Brefeldin-A Inhibits the Delivery of the Polymeric Immunoglobulin Receptor to the Basolateral Surface of MDCK Cells*

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We have studied the effects of brefeldin A (BFA) on the polarized delivery of the polymeric immunoglobulin receptor to the basolateral surface of MDCK cells. Unlike the delivery of several other basolateral membrane and secretory proteins, the delivery of the polymeric immunoglobulin receptor from the trans-Golgi network to the cell surface was inhibited by BFA. The effect of BFA treatment was apparent at 1.0 μg/ml (36% inhibition), and maximal inhibition was observed at 10 μg/ml (70% inhibition). The delivery of the receptor from the endoplasmic reticulum to the basolateral surface was even more sensitive to the effect of BFA; delivery was inhibited 95% in cells treated with 1 μg/ml BFA. The selective action of BFA on the basolateral delivery of the polymeric immunoglobulin receptor suggests that there may be multiple pathways for delivery of proteins to the basolateral cell surface of MDCK cells.

Brefeldin A (BFA) is a fungal metabolite that has dramatic effects on the structure and function of the cellular biosynthetic and endocytic pathways. In many cells, BFA treatment causes the Golgi apparatus to disperse and fuse with the ER (1–3). One result of this fusion is that protein secretion is inhibited in drug-treated cells (4, 5). An additional effect is that the N-linked, high-mannose sugars of ER resident proteins are processed by enzymes normally residing in the Golgi apparatus (2, 6). As a result, these branched sugar molecules become insensitive to the action of endoglycosidase H, an enzyme that selectively removes high-mannose sugars present on newly synthesized proteins or resident ER proteins. BFA treatment also alters the distribution of Golgi-associating proteins. One of the earliest events in the disassembly of the Golgi apparatus is the redistribution of β-Cop, the 110-kDa subunit of the coat protein complex, from the Golgi stacks to the cytosol (7, 8). Similarly, BFA causes the rapid and reversible redistribution of a 200-kDa protein from the Golgi apparatus to the cytosol (9) and of γ-adaptin from the trans-Golgi network (TGN) to the cytosol (10, 11). The action of BFA is not limited to the Golgi apparatus as it also alters the endosomal compartment. In many cell types, an extensive endosomal tubular network is formed that fuses with the TGN (12, 13). Additionally, lysosomes extend long tubular processes in drug-treated cells (12).

The effect of BFA on membrane trafficking in epithelial cells is now mostly being explored. Polarized epithelial cells, e.g. Madin-Darby canine kidney (MDCK) cells, function to regulate the exchange of molecules between an underlying tissue and the external milieu. One of the hallmarks of epithelial cells is that they have apical and basolateral plasma membrane domains which maintain distinct protein and lipid compositions. At least two pathways are used by epithelial cells to achieve this polarity (reviewed in Ref. 14). The first mechanism allows for the direct delivery of membrane proteins and lipids from the TGN to the appropriate plasma membrane domain. The second mechanism is the sorting of membrane proteins after endocytosis from either cell surface. Endocytosed proteins can either be recycled, delivered to lysosomes, or transcytosed to the opposite cell surface. In hepatocytes, transcytosis is the only known mechanism for the apical delivery of membrane proteins. In other cell types, e.g. the intestinal cell line CaCo-2, some proteins are delivered directly to the apical cell surface while other proteins are transcytosed from the basolateral to the apical plasma membranes.

Many of the known effects of BFA on polarized epithelia come from work on MDCK cells. Interestingly, in MDCK cells and in the kangaroo rat cell line PTK-1, the Golgi stacks remain morphologically intact in BFA-treated cells (15, 16). However, the function of this organelle may be altered. In MDCK cells, BFA selectively inhibits the apical secretion of a number of endogenous soluble proteins, while secretion of basolateral proteins is unaffected or in some cases increased (5, Low et al. (17) have recently reported that in BFA-treated MDCK cells dipeptidyl peptidase IV (DPPIV), a membrane protein that is normally delivered to the apical surface of these cells, is mis-targeted to the basolateral cell surface. In contrast, the delivery of another membrane protein, urokinase, to the basolateral cell surface is unaffected by similar treatment (17). Further evidence for the selective action of BFA on delivery to the apical cell surface comes from work with the influenza hemagglutinin (HA). When expressed in MDCK cells, this protein is delivered directly apically (18). BFA treatment reduces apical delivery of wild-type HA and causes increased expression of this protein at the basolateral cell surface. In contrast, BFA has little effect on the delivery to the basolateral cell surface of a mutant HA in which Cys543 has been mutated to a tyrosine residue (19). BFA also

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The abbreviations used are: BFA, brefeldin-A; HA, influenza hemagglutinin; MDCK, Madin-Darby canine kidney cells; pGK, polymeric immunoglobulin receptor; SC, secretory component; TGN, trans-Golgi network; ER, endoplasmic reticulum; MEM, minimum essential medium; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis.

m. Roth, personal communication.
affects the morphology and function of the endosomal compartments of MDCK cells (20). Tubular endosomes are formed both apically and basolaterally in drug-treated cells, and, while the apical endocytosis of horseradish peroxidase and ricin are stimulated, basolateral endocytosis is unaffected. Transcytosis of these two proteins is stimulated in the basolateral to apical direction (20). In contrast, transcytosis of IgA by the polymeric immunoglobulin receptor (pIgR) in the basolateral to apical direction is inhibited by drug treatment (16).

The transcytotic pathway of the pIgR in epithelial cells has been well characterized (21). This protein functions in transport of polymeric immunoglobulins (IgA and IgM) from the underlying tissues of epithelia into secretions, and, when expressed in MDCK cells, the pIgR functions as in vivo. The receptor is initially synthesized in the ER and then transported to the Golgi apparatus. From the TGN, the pIgR is delivered directly to the basolateral cell surface. The sorting signal for this step resides in a 17-amino acid stretch of the cytoplasmic domain of the pIgR (22). At the basolateral plasma membrane, the pIgR binds its ligand. The ligand-receptor complex is endocytosed and subsequently delivered to the apical plasma membrane where the ligand binding domain of the receptor is proteolytically cleaved and released with its bound ligand into the apical secretions. This cleaved form of the receptor is termed secretory component (SC).

The effect of BFA on the polarized delivery of the pIgR to the basolateral surface of MDCK cells has been explored. We chose to study this particular protein because it has a well characterized basolateral sorting signal and because its intracellular-trafficking is well understood. We report that unlike the delivery of other basolateral membrane proteins, the basolateral delivery of the pIgR is inhibited by BFA. This result suggests that there may be distinct pathways for delivery of proteins to the basolateral cell surface of MDCK cells, and these pathways may be distinguished by their sensitivity to BFA.

**Experimental Procedures**

**Cells and Cell Culture**—MDCK cells expressing the wild-type pIgR have been described (23). The cells were maintained in MEM (CellGro; Medistetch, Washington, D.C.) supplemented with 10% (v/v) fetal bovine serum (Hyclone), 100 units/ml penicillin, and 100 μg/ml streptomycin in 5% CO₂, 95% air. For all experiments, cells were cultured on 0.4-μm Transwells (Costar) as described (23) and used 3–4 days post culture. Cells were split 1:10 and passed at least once weekly.

**Metabolic Labeling**—Filter-grown cells were washed with warm (37 °C) PBS* (PBS containing 0.5 mM MgCl₂ and 0.9 mM CaCl₂) and then starved for 15 min at 37 °C in MEM, supplemented with Hanks’ balanced salts, 20 mM Hepes, pH 7.4, 5% (v/v) dialyzed fetal bovine serum. Cells were then pulse-labeled for 15 min in the same medium containing 3 μCi/ml [³⁵S]methionine (1130 Ci/mmol; Du Pont-New England Nuclear). Chases were performed in MEM/BSA (MEM containing Hank’s balanced salt solution, 20 mM Hepes, pH 7.4, 0.6% BSA).

**Immunoprecipitation**—Metabolically labeled SC was immunoprecipitated from SDS-treated and boiled media, and full-length pIgR was immunoprecipitated from SDS-treated and boiled cell lysates using sheep anti-rabbit SC antibodies coupled to protein G-Sepharose as described (23). Immunoprecipitates were washed and resolved by SDS-PAGE, and the gel was impregnated with 2,5-diphenyloxazole (Sigma) prior to dry-fluorography, and exposed to x-ray film.

**Endo H Digestion**—Radiolabeled pIgR was immunoprecipitated as described above. Following the washes, the immunoprecipitates were resuspended in 60 μl of 100 mM sodium citrate, pH 6.5, 1.0% (w/v) SDS and boiled for 5 min. Three microliters of Endo H (1000 units/ml; Boehringer Mannheim) were added to one-half of the supernatant, and aliquots of Endo H-treated and nontreated samples were incubated overnight at 37 °C. An equal volume of sample buffer was added to the samples, and proteins were resolved by SDS-PAGE. The gel was dried, fluorographed, and exposed to x-ray film.

**Cell Surface Delivery Assay**—To measure TGN to cell surface delivery of the pIgR, filter-grown cells were metabolically labeled as detailed above, washed with MEM/BSA equilibrated at 18.5 °C, and incubated for 90 min at 18.5 °C. Cells were then treated with 0–20 μg/ml BFA (stored as a 10 mg/ml stock in dimethyl sulfoxide at –20 °C; EpiCenter Technologies, Madison, WI) for an additional 15 min at 18.5 °C. Cells were subsequently incubated at 37 °C in MEM/BSA containing the appropriate BFA concentration for 60 min in the presence or absence of 25 μg/ml V8 protease (Boehringer Mannheim) added to the basolateral compartment of the Transwell. To measure ER to cell surface delivery of the pIgR, filter-grown cells were metabolically labeled in the presence of BFA and then chased in the presence of BFA and basolateral V8 as described above. At the end of the proteinase treatment, the media were collected and the cells were washed three times with ice-cold MEM/BSA containing 10% horse serum (Sigma). SC and full-length pIgR were immunoprecipitated as described above. Radiolabeled pIgR and SC was resolved by SDS-PAGE, and the amount of radioactive pIgR and SC was quantitated in a Molecular Dynamics PhosphorImager using Image-Quant software.

The total amount of labeled pIgR is obtained by summing the amount of cell-associated pIgR and that cleaved to SC in the apical and basolateral media in cells not treated with proteinase. In non-BFA-treated cells, the total amount of immunoprecipitable pIgR is reduced in V8-treated cells. This is a result of radiolabeled receptor being digested by the protease as it is delivered to the basolateral cell surface. The percent of basolateral delivery of pIgR can be derived from the reduced pIgR signal using Equation 1.

\[
\left(1 - \frac{\text{the amount of SC in the apical media and pIgR present in cells following treatment with V8}}{\text{the total amount of labeled pIgR and SC in non-proteinase-treated cells}}\right) \times 100 \\
\text{(Eq. 1)}
\]

Upon arrival at the apical surface of cells, the pIgR is cleaved by an endogenous proteinase to SC. This is an efficient process as the amount of cell-associated pIgR is not altered by the addition of V8 proteinase to the apical chamber of the Transwell (see Fig. 2A). The amount of SC released into the apical medium of cells treated with basolateral V8 reflects pIgR molecules that are delivered directly apically. The percent of direct apical delivery can be derived from Equation 2.

\[
\left(\frac{\text{the amount of SC present in the apical media following basolateral V8 treatment}}{\text{the total amount of labeled pIgR in non-proteinase-treated cells}}\right) \times 100 \\
\text{(Eq. 2)}
\]
grids, stained with 8.0% (w/v) lead citrate, and viewed in a Zeiss (West Germany) EM-10 electron microscope.

RESULTS

The pIgR is delivered in a signal-dependent fashion directly from the TGN to the basolateral surface of MDCK cells (22). We were interested in determining if BFA alters the polarized delivery of this protein to the basolateral cell surface. To assay for cell surface delivery, MDCK cells expressing the pIgR were pulse-labeled with [35S]cysteine, and then chased for 90 min at 18.5 °C to allow newly synthesized pIgR molecules to accumulate in a post-ER compartment, presumably the TGN. To assess the intracellular location of the pIgR in the biosynthetic pathway following this treatment, labeled receptor was treated with endo H. The reduced mobility of newly synthesized receptor following endo H treatment was small but reproducible (Fig. 1). Following the 90-min incubation at 18.5 °C, approximately 85% of the receptor was insensitive to the action of endo H (Fig. 1). These data confirm that under these conditions the pIgR had exited from the ER and entered the Golgi apparatus where high mannose sugars are converted to their endo H-insensitive form.

Following the 90-min incubation at 18.5 °C, cells were pre-treated for 15 min at 18.5 °C with 0–20 µg/ml BFA. Delivery to the basolateral cell surface was assessed by the addition of V8 proteinase to the medium bathing the basolateral surface of the cells during a 1-h incubation at 37 °C in the continuous presence of 0–20 µg/ml BFA.

The delivery of the pIgR in non-BFA treated cells is shown in lanes 1–6 of Fig. 2A. In the presence of basolateral V8, receptor molecules delivered to the basolateral cell surface were rapidly proteolyzed and could no longer be immunoprecipitated as full length pIgR (compare lanes 1 and 3 in Fig. 2A). Under these conditions, approximately 77% of the pulse-labeled pIgR was delivered directly to the basolateral surface (Fig. 2B). In contrast, receptors delivered to the apical cell surface were cleaved to SC by an endogenous apical proteinase and released into the apical medium. In control experiments, 17% of newly synthesized pIgR was delivered directly apically (Fig. 2C). V8 proteinase was added to the apical compartment of the Transwell in some experiments to detect pIgR molecules that had reached the apical plasma membrane but had not yet been cleaved to SC (Fig. 2A, lane 2). In non-BFA-treated cells, there was no decrease in the amount of immunoprecipitable pIgR upon addition of apical V8, indicating that cleavage of the receptor to SC at the apical surface was efficient.

The effect of BFA on the delivery of the pIgR from the TGN to the basolateral cell surface is shown in lanes 7–12 of Fig. 2A. In cells treated with 10 µg/ml BFA, there was a marked inhibition of receptor delivery to the basal cell surface (Fig. 2A, compare lanes 7 and 9). Inhibition of pIgR basolateral delivery was not coupled with a corresponding increase in apical delivery (Fig. 2C). In addition, if V8 proteinase was added to the apical medium of BFA-treated cells during the delivery assay, no additional apical delivery was detected (compare lanes 7 and 8 in Fig. 2A), confirming that pIgR molecules were not being

![Fig. 1. Endo H digestion of newly synthesized, and TGN accumulated pIgR.](image-url)
redirected to the apical cell surface. The effect of increasing concentrations of BFA on the delivery of the pIgR to the apical and basolateral cell surfaces is quantified in Fig. 2, B and C, respectively. Even at a 1 µg/ml concentration of the drug, delivery of the pIgR to the basolateral cell surface was inhibited by 36% (Fig. 2B). Maximal inhibition of basolateral delivery was achieved in cells treated with 10–20 pg/ml BFA (Fig. 2B); at these concentrations, basolateral delivery was inhibited by approximately 70%. Under these conditions, there was no significant increase in apical delivery of pIgR in BFA-treated cells (Fig. 2C), again confirming that receptor was not being mis-sorted to the apical cell surface. Similar results were found when we tested other clones of MDCK cells expressing the pIgR (data not shown).

We have also monitored the effect of BFA on the delivery of the pIgR from the ER to the basolateral cell surface. In these experiments, the TGN block at 18.5 °C was omitted and BFA was included during the 15-min pulse at 37 °C and during the chase in the presence of V8 proteinase at 37 °C (Fig. 3A). In the absence of BFA, 80% of the pIgR was delivered to the basolateral cell surface under these conditions (Fig. 3B). In the presence of BFA, ER to cell surface delivery of the pIgR was markedly inhibited by the drug; 1 µg/ml BFA was sufficient to inhibit delivery of 95% of the pIgR molecules to the basolateral cell surface (compare lanes 2 and 7 in Fig. 3A). Again, there was no corresponding increase in apical SC release (<5% of the total receptor was delivered apically; Fig. 3B), indicating that the receptor was not being redirected to the apical cell surface in BFA-treated cells.

The inhibition of pIgR delivery to the basolateral cell surface was not a result of BFA causing a general inhibition of basolateral secretion. As previously reported by Low et al. (5), the secretion of gp80 into the apical medium of pIgR-expressing cells was inhibited by BFA (Fig. 4A), while secretion of gp80 into the basolateral medium was stimulated at 1 or 10 µg/ml BFA (Fig. 4B). The total amount of gp80 secretion increased slightly (+12%) in cells treated with 1 µg/ml BFA. There was no inhibition of total gp80 secretion in cells treated with a 10 µg/ml concentration of the drug, but secretion of gp80 was inhibited 30% in cells treated with a 20 µg/ml concentration of the drug.

To determine if the BFA-mediated inhibition of pIgR to cell surface delivery was reversible, the following experiment was performed: cells were pretreated with 10 µg/ml BFA for 75 min at 37 °C, washed rapidly in BFA-free medium, and pulse-labeled, and the delivery of the pIgR was assessed in the absence of BFA. Under these conditions, 67% of pIgR molecules were...
delivered basolaterally, and 20% were delivered apically (Fig. 3B). These data indicate that the effect of BFA on basolateral delivery of the pIgR was rapidly reversible.

Finally, several groups have reported that the Golgi stacks of MDCK cells remain intact when cells are treated with low concentrations (1–3 μg/ml) of BFA (5, 16). We were interested in determining if the Golgi remained intact, even when treated for 75 min at 37 °C with 10 μg/ml BFA. It is clear in Fig. 5, that at least morphologically, the Golgi stacks remain intact, even in cells treated with this high concentration of BFA. This result suggests that the inhibition of pIgR delivery in MDCK cells treated with 10 μg/ml BFA was not the result of the receptor being unable to exit from the ER and/or Golgi because of the formation of a fused ER-Golgi network.

**DISCUSSION**

BFA has numerous effects on membrane trafficking pathways. In many, but not all, cell types, the Golgi apparatus disperses and fuses with the ER (1–3). This step is preceded by the dissociation of β-Cop from the Golgi stacks (7, 8). The TGN is similarly affected, but instead of fusing with the ER this organelle fuses with endosomes (12, 13). Similar to the Golgi, this step is preceded by the dissociation of γ-adaptin from the TGN (10, 11). It has recently been argued by Klausner et al. (24) that organelles in the biosynthetic and endocytic pathways are dynamic structures whose identity at steady state is regulated by the binding of cytosolic coat proteins like β-Cop. In BFA-treated cells, these coat proteins are unable to bind to their target organelles, and, in their absence, homotypic organelles (ones that fuse in the presence of BFA, e.g. Golgi and ER) coalesce with one another to form dynamic tubular networks. In addition to their hypothesized role in maintaining the architecture of organelles at steady state, coat proteins may have other functions as well. For example, the TGN of epithelial cells is thought to play a key role in achieving the polarized state of MDCK cells and other epithelia. At the level of the TGN, coat proteins could be involved in the segregation of proteins into forming apical or basolateral transport vesicles, or even on the formation of the transport vesicles themselves. BFA could exert an effect on these processes by preventing the binding of these coat proteins.

Recent evidence suggests that BFA may act selectively on the pathways for apical delivery in epithelial cells. In particular, Low et al. (5) have demonstrated that the apical secretion of endogenous proteins is affected by concentrations of BFA as low as 0.5 μg/ml. In contrast, secretion of proteins delivered basolaterally is not substantially altered by BFA concentrations as high as 10 μg/ml. Similarly, the same laboratory has reported that apical delivery of the membrane protein dipeptidyl peptidase IV in MDCK cells is selectively inhibited at 1 μg/ml BFA (17). This inhibition results in misrouting of the normally apical dipeptidyl peptidase IV to the basolateral cell surface. Under identical conditions, delivery of uvomorulin, a protein delivered exclusively to the basolateral surface of MDCK cells, is unaffected by concentrations of BFA as high as 10 μg/ml (17). In addition, recent work in the laboratory of M. Roth has shown that 1 μg/ml BFA inhibits direct delivery of HA to the apical cell surface of MDCK cells. Like dipeptidyl peptidase IV, the HA is misrouted to the basolateral cell surface. In contrast, delivery of a mutant form of HA, in which Cys543 has been mutated to a Tyr, is delivered basolaterally regardless of drug treatment. The above results suggest that BFA selectively inhibits the apical delivery of secretory and membrane proteins in MDCK cells, whereas basolateral targeting of these proteins is not affected.

![Morphology of the Golgi apparatus in BFA-treated MDCK cells.](image)

Filter-grown cells were incubated for 60 min at 37 °C with 10 μg/ml BFA. Cells were processed as described under “Experimental Procedures” and viewed in an electron microscope. Bar = 0.5 μm.
We now report that delivery of the pIgR from the TGN to the basolateral cell surface of MDCK cells is markedly inhibited (70%) by 10 \( \mu \)g/ml BFA. In our pIgR-expressing MDCK cells, the basolateral secretion of gp80 was not inhibited but was stimulated in cells treated with 1–10 \( \mu \)g/ml BFA. These results, which are similar to those observed by Low et al. (5), demonstrate that under our experimental conditions BFA at 10 \( \mu \)g/ml has no general inhibitory effect on basolateral secretion. These results also rule out the possibility that the differences observed in this study and those reported by Low et al. (5) are the result of the different variants of the MDCK cell line used or the different sources of BFA. We have also monitored the effect of BFA on the delivery of the pIgR from the ER to basolateral cell surface, by omitting the TGN block and including BFA during the pulse, and the chase of our delivery assay. Under these conditions, delivery of the pIgR to the basolateral cell surface was found to be even more sensitive to the effect of BFA as even 1 \( \mu \)g/ml BFA inhibited delivery of the pIgR >90%. This effect was rapidly reversible. One interpretation of these data is that there is a step in the transit of the pIgR from the ER to TGN that is more sensitive to the action of BFA than the delivery of the pIgR from the TGN to basolateral cell surface.

The selective inhibition of pIgR delivery to the basolateral surface of BFA-treated MDCK cells under conditions where the delivery of other basolateral proteins is unaffected suggests that MDCK cells may have more than one pathway to the basolateral cell surface. Several models are possible. In the TGN, the pIgR could be sorted into a biochemically and/or morphologically distinct set of basolateral carrier vesicles from those used by other basolateral proteins. One possibility is that some proteins would be delivered from the TGN to early endosomes and from there to the cell surface, while other proteins would be delivered directly from the TGN to the cell surface. BFA could act by selectively blocking one of these pathways. The possibility of distinct basolateral carrier vesicles has been proposed by Boll et al. (25). Based on differential sensitivity to microtubule disrupting drugs, they have suggested that basolateral secretory proteins may enter carrier vesicles that are distinct from those occupied by basolateral membrane proteins. Another possible model is that BFA selectively alters the entry of the pIgR into a pool of basolateral transport vesicles shared by all basolateral proteins. The effect of BFA in this case might be to cause the dissociation of a cytosolic coat protein or sorting protein required for the entry of the pIgR into basolateral transport vesicles. An alternative possibility is that BFA might exert its effect by preventing pIgR-containing basolateral transport vesicles from fusing with the plasma membrane.

The differential sensitivity of protein delivery to the basolateral surface of BFA-treated MDCK cells may reflect the diverse sorting mechanisms used by these proteins. Recent studies in our laboratory reveal that basolateral delivery of the pIgR is primarily dependent on residues His\(^{605}\), Arg\(^{657}\), and Val\(^{660}\). This signal acts independently of endocytosis (22). In contrast, other proteins such as HA-Tyr\(^{642}\), the lysosomal membrane protein lgp 120, and the Fc receptor II contain basolateral sorting signals that overlap with their signals for endocytosis (18, 26, 27). Moreover, the cytoplasmic tail of the low density lipoprotein-receptor contains two tyrosine-dependent signals for basolateral sorting, one endocytosis-dependent, and the other endocytosis-independent (28). These signals differ in their capacity to mediate basolateral targeting. On the basis of these observations, these authors suggest that the different features of these two determinants may reflect the existence of two distinct pathways for delivery of the low density lipoprotein-receptor to the basolateral surface of MDCK cells (28).

It is entirely plausible that proteins with different types of basolateral sorting signals utilize distinct pathways to the basolateral cell surface of MDCK cells and one of these pathways is sensitive to BFA. The pIgR utilizes an endocytosis-independent basolateral sorting signal and may explain why basolateral delivery of the pIgR is sensitive to BFA. In contrast, other proteins, such as HA-Tyr\(^{642}\), have basolateral sorting signals that overlap with their endocytosis signal and may utilize a pathway for basolateral delivery that is unaffected by BFA. As a test of this hypothesis, it would be interesting to compare the effect of BFA on the basolateral delivery of other proteins containing either endocytosis-dependent or independent basolateral sorting signals.

REFERENCES

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\(^3\) B. Arisue and K. Mostov, unpublished results.