Mechanisms and Functions of Vinculin Interactions with Phospholipids at Cell Adhesion Sites

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The cytoskeletal protein vinculin is a major regulator of cell adhesion and attaches to the cell surface by binding to specific phospholipids. Structural, biochemical, and biological studies provided much insight into how vinculin binds to membranes, what components it recognizes, and how lipid binding is regulated. Here we discuss the roles and mechanisms of phospholipids in regulating the structure and function of vinculin and of its muscle-specific metavinculin splice variant. A full appreciation of these processes is necessary for understanding how vinculin regulates cell motility, migration, and wound healing, and for understanding of its role in cancer and cardiovascular diseases.

Inositol phospholipids are crucial regulators of cell physiology, and their head group interactions play fundamental roles in controlling membrane/cytosol interfaces. In addition to signal transduction at the cell surface, these lipids regulate a range of cellular processes including membrane trafficking, polarity, nuclear events, the permeability and transport functions of membranes, and cytoskeletal organization. Phosphoinositides are spatially and temporally regulated at sites of actin assembly and cytoskeleton remodeling. Phosphatidylinositol 4,5-bisphosphate (PIP$_2$) is a precursor of the second messengers diacylglycerol, inositol 3,4,5-trisphosphate, and phosphatidyl 3,4,5-trisphosphate and is crucial in the organization of the actin and cytoskeleton at the plasma membrane (1). PIP$_2$ participates in the recruitment and activation of a wide variety of adaptor proteins and actin regulatory proteins. During the assembly of focal adhesions (FAs), the cytoskeletal protein talin recruits an isoform of phosphatidylinositol 4-phosphate 5-kinase (PIP$_2$K$_{790}$) that catalyzes the phosphorylation of phosphatidyl 4-phosphate to generate a local enrichment of PIP$_2$ (2–4). This localized pool of PIP$_2$ is critical for the subsequent recruitment of FA proteins and maturation of the FAs (5, 6). Vinculin is an early and essential component of nascent cell-matrix adhesion and acts as a scaffold by binding to several actin-organizing proteins. In mature FAs, vinculin is a key component of the “molecular clutch” that mediates the transmission of force from cytoplasmic F-actin to membrane-bound integrins (7–9). Cytosolic vinculin mainly adopts a closed conformation that is kept inactive through extensive hydrophobic interactions of its globular 91-kDa head (VH, residues 1–840) that harbors binding sites for talin, α-actinin, or α-catenin to VH (10–13) and its 21-kDa tail (Vt, residues 879–1066) domains (14, 15), which are connected via a proline-rich linker (Fig. 1, A and B). The intramolecular VH-Vt interaction masks the binding sites of F-actin (16, 17), PIP$_2$ (18), and raver1 (19, 20) to Vt. Cytosplasmic vinculin must be recruited to FAs and activated to expose ligand-binding sites through a complex process that is not completely understood (21).

Vinculin is essential during development due to its role in regulating adhesion and motility, as well as cell spreading (22–24). Mice lacking both isoforms of the vinculin gene die during embryogenesis by embryonic day 10 (22). Vinculin-null mouse embryo fibroblasts are viable but generate small non-functional FAs and display marked defects in the organization of the actin cytoskeleton and in cell spreading (22). These defects can be rescued by the exogenous introduction of wild-type vinculin fused to the GFP. However, GFP-vinculin constructs containing specific mutations that specifically prevent PIP$_2$ binding without compromising other vinculin functions are unable to rescue any of the vinculin-null phenotypes (25). Thus, PIP$_2$ binding by vinculin is critical for the assembly of functional FAs

Identification of the PIP$_2$-binding Site

Despite considerable efforts, determination of the lipid binding specificity of vinculin had been elusive until recently. Part of the problem is that because intact full-length vinculin does not bind phospholipids effectively, it was necessary to use truncated vinculin fragments, and many of these were unstable (26–32). In particular, deletion of the last 15 residues destabilizes Vt (31), probably because it disrupts the interaction of strictly conserved Trp-1058 with Trp-912. Furthermore, pure lipid micelles might act as detergents, and other artifacts might have convoluted interpretation (33). Identification of the PIP$_2$-binding site on vinculin was further complicated by the fact that the known conserved phosphoinositide- and PIP$_2$-binding motifs are not present in vinculin. Originally, vinculin residues...
on α-helices H3 (935–978; Fig. 1C) and H5 (1020–1040) were proposed to interact with acidic phospholipids (34), and the N terminus (residues 893–985) and C terminus (residues 1016–

FIGURE 1. Structure and sequence alignment of vinculin and MV. A, the vinculin head domain, Vh, is composed of three structurally similar domains (Vh1, residues 1–258; Vh2, 257–486; and Vh3, 492–715) and Vt (residues 719–1066). Vh1 and the five-helix bundle tail domain, Vt (879–1066) with deviations in that region. For the remainder, Vt residues 911–1066 with deviations from human Vt 

FIGURE 2. Sequential lipid and F-actin-binding site activation on vinculin. Vt (α-helices H1 through H5 are colored spectrally as in Fig. 1B; PIP_2 is shown as spheres) has two PIP_2-binding sites: the dimerization site 1 involving the H1-H2 loop (residues Ser-913, Lys-915) and the C terminus (Lys-1061), and site 2 involving H3 (Lys-944 and Arg-945). Hydrolysis of PIP_2 bound to the second site exposes the actin-binding site while still keeping the lipid-induced Vt dimer bound to the plasma membrane. F-actin subunits, based on the cryo-electron microscopy Vt-F-actin structure (77), are shown in gray or black.
on vinculin (17, 18, 35, 37–40) including that for PIP$_2$ are blocked by the nanomolar head-tail interaction of cytoplasmic vinculin. Further, recombinant vinculin only binds PIP$_2$ after activation by talin (25), and native full-length vinculin does not bind acidic phospholipids spontaneously (27). More recently, three independent studies have shown that mutant vinculin molecules deficient in PIP$_2$ binding are recruited to FAs, although these FAs are non-functional (33, 41, 42). It has recently been proposed that inactive vinculin is recruited to FAs at the plasma membrane by phosphorylated paxillin (8, 43) where it is activated via a complex process that probably involves binding of multiple ligands including talin and actin (44).

Talin is often cited as the master activator of vinculin. However, talin is also recruited to FAs in an auto-inhibited state that must be activated by binding of ligands, which may include PIP$_2$, vinculin, and actin, and requires the engagement of the actomyosin contractile machinery (6, 21). Thus, talin and vinculin probably work in concert to activate one another through pathways that are not clearly understood. Building on previous work, a recent study (21) led to the following proposal: Auto-inhibited talin is recruited to FAs through binding of actin to the actin-binding site (ABS3) at the C terminus or through interactions of the N-terminal domain to integrins, PIP$_2$, or other FA components. The head-rod interaction is then at least partially disrupted, perhaps by PIP$_2$, to allow the N-terminal FERM domain to activate integrins and tether talin to the plasma membrane. Actin binding to the ABS3 domain and the application of actomyosin-mediated tension then stretch talin to expose cryptic high affinity vinculin-binding sites and additional actin-binding domains. Binding of vinculin to these newly exposed sites would contribute to vinculin activation as well as stabilizing the extended talin to allow further stretching and vinculin binding in a self-reinforcing manner. In this study, several additional observations were made that are germane to the topic of this review. Cells expressing talin with mutations in the ABS3 domain that compromise actin binding do not develop functional FAs, and the mutant talin is not associated with actin filaments. However, co-expression of a constitutively active T12 vinculin mutant (D974A,K975A,R976A,R978A) (45) fully rescued FA formation (46). Inclusion of the vinculin A50I mutation, which inhibits vinculin-talin binding, prevented this rescue. The authors proposed that alternative pathways that (partially) activate vinculin, such as those involving PIP$_2$ binding, may bypass the role of actin binding to the talin ABS3 domain to contribute to FA formation and stabilization. Thus, although PIP$_2$ binding has been assigned an auxiliary role in vinculin activation, it may be more important than currently appreciated.

**Possible Roles of PIP$_2$ Binding in FA Turnover**

The regulation of the connections of the actin cytoskeleton with the cell membrane and the adhesion of neighboring cells or cells with the extracellular matrix (ECM) are crucial to many physiological and pathological processes including migration, differentiation, proliferation, and survival, as well as tissue organization, wound healing, and tumorigenesis. These are dynamic processes, and the responsible macromolecular complexes, such as FAs, display a dynamic assembly/disassembly behavior that is critical for proper function. Fluorescence recovery after photobleaching (FRAP) and related techniques have shown that most FA components are rapidly exchanging with a cytoplasmic pool in a highly regulated fashion (47, 48). Many, if not most, of the protein components of FAs display residence times within the FA of less than a minute. Recent studies (49, 50) have demonstrated that the exchange process is regulated to a large degree by actomyosin-generated tension and that vinculin is a key component that integrates both the recruitment and the release of other FA proteins in response to force.

Treatment of cells with blebbistatin, which inhibits actin-dependent myosin II activity, causes disassembly of FAs and the release of vinculin, zyxin, and paxillin (48). A follow-up study showed that treatment with the Rho kinase inhibitor Y-27632 resulted in disruption of the FA association actin network and the release of eight different FA components, including vinculin (49). FRAP assays from both studies revealed that release of vinculin following actomyosin relaxation is a first-order process mediated by an increased $k_{off}$ value.

Using vinculin-null mouse embryonic fibroblasts (MEFvin$^{-/-}$) cells, it was recently shown that treatment with the actin-disrupting drugs blebbistatin, Y-27632, or cytochalasin D resulted in the rapid release of full-length wild-type vinculin but not that of mutant vinculin constructs containing C-terminal deletions (vin258, residues 1–258; and vin880, residues 1–880) or mutations that render vinculin constitutively active (T12) (50). The “stabilization” of FA in the presence of actin-disrupting drugs by vin880, which lacks the actin-binding domain, including the PIP$_2$-binding sites, was shown to prevent the dissociation of a number of other FA components. This was interpreted to indicate that wild-type vinculin in high tension areas of the cell plays a major role in the recruitment and retention of core FA components. The constitutively active vinT12 mutant that retains actin binding properties is also stabilized within FA under conditions that disrupt the force-generating machinery via a mechanism that requires binding to talin. It should be appreciated that these “stabilized” FAs are not fully functional. Expression of these mutant forms of vinculin in B16F10 melanoma cells or MEFvin$^{-/-}$ cells impaired polarized migration (50). These results support the view that the spatio-temporal cycling of vinculin from an activated to an inactivated form, in response to differences in the magnitude of actomyosin-generated force, may be critical for efficient cell migration.

Three independent studies have directly addressed the role of lipid binding by vinculin in recruitment to FAs and FA turnover (33, 41, 42). Introducing a lipid binding-deficient mutant vinculin-LD (K952Q,K956Q,R963Q,K966Q,R1060Q,K1061Q) in B16-F1 mouse melanoma cells expressing endogenous wild-type vinculin resulted in vinculin-LD recruitment to FAs but acted in a dominant negative manner to significantly reduce FA turnover, resulting in impaired cell spreading and migration (41). Elevation of PIP$_2$ levels by the overexpression of PIP$_2$Ko in B16–F1 cells induces release of wild-type vinculin from FAs, a behavior that was significantly suppressed by the expression of vinculin-LD. Thus, PIP$_2$ binding by vinculin might function as a mechanism for vinculin release from FA. In another study, a
lipid binding-deficient mutant of vinculin lacking the last 14 amino acids of the C terminus was expressed in MEFvin−/− cells (42). The mutant vinculin was recruited to FA-like structures, but these structures were much more stable than FAs formed upon reintroduction of wild-type vinculin, and they were not able to rescue the spreading defect of MEFvin−/− cells. These two studies led to similar conclusions, although there is some concern that the mutations in these constructs might compromise other aspects of vinculin function. Based on the identification of the PIP2-binding site by x-ray crystallography, we generated vinculin mutants that are specifically impaired in PIP2 binding (K944Q,K945Q and K1061Q) (25). We were able to demonstrate that the expression of wild-type GFP-tagged vinculin was able to rescue the cell spreading and migration defects of MEFvin−/− cells and to re-establish an organized actin network. However, when the lipid binding-deficient mutants where expressed to comparable levels, they were recruited effectively to FA structures, but they did not lead to rescue of any of the MEFvin−/− phenotypes. Furthermore, FRAP studies revealed that a large fraction of mutant vinculin in these FAs was completely immobile, i.e., not exchanging with the cytoplasmic pool. Collectively, the results from these studies indicate that in the absence of the ability to bind PIP2, vinculin becomes irreversibly “stuck” in FAs, rendering them non-functional.

Vinculin-Lipid-Actin Interactions in the Context of the FA Three-dimensional Nanostructure

Several recent studies using super-resolution microscopy have provided insight into the nanoscale structure of FAs (8, 51, 52). These studies show that FAs are stratified in the Z-axis into three layers: a membrane-proximal integrin signaling layer (ISL), an actin regulatory layer (ARL) located ~60 nm higher, and an intermingled force-transducing layer between the ISL and ARL. Among the major FA components, only talin in the unfolded state is long enough to simultaneously engage integrins and actin. A recent study provided evidence that talin is the primary determinant of FA nanoscale structure, acting as a molecular “ruler” to span the FA core (52). Vinculin was shown to be non-uniformly distributed between all three of the axial FA layers, and it was suggested that vinculin “climbs” talin to reach the ARL and bind actin (8). Although numerous observations indicate that vinculin-actin binding is crucial for force transmission and for FA assembly and maturation (50,53), vinculin mutants deficient in actin binding are distributed within the FA structure similarly to wild type (8).

Inspection of the PIP2-binding site of vinculin reveals that simultaneous binding of actin and PIP2 is possible (33, 54). Early FRAP experiments suggested that vinculin interactions with cell junction components induce binding of vinculin to the plasma membrane independent of actin binding and that the vinculin-membrane attachment site serves as a nucleation center for the assembly of actin bundles (55). In vitro binding of F-actin to vinculin was shown to be minimal in the absence of lipids, but PIP2 increased binding 3-fold (36). Furthermore, VH-Vt bound F-actin in the presence but not the absence of PIP2, suggesting that PIP2 binding promotes adhesion site targeting of vinculin (36). In contrast, another study suggested that the earlier work was flawed due to the effect of magnesium on aggregation of PIP2 micelles and instead showed that polyphosphoinositides binding to vinculin block interactions with F-actin (56).

In FAs, only about 35% of vinculin molecules and 15% of actin molecules are found in the ISL. Most of the actin is found in the ARL located ~60 nm higher, which would preclude a single vinculin molecule from binding simultaneously with PIP2 in the membrane and F-actin in the ARL. However, many FA proteins are enriched in a particular layer but also rather broadly distributed, consistent with these proteins moving between layers as FAs mature or mediate translocation. Presumably, PIP2 remains in the membrane bilayer, so simultaneous binding of PIP2 and actin by vinculin might be a transient event that occurs in the ISL to contribute to vinculin activation. One might imagine a scenario in which localized PIP2 levels tether partially activated vinculin in the distal tip. Subsequent binding by actin might stimulate release of vinculin from PIP2 to promote full activation and transfer to the ARL. This model is not consistent with the observation that actin binding-deficient vinculin mutants still localize to the ARL, although data from super-resolution microscopy do not provide direct information on the dynamic behavior of proteins at the nanoscale level. Thus, actin binding might contribute to the efficiency of vinculin activation and transport to the ARL but not be absolutely required. An alternative model is that PIP2 binding by vinculin is a critical component for the release of vinculin from FAs. Actomyosin-generated force stabilizes the interaction of vinculin with actin and talin to engage the “molecular clutch” (57). If PIP2 and actin binding by vinculin is antagonistic, then in regions of the FA with reduced tension such as the FA-proximal tip, the clutch might become disengaged and favor PIP2 binding and perhaps subsequent auto-inhibition and release. This model is consistent with the observation that the vinculin exchange rate is significantly faster at the proximal versus the distal end of FAs (48) and with the effects of drug-induced disruption of actomyosin-generated force on mutant forms of vinculin (50). The demonstration that the expression of specific lipid binding-deficient mutant vinculin in MEFvin−/− cells leads to a marked inhibition of exchange, and the development of hyperstable FAs is also consistent with this model (25).

Vinculin-Lipid Binding in the Regulation of Specific Physiological Processes

Communication between the ECM and the contractile cytoskeleton is critical for most regulated processes related to cell movement. FAs are one type of macromolecular structure that mediate this communication by responding to external cues (outside-in signaling) and also by applying regulated changes in force to the ECM to mediate directed cell movements (inside-out signaling). Vinculin is essential for both types of signaling by coupling changes in tension within the contractile cytoskeleton to force applied to the ECM via its role as part of the molecular clutch that links talin to actin in the cytoskeleton and integrins in the cell membrane. Here we will discuss two interesting situations in which the interaction of vinculin with phosphoinositide lipids has been specifically implicated in regulating a physiological response.
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The endogenous intrarenal hormone bradykinin restructures FAs transiently in renal papillary collecting duct cells, causing a reversible dissociation of vinculin but not talin from FAs and inducing the reorganization of the actin cytoskeleton while preserving cell attachment to the ECM and neighboring cells (58). Subsequent studies provided evidence that some of the vinculin released upon bradykinin stimulation was located in cytoplasmic vesicles containing PIP2 (59). This led to the novel idea that activated or partially activated vinculin might be transported to FA reassembly sites via a vesicular endocytic recycling pathway (60).

Neurite remodeling is regulated by low molecular weight GTPases of the Rho family. In N1E-115 cells, neurite retraction in response to lysophosphatidic acid stimulation is associated with an FA-localized increase in PIP2 due to a Rho GTPase-mediated increase in PIP2K (61). Using mutants of PIP2K and lipid-binding-deficient mutants, a causal link between PIP2 levels and vinculin levels in FAs in directed neurite migration was established. Interestingly, the authors propose that a localized activation of PIP2K leads to increased PIP2 levels at the trailing edge of migrating cells to destabilize FAs by promoting vinculin release (61).

ECM stiffness has been identified as a factor contributing to malignant transformation and tumor metastasis. The ECM stiffness was recently shown to activate and stabilize vinculin at the invasive border of human breast cancer tumors (62). Using vinculin mutants, these authors present evidence that stabilized vinculin enhances the conversion of PIP2 to PIP3 by phosphorylation of Tyr-1065, leading to enhanced Akt signaling and thereby promoting tumor progression.

Metavinculin

The larger (1134-residue) vinculin splice variant, termed MV, is an integral component of adhesion complexes and of intercalated discs in cardiac muscle and is essential for proper cardiac development and homeostasis. Mutations of MV in patients with dilated cardiomyopathy and hypertrophic cardiomyopathy, an acute cause of heart attack, have been shown to occur in the unique 68-residue insert present in the MV tail domain, MVt (Fig. 1A). MV has a specific function in filament attachment in muscle cells because mutations or loss correlates with hereditary dilated cardiomyopathy (63, 64) and disrupted intercalated discs in those patients (64). It was also proposed that the site of the MV insert, between residues 915 and 916, would be strongly involved in lipid binding, and indeed in dimerized vinculin, PIP2 is sandwiched between two Arg-915 residues, one from each of two Vt molecules (25).

MV is co-expressed with vinculin at different molar ratios (65–67) in vertebrate smooth muscle, where it is expressed highly (65, 66, 68), and heart muscle, where its expression varies (65, 66). More recently, MV expression was shown in skeletal muscle where it co-localizes with vinculin in costameres, the adhesion site that links sarcomeric Z-lines to the sarcolemma (69), and the MV-vinculin heterodimer was confirmed in vitro from muscle extract precipitates (69). Vinculin was also suggested to co-activate MV, which could explain their co-expression (69). Significantly, relative vinculin expression levels remain constant when compared with actin and three times the amount in smooth and cardiac muscle when compared with skeletal muscle. This suggests that MV expression levels in different muscle types might modulate microfilament organization and attachment according to specific cellular needs (69).

MV heterodimer formation was confirmed in vivo by immunoprecipitation with murine skeletal muscle extracts where both isoforms were present and with predominant amounts of vinculin (69). In vitro, PIP2-adsorbed Vt bound to MVt strongly but not vice versa (69). However, all lipid-binding Vt residues (Ser-913, Lys-915, Lys-944, Arg-945, Lys-1061) and those involved in lipid-induced dimerization (His-906, Trp-1064, Tyr-1065) (25) are strictly conserved among all 26 known vinculin and MV species (Fig. 1C). Thus, it is unclear why MV should not homodimerize akin to vinculin. It is possible that the earlier results might be more complicated to interpret because their MVt construct harbored 68 additional unstructured residues at its N terminus (69) that might interfere.

The structures (and polypeptide chains) of MV and vinculin are identical for the first ~840 and last ~150 residues, whereas the N termini of their respective tail domains are distinct where an α-helix (H1t) and extended coil from the MV insert replace these same structural elements of the vinculin tail domain to form a distinct five-helix bundle tail domain (Fig. 1A) (70). The structural differences correlate with distinct functions, where Vt and MVt have different effects on F-actin bundling (71) and the ribonucleoprotein raver1 has a higher affinity for MVt than Vt (72) (which we quantified as 1.8 μM versus 12.6 μM) and binds to inactive MV (19). We obtained similar binding constants $K_d$ for the cardiomyopathy-associated MVt (A934V, ΔL954, R975W) for binding to raver1 (0.8, 0.6, and 1.6 μM, respectively). Raver1 binds to full-length MV but not full-length vinculin (20, 72), and PIP2 significantly enhances binding to full-length proteins (72).

Two recent studies have provided additional insight into MVt function. From data derived from a variety of in vivo and in vitro techniques, it was concluded that MVt “tunes” actin filament bundles by altering the architecture and flexibility (73). Using high resolution cryo-electron microscopy, a sub-nanometer three-dimensional reconstruction of the vinculin tail-actin complex was obtained (74). This study suggests that upon binding to actin, the N-terminal helix H1 of Vt unfurls, leading to vinculin dimerization and actin bundling. Indeed, H1 (or H1’ in MV) has previously been shown to direct differential actin-organizing functions (70).

Surprisingly, because of the importance of MV in the heart, the role of acidic phospholipids in binding to MV is completely understudied. Interestingly, cardiac-specific vinculin overexpression reinforced the cortical cytoskeleton, thereby enhancing the organization of the myofilament and improving contractility and hemodynamic stress tolerance (75). Indeed, understanding the effects of post-translational modifications such as vinculin palmitoylation and myristoylation could lead to the use of vinculin as a biomarker of aging or heart failure (76).

Conclusions and Future Directions

The role of vinculin as a key component of the molecular “clutch” that regulates the mechanosensitivity machinery of
FA functions. Although not strictly required for vinculin recruitment to FAs, PIP2 binding at the distal tip of FAs might stabilize a partially open vinculin molecule and facilitate a local concentration of vinculin and its co-activator talin, which also binds PIP2. Vinculin-PIP2 binding might then stabilize vinculin-talin binding interactions during early stages of activation of both proteins; subsequent binding of actin to Vt might sever the vinculin-PIP2 interaction to complete activation. At the proximal end of FAs, these steps might be reversed to promote release of vinculin.

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