Targeting of an Intestinal Apical Endosomal Protein to Endosomes in Nonpolarized Cells

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Abstract. Polarized cells such as epithelial cells and neurons have distinct endosomal compartments associated with different plasma membrane domains. The endosomes of the neuronal cell body and the basolateral cytoplasm of epithelial cells are thought to perform cellular “housekeeping” functions such as the uptake of nutrients and metabolites, while the endosomes in the apical cytoplasm or axons are thought to be specialized for the sorting and transcytosis of cell type–specific ligands and receptors. However, it is not known if nonpolarized cells such as fibroblasts contain a specialized endosomal compartment analogous to the specialized endosomes found in neurons and epithelia. We have expressed a protein that is normally found in the apical early endosomes of developing intestinal epithelial cells in normal rat kidney fibroblasts. This apical endosomal marker, called endotubin, is targeted to early endosomes in transfected fibroblasts, and is present in peripheral as well as perinuclear endosomes. The peripheral endosomes that contain endotubin appear to exclude transferrin, fluid phase markers, and the mannos-6-phosphate receptor, although in the perinuclear region colocalization of endotubin and these markers is present. In addition, endotubin positive structures do not tubulate in response to brefeldin A and instead redistribute to a diffuse perinuclear location. Since this endosomal compartment has many of the characteristics of an apical or axonal endosomal compartment, our results indicate that nonpolarized cells also contain a specialized early endosomal compartment.

Endocytosis is the process by which surface bound ligands and fluid phase macromolecules are internalized by eukaryotic cells. After endocytosis, internalized macromolecules must be sorted and targeted to their next cellular destination (Trowbridge et al., 1993). Sorting, recycling, and targeting are mediated by a series of morphologically and functionally heterogeneous membrane-bound compartments known collectively as endosomes (Geuze et al., 1984; Schmid et al., 1988; Dunn and Maxfield, 1992). Much of our understanding of endosomal dynamics has resulted from studies of nonpolarized cells. In these cells, sorting endosomes are located in the peripheral of the cell and contain internalized ligands and receptors (Yamashiro et al., 1984; Dunn et al., 1989; Ghosh et al., 1994). Recycling endosomes are a pericentriolar network of tubules and vesicles that are distinct from the sorting endosomes and contain recycling receptors and lipids (Dunn et al., 1989; Mayor et al., 1993; Tooze and Hollinshead, 1991). Late endosomes contain lysosome-directed receptors and ligands, and are thought to mature from the sorting endosomes (Stoorvogel et al., 1991; Dunn and Maxfield, 1992).

The distribution of different types of endosomal compartments in polarized cells remains controversial. Distinct early endosomal populations in the apical and basolateral cytoplasm of epithelial cells have been identified in tracer studies (Bomsel et al., 1989; Parton et al., 1989; Fujita et al., 1990). Basolateral endosomes are involved in the uptake and recycling of receptors and ligands involved in cell maintenance, and are sometimes referred to as “housekeeping endosomes” (Kelly, 1993). In contrast, apical endosomes were thought to be involved in epithelial cell type–specific processes such as transcytosis and therefore specialized for epithelial cells (Simonoski et al., 1986; Barroso and Sztul, 1994). However, recent work in Madin-Darby Canine Kidney (MDCK) cells and the intestinal cell line, Caco-2, has shown that some apical endosomes contain recycling transferrin (Apodaca et al., 1994; Hughson and Hopkins, 1990; Knight et al., 1995) and has led to the suggestion that no “specialized” apical endosomal compartment exists (Apodaca et al., 1994). However, the fact that some endosomes of the apical cytoplasm fail to label with internalized transferrin leaves open the possibility that portions of the apical endosomal compartment are unique in composition and function (Hughson and Hopkins, 1990; Knight et al., 1995). Also, we have previously
identified a glycoprotein, called endotubin, that is highly enriched in the apical early endosomal tubules of epithelial cells of the neonatal rat ileum (Wilson et al., 1987) and serves as a marker for this specialized endosomal compartment. Neurons represent another type of polarized cell that contains different endosomal populations (Rodriguez-Boulan and Powell, 1992; Kelly, 1993). Endosomes of the cell body and dendrites perform housekeeping functions, whereas endosomes located in the axons are specialized for recycling of synaptic vesicle proteins (Parton et al., 1992; Cameron et al., 1991; Mundigil, 1993; Bonzelius et al., 1994). These endosomal populations contain different synaptic vesicle proteins and have been shown to have differing sensitivity to the fungal metabolite brefeldin A (BFA) (Mundigil et al., 1993). Therefore, it seems clear that polarized cells contain endosomal compartments that are functionally and biochemically distinct.

The question remains open, however, whether nonpolarized cells such as fibroblasts contain a specialized endosomal compartment comparable to the apical endosomes of polarized cells (Rodriguez-Boulan and Powell, 1992; Matter and Mellman, 1994). Progress on this question has been hampered by a lack of morphological or biochemical markers for these membranes. Because endotubin is found in a specialized endosomal compartment in epithelial cells, we wished to determine if it would be targeted to an endosomal compartment when expressed in nonpolarized cells. Expression of the cDNA encoding endotubin in nonpolarized cells (Rodriguez-Boulan and Powell, 1992; Matter and Mellman, 1994) is described above. For transferrin uptake, cells were incubated in serum-free medium for 1 h at 37°C, and then transferred to medium containing 1 mg/ml ovalbumin, 100 μg/ml iron saturated human transferrin (Polysciences Inc., Warrington, PA) for 1 h at 4°C, and then warmed for 2 or 60 min to 37°C. After the 2-min incubation, cells were washed at 4°C in buffer containing 25.5 mM citric acid, 24.5 mM sodium citrate, 280 mM sucrose, pH 4.6, to remove surface bound transferrin. Cells were fixed on ice and processed as described above. Anti–human transferrin antibody (Boehringer Mannheim Biochemicals, Indianapolis, IN) was diluted 1:200 in blocking buffer (Miltenyi Biotec, Auburn, CA) and was diluted 1:100 in blocking buffer. After incubation with the monoclonal antibody and secondary antibody conjugated to Texas red, the cells were permeabilized with 0.05% saponin and incubated again with the monoclonal antibody in blocking buffer followed by secondary antibody conjugated to FITC.

**Uptake Studies**

For ricin uptake, transfected cells were incubated for 30 min at 4°C in 100 μg/ml ricinα-FITC (Sigma Chem. Co., St. Louis, MO) in serum-free medium. Cells were warmed to 37°C for 2–20 min, placed back on ice, rinsed with 0.1 M lactose to remove surface bound ligand, fixed on ice, and blocked as described above. For transferrin uptake, cells were incubated in serum-free medium for 1 h at 37°C, and then transferred to medium containing 1 mg/ml ovalbumin, 100 μg/ml iron saturated human transferrin (Polysciences Inc., Warrington, PA) for 1 h at 4°C, and then warmed for 2 or 60 min to 37°C. After the 2–min incubation, cells were washed at 4°C in buffer containing 25.5 mM citric acid, 24.5 mM sodium citrate, 280 mM sucrose, pH 4.6, to remove surface bound transferrin. Cells were fixed on ice and processed as described above. Anti–human transferrin antibody (Boehringer Mannheim Biochemicals, Indianapolis, IN) was diluted 1:200 in blocking buffer. For ovalbumin uptake experiments, ovalbumin conjugated to Texas red (Molecular Probes, Eugene, OR) was reconstituted in media at 600 μg/ml, centrifuged for 5 min at 14,000 g and placed on the cells for 1 h at 37°C. Cells were rinsed in PBS and fixed in methanol for 5 min followed by processing for immunofluorescence.

**Confocal Microscopy**

Fluorescent images were obtained using a Leica TCS 4D laser scanning confocal microscope (Arizona Research Laboratory, Division of Biotechnology, University of Arizona, Tucson, AZ) using a 100×, NA 1.3 oil immersion objective. Simultaneous two-channel recording was performed.

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1. Abbreviations used in this paper: BFA, brefeldin A; lgp, lysosomal glycoprotein; M6PR, mannose-6-phosphate receptor; NRK, normal rat kidney.

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with a pinhole size of 90 μm using excitation wavelengths of 488/588 nm, a 510/580 double dichroic mirror, and a 515–545 band pass FITC filter together with a 590-nm-long pass filter. All figures are derived from a single optical section which is estimated to be 0.35 μm in thickness. Images were processed and merged using Adobe Photoshop software and printed using a Codonics NP1600 dye sublimation printer.

To quantitate the proportions of peripheral endosomes containing endotubin immunoreactivity and the different internalized tracers or ligands, immunofluorescent images were merged and the peripheral cytoplasm more than 5 μm from the nucleus was divided into sections. Endosomes were scored for the presence of endotubin staining, tracer (ricin or transferrin) staining, or colocalization of fluorescent staining. A minimum of 400 endosomes were scored for each time point.

Western Blotting

Cells were treated overnight with 0.01 M butyric acid and then scraped into buffer containing 0.25 M sucrose, 10 mM Hepes, pH 7.4, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.25 M phenylmethylsulfonyl fluoride. The cells were homogenized with a Teflon pestle and breakage was monitored by phase contrast microscopy. The homogenate was spun at 500 g for 5 min and the resulting supernatant respun for 1 h at 100,000 g. The crude membrane pellet was solubilized by boiling in SDS-PAGE sample buffer, separated by SDS-PAGE, and electroblotted to nitrocellulose. Blots were blocked using 5% milk in Tris buffered saline containing 0.1% Tween 20 and then incubated with the monoclonal antibody followed by anti–mouse antibody conjugated to alkaline phosphatase (Sigma). Blots were developed using Western blue alkaline phosphatase substrate (Promega, Madison, WI).

Results

Endotubin Expression in NRK Cells

In the epithelial cells of the neonatal rat ileum, endotubin is present in an apical endocytic complex of tubules and vesicles and is excluded from the large supranuclear lysosomal vacuole (Fig. 1; Cornell and Padykula, 1969; Wilson et al., 1987). To determine the distribution of endotubin in nonpolarized fibroblasts after transfection with the endotubin cDNA, we analyzed both the cell surface and subcellular staining patterns. Many proteins that are markers of intracellular membranes are present in small amounts on the plasma membrane (Lippincott-Schwartz and Fambrdings, 1987; Reaves et al., 1993), and, in neonatal rat ileum, some endotubin is detected on the apical plasma membrane (Wilson et al., 1987). To determine if endotubin was present on the plasma membrane of NRK cells, unpermeabilized cells were stained using either a monoclonal antibody (Fig. 2 A) or a polyclonal antibody (not shown) against the ectoplasmic domain of the protein. After binding of antibody to the cell surface, the cells were permeabilized and re-incubated with antibody to label intracellular populations of endotubin and to ensure that the cells stained were expressing endotubin at significant levels (Fig. 2 B). These experiments showed that endotubin was targeted almost exclusively to intracellular compartments, as fluorescence was barely detected on the cell surface. To determine if endotubin is targeted to lysosomes in NRK cells, we performed double label immunofluorescence with antibodies against both endotubin and the lysosomal membrane protein lgp120 (Lewis et al., 1985; Dunn, 1990). Although endotubin is normally excluded from the lysosomes of intestinal epithelial cells, if NRK cells lacked the correct endosomal compartment for endotubin targeting, it might be targeted to lysosomes for degradation. Endotubin staining was distributed throughout the cytoplasm in small punctate spots, with larger, brightly stained spots concentrated in the perinuclear region (Fig. 2 C). The lysosomal staining was present in large spots both in the perinuclear region and in the peripheral cytoplasm (Fig. 2 D). Merging of these images showed that most of the endotubin and lgp 120 staining was distinct, although there may be some colocalization in the perinuclear region (Fig. 2 E). These results indicate that endotubin is not being targeted to lysosomes by default, and is instead targeted to another intracellular compartment.

We next compared the size of the protein expressed in NRK cells with the protein found in neonatal rat intestine. In the neonatal rat intestine, endotubin is synthesized as a high molecular weight precursor of approximately 140 kD that is cleaved and glycosylated to a 55–60-kD form (Allen, K.A, K.E. Gokay, B.A. Speelman, and J.M. Wilson, manuscript submitted for publication). To determine if endotubin is processed in a similar fashion in NRK cells, we performed Western blot analysis on membranes isolated from transfected NRK cells. Western blotting with the monoclonal antibody showed that, in these cells, the expressed protein is also 55–60 kD, indicating that synthesis and processing in NRK cells is similar to that found in intestinal epithelial cells (Fig. 3).

Endotubin Is Present in Early Endosomes of NRK Cells

As mentioned above, endotubin is present in the apical endosomes of ileal epithelial cells, and short incubation with exogenous tracer labels these tubules and vesicles (Gonnella and Neutra, 1984; Wilson et al., 1987), indicating that these endosomes are early in the endocytic pathway. To
determine if endotubin was being targeted to an early endosomal compartment in NRK cells, transfected cells were incubated with fluorescent ricin for 2 min at 37°C, processed for immunofluorescence using the monoclonal antibody against endotubin, and imaged using confocal microscopy. After 2 min of ricin uptake, many stained spots ricin-FITC and endotubin staining were seen to colocalize in the periphery of the cell (Fig. 4, A–C). There were also many spots that contained endotubin staining that did not contain ricin staining, and some ricin positive spots that did not stain for endotubin. The colocalization seen after 2 min of uptake was predominantly in the periphery of the cell, although some colocalization was evident in the perinuclear area. After 20 min of ricin uptake, there was extensive colocalization of ricin and endotubin fluorescence in endosomes in the periphery as well as in the perinuclear region of the cell (Fig. 4, D–F). When the endosomes in the periphery of the cell were counted to determine the proportions that contained endotubin staining alone, endotubin and ricin colocalization, or ricin staining alone, it was found that the proportion of endosomes containing both labels increased between 2 and 20 min of uptake (Table I). These results may indicate that this compartment continues to fill with tracer during this time and that exit from the compartment is not as rapid as entry into these endosomes.

**Ricin and Endotubin Colocalization Is Not Due to Nonclathrin Mediated Endocytosis**

Ricin can be taken up by both clathrin-mediated and nonclathrin-mediated endocytosis (Moya et al., 1985), and these pathways can be distinguished by depleting intracel-

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**Figure 2.** Distribution of endotubin staining in transfected NRK cells. There is little expression of endotubin on the cell surface, as endotubin staining is not detected on nonpermeabilized cells (A), even though intracellular expression is high (B). In C and D, and E, NRK cells transfected with endotubin cDNA were double labeled with antibodies against endotubin (C) and lgp120 (D). The merged image (E) shows the distinct staining patterns, although there may be some colocalization in the perinuclear region (arrows). N, nucleus. Bar equals 5 μm.

**Figure 3.** Western blot of endotubin in NRK cells and neonatal rat intestine. Western blots of membrane from rat intestinal epithelial cells (lane 1) and transfected cells (lane 2) were incubated with monoclonal antibody against endotubin. The molecular weight of endotubin is the same in NRK cells as in neonatal rat intestine. The second band seen in lane 2 is likely due to differences in glycosylation in NRK cells.
lular K+ before tracer internalization (Larkin et al., 1983; Moya et al., 1985; Hansen et al., 1993). K+ depletion has been shown to cause disassembly of clathrin lattices and inhibition of clathrin-mediated endocytosis (Larkin et al., 1983; Hansen et al., 1993). Therefore, to examine if the colocalization of endotubin and ricin could be explained by uptake via nonclathrin-mediated endocytosis, the ricin uptake experiments were repeated using K+ depleted cells. When the cells were K+ depleted and allowed to take up ricin for 2 min at 37°C followed by fixation and immunolabeling using anti-endotubin antibodies, no colocalization of the ricin and endotubin staining was observed (Fig. 5, A–C). Even after 20 min of ricin uptake, no colocalization of ricin and endotubin was seen (Fig. 5, D–F), suggesting that endotubin was not targeted to an endosomal compartment associated with the nonclathrin-mediated pathway. Interestingly, there was a redistribution of much of the endotubin staining to a diffuse perinuclear location, similar to that seen after brefeldin A treatment (see below).

### Localization of Endotubin and Transferrin

Ricin serves as a general marker of the endocytic complex and can label early and late endosomes, as well as lysosomes. We next wanted to determine if transferrin, a marker of basolateral and recycling endosomes, colocalized with endotubin. Transferrin has been used in many studies of endosomal dynamics and is considered a classical marker of the early endosomal compartment (Hopkins, 1983; Schmid et al., 1988). In epithelial cells, transferrin is internalized at the basolateral plasma membrane to basolateral endosomes before recycling to the basolateral plasma membrane. However, an endosomal compartment in the apical cytoplasm has also been implicated in the recycling of transferrin to the basolateral surface of epithelial cells (Hughson and Hopkins, 1990; Apodaca et al., 1994;
To further characterize the endosomal population that contains endotubin, cells were incubated with iron-saturated transferrin and processed for double label immunofluorescence with anti-endotubin and anti-transferrin antibodies. After 2 min of uptake, endotubin and transferrin staining was present in small, punctate dots throughout the cells. However, there was little colocalization of endotubin and transferrin immunostaining (Fig. 6, A–C). Quantitation of endosomes containing either or both markers indicated that there was a much lower proportion of endosomes with transferrin and endotubin colocalization compared to the proportion of endosomes with endotubin and ricin colocalization after 2 min of ricin uptake (Table I). In these experiments, 2 min of uptake will label early sorting endosomes; however, it is possible that this short pulse may not label all sorting endosomes through which transferrin would normally traffic. To saturate the transferrin endocytic pathway, cells were incubated for 1 h in the continuous presence of transferrin. Under these conditions, both the sorting and recycling endosomes will be labeled (Hopkins, 1983; Dunn et al., 1989). After 1 h of continuous transferrin uptake, colocalization of transferrin and endotubin in the peripheral endosomes remained low (Fig. 6, D–F and Table I); however, there was colocalization of these markers in the perinuclear region (Fig. 6, G–I).

**Uptake of Fluid Phase Markers**

Both ricin and transferrin bind to the cell surface and are taken up by adsorptive endocytosis. Fluid phase markers will also label endocytic structures; however, tubular apical endosomes in neonatal, intestine and MDCK cells remain largely unlabeled after uptake of these markers (Gonnella and Neutra, 1984; Barroso and Sztul, 1994; Apodaca et al., 1994). To determine if the endotubin containing compartment could be labeled by molecules taken up by fluid phase endocytosis, we incubated transfected cells with ovalbumin conjugated to Texas red for 1 h at 37°C to label the endocytic pathway followed by anti-endotubin staining. After this incubation, the labels were in a distinct punctate pattern in the periphery of the cell (Fig. 7, A–C); although there was some colocalization of endotubin staining and ovalbumin in the perinuclear region. These results indicate that peripheral endosomes have similar properties to the tubular apical endosomes of epithelial cells whereas the perinuclear endosomes are more like a late endosomal compartment.

**Endotubin Containing Endosomes Do Not Tubulate in Response to BFA**

Endosomes in NRK cells have been shown to be sensitive to the fungal metabolite BFA, forming long membrane tu-
bulbs after incubation with this drug (Lippincott-Schwartz et al., 1991; Wood et al., 1991). To compare the effect of BFA on the endotubin containing endosomal compartment with the transferrin containing endosomal compartment, cells were incubated with transferrin for 1 h at 37°C and then exposed to 1 or 5 μg/ml BFA for 10 min at 37°C followed by fixation and double label immunofluorescence. Under these conditions, the differences between the transferrin and endotubin containing endosomes were striking (Fig. 8). At 1 μg/ml BFA, the transferrin containing endosomes had started to tubulate. However, the endotubin containing endosomes do not tubulate, but the strong perinuclear staining has disappeared and staining was present in a diffuse punctate pattern throughout the cell (Fig. 8, A–B). In the presence of 5 μg/ml BFA, the transferrin containing endosomes were even more extensively tubulated (Fig. 8 D). The endotubin staining was also redistributed, but to a predominantly perinuclear localization (Fig. 8 C). Under these conditions, there was no colocalization of the transferrin and endotubin immunostaining.
Because the endotubin containing compartment of NRK cells did not tubulate in response to BFA, we next wished to determine if the apical endosomes of neonatal rat intestine were affected by BFA. Incubation of explants of ileum in 10 μg/ml BFA for 15 min did not change the distribution of endotubin staining (Fig. 9 A). However, because of the intensity of the endotubin staining, it was possible that we would not detect any tubulation if it were present. To rule this out, we determined if tubulation of these endosomes could be detected at the ultrastructural level. Intestinal explants were incubated in BFA and processed for electron microscopy. The apical endosomes from neonatal intestine normally have a tubular-vesicular morphology (Wilson et al., 1987; Fig. 9 B), and this morphology was unaltered by BFA treatment (Fig. 9 C), suggesting that, at least in these cells, apical endosomes are not affected by BFA.

Comparison of Endotubin Distribution with Other Organelle Markers

To further characterize the compartment that contained endotubin, cells were double-labeled with antibodies against endotubin and the trans-Golgi network marker TGN38/41 (Luzio et al., 1990) or the mannose-6-phosphate receptor (M6PR). TGN38/41 staining was present in a cisternal pattern of the perinuclear area and was distinct from the perinuclear endotubin staining (Fig. 10, A–C). The M6PR is concentrated in the trans-Golgi network and late endosomes (Pfeffer, 1987; Braulke et al., 1987; Geuze et al., 1988); however, it is also present on the cell surface and throughout the endocytic pathway (Duncan and Kornfeld, 1988; Jin et al., 1989). When cells were double-labeled for endotubin and the M6PR, the staining of these markers was distinct in the periphery of the cell (Fig. 10, D–F). Some colocalization of these markers was evident in the perinuclear region, but distinct staining was also present. These results, together with the results of the TGN38/41 staining and previous studies of M6PR in NRK cells (Wood et al., 1991), suggest that endotubin and the M6PR that are colocalized represent a late endosomal compartment.

One possible explanation for the different characteristics of the peripheral endotubin containing compartment is that expression of this protein induced the formation of a new endosomal compartment in NRK cells. Although we cannot rule this out, the pattern and extent of uptake of exogenous tracers was indistinguishable between expressing and non-expressing cells, suggesting that no change in endosomal populations had occurred.

Discussion

In this study, we have expressed the apical endosomal protein endotubin (Wilson et al., 1987) in a fibroblast cell line to determine if this protein could be targeted to an endosomal compartment in nonpolarized cells. We found that endotubin is targeted both to an early endosomal compartment in the periphery of NRK fibroblasts and a perinuclear endosomal compartment. The peripheral endosomes are distinct from transferrin and M6PR containing peripheral endosomes, although the perinuclear endosomes may contain both markers. However, unlike transferrin containing endosomes, the endotubin containing endosomes do not tubulate in response to BFA and instead redistribute to a diffuse perinuclear location. Also, like the tubal apical endosomes of the neonatal rat ileum and MDCK cells (Gonnella and Neutra, 1984; Barroso and Sztul, 1994; Apodaca et al., 1994), the endotubin containing peripheral endosomes failed to label with fluid phase markers. Table II lists the characteristics of early endosomes in polarized and nonpolarized cells. As illustrated in this table, endotubin containing peripheral endosomes demonstrate many characteristics of an apical or axonal endosomal compartment. In contrast, the perinuclear endosomes that contain endotubin, M6PR, transferrin, and ovalbumin may represent a common recycling compartment. Alternatively, endotubin may be present in both a transferrin containing recycling compartment and a late endosomal compartment labeled by fluid phase markers and the M6PR.

Together, our results are consistent with the idea that there are multiple populations of early endosomes in nonpolarized cells and that endosomes analogous to the specialized apical or axonal endosomes exist in nonpolar-
ized cells (Rodriquez-Boulan and Powell, 1992; Kelly, 1993; Matter and Mellman, 1994). Although we cannot rule out that expression of endotubin has induced a novel compartment in these cells, these results nevertheless indicate that nonpolarized cells contain the sorting machinery necessary for targeting endotubin to a specialized compartment. In addition, these results are also consistent with recent studies showing that post-Golgi signals and machinery for sorting and targeting of apical and basolateral proteins in nonpolarized cells are similar to those in polarized cells (Musch et al., 1996; Yoshimori et al., 1996), suggesting that membrane trafficking pathways in polarized and nonpolarized cells have many elements in common.

In polarized cells, specialized endosomes have been suggested to have a variety of functions. Specialized endosomes of epithelial cells may be involved in the transcytosis of polymeric IgA, IgG, or growth factors (Abrahamson and Rodewald, 1980; Simonoski et al., 1987; Barroso and Sztul, 1994), and the insertion of water channels into the apical plasma membrane in response to stimulation by vasopressin (Brown and Sabolic, 1993). In addition, specialized endosomes in neurons may be important in the sorting of synaptic vesicle proteins after a cycle of exocytosis at the synapse (Heuser, 1989; Linstedt and Kelly, 1991; Sulzer and Holtzman, 1989). In nonpolarized cells, a novel endosomal compartment is involved in the association of MHCII with peptide antigen for presentation by B lymphocytes (Amigorena et al., 1994; West et al., 1994) and may mediate the cycling of the glucose transporter GLUT4 to the adipose cell surface in response to insulin (Slot et al., 1991, 1992; James and Piper, 1994). In addition, different endosomal populations have been identi-
Figure 9. Brefeldin A does not cause tubulation of apical endosomes of developing rat intestine. Explants of neonatal rat intestine were incubated for 15 min at 37°C in 10 μg/ml BFA, fixed, and processed for immunofluorescence with anti-endotubin antibodies (A) or electron microscopy (C). There was no redistribution of endotubin staining after BFA treatment (compare with Fig. 1). Electron microscopy of control incubated (B) and BFA-treated (C) intestine shows the tubular vesicular morphology of these endosomes was unaffected by BFA treatment. N, nucleus.

Figure 10. Immunostaining with antibodies against the trans-Golgi network and mannose-6-phosphate receptor. Cells were double labeled to localize endotubin (A) and TGN38/41 (B). Merging of the images (C) shows no colocalization of these markers. Staining for endotubin (D) and M6PR (E) results in some colocalization in the perinuclear region (C). However, staining in the periphery is distinct. Bar equals 5 μm. N, nucleus.
fied in the macropinocytic and clathrin-mediated pathways in A431 cells (Hewlett et al., 1994). It is not yet known what role specialized endosomes might play in fibroblasts. However, polarized insertion of recycling receptors into the leading lamellae of fibroblasts has been reported (Hopkins et al., 1994), and disruption of endocytosis in fibroblasts inhibits the development of a polarized morphology (Altankov and Grinnell, 1993).

The biochemical composition of specialized endosomes is still largely unresolved. In epithelial cells, the early endosomes in the apical and basolateral portions of the cell share small GTP-binding proteins (Bucci et al., 1994), but are not able to fuse together in in vitro fusion assays (Bomsel et al., 1990), evidence of their distinct compositions. In neurons, endosomes located in the dendrites and cell body contain the transferrin receptor whereas endosomes located in the axons exclude this receptor (Cameron et al., 1991; Mundigl et al., 1993), and immunostaining of synaptic vesicle proteins showed that different populations of vesicles contain synaptophysin, synaptotagmin, and SV2 (Mundigl et al., 1993). It is of interest to note that when the synaptic vesicle proteins synaptophysin and SV2 are expressed in CHO fibroblasts, synaptophysin colocalizes with transferrin immunoreactivity (Cameron et al., 1991; Feany et al., 1993) but SV2 did not colocalize with transferrin or other intracellular markers (Feany et al., 1993). It may be that in CHO fibroblasts, SV2 was targeted to a compartment similar to the endotubin containing endosomal compartment described in this study.

In MDCK cells, the fungal metabolite brefeldin A has been shown to cause tubulation of transferrin and IgA containing endosomes (Hunziker et al., 1991). BFA inhibited transepithelial transport of polymeric immunoglobulin A, and may have effects at both the level of sorting from the basolateral endosome or exit from the apical endosome (Hunziker et al., 1991; Barroso and Sztul, 1994). In neurons, BFA caused tubulation of transferrin and synaptotagmin containing endosomes, but endosomes of the axon failed to tubulate (Mundigl et al., 1993). Our results indicate that, in fibroblasts, BFA has differential effects on transferrin containing endosomes and endotubin containing endosomes, but both compartments undergo a redistribution after BFA treatment. These results suggest that both populations of endosomes contain coat proteins that are sensitive to BFA treatment and may explain the effect of this drug at both the basolateral and apical points in the transcytotic pathway in epithelial cells.

The redistribution of the endotubin staining to a diffuse, perinuclear localization after BFA treatment strongly resembled the redistribution of endotubin seen after K+ deploitation. BFA is known to prevent the binding of coat proteins ARF, AP-1, and B-COP to Golgi membranes (Donaldson et al., 1992a,b; Helms and Rothman, 1992; Traub et al., 1993), and members of the COP-1 and ARF families have been shown to bind to endosomal membranes in a BFA dependent fashion (Whitney et al., 1995). In addition, endosomes have been shown to contain clathrin-coated membranes (Stoorvogel et al., 1996). Since K+ deploitation has been shown to affect the ability of clathrin coats to bind to membrane (Larkin et al., 1983; Hansen et al., 1993), it is possible that the endotubin containing endosomes contain a clathrin coat that is sensitive to BFA, but this sensitivity is not manifested by tubulation of the membranes.

This manuscript is dedicated to the memory of Dr. Barry King.

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Table 2. Characteristics of Early Endosomes in Polarized and Nonpolarized Cells

|                | BFA sensitive | T/Trf-R | M6PR | Fluid phase markers |
|----------------|--------------|--------|------|--------------------|
| Apical/axonal  | –            | + and –| –    | –                  |
| Basolateral/dendritic | +          | +      | +    | +                  |
| Fibroblasts/previous work | +          | +      | +    | +                  |
| Fibroblasts/endotubin containing endosomes (this study) | –          | –      | –    | –                  |

For specific references, see text.
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