Mediation, Modulation, and Consequences of Membrane-Cytoskeleton Interactions

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Annu. Rev. Biophys. 2008. 37:65–95

First published online as a Review in Advance on December 3, 2007

The *Annual Review of Biophysics* is online at biophys.annualreviews.org

This article's doi: 10.1146/annurev.biophys.37.032807.125912

1936-122X/08/0609-0065$20.00

Key Words

BAR domain, disease, endocytosis, filopodia, membrane deformation, migration

Abstract

Elements of the cytoskeleton interact intimately and communicate bidirectionally with cellular membranes. Such interactions are critical for a host of cellular processes. Here we focus on the many types of interactions that exist between the cytoskeleton and the plasma membrane to illustrate why these cellular components can never truly be studied in isolation in vivo. We discuss how membrane-cytoskeleton interactions are mediated and modulated, and how many proteins involved in these interactions are disrupted in human disease. We then highlight key molecular and physical variables that must be considered in order to mechanistically dissect events associated with changes in plasma membrane morphology. These considerations are integrated into the context of cell migration, filopodia formation, and clathrin-mediated endocytosis to show how a holistic view of the plasma membrane-cytoskeleton interface can allow for the appropriate interpretation of experimental findings and provide novel mechanistic insight into these important cellular events.

Contents

INTRODUCTION

From one perspective, the plasma membrane of mammalian cells can be considered a barrier to diffusion that is sufficiently flexible to be permissive to changes in its shape that are induced by the regulated assembly of intracellular cytoskeletal elements. From another perspective, the plasma membrane can be viewed as a highly regulated, heterogeneous environment that functions as an active participant in

interactions with the outside world and provides a highly dynamic platform that controls its own local morphology through the regulated assembly of permissive, stabilizing cytoskeletal elements. To appreciate how cells regulate their myriad morphologies and communicate with the outside world (e.g., through adhering to surrounding cells or extracellular matrix, sensing and responding to the local environment through the exo-endocytic cycle, or migrating in response to chemotactic stimuli), a thorough understanding of the nature of the interface between the cell membrane and the cytoskeleton is of paramount importance. A great deal of work has examined this interface to attempt to understand, at a mechanistic level, how processes that require maintenance of, or changes in, plasma membrane morphology are mediated. These studies have discovered that cytoskeletal elements interact intimately and bidirectionally with the plasma membrane and that neither can therefore truly be studied in isolation in vivo.

Enormous bodies of literature have fostered our current understanding of membrane biology, the cytoskeleton, and signaling pathways that regulate membranes and cytoskeleton components, and we draw the reader's attention to numerous excellent reviews regarding the mechanisms and regulation of cytoskeleton polymerization, the functions and regulation of plasma membrane lipids, and small G-protein signaling pathways (26, 45, 59, 61, 114, 122, 146). Here we focus predominantly on the mechanisms by which plasma membrane shape changes can occur and be maintained through the regulation of membrane-cytoskeleton interactions. We begin with a discussion of the interplay that occurs between the plasma membrane, small G-proteins, and cytoskeletal elements before focusing on the nature and then the consequences of membrane-cytoskeleton interactions. We then consider at the mechanistic level how an appreciation of these interactions can be integrated with biochemical and cell biological findings in events where plasma membrane deformation occur, with emphasis on clathrin-mediated endocytosis. This review is intended to emphasize something of the complexity of the network of interactions that cell biologists study, to illustrate the importance of studying the membrane and the cytoskeleton together as part of a single system to decode mechanistic information, and to provide a framework within which certain experiments can be appropriately interpreted and approached.

INTERPLAY BETWEEN CYTOSKELETAL ELEMENTS, SMALL G-PROTEINS, AND THE PLASMA MEMBRANE

Cytoskeletal Elements Interact with the Plasma Membrane

Filamentous cytoskeletal elements interact intimately with the plasma membrane at a wide variety of cellular locations, including very highly curved regions of the plasma membrane and sites of adhesion to the cellular surroundings (**Figure 1**). There are many types of actin-based superstructures (both at the morphological and molecular levels), including a mesh of filaments underlying and tightly apposed to the plasma membrane, a dense network of highly branched actin filaments at the leading edge of migratory cells, long bundles of actin cables (stress fibers) that are usually anchored to sites of adhesion, actin-rich structures associated with membrane invaginations in endocytic and phagocytic structures, and long unbranched actin filaments found in filopodia. These structures are often highly dynamic and regulate interactions of cells with their environments.

In fibroblasts, microtubule (MT) minus ends are located at a centrosome or MT organizing center, usually found in a juxtanuclear location. In polarized epithelia, MTs are predominantly oriented in an apico-basal axis, with their minus ends located toward the apical surface and their plus ends toward the basal surface. Some MTs are also oriented

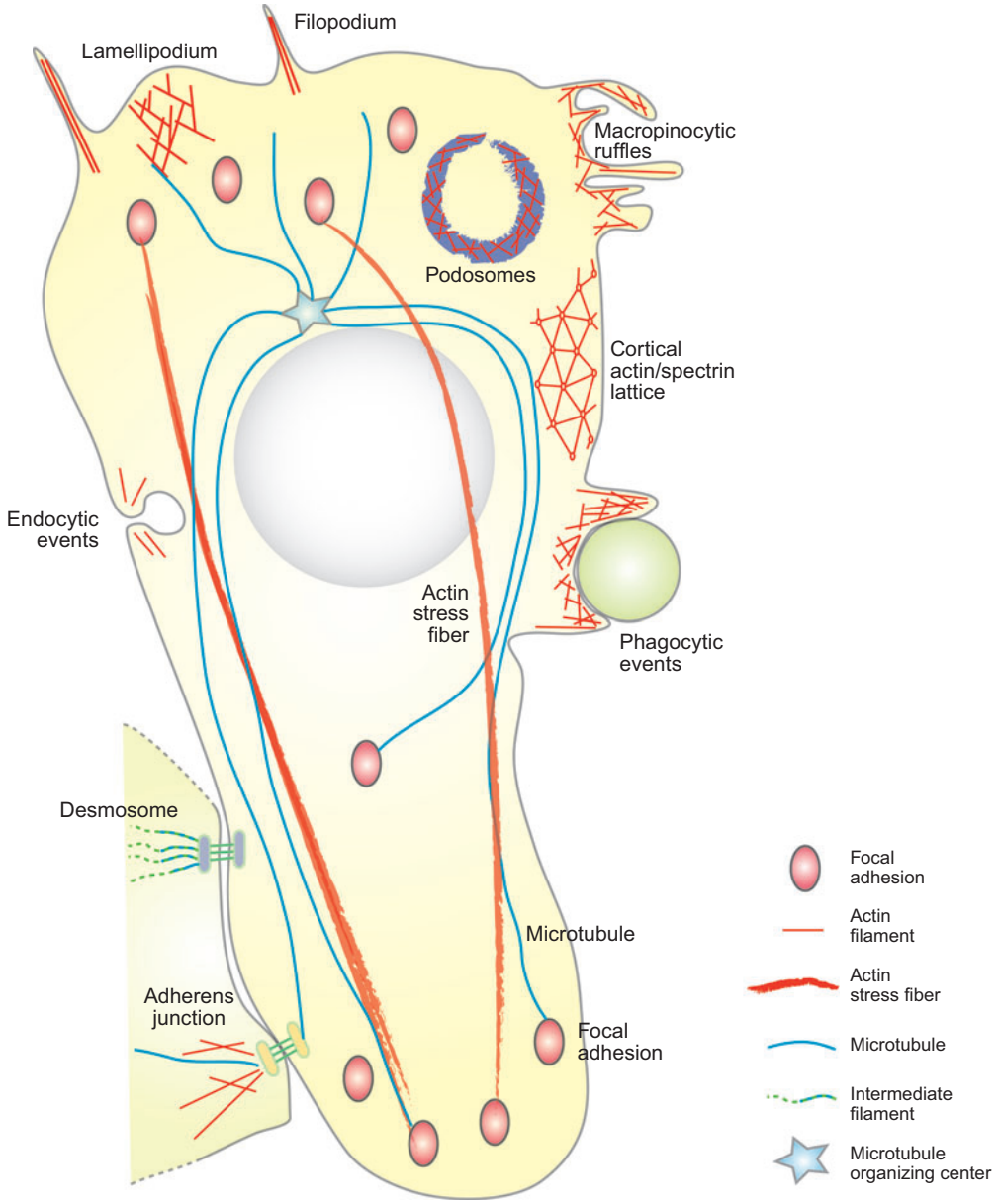

Figure 1

The array of membrane-cytoskeleton interactions in mammalian cells. Schematic diagram illustrating the main types of cellular locations where membrane-cytoskeleton interactions are formed.

roughly normal to this axis at the apices and bases of columnar epithelia. A variety of MT-associated proteins regulate the assembly and disassembly of MTs (22, 25, 51), and some of these proteins interact with membranes or membrane-localized proteins to anchor MTs. In addition to important roles of MTs in the trafficking of intracellular organelles to and from the plasma membrane, MTs communicate extensively with the plasma membrane,

where they can be captured and stabilized. This has been studied in most detail at sites of adhesion to the cell's surroundings (36, 65).

Intermediate filaments (IFs), of which there are many differentially expressed subtypes, are dynamic structures but turn over much more slowly than do actin filaments or MTs. In addition to their important roles in intracellular organelle positioning and dynamics (151), IFs can interact tightly with the plasma membrane, particularly at sites of cell-cell and cell-matrix adhesion at desmosomes and hemidesmosomes (73). Each IF type likely participates in the production and maintenance of particular cellular morphologies through their membrane attachments and their resistance to mechanical stress.

Interplay Between Cytoskeletal Filaments

While actin, MT, and IF cytoskeletal elements are structurally distinct and are honed for specific functions, they are also interdependent. Depolymerization of MTs disrupts the polarity of the actin cytoskeleton and promotes stress fiber and focal adhesion formation (6). Actin depolymerization has little gross effect on MT polarity (87), but because MTs appear to be guided by actin stress fibers toward cell-matrix adhesion sites (75) and because actin is necessary for the formation of such sites, actin depolymerization affects MT targeting. Both actin filaments and IF components such as vimentin fibrils appear to undergo transport by motors along MTs (117, 137), and several MT-associated proteins bind to actin. The extension of IFs is dependent upon the integrity of the MT cytoskeleton (50), the distribution of which is commonly mimicked by the IF cytoskeleton. Plectin can also directly link IFs to actin and MTs (139) and is mutated in a subset of patients with the blistering skin condition epidermolysis bullosa and a concomitant muscular dystrophy (41), underlining its important role in maintaining tissue integrity.

Such cross talk makes it difficult to fully ascribe the primary nature of the dependence of particular events at the plasma membrane to any single cytoskeletal component because even acute ablation of one type of filament can functionally affect another. These problems are compounded by the fact that acute disruption of one type of cytoskeleton can alter the activation states of small G-proteins (121), which can alter the dynamics of more than one cytoskeletal element. Thus great care should be taken when interpreting results from experiments in which even an acute and pharmacologically specific cytoskeleton disruption has been made.

Small G-Proteins at the Plasma Membrane Regulate the Cytoskeleton

Small G-protein regulation is tightly coupled to membrane association. At least 14 members of the Rho family of small G-proteins (including the canonical family members RhoA, Cdc42, and Rac1) may act at the plasma membrane (122). RhoGEFs, which stimulate GDP-GTP exchange by Rho family members, activate these proteins on membranes. Their activity is downregulated by RhoGAPs (which stimulate nucleotide hydrolysis), many of which can also associate with membranes. Rho family members mediate their functions in cytoskeletal regulation through activation of a variety of effector proteins (59). These include WASP and WAVE family proteins (which stimulate actin nucleation), formins (which bind to the barbed ends of actin filaments and promote their elongation, inhibit actin filament branching, and regulate MT dynamics), and kinases such as PAKs and Rho kinases (which can regulate cytoskeletal dynamics in a variety of manners).

Communication between small G-proteins and the cytoskeleton is bidirectional and complex. For example, MTs can modulate RhoGEFs (35, 59), and upon MT depolymerization, RhoA becomes rapidly activated (121) (which leads to actin stress fiber production and maintenance), although it is not known precisely how this occurs. Further,

RacGTP binds to dimers of tubulin, but not to polymerized tubulin (7), so it may become released upon tubulin polymerization. Because MTs are necessary for many processes, including the trafficking of intracellular membranes, and less-well-understood processes such as focal adhesion disassembly (65), many factors likely contribute to such effects.

Regulation of the Plasma Membrane by Small G-Proteins

Rho family members can modify plasma membrane phospholipids locally through the regulation of lipid-modifying enzymes, thereby indirectly influencing cytoskeletal dynamics (26, 59). For example, these proteins can control PtdIns(4,5)P_2 levels at the plasma membrane by inducing the recruitment of PIP5K [which catalyzes the formation of PtdIns(4,5)P_2] to the plasma membrane, which may be directly activated at this site by Arf6, which functions with RhoA (and Rho kinase) in its recruitment (55, 106).

Of the six Arf small GTPases, only Arf6 is found at the plasma membrane (31), where it regulates a variety of sites of active membrane deformation primarily through the regulation of local lipid composition (8). In addition to PIP5K, Arf6 effectors also include phospholipase D (PLD), which catalyzes the formation of phosphatidic acid. Because phosphatidic acid stimulates PIP5K (55), and because PtdIns(4,5)P_2 activates PLD (111), these effectors likely synergistically favor PtdIns(4,5)P_2 formation. Arf6 activation can thereby induce the activation of actin-nucleating factors and recruit a multitude of other PtdIns(4,5)P_2-binding proteins (see below). Arf6 can also recruit Rac1 to the plasma membrane, where it regulates Rac1 activity (through proteins such as ARNO, NM23-H1, and arfaptin2) (18). Arf6 activity is also regulated by a variety of GAP and GEF proteins, many of which can interact with membranes (45, 118).

The regulation of Rho family and Arf6 small G-proteins is centered on the plasma membrane, where they undergo activation and complex cross-regulation in response to extracellular cues. Such regulation ultimately allows for the regulation of membrane and cytoskeletal dynamics, and small G-proteins are already implicated in most cellular events where plasma membrane-cytoskeleton interactions or plasma membrane shape changes (plasma membrane deformations) occur.

Plasma Membrane Lipids Provide Directionality to Actin Polymerization

In addition to the local activation of small G-proteins and their effectors at the plasma membrane, a number of other crucial proteins that stimulate the nucleation and regulation of actin filaments are activated/inactivated directly by certain plasma membrane phospholipids. This allows for actin polymerization to occur at appropriate sites of the plasma membrane. These phospholipids are turned over locally in response to extracellular cues and are critical for the regulation of membrane-cytoskeleton interactions. These lipids have many direct and indirect effects on the control of actin dynamics at the plasma membrane. The most important lipids that perform this role are PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 [formed from PtdIns(4,5)P_2 by PI3K, which is recruited to the plasma membrane upon activation of certain transmembrane receptors (52)], which are bound by a variety of cytoskeletal proteins and regulators (61, 146).

WASP and WAVE Act as Coincidence Detectors to Control Actin Nucleation at the Plasma Membrane

WASP and WAVE family proteins (which include WASP, N-WASP, and WAVE1–3) are small G-protein effectors that are important stimulators of actin nucleation. WASP family proteins become activated by PtdIns(4,5)P_2 at the plasma membrane (146). N-WASP is widely expressed, whereas WASP expression

is found predominantly in white blood cells, in which it is required for the majority of actin-associated plasma membrane deformation events (24, 66). WASP family proteins can bind to the Arp2/3 complex, leading to its activation. The activated Arp2/3 complex binds to a mother actin filament and nucleates branching of this filament. Because actin nucleation requires the oligomerization of three actin monomers, and the Arp2/3 complex contains two actin monomer-like globular proteins, the binding of a single actin monomer (which is supplied by WASP) by Arp2/3 can stimulate actin nucleation (146).

The N-terminal regions of WASP proteins sterically inhibit their Arp2/3 complex binding ability (123). This inhibition can be stabilized in vitro by binding to WIP (91) and released by the cooperative binding of the membrane-associated small G-protein Cdc42 and PtdIns(4,5)P_2 (146). The SH3 domains of membrane-binding proteins such as Toca1 and SNX9 can also bind to N-WASP (54, 161). SH3 domain binding synergizes with PtdIns(4,5)P_2 in N-WASP activation (124). All these releasing proteins likely allow coincidence detection at specific target sites in vivo to ensure that only at a membrane with an appropriate lipid and protein microenvironment, produced in response to extracellular cues, are WASP proteins able to stimulate actin nucleation. Actin nucleated by such mechanisms has been shown to remain dynamically associated with membranes in a N-WASP-dependent manner (14).

WAVE proteins are homologous to WASP proteins and are widely expressed, with WAVE1 and WAVE3 being brain-enriched (146). WAVE proteins preferentially and directly associate with PtdIns(3,4,5)P_3 (104). WASP and WAVE proteins differ at their N termini, and WAVE proteins interact with other proteins to form a fully functional and interdependent pentameric WAVE complex. WAVE proteins lack the small G-protein-binding domain present in WASP proteins and do not appear to interact with small G-proteins directly, but the complex is capable of binding to activated Rac through one of the complex components, SRA1 (72). The complex can also activate Arp2/3 in a phosphorylation-dependent manner (70).

The differential promotion of WASP/WAVE family-stimulated actin nucleation by PtdIns(4,5)P_2/PtdIns(3,4,5)P_3 is likely critical for the spatiotemporal restriction of their nucleating activities. WASP and WAVE proteins are implicated in a wide variety of plasma membrane deformation events, but the plasma membrane is also important in controlling many other elements that command actin polymerization events.

Regulation of Other Cytoskeletal Regulators by the Plasma Membrane

Other actin regulatory proteins are regulated by phospholipid binding in addition to nucleating factors. Binding of gelsolin, capping protein, profilin, and cofilin to PtdIns(4,5)P_2 releases these proteins from actin (26, 61). Such regulation likely permits rapid polymerization at the membrane-filament interface. PtdIns(4,5)P_2 also releases the autoinhibition of the actin cross-linking α-actinin homodimer (60). This allows it to interact with other proteins and releases its actin-binding site. This form of release is similar to that which occurs in WASP family proteins and is also found in vinculin, talin, and ERM (ezrin, radixin, and moesin) family proteins (60). These proteins might link F-actin filaments to PtdIns(4,5)P_2, each other, or both. It is not known, however, if phosphoinositides and actin can bind simultaneously to these proteins in vivo. Filamin cross-links actin filaments and binds membranes strongly in a phosphoinositide-independent manner. Its cross-linking activity is negatively regulated by phosphoinositides (40). Phospholipids can also influence small G-protein activity directly and indirectly, for example, by changing the preferred small G-protein targets of RhoGAPs (83).

Overall, increased PtdIns(4,5)P_2 promotes net actin polymerization, and upon

PtdIns(4,5)P_2 depletion, net actin disassembly and detachment of actin from membranes occur (162). Lipids regulate other cytoskeletal elements in less-well-understood manners. For example, MTs are captured by domains rich in PtdIns(4,5)P_2 in vivo (47), but how this is precisely mediated by proteins that associate with MT tips is unclear.

MEDIATION AND MODULATION OF MEMBRANE-CYTOSKELETON INTERACTIONS AND THEIR IMPORTANCE IN HUMAN DISEASE

Myriad Links Between the Membrane and the Cytoskeleton

Many proteins link cellular membranes to the cytoskeletal machinery. Given the range of the types of membrane-cytoskeleton interactions that occur, the functions of these proteins must be controlled by regulated networks of interaction that precisely orchestrate their assembly and activity. Although there are direct linkages between cytoskeletal components and the membrane, many proteins can function as adaptors between cytoskeletal components and the membrane. Such an arrangement usually results in additional layers of regulation and specificity, and complexes of adaptor proteins allow tighter interactions between cytoskeletal components and the plasma membrane. The main types of interactions that occur are summarized in **Figure 2**.

Some Cytoskeletal Elements and Motor Proteins May Interact with Membranes Directly

Cytoskeletal components with domains (such as PH superfamily domains) that interact with specific phosphoinositides are strong candidates for mediating important membrane-cytoskeleton linkages, and these include filament components such as high-molecular-weight forms of β-spectrin/fodrin as well as unconventional myosins. MyosinVII interacts with the focal adhesion component talin with which it appears to participate in adhesion (154), while myosinX binds β-integrins, can link these to MTs, and appears to be required for integrin-mediated cell-matrix adhesion (140). MyosinX is also involved in filopodial production and the extension of pseudopods around a phagocytic cup. MyosinXVa brings whirlin to the tips of hair cell stereocilia in the ear (3) and is required for hearing (82). It is not known whether these proteins can perform functions distinct from intracellular membrane trafficking, but they are often concentrated at sites of membrane-cytoskeleton interactions, implying that they are not simply transporters.

Transmembrane Proteins Link Membranes to the Cytoskeleton

Transmembrane proteins likely have major roles in regulating the nature of their surrounding lipids by preferential interactions with certain lipids and, if locally concentrated, may also contribute to local membrane curvature (93) (and therefore membrane deformation). Moreover, transmembrane proteins transduce signals from the cellular exterior into signaling cascades, which can indirectly affect cytoskeletal elements. The importance of posttranslational modifications of membrane-cytoskeleton interactions through signaling pathways should not be ignored but falls beyond the scope of this review. Here we consider roles for transmembrane proteins in mediating membrane-cytoskeleton interactions either directly or though associated adaptor proteins or adaptor complexes.

The EGF receptor can bind to actin directly (and this appears to negatively regulate its activation), and actin binding is dramatically enhanced by adaptor proteins (147), and indirect interactions likely allow for greater regulation. Commonly, transmembrane transporters and channels are tightly coupled to the actin cytoskeleton.

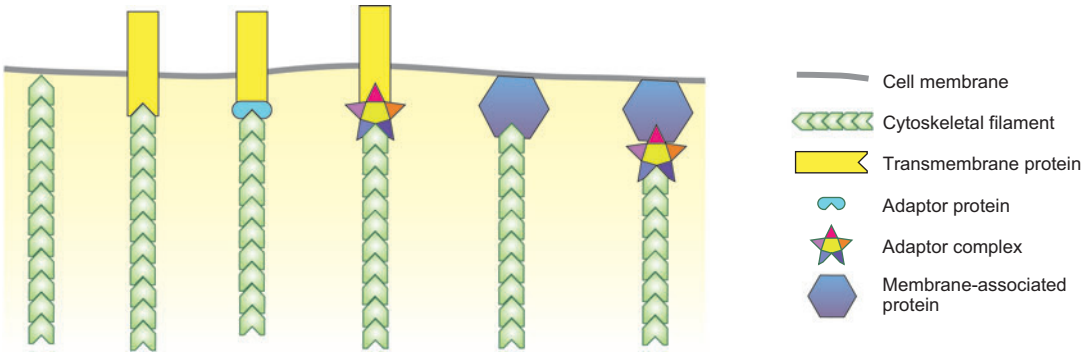

Figure 2

Schematic diagram illustrating how the main types of membrane-cytoskeleton interactions in mammalian cells are mediated.

For example, Band3 (a Cl^-/HCO_3^- counter-transporter that is the most common integral protein of the erythrocyte membrane) is intimately linked to the spectrin-actin lattice that constitutes the erythrocyte's highly flexible cytoskeleton. A major mediator of this interaction is ankyrin-R, which directly binds spectrin and Band3. Membrane-cytoskeleton linkages are likely stabilized here by pallidin, which binds Band3, spectrin, and ankyrin-R, and by the FERM domain-containing Band 4.1, which stabilizes the spectrin-actin interaction and interacts with another transmembrane protein, glycophorinC. These interactions are reviewed in Reference 5.

The nonerythrocytic spectrin homolog fodrin also appears to be intimately linked to both other cytoskeletal components and membranes. Fodrin can bind actin, ankyrins, transmembrane proteins including various glutamate receptors and polycystin1, as well as calpactin (which binds both membranes and actin) and plectin (a plakin that binds actin, IFs, MTs, spectrins, integrins, and the EGF receptor). Ankyrins likely play important roles as adaptors for membrane-cytoskeleton interactions globally (reviewed in Reference 100). These can link the plasma membrane to the cytoskeleton in epithelial cells and can bind tubulin and membrane proteins including the cell-cell adhesion protein E-cadherin and the Na^+/K^+ ATPase. The Na^+/K^+ ATPase redistributes to cell-cell contacts in the presence of the E-cadherin uvomorulin (94), and ankyrin-G also binds Na^+ channel β-subunits, which have roles in cell-cell adhesion (88). IFs may bind both spectrin and actin and therefore communicate directly with the spectrin-actin cytoskeleton.

Given the abundance and strength of cell-cell and cell-matrix adhesion sites, they contribute extensively to membrane-cytoskeleton interactions in vivo. Cadherins mediate cell-cell contact at adherens junctions. The classical view is that ligation of cadherins results in the recruitment of β-catenin, which through α-catenin may link these sites to the actin cytoskeleton [which is required for these sites to grow and to be maintained and which is controlled by Rac1/Cdc42 (13, 80)]. This view is likely too simplistic (157), and many other proteins have been implicated in the formation of these sites. Focal adhesions are major mediators of cell-matrix interactions and are formed upon integrin ligation by the extracellular matrix. These mature in a RhoA-dependent manner. A panoply of proteins (including vinculin and others discussed above) mediate the linkage of the membrane at these sites to actin stress fibers (163).

It is important to study membrane-cytoskeleton interactions not only because of their critical roles in cell biology, but also because many of these interactions are

disrupted or modified in disease. For example, the importance of the myriad links mediating spectrin-actin-membrane interactions in erythrocytes is demonstrated by the variety of defects in these interactions that result in hereditary spherocytosis (HS) (33). In this condition, erythrocytes lose their characteristic biconcave disc morphologies and instead are roughly spherical with an increased surface area/volume ratio. The morphologies of erythrocytes from patients with HS mimic those adopted by erythrocytes in hypotonic solutions, which likely also disrupt membrane-cytoskeleton interactions.

Cells that are exogenously forced to change their morphology ordinarily revert back to their former morphology after removal of the force. This is dependent on the presence of membrane-cytoskeleton interactions that revert the cell to its most relaxed state after deformation, thereby providing a morphological memory. When these interactions are disrupted, such as occurs in hereditary elliptocytosis, this memory is lost. Mutations resulting in this disease are found in a set of proteins similar to those that result in HS, but at different sites (23). Erythrocytes carrying these mutated proteins cannot reform their biconcave disc morphologies after passing through capillaries (in which erythrocytes ordinarily assume elliptical shapes). Both spherical and elliptical erythrocytes are broken down in the spleen much faster than erythrocytes with normal morphologies.

Another example of the disruption of membrane-cytoskeleton interactions in disease is that which occurs upon cell insult, for example, after axonal injury. Calpain is an important Ca^{2+}-activated protease that has roles in the disruption of membrane-cytoskeleton interactions. Upon axonal injury, this protease appears to cleave spectrin (9), and this may result in membrane blebbing and cell death in injured neurons.

Our final examples discuss diseases associated with defects in cell-cell and cell-matrix adhesion. Antibodies in a group of autoimmune disorders known as pemphigus attack specific cadherins (desmogleins) in epithelial desmosomes, leading to blistering of the skin (46). Mutations in desmoplakin (which links IFs to the desmosome) cause skin, hair, and heart conditions, including a variety of epidermolysis bullosa (OMIM: 125647). In skeletal muscle, the dystrophin-associated protein complex (or costamere) links extracellular matrix to intracellular actin cables and is critical for skeletal muscle integrity (reviewed in Reference 34). In these complexes, α-dystroglycan binds to basement membrane matrix, as well as to the transmembrane protein β-dystroglycan, which also binds to dystrophin. Dystrophin binds directly to actin, and this complex (and many other proteins present) couples the Z-disc of the sarcomere to the extracellular basement membrane. Mutations in dystrophin lead to muscular dystrophy.

Membrane-Associated Proteins and the Cytoskeleton

We have discussed the importance of transmembrane proteins in mediating membrane-cytoskeleton interactions, but many multidomain proteins can also mediate such linkages. For example, many proteins have regions that can dynamically interact with membranes (e.g., through PH superfamily domains, FYVE domains, C2 domains, ANTH/ENTH domains, or basic regions) and regions that can interact with cytoskeletal components. Some of these proteins are important regulators of membrane-cytoskeleton interactions at sites of distinct lipid composition (and bilayer curvature). Proteins such as vinculin, talin, and ERM (ezrin/radixin/moesin) family proteins are found at sites of membrane-cytoskeleton interaction, where they bind both phosphoinositides [usually PtdIns(4,5)P_2] and actin. Ena/VASP family proteins can also bind to membranes and actin and are also important regulators of actin polymerization (130). All these proteins have other interactions with mediators and modulators of actin

polymerization, and mutations in some of these proteins are strongly linked to human disease. Mutations in vinculin lead to dilated cardiomyopathy (105), radixin is essential for normal cochlear stereocilia (71) [and mutations in this protein result in hereditary deafness (69)], and mice lacking ezrin have abnormal morphogenesis of intestinal villi (127).

A large group of proteins (>70) in the human proteome have both canonical lipid-binding domains and GAP/GEF domains active against Rho/Arf family small G-proteins. Proteins of this type that act at the plasma membrane (as opposed to those targeted to other intracellular membranes) are prime candidates for modulating membrane-cytoskeleton interactions through regulation of plasma membrane-associated small G-proteins. These include the RhoGEF intersectin proteins and various proteins of the ArfGAP centaurin family. Many proteins of this type are mutated in human disease, including BCR [the fusion partner of Abl in the leukemogenic Philadelphia chromosome (136)] and FGD proteins [FGD1 is mutated in faciogenital dysplasia, which comprises a variety of facial and other malformations (OMIM: 300546); FGD4 is mutated in a demyelinating form of Charcot-Marie Tooth neuropathy (OMIM: 611104)]. Other proteins of this type include oligophrenin [which is required for dendritic spine morphology (49) and is commonly mutated in syndromic mental retardation (OMIM: 300127)] and the vav oncogenes and sos family proteins [mutations in sos1 are found in gingival fibromatosis and a subset of Noonan syndrome patients—this latter disease is characterized by facial dysmorphism and a short stature, with heart and skeletal muscle abnormalities (OMIM: 182530)]. Although some of these diseases may be due to the loss of other regions of these proteins, the potential for these proteins to modulate important membrane-cytoskeleton interactions must not be overlooked. A large number of other proteins with GAP/GEF activity (or cytoskeleton-binding regions) may interact with membranes through noncanonical lipid-binding regions, through posttranslational modifications, or via adaptor proteins or complexes. These interactions should be identified and investigated for their effects on membrane-cytoskeleton linkages.

Membrane Blebbing and Tether Formation Might Be Used to Identify Mediators/Modulators of Local Membrane-Cytoskeleton Interactions

Membrane-cytoskeleton linkage is difficult to study, not least because of the cross talk between cytoskeletal elements, and the important consequences of membrane-cytoskeleton interactions, that confound certain routine experimental approaches. Other novel approaches must be developed to more precisely study these interactions. Membrane blebbing is an important phenotype of the local loss of membrane cytoskeletal interactions. Membrane blebbing is an indicator of adverse cellular health and is mediated through cleavage and activation of ROCK1 by caspases during apoptosis (15), but it is also found physiologically. Moreover, membrane blebbing might be exploited experimentally to determine the identity and mechanism of action of important proteins mediating membrane-cytoskeleton linkages (133). Blebs form when there is a loss of membrane-cytoskeletal adhesion larger than ~1 μm. These blebs are reversible, and reversal requires actin polymerization into these sites to provide new membrane-cytoskeleton linkage. Blebs occur upon PtdIns(4,5)P_2 hydrolysis or sequestration, reflecting the importance of this lipid in mediating membrane-cytoskeleton interactions (120). Blebs also occur when other important proteins mediating membrane-cytoskeleton interactions are depleted, including spectrin and ankyrin (which are found mutated in HS) and filamin (133). The study of membrane blebbing could be used after manipulation of proteins to identify their roles in mediating/modulating local membrane-cytoskeleton interaction, so long

as functional targeting is sufficiently acute so as to minimize the contribution of confounding effects.

Another powerful technique for studying local membrane-cytoskeleton interaction is tether formation using optical tweezers. The energy requirements for loss of membrane-cytoskeleton adhesion can be indirectly probed by pulling membrane tethers away from the cell surface, as this requires the disruption of membrane-cytoskeleton bonds (132). This approach could also be applied after molecular manipulation. This technique has been validated by several findings. For example, PtdIns(4,5)P_2 sequestration results in a profound decrease in the force required for tether formation (120), consistent with its central role in the mediation of membrane-actin communication. PtdIns(4,5)P_2 is rapidly hydrolyzed upon stimulated secretion, resulting in reduced adhesion, and this is also coupled with a reduction in the force required for tether formation (20). Further, sites where actin-membrane interactions are undergoing constant turnover more easily allow tether formation, such as occurs at lamellipodia (119).

CONSEQUENCES OF MEMBRANE-CYTOSKELETON INTERACTIONS

Membrane-Cytoskeleton Interactions Control Membrane Diffusion and Order

The cytoskeleton has profound effects on the plasma membrane. Lateral diffusion of lipids and proteins in the plasma membrane is not always uninhibited and can be regulated by membrane-cytoskeleton links that provide obstacles to free diffusion, producing distinct diffusion-limited lipid domains (corrals) enclosed by an actin perimeter (76, 101). Precisely how this relates to the formation of cholesterol-dependent liquid-ordered (lipid rafts) and liquid-disordered domains of the plasma membrane, which would also limit lateral diffusion, is not clear. Certainly, the mobility of (liquid-ordered) Caveolin1- and Flotillin2-positive membrane microdomains is regulated by actin (79, 149). Focal adhesions, which mediate strong membrane-cytoskeleton interactions, have a high degree of membrane order. Further, upon deligation of cells from their surrounding matrix a large portion of liquid-ordered plasma membrane is lost [as assessed using the fluorescent Laurdan dye, which emits distinctly in liquid-ordered and liquid-disordered membranes (43)]. These results strongly suggest that the formation of membrane-cytoskeleton linkages at cell-matrix contacts is necessary for the stabilization of these membrane regions. This may be due to many factors, including a high concentration of transmembrane and membrane-associated proteins mediating membrane-cytoskeleton interactions at these sites, which could associate preferentially with specific lipids, as well as local changes in membrane tension/shape, which might best be accommodated by the assembly of liquid-ordered regions. Actin polymerization in large PtdIns(4,5)P_2 synthetic vesicles can phase-separate the membrane in a manner dependent on membrane-cytoskeleton interactions (85). Membrane-cytoskeleton interactions can also induce membrane curvature, which results in phase separation (107).

Liquid-ordered regions are thought by some to be insoluble and float after extraction with cold detergent. It is possible that at least a subset of plasma membrane regions is insoluble due to tight interactions with cytoskeleton components, and this should be considered when performing such manipulations. Liquid-ordered regions of the plasma membrane are enriched in cholesterol and sphingolipids, on which they are dependent for their formation. The exact relationship between liquid-ordered domains and PtdIns(4,5)P_2-enriched domains is enigmatic, but PtdIns(4,5)P_2 and cholesterol depletion have similar phenotypes on the actin cytoskeleton, and cholesterol

depletion reduces PtdIns(4,5)P_2 levels at the plasma membrane (78). Such observations complicate the determination of the primary cause of phenotypes observed after lipid, lipid-organizing protein, or cytoskeleton manipulation. For example, the observed cholesterol/PtdIns(4,5)P_2 dependence of particular endocytic pathways has been assessed using lipid depletion/sequestration. However, such techniques may perturb other lipids or have indirect effects on the cytoskeleton and/or membrane-cytoskeleton interactions. Likewise, actin depolymerization may produce profound changes in lipid composition and lateral diffusion in the plasma membrane, which could change membrane permissivity for endocytic events and recruitment of important pathway effector proteins. A more speculative role for actin may be in controlling plasma membrane lipid asymmetry. For example, in apoptotic cells many membrane-cytoskeleton interactions are lost, and phosphatidylserine (usually confined to the inner leaflet) is found on the outer plasma membrane leaflet.

Membrane-Cytoskeleton Interactions Control Membrane Tension

The cytosol exerts a pressure on the plasma membrane that contributes to its apparent tension, and this is modulated not only by changes in membrane area, but importantly by the strength of membrane-cytoskeleton interactions (19). The need to overcome the resistance provided by membrane tension in any plasma membrane deformation event is critical. This is exemplified by the importance of membrane tension in the exo-endocytic cycle. Exocytosis to the plasma membrane can stimulate rapid compensatory endocytosis which recovers the membranes delivered to the surface and likely restores the pre-existing membrane tension (148). An increased surface tension reduces endocytosis (20). Exocytosis decreases membrane tension by increasing local surface area and reducing cytosolic pressure. The plasma membrane is usually resistant to membrane deformation and the force exerted by the endocytic machinery must overcome the resistance provided by plasma membrane tension. Any endocytosis that occurs will have the reverse effect to exocytosis, increasing cytosolic pressure and reducing surface area to inhibit further endocytic events. Actin ordinarily imposes physiologically relevant membrane tension, illustrated elegantly by the observations that actin depletion obviates the need for secretion to reseal punctured membranes (150), and that actin depolymerization allows proteins capable of deforming the plasma membrane to do so more readily in vivo (57). The rigidity of the substrate upon which cells are grown is likely a critical modulator of membrane tension and must be borne in mind when performing cell biological experiments.

Membrane-Cytoskeleton Interactions in Global Cell Shape/Volume Regulation

The morphology of the plasma membrane is dependent upon the integrity of the cytoskeleton, and its overall shape is determined by the conformation of its components. The surface area/volume ratio of cells is related to their morphologies. This is high in most cells, coupled to significant levels of plasma membrane invaginations and evaginations, allowing them to significantly increase their internal volumes before the membrane's elastic limit is reached and cell lysis occurs. The cytoskeleton does not regulate surface area/volume ratio in isolation, because the surface area and volume of the cell are independently controlled by the exo-endocytic cycle and transmembrane channels, respectively. When cell volume is increased by bathing cells in hypotonic solutions, the surface area/volume ratio decreases until a spherical cell is formed. Upon recovery from hypotonic stretch, cells shrink and new invaginations from the membrane are formed (133). The formation of new invaginations may

reflect two parameters that are altered upon stretch. First, an increase in membrane surface area occurs due to exocytosis (and relaxation of membrane invaginations into the predominant plane of the plasma membrane), which occurs in order to relieve the highly tensed membrane. Upon recovery from stretch, the pre-existing cell membrane relaxes onto the preformed cytoskeleton, and the newly delivered lipids (which are not attached to the cytoskeleton) are no longer pushed out owing to the drop in cytosolic pressure. Second, and coupled with this phenomenon, the rupture of cytoskeletal links with pre-existing membrane results in membrane tension changes, as well as the loss of a relaxation barrier. Cells usually recover to a point at which cytoskeletal strain is minimal.

Perhaps surprisingly the role of actin in cell volume maintenance is controversial, and in most cases actin disruption in isosmotic conditions does not result in cell swelling (84, 108), which might be expected if actin was holding the plasma membrane in. Cell volume changes (which are common in vivo, e.g., when local osmolarity is increased through anaerobic muscle respiration), however, can have large effects on cellular filamentous actin content (108), suggesting that the membrane-cytoskeleton interactions have been profoundly changed and actin is certainly important during responses to changes in cell volume. It is unknown whether volume regulation is at all linked to the direct interactions observed between ion channels and actin (108). MTs act together with actin filaments in volume regulation under hypotonic conditions, at least in kidney epithelia (84) and some leukocytes (32), but whether MTs supply mechanical or membrane trafficking (allowing exocytosis) support is unknown. Given what is known about cell volume control, it is likely that membrane redistribution is the major contributing factor, and the importance of the exo-endocytic cycle, e.g., in controlling leading edge dynamics, is becoming better appreciated (152, 164).

Membrane-Cytoskeleton Interactions Dynamically Control Plasma Membrane Morphology

To appreciate how cells interact with their environments, it is important that we understand at a mechanistic level exactly how bidirectional communication between lipids and cytoskeletal elements regulates the maintenance and dynamics of adhesion sites and plasma membrane morphology. In this section we focus on how membrane-cytoskeleton interactions are critical for understanding cell biological events where changes in plasma membrane morphology occur, with particular emphasis on the membrane deformation that occurs at the leading edge of migratory cells, during filopodial production, and during endocytosis. Plasma membranes are predominantly flat structures, and because a flat bilayer represents its lowest energy state, energy must be supplied to allow membrane deformation. The three major molecular variables in membrane deformation are the cytoskeleton, soluble intracellular proteins, and intrinsic components of the membrane itself. All these contribute to membrane deformation.

The polymerization of actin and tubulin within lipid vesicles can result in dramatic protrusive shape changes, which are most exaggerated upon tubulin polymerization (16, 56). Membrane deformation by cytoskeletal elements in such systems requires vectorial displacement of membranes apposed to the filaments in the direction of polymerization, which could occur by either of two mechanisms (or indeed both). The first mechanism is through direct provision of force, whereby filament polymerization actively pushes on a closely apposed membrane region, forcing it to adopt a new conformation around the morphology of the filament. The second, more passive mechanism is via the rectification of thermally induced bending fluctuations of the lipid bilayer. This would be simply provided by mechanical resistance of a newly elongated filament to backward fluctuation of the membrane.

A close analysis of the actin filament-membrane interface is important. If an actin filament abuts a membrane, this would sterically inhibit further ATP-actin addition, which would occur in a load-sensitive manner. It remains unknown whether the filament itself might then transiently bend away from the membrane, or whether fluctuations in the membrane itself (either through thermal fluctuation or induced by cellular proteins) would provide a space for further filament elongation.

The actoclampin filament end-tracking model offers an interesting hypothesis to explain how actin-mediated membrane deformation might occur. In this model, proteins such as N-WASP, Ena/VASP family members, and formins (so-called actoclampins) are constantly tethered to the membrane and processively supply actin monomers for filament elongation (27, 28). In so doing these proteins act as potential motors for elongation under tension. In other models, untethered filaments must overcome tensile stresses by other means. Under both models, the elongation rate declines as load increases, but end-tracking motors would allow the half-maximum rate of elongation to occur at a higher load. This model is attractive, but experimental evidence for this is currently lacking.

A pushing force for cytoskeletal elements is implicated in protrusion from the plasma membrane at, for example, lamellipodia, membrane ruffles, and filopodia. This pushing force has also been suggested to play roles at other sites, for example, in pushing membranes of the necks of endocytic vesicles together to promote their fission from the plasma membrane, and the propulsion of intracellular vesicles and bacteria.

Membrane Deformation at the Leading Edge

To effectively deform membranes, cytoskeletal elements must be sufficiently rigid and interact closely with the membrane. Although actin filaments appear stiff under electron microscopy, over micron distances they are flexible, and shorter actin filaments are stiffer than longer ones. The steady-state treadmilling of actin filament turnover is slow (and generates no mechanical force), and barbed ends of actin filaments are capped rapidly by proteins that inhibit further subunit addition. Such capping would act as a strong barrier to rapid membrane deformation, yet many cells can elongate rapidly in actin-dependent manners. To overcome these limitations at the leading edge of cells, new barbed ends are constantly produced by severing proteins (which break polymerized filaments), by the removal of capping proteins, by the nucleation of new filaments, or as branches from pre-existing filaments by the Arp2/3 complex (114, 115). Arp2/3 nucleates branches at ~70° angles in vitro (102) and in vivo (145) and is found at filament branch points (144). At the leading edge, these branches are polarized in the direction of growth, with filament long axes oriented at roughly +/–35° to the normal of the bilayer (89). These mechanisms allow for the production of a network of short, stiff actin filaments at the leading edge, and with certain assumptions this can be successfully modeled in silico because a large number of important kinetic parameters in this process are already known (128). A complex array of interconnected actin filaments is produced that induces membrane deformation by constant cycles of assembly and turnover at the membrane, concomitant with dismantling of filament ends proximally rather than relying on the turnover of single monomers at single filament ends. This directionality is provided by cues from the plasma membrane, whose components transduce stimulatory cues from the exterior into PtdIns(4,5)P_2/PtdIns(3,4,5)P_3 accumulation, and activation of Rac/Cdc42 at the leading edge, leading to polarized actin assembly.

A model for membrane advancement by actin must also account for other parameters, including lipid diffusion. Also, because the cell is bound to surrounding matrix and other cells, significant advancement in one

direction (such as occurs at the leading edge of a migrating cell) is likely not permitted without directional membrane trafficking to the leading edge and the disassembly of adhesion interactions at distant sites.

MTs are stiffer than single actin filaments and likely also provide some rigidity to the cell. Both actin and MT filaments are more fragile than IFs and break under low strains (60). IFs are likely the most rigid components of the cytoskeleton in vivo and they demonstrate strain hardening in vitro, such that they become stronger upon deformation. This, coupled with their relatively slow turnover, suggests that IFs therefore likely play important roles in the maintenance of cellular morphologies. Little is known about how the myriad cell morphologies that exist are actively produced and maintained, and further study of IFs should provide important insights into the mechanics behind these often remarkable events.

Not Just the Cytoskeleton: There Are Other Ways to Bend a Membrane

In addition to important roles for the regulated assembly of cytoskeletal elements in plasma membrane deformation and maintenance of its morphology, some protein domains can also directly deform cellular membranes and some can sense and stabilize certain membrane curvatures. The existence of such domains in proteins strongly suggests a role for these proteins in membrane sculpting. BAR superfamily domains, including BAR, F-BAR, and I-BAR (IRSp53 homology domain, or IMD) domains, form dimeric coiled-coil modules (53, 99, 112, 134). Such modules can also deform cellular membranes both in vitro and in vivo and, at the plasma membrane, are sufficient to induce plasma membrane invaginations (BAR/F-BAR) (57, 112, 153) and evaginations (I-BAR) (92). This superfamily encompasses a wide variety of effector domains (such as GAP/GEF domains) that would be delivered to sites of highly curved membranes deformed either by other proteins or by themselves.

Other multidomain proteins can also deform membranes, including those proteins with Epsin N-terminal Homology (ENTH) domains (37) and C2 domains (90), each of which inserts an amphipathic helix or bulky hydrophobic residues, respectively, into a juxtaposed monolayer, resulting in the splaying of headgroups in this layer and curvature induction. These are short-range effects, but some of these proteins oligomerize on membranes or become concentrated by avidity interactions. For example, epsin (which contains an ENTH domain) can become concentrated upon clathrin polymerization (37), which also stabilizes the induced curvature. Other proteins may have membrane-deforming modules [e.g., amphipathic helices such as those that are found in the N-BAR domain subfamily (42)]. Polymeric scaffolds also help deform membranes, including those that directly (e.g., dynamin) and indirectly (e.g., clathrin) interact with membranes. Transmembrane domains may also be capable of inducing membrane deformation, and some likely preferentially associate with membrane regions of particular curvatures. For a review of membrane deformation see Reference 93. Membrane deformation by such elements and cytoskeletal structures is illustrated in **Figure 3*a***.

Proteins that can directly interact with and deform membranes in vitro are candidates for plasma membrane deformation events in vivo, and many of these proteins are found at actively deforming plasma membrane sites together with cytoskeletal proteins. These may function together in several ways. (*a*) They may be recruited to sites where the cytoskeleton has directly induced membrane deformation through the proteins' abilities to sense membrane curvature or bind cytoskeletal elements, bringing to these sites effector domains that locally modulate the cytoskeleton. (*b*) They may stabilize membrane curvature induced by cytoskeletal elements. (*c*) They may synergize with cytoskeletal elements in

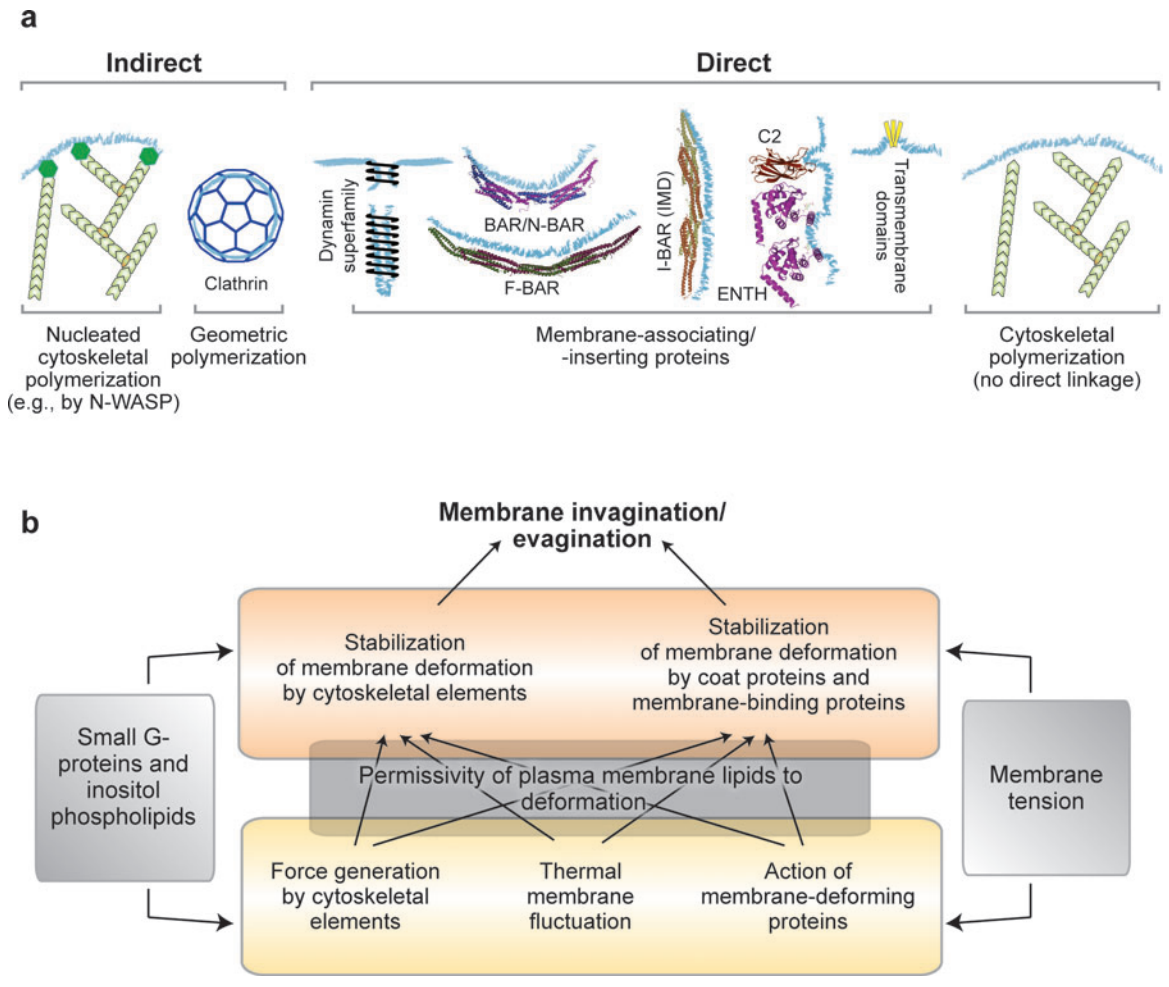

Figure 3

Membrane deformation. (*a*) Routes to membrane deformation. Diagram illustrates direct and indirect methods by which membrane deformation can be effected by cellular proteins. (*b*) Considerations for the study of membrane invaginations and evaginations. Diagram illustrates how membrane deformation and stabilization of deformed membranes can occur under the modulation of small G-proteins, membrane inositol phospholipids, membrane tension, and membrane permissivity.

membrane deformation. (*d*) They may be primary mediators of membrane deformation and stimulate nucleation/polymerization of cytoskeletal elements, which then stabilize the curvature induced by membrane-deforming proteins.

The finding that many BAR superfamily proteins possess SH3 domains that interact with N-WASP (and less to WAVE) provided a potential explanation for the specific recruitment and activation of the actin polymerization machinery at sites of distinct membrane curvature (12, 21, 53). Many of these proteins preferentially bind to PtdIns(4,5)P_2-enriched membranes, which would promote the activation of recruited N-WASP proteins. N-WASP might be activated locally through coincidence detection of PtdIns(4,5)P_2 and membrane curvature that is sensed and transduced through BAR superfamily proteins. N-WASP might also be activated at these sites to allow stabilization of membrane

deformation induced by BAR superfamily domains. Many BAR superfamily proteins also bind to the large GTPase dynamin, which has its own ways to modulate actin polymerization. Dimerization/oligomerization of some of these proteins may allow them to interact with many binding partners in the same area. For example, syndapin is found in a large complex with dynamin and N-WASP in a manner dependent on its ability to oligomerize (68). Syndapin also binds Cordon bleu (Cobl), which nucleates the growth of long, unbundled actin filaments independently of formins and the Arp2/3 complex [the other known actin nucleators (1)]. Cobl is highly conserved from fish to human and is necessary for normal neuronal arborization. However, the mechanism by which Cobl acts in vivo remains to be established. Cobl activation is presumably tightly linked to membranes and, given its interaction with syndapin, may also be coupled to membrane deformation by syndapin and/or other BAR superfamily proteins.

Further, many BAR superfamily proteins have domains involved in the regulation of small G-proteins of the Arf and Rho families, and thereby have complex roles in cytoskeletal regulation. BAR superfamily proteins may have universal roles in the spatiotemporal coordination of actin polymerization, and it is important that we determine their precise mechanistic contributions.

Filopodia

Filopodia are an elegant example of how soluble membrane-deforming proteins and the cytoskeleton can functionally synergize. These long and thin plasma membrane evaginations are involved in the sensing of the extracellular environment and are commonly found in association with lamellipodia at the leading edge of cells, although lamellipodia are not necessary precursor structures. In contrast to lamellipodia, which are dense networks of branched actin filaments, in order to produce a filopodium, the genesis of unbranched bundles of actin filaments is required. Whereas Arp2/3 induces branched actin filaments in lamellipodia, Arp2/3 is generally absent from filopodia, and Arp2/3, WAVE, and N-WASP activities are not necessary for their formation (141), although overexpression of N-WASP induces their formation (98), perhaps by increasing the pool of upstream elements. Unbranched filaments are produced by formins, which promote actin nucleation and inhibit filament branching. The formin mDia2 localizes to filopodial tips (98), where it likely nucleates the growth of filament barbed ends and inhibits their capping. Cdc42 appears to activate mDia2 to induce filopodial extension (109). Cdc42 itself is not necessary for filopodial production and it appears that the small G-protein Rif (Rho in filopodia) is an alternative activator of mDia2 (98).

Ena/VASP family members are also found at filopodial tips, where they turn over slowly (2), and may have important actin nucleation and actin-bundling activities (77). They might also function as actoclampin motors to enhance actin polymerization. The bundled actin produced by these proteins appears to be stabilized by fascin, which is found throughout the filopodial shaft (156). Fascin also binds the NGF receptor (135), which controls filopodial dynamics in neuronal growth cones (44). It is unclear whether the production and maintenance of bundled actin filaments by proteins such as fascin and Ena/VASP are sufficient to drive filopodial protrusions in vivo.

IRSp53 is required for filopodial formation and is found at the interface between F-actin filaments and the plasma membrane at filopodial tips (103) and requires binding to PtdIns(4,5)P_2 in order to promote filopodia production (92). The I-BAR domain of IRSp53 can bundle actin filaments in vitro but has only weak actin-bundling activities at physiological ionic conditions, necessitating a different explanation for its function at these sites. The I-BAR domain of IRSp53 can deform membranes and produce membrane evaginations such as those found in

filopodia (92). The I-BAR domain alone of IRSp53 is sufficient to induce filopodial formation. Furthermore, IRSp53 overexpression can induce membrane protrusions that do not contain actin (143). This suggests that IRSp53 may be the main driving force for filopodial membrane protrusion, and the membrane deformation that this induces may then be stabilized by the ordinarily concomitant assembly of actin filaments. IRSp53 associates with Cdc42 and Eps8 (39) (which mediates actin cross-linking and filament capping and modulates Rac activity). Eps8 and IRSp53 synergize in filopodial formation, and both are required for Cdc42-stimulated filopodial production (29). Cdc42 binds and controls the distribution of the Eps8/IRSp53 complex. IRSp53 also binds Ena/VASP family proteins and Cdc42 (74). How these interactions mechanistically lead to regulated and robust filopodial protrusion is unknown. Filopodial formation is an exciting model for the study of the membrane-cytoskeleton interface, and deciphering the network of interactions here, where relatively few protein players are implicated in the process, may have far-reaching consequences for our understanding of the biophysics of membrane protrusion.

Integrating Theory and Experiment to Understand Endocytosis

The molecular machinery involved in and the mechanistic detail of clathrin-mediated endocytosis have largely been established, yet many questions surrounding the role of actin in this process remain unanswered. It is important that we understand how the core molecular components of this pathway integrate with actin polymerization. As with any event that involves membrane deformation, there are many routes that may provide the directional changes in morphology observed at endocytic sites (**Figure 3*b***). At certain cellular locations, membrane protrusions or invaginations may be provided either by cytoskeletal filament polymerization or by membrane-associated or membrane-inserting proteins only. At other sites, both mechanisms may synergize, as appears to be the case for filopodial protrusion. These mechanisms are modulated by membrane inositol phospholipids and small G-proteins and must overcome the resistance to deformation provided by membrane tension. In addition, the lipids at a deforming site must be permissive for the extent and directionality of membrane deformation that occurs, i.e., lipids with shapes appropriate for accommodating the change in bilayer curvature must be concentrated at these sites.

The actin mesh covering the cytoplasmic surface of the plasma membrane provides mechanical resilience to the cell. Observers have argued for roles in both actin polymerization (in the provision of force for membrane deformations and in specification of endocytic sites) and depolymerization (to remove actin that would otherwise form a steric or tensive barrier) to allow endocytic vesicle formation (138). Total internal reflection-fluorescence microscopy (TIR-FM) has shown that actin polymerization does occur at a developing clathrin-coated pit. Although this can be an early event, it appears to occur predominantly late in the process, when the pit is invaginating and the nascent vesicle's neck is constricting and undergoing scission (97, 160).

Endocytic events might require actin for many reasons. Connection of the rim of a developing pit to actin may allow the actomyosin apparatus to help pull in deforming membranes [perhaps using motors such as myosinVI (10)], as well as provide a tract to allow the subsequent trafficking of vesicles postscission (or even for the localization of endocytic proteins). Further, actin polymerization at the neck of a coated pit may help push the membranes of this site together in order to promote membrane fission. Actin polymerization is certainly required for some clathrin-mediated endocytic events. The number of coated pits at the apical surface of epithelial cells is increased by actin depolymerization (48). A direct role for actin in their scission is suggested by the long necks of clathrin-coated pits observed in such cells when the

actin cytoskeleton is disrupted. Actin polymerization appears to be dispensable for other clathrin-mediated endocytic events, and this varies with the cell type studied (38). However, because it is often found tightly associated with endocytic events, actin polymerization therefore likely facilitates at least a subset of these, increasing their speed of formation and/or fidelity, but is unlikely a global driving force for constriction of endocytic necks. What actin polymerization may provide at these and other sites is directionality to a specific process by providing mechanical resistance to relaxation of newly deformed membranes. Such a facilitatory role for actin might be suggested by the presence of a variety of proteins at such sites that are capable of both deforming membranes and stimulating actin polymerization.

Dynamin oligomerizes in a helical manner around, and mediates the scission event at, nascent vesicle necks upon GTP hydrolysis (116). Its recruitment is roughly concomitant with an increase in actin polymerization (97). Cortactin binds to dynamin (95) and F-actin (159) and is recruited to coated pits maximally (with N-WASP and Arp2/3) at the time of scission (for reviews on the temporal recruitment of these proteins see References 63, 96). Cortactin binds to and activates Arp2/3 synergistically with N-WASP and therefore likely stimulates actin nucleation at clathrin-coated pits. Dynamin also binds profilin (158) and this interaction may also promote local actin polymerization. Actin polymerization at the pit neck may promote local membrane tension, which may promote fusion of the apposed bilayers at this site. This could occur by providing longitudinal increases in tension (stretching of the neck), which aids the ability of dynamin to induce scission in vitro (125) or to bring these bilayers closer through the direct provision of force in an axis normal to these bilayers. Neck formation is a thermodynamically unfavorable event due to the high curvature of such regions. In addition to dynamin, many proteins might aid the formation of the curvature of this region by membrane deformation in these and other endocytic events. BAR superfamily proteins such as endophilin, SNX9, CIP4, and syndapin, which can bind membranes and N-WASP, have been implicated in both membrane deformation and actin remodeling during endocytosis (67, 86, 110, 134, 153, 161). Such proteins also bind dynamin and are likely recruited to or involved in the formation of endocytic necks and may stimulate actin polymerization at these sites. It is likely that actin polymerization cooperates with these proteins and dynamin in neck constriction.

HIP1 and intersectin interact with the cortical actin cytoskeleton. HIP1R promotes clathrin-mediated endocytosis and binds to PtdIns(4,5)P_2 (via its ENTH domain) (58) and clathrin (11), as well as to F-actin directly through a talin-like (I/LWEQ) module (131) and to cortactin; this latter interaction produces filament capping and inhibits the ability of cortactin to bind to dynamin (81). Such regulation may provide the pit neck with polymerizing actin, while the rest of the pit may be connected to assembled actin filaments along which they can then be trafficked. Indeed, there may also exist feedback to the scission machinery such that scission occurs only if the nascent vesicle is already linked to these filaments and can therefore be appropriately trafficked.

Many factors control membrane tension, and adhesive links between the membrane and cytoskeleton are major contributors to this. Tension is not homogeneous over the plasma membrane owing to the nonuniform spatial location of membrane-cytoskeleton interactions and their associated adhesive strength. Local membrane tension is the important parameter that must be considered. The molecular nature, rigidity, and spatial location of the matrix upon which cells are grown affect membrane tension and have profound effects on membrane deformability. For example, neurons branch more extensively on softer substrates than on more rigid ones. Furthermore, cells cultured on two-dimensional substrates have

membrane-cytoskeleton interactions distinct from those on three-dimensional substrates, on which adhesion sites are smaller and more dynamic (30). As the rigidity of the matrix is increased, adhesions that are formed on three-dimensional substrates mimic those grown on two-dimensional substrates such as fibronectin or glass (17). In order to interpret experiments using cells on two-dimensional substrates (which are more easily subjected to experimental interrogation, including by TIR-FM, which is a powerful tool for studying endocytosis), we must use substrates with rigidities closely aligned to those of in vivo correlates to more closely mimic the situation in a tissue of interest.

Consistent with this, the dependence of certain cells on actin for endocytosis relies on the substrate on which they are grown and differs if cells are cultured in suspension (38). Furthermore, although treatment of polarized epithelial cells with cytochalasinD abrogated apical endocytosis, it did not affect basolateral endocytic ability (48), suggesting that these sides of the cell have differential requirements for actin polymerization in endocytosis. This may be due to distinct membrane tension at these sides of the cell.

Actin disruption can have no effect on or actually stimulate fluid-phase endocytosis (113, 155). These findings suggest both that certain actin-independent endocytic pathways can be upregulated to an extent to compensate for the loss of actin-dependent endocytosis and that actin is only important in endocytic events under certain conditions of plasma membrane tension. While actin may be necessary for endocytosis endogenously, complete actin depolymerization (which will change plasma membrane tension globally) may even obviate the need for localized actin polymerization. Given heterogeneous membrane tension, certain regions of the membrane may require actin for endocytosis whereas others may not. Indeed, a study that used a single-cell type and actin depolymerization protocol to assess clathrin-mediated endocytosis globally, as well as on the basal surface on a two-dimensional substrate, found that whereas basal endocytosis was almost completely inhibited upon actin disassembly, total clathrin-mediated endocytosis was affected much less severely (160).

A number of clathrin accessory proteins have the potential to bind or regulate actin assembly mediators. From network analyses, the actin polymerization machinery can be appreciated as a modular component within the endocytic interactome (129). How the decision is made whether to recruit/activate this module at specific endocytic sites is unknown, but there is likely variation in accessory component recruitment to clathrin-mediated endocytic events. This variation may depend on the cellular location/cargo incorporated (4), and this may allow for precise regulation of actin recruitment to certain subtypes of events. Actin is required for endocytosis in yeast (62). As a high-speed exo-endocytic cycle becomes required, such as occurred with the development of the synapse, the role for actin in endocytosis appears to be less important (126). This finding suggests that there may be a continuum depending on the evolutionary incorporation of additional layers of complexity.

Depletion of actin and certain phosphoinositides can have similar phenotypes in abrogating plasma membrane deformation events. This is not due solely to the inhibition of actin polymerization. For example, many of the core molecular components (including BAR superfamily proteins) that drive clathrin-mediated endocytosis bind preferentially to PtdIns(4,5)P_2, which is necessary for coated-pit assembly. That these components and actin polymerization are stimulated by PtdIns(4,5)P_2 suggests that they can be closely coupled by this phosphoinositide, likely through coincidence detection by actin nucleators of the activating lipid and activating proteins such as appropriate SH3 domains. Plasma membrane deformation events usually have specific phosphoinositide requirements, which may couple distinct forms of actin polymerization to distinct

core machineries. Several BAR and F-BAR domain-containing proteins also bind synaptojanin (21), which hydrolyzes PtdIns(4,5)P_2, and this may be important in locally limiting actin polymerization. Overproduction of PtdIns(4,5)P_2 inhibits clathrin-mediated endocytosis (64), perhaps by inducing overzealous actin polymerization.

MTs are involved in the swift trafficking of endosomes toward the center of the cell after their relatively slow travels on the cortical actin network. MTs are also involved in endocytosis. MT depolymerization reduces transferrin uptake in cells grown in suspension but not in cells on a two-dimensional substrate (142). This may reflect differences in membrane tension in these systems and their distinct needs for exocytosis of MT-delivered vesicles to modulate this.

Other endocytic events are also associated with extensive actin remodeling such as caveolar-type and membrane ruffle-associated endocytic events. Whether these employ similar mechanisms to allow membrane fission is not known. Although some clathrin-independent endocytic pathways have been defined by their dependence on certain lipids, small G-proteins, and cytoskeletal elements, the myriad events that these components regulate make their roles in such pathways difficult to discern.

In addition to the energy required to disrupt membrane-cytoskeleton linkages, resistance to membrane tether formation also comes from the production of highly curved membranes by the optical tweezers and thus might be used as a surrogate marker for the ease with which local membrane deformation events such as endocytosis can occur (132) (although lipid asymmetry and other factors might confound such extrapolation). An increase in exocytosis is coupled with a drop in the amount of force required to produce a membrane tether consistent with the rise in endocytosis that normally compensates for this. After decades of research on defining the molecular components of endocytosis by biochemical and cell biological approaches, the systems biology/biophysical era of endocytosis is upon us.

FUTURE ISSUES

1. How exactly does actin polymerization at the plasma membrane generate force? How do proteins mechanistically and spatiotemporally regulate this? How does this occur in concert with membrane deformation by other proteins? How does the cell decide at which endocytic events actin is required, and to what extent?
2. What are the core features of membrane-cytoskeleton interactions? How do these interactions differ between sites and cells to allow specialization of function?
3. How can we identify novel proteins that mediate and modulate membrane-cytoskeleton interactions? How can we get around confounding factors to probe these interactions experimentally in vivo?
4. How are the large number of dynamic changes in membrane-cytoskeleton interactions that occur in migrating cells coordinated? How are these coupled with directional membrane trafficking?
5. How does membrane tension affect the need for actin polymerization at endocytic sites? What precise roles does actin polymerization perform here?

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

We thank all researchers who have contributed invaluably to the understanding that has led to this review, and we sincerely apologize that space restrictions have not allowed us to highlight all their important findings. We thank Rohit Mittal for his careful reading of this manuscript and for his comments and suggestions.

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Errata

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