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Topical Review

Role of Actin Cytoskeleton in Regulation of Ion Transport: Examples from Epithelial Cells

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Introduction

"Cytoskeleton-membrane interactions" go far beyond simple structural or mechanical linkages between the cytoskeleton and the plasma membranes and are now recognized as dynamic interactions that include newly recognized functions involving signal transduction pathways. Results from diverse studies such as signal transduction, membrane trafficking, channel and ion transport proteins suggest that the cytoskeleton may play a key role not only in the stabilization and delivery but also activation of the membrane proteins mediating ion transport events. The mammalian cell cytoskeleton consists of three major protein families: microfilaments, microtubules and intermediate filaments as well as numerous associated proteins. This review will focus on the microfilament structure and proteins associated with the microfilaments and will use the example of epithelial cells, which demonstrate a unique cytoskeletal architecture, to elucidate the role of the actin cytoskeleton in the regulation of ion transport proteins.

Architecture of an Epithelial Cell

Polarized epithelial cells display a cell surface organization well adapted to their specialized functions of absorption, secretion, ion transport and forming a barrier between the external milieu and the organism's interior. Both the vectorial transport and barrier functions of epithelia are dependent on the polarized distribution of proteins and lipids between the apical plasma membrane domain and the basolateral domain. The apical membrane has a unique protein composition specific to the epithelial cell type and function. The apical membrane of epithelial cells is described as "less fluid" than the basolateral membrane due to: (i) a higher cholesterol/protein ratio, (ii) a lower phospholipid/protein ratio, (iii) a higher sphingomyelin/choline glycerophospholipid ratio, and (iv) a lower degree of unsaturated fatty acids [115]. It is also believed that this lower fluidity restricts the movement of apical membrane proteins. The protein and lipid composition of the basolateral plasma membrane resembles that of nonpolarized cells. Since the membrane fluidity is an important determinant of membrane function, the difference between the apical and basolateral membrane fluidity has been demonstrated to have physiological effects including regulation of ion transport proteins. The apical membrane of epithelial cells demonstrates three unique structures consisting of actin and actin-binding proteins. These include the microvillus core at the apical surface, the elaborate cytoskeleton of the terminal web (TW) that underlies the apical plasma membrane and the tight junction complex that mediates cell-cell adhesion and participates in the organization of the cytoskeleton at cell-cell contacts.

Tilney first recognized the intestinal brush border (BB) as a simple model system for the study of the cytoskeleton and its association with the membrane [135]. However, Hopfer [67] recognized the use of isolated membrane vesicles as tools for analysis of epithelial transport. Through a combined morphological and

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biochemical analysis, the molecular arrangement of many components of the BB have been elucidated. The BB of the intestine, for example, is comprised of numerous surface microvilli. The actin filaments in the microvillus core are bundled by at least three proteins, villin, fimbrin and espin. The actin bundles of the microvilli are attached to the plasma membrane by a brush border specific isoform of myosin, myosin I, and multiple associated calmodulin molecules. It is suggested that the upright position of BB type microvilli depends on myosin filaments [134]. Myosin I is a mechanoenzyme [105] and its ATPase activity and actin-binding properties are regulated by a 28-kDa-zipper protein that resembles tropomyosin. Several nonconventional myosins and actin-membrane cross-linking proteins have also been identified in the BB [64]. The actin cores of the microvilli terminate as rootlets in the TW of the brush border. The TW is comprised largely of narrow strands that interconnect adjacent actin bundles in the rootlets with one another, with the tight junction complex, and intermediate filaments underlying the TW. The actin-binding proteins identified in this region include myosin II, V, VI, tropomyosin, filamin and α-actinin, β-spectrin, cortactin, fodrin, TW-260/240, isoforms of titin, caldesmon, and gelsolin. These molecules exist along the whole length of the rootlets, and can be seen to connect not only the rootlets with each other but also to the overlying cell membrane and the vesicular elements in the TW.

Since epithelial cells perform vectorial transport, the space between individual epithelial cells must be sealed so that transepithelial osmotic and electrical gradients are maintained. The structure that creates this paracellular barrier, is the tight junction. The tight junction complex represents the apical element of a tripartite junctional complex consisting of the apical most tight junction or zona occluden (ZO), the zonula adhaerens (ZA) and the desmosome. The cytoskeleton of the TW domain also intermingles with elements of ZO and ZA. It is apparent that the BB cytoskeleton is intimately tied into the overall cytoskeletal network of the cell. Other BB-containing cell types show some differences, at least in structural organization, but this is limited to microvillar dimensions and the complexity of the TW. Cells of the kidney proximal tubule generally have longer, thinner microvilli than those of the intestine, but lack an extensive TW domain. Colonic cells generally have shorter microvilli and abundant intermediate filaments compared to small intestinal cells.

How Does the Actin Cytoskeleton Regulate Ion Transporting Proteins?

Intestinal and renal epithelial cells can rapidly and reversibly alter solute transport rates by changing the kinetics of transport proteins. At least two molecular mechanisms may be involved in this process: (i) regulation of the transporter kinetics itself such as by phosphorylation of the transporter i.e., the turnover rate (mol/sec) of the individual transporter or alternatively; (ii) by altering the number of transport proteins per unit area of the plasma membrane. These two mechanisms may not be mutually exclusive. Both these mechanisms can be regulated by the actin cytoskeleton. Actin and the actin-binding proteins can modulate ion transport function by either altering the membrane fluidity, cell morphology, cell motility, and/or by regulating signaling pathways that may influence the ion transporters. Additionally, actin-binding proteins with motor activity may regulate the movement of vesicles containing these ion transport proteins thus changing the number of transport proteins present at the membrane surface.

Cytoskeleton-Membrane Interaction: Changes in Membrane Fluidity and Cell Morphology

The plasticity of the plasma membrane is correlated with changes in its dynamic behavior and biological functions. For over a decade it has been known that the lipid composition and fluidity of epithelial cells is markedly different at the apical and basolateral domains. This difference was observed in both membrane preparations as well as liposomes made from total lipid extracts from the apical and basolateral domains [82]. Furthermore, this pattern is also demonstrated along the various regions of the gut [43]. For instance there is decreased BB membrane fluidity in the distal *vs.* the proximal colonocytes [15]. More significantly, the regional variations in rat colonic luminal membrane lipid fluidity and composition are suspected to be responsible for differences in the sodium and water absorption along the length of the colon [15]. Agents that alter membrane fluidity have been used to demonstrate that changes in colonic BB membrane fluidity can influence Na^+H^+ exchange and osmotic water flow across BB membrane vesicles [16]. Alterations in fluidity and/or lipid composition have also been demonstrated to modulate transport of D-glucose and L-glutamate [43]. The interdependence of membrane fluidity and ion transport is physiologically significant. It has been demonstrated that variations in essential fatty acids [33] and dietary restrictions such as starvation [55] alter the lipid composition and fluidity of rat intestinal plasma membrane and this results in an increase in the basolateral membrane specific activity of the Na^+,K^+-ATPase pump [22]. Intestinal calcium absorption and its regulation by vitamin D is mediated *via* changes in the polyunsaturated fatty acid content of the intestinal membranes [35]. Small intestinal BB from vitamin E deficient animals display changes in membrane fluidity and this results in hypersecretion which predisposes or perpetuates protracted diarrhea [83]. Similarly

in renal proximal tubule cells dietary phosphate restriction involves a reduction in BB membrane cholesterol, which increases membrane fluidity and tubular reabsorption of P_i [101].

There is growing evidence for an involvement of microfilaments in the organization and function of membranes and *vice versa*. The cortical cytoskeleton can regulate the membrane structure and the lateral mobility of lipids in a bilayer. Alterations in membrane fluidity [104] accompanied by changes in the actin membrane cytoskeleton have been demonstrated to elevate an already increased Na^+,K^+-ATPase activity in the basolateral membrane isolated after ischemia [102]. Cytoskeletal modulation of the physical properties of the cell membrane lipids by proteins has been found to be abnormal in subjects with diabetic nephropathy [73]. Interaction of the pericanalicular cytoskeleton and modifications in membrane fluidity has been demonstrated to affect bile acid transport in isolated rat hepatocytes [123]. In phalloidin-induced cholestasis in rat, changes in membrane fluidity were associated with decreased Na^+,K^+-ATPase and Mg^{2+}-ATPase activities in membrane fractions [86].

Membrane-bound proteins and lipids can act to regulate the microfilament structure. Most actin-regulatory proteins directly bind to phospholipids and include: villin [72], myosin 1 [150], ezrin [111], gelsolin [148], profilin [128], and vinculin [54]. Binding of phospholipids to BB myosin I inhibits actin filament motility [150]. Mutating the phosphatidylinositol 4,5 bisphosphate (PIP_2) binding site in profilin decreases the inhibition of PLC-γ_1 activity by profilin perhaps by inhibiting binding of G-actin monomers [128]. PIP_2 dissociates vinculin's head-tail interaction unmasking its talin- and actin-binding sites, thus disrupting the focal adhesions and adherens junctions [131]. The binding of PIP_2 to villin and gelsolin dissociates them from filament ends thus promoting actin assembly [72]. PIP_2 association with ezrin promotes ezrin's interaction with plasma membrane components such as intercellular adhesion molecules [65]. While these examples demonstrate the role of lipid components in the membrane to regulate the microfilament structure *via* the actin-binding proteins, there is evidence that actin itself may interact with membrane lipids. Studies done using pure actin and liposomes demonstrate that direct interaction of actin with membrane lipids results in drastic alterations in the conformation of actin [56]. Membrane-cytoskeleton interactions thus are not mutually exclusive. The association of membrane proteins and lipids with the cortical cytoskeleton can modulate both the membrane dynamics as well as the cortical cytoskeleton.

Changes in ion transport require spatially and temporally coordinated changes in cell morphology and cell motility, and these changes can be brought about by changes in actin dynamics. Changes in the cortical cytoskeleton can alter cell morphology drastically, and the proteins associated with actin regulate such changes. Actin-binding proteins that can either sever, cap, nucleate, or bundle actin filaments or sequester monomeric G-actin, thus altering the gel-sol state of the cortical structure, initiate changes in the cortical cytoskeleton. Several such proteins are identified in epithelial cells and include: villin, gelsolin, profilin, fimbrin, plastin, α-actinin, annexins, and MARCKS protein amongst others [71]. The roughly opposite effects of actin bundling and actin severing proteins could lead to reorganization of the cytoskeleton when cells are activated to be motile or solate the cell cortex and allow deformation in response to mechanical forces generated either by motor proteins or other extracellular stimuli such as changes in osmolarity. These actin-binding proteins may determine the cell shape and thus plasma membrane surface available for the ion transport proteins or their interaction with the plasma membrane may help stabilize the lipid bilayer, which contains the ion transporting molecules.

Cytoskeleton and Vesicle Movement

The second mode of regulating plasma membrane solute transport involves changes in the number of transport proteins per unit area. Acute regulation of transport proteins involves recruitment of preexisting transport proteins or endocytic retrieval from the cell surface. Exocytic insertion and endocytic retrieval are dynamic processes in which the steady-state distribution of transport proteins is dependent on the relative rates of both these processes. Both the endocytic and exocytic processes have been demonstrated to require the actin cytoskeleton [80, 106]. Actin regulatory proteins have also been shown to directly bind to proteins involved in vesicular trafficking [46] or to signaling molecules known to regulate vesicle trafficking. Signals originating at the plasma membrane control the formation and dissolution of diverse protrusions of the cell surface such as microvilli, membrane ruffles, both of which are recognized in epithelial cells. Agonists that bind to the plasma membrane may rearrange the microvilli cytoskeleton such that it expands elastically in some regions to form protrusions and contracts, liquefies, and attenuates in others to permit fusion of the vesicles with the cell membrane, whereupon insertion of the transport proteins into the plasma membrane may occur.

BB myosin I is believed to participate in the biogenesis and recycling of the apical membrane by transporting vesicles up through the TW. BB myosin I has been shown to associate in vivo with vesicles within the perinuclear region, an area containing numerous mitochondria, acidic, lysosomal and golgi organelles, again suggesting that BB myosin I may be the motor protein driv-

ing vesicle translocation [37]. More recently the ubiquitous subclass of myosin's, myosin I-α has been shown to associate with endosomes and lysosomes in mammalian cells [119]. Truncations in the BB myosin I have been shown to impair the distribution and the function of endocytic compartments [45]. Cell surface ruffling in response to epidermal growth factor (EGF) phosphorylates myosin VI and recruits it into newly formed ruffles [23]. Thus myosin VI could play a role in membrane traffic in secretory pathways. These results are consistent with a role for myosin I and VI as an apically targeted motor for vesicle translocation in epithelial cells.

The favored mechanism for regulation of most epithelial ion transport proteins, including several described in this review, is *via* clathrin-dependent vesicular trafficking. In the kidney vasopressin induces cycling of vesicles carrying the water channels and the proton pumps to and from the plasma membrane. In both instances, clathrin-coated carrier vesicles are involved [19]. The apical plasma membrane of kidney proximal tubule cells has an elaborate and extensive clathrin coat and is far more developed and elaborate than those seen in cultured cells [122]. It appears that cell types that have amplified levels of endocytosis exhibit this rather elaborate distribution of clathrin such as the oocyte [99] and the phagocytosing macrophages [1]. Clathrin vesicles containing ion transport proteins have also been isolated from intestinal epithelial cells [32]. Examples of epithelial ion transport proteins regulated by clathrin mediated trafficking include the renal Na^+,K^+-ATPase [112]; the renal water channel and the proton pump [59, 75]; renal ClC-5 chloride channel [58], the epithelial sodium channel [126]; the epithelial Cl^- channel CFTR has been shown to bind to the clathrin adaptor protein AP-2 [145] and to be internalized *via* clathrin-coated vesicles [89]; the intestinal Na^+ cotransporter, NHE3 [32] and, the gastric H^+-K^+-ATPase [113]. Relevant to this is the observation that non-clathrin vesicles such as caveolin are not found in the renal and intestinal epithelial cells [17, 142] with one exception, the kidney cell line, MDCK [142].

Regulation of Signaling Pathways by the Cytoskeleton

The chemical and physical properties of the cytoskeleton suggest that it may have the ability to pass information from cell surface receptor activation to ultimately initiate changes in cell structure, cell function or gene expression. Since the cytoskeleton forms a continuous, dynamic, connection between most cellular structures it may also provide an enormous surface area where proteins and other cytoplasmic components can dock. Alternatively it may function for mechanical coupling through the cell, changing the gel-sol state of the cell in response to mechanical stimuli sensed and transmitted from the cell surface.

PIP_2 is a membrane phospholipid that regulates many important cellular processes, including organization of the cytoskeleton, vesicular trafficking and regulation of ion transport proteins. The discovery of proteins whose activities are modified by interaction with PIP_2 has helped redefine the role of this lipid both in cytoskeletal rearrangement and signal transduction pathways. Previous studies demonstrated that several phospholipases and lipid kinases were recruited to the cytoskeleton in response to extracellular stimuli. The association of PIP_2 with the actin-regulatory proteins not only modulates the actin modifying properties of these proteins but also suggests regulation of phosphoinositide-mediated signal transduction pathways by the microfilament. For instance, binding of PIP_2 to both villin and gelsolin disassociates them from the actin network and inhibits their actin-severing property [72]. The calcium-dependent, phospholipid and membrane-binding protein annexin interacts with profilin and regulates the inhibitory effect of profilin on actin polymerization. The binding of annexin to the membrane is decreased by profilin but restored when PIP_2 is added in the liposomes [5]. Gelsolin regulates the cation (such as Ca^{2+})-induced aggregation of PIP_2, suggesting that gelsolin interaction with PIP_2 may also have the ability to modify the plasma membrane structure itself [48]. Ezrin, a cytoplasmic linker molecule between plasma membrane and the actin cytoskeleton, associates with intercellular adhesion molecules and this interaction is induced and/or enhanced by PIP_2 [65]. Several of these actin-binding proteins are also known to either interact directly with phospholipases or regulate their activity. Both villin and gelsolin have been shown to interact with Phospholipase C-γ_1 and regulate its catalytic activity [7, 76]. Both enzymes inhibit Phospholipase C-γ_1 activity by competing for PIP_2, the substrate for this enzyme [132] (S. Khurana, *unpublished observation*). Regulation of Phospholipase C-γ_1 activity by profilin is well-documented [57]. The membrane-bound G protein-coupled isoform (PLC-β) also depends on the actin cytoskeleton for its localization and coupling to the G protein [136]. While the actin-regulatory proteins described above are involved in the hydrolysis of PIP_2, high concentrations of PIP_2 itself have been shown to regulate the actin cytoskeleton. It is suggested that high levels of PIP_2 may promote actin filament growth by inhibiting capping, severing and removing capping proteins from the barbed ends [40]. Studies using fluorescent PIP_2 in liposomes have revealed that phosphoinositide-binding peptides can stabilize membrane microdomains in which both protein and phospholipid components are segregated [147]. The cytoskeleton also has the ability to regulate the content of

other phospholipids of the plasma membrane such as phosphatidylethanolamine [41]. Activities of other phospholipid-regulating enzymes known to be modulated by actin-regulatory proteins include: Phospholipase D and Phospholipase A2 by fodrin and gelsolin [90, 130]. The cross-talk between actin-regulatory proteins and lipid-modifying enzymes suggests that the interaction of actin-binding proteins with these enzymes has the ability to modulate both the cortical cytoskeleton and phosphoinositide-mediated signal transduction pathways.

Other phosphoinositide-mediated second messengers regulated by the microfilament structure include Protein Kinase C (PKC) and Ca^{2+}. Remodeling of the actin cytoskeleton has been demonstrated to be regulated by PKC [52]. Actin-regulatory proteins known to be phosphorylated by the proteins kinases include annexin II [74] and ezrin [18] among others. In epithelial cells, the assembly of the tight junctions has been reported to be modulated by protein kinase C [6]. It is believed that PKC may be in the signaling pathway activated by E-cadherin mediated cell-cell adhesion. PKC can also regulate vesicle trafficking *via* its link with the GTPases and the actin cytoskeleton. In cells treated with the PKC agonist, phorbol 12-myristate 13-acetate, there is a dramatic redistribution of ARF6 and the actin network into membrane protrusions resembling lamellipodia. Protein kinase N, a downstream regulator of the small GTPase Rho has been shown to be associated with the cytoskeletal protein α-actinin [107].

Myristoylated alanine-rich C-kinase substrate (MARCKS) is a calmodulin, actin, and anionic phospholipid binding protein that is the predominant and specific substrate of PKC activation. Phosphorylation of MARCKS by PKC leads to its redistribution from the plasma membrane to the cytoplasm, and eliminates the actin filament cross-linking activity of MARCKS. It has long been suggested that MARCKS may be a regulated crossbridge between actin and the plasma membrane, and modulation of the actin crosslinking activity of MARCKS by calmodulin or phosphorylation by PKC may represent a potential convergence of the calcium-calmodulin and PKC signal transduction pathways in the regulation of the actin cytoskeleton. More recently it has been shown that cytoskeletal integrity is not required for either phosphorylation or translocation of MARCKS in response to activated PKC, but that the interaction of both F-actin and calmodulin can modulate PKC-regulated localization and function of MARCKS at cellular membranes [42]. Adducin is a heterotrimeric protein with subunits containing a COOH-terminal MARCKS-related domain that caps and preferentially recruits spectrin to the fast-growing ends of actin filaments. Phosphorylation of the MARCK-related domain in adducin by PKC alters the activities of adducin involving actin and spectrin. This is relevant since adducin is known to regulate the Na^+,K^+-ATPase pump in the kidney. Some isoforms of PKC directly associate with F-actin, these include PKC-ε and PKC β-II [13, 118]. A specific actin-binding domain within the structure of PKC-ε is suggested to anchor it to actin filaments and even facilitate specific ion transport functions [118].

Several lines of evidence point to a direct link between cytoskeletal structure and intracellular Ca^{2+} concentrations. It is speculated that the actin cytoskeleton may not only regulate Ca^{2+} release but may itself be part of the intracellular calcium stores [81]. Thus F-actin is seen as an ATP-dependent and IP_3-sensitive Ca^{2+} store (*see* Fig. 2). The probability that actin subunits near the cell membrane may bind Ca^{2+} and then incorporate it into filaments would allow accumulation of several micromoles of Ca^{2+} in the pool of actin. This pool of Ca^{2+} would be released when the actin filaments depolymerize or treadmill. Actin-binding proteins that nucleate, cap, bundle or sever actin filaments mediate changes in the polymerization of actin. The actin-regulatory properties of these proteins has been demonstrated to be regulated by Ca^{2+} and several calcium binding sites have been identified in these proteins [71].

PIP_2-binding proteins can regulate other signaling pathways such as signaling by tyrosine kinases by modulation of their association with PIP_2. For instance α-actinin and vinculin, two PIP_2 binding proteins, demonstrate decreased binding to PIP_2 in response to Platelet-derived growth factor [54]. This suggests that tyrosine kinase-activated Phospholipase C may hydrolyze PIP_2 bound to α-actinin and vinculin thus leading to the simultaneous generation of second messengers and reorganization of the cytoskeleton. In epithelial cells, the E-cadherin complex contains the src kinase substrate p120 [2]. Treatment of monolayers of MDCK cells with tyrosine phosphatase inhibitors results in a rapid increase in paracellular permeability [36]. In vitro studies have revealed that EGF receptor kinase phosphorylates the β-subunit of fodrin on tyrosine residues and this is markedly inhibited by F-actin. In contrast src kinase phosphorylates the α-subunit of fodrin and this is not inhibited by F-actin [4]. Calpactin is a phospholipid-dependent Ca^{2+} binding protein that is a component of the membrane skeleton where it has been shown to co-localize with spectrin. Calpactin is a major substrate for src kinase [63]. Actin regulatory proteins themselves can be substrates for tyrosine kinases, this includes ezrin [47], gelsolin [39], and villin [76]. Tyrosine phosphorylation of these proteins regulates both their actin-regulatory properties [38] and their ability to regulate phosphoinositide-mediated signal transduction pathways (A. Panebra, M. Shu-Xa, S.G. Rhee, Y.S. Bae, X.-T. Wang, and S. Khurana, *unpublished observation*).

In recent years the inositol phospholipids have been

found to have signaling roles that do not require their hydrolysis. In this role the phosphoinositides serve as site-specific signals on membranes that recruit protein complexes leading to membrane budding and fusion processes that are spatially restricted to specific membrane domains. A role for PIP_2 in membrane ruffling requiring actin reorganization is known [66]. Several proteins that regulate the formation and function of clathrin-coated vesicles in endocytosis, including AP-2 adaptor, and dynamin, bind PIP_2. Regulated exocytosis is also PIP_2-dependent as documented by the requirement for enzymes that direct its synthesis in the priming/fusion step at the plasma membrane [87]. Direct interaction between PIP_2 molecules on the inner leaflet of the plasma membrane and anchoring proteins of the microfilament structure are known. Other evidence to suggest this comes from studies where expression of 5′ PIP_2 phosphatase led to reduced total cellular F-actin [120]. Furthermore, the actin polymerizing drug jasplakinolide increases adhesion energy in these experiments, while cytochalasin D has the opposite effects. These studies suggest that PIP_2 could regulate cell shape in a localized domain when plasma membrane and cytoskeleton separate and the adhesion energy is lowered, leading to the formation of membrane blebs. Several high affinity inositol hexakisphosphate (IP_6) binding proteins involved in vesicular trafficking have been identified. The IP_6 binding protein identified first was the clathrin assembly protein, AP-2 [143]. In vitro binding studies have shown the IP_6 binding inhibits the clathrin assembly catalyzed by AP-2, suggesting that IP_6 is an inhibitor of endocytosis. Coatomer involved in *cis-to-trans* vesicle transport through Golgi stacks and probably in retrograde transport from golgi to ER. Coatomer has also been shown to bind IP_6 and other phosphoinositides [49].

The lipid kinase, Phosphatidylinositol 3-kinase (PI3-kinase) and its product have been shown to regulate constitutive vesicle traffic in both epithelial and nonepithelial cells [53]. The immediate downstream effector for phosphatidylinositol 3-phosphate (PI 3-P), the product of PI3-kinase activation remains to be identified. However, PI 3-P is speculated to promote the recruitment of guanine nucleotide exchange factors to specific sites on the membrane for activation of Rho family GTPases. It is also speculated that PI 3-P could alter the structure of the lipid bilayer to facilitate budding, recruit coat proteins or adaptors to the site of budding or function as a vesicle membrane component essential for subsequent docking or fusion reactions. The actin-regulatory proteins profilin and gelsolin are known to stimulate PI 3-kinase activity [11, 28]. PI3-kinase has been demonstrated to regulate several ion-transporting proteins [77, 133]. Some phosphoinositide isomers have been proposed to have specific functions. One of them, [3,4,5,6]-IP_4 is reported to inhibit Cl^- secretion in epithelial cells, and suggested to be involved in osmoregulation [149]. Other known signaling molecules involved in vesicular trafficking and which also function as phosphoinositide effectors, include the small GTPases and Phospholipase D (PLD). The actin cytoskeleton is regulated by heterotrimeric G proteins and small GTPases of the rho family [60]. The role of Phospholipase D in vesicle-membrane fusion is well documented [84]. PLD itself is an effector for phosphoinositides in membrane budding reactions since PLD activity and its subsequent stimulation of Arf requires phosphoinositides [21, 85]. The phosphoinositide dependence of PLD activity is mediated by direct interaction with the phosphoinositides [62]. Gelsolin has been shown to stimulate Phospholipase D activity while fodrin has been shown to inhibit the activity of this enzyme [91, 130]. The numerous effects of phosphoinositide-regulated cytoskeletal proteins on inositol lipid kinases and phospholipases suggest many possibilities for stimulation or inhibition of lipid-cytoskeletal interactions. The common denominator in signal transduction pathways and the distribution of specific actin-regulatory proteins in epithelial cells suggests that localized signal generation and the spatially localized recruitment of actin-regulatory proteins may be a mechanism employed in signal transduction, cytoskeletal, and membrane trafficking events and even regulation of ion transport proteins. Since a large number of actin-regulatory proteins have been identified as possible effectors of phosphoinositide signals, the microfilament structure may act not only to localize these signaling proteins but promote their activation at specific sites.

Ion Transport in Epithelial Cells

Ion Pumps

The Na^+,K^+-ATPase Pump

A principal cytoskeletal protein whose distribution has been correlated with that of Na^+,K^+-ATPase is spectrin. A role for spectrin in regulating the distribution of the pump was suggested by the exogenous expression of E-cadherin in fibroblasts and in transformed RPE cell line, which mediated lateral membrane interactions, formation of junctional complexes and recruitment of the pump to the lateral surface [96, 98]. In cultured epithelial cell lines the pump colocalizes in vivo with spectrin and ankyrin [78, 110]. Using a direct biological and chemical approach, ankyrin has been shown to bind to the Na^+,K^+-ATPase pump [109, 110]. The fact that the recruitment of Na^+,K^+-ATPase could be mimicked in nonepithelial fibroblasts that express exogenous E-cadherin suggested that recruitment and assembly of the membrane skeleton at E-cadherin-mediated cell-cell con-

tacts directly facilitates the restricted distribution of the pump in the lateral membrane. It is also suggested that the membrane cytoskeleton restricts its access to the endocytic pathway, thus facilitating its retention within this domain [61].

Changes in the membrane cytoskeleton such as in receptor-mediated decrease in proximal tubule Na^+,K^+-ATPase activity induced by dopamine is associated with internalization of the α- and β-subunits of the pump into early and late endosomes *via* a clathrin-dependent pathway that has also been shown to be PKC dependent [29, 116]. The role of PKC in the regulation of epithelial cell cytoskeleton is correlated with translocation of MARCKS protein, redistribution of fodrin, and disintegration of the membrane cytoskeleton [140]. A PI 3-kinase mediated endocytosis of α-subunit of the pump in response to dopamine has also been reported [31]. In the lung alveolar cells, isoproterenol has been shown to increase Na^+,K^+-ATPase activity by recruiting α-subunits into the plasma membrane from an intracellular compartment in a Na^+-independent manner [10]. A functional role of actin on the pump activity is suggested by studies where addition of actin was associated with an increase in the affinity of the pump for Na^+ but not other enzymatic substrates [25]. Using Western analysis, purified Na^+,K^+-ATPase pump was shown to associate with actin suggesting that the pump may be directly linked to the microfilament structure *via* actin. This hypothesis is supported by the observation that the α-subunit of the pump contains a putative actin-binding domain.

Pathophysiology of epithelial cells helps shed some more light on the regulation of the pump by the actin cytoskeleton. Ischemia results in the duration-dependent loss of apical and basolateral surface membrane lipid and protein polarity. Loss of polarity is preceded by disruption of the microfilament network and opening of the tight junctions. Functionally, ischemia-induced loss of epithelial polarity has been shown to be responsible for reduced sodium and glucose reabsorption. Reduced Na^+ reabsorption has been related to redistribution of Na^+,K^+-ATPase pump into the apical membrane [103]. Concurrently, during recovery from ischemic injury the proximal tubule undergoes remodeling of the surface membrane including the establishment of polarity and redistribution of the pump to the basolateral surface. The kidney is believed to play a major role in the initiation and maintenance of hypertension. Some forms of hypertension are now attributed to a polymorphism in the basolateral cytoskeletal protein adducin. Adducin contains binding sites for both actin and spectrin and can recruit spectrin molecules to actin filaments [68], thereby stabilizing the actin-spectrin membrane skeleton. Recent studies suggest that adducin polymorphism affects kidney function by modulating the overall capacity of tubular epithelial cells to transport ions, including the regulation of the pump by modifying the assembly of the actin cytoskeleton [95]. This idea is supported by transfection studies of KE52 cells in which mutated α-adducin was reported to be associated with an increase of the ouabain-sensitive $86Rb^+$-uptake, indicating an increase of the Na^+,K^+-ATPase-dependent transport capacity [137].

The H^+-K^+-ATPase Pump

H^+-K^+-ATPase, a pump of parietal cells is exocytically inserted into and endocytically retrieved from the plasma membrane in response to stimuli for gastric acid secretion. The role of the actin cytoskeleton and specifically ezrin, is well documented in the regulation of this pump [3]. Existing data support the recycling/recruitment hypothesis of HCl secretion where tubulovesicles containing the pump are recruited from a cytoplasmic domain to the apical surface. cAMP-induced increase in the activity of the pump in parietal cells has been shown to be associated with an increase in the number of pump units in the apical membrane as well as the phosphorylation of the cytoskeletal protein ezrin [138]. These studies also suggest that ezrin is a substrate for kinases and that phosphorylation of ezrin may be involved in regulating the translocation of the proton pump. Phosphorylation of ezrin is increased by both histamine and cAMP pathways, both of which are known to regulate acid secretion in parietal cells [50]. Antiulcer drugs have been shown to inhibit acid secretion by inhibiting the redistribution of the pump from microsomes into the apical membrane and delocalizing ezrin from the apical membrane [139]. Immunofluorescence studies have demonstrated the colocalization of the pump with ezrin [129]. The role of GTPases such as rab11a in parietal cell activation is also known [44]. Stimulated acid secretion from parietal cells is inhibited by cytochalasin in a dose-dependent manner, presumably by disrupting the actin cortex, which may provide the tracks for the movement of the vesicles containing the pump [51].

Ion Cotransporters

The Sodium-Hydrogen Exchanger Isoform 3

Epithelial cells express several isoforms of the Na^+/H^+ exchanger (NHE), the epithelial cell specific isoform involved in Na^+ absorption is NHE3. In studies done in the intestinal cell line Caco-2 serum deprivation was shown to inhibit an apical Na^+/H^+ exchanger [144]. This inhibition was reversible and basal activities could be restored by the readdition of serum. The addition of cytochalasin D was shown to block the inhibition of Na^+/H^+ exchange by serum deprivation. Interestingly cytochalasin D did not alter basal NHE3 activity, which is in

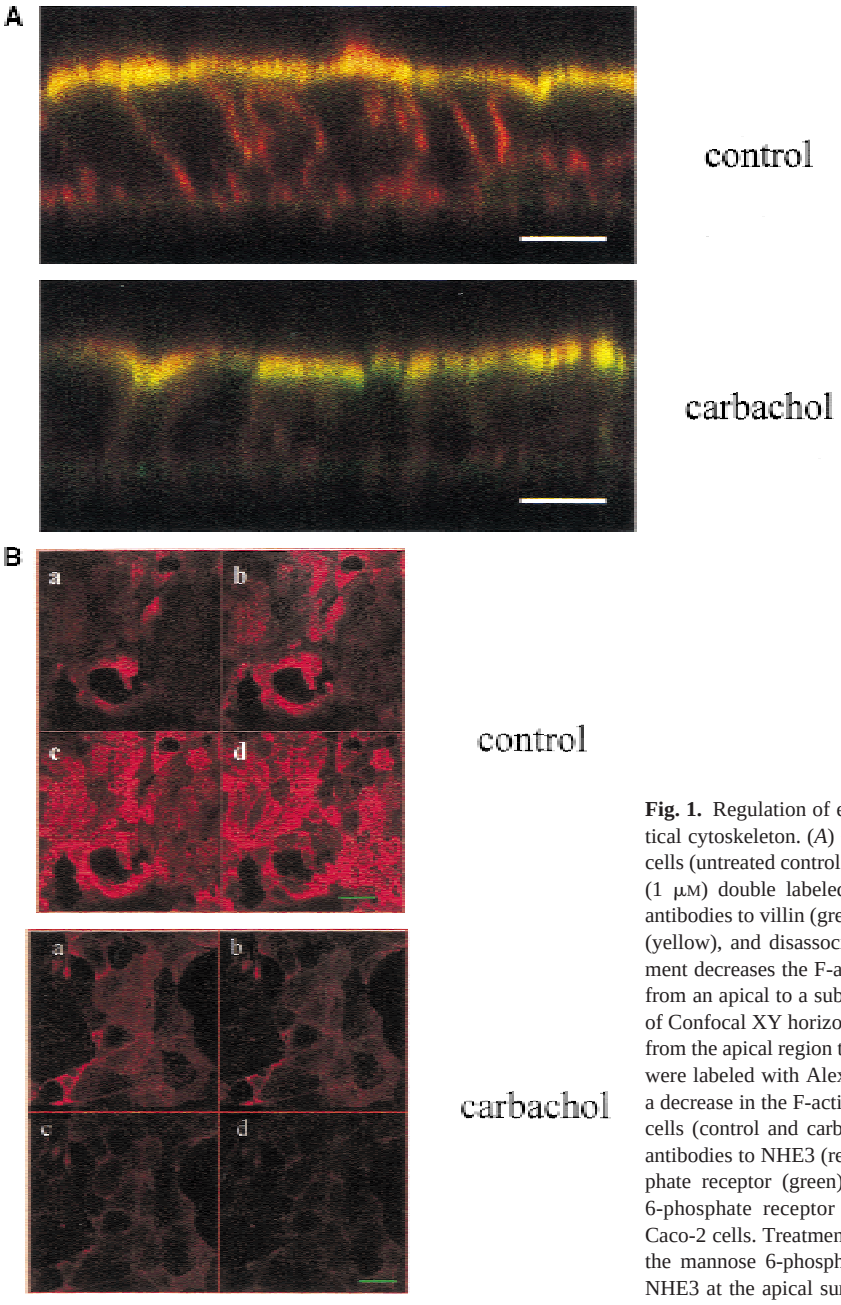

Fig. 1. Regulation of epithelial Na^+/H^+ exchanger, NHE3, by the cortical cytoskeleton. (*A*) Confocal laser scanning micrographs of Caco-2 cells (untreated control cells and cells treated for 10 min with carbachol (1 μM) double labeled with Alexa-phalloidin (red) and monoclonal antibodies to villin (green). F-actin and villin colocalize in control cells (yellow), and disassociate in carbachol-treated cells. Carbachol treatment decreases the F-actin staining as well as the distribution of villin from an apical to a sub-apical domain. Scale bar = 10 μm; (*B*) Series of Confocal XY horizontal sections collected at 1 μm intervals starting from the apical region towards the cell body (a to d, respectively). Cells were labeled with Alexaphalloidin (red). Carbachol treatment leads to a decrease in the F-actin staining; (*C*) Confocal micrographs of Caco-2 cells (control and carbachol treated) double-labeled with monoclonal antibodies to NHE3 (red) and the cation-independent mannose 6-phosphate receptor (green). NHE3 and the cation-independent mannose 6-phosphate receptor colocalize (yellow) at the apical surface of Caco-2 cells. Treatment of cells with carbachol leads to endocytosis of the mannose 6-phosphate receptor and a decrease in the amount of NHE3 at the apical surface.

contrast to other studies done in Caco-2 cells which demonstrate that cytochalasin D treatment alters microvilli actin filament architecture [70] and induces abnormalities in the ZO structure and function which coincides with contraction of the perijunctional actin-myosin ring [92, 93]. In our own studies treatment of Caco-2 cells with either cytochalasin D or B leads to redistribution of actin at the apical membrane surface including the distribution of NHE3 (S. Khurana, X.-T. Wang, *unpublished observation*). Regulation of NHE3 by the actin cytoskeleton has been demonstrated in the native tissue. Receptor-mediated (the cholinergic-muscarinic agonist, carbachol) inhibition of NHE3 can be prevented by the actin-stabilizing drug, jasplakinolide, suggesting that carbachol-induced inhibition of NHE3 involves depolymerization of actin. This process also involves the tyrosine phosphorylation of villin and its redistribution between an actin-bound and -free form in the native tissue [76]. In the intestinal cell line Caco-2, the actin-regulatory protein villin and F-actin colocalize under basal conditions, in contrast treatment of Caco-2 cells with carbachol leads to a redistribution of villin and F-actin, such that most of the villin now appears to redistribute to a subapical domain and does not colocalize

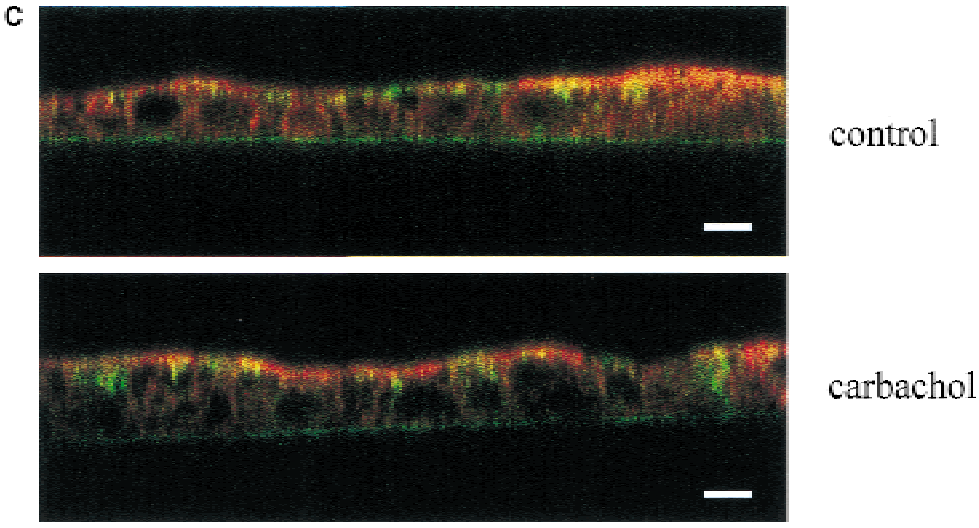

Fig. 1. Continued.

with F-actin (Fig. 1*A*). There is also decrease in the amount of F-actin suggesting actin-severing in response to carbachol (Fig. 1*B*). Interestingly, NHE3 colocalizes with the late endosomal marker, cation-independent mannose 6-phosphate receptor, and carbachol treatment leads to a decrease in the amount of apical NHE3 (Fig. 1*C*). Based on our observation and recent studies on calcium stores in microvilli, a simple model to explain the receptor-mediated inhibition of NHE3 in intestinal cells could be speculated as shown in Fig. 2. Receptor activation leads to actin depolymerization by villin. It is possible that other actin-severing proteins identified in the apical surface of epithelial cells such as gelsolin are also involved in this mechanism. Functional RhoA (a small GTP-binding protein) and Rho associated kinase have been found to be essential for the effective Na^+/H^+ exchange by NHE3 (Dr. J. Orlowski, McGill University, Montreal, Canada, *personal communication*). The significance of this observation is that Rho-associated kinase is a known regulator of actin assembly, which is mediated *via* phosphorylation of the actin severing protein cofilin [94]. This observation suggests that regulation of actin-severing proteins (and hence the state of actin assembly) may be a significant mechanism involved in the regulation of NHE3. Yet another mechanism for the regulation of NHE3 by the actin cytoskeleton is *via* exocytic insertion of NHE3 in response to growth factors. Epidermal growth factor stimulates NHE3 activity by a mechanism involving the activation of PI 3-kinase [77]. That NHE3 is regulated by vesicular trafficking is further demonstrated by the presence of the cotransporter in isolated clathrin-coated vesicles from ileal villus cells [32].

While redistribution of NHE3 by the actin cytoskeleton is described in the studies above, regulation of the intrinsic activity of the antiporter without changes in the number of transporters at the cell surface are also reported [79]. In these studies cytoskeletal disorganization was associated with a subcellular redistribution of NHE3 to the sites of actin aggregation suggesting an interaction of the exchanger with the cortical cytoskeleton. This interaction was further supported by the cosedimentation of the transporter with the actin cytoskeleton [77].

Sodium-dependent Cotransporters

Other Na^+-dependent transport processes have also been demonstrated to be regulated by the actin cytoskeleton. The Na^+-glucose cotransporter (SGLT1) expression and activity are increased in response to EGF treatment, which can be abolished by cytochalasin D treatment. This acute regulation of SGLT1 by EGF involves exocytic insertion of the transporter into the BB by a mechanism requiring actin polymerization [34]. SGLT1 is regulated by PKA and PKC *via* a mechanism involving exocytosis and/or endocytosis [146]. The basolateral Na-K-2Cl cotransporter of epithelial cells is activated by cAMP in the human intestinal cell line, T84 and is associated with remodeling of the basolateral F-actin distribution [125]. Forskolin stimulates the Na-K-2Cl pump, and this activity is markedly decreased in T84 cells loaded with phalloidin. This effect was shown to be specific for the transporter since phalloidin treatment did not affect either the Na^+,K^+-ATPase pump or the regulated Cl^- and K^+ channels [97]. It is suggested that the cotransporter may be functionally linked to the cytoskeleton and be involved in cAMP-induced electrogenic Cl^- secretion in intestinal cells. While the mechanism for this regulation is not well elucidated, it is suggested that interaction of Na-K-2Cl with yet unidentified membrane proteins may be influenced by the actin cytoskeleton, and that this association between the pump and the membrane proteins regulates its activity. More recent studies

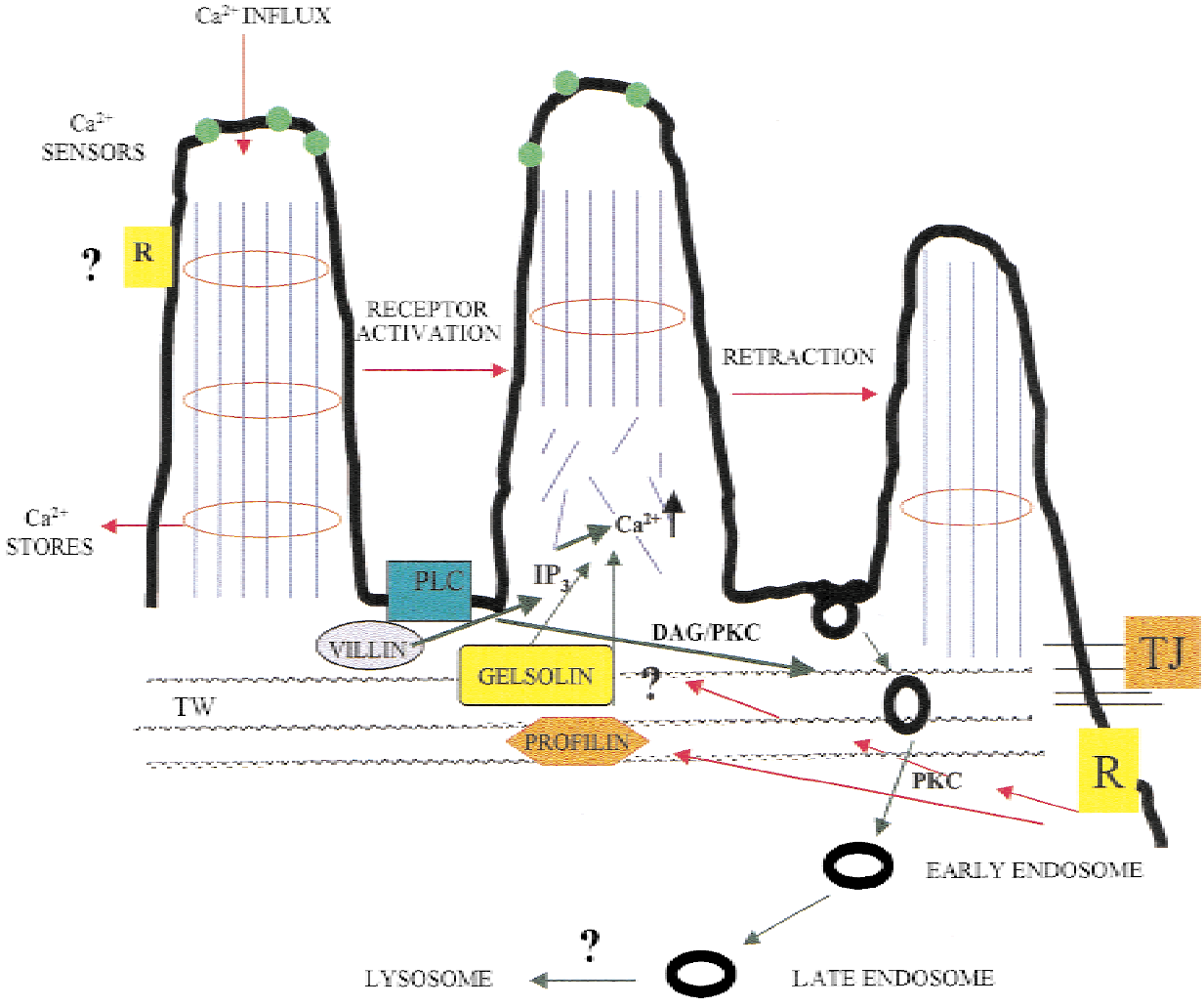

Fig. 2. Proposed model for the regulation of intestinal and renal apical transport protein, NHE3, by the cortical cytoskeleton. Receptor activation following carbachol treatment leads to tyrosine phosphorylation of villin, its association with the SH2 domain of PLC-γ_1, and activation of PLC-γ_1 catalytic activity. The products of PLC-γ_1 activation, i.e., IP_3 releases Ca^{2+} from intracellular stores, while DAG activates PKC. Elevated intracellular Ca^{2+} increases the actin-severing property of villin. Disruption of the microvillar F-actin leads to endocytic retrieval of apical membrane including that containing NHE3. PKC may be involved in facilitating the movement of apical membrane containing vesicles across the terminal web. The source of calcium may be the Ca^{2+} sequestered by microvillar actin filaments. Other actin-regulatory proteins that may be involved in this process may include gelsolin and profilin, both of which are known to be associated with PLC-γ_1-mediated signal transduction pathways. The colocalization of NHE3 with the late endosomal marker, cation-independent mannose 6-phosphate receptor (*see* Fig. 1*B*) suggests that endocytic retrieval of NHE3 would direct the protein towards lysosomal degradation. Removal of the ligand and recovery from receptor activation would involve repolymerization of actin and insertion of pre-existing apical membrane proteins, thus restoring the apical membrane surface area and number of apical membrane transport proteins (such as NHE3).

suggest that phosphorylation of myosin light chain and subsequent contraction of basal actin-myosin bundles are crucial to the cAMP-driven activation of Na-K-2Cl and subsequent apical Cl^- efflux in T84 cells.

The actin cytoskeleton is also known to participate in the regulation of the Na^+/P_i cotransporter. Reabsorption of P_i by renal tubular epithelia is performed by at least two Na/P_i cotransporters located in the apical membrane. The activity of the cotransporter is regulated by alterations in the number of cotransporter units contained in the luminal membrane [108]. Brief incubation of opossum kidney cells with a low concentration (0.1 mM) of P_i causes a rapid F-actin depolymerization and significant increase in the rate of cellular P_i uptake. Measurement of P_i uptake in the presence of agents known to stabilize microfilament structure (phallacidin) leads to a marked decrease in P_i uptake under these conditions [114]. These results suggest that acute alteration of the microfilament structure is involved in the modulation of Na/P_i cotransport activity and this may be related to the

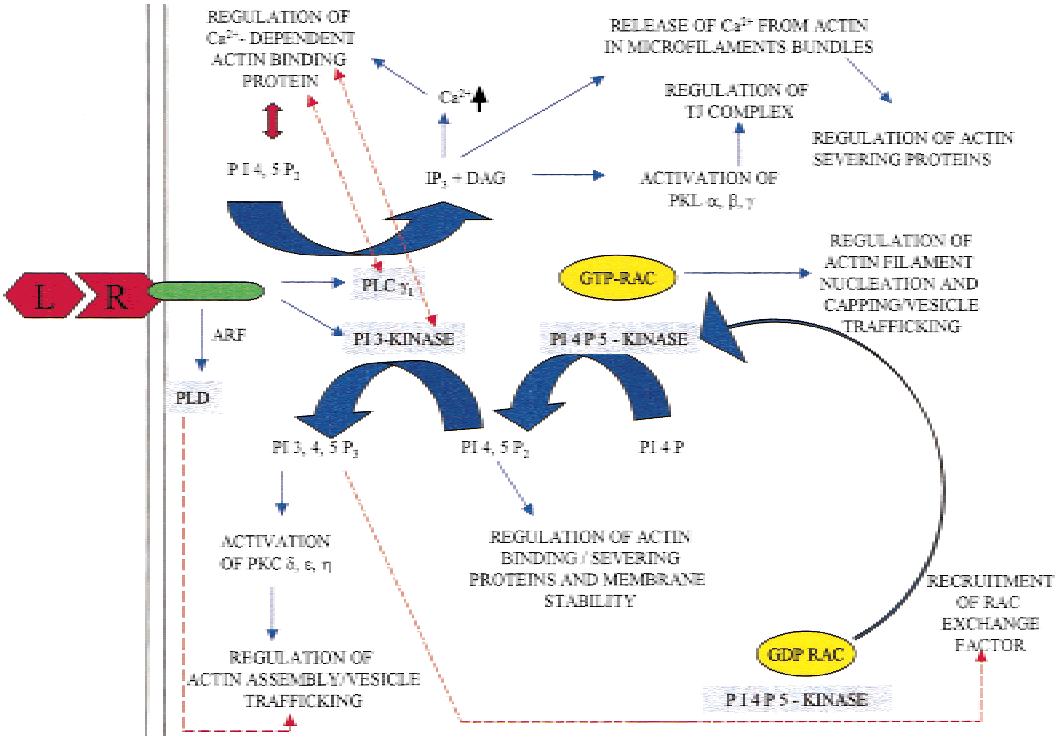

Fig. 3. Cross-talk between signal transduction pathways and the microfilament structure. Receptor$^{(R)}$ activation following the binding of a specific extracellular ligand (L) leads to the activation of several signaling molecules including the lipid kinases and phospholipases. Integrated into these signal transduction pathways are other signaling molecules such as small GTPases of the Rho-Rac family. Products of the various signal transduction cascades lead to rearrangement of the microfilament structure by regulating the actin-regulatory proteins that bundle, sever, cap or nucleate actin filaments.

vesicular movement involved in the apical insertion of the cotransporter. This hypothesis is supported by previous work showing both changes in the abundance of cotransporter at the apical surface without parallel changes in mRNA expression for the symporter, and the existence of functional cotransporters in renal cortical endosomes. Furthermore, parathyroid hormone has been shown to lead to the endocytic retrieval of the cotransporter followed by lysosomal degradation, and this is mediated *via* PKA and/or PKC [12].

Ion Channels

The Epithelial Na^+ Channel

One of the best studied examples of ion channels regulated by the actin cytoskeleton is the epithelial Na^+ channel, ENaC. The apical Na^+ channel is the distal regulated pathway for Na^+ reabsorption in the kidney and colon. Sodium reabsorption by renal epithelia is initiated by the activation of the Na^+ channel whose activity in turn is controlled by hormones including vasopressin and aldosterone. The apical Na^+ channel has been shown to be associated with spectrin-based membrane cytoskeleton and this association is believed to restrict its mobility [124]. It has also been demonstrated that the linkage of the Na^+ channel to the membrane cytoskeleton is the site for modulation of Na^+ channel activity by vasopressin and aldosterone [127].

The actin filaments have been demonstrated to colocalize with and regulate the channel activity in the amphibian distal nephron cell line, A6 [27]. PKA activates the Na^+ channel and addition of DNAse I, which prevents actin polymerization, inhibits the PKA-mediated activation of the channel [117]. ENaC has also been shown to be regulated by G proteins and this effect has been shown to occur only in the presence of actin [9]. The actin depolymerizing drug cytochalasin D activates the Na^+ channel activity [27]. DNAse I inhibits the cytochalasin D induced Na^+ channel activity leading to the hypothesis that short actin filaments may be involved in the regulation of the Na^+ channel. The addition of purified polymerized actin (>5.0 μM) to the cytoplasmic side of excised inside out patches increases the Na^+ channel activity by increasing the number of channels without any change in the open time of active Na^+ channel [27]. To determine the actual length of actin filaments necessary to regulate the Na^+ channel activity, the actin depolymerizing protein gelsolin was used, since actin poly-

merization in the presence of varying concentrations of gelsolin is known to elicit filaments of varying lengths. Using this approach an actin/gelsolin ration of <32:1 evoked a sustained activation of the Na^+ channel, with a maximal effect at an actin/gelsolin ratio of 2:1. These results are consistent with the interpretation that interaction of the Na^+ channel with short actin filaments regulates channel activity [8]. Whether there is a direct interaction between the channel and the actin filaments remains to be determined. Other mechanisms suggested for the regulation of the Na^+ channel include phosphorylation of G-actin by PKA, which facilitates the activation of the Na^+ channel [117].

More recently actin has been shown to play a role in the regulation of ENaC by CFTR (cystic fibrosis transmembrane conductance regulator). Immunopurified CFTR coreconstituted into a lipid bilayer with the Na^+ channel decreased the open probability of ENaC in the presence of actin by over 60% but only 20% in the absence of actin [69]. These studies suggest that CFTR can directly downregulate single channel activity and that the presence of actin confers an enhanced modulatory ability of CFTR on the Na^+ channel. Yet another mechanism proposed for the regulation of the Na^+ pump in A6 cells is vesicular trafficking. Using various pharmacological agents, it has been shown that the cytoskeleton is dominantly involved in osmotic channel regulation at the apical membrane of A6 cells, and that actin filaments and molecular motors are involved in the recruitment of additional Na^+ channels [121]. Antidiuretic hormone regulates apical Na^+ channel in A6 cells by regulation of apical vesicular membrane movement and expression of Na^+ channels at the apical membrane [141].

CFTR

In T84 cells and other secretory epithelia, cAMP-mediated chloride transport is associated with the expression of the CFTR protein. CFTR acts as a cAMP/PKA-sensitive chloride channel. Moreover, CFTR has been shown to have additional roles such as in regulating other ion transport pathways such as the Na^+ channel described above. CFTR has been shown to be critical for cAMP-dependent regulation of membrane recycling in epithelial cells [14], and is present and functional in endosomes [88]. Phalloidin blocks cAMP-activated Cl^- conductance and cAMP-induced rearrangement of F-actin in T84 cells [125]. Cytochalasins and cAMP have been shown to depolymerize F-actin in MDCK kidney cells and decrease cell volume by activating a Cl^- conductance [100]. In patch-clamp studies done in mouse mammary adenocarcinoma cells expressing the human epithelial CFTR and in cultured neonatal rat ventricular myocytes, CFTR activity is regulated by the actin cytoskeleton [26]. Cytochalasin D activates CFTR, so does the addition of actin to excised, inside-out patches.

Summary

Actin is one of the most abundant proteins expressed in a cell and is also one of the most highly conserved proteins. These facts point to the fundamental importance of actin in biology. The actin cytoskeleton may be seen as a mechanism that allows extracellular signals to be relayed from the cell membrane to specific intracellular targets including ion transport proteins. The microfilament structure can also be viewed as specialized proteins that participate in the assembly of signaling proteins into biochemical pathways. A simple model summarizing these observations is depicted in Fig. 3. Integration of information from such diverse fields as signal transduction, microfilament assembly and regulation of vesicle trafficking, will help us in the coming years to better understand the molecular and cellular mechanisms involved in the regulation of ion transport proteins by the actin cytoskeleton.

The Confocal data on the role of villin in the regulation of NHE3 in the intestinal cell line Caco-2 was generated in collaboration with Dr. Xi-Tao Wang (Johns Hopkins University School of Medicine, Baltimore, MD). The author would like to thank Dr. S.E. Guggino for her assistance with these studies and her excellent comments in editing this review; Dr. W.J. Brown (Cornell University, Ithaca, NY) for the antibodies to the cation-independent Mannose 6, phosphate receptor. Research on the role of actin cytoskeleton and the regulation of NHE3 is funded by grants from the American Heart Association (9950941U), the American Digestive Health Foundation (Industry Research Scholar Award), and the NIH (RO1DK54755).

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