The Vinculin Binding Sites of Talin and α-Actinin Are Sufficient to Activate Vinculin*§

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Vinculin regulates both cell-cell and cell-matrix junctions and anchors adhesion complexes to the actin cytoskeleton through its interactions with the vinculin binding sites of α-actinin or talin. Activation of vinculin requires a severing of the intramolecular interactions between its N- and C-terminal domains, which is necessary for vinculin to bind to F-actin; yet how this occurs in cells is not resolved. We tested the hypothesis that talin and α-actinin activate vinculin through their vinculin binding sites. Indeed, we show that these vinculin binding sites have a high affinity for full-length vinculin, are sufficient to sever the head-tail interactions of vinculin, and they induce conformational changes that allow vinculin to bind to F-actin. Finally, microinjection of these vinculin binding sites specifically targets vinculin in cells, disrupting its interactions with talin and α-actinin and disassembling focal adhesions. In their native (inactive) state, the vinculin binding sites of talin and α-actinin are buried within helical bundles present in their central rod domains. Collectively, these results support a model where the engagement of adhesion receptors first activates talin or α-actinin, by provoking structural changes that allow their vinculin binding sites to swing out, which are then sufficient to bind to and activate vinculin.

Vinculin is a 117-kDa modular protein that is comprised of five helical bundle domains (Vh1, Vh2, Vh3, Vt2, and Vt, Ref. 12). Intramolecular interactions of its N-terminal seven-helical bundle (Vh1) domain with its C-terminal five-helical bundle tail (Vt) domain clamp vinculin in its inactive closed conformation (12–15). Biochemical studies have shown high affinity interactions of isolated Vh1 and Vt domains (14, 16), and additional interdomain interactions of Vt with the Vt2 domain have been proposed to further lower the Kd of the head-tail interaction (to 50–90 nM; Refs. 17, 18). This intramolecular interaction has also been suggested to mask cryptic binding sites for other partners of vinculin, including those that mediate the binding of talin and α-actinin to the Vh1 domain (15–21), and of F-actin and paxillin to the Vt domain (21–24). Thus, severing the head-tail interaction is necessary for vinculin activation.

Acidic phospholipids such as phosphatidylinositol-4,5-bisphosphate (PIP2)² have been thought to serve as the triggers that activate vinculin (21, 25), and indeed PIP2 can bind to and alter the conformation of the Vt domain (26). However, PIP2 interactions with vinculin are disrupted at physiological salt concentrations, and its binding to vinculin requires high levels of PIP2 in lipid micelles (27, 28). Furthermore, PIP2 competes with F-actin for binding to vinculin (29), and the PIP2 binding site is occluded in the full-length structure of inactive vinculin (15). Finally, vinculin mutants selectively defective in PIP2 binding still recruit to sites of focal adhesions (28). Thus, although PIP2 may play some role in regulating vinculin, other triggers likely activate vinculin.

Mounting evidence suggests that talin and α-actinin may directly activate vinculin. During outside-in integrin signaling, the first event detected is PIP2-mediated changes in the conformation of the head domain of talin (30), which then rapidly associates with the cytoplasmic tails of β-integrin receptors (31–33). Talin then interacts with vinculin through several high-affinity vinculin binding sites (VBS) present in its central rod domain (16, 34–36), which could allow talin to bind to multiple vinculin molecules and amplify outside-in integrin signaling (35). These interactions are likely essential, as targeted deletion of talin abolishes the formation of focal adhesions (37). Similarly, α-actinin plays an important role in the maturation of adhesion complexes (38, 39) and binds to vinculin through a single high affinity VBS (aVBS) present in the R4 spectrin repeat at the end of its rod domain (19, 20, 40). Notably, the αVBS and VBSs of talin efficiently disrupt the Vt domain from pre-existing Vh1-Vt complexes (24, 35, 41). Moreover, the crystal structure of inactive human vinculin (12), those of Vh1-talin-VBS complexes (24, 35, 36, 41), and that of the Vh1-αVBS complex (40), have demonstrated that the binding site for talin or α-actinin in vinculin is

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2 The abbreviations used are: PIP2, phosphatidylinositol-4,5-bisphosphate; TRITC, tetramethylrhodamine isothiocyanate; SPR, surface plasmon resonance; VBS, vinculin binding site; Vh, vinculin head (residues 1–840); Vh1, N-terminal seven-helical bundle domain of vinculin (residues 1–258); Vt, C-terminal (tail) five-helical bundle domain of vinculin (residues 879–1,066); Vt2, the four-helical bundle domain of vinculin (residues 719–835), which is structurally similar to Vt.
not masked by the head-tail interactions of vinculin; rather, it is readily accessible to the VBSs of talin or α-actinin (12). Finally, the structures of talin-VBS- and αVBS-bound to Vh1 have demonstrated that these amphipathic α-helices disrupt the Vh1-Vt interaction from a distance, by provoking conformational changes in the N-terminal helical bundle of vinculin, by a process coined helical bundle conversion (24, 35, 40).

The structures of native, inactive talin and α-actinin have revealed that their VBSs are normally buried within helical bundles that comprise their rod domains (40–43). Thus, in their resting state, the rod domains of talin or α-actinin have a rather low affinity for vinculin (15, 20), and this has suggested a combinatorial model for vinculin activation, where simultaneous binding of two or more ligands are required to provide the free energy necessary to break the head-tail interactions of vinculin (15, 18). However, this model only considers talin or α-actinin in their inactive states and the structure of the helical bundle domains of talin or α-actinin may also be dynamic as, for example, atomic force microscopy has shown that helical bundle domains that comprise spectrin repeats (also found in α-actinin) can form stable, unfolded intermediates when exposed to mechanical stress (44). Thus, when exposed to tension forces from within or from outside the cell, such as occurs during the formation of adhesion complexes (45–48), the VBSs present in the helical bundle domains of talin and α-actinin might become exposed to bind to and activate vinculin, without the need for co-stimulatory signals.

Because talin and α-actinin do associate with vinculin at adhesion sites in cells (49–51), and αVBS has a comparably high affinity for full-length vinculin versus the isolated Vh1 domain (Kₐ of ~2 nM; Ref. 40), we predicted that physiological levels of the VBSs of talin and α-actinin might be sufficient to activate vinculin. Indeed, here we present biochemical and biological tests of this hypothesis, which support the notion that the VBSs of talin and α-actinin function as physiological triggers of vinculin activation in adhesion complexes.

MATERIALS AND METHODS

Protein Expression and Purification—The octahistidine-tagged human full-length vinculin (residues 1–1,066), Vh1 (residues 1–258), VH (residues 1–840), and Vt (residues 879–1,066) proteins were expressed and purified as previously described (24, 40). Human α-actinin VBS (residues 731–760; αVBS) and VBSs of human talin (VBS1, residues 607–636; VBS2, residues 852–875; VBS3, residues 1,945–1,970) were synthesized and high performance liquid chromatography-purified in our in-house facility.

Surface Plasmon Resonance Assays—Binding studies were performed by surface plasmon resonance (SPR) using a Biacore 2000 biosensor equipped with a carboxymethyl dextran-coated gold surface (CM-5) sensor. The carboxymethyl groups on the chip were activated with EDC and N-hydroxysuccinimide to form the N-hydroxysuccinimide ester of carboxymethyl dextran. Vinculin protein was attached to this activated surface by reaction of the carboxyl groups of dextran with the primary amines of vinculin to form an amide linkage. Any remaining reactive sites on the surface were blocked by a reaction with ethanolamine. Reference cells were prepared similarly except that no vinculin protein was added. The interaction of vinculin with the VBSs of talin was analyzed at 25 °C in HPBS-P buffer (10 mM HEPES (pH 7.4), 150 mM NaCl, 0.1 mg/ml bovine serum albumin, 0.005% P-20). Talin-VBS1, -VBS2, and -VBS3 peptides were solubilized in water and then diluted in HPBS-P buffer. Binding was measured by flowing the individual talin-VBS peptides at a flow rate of 15 μL/min through the reference and vinculin-containing flow cells in sequence. Blanks were also run consisting of only buffer. Association was measured for 10 min, and dissociation was measured for 15 min. Data reported are the difference in SPR signals between the flow cells containing vinculin and the reference cells. Any contribution to the signal was removed by subtraction of the blank (buffer) injection from the reference-subtracted signals. Responses at equilibrium were then plotted as a function of the peptide concentration to calculate the affinities of the interactions of the VBSs of talin for full-length vinculin using Scrubber software.

Vinculin-VBS Binding Assays—Recombinant protein comprising the entire vinculin head domain (VH, residues 1–840), which includes residues in the Vt2 domain that have been suggested to contribute to the head-tail interactions of vinculin (18), was incubated with Vt (residues 879–1,066) for 20 min. To ensure that all VH was driven into a complex with Vt, the VH-Vt complex was formed using a 1:2 molar ratio of VH:Vt. An analysis on native polyacrylamide gels confirmed the rapid and complete formation of the VH-Vt complex under these conditions, which was distinguishable from free VH protein. αVBS or talin-VBS3 peptides were then added to the VH-Vt complex at the indicated molar ratios (from 1:1 to 20:1, VBS:vinculin) and allowed to incubate for 20 min. Complexes formed were then resolved on 8–25% gradient native polyacrylamide gels. The identity of components of the complexes in native gels was confirmed by cutting out the bands and analyzing on SDS-polyacrylamide gels and by immunoblotting with antibody specific for the histidine tag on vinculin (data not shown).

Vinculin-Actin Binding Assays—Vinculin was first incubated for 20 min at ambient temperature with talin-VBS3 or αVBS peptide (in 20 mM Tris-HCl (pH 8.0), 0.2 mM CaCl₂, 0.2 mM ATP, 2 mM MgCl₂ and 100 mM KCl) at the indicated molar ratios (from 1:1 to 20:1, VBS:vinculin) in a final volume of 580.8 μL. F-actin was polymerized as described (29) in a 100-μL total volume of the same solution, supplemented with 5% (w/v) sucrose and 1% (w/v) dextran. 19.2 μL of polymerized F-actin (final concentration of 7.5 μM) was then added. These complexes were then incubated for 1 h (at ambient temperature, a final total reaction volume of 600 μL), and the samples were then sedimented at 100,000 × g at 25 °C in a Beckman ultracentrifuge for 15 min. Equal volumes of reaction pellets (P) and supernatants (S) were resolved on 7.5% SDS-polyacrylamide gels and the gels were stained with Coomassie Blue.

NMR Spectroscopy—All NMR experiments were carried out at 25 °C on a Bruker DRX800 spectrometer equipped with a 5-mm HCN/z probe. Each 1H,15N TROSY experiment was recorded for 24 h. Product ion (MS2) spectra were subjected to search using the SEQUEST program of Eng and Yates (ThermoQuest), 98% 1H,15N-labeled, octahistidine-tagged, full-length human vinculin (residues 1–1,066) protein was expressed in Escherichia coli strain BL-21 in a culture of M9 minimal medium (D₂O) supplemented with 1 g of 15N-labeled ammonium chloride and 4 g/liter glucose. 2H,15N-labeled vinculin was purified as previously described (12) and was dialyzed against 20 mM potassium phosphate buffer (pH 7.6) and was concentrated to 0.2 mM. NMR spectroscopy of the vinculin-αVBS and the vinculin-talin-VBS3 complexes were obtained following mixing of a 1:4.1 molar ratio of these VSBS to vinculin.

Cell Culture—Swiss-3T3 cells were obtained from the American Type Culture Collection (Manassas, VA) and were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, penicillin, streptomycin, and l-glutamate in 9% CO₂. Vinculin⁻/⁻ and vinculin⁻/⁻ cells were the generous gift of Eileen Adamson (The Burnham Institute, La Jolla, CA) and were cultured as previously described (7).

Microinjection, Immunofluorescence, and Video Microscopy—For microinjection, cells were plated onto poly(d-lysine)-coated 35-mm glass bottom dishes (MatTek Corp., Ashland, MA) the day before injec-
Vinculin Activation

![Image](50x437 to 300x732)

FIGURE 1. The VBSs of talin have high affinity for full-length vinculin. Biacore surface plasmon resonance was used to measure the affinity of the VBSs of talin. Biotinylated full-length human vinculin was captured on a carboxymethyl-dextran-coated gold surface (CM-5 chip). Talin-VBS1, -VBS2, and -VBS3 peptides were injected over the reference cell and vinculin-immobilized cells in sequence, and the release of the bound VBS peptides were determined. Results for parallel injections of each sample over the reference cell (CM-5 chip). Talin-VBS1, -VBS2, and -VBS3 peptides were injected over the reference cell and the reference cell was incubated with Vt (residues 879–1,066) protein (at a 1:2 molar ratio, to assure complete association of Vt with Vt; the resulting Vt-Vt complex detected in native gels (lane 2) was confirmed to contain the ~90 and 30-kDa tail domains by SDS-PAGE analysis (data not shown) and was distinguishable from free Vt (lane 1). Note that free Vt is not detectable on native gels because of its high pl. αVBS (A and B) or talin-VBS3 (C and D) peptides were then titrated into the VH-Vt complex and allowed to incubate for 20 min. Even relatively low molar ratios of these VBSs were effective at displacing Vt to form the indicated VH-VBS complexes, which were distinct from those of the VH-Vt complex or free Vt on native gels. Again, the identity of the VH-VBS complexes were confirmed by SDS-PAGE analyses (data not shown). B and D, quantitation of the dissociation of the VH-Vt complex (solid lines) and the formation of the VH-αVBS (B) and VH-talin-VBS3 (D) complexes (dotted lines) are also shown.

RESULTS

The VBSs of Talin Have High Affinity for Full-length Vinculin—The binding affinity of αVBS for full-length vinculin is similar to that for the Vh1 domain alone ($K_d$ of both are ~2 nM; Ref. 40). The affinity of talin-VBS3 for the Vh1 domain (residues 1–258) was in the same range ($K_d$ of 3 nM) and is greater than that of talin-VBS1 ($K_d$ of ~15 nM) or talin-VBS2 ($K_d$ of 32 nM) for Vh1 (35). However, additional interdomain contacts of Vt with Vt2 have been shown to compromise the binding of the entire rod domain of talin (residues 397–2,541; Ref. 18). To define the affinity of the VBSs of talin for full-length vinculin, we performed SPR binding assays for talin-VBS1, -VBS2, and -VBS3 to full-length human vinculin protein immobilized on a Biacore chip. Similar to its lower affinity interaction for the isolated Vh1 domain (35), talin-VBS2 had a relatively low affinity for full-length vinculin (Fig. 1, C and D).

To visualize the actin cytoskeleton and the localization of cytoskeletal proteins, Swiss-3T3 cells were plated and microinjected on Permanox lab-tek chamber slides (Nalge Nunc Corp., Naperville, IL). Immediately after injection, the cells were fixed for 10 min in 4% paraformaldehyde 1 × phosphate-buffered saline. The cells were then permeabilized for 10 min with 0.1% Triton X-100 in phosphate-buffered saline and were then blocked with 3% milk in phosphate-buffered saline prior to applying the primary antibodies. Actin filaments were identified by staining the cells with TRITC-coupled phalloidin (Sigma). Focal adhesion complexes were identified by staining cells with antibodies that detect mouse vinculin, talin, and α-actinin (all from Sigma).
of talin for full-length vinculin were lower than that for the isolated Vh1 domain (24) additional intramolecular interactions within the full-length molecule do impair talin-VBS binding to the Vh1 domain. However, their affinities for full-length vinculin are still quite high, and in the physiological range expected to effectively compete with the intramolecular interactions of the head and tail domains of vinculin.

The VBSs of Talin and α-Actinin Displace the Head-Tail Interactions of Vinculin—All of the VBSs of talin and α-Actinin bind to the Vh1 domain of vinculin in a mutually exclusive fashion, by inserting between helices α1 and α2 of the N-terminal helical bundle of the Vh1 domain of vinculin, and all are capable of displacing Vt from pre-existing Vh1-Vt complexes (24, 35). The high affinity of the VBSs of talin and α-Actinin for full-length vinculin in SPR binding assays (Fig. 1 and Ref. 40) suggested that the same would hold true in solution. However, others have shown that the interactions of Vt with both the Vh1 and Vt2 domains compromises binding of the entire talin rod to full-length vinculin in solution (18). To test whether talin-VBS3 and αVBS were sufficient to displace Vt from complexes formed between the entire head domain of vinculin (VH, residues 1–840) with Vt, recombinant VH and Vt proteins were incubated together (at a 1:2 molar ratio of VH:Vt); native gel

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FIGURE 3. The binding of the VBSs of talin and α-actinin induces unique conformational changes in full-length vinculin. The $^1$H,$^15$N TROSY spectra of 98% deuterated $^15$N-labeled vinculin when in complex with a 1.4-fold molar excess of talin-VBS3 (A, blue), vinculin alone (B, black), or vinculin in complex with αVBS (C, red). Subtraction of the $^1$H,$^15$N TROSY spectra of uniformly $^15$N-labeled vinculin-VBS3 complex (D) and of the vinculin-αVBS complex (E) (both in red) from the $^15$N-labeled vinculin (black) highlights the unique conformations that vinculin adopts when bound by these VBSs.
analyses established the rapid and complete formation of the VH-Vt complex, which was distinguishable from free VH on these gels (Fig. 2, A, lane 2 versus lane 1, and C, lane 1). To then test whether the VBSs of α-actinin and talin were sufficient to disrupt Vt from this complex, we titrated these VBS peptides into the head-tail complex. Even relatively low molar ratios of αVBS or talin-VBS3 were sufficient to displace Vt and to form novel VH-VBS complexes (Fig. 2, A–D). Similar findings were evident when testing the ability of talin-VBS1 to disrupt Vt from the VH-Vt complex (data not shown). Further, consistent with its higher affinity by SPR assays (40), αVBS was more effective at displacing Vt from the VH-Vt complex than talin-VBS3 or talin-VBS1 (Fig. 2, and data not shown). Therefore, in solution, the VBSs of α-actinin or talin are sufficient to disrupt the intramolecular head-tail interactions of vinculin, the initiating event of vinculin activation.

The VBSs of Talin and α-Actinin Alter the Conformation of Full-length Vinculin—The binding of the VBSs of talin or α-actinin to full-length vinculin alters its sensitivity to proteases, suggesting that these VBSs open up regions that are buried in inactive vinculin (40). To directly assess the effects of the VBSs of talin and α-actinin on the overall conformation of vinculin, we performed 1H,15N TROSY NMR spectroscopy of full-length vinculin (Fig. 3B) and of vinculin when bound by talin-VBS3 (Fig. 3A) or αVBS (Fig. 3C), at a molar ratio of 1:4:1, VBS:vinculin. Lack of the full NMR resonance assignment of the very large vinculin protein precludes any residue by residue-based interpretation of the data, allowing us to assess only the global changes in the spectra. Nonetheless, superposition of the NMR data revealed significant shift changes and the appearance of numerous new cross-peaks in talin-VBS3- and αVBS-bound [1H,15N]vinculin versus unbound [1H,15N]vinculin (Fig. 3, A–C). This implies that vinculin undergoes significant changes in its overall conformation when bound to these VBSs. The extent of the changes upon binding was especially evident in the superpositions of αVBS- versus talin-VBS3-bound full-length [1H,15N]vinculin, which suggests that talin-VBS3 and αVBS also provoke at least some unique conformational changes in the overall structure of vinculin (Fig. 3, D and E). Therefore, the VBSs of α-actinin or talin are sufficient to disrupt intramolecular interactions that hold vinculin in its inactive state, and appear to do so by provoking novel changes in the overall conformation of full-length vinculin.

The VBSs of Talin and α-Actinin Promote Binding of Vinculin to F-actin—When vinculin is activated it becomes competent to bind to F-actin, which requires severing of the head-tail interactions of vinculin (21, 23). The ability of αVBS and talin-VBS3 to provoke significant changes in the structure of vinculin, and to disrupt the VH-Vt interactions, suggested that binding of these VBSs might also facilitate the binding of vinculin to F-actin. To test this hypothesis, αVBS and talin-VBS3 were incubated with full-length vinculin (at molar ratios ranging from 1:1 to 20:1, VBS:vinculin), and these complexes were then incubated with F-actin. As expected, full-length vinculin bound poorly to F-actin (Fig. 4). By contrast, a large proportion of αVBS- or talin-VBS3-bound vinculin was capable of binding to and co-sedimenting with F-actin, such that as much as 55% (αVBS-bound, Fig. 4A) or 45% (talin-VBS3-bound, Fig. 4B) of vinculin became competent to bind to F-actin. Again, the increased F-actin binding potential of αVBS-bound vinculin correlates with its higher affinity for full-length vinculin versus talin-VBS3. Regardless, these findings establish that the VBSs of α-actinin and talin are indeed sufficient to activate the latent F-actin binding potential of vinculin, a hallmark of vinculin activation in adhesion complexes.

The VBSs of Talin and α-Actinin Selectively Compromise the Contacts of Vinculin at Sites of Focal Adhesions—The ability of the VBSs of talin and α-actinin to sever the head-tail interactions of vinculin suggested that these VBSs would also bind to vinculin in cells and would thus disrupt its ability to link to talin and α-actinin at sites of focal adhesions. To test this notion, Swiss-3T3 cells were microinjected with ~1,000 molecules of talin-VBS3 or αVBS peptides per cell and were then assessed for changes in the localization of endogenous vinculin, talin, and α-actinin, which concentrate at sites of focal adhesions, and for changes in the actin cytoskeleton by staining with phalloidin. As
expected, in control microinjected Swiss-3T3 cells there were pronounced actin stress fibers and intense staining of vinculin, talin, and α-actinin at sites of focal adhesions (Fig. 5). By contrast, in cells microinjected with talin-VBS3 or with αVBS peptides, vinculin, talin, and α-actinin were rapidly relocalized to the cytosol or were diffusely distributed to the margins of cell membranes and were no longer associated with focal adhesions (Fig. 5). Further, VBS-microinjected cells essentially lost nearly all of their focal adhesions, and there was also depolymerization and a collapse of the actin cytoskeleton (Fig. 5), consistent with the role of vinculin in stabilizing cell adhesion contacts (5, 6). As a net result there were large reductions in the overall volume and size of VBS-microinjected cells (Fig. 5). Collectively, these data suggest that these VBS peptides can function as efficient sinks for vinculin in cells, disrupting the associations of vinculin with talin and α-actinin and then with the actin cytoskeleton.

The profound effects of the VBSs of talin and α-actinin on the actin cytoskeleton of Swiss-3T3 cells suggested that they should rapidly compromise cell-matrix contacts. As expected, video microscopy demonstrated that control fibroblasts lacked any noticeable changes in their shape or attachments following microinjection (Fig. 6, top panels, and supplemental data, movie 1). By contrast, there was a rapid loss of focal adhesions and a marked retraction and reduction in cell size and volume in αVBS-microinjected fibroblasts (Fig. 6, middle panels, and supplemental data, movies 2 and 3) and in talin-VBS3-microinjected cells (Fig. 6, bottom panels, and supplemental data, movies 4 and 5). Again, microinjection of as little as ~1,000 molecules of VBS peptides/cell was sufficient to induce collapse of the cytoskeleton, suggesting efficient disruption of nearly all focal contacts. With time these rather catastrophic events lead to the deaths of all talin-VBS3- or αVBS-microinjected cells, whereas control-microinjected cells remained viable and continued to proliferate (data not shown). Therefore, disruption of the contacts of vinculin and subsequent collapse of the actin cytoskeleton triggers fibroblast cell death.

Collectively, these findings suggested that αVBS and talin-VBS3 peptides target vinculin and disrupt its functions at sites of focal adhesions. However, to prove this was not because of off-target effects, we evaluated the consequences of microinjection of the VBSs of talin and α-actinin into isogenic vinculin−/− and vinculin+/+ cells (7). Vinculin-deficient cells have similar numbers of focal contacts but are less spread and less adhesive than their wild type counterparts (7). As expected, microinjection of talin-VBS3 or αVBS peptides provoked profound cell retraction, a reduction in cell size and volume, and loss of focal adhesions in vinculin−/− cells (Fig. 7A, and supplemental data, movies 6 and 7), and ultimately these cells died (data not shown). By contrast, microinjection of talin-VBS3 or αVBS peptides failed to induce cell retraction or affect the size of vinculin+/+ cells (Fig. 7B, and supplemental data, movies 9 and 10), and indeed these cells remained viable and continued to proliferate (data not shown). Therefore, the VBSs of talin and α-actinin selectively target vinculin functions in adhesion complexes.
Other, their ability to disrupt focal adhesions and sever contacts with the actin cytoskeleton abolishes the tensive forces that anchor the cell to the extracellular matrix, which are required for cell survival.

**DISCUSSION**

Vinculin binding to talin or α-actinin provides essential links for adhesion receptors with the actin cytoskeleton and, as underscored by the studies presented here, vinculin plays essential roles in stabilizing adhesion complexes. The binding of PIP2 was thought to activate vinculin to allow it to bind to its other partners, by altering the conformation of vinculin head-tail interactions of vinculin (21, 25, 26). However, PIP2 impairs the associations of vinculin with actin (29), and the structures of inactive vinculin revealed that the PIP2 binding site is occluded in its inactive state (12, 15). Furthermore, mutants of vinculin defective in PIP2 binding have no defects in recruitment to focal adhesions (28). Thus, it follows that other regulators must activate vinculin, and the studies presented here suggest that the VBSs of talin and α-actinin fulfill this role.

Several lines of evidence support a direct role for talin and α-actinin in activating vinculin. First, both talin and α-actinin avidly interact with vinculin at sites of cell adhesions (49, 51, 52). Further, both talin and α-actinin harbor VBSs within their central rod domains that bind to full-length vinculin with affinities that are higher than (αVBS, 2 nM; Ref. 40) or are comparable to (talin-VBS1 and -VBS3, ~70 nM; Fig. 1) those of the vinculin head-tail interaction (~50–90 nM; Refs. 17, 18). In accord with these findings, the crystal structures of inactive human vinculin (12) and those of the Vh1-talin-VBS and Vh1-αVBS complexes (24, 35, 36, 40, 41) revealed that the binding site in the Vh1 domain of vinculin that interacts with the VBSs talin and α-actinin is not "cryptic" nor occluded by the head-tail interaction; rather it is readily accessible to bind to these VBSs. Finally, these VBSs fulfill all the criteria one would expect for triggers that activate vinculin as they 1) efficiently displace the head-tail interactions of vinculin (Fig. 2), 2) induce significant alterations in the conformation of vinculin (Fig. 3 and Ref. 40), 3) promote vinculin binding to F-actin (Fig. 4), and 4) specifically target vinculin in cells and have profound effects on focal adhesions and the actin cytoskeleton (Figs. 5–7).

The binding affinities of talin and α-actinin for vinculin in their native, inactive states is low (15, 18, 20), and this has suggested a combinatorial model of vinculin activation, where two or more ligands are
needed to sever the head–tail interaction (15, 18, 53). Indeed, the hydrophobic faces of the VBSs that insert between the α1 and α2 helices of the Vh1 domain of vinculin are buried in the cores of the helical bundles present in the rod domains of talin and α-actinin (40, 41, 43). Thus, we predict that structural changes in these bundles occur following the interactions of talin or α-actinin with integrin receptors and that these alterations would include an unfolding of the bundles to release the VBSs to allow binding to vinculin (Fig. 8). Indeed, the α-helices within the three-helical bundles of spectrin repeats that are found in α-actinin are known to unfurl and form stable intermediates following their exposure to mechanical stress (44). We speculate that a similar scenario also applies to the helical bundles of the rod domain of talin when they are exposed to mechanical stress, which is sufficient to induce the formation of focal adhesions, and to recruit vinculin, within seconds of the applied stress (45, 46). Such models would then allow for rapid, high-affinity binding of these VBSs to vinculin, and their severing of the head–tail interactions of vinculin would then allow vinculin to bind to its other partners, in particular F-actin (Fig. 8). Further, such talin-vinculin and α-actinin-vinculin interactions appear required to stabilize focal adhesions, as displacement of their interactions by the free VBSs of talin or α-actinin leads to catastrophic effects on the actin cytoskeleton and rapid cell retraction (Figs. 5–7).

The notion that the VBSs of talin and α-actinin are sufficient to trigger vinculin activation appears, at first glance, to contradict the studies of others that have shown that the talin rod has a low affinity for full-length vinculin (15) and that the Vh1-talin-rod complex is effectively displaced by the Vt domain (18). Our studies also challenge the notion that vinculin activation, and specifically its ability to bind to F-actin, requires two or more ligands to efficiently disrupt its head–tail interactions (15, 53), which has been proposed to include both Vh1-Vt and Vt2-Vt interdomain contacts (18). However, these studies evaluated the interaction of the entire native talin rod with vinculin, with the bias that the structure of the rod of talin changes very little in its native versus vinculin-bound state. The crystal structures of the native helical bundles of talin (41, 43) and of α-actinin (42), versus those of the VBS-vinculin complexes (24, 35, 36, 40, 41), have revealed that this is not the case, where the VBSs of talin and α-actinin must unravel from their buried locations to bind to vinculin. Given that at least talin avidly interacts with vinculin in cells (52), we therefore propose that structural alterations of the VBSs of talin and α-actinin are initial and necessary events that allow these α-helices to swing out to bind to and activate vinculin (Fig. 8). We recognize that the ability of the VBS peptides to efficiently disrupt the contacts of vinculin with talin and α-actinin in cells (Fig. 5) demonstrates that their affinity is higher than that of endogenous talin- and α-actinin-VBS-vinculin interactions in cells. Nonetheless, the fact that this displacement occurs establishes central roles for these VBSs in mediating these interactions and in regulating actin dynamics in cells.

In cells, vinculin associates with actin at sites of focal adhesions or adherens junctions (54, 55). In its native state, the affinity of vinculin for actin is rather low (∼1 μM; Ref. 23), and others have suggested that talin-VBS3 (34) and pVR, a VBS of unknown significance (29), are not sufficient to activate the latent actin-binding potential of vinculin. However, these studies were performed by first incubating G-actin with vinculin and then testing the effects of talin-VBS3 or pVR, whereas ours tested the ability of talin-VBS3- or αVBS-bound vinculin to then bind to F-actin. We believe the latter schema more accurately recapitulates the chain of events in cells in vivo, and our studies clearly indicate that these VBSs can indeed activate the latent actin binding potential of vinculin. Evaluating the affinities of talin-vinculin-actin and α-actinin-vinculin-actin interactions at sites of focal adhesions in cells, for example by fluorescence resonance energy transfer, should resolve the order and affinities of these interactions.

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Note Added in Proof—Our model suggests that the VBSs present in the rod domains of α-actinin and talin would swing out from their buried locations in helical bundle domains following the receipt of signals that unravel these bundles. In accord with our model, Patel et al. (Patel, B. C., Gingras, A. R., Bobkov, A. A., Fujimoto, L. M., Zhang, M., Liddington, R. C., Mazzeo, D., Emsley, J., Roberts, G. C. K., Barsukov, I. L., and Critchley, D. R. (January 8, 2006) J. Biol. Chem. 10.1074/jbc.M508058200) have recently shown that one of the helical bundle domains of talin is inherently unstable and that at least one of the VBSs in this helical bundle domain confers much higher affinity for the N-terminal helical bundle domain of vinculin. We propose that such a VBS may provide initial contacts with vinculin and elicit the helical bundle conversion events that sever the head–tail interactions of vinculin and that lead to vinculin activation.

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