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Nocodazole-Dependent Transport, and Brefeldin A–Sensitive Processing and Sorting, of Newly Synthesized Membrane Proteins in Cultured Neurons

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The envelope glycoproteins of Semliki Forest virus (SFV), Vesicular Stomatitis virus (VSV), and Influenza Fowl Plague virus (FPV) are vectorially targeted in neurons to the plasma membrane of dendrites (SFV and VSV) and axons (FPV). To gain insight into the mechanisms responsible for such polarized delivery we have examined the effects on neurons of nocodazole and brefeldin A (BFA), which are known to cause microtubule depolymerization and disassembly of the Golgi apparatus, respectively. Nocodazole treatment blocked transport of all viral glycoproteins to both axons and dendrites. BFA treatment induced disruption of the Golgi complex, including the *trans*-Golgi network (TGN), and tubulation of endosomes. However, the delivery of the SFV and FPV glycoproteins to the cell surface was not affected significantly by BFA, although processing and sorting were altered, as revealed by surface biotinylation and immunofluorescence microscopy of fixed nonpermeabilized cells. These results demonstrate the involvement of microtubules in axonal and dendritic transport of integral membrane glycoproteins, and the existence of a BFA-sensitive component in the sorting but not in the transport machinery.

[Key words: microtubules, sorting, virus, hippocampus, glycoproteins, neurons, intracellular transport, Golgi apparatus, endosomes]

Neurons are characterized by a polarized cell surface organization that segregates and maintains proteins into two discrete functional domains: the axonal and the somatodendritic plasma membrane. This molecular segregation is essential for the efficiency of the vectorial functions of nerve cells. In previous work on the mechanisms governing protein sorting in neurons (Dotti and Simons, 1990; Kobayashi et al., 1992; Dotti et al., 1993), we have applied experimental strategies such as infection with enveloped viruses that have proven useful to study the mechanisms of sorting of apical and basolateral proteins in epithelial cells. In these cells, the influenza virus hemagglutinin (HA) is routed to the apical surface while the spike glycoproteins of VSV and SFV are delivered to the basolateral surface (Rodriguez-Boulan and Pendergast, 1980; Fuller et al., 1985; Roman and Garoff, 1986). When sorting of these viral glycoproteins was studied in hippocampal cells cultured from 18-d-old rat embryos, a well-established neuronal culture system (Goslin and Banker, 1991), we found that the influenza HA was routed to the axon while the VSV and SFV glycoproteins were distributed to the somatodendritic cell surface (Dotti and Simons, 1990; Dotti et al., 1993). These observations suggested that, at least in the way viral glycoproteins are sorted, the axonal and dendritic domains of neurons are equivalent to the apical and basolateral domains of epithelial cells, respectively.

Microtubules have been shown to be involved in protein delivery to the apical surface in epithelial cells (Rindler et al., 1987; Breitfeld et al., 1990; Gilbert et al, 1991) as well as in axonal transport in neurons (Kelly, 1990). However, it is likely that different motors drive apical and axonal delivery, since microtubules are oriented in opposite ways in the two cell types: with their minus ends toward the apical membrane in epithelial cells (Bacallao et al., 1989) and toward the cell body in neurons (Baas et al., 1988). In epithelial cells, microtubules seem not to be required for basolateral delivery of viral glycoproteins (Salas et al., 1986; Rindler et al., 1987), although in an *in vitro* assay both apical and basolateral vesicles bound specifically to microtubules (van der Sluijs et al., 1990). In neurons, intact microtubules are required for membrane transport in the axons (reviewed in Hammerschlag and Brady, 1988; Brady et al., 199). Little is known, however, about the requirement for microtubules in dendritic transport of exocytic vesicles.

In this work, we have extended the analysis of the mechanisms of protein targeting to the surface of polarized hippocampal neurons in culture. To this end we used viral glycoproteins as markers for either axonal or somatodendritic sorting, and have studied the effects on their transport to the cell surface of two different inhibitors of intracellular membrane traffic: nocodazole, a microtubule depolymerizing agent; and BFA, a fungal metabolite that induces disassembly of the Golgi stacks and impairs intracellular transport beyond the Golgi complex (Takatsuki and Tamura, 1985; Misumi et al., 1986; Oda et al., 1987; Lippincott-Schwartz et al., 1989; Low et al., 1991).

Our results indicate that depolymerization of the microtubular network with nocodazole leads to inhibition of both axonal and dendritic delivery of newly synthesized viral glycoproteins. On the other hand, transport of the SFV and FPV glycoproteins to the cell surface was not impaired by BFA, although their polarized distribution was altered.

Materials and Methods

Hippocampal cultures. Cultures of hippocampal cells were prepared from the brains of 18-d-old fetal Sprague-Dawley rats as described pre-

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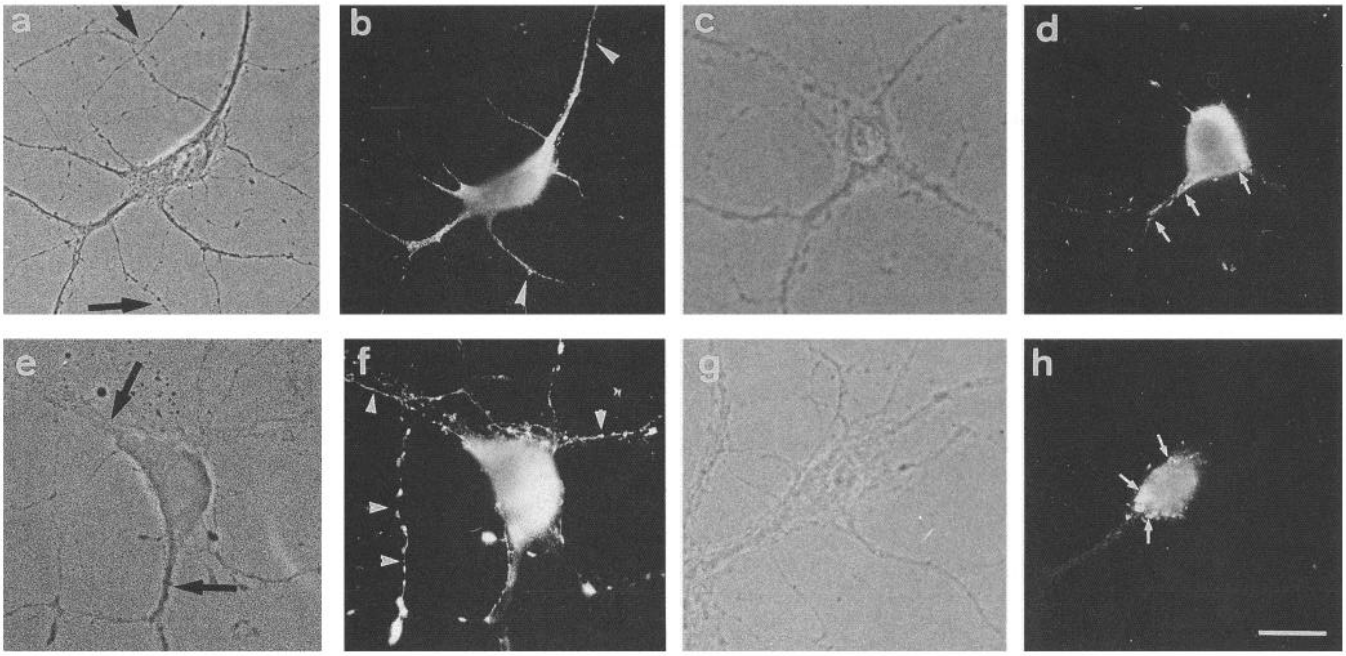


Figure 1. Effect of nocodazole on the distribution of the viral glycoproteins VSV-G and influenza HA. VSV-infected cells were fixed after 1.5 hr at the permissive temperature of 32°C in the absence (*a, b*) or presence (*c, d*) of 30 μM nocodazole. In the absence of nocodazole the glycoprotein is present exclusively in the dendrites and cell body (*arrowheads in b*) but not in the axons (only seen in *a, arrows*). Fixation of infected cells that had been incubated with 30 μM nocodazole resulted in the accumulation of the glycoprotein in the cell body (*arrows in d*). Nocodazole treatment also affected the delivery of the FVP HA glycoprotein to the axon. In untreated cells (*e, f*) the glycoprotein is present in the cell body and axons (*arrowheads in f*) but not in dendrites (*arrows in e*). In nocodazole-treated neurons the HA protein accumulates in the cell body (*arrows in h*). Some of the cell body labeling may represent disrupted Golgi cisternae. Scale bar, 10 μm.

viously (Banker and Cowan, 1977; Goslin and Banker, 1991). Briefly, after chemical and mechanical dissociation of the hippocampi, the cells were plated onto polylysine-coated glass coverslips (1.5 cm diameter). These neurons survive for several weeks and undergo full polarization when cultured in serum-free medium (SFM) in the presence of a supporting layer of astrocytes (Goslin and Banker, 1991).

Virus infection and immunofluorescence. Cultured hippocampal cells were infected with VSV, SFV, or FPV as described elsewhere (Dotti et al., 1993) for 1 hr at 37°C with the appropriate virus (80–100 pfu/cell), the coverslips rinsed in SFM, and infection continued for 3–8 hr at 37°C (FPV and SFV), or for 2 hr at 39°C followed by 2 hr at 19.5°C and 1.5–6 hr at 32°C (VSV O-45 thermosensitive mutant). Intracellular

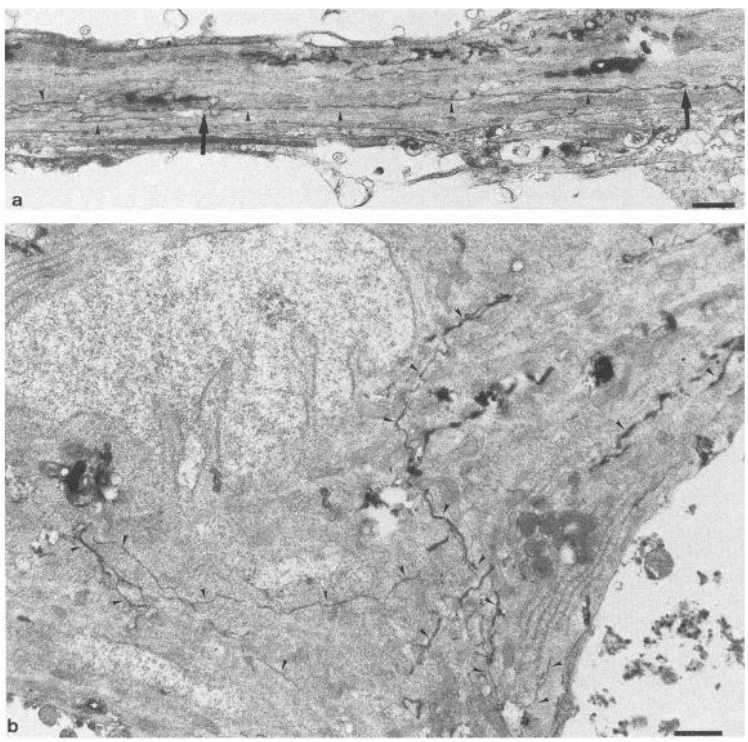


Figure 2. Effect of BFA on the organization of early endosomes. Hippocampal neurons were incubated with high activity HRP (10 mg/ml) in the presence of 5 μg/ml BFA for 30 min at 37°C. Semithick sections were cut parallel to the culture dish. The length of HRP-labeled endosomes (*arrowheads*) in both the dendritic domain (*a*) and cell body (*b*) is dramatically increased (the *two arrows in a* point to the possible ends of a single endosome). Scale bars, 1 μm.

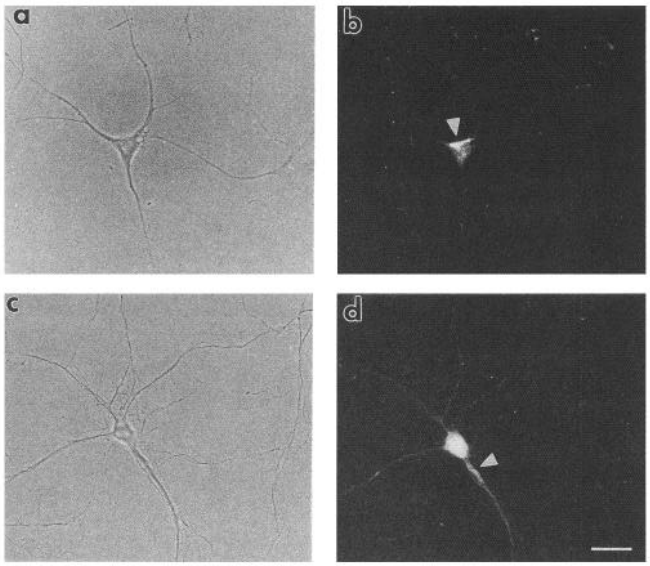


Figure 3. Immunofluorescence analysis of the effect of BFA on the distribution of mannosidase II in cultured hippocampal neurons. Fully polarized cells were incubated in the absence of BFA (*a, b*) or in the presence of 10 μg/ml of BFA for 15 min (*c, d*). After fixation (see Materials and Methods) immunofluorescence labeling was performed using an antibody against the *cis*/medial Golgi marker mannosidase II and a fluorescein-conjugated secondary antibody. Note the discrete tubular appearance of the Golgi stacks close to a nuclear pole in control cells (*b*), whereas in the BFA-treated neurons the labeling is intense throughout the entire cell body and the initial segment of the neurites (*arrowhead in d*). Scale bar, 10 μm.

detection of the viral glycoproteins was performed as previously described (Dotti and Simons, 1990). Surface appearance was done by incubating living infected cells with the appropriate antibodies (see below) at 4°C for 30 min, then rinsing and fixing. Immunofluorescence analysis and photography were performed with an Axiophot (Zeiss, Germany) microscope.

Antibodies. The following primary antibodies were used: rabbit anti-VSV glycoprotein (Matlin et al., 1982), rabbit anti-FPV HA (Matlin et al., 1981), mouse monoclonal (9AB4) anti-SFV glycoprotein (Burke et al., 1983), rabbit polyclonal anti-mannosidase II (Moremen and Touster, 1985), and rabbit peptide serum anti-β-COP (Pepperkok et al., 1993). Species-specific secondary antibodies were from Dianova GmbH (Germany).

Microtubule depolymerization. The microtubules were depolymerized in cells infected with the VSV O-45 thermosensitive mutant by adding 30 mM nocodazole (Aldrich Chemical Co., Milwaukee, WI) to the culture dishes during the last 90 min at 19.5°C. The coverslips were next transferred for 15 min to dishes containing ice-cold buffer and 30 μM nocodazole and, finally, to dishes containing warm (31°C) SFM and 30 μM nocodazole for 1.5–6 hr. In FPV-infected cells, the 15 min cold-nocodazole treatment was performed immediately after viral absorption. Nocodazole was maintained in the culture medium (37°C) during the following 9 hr of incubation.

BFA treatment and immunofluorescence. BFA (Sigma, St. Louis, MO) was prepared as a stock solution of 5 mg/ml in methanol and stored at −20°C. Neurons infected with either SFV or FPV were treated with 10 μg/ml at 3 hr postinfection. The drug was maintained in the culture medium for the subsequent 2 hr. The infected–treated cells were then incubated for the last 30 min of infection with the corresponding primary antibodies for the surface detection of the viral glycoproteins, and immunofluorescence microscopy performed as described above.

Metabolic labeling. Neurons (14 d cultures) plated onto coverslips (2000 cells/coverslip on average) were infected for 1 hr with SFV and rinsed briefly at 37°C in Hank's balanced salt solution (HBSS), and viral expression let to proceed for 3 hr in SFM. At this time, the cells were first washed briefly in HBSS and incubated for 30 min at 37°C in methionine-free SFM. Labeling was for 30 min with ^{35}S-methionine (1 mCi/ml) in HBSS (metabolic pulse). After the metabolic pulse, the coverslips were rinsed in HBSS, and infection continued for 2.5 hr (metabolic chase). BFA (10 μg/ml) was added (1) at the beginning of the infection, (2) 15 min before the metabolic labeling, and (3) at the end of the metabolic labeling. BFA was then maintained in the chase medium until the end of the experiments, even during the biotinylation at 4°C (see below). The viral glycoprotein that reached the neuronal plasma membrane was detected by surface biotinylation.

Cell surface biotinylation. Selective biotinylation of plasma membrane proteins was carried out essentially as described (Pimplikar and Huttner, 1992). Tissue culture dishes containing SFV-infected, ^{35}S-methionine–labeled cells, treated or not with BFA, were rinsed twice in cold Ca^{2+}/Mg^{2+}-PBS (1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ in PBS), and biotinylation performed by incubating for 30 min at 4°C in Ca/Mg-PBS containing 1 mg/ml S-NHS-SS-biotin. As a negative control, six coverslips of SFV-infected/BFA-untreated cells were processed in the same way except that biotin was omitted. Biotinylation was ended by washing the cells in cold PBS followed by an incubation for 10 min at 4°C in Ca/Mg-PBS containing 0.1 M glycine and 0.3% BSA. The cells were then solubilized in PBS containing 2% Nonidet P-40, 0.2% SDS, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The lysates were centrifuged for 10 min to remove insoluble material and then used for immunoprecipitation.

Immunoprecipitation. SFV glycoprotein was immunoprecipitated for 3 hr at room temperature from cell lysates (1 ml) with the monoclonal antibody 9AB4 (ascites fluid, diluted 1:100), which recognizes the p62 and E2 glycoproteins of SFV (Burke et al., 1983). The cell lysate was then incubated with 50 μl of 50% (v/v) protein A sepharose CL-4B (Pharmacia, Sweden) in distilled water. After 1 hr, the unbound material was removed by centrifugation, and the beads washed three times with NET buffer (200 mM NaCl, 1% NP-40, 2 mM EDTA, and 50 mM Tris-HCl, pH 7.5). The bound material was eluted with SDS-PAGE sample buffer (Laemmli, 1970) devoid of β-mercaptoethanol, and then diluted with 1 ml of lysis buffer (250 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% BSA, 25 mM Tris-HCl pH 7.5, and 0.5 mM PMSF). After removing the protein A sepharose by centrifugation, the supernatant was incubated with 35 μl of slurry (1:1) of streptavidin-agarose (Pierce, Rockford, IL) overnight at 4°C. The unbound material was then removed by centrifugation and the resin washed twice with PBS/1% Nonidet P-40, and twice with PBS/0.1% Nonidet P-40, 0.5 M NaCl. Bound protein was eluted by boiling with SDS-PAGE sample buffer and analyzed by SDS-PAGE (Laemmli, 1970) followed by fluorography. The densitometric analysis of the autoradiograms was performed with the program NIH-IMAGE (version 1.40).

Electron microscopy. Analysis of the endocytic pathway was performed as described in early work (Parton et al., 1992). Briefly, the cells were rinsed in HBSS and incubated in neuronal medium containing high-activity horseradish peroxidase (HRP; Serva Biochemicals, Heidelberg, Germany) at a concentration of 10 mg/ml for 30 min at 37°C in the presence of brefeldin A (10 μg/ml). At the end of the incubation the cells were rinsed and fixed in 0.1% glutaraldehyde in 100 mM cacodylate buffer. After embedding in Epon, semithick sections (200 nm) were prepared and analyzed at an accelerating voltage of 60 kV.

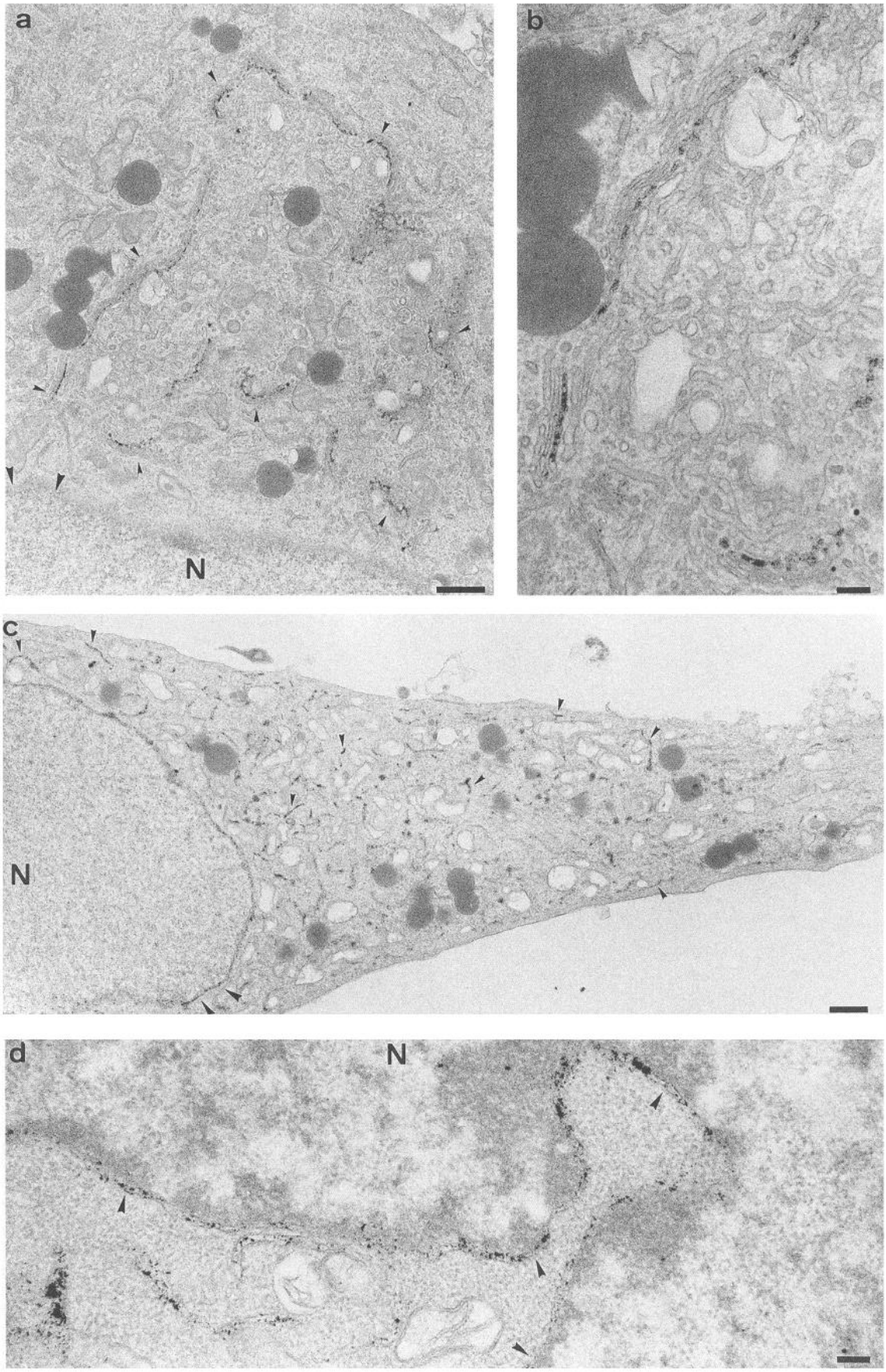
For the ultrastructural analysis of the Golgi apparatus untreated rat hippocampal neurons or neurons treated for 30 min with 10 μg/ml BFA were stained for thiamine pyrophosphatase (TPPase) as described by Griffiths et al. (1983). After Epon embedding, sections were cut parallel to the substratum and were viewed without further contrasting.

Protein secretion analysis. Hippocampal neurons grown on 3 cm tissue culture plastic dishes (150,000 cells/dish) were washed twice with HBSS and then incubated for 30 min at 37°C with methionine-free SFM. The cells were then labeled with ^{35}S-methionine (1 mCi/ml) as described above (metabolic pulse), washed, and incubated for various times in 1 ml of medium containing excess cold methionine (metabolic chase). BFA (5–10 mg/ml) was included in the pulse and chase media. At the times indicated in Figure 8, aliquots of medium were taken and precipitated in 10% trichloroacetic acid, and radioactivity measured in a scintillation counter.

Results

Nocodazole blocks transport of newly synthesized viral glycoproteins

The effect of microtubule depolymerization on dendritic delivery of viral glycoproteins was first studied in hippocampal neu-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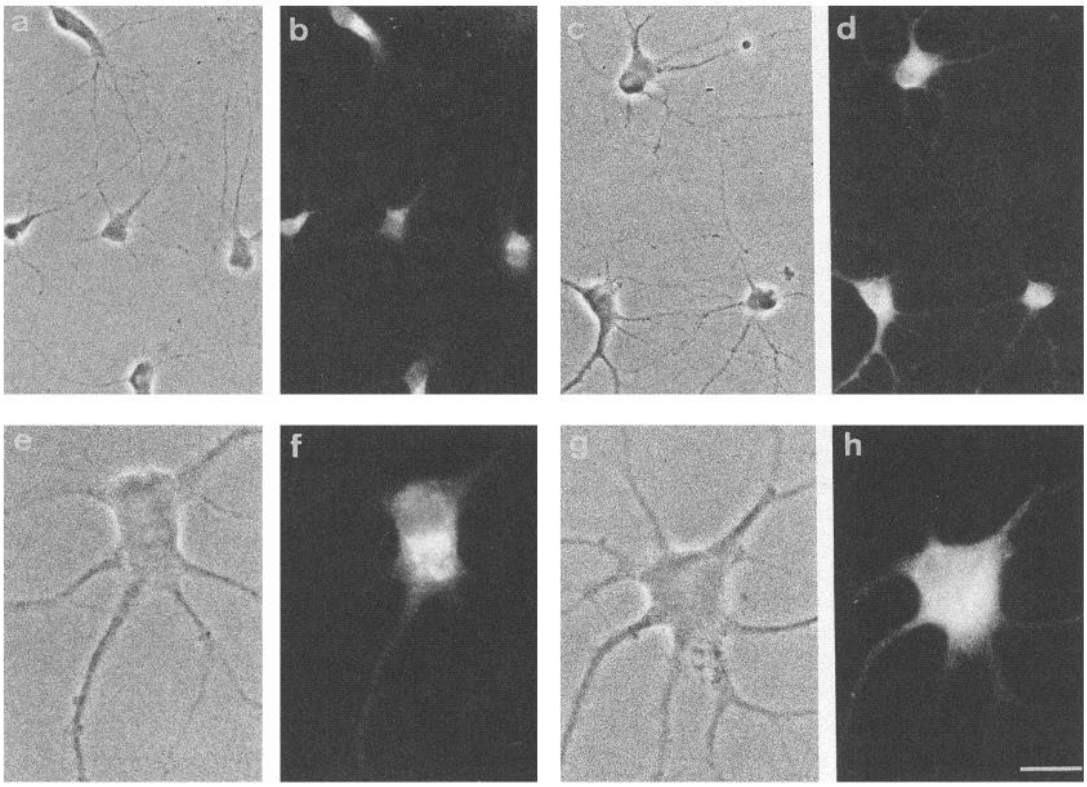

Figure 5. Effect of BFA on the distribution of β-COP. *a* and *b*, and *e* and *f* are low and high magnification, respectively, of untreated cultured hippocampal neurons stained with a anti-β-COP antibody. Note that the labeling is restricted in all cells to one pole of the cell body. After treatment with 10 μg/ml of BFA for 15 min (*c* and *d*, low magnification, and *g* and *h*, high magnification) the staining is diffuse throughout the entire cell body and also extends into some of the neurites.

rons that were infected with the VSV O-45 thermosensitive mutant and incubated in the presence or absence of nocodazole. For this strain, synchrony of delivery of newly synthesized viral glycoproteins from the rough endoplasmic reticulum to the plasma membrane can be achieved by successive temperature shifts (Bergmann et al., 1981; Bergmann and Singer, 1983; Griffiths et al., 1985). At 39°C the VSV glycoprotein accumulates in the rough endoplasmic reticulum, while it moves to the Golgi complex at 20°C and only at 32°C does occur detectable transport to the plasma membrane. As shown previously (Dotti and Simons, 1990), the VSV glycoprotein is selectively transported to the somatodendritic territory of infected hippocampal cells.

Immunofluorescence analysis using specific antibodies showed that microtubule depolymerization (see Materials and Methods) during incubations of infected cells either at 20°C or at 32°C resulted in suppression of dendritic delivery of the VSV glycoprotein (Fig. 1*a–c*). As determined by counting labeled neurons (about 150 of each condition) in representative fields of infected cells, in 71% of them dendritic transport of the VSV glycoprotein was abolished by the nocodazole treatment. Approximately the same degree of inhibition was observed when incubation with nocodazole at 32°C was extended for 6 hr. Since these cells are highly sensitive to nocodazole (Dotti and Banker, 1991), a likely explanation for the remaining 30% of cells in which dendritic labeling was observed might be that the protein had already been transported to the surface before the drug was added. In all cells in which dendritic transport of the VSV glycoprotein was absent, a more intense labeling was seen in the cell body, as compared to untreated cells.

The effect of nocodazole was next studied in hippocampal cells infected for 9 hr with FPV. In untreated cells, the FPV HA is delivered to the axons within this time (Dotti and Simons, 1990). However, immunofluorescence detection of the FPV HA in infected cells incubated with nocodazole showed that microtubule depolymerization had a dramatic effect on the axonal transport of the HA glycoprotein, which was completely abolished and the HA-specific labeling restricted to the cell body (Fig. 1*d–f*).

In both cases the effect of nocodazole was reversible, since washing out the drug and continuing infections with the VSV O-45 mutant or FPV for various times resulted in progressive reappearance of the corresponding viral glycoproteins in the dendritic or axonal territories, respectively (data not shown). In addition, plaque titration assays showed that formation of infec-

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Figure 4. Staining of TPPase in control and BFA-treated cells. Untreated rat hippocampal neurons (*a, b*) or neurons treated for 30 min with BFA (*c, d*) were fixed and stained for TPPase. In control cells (*a, b*) staining is evident in the cell body within a single cisterna of the Golgi complex (*small arrowheads*) as shown at low magnification in *a* and at higher magnification in *b*. No staining is evident within the nuclear envelope (*large arrowheads*). After BFA treatment (*c, d*) staining is evident within tubular profiles, characteristic of cisternae of the endoplasmic reticulum, which extend into the cell periphery. The nuclear envelope (*large arrowheads*) is also labeled as shown at higher magnification in *d*. No stacked Golgi cisternae were observed under these conditions. *N*, nucleus. Scale bars: *a* and *c*, 1 μm; *b* and *d*, 200 nm.

tive VSV was not affected by nocodazole (data not shown), suggesting that efficient viral budding was taking place from the cell body. Collectively, these results indicate that, as shown previously for other cell types (Rogalski et al., 1984), the inhibitory effect of nocodazole on viral glycoprotein delivery is due to an overall reduction of protein transport to the processes corresponding to the dispersal of the Golgi apparatus rather than to a complete disruption of the exocytic pathway.

BFA affects the intracellular organization of cultured hippocampal neurons

Previous studies using a number of cell types, such as hepatocytes, rat kidney, AtT20, and HeLa cells, have shown that BFA, at a concentration ranging from 1 to 10 μg/ml, induces redistribution of Golgi proteins into the endoplasmic reticulum via long, tubulovesicular structures extending from the Golgi apparatus along microtubules (Fujiwara et al., 1988; Lippincott-Schwartz et al., 1989, 1990; Tooze and Hollinshead, 1992). Prior to examination of the effect of BFA on viral glycoprotein transport/sorting in hippocampal neurons, we determined whether BFA induced in these cells morphological changes in the tubular endosomes and the Golgi cisternae similar to those described previously for established cell lines.

The organization of the neuronal endocytic compartment after BFA treatment was analyzed by electron microscopy. By using the fluid phase marker horseradish peroxidase (HRP), we showed previously (Parton et al., 1992) that the neuronal early endosomes have a homogeneous tubulovesicular appearance. After incubating the cells in the presence of 10 μg/ml of BFA, the tubulovesicular endosomes became progressively longer and less vesicular, extending up to 10 μm (Fig. 2).

The effect of BFA on the Golgi apparatus in neurons was demonstrated for the *cis*/medial Golgi marker mannosidase II by immunofluorescence microscopy (Fig. 3). In untreated neurons the mannosidase II labeling is localized to the Golgi apparatus, which in these cells appears as a network of tubules close to a nuclear pole (Fig. 3*a,b*). After 15 min of BFA treatment, the staining became more diffuse and a dense punctate staining pattern appeared throughout the cell body and the initial segment of the dendrites (Fig. 3*c,d*), suggesting the disassembly of the Golgi apparatus.

We next used TPPase, a marker of medial/*trans*-Golgi cisternae (Griffiths et al. 1983), to analyze the effect of BFA on the organization of the Golgi apparatus. As in other cell types TPPase staining was restricted to one or two cisternae of the Golgi in control cells (Fig. 4*a,b*). In BFA-treated cells stacked Golgi cisternae were no longer observed and the TPPase staining was observed within the endoplasmic reticulum that surrounds the nucleus and extends into the cell periphery (Fig. 4*c,d*). Some groups of tubular/vesicular elements also showed weak TPPase reactivity after BFA treatment (results not shown). Whether these represented Golgi remnants as described by Hidalgo et al. (1992), elements of the TGN, or both is not clear. However, we can conclude from these experiments that the stacked Golgi cisternal organization is disrupted upon BFA treatment and that, as in other cells, there must be fusion of Golgi elements with the ER.

Consistent with the change of Golgi organization, BFA treatment also changed the distribution of β-COP, a molecule involved in the transport of membrane proteins from the endoplasmic reticulum to the Golgi apparatus (Pepperkok et al., 1993). In untreated cells the β-COP staining was clustered to one pole of the cell body (Fig. 5*a,b; e,f*), agreeing with the localization of the rough endoplasmic reticulum and Golgi apparatus of these cells (J. Krijnse-Locker, R. G. Parton, G. Griffiths, S. Fuller, and C. Dotti, unpublished observations). However, after 15 min of BFA treatment the staining appeared more diffuse in the cell body and was also evident in the proximal segment of the processes (Fig. 5*c,d; g,h*).

BFA does not block transport of viral glycoproteins to the plasma membrane but affects their normal distribution

The effect of BFA on protein transport and sorting in neurons was first studied by immunofluorescence microscopic analysis of SFV and FPV glycoprotein expression. The intracellular and surface-located glycoproteins were separately detected by using different fixation methods including or not permeabilization, respectively (see Materials and Methods). At short times after infection (1–3 hr) the SFV glycoprotein was found intracellularly, mainly restricted to the cell body (data not shown). However, at 5 hr postinfection the SFV glycoprotein was present on the cell body and dendritic surfaces (Fig. 6*b*). Addition of BFA at the third hour postinfection and further incubation for 2 hr more did not reveal any change in the surface distribution of the SFV glycoproteins, although the intensity of labeling was somewhat lower than in control untreated cells. Besides labeling on the dendritic surface, however, in 30–40% of the BFA-treated cells a punctate staining was also observed on processes with clear axonal characteristics (Fig. 6*d*).

As observed for the SFV glycoprotein, transport of the FPV HA glycoprotein to the plasma membrane was not blocked by BFA treatment, yet the normal surface distribution of the protein was altered. In untreated neurons, the HA glycoprotein was found in the cell body and axonal membrane (Fig. 7*b*). In BFA-treated neurons, the HA-specific labeling was not only seen associated with the axonal surface but also on the dendritic surface (Fig. 7*d*).

The overall effect of BFA on protein transport was further determined by measuring total protein secretion. Neurons cultured on dishes were metabolically labeled for 30 min in the presence of 5 μg or 10 μg/ml of BFA and, in the continuous presence of the drug, the radioactivity released to the medium was measured at different times postlabeling. As shown in Figure 8, protein secretion was only partially inhibited (20%) by BFA, irrespective of the concentration of BFA used.

The intracellular processing of SFV glycoproteins is altered by BFA

The above results suggested that sorting but not transport of newly synthesized viral glycoproteins is sensitive to BFA. Since BFA induces profound changes in the organization of the Golgi apparatus and the endoplasmic reticulum, we analyzed whether this drug may alter the normal processing of newly synthesized SFV glycoproteins.

All the structural proteins of SFV are synthesized as a polyprotein whose major component, p62, is the precursor form of the E2 and E3 glycoproteins. The p62 protein undergoes extensive posttranslational modifications. During chain elongation in the endoplasmic reticulum high mannose oligosaccharides are added that, in the different stacks of the Golgi apparatus, are trimmed and further modified to form complex glycoproteins containing *N*-acetylglucosamine, galactose, sialic acid, and fucose. Either in the TGN or during transport to the plasma membrane, the p62 protein is cleaved to yield E2 and E3 (de Curtis

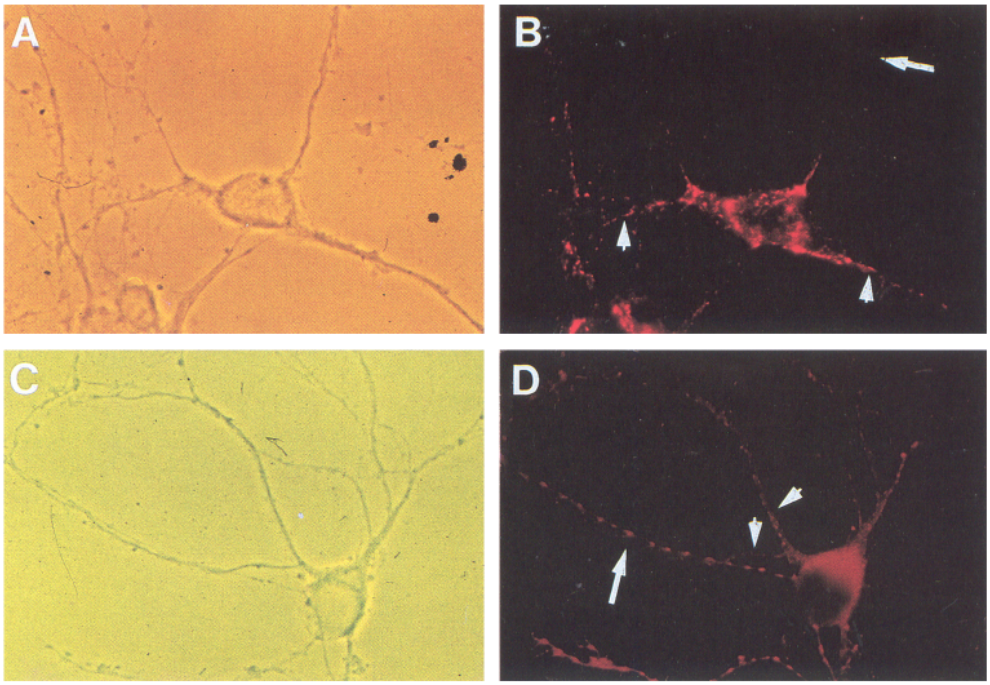

Figure 6. Effect of BFA on the dendritic distribution of the SFV-glycoprotein. *A* and *B*, Untreated cells. Incubation of infected cells with the 9AB4 antibody at 4°C before fixation and immunofluorescence microscopy shows the viral glycoprotein in the cell body and the dendrites (*arrowheads in B*). Processes with axonal appearance are unlabeled (*arrow in B*). *C* and *D*, BFA-treated cells. Incubation with 10 μg/ml of BFA in the last 2 hr of infection results in a similar pattern: dendritic labeling in the form of dispersed clusters on the dendrites (*arrowheads in D*). Some axonal labeling is also evident (*arrow*). The nature of the processes as axons and dendrites, thin and of uniform diameter and tapering with distance from the cell body, respectively, is unequivocal from the corresponding phase contrast photographs (*A, C*). Scale bar, 10 μm.

and Simons, 1988). Once in the plasma membrane the cytoplasmic domains of E1, E2, and E3 are recruited by the nucleocapsid and budding of a mature virus is initiated.

Hippocampal neurons were infected with SFV for 2 hr and the newly synthesized viral glycoproteins labeled with ^{35}S-methionine. Either at the time of infection or just before addition of the radioactive precursor BFA was added to the medium at 10 μg/ml. The presence of labeled viral glycoproteins at the neuronal surface was determined 2.5 hr later by surface biotinylation at 4°C followed by immunoprecipitation, recovery of the biotinylated immunoprecipitates with streptavidin-agarose, SDS-PAGE, and fluorography. The antibody used for the immunoprecipitation (mAb 9AB4) recognizes both the p62 and the E2 glycoproteins (Burke et al., 1983). As shown in Figure 9, in infected non-BFA-treated cells only the E2 glycoprotein reached the plasma membrane (lane 1b), indicating that efficient cleavage of p62 takes place before or during transport to the plasma membrane. In contrast, in SFV-infected cells, treated with BFA either 15 min before the metabolic pulse (lane 2b) or from the beginning of infection (lane 3b), both p62 and E2 were evident

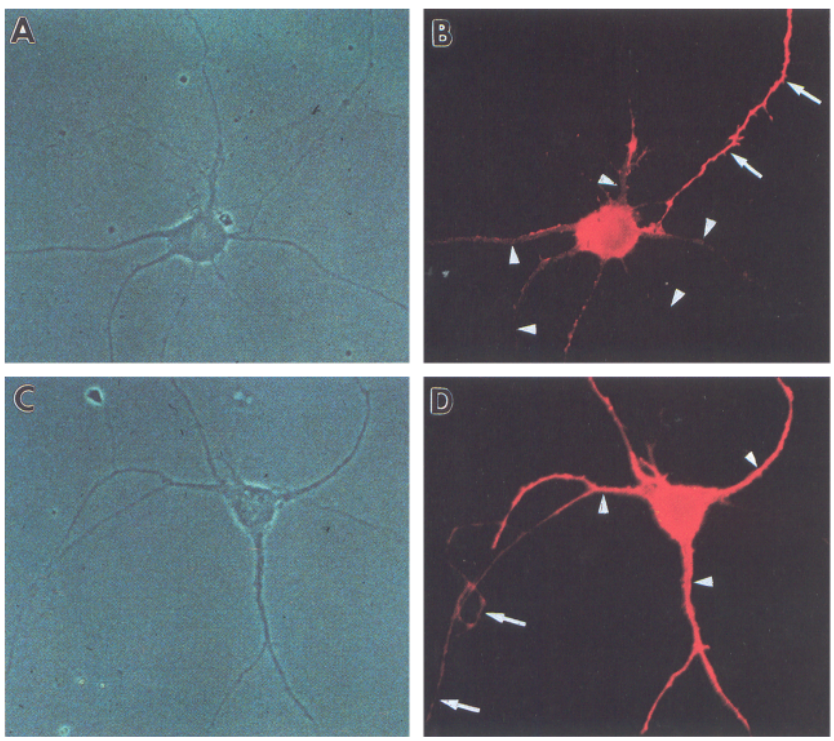

Figure 7. BFA induces redistribution of the FPV HA glycoprotein to the dendrites of infected hippocampal neurons. *A* and *B*, Untreated cells. The HA glycoprotein is observed on the surface of the cell body and one process of fixed, unpermeabilized cells. It ended in a noninfected cell. The thick processes (*arrowheads*) are the cell's dendrites and appear devoid of labeling except at sites of axonal contacts, probably from other infected cells. *C* and *D*, BFA-treated neurons. Addition of 10 μg/ml of BFA during the last 3 hr of infection induces appearance of HA labeling on the dendritic surface (*arrowheads*). Axonal labeling persists (*arrows*). Scale bar, 10 μm.

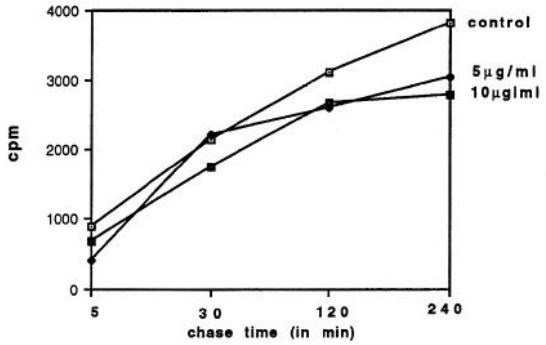

Figure 8. Effect of BFA on total protein secretion. Hippocampal cells were pulse-labeled with ^{35}S-methionine in 1 ml of neuronal medium for 30 min. The cells were then washed in medium containing excess cold methionine and chased for 30 min, 2 hr, and 4 hr in the presence of 5 µg/ml or 10 µg/ml of BFA. Aliquots were taken at the end of the pulse and at different chase intervals, proteins precipitated with trichloroacetic acid (10%), and radioactivity counted. The average data from two experiments is shown. A reduction of 20% in the amount of radioactive protein occurred at both BFA concentrations.

on the plasma membrane. Densitometric tracings of the autoradiographic bands of representative blots like that of Figure 9 revealed that BFA induced a reduction of 20% (when BFA was added 15 min before the pulse) and 35% (BFA added at the time of infection) in the total amount of viral protein on the plasma membrane. In addition, the BFA treatment changed the relative amount of p62 that reached the plasma membrane, from less than 5% of the total biotinylated protein in untreated cells (lane 1b) to nearly 60% in BFA-treated cells (lane 2b). Finally, in BFA-treated cells the E2 glycoprotein appeared of lower molecular weight than in untreated cells, probably as a result of defective glycosylation.

These results demonstrate that BFA is able to change the intracellular processing of the glycoproteins (shift in E2 molecular weight and presence of p62 on the membrane), but not to abolish their transport to the plasma membrane.

Discussion

BFA has been widely used to dissect the machinery involved in several steps of intracellular traffic (Lippincott-Schwartz et al., 1991). Different cell types react in a different manner to the drug. In most cases, BFA disassembles the Golgi stacks and this perturbs membrane traffic and leads to protein accumulation in the endoplasmic reticulum (Armstrong and Warren, 1990; Klausner et al., 1992). There are, however, some exceptions to this effect of BFA. In kangaroo rat (PtK_1) cells the Golgi complex is resistant to the drug at concentrations that normally have a dramatic effect in other cell types (Kistakis et al., 1991). In MDCK epithelial cells, depending on the concentration of BFA used, the TGN is unaffected or completely disassembled (Hunziker et al., 1991; Low et al., 1991; Wagner et al., 1994). In hypothalamic neurons, large concentrations of intracerebrally injected BFA perturb the structure of the Golgi complex but do not affect the secretion of vasopressin and oxytocin (Pow and Morris, 1992).

In addition, BFA treatment has been shown to affect protein sorting in MDCK cells (Low et al., 1991, 1992). Apical delivery of secretory proteins and dipeptidyl peptidase IV, a transmembrane apical protein, is impaired by the drug. This decrease in apical exocytosis leads to enhanced basolateral secretion and to increased delivery of dipeptidyl peptidase to the basolateral medium. Matter et al. (1993) showed that BFA affects the sorting in the TGN to both the basolateral and apical surfaces, although transport to the plasma membrane still occurs (Matter et al., 1993). Missorting without an effect on protein transport has also

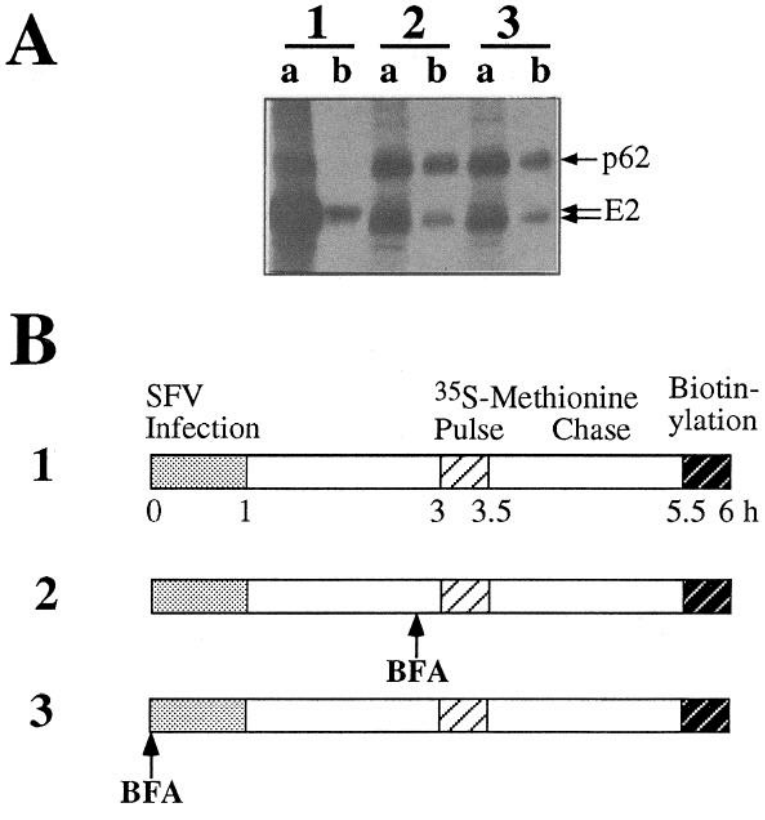

Figure 9. Fluorography of streptavidin-agarose precipitated biotinylated SFV E2 glycoprotein. *Lanes 1*, control untreated neurons. In *lanes 2*, BFA (10 µg/ml) was added at the time of infection and maintained throughout the entire experiment. In *lanes 3*, BFA was added 15 min before metabolic labeling. *Lanes a*, total immunoprecipitated material; *lanes b*, eluates of the streptavidin-agarose precipitation. In control cells only the E2 viral protein is present on the plasma membrane. In BFA-treated neurons p62 is also detected. The *arrows* point to the E2 glycoprotein and to p62. Note the slight shift in E2 molecular weight in the BFA-treated cells.

been found for the transcytosis of mutated LDL receptors and for the exocytosis of the HA glycoprotein in MDCK cells (Matter et al., 1993; Wagner et al., 1994).

In the present study we examined the effects of BFA in a model system, the rat hippocampal neuron culture, and found that transport to the plasma membrane of newly synthesized HA and E2 viral glycoproteins takes place normally, although their polarized distribution is changed by BFA.

Our data leave little doubt that the viral glycoproteins had reached the plasma membrane despite the fact that BFA disassembled the Golgi stacks and induced the dissociation of β-COP. The appearance of the viral glycoproteins in the plasma membrane was demonstrated by immunofluorescence microscopy of fixed nonpermeabilized neurons and by surface biotinylation. By the latter means we detected in the plasma membrane of SFV-infected/BFA-treated cells not only the E2 glycoprotein but also its precursor, p62. Moreover, the E2 glycoprotein displayed a molecular weight slightly lower than normal. This could result from incomplete glycosylation due to the BFA effects on the organization of the Golgi apparatus. Such change in molecular size, however, does not seem to be caused by a failure in sialic acid addition, because neuraminidase treatment of biotinylated E2 from BFA-treated and untreated cells changed its electrophoretic mobility in a similar manner (data not shown). On the other hand, the appearance of p62 in the plasma membrane is a very characteristic feature induced by BFA. Since the cleavage of p62 into E2 normally takes place in the TGN and during transport to the plasma membrane, the presence of p62 in the surface might result from a shift in distribution of the responsible proteases, that is, from the Golgi apparatus to the plasma membrane, induced by BFA. Alternatively, BFA might be altering the optimal conditions for cleavage in the exocytic vesicles, for example, by modifying their pH. The formal possibility also exists that some of the p-62 and E2 present in the plasma membrane of the BFA-treated neurons might result from direct transport from the rough endoplasmic reticulum.

Altogether, these results show that although BFA alters three important functions of the TGN (sorting, glycosylation, and proteolytic cleavage), the secretory pathway remains functional and transport to the plasma membrane is still possible.

What are the molecular targets of BFA? In non-neuronal cells BFA treatment inhibits nucleotide exchange in ARF proteins (Donaldson et al., 1991a,b), small GTP-binding proteins involved in regulating coat assembly in the Golgi complex during intra-Golgi transport (Stearns et al., 1990; Serafini et al., 1991). Due to this inhibition, β-COP and other coat components cannot associate with the Golgi membranes, leading to the disassembly of the Golgi apparatus (Robinson and Kreis, 1992). BFA treatment has also been shown to induce release of γ-adaptin and clathrin from the TGN (Wagner et al., 1994). Therefore, BFA may have altered protein sorting in the neurons by a mechanism similar to that operating in non-neuronal cells. The effect of BFA on protein sorting may also involve trimeric GTP-binding proteins. For example, AlF_4, which activates trimeric G proteins, inhibits the release of γ-adaptin by BFA treatment (Wagner et al., 1994). Apical and basolateral sorting in perforated epithelial cells (Pimplikar and Simons, 1993) as well as transcytosis (Bomsel and Mostov, 1992) also seem to be under control of these proteins.

The data obtained with BFA on protein sorting in hippocampal neurons further confirm the involvement of different sets of BFA targets for protein sorting and transport in the Golgi apparatus and, in view of the similar effects in different polarized cells, strengthen the idea that the sorting and transport machinery is conserved among different cell types. The elucidation of the specific components have to be explained in molecular terms by future work.

The similarities in the membrane sorting pathway do not extend to the role of microtubules in MDCK cells and neurons. In MDCK cells, the minus ends of the microtubules are mainly underneath the apical membrane while the plus ends are toward the basal membrane (Bacallao et al., 1989). Apically, there are also microtubules running parallel to the surface with mixed polarity. In the hippocampal neurons the axonal microtubules are uniformly oriented with their plus ends distal to the cell body. The microtubules in the dendrites close to the cell body are of mixed polarity while the microtubule extending into the distal regions of the dendrites are of uniform polarity with their plus ends distally like in the axons (Baas et al., 1988). From previous work it is well known that microtubules play a role in the delivery of axonal proteins from the Golgi complex to the axonal surface (Hammerschlag and Brady, 1988). Newly synthesized axonal proteins use kinesin as a motor to move distally (Brady et al., 1990; Hirokawa et al., 1991). Transport of the influenza HA was blocked in the cell body as expected by treating the hippocampal cultures with nocodazole. Also, transport of apical proteins in MDCK cells from the TGN to the apical side is facilitated by microtubules (Rindler et al., 1987). The microtubular motor employed in MDCK cells has not yet been identified but in contrast to axons, the movement is toward the minus ends. In MDCK cells the basolateral transport of the VSV glycoprotein from the TGN is not affected by depolymerization of the microtubules (Salas et al., 1986). However, we show here that dendritic transport from the Golgi complex is clearly inhibited by nocodazole treatment of the neurons. Most likely, the viral glycoproteins remain in the cell body because the axonal and the dendritic carrier vesicles require microtubules for movement out into the neurites. We assume, in analogy with MDCK cells, that sorting into carriers occurs in the absence of microtubules and that microtubules are involved in providing tracks for moving vesicles to their final destination. It has been proposed that dendritic carrier vesicles would use microtubules having their plus ends in the cell body to differentiate them from axonal microtubules. However, these tracks would not carry the vesicles all along the way toward the distal part of the dendrites. Therefore, it would be possible that the microtubule tracks are marked by specific proteins (e.g., MAP2, dendritic) and that the putative carrier vesicles carry tags that position them on the right tracks. Alternatively, plus end–directed motor proteins different from the kinesin family (Gho et al., 1992) could determine the correct pathway of the exocytic vesicles.

The sorting and targeting of cell surface proteins in neurons is a fascinating subject that has now become accessible to experimental analysis. It is encouraging that strategies that have been useful in dissecting epithelial polarity are proving their worth once again. The future challenge will be to describe in molecular terms the mechanisms responsible for generating and maintaining cell polarity of both neurons and epithelial cells.

References

Armstrong J, Warren G (1990) Violating the one-way system. Nature 344:383–385.

Baas P, Deitch J, Black M, Banker G (1988) Polarity orientation of microtubules in hippocampal neurons: uniformity in the axon and

nonuniformity in the dendrites. Proc Natl Acad Sci USA 85:8335–8339.

Bacallao R, Antony C, Dotti C, Karsenti E, Stelzer E, Simons K (1989) The subcellular organization of Madin-Darby canine kidney cells during the formation of a polarized epithelium. J Cell Biol 109:2817–2832.

Banker G, Cowan W (1977) Rat hippocampal neurons in dispersed cell culture. Brain Res 126:397–425.

Bergmann J, Singer S (1983) Immunoelectron microscopic studies of the intracellular transport of the membrane glycoprotein (G) of vesicular stomatitis virus in infected Chinese hamster ovary cells. J Cell Biol 97:1777—1787.

Bergmann J, Tokayasu K, Singer S (1981) Passage of an integral membrane protein, the Vesicular Stomatitis virus glycoprotein, through the Golgi apparatus en route to the plasma membrane. Proc Natl Acad Sci USA 78:1746–1750.

Bomsel M, Mostov K (1992) Role of heterotrimeric G proteins in membrane traffic. Mol Biol Cell 3:1317–1328.

Brady S, Pfister K, Bloom G (1990) A monoclonal antibody against the heavy chain of kinesin inhibits both anterograde retrograde axonal transport in isolated squid axoplasm. Proc Natl Acad Sci USA 87: 1061–1065.

Breitfeld PP, McKinnon WC, Mostov KE (1990) Effect of nocodazole on vesicular traffic to the apical and basolateral surfaces of polarized Madin-Darby canine kidney cells. J Cell Biol 111:2365–2373.

Burke B, Walter C, Griffiths G, Warren G (1983) Viral glycoprotcins at different stages of intracellular transport can be distinguished using monoclonal antibodies. Eur J Cell Biol 31:315–324.

Cameron P, Sudhof T, Jahn R, de Camilli P (1991) Colocalization of synaptophysin with transferrin receptors: implications for receptor biogenesis. J Cell Biol 115:151–164.

de Curtis, Simons K (1988) Dissection of Semliki Forest virus glycoprotein delivery from the *trans*-Golgi network to the cell surface in permeabilized BHK cells. Proc Natl Acad Sci USA 85:8052–8056.

Donaldson J, Kahn R, Lippincott-Schwartz J, Klausner R (1991a) Binding of ARF and B-COP to Golgi membranes: possible regulation by a trimeric G protein. Science 254:1197–1199.

Donaldson J, Lippincott-Schwartz J, Klausner R (1991b) 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.

Dotti C, Banker G (1991) Intracellular organization of hippocampal neurons during the development of neuronal polarity. J Cell Sci 15: 75–84.

Dotti C, Simons K (1990) Polarized sorting of viral glycoproteins to the axon and dendrites of hippocampal neurons in culture. Cell 62: 63–72.

Dotti C, Parton R, Simons K (1991) Polarized sorting of glypiated proteins in hippocampal neurons. Nature 349:158–161.

Dotti C, Kartenbeck J, Simons K (1993) Polarized delivery of the viral glycoproteins of Vesicular Stomatitis, Fowl Plague, and Semliki Forest viruses in hippocampal neurons in culture: a light and electron microscopy study. Brain Res 610:141–147.

Fujiwara T, Oda K, Yokota S, Takatsuki A, Ikehara Y (1988) Brefeldin A causes disassembly of the Golgi complex and accumulation of the secretory proteins in the endoplasmic reticulum. J Biol Chem 263: 18545–18552.

Fuller S, von Bonsdorf K-H, Simons K (1985) Cell surface influenza hemagglutinin can mediate infection by other animal viruses. EMBO J 4:2475–2485.

Gho M, McDonald K, Ganetzky B, Saxton W (1992) Effect of kinesin mutations on neuronal functions. Science 258:313–316.

Gilbert T, Le Bivic A, Quaroni A, Rodriguez-Boulan E (1991) Microtubular organization and its involvement in the biogenetic pathways of plasma membrane proteins in Caco-2 intestinal epithelial cells. J Cell Biol 113:275–288.

Goslin K, Banker G (1991) Rat hippocampal neurons in low density cultures. In: Culturing nerve cells (Banker G, Goslin F, eds), pp 251–281. Cambridge, MA: MIT Press.

Griffiths G, Quinn P, Warren G (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.

Griffiths G, Pfeiffer S, Simons K, Matlin K (1985) Exit of newly synthesized membrane proteins from the trans cisterna of the Golgi complex to the plasma membrane. J Cell Biol 101:949–964.

Hammerschlag R, Brady S (1988) The cytoskeleton and axonal transport. In: Basic neurochemistry (Siegel G, Alberts B, Agranoff B, Molinof P, eds), pp 457–478. New York: Raven.

Hidalgo J, Garcia-Navarro R, Gracia-Navarro F, Perez-Vilar J, Velasco A (1992) Presence of Golgi remnant membranes in the cytoplasm of brefeldin-A treated cells. Eur J Cell Biol 58:214–227.

Hirokawa N, Sato-Yoshitake R, Kobayashi N, Pfister K, Bloom G, Brady S (1991) Kinesin associates with anterogradely transported organelles *in vivo*. J Cell Biol 114:295–302.

Hunziker W, Whitney JA, Mellman I (1991) Selective inhibition of transcytosis by brefeldin A in MDCK cells. Cell 67:617–628.

Kelly RB (1990) Microtubules, membrane traffic and cell organization. Cell 61:5–7.

Kistakis N, Roth M, Bloom G (1991) PtK1 cells contain a nondiffusible, dominant factor that makes the Golgi apparatus resistant to the effect of brefeldin A. J Cell Biol 113:1009–1023.

Klausner R, Donaldson J, Lippincott-Schwartz J (1992) Brefeldin A: insights into the control of membrane traffic and organelle structure. J Cell Biol 116:1071–1080.

Kobayashi T, Storrie B, Simons K, Dotti C (1992) A functional barrier to movement of lipids in polarized neurons. Nature 359:647–650.

Laemmli UK (1970 Cleavage of structural proteins during the assembly of the head bacteriophage T4. Nature 227:680–685.

Lippincott-Schwartz J, Yuan L, Bonifacino J, Klausner R (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.

Lippincott-Schwartz J, Donaldson JG, Schweizer A, Berger EG, Hauri HP, Yuan LC, Klausner RD (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.

Lippincott-Schwartz J, Yuan LC, Tipper C, Amherdt M, Orci L, Klausner RD (1991) Brefeldin A's effects on endosomes, lysosomes and the TGN suggests a general mechanism for regulating organelle structure and membrane trafficking. Cell 67:601–616.

Low SH, Tang BL, Wong SH, Hong W (1992) Selective inhibition of protein targeting to the apical domain of MDCK cells by brefeldin A. J Cell Biol 118:51–62.

Low S, Wong S, Tang B, Tan P, Subramanian V, Hong W (1991) Inhibition by brefeldin A of protein secretion from the apical cell surface of Madin-Darby canine kidney cells. J Biol Chem 266:17729–17732

Matlin KS, Reggio H, Helenius A, Simons K (1981) Infection entry pathway of influenza virus in a canine kidney cell line. J Cell Biol 91:601–613.

Matlin KS, Reggio H, Helenius A, Simons K (1982) Pathway of Vesicular Stomatitis virus entry leading to infection. J Mol Biol 156: 609–631.

Matter K, Whitney JA, Yamamoto EM, Mellman I (1993) Common signals control low density lipoprotein receptor sorting in endosomes and the Golgi complex of MDCK cells. Cell 74:1053–1064.

Misumi Y, Miki A, Takatsuki A, Tamura G, Ikehara Y (1986) Novel blockade by brefeldin A of intracellular transport of secretory proteins in cultured rat hepatocytes. J Biol Chem 261:11389–11403.

Moremen K, Touster O (1985) Biosynthesis and modification of Golgi mannosidase II in HeLa and 3T3 cells. J Biol Chem 260:6654–6662.

Oda K, Hirose S, Takami N, Misumi Y, Takatsuki A, Ikehara Y (1987) Brefeldin A arrests the intracellular transport of a precursor of complement C3 before its conversion site in rat hepatocytes. FEBS Lett 214:135–138.

Parton R, Simons K, Dotti C (1992) Axonal and dendritic endocytic pathways in cultured neurons. J Cell Biol 119:123–137.

Pepperkok R, Scheel R, Horstmann H, Hauri H-P, Griffiths G, Kreis T (1993) Cell 74:1–12.

Pietrini G, Matteoli M, Banker G, Caplan M (1992) Isoforms of the Na, K-ATPase are present in both axons and dendrites of hippocampal neurons in culture. Proc Natl Acad Sci USA 89:8414–8418.

Pimplikar SW, Huttner WB (1992) Chromogranin B (secretogranin I), a secretory protein of the regulated pathway, is also present in a tightly membrane-associated form in PC12 cells. J Biol Chem 267: 4110–4118.

Pimplikar SW, Simons K (1993) Regulation of apical transport in ep-

ithelial cells by a Gs class of heterotrimeric G protein. Nature 362: 456–458.

Pow D, Morris J (1992) Tunicamycin, puromycin, and brefeldin A influence the subcellular distribution of neuropeptides in hypothalamic magnocellular neurones of the rat. Cell Tissue Res 269:547–560.

Rindler M, Ivanov I, Sabatini D (1987) Microtubule-acting drugs lead to the non-polarized delivery of the influenza hemagglutinin to the cell surface of polarized Madin-Darby canine kidney cells. J Cell Biol 104:231–241.

Robinson M, Kreis T (1992) Recruitment of coat proteins onto Golgi membranes in intact and permeabilized cells: effects of Brefeldin A and G protein activators. Cell 69:129–138.

Rodriguez-Boulan E, Pendergast M (1980) Polarized distribution of viral envelope glycoproteins in the plasma membrane of infected epithelial cells. Cell 20:45–54.

Rogalski AA, Bergmann JE, Singer SJ (1984) Effect of microtubule assembly status on the intracellular processing and surface expression of an integral protein of the plasma membrane. J Cell Biol 99:1101–1109.

Roman L, Garoff H (1986) Alteration of the cytoplasmic domain of the membrane spanning glycoprotein p62 of Semliki Forest virus does not affect its polar distribution in established lines of Madin-Darby canine kidney cells. J Cell Biol 103:2603–2618.

Salas P, Misek D, Vega-Salas D, Gundersen D, Cereijido M, Rodriguez-Boulan E (1986) Microtubules are not critically involved in the biogenesis of epithelial cell surface polarity. J Cell Biol 102:1853–1867.

Serafini T, Orci L, Amherdt M, Brunner M, Kahn R, Rothman J (1991) ADP-ribosylation factor (ARF) is a subunit is a subunit of the coat of Golgi-derived COP-coated vesicles: a novel role for a GTP-binding protein. Cell 67:239–253.

Stearns T, Willingham M, Botstein D, Kahn R (1990) ADP ribosylation factor is functionally and physically associated with the Golgi complex. Proc Natl Acad Sci USA 87:1238–1242.

Takatsuki A, Tamura G (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. Agr Biol Chem 49:899–902.

Tooze J, Hollinshead M (1992) In AtT20 and HeLa cells brefeldin A induces the fusion of tubular endosomes and changes their distribution and some of their endocytic properties. J Cell Biol 118:813–830.

van der Sluijs P, Bennett M K, Antony C, Simons K, Kreis TE (1990) Binding of exocytic vesicles from MDCK cells to microtubules *in vitro*. J Cell Sci 95:545–553.

Wagner M, Rajasekaran AK, Hanzel DK, Mayor S, Rodriguez-Boulan E (1994) Brefeldin A causes structural and functional alterations of the *trans*-Golgi network of MDCK cells. J Cell Sci 107:933–943.