The Rubella Virus E1 Glycoprotein Is Arrested in a Novel Post-ER, Pre-Golgi Compartment

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Abstract. Evidence is accumulating that a distinct compartment(s) exists in the secretory pathway interposed between the rough ER (RER) and the Golgi stack. In this study we have defined a novel post-RER, pre-Golgi compartment where unassembled subunits of rubella virus (RV) E1 glycoprotein accumulate. When RV E1 is expressed in CHO cells in the absence of E2 glycoprotein, transport of E1 to the Golgi complex is arrested. The compartment in which E1 accumulates consists of a tubular network of smooth membranes which is in continuity with the RER but has distinctive properties from either the RER, Golgi, or previously characterized intermediate compartments. It lacks RER and Golgi membrane proteins and is not disrupted by agents which disrupt either the RER (thapsigargin, ionomycin) or Golgi (nocodazole and brefeldin A). However, luminal ER proteins bearing the KDEL signal have access to this compartment. Kinetically the site of E1 arrest lies distal to or at the site where palmitylation occurs and proximal to the low temperature 15°C block. Taken together the findings suggest that the site of E1 arrest corresponds to, or is located close to the exit site from the ER. This compartment could be identified morphologically because it is highly amplified in cells overexpressing unassembled E1 subunits, but it may have its counterpart among the transitional elements of non-transfected cells. We conclude that the site of E1 arrest may represent a new compartment or a differentiated proximal moiety of the intermediate compartment.

PROTEINS following the exocytic pathway first enter the rough ER (RER)[1] and are then transported to the Golgi complex where they are sorted and directed to their final destinations (16, 44). It is widely believed that no specific signals are required for exit from the ER or intra-Golgi transport (69); however, proteins must undergo proper folding and in many cases oligomerize to become transport competent (22, 43, 50). Proteins exit the ER system in vesicular carriers derived from transitional elements (42) and pass through pre-Golgi elements before entering the Golgi complex (52). Pre-Golgi structures appear to represent the site of budding of certain viruses (61, 63), the site of the low temperature (15°C) (52) block and the site of accumulation of certain transmembrane and secretory proteins (48, 54, 66).

Currently a good deal of interest is focused on characterizing these intermediate structures involved in ER to Golgi transport, and the overexpression of viral membrane glycoproteins has been invaluable in these studies. To further address aspects of ER to Golgi transport, we have studied the expression and intracellular transport of the structural proteins of rubella virus (RV). RV, a Togavirus that assembles on intracellular membranes (2, 68), contains two type I membrane envelope glycoproteins E2 and E1 (12, 40, 67) which are thought to form a heterodimeric complex within the ER. Transient expression of RV E1 and E2 together or E1 alone in COS cells resulted in accumulation of the proteins in a post-RER, juxtanuclear compartment believed to be part of the Golgi complex (20). In the present study we have further characterized the site of arrest of E1 by analysis of stably transfected CHO cells expressing E1. We found that when RV E1 and E2 are expressed together in CHO cells they are targeted to the Golgi; however, when E1 is expressed alone it is arrested in a novel, post-RER/pre-Golgi compartment composed of tubular smooth membrane elements that appear to be located near the site of exit from the ER. In this paper, we have characterized this compartment and found that it has distinctive properties from either the RER or the Golgi complex.

Materials and Methods

Materials

Reagents and supplies were from the following sources: MEM alpha and dialyzed fetal bovine serum (DFBS) were purchased from Irvine Scientific (Santa Ana, CA). Brefeldin A (BFA) was from Epicenter Technologies (Madison, WI). Fibronectin, calcium ionophores A23187 and ionomycin, SDS, hypoxanthine–thymidine supplement, chloroquine, nocodazole, and

1. *Abbreviations used in this paper*: BFA, Brefeldin A; BiP, binding protein; DFBS, dialyzed fetal bovine serum; dhfr, dihydrofolate reductase; DSP, dithiobis succinimidylpropionate; PDI, protein disulfide isomerase; RER, rough ER; RV, rubella virus.

BSA were purchased from Sigma Chemical Co. (St. Louis, MO). [^{35}S]cysteine (800 Ci/mM) was from ICN Biomedicals, Inc. (Irvine, CA) and ^{3}H-[9, 10] palmitate (30–60 Ci/mM) was from New England Nuclear (Boston, MA). ^{14}C-labeled protein standards and goat anti-rabbit IgG-gold conjugate (5 nm) were purchased from Amersham Corp. (Arlington Heights, IL). Omnibind A/G agarose was from Infergen (Benecia, CA). Endoglycosidases D and H were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN), and thapsigargin was from Calbiochem-Behring Corp. (San Diego, CA). Dithiobis succinimidylpropionate (DSP) was obtained from Pierce Chemical Co. (Rockford, IL). Rhodamine (TRITC)-conjugated goat anti-rabbit IgG was purchased from Tago (Burlingame, CA). Rabbit anti-mouse IgG and fluorescein (FITC)-conjugated goat anti-human and goat anti-mouse IgG were purchased from Zymed (San Francisco, CA). FITC-goat anti-rat IgG (with no cross-reactivity to mouse IgG) was obtained from Organon Technika Corp. (Durham, NC).

Cell Culture

CHODG44 cells which are deficient in dihydrofolate reductase (dhfr) (64) were grown in MEM alpha supplemented with 10% FBS, 0.5× hypoxanthine-thymidine supplement, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine at 37°C in a 5% CO_2 incubator.

Antibodies

Human anti-rubella virus serum was obtained from Dr. Aubrey Tingle (University of British Columbia, Vancouver, Canada) or was purchased from Vital Blood Products (Calabasa, CA). Mouse monoclonals H2C213 and H4C52 to RV E1 were kindly provided by Dr. John Safford (Abbott Laboratories), and 21B9H and 3D9F (21) were produced and characterized in the lab of Dr. Shirley Gillam (University of British Columbia, Vancouver). Rabbit anti-ER serum (33) was from Dr. Daniel Louvard (Pasteur Institute, Paris), and rabbit anti-lgp120 serum (15) was provided by Dr. W. A. Dunn (University of Florida, Gainesville, Florida). Rabbit antibodies to mannose-6-phosphate receptor were characterized previously (9). Rat monoclonal anti-BiP (5) was provided by Dr. Linda Hendershot (St. Judes Hospital), and affinity-purified rabbit anti-cathepsin D was from Dr. Keitaro Kato (Kyushu University, Kyushu, Japan). Dr. David Meyers (University of California, Los Angeles, CA) kindly supplied us with anti-160-kD ER membrane protein serum. Rabbit anti-PDI antibody (62) was obtained from Dr. Stephen Fuller (European Molecular Biology Laboratory, Heidelberg, Germany). Rabbit anti-serum to gp58 was obtained from Jaakko Saraste (Ludwig Cancer Institute, Stockholm, Sweden), and mouse polyclonal antibody to gp58 was prepared by Dr. Linda Hendricks (this lab). Rabbit antiserum to α-mannosidase II was prepared by Dr. Linda Hendricks (this lab) and Dr. Kelley Moremen (University of Georgia, Athens, GA) using antigen prepared as described (38). Mouse mAb to VSV G protein was a kind gift from Dr. William Balch (Scripps Clinic and Research Foundation, La Jolla, CA).

Transfections

CHODG44 cells (2×10^5) were cotransfected with 20 μg pCMV5-E1 or pCMV5-E2E1 (20) and 2 μg pFR400 (57) using Lipofectin (Bethesda Research Laboratories, Gaitherburg, MD). 2 d later cells were trypsinized and plated into MEM alpha containing 10% DFBS, penicillin, streptomycin, and glutamine. Media was changed every 4 d and colonies were picked after 12 to 14 d. Clones were expanded and screened for RV antigen by indirect immunofluorescence and radioimmunoprecipitation. Since adequate levels of expression were observed without methotrexate amplification, the amplification process was omitted. CHO cells expressing RV E1 are referred to as CHOE1, whereas CHOE2E1 cells express both E2 and E1.

Immunofluorescence Microscopy

Cells were plated onto fibronectin-coated chamber slides or 12-mm glass coverslips (10 μg/ml) at a density of 2×10^4 cells/cm^2. After 2 d, cells were washed (four times) in PBS containing 0.5 mM magnesium and then either fixed and permeabilized with 100% methanol at −20°C for 6 min, or fixed with 2% paraformaldehyde 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 non-specific binding of primary antibodies. Primary and secondary antibodies were diluted in PBS/1% BSA, and incubations were for 1 h. Cells were mounted in 90% glycerol/PBS and 1 mg/ml paraphenylinediamine, examined, and photographed with a Zeiss Axiophot (Carl Zeiss, Oberkochen, Germany). Where indicated, cells were incubated with media containing 2 μg/ml BFA (from methanol stock), 25 μg/ml nocodazole (from DMSO stock), 250 nM thapsigargin, 5 μM A23187, or 5 μM ionomycin (from ethanol stocks) for the indicated time periods prior to 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 100–200 μCi/ml [^{35}S]cysteine for 30–60 min. Post pulse the cells were incubated with complete media containing 25× excess unlabeled cysteine. For chloroquine experiments, only the chase medium contained chloroquine (100 μM). Labeling with ^{3}H-[9, 10] palmitate (250 μCi/ml) was for 6 h at 37°C in complete medium. Cells were washed (three times) with cold PBS and lysed in 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 1 μg/ml of chymostatin, leupeptin, antipain, and pepstatin A by incubation on ice for 15 min. The lysates were centrifuged for 3 min in a microfuge, and the supernatants were incubated with protein A/G agarose beads coated with human anti-RV serum at 4°C for at least 2 h on a rotating device. Immunoprecipitates were washed (three times) with RIPA buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) and once with water. Proteins were eluted by heating at 100°C in SDS-gel sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 2% β-mercaptoethanol, 10% glycerol, 0.1% bromophenol blue).

Endo H and Endo D 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 (19). For endo D digestions, samples 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 (63).

Cross-linking with DSP

Chemical cross-linking was done as previously described (18) with slight modifications. CHOE1 cells were radiolabeled as above and incubated with 500 μg/ml DSP (from a fresh DMSO stock) on ice for 30 min before lysis in PBS (pH 8.0) continuing 1% NP-40. Where indicated, labeled proteins were also cross-linked during cell lysis by including cross-linking agent in the lysis buffer. After 30 min lysates were adjusted to 60 mM glycine to inactivate the cross-linker. Protease inhibitors were also added after the cross-linking reaction. The postnuclear lysates were immunoprecipitated as above.

SDS-PAGE and Autoradiography

Proteins were separated on 5–10% gradient or 10% polyacrylamide gels (27). 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 (Eastman Kodak Co., Rochester, NY) at −80°C. Protein bands were quantitated using a BioRad densitometer.

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 N_2 according to Tokuyasu (60). Ultrathin cryosections prepared from the pellets were collected on carbon/formvar-coated nickel grids and incubated for 1 h in mouse monoclonal anti-E1 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% carbowax.

Infection of CHOE1 Cells with VSV ts045

CHOE1 cells grown on coverslips were infected 2 to 3 d after seeding with VSV ts045 (kindly provided by Dr. William Balch, Scripps Research Institute) such that 50% of the cells were infected at the time of assay. Cells were washed twice with serum-free MEM alpha containing 1/6 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 then removed and replaced with the same media containing 10% FBS. Cells were incubated in a 39.5°C water bath for 2.5 h to accumulate G protein in the RER, after which cycloheximide (500 μM) was added, and the cells were transferred to 15°C and/or 32°C water baths for various chase periods before assay by indirect immunofluorescence.

Results

Isolation of CHO Cells Expressing RV Glycoproteins

It was previously shown that transient transfection of RV cDNAs in COS cells resulted in adequate levels of expression for biochemical analysis of RV proteins (19); however, when the cells were examined by EM, numerous autophagic vacuoles and lysosomes were present most likely due to uptake of the DEAE-dextran used in the transfection procedure. To circumvent this and other possible cellular perturbations associated with transient transfection, we constructed stably transfected cells lines expressing moderate levels of E1 and E2 together or E1 alone. Stable cell lines were obtained by cotransfecting *dhfr*−CHO cells with plasmids containing genes for RV structural proteins and the plasmid pFR400 which contains the selectable marker *dhfr* (57). Selection was based on the ability of transfectants to grow in nucleoside-free medium. Clones were isolated and screened for expression of RV antigens by indirect immunofluorescence and radioimmunoprecipitation. E1 glycoprotein in CHO cells expressing both RV E2 and E1 (CHOE2E1 cells) was localized in the Golgi region (Fig. 1, *A* and *B*), whereas in CHO cells expressing E1 alone (CHOE1 cells), the glycoprotein was localized in large compact structures often located adjacent to the nucleus (Fig. 1 *C*). In some cells only a single large structure (up to 2–3 μm) was observed, while in others, two or more larger staining masses were evident in addition to many smaller ones. These structures were not part of the Golgi complex since they were not stained by antibodies to α-mannosidase II (Fig. 1 *D*). Incubating cells in cycloheximide for up to 6 h did not affect the distribution of

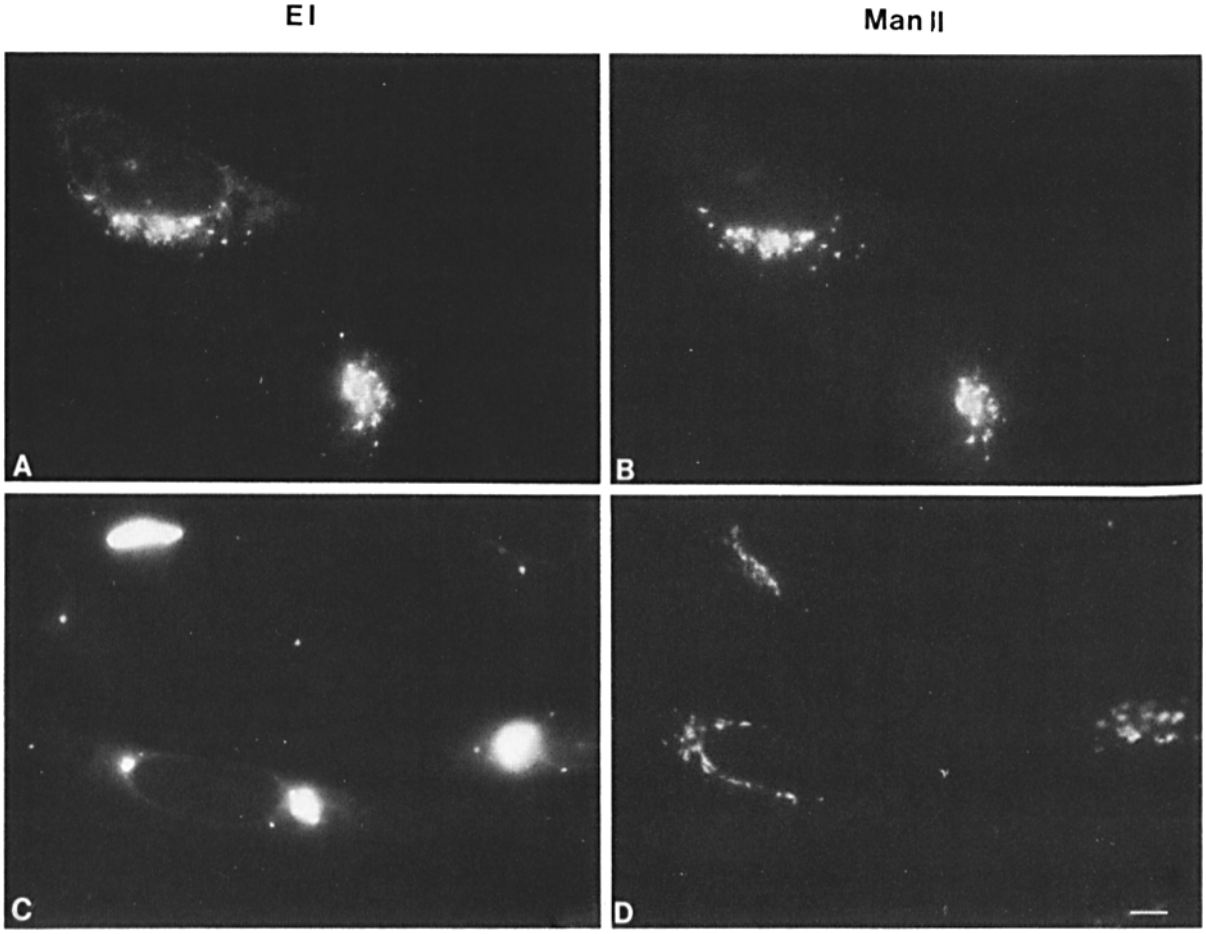
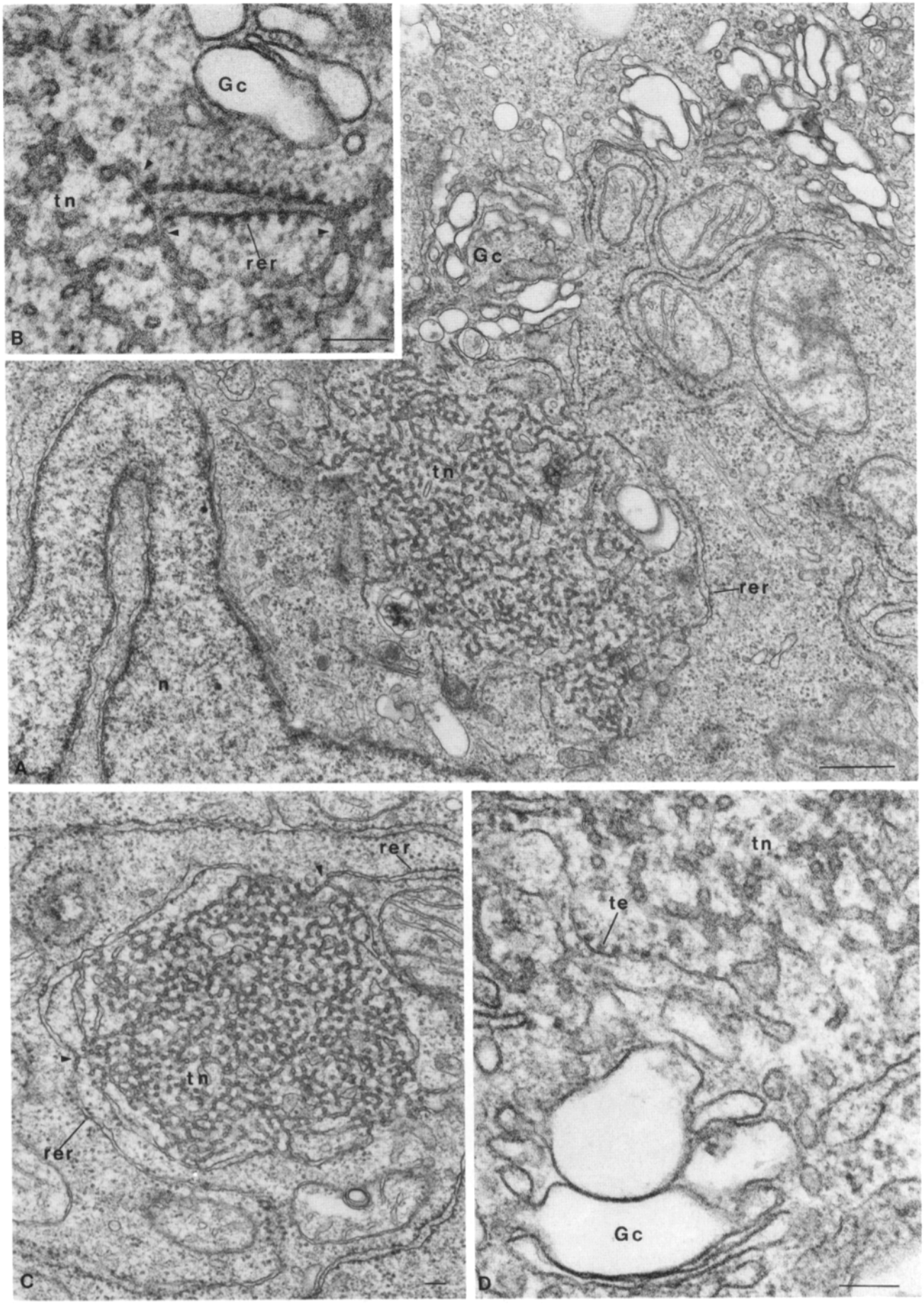


Figure 1. E1 colocalizes with the Golgi marker α-mannosidase II in CHOE2E1 cells but not in CHOE1 cells. Transfected cells were seeded onto fibronectin-coated chamberslides. 40 h later, cells were fixed and permeabilized with 100% methanol at −20°C, and double labeled for E1 and α-mannosidase II (*Man II*)). (*A*) CHOE2E1 cells stained with mouse anti-E1; (*B*) same cells labeled with rabbit anti-Man II; (*C*) CHOE1 cells stained with mouse anti-E1; (*D*) same cells labeled with rabbit anti-Man II. Bar, 5 μm.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E1 (not shown). CHO cells constitutively expressing E2 alone also did not contain the heavily stained cytoplasmic structures found in CHOE1 cells. Instead E2 antigen was distributed throughout the RER and Golgi membranes and at the cell surface (not shown). The results indicated that when E2 and E1 are expressed together they are targeted to Golgi elements, whereas E1 accumulates in a compartment of unusual morphology when expressed without E2.

E1 Is Located in Smooth Membrane Tubular Structures Contiguous with the RER

To obtain further information on the nature of the site of E1 accumulation, we examined CHOE1 cells by EM. In routine Epon sections, structures composed of masses of tubular, smooth membranes were evident that were not present in non-transfected controls or in CHOE2E1 cells. These masses were surrounded by RER (Fig. 2, *A* and *C*) and were located adjacent to Golgi stacks (Fig. 2, *A* and *D*). In favorable sections continuity with RER membranes was seen (Fig. 2 *B*). The smooth membranes differ from RER cisternae in that they are more tubular, have a narrower lumen, and form complicated networks. The tubular networks were relatively homogeneous in that other organelles were excluded from these areas (Fig. 2 *C*). After immunogold labeling of ultrathin cryosections of CHOE1 cells, the membranes of the tubular network were heavily labeled for E1, whereas the RER had a very low level of labeling and the Golgi complex was not significantly labeled above background (Fig. 3, *A* and *B*).

These ultrastructural findings demonstrate that the site of E1 arrest consists of a smooth membrane compartment with distinct morphologic properties which is located near the Golgi complex and is in direct continuity with the RER.

E1 Remains Endo H-Sensitive and Endo D-Insensitive in CHOE1 Cells

The absence of α-mannosidase II together with the ultrastructural findings suggested that E1 was arrested in a smooth membrane compartment that did not correspond to the Golgi complex. To determine whether any E1 entered the Golgi complex immunoprecipitates from radiolabeled CHOE1 cells were digested with endo H. Consistent with previous work (20), only a small fraction of E1 entered the Golgi as evidenced by the time-dependent appearance of an endo H–resistant form (Fig. 4, *arrowhead*); most of the E1 (>80%) remained endo H-sensitive throughout the chase period, indicating that the bulk of the glycoprotein did not reach the middle Golgi complex. Beyond 2-h chase periods, significant turnover of E1 was observed (see below), rendering it difficult to accurately determine how much of the protein had reached the Golgi complex.

To rule out the possibility that E1 was accumulating in a region of the Golgi complex proximal to the site where endo H resistance is acquired, immunoprecipitates were digested with endo D. At no time could any endo D–sensitive forms of E1 be detected (Fig. 4). Since glycoproteins become endo D sensitive after modification by α-mannosidase IA, assumed to be located in the *cis*-Golgi in CHO cells (3), these biochemical results together with the immunofluorescence and electron microscopic findings indicated that E1 is arrested in a pre-Golgi, not a Golgi compartment.

E1 Glycoprotein Is Not Misfolded and Does Not Form Large Aggregates

There are a number of examples where unassembled subunits of oligomeric proteins or misfolded proteins are transport incompetent and cannot leave the RER. Typically misfolded proteins form large aggregates that are frequently permanently associated with BiP (13, 22, 35). In contrast, unassembled subunits do not form stable aggregates and are usually not permanently associated with BiP (22). To address whether the E1 had accumulated as misfolded aggregates, immunoprecipitates from CHOE1 cells were subjected to SDS-PAGE under nonreducing conditions. In the presence of reducing agent (Fig. 5 *A*, *+βME*) E1 migrates at 57 kD as expected, whereas in the absence of reducing agent (Fig. 5 *A*, *−βME*), the mobility of E1 was increased substantially. Since E1 is very cysteine rich (12), the mobility shift is most likely due to formation of intramolecular disulfide bonds. Species with molecular weights expected of E1 dimers and trimers were also observed in nonreduced samples (Fig. 5 *A*, *2×*, *3×*); however, no large aggregates which would be expected to be retarded at the top of the separating gel (34) were evident before or after the 2 h chase.

Intact cells were treated with the membrane-permeable, thiol-cleavable cross-linker DSP to determine if E1 was arranged in noncovalent aggregates. A 60-min labeling period was used to ensure that endogenous proteins that might be associated with E1 were labeled as well. When immunoprecipitates were separated by SDS-PAGE, slightly less monomeric and dimeric E1 was seen after cross-linking due to formation of larger oligomers with an apparent molecular weight >200 kD (Fig. 5 *A*, *arrowhead*). Crosslinking efficiency in intact cells was not improved substantially by increasing the concentration of DSP up to 1 mg/ml (not shown). Cross-linking efficiency was improved greatly by adding DSP to radiolabeled cells at the time of lysis. Cells were lysed in the presence of the nonionic detergent NP-40 (without SDS) so as not to disrupt protein–protein interactions. Under these conditions, cross-linking efficiency was much greater as very little monomeric E1 remained; however, no large aggregates were seen at the top of the resolving

Figure 2. Routine epon sections demonstrating the morphology of the compartment that represents the site of E1 arrest in CHO cells expressing E1. (*A*) Low magnification view showing that the compartment consists of a mass (*tn*) of tubular membranes often located adjacent to the nucleus (*n*) and in close proximity to Golgi cisternae (*Gc*). The smooth membranes form a reticular, tubular network (*tn*) with lumens of smaller dimensions than those of the rough ER cisternae (*rer*) seen at their periphery. (*B*) Points of continuity between RER cisternae (*rer*) and the tubular smooth network can be seen in favorable sections. Three such points of continuity are visible here (*arrowheads*). (*C*) Another example of the site of E1 arrest illustrating the exclusion of other organelles from the network of tubular, smooth membranes and probable connections with the surrounding ER cisternae (*arrowheads*). (*D*) Higher magnification view of the periphery of the tubular membranes, demonstrating their proximity to Golgi cisternae and transitional elements (*te*) of the ER. Bars: (*A–C*), 0.5 μm; (*D*) 0.1 μm.

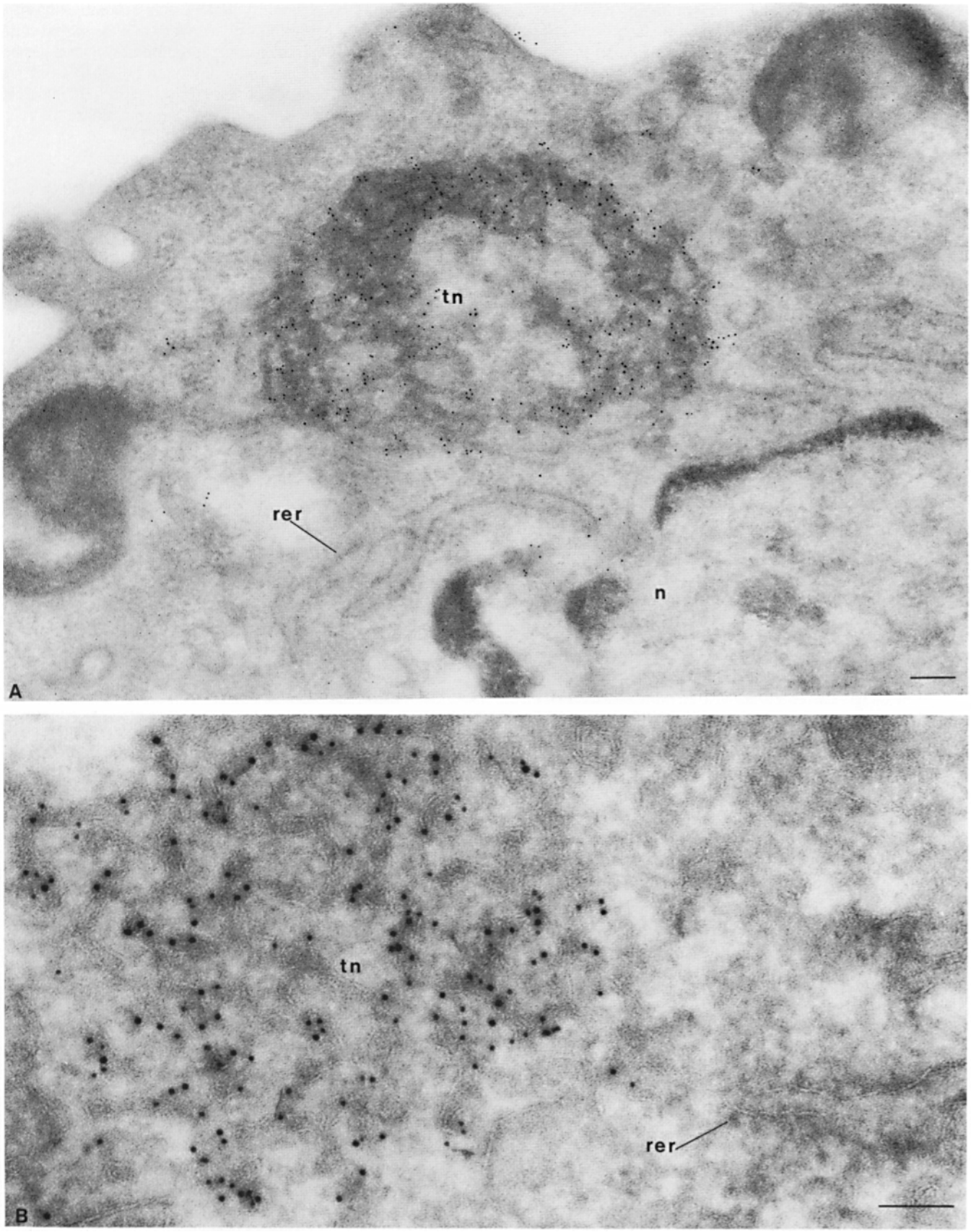


Figure 3. Immunogold labeling of CHOE1 cells for E1. Ultrathin cryosections were incubated sequentially with mouse monoclonal anti-E1, rabbit anti-mouse IgG, and goat anti-rabbit IgG coupled to 5 nm gold. (*A*) E1 is present in high concentration in the network of tubular membranes, but little labeling of the rough ER (*rer*) is seen. (*B*) Higher magnification showing the high density of gold particles associated with the tubular, smooth membranes (*tn*) that represent the site of E1 arrest. Note that an RER cisterna present nearby is not labeled for E1. This suggests that E1 rapidly leaves the rough ER and moves to the smooth, tubular membrane compartment where it accumulates. *n*, nucleus. Bar, 0.1 μm.

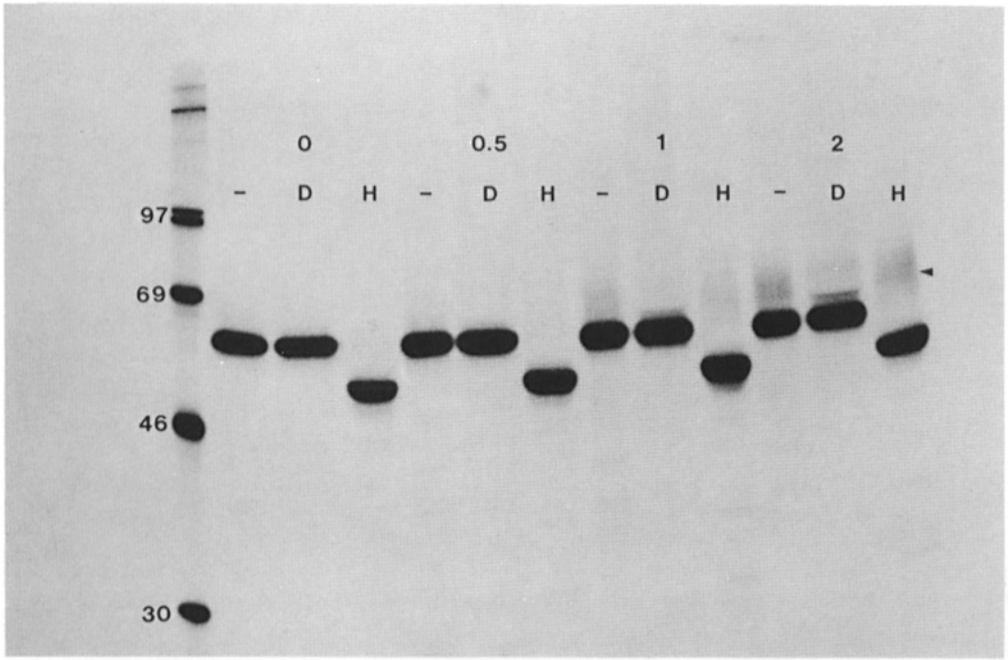


Figure 4. Transport of E1 is blocked in a pre-Golgi compartment. Subconfluent monolayers were pulse labeled with [^{35}S]cysteine for 30 min and then chased in complete media containing excess unlabeled cysteine for the indicated time periods (*h*). Cells were lysed and E1 was recovered from the postnuclear supernatants by incubation with human anti-RV serum and Omnibind agarose beads. Samples were divided into three aliquots and treated with or without endo H (*H* lanes) or endo D (*D* lanes) or incubated without enzyme (− lanes). The proteins were separated by SDS-PAGE on 10% polyacrylamide gels and fluorographed. Golgi-specific processing of E1 glycans is indicated by an arrowhead. ^{14}C-labeled protein standards (*kD*) are included for reference. In the endo D lanes the E1 bands appeared to be compressed in the chased samples. This flattening effect on E1 was most likely due to BSA (in the endo D preparation) which migrates slightly above E1.

gel (Fig. 5 *B*, $-\beta ME$). Rather, the E1 monomers, dimers, and trimers were cross-linked into oligomers >200 kD that possibly represent pentamers and hexamers (Fig. 5 *B*, *arrowheads*). These oligomers presumably consisted mainly of E1, since no other proteins including BiP (78 kD) specific to the cross-linked samples were evident after cleaving the cross-linker with β-mercaptoethanol (Fig. 5 *B*, $+\beta ME$). Since CHO BiP can be radiolabeled with cysteine and contains 60 lysine residues (59) (some of which would be expected to be available for cross-linking with DSP), we would expect to be able to detect BiP under these conditions if it were associated with E1. Furthermore, E1 from CHOE1 cells was recognized by four different mAbs which specify important immunologic epitopes, suggesting that E1 is properly folded (not shown).

Together these results suggest that (*a*) transport-arrested E1 is not misfolded and does not form large covalent aggregates; (*b*) it may exist as disulfide linked dimers and trimers and/or noncovalently associated homooligomers consisting of five to six molecules; and (*c*) E1 does not appear to be associated with BiP at the site of arrest.

The Site of E1 Arrest Does Not Contain ER Membrane Proteins but Is Accessible to Luminal Proteins

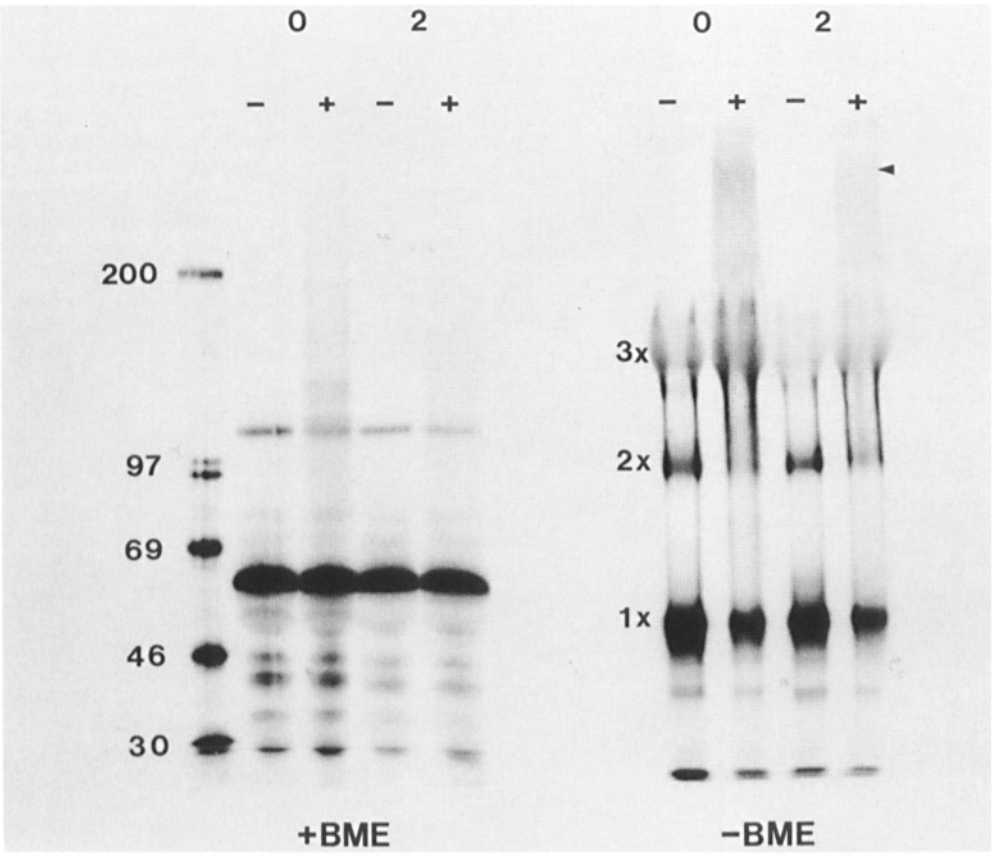
The collective ultrastructural and biochemical findings suggested that unassembled E1 subunits accumulate at a pre-Golgi site which is in continuity with the RER but morphologically does not resemble either RER or Golgi complex. To determine if ER markers were present at the site of E1 arrest, we used antibodies to various resident proteins of this organelle in double immunofluorescence experiments. A polyclonal antiserum that recognizes four RER membrane proteins (33) gave a reticular cytoplasmic staining pattern typical of the RER; however, no staining of the E1-containing compartment was evident (Fig. 6, *A* and *B*). Similarly, a polyclonal antibody to a 160-kD ER membrane protein (obtained from D. Meyer) and another antiserum to three other integral membrane proteins of the RER (15) did not label the site of E1 accumulation (not shown). The fact that very little reticular ER staining for E1 was evident (Fig. 6 *A*) suggests that E1 rapidly leaves the RER after translocation. In contrast to that of RER membrane proteins, the distribution of two KDEL-containing content proteins, protein disulfide isomerase (PDI), and BiP, partially overlapped with that of E1 (Fig. 6, *C–F*). However, these proteins were clearly not concentrated in this compartment.

From these immunofluorescence results, we conclude that membrane components of the RER are excluded from the site of E1 arrest, whereas KDEL-containing proteins and possibly other ER content proteins have access to this compartment.

The Pre-Golgi Site of E1 Accumulation Is Not Affected by Agents That Disrupt Golgi or ER Organization

To obtain further information on the properties of the tubular E1–containing membranes, we carried out experiments with agents known to differentially affect ER and Golgi membranes. For example, the Golgi complex is dispersed upon treatment with microtubule-depolymerizing agents such as nocodazole (49) and BFA (17, 32) whereas the RER is unaffected. In contrast, thapsigargin, a specific inhibitor of ER Ca^{2+} ATPase which causes a rapid depletion of ER Ca^{2+} stores via an inositol triphosphate–independent mechanism (58), results in vesiculation of ER membranes and subsequent secretion of luminal ER proteins while the Golgi is unaffected (8, 25).

Treatment of CHOE1 cells with 25 mM nocodazole or 2 μg/ml BFA, resulted in dispersion of the Golgi complex as evidenced by the drastic change in α-mannosidase II distri-



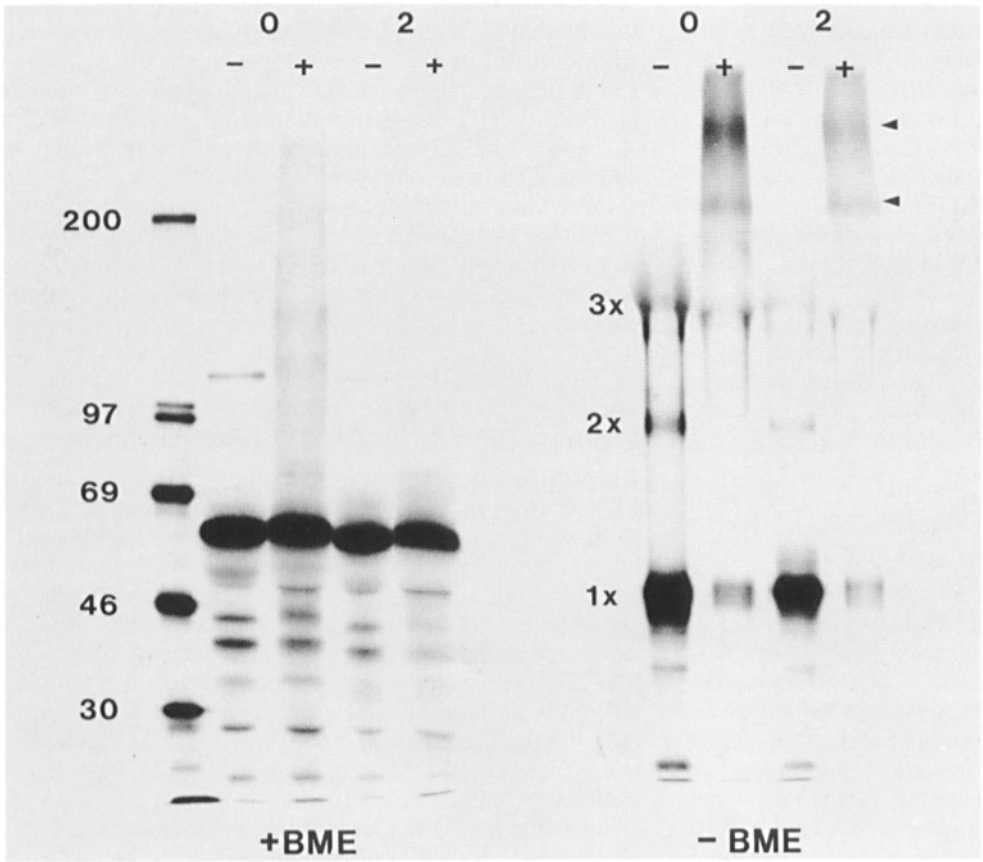


Figure 5. E1 does not form disulfide-linked aggregates and is not associated with BiP. Cells were pulse labeled for 60 min with 100 μCi [^{35}S]cysteine and chased for 0 and 2 h as indicated before cross-linking with 500 μg/ml of DSP (+ lanes) before (*A*) or during lysis (*B*) in the presence of NP-40. Immunoprecipitates were prepared and subjected to SDS-PAGE on 5–10% polyacrylamide gels. Reduced samples (*+βME*) were denatured in the presence of 2% β-mercaptoethanol before loading, while nonreduced samples did not contain reducing agent (*−βME*). Positions of monomers (*1X*) and putative dimers (*2X*) and trimers (*3X*) are indicated. The identity of the proteins of 30–40 kD in the first two lanes is unknown, but they are only observed when cells are lysed in the absence of 0.1% SDS.

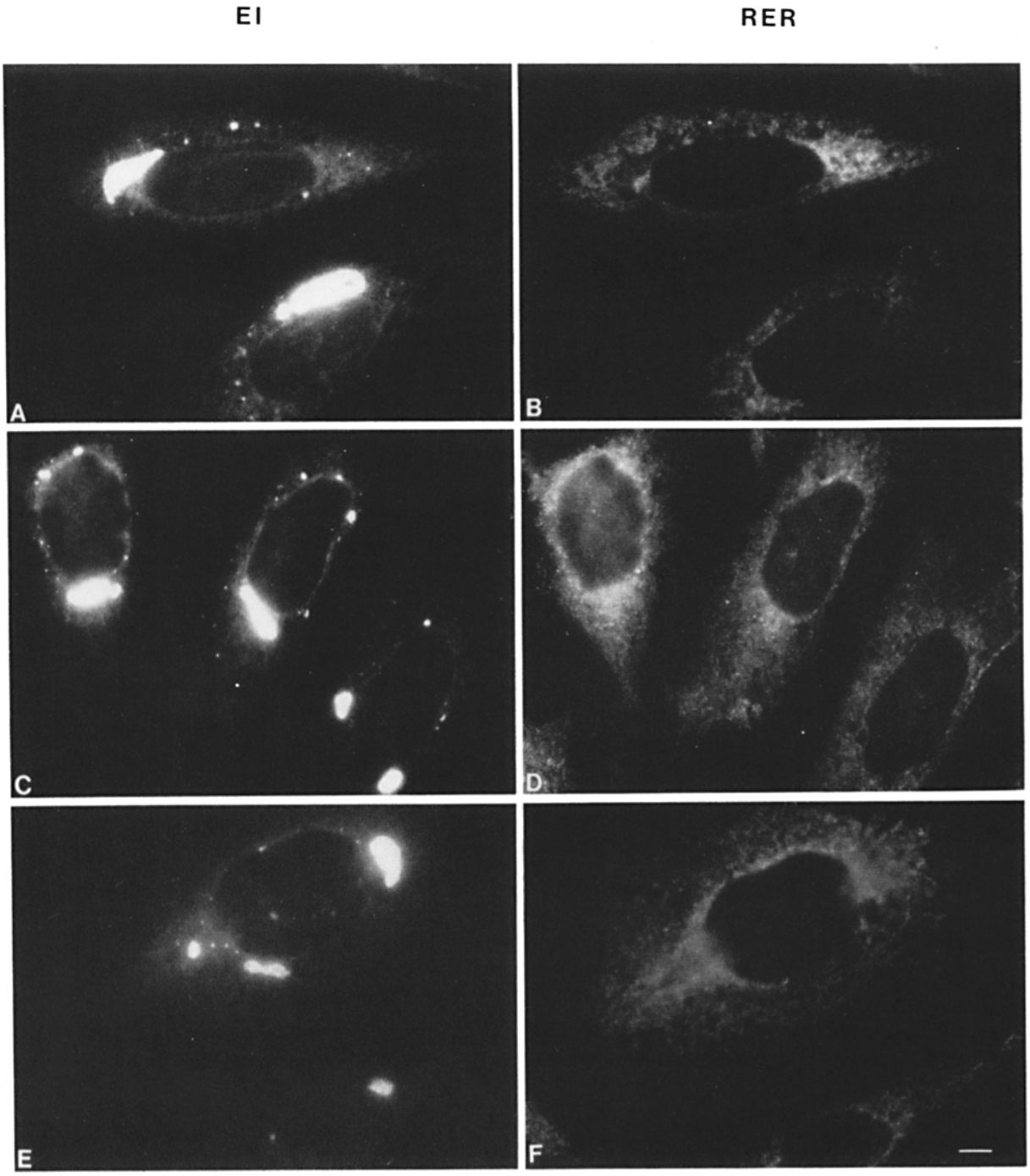


Figure 6. The site of E1 arrest does not contain RER membrane proteins but is accessible to KDEL-containing proteins. CHOE1 cells were fixed and permeabilized as above and incubated with mouse anti-E1 and rabbit or rat antibodies to RER marker proteins. (*A*) Mouse anti-E1; (*B*) same cells with anti-RER serum (33); (*C*) mouse anti-E1; (*D*) same cells with rabbit anti-PDI; (*E*) mouse anti-E1; (*F*) same cells with rat anti-BiP. Bar, 5 μm.

bution from the typical compact perinuclear staining (Fig. 7 *B*) to widely dispersed vesicular and diffuse cytoplasmic staining patterns (Fig. 7, *D* and *F*), but the staining pattern of E1 was not altered significantly under these conditions (Fig. 7, *A*, *C*, and *E*). In contrast, very little colocalization of α-mannosidase II and E1 was evident in nocodazole or BFA-treated cells (Fig. 7, *C–F*). Treatment of CHOE1 cells with BFA for shorter time periods (5, 10, 15, 20, and 30 min) indicated that Man II does not pass through the tubular elements (data not shown) before redistributing to the RER (14, 32).

When CHOE1 cells were incubated with 250 nM thapsigargin for up to 5 h and then examined by double indirect immunofluorescence with anti-E1 and anti-RER antibodies, some disassembly of the RER was evident after 1 h (not shown), and massive vesiculation had occurred at 2 h and later (Fig. 8, *D* and *F*). Furthermore the size of the RER-derived vesicles increased with time (Fig. 8, *D* and *F*). By

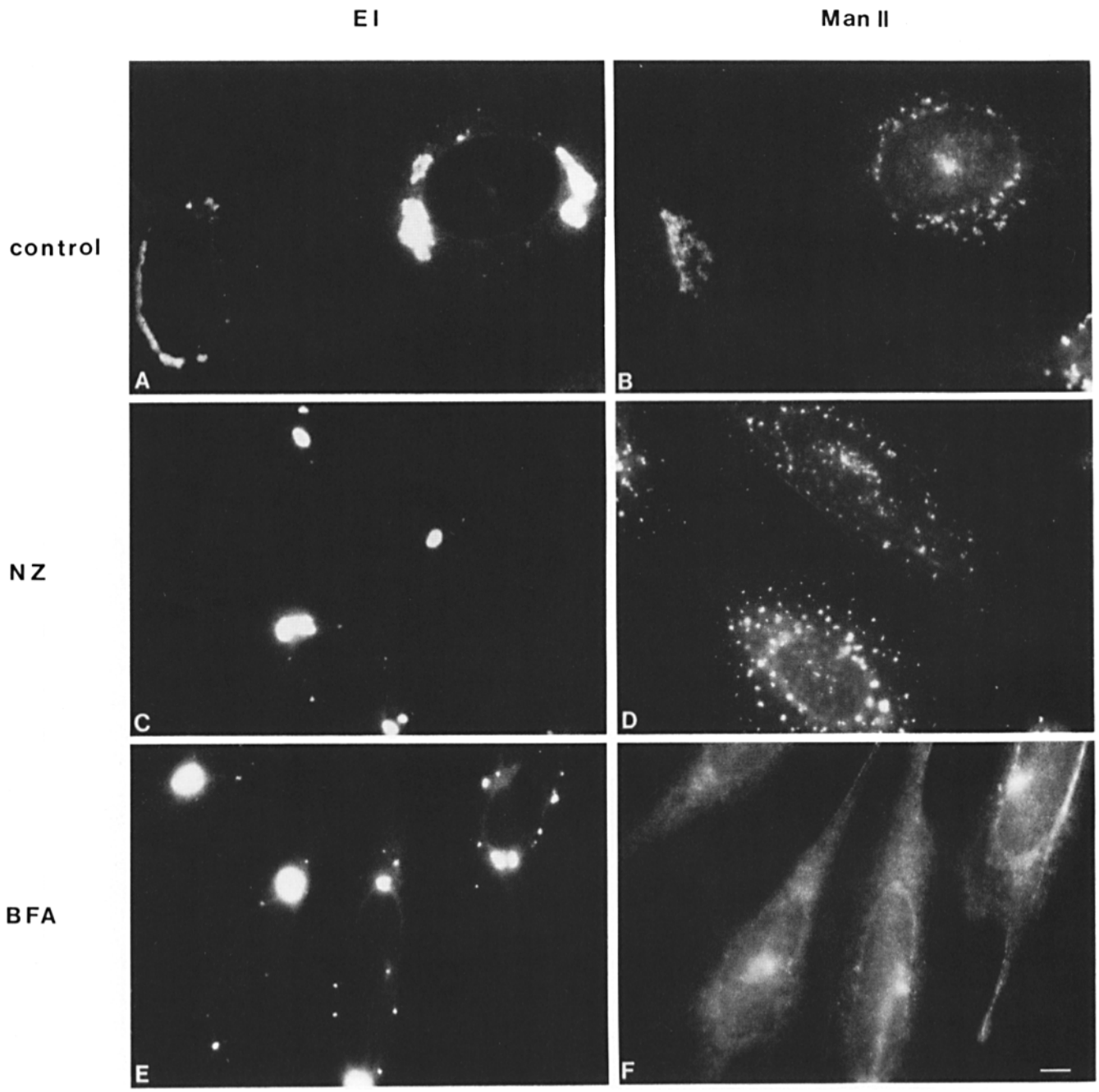


Figure 7. Golgi perturbants do not affect the distribution of E1. CHOE1 cells were incubated for 60 min with 25 μg/ml (*NZ*) nocodazole (*C* and *D*) or 2 μg/ml BFA (*E* and *F*). Untreated cells (*control*) are shown in *A* and *B*. *A*, *C*, and *E* show the distribution of E1 using mouse anti-E1 while *B*, *D*, and *F* show the Golgi marker α-mannosidase II (*Man II*). Bar, 5 μm.

contrast, the E1-containing elements were largely unaffected by thapsigargin treatment (Fig. 8, *C* and *E*). Similar results were obtained using the calcium-specific ionophores ionomycin (Fig. 8, *G* and *H*) and A23187 (not shown).

These results indicate that the site of E1 arrest is resistant to agents that disrupt the integrity of the Golgi complex and RER and suggest that the compartment is not stabilized by either calcium ions or microtubules.

Arrested E1 Is Palmitylated

Previously we reported that RV E1 efficiently incorporated [^{3}H]palmitate when expressed in COS cells in the absence of E2 (20). To determine whether E1 reaches the site where palmitylation occurs in CHO cells, CHOE1 cells were labeled with [^{3}H]palmitic acid. The results in Fig. 9 indicate that palmitic acid is incorporated into E1 in CHOE1 cells. Palmitylated E1 was sensitive to endo H (Fig. 9), ruling out the possibility that only the fraction of E1 that reaches the Golgi complex becomes acylated.

VSV G Protein Can Enter and Exit the Site of E1 Arrest

The previous experiments indicated that newly synthesized E1 leaves the RER and accumulates in the smooth membrane

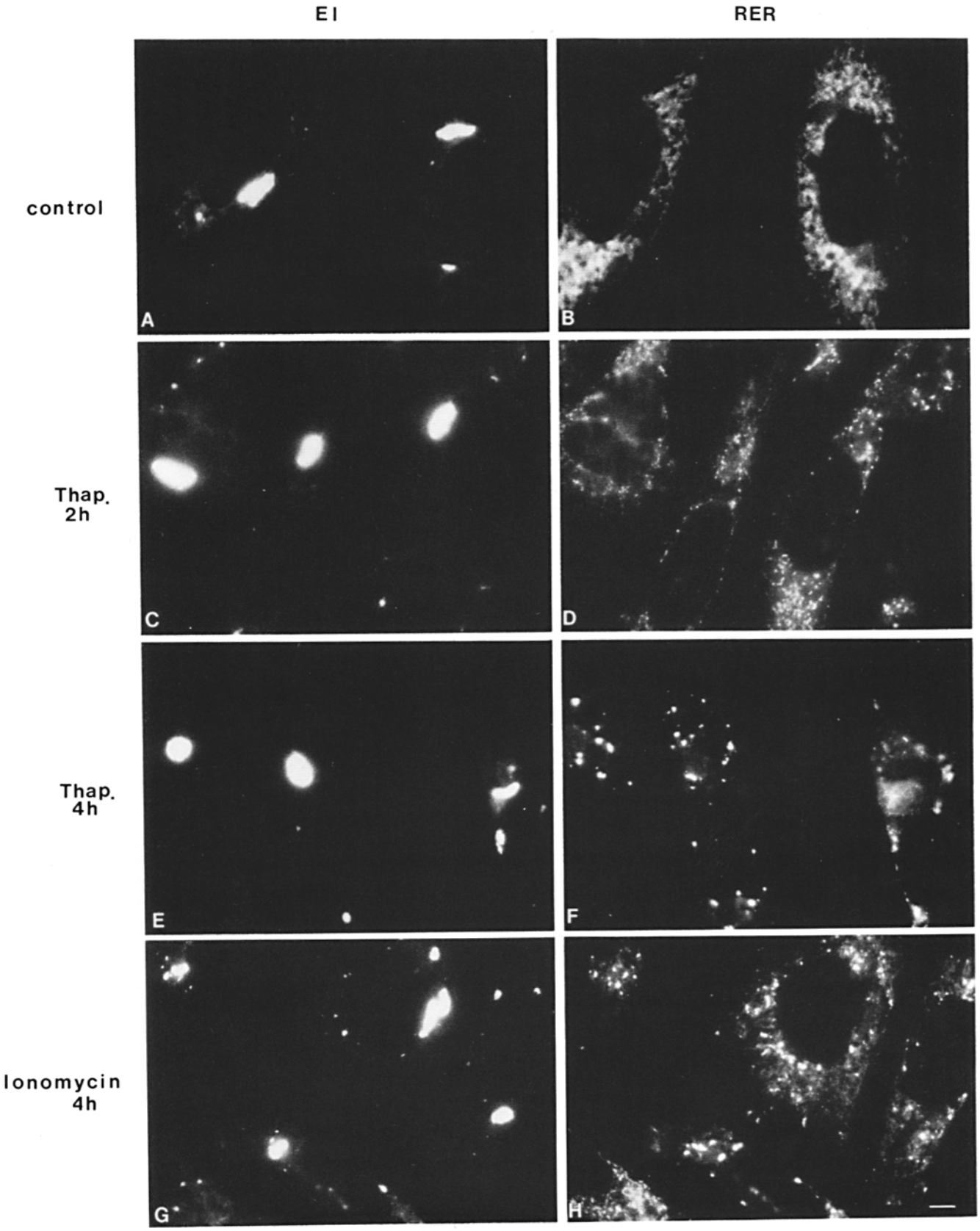


Figure 8. Ca^{2+}-depleting agents do not affect the E1 containing structures. CHOE1 cells were incubated with thapsigargin [Thap] (250 nM) for 2 (*C* and *D*) and 4 h (*E* and *F*) or with 5 nM ionomycin (*G* and *H*). *A* and *B* show untreated cells (*control*) for reference. *A*, *C*, *E* and *G* show the distribution of E1 using mouse anti-E1 while cells in *B*, *D*, *F*, and *H* were stained using rabbit anti-RER serum (*RER*). Bar, 5 μm.

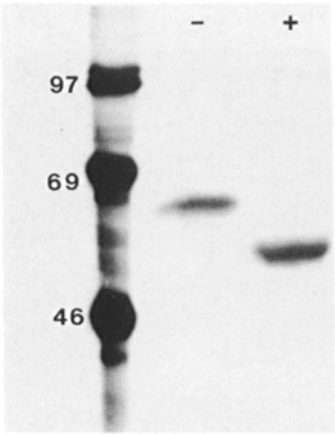


Figure 9. Palmitylated E1 is sensitive to endo H. Cells were labeled with [^{3}H]palmitate for 6 h and E1 was immunoprecipitated as above and subjected to SDS-PAGE and fluorography. Samples were divided into two halves and incubated with (+) or without (−) endo H. Radiolabeled protein standards (*kD*) are included for reference.

tubular network, but it was not clear if this compartment is on the main line or represents a site of diversion of protein traffic from the exocytic pathway. To determine if proteins can enter and leave these structures and reach the Golgi complex, CHOE1 cells were infected with the ts045 strain of VSV. At 39.5°C, the G protein of ts045 does not enter the Golgi complex due to a temperature-sensitive transport defect, but when shifted to 32°C the protein rapidly exits the ER and is transported through the Golgi to the cell surface (24, 28). When CHOE1 cells were kept at 39.5°C, VSV G protein was present in both the RER and the E1 tubular network (Fig. 10, *A* and *B*). This indicates that although unable to enter the Golgi complex, G protein had access to the site of E1 arrest at the restrictive temperature. To follow the movement of G protein out of the ER system, infected cells were first incubated at 39.5°C and subsequently shifted to 32°C with cycloheximide present. By 5 min after shift to the permissive temperature, G protein could be seen within vesicular structures surrounding the tubular network and throughout the cytoplasm (Figs. 10, *C* and *D*). G protein was seen in similar vesicular structures when infected cells were held at 15°C following G protein buildup at 39.5°C (Fig. 10 *L*). Continued incubation at 32°C allowed complete egress of G from the E1-containing compartment and movement of the G-containing vesicles to the Golgi region (Fig. 10, *E–H*). By 40 min, much of the G protein was present at the cell surface while E1 remained arrested in the tubular compartment (Fig. 10, *I* and *J*). These experiments indicate that: (*a*) VSV G, a plasma membrane protein, passes through the tubular network and is transported to the Golgi complex en route to the cell surface; and (*b*) G protein enters structures corresponding in morphology to the 15°C compartment after passing through the site of E1 arrest.

We conclude that the pre-Golgi site of E1 arrest lies along the main route of exocytic traffic and is proximal to the 15°C compartment. Attempts to localize gp58 in relationship to the site of E1 arrest were not successful, as no gp58 signal was obtained in CHOE1 cells using rabbit and mouse polyclonal antibodies (not shown).

Turnover of E1

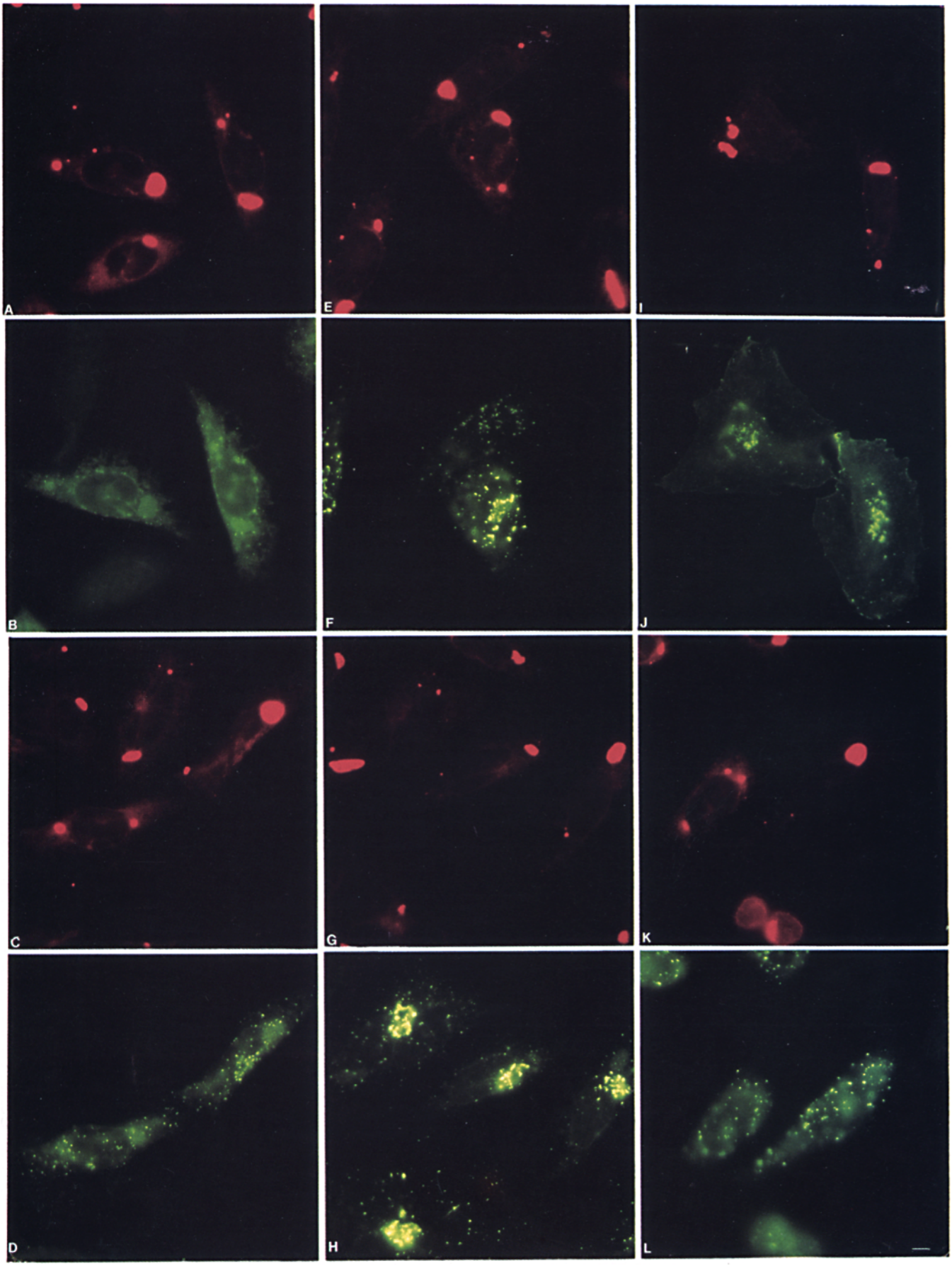
Recently it has been shown that unassembled subunits of multimeric protein complexes expressed in transfected cells are rapidly degraded in non-lysosomal, pre-Golgi compartments (1, 7, 31). Biosynthetic labeling experiments with extended chase periods were used to determine the stability of E1 in its pre-Golgi site of accumulation. Subconfluent monolayers of CHOE1 cells were pulse labeled for 60 min with [^{35}S]cysteine and chased for various time periods. E1 was immunoprecipitated and subjected to SDS-PAGE and fluorography (Fig. 11). Bands were quantitated by scanning densitometry and plotted as a function of time (not shown). The half-life of E1 was determined to be ~6 h which is comparable to that of unassembled subunits of many other proteins which typically ranges from 6–10 h (22). A putative 14-kD degradation product accumulated during increased chase periods (Fig. 11, *arrowhead*). When pulse-chase experiments were carried out in the presence of chloroquine, the drug had very little if any effect on the rate of E1 proteolysis (not shown) rendering it unlikely that E1 is degraded in an acidic compartment such as lysosomes or endosomes. Double immunofluorescence experiments indicated that the tubular networks did not contain the lysosome markers lgp120 (30) and cathepsin D, or the endosome marker, mannose-6-phosphate receptor (not shown). Similarly, E1 was not detected in these endocytic structures. These results suggest that E1 may undergo degradation at its site of accumulation.

Discussion

The Site of E1 Arrest Constitutes a Novel Pre-Golgi Compartment

The results in this paper demonstrate that RV E1 glycoprotein which does not assemble with E2 is unable to be transported to the Golgi complex and accumulates in a novel pre-Golgi compartment consisting of a tubular network of smooth membranes distinct from, but in continuity with the RER. Kinetically, the compartment is located distal to, or at the site where palmitylation of E1 occurs, but proximal to the 15° block which corresponds to the so-called intermediate compartment (43, 52). The site of E1 arrest has distinct properties from the RER, intermediate compartment, or Golgi: (*a*) membrane proteins that serve as markers of RER or Golgi are excluded from this compartment; (*b*) agents which disrupt RER (thapsigargin, ionomycin) or Golgi (nocodazole and BFA) integrity did not affect it; and (*c*) its morphologic organization (network of smooth membranes)

Figure 10. Transport of VSV G protein through the tubular network. CHOE1 cells were infected with VSV ts045 at 32°C for 60 min. Cells were then transferred to 39.5°C for 2.5 h after which they were immediately processed by indirect immunofluorescence (*A* and *B*) or transferred to 32°C for 5 (*C* and *D*), 10 (*E* and *F*), 15 (*G* and *H*), or 40 (*I* and *J*) min before assay. Cycloheximide (500 μM) was added to samples immediately before the temperature down shift. In *K* and *L*, cells were placed at 16°C for 2 h after incubation at 39°C. *A*, *C*, *E*, *G*, *I*, and *K*) show the distribution of E1 glycoprotein as revealed by human anti-RV serum. The distribution of VSV G protein in the corresponding cells is shown in *B*, *D*, *F*, *H*, *J*, and *L*). Bar, 5 μm.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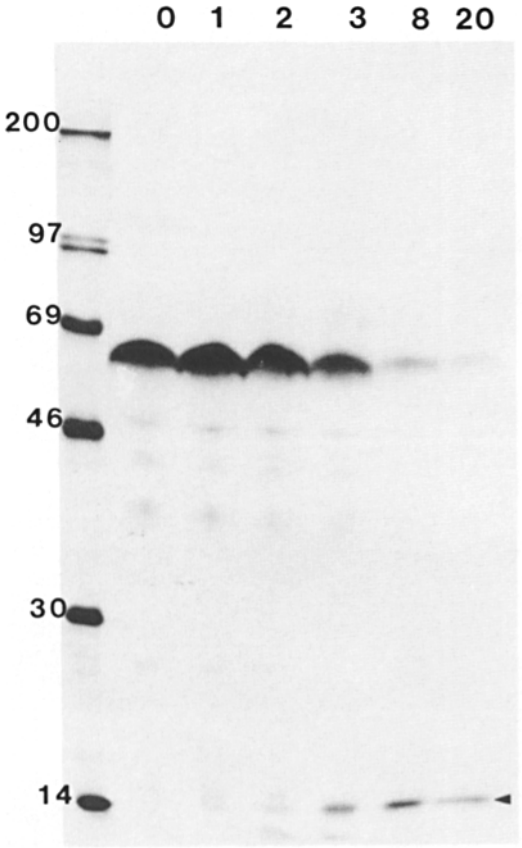

Figure 11. Degradation of E1 glycoprotein. Subconfluent CHOE1 cells were pulse labeled for 60 min with 50 μCi of [^{35}S]cysteine and chased for the indicated time periods (*h*) with medium containing excess unlabeled cysteine before lysis. E1 proteins were recovered by immunoprecipitation as above and separated by SDS-PAGE on 10% polyacrylamide gels followed by fluorography. ^{14}C-labeled protein standards (*kD*) were included for reference. The 14-kD band (*arrowhead*) presumably represents a degradation product of E1.

as seen by EM is entirely different from that of the RER, Golgi complex, or the intermediate compartment (54, 55). However, KDEL-containing luminal ER proteins, BiP and PDI, seem to have access to this region.

That the site of E1 arrest is not a dead end and presumably lies along the main route of exocytic traffic, was demonstrated by the finding that VSV G protein synthesized by the ts045 mutant which is defective in ER to Golgi transport at 39.5°C, could enter the tubular network at the restrictive temperature, and then exit this compartment when shifted to the permissive temperature. Taken together, the collective findings suggest that the site of E1 arrest corresponds to, or is located in close proximity to the exit site from the ER system which is highly amplified under these conditions, i.e., overexpression of unassembled E1 subunits. It appears that E1 subunits travel to the extreme distal extensions of the ER where they can go no further in the absence of E2, accumulate, and are degraded with a half-life of 6 h.

It was surprising to find that although physical connections between the RER and tubular elements were present, E1 rapidly leaves the RER after biosynthesis (based on negligible immunogold and immunofluorescence labeling for E1 in the RER) and becomes concentrated in the tubular network. A question that remains unanswered is: What features of RV E1 render it competent to leave the RER? —a property that distinguishes it from many other unassembled protein subunits. Another key question that remains unanswered is: How is E1 retained and concentrated at the exit site? We can rule out that arrest is due to formation of covalent aggregates due to misfolding of E1 since no large disulfide-linked complexes were detected, and the glycoprotein was recognized by a number of mAbs that bind conformation-dependent epitopes. Also, it is unlikely that retention is due to stable interaction with BiP, and associations with other proteins were not detected. It is nevertheless possible that E1 may be retained by interaction with other proteins not detected by our methods.

The Site of E1 Arrest May Correspond to a Hypertrophied Compartment Present in Nontransfected Cells

We assume that the elaborate smooth membrane structures found in CHOE1 cells do not represent an aberrant response of a single cell type to transfection because stably transfected BHK-21 and murine fibrosarcoma cell lines expressing E1 contained comparable structures by indirect immunofluorescence (unpublished observations). Also, these structures are not formed simply as a consequence of overexpression of membrane proteins, because they were not found in cells expressing both E2 and E1 or E2 alone or cells expressing other Togavirus E1 glycoproteins (26, 36, 37).

Although the tubular networks found in CHOE1 cells are not seen in non-transfected CHO cells, it is quite possible that this compartment has a less-developed counterpart in wild type cells. It is the part rough/part smooth transitional elements of the ER that are presumed to represent the exit site for vesicles shuttling between the ER and Golgi complex or the ER and the intermediate compartment; however, the morphology of this region varies considerably from one cell type to another as detailed below. One possibility is that the E1-containing tubular networks may arise as a consequence of extensive proliferation of smooth regions of the ER which correspond to transitional elements. The fact that the E1-containing compartment is not destabilized by Ca^{2+} removal is in keeping with the model proposed by Sambrook (51) which predicts that transport vesicles bud from regions of the ER that are not sensitive to fluctuations in calcium ions. Our findings are compatible with the assumption that proteins in transit to these specialized extensions of the ER may still be associated with molecular chaperones and enzymes required for attainment of correct tertiary structure such as BiP and PDI, respectively. Upon arrival at this juncture, properly assembled transport-competent protein complexes would presumably then enter transport vesicles, and the chaperone proteins could be returned to the RER by diffusion, or they may enter the vesicles, leave the ER system, and be returned via receptor-mediated retrieval from a salvage compartment (39). By contrast unassembled subunits that are unable to enter the transport vesicles are retained by some unknown mechanism and are ultimately degraded. Alternatively, these membranes may develop from other as yet uncharacterized subregions of the ER. The situation will be clarified only when antibodies to resident proteins of the E1-containing

compartment are generated which should allow identification of these membranes in nontransfected cells.

Other Pre-Golgi Smooth Membrane Compartments

The E1-containing tubular elements are morphologically reminiscent of tubular smooth ER seen in phenobarbital-treated hepatocytes where the volume of the system increases greatly in order to accommodate the increased concentration of detoxifying enzymes (41, 46). Unlike the structures described in this paper which form relatively homogeneous tubular networks, smooth ER of this type is not arranged in tubules and is often interspersed with other organelles such as mitochondria (41). Several other ER-associated specialized pre-Golgi smooth membrane compartments have been described previously including, dilated smooth membrane cisternae that are the intracellular site of mouse hepatitis virus (61), spleen focus forming virus, and murine leukemia virus (63) budding in certain cell types; a smooth ER subcompartment consisting of anastamosing tubules where concentration and assembly of chondroitin sulfate proteoglycan precursors is believed to take place in chondrocytes (66); "crystalloid smooth ER" found in mutant CHO cells synthesizing massive amounts of the resident ER membrane protein HMG CoA reductase, (11), which ts045 G protein can also enter and exit (4); flattened smooth cisternae postulated to be amplified transitional elements in continuity with the RER in transfected COS cells expressing a rat growth hormone–influenza hemagglutinin chimeric protein (48). None of these structures resembles morphologically the E1-containing compartment described here; however, the latter compartment may be functionally equivalent since the growth hormone–influenza hemagglutinin protein remained endo H-sensitive and was modified with palmitate (47, 48). The only other structures described to date that morphologically resemble the E1-containing tubules are those that develop after endocytosis of SV-40 in CV-1 cells; however, these tubules do not appear to be involved in exocytic processes, because newly synthesized virus particles do not enter them (23).

Relationship between the E1-containing Tubular Network and the 15°C Intermediate Compartment

The intermediate compartment was originally defined as a system of vacuoles located between the ER and *cis*-Golgi which newly synthesized viral proteins could enter at 15°C (52). Although the intermediate compartment is still poorly defined, a number of properties and functions have been ascribed to it including; (*a*) palmitylation of viral membrane proteins (6); (*b*) budding of mouse hepatitus virus coronavirus (61); and (*c*) site of localization of certain membrane proteins (53, 54). Originally, it was proposed that the intermediate compartment was the site where retrieval of KDEL-bearing proteins occurred. However, more recently this process has been suggested to occur in the Golgi complex (29). An intermediate compartment would be expected to have physical properties and resident proteins distinct from the RER and *cis*-Golgi, and results obtained by Schweizer and colleagues demonstrate that such is the case (56). The structure described in this paper shares some of the characteristics attributed to the intermediate compartment, such as, (*a*) palmitylation which occurs either proximal to or at the site of E1 accumulation; (*b*) it is not affected by agents which disrupt the Golgi or ER; and (*c*) it lacks RER and Golgi resident membrane proteins. It differs, however, in that transport of E1 is arrested proximal to the 15°C compartment as defined by transport of VSV G protein. This raises the question of how the compartment we have partially characterized and the intermediate compartment interact. The presence of physical connections between them seems unlikely. Rather, it seems more likely that a round of vesicular transport is required for passage of proteins from the site of E1 arrest to the pre-Golgi vacuoles that constitute the 15°C intermediate compartment.

Lack of Marker Proteins for Pre-Golgi Structures

Except for PDI and BiP which only partially overlap with E1, no other marker proteins have been found that colocalize to the site of E1 arrest. HMG CoA reductase remains a potential marker; however, we were unable to detect this protein by immunofluorescence in our CHOE1 cells, presumably because expression levels of this enzyme are low except in these cells containing amplified copies of the gene. The GTP-binding protein rablb, which colocalizes with rab2 to the intermediate compartment (10) and is found predominantly in smooth ER fractions of tissue homogenates (45) also represents a potential marker, but we were not able to ascertain by immunofluorescence whether or not it was present at the site of E1 arrest. Similarly, our attempts to label CHOE1 cells using antibodies to the 72-kD KDEL receptor (65) or gp58 (53) were unsuccessful. We did not try p53 (54) because this intermediate compartment marker reacts only with human or primate cells (H.-P. Hauri, personal communication).

The results presented here as well as those reported by others (11, 61, 63, 66) demonstrate that the smooth membrane pre-Golgi compartments that lie between the RER and *cis*-Golgi are diversified in organization and function and have the capacity to undergo amplification. They also raise the possibility that the smooth membrane pre-Golgi compartments may consist of two or more distinct subcompartments with different specialized functions. We consider that the site of E1 arrest may represent a new compartment or alternatively a differentiated proximal moiety of the intermediate compartment (54, 55). Without appropriate markers it is difficult to know where to draw the line between the ER and intermediate compartments. The high level of expression of E1 in the CHOE1 cells should facilitate the isolation of the E1-containing tubular elements by cell fractionation and immunoisolation, and make it possible to characterize the resident proteins of this novel compartment.

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