# Golgi-derived Vesicles from Developing Epithelial Cells Bind Actin Filaments and Possess Myosin-I As a Cytoplasmically Oriented Peripheral Membrane Protein

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***Abstract.*** In the intestinal brush border, the mechanoenzyme myosin-I links the microvillus core actin filaments with the plasma membrane. Previous immunolocalization shows that myosin-I is associated with vesicles in mature enterocytes (Drenckhahn, D., and R. Dermietzel. 1988. *J. Cell Biol.* 107:1037–1048) suggesting a potential role mediating vesicle motility. We now report that myosin-I is associated with Golgi-derived vesicles isolated from cells that are rapidly assembling brush borders in intestinal crypts. Crypt cells were isolated in hyperosmotic buffer, homogenized, and fractionated using differential- and equilibrium-density centrifugation. Fractions containing 50–100-nm vesicles, a similar size to those observed in situ, were identified by EM and were shown to contain myosin-I as demonstrated by immunoblotting and immunolabel negative staining. Galactosyltransferase, a marker enzyme for *trans*-Golgi membranes was present in these fractions, as was alkaline phosphatase, which is an apical membrane targeted enzyme. Galactosyltransferase was also present in vesicles immunopurified with antibodies to myosin-I. Villin, a marker for potential contamination from fragmented microvilli, was absent. Myosin-I was found to reside on the vesicle "outer" or cytoplasmic surface for it was accessible to exogenous proteases and intact vesicles could be immunolabeled with myosin-I antibodies in solution. The bound myosin-I could be extracted from the vesicles using NaCl, KI and $Na_2CO_3$, suggesting that it is a vesicle peripheral membrane protein. These vesicles were shown to bundle actin filaments in an ATP-dependent manner. These results are consistent with a role for myosin-I as an apically targeted motor for vesicle translocation in epithelial cells.

THE plasma membrane of intestinal epithelial cells is differentiated into two domains, the apical or brush border (BB)[1] and the basolateral membranes. These domains are separated by a circumferential band of tight junctions and actin filaments near the cell apex. Many of the proteins and phospholipid constituents of these membranes, as well as the proteins forming the underlying cortical actin cytoskeleton, are unique for each domain. The mechanisms of sorting and pathways by which constituents reach the apical or basolateral plasma membranes varies between types of polarized epithelial cells; (see *Seminars in Cell Biology*, vol 2, 1992 for a collection of papers reviewing epithelial cell protein trafficking). In the intestinal epithelia, the sorting of many newly synthesized proteins targeted for the apical and basolateral compartments is thought to occur in the *trans*-Golgi network (Danielsen and Cowell, 1985; Griffiths and Simons, 1986). Once sorted, proteins destined for each domain apparently translocate to the plasma membrane by different mechanisms.

It is generally believed that efficient vectorial transport of proteins to the apical membrane in epithelial cells requires intact microtubules (Achler et al., 1989; Bennett et al., 1984; Breitfeld et al., 1990; Hugon et al., 1987; Quaroni et al., 1979), although see Salas et al. (1986) for a dissenting view. Furthermore, a direct binding of isolated Golgi vesicles (Allan and Kreis, 1986; Bloom and Brashear, 1989; Coffe and Raymond, 1990) and exocytotic vesicles from MDCK cells (van der Sluijs et al., 1990) to microtubules has been demonstrated. The microtubules in intestinal epithelia are oriented with their minus ends at the cell apex and their plus ends directed basally (Achler et al., 1989). This orientation suggests that a cytoplasmic dynein (a "minus end"-directed motor) drives apical vesicle translocation in epithelia (Bloom, 1992; Schroer and Sheetz, 1991). Although microtubules are required for the efficient movement of vesicles to the cell apex, microtubules do not extend to the apical surface, but terminate below or rarely within the actin-rich BB-terminal web (Sandoz et al., 1985). Because the highly cross-linked-terminal web appears to exclude organelles from the apical membrane, it is unlikely that vesicles can simply diffuse from the ends of microtubules through the terminal web to reach the plasma membrane (Achler et al.,

1. *Abbreviations used in this paper*: BB, brush border; GalTase, galactosyltransferase; MV, microvilli; RT, room temperature.

1989). It has been proposed that another motor, perhaps myosin-I, is required to move vesicles through this meshwork to the membrane (Achler et al., 1989).

The myosin-I's are a class of actin-activated ATPases and mechanochemical motors directed toward the barbed ends of actin filaments that are likely associated with both intracellular and plasma membranes (Adams and Pollard, 1989; Baines and Korn, 1990; Hayden et al., 1990; Korn and Hammer, 1988; Miyata et al., 1989). The members of the myosin-I family all contain a conserved head domain with an ATP-sensitive actin binding site that is similar to myosin-II, or conventional myosin. Myosin-I's from different species, and different isoforms from the same species, contain a variable tail domain that may contain a positively charged membrane-binding domain and/or another actin binding site (Cheney and Mooseker, 1992; Pollard et al., 1991). Because purified myosin-I can translocate vesicles comprised of isolated membranes (Adams and Pollard, 1986) and can move actin filaments on planar phospholipid substrates (Zot et al., 1992), it is thought to mediate intracellular vesicle movement. Furthermore, the movement of organelles isolated from *Acanthamoeba* in in vitro motility assays is inhibited by an antibody to myosin-I (Adams and Pollard, 1986). More direct evidence for a role of myosin-I in vesicle motility comes from work with *Saccharomyces cerevisiae* mutants which lack myosin-I (the MYO2 gene product) (Johnston et al., 1991). These studies conclude that myosin-I serves as a motor transporting secretory vesicles along actin substrates to the site of bud formation during cell replication.

Myosin-I in the intestinal BB, previously termed the 110-kD protein, forms radial links connecting the core bundle of actin filaments in microvilli (MV) with the plasma membrane (Matsudaira and Burgess, 1979; Matsudaira and Burgess, 1982; Mooseker and Tilney, 1975). BB myosin-I possesses three distinct domains: (*a*) a head domain with an ATP-regulated actin-binding site; (*b*) a neck domain containing multiple calmodulin-binding sites; and (*c*) a tail domain expressing a membrane-binding region (Coluccio and Bretscher, 1988; Hayden et al., 1990). The tail domain of BB myosin-I can be phosphorylated by protein kinase C in vitro (Swanljung-Collins and Collins, 1992). This phosphorylation is regulated by a $Ca^{2+}$-stimulated binding of myosin-I to phosphatidylserine-containing vesicles. It is not known if phosphorylation occurs in vivo, however these results suggest that BB myosin-I might be regulated by both a $Ca^{2+}$ and a phosphoinositide-mediated pathway. Although purified BB myosin-I has been shown to be a mechanoenzyme using in vitro motility assays (Collins et al., 1990), no mobility of MV or their components has been observed (Mooseker and Coleman, 1989). BB myosin-I has been proposed to move membranous vesicles transporting proteins and lipids from the Golgi to their sites of incorporation into the apical plasma membrane (Collins et al., 1990; Conzelman and Mooseker, 1987; Fath et al., 1990; Shibayama et al., 1987). Immunolocalization of myosin-I on vesicles in the apical cytoplasm of mature chicken and human enterocytes (Drenckhahn and Dermietzel, 1988) is consistent with a membrane translocation role.

The assembly of BBs in immature epithelial cells in the lower zones of the crypts of Lieberkühn requires the transport of tremendous amounts of membrane from the Golgi to the plasma membrane of forming MV. These cells contain a large number of membranous vesicles in the apical cytoplasm (Fath et al., 1990) many of which are destined for the plasma membrane. Previous immunolocalization of enterocyte vesicles with associated myosin-I (Drenckhahn and Dermietzel, 1988) led us to explore a potential role of BB myosin-I in the translocation of vesicles along polarized actin bundles to the forming MV. Here we report the isolation of Golgi-derived vesicles with myosin-I on their external or cytoplasmic surfaces and propose a role for these vesicles in the delivery of membrane proteins to the apical plasma membrane and in MV assembly. These data further support the hypothesis that myosin-I is a motor translocating vesicles from the Golgi to the apical plasma membrane in polarized epithelial cells.

## Materials and Methods

### Isolation of Golgi Vesicles from Chicken Intestinal Crypts

Crypt cells were isolated from adult White Leghorn chickens (Truslow Farms, MD) as described previously (Burgess et al., 1989) except the cells were stirred in PBS-sucrose-EDTA (100 mM sucrose, 137 mM NaCl, 27 mM KCl, 1.47 mM $KH_2PO_4$, 8 mM $Na_2HPO_4$, 20 mM EDTA, pH 6.9) on ice with crypts sloughing off the lamina propria between 90 and 140 min. To inhibit proteolysis, the following inhibitors were added to all solutions: 0.5 μg/ml leupeptin, 1.5 μg/ml aprotinin, 0.3 mM PMSF, $4 \times 10^{-4}$ U/ml $\alpha_2$-macroglobulin, 15 μg/ml soybean trypsin inhibitor, and 10 μg/ml benzamadine. During cell isolation and homogenization, diisopropyl fluorophosphate (DIFP) was added to 0.1 mM. Unless noted, all steps were performed at 4°C. Isolated crypt cells were washed with PBS-sucrose-EDTA by pelleting at ~500 $g_{max}$ and homogenized in 2.5 ml ice-cold PEMS (0.25 M sucrose, 10 mM Pipes, 1 mM EGTA, 2 mM $MgCl_2$, pH 7.0) with a hand-driven glass Dounce homogenizer. Intact BBs, unbroken cells and nuclei were removed by centrifugation at 3,000 $g_{ave}$ for 15 min. To release trapped vesicles, the pellet was resuspended in 1 ml additional PEMS and recentrifuged at 3,000 $g_{ave}$ for 15 min. The supernatants were combined and centrifuged at 10,000 $g_{ave}$ for 30 min to pellet any free MV and large organelles. The vesicles in the 10,000 $g_{ave}$ supernatant were layered over a 40% (wt/wt) sucrose pad in PEMS and centrifuged at 166,000 $g_{ave}$ for 60 min in a TLS-55 rotor (Beckman Instruments, Inc., Palo Alto, CA). 40% sucrose was chosen because intact MV and vesicles from fragmented MV pellet through this pad (Gratecos et al., 1978), while microsomes and other cytoplasmic organelles being less dense will concentrate at the interface. The material concentrated at the 40% interface was layered onto a sucrose step gradient containing 15, 28, and 35% (wt/wt) sucrose and centrifuged to equilibrium density at 200,000 $g_{ave}$ for 90 min using a SW 50.1 rotor (Beckman Instruments, Inc.). All sucrose solutions were formed with PEM and their densities confirmed with a refractometer. Gradient fractions were collected, diluted severalfold in PEM and pelleted at 200,000 $g_{ave}$ for 30 min. Pellets were resuspended in PEMS and stored on ice. Samples were analyzed for the presence of vesicles by negative staining and for myosin-I by immunoblotting. The vesicles were generally used in experiments 12–24 h after isolation.

### Electron Microscopy

Conventional transmission EM of intact intestines was performed as previously described (Fath et al., 1990). Gradient fractions were fixed and osmicated as described by Storrie (Storrie and Madden, 1990), dehydrated through graded ethanols into propylene oxide, and embedded in Epon-Araldite. For negative staining, carbon-over-Formvar-coated grids were floated on a drop of sample, then rinsed by quickly touching to the surface of two drops of PEMS and fixed on a drop of 1% glutaraldehyde in PEMS. The fixed material was rinsed by flotation on two successive drops of PEMS, then stained with 1% aqueous uranyl acetate. Micrographs were taken on either a Philips 301 (Philips Electronic Instruments Co., Mahwah, NJ) or a Zeiss EM902 (Carl Zeiss, Inc., Thornwood, NY) transmission electron microscope.

### Immunological Techniques

The polyclonal antiserum monospecific for the 110-kD heavy chain of BB myosin-I (Collins et al., 1990) and affinity purified myosin-I antibodies were the generous gift of K. Collins and P. Matsudaira (Whitehead Institute, M.I.T., Cambridge, MA). We confirmed that these antibodies were monospecific for myosin-I in isolated BBs (data not shown). Polyclonal antisera elicited against villin has been previously characterized (Fath et al., 1990).

Samples were electrophoresed on 10% SDS-PAGE microslab gels (Matsudaira and Burgess, 1978) and electrophoretically transferred to 45 μm Immobilon-P membranes (Millipore Corp., Bedford, MA). The membranes were "blocked" to alleviate nonspecific binding with 3% (wt/vol) nonfat dry milk (Carnation Co., Los Angeles, CA), 0.2% (vol/vol) Tween 20 in TBS (150 mM NaCl, 50 mM Tris, 0.1% $NaN_3$, pH 7.4) for at least 1 h at room temperature (RT). The blots were then reacted with primary antibodies diluted in 0.75% nonfat milk, 0.05% Tween 20, TBS overnight at 4°C or 2 h at RT and washed 3 × 15 min in TBS/0.05% Tween 20. The bound primary antibodies were detected with $^{125}$I-goat anti-rabbit IgG (ICN Radiochemicals, Costa Mesa, CA) at $10^6$ dpm/ml in 0.75% nonfat milk, 0.05% Tween 20, TBS for 1 h at RT and washed in the same manner as the primary antibodies. Autoradiography was performed with or without intensifying screens on X-OMAT AR film (Eastman Kodak Co., Rochester, NY) and developed with GBX (Eastman Kodak Co.).

To determine the relative amount of myosin-I in selected fractions from the vesicle isolation, samples were solubilized in SDS-PAGE sample buffer and either spotted onto nitrocellulose or separated on 10% gels and blotted. The blots were immunostained as described above and exposed to X-OMAT AR film. The myosin-I band or dotblot spot was excised from the membrane using the autoradiograph as a reference, and the amount of $^{125}$I-goat anti-rabbit IgG bound determined in a liquid scintillation counter. Serial dilutions of the whole homogenate were used to demonstrate a linear relationship between the amount of protein applied with the amount of radioactivity bound. The experimental samples were within the linear range of the assay.

The distribution of myosin-I was determined in 7-μm-thick frozen sections of adult chicken duodena by indirect immunofluorescent light microscopy as previously described (Fath et al., 1990), the only modification being the inclusion of 0.5% normal goat serum in the blocking buffer.

Isolated vesicles from the 15/28% interface were incubated with affinity-purified myosin-I antibody in PEMS for 90 min at room temperature. Then, 15 nm gold-labeled goat anti-rabbit IgG (Amersham Corp., Arlington Heights, IL) was added for an additional 60 min at room temperature. The sample was layered onto a 66% sucrose cushion and centrifuged for 20 min at 96,600 $g_{ave}$. Unlabeled vesicles remained in the supernatant, while labeled vesicles, being made heavier by the bound gold, entered the sucrose cushion. The primary antibody was omitted from some samples to ascertain that the gold label was not artifactually binding to vesicles. The labeled fraction was then negatively stained with 1% uranyl acetate.

### Extraction of Myosin-I from Vesicles

5 μg of total vesicle protein in PEMS was reacted with either 0.6 M KI/5 mM ATP, 1.0 M NaCl/5 mM ATP, or PEMS buffer alone for 30 min at 4°C. For the alkali stripping, 5 μg of vesicle protein was diluted 20-fold with 100 mM $Na_2CO_3$, pH 11.5, and incubated for 30 min at 4°C (Fujiki et al., 1982). The salt- and alkali-treated vesicles were then pelleted at 279,000 $g_{ave}$ for 30 min at 4°C in a TLA 100 rotor (Beckman Instruments, Inc.). Supernatants were aspirated, precipitated with 10% (vol/vol) TCA on ice, washed with 100% ethanol, then resuspended in 20 μl SDS sample buffer. Pelleted vesicles were solubilized directly with 20 μl SDS sample buffer and immunoblotted to determine the relative distribution of myosin-I in the supernatants and pellets.

### α-Chymotrypsin Treatment of Vesicles

5 μg of total vesicle protein were reacted with 0.6 μg α-chymotrypsin (Type I-S, Sigma Chemical Co., St. Louis, MO) in PEMS for 30 min at RT. Further proteolysis was stopped with 15% (vol/vol) TCA and tubes set on ice. The precipitates were collected, washed with 100% ethanol, and solubilized in SDS sample buffer. The samples were then immunoblotted as described above to assay for the proteolysis of myosin-I.

### Characterization of Vesicle Interactions with Actin Filaments

Vesicles were fluorescently labeled with 2.5 μg/ml lipophilic dye $DiOC_6(3)$ (Molecular Probes Inc.; Eugene, OR) and mixed with 2 μM F-actin in PEMS either in the presence or absence of ATP. After several minutes at RT, the fractions were either analyzed by fluorescent light microscopy or negatively stained for transmission electron microscopy. Actin was purified from chicken skeletal muscle (Pardee and Spudich, 1982).

### Enzyme Assays

To determine if membranes from the *trans*-cisternae of the Golgi complex were present in the vesicle fraction, the activity of galactosyltransferase (GalTase) was determined in the sucrose fractions using $^3$H-UDP-galactose (47.5 Ci/mmol; Dupont, NEN, Boston, MA) with *N*-acetylglucosamine as acceptor (Aoki et al., 1990). To determine if the same vesicles contained both surface myosin-I and GalTase, vesicles from three preparations of nine chickens each were immunoisolated with myosin-I antibodies and associated GalTase activity determined. Gradient-purified vesicles in PEMS were divided into three aliquots. One aliquot was incubated with 4 μl of affinity-purified myosin-I antibody (0.5 μg/ml) and a second aliquot was incubated with 4 μl of nonimmune rabbit serum (0.5 μg/ml). The third aliquot received no additions and served as the starting material reference. The samples were shaken gently on ice overnight, then 10 μl of protein A-agarose (Sigma Chemical Co., St. Louis, MO) was added and the samples incubated an additional 2 h on ice. To alleviate nonspecific binding, the protein A-agarose had been pretreated with vesicle membranes and washed extensively in PEMS. The immunocomplexes were collected by centrifugation at 500 *g* (model HSC 10K; Savant Instruments, Inc., Farmingdale, NY) for several seconds. The supernatant was aspirated and the GalTase activity remaining in the supernatant determined.

The presence of apical membrane proteins was determined by assay for the marker enzyme alkaline phosphatase (Weiser, 1973) adapted for 96-well microtiter plates.

### Miscellaneous

Protein concentrations were determined with the Bicinchoninic Acid Assay (Pierce Chemical Co., Rockford, IL) using BSA as a standard.

## Results

### Developing Intestinal Epithelia Contain Many Apical Cytoplasmic Vesicles

Immature cells in the lower zones of the intestinal crypts of Lieberkühn that are rapidly assembling MV, require the synthesis of large amounts of membrane and other MV components and their transport to the apical membrane (Weiser et al., 1986). These developing cells initially form short MV-containing long bundles of actin filaments that extend ~1 μm into the apical cytoplasm (Fig. 1). Because the total length of the actin bundle in immature and mature MV is approximately the same, MV development proceeds by the elongation of the MV at the expense of a shortening rootlet, presumably through the addition of membrane at the MV base (Fath et al., 1990). The apical cytoplasm of these cells also contained a high concentration of 50–100-nm vesicles (Fig. 1), relative to differentiated enterocytes, that previous immuno- and $^3$H-fucose labeling studies suggest are targeted for the apical plasma membrane (Achler et al., 1989; Bennett et al., 1984).

### BB Myosin-I Is Diffusely Distributed in the Apical Cytoplasm of Developing Crypt Cells

To determine if myosin-I was localized to the apical cytoplasm where cytoplasmic vesicles concentrate in de-

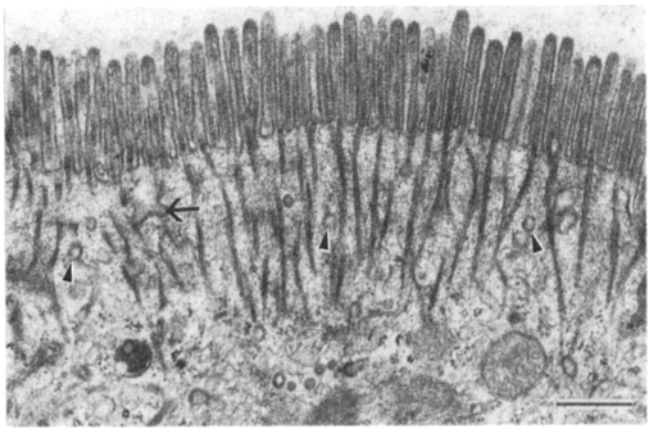

*Figure 1.* The apical cytoplasm of crypt cells contains many small vesicles. Transmission electron micrograph of the apical cytoplasm of a crypt cell that was likely assembling a BB. There are many 50–100-nm vesicles (*arrowheads*) among the long actin-containing rootlets that extend from the MV core. The arrow indicates a possible vesicle–plasma membrane fusion profile. Bar, 0.5 μm (length of mature rootlets).

veloping crypt cells, the distribution of myosin-I was determined by immunofluorescence microscopy. Myosin-I was diffusely distributed in the apical cytoplasm of cells in the lower regions of the crypt (Fig. 2 *A*). Myosin-I was also observed at lower concentrations at the lateral plasma membrane as previously reported by others (Heintzelman and Mooseker, 1990). By contrast, in mature enterocytes residing on the villus (Fig. 2 *B*), myosin-I was not diffusely distributed in the apical cytoplasm, but was concentrated in the BB. Transmission electron microscope images suggest that mature enterocytes with a fully assembled BB contain fewer apical vesicles than do the cells in the crypts (Drenckhahn and Dermietzel, 1988; Fath et al., 1990). Therefore, the distribution of membranous vesicles in the apical cytoplasm of crypt and villus cells parallels the distribution of myosin-I as detected by immunofluorescence.

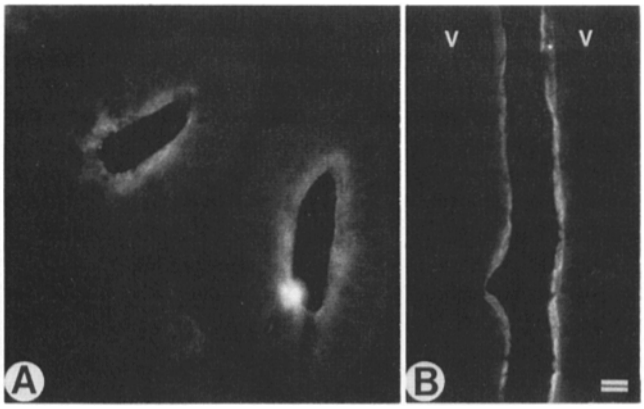


*Figure 2.* BB myosin-I is diffusely distributed in the apical cytoplasm of crypt epithelial cells. Fluorescent light micrographs of the distribution of myosin-I in intestinal crypts (*A*), or villi (*B*). 7-μm-thick frozen sections of adult chicken duodena were immunostained with polyclonal antibodies that were affinity purified using myosin-I. The bound antibody was detected with FITC-goat anti-rabbit IgG. (*A*) The distribution of myosin-I in cross sections of two intestinal crypts. Myosin-I is diffusely distributed in the apical cytoplasm of the crypt epithelia and is weakly present at the lateral plasma membrane. (*B*) In the more developed enterocytes residing on the villus, myosin-I is restricted to the brush border. This section shows the lateral edges of two adjacent villi whose centers are indicated with a *V*. Bar, 10 μm.

### *Isolation of Vesicles with Associated BB Myosin-I: Immunological, Enzymatic, and Morphological Characterization*

To determine if crypt cells contained vesicles with associated myosin-I, we isolated Golgi-derived vesicles from intestinal crypt epithelia of adult chickens and assayed for the presence of myosin-I in the fractions. Intact intestinal crypts were isolated in hyper-osmotic buffer to ~95% purity as assayed with phase-contrast microscopy (data not shown). The remaining 5% were immature enterocytes from the lower villus. Isolated crypts were homogenized in buffered 0.25 M sucrose (PEMS) and vesicles isolated by differential- and equilibrium-density centrifugation. The isolation protocol was adapted from previously published methods for isolating enterocyte Golgi membranes (Weiser et al., 1978). Since it was crucial that any vesiculated MV, which would contain myosin-I, did not contaminate the cytoplasmic vesicle fractions, a pregradient centrifugation using a 40% sucrose cushion was included to separate any denser fragmented MV from Golgi vesicles. We determined the distribution of myosin-I on the sucrose gradients by assaying the fractions using immunoblotting. We identified a membrane fraction banding at the 15/28% sucrose interface that contained myosin-I (Fig. 3, lane *B*). By quantifying immunoblots we determined that this fraction contained a twofold enrichment of myosin-I when compared with the crude homogenate and represented ~0.03% of the total cellular myosin-I (Table I). ~60% of the total myosin-I were recovered with intact BBs (3,000 *g* pellet) and with MV (10,000 *g* pellet). Nearly 2% of the myosin-I pelleted through the 40% sucrose pad (166,000 *g* pellet) and probably represent fragmented MV since this fraction contained the microvillar protein villin (data not shown) and a sixfold enrichment of myosin-I when compared with the crude homogenate.

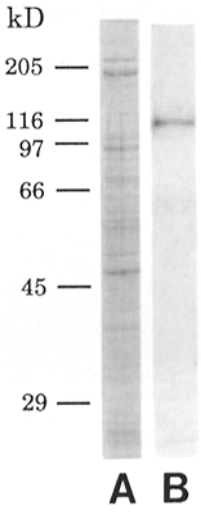


*Figure 3.* Detection of myosin-I associated with Golgi-derived vesicles. Gradient-isolated vesicles were analyzed by SDS-PAGE and the proteins blotted onto Immobilon membrane. The blot was probed with an antibody to myosin-I, washed and the bound antibody detected with $^{125}$I-goat anti-rabbit IgG and autoradiography. (*A*) Coomassie blue–stained gel showing proteins found in the vesicle fraction. (*B*) Immunoblot showing the presence of myosin-I. The mobilities of molecular weight standards ($\times 10^{-3}$) are indicated.

*Table I. Comparison of the Amount of Myosin-I in Fractions from a Representative Vesicle Preparation Relative to Total Protein in Each Fraction and As a Percentage of Myosin-I in the Initial Homogenate*

| Fraction | cpm/μg | % of homogenate | Contents |
|---|---|---|---|
| Homogenate | 281 | 100.0 | Crypt cells |
| 3,000 *g* pellet | 253 | 54.0 | Brush borders |
| 10,000 *g* pellet | 1412 | 2.4 | Intact microvilli |
| 166,000 *g* supernatant | 6 | 0.2 | Soluble proteins |
| 166,000 *g* pellet | 1674 | 1.7 | Fragmented microvilli |
| 15/28% interface | 472 | 0.03 | Golgi membranes |

Samples of fractions taken from a vesicle preparation were solubilized in SDS-PAGE sample buffer and relative amounts of myosin-I quantified on dotblots with affinity-purified myosin-I antibodies and $^{125}$I-goat anti-rabbit IgG. Dots were cut from the nitrocellulose and the amount of antibody bound determined in a liquid scintillation counter. Data are expressed as counts per min of $^{125}$I-goat anti-rabbit IgG bound per μg total protein (*cpm/μg*) and as percentage of total myosin-I in each fraction relative to that in the total homogenate (*% of homogenate*).

The density of the myosin-I–associated membranes that concentrated at the 15/28% sucrose ranged between 1.09 and 1.12 g/ml, the same density as Golgi vesicles isolated from rat crypt cells (Weiser et al., 1978). To verify that this fraction contained Golgi vesicles, we measured the activity of GalTase, a marker enzyme for the *trans*-Golgi network. The specific activity of GalTase was approximately twofold higher at the 15/28% interface when compared with the crude homogenate (Table II), suggesting an enrichment in Golgi membranes in this fraction. Because this membrane fraction contained a heterogeneous population of Golgi vesicles, we wished to determine if the same vesicles with myosin-I on their cytoplasmic surface also contained GalTase. We immunoisolated intact Golgi vesicles by pelleting using myosin-I antibodies and protein A–agarose, then measured the GalTase remaining in the supernatant. Compared with the GalTase activity in the initial vesicle fraction, myosin-I antibody immunopelleted an average of 42% of the GalTase activity, while nonimmune serum pelleted only an average of 14% of the activity ($n$ = 3 experiments). These results suggest that the vesicles expressing myosin-I are Golgi-derived vesicles.

We also wished to establish if alkaline phosphatase, an apical plasma membrane-specific marker enzyme that should be present on apically targeted membranes, was also present in this vesicle fraction. The specific activity of alkaline phosphatase was three- to fivefold higher in the 15/28% interface than it was in the crude homogenate (Table II).

*Table II. Specific Activity of GalTase and Alkaline Phosphatase in Cell Fractions*

| Fraction | GT* | AP‡ |
|---|---|---|
| Homogenate | 0.5 | 12 |
| 15/28% interface | 0.8 | 38 |

The specific activity of a marker enzyme for the *trans*-Golgi network (galactosyltransferase) and the apical plasma membrane (alkaline phosphatase) in whole crypt homogenates and isolated vesicles. Because the actual numbers varied between experiments, but the relative ratio between the two fractions remained constant, the values from a representative experiment are shown.
* Activity of GalTase, expressed as nmol × min$^{-1}$ × mg$^{-1}$.
‡ Activity of alkaline phosphatase, expressed as the change in $OD_{405}$ × min$^{-1}$ × mg$^{-1}$.

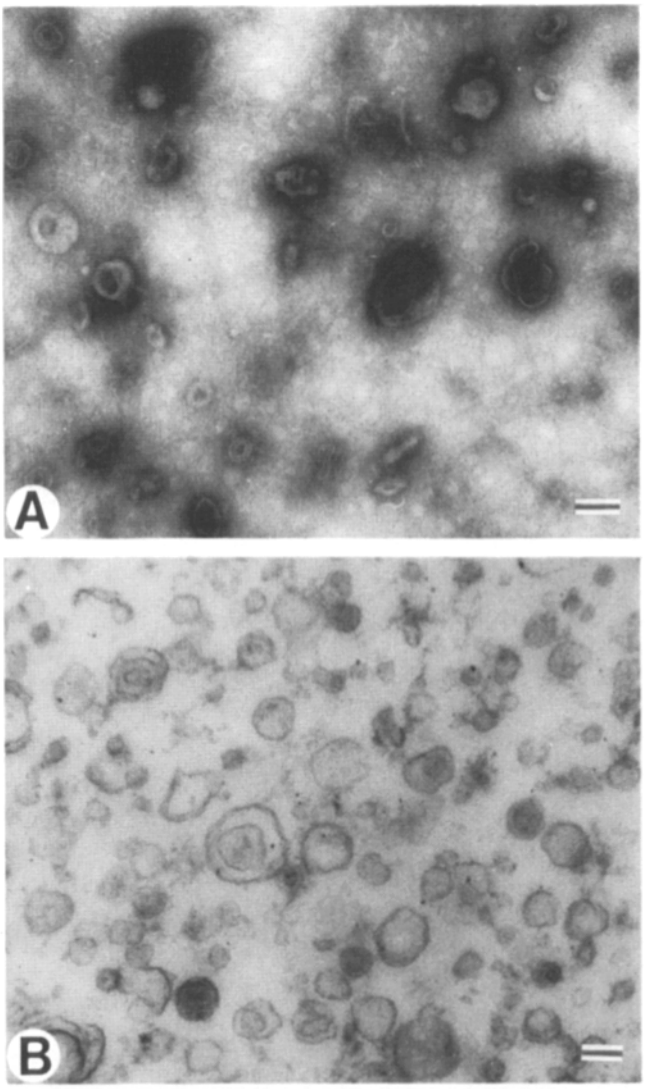


*Figure 4.* Ultrastructure of isolated vesicles. (*A*) Negative stain electron micrograph of the material concentrating at the 15/28% sucrose interface after equilibrium-density centrifugation. This fraction contains a heterogeneous population of vesicles with diameters ranging from 50 to 150 nm. Neither fragmented MV nor actin filaments are observed. (*B*) Thin section of the same material as in *A* showing that the vesicles contain primarily a single limiting membrane. Note the absence of mitochondria and fragmented MV. Bars, 0.1 μm.

By negative stain EM the membrane fraction contained a heterogeneous population of vesicles with diameters ranging from 50 to 150 nm (Fig. 4 *A*), the same diameters as those observed in the apical cytoplasm of intact crypt cells (Fig. 1). Fragmented MV or actin filaments were not seen in these samples. To further characterize the constituents of these fractions, gradient samples were analyzed by conventional thin section EM. The membranes were closed vesicles without lumenal contents and were generally bounded by a single limiting membrane (Fig. 4 *B*). Absent from these fractions were profiles characteristic of plasma membrane sheets, in-

tact Golgi stacks, and mitochondria. Also absent were MV vesicles that characteristically contain a fibrous filling and a fuzzy coat (Colas and Maroux, 1980; Louvard et al., 1973).

The vesicle fractions contained a number of proteins in addition to myosin-I (Fig. 3, lane *A*). To ascertain potential contamination of the membrane preparation by vesiculated MV, although none were observed in the electron microscope, the fractions were immunoblotted for villin, a MV actin-bundling protein (Bretscher and Weber, 1979). Although not illustrated, villin was not found in these fractions, but was easily detected in the 10,000 $g_{ave}$ pellet (data not shown). These results strongly suggested that the isolated membranes with associated myosin-I were not fragmented MV, but were Golgi-derived vesicles.

### *BB Myosin-I Is on the Cytoplasmic Face of Isolated Golgi-derived Vesicles*

To determine if the myosin-I was located on the vesicle "outer" or cytoplasmic surface, the vesicles were incubated with the protease α-chymotrypsin, then immunoblotted to detect any myosin-I proteolysis. If the myosin-I was on the outside of the vesicle, it would be cleaved by the protease; however, if on the inside of an intact vesicle, it would be resistant to proteolysis. Vesicles were incubated with α-chymotrypsin in PEMS for 30 min at RT, then TCA-precipitated to inhibit further proteolysis and immunoblotted. Fig. 5 (lane *C*) shows that myosin-I was cleaved from its native SDS-PAGE molecular mass of 110 kD into a major fragment of ~76 kD. This fragment corresponds in molecular mass to that of one previously identified by the cleavage of purified BB myosin-I with α-chymotrypsin (Coluccio and Bretscher, 1988). Vesicles incubated without protease exhibited no degradation (Fig. 5, lane *B*) and served to assess the degree of endogenous proteolysis. The validity of this assay requires that the vesicle membrane be intact, therefore the integrity of the vesicle membranes was determined by assaying GalTase activity. Since GalTase is found on the inside of *trans*-Golgi vesicles, an intact membrane would prevent access of the added exogenous substrate to the lumenal enzyme. Therefore, we compared GalTase activity of these vesicles in the presence or absence of 0.5% Triton X-100 to determine the degree of intactness. We determined that isolated vesicles had a latency of ~94% as defined by: [(activity in the presence of detergent − activity in the absence of detergent)/(activity in the presence of detergent)] × 100 (Storrie and Madden, 1990). Therefore, accessibility of myosin-I to α-chymotrypsin in these intact vesicles suggested that the myosin-I was on the vesicle cytoplasmic face. Furthermore, the external positioning of the myosin-I suggests that these vesicles are not fragmented MV which would contain "internal" myosin-I (Haase et al., 1978).

The presence of myosin-I on the cytoplasmic face of the vesicles was also confirmed by immunolabeling with myosin-I antibodies. Intact vesicles were incubated with affinity-purified myosin-I antibody followed by gold-labeled secondary antibody. We found that a portion of the vesicles was labeled by the antibody (Fig. 6), while labeling was not observed when the primary antibody was omitted (data not shown). Although our methods were not designed to be quantitative, we found that only a small percentage of the vesicles was immunostained. The access of the antibody to myosin-I on intact vesicles also supports the contention that the myosin-I is on the vesicle surface.

### *BB Myosin-I Is a Peripheral Membrane Protein in the Isolated Vesicles*

When the vesicles were centrifuged at 279,000 $g_{ave}$ for 30 min, all of the myosin-I was found in the pellet (Fig. 7, *A* and *B* lanes *C*) as determined by immunoblotting. That the myosin-I pellets with the vesicles and bands on sucrose gradients at densities characteristic of membranes, is consistent with the association of myosin-I with the membranes. The topology of myosin-I in the membrane was determined by salt extraction of the vesicles, followed by centrifugation and immunoblotting of the pellet and TCA-precipitated supernatant. When incubated with 0.6M KI (Fig. 7 *A*) or 1.0 M NaCl (Fig. 7 *A*) for 30 min at 4°C, a portion of myosin-I was

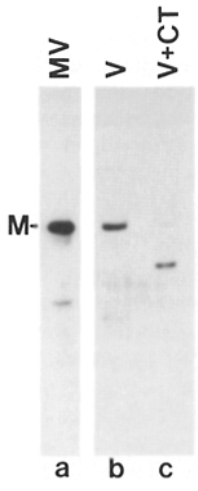


*Figure 5.* Myosin-I resides on the external vesicle surface and is accessible to exogenous α-chymotrypsin. To determine if the vesicle-bound myosin-I was on the inner or outer surface, vesicles were incubated with exogenous α-chymotrypsin, then immunoblotted to determine if the myosin-I was on the outside of the vesicle and thus proteolyzed. (*A*) Immunoblot showing the mobility of myosin-I from isolated MV. This lane serves to show the relative mobility of the myosin-I heavy chain (*M*). (*B*) Immunoblot of isolated vesicles incubated for 30 min at RT without added protease, then TCA precipitated and analyzed. Note that the myosin-I heavy chain was not proteolyzed by endogenous proteases for it has the same relative mobility as myosin-I from MV. (*C*) Immunoblot of vesicles incubated with α-chymotrypsin for 30 min at RT, then TCA precipitated and analyzed. The myosin-I heavy chain was proteolyzed to a major polypeptide of ~76 kD.

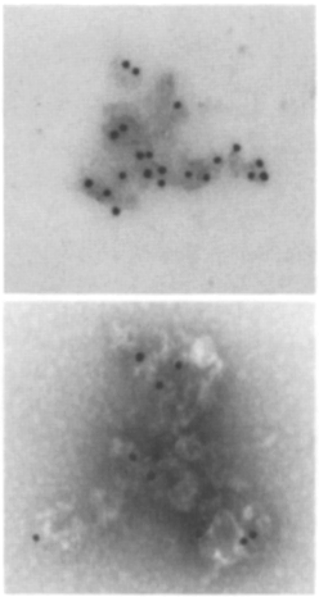

*Figure 6.* Immunostaining of isolated vesicles with myosin-I antibodies. To further confirm the topology of myosin-I in the vesicle membrane, isolated vesicles were incubated with affinity-purified myosin-I antibody followed by gold-labeled goat anti-rabbit IgG and negative stained. These micrographs show that a subpopulation of isolated vesicles bound antibody and suggests that the myosin-I in these fractions is bound to the vesicle and is on the external membrane face. Gold particles, 15 nm.

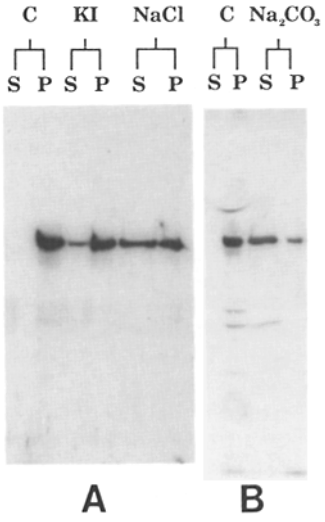


*Figure 7.* Myosin-I is a vesicle peripheral membrane protein. Isolated vesicles incubated with high salt or alkaline buffers then centrifuged at 279,000 $g_{ave}$ for 30 min. The subsequent supernatants (*S*) and pellets (*P*) were immunoblotted for myosin-I. (*A*) Salt extraction: vesicles incubated with PEMS (*C*, control) results in all myosin-I in the pellet. A portion of the myosin-I was extracted into the soluble phase by 0.6 M KI, whereas a larger percentage was extracted by 1.0 M NaCl. (*B*) Alkali extraction: vesicles incubated with 100 mM $Na_2CO_3$, pH 11.5, results in the release of the majority of the myosin-I from the vesicles. In PEMS, all of the myosin-I remained in the membrane pellet. The lower molecular weight bands are proteolytic fragments of myosin-I that are also stripped from the membrane under these conditions.

released from the vesicles into the soluble phase. More myosin-I was released by NaCl than by KI. Although 5 mM ATP was included with the NaCl and KI in the experiment shown in Fig. 7 *A*, in other experiments extractions in PEMS containing either NaCl or KI, but lacking ATP, confirmed that the nucleotide was not responsible for the release of myosin-I from the vesicles (data not shown). When vesicles were washed with 100 mM $Na_2CO_3$, pH 11.5, and the soluble and insoluble material analyzed by immunoblotting, myosin-I was found predominantly in the soluble fraction (Fig. 7 *B*; $Na_2CO_3$, *S*) with little in the insoluble pellet (Fig. 7 *B*; $Na_2CO_3$, *P*). These salt and alkali extraction data suggest that myosin-I is a vesicle peripheral membrane protein.

### *Golgi-derived Vesicles Aggregate F-actin in an ATP-dependent Manner*

If these Golgi-derived vesicles with surface myosin-I translocate on actin filaments in vivo as we propose, then they should be able to interact with purified actin in an ATP-sensitive fashion. Vesicles were fluorescently labeled with the lipophilic dye $DiOC_6(3)$ and mixed with 2 $\mu$M F-actin in PEMS either in the presence or absence of 5 mM ATP. Samples incubated without ATP contained aggregates of vesicles as seen in the fluorescent light microscope (Fig. 8 *A*). These aggregates were not observed when vesicles were incubated with F-actin in PEMS containing 5 mM ATP (Fig. 8 *B*). Negative stain transmission EM confirmed that these aggregates correspond to loose bundles of actin filaments associated with membranous vesicles (Fig. 8 *C*). In the presence of ATP these bundles were absent; only free vesicles and unbundled filaments were observed (data not shown).

## *Discussion*

Using established methods of Golgi vesicle purification, we have isolated membranes from intestinal epithelial crypt cells that possess myosin-I as a cytoplasmically oriented peripheral membrane protein. These vesicles also contain the *trans*-Golgi membrane marker enzyme GalTase and the apically targeted enzyme alkaline phosphatase. Therefore, we propose that these vesicles represent a population of Golgi-

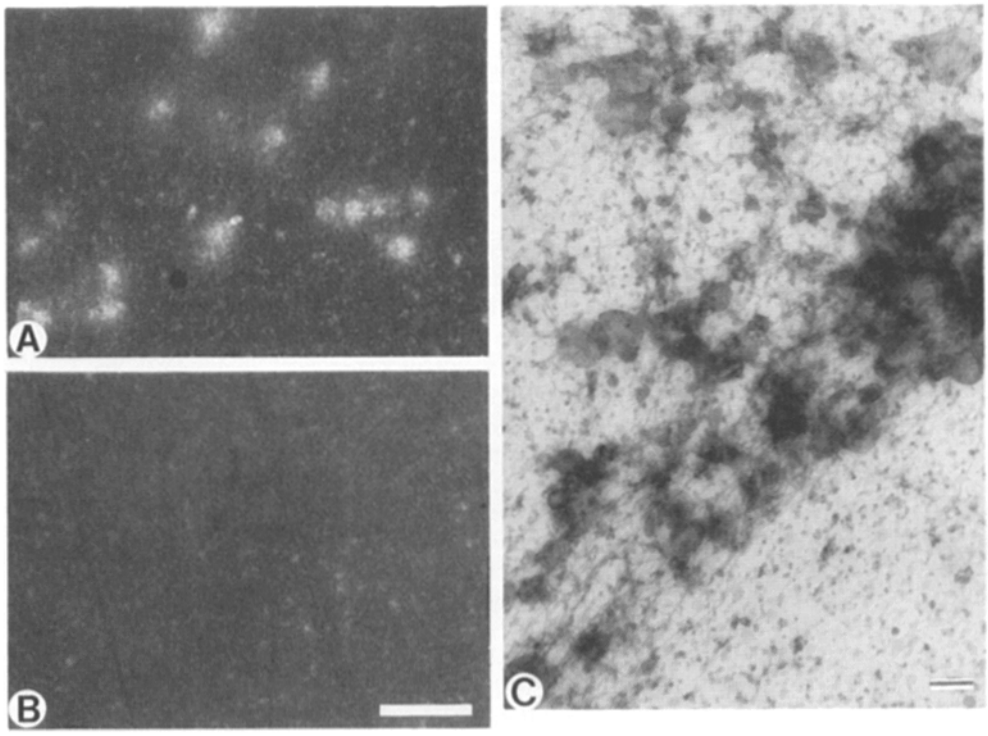


*Figure 8.* Isolated vesicles aggregate F-actin in an ATP-dependent manner. Micrographs of vesicles labeled with $DiOC_6(3)$ and mixed with 2-$\mu$M actin filaments in PEMS either in the absence (*A* and *C*) or presence of 5 mM ATP (*B*). (*A*) Fluorescent vesicles formed large aggregates when incubated with F-actin. (*B*) In the presence of 5 mM ATP the vesicles remained monodisperse and did not aggregate F-actin. (*C*) Negative stain electron micrograph showing that the fluorescent aggregates visible in (*A*) were loose bundles of vesicles and actin filaments. Bars: (*A* and *B*) 10 $\mu$m; (*C*) 0.2 $\mu$m.

derived vesicles carrying apically directed lipid and protein. In that only a subpopulation of the vesicles in this fraction was immunolabeled with myosin-I antibody, it is likely that this fraction contains a heterogeneous population of vesicles. The limited number of vesicles labeled with myosin-I antibody is not surprising since carrier vesicles should represent only a small percentage of Golgi membranes and not all classes of carrier vesicles may require myosin-I.

Because purified myosin-I can bind to acidic phospholipids (Adams and Pollard, 1989; Hayden et al., 1990; Zot et al., 1992), it was important that cell homogenization not induce potential soluble myosin-I to attach artifactually to all membranes. Our observations that only a portion of the Golgi-derived vesicles immunolabeled with myosin-I antibody and only 42% of the GalTase activity partitioned with vesicles immunoisolated with antibodies to myosin-I, suggest that it is unlikely that myosin-I was artifactually binding to cell membranes. We were also concerned that the vesicle-induced aggregation of actin filaments that we observed was a result of potential contaminating myosin-II. We suggest that the binding of Golgi vesicles to actin filaments was not an effect of myosin-II for the following reasons. First, myosin-II should not be active in our preparations. Previous studies using myosin-II isolated from chicken enterocytes found that myosin-II, as isolated, was not functionally active in the *Nitella* motility assay unless it was first phosphorylated by exogenous myosin light chain kinase (Mooseker et al., 1989). Second, myosin-II forms large filamentous aggregates at the ionic strength of our buffer (Broschat et al., 1983). These filaments, which would be easily visible in the electron microscope, were never observed. The positioning of myosin-I on the external or cytoplasmic surface of the vesicles, and the ATP-dependent bundling of actin filaments by vesicles in vitro, is consistent with the idea that myosin-I may be able to bind actin filaments in vivo and may serve as a motor for vesicle translocation.

It was crucial that fragmented MV, which contain a large amount of myosin-I, were not contaminating the Golgi-derived vesicle preparation. We propose that the isolated vesicles are not vesiculated MV for the following reasons: they do not contain villin, an abundant MV protein; their apparent densities on sucrose gradients ranged between 1.09 and 1.12 g/ml, while MV vesicles band at ~1.197 g/ml (Gratecos et al., 1978); myosin-I was on the outside of these vesicles, whereas it is on the inside of MV vesicles (Haase et al., 1978); the morphology, by thin section EM, is that of intracellular vesicles and not of vesiculated plasma membrane. Furthermore, the vesicles were not basolateral membranes, which are denser and band at 1.15 g/ml sucrose (Colas and Maroux, 1980; Weiser et al., 1978).

Golgi-derived carrier vesicles with surface myosin-I may be important in the assembly of MV in developing enterocytes. Previously, we proposed that vesicles with myosin-I on their cytoplasmic surfaces supply both membrane and myosin-I to elongating MV in maturing enterocytes (Fath et al., 1990; see also Collins et al., 1990; Conzelman and Mooseker, 1987; Shibayama et al., 1987). From transmission electron micrograph images we determined that the running length of the core bundle of microvillar actin filaments (i.e., that in the MV and in the rootlets) is approximately the same in immature as in mature enterocytes. As cells mature, the percentage of actin filament length encompassed by

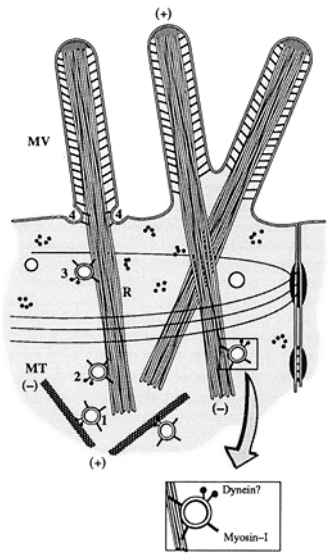


*Figure 9.* Drawing illustrating possible steps in the transport of Golgi-derived vesicles to the apical plasma membrane in developing intestinal epithelial cells. (*1*) Vesicles move from the Golgi to the apical cytoplasm along microtubules (*MT*) using a minus end–directed motor (*Dynein?*). (*2*) Upon reaching the end of the microtubules, the vesicles bind to actin filaments in the MV rootlet (*R*) through their surface myosin-I. (*3*) The vesicles translocate apically through the terminal web towards the plus end of the actin filaments using the myosin-I motor. (*4*) Upon reaching the base of the MV, the vesicles fuse with the plasma membrane and link the membrane with the actin core through the attached myosin-I.

membrane (i.e., in a MV) increases with MV elongation. That is to say, immature BBs express short MV with long rootlets, while mature BBs express long MV with short rootlets. From these measurements, we proposed that MV elongation is driven by the addition of membrane at the MV base (Fath et al., 1990). This membrane, we propose, is supplied to the apical plasma membrane by the mechanism illustrated in Fig. 9. The model is as follows: vesicles targeted for the apical plasma membrane pinch off from the *trans*-Golgi network and translocate to the apical cytoplasm using a minus end–directed microtubule-based motor (Fig. 9, step *1*). Cytoplasmic dynein is a likely candidate for this motor (Schroer et al., 1989). Upon reaching the apical extent of microtubules, vesicles bind to and translocate along actin filaments in the MV rootlet through their surface myosin-I (Fig. 9, step *2*). Because the actin filaments in the MV are oriented with their plus or barbed ends at the tip (Mooseker and Tilney, 1975), the vesicles move apically towards the plasma membrane (Fig. 9, step *3*). When the vesicles reach the MV base, they fuse with the plasma membrane (Fig 9, step *4*), thus serving to link the membrane to the actin core through the attached myosin-I. The fusion of many vesicles would "zip" the membrane down around the core with myosin-I cross-bridges and serve to lengthen the MV.

As in the developing enterocyte, an uninterrupted supply of membranes and myosin-I to the apical membrane is also necessary in mature enterocytes. In the presence of protein synthesis inhibitors (LeCount and Grey, 1972), starvation, or microtubule depolymerization agents (Achler et al., 1989; Pavelka et al., 1983), MV rapidly shorten. This shortening is indicative of the rapid turnover of BB cytoskeletal constituents that was detected in metabolic labeling studies in adult animals (Cowell and Danielsen, 1984; Stidwill et al., 1984). Therefore, the movement of proteins and lipids to the apical cytoplasm, perhaps by a myosin-I–facilitated transport, is required throughout the life of an intestinal enterocyte. Once incorporated into a MV, the myosin-I may

serve to pull the core actin bundle downward, away from the membrane or some plus-end actin filament capping region at the apical MV tip. This displacement may expose the plus ends of the actin filaments, thus facilitating the addition of actin monomer required for maintaining actin filament length in the presence of cytoskeletal protein turnover. This role of myosin-I in motor-dependent actin assembly is adapted from a model proposed for filopodia extension (Sheetz et al., 1992).

In addition to being a motor translocating their vesicle cargo, myosin-I may assist in the targeting of apically directed proteins. As presented in the introduction, intact microtubules are required for the efficient transport of products to the apical plasma membrane in polarized epithelial cells. However, when microtubules are depolymerized by nocodazole or colchicine, apical targeting is delayed or decreased, not eliminated (Achler et al., 1989; Bennett et al., 1984; Breitfeld et al., 1990; Hugon et al., 1987; Quaroni et al., 1979). Because the average pore size of cytoplasm is smaller than vesicles (Luby-Phelps et al., 1987), passive diffusion alone cannot account for movement of vesicles from the Golgi to the apical plasma membrane; some active mechanism is required. The continued delivery of membrane in the absence of microtubules suggests that either some stable microtubules remain intact in the presence of microtubule-disrupting drugs, or that an alternative transport system is used. Since microtubules rarely extend into the highly cross-linked terminal web separating the apical membrane from the remainder of the cytoplasm (Sandoz et al., 1985), another motor, perhaps attached to the same vesicle translocated first along microtubules, may be required to move vesicles through the terminal web to the plasma membrane. This translocation of vesicles to the apical membrane in mature enterocytes might occur by a mechanism similar to that shown in Fig. 9 for developing enterocytes. The MV rootlets in the mature BB, while not as long as those in the developing enterocyte (Fath et al., 1990), extend into the terminal web and approach the microtubule ends (Sandoz et al., 1985). Support for such a proposal comes from microtubule disruption studies in intestinal epithelial cells (Achler et al., 1989; Pavelka et al., 1983) and in an intestinal epithelial cell line (Eilers et al., 1989). When microtubules are disrupted, nearly 50% of the newly synthesized proteins normally targeted to the apical membrane is misdirected to the basolateral membrane where they form BBs. These ectopic BBs contain fully formed MV that possess myosin-I cross-bridges and extend into the intercellular space (Achler et al., 1989). Basolaterally directed membranes were not detected fusing with the apical membrane in control cells or with the aberrant BB domains formed in the lateral membrane in microtubule-disrupted cells. This apparent inability of basolaterally targeted vesicles to penetrate the terminal web of the ectopic BBs has led to the proposal that because these vesicles lack associated myosin-I, they cannot translocate on the rootlet actin filaments to contact the apical bilayer (Achler et al., 1989). Therefore, myosin-I on the surface of vesicles may not only serve as a motor, but may serve as a mechanism whereby selected vesicles are targeted to their correct destination at the apical plasma membrane.

In the past several years it has become well accepted that members of the kinesin and dynein families are responsible for the transportation of vesicles along microtubule substrates in many cells (Bloom, 1992; Schroer and Sheetz, 1991). Other work has suggested that motors using actin filament substrates can also move membranes within cells (Adams and Pollard, 1986; Kachar, 1985). Quite surprisingly, recent studies have suggested that there may be a functional redundancy in motor mechanisms such that a single membrane may translocate by either an actin- or a microtubule-based motor (for review see Atkinson et al., 1992). The translocation of vesicles to forming bud sites in dividing *S. cerevisiae*, requires MYO2p, which is a member of the myosin-I family (Johnston et al., 1991). Temperature-sensitive mutants not expressing MYO2p accumulate vesicles in the cytoplasm; vesicles do not move to the plasma membrane to form a mitotic bud. These mutants can be rescued by the overexpression of the SMY1 gene that encodes for a protein sharing sequence similarity with the ATP-binding regions of kinesins (Lillie and Brown, 1992). Lillie and Brown (1992) conclude that the SMY1 protein may normally provide a minor microtubule-based pathway working in concert with MYO2p; only when MYO2p is not expressed is the microtubule-based motility functionally apparent. Further evidence for functional redundancy in vesicle motor systems comes from recent studies identifying actin-dependent organelle movement in squid axoplasm (Kuznetsov et al., 1992). These workers found that at the edge of isolated axoplasm, where microtubules and microfilaments are splayed out, organelles could translocate on both microfilaments and microtubules. Moreover, they observed single moving organelles apparently jumping from a microtubule to a microfilament track suggesting that a given organelle can use both motility systems.

Could the translocation of membranous vesicles using myosin-I be a general mechanism in many cells? In that most eukaryotic cells do not contain regular arrays of actin filaments in the deep cytoplasm, it is likely that microtubule-based motility is used in these regions. However, myosin-I–mediated motility may be important in the cell cortex and other areas that contain many actin filaments and relatively few microtubules. For example, as in the enterocyte, a subcortical actin network may form a physical barrier at the apical membrane in chromaffin cells (Aunis and Dader, 1988) and at the active site in presynaptic terminals of axons (Trimble et al., 1991). Perhaps a myosin-I–facilitated transport of chromaffin granules and synaptic vesicles through this actin network is required to allow vesicle fusion with the plasma membrane. These ideas are consistent with the proposal that individual vesicles possess multiple types of motor proteins, each of which is active or differentially regulated in different regions of the cell.

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### *References*

Achler, C., D. Filmer, C. Merte, and D. Drenckhahn. 1989. Role of microtubules in polarized delivery of apical membrane proteins to the brush border of the intestinal epithelium. *J. Cell Biol.* 109:179–189.

Adams, R. J., and T. D. Pollard. 1986. Propulsion of organelles isolated from *Acanthamoeba* along actin filaments by myosin-I. *Nature (Lond.).* 322:754–756.

Adams, R. J., and T. D. Pollard. 1989. Binding of myosin I to membrane lipids. *Nature (Lond).* 340:565–568.

Allan, V. J., and T. E. Kreis. 1986. A microtubule-binding protein associated with membranes of the Golgi apparatus. *J. Cell Biol.* 103:2229–2239.

Aoki, D., H. E. Appert, D. Johnson, S. S. Wong, and M. N. Fukuda. 1990. Analysis of the substrate binding sites of human galactosyltransferase by protein eingineering. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:3171–3178.

Atkinson, S. J., S. K. Doberstein, and T. D. Pollard. 1992. Moving off the beaten track. *Current Biology.* 2:326–328.

Aunis, D., and M.-F. Dader. 1988. The cytoskeleton as a barrier to exocytosis in secretory cells. *J. Exp. Biol.* 139:253–266.

Baines, I. C., and E. D. Korn. 1990. Localization of myosin IC and myosin II in *Acanthamoeba castellanii* by indirect immunofluorescence and immunogold electron microscopy. *J. Cell Biol.* 111:1895–1904.

Bennett, G., E. Carlet, G. Wild, and S. Parsons. 1984. Influence of colchicine and vinblastine on the intracellular migration of secretory and membrane glycoproteins. III. Inhibition of intracellular migration of membrane glycoproteins in rat intestinal columnar cells and hepatocytes as visualized by light and electron-microscope radioautography after $^3$H-fucose injection. *Am. J. Anat.* 170:545–566.

Bloom, G. S. 1992. Motor proteins for cytoplasmic microtubules. *Curr. Opin. Cell Biology.* 4:66–73.

Bloom, G. S., and T. A. Brashear. 1989. A novel 58-kDa protein associates with the Golgi apparatus and microtubules. *J. Biol. Chem.* 264:16083–16092.

Breitfeld, P. P., W. C. McKinnon, and K. E. Mostov. 1990. Effect of nocodazole on vesicular traffic to the apical and basolateral surfaces of polarized MDCK cells. *J. Cell Biol.* 111:2365–2373.

Bretscher, A., and K. Weber. 1979. Villin: the major microfilament-associated protein of the intestinal microvillus. *Proc. Natl. Acad. Sci. USA.* 76:2321–2325.

Broschat, K. O., R. P. Stidwill, and D. R. Burgess. 1983. Phosphorylation controls brush border motility by regulating myosin structure and association with the cytoskeleton. *Cell.* 35:561–571.

Burgess, D. R., W. Jiang, S. Mamajiwalla, and W. Kinsey. 1989. Intestinal crypt stem cells possess high levels of cytoskeletal-associated phosphotyrosine-containing proteins and tyrosine kinase activity relative to differentiated enterocytes. *J. Cell Biol.* 109:2139–2144.

Cheney, R. E., and M. S. Mooseker. 1992. Unconventional myosins. *Curr. Opin. Cell Biol.* 4:27–35.

Coffe, G., and M.-N. Raymond. 1990. Association between microtubules and Golgi vesicles isolated from rat parotid glands. *Biol. Cell.* 70:143–152.

Colas, B., and S. Maroux. 1980. Simultaneous isolation of brush border and basolateral membrane from rabbit enterocytes. Presence of brush border hydrolases in the basolateral membrane of rabbit enterocytes. *Biochim. Biophys. Acta.* 600:406–420.

Collins, K., J. R. Sellers, and P. Matsudaira. 1990. Calmodulin dissociation regulates brush border myosin I (110-kD-calmodulin) mechanochemical activity in vitro. *J. Cell Biol.* 110:1137–1147.

Coluccio, L. M., and A. Bretscher. 1988. Mapping of the microvillar 110K-calmodulin complex: calmodulin-associated or -free fragments of the 110-kD polypeptide bind F-actin and retain ATPase activity. *J. Cell Biol.* 106: 367–374.

Conzelman, K. A., and M. S. Mooseker. 1987. The 110-kD protein-calmodulin complex of the intestinal microvillus is an actin-activated MgATPase. *J. Cell Biol.* 105:313–324.

Cowell, G. M., and E. M. Danielsen. 1984. Biosynthesis of intestinal microvillar proteins: rapid expression of cytoskeletal components in microvilli of pig small intestinal mucosal explants. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 172:309–314.

Danielsen, M., and G. M. Cowell. 1985. Biosynthesis of intestinal microvillar proteins: evidence for an intracellular sorting taking place in, or shortly after, exit from the Golgi complex. *Eur. J. Biochem.* 152:493–499.

Drenckhahn, D., and R. Dermietzel. 1988. Organization of the actin filament cytoskeleton in the intestinal brush border: a quantitative and qualitative immunoelectron microscope study. *J. Cell Biol.* 107:1037–1048.

Eilers, U., J. Klumperman, and H.-P. Hauri. 1989. Nocodazole, a microtubule-active drug, interferes with apical protein delivery in cultured intestinal epithelial cells (Caco-2). *J. Cell Biol.* 108:13–22.

Fath, K. R., S. D. Obenauf, and D. R. Burgess. 1990. Cytoskeletal protein and mRNA accumulation during brush border formation in adult chicken enterocytes. *Development (Camb.).* 109:449–459.

Fujiki, Y., A. L. Hubbard, S. Fowler, and P. B. Lazarow. 1982. Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. *J. Cell Biol.* 93:97–102.

Gratecos, D., M. Knibiehler, V. Benoit, and M. Sémériva. 1978. Plasma membranes from rat intestinal epithelial cells at different stages of maturation. I. Preparation and characterization of plasma membrane subfractions originating from crypt cells and from villous cells. *Biochim. Biophys. Acta.* 512:508–524.

Griffiths, G., and K. Simons. 1986. The *trans* Golgi network: sorting at the exit site of the Golgi complex. *Science (Wash. DC).* 234:438–443.

Haase, W., A. Schäfer, H. Murer, and R. Kinne. 1978. Studies on the orientation of brush-border membrane vesicles. *Biochem. J.* 172:57–62.

Hayden, S. M., J. S. Wolenski, and M. S. Mooseker. 1990. Binding of brush border myosin I to phospholipid vesicles. *J. Cell Biol.* 111:443–451.

Heintzelman, M. B., and M. S. Mooseker. 1990. Assembly of the brush border cytoskeleton: changes in the distribution of microvillar core proteins during enterocyte differentiation in adult chicken intestine. *Cell Motil. Cytoskeleton.* 15:12–22.

Hugon, J. S., G. Bennett, P. Pothier, and Z. Ngoma. 1987. Loss of microtubules and alteration of glycoprotein migration in organ cultures of mouse intestine exposed to nocodazole or colchicine. *Cell Tissue Res.* 248:653–662.

Johnston, G. C., J. A. Prendergast, and R. A. Singer. 1991. The *Saccharomyces cerevisiae* MYO2 gene encodes an essential myosin for vectorial transport of vesicles. *J. Cell Biol.* 113:539–551.

Kachar, B. 1985. Direct visualization of organelle movement along actin filaments dissociated from Characean algae. *Science (Wash. DC).* 227:1355–1357.

Korn, E. D., and J. A. I. Hammer. 1988. Myosins of nonmuscle cells. *Annu. Rev. Biophys. Biophys. Chem.* 17:23–45.

Kuznetsov, S. A., G. M. Langford, and D. G. Weiss. 1992. Actin-dependent organelle movement in squid axoplasm. *Nature (Lond.).* 356:722–725.

LeCount, T. S., and R. D. Grey. 1972. Transient shortening of microvilli induced by cycloheximide in the duodenal epithelium of chicken. *J. Cell Biol.* 53:601–605.

Lillie, S. H., and S. S. Brown. 1992. Suppression of a myosin defect by a kinesin-related gene. *Nature (Lond.).* 356:358–361.

Louvard, D., S. Maroux, J. Baratti, P. Desnuelle, and S. Mutaftschiev. 1973. On the preparation and some properties of closed membrane vesicles from hog duodenal and jejunal brush border. *Biochim. Biophys. Acta.* 291:747–763.

Luby-Phelps, K., P. E. Castle, L. Taylor, and F. Lanni. 1987. Hindered diffusion of inert tracer particles in the cytoplasm of mouse 3T3 cells. *Proc. Natl. Acad. Sci. USA.* 84:4910–4913.

Matsudaira, P. T., and D. R. Burgess. 1978. SDS microslab linear gradient polyacrylamide gel electrophoresis. *Anal. Biochem.* 87:386–396.

Matsudaira, P. T., and D. R. Burgess. 1979. Identification and organization of the components in the isolated microvillus cytoskeleton. *J. Cell Biol.* 83:667–673.

Matsudaira, P. T., and D. R. Burgess. 1982. Organization of the cross-filaments in intestinal microvilli. *J. Cell Biol.* 92:657–664.

Miyata, H., B. Bowers, and E. D. Korn. 1989. Plasma membrane association of *Acanthamoeba* myosin I. *J. Cell Biol.* 109:1519–1528.

Mooseker, M. S., and T. R. Coleman. 1989. The 110-kD protein-calmodulin complex of the intestinal microvillus (brush border myosin I) is a mechanoenzyme. *J. Cell Biol.* 108:2395–2400.

Mooseker, M. S., and L. G. Tilney. 1975. The organization of an actin filament-membrane complex: filament polarity and membrane attachment in the microvilli of intestinal epithelial cells. *J. Cell Biol.* 67:725–743.

Mooseker, M. S., K. A. Conzelman, T. R. Coleman, J. E. Heuser, and M. P. Sheetz. 1989. Characterization of intestinal microvillar membrane disks: detergent-resistant membrane sheets enriched in associated brush border myosin I (110K-calmodulin). *J. Cell Biol.* 109:1153–1161.

Pardee, J. D., and J. A. Spudich. 1982. Purification of muscle actin. *In* The Cytoskeleton Part. A. L. Wilson, editor. Academic Press, New York. 24. 271–289.

Pavelka, M., A. Ellinger, and A. Gangl. 1983. Effect of colchicine on rat small intestinal absorptive cells. *J. Ultrastruct. Res.* 85:249–259.

Pollard, T. D., S. K. Doberstein, and H. G. Zot. 1991. Myosin-I. *Annu. Rev. Physiol.* 53:653–681.

Quaroni, A., K. Kirsch, and M. M. Weiser. 1979. Synthesis of membrane glycoproteins in rat small-intestinal villus cells. Effect of colchicine on the redistribution of L-[1,5,6-$^3$H]fucose-labelled membrane glycoproteins among Golgi, lateral basal and microvillus membranes. *Biochem. J.* 182:213–221.

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

Sandoz, D., M.-C. Lainé, and G. Nicolas. 1985. Distribution of microtubules within the intestinal terminal web as revealed by quick-freezing and cryosubstitution. *Eur. J. Cell Biol.* 39:481–484.

Schroer, T. A., and M. P. Sheetz. 1991. Functions of microtubule-based motors. *Annu. Rev. Physiol.* 53:629–652.

Schroer, T. A., E. R. Steuer, and M. P. Sheetz. 1989. Cytoplasmic dynein is a minus end-directed motor for membranous organelles. *Cell.* 56:937–946.

Sheetz, M. P., D. B. Wayne, and A. L. Pearlman. 1992. Extension of filopodia by motor-dependent actin assembly. *Cell Motil. Cytoskeleton.* 22:160–169.

Shibayama, T., J. M. Carboni, and M. S. Mooseker. 1987. Assembly of the intestinal brush border: appearance and redistribution of microvillar core proteins in developing chick enterocytes. *J. Cell Biol.* 105:335–344.

Stidwill, R. P., T. Wysolmerski, and D. R. Burgess. 1984. The brush border cytoskeleton is not static: in vivo turnover of proteins. *J. Cell Biol.* 98:641–654.

Storrie, B., and E. A. Madden. 1990. Isolation of subcellular organelles. *Methods Enzymol.* 182:203–225.

Swanljung-Collins, H., and J. H. Collins. 1992. Phosphorylation of brush bor-

der myosin I by protein kinase C is regulated by $Ca^{++}$-stimulated binding of myosin I to phosphatidylserine concerted with calmodulin dissociation. *J. Biol. Chem.* 267:3445–3454.

Trimble, W. S., M. Linial, and R. H. Scheller. 1991. Cellular and molecular biology of the presynaptic nerve terminal. *Annu. Rev. Neurosci.* 14:93–122.

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

Weiser, M. M. 1973. Intestinal epithelial cell surface membrane glycoprotein synthesis. I. An indicator of cellular differentiation. *J. Biol. Chem.* 248:2536–2541.

Weiser, M. M., M. M. Neumeier, A. Quaroni, and K. Kirsch. 1978. Synthesis of plasmalemmal glycoproteins in intestinal epithelial cells. Separation of Golgi membranes from villus and crypt cell surface membranes; glycosyltransferase activity of surface membrane. *J. Cell Biol.* 77:722–734.

Weiser, M. M., J. Walters, and J. R. Wilson. 1986. Intestinal cell membranes. *Int. Rev Cytol.* 101:1–57.

Zot, H. G., S. K. Doberstein, and T. D. Pollard. 1992. Myosin-I moves actin filaments on a phospholipid substrate: implications for membrane targeting. *J. Cell Biol.* 116:367–376.