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Assembly and Polarized Release of Punta Toro Virus and Effects of Brefeldin A

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Punta Toro virus (PTV), a member of the sandfly fever group of bunyaviruses, is assembled by budding at intracellular membranes of the Golgi complex. We have examined PTV glycoprotein transport, assembly, and release and the effects of brefeldin A (BFA) on these processes. Both the G1 and G2 proteins were transported out of the endoplasmic reticulum (ER) and retained in the Golgi complex in a stable structure, either during PTV infection or when expressed from a vaccinia virus recombinant. BFA treatment causes a rapid and dramatic change in the distribution of the G1 and G2 proteins, from a Golgi pattern to an ER pattern. The G1 and G2 proteins were found to be modified by medial but not *trans* Golgi network enzymes, in the presence or absence of BFA. We found that BFA blocks PTV release from cells but does not interfere with the intracellular assembly of infectious virions. Further, the BFA block of virus release is fully reversible, with high levels of virus release occurring upon removal of the inhibitor. It was also found that the release of PTV virions is polarized, occurring exclusively from the basolateral surfaces of the polarized Vero C1008 epithelial cell line.

Membrane glycoproteins of enveloped viruses have been used as model systems to study intracellular transport and posttranslational modification of cellular glycoproteins. Most of these glycoproteins, such as the vesicular stomatitis virus (VSV) G protein and influenza virus hemagglutinin (HA) protein, are rapidly transported from their sites of synthesis, the rough endoplasmic reticulum (ER), to the plasma membrane via the Golgi complex (3, 9). However, enveloped viruses of some other families, i.e., bunyaviruses (35), coronaviruses (53), flaviviruses (24), and toroviruses (12), mature intracellularly rather than at the plasma membrane. Punta Toro virus (PTV), a member of the phlebovirus serogroup of bunyaviruses, consists of three major structural proteins, a lipid bilayer, and a tripartite, single-stranded, negative-sense RNA genome (4). Two structural proteins, G1 and G2, are glycoproteins located on the surface of the virion. The M RNA segment possesses a single open reading frame in the virus-complementary sequence that is presumed to code for a polyprotein precursor which is cleaved into the G1 and G2 glycoproteins, and probably a nonstructural (NS_M) protein (16, 47). PTV glycoproteins accumulate in the Golgi region, and virions are formed by budding at smooth-surfaced membranes in this region (48). Recently, it was shown that PTV glycoproteins expressed from a vaccinia virus recombinant are properly processed, transported from the ER to the Golgi complex, and retained there (32). Therefore, PTV glycoproteins appear to be a good system for studying the retention mechanism of membrane proteins in the Golgi complex.

The transport of membrane and secretory proteins is mediated by the central vacuolar system. Newly synthesized proteins are transported from the rough ER through the *cis*, medial, and *trans* Golgi cisternae to a complex organelle referred to as the *trans* Golgi network (18, 42). Each of these organelles also possesses characteristic resident membrane proteins that define its unique structure and function. The mechanisms by which the individual organelles along the exocytic pathway distinguish and sort the massive quantities of transitory proteins, while maintaining their distinctive properties, have not been clearly elucidated. It has been found that a number of soluble ER proteins possess the carboxy-terminal sequence KDEL, which is involved in their retention (34, 36, 37). It has been proposed that a KDEL receptor in an early Golgi compartment recognizes the KDEL sequence and recycles the protein to the ER. An alternative mechanism for determining intracellular retention does not involve a specific retention sequence. Studies with influenza virus HA and VSV G proteins have demonstrated that correct folding and assembly into oligomers is required for transport from the ER to the Golgi complex (11, 15, 20). Failure to attain the proper three-dimensional structure would thus result in retention by default.

Brefeldin A (BFA) is a fungal metabolite with a 13-membered macrocyclic lactone ring, which has been reported to inhibit the transport of proteins out of the ER (13, 31, 33, 50). Recently, Lippincott-Schwartz et al. (26, 27) reported a rapid and dramatic redistribution to the ER of resident proteins of the Golgi complex in response to treatment with BFA. In this study, we examined the effects of BFA on PTV glycoprotein transport, assembly, and release. We also investigated the release of PTV from polarized epithelial cells grown on permeable supports and the effects of BFA on this release process.

MATERIALS AND METHODS

Chemical and antibodies. BFA was a gift from H. Stahelin and J. Brunner (Sandoz Ltd., Basel, Switzerland), and was kept as a 10-mg/ml stock solution at −20°C in methanol. Hyperimmune mouse ascitic fluids and monoclonal antibodies to PTV G1 and G2 glycoproteins were kindly provided by J. F. Smith and D. Pifat (USAMRIID, Fort Detrick, Md.). Monoclonal antibody to immunoglobulin heavy-chain binding protein (BiP) was kindly supplied by L. M. Hendershot (5). Polyclonal antibodies to VSV G glycoprotein were prepared as described previously (45). Endoglycosidase H (endo H) and neuraminidase were purchased from Boehringer Mannheim (Indianapolis, Ind.). Cycloheximide was

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from Sigma (St. Louis, Mo.). [^{35}S]methionine was purchased from New England Nuclear (Wilmington, Del.). [^{3}H]inulin was purchased from Amersham Corp. (Arlington Heights, Ill.).

Viruses and cells. The vaccinia virus recombinant (VV-G) containing the PTV M genomic cDNA sequences coding for the G1 and G2 glycoproteins was described previously (32). PTV was obtained from USAMRIID and passaged in Vero cells. The stock of PTV had a titer of 10^8 PFU/ml. A vaccinia virus recombinant expressing the VSV (Indiana strain) G glycoprotein was obtained from B. Moss (29). Vero C1008 cells were obtained from the American Type Culture Collection, Rockville, Md. These cells were previously shown to form tight epithelial monolayers in which maturation of enveloped viruses and expression of virus glycoproteins as well as release of simian virus 40 were found to be polarized (8, 49). Vero, Vero C1008, and HeLa T4$^+$ cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn bovine serum.

Plaque Assay. PTV infectivity titers were measured by plaque assays on confluent monolayer of Vero cells in six-well plates. The monolayers were washed twice with phosphate-buffered saline, pH 7.2 (PBS), and inoculated with 0.2 ml of serial dilutions of PTV. After an adsorption period of 2 h at 37°C, the cells were washed once with PBS, and 3 ml of overlay medium (0.6% agarose in 5% serum DMEM) was added. Plaques started to appear on day 3 postinfection (p.i.). At 4 days p.i., cells were stained by the addition of a second overlay containing 0.01% neutral red in the overlay medium and incubated at 37°C; plaques were counted the next day.

Virus release curves. Vero C1008 cell monolayers were grown to confluence on 24.5-mm-diameter, 0.40-μm-pore-size Costar-Transwell filters (Costar Corp., Cambridge, Mass.) placed in six-well plates. PTV was added to the apical and basal chambers at a multiplicity of infection (MOI) of 10 and allowed to absorb for 1 h. The inoculum was removed, and the monolayers were rinsed with PBS three times prior to addition of 2.0 ml of DMEM supplemented with 2% fetal bovine serum to both apical and basal chambers. At intervals postinfection, the apical and basal media were collected separately, clarified by centrifugation, and frozen at −70°C. The virus titers in each sample were determined by plaque assay.

Labeling of cells and analysis of viral proteins. HeLa T4$^+$ cells were infected with vaccinia virus recombinants at an MOI of 10. Cells were washed once with PBS at 16 h p.i. and incubated in methionine-free medium for 3 h. Cells were then labeled with [^{35}S]methionine (100 μCi/ml) in methionine-free medium for 15 min and chased in Eagle's medium supplemented with 10 mM methionine for the indicated periods. Cells were washed three times in ice-cold PBS and lysed with 0.3 ml of cell lysis buffer (50 mM Tris-HCl [pH 7.5], 0.15 M NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 20 mM EDTA). Nuclei were removed by centrifugation at 13,000 × *g* for 5 min at 4°C. Antibodies were incubated with samples for 90 min at 37°C or overnight at 4°C with protein A-Sepharose CL-4B (Pharmacia Inc., Piscataway, N.J.). The precipitates were pelleted, washed three times with cold lysis buffer, and resuspended in Laemmli sample buffer with β-mercaptoethanol for SDS-polyacrylamide gel electrophoresis (PAGE) (23).

Endo H and neuraminidase digestion. For endo H digestion, immunoprecipitated samples were resuspended in 200 μl of 0.1 M sodium acetate (pH 5.5) and then divided into two equal aliquots. One aliquot was incubated with 8 mU of endo H per ml for 16 h at 37°C. Samples were centrifuged at 15,000 × *g* for 5 min; the precipitate was resuspended in Laemmli sample buffer and boiled for 5 min before the Sepharose CL-4B beads were spun down and samples were loaded for SDS-PAGE. For neuraminidase digestion, the precipitate was resuspended in 300 μl of 0.1 M sodium acetate (pH 6.0), and half aliquots were digested with 20 mU of neuraminidase for 2 h at 37°C. Samples then were pelleted, resuspended in Laemmli sample buffer, boiled for 5 min, centrifuged at 15,000 × *g* for 5 min, and analyzed by SDS-PAGE.

Indirect immunofluorescence. HeLa T4$^+$ cells or Vero cells grown on glass coverslips were infected with vaccinia virus, vaccinia virus recombinant or PTV at an MOI of 10. At indicated times postinfection, cells were washed with PBS and fixed with ethanol containing 5% acetic acid for 20 min at −20°C for intracellular staining or with 1% formaldehyde for 5 min at room temperature for surface staining. Cells were then washed with PBS and reacted with monoclonal or polyclonal antibodies for 30 min at 37°C, followed by a fluorescein- or rhodamine-conjugated goat anti-mouse immunoglobulin G (IgG). After a final washing, the cells were mounted and observed with a Nikon Optiphot microscope equipped with a modified B2 tube.

Electron microscopy. Cell monolayers grown in plastic dishes were infected with PTV at an MOI of 15 for 60 min, washed with PBS, and then incubated in the DMEM supplemented with 5% serum at 37°C with 5% CO_2. BFA at a final concentration of 10 μg/ml was added into the culture medium at 4-h intervals. The cell monolayers were fixed at 18 h p.i. and then embedded as described previously (46).

RESULTS

Effects of BFA on transport of viral G1 and G2 glycoproteins. Since BFA was reported to cause components in the Golgi cisternae to redistribute to the ER (10, 26, 27), we examined the intracellular location of the G1 and G2 proteins by indirect immunofluorescence after BFA treatment. At 20 h p.i., the G1 and G2 proteins were found to be distributed throughout the cytoplasm (Fig. 1a). When cycloheximide was added at a final concentration of 50 μg/ml and incubation continued, the immunofluorescence staining patterns gradually changed to a well-defined perinuclear Golgi pattern, indicating accumulation of the PTV glycoproteins in the Golgi complex (Fig. 1b and c), as described previously (32). A low level of immunofluorescence was detected on the cell surface (data not shown). Upon addition of BFA to cells that had been treated with cycloheximide, the immunofluorescence staining pattern changed dramatically, from a Golgi pattern into a dispersed ER pattern, within 10 min (Fig. 1d). To further define the BFA-induced redistribution of the G1 and G2 proteins, the immunofluorescence staining pattern was compared with that of a cellular resident ER protein, BiP. Prior to BFA treatment, BiP-specific immunofluorescence was observed dispersed throughout the cytoplasm in the presence or absence of cycloheximide (Fig. 1e). After BFA treatment, BiP-specific immunofluorescence was still observed throughout the cytoplasm, in a pattern similar to that of the G1 and G2 proteins (Fig. 1f). After removal of BFA, PTV G1 and G2 proteins almost completely redistributed from the diffuse reticular pattern to a perinuclear localization within 2 h (not shown). After 3 h after removal of BFA, the pattern of G1 and G2 protein localization was indistinguishable from that observed in PTV-infected cells treated with cycloheximide. These results indicate that the

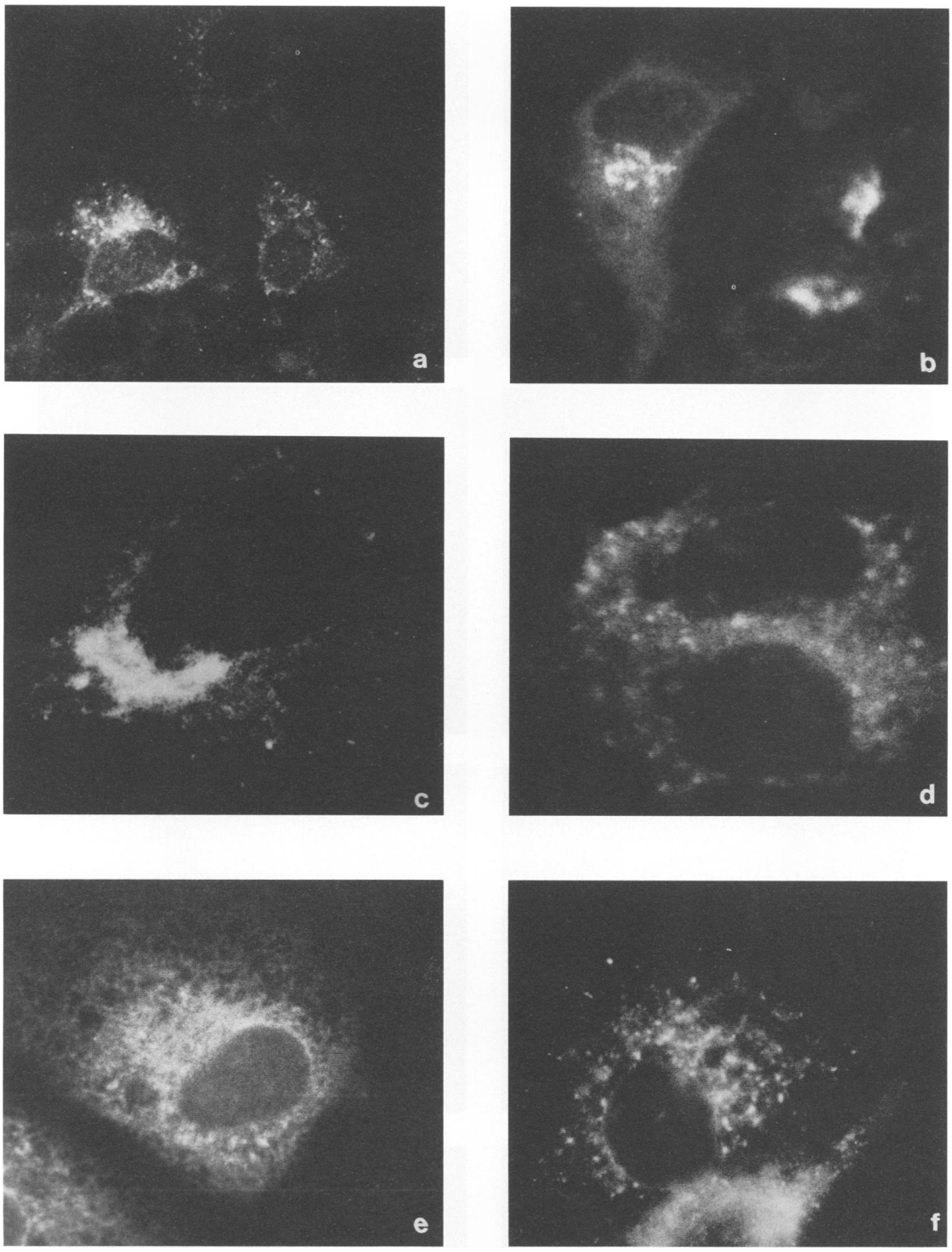
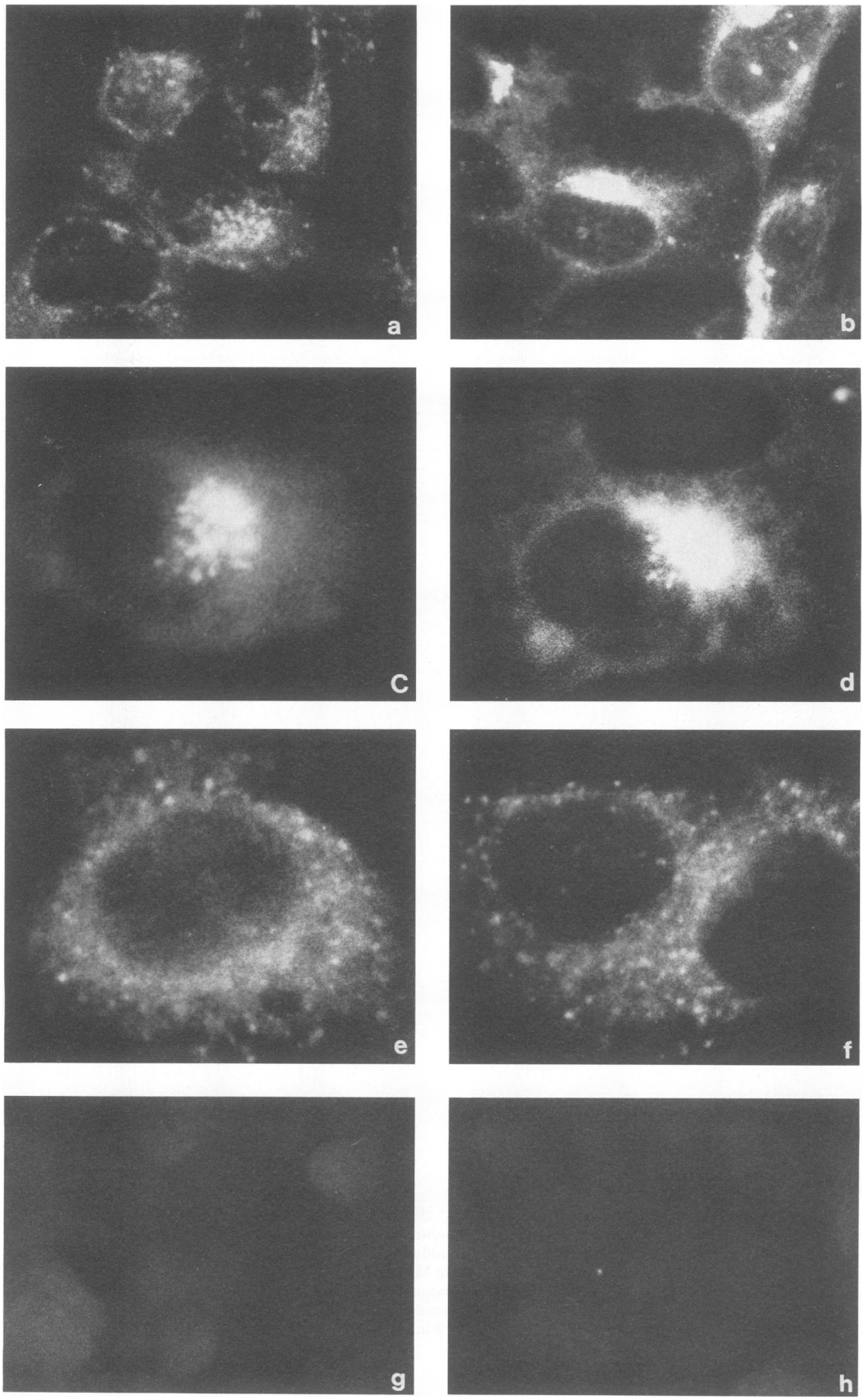


FIG. 1. Intracellular localization of G1 and G2 glycoproteins and effects of BFA on their distribution during PTV infection. Vero cells grown on coverslips were infected with PTV at an MOI of 10 and incubated for 20 h, after which cells were either fixed or incubated with cycloheximide (50 μg/ml) at 37°C for 2 h. BFA (10 μg/ml) was then added to some cultures for 20 min prior to fixation. The cells were fixed and prepared for indirect immunofluorescence staining as described in Materials and Methods. Cells were stained with mixed anti-G1 and G2 or with anti-BiP monoclonal antibodies. After a wash to remove unbound IgG, cells were incubated with rhodamine- or fluorescein-labeled goat anti-mouse IgG. (a) G1 and G2 glycoproteins without cycloheximide treatment; (b and c) G1 and G2 glycoproteins after cycloheximide treatment; (d) G1 and G2 glycoproteins after BFA treatment; (e) distribution of BiP after cycloheximide treatment; (f) distribution of BiP after BFA treatment.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G1 and G2 proteins synthesized during PTV infection are reversibly redistributed from a perinuclear location into a pattern closely resembling that of a resident ER protein in BFA-treated cells.

Effects of BFA on transport of recombinant G1 and G2 glycoproteins. To determine whether the transport characteristics of G1 and G2 proteins might depend on other proteins produced during PTV infection, we examined the localization and effects of BFA on the distribution of G1 and G2 proteins expressed from a vaccinia virus recombinant designated VV-G. Since HeLa $T4^+$ cells are relatively resistant to the cytopathic effects of vaccinia virus infection (32), these cells were used to study the transport of PTV proteins expressed from the vaccinia virus recombinant. HeLa $T4^+$ cells grown on coverslips were infected with the VV-G recombinant, fixed at 16 h p.i., and examined by indirect immunofluorescence. The G1 and G2 proteins were found to be distributed throughout the cytoplasm at that time (Fig. 2a). When cycloheximide was added at a final concentration of 50 μg/ml and incubation continued, the cells revealed increasingly intense labeling of both G1 and G2 proteins in the perinuclear region characteristic of the Golgi organelle; even after 8 h of treatment, an intense typical Golgi staining pattern was observed (Fig. 2b to d). Neither G1 nor G2 protein was found on the cell surface by cell surface immunofluorescence (Fig. 2g and h) or surface immunoprecipitation (data not shown) during this period. Addition of BFA to the culture medium of HeLa $T4^+$ cells that had been treated with cycloheximide for 8 h caused a dramatic change in the overall cellular distribution of G1 and G2 proteins. Immunofluorescence revealed that the G1 and G2 protein distribution changed from a predominant Golgi-like pattern to a fine, reticular ER labeling pattern throughout the cytoplasm upon BFA treatment (Fig. 2e and f). This change in distribution occurred very rapidly. Within 5 min of BFA treatment, most of the G1 and G2 proteins had redistributed from a perinuclear Golgi staining pattern into a reticular ER pattern. After 20 min of BFA treatment, no further change was observed. Thus, the G1 and G2 proteins expressed from VV-G are transported out of the ER, retained in the Golgi region, and redistributed by BFA similarly to the proteins synthesized in PTV-infected cells.

To further define the intracellular localization of PTV proteins, double immunofluorescence staining with rhodamine-conjugated wheat germ agglutinin (WGA) was carried out to compare the change in distribution of G1 and G2 proteins upon BFA treatment. A specific labeling of cisternae along the *trans* Golgi stack and associated vesicles may be achieved by using rhodamine-WGA, following preincubation of intact cells with unlabeled WGA to block plasma membrane staining (54), since WGA binds to clustered terminal *N*-acetylneuraminic acid residues as well as *N*-acetylglucosamine-containing oligosaccharide chains (51). Little change in the staining pattern for WGA occurred upon BFA treatment in VV-G-infected HeLa $T4^+$ cells (data not shown), as also was reported by Lippincott-Schwartz et al. (26) for normal rat kidney cells. In contrast, G1 and G2 proteins were dramatically redistributed from a Golgi pattern to an ER pattern in the same cells (data not shown). Thus, G1 and G2 proteins expressed by the vaccinia virus recombinant differ in localization from that of either *trans* Golgi network or ER proteins, indicating that they are localized in an intermediate compartment, which presumably represents the *cis* and medial cisternae of the Golgi complex.

Modification of G1 and G2 glycoprotein oligosaccharide chains and the effects of BFA. To determine the transport kinetics of G1 and G2 proteins expressed from the VV-G recombinant, we monitored their acquisition of endo H resistance. Both G1 and G2 proteins became endo H resistant at a slow rate (Fig. 3A). Although both proteins became completely endo H resistant at roughly the same time (180 min), G2 showed several intermediate forms after endo H digestion in the 120-min chase sample, suggesting that it is heterogeneously glycosylated. The G1 protein appeared as only two bands, which were endo H resistant or sensitive. Neuraminidase treatment was used to determine whether the G1 and G2 proteins were transported to the *trans*-Golgi network and modified by sialyltransferase, which is localized in the *trans* Golgi cisternae or *trans* Golgi network (19, 44). HeLa $T4^+$ cells infected with VV-G recombinants were labeled for 15 min with [^{35}S]methionine and chased in the presence of excess unlabeled methionine for various periods of time. A recombinant expressing the VSV G protein, which is known to be transported to the cell surface and modified by sialic acid addition, served as a control. No change in electrophoretic mobility of G1 and G2 proteins due to the addition of sialic acid was seen during the chase period, and no down-shift in molecular weight was found after neuraminidase digestion, whereas the VSV G protein showed an obvious increase in mobility after neuraminidase digestion (Fig. 3B). Thus, the G1 and G2 proteins are not modified by sialyltransferase. These results also support the conclusion that the G1 and G2 proteins are not transported to the *trans* Golgi network but are predominantly retained in the *cis* and medial Golgi cisternae.

Lippincott-Schwartz et al. (26, 27) and Doms et al. (10) recently reported that mannosidase II was redistributed from the medial Golgi cisternae to the ER in BFA-treated cells. To investigate the possibility of such a redistribution, we examined the oligosaccharide chains of the PTV G1 and G2 glycoproteins in BFA-treated cells. A fraction of G1 and G2 proteins become endo H resistant at 120 min, and at 180 min most protein molecules were endo H resistant (Fig. 4). Addition of sialic acid to PTV G1 and G2 proteins was not observed, even after 180 min of chase (data not shown). Thus, in contrast to the results of immunofluorescence, biochemical analysis of the oligosaccharide side chains of G1 and G2 in BFA-treated cells indicates that they are processed by exposure to the medial Golgi enzymes. These results are consistent with those of previous reports (10, 26, 27) which indicate that Golgi enzymes are redistributed to the ER upon BFA treatment. The *trans* Golgi network,

FIG. 2. Intracellular localization of G1 and G2 glycoproteins expressed from vaccinia virus recombinant VV-G and effects of BFA on their distribution. HeLa $T4^+$ cells grown on coverslips were infected with VV-G at an MOI of 10 and incubated for 16 h, after which cells were either fixed or incubated with cycloheximide (50 μg/ml) at 37°C for 8 h prior to fixation. BFA (10 μg/ml) was then added to the culture medium for 20 min. Cells were fixed and prepared for indirect immunofluorescence staining as described in Materials and Methods. (a) G1 and G2 glycoproteins without cycloheximide treatment; (b) G1 and G2 glycoproteins after cycloheximide treatment; (c) G1 glycoprotein after cycloheximide treatment; (d) G2 glycoprotein after cycloheximide treatment; (e) G1 glycoprotein after BFA treatment; (f) G2 glycoprotein after BFA treatment; (g) Cell surface staining for G1 glycoprotein after cycloheximide treatment; (h) Cell surface staining for G2 glycoprotein after cycloheximide treatment.

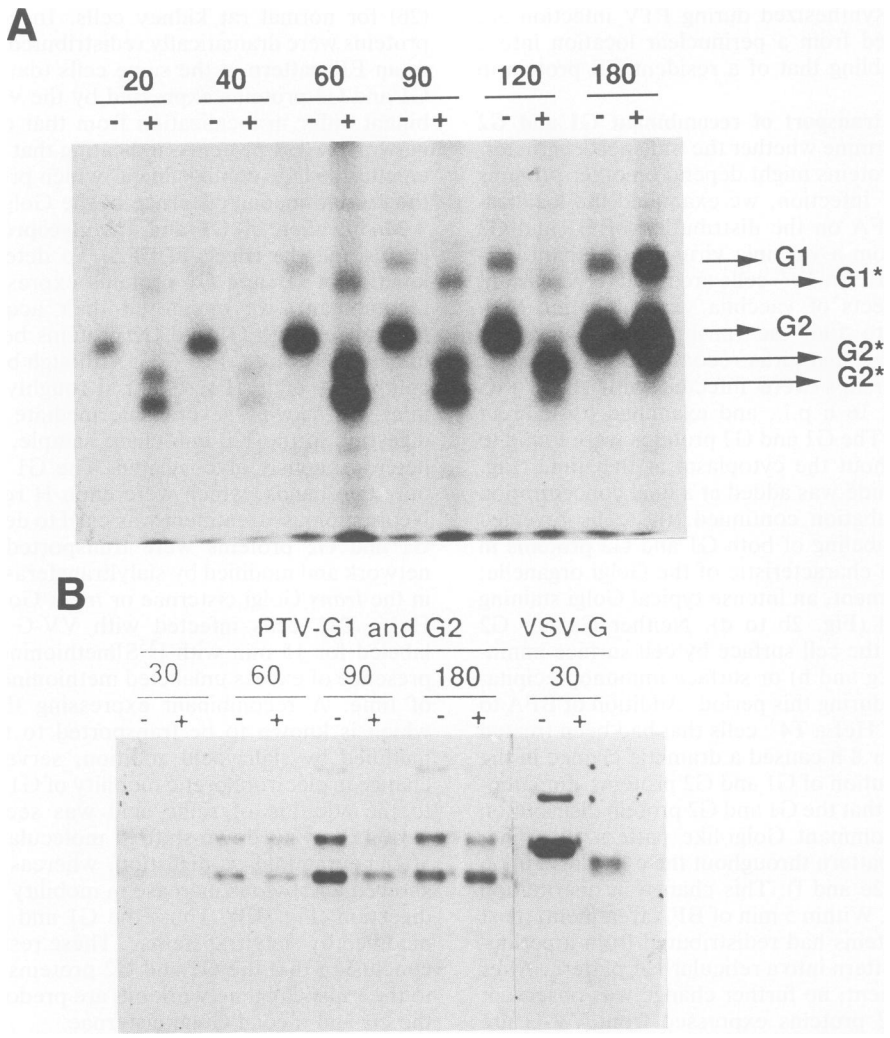

FIG. 3. Endo H and neuraminidase digestion of G1 and G2 proteins. HeLa T4$^+$ cells were infected with recombinant VV-G at an MOI of 10, incubated for 16 h, and labeled with [^{35}S]methionine for 15 min at 37°C. The cells were then chased in the presence of excess methionine for 20, 40, 60, 90, 120, or 180 min for endo H digestion and 30, 60, 90, or 180 min for neuraminidase digestion. The cells were lysed and immunoprecipitated with polyclonal antibodies to G1 and G2 proteins. The samples were incubated with (+) or without (−) 8 mU of endo H at 37°C for 16 h (A) or with 20 mU of neuraminidase (+) or without neuraminidase (−) at 37°C for 2 h (B) and then analyzed by SDS-PAGE. G1* and G2* indicate the endo H-sensitive forms of the glycoproteins.

which is an important branchpoint of protein sorting, seems to be excluded from this redistribution process.

BFA blocks PTV release but not virus assembly. To investigate the effects of BFA on virus assembly and release, we first measured the effects of various concentrations of BFA on the yields of released and cell-associated PTV in Vero cells. Extracellular virus was nearly undetectable at BFA concentrations of 5 or 10 μg/ml (Fig. 5A). In contrast, titers of cell-associated virus of about 5×10^6 PFU/ml were only slightly lower than those of infected cells assayed without BFA treatment. At a BFA concentration of 2.5 μg/ml virus release was also much lower than in control cells. Thus, BFA is able to block virus release from cells. We further observed the time course of the yields of released and cell-associated viruses during BFA treatment. Infected cells were treated with 10 μg of BFA per ml at 4-h intervals, and virus yields were determined at selected intervals. Extracellular virus was nearly undetectable from 6 to 22 h p.i. during BFA treatment (Fig. 6B), whereas cell-associated virus titers were only slightly lower than those of infected cells assayed without BFA treatment (Fig. 6A). We also observed that viral protein synthesis was not inhibited in infected cells treated with BFA at 2.5, 5, or 10 μg/ml (data not shown). Thus, BFA completely blocks PTV release from cells but does not prevent intracellular viral assembly or viral protein synthesis. After the medium was replaced with BFA-free medium, cell-associated virus began to be released into the medium, and high levels of released virus were found by 4 h after removal of BFA (Fig. 6C). We also found that the block of virus release was gradually lost if BFA was not added to cells repeatedly (data not shown). At a BFA concentration of 5 μg/ml, virus began to release after 4 h of treatment; at a BFA concentration of 10 μg/ml, virus was released after 8 h of treatment. After 16 h of treatment with 10 μg of BFA per ml, yields of released virus reached the same level as that of untreated cells. Reappearance of the Golgi structure was

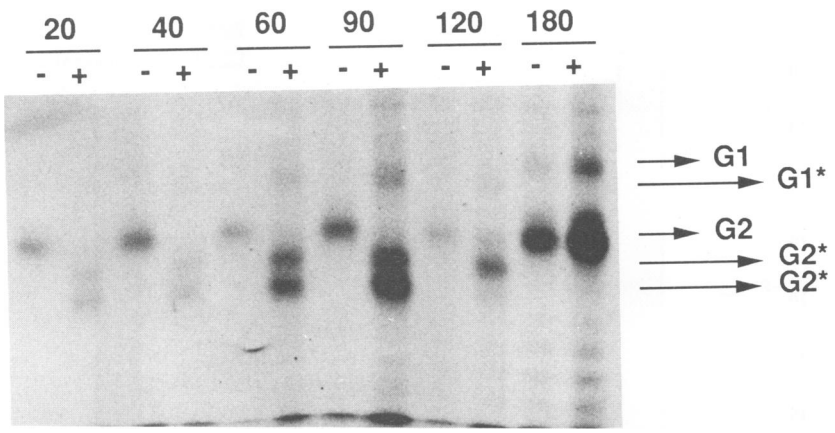

FIG. 4. Endo H digestion of G1 and G2 proteins synthesized during BFA treatment. HeLa T4$^+$ cells were infected with recombinant VV-G at an MOI of 10, incubated for 16 h, treated with BFA (10 μg/ml), and labeled with [^{35}S]methionine for 15 min at 37°C. The cells were then chased in the presence of excess methionine for 20, 40, 60, 90, 120, or 180 min in the presence of BFA. The cells were lysed and immunoprecipitated with polyclonal antibodies to G1 and G2 proteins. The samples were incubated with (+) or without (−) 8 mU of endo H at 37°C for 16 h and then analyzed by SDS-PAGE. Arrows to G2* indicate that the bulk of G2 is heterogeneously glycosylated.

also observed after 8 h of treatment by electron microscopy (data not shown). These results indicate that BFA is metabolized by cells and that repeated addition of BFA is needed to maintain its effects.

We further compared the effect of BFA with that of monensin, another inhibitor of vesicular transport (1), on PTV assembly and release. It was observed that monensin decreased the yields of both cell-associated and released virus as the concentration was increased (Fig. 5B). Thus, the effect of BFA on virus assembly and release is different from that of monensin.

The site of virus assembly in BFA-treated cells was examined by electron microscopy (Fig. 7). In the absence of BFA (Fig. 7A), virus particles were found in the lumen of smooth-surfaced intracytoplasmic membranes in the Golgi region, as reported previously (48). In BFA-treated cells (Fig. 7B), marked dilation of the rough ER was evident, and particles of similar morphology were localized in distended vesicles which may represent fused Golgi-ER vesicles. The particles seen in Fig. 7B are associated with a surrounding layer of amorphous electron-dense material that was not observed in untreated cells.

Polarized release of PTV from epithelial cells. Epithelia, which serve as selective barriers, are composed of cells with two distinct surface domains, designated apical and basolateral, that are separated by a continuous belt of tight junctions, the zonula occludens. These two surface domains are characterized by markedly different protein compositions, reflecting the ability of the cells to target newly synthesized membrane proteins to specific cell surface domains (6). Enveloped viruses which form by budding at the cell surface are released exclusively at the apical or basolateral membranes in such cells, depending on the virus type (43, 46). However, not much information is available about the release of viruses that are assembled by budding intracellularly in epithelial cells, and we therefore investigated PTV release in the polarized epithelial Vero C1008 cell line. To quantitate the release of PTV from apical and basolateral surfaces of polarized cells, release curves were determined by using confluent monolayers of Vero C1008 cells grown on nitrocellulose filters. Released virus titers in apical and basolateral chambers were determined separately by plaque assay. Prior to determining virus release, we examined the integrity of Vero C1008 cell monolayers during PTV infection and BFA treatment by determining the transepithelial permeability of [^{3}H]inulin at selected time points. The results showed that [^{3}H]inulin diffused across the cell monolayer at background levels during either PTV infection or BFA treatment at the time periods studied. In contrast, [^{3}H]inulin freely diffused into the apical chamber after EGTA treatment, which disrupts the tight junctions (Fig. 8D). This finding demonstrated that the integrity of the epithelial monolayer had not been disrupted by virus infection or BFA treatment. The virus release curves showed that more than 99% of virions were recovered from the basolateral chamber of the polarized epithelial Vero C1008 cells from 12 to 60 h p.i., whereas nearly equal titers of virus were released into the apical and basolateral chambers in nonpolarized Vero cells at the same time periods (Fig. 8A and B). Virus particles associated with basolateral membranes of Vero C1008 cells were also observed by electron microscopy (data not shown).

Since drugs affecting protein transport, such as ammonium chloride and tunicamycin, affect the polarized release of secreted proteins in epithelial cells (6, 7), we investigated the polarized release of PTV after reversal of BFA treatment. It was observed that the virus was still preferentially released into basolateral chambers after BFA-containing medium was removed (Fig. 8C). From these results, we conclude that the release of PTV from epithelial cells is polarized at basolateral membranes and that cells rapidly resume their directional release of virus upon reversal of BFA treatment.

DISCUSSION

Important knowledge about viral protein transport, assembly, and release has been obtained from studies using drugs that interfere with vesicular transport (1, 17). Recent results indicate that BFA interferes with vesicular transport in a novel manner which may reveal a new process of retrograde transport (26, 27). Bunyavirus particles are formed by budding into Golgi cisternae or Golgi-related vesicles and are believed to be transported by vesicles to the cell surface, where they are released by vesicle fusion with the cell membrane (47). Our results show that infectious virus is

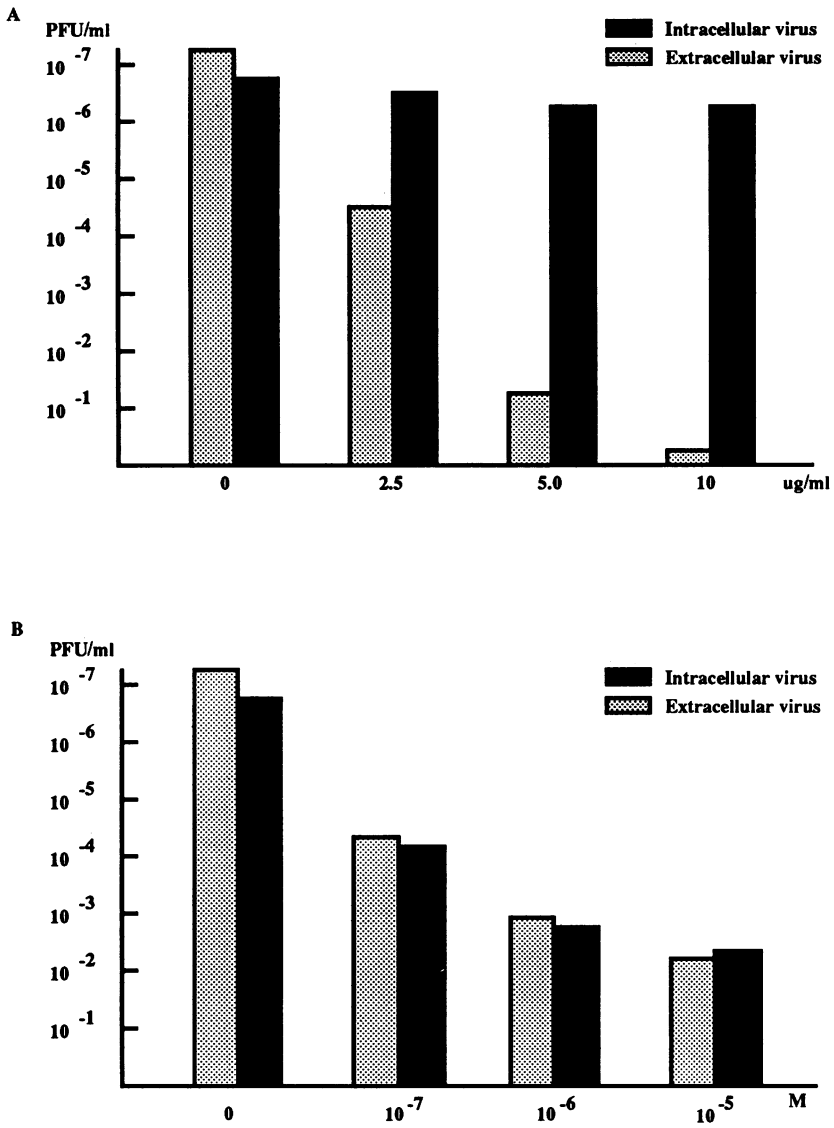

FIG. 5. Effects of different concentrations of BFA and monensin on PTV assembly and release in Vero cells. Vero cells were infected with PTV at an MOI of 10. (A) BFA was added to culture medium at a final concentration of 0, 2.5, 5, or 10 μg/ml for 6-h intervals prior to harvest of each sample. (B) Monensin was added to culture medium at a final concentration of 10^{-5}, 10^{-6}, or 10^{-7} M from the onset of infection. Culture medium and cells were harvested at 18 h p.i., and cells were frozen-thawed three times. Virus yields were determined by plaque assay.

produced in BFA-treated cells but remains cell-associated rather than being released during drug treatment. Since presumably PTV membrane glycoproteins accumulate in fused vesicles of the Golgi and ER as a result of BFA treatment (27), our results suggest that PTV is capable of budding into such vesicles under these conditions. We also found that the BFA block of virus release is reversible and that cells appear to metabolize BFA, since periodic replenishment is needed to maintain its inhibitory effects.

Studies of protein transport in polarized epithelial cells have been greatly facilitated by the finding that enveloped viruses are assembled by budding either at the apical or the basolateral membrane in such cells (43, 46). It is known that plasma membrane proteins, including viral glycoproteins, that are to be segregated in basolateral or apical domains are sorted intracellularly, and the polarized virus budding reflects the polarized distribution of viral envelope proteins in the plasma membrane (6). In contrast, bunyavirus glycoproteins are retained in the Golgi complex; virions are assembled there and released by an exocytosis process (47). In many epithelial cells, protein secretion also takes place in a polarized fashion (7). We observed that PTV can infect the polarized epithelial cell line Vero C1008 and does not disrupt the integrity of the polarized monolayers during the time periods studied. Our results reveal that the release of PTV is polarized, occurring exclusively from the basolateral surfaces of the polarized epithelial cells. Polarized release was also found to resume rapidly when a BFA block was removed. The polarized release of bunyaviruses from basolateral surfaces of epithelial cells may facilitate the spread of virus during natural infection to produce a systemic disease (52).

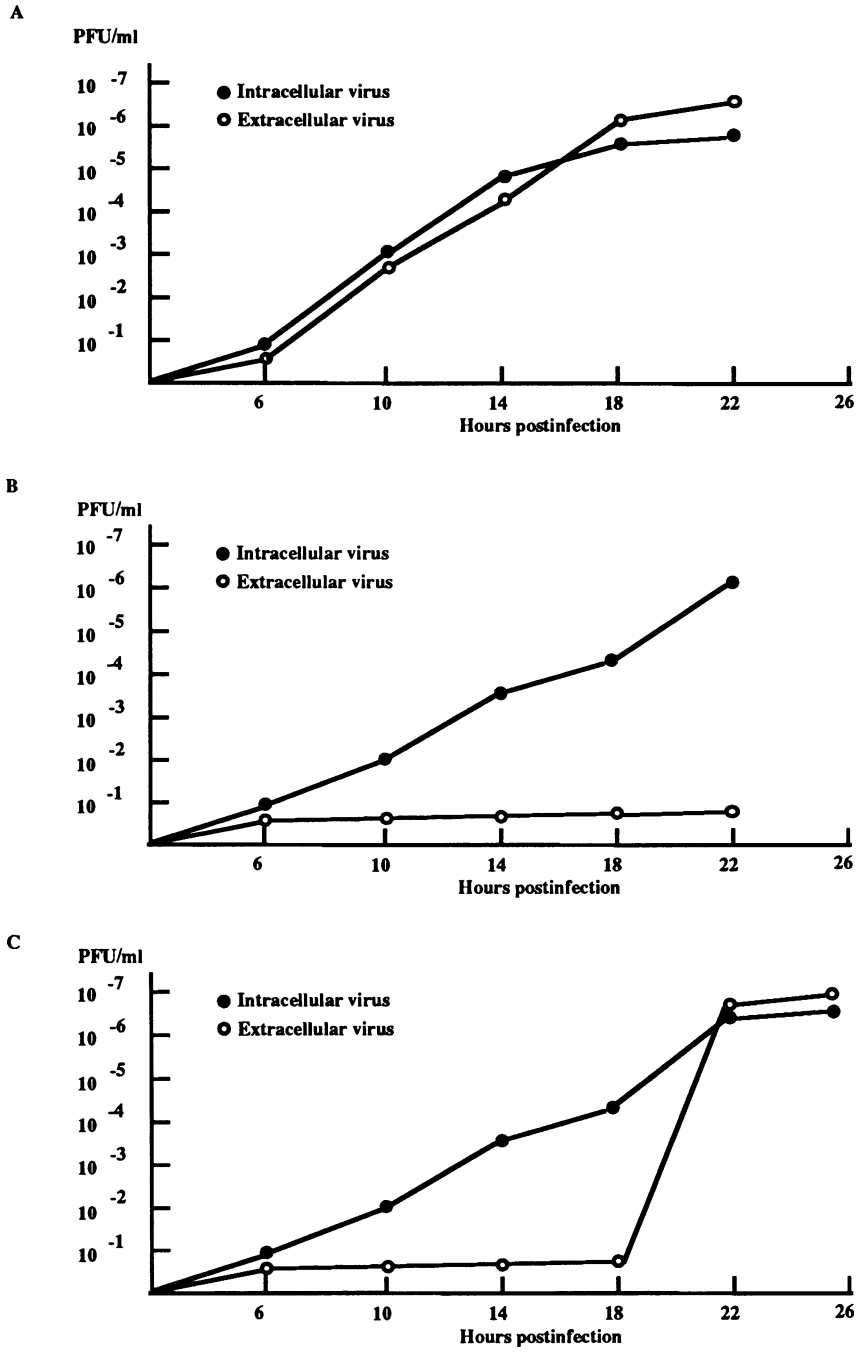

FIG. 6. Effects of BFA on virus assembly and release. Vero cells were infected with PTV at an MOI of 10. (A) An aliquot of methanol without BFA was added for 4 h-intervals prior to harvest. (B) BFA was added to culture medium at a final concentration of 10 μg/ml for 4-h intervals prior to harvest of each sample. (C) Culture medium of infected cells was supplemented with BFA at a final concentration of 10 μg/ml for 4-h intervals until 18 h p.i. and then washed with PBS three times, after which medium without BFA was added. Culture medium and cells were harvested at intervals, cells were frozen-thawed three times, and virus yields were determined by plaque assay.

It has been observed that bunyavirus glycoproteins are transported slowly out of the ER and accumulate in the Golgi region, from which virion budding occurs, although a relatively small fraction of the viral glycoproteins and low levels of budding of virus have been reported on the cell surface (2, 21, 41). Two alternative possibilities were postulated for the mechanism of protein accumulation in the Golgi region. One possibility is that inefficient (slow) intracellular transport of bunyavirus glycoproteins from the ER to the plasma membrane results in protein accumulation in the Golgi complex, thereby increasing the probability of virus budding at this site (21, 22, 30). Alternatively, bunyavirus glycoproteins may contain specific retention signals that ensure their retention in the Golgi region (32, 56, 58). To distinguish these

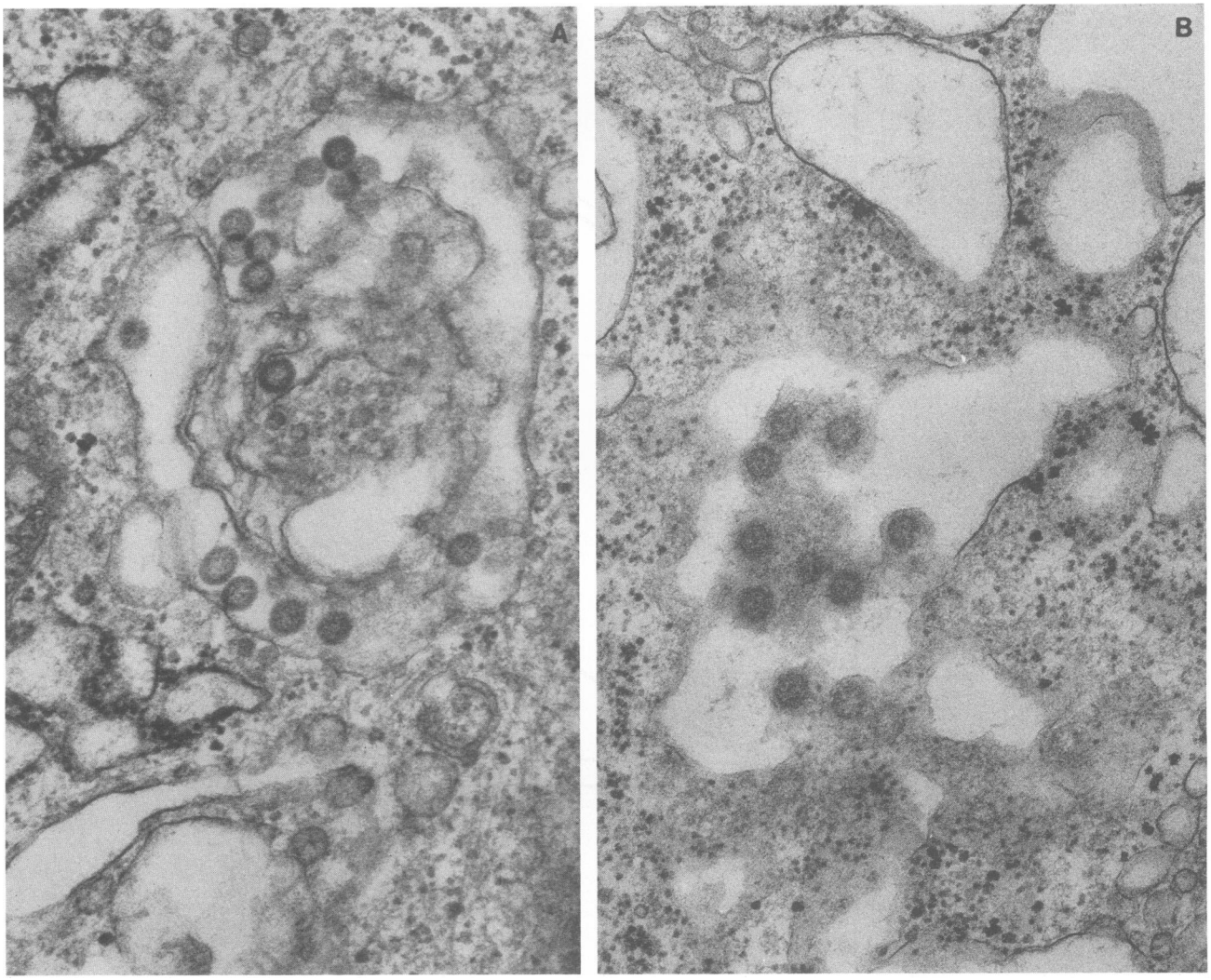

FIG. 7. Electron microscopy of virus assembly in BFA-treated cells. Monolayers of Vero cells were infected with PTV at an MOI of 15, and some were treated with BFA at 10 μg/ml at 4-h intervals. At 18 h p.i., cells were fixed and prepared for transmission electron microscopy. (A) PTV-infected cells without BFA treatment showing intracellular viral maturation; (B) PTV-infected cells after BFA treatment showing dilated ER vesicles and particles within dilated vesicles. Magnification, ×19,000.

possibilities, it is important to determine whether between PTV G1 and G2 glycoproteins are exclusively retained in a particular cellular compartment. In this study, since the VV-G recombinant was used, we can exclude the possibility that any glycoproteins appearing on the surface of recombinant-infected cells may represent cell-associated virions that mature at the Golgi complex and are subsequently released at the cell surface. Our results clearly show that the distribution of PTV G1 and G2 proteins, whether during PTV infection or when expressed from a vaccinia virus recombinant, gradually changes from a more diffuse pattern characteristic of the ER and Golgi into a typical Golgi pattern and that proteins are stably retained in the Golgi complex following cycloheximide treatment. G1 and G2 proteins were not observed on the cell surface by either surface immunofluorescence or surface immunoprecipitation in VV-G-infected cells. In BFA-treated cells, the G1 and G2 protein distribution changed dramatically from a perinuclear Golgi pattern to a dispersed ER pattern, whereas *trans* Golgi network proteins or a resident ER protein (BiP) changed only slightly upon BFA treatment. Since BFA causes a marked redistribution of components of the Golgi cisternae, but not the *trans* Golgi network (26, 27), the observed redistribution of the G1 and G2 proteins indicates that they are primarily retained in the *cis*-medial Golgi complex. Further evidence in support of a *cis*-medial Golgi localization of the G1 and G2 proteins is provided by our observations that both proteins lack sialic acid modification, a modification that occurs in the *trans* Golgi or *trans* Golgi network. Some sialic acid was reported in G1 and G2 proteins in Uukuniemi virus-infected cells (21). Perhaps, in this case, the modification occurred following budding from the Golgi network as a result of virion transport through the *trans* Golgi cisternae prior to release from the cell surface. The observations thus indicate that PTV G1 and G2 proteins are transport competent and are specifically retained in the *cis* and medial Golgi cisternae as a result of specific sorting signals that direct their localization rather than as the result of inefficient transport from the ER to the plasma membrane. It was also reported that Hantaan virus and Rift Valley fever

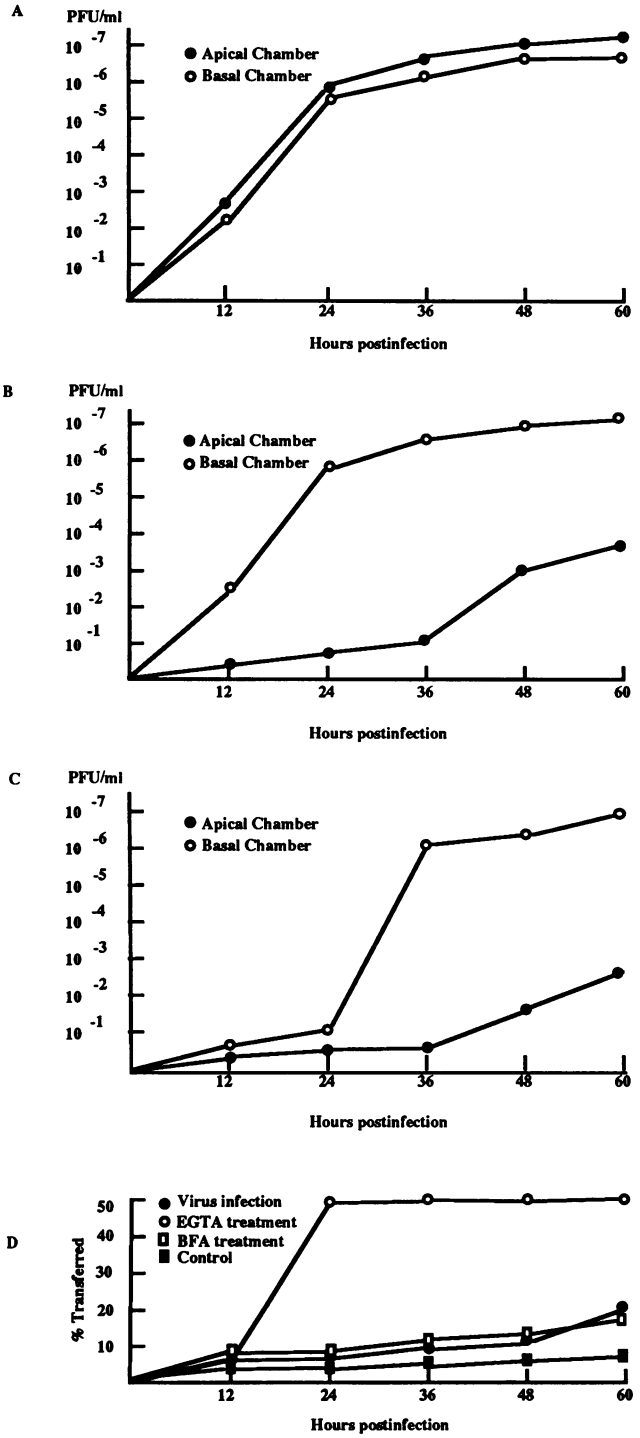

FIG. 8. Evidence that release of PTV is polarized. Vero C1008 cells or nonpolarized Vero cells were grown on nitrocellulose filters and infected with PTV at an MOI of 10. Culture medium from both the apical and basal chambers was harvested separately at intervals postinfection, and virus release was measured by plaque assay. (A) Virus release from nonpolarized Vero cells. (B) Virus release from polarized Vero C1008 cells. (C) Virus release from polarized Vero C1008 after reversal of BFA treatment. BFA was added to culture medium at a concentration of 5 μg/ml every 6 h until 24 h p.i.; the medium was then rinsed with PBS and replaced with BFA-free medium. (D) Diffusion of [^{3}H]inulin across cell monolayers. [^{3}H]inulin (1 μCi) was added to the basal chamber at time zero; at the times indicated, the presence of [^{3}H]inulin in the apical and basal chambers was determined by liquid scintillation spectrometry. Plotted is the percentage of total [^{3}H]inulin present in the apical chamber. Symbols: ■, Diffusion of [^{3}H]inulin to the apical chamber in control monolayers; ●, diffusion of [^{3}H]inulin to the apical chamber in monolayers infected with PTV; □, diffusion of [^{3}H]inulin to the apical chamber in monolayers treated with BFA at a concentration of 5 μg/ml every 6 h until 24 h p.i.; ○, diffusion of [^{3}H]inulin to the apical chamber in monolayers treated with 50 mM EGTA at 12 hp.i.

virus glycoproteins expressed from vaccinia virus recombinants appeared exclusively to be Golgi associated and were not found on the cell surface (38, 56). It was noted that some members of the bunyaviruses, such as Hantaan virus, have the ability to persistently infect their natural host, even in the presence of high levels of antibodies (25). Thus, the intracellular retention of bunyavirus glycoproteins and budding of virions from intracellular membranes may provide an important advantage to the virus in evasion of the host immune system. It has been suggested (28) that the conversion of an envelope protein from a plasma membrane protein to one that is retained intracellularly may be an important step in the successful evolution of viruses.

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REFERENCES

1. **Alonso, F. V., and R. W. Compans.** 1981. Differential effect of monensin on enveloped viruses that form at distinct plasma membrane domains. J. Cell Biol. **89**:700–705.
2. **Anderson, G. W., Jr., and J. F. Smith.** 1987. Immunoelectron microscopy of Rift Valley fever viral morphogenesis in primary rat hepatocytes. Virology **161**:97–100.
3. **Bergman, J. E., K. T. Tokuyasu, and S. J. Singer.** 1981. Passage of an integral membrane protein, the vesicular stomatitis glycoprotein, through the Golgi apparatus en route to the plasma membrane. Proc. Natl. Acad. Sci. USA **78**:1746–1750.
4. **Bishop, D. H. L.** 1990. Bunyaviridae, p. 1155–1174. *In* B. N. Fields et al. (ed.), Virology. Raven Press, New York.
5. **Bole, D. G., L. M. Hendershot, and J. F. Kearney.** 1986. Posttranslational association of immunoglobulin heavy chain binding protein with nascent heavy chains in nonsecreting and secreting hybridomas. J. Cell Biol. **102**:1558–1566.
6. **Caplan, M., and K. S. Matlin.** 1989. Sorting of membrane and secretory proteins in polarized epithelial cells. Mod. Cell Biol. **8**:71–127.
7. **Caplan, M. J., J. L. Stow, A. P. Newman, J. Madri, H. C. Anderson, M. G. Farquhar, G. E. Palade, and J. D. Jamieson.** 1987. Dependence of pH of polarized sorting of secreted proteins. Nature (London) **329**:632–635.
8. **Clayson, E. T., L. V. Brando, and R. W. Compans.** 1989. Release of simian virus 40 virions from epithelial cells is polarized and occurs without cell lysis. J. Virol. **63**:2278–2288.
9. **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.
10. **Doms, R. W., G. Russ, and J. W. Yewdell.** 1989. Brefeldin A redistributes resident and itinerant Golgi proteins to the endoplasmic reticulum. J. Cell Biol. **109**:61–72.
11. **Doms, R. W., A. Ruusala, C. Machamer, J. Helenius, A. Helenius, and J. K. Rose.** 1988. Differential effects of mutation in three domains on folding, quaternary structure, and intracellular transport of vesicular stomatitis virus G protein. J. Cell Biol. **107**:89–99.
12. **Fagerland, J. A., J. F. L. Pohlenz, and G. N. Woode.** 1986. A morphologic study of the replication of Breda virus (proposed family Toroviridae) in bovine intestinal cells. J. Gen. Virol. **67**:1293–1304.
13. **Fujiwara, T., K. Oda, S. Yokota, A. Takatsuki, and Y. Ikehara.** 1988. Brefeldin A causes disassembly of the Golgi complex and accumulation of secretory proteins in the endoplasmic reticulum. J. Biol. Chem. **263**:18545–18552.
14. **Griffiths, G., and K. Simons.** 1986. The trans Golgi network: sorting at the exit site of the Golgi complex. Science **234**:438–443.
15. **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.
16. **Ihara, T., J. Smith, J. M. Dalrymple, and D. H. L. Bishop.** 1985. Complete sequences of the glycoproteins and M RNA of Punta Toro phlebovirus compared to those of Rift Valley fever virus. Virology **144**:246–253.
17. **Johnson, D. C., and M. Schlesinger.** 1980. Vesicular stomatitis virus and Sindbis virus glycoprotein transport to the cell surface is inhibited by ionophores. Virology **103**:407–424.
18. **Klausner, R. D.** 1989. Sorting and traffic in the central vacuolar system. Cell **57**:703–706.
19. **Kornfeld, R., and S. Kornfeld.** 1985. Assembly of asparagine-linked oligosaccharides. Annu. Rev. Biochem. **54**:631–664.
20. **Kreis, T. E., and H. F. Lodish.** 1986. Oligomerization is essential for transport of vesicular stomatitis virus viral glycoprotein on the cell surface. Cell **46**:929–937.
21. **Kuismanen, E.** 1984. Posttranslational processing of Uukuniemi virus glycoprotein G1 and G2. J. Virol. **51**:806–812.
22. **Kuismanen, E., K. Hedman, J. Saraste, and R. F. Pettersson.** 1982. Uukuniemi virus maturation: accumulation of virus particles and viral antigens in the Golgi complex. Mol. Cell. Biol. **2**:1444–1458.
23. **Laemmli, U. K.** 1970. Cleavage of the structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
24. **Leary, K., and C. D. Blair.** 1980. Sequential events in the morphogenesis of Japanese encephalitis virus. J. Ultrastruct. Res. **72**:123–129.
25. **Lee, H. W.** 1982. Korean hemorrhagic fever. Prog. Med. Virol. **28**:96–113.
26. **Lippincott-Schwartz, J., J. G. Donaldson, A. Schweizen, 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.
27. **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 Golgi to ER. Cell **56**:801–813.
28. **Machamer, C. E., and J. K. Rose.** 1987. A specific transmembrane domain of a coronavirus E1 glycoprotein is required for its retention in the Golgi region. J. Cell Biol. **105**:1205–1214.
29. **Mackett, M., T. Yilma, J. K. Rose, and B. Moss.** 1985. Vaccinia virus recombinants: expression of VSV genes and protective immunization of mice and cattle. Science **227**:433–435.
30. **Madoff, D. H., and J. Lenard.** 1982. A membrane glycoprotein that accumulates intracellularly: cellular processing of the large glycoprotein of LaCrosse virus. Cell **28**:821–829.
31. **Magner, J. A., and E. Papagiannes.** 1988. Blockade by Brefeldin A of intracellular transport of secretory proteins in mouse pituitary cells: effects on the biosynthesis of thyrotropin and free alpha-subunits. Endocrinology **122**:912–920.
32. **Matsuoka, Y., T. Ihara, D. H. L. Bishop, and R. W. Compans.** 1988. Intracellular accumulation of Punta Toro virus glycoproteins expressed from cloned cDNA. Virology **167**:251–260.
33. **Misumi, Y., Y. Misumi, K. Miki, A. Takatsuki, G. Tamura, and Y. Ikehara.** 1986. Novel blockade by brefeldin of intracellular transport of secretory protein in culture rat hepatocytes. J. Biol. Chem. **261**:11398–11403.
34. **Munro, S., and H. R. B. Pelham.** 1987. A C-terminal signal prevents secretion of luminal ER proteins. Cell **48**:899–907.
35. **Murphy, F. A., A. K. Harrison, and S. G. Whitfield.** 1973. Bunyaviridae: morphologic and similarities of Bunyamwera serologic supergroup viruses and several other arthropod-borne viruses. Intervirology **1**:297–316.
36. **Pelham, H. R. B.** 1988. Evidence that luminal ER proteins are sorted from secreted proteins in a post-ER compartment. EMBO J. **7**:913–918.
37. **Pelham, H. R. B.** 1989. Heat shock and the sorting of luminal ER proteins. EMBO J. **8**:3171–3176.
38. **Pensiero, M. N., G. B. Jennings, C. S. Schmaljohn, and J. Hay.** 1988. Expression of the Hantaan virus M genome segment by using a vaccinia virus recombinant. J. Virol. **62**:696–702.
39. **Persson, R., L. Wikstrom, and R. F. Pettersson.** 1989. Dimerization and intracellular transport of two Golgi-specific bunyavirus membrane glycoproteins, p. 229–242. *In* R. W. Compans et al. (ed.), Cell biology of virus entry, replication and pathogenesis. Academic Press, Inc., Orlando, Fla.
40. **Pesonen, M., E. Kuismanen, and R. F. Pettersson.** 1982. Monosaccharide sequence of protein-bound glycans of Uukuniemi virus. J. Virol. **41**:390–400.
41. **Pettersson, R. F., N. Gahmberg, E. Kuismanen, L. Kaariainen, R. Ronnholm, and J. Saraste.** 1988. Bunyavirus membrane glycoproteins as models for Golgi-specific proteins. Mod. Cell Biol. **6**:65–96.
42. **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.
43. **Rodriguez-Boulan, E., and D. D. Sabatini.** 1978. Asymmetric budding of viruses in epithelial monolayers: a model system for the study of epithelial polarity. Proc. Natl. Acad. Sci. USA **75**:5071–5075.
44. **Roth, J., D. J. Taatjes, J. M. Lucocq, J. Weinstein, and J. C. Paulson.** 1985. Demonstration of an extensive trans-tubular network continuous with the Golgi-apparatus stack that may function in glycosylation. Cell **43**:287–295.
45. **Roth, M. G., and R. W. Compans.** 1980. Antibody-resistant spread of vesicular stomatitis virus infection in cell lines of epithelial origin. J. Virol. **35**:547–550.
46. **Roth, M. G., R. V. Srinivas, and R. W. Compans.** 1983. Basolateral maturation of retroviruses in polarized epithelial cells. J. Virol. **45**:1065–1073.
47. **Schmaljohn, C. S., and J. L. Patterson.** 1990. Replication of Bunyaviridae, p. 1175–1194. *In*: B. N. Fields et al. (ed.), Virology. Raven Press, New York.
48. **Smith, J. F., and D. Y. Pifat.** 1982. Morphogenesis of sandfly fever viruses (Bunyaviridae family). Virology **121**:61–81.
49. **Srinivas, R. V., N. Balachandran, F. V. Alonso-Caplan, and R. W. Compans.** 1986. Expression of herpes simplex virus glycoproteins inpolarized epithelial cells. J. Virol. **58**:689–693.
50. **Takatsuki, A., and G. Tamura.** 1985. Brefeldin A, a specific inhibitor of intracellular translocation of vesicular stomatitis virus G protein: intracellular accumulation of high mannose type G protein and inhibition of its cell surface expression. Agric. Biol. Chem. **48**:899–902.
51. **Tartakoff, A., and P. Vassalli.** 1983. Lectin-binding sites as markers of Golgi subcompartment: proximal-to-dismal maturation of oligosaccharides. J. Cell Biol. **97**:1243–1248.
52. **Tashiro, M., M. Yamakawa, T. Tobita, J. T. Seto, H.-D. Klenk, and R. Rott.** 1990. Altered budding site of a pantropic mutant of Sendai virus, F1-R, in polarized epithelial cells. J. Virol. **64**: 4672–4677.
53. **Tooze, J., and S. A. Tooze.** 1985. Infection of AtT20 murine pituitary tumor cells by mouse hepatitis virus strain A59: virus budding is restricted to the Golgi region. J. Cell Biol. **33**:281–

293.
54. **Virtanen, I., P. Ekblom, and P. Laurila.** 1980. Subcellular compartmentalization of saccharide moieties in culture normal and malignant cells. J. Cell Biol. **85**:429–434.
55. **Warren, G.** 1987. Signals and salvage sequences. Nature (London) **327**:17–18.
56. **Wasmoen, T. L., L. T. Kaleach, and M. S. Collett.** 1988. Rift Valley fever M segment: cellular localization of M segment-encoded proteins. Virology **166**:275–280.
57. **Wieland, F. T., M. L. Gleason, T. A. Serafini, and J. E. Rothman.** 1987. The rate of bulk flow from the endoplasmic reticulum to the cell surface. Cell **50**:289–300.
58. **Wikstrom, L., R. Persson, and R. F. Pettersson.** 1989. Intracellular transport of the G1 and G2 membrane glycoproteins of Uukuniemi virus, p. 33–41. *In* D. Kolakofsky and R. Mahy (ed.), Genetics and pathogenicity of negative strand viruses. Elsevier Science Publications, Amsterdam, The Netherlands.