Retention of Secretory Proteins in an Intermediate Compartment and Disappearance of the Golgi Complex in an END4 Mutant of Chinese Hamster Ovary Cells

Chia-Yi Kao and Rockford K. Draper

The Molecular and Cell Biology Program, FO3.1, The University of Texas at Dallas, Richardson, Texas 75083-0688

Abstract. Mutant V.24.1, a member of the End4 complementation group of temperature-sensitive CHO cells, is defective in secretion at the restrictive temperature (Wang, R.-H., P. A. Colbaugh, C.-Y. Kao, E. A. Rutledge, and R. K. Draper. 1990. *J. Biol. Chem.* 265:20179–20187; Presley, J. F., R. K. Draper, and D. T. Brown. 1991. *J. Virol.* 65:1332–1339). We have further investigated the secretory lesion and report three main findings. First, the block in secretion is not due to aberrant folding or oligomerization of secretory proteins in the endoplasmic reticulum because the hemagglutinin of influenza virus folded and oligomerized at the same rate in mutant and parental cells at the restrictive temperature. Second, secretory proteins accumulated in a compartment intermediate between the ER and the Golgi. Several lines of evidence support this conclusion, the most direct being the colocalization by immunofluorescence microscopy of influenza virus hemagglutinin with a 58-kD protein that is known to reside in an intermediate compartment. Third, at the resolution of fluorescence microscopy, the Golgi complex in the mutant cells vanished at the restrictive temperature.

SECRETORY material in mammalian cells first enters transport vesicles in the ER. Recent evidence suggests that material is delivered next to a compartment intermediate between the ER and the Golgi where proteins may then be either recycled back to the ER or pass on to the *cis*-Golgi (3, 37, 43, 44, 49, 50). Material then traverses the Golgi complex and finally enters the *trans*-Golgi network where contents are sorted and eventually delivered either to the plasma membrane or to lysosomes (for reviews see 2, 17, 38). The general mechanism of membrane movement from one organelle to another during secretion is believed to be vesicular transport, which involves four main steps: (*a*) the sorting of proteins to be transported from those to be left behind; (*b*) vesicle formation; (*c*) vesicle targeting to the specific acceptor membrane; and (*d*) vesicle fusion with the acceptor membrane. The molecular mechanisms of some of these steps are just now beginning to emerge.

Major contributions to understanding secretory transport at the molecular level have been made by combining the genetic analysis of the secretory pathway in yeast (21, 32, 46) with the in vitro biochemical study of secretory transport in both mammalian cells (for example, see 2, 6, 58) and yeast (for example, see 1, 42, 51). Progress in understanding secretion would also be advanced by isolating and analyzing secretion-defective mutants in animal cells. We previously isolated and studied a temperature-sensitive, conditional-lethal mutant of CHO cells, termed V.24.1, that defined the End4 complementation group (7) and which had aberrant lysosomes at the restrictive temperature (8). Subsequent studies demonstrated that V.24.1 cells were defective in secretion (39, 56). In the present work, we used immunofluorescence microscopy and biochemical assays to demonstrate that the secretory block in the V.24.1 cells prevents the export of material from a compartment that is intermediate between the ER and the Golgi. Interestingly, the block in secretion is correlated with the disappearance of the Golgi complex. A preliminary report of this work has appeared (Kao, C.-Y., P. A. Colbaugh, and R. K. Draper. 1990. *J. Cell Biol.* 111:199a).

Materials and Methods

Reagents

TPCK-trypsin, soybean trypsin inhibitor, protein A-sepharose CL-4B, polyacrylamide, Tris base, MES, PMSF, aprotinin, sucrose, Triton X-100, nocodazole, NP-40, and bicinchoninic acid (BCA) protein assay reagent were purchased from Sigma Chemical Co. (St. Louis, MO). Endoglycosidase D (endo D) and endoglycosidase H (endo H) were from Boehringer Mannheim Biochemicals (Indianapolis, IN). Monensin was from Calbiochem (La Jolla, CA). AcrylAide was from FMC Corporation (Rockland, ME). Brefeldin A (BFA) was purchased from Epicentre Technologies (Madison, WI), dissolved at 1 mg/ml in ethanol, and stored at −70°C before use. GammaBind G Agarose was from Genex Corporation (Gaithersberg, MD). Entensify was from Du Pont-New England Nuclear (Boston, MA). Tran^{35}S-label was from ICN Radiochemicals (Irvine, CA).

1. *Abbreviations used in this paper*: BFA, brefeldin A; endo D, endoglycosidase D; endo H, endoglycosidase H; GlcNAc, N-acetylglucosamine; HA, hemagglutinin; Man, mannose.

Antibodies

Goat anti-HA (strain X31) antiserum was from Dr. R. Webster (Saint Jude Children's Hospital, Memphis, TN). Rabbit antimannosidase II (30) was provided by Drs. N. T. Ktistakis, G. Bloom, and M. Roth (University of Texas Southwestern Medical Center, Dallas, TX). Rat mAb ABL-70 (anti-Golgp125; Storrie, B., Y. Cha, and J. T. August. 1988. *J. Cell Biol.* 107:760a; Cha, Y., L. M. Arterburn, M. C. Willingham, and J. T. August. 1990. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 4:A1979.) to a 125-kD Golgi protein, termed here anti-gp125, was donated by Dr. T. August (Johns Hopkins University, School of Medicine, Baltimore, MD). Rabbit anti-p58 serum (44, 45) was from Dr. J. Saraste (Ludwig Institute for Cancer Research, Stockholm, Sweden). Rabbit anti-BiP was the gift of Dr. M.-J. Gething (University of Texas Southwestern Medical Center, Dallas, TX). Mouse mAb 3D1 (antiribophorin II) was provided by Dr. D. Meyer (UCLA Medical School, Los Angeles, CA). Mouse antityrosinated tubulin was from Sigma Chemical Co. Fluorescent labeled secondary antibodies were from Jackson Immuno Research Laboratories, Inc. (West Grove, PA).

Cells and Virus

The isolation and culture of mutant cell line V.24.1 was described by Colbaugh et al. (7). The propagation and use of recombinant influenza virus X31 was as described previously by Wang et al. (56).

Folding of Influenza Virus HA

The extent of hemagglutinin (HA) folding within the ER was measured by its resistance to trypsin upon extraction from cells (9, 10). Cells were seeded in 35-mm culture dishes for 1–2 d before the experiment. For virus infections at the restrictive temperature, cells were incubated at 41°C for 4 h, shifted to 39.5°C and medium containing virus was added. After 1 h, the medium was changed to remove free virus and the incubation was continued at 39.5°C for 4 h. Viral proteins were radiolabeled with Tran^{35}S-label for 5 min as described by Wang et al. (56). At different times after the pulse, the cells were lysed with 350 μl of lysis buffer (1% NP-40, 50 mM Tris, 150 mM NaCl, 10 mM EDTA, pH 8.0). The lysates were clarified by centrifugation in a Brinkman Microfuge for 10 min and the protein concentration was determined by BCA assay (Sigma Chemical Co.). 75 μg of protein from each lysate were treated with 100 μg/ml of TPCK trypsin for 30 min at 0°C. Soybean trypsin inhibitor (final concentration 1 mg/ml) and aprotinin (0.1 TIU/ml) were added to stop the trypsin digestion. HA was immunoprecipitated with goat anti-HA antibody and GammaBindG Agarose. The immunoprecipitated proteins were then analyzed on 12% SDS-polyacrylamide gels (22) with AcrylAide as cross-linker. The gels were treated with Entensify, dried and exposed to preflashed (23) Kodak X-Omat AR film.

Trimerization of HA

The trimerization of HA within the ER was determined by the rate of HA sedimentation in sucrose gradients, as described by Copeland et al. (9) with minor modifications. Virus infection at the restrictive temperature and radiolabeling were as in the previous section. At times after radiolabeling, cells were lysed with 500 μl of 1% Triton X-100, 20 mM MES, 30 mM Tris, and 100 mM NaCl, pH 7.4, with protease inhibitors (1 mM PMSF, 0.1 TIU/ml aprotinin, and 100 μg/ml soybean trypsin inhibitor). The lysates were clarified by centrifugation in a microfuge and layered on 5–25% (wt/vol) sucrose gradients in the lysis buffer without 1% Triton X-100. The gradients were centrifuged in a rotor (model SW41; Beckman Instruments, Inc., Palo Alto, CA) at 40,000 rpm for 16.5 h at 20°C. After centrifugation, 0.5-ml fractions were collected from the top of the tubes. HA was immunoprecipitated from alternate fractions and electrophoresed in 7.5% SDS-polyacrylamide gels.

Endo H Digestion

Cells were grown on 35-mm plates for 1–2 d before the experiment. After virus infection and radiolabeling, as described in the legend to Fig. 1, the cells were lysed for 20–30 min on ice in 500 μl of lysis buffer containing protease inhibitors as described in the preceding paragraph. The lysates were clarified by centrifugation in a Brinkman Microfuge for 10 min at 4°C and HA was immunoprecipitated with anti-HA antibodies and protein A-Sepharose. 30 μl of 0.1% SDS in 50 mM sodium citrate, pH 5.5, was added to each sample and boiled for 5 min. Two 12-μl aliquots were taken from each sample. One aliquot was mixed with 12 μl of 50 mM citrate, pH 5.5 containing 0.2 mU endo H, and the other was mixed with citrate buffer only. Digestion was for 16 h at 37°C. Laemmli sample buffer was added to stop the digestion and HA was electrophoresed in 7.5% SDS-polyacrylamide gels. Different forms of HA on the gel fluorographs were quantitated using a laser scanning densitometer (model 300K; Molecular Dynamics, Sunnyvale, CA).

Endo D Digestion

Virus-infected cells were prepared and lysed as for endo H digestion except 350 μl of lysis buffer was used. Two 100-μl aliquots of each lysate were added to 100 μl of 2.5× digestion buffer (0.5 M sodium citrate, 6.25 mM EDTA, pH 6.35). 50 μl of 10 mM Tris-HCl, pH 7.2, containing 200 mM NaCl, either with or without 1 mU Endo D were then added. The tubes were tightly sealed and incubated at 37°C for 20 h. HA was immunoprecipitated and electrophoresed as for endo H digestion.

Immunofluorescence Microscopy

Cells were grown on 12-mm round No. 1 glass coverslips for 2–3 d before an experiment. Except when staining for the p58 antigen, cells were rinsed twice with PBS containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ and incubated in methanol at −20°C for 5–10 min to simultaneously fix and permeabilize the cells. After the methanol treatment, cells were rinsed with PBS and incubated with 1% BSA (in PBS) for 15 min to block nonspecific protein binding sites. Primary antibodies diluted in 1% BSA were added for 30 min at room temperature. The cells were then rinsed with 1% BSA three times during a 20-min period to wash away unbound primary antibodies. The same incubation and washing procedures were used for fluorescent-labeled secondary antibodies. The cells were rinsed finally in double-distilled water and mounted in 4 μl of Mowiol (Polyscience Inc., Warrington, PA).

Cells stained for p58 were rinsed twice with PBS containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$, fixed with 3% paraformaldehyde (in PBS) for 15–20 min at room temperature, the aldehyde was quenched with 50 mM NH_4Cl for 10 min, and cells were permeabilized with 0.05% Triton X-100 in NET/gel (150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.4, 0.05% NP-40, 0.25% gelatin, 0.02% sodium azide) for 15 min. The cells were then rinsed three times with NET/gel solution and incubated for 45 min at room temperature with anti-p58 antiserum diluted 1:100 in NET/gel. After incubation with the antibody, the cells were washed with NET/gel five times during a 30-min period with agitation, rinsed three times with PBS, and treated with methanol at −20°C for 5 min. Staining for a second antigen, and adding secondary antibodies, was as described at the beginning of this section. Samples were viewed with a Zeiss photoscope equipped with epi-fluorescence illumination and a 63× Apochromat lens. Photography was with Kodak TMAX p3200 film (developed as EI 1,600) or Kodak TMAX 400 film. The bars shown on the prints represent 5 μm.

Results

Time at the Restrictive Temperature Required to Block ER to Golgi Transport in V.24.1 Cells

In V.24.1 cells, glycoproteins containing N-linked oligosaccharides do not acquire resistance to endo H at the restrictive temperature, evidence that transport from the ER to the Golgi is blocked (39, 56). To see if this block was an early response to elevating the temperature, we measured the time at 41°C required to inhibit the acquisition of endo H resistance by HA. A summary of the procedure is diagrammed in Fig. 1 *a*: Cells were infected with influenza virus at 34°C, shifted to 41°C for different times (ΔT), and labeled with Trans^{35}S-label for 5 min followed by a 30-min chase period. The sensitivity of HA to endo H was then determined (Fig. 1 *b*). With wild-type cells, HA was almost completely resistant to endo H when cells were labeled immediately after raising the temperature to 41°C (ΔT = 0). This indicated that the 35 min used for the pulse and chase was sufficient time for HA to be transported from the ER to the Golgi or beyond. HA from the wild-type cells remained endo H resistant at ΔT = 4 h. With V.24.1 cells, only 62% of the HA

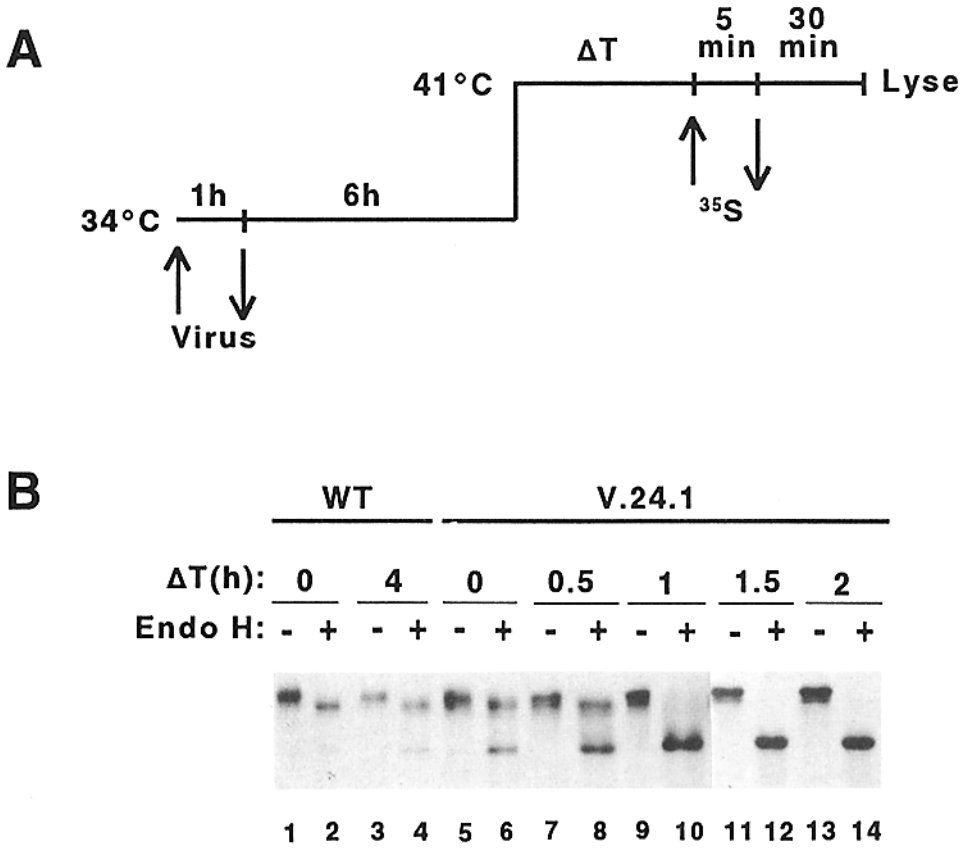

Figure 1. Time at 41°C required to block ER to Golgi transport in V.24.1 cells. A summary of the procedure is shown in *A*. Cells at 34°C were infected with influenza virus and the temperature was increased to 41°C. Tran^{35}S-label was added for 5 min at different times after the temperature change, the radiolabel was removed, and the cells were incubated a further 30 min in medium containing excess cold methionine and cysteine before they were lysed and HA was extracted. Endo H sensitivity was determined as described in Materials and Methods. The percentage of total HA that was endo H sensitive was measured by densitometric scanning and was: (lane *2*) 12%; (lane *4*) 22%; (lane *6*) 38%; (lane *8*) 52%; (lane *10*) 94%; (lane *12*) 96%; (lane *14*) 97%.

was endo H resistant by 35 min at 41°C ($\Delta T = 0$), decreasing to 48% by 65 min ($\Delta T = 0.5$ h) and falling to 6% by 95 min ($\Delta T = 1$ h). This relatively rapid inhibition of the acquisition of endo H resistance in V.24.1 cells indicates that the block of ER to Golgi transport is an early response to high temperature, consistent with the possibility that this is closely related to the primary lesion in the cells.

Influenza Virus HA Remains Resistant to Digestion by Endo D in V.24.1 Cells at the Restrictive Temperature

N-linked oligosaccharides of glycoproteins in the Mannose$_9$ *N*-acetylglucosamine$_2$ ($Man_9GlcNAc_2$) form within the ER are resistant to digestion by endo D. However, once in the Golgi the oligosaccharide chains are trimmed by α-1,2-mannosidase I to the Man_5 $GlcNAc_2$ species (4), which is sensitive to digestion by endo D (29, 52). In addition, after oligosaccharide side chains acquire more GlcNAc by the action of N-acetylglucosamine transferase I, they are again resistant to endo D, and upon removal of more mannose by Golgi mannosidase II, they become resistant to endo H. Since HA did not become resistant to endo H, it was presumably not modified by *N*-acetylglucosamine transferase I and mannosidase II, but could have been present in the $Man_5GlcNAc_2$ form within the Golgi. If the HA were present in the $Man_5GlcNAc_2$ form, it should be sensitive to digestion by endo D, and we assessed this possibility. As a positive control for the activity of endo D, the sensitivity of HA to endo D in the CHO 15B cell line, which fails to add GlcNAc to glycoproteins in the medial Golgi, was measured. In CHO 15B cells, all N-linked glycoproteins should retain sensitivity to endo D once in or past the *cis*-Golgi. CHO 15B and V.24.1 cells were incubated at high temperature as indicated in the legend to Fig. 2, infected with influenza virus, pulsed with Tran^{35}S-label for 5 min, and chased for 0.5 h. Before the chase (Fig. 2, lanes *1* and *2*), HA extracted from CHO 15B cells was resistant to endo D, and was primarily in the

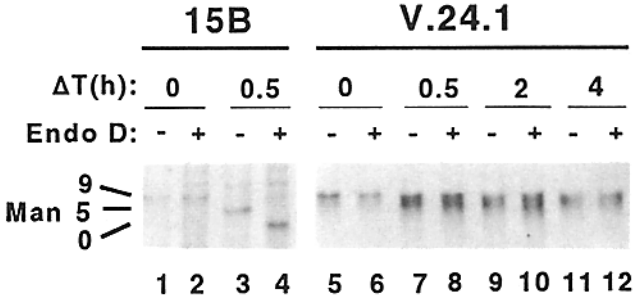

Figure 2. HA remains resistant to digestion by endo D in V.24.1 cells at the restrictive temperature. Cells were incubated at 41°C for 2 h, placed at 39.5°C, infected with influenza virus, pulsed for 5 min with Tran^{35}S-label at 4 h postinfection, and lysed after indicated chase times (ΔT). Cell lysates were treated with endo D as described in Materials and Methods. HA was immunoprecipitated, electrophoresed in 7.5% SDS-polyacrylamide gels, and radiolabeled proteins were detected by fluorography. HA synthesized in CHO 15B cells (lanes *1–4*) served as a positive control for the activity of endo D. The inferred mannose content of the HA species is listed on the left.

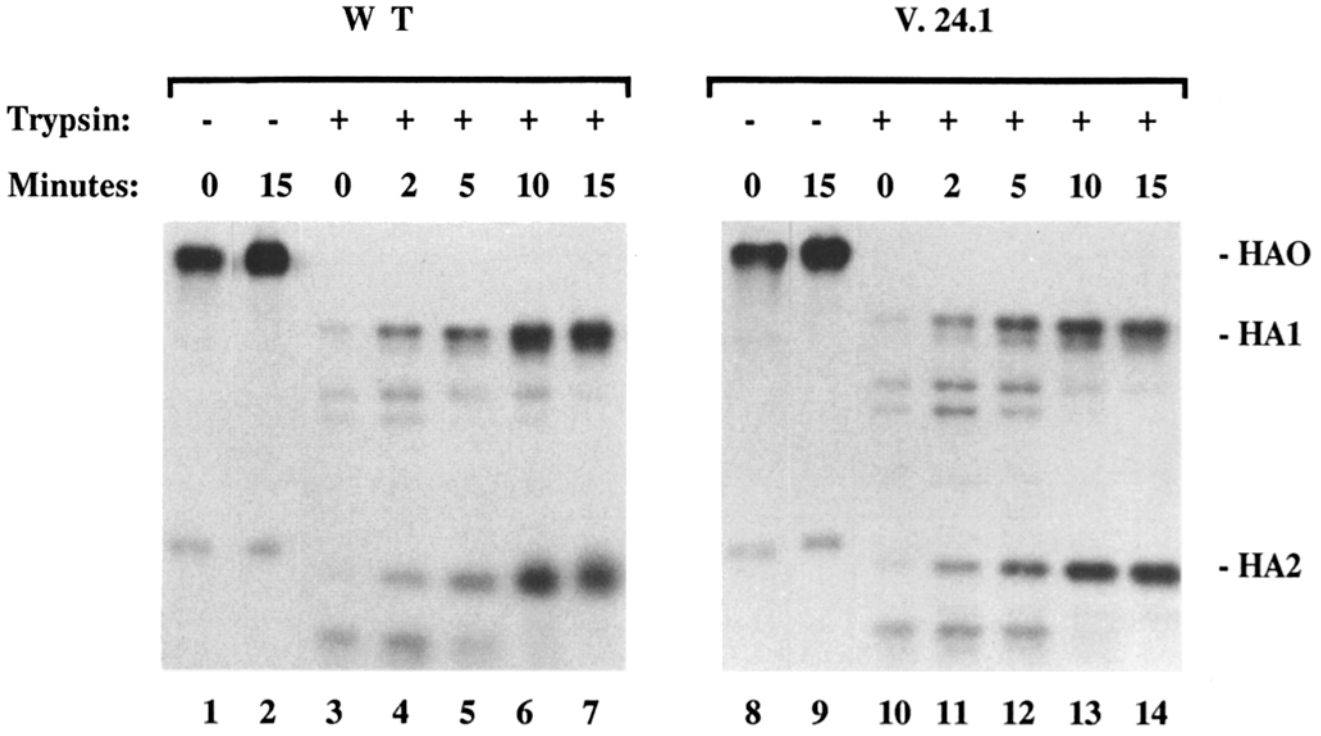

Figure 3. Folding of HA in V.24.1 cells assessed by sensitivity to trypsin. The folding of newly synthesized HA was determined by testing its sensitivity to trypsin. Trypsin cleaves the initial form of properly folded HA (HA0, $M_r \approx 78{,}000$) into HA1 ($M_r \approx 55{,}000$) and HA2 ($M_r \approx 27{,}000$), whereas unfolded and misfolded HA is completely degraded. Cells were infected at the restrictive temperature with virus, radiolabeled, and lysed at the indicated times after radiolabeling as described in Materials and Methods. 75 μg of protein from each lysate was treated with TPCK-trypsin. Trypsin digestion was then stopped by the addition of soybean trypsin inhibitor. HA was immunoprecipitated with anti-HA antibodies, and electrophoresed in 12% SDS-polyacrylamide gels. Radiolabeled proteins were detected by fluorography.

Man_9 form. After a chase of 0.5 h, HA appeared in the Man_5 form (Fig. 2, lane *3*) and was sensitive to endo D, as seen in lane 4 (Man_0 is the endo D digestion product). This control experiment demonstrated that the endo D was active.

With V.24.1 cells, all of the HA was completely resistant to endo D digestion, even at 4 h after radiolabeling (Fig. 2, lanes *5–12*), indicating that the $Man_5GlcNAc_2$ form was not accumulating. Schwaninger et al. (47) recently presented evidence that the *cis*-Golgi compartment in CHO cells contained both *N*-acetylglucosamine transferase I and α-1,2-mannosidase II in addition to α-1,2-mannosidase I. The failure of N-linked oligosaccharides to acquire resistance to endo H or to accumulate in the $Man_5GlcNAc_2$ form suggests that glycoproteins are not reaching even the *cis* aspect of the Golgi complex in the mutant cells.

Folding and Trimerization of HA Is Normal in V.24.1 Cells at the Restrictive Temperature

One explanation for the failure of glycoproteins to reach the Golgi in V.24.1 cells is that the lesion somehow impairs protein folding or oligomerization within the ER. It is well-documented that the transport of proteins out of the ER to the Golgi apparatus requires proper folding and oligomerization (10, 16, 37). To investigate the conformation of a secretory glycoprotein within the ER, we used established procedures (10, 16) to monitor the folding and trimerization of HA. Before folding, HA (referred to as HA0) is sensitive to digestion by trypsin but becomes resistant as it folds and trimerizes. Trypsinization of folded HA0 results in only two fragments, HA1 and HA2. To assess folding, mutant and parental cells were infected with influenza virus at the restrictive temperature and pulsed for 3 min with Tran^{35}S-label. At times up to 15 min after the pulse, cells were lysed and extracts were incubated with trypsin. Trypsin inhibitor was added and immunoprecipitated HA was electrophoresed in SDS-polyacrylamide gels. Samples without trypsin treatment were included at times 0 and 15 min to assess the recovery of HA in the absence of trypsin (Fig. 3, lanes *1*, *2* and *8*, *9*). In the presence of trypsin, the recovery of HA1 and HA2 was very low at time 0, indicating that HA had not yet folded and was digested by trypsin (Fig. 3, compare lane *1* with *3* and lane *8* with *10*). Maximum recovery of HA1 and HA2 was observed by 10 min after the pulse, revealing the acquisition of trypsin resistance that accompanies folding. There was no apparent difference between the mutant and parental cells in the rate at which HA became trypsin resistant.

HA trimerization in pulse-labeled cells was assessed by sedimenting cell lysates in sucrose gradients (9). As shown in Fig. 4, monomers of HA sedimented in fractions 6–8 of the gradients while trimers (centered around fraction 14) from the parental cells were observed within 5 min of the chase and predominant by 10 min of the chase period. There was no apparent difference in the rate of HA trimerization with the mutant cells (Fig. 4, *right side*). Altogether, the data in Figs. 3 and 4 suggest that the secretory block in V.24.1 cells is not caused by a defect in the folding or oligomerization of proteins in the ER.

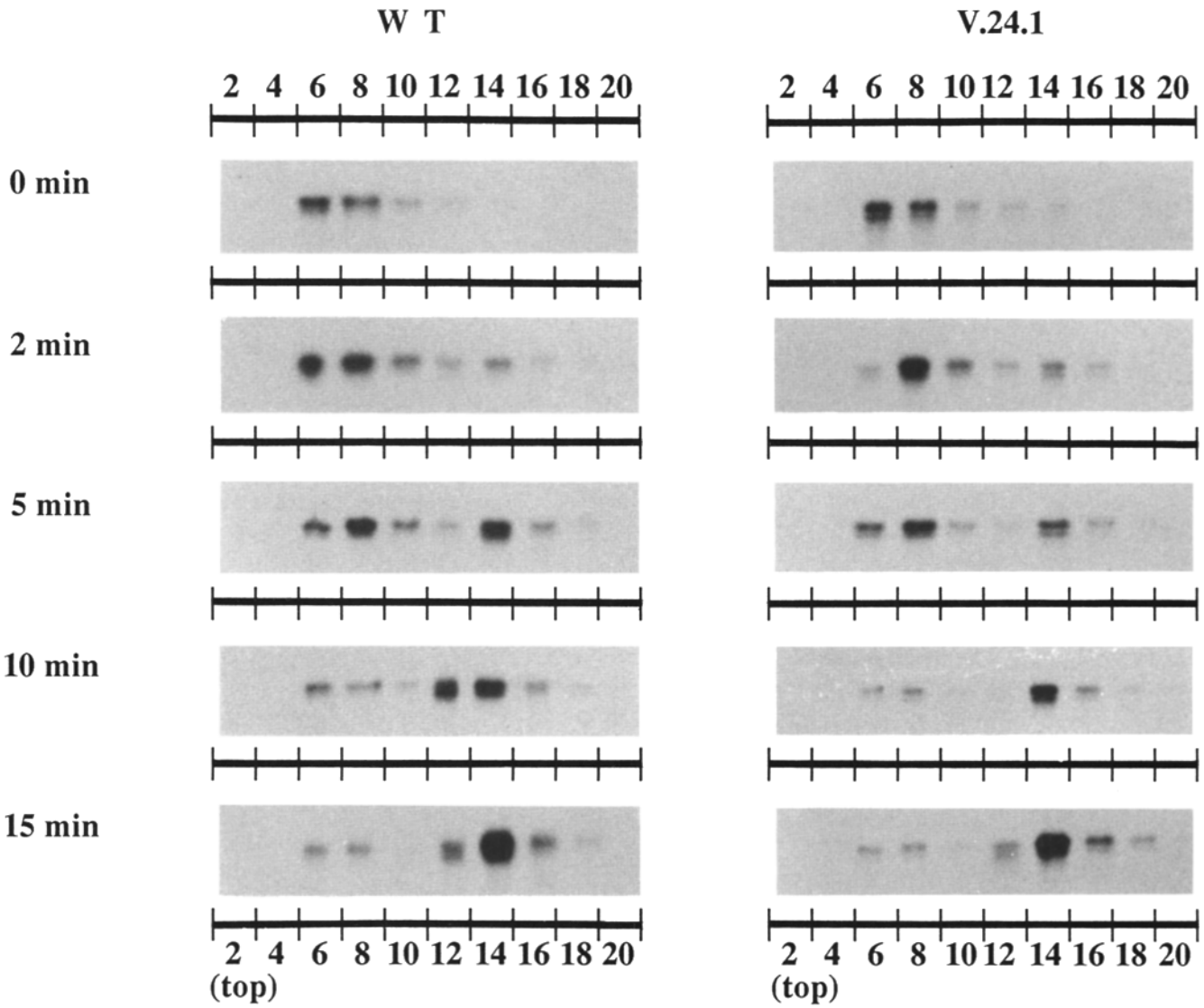

Figure 4. Rate of HA trimerization in wild-type and mutant cells at the restrictive temperature. Cells were incubated at the restrictive temperature, infected with influenza virus and radiolabeled as described in Materials and Methods. The cells were lysed at different times after radiolabeling as indicated on the left side of the figure. The monomeric and trimeric forms of HA were separated in 5–25% sucrose gradients. HA was then immunoprecipitated from every other fraction collected from the gradients (fraction numbers are at the bottom of the figure), electrophoresed in 7.5% SDS-polyacrylamide gels, and proteins were detected by fluorography.

HA Accumulates in a Post-ER Pre-Golgi Compartment

Considering that HA does not appear to reach the Golgi, it should either be retained within the ER itself or be transported as far as a compartment intermediate between the ER and the Golgi. To investigate this, we determined whether HA colocalized with a marker for the ER or with a marker for a post-ER compartment by immunofluorescence microscopy. The ER was identified using antibodies to BiP, a soluble protein located primarily within the ER (37). The intermediate compartment was identified with antibody to p58, a 58-kD protein reported by Saraste and co-workers to reside in a reticular compartment intermediate between the ER and the *cis*-Golgi and in the *cis*-Golgi (44, 45).

Wild-type and mutant cells were incubated at 41°C to initiate the lesion in the mutant cells and infected with influenza virus. The cells were then prepared for immunofluorescence microscopy to simultaneously visualize either HA and BiP (Fig. 5, *a–d*), or HA and p58 (Fig. 5, *e–h*). In wild-type cells, HA was predominantly in the Golgi and on the cell surface (Fig. 5, *a* and *e*). In the mutant cells, HA appeared to be mainly in discrete vesicular structures concentrated in the nuclear area, but which were also present towards the cell periphery (Fig. 5, *c* and *g*). The nuclear envelope in the mutant cells appeared to contain HA and HA also was observed in the ER. The morphology of the ER, shown by the staining for BiP, was similar in mutant and parental cells (Fig. 5, *b*

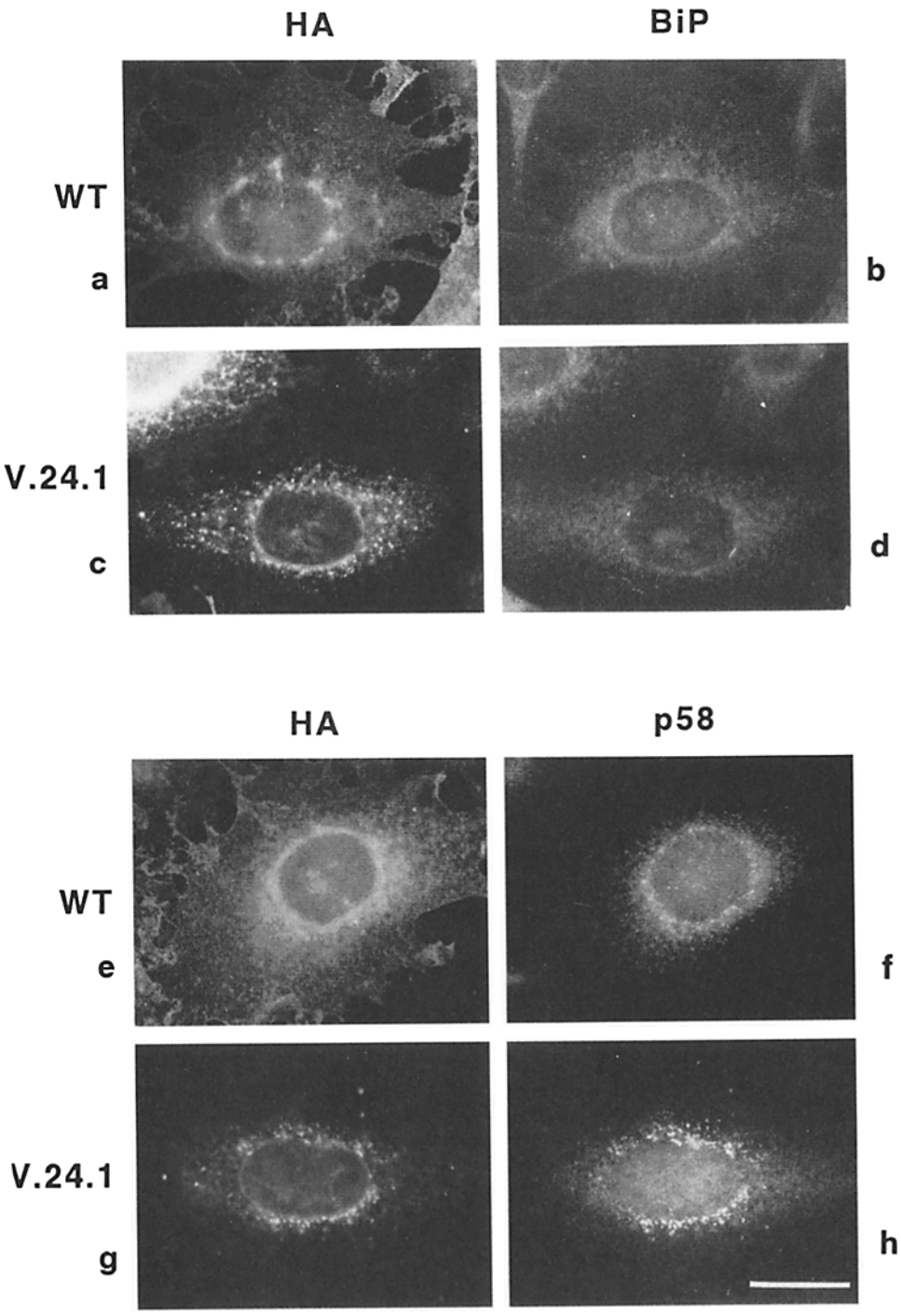

Figure 5. Localization of HA, BiP, and p58 in mutant and wild-type cells infected with virus at the restrictive temperature. Cells grown on glass coverslips were incubated at 41°C for 2 h to elicit the temperature sensitive lesion, shifted to 39.5°C, infected with virus, and fixed at 4 h after infection. Cells were then incubated with goat anti-HA (*a*, *c*, *e*, *g*), and either rabbit anti-BiP (*b* and *d*) or rabbit anti-p58 (*f* and *g*) primary antibodies. Secondary antibodies were Texas red-labeled mouse anti-goat IgG and FITC-labeled mouse anti-rabbit IgG.

and *d*), indicating that the ER was intact in V.24.1 cells (see also Fig. 7 *d*). In wild-type cells, most of the p58 appeared to be within small vesicles concentrated primarily around the nucleus (Fig. 5 *f*). Most interesting, however, was the colocalization of p58 and HA in vesicles of the mutant cells (Fig. 5, *g* and *h*). Considering that HA may not reach the *cis*-Golgi, and that anti-p58 could stain both the intermediate compartment and the *cis*-Golgi, vesicles containing both HA and p58 may be the intermediate compartment between the ER and the *cis*-Golgi.

A Resident Golgi Protein Is Not Transported beyond the p58-containing Compartment in V.24.1 Cells at the Restrictive Temperature

To see whether the transport of an endogenous cell protein, in addition to a viral glycoprotein, was arrested at the compartment defined by p58, we treated mutant and parental cells with BFA for 1 h at 34°C to redistribute Golgi proteins to the ER. The cells were then incubated for 2 h at 41°C to induce the lesion in the mutant cells and a control sample

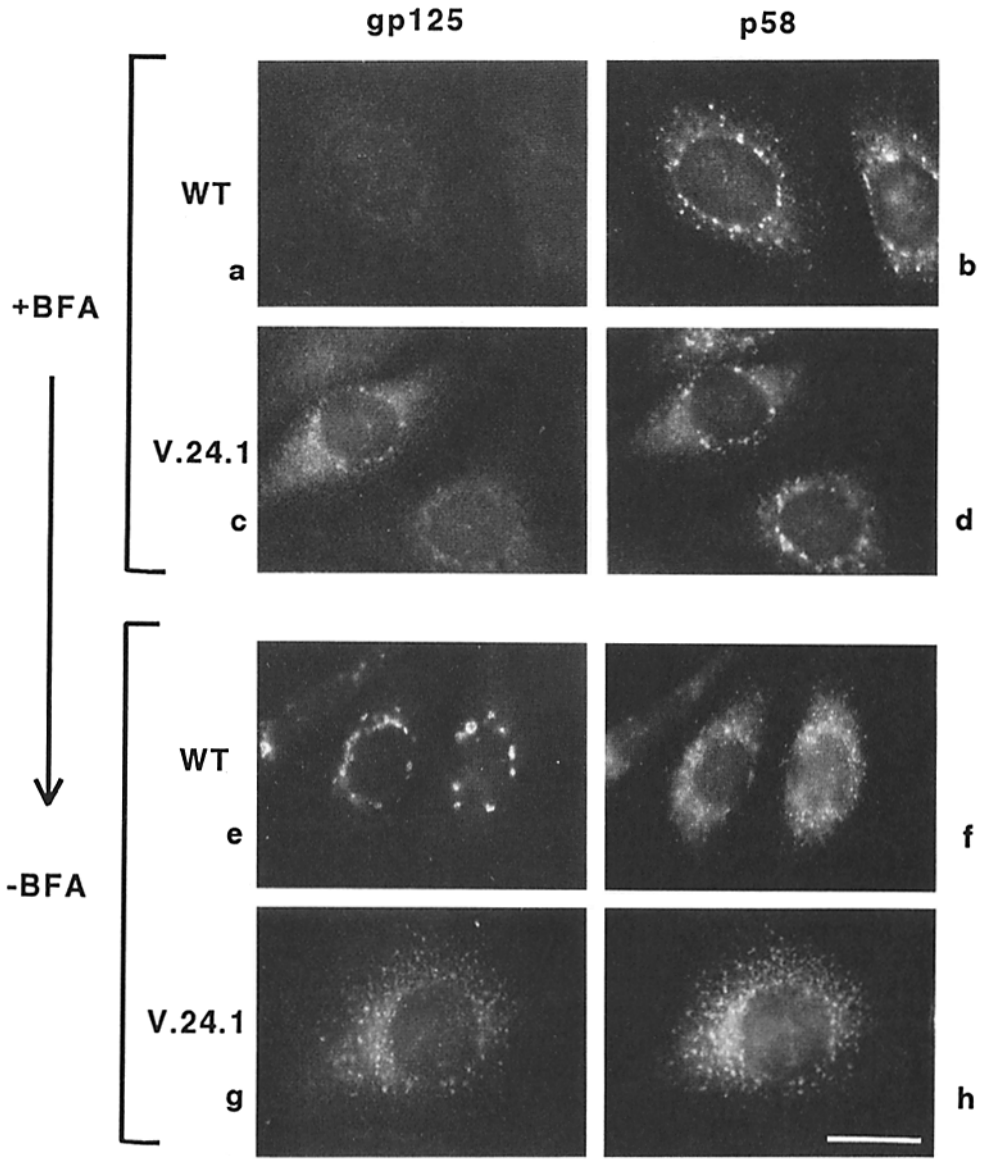

Figure 6. A Golgi protein induced to enter the ER by BFA accumulates in the intermediate compartment in V.24.1 cells upon removal of BFA at 41°C. Cells grown on coverslips were treated with 5 μg/ml BFA at the permissive temperature for 1 h and then incubated for 2 h in the presence of the drug at 41°C. BFA was then removed by extensive washing and the cells were either fixed immediately (*a–d*) or incubated for an additional 0.5 h at 41°C in the presence of cycloheximide before fixation (*e–h*). The cells were stained for double immunofluorescence using rat anti-gp125 and rabbit anti-p58 followed by FITC-conjugated donkey anti-rat IgG and Texas red–conjugated mouse anti-rabbit IgG.

of each cell type was fixed at this time. The remaining cells were washed to remove BFA and incubation at 41°C was continued for 0.5 h to allow transport of Golgi proteins in the ER back to the Golgi. Cells were then prepared for double immunofluorescence staining to simultaneously detect p58 and gp125, an integral membrane protein associated with the *cis*/medial-Golgi (Fig. 6). In the presence of BFA, a coherent perinuclear Golgi complex was not evident in either wild-type (Fig. 6 *a*) or mutant cells (Fig. 6 *c*) as expected. The intermediate compartment defined by p58 was present in both cell types in the presence of the drug (Fig. 6, *b* and *d*), consistent with observations by Lippincott-Schwartz et al. (25) that the intermediate compartment persists in the presence of BFA. When BFA was removed and the cells were incubated at the restrictive temperature for an additional half hour, the Golgi complex reassembled in the wild-type cells (Fig. 6 *e*) but not in the V.24.1 cells (Fig. 6 *g*). In the mutant cells, gp125 colocalized with p58 in discrete vesicular structures (compare Fig. 6, *g* and *h*), similar to the colocalization of HA with p58 in Fig. 5. This indicates that the failure to be transported beyond the p58-containing compartment is not specific to a viral glycoprotein, but also occurs with an endogenous Golgi protein, gp125.

Disappearance of the Golgi Complex in V.24.1 Cells at the Restrictive Temperature

To see what effect the lesion in the mutant cells had on the morphology of the Golgi complex, mutant and wild-type cells were incubated at 41°C for 2 h and were prepared for immunofluorescence microscopy to simultaneously observe gp125 and either ribophorin II (an ER resident protein) or p58. The morphology of the ER (compare Fig. 7, *b* and *d*) was similar in the mutant and wild-type cells, as was the morphology of the intermediate compartment (compare Fig. 7, *f* and *h*), suggesting that the structure of these two compartments was not severely affected by the lesion in the mutant cells. However, the Golgi complex, readily identified by

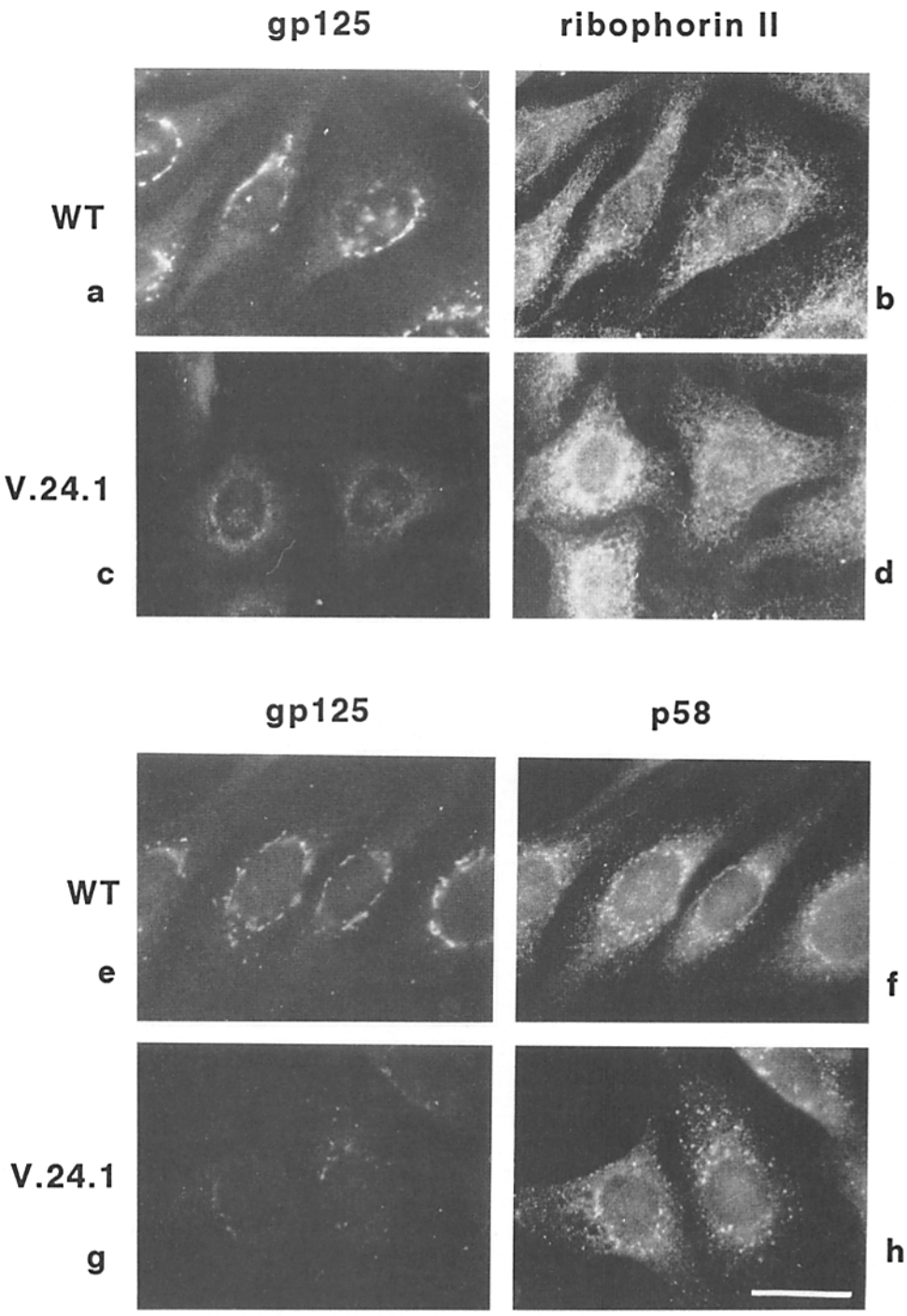

Figure 7. Localization of gp125, ribophorin II, and p58 in mutant and wild-type cells at the restrictive temperature. Cells grown on glass coverslips were incubated at 41°C for 2 h, fixed, permeabilized, and then stained for double immunofluorescence using antibodies as indicated. (*a–d*) Cells incubated with rat anti-gp125 and mouse antiribophorin II primary antibodies followed by FITC-labeled donkey anti-rat IgG and Texas red-labeled goat anti-mouse IgG as secondary antibodies. (*e–h*) Cells incubated with rat anti-gp125 and rabbit anti-p58 primary antibodies followed by FITC-labeled donkey anti-rat IgG and Texas red-labeled mouse anti-rabbit IgG as secondary antibodies.

staining for gp125 in the wild-type cells (Fig. 7, *a* and *e*), was hardly visible above the background in V.24.1 cells (Fig. 7, *c* and *g*). Within the resolution of fluorescence microscopy, it did not appear as if gp125 had left the Golgi and distributed to either the ER or the p58-containing compartment in the mutant cells.

To verify the apparent absence of the Golgi complex in V.24.1 cells, we used a different marker, mannosidase II (another Golgi-associated integral membrane protein) (25), and monitored the rate of the Golgi disappearance upon shifting cells to the restrictive temperature. As seen in Fig. 8 (*a* and *b*), the morphology of the wild-type Golgi complex stained for mannosidase II was similar to that when stained for gp125 in Fig. 7 (*a* and *e*). In addition, the Golgi in the wild-type cells looked similar at 34 and 41°C. There was also little difference between the Golgi in V.24.1 cells and wild-type cells at 34°C (Fig. 8, *a* and *c*). However, after 15 min at the restrictive temperature the Golgi complex in V.24.1 cells appeared to fragment into vesicles that with time became smaller and more dispersed throughout the cytoplasm until by 120 min there was little visible evidence of the Golgi (Fig. 8, *d–h*). The 58-kD protein did not colocalize in vesi-

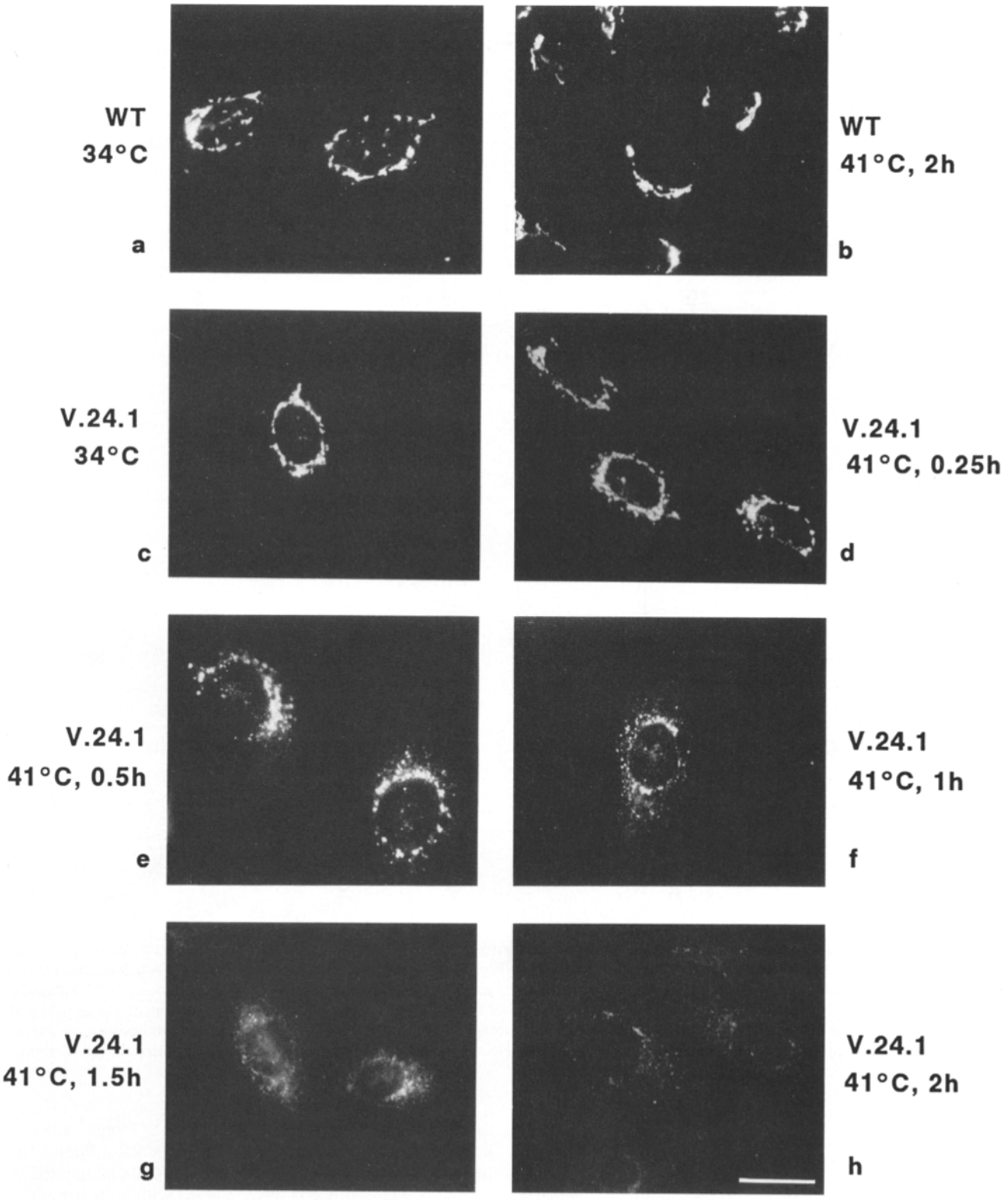

Figure 8. The rate of Golgi complex disappearance in V.24.1 cells at the restrictive temperature. Cells grown on glass coverslips were incubated at 41°C for the times indicated. The cells were then fixed, permeabilized, and stained for immunofluorescence using rabbit anti-mannosidase II followed by rhodamine-labeled goat anti-rabbit IgG.

cles with gp125 during the fragmentation process (data not shown), evidence that Golgi proteins did not accumulate in an intermediate compartment occupied by p58 as the fragmentation process progressed. The Golgi complex was also not visible by 2 h at 41°C when stained with the fluorescent sphingolipid C_6-NBD-ceramide (34), suggesting that both Golgi proteins and Golgi lipids dispersed in the mutant cells (data not shown).

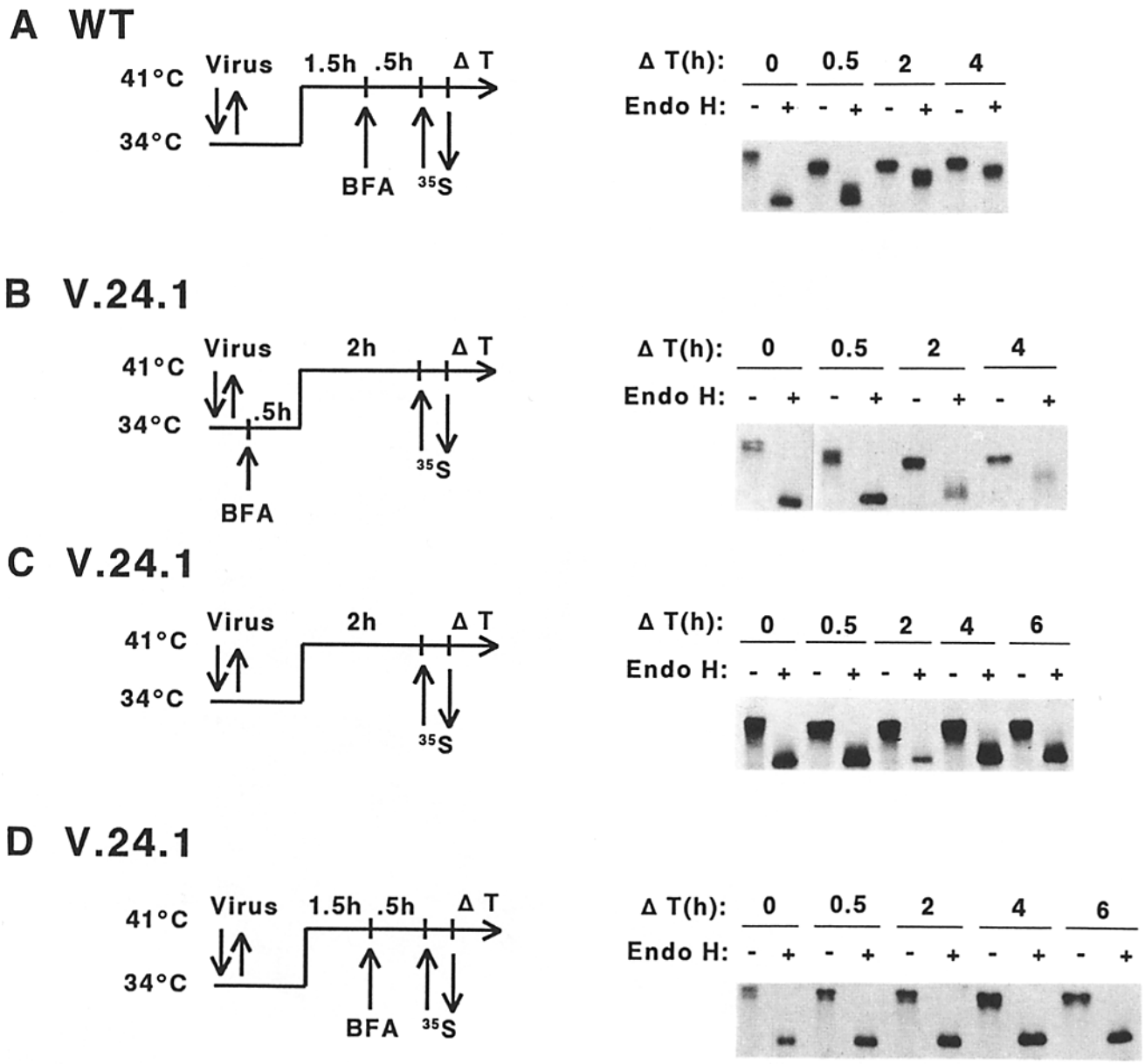

Figure 9. Golgi carbohydrate processing enzymes do not appear to return to the ER in V.24.1 cells at the restrictive temperature. Wild-type and mutant cells were infected with influenza virus at 34°C and incubated at 34°C for an additional 6 h to express HA before temperature shift (*a*, *c*, *d*) or the addition of BFA (*b*). BFA (final concentration 5 μg/ml) was added either at 41°C (*a* and *d*), or at 34°C before the shift to 41°C (*b*), or not at all (*c*). Cells were pulsed with Tran^{35}S-label for 5 min and chased for the times indicated. For *b*, 100 μM of cycloheximide was added with BFA and was kept in the medium throughout the experiment to stop new protein synthesis except during pulse labeling. HA was immunoprecipitated, digested with endo H, electrophoresed and detected as described in Materials and Methods.

Golgi Carbohydrate-processing Enzymes Fail to Process N-linked Oligosaccharide Chains of HA in the ER of V.24.1 Cells at High Temperature

The disappearance of the Golgi complex in the mutant cells is reminiscent of the loss of the Golgi in cells caused by BFA (12–14, 24, 25, 33). However, it did not appear that gp125 or mannosidase II were redistributing to the ER by immunofluorescence microscopy. Nevertheless, the two Golgi proteins may have been too dilute in the ER to detect. Therefore, we checked the return of Golgi carbohydrate processing enzymes to the ER by their ability to modify the N-linked oligosaccharides of HA within the ER. As one control, we measured the capacity of BFA to induce Golgi carbohydrate-processing enzymes to return to the ER at high temperature in wild-type cells. The procedure is summarized at the left of Fig. 9 *a*. Cells were infected with influenza virus at 34°C and incubated for an additional 6 h to express HA, placed at 41°C, treated with BFA, pulsed for 5 min with Tran^{35}S-label, and chased for different times. HA was immunoprecipitated and treated with endo H to see if carbohydrate processing of HA had occurred in the ER. As seen in the right portion of Fig. 9 *a*, by 4 h of the chase, almost all of the HA had been processed to the endo H–resistant form, demonstrating the validity of the assay. As a second control,

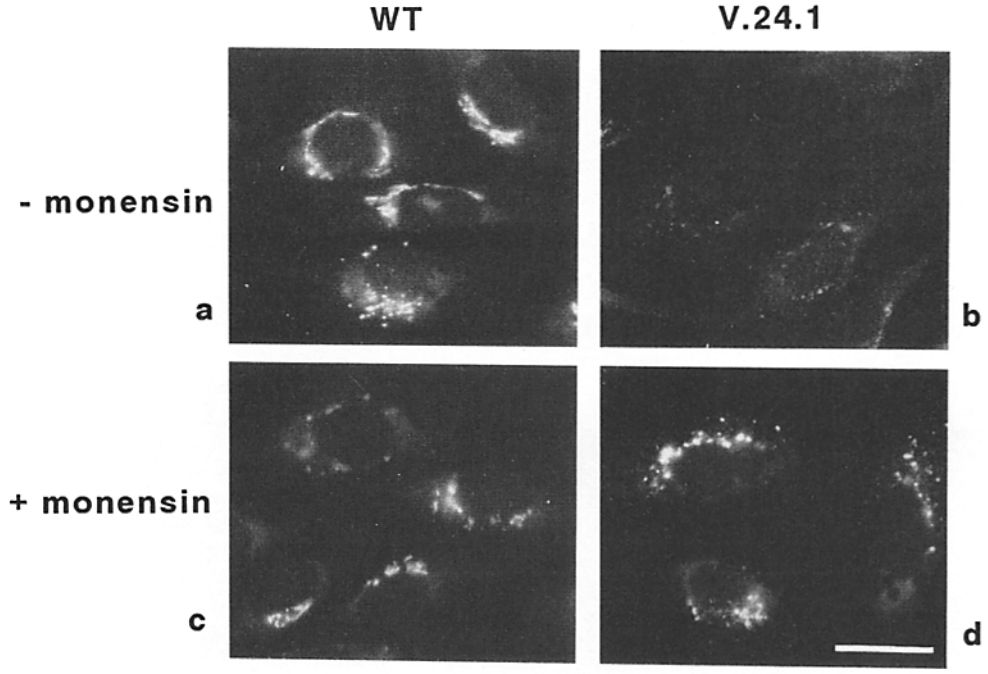

Figure 10. Monensin delays the disappearance of the Golgi complex in V.24.1 cells. Cells were incubated 2 h at 41°C in the absence (*a* and *b*) or the presence (*c* and *d*) of monensin (10 μM), fixed, permeabilized, and stained for gp125 as in Figs. 6 and 7. Cycloheximide was present in all media during 41°C incubation to block the synthesis of new gp125.

we added BFA to induce Golgi enzymes to return to the ER in V.24.1 cells at 34°C, and then raised the temperature and assessed the effects on the endo H sensitivity of HA (Fig. 9 *b*). Cycloheximide was added together with BFA and was present throughout the experiment except during pulse labeling to eliminate the possibility of HA being processed by newly synthesized Golgi carbohydrate processing enzymes retained in the ER by BFA treatment. HA became resistant to endo H by 4 h of chase, demonstrating that Golgi carbohydrate processing enzymes were not inactivated in the mutant cells simply by raising the temperature to 41°C. Next, we determined whether incubating V.24.1 cells at 41°C in the absence of BFA resulted in processing of HA to endo H resistance. As shown in Fig. 9 *c*, HA remained sensitive to endo H, even after 6 h at 41°C, strongly suggesting that the lesion in V.24.1 cells was not causing Golgi enzymes to enter the ER. Furthermore, there was no processing of HA oligosaccharides even when BFA was added to V.24.1 cells after they had been incubated at high temperature (Fig. 9 *d*). Altogether, this data indicates that the effects of the lesion on the Golgi complex in the mutant cells is not identical to the effects of BFA on the Golgi.

Monensin Retards the Disappearance of the Golgi Complex

Two different kinds of mechanism could underlie the apparent disappearance of the Golgi complex in the mutant cells. One possibility is that some aspect of membrane transport between Golgi cisternae participates in dissipating Golgi membrane. Alternatively, the Golgi might somehow dissipate without need for any specific membrane transport process. If transport between Golgi cisternae is necessary for the Golgi to disappear, then inhibiting transport should result in the persistence of the Golgi at the restrictive temperature. The proton ionophore monensin, which exchanges protons for sodium across membranes, inhibits transport between cisternae of the Golgi (18, 53). To see whether monensin had an effect on the disappearance of the Golgi complex, wild-type and V.24.1 cells were incubated at 41°C for 2 h without (Fig. 10, *a* and *b*) or with (Fig. 10, *c* and *d*) monensin and stained with antibody to gp125. The Golgi complex was still visible in mutant cells treated with monensin, but not in the control cells in the absence of the drug. This result suggests that whatever process underlies the disappearance of the Golgi complex, membrane transport between Golgi cisternae is involved. In further experiments we extended the incubation at 41°C in the presence of monensin up to 4 h and observed that, given the additional time, the Golgi complex did eventually disappear (data not shown). Thus, monensin delays, but does not completely stop, the disappearance of the Golgi complex in V.24.1 cells.

Another activity of monensin is to raise lysosomal pH, which blocks degradation of material within lysosomes (28). If Golgi membrane were aberrantly entering lysosomes in the mutant cells, then undegraded Golgi proteins might accumulate in lysosomes in the presence of monensin. However, we observed no accumulation of gp125 within lysosomes during prolonged treatment with the drug (data not shown). Treating cells with the lysosomotropic drug chloroquine, which also inhibits digestion in lysosomes (11), also did not result in visible accumulation of gp125 in lysosomes (data not shown).

The Disappearance of the Golgi Complex in V.24.1 Cells Is Not a Consequence of Microtubule Depolymerization

The depolymerization of microtubules in intact cells causes the Golgi complex to fragment and then disperse throughout the cytoplasm (35, 40, 41, 55). It was possible, therefore, that the fragmentation of the Golgi in the mutant cells could be

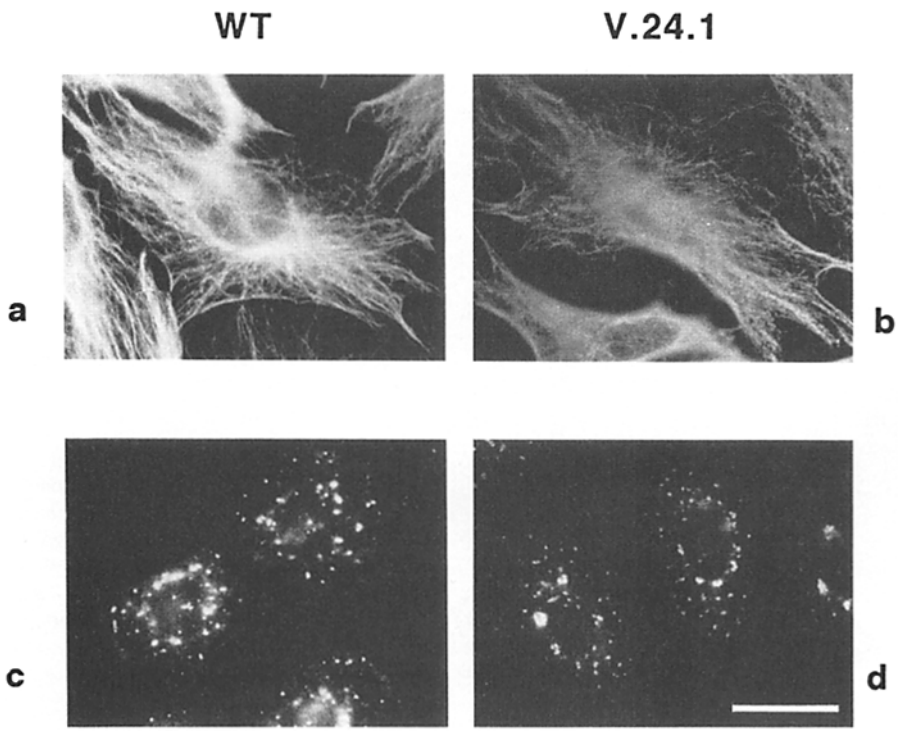

Figure 11. The role of microtubules in the disappearance of the Golgi complex in V.24.1 cells. In *a* and *b*, cells were incubated at 41°C for 2 h, fixed, and incubated with mouse antityrosinated tubulin followed by a secondary antibody of RITC-conjugated goat antimouse. In *c* and *d*, cells were incubated at 34°C with nocodazole (20 μg/ml) for 2 h and shifted to 41°C for an additional 2 h in the presence of nocodazole. The cells were stained to detect Golgi mannosidase II as in Fig. 8.

caused by a loss in the integrity of microtubules. To investigate the possibility that microtubules were depolymerized in V.24.1 cells at the nonpermissive temperature, mutant and wild-type cells were incubated at 41°C and stained for tubulin. The microtubular network looked the same in both cells (Fig. 11, *a* and *b*), evidence that the lesion in V.24.1 cells was not correlated with any gross depolymerization of microtubules. In addition, we compared the morphology of the Golgi complex in mutant and wild-type cells that were pretreated at 34°C with nocodazole to depolymerize microtubules, incubated at high temperature for 2 h with nocodazole, and then stained for mannosidase II (Fig. 11, *c* and *d*). In wild-type cells, the Golgi complex fragmented when microtubules were depolymerized, but the fragments were large and visible at all times. Interestingly, when microtubules were depolymerized in V.24.1 cells before incubation at the restrictive temperature for 2 h, the Golgi complex fragmented to the extent seen in wild-type cells with nocodazole, but did not disappear as when nocodazole was absent (compare Fig. 11, *d* and 8 *h*). Upon longer incubation at the restrictive temperature, the Golgi complex did eventually disappear (data not shown), analogous to results with monensin, indicating that nocodazole retarded, but did not completely inhibit, the loss of the Golgi. The disappearance of the Golgi complex in V.24.1 cells at high temperature appears to be fundamentally different than the fragmentation of the Golgi complex in cells that have depolymerized microtubules.

Discussion

V.24.1 cells define the End4 complementation group of temperature-sensitive CHO cell mutants selected for resistance to protein toxins (7). Previous studies indicated that the properties of mutant V.24.1 are consistent with the presence of a single and revertible genetic lesion that creates a temperature-sensitive protein (8). We recently reported that secretion in V.24.1 cells was blocked at a step before the medial Golgi (39, 56). In 1985, Nakano et al. (31) described a temperature-sensitive mutant of CHO cells, termed DS28-6, that had a similar secretion-defective phenotype. We have recently analyzed somatic cell hybrids made by fusing V.24.1 and DS28-6 cells and found that the hybrids have the mutant phenotype, indicating that the two mutants are in the same complementation group (Wang, R.-H., P. A. Colbaugh, and R. K. Draper, unpublished work). Another mutant, termed HRP-1, independently isolated in this lab using a new selection scheme, also failed to complement the lesion in V.24.1 and DS28-6 cells (Wang, R.-H., P. A. Colbaugh, and R. K. Draper, unpublished work). Thus, there are now at least three independently isolated members of the End4 complementation group.

A failure to secrete glycoproteins could be a consequence of aberrant folding or oligomerization in the ER, leading to retention of misfolded proteins. However, this does not explain the defect in End4 mutants, because HA folded and trimerized at the same rate in V.24.1 and wild-type cells at the restrictive temperature. The failure to secrete could also be related to unusual protein glycosylation, since Hearing et al. (19, 20) reported that addition of truncated N-linked oligosaccharides to HA impaired secretion in a temperature-sensitive mutant of CHO cells. However, in the present work, and in previous studies (39, 56), we have not observed any unusual electrophoretic migration of newly synthesized glycoproteins in SDS-polyacrylamide gels that would suggest aberrant or heterogeneous oligosaccharides were present on glycoproteins. There is no evidence that the failure of End4 mutants to secrete is related to structural or conformational abnormalities of secretory proteins.

The Intermediate Compartment

Morphological (43, 44, 49) and biochemical (3, 50, 54) evidence in recent years has supported the presence of a compartment intermediate between the ER and the *cis*-Golgi in mammalian cells. The intermediate compartment is proposed to function in the retrieval of resident ER proteins back to the ER (36, 37, 57), in O-glycosylation (54) and in phosphorylation of lysosomal enzymes (36, 37). Several lines of evidence support the idea that secretory material enters a compartment intermediate between the ER and the Golgi in V.24.1 cells at the restrictive temperature, but is not transported on to the Golgi apparatus. HA from mutant cells infected with influenza virus at the restrictive temperature did not become resistant to digestion by endo H or sensitive to digestion by endo D. Since all of the enzymes needed to convert N-linked oligosaccharides to either endo H resistance or endo D sensitivity are probably present to some extent in the *cis*-Golgi of CHO cells (47), this implies that the block in secretion is before the *cis*-Golgi, either in the ER or in a compartment intermediate between the *cis*-Golgi and the ER. Observation by immunofluorescence microscopy of HA in mutant cells showed that most of the HA was within vesicles that did not resemble the ER (or the Golgi), but were similar to the intermediate compartment others have observed by immunofluorescence microscopy (44, 48). The HA-containing vesicles also contained p58, a protein reported to reside in the intermediate compartment and the *cis*-Golgi (44, 45), consistent with the possibility that HA was in the intermediate compartment since it is unlikely that HA reached the *cis*-Golgi. Vesicles containing HA and p58 were not a collapsed form of the ER caused by the mutation in V.24.1 cells because the morphology of the ER was normal as assessed by staining for the ER proteins BiP and ribophorin II. A Golgi protein, gp125, that had been induced to return to the ER by addition of BFA at the permissive temperature, also accumulated in the p58-containing vesicles when BFA was removed at the restrictive temperature. This demonstrated that viral infection is not a factor in the accumulation of proteins between the ER and the Golgi.

The simplest interpretation of our observations is that secretion is blocked in V.24.1 cells at the level of export from the intermediate compartment defined by others. However, there are alternative possibilities. The compartment in which HA accumulates could represent a collection of transport vesicles en route from the ER to the intermediate compartment, or en route from the intermediate compartment to the Golgi. It is also possible that HA accumulates in a special subdomain of the ER, such as the transitional ER, especially if p58 were to collect in this subdomain in the mutant cells but not parental cells.

The intermediate compartment consists of vesicles and tubules that are frequently, although not always, very close to the *cis*-Golgi (43–45, 49). As a result, the relationship between the *cis*-Golgi and the intermediate compartment has been difficult to define. When cells are treated with nocodazole to depolymerize microtubules and disperse the Golgi, the intermediate compartment appears to go with the Golgi as if they were one organelle (5, 25, 44). However, in BFA-treated cells, the Golgi merges with the ER, but the intermediate compartment retains at least some structural identity (25, 44), suggesting the two compartments are different. To the extent that p58 marks the intermediate compartment in the mutant cells, the lesion in End4 mutants distinguishes between the Golgi and the intermediate compartment since the Golgi complex vanishes at the restrictive temperature while the p58-containing compartment persists.

Disappearance of the Golgi Complex

The Golgi complex could not be detected by immunofluorescence microscopy in V.24.1 cells after 2 h at 41°C when stained with anti-gp125, antimannosidase II, or C_6-NBD-ceramide. It should be emphasized that the Golgi complex disappears at the resolution of fluorescence microscopy: ultrastructural studies are in progress and it is possible that Golgi remnants may be identified at high resolution. The absence of the Golgi agrees with the recent report of Zuber et al. (59) that the Golgi complex disappeared at high temperature in mutant DS28-6, which is in the same complementation group as mutant V.24.1. Zuber et al. (59) further reported several aspects of the disappearing Golgi in DS28-6 cells that were similar to the effects of BFA on the Golgi. (*a*) The redistribution of the Golgi enzymes mannosidase II and galactosyl transferase to the ER by immunofluorescence microscopy. (*b*) The redistribution of a Golgi lipid marker to the ER by immunofluorescence microscopy. (*c*) The presence of complex carbohydrates in the ER detected with lectin-gold labeling, consistent with the possibility of Golgi-associated carbohydrate-processing enzymes being returned to the ER. Our data differ regarding the analogy between BFA and the lesion in End4 mutants. While we have no ultrastructural data to indicate where the Golgi is going, we did not observe a clear accumulation of Golgi protein or lipid in the ER by immunofluorescence. This could be explained if our experiments were not sensitive enough to detect Golgi markers diluted into the ER. However, we also did not biochemically detect processing of N-linked oligosaccharides of HA in the ER by Golgi enzymes.

One explanation to account for the disappearance of the Golgi is that Golgi components might be returning to the ER as in BFA-treated cells, but in a fashion that somehow does not result in the processing of N-linked oligosaccharides by Golgi enzymes in the ER. The complex carbohydrate within the ER observed by Zuber et al. (59) using lectin-gold cytochemistry could have come from preexisting glycoproteins transported from the Golgi to the ER rather than by processing of oligosaccharide chains in the ER. However, the possibility that Golgi components are not returning to the ER cannot be dismissed and we can think of three explanations that might account for this. First, Golgi membrane might fragment into vesicles too small to be detected by immunofluorescence microscopy if vesicles involved in transport between Golgi cisternae formed but did not bind to or fuse with their target membranes. Conceivably, the vesicles might require microtubules to disperse throughout the cytoplasm. This mechanism is similar to that proposed to explain the fragmentation of the Golgi during mitosis (15, 26, 27) and it is possible that the Golgi undergoes premature mitotic breakdown in the mutant cells. Second, the Golgi might disappear if secretory transport out of the Golgi were to continue while compensatory replacement of secreted membrane was blocked due to the lesion. Secretion would be delayed by monensin and nocodazole, accounting for the persistence of the Golgi in the presence of the drugs. If the secretion model were correct, we might have expected in our

immunofluorescence studies to have seen gp125 and mannosidase II in the plasma membrane, or possibly in vesicles of the endocytic apparatus, but we did not. However, the Golgi proteins might not have been detected if they were highly diluted in cell surface and endocytic membranes. Third, Golgi membrane might be degraded by a process that requires membrane transport and microtubules. Although we did not observe any accumulation of gp125 in lysosomes in the presence of monensin or chloroquine to inhibit lysosomal degradation, there may not have been enough gp125 in lysosomes to detect. Moreover, it is possible that degradation is by a nonlysosomal process. Further work is necessary to understand what happens to the Golgi in V.24.1 cells.

The Primary Lesion

Considering that high temperature causes both the disappearance of the Golgi complex and blocks transport to the Golgi, what is the primary lesion in the mutant cells? Does the Golgi disappear because transport is blocked, or does the Golgi complex first disappear, which somehow blocks secretory transport because there is no recipient Golgi? When cells were treated with BFA at 34°C to insure retraction of Golgi components into the ER, and then incubated at the restrictive temperature in the absence of BFA, the Golgi did not reform; instead, gp125 accumulated in the p58-containing compartment. This argues that the primary lesion has a direct effect on the export of secretory material from the p58-containing compartment. This does not exclude the possibility that a single lesion might block secretory transport at two steps, from the ER to the Golgi as well as transport between Golgi cisternae, and that Golgi membrane somehow disperses as a consequence. However, this idea is difficult to reconcile with the observation that monensin retarded disappearance of the Golgi, suggesting that transport between Golgi cisternae might be necessary for Golgi disappearance. A primary effect of the lesion on transport to the Golgi is also consistent with the observation that the block in ER to Golgi transport was complete within 60–90 min of elevating the temperature, a time at which the Golgi was still morphologically visible.

In summary, our current model is that End4 mutants contain a temperature-sensitive mutation affecting a protein necessary for transport of material between the ER and the Golgi, probably at the level of export from a compartment intermediate between the ER and the Golgi. The Golgi complex apparently disappears as a result of this block, but what happens to the Golgi is not yet clear. We are presently investigating the defect in End4 mutants using in vitro assays of membrane transport and morphological approaches.

We are grateful to Drs. R. Webster, N. T. Ktistakis, G. Bloom, M. Roth, T. August, J. Saraste, M.-J. Gething, T. E. Kreis, and D. Meyer for providing antibodies. We thank Drs. N. T. Ktistakis and M. Roth for their technical help in photography and helpful discussions and suggestions throughout the work. We also thank Drs. C. Mikoryak and P. Colbaugh for discussions and critically reading the manuscript.

This work was supported in part by grant GM34297 from the National Institutes of Health and by Research Partners Program grant 10689-072 from the State of Texas. This work is in partial fulfillment for the requirements for the Ph.D. Degree in Molecular and Cell Biology for C.-Y. Kao.

Received for publication 28 October 1991 and in revised form 30 January 1992.

References

1. Baker, D., L. Hicke, M. Rexach, M. Schleyer, and R. Schekman. 1988. Reconstitution of *SEC* gene product-dependent intercompartmental protein transport. *Cell.* 54:335–344.
2. Balch, W. E. 1989. Biochemistry of interorganelle transport. *J. Biol. Chem.* 264:16965–16968.
3. Beckers, C. J. M., and W. E. Balch. 1989. Calcium and GTP: essential components in vesicular trafficking between the endoplasmic reticulum and Golgi apparatus. *J. Cell Biol.* 108:1245–1256.
4. Beckers, C. J. M., D. S. Keller, and W. E. Balch. 1987. Semi-intact cells permeable to macromolecules: use in reconstitution of protein transport from the endoplasmic reticulum to the Golgi complex. *Cell.* 50:523–534.
5. Chavrier, P., R. G. Parton, H. P. Hauri, K. Simons, and M. Zerial. 1990. Localization of low molecular weight GTP binding proteins to exocytic and endocytic compartments. *Cell.* 62:317–329.
6. Clary, D. O., I. C. Griff, and J. E. Rothman. 1990. SNAPs, a family of NSF attachment proteins involved in intracellular membrane fusion in animals and yeast. *Cell.* 61:709–721.
7. Colbaugh, P. A., C.-Y. Kao, S.-P. Shia, M. Stookey, and R. K. Draper. 1988. Three new complementation groups of temperature-sensitive Chinese hamster ovary cell mutants defective in the endocytic pathway. *Somatic Cell Mol. Genet.* 14:499–507.
8. Colbaugh, P. A., M. Stookey, and R. K. Draper. 1989. Impaired lysosomes in a temperature-sensitive mutant of Chinese hamster ovary cells. *J. Cell Biol.* 108:2211–2219.
9. Copeland, C. S., R. W. Doms, E. M. Bolzau, R. G. Webster, and A. Helenius. 1986. Assembly of influenza hemagglutinin trimers and its role in intracellular transport. *J. Cell Biol.* 103:1179–1191.
10. Copeland, C. S., K. P. Zimmer, K. R. Wagner, G. A. Healey, I. Mellman, and A. Helenius. 1988. Folding, trimerization, and transport are sequential events in the biogenesis of influenza virus hemagglutinin. *Cell.* 53:197–209.
11. de Duve, C., T. de Barsey, B. Poole, A. Trouet, P. Tulkens, and F. van Hoff. 1974. Lysosomotropic agents. *Biochem. Pharmacol.* 23:2495–2531.
12. Donaldson, J. G., J. Lippincott-Schwartz, G. S. Bloom, T. E. Kreis, and R. D. Klausner. 1990. Dissociation of a 110-kD peripheral membrane protein from the Golgi apparatus is an early event in brefeldin A action. *J. Cell Biol.* 111:2295–2306.
13. Donaldson, J. G., J. Lippincott-Schwartz, and R. D. Klausner. 1991. Guanine nucleotides modulate the effects of brefeldin A in semipermeable cells: regulation of the association of a 110-kD peripheral membrane protein with the Golgi apparatus. *J. Cell Biol.* 112:579–588.
14. Duden, R., G. Griffiths, R. Frank, P. Argos, and T. E. Kreis. 1991. β-COP, a 110 kd protein associated with non-clathrin-coated vesicles and the Golgi complex, shows homology to β-adaptin. *Cell.* 64:649–665.
15. Featherstone, C., G. Griffiths, and G. Warren. 1985. Newly synthesized G protein of vesicular stomatitis virus is not transported to the Golgi complex in mitotic cells. *J. Cell Biol.* 101:2036–2046.
16. Gething, M. J., K. McCammon, and J. Sambrook. 1986. Expression of wild-type and mutant forms of influenza hemagglutinin: the role of folding in intracellular transport. *Cell.* 46:939–950.
17. Griffiths, G., and K. Simons. 1986. The trans Golgi network: sorting at the exit site of the Golgi complex. *Science (Wash. DC).* 234:438–443.
18. Griffiths, G., P. Quinn, and G. Warren. 1983. Dissection of the Golgi complex I: Monensin inhibits the transport of viral membrane proteins from *medial* to *trans* Golgi cisternae in baby hamster kidney cells infected with Semliki Forest virus. *J. Cell Biol.* 96:835–850.
19. Hearing, J., M. J. Gething, and J. Sambrook. 1989. Addition of truncated oligosaccharides to influenza virus hemagglutinin results in its temperature-conditional cell-surface expression. *J. Cell Biol.* 108:355–365.
20. Hearing J., E. Hunter, L. Rodgers, M. J. Gething, and J. Sambrook. 1989. Isolation of Chinese hamster ovary cell lines temperature conditional for the cell surface expression of integral membrane glycoproteins. *J. Cell Biol.* 108:339–353.
21. Hicke, L., and R. Schekman. 1990. Molecular machinery required for protein transport from the endoplasmic reticulum to the Golgi complex. *BioEssays.* 12:253–258.
22. Laemli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
23. Laskey, R. A., and A. D. Mills. 1975. Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. *Eur. J. Biochem.* 56: 335–341.
24. Lippincott-Schwartz, J., L. C. Yuan, J. S. Bonifacino, and R. D. Klausner. 1989. Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from the Golgi to ER. *Cell.* 56:801–813.
25. Lippincott-Schwartz, J., J. G. Donaldson, A. Schweizer, E. G. Berger, H. P. Hauri, L. C. Yuan, and R. D. Klausner. 1990. Microtubule-dependent retrograde transport of proteins into the ER in the presence of brefeldin A suggests an ER recycling pathway. *Cell.* 60:821–836.
26. Lucocq, J. M., and G. Warren. 1987. Fragmentation and partitioning of the Golgi apparatus during mitosis in HeLa cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:3239–3246.

27. Lucocq, J. M., J. G. Pryde, E. G. Berger, and G. Warren. 1987. A mitotic form of the Golgi apparatus in HeLa cells. *J. Cell Biol.* 104:865–874.
28. Mellman, I., R. Fuchs, and A. Helenius. 1986. Acidification of the endocytic and exocytic pathways. *Annu. Rev. Biochem.* 55:663–700.
29. Mizuochi, T., J. Amano, and A. Kobata. 1984. New evidence of the substrate specificity of endo-beta1-N-acetylglucosaminidase D. *J. Biochem. (Tokyo).* 95:1209–1213.
30. Moreman, K., and O. Touster. 1985. Biosynthesis and modification of Golgi mannosidase II in HeLa and 3T3 cells. *J. Biol. Chem.* 260:6654–6662.
31. Nakano, A., M. Nishijima, M. Maeda, and Y. Akamatsu. 1985. A temperature-sensitive Chinese hamster ovary cell mutant pleiotropically defective in protein export. *Biochem. Biophys. Acta.* 845:324–332.
32. Novick, P., C. Field, and R. Schekman. 1980. Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell.* 21:205–215.
33. Orci, L., M. Tagaya, M. Amherdt, A. Perrelet, J. G. Donaldson, J. Lippincott-Schwartz, R. D. Klausner, and J. E Rothman. 1991. Brefeldin A, a drug that blocks secretion, prevents the assembly of non-clathrin coated buds on Golgi cisternae. *Cell.* 64:1183–1195.
34. Pagano, R. E., M. A. Sepanski, and O. C. Martin. 1989. Molecular trapping of a fluorescent ceramide analogue at the Golgi apparatus of fixed cells: interaction with endogenous lipids provides a *trans*-Golgi marker for both light and electron microscopy. *J. Cell Biol.* 109:2067–2079.
35. Pavelka, M., and A. Ellinger. 1983. Effect of colchicine on the Golgi complex of rat pancreatic acinar cells. *J. Cell Biol.* 97:737–748.
36. Pelham, H. R. B. 1988. Evidence that luminal ER proteins are sorted from secreted proteins in a post-ER compartment. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:913–918.
37. Pelham, H. R. B. 1989. Control of protein exit from the endoplasmic reticulum. *Annu. Rev. Cell Biol.* 5:1–23.
38. Pfeffer, S. R., and J. E. Rothman. 1987. Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi. *Annu. Rev. Biochem.* 56:829–852.
39. Presley, J. F., R. K. Draper, and D. T. Brown. 1991. Defective transport of Sindbis virus glycoproteins in End4 mutant of Chinese hamster ovary cells. *J. Virol.* 65:1332–1339.
40. Robbins, E., and N. K. Gonatas. 1964. Histochemical and structural studies on HeLa cell cultures exposed to spindle inhibitors with special reference to the interphase cell. *J. Histochem. Cytochem.* 12:704–711.
41. Rogalski, A. A., and S. J. Singer. 1984. Association of elements of the Golgi apparatus with microtubules. *J. Cell Biol.* 99:1092–1100.
42. Ruohola, H., A. K. Kabcenell, and S. Ferro-Novick. 1988. Reconstitution of protein transport from the endoplasmic reticulum to the Golgi complex in yeast: the acceptor Golgi compartment is defective in the *sec*23 mutant. *J. Cell Biol.* 107:1465–1476.
43. Saraste, J., and E. Kuismanen. 1984. Pre- and post-Golgi vacuoles operate in the transport of Semliki Forest virus membrane glycoproteins to the cell surface. *Cell.* 38:535–549.
44. Saraste, J., and K. Svensson. 1991. Distribution of the intermediate elements operating in ER to Golgi transport. *J. Cell Sci.* 100:415–430.
45. Saraste, J., G. E. Palade, and M. G. Farquhar. 1987. Antibodies to rat pancreas. Golgi subfractions: identification of a 58 kD *cis* Golgi protein. *J. Cell Biol.* 105:2021–2030.
46. Schekman, R. 1985. Protein localization and membrane traffic in yeast. *Annu. Rev. Cell Biol.* 1:115–143.
47. Schwaninger, R., C. J. M. Beckers, and W. E. Balch. 1991. Sequential transport of protein between the endoplasmic reticulum and successive Golgi compartments in semi-intact cells. *J. Biol. Chem.* 266:13055–13063.
48. Schweizer, A., J. Fransen, T. Bachi, L. Ginsel, and H. Hauri. 1988. Identification, by a monoclonal antibody, of a 53 kD protein associated with a tubulo-vesicular compartment at the cis-side of the Golgi apparatus. *J. Cell Biol.* 107:1643–1653.
49. Schweizer, A., J. A. M. Fransen, K. Matter, T. E. Kreis, and L. Ginsel. 1990. Identification of an intermediate compartment involved in protein transport from endo-plasmic reticulum to Golgi apparatus. *Eur. J. Cell Biol.* 53:185–196.
50. Schweizer, A., K. Matter, C. M. Ketcham, and H.-P. Hauri. 1991. The isolated ER-Golgi intermediate compartment exhibits properties that are different from ER and *cis* Golgi. *J. Cell Biol.* 113:45–54.
51. Segev, N. 1991. Mediation of the attachment or fusion step in vesicular transport by the GTP-binding Ypt1 protein. *Science (Wash. DC).* 252: 1553–1556.
52. Tai, T., K. Yamashita, M. Ogata-Arakawa, N. Koide, T. Muramatsu, S. Iwashita, Y. Inoue, and A. Kobata. 1975. Structural studies of two ovalbumin glycopeptides in relation to the endo-beta1-N-acetylglucosaminidase specificity. *J. Biol. Chem.* 250:8569–8575.
53. Tartakoff, A. M. 1983. Perturbation of vesicular traffic with the carboxylic ionophore monensin. *Cell.* 32:1026–1028.
54. Tooze, S. A., J. Tooze, and G. Warren. 1988. Site of addition of N-acetylgalactosamine to the E1 glycoprotein of a mouse hepatitis virus-A59. *J. Cell Biol.* 106:1475–1487.
55. Turner, J. R., and A. M. Tartakoff. 1989. The response of the Golgi complex to microtubule alterations: the roles of metabolic energy and membrane traffic in Golgi complex organization. *J. Cell Biol.* 109:2081–2088.
56. Wang, R.-H., P. A. Colbaugh, C.-Y. Kao, E. A. Rutledge, and R. K. Draper. 1990. Impaired secretion and fluid-phase endocytosis in the End4 mutant of Chinese hamster ovary cells. *J. Biol. Chem.* 265:20179–20187.
57. Warren, G. 1987. Signals and salvage sequences. *Nature (Lond.).* 327: 17–18.
58. Wilson, D. W., C. A. Wilcox, G. C. Flynn, E. Chen, W.-J. Kuang, W. J. Henzel, M. R. Block, A. Ullrich, and J. E. Rothman. 1989. A fusion protein required for vesicle-mediated transport in both mammalian cells and yeast. *Nature (Lond.).* 339:355–359.
59. Zuber, C., J. Roth, T. Misteli, A. Nakano, and K. Moreman. 1991. DS28-6, a temperature-sensitive mutant of Chinese hamster ovary cells, expresses key phenotypic changes associated with brefeldin A treatment. *Proc. Natl. Acad. Sci. USA.* 88:9818–9822.