/ml streptomycin and 2 mM glutamine at 37°C in a 5% CO $_2$ incubator. Vero cells and BHK-21 cells were grown in MEM supplemented with 10% FBS, antibiotics and glutamine. Stable BHK-21 lines expressing RV E2 and E1 were obtained by transfecting (using Lipofectin) the cells with a cDNA encoding RV E2 and E1 (18) subcloned downstream from the MLV long terminal repeat in the vector MLV-neo. Transfectants were selected by culturing in 700 μ g/ml G418 for 12–14 d after which colonies were picked and expanded. Clones were screened by indirect immunofluorescence using human anti-RV serum. For transient transfection of Vero and BHK-21 cells, experiments were performed 2 d after transfection (Lipofectin) with pCMV5E2E1 (18).

Immunofluorescence Microscopy

Cells were plated onto fibronectin-coated (10 μ g/ml) chamber slides or 12 mm glass coverslips at a density of 2×10^4 cells/cm 2 . After 2 d, cells were washed four times in PBS containing 0.5 mM Mg $^{2+}$ and then either fixed and permeabilized with 100% methanol at -20°C (6 min), or fixed with 2% paraformaldehyde in PBS (15–20 min) and permeabilized with 0.1% Triton X-100. They were then washed three times with PBS followed by incubation with PBS/1% BSA to block nonspecific binding. Primary and secondary antibodies were diluted in PBS/1% BSA, and incubations were for 1 h at room temperature. Cells were mounted in 90% glycerol/PBS containing 1 mg/ml paraphenylenediamine, examined and photographed with a Zeiss Axiophot. Where indicated, cells were incubated with media containing 2 μ g/ml BFA, 25 μ g/ml nocodazole, or 250 μ M cyclohexamide for the indicated time periods before fixation.

Metabolic Labeling and Radioimmunoprecipitation

Subconfluent monolayers in 35-mm dishes were washed (two times) in PBS, incubated in cysteine-free MEM/10% DFBS for 30 min at 37°C, and pulse

labeled with 200–1,000 μ Ci/ml [35 S]cysteine for the indicated time periods. Post pulse, the cells were incubated with complete media containing 50 \times excess unlabeled cysteine. Cells were washed (three times) with cold PBS and lysed by incubation in 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS containing protease inhibitors (1 μ g/ml chymostatin, leupeptin, antipain and pepstatin A) on ice for 15 min. The lysates were centrifuged at top speed for 3 min in a microfuge, and the supernatants were incubated with primary antibodies at 4°C for at least 3 h before addition of protein A or G-agarose beads. Immunoprecipitates were washed (three times) with RIPA buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1% NP40; 0.5% sodium deoxycholate; 0.1% SDS) and once with water, and subjected to SDS-PAGE and fluorography as described (20).

When coprecipitation of E2 and E1 was being examined, cells were solubilized in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA and 1% NP40, and immunoprecipitates were washed twice in NET buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% NP40, 0.25% gelatin) and once with 10 mM Tris-HCl (pH 7.4) and 0.1% NP40.

To examine folding of E1 and E2, CHO2E1 cells were pulse labeled for 2.5 min with [35 S]cysteine (1,000 μ Ci/ml) and chased with 100 \times excess cysteine. Before lysis, cells were washed with PBS containing 20 mM N-ethylmaleimide (NEM) to alkylate free sulfhydryl groups (8, 35). Immunoprecipitates were prepared using rabbit anti-RV serum or human anti-RV as above and subjected to SDS-PAGE under reducing or nonreducing conditions. In nonreduced samples, β -mercaptoethanol was omitted from the SDS-gel sample buffer.

Cell Surface Immunoprecipitation

Cells were pulse labeled with [35 S]cysteine and chased with excess unlabeled cysteine for the indicated time periods. Afterwards the cells were washed with ice-cold PBS, and cell surface proteins were biotinylated by incubation with Sulfo-NHS-biotin (1 mg/ml in PBS) for 30 min at 4°C with occasional rocking (29). Biotinylation reactions were terminated by quenching with PBS containing 50 mM lysine, and RV glycoproteins were immunoprecipitated from cell lysates as described above. Immune complexes were dissociated by successive boiling in 2×100 μ l aliquots of 200 mM Tris-HCl (pH 8.8); 1% SDS; 5 mM EGTA. Biotinylated RV proteins were recovered by incubating one half of the eluate with 50 μ l of a 50% slurry of streptavidin agarose at 4°C for 30 min. The beads were washed as above and the biotinylated RV antigens were eluted in SDS gel sample buffer and subjected to SDS-PAGE and fluorography. The fraction of RV glycoproteins present in intracellular and cell surface pools was estimated by liquid scintillation counting of aliquots of the immunoprecipitates.

Endo H, Endo D and Neuraminidase Digestions

For endo H digestions, immunoprecipitates were released from the protein A-Sepharose beads by heating in 0.5% SDS, 100 mM sodium citrate (pH 5.5) at 100°C for 5 min. The eluates were diluted with an equal volume of 100 mM sodium citrate buffer (pH 5.5) and incubated with or without 2.5 mU endo H for at least 8 h at 37°C (17). For endo D digestions, eluates were adjusted to 50 mM sodium citrate (pH 6.5), 10 mM EDTA, 2% Triton X-100, 0.25% SDS and incubated with 2.5 mU endo D for at least 8 h at 37°C as described (20). Neuraminidase digestions were performed by resuspending immune complexes (still attached to protein A-Sepharose) in 50 mM sodium acetate (pH 5.4) containing protease inhibitors (see above) followed by incubation at 37°C for 4 h with or without neuraminidase (1 U/ml) from *A. ureafaciens*. Immune complexes were then dissociated in sample buffer and separated by SDS-PAGE.

SDS-PAGE and Autoradiography

Proteins were separated on 10% polyacrylamide gels (26). Gels were fixed in isopropanol:water:acetic acid (25:65:10) and soaked in Amplify (Amersham Corp.) before drying and exposure to Kodak XAR film at -80°C.

Electron Microscopy and Immunogold Labeling

For routine morphology studies, cells were fixed (1 h) with 2% glutaraldehyde, 3% paraformaldehyde in 100 mM cacodylate-HCl buffer, pH 7.2, scraped from the culture dish, and pelleted in a microfuge. The cell pellets were then postfixed (1 h) in 2% OsO $_4$ in the same buffer, stained in block (2 h) with 2% uranyl acetate (pH 6.0), dehydrated in graded ethanols and embedded in Epon.

For immunogold labeling, cells were fixed with 3% paraformaldehyde, 0.05% glutaraldehyde in 100 mM cacodylate-HCl-buffer, pH 7.4 (1 h), after which they were scraped from the culture dish, pelleted in a microfuge and cryoprotected by infiltration with 2.3 M sucrose in 0.1 M phosphate buffer, pH 7.4, containing 20% polyvinylpyrrolidone, and then mounted on aluminum nails and frozen in liquid N₂ (49). Ultrathin cryosections prepared from the pellets were collected on carbon/formvar coated nickel grids and incubated for 1 h in mouse monoclonal anti-E1 or E2 ascites (diluted 1/300 in 10% FCS/PBS), followed by rabbit anti-mouse IgG (1 h) and goat anti-rabbit IgG-gold (5 nm) conjugate (diluted 1/50) for 1 h. Grids were stained in 2% neutral uranyl acetate (20 min) and absorption stained with 0.2% uranyl acetate (10 min) in 0.2% methylcellulose, and 2% carboxax.

Infection of CHO_{E2E1} Cells with VSV ts045

CHO_{E2E1} cells grown in 35 mm dishes were infected 2–3 d after seeding with VSV ts045 (provided by Dr. William Balch, Scripps Research Institute). Cells were washed twice with serum-free MEM α containing one sixth the normal amount of sodium bicarbonate plus 10 mM Hepes (pH 7.2) before addition of virus. Virus was allowed to bind to cells for 60 min at 32°C. The virus-containing media was removed and replaced with the same media containing 10% FBS, and the cells were incubated at 32°C for 4 h. They were then incubated in cysteine-free media for 30 min, labeled for 10 min at 37°C with [³⁵S]cysteine, chased for 0 or 60 min at 32°C followed by treatment with sulfo-NHS-biotin as above.

Results

Rubella Virus Glycoproteins Are Localized to the Golgi Complex

RV has been reported to bud from either intracellular membranes (BHK-21 cells) or the plasma membrane (Vero cells) (4). The site of virus assembly is likely determined by the targeting of the E2-E1 spike complex. To determine if differential targeting occurred, pCMV5E2E1 (18) was used to transfect these two cell lines. In stably transfected BHK-21 cells, both E2 and E1 colocalized with the resident Golgi glycoprotein α -mannosidase II (man II) by indirect immunofluorescence (Fig. 1, A–D). Similar results were obtained in transiently transfected cells (not shown). Next we examined transiently transfected Vero cells and found, surprisingly, that E2 and E1 glycoproteins also appear to be retained in the Golgi complex (Fig. 1 E). It should be noted, however, that we used a cDNA from a different strain of RV than that used in the previous study (4). Our results suggest that RV E2 and E1 are targeted to the Golgi of both BHK-21 and Vero cells.

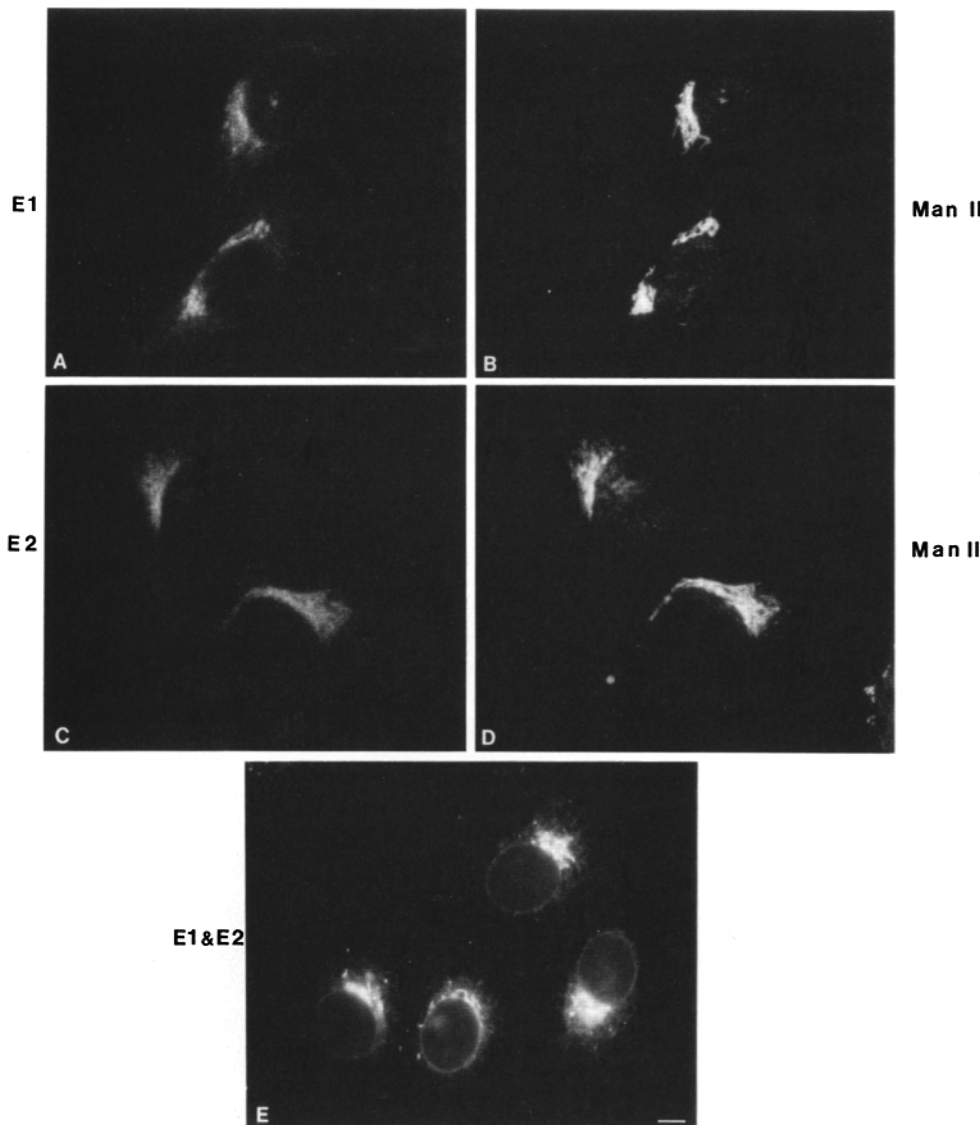
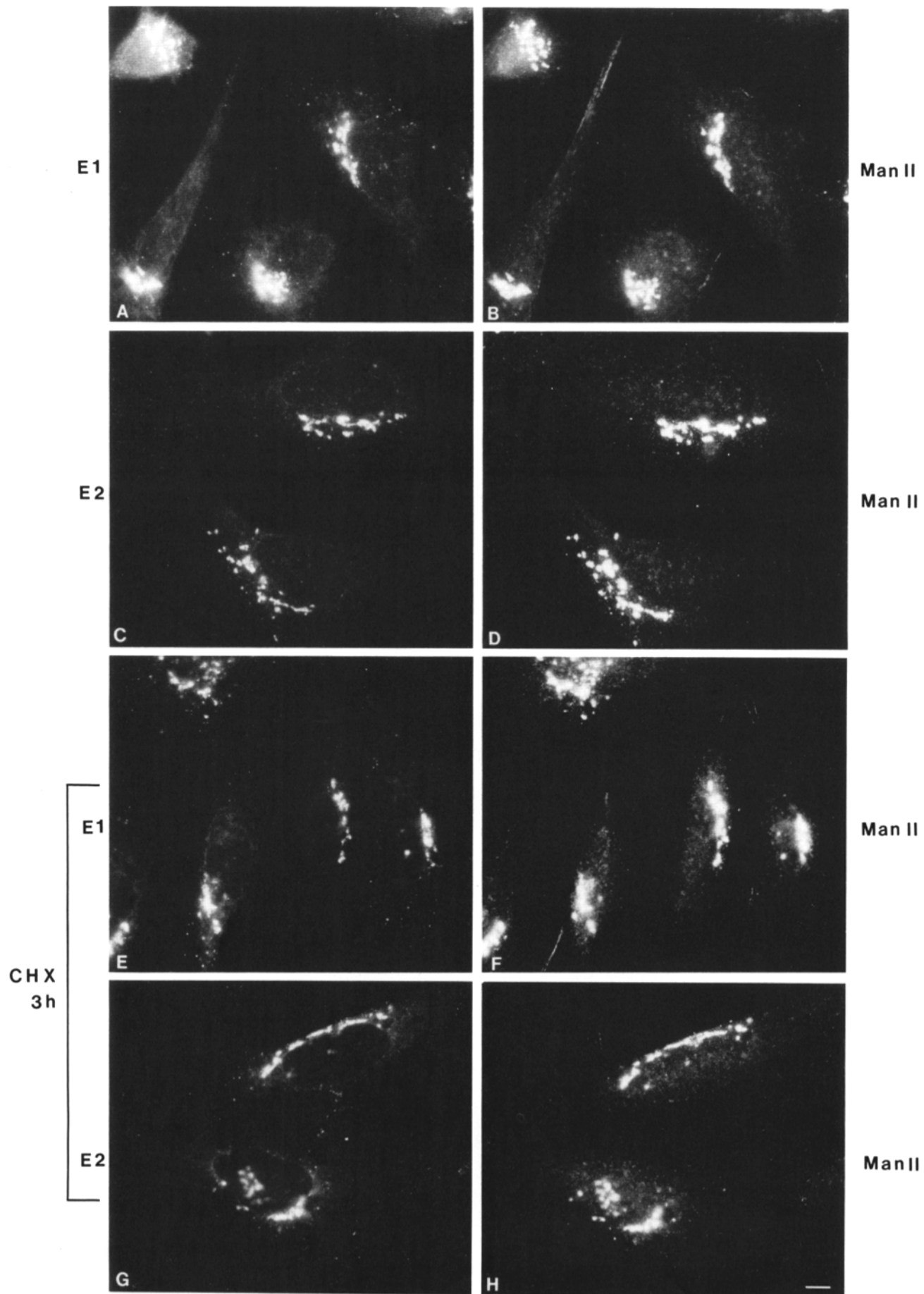


Figure 1. RV E2 and E1 glycoproteins are targeted to the Golgi complex of both BHK-21 and Vero cells. Cells transfected with pCMV5E2E1 were grown on coverslips, fixed and permeabilized with methanol at -20°C , and double-labeled with mouse monoclonal to either E1 or E2 (left) and rabbit anti-man II (right) to visualize the Golgi complex followed by TRITC-goat anti-mouse IgG and FITC-donkey anti-rabbit IgG. (A–D) Stably transfected BHK-21 were stained for either E1 (A) or E2 (C) and man II (B and D). Staining for E1 and E2 coincides with that for the Golgi marker man II. (E) Vero cells were transiently transfected and stained for E2 and E1. The viral glycoproteins are concentrated in the Golgi region where they overlap with man II. Bar, 5 μm .



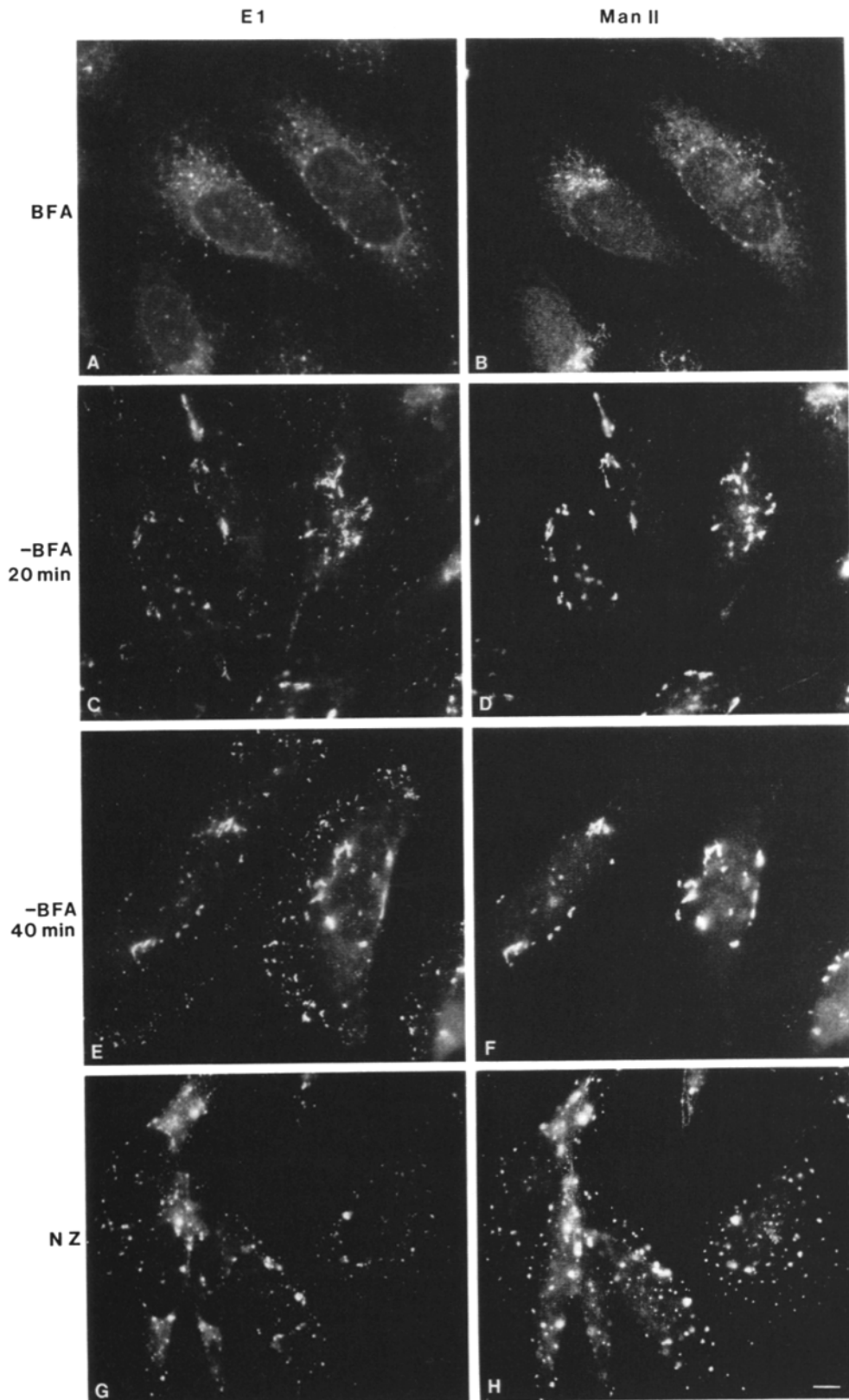


Figure 3. Golgi distribution of RV E2 and E1 glycoproteins is disrupted by BFA and nocodazole. (A and B) CHO2E1 cells grown on coverslips were incubated with BFA (2 $\mu\text{g/ml}$) for 60 min and processed for double staining with either E1 (left) or man II (right) by indirect immunofluorescence. Staining for both RV glycoproteins and man II is dispersed into a diffuse vesicular pattern. (C–F) Cells incubated with BFA for 60 min, after which BFA was removed and replaced with medium containing 500 μM cycloheximide for 20 (C, D) or 40 (E, F) min. Staining for man II has relocated to the Golgi region as has most of the E1. Some vesicles containing only E1 are dispersed throughout the cytoplasm. (G–H) CHO2E1 cells were incubated with nocodazole (25 $\mu\text{g/ml}$) for 60 min at 37°C before processing for double immunofluorescence. Staining for both E1 and man II is dispersed after this treatment. Identical results were observed when cells were stained for E2 (not shown). Bar, 5 μm .

Figure 2. RV E2 and E1 are retained in the Golgi complex of CHO cells. Stably transfected CHO cells (CHO2E1) expressing E2 and E1 were grown on coverslips and processed for indirect immunofluorescence as described for Fig. 1. (A and B) Cells doubly stained for E1 and man II. (C and D) Cells doubly stained for E2 and man II. (E and F) Cells were treated with 500 μM cycloheximide for 3 h before fixation (CHX 3 h) and doubly stained for E1 and man II. (G and H) Cells similarly treated and doubly stained for E2 and man II. In all cases staining for RV glycoproteins coincides with that of man II. Bar, 5 μm .

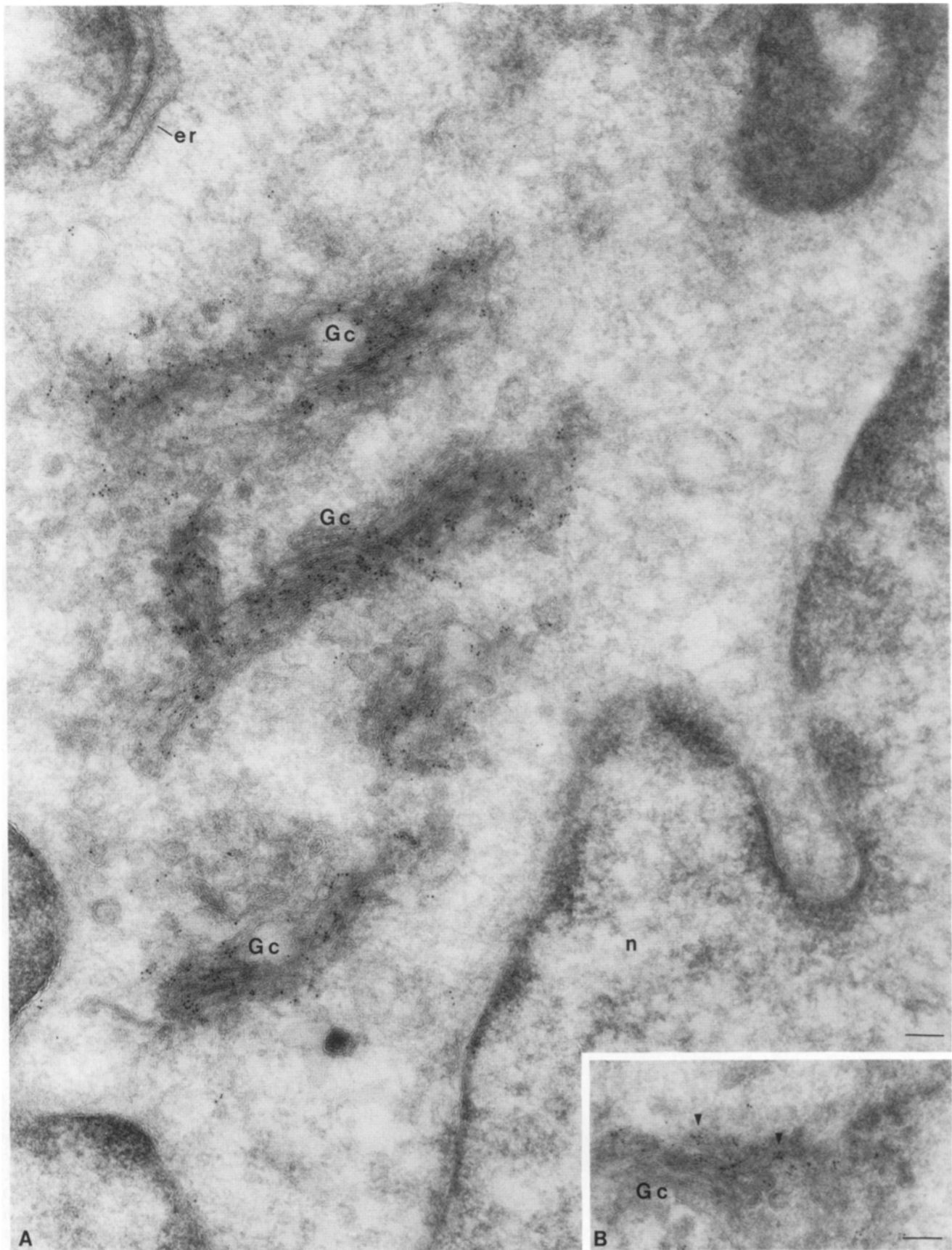


Figure 4. RV glycoproteins are distributed across the Golgi stack. Ultrathin cryosections were incubated with either mouse anti-E1 (A) or anti-E2 (B), followed by rabbit anti-mouse IgG (used as a bridging antibody) and goat anti-rabbit IgG coupled to 5 nm gold. Both E1 and E2 are detected across the Golgi stack (Gc) and within associated vesicles, but they are not detected in rough ER (er) membranes. Gold particles are more concentrated on one side of the stack (arrowheads) than the other. n = nucleus. Bar, 0.5 μ m.

Although BHK-21 cells were our initial choice for studying transport of E2 and E1 to the Golgi, biochemical analysis of the RV glycoproteins by radioimmunoprecipitation from transfected BHK-21 cells indicated relatively low levels of expression in this cell line. To facilitate ultrastructural and biochemical analysis of these glycoproteins, we used stably transfected CHO cells expressing RV E2 and E1 (CHOE2E1) because moderate levels of expression are easily achieved in CHO cells (20). CHOE2E1 cells contained E2 and E1 primarily in the Golgi region as indicated by colocalization with man II (Fig. 2, A-D). No increase in cell surface expression of E2 and E1 was seen after treating CHOE2E1 with cycloheximide for 3 h (Fig. 2, E-H), suggesting that the bulk of these proteins is stably retained in the Golgi.

Distribution of E2 and E1 Glycoproteins Is Disrupted by Agents which Perturb the Golgi

BFA and nocodazole cause rapid, reversible disassembly of the Golgi in many cell types (23, 45). To determine their effects on the distribution of RV glycoproteins, CHOE2E1 cells were treated with nocodazole (25 μ g/ml) or BFA (2 μ g/ml).

BFA treatment led to dispersal of both man II and E1 to a diffuse localization (Fig. 3, A and B). Similar results were obtained when cells had been treated with cycloheximide for 3 h before BFA treatment (not shown). Nocodazole treatment resulted in dispersal of the Golgi into vesicular structures, most of which contained both RV glycoproteins and man II (Fig. 3, G and H). Identical results were obtained using an anti-E2 monoclonal antibody. These results indicate that E2 and E1 behave as integral membrane proteins of the Golgi complex.

To determine the fate of RV glycoproteins during reassembly of the Golgi, BFA-containing media were removed and

replaced with media containing cycloheximide. 20 min after BFA washout, E1 was found mainly in the Golgi region where it largely colocalized with man II (Fig. 3, C and D). Interestingly, between 40 (Fig. 3, E and F) and 60 min after BFA removal, E1 persisted in vesicular structures found in peripheral regions that did not contain man II (Fig. 3, E and F). These structures were no longer seen at 2 h after BFA washout (not shown). Similar results were obtained with E2. Increased levels of E2 and E1 were also seen at the cell surface 60 min after BFA washout (not shown). The nature of these E2E1-containing structures remains unclear; they did not colocalize with markers for lysosomes (Igp 120), or early (transferrin receptor) or late (mannose-6-phosphate receptor) endosomes, and they did not stain for β -COP or clathrin suggesting that they are not pre- or post-Golgi transport vesicles (not shown). Furthermore, they are not of RER origin because they were not stained by a polyspecific antiserum that recognizes four ER membrane proteins (not shown) (31). The results of the BFA washout experiments suggest that although most of the RV glycoproteins may reside in the same intraGolgi location as man II, a fraction of the E2 and E1 pool may reside in a different region of the Golgi.

Rubella Virus E2 and E1 Are Distributed across the Golgi Stack

To determine precisely where in the Golgi complex E2 and E1 are localized, E2 and E1 were localized by immunogold labeling of ultrathin cryosections. The RV glycoproteins were detected in all cisternae throughout the Golgi stack as well as within vesicles of uniform size in the vicinity of this organelle (Fig. 4, A and B). Very little labeling of the rough ER was detected. In favorable sections, a gradient of gold particles was seen across the stack with labeling on one side greater than that on the other. Similar results were obtained

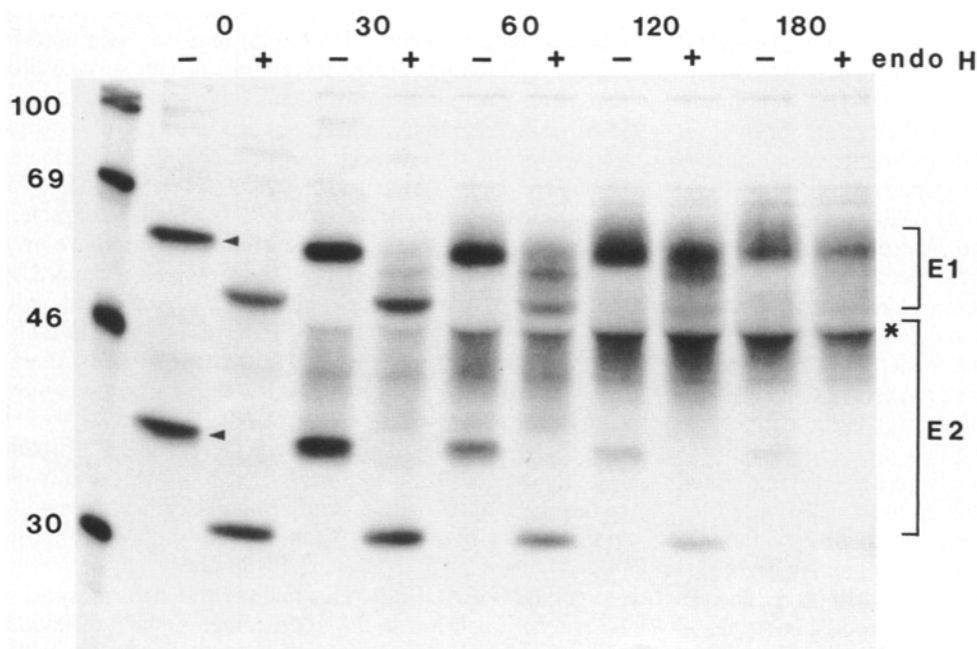


Figure 5. E2 and E1 become resistant to endo H. CHO-E2E1 cells were pulse labeled for 30 min with [35 S]cysteine followed by chase for the indicated time periods (in minutes). E2 and E1 were immunoprecipitated from cell lysates with human anti-RV serum (precipitates both E2 and E1) and digested with (+) or without (-) endo H. Samples were subjected to SDS-PAGE and fluorography. 14 C-labeled protein standards (kD) were included for reference. Immediately postpulse E2 and E1 have apparent molecular weights of 39 and 57 kD, respectively (arrows), and are endo H sensitive. By 30 min chase endo H resistant forms, 45 and 60 kD, are detected, and by 180 min >90% of the E2 and E1 become endo H resistant. The asterisk marks the position of the Golgi-processed (endo H resistant) form of E2.

when cycloheximide was added to the culture media. It was not possible to determine which side was more heavily labeled due to the lack of markers indicating the *cis/trans* polarity. These results demonstrate that E2 and E1 accumulate in bona fide Golgi elements.

Rubella Virus Glycoproteins Become Endo H Resistant and Acquire Sialic Acid

E2 contains three N-linked (10, 44) as well as O-linked glycans (32), whereas E1 contains only three N-linked glycans (19, 32). To obtain information on the extent of processing of E1 and E2 by Golgi enzymes, CHO2E1 cells were pulse labeled with [³⁵S]cysteine and chased for various time periods before lysis and immunoprecipitation with human anti-RV sera that recognize both E2 and E1. Immunoprecipitates were divided into two and incubated with or without endo H before separation by SDS-PAGE and autoradiography (Fig. 5). The apparent molecular weights of E2 and E1 immediately after the labeling period are 39 and 57 kD, respectively (18). Their sensitivity to endo H digestion demonstrated that they correspond to high-mannose, ER forms of the glycoproteins. Chase with media containing excess unlabeled cysteine resulted in acquisition of endo H resistance with a *t*_{1/2} of 1–1.5 h indicating transport of E2 and E1 to the middle Golgi. Processing of E2 and E1 was detectable at 0.5 h chase and by 3 h chase >90% of E1 and E2 were resistant to endo H. The Golgi processed, endo H-resistant forms of E2 and E1 were 42–47 and 57–66 kD, respectively (Fig. 5). The large shift in size of processed E2 seen by SDS-PAGE is at least in part due to the presence of O-linked oligosaccharides (32). The fact that Golgi processing of N-linked glycans on E2 and E1 proceeded at similar rates suggests that these proteins may be transported to the Golgi as a complex. Endo D sensitive forms of E2 and E1 were not detected at any time point (not shown) suggesting that transport is not delayed in the *cis* Golgi.

Immunoprecipitates were also digested with a neuraminidase (*A. ureafaciens*) which has a broad-range specificity and cleaves sialic acid from both N- and O-linked glycans (30). The endo H resistant forms of both E2 and E1 became partially sensitive to neuraminidase in a time-dependent manner (Fig. 6) indicating that at least a portion of these glycoproteins were modified by sialyltransferases. Neuraminidase digestion affected only the endo H-resistant forms (>60 min chase) of RV glycoproteins indicating that some but not all of their electrophoretic heterogeneity was due to sialic acid. The neuraminidase-sensitivity of E2 was more difficult to discern (Fig. 6) due to the heterogeneous nature of neuraminidase-digested E2. It has been recently reported that sialylation of O-linked oligosaccharides may occur in the medial Golgi (30), whereas sialylation of N-linked glycans occurs in the TGN. The finding that E1, which contains only N-linked glycans, was partially sensitive to neuraminidase suggests that a significant proportion of these glycoproteins reach trans Golgi cisternae.

Biosynthetic labeling experiments were also carried out on CHO2E1 cells expressing only E2. In these cells, very little processing of N-linked glycans on E2 was observed after a 3 h chase (not shown), and by indirect immunofluorescence the majority of the glycoprotein appeared to be retained in the RER. However, some antigen was detected in the Golgi

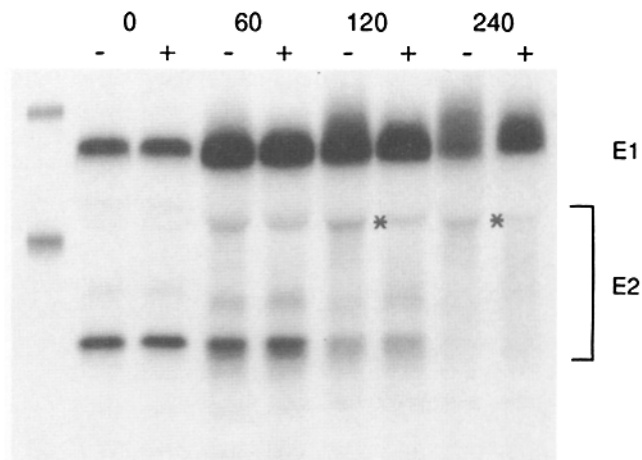


Figure 6. E2 and E1 undergo sialylation. CHO2E1 cells were pulse-labeled and processed for immunoprecipitation as in Fig. 5. E2 and E1 immunoprecipitates were digested with (+) or without (-) neuraminidase from *A. ureafaciens*. Neuraminidase-sensitive forms of E2 and E1 are first clearly evident at 120 min. Neuraminidase sensitivity of E2 is indicated by the decrease in intensity of the 47-kD species (*asterisk*) after treatment, and neuraminidase sensitivity of E1 is indicated by a reduction in the size of E1.

based on colocalization with man II, and extremely low levels of E2 could be detected on the cell surface of nonpermeabilized cells (not shown). We conclude that the majority of E2 expressed in the absence of E1 remains in the ER, but a minor fraction is transported to the Golgi and cell surface.

The Majority of the Rubella Virus Spike Complex Is Retained in the Golgi

The results obtained by indirect immunofluorescence and immunogold labeling of CHO2E1 cells suggested that the bulk of the E2 and E1 remains in the Golgi. To confirm by an alternative approach that the majority of these proteins do not reach the plasma membrane, we utilized a commercially available reagent (sulfo-NHS-biotin) that has been used to selectively biotinylate primary amines of cell surface proteins (27). The biotinylated proteins subsequently can be isolated using streptavidin-agarose and thus, easily distinguished from the intracellular pool. When radiolabeled CHO2E1 cells were treated with sulfo-NHS-biotin to biotinylate cell surface proteins, <10% of either glycoprotein was detected at the cell surface after 180-min chase even though they had clearly entered the Golgi as evidenced by their increased molecular weight (Fig. 7). CHO2E1 cells which contain large amounts of E1 arrested in a pre-Golgi compartment (20) were used as a negative control. E1 was not detected at the surface of CHO2E1 cells using the biotinylation protocol (Fig. 7). As a positive control, when CHO2E1 cells were infected with VSV ts045, G protein was transported to the cell surface at 32°C where it could be biotinylated and subsequently isolated using streptavidin-agarose (data not shown). From these results we conclude that (a) the majority of E2 and E1 do not reach the plasma membrane in CHO2E1 cells, and (b) although transport of E2 and E1 is arrested in the Golgi complex, their accumula-

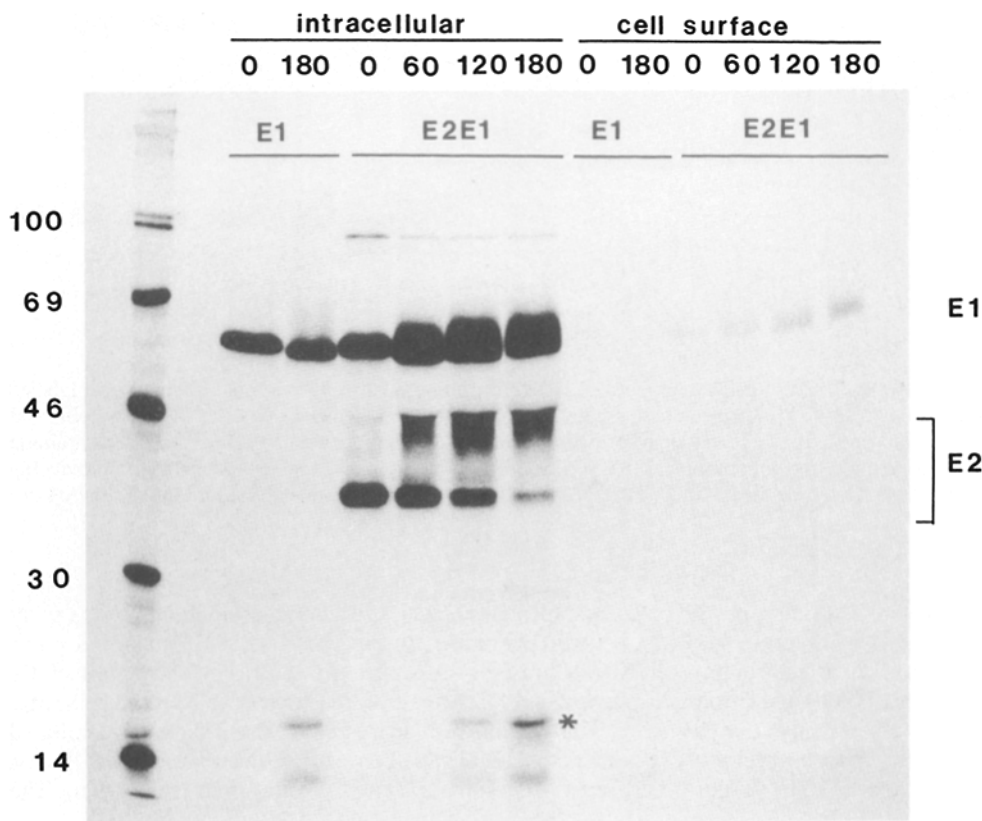


Figure 7. The majority of the RV spike complex is not transported to the cell surface. CHO-E2E1 (E2E1) and CHO-E1 (E1) cells were radiolabeled for 30 min and chased for the indicated times (in minutes). Cell surface proteins were biotinylated with sulfo-NHS-biotin. RV proteins were immunoprecipitated from cell lysates using human anti-RV serum and divided into two aliquots. Biotinylated cell surface proteins were recovered from one half using streptavidin agarose, and the remaining half constituted the intracellular sample. Samples were then subjected to SDS-PAGE and fluorography. The protein marked by the asterisk is believed to be a degradation product of E1. Very little (<10%) of the E2 or E1 reaches the cell surface in cells expressing both proteins. As a negative control, E1 is not detected at the cell surface in cells expressing E1 alone. (Note: because so little of the RV glycoproteins was recovered in the cell surface fractions, the entire cell surface samples were loaded onto gels whereas only one half of the intracellular fractions were loaded).

tion does not prevent the egress of other glycoproteins from this organelle.

Folding of E2 and E1 Occur at Different Rates

The data presented here and in previously published work (5, 6, 18) are consistent with the assumption that E2 and E1 form a heterodimer in the ER that is transported as such to the Golgi complex. Furthermore, the fact that the $t_{1/2}$ for acquisition of endo H resistance is 1–1.5 h indicates that transport to the medial/*trans* Golgi is slow. The simplest explanation for this delay in exiting the ER is that considerable time is required for folding and acquisition of correct tertiary and quaternary structures by RV glycoproteins in the ER. The folding of glycoproteins and formation of intramolecular disulfide bonds in the ER can be monitored by SDS-PAGE of the proteins under nonreducing conditions. Proteins that have not yet completed the formation of their intrachain disulfide bonds typically migrate with a slower electrophoretic mobility, presumably because they are in an extended conformation (8, 35). To compare the folding rates of E2 and E1, CHO-E2E1 cells were pulse labeled for 2.5 min, chased for various times with excess unlabeled cysteine, and treated with NEM before lysis (8, 35). Immunoprecipitates were subjected to SDS-PAGE under non-

reducing (Fig. 8 A) and reducing (Fig. 8 B) conditions. Immediately after the pulse, one prominent E1 species and two faint bands were evident when samples were separated under nonreducing conditions. However, very little E2 was recognized by the polyclonal anti-RV serum at this time point (Fig. 8 A). After a 2 min chase, a faint homogeneous E2 band was detected, whereas E1 still migrated as at least two distinct species in the absence of reducing agent. In contrast, under reducing conditions E1 did not display electrophoretic heterogeneity (Fig. 8 B). With increasing chase time, the mobility of nonreduced E2 did not change, whereas the mobility of E1 increased (Fig. 8 A). The two slower migrating forms of E1 present at early time points likely represent forms of the protein that have not attained their full complement of intrachain disulfide bonds. After 60-min chase, most of the E1 was present as the faster migrating compact form. Using a human anti-RV serum that recognizes only mature forms of E1 and E2, we also found that maximal expression of epitopes on E2 occurred between 5 and 15 min whereas E1 required up to 60 min (not shown). These results suggest that formation of intramolecular disulfide bonds and folding of E1 (which has 24 cysteine residues), occurs much more slowly than E2 which has only 13 cysteine residues (10).

The quaternary structure of the RV spike complex was examined via coprecipitation experiments. Radiolabeled

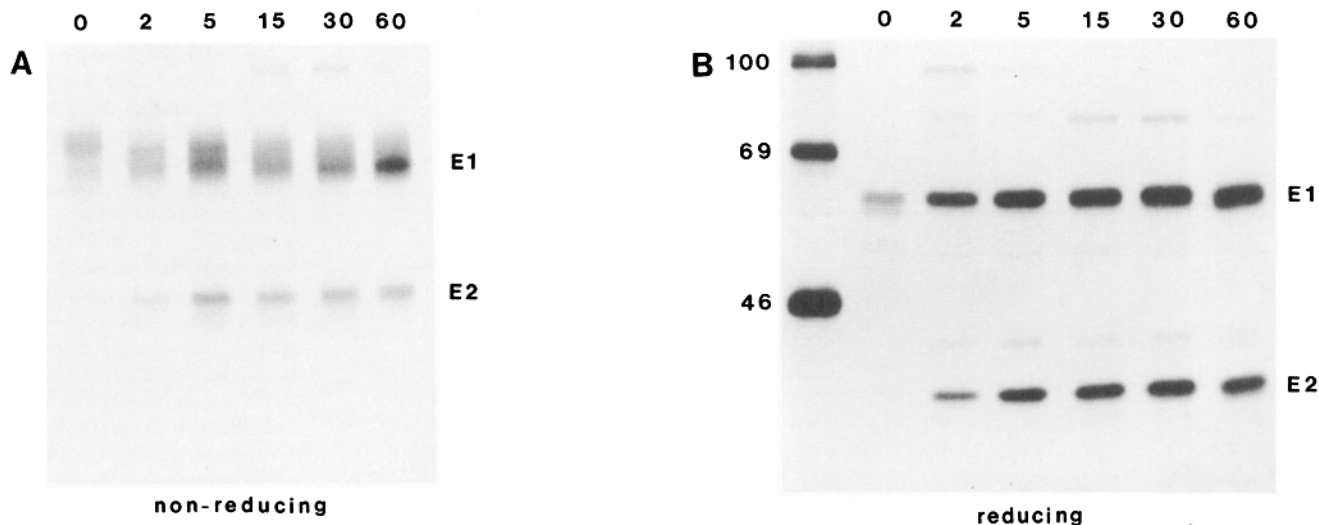


Figure 8. Disulfide bond formation occurs more rapidly in E2 than in E1. CHO2E1 cells grown in 35-mm dishes were pulse labeled with [³⁵S]cysteine (1,000 μCi/ml) for 2.5 min at 37°C. Prewarmed chase medium (2.5 ml) containing 100X excess unlabeled cysteine was added after the pulse, and the cells were incubated for the indicated time periods (in minutes). Cells were washed in ice cold PBS containing 20 mM NEM before lysis and radioimmunoprecipitation with rabbit anti-RV serum. Immunoprecipitates were separated under nonreducing (A) and reducing (B) conditions by SDS-PAGE. Disulfide formation in E2 appears to be completed by 5 min whereas folding of E1 requires up to 60 min.

CHOE2E1 cells were solubilized under mild conditions (1% NP-40 in isotonic Tris-saline) to preserve protein-protein interactions between E2 and E1. A mAb to E2 was used to prepare immunoprecipitates from CHO2E1 cells at various chase times after a short (5 min) pulse. Immediately after the pulse, a faint E2 band was seen in addition to a protein of ~95 kD which likely represents the uncleaved E2E1 precursor

polyprotein (Fig. 9). E1 coprecipitated with E2 as early as 5 min postpulse, and the amount of E1 increased up to 30–60 min (Fig. 9, right). In contrast, the amount of E2 precipitated reached a maximum between 5 and 15 min (Fig. 9). Half of the cell lysates were also immunoprecipitated with a polyclonal anti-RV serum to demonstrate that E1 was present in the samples at all the time points (Fig. 9, left). The

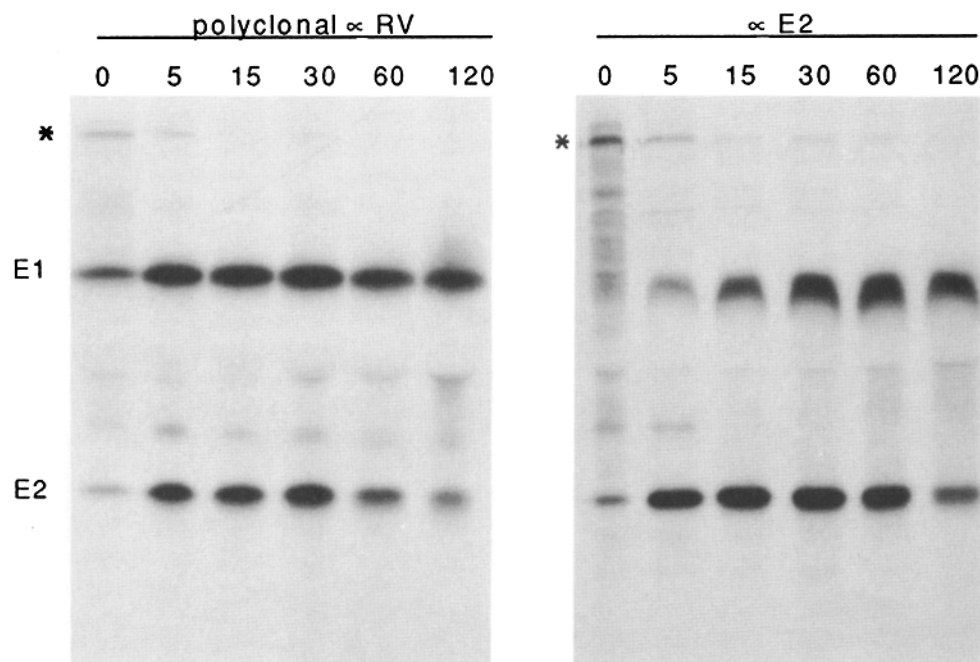


Figure 9. E2 and E1 form a heterodimeric complex in the ER. CHO2E1 cells were pulse-labeled for 5 min with [³⁵S]cysteine (1,000 μCi/ml) followed by incubation with chase medium for the indicated time periods (in minutes). Cells were solubilized in buffer containing 1% NP-40 and immunoprecipitated with mouse anti-E2 (right) or human anti-RV serum (left) followed by SDS-PAGE and fluorography. E1 is coprecipitated by mouse monoclonal anti-E2 (αE2) beginning at 5 min postpulse (right) indicating that E2 and E1 form a heterodimer in the ER. Results obtained with polyclonal anti-RV serum (polyclonal αRV) indicate that the amount of E1 does not increase during the chase period (after 5 min), whereas the amount of E1 that coprecipitates

with E2 increases up to 30–60 min. The large protein marked by an asterisk likely represents the 95 kD E2-E1 polyprotein precursor before cleavage by signal peptidase. The intensity of the E2 (39 kD) and E1 (57 kD) bands decreases after 60 min due to processing by Golgi enzymes and conversion to the larger endo H-resistant species.

fact that E2 and E1 coprecipitate at very early (5 min) time points, i.e., before acquisition of endo H resistance (see Fig. 5), indicates that heterodimer formation precedes exit from the ER. Because transport of RV glycoproteins to the Golgi (based on acquisition of endo H resistance [Fig. 5]) appears to occur at a similar rate, our results suggest that the folding of E1 may be the rate limiting step in heterodimer formation and subsequent transport to the Golgi.

Discussion

In this paper we have undertaken a comprehensive study of the assembly and transport of the RV E2 and E1 glycoproteins in transfected cells and have shown that when these two proteins are expressed together they are targeted to the Golgi and behave essentially as integral Golgi membrane proteins. This conclusion is based on the findings that: (a) E2 and E1 colocalized with the Golgi marker man II by immunofluorescence in a number of cell types; (b) their Golgi localization was sensitive to agents that disrupt the Golgi such as nocodazole and BFA; (c) they were localized to the stacked Golgi cisternae by immunogold labeling; and (d) they became largely resistant to endo H and were at least partially sialylated but were not detected (<10%) on the plasma membrane, indicating that for the most part they were retained in the Golgi. We found that the time required for E2 and E1 to mature, leave the RER and become endo H resistant was relatively long ($t_{1/2}$ = 1–1.5 h). This presumably was due to the time required for folding of these proteins and maturation of the heterodimeric complex. Biosynthetic labeling experiments indicated that E1 glycoprotein folded much more slowly than E2. Coincidentally, processing of RV glycoproteins by Golgi enzymes (detected after 30-min chase) was not observed until heterodimer formation (as detected by coprecipitation of E1 with anti-E2 monoclonal) reached a maximum (30–60 min). These results suggest that the maturation of E1 is the limiting factor in transport of the RV spike complex to the Golgi.

IntraGolgi Location of E2 and E1

At the light microscopic level, the location of E2 and E1 was indistinguishable from the medial Golgi marker man II except that occasionally the RV glycoproteins were detected in peripheral vesicles which did not contain man II. The fact that the majority of the E2 and E1 glycoprotein became resistant to endo H and partially sialylated suggests that these glycoproteins were transported to the medial and *trans* regions of the Golgi complex where the enzymes responsible for trimming (man II) and addition of sialic acid (sialyltransferases) to N-linked oligosaccharides are assumed to reside. Unlike glycoproteins which traverse the Golgi completely, only a fraction of E2 and E1 appear to be modified by the addition of sialic acid. The fact that after BFA washout, increased levels of E2 and E1 were observed on the cell surface of nonpermeabilized cells could be explained if a fraction of the E2 and E1 complex reaches the TGN which has been reported to fuse with the endosomal network after BFA treatment (28, 52). Thus, the structures observed during BFA washout that did not contain man II may represent *trans* Golgi/endosomal elements which recycle to the cell surface. Moreover, in favorable sections, immunogold labeling revealed a polarized distribution or gradient of E2 and E1

across the Golgi cisternae in CHO/E2/E1 cells. Although it is difficult to determine the *cis-trans* polarity of the Golgi in CHO cells by morphology alone, the fact that the majority of the E2 and E1 glycoproteins become endo H resistant and at least some of the N-linked glycans on E1 are sialylated support the conclusion that these proteins have a predominantly medial/*trans* Golgi localization.

Structure and Assembly of the E2/E1 Heterodimer

Generally, only properly assembled oligomeric complexes are able to be efficiently transported from the ER (21, 47) whereas unassembled subunits are frequently retained and ultimately degraded (7, 24). In the case of RV glycoproteins, expression of E1 in the absence of E2 results in transport from the RER to a tubular pre-Golgi compartment which is physically connected to the RER (20). When E2 was expressed alone in CHO cells, the majority of the protein was retained in the ER and to a lesser extent in the Golgi. Only trace amounts of E2 were detected on the cell surface by indirect immunofluorescence. These observations suggest that E2 and E1 must be expressed together to exit the ER and to be efficiently transported to the Golgi. Indeed, coprecipitation experiments indicated that E2 and E1 assemble into a heterodimeric complex in the ER. Because E2 and E1 associate shortly after synthesis (within 5 min) it is possible that the glycoproteins attain their tertiary structures by using each other as a scaffold. The rate limiting step in heterodimer maturation is likely the folding rate of E1 which is much slower than that of E2. The situation appears to be similar in the case of the Uukuniemi virus G1 and G2 spike glycoproteins which also form a heterodimer in the ER and are targeted to the Golgi (42).

We were not able to detect oligomer formation comparable to that seen with other viral proteins (15, 50) in sucrose gradient velocity experiments; however, it is possible that any higher order structures (e.g., trimers) formed may dissociate during centrifugation (16).

Other Golgi-specific Glycoproteins

Other viral glycoproteins that localize to the Golgi include the coronavirus E1 glycoproteins (avian infectious bronchitis virus [37] and mouse hepatitis virus [2, 30, 48]) which have three membrane-spanning domains, and bunyavirus glycoprotein G1/G2 spike complexes which have only one membrane-spanning domain (11, 22, 46). Bunyavirus glycoproteins, like RV glycoproteins, are type I membrane proteins, whereas the integral Golgi membrane proteins that have been cloned and sequenced are type II membrane proteins (34). The only exception to date is TGN38 which is found in the TGN (33). When the avian and mouse coronavirus E1 glycoproteins are expressed using vaccinia virus recombinants, they have different intraGolgi sites of residence, i.e., they are localized to the *cis* Golgi (36) and TGN (30), respectively. In the case of the avian E1 glycoprotein, retention signals responsible for targeting to the Golgi have been localized to its first membrane spanning domain (37), whereas in the mouse hepatitis virus E1 glycoprotein, the cytoplasmic tail appears to be important but not sufficient for Golgi retention (3). Therefore, it is possible that the mechanism of retention of the two coronavirus E1 glycoproteins may be different. Golgi retention signals for type II Golgi

membrane proteins are invariably located in the signal/anchor domains and flanking sequences (34).

RV is perhaps most similar to bunyaviruses in that a heterodimer is formed between two type I membrane proteins which are then retained in the Golgi as a complex. Interestingly, both RV and bunyavirus glycoproteins are unusually rich in cysteine residues (10, 11, 13, 14, 22, 46). The intraGolgi location of the various bunyavirus glycoproteins expressed from cDNAs has not yet been determined by immunoelectron microscopy. We have detected trace amounts of E2 and E1 glycoproteins on the cell surface of transfected cells with significantly more being detected on the surface of transiently transfected COS cells (18) than in stably transfected CHO cells even though the ratio of intracellular to cell surface label was the same (i.e., >90% of labeled RV glycoproteins retained intracellularly after 3-h chase). Typically, low levels of bunyavirus glycoproteins are also found on the surface of infected cells or cells expressing G1 and G2 from cDNA (25, 38).

RV Glycoproteins as Models for Golgi Membrane Proteins

CHOE2E1 cells should prove to be very useful for further study of the synthesis, targeting and turnover of Golgi membrane proteins. Expression of high levels of RV glycoproteins does not significantly impair the growth of these cells and did not cause any obvious cellular abnormalities (i.e., autophagic vacuoles, vesiculated Golgi) in CHO cells. Thus, for all intents and purposes, RV E2 and E1 behave as endogenous Golgi membrane proteins in CHO cells and should prove to be ideal for analysis of Golgi targeting signals. According to prevailing concepts, for proteins to be retained in the Golgi they must contain a Golgi retention signal (34). It follows that E2 and/or E1 must contain such a retention signal for Golgi targeting to occur. We are now attempting to localize the Golgi retention signal(s) on the RV glycoproteins by constructing chimeras of E2 and E1 with proteins that are normally transported to the cell surface.

Finally, the availability of a biochemically and morphologically well defined cell line expressing high levels of E2 and E1 together with their relatively slow rate of transport from the ER to the Golgi make CHOE2E1 cells an excellent alternative to cells infected with VSV G as a model system for further biochemical studies of ER to intermediate compartment and Golgi transport.

This research was supported by National Institutes of Health grant DK17780 (to M.G. Farquhar) and a postdoctoral fellowship from the Medical Research Council of Canada (to T. C. Hobman).

Received for publication 16 November 1992 and in revised form 28 January 1993.

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