Actin Is Required for Endocytosis at the Apical Surface of Madin-Darby Canine Kidney Cells where ARF6 and Clathrin Regulate the Actin Cytoskeleton

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In epithelial cell lines, apical but not basolateral clathrin-mediated endocytosis has been shown to be affected by actin-disrupting drugs. Using electron and fluorescence microscopy, as well as biochemical assays, we show that the amount of actin dedicated to endocytosis is limiting at the apical surface of epithelia. In part, this contributes to the low basal rate of clathrin-dependent endocytosis observed at this epithelial surface. ARF6 in its GTP-bound state triggers the recruitment of actin from the cell cortex to the clathrin-coated pit to enable dynamin-dependent endocytosis. In addition, we show that perturbation of the apical endocytic system by expression of a clathrin heavy-chain mutant results in the collapse of microvilli. This phenotype was completely reversed by the expression of an ARF6-GTP-locked mutant. These observations indicate that concomitant to actin recruitment, the apical clathrin endocytic system is deeply involved in the morphology of the apical plasma membrane.

INTRODUCTION

Endocytosis is an essential process in eukaryotic cells that is used for a variety of cellular functions, such as nutrient uptake and cell-surface-receptor internalization. Clathrin-mediated endocytosis (CME) is the most well-characterized pathway for the internalization of soluble macromolecules and integral membrane proteins from the plasma membrane (PM; da Costa *et al.*, 2003).

Actin has been implicated in the endocytic process in a number of studies using a wide range of approaches. The most conclusive evidence coupling the endocytic machinery with the actin cytoskeleton first came from genetic analyses in yeast that identified actin-associated proteins required for endocytosis. Proof of this association has been strengthened by the observation that a continuous turnover of actin filaments is essential for yeast endocytosis (Geli and Riezman, 1998; Munn, 2001). In yeast, proteins that directly or indirectly interact with actin and that are essential for endocytosis have recently been identified: Sla2p/End4p (Wesp *et al.*, 1997; Iwanicki *et al.*, 2002) binds directly to actin through a talinlike domain and participates in endocytosis through its N-terminal domain, whereas the Pan1p scaffolding protein blocks endocytosis and aggregates actin when overexpressed (Duncan *et al.*, 2001; Miliaras *et al.*, 2004). Further, Abp1p, which is known to affect nucleation and branching

by the Arp2/3 complex, has been shown to interact indirectly with dynamin (Goode *et al.*, 2001; Fenster *et al.*, 2003). Studies utilizing pharmacological agents have yielded conflicting results with respect to actin's involvement in mammalian endocytosis (Fujimoto *et al.*, 2000; Yazar *et al.*, 2005). Nevertheless, proteins that both modulate actin dynamics and interact with endocytic components have been identified (Jeng and Welch, 2001; Schafer, 2002). For example, the mammalian homologue of Sla2p, the mouse actin-binding protein Hip1R (Engqvist-Goldstein *et al.*, 1999), has been shown to bind both actin and clathrin light chain (Bennett *et al.*, 2001; Engqvist-Goldstein *et al.*, 2001). The mouse protein Abp1 has been found to recruit Arp2/3 complex to the sides of actin filaments and link the growing filaments to endocytic events (Goode *et al.*, 2001). Two other proteins, intersectin (Hussain *et al.*, 2001) and syndapin (Qualmann and Kelly, 2000), both bind N-WASP, which in turn induces actin polymerization via Arp2/3. A more mechanistic study has provided evidence for the transient accumulation of actin at the clathrin-coated pit (CCP) after the appearance of dynamin, just before internalization (Merrifield *et al.*, 2002).

Several actin-targeted pharmacological agents have been used to investigate the relationship between actin filaments and endocytosis, generating results specific to either a cell line (Fujimoto *et al.*, 2000) or a PM domain (Gottlieb *et al.*, 1993). In polarized epithelial cells, discerning the role of actin microfilaments in endocytosis is complicated, because macromolecules can be internalized by CME from apical as well as basolateral domains (Gottlieb *et al.*, 1993). Previous work in epithelia suggests that actin filaments play a more important role in apical than basolateral PM endocytosis. Cytochalasin D inhibits receptor-mediated and fluid-phase endocytosis at the apical surface of polarized Caco2 (Jackman *et al.*, 1994) and Madin-Darby canine kidney (MDCK) cells with a concomitant increase in clathrin-coated pits (CCPs) in this membrane (Gottlieb *et al.*, 1993), but has no effect on endocytosis from the basolateral surface. An in-

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Abbreviations used: pIgA, polymeric IgA; PM, plasma membrane; CME, clathrin-mediated endocytosis; CCP, clathrin-coated pit; GFP, green fluorescent protein; MDCK, Madin-Darby canine kidney; ARF, ADP ribosylation factor; Dx, doxycycline; WT, wild type.

depth study of the effects of actin-perturbing drugs on endocytosis gave variable results, depending on the cell type used. It was concluded that actin may play a key, but not obligatory role in receptor-mediated endocytosis in mammalian cells (Fujimoto *et al.*, 2000). However, the roles of actin filaments at different stages of endocytosis remain to be clarified. Myosin VI is the first actin-based motor protein identified to play a specific role in polarized epithelial endocytosis, its C-terminal tail conferring its apical localization. The alternatively spliced isoform of myosin VI, containing a 30-aa insert at this tail, localizes to CCPs in the apical PM. Expression of this C-terminal tail in fibroblasts inhibits transferrin endocytosis (Buss *et al.*, 2001).

The ADP ribosylation factor (ARF) subfamily of small GTPases has been shown to play critical roles in vesicular transport (Donaldson and Klausner, 1994; D'Souza-Schorey *et al.*, 1995). ARF6, the least conserved of the human ARF proteins, is localized at the cell periphery and cycles between the PM and intracellular endosomal vesicles, depending on its nucleotide status (D'Souza-Schorey *et al.*, 1995; Peters *et al.*, 1995). Expression of the ARF6-GTP-locked mutant, Q67L, results in extensive F-actin-rich protrusions and ruffles at the PM, as well as redistribution of endosomal membrane to the cell periphery. This suggests that ARF6 coordinates membrane-traffic regulation and actin assembly (D'Souza-Schorey *et al.*, 1997; Song *et al.*, 1998; Radhakrishna *et al.*, 1999). In support of this, in nonpolarized cells, inhibitors of actin assembly block the recycling of membrane components back to the PM (Radhakrishna and Donaldson, 1997), and expression of the putative ARF guanine nucleotide exchange factors, ARNO and EFA6, induces the formation of F-actin-rich ruffles and promotes cell spreading (Frank *et al.*, 1998; Franco *et al.*, 1999). Expression of ARF6-Q67L results in remodeling of the actin cytoskeleton by inducing actin polymerization at the cell periphery. ARF6 regulates actin cytoskeletal organization by a mechanism independent of Rac1 and suggests a role for POR1 in ARF6-mediated signal transduction (D'Souza-Schorey *et al.*, 1997). ARF6 may stimulate actin assembly via a tyrosine kinase-signaling cascade similar to that used by the vaccinia virus (Schafer *et al.*, 2000). We have shown that ARF6 is localized at and regulates clathrin-dynamin-dependent endocytosis at the apical surface of epithelial cells, where actin plays a critical role in endocytosis and microvillus morphology (Altschuler *et al.*, 1999).

Here, we present biochemical and morphological data showing that ARF6 couples the actin cytoskeleton to CCPs to stimulate apical endocytosis in polarized MDCK epithelial cells. Actin is shown to be the limiting factor for this process. Moreover, correctly functioning apical endocytic machinery, dependent on ARF6 and clathrin, is required for the actin-dependent structure of microvilli.

MATERIALS AND METHODS

Materials

Cytochalasin D and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. Growth media were from Biological Industries (Beit Haemek, Israel). All fluorescent secondary antibodies and Alexa Fluor 488 phalloidin were from Molecular Probes (Eugene, OR). T7 tag antibody was from Novagen (San Diego, CA). X22 antibody against clathrin heavy chain was kindly provided by Frances Brodsky (UCSF, San Francisco, CA). All images were compiled using Adobe Photoshop (San Jose, CA), and/or Canvas software (ACD Systems International, Saanichton, British Columbia, Canada), and are representative of the original data.

Methods

MDCK cells were grown as previously described (Altschuler *et al.*, 1999). Adenovirus-expressing actin-green fluorescent protein (GFP) chimera was a

gift from D. Kalman (Emory University, Atlanta, GA). The gene for actin was amplified and fused to GFP according to Ballestrem *et al.* (1998) and then subcloned into a pADlox vector and used for adenovirus production (Hardy *et al.*, 1997). Recombinant adenoviruses encoding ARF6, dynamin and their corresponding mutants, as well as the clathrin hub, were produced as described previously (Altschuler *et al.*, 1998, 1999). Protein levels were regulated by the concentration of doxycycline (Dx), the amount of virus, and the length of time after infection and/or Dx removal, because very high levels of expression produce toxic effects. For instance, 0.1 ng/ml Dx was included to partially repress ARF6 production. Cells infected by recombinant adenovirus were incubated for 16–18 h to express recombinant proteins (ARF6 and mutants, dynamin I and mutants, and actin-GFP), except for the clathrin hub, which took 24–26-h incubation. Unless stated otherwise, we used 60–70 pfu/cell for ARF6-WT and ARF6-Q67L or 90–100 pfu/cell for ARF6-T27N. These produced equal amounts of ARF6 protein, as determined by immunoblot assay. Using antibodies that specifically recognize endogenous ARF6 (kind gift of V. Hsu), the level of exogenous ARF6 was determined to be about fivefold that of endogenous ARF6. For immunofluorescence studies in ARF6-expressing cells, we omitted the Dx to produce ninefold overexpression relative to endogenous levels. These conditions minimized the possibility of toxic effects and produced an adequate signal for our localization and functional studies. Controls in all experiments included cells that were 1) not infected, 2) infected but the expression of ARF6, dynamin I K44A, actin GFP, or clathrin hub was fully repressed by 20 ng/ml Dx, or 3) infected with a control virus encoding β -galactosidase (gal). These caused complete loss of the ARF6-, dynamin I-, actin-GFP-, or clathrin-hub-specific signal in immunofluorescence and biochemical studies. In immunofluorescence experiments containing cytochalasin D, cells were preincubated with the drug (1 μ M) for 60 min before fixation. Images were taken using a Bio-Rad (Richmond, CA) confocal or Nikon TE-2000S (Nikon, Melville, NY) inverted fluorescence microscope with a plan Apo 60X objective lens (Nikon), equipped with a Z stepper and a Hamamatsu CCD ORCAII camera (Hamamatsu, Tucson, AZ). Images were all deconvolved with SimplePCI software (Improvision, Coventry, United Kingdom).

Subcellular fractionation was modified from a previously described protocol to maintain actin in polymerized form (Huttner *et al.*, 1983). Briefly, MDCK cells were grown on 10-cm dishes for 3 d, infected with appropriate recombinant adenoviruses, and incubated for 18 h. Cells were rinsed with cold phosphate-buffered saline (PBS; containing 1 mM CaCl₂ and 1 mM MgCl₂), scraped, and resuspended in ice-cold homogenization buffer (100 mM NaCl, 3 mM imidazole, 300 mM sucrose, 20 mM Tris, 1 mM DTT protease inhibitor cocktail, pH 8.0). Cells were homogenized by 80–90 passages through 27-gauge syringe. Subsequently, the homogenate was centrifuged at 1000 $\times$ g to remove nuclei and large debris. The supernatant was centrifuged at 10,000 $\times$ g to obtain a crude PM fraction. The resulting supernatant was centrifuged at 165,000 $\times$ g to obtain a light membrane fraction (endoplasmic reticulum and Golgi) and the supernatant (Cyt) containing the cell cytosol. After each centrifugation the resulting pellet was rinsed briefly with ice-cold PBS before subsequent fractionations to avoid possible cross-over contamination.

Processing for electron microscopy was as described previously (Apodaca *et al.*, 1993). Cells were observed at a magnification of 19,000 $\times$ g and every third cell was photographed and viewed at 80 kV. A total of 30 randomly selected cell profiles were photographed. The negatives were scanned using Adobe Photoshop at a resolution of 1000 dpi.

Endocytosis was assayed as described previously (Altschuler *et al.*, 1999). In endocytosis experiments containing cytochalasin D, cells were preincubated with the drug (1 μ M) for 60 min before the beginning of the experiment, as well as throughout the experiment.

RESULTS

The Endocytic Machinery Influences the Actin Cytoskeleton and Cell Morphology

Pharmacological or biochemical disruption of the actin cytoskeleton is known to disrupt polarized cells' structure (Apodaca, 2001). We examined the previously proposed hypothesis that the actin cytoskeleton and endocytic machinery exclusively interact at the apical PM in polarized cell endocytosis (Gottlieb *et al.*, 1993). Moreover, because the molecular mechanism underlying actin's involvement in apical endocytosis has not been examined, we investigated the effect of cytochalasin D-mediated actin disruption on clathrin- and ARF6-dependent apical endocytosis, as well as on clathrin basolateral endocytosis of polymeric pIgA receptor by the IgA receptor. IgA endocytosis was quantified at both the apical and basolateral surfaces of filter-grown polarized MDCK cells after their exposure to growth media with or without cytochalasin D.

Figure 1. Effect of cytochalasin D on basolateral and ARF6-stimulated apical endocytosis. (A) MDCK tet off cells were grown for 3 d on Corning transwells. Control (uninfected) and ARF6-Q67L-expressing cells were then preincubated for 60 min with or without 1 μ M cytochalasin D and incubated for 18 h. Cells were then assayed for IgA endocytosis from the apical surface for 5 min at 37°C. (B) As in A, except that the cells were assayed for IgA endocytosis from the basolateral surface for 5 min at 37°C. All assays were performed in triplicate and repeated at least three times.

In agreement with previous studies (Gottlieb *et al.*, 1993; Jackman *et al.*, 1994), basolateral endocytosis of pIgA was unaffected by cytochalasin D (Figure 1B), whereas its apical endocytosis was inhibited by 60–80% (Figure 1A). The apical endocytic machinery requires the activity of the small GTPase ARF6 (Altschuler *et al.*, 1999). Expression of an ARF6-GTP-locked mutant (ARF6-Q67L) causes a sixfold increase in apical endocytosis of pIgA (Altschuler *et al.*, 1999; Figure 1A). Actin disruption with cytochalasin D reduces ARF6-Q67L-stimulated endocytosis by ~70%, indicating that polymerized actin is essential for clathrin- and ARF6-dependent apical endocytosis, but not basolateral endocytosis, in polarized MDCK cells.

We assessed whether redistribution of the actin cytoskeleton occurs when either WT or GTP-locked ARF6 is expressed in MDCK cells. The apical PM was visualized by antibodies to the apical marker gp135 (Ojakian *et al.*, 1990).

Figure 2. ARF6 stimulates translocation of cortical actin to the apical PM. Confocal images of MDCK tet off uninfected (control) cells, cells expressing ARF6-WT and those expressing the ARF6-Q67L for 18 h were costained for apical PM marker with anti-gp135 (green) followed by donkey anti-mouse Alexa 488 and for polymerized actin (red; phalloidin-Alexa 594). Sections were taken above the tight junction, through the apical domain. Arrows indicate plasma membrane (PM). Bar, 10 μ m.

The actin cytoskeleton was visualized using phalloidin staining of filter-grown MDCK cells. In both control MDCK cells and those expressing ARF6-WT, actin was localized to the subapical actin network. However, expression of ARF6-Q67L caused extensive recruitment of actin from the subapical network to the apical PM (Figure 2C). gp135 apical localization was unaffected in cells in which actin was recruited to the membrane by the GTP-locked ARF6 mutant (Figure 2).

ARF6-Q67L Expression Induces Colocalization of Actin and Clathrin at the Apical Cell Surface

Expression of a functional actin-GFP chimera (Morales *et al.*, 2000) had no apparent effect on actin organization, as determined by red fluorescent phalloidin staining of fixed cells (unpublished data). Expression of actin-GFP resulted in higher-resolution labeling of the subapical cytoskeletal meshwork (Figure 3, A and B) than phalloidin staining (Figure 2, A and B). In contrast to actin fibers that localize along the basolateral PM (Figure 3, E and F), actin at the apical surface forms rods that are perpendicular to the apical membrane. Coimmunofluorescence staining with antibodies against clathrin heavy chain (X22 antibodies) demonstrated a typical apical PM localization (Figure 4A) and minor colocalization with actin. However, both actin and clathrin exhibited consistent proximity: areas that contained a high prevalence of actin also contained a high prevalence of clathrin. Expression of ARF6-WT did not alter the localization of actin-GFP with respect to clathrin (unpublished data). In contrast, expression of ARF6-Q67L redistributed both clathrin and actin-GFP into a fine meshwork, with a significant increase in their colocalization (Figures 4A and 2C). Exposure of cells to cytochalasin D resulted in diffuse actin staining (Figure 4B). In cells expressing ARF6-Q67L, both actin and clathrin showed increasingly diffuse staining (Figure 4B). Cytochalasin D exclusively reversed the colocalization of actin-GFP and clathrin but did not have any

Figure 3. Actin-GFP expression enables high-resolution actin tracing at the apical PM. MDCK tet off cells grown for 3 d on Corning transwells were infected with 150 pfu/cell of adenovirus expressing actin-GFP and expressed for 18 h. Cells were fixed, and XY sections were taken every micron through the apical domain. (A and B) The longitudinal extension of apical actin, creating the terminal web and microvilli; sections through the tight junction (C and D) and sections through the lateral PM (E and F) show actin stress fibers. Images were collected through GFP and RFP filters followed by deconvolution. Arrowheads indicate longitudinal actin extensions; arrows indicate horizontal actin extensions. Bar, 10 μ m.

apparent affect on the meshwork of actin and clathrin (Figure 4B).

The Level of Actin Tightly Regulates Endocytosis at the Apical PM

Our results indicated that maintenance of the apical actin cytoskeleton is essential for endocytic processes at the apical PM. The rate of endocytosis at the apical PM has been shown to be approximately one-fifth its rate at the basolateral surface (Naim *et al.*, 1995). We reasoned that the actin required for this process was in limiting concentrations and that increased actin might enhance the rate of actin-dependent apical endocytosis. Therefore, we expressed increasing concentrations of actin-GFP in MDCK cells by applying increasing amounts of the recombinant adenovirus particles encoding it. This resulted in an increased rate of apical pIgA endocytosis. Moreover, even at low levels of actin-GFP expression, IgA endocytosis increased significantly (Figure 5A). In addition, IgA actin-dependent endocytosis was analyzed by immunofluorescence microscopy, using GFP expression to identify cells that express actin and IgA (red) to trace endocytosed IgA. We found a correlation between increased internalization of IgA to expression of actin within cells that overexpress actin (Figure 6B). In bottom panel of Figure 6B we show two cells, one of which expresses actin-GFP and reveals much higher amount of endocytosed IgA than the adjacent cell that does not overexpress actin reveals reduced-control level of endocytosed IgA. This indicated that despite the abundance of actin at the apical cortex, the pool of actin devoted to endocytosis is limiting.

Figure 4. Expression of ARF6-Q67L stimulates colocalization of actin and clathrin on the apical PM. (A) MDCK tet off cells were grown for 3 d on Corning transwells. Uninfected (control) cells, and cells expressing ARF6-WT or ARF6-Q67L, all coexpressing actin-GFP, were stained for clathrin using X22 antibody (Alexa 594). Sections were taken at the top of the apical domain. Control and ARF6-WT cells show no colocalization. ARF6-Q67L cells show increased colocalization and the generation of a clathrin network above that of actin. (B) Uninfected cells and those expressing ARF6-Q67L were infected with actin-GFP and expressed for 18 h. They were then treated with cytochalasin D for 60 min. Cells were stained for clathrin using X22 antibody (Alexa 594). Sections were taken at the top of the apical domain. Cytochalasin D abolished colocalization of clathrin and actin, creating diffuse staining of both. Bar, 2 μ m.

To determine whether actin-driven endocytosis is dynamin-dependent (Altschuler *et al.*, 1998, 1999), we coexpressed actin-GFP with the dominant-negative dynamin I K44A mutant: we found actin-stimulated apical endocytosis was inhibited (Figure 5B), supporting actin's participation in a dynamin-dependent endocytic process. To further characterize the role of ARF6 in actin-dependent apical endocytosis, we coexpressed both actin-GFP and the GTP-locked ARF6 mutant. Because of the high rate of apical endocytosis obtained in cells expressing each of these constructs, we expressed reduced amounts of recombinant adenovirus of both ARF6-Q67L and actin-GFP and measured endocytosis during a 1-min uptake. When both ARF6-Q67L and actin were expressed together, there was additive stimulation

Figure 5. Actin-GFP is a limiting factor for clathrin-dynamin-dependent apical endocytosis. MDCK tet off cells were grown for 3 d on Corning transwells. (A) Control cells (uninfected) and cells expressing 1× (50 pfu/cell) and 3× (150 pfu/cell) actin-GFP for 18 h were used in a 5-min assay of IgA endocytosis from the apical surface. Actin-GFP stimulates apical endocytosis. (B) Control cells (uninfected), cells expressing 1× (50 pfu/cell) actin-GFP, and 1× actin-GFP together with the dynamin I K44A mutant for 18 h were used in a 5-min assay of IgA endocytosis from the apical surface. Dynamin I K44A mutant inhibits actin-stimulated apical endocytosis. Data are shown relative to control cells. (C) Control cells (uninfected), cells expressing 0.5× (25 pfu/cell) ARF6-Q67L and cells coexpressing reduced ARF6-Q67L and 0.5× actin were used in a 1-min assay of IgA endocytosis from the apical surface. ARF6-Q67L and actin additively increase apical endocytosis. (D) Control cells (uninfected), cells expressing clathrin hub mutant (150 pfu/cell) for 24 h, cells expressing 0.5× actin-GFP (25 pfu/cell) for 18 h and cells coexpressing the two were used in a 5-min assay of IgA endocytosis from the apical surface. Clathrin hub mutant is inhibitory to actin-dependent apical endocytosis.

(Figure 5C), indicating the ARF6 dependence of actin's involvement in apical endocytosis. Finally, we confirmed that this actin-ARF6-stimulated endocytosis process was mediated by clathrin. Expression of a clathrin hub mutant has a dominant-negative effect on CME in nonpolarized cells, as well as in polarized epithelial cells (Lu *et al.*, 1998; Altschuler *et al.*, 1999; Trejo *et al.*, 2000). Here we show that clathrin hub inhibits the stimulatory effect of actin-GFP (Figure 5D). To

obtain a better insight into clathrin participation in actin dependent endocytosis at the apical surface, we have performed analyses with immunofluorescence microscopy. In Figure 6C we have expressed both actin-GFP and clathrin hub and examined actin and endocytosed IgA. Cells that do not show actin expression show modest amount of endocytosed IgA, cells that reveal actin expression through GFP show reduced amount of endocytosed IgA in comparison to

Figure 6. Both actin and clathrin affect apical endocytosis MDCK tet off cells grown for 3 d on Corning transwells were either not infected (A), infected with actin-GFP (B), both actin GFP and clathrin hub (C), or clathrin hub only (D) and expressed for 18 h for actin-GFP and 24 h for clathrin hub. Cells were then incubated in the

cells that express actin alone (Figure 6B). In Figure 6D, we have expressed only clathrin hub and found very modest IgA endocytosis. In the bottom panel of Figure 6D we show enlarged view of two cells, of which only one expressed clathrin hub (Figure 6D, bottom panel, left cell) and observe no IgA endocytosis in this cell compared with the adjacent cell that do not express clathrin hub (right cell) and reveal endocytosed IgA. These set of results indicate that IgA endocytosis at the apical surface is clathrin dependent in both the basal endocytosis and when actin is present in excess and stimulates endocytosis. This demonstrated actin involvement in the ARF6-clathrin-dynamin endocytosis pathway at the apical surface of MDCK cells.

Newly Synthesized Actin Binds the PM in a Clathrin-dependent Manner

Our results show that actin tightly affects endocytosis through a direct or indirect association with clathrin. To establish if there was a role for clathrin in actin distribution, we have performed cell fractionation to obtain cytosol and PM fractions devoid of light membranes (endoplasmic reticulum, Golgi) without altering the polymerization state of actin. In Figure 7 we show actin is distributed equally between the PM and cytosol (54:46 PM to cytosol, respectively). Expression of clathrin hub did not alter endogenous actin distribution. Expression of actin-GFP for 18 h before cell fractionation increased actin by as little as 17–25% over endogenous actin. Although this expression did not alter the distribution of endogenous actin, it did not follow the equal PM::cytosol distribution but significantly ($p = 0.021$; t test) favored the PM fraction (Figure 7). Interestingly, when Actin GFP and clathrin hub are coexpressed both actin GFP and endogenous actin fractionation into the PM drops by an average of 38% ($p = 0.003$, $p = 0.02$, respectively) to less than half of the control level. These results indicate that clathrin is a major factor involved in actin binding to the apical PM.

Actin Cytoskeleton Organization Requires Functional Endocytic Machinery

Microvilli are one of the core characteristics of epithelial morphology known to depend on the actin cytoskeleton. We previously expressed a dominant-negative clathrin hub mutant, which is known to disrupt endocytosis in nonpolarized cells, as well as the apical and basolateral PM endocytic system (Liu *et al.*, 1998; Lu *et al.*, 1998; Altschuler *et al.*, 1999; Trejo *et al.*, 2000). Electron microscopy of cells expressing this dominant-negative clathrin hub showed the collapse of all apical microvilli. In contrast to the partial inhibitory effect of clathrin hub on endocytosis (Altschuler *et al.*, 1999), the effect on microvilli was complete, manifested by their full collapse. This indicated that it had affected cells expressing both low and high levels of the dominant-negative clathrin:

cold with IgA placed in the apical chamber for 60 min, excess IgA was washed, and cells were warmed up for 5 min to allow endocytosis to take place. Next cells were cooled to 4°C, and IgA remaining at the apical PM (not endocytosed) was removed by treatment with trypsin. Subsequently cells were fixed and processed for indirect immunofluorescence. Actin is labeled by GFP (A–C) and clathrin hub labeled by T7 tag antibody followed by goat anti-mouse Alexa 488 (D), endocytosed IgA is labeled by sheep anti-human IgA followed by goat anti-sheep Alexa 594. Cells that express actin show increase in endocytosed IgA (B). Clathrin hub expression reduces IgA endocytosis in cells expressing clathrin hub only (D) as well as in cells coexpressing actin and clathrin hub (C). Bar, 10 μ M.

Figure 7. Clathrin hub mutant dramatically reduces actin association with the PM. Subcellular fractionation of MDCK cells expressing clathrin hub, actin GFP, both clathrin hub and actin GFP, or none of the above was performed as described in *Materials and Methods*. Crude PM fraction (PM) and cytosolic fraction (Cyt) were resolved on 10% SDS-PAGE, and membranes were probed with anti-actin antibodies. Representative Western blot reveals actin and actin-GFP when appropriate (A). To ensure the integrity of fractionation, membranes were probed with anti-pIgR (SC166) antibodies correct fractionation (unpublished data). Western blots of three different experiments were scanned, and densitometry performed using NIH Image software. (B) Histogram illustrates the ratio between actin and actin GFP appearing in the crude PM compared with cytosol fraction; mean $\pm$ SD ($n = 3$). ** $p < 0.01$ and * $p < 0.05$ indicate significantly different from control (t test). Actin GFP expression results in distribution of the newly synthesized actin to associate with the PM. Expression of both actin GFP and clathrin hub dramatically reverses the actin distribution toward the cytosol.

all microvilli were present, but collapsed on one another or onto the apical PM (Figure 8B).

Remarkably, expression of ARF6-Q67L completely restored microvillus morphology in all cells expressing the clathrin hub (Figure 8C). Expression of dynamin I K44A, a potent inhibitor

of endocytosis, had no effect on microvilli structure, indicating that endocytosis is not required for the microvilli structure (Figure 8D). Thus, clathrin is required for microvilli structure; the CCPs are not present on microvilli, but rather within the apical membrane between the microvilli.

Figure 8. Clathrin and ARF6 positively regulate microvillus structure as shown by EM. MDCK tet off cells grown for 3 d on Corning transwells were either not infected, infected with adenovirus expressing clathrin hub, and expressed for 24 h or infected with both clathrin hub (24 h expression) and ARF6-Q67L (18-h expression). Cells were then processed for EM and a representative cell is shown. (A) Typical example of apical microvillus structure at the apical PM of MDCK control cells. (B) Typical apical microvilli of cells expressing the clathrin hub mutant have collapsed. (C) Typical apical microvilli of cells coexpressing clathrin hub and ARF6-Q67L. The latter rescues the microvillus collapse resulting from clathrin mutant expression. (D) Typical apical microvilli of cells expressing Dynamin I K44A no effect on microvilli structure and indicate that the endocytic process is not involved in maintenance of microvilli.

DISCUSSION

Cell polarity is a fundamental aspect of higher eukaryotic life. This feature is achieved in epithelia by a polarized cytoskeletal organization: actin forms a basolateral fringe, as well as a subapical terminal web and microvilli at the apical domain.

Recent findings highlight the complexity of actin's involvement in endocytosis (Fujimoto *et al.*, 2000; Duncan *et al.*, 2001; Goode *et al.*, 2001; Merrifield *et al.*, 2002; Schafer, 2002; Fenster *et al.*, 2003; Merrifield, 2004; Yazar *et al.*, 2005). Actin is associated via Hip1R to clathrin (Bennett *et al.*, 2001; Engqvist-Goldstein *et al.*, 2001; Chen and Brodsky, 2005; Legendre-Guillemin *et al.*, 2005), which may support the invagination step in which actin facilitates the withdrawal of the CCP away from the PM; the second occurs later, during the scission step, through an association with dynamin. The latter association may help in the constriction and release of the vesicle from the membrane and is achieved through syndapin (Qualmann and Kelly, 2000), Pan1 (an Eps15 homologue; Duncan *et al.*, 2001; Miliaras *et al.*, 2004), cortactin (Weaver *et al.*, 2002; Cao *et al.*, 2003), and Abp1 (Wesp *et al.*, 1997; Fenster *et al.*, 2003).

We examined the potential interaction between actin organization and differential endocytosis in polarized MDCK cells.

Actin Is an Obligatory and Limiting Factor for Endocytosis at the Apical PM

Cytochalasin D is a fungal metabolite that inhibits actin microfilament formation by capping the growing filament's

barbed end and thus preventing its growth, resulting in shortened microfilaments. Treatment of polarized MDCK monolayers with cytochalasin D has a cell-surface-specific effect on endocytosis, whereby apical endocytosis is over 80% reduced, whereas basolateral endocytosis is unaffected (Gottlieb *et al.*, 1993; Figure 1). Thus, growth of the actin filament is required for apical endocytosis. In other forms of endocytosis, such as basolateral, actin may have already reached the CCP: its polarization would then no longer be required, making the process insensitive to cytochalasin D.

We utilized the fusion protein actin-GFP to image actin. This chimera has been shown to provide staining similar to phalloidin in hippocampal neurons in a study that provided evidence of actin's involvement in the release of neurotransmitter (Morales *et al.*, 2000) and to resolve the transient incorporation of actin in CCP movement (Merrifield *et al.*, 2002). It should be noted that the effect of GFP on the polymerization rate of actin has never been examined. Nevertheless, actin-GFP remains a reliable and well-characterized tool for examining actin dynamics and function during endocytosis.

Actin was resolved as a cortical pellicular web in polarized MDCK cells. Actin and clathrin were found in the apical sections of control cells. The images of actin distribution, which were more punctuate and therefore detailed than phalloidin staining, showed vertical actin fibers that adjoin the cortical apical terminal web and microvilli (Figure 3, A and B). Actin activity and localization coincided with previous determinations by immunoelectron microscopy and phalloidin staining.

Actin is concentrated at the apical cortex to serve the many functions of the apical domain within epithelial cells. We tested whether actin is a limiting factor for apical endocytosis. Surprisingly, when we moderately overexpressed actin, we found five- to sixfold stimulation of apical endocytosis, indicating that despite the amount of actin at the apical cortex, that dedicated to endocytosis is limited. The discovery of myosin VI, which specifically localizes to the CCPs at the apical PM, deserves special attention. This actin motor protein, unlike all other myosins, moves toward the minus end of the actin filament, i.e., it moves away from the membrane; this may support the hypothesis that actin together with myosin VI participate in pulling the growing pit away from the membrane as well as in constricting the CCP at its base (Buss *et al.*, 2004). This hypothesis is bolstered by the interaction between myosin VI and Dab2, the latter linking myosin VI to receptors on the membrane (Morris and Cooper, 2001) and thus supports its involvement in the early steps of endocytosis. In agreement with the published data, we speculate that myosin VI is positioned at the apical shallow CCPs and that actin's growth into their vicinity (in an ARF6-GTP-dependent manner) results in their interaction: together, they participate in further invagination of the shallow coated pit to a deeply constricted one.

Actin Affects the ARF6-dependent Apical Endocytosis Pathway

We previously demonstrated a regulatory role for ARF6 in apical endocytosis: expression of an ARF6-GTP-locked mutant resulted in a sixfold increase in apical, but not basolateral, endocytosis. This stimulation was accompanied by a significant increase in the fraction of invaginated CCPs at the apical PM (Altschuler *et al.*, 1999). ARF6 is also implicated in the stability of cell morphology, by permitting congruent remodeling of the cell surface and actin cytoskeleton (Frank *et al.*, 1998; Song *et al.*, 1998; Boshans *et al.*, 2000). Moreover,

it is involved in regulating actin dynamics during exocytosis and macrophage phagocytosis (Niedergang *et al.*, 2003).

Expression of a GTP-locked ARF6 mutant in NRK cells localized the endogenous ARF6 to cortical actin sites involved in cell spreading (Song *et al.*, 1998). Further, agonist treatment causes the redistribution of ARF6 and Rac1 to the cell surface, accompanied by cortical actin rearrangements (Boshans *et al.*, 2000), indicating ARF6's involvement in actin activity at the PM. Expression of ARF6-Q67L caused a massive translocation of actin to the apical PM, in agreement with our previous observation that under expression of this mutant, the invaginated CCPs are two- to threefold more prevalent on the apical PM than in control cells (Altschuler *et al.*, 1999).

We next analyzed the sensitivity of ARF6-regulated apical endocytosis to cytochalasin D. GTP-locked ARF6 expression increased apical endocytosis sixfold over controls and this was >80% inhibited by cytochalasin D. Thus, an intact polymerizing actin cytoskeleton is an obligate requirement for endogenous as well as ARF6-dependent endocytosis at the apical surface.

To determine whether ARF6 directly regulates the organization of the actin microfilament network, the distribution of actin was analyzed in polarized MDCK monolayers expressing either the WT or mutant ARF6. Similar to uninfected cells, ARF6-WT expression had no effect on actin distribution. However, ARF6-Q67L expression caused a marked redistribution of actin from the apical cell cortex to the apical PM where it localized in close association with the apical marker gp135. Therefore, activation of apical endocytic transport shows a concomitant increase in the local density of actin at this membrane.

Expression of the ARF6 Q67L mutant caused reorganization of both actin and clathrin and increased their colocalization at the apical PM. Thus, increased ARF6-dependent apical endocytosis requires the reorganization of actin and its proximity to the CME machinery. We speculate that ARF6 acts to shift actin to its polymerized form and thus enhance cellular functions that are dependent on actin polymerization, such as apical endocytosis (Altschuler *et al.*, 1999), microvilli structure, and membrane protrusions for cell spreading (Song *et al.*, 1998) or ruffling (Radhakrishna *et al.*, 1999).

In nonpolarized cells, the alignment and colocalization of the actin cytoskeleton with CCP is required at all times to enable a high, constitutive rate of endocytosis (Bennett *et al.*, 2001). In contrast, at the apical PM, where the rate of endocytosis is very slow, a considerable amount of actin is positioned further away at the apical cortex-terminal web and when triggered by ARF6, it is translocated to the CCP for endocytosis. The actin translocation (by polymerization) triggered by ARF6 is revealed by massive phalloidin staining at the PM (Figure 2), as well as by actin-GFP tracing a mesh network of actin under the CCP network with increased colocalization compared with control cells. Thus, ARF6 may stimulate transient alignment of the actin network with the CCP before actin physically associates with the endocytic machinery. This network alignment is abolished upon treatment with cytochalasin D.

Actin Stimulation Affects the Clathrin-dynamain-dependent Pathway

Because we found actin to be limiting for apical endocytosis in an ARF6-dependent pathway, we tested whether actin-induced endocytosis is clathrin-dynamain-dependent (Altschuler *et al.*, 1999). In Figures 1A and 5C, we show that this pathway is ARF6-dependent, and in Figure 5B we show that

it is dynamain-dependent, as a well-characterized dynamain mutant dramatically inhibited actin-induced apical endocytosis. As dynamain has been shown to be involved in both clathrin-mediated and caveolae-mediated endocytosis, we had to confirm that this pathway is only clathrin-dependent (Nabi and Le, 2003). We therefore expressed actin-GFP along with a clathrin hub dominant-negative mutant that has been previously shown to specifically inhibit only CME (Altschuler *et al.*, 1998; Lu *et al.*, 1998; Trejo *et al.*, 2000). Their expression resulted in an inhibitory effect similar to that obtained with clathrin hub in cells expressing only the latter (Figure 5D) or to clathrin hub inhibition of ARF6-stimulated endocytosis (Altschuler *et al.*, 1999), despite the clathrin hub's ability to bind clathrin light chain and to assemble into CCPs at the PM (Liu *et al.*, 1998), generating many more actin-binding sites within the CCP. This property could have resulted in rescue of the dominant-negative effect of the clathrin hub by overexpressed actin. Therefore, the inhibitory effect of dominant-negative clathrin on actin-stimulated endocytosis indicates that this pathway is clathrin-dependent (Bennett *et al.*, 2001; Duncan *et al.*, 2001; Schafer, 2002; Chen and Brodsky, 2005; Legendre-Guillemin *et al.*, 2005).

Clathrin Affects Actin Association with the PM and Thus Affects Microvilli Structure

We analyzed the effect of the dominant-negative clathrin hub mutant expression on the ability of actin to associate with the PM (Figure 7) and its effect on apical microvilli in MDCK monolayers (Figure 8). The observation that newly synthesized actin-GFP preferentially associates with the plasma membrane fraction indicates that it has an abundance of actin binding sites. The dramatic drop in actin appearance in the PM fraction when we coexpress clathrin hub with actin-GFP indicates that the N-terminal domain of clathrin heavy chain (not present in clathrin hub) may be the binding site for actin at the apical PM. Recently clathrin triskelia were shown to associate with microtubules through the N-terminal clathrin heavy chain, supporting a role for this domain in binding the cytoskeleton (Royle *et al.*, 2005).

Electron microscopy demonstrated that overexpression of the clathrin hub collapsed apical microvilli. Because expression of GTP-locked ARF6 resulted in recruitment of actin to the apical surface, we analyzed whether coexpression of both would restore microvilli. Expression of the ARF6 mutant dramatically rescued microvilli structure in all cells compared with those expressing the clathrin hub alone (Figure 8C), and the width and length of the rescued microvilli were fundamentally identical to control cells. Clathrin hub expression resulted in a collapse of all microvilli within all cells infected. Nevertheless, Dynamain I K44A mutant expression had no effect on microvilli structure indicating that the endocytosis event is not required. Taken together the drop of actin deposition in PM fraction due to clathrin hub expression concomitantly with microvilli collapse, we hypothesize that microvilli structure requires actin binding to CCP's outside the microvilli (Figures 8 and 9, C and D). If correct, then the distal arm (N-terminal domain) of clathrin heavy chain (not present in the clathrin hub mutant) is another site required to anchor actin to the CCP. ARF6 may rescue the collapse by increasing actin polymerization in parallel to stimulating an increase in the abundance of CCP at the apical PM (Figure 9, C and D).

In summary, apical endocytosis in polarized MDCK monolayers requires an active actin cytoskeleton and that polymerizing actin is a limiting factor for this process. Our results show that actin polymerization to the CCP is stimulated by ARF6 in its GTP form, to induce the invagination of

Figure 9. ARF6 regulates actin's involvement in apical endocytosis. (A) In nonstimulated epithelial cells, ARF6 is bound to GDP, the clathrin-coated pit (CCP) is shallow and actin is distal at the terminal web. (B) When cells are stimulated for apical endocytosis, ARF6 releases the GDP, binds GTP, and several events take place concomitantly that facilitate clathrin coat assembly and fission from the membrane. Actin polymerizes toward the CCP. Actin associates with the coated pits at the neck through a complex containing dynamin and also at the coat surface through a complex containing clathrin light chain and Hip1R. Additionally, actin associates with the N-terminal of the clathrin heavy chain. All together, these associations facilitate the deep invagination of the coated pit as well as narrowing of the neck. (C) Microvillus structure is dependent on actin's association with the plasma membrane (PM) as an anchor to produce a microvilli upright structure. To perform its function, actin must bind to the PM somewhere outside the microvilli. Our central hypothesis is that the N-terminal of the clathrin heavy chain within the assembled CCP (arrows) is the site of association for actin to serve other functions of actin apart from endocytosis such as microvilli structure. (D) Expression of clathrin hub (lacking actin-binding site) and its subsequent assembly into CCP, significantly reduces the number of actin binding sites within CCP results in reduced actin binding at the PM through the CCP, causing microvilli to lose their straight, upright structure (arrowheads). Symbols from top to bottom: (A) receptor, 4 symbols above black line; AP2, blue; clathrin, 9 shaded symbols below blue line; actin, red; (B) dynamin, green; receptor, 4 symbols above black line; AP2, blue; clathrin, 9 shaded symbols below blue line; actin, red.

a CCP containing the clathrin-dynamin endocytic system. Moreover, just as important as the requirement of actin for endocytosis is that of CCP's regulated by ARF6 for other actin functions, as demonstrated by collapse and rescue of microvillus structure.

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