Structural Basis for Amplifying Vinculin Activation by Talin*

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Talin interactions with vinculin are essential for focal adhesions. Curiously, talin contains three noncontiguous vinculin binding sites (VBS) that can bind individually to the vinculin head (Vh) domain. Here we report the crystal structure of the human Vh/VBS1 complex, a validated model of the Vh-VBS2 structure, and biochemical studies that demonstrate that all of talin VBSs activate vinculin by provoking helical bundle conversion of the Vh domain, which displaces the vinculin tail (Vt) domain. Thus, helical bundle conversion is a structurally conserved response in talin-vinculin interactions. Furthermore, talin VBSs bind to Vh in a mutually exclusive manner but do differ in their affinity for Vh and in their ability to displace Vt, suggesting that the strengths of these interactions could lead to differences in signaling outcome. These findings support a model in which talin binds to and activates multiple vinculin molecules to provoke rapid reorganization of the actin cytoskeleton.

Talin and vinculin are essential components and regulators of cell-matrix (focal adhesion) complexes where they direct the assembly and reorganization of the actin cytoskeleton (1). Both talin and vinculin are critical for cell growth, morphogenesis, and cell migration during the differentiation and organization of tissues, and loss of either talin or vinculin in mice results in marked defects in focal adhesions and embryonic lethality (2, 3). Furthermore, both talin and vinculin play important roles in pathophysiological scenarios, including wound healing, ischemia, apoptosis, and metastasis of cancer cells (4–7).

Focal adhesion signaling is initiated after the specific interactions of components of the extracellular matrix with the ectodomains of various integrin receptors, which are unique in their ability to direct both outside-in and inside-out signaling (8–10). Connections to the actin cytoskeleton are mediated in part by activation of focal adhesion kinase, which phosphorylates downstream substrates on tyrosine residues and which directly associates with paxillin, which is linked to the actin cytoskeleton via its interactions with vinculin (for reviews, see Refs. 11 and 12). In addition, there are direct interactions of the cytoplasmic domains of integrin receptors with components of the cytoskeleton. For example, an NPXY/F variant of the canonical phosphotyrosine binding domain of integrin receptors mediates its interaction with the FERM (four point one, ezrin, radixin, and moesin) motif present in the head domain of talin (13, 14). In turn, talin binds to vinculin through vinculin binding sites (VBSs) present in its central rod domain, and this interaction contributes to reorganization of the actin cytoskeleton (15–17).

Until recently it was thought that talin largely played a passive role in focal adhesions, as a scaffold protein that simply bridged integrin receptors to the F-actin cytoskeleton via its association with other actin-binding proteins, in particular vinculin (18). However, binding of the talin FERM domain to the cytoplasmic tails of integrin receptors activates focal adhesion signaling pathways (13, 14). For inside-out signaling this occurs through FERM-induced conformational changes in the receptor chains, which increase their affinities for ligands (19). Furthermore, the talin FERM domain can also interact with and activate phosphatidylinositol 4,5-bisphosphate kinase-1 (20–22), a signaling effector that generates the second messenger phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2). Finally, the VBSs of talin have been shown to facilitate the in vitro binding of vinculin to F-actin (23).

Vinculin contains a highly conserved head (Vh) domain (residues 1–258) that interacts, in an intramolecular fashion, with its tail (Vt) domain (residues 879–1066; 24–26). This interaction is thought to hold vinculin in an inactive conformation because biochemical studies have demonstrated that binding of Vh with Vt masks cryptic binding sites for talin, α-actinin, and α-catenin to Vh, and for F-actin to Vt (25, 27–29). Thus, vinculin activation is generally agreed to involve structural changes that sever the Vh-Vt interaction, yet the signals that disrupt these intramolecular contacts are still unresolved. The conventional model has suggested that these constraints are simply relieved by the binding of PI(4,5)P2 to the Vt domain (25), which can insert into acidic phospholipid bilayers (30). Indeed, binding of PI(4,5)P2 changes the conformation of Vt, and this facilitates the in vitro interaction of Vt with F-actin (31) and, by disrupting the Vh-Vt interaction, has been suggested to allow talin binding to Vh (25, 31). However, other models are now equally plausible because talin VBS3 and the vinculin binding site of α-actinin can displace Vt from preexisting Vh-Vt complexes (32). Furthermore, the crystal structure of the Vh/VBS3 complex established that talin VBS3 distorts the binding site for Vt in Vh from a distance, by provoking dramatic alterations in the structure and positions of the α-helices of the N-terminal

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¶ The abbreviations used are: FERM, four point one, ezrin, radixin, and moesin motif; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; GST, glutathione S-transferase; PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate; SPR, surface plasmon resonance; VBS(s), vinculin binding site(s); VBS2e, VBS2-enhanced; Vt, vinculin tail domain (residues 1–258); Vt, vinculin tail domain (residues 879–1066).
helical bundle of Vh, by a process coined as helical bundle conversion (32).

A long-standing mystery in focal adhesion signaling is how the structural proteins of the actin cytoskeleton undergo such rapid changes in their localization and organization. One possible mediator is talin, which harbors three separate high affinity vinculin binding sites (VBS1, VBS2, and VBS3) that are located in its central rod domain (residues 607–636 (VBS1), 852–879 (VBS2), and 1944–1969 (VBS3); see Refs. 15, 17, and 23). Each of the talin VBSs have been predicted to form an amphipathic α-helix (17), and larger portions of talin containing these VBSs can bind individually to Vh (17) and act as competitive inhibitors that prevent the binding of Vt to Vh (23). However, it was not known whether these interactions were indeed mediated by the refined sequences of talin VBSs. We reasoned that if all of the more highly defined VBSs of talin indeed bound to Vh in a mutually exclusive manner and each was individually capable of activating vinculin, this would allow one molecule of talin to activate three molecules of vinculin effectively, a scenario that would amplifying integrin signaling and promote rapid changes in the actin cytoskeleton. To test this hypothesis we assessed the ability of talin VBS1 and VBS2 to displace Vt from preexisting Vh/Vt complexes and solved the crystal structure of the Vh/VBS1 complex to 2.4 Å resolution.

**EXPERIMENTAL PROCEDURES**

Vh/VBS1 Crystallization and X-ray Data Collection—The His6-tagged human Vh (residues 1–258) and Vt (residues 879–1066) expression constructs and the purification of Vh and Vt proteins have been described previously (32). Native Vh/VBS1 crystals were obtained from conditions similar to those for Vh/VBS3 crystals (32). These crystals belong to space group R32 with one heterodimer in the asymmetric unit, a solvent content of 59%, and a volume to mass ratio of 3.04 Å³/Da. The Vh/VBS1 crystals were cryoprotected in paratone oil. Vh/VBS1 x-ray data were collected at the Advanced Photon Source, SBC-CAT ID beamline. The Rmerge to 2.42 Å
was 0.179 (0.485 in the 2.51–2.42 Å shell) with a completeness of 97.6% (80.8%), a redundancy of 18, and an average $F^2/|F|^2$ of 8.3 (3 in the last shell; 33).

**Vh-VBS1 Structure Determination and Refinement**—The human Vh-VBS1 crystal structure was solved by molecular replacement using the Vh-VBS3 structure (32) as a search model in the program AmoRe (34). The VBS3 peptide was omitted from the model. An electron density map based on the phases from the molecular replacement solution immediately showed the position of the entire backbone and side chains of the C-terminal helical bundle of Vh but gave poor initial definition of the N-terminal helical bundle of Vh. The helices in this bundle were first placed manually and refined as rigid bodies and then improved by extensive rebuilding with the O interactive graphics program (35). The final stages were the interactive model building of the VBS1 peptide, and of the $\alpha_1-\alpha_2$ loop, where the electron density was weaker in the 2.7 Å Vh-VBS3 structure (32). The calculation of $\sigma^2$-weighted $2F_\alpha - F_\sigma$ maps and the restrained refinement of positions and $B$-factors were carried out with BUSTER/TNT (36, 37). Subsequent positional and
B-factor refinement with BUSTER/TNT and interactive model building with O (35) allowed further improvements in the model. A Ramachandran plot analysis by the program PROCHECK (38) indicates that 95.7% of all of the residues lie in the most favorable region, that the remaining 4.3% lie in the additional allowed regions, and that there are no residues in the generously allowed or disallowed regions. This structure analysis also showed that all stereochemical parameters are better than expected at the given resolution (see Table II).

Talin VBS Binding Assays—0.26 nM Vh and 0.52 nM Vt were incubated for 20 min at room temperature to form the Vh-Vt complex. Talin VBS1, VBS2, and VBS2e peptides were then titrated into the sample at molar ratios ranging from 0 to 5:1 and allowed to incubate for 20 min. Complexes were then analyzed by native PAGE.

Binding of Talin VBS1, VBS2, VBS2e, and VBS3 to Vh—Binding studies were performed on a Biacore 3000 surface plasmon resonance (SPR) instrument. Vh was covalently attached to a carboxymethyl-dextran coated gold surface (CM-5 chip, Biacore). The carboxymethyl groups on the chip were activated with EDC and N-hydroxysuccinimide to form the N-hydroxysuccinimide ester of carboxymethyl-dextran. Vh was attached at pH 5.0 to this activated surface by reaction of the carboxyl groups of dextran with the primary amines of Vh to form an amide linkage. Any remaining reactive sites on the surface were blocked by reaction with ethanolamine. A reference cell was prepared similarly except that no Vh was added. Binding was measured by flowing the VBS peptides in 20 mM HEPES, 0.1 mM EGTA, 0.1 mM EDTA, 0.5 mM dithiothreitol at a flow rate of 20 μl/min through the reference and Vh-containing flow cells in sequence. A blank was also run consisting of only buffer. After the injection, release of the bound VBS peptides was measured by flowing only buffer through the flow cells. Regeneration of the chip surface to remove bound VBS peptides was accomplished by injecting 20 μl of 10 mM glycine, pH 1.5, through the flow cells followed by a 7-min equilibration in buffer. Data reported are the difference in SPR signal between the flow cell containing Vh and the reference cell. Any contribution to the signal was removed by subtraction of the blank (buffer) injection from the reference-subtracted signal.

RESULTS

Structure of the Human Vh-VBS1 Complex—The structure of the Vh domain in its inactive conformation when complexed with Vt defined a two four-helical bundle structure linked by a long shared α-helix (α4) that is remarkably similar to the structure of the β-catenin-α-catenin complex (39). Strikingly, the structure of the Vh-VBS3 complex revealed that VBS3 binding provoked remarkable changes in the N-terminal helical bundle of Vh (helices α1–α4), creating an entirely new five-helical bundle structure; hence the idiomy, helical bundle conversion. In contrast, the structure of the C-terminal helical bundle of Vh (helices α4–α7) is essentially unchanged by binding of VBS3, suggesting that the C-terminal helical bundle of Vh functions as a rigid scaffold that allows the N-terminal helical bundle to bind to its various partners (32).

Talin is unique among the vinculin-binding partners because...
it harbors, curiously, three noncontiguous VBSs, VBS1 (residues 607–636), VBS2 (residues 852–879), and VBS3 (1944–1969; Refs 15, 17, and 23). Based upon their \( \alpha \)-helical amphipathic nature (17) and in part from competition studies using larger portions of talin harboring VBS1 (residues 498–636; Ref. 23), we reasoned that VBS1 and VBS3 would have similar or overlapping binding sites in Vh and would share in their ability to trigger structural changes in the N-terminal helical bundle of Vh. As expected from the Vh-VBS1 structure (32) and binding studies with VBS1 (498–636) (23), in the Vh-VBS1 crystal structure one molecule of VBS1 binds to one molecule of Vh (Fig. 1A). Superimposition of the Vh-VBS1 structure with that of the inactive Vh-Vt complex established that VBS1 is very similar to VBS3 in its ability to activate vinculin. Specifically, insertion of the amphipathic \( \alpha \)-helix of talin VBS1 between Vh helices \( \alpha 1 \) and \( \alpha 2 \) generates a totally new five-helical N-terminal bundle structure and stretches the loop linking Vh helices \( \alpha 1 \) and \( \alpha 2 \) by unwinding the first two \( \alpha \)-helical turns of \( \alpha 1 \) which are present in the Vh-Vt structure (Fig. 1B). As in the Vh-VBS3 structure (32), all VBS1 interactions are restricted to the N-terminal helical bundle of Vh, with little effect on the structure of the C-terminal helical bundle (Fig. 1, A and B). Thus, binding of either talin VBS1 or VBS3 has dramatic effects on the structure of the N-terminal helical bundle of Vh, yet leaves the structure of its C-terminal helical bundle essentially unchanged.

The shared similarities in the structures of the Vh-VBS1 and Vh-VBS3 complexes were striking (Fig. 2), especially because talin VBS1 and VBS3 share only 16% identity and 46% similarity (Fig. 3A and Ref. 17). This similarity in structure also occurs even though VBS3 has three additional ordered residues that add an extra turn to its N terminus and that VBS1 has six additional ordered residues on its C terminus which bend away at \( \sim 40^\circ \) from its position in the Vh-VBS3 structure. Nonetheless, the binding of VBS1 to Vh is similar to that of VBS3, where Van der Waals interactions between the hydrophobic face of VBS1 with the hydrophobic core of the N-terminal helical bundle of Vh (Fig. 1C and Table I) provoke dramatic changes in the structures of Vh helices \( \alpha 1, \alpha 2, \) and \( \alpha 4 \) (Fig. 1B). Therefore, like talin VBS3, talin VBS1 is remarkable in its ability to provoke helical bundle conversion of the N-terminal bundle of Vh. Further, the residues of Vh interacting with VBS1, and the position of the VBS1 amphipathic \( \alpha \)-helix between Vh helices \( \alpha 1 \) and \( \alpha 2 \), indicate that talin VBS1 and VBS3 must bind to the N-terminal helical bundle of Vh in a mutually exclusive fashion.

**Helical Bundle Conversion of Vh by Talin Is a Conserved Response**—The structure of the Vh-VBS1 (Table II) complex was similar in its overall architecture to that of the Vh-VBS3 complex. Superimposition of the Vh-VBS1 and Vh-VBS3 structures demonstrated that, despite considerable differences in their sequences, VBS1 and VBS3 fit into a similar cavity created by their insertion between Vh helices \( \alpha 1 \) and \( \alpha 2 \) (Fig. 2). The \( \alpha \)-carbon positions of VBS1-bound Vh (residues 3–18, 48–209, and 230–248) can be superimposed onto the 197 equivalent \( \alpha \)-carbon positions of VBS3-bound Vh with root mean square deviation of 1.06 Å. The largest differences in the structures of the N-terminal helical bundle of the two complexes were in the loop between Vh helices \( \alpha 1 \) and \( \alpha 2 \). This shift begins in the vicinity of Vh residue 19 and extends through to residue 47, with movements of more than 6 Å at residue 34 being accompanied by a rotation of the C terminus of helix \( \alpha 1 \) by \( \sim 15^\circ \) (Fig. 2).

As in the Vh-VBS3 complex (32) the interactions of Vh with talin VBS1 are largely hydrophobic in nature, with 24 residues of Vh participating in hydrophobic interactions with 16 residues of VBS1, and Gln\(^{19} \) of Vh is within hydrogen bonding distance to Ser\(^{20} \) of VBS1 (Table I). VBS1 buries 53% (more than 1,200 Å\(^2 \)) of its entire solvent-accessible surface area upon binding to Vh. The binding of the N-terminal half of either VBS1 or VBS3 to the N-terminal portion of Vh helix \( \alpha 1 \) and to the C-terminal half of Vh helix \( \alpha 2 \) is nearly identical. However, the C terminus of each talin peptide distorts the C-terminal half of \( \alpha 1 \), the N-terminal half of \( \alpha 2 \), and the connecting loop in quite different ways (Fig. 2). Nonetheless, the striking structural alterations leading to helical bundle conversion in Vh provoked by the binding of VBS1 or VBS3, and the architecture of the binding cavity for these VBSs, are remarkably similar in the two complexes; therefore, the binding of VBS1 and VBS3 results in a similar and profound outcome in vinculin structure.

**Vh-VBS2 Model**—Human talin VBS2 (residues 852–879) has 38% identity and 57% similarity with VBS1 and 15% identity and 46% similarity with VBS3 (Fig. 3A and Ref. 17). Based on the sequence alignment and the Vh-VBS1 crystal structure, we modeled the interaction of talin VBS2 (Fig. 3B) with Vh. The Vh-VBS2 model moved only slightly after 200 cycles of coordinate energy minimization, as the 279 \( \alpha \)-carbon positions of the Vh-VBS2 starting model can be superimposed onto those of the energy-minimized Vh-VBS2 model with a root mean square deviation of 0.367 Å. Again, the modeled structure of the Vh-VBS2 complex predicts little change in the structure of the C-terminal helical bundle of Vh as seen in its inactive state. However, VBS2 binding is predicted to cause alterations in Vh helices \( \alpha 1, \alpha 2, \) and \( \alpha 4 \) which lead to helical bundle conversion. The side chains of Vh that are predicted to contribute to the binding of all three VBSs are conserved, and the minimal binding site includes several residues of Vh helices \( \alpha 1 \) and \( \alpha 2 \) and the loop connecting \( \alpha 1-\alpha 2 \), and some residues on Vh helix \( \alpha 4 \) (Table I).

Finally, the architecture of the modeled VBS2 structure (Fig. 3C) is remarkably similar to the structure of VBS1 (Fig. 3D) when bound to Vh. Notable features include a string of hydrophobic residues on the face of VBSs which interact with Vh (left images in Fig. 3, C and D) and a conservation of polar residues along the face of these amphipathic \( \alpha \)-helices which is exposed to the solvent (right images in Fig. 3, C and D).

**Binding Affinities of Talin VBSs for Vh**—Chicken talin VBS3 has been reported to bind Vh with fairly high affinity (\( K_d \) of 39 nm; Ref. 23). However, these experiments were performed using a larger domain, talin residues 1943–2157, and the Vh used in these assays was a glutathione S-transferase fusion protein (23). Furthermore, the binding constants for talin VBS1 and VBS2 are unknown. Given the surface areas of talin VBSs buried in the Vh-VBS structures we reasoned that the affinities of the refined talin VBSs would be significantly higher than appreciated previously and that this should correlate with their degree of interaction with Vh helices \( \alpha 1, \alpha 2, \) and \( \alpha 4 \). We therefore determined the binding affinities of talin VBS1 (residues 607–636), VBS2 (residues 852–879), and VBS3 (residues 1944–1969) for Vh using Biacore 3000 SPR. Under these conditions, binding of VBS3 in the fit model was determined with an estimated \( K_d \) of \( \sim 3.1 \) nm. In agreement with their actual or modeled structures in the binding cleft of Vh, the affinities of talin VBS1 and VBS2 were somewhat reduced relative to that of VBS3, with estimated \( K_d \) for VBS1 and VBS2 of \( \sim 14.7 \) and \( 32.8 \) nm, respectively (Fig. 4).

**Talin VBS1 and VBS2 Displace Vt from Vh-Vt Complexes**—The similar talin VBSs in Vh and the ability of talin VBS1 to provoke helical bundle conversion in Vh suggested that VBS1 and VBS2 would also distort the binding site for Vt in Vh from a distance. We therefore tested whether VBS1 and VBS2 could displace Vt from preexisting Vh-Vt complexes. Free Vh and complexes containing Vh bound to Vt, VBS1, VBS2, or VBS3...
were easily distinguishable on native gels on the basis of their migration (Fig. 5A). Vh-Vt complexes were formed at a molar ratio of 2 mol of Vt to 1 mol of Vh, and these complexes were then incubated with increasing concentrations of talin VBS1 or VBS2 which reduce its ability to displace Vt from Vh-Vt complexes. VBS1 peptide began to displace Vt from preexisting Vh-Vt complexes even at ratios as low as 1:20, and Vt was displaced from all Vh complexes at a 1:2 ratio of VBS1 to Vt (Fig. 5, B and C). VBS2 was also capable of displacing Vt from preexisting Vh-Vt complexes, but much higher levels of VBS2 were necessary to see these effects, and Vt was not totally displaced from Vh even at a 2.5:1 ratio of VBS2 to Vt (Fig. 5, D and E). Thus, although both VBS1 and VBS2 are capable of displacing Vt from Vh-Vt complexes, VBS1 was clearly more effective at displacing Vt.

Our modeled Vh-VBS2 structure suggests an explanation for the reduced ability of VBS2 to displace Vt versus VBS1 (Fig. 5) and VBS3 (32). First, even when considering equivalent residues (VBS1 607–626 versus VBS2 855–874) as modeled when bound to Vh (not shown) versus VBS1 (in teal) as seen in the Vh-VBS1 crystal structure. Residues contributing to the interactions of talin VBS1 and Vh are underlined. Italicized residues are those proposed to reduce the ability of VBS2 to displace Vt compared with VBS1. C, electrostatic surface potential (51) of VBS2 (residues 855–874) as modeled when bound to Vh (red, negative; blue, positive; white, uncharged). Left and right images are presented in the same orientation as shown in D for VBS1. D, electrostatic surface potential of VBS1 (only equivalent residues 607–626 are shown) as seen in the Vh-VBS1 crystal structure. The right image shows the solvent-exposed VBS1 surface, and the left image shows the surface buried upon binding to Vh.
FIG. 3—continued

Talin Binding Amplifies Vinculin Activation

B

A873 K875
M870 V871
A866 T867
L863 A864
I862 A860 K861
A859 L856 L857
K855

K875 A873
E872 A868 D865
I862 K861 S858
L857 K855

180°

S625 A626
L622 L623
A618 V619
L615 A616
A611 A612
L608 L609
P607

R624 S620 E621
A619 A616 G617
G614 K613 Q610
L609 P607

180°
several Vh residues (Gln19, Ile20, and Leu23; Table I), is replaced by a valine in VBS2, and the smaller side chain can only interact with Leu23 but not with Gln19 or Ile20. Collectively, these alterations would be predicted to impair some of the hydrophobic interactions of VBS2 with Vh.

Previous studies evaluating the ability of a larger domain containing talin VBS3 (residues 1943–2157) to bind to a GST-Vh fusion protein demonstrated that larger portions of talin containing VBS1 (residues 498–636) and VBS2 (residues 727–926) could compete with talin VBS3 for binding to Vh (23). However, these experiments did not definitively pinpoint the regions that compete for binding to Vh to the more refined sequences of talin VBSs. The binding site for talin VBS1 (residues 607–626) in our Vh/VBS1 complex and the crystal structure suggests that these alterations would be predicted to impair some of the hydrophobic interactions of VBS2 with Vh.

This notion, we synthesized this modified VBS2 (VBS2e, for “VBS2 enhanced”; Fig. 7A) and addressed its ability to displace Vt from preexisting Vh/Vt complexes (Fig. 7, B and C), and to displace VBS1 from preexisting Vh/VBS1 complexes (Fig. 7, D and E). As predicted, these mutations in VBS2 converted this talin peptide into one that behaved as VBS1 and VBS2, thus validating the model of the Vh-VBS2 structure. Finally, we performed binding studies using Biacore 3000 SPR and determined the binding of VBS2e in the fit model with an estimated $K_d$ of $-7.65 \text{ nM}$ (Fig. 7F), confirming the importance of these three residues in establishing hydrophobic contacts in the binding cleft of the N-terminal helical bundle of Vh.

### DISCUSSION

Talin loss abolishes the formation of focal adhesions (3), consistent with its accepted role as a structural scaffold that bridges integrin receptors with components of the actin cytoskeleton, in particular with vinculin (1). However, talin has recently been shown to play an active role in inside-out signaling directed by these receptors (19). Here, our structures and binding-displacement assays establish that the three VBSs of talin bind to the same site in Vh and share in their ability to displace Vt from preexisting Vh/Vt complexes, suggesting that they play a direct role in activating vinculin and outside-in integrin signaling. The differences in the affinity of the three talin VBSs for Vh also establish a hierarchy in the binding of these recognition elements to vinculin (VBS3 > VBS1 > VBS2), and we propose that this property may allow talin to function as a rheostat, where its progressive binding to vinculin molecules would allow it to regulate signaling outcome.

One of the earliest events detected following the interaction of integrin receptors with the extracellular matrix is the binding of Pf(4,5)P2 to the talin head domain (40, 41). In turn, binding of Pf(4,5)P2 provokes conformational changes in the talin head which allow the rapid association of its FERM domain with the NPXY/F motif present in the cytoplasmic tails of β-integrin receptors (14, 42, 43). In their resting state, the α and β subunits of integrin receptors are clamped together through multiple hydrophobic and electrostatic contacts within their membrane-proximal helices (44). However, binding of the talin FERM domain to β-integrin receptors pushes the α and β subunits apart (9), and this structural alteration is transferred down the length of the receptor subunits to their ectodomains.

### TABLE I

| Vh residues interacting with talin VBS1 residues as seen in the Vh-VBS1 crystal structure |
|-------------------------------------|---------------------------------|----------------|
| Vh residues                          | VBS1 residues                   | Distance (Å) |
| Hydrophobic interactions             |                                 |              |
| Thr4 CG1                            | Leu609 CD2                      | 3.4          |
| Ile12 CG1                           | Lys613 CG                        | 3.8          |
| Val46 CG1                           | Ala616 CB                        | 3.8          |
| Gln19 CB                            | Leu620 CD1                      | 3.9          |
| Ile20 CG1                           | Leu620 CD1                      | 3.5          |
| Leu23 CD2                           | Leu620 CD2                      | 3.3          |
| Met26 CE                            | Gln627 CG                        | 3.9          |
| Met28 CE                            | Pro629 CD                        | 3.5          |
| Met30 CE                            | Ala630 C                         | 3.3          |
| Lys35 CD                            | Pro629 CD                        | 3.4          |
| Pro35 CD                            | Ala628 C                         | 3.8          |
| Leu46 CD2                           | Ser620 C                         | 3.8          |
| Leu46 CD2                           | Ala628 CA                        | 3.7          |
| Pro43 CB                            | Leu628 CD1                      | 3.8          |
| Val44 CG1                           | Ala628 CD1                      | 3.8          |
| Val47 CA                            | Ala628 CB                        | 3.8          |
| Val47 CG1                           | Ala628 CD1                      | 3.8          |
| Ala50 CB                            | Leu628 CD1                      | 3.7          |
| Val51 CG2                           | Leu628 CD1                      | 3.6          |
| Leu54 CD1                           | Leu628 CD1                      | 3.7          |
| Leu54 CD1                           | Ala613 CB                        | 3.9          |
| Leu54 CD2                           | Ala614 CD1                      | 3.5          |
| Met54 CE                            | Pro629 C                         | 3.8          |
| Met57 CG1                           | Ala628 CA                        | 3.7          |
| Val59 CG1                           | Leu628 CD2                      | 3.9          |
| Met62 CE                            | Leu628 CD2                      | 3.4          |
| Leu66 CG                            | Leu628 CD2                      | 3.8          |
| Ile85 CG2                           | Leu628 CD2                      | 3.8          |
| Val115 CG2                          | Val619 CG                        | 3.8          |
| Thr119 CG2                          | Ala612 CB                        | 4.0          |
| Leu122 CD2                          | Ala612 CB                        | 3.7          |
| Phe126 CG                           | Leu628 CD2                      | 3.3          |
| Polar interactions                  |                                 |              |
| Gln19 NE2                           | Ser620 OG                        | 2.6          |

### TABLE II

| Vh-VBS1 crystallographic refinement statistics |
|------------------------------------------------|
| Resolution range                               | 80.0–2.42 Å                        |
| Last shell                                     | 2.57–2.42 Å                        |
| No. of reflections (working set)               | 14,289                              |
| No. of reflections (test set)                  | 771                                 |
| $R$-factor (overall)                           | 0.188                               |
| $R$-factor (last shell)                        | 0.236                               |
| $R_{free}$ (overall)                           | 0.258                               |
| $R_{free}$ (last shell)                        | 0.258                               |
| No. of amino acid residues                     | 2,984                               |
| No. of protein atoms                           | 2,199                               |
| No. of solvent molecules                       | 190                                 |
| Average B-factor (main chain)                  | 51.9 Å                              |
| Average B-factor (side chain)                  | 63.5 Å                              |
| Average B-factor (solvent)                     | 71.4 Å                              |
| Root mean square deviation                     | from ideal geometry                 |
| Covalent bond lengths                          | 0.011 Å                             |
| Bond angles                                    | 1.15°                               |

- $R$-factor = $\sum_{i,j} [F_{OBS} - (F_{OBS})/\sum_{i,j} |F_{OBS}|$ where $(F_{OBS})$ is the expectation of $F_{OBS}$ under the error model used in maximum likelihood refinement.
- The free $R$-factor is a cross-validation residual calculated by using 5% of the native data, which were randomly chosen and excluded from the refinement.

This table presents the crystallographic refinement statistics for the Vh-VBS1 complex. The resolution range is from 80.0 to 2.42 Å, with the last shell of 2.57–2.42 Å. The number of reflections for the working set is 14,289, and for the test set is 771. The $R$-factor (overall) is 0.188, and the free $R$-factor (last shell) is 0.258. The average B-factor for the main chain is 51.9 Å, and for the side chain is 63.5 Å. The root mean square deviation from ideal geometry is 0.011 Å, and the bond angles are 1.15°.
where it changes the affinity of these receptors for extracellular matrix ligands (19). Furthermore, talin plays an active role in amplifying the inside-out signal by also binding to and triggering the activity of phosphatidylinositol phosphate kinase-1 (20–23), which generates more PI(4,5)P₂, which would then bind to talin to amplify and sustain the response.

Talin is also essential for outside-in signaling triggered by focal adhesions (3), but here its role has been generally thought to be passive, by acting as a scaffold protein that binds to other proteins of the actin cytoskeleton. Furthermore, it has been suggested that talin acts downstream of vinculin in this signaling pathway, where the binding of PI(4,5)P₂ to the Vt domain of vinculin has been proposed to "unfurl" its five-helical bundle, thus severing its intramolecular interaction with Vh and allowing Vh then to bind to talin and other partners (25, 31). Our structures of the Vh/VBS1 and Vh/VBS3 complexes, the validated model of the Vh/VBS2 complex, and the ability of all three VBSs to displace Vt from preexisting Vh/Vt complexes now suggest a different model. Specifically, we propose that talin is positioned at the proximal end of the outside-in signaling pathway and acts as an effector that activates vinculin (Fig. 8).

The structures of the Vh/VBS1 and Vh/VBS3 complexes, along with the validated modeled structure of the Vh/VBS2 complex, suggest that helical bundle conversion may occur in other scenarios as well. In the case of vinculin the amphipathic α-helices of all three talin VBSs insert between Vh helices, where it changes the affinity of these receptors for extracellular matrix ligands (19). Furthermore, talin plays an active role in amplifying the inside-out signal by also binding to and triggering the activity of phosphatidylinositol phosphate kinase-1 (20–23), which generates more PI(4,5)P₂, which would then bind to talin to amplify and sustain the response.

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**Fig. 4. Binding affinities of talin VBSs for Vh.** Biacore surface plasmon resonance was used to measure the affinity of talin VBSs. Biotinylated Vh was captured on a carboxymethyl-dextran-coated gold surface (CM-5 chip, Biacore). Talin VBS1, VBS2, and VBS3 peptides were injected over the reference and Vh-immobilized cells in sequence, and the release of bound VBS peptides was determined. Results from parallel injections of each sample over a reference cell were subtracted from the experimental data. Representative sensorgrams are shown. A, affinity of talin VBS1 for Vh. The calculated $K_d$ is shown in the inset. B, affinity of talin VBS2 for Vh. The calculated $K_d$ is shown in the inset. C, affinity of talin VBS3 for Vh. The calculated $K_d$ is shown in the inset.

**Fig. 5. Binding of talin VBS1 and VBS2 peptides to Vh actively displaces Vt from preexisting Vh-Vt complexes.** A, native PAGE analysis of free Vh and of Vh bound to Vt, VBS1, VBS2, and VBS3. B and D, talin VBS1 (B) and VBS2 (D) peptides were titrated into preexisting Vh-Vt complexes (closed circles) at molar ratios of the respective VBS-Vt of 1:20, 1:7, 1:2, 0.75:1, 1:5:1, and 2.5:1, and displaced Vt to form distinct Vh-VBS1 (open circle) and Vh-VBS2 complexes (gray circle). Free Vh is indicated with an asterisk. Free Vt is not visible in native gels because of its high pI. C and E, quantitation of the dissociation of the Vh-Vt complex (solid lines) and the formation of the Vh-VBS1 (C) and Vh-VBS2 (E) complexes (dotted lines) are shown.
and α2, causing dramatic movements and distortions of these helices, and that of helix α4, and collectively these alterations create a hydrophobic cavity that literally swallows the talin VBSs. In addition, in all three structures the C-terminal helical bundle of Vh is impervious to change, supporting the notion that it serves as an inflexible scaffold that supports the dynamic structural changes that occur in the Vh N-terminal helical bundle when vinculin transitions from its inactive to active state. Thus, there is a high degree of specificity to these interactions, and these structures have defined the talin ligand binding sites as residing in the cavity created by the conversion of Vh helices α1, α2, and α4.

Another insight coming from these studies is that there are indeed appreciable differences in the interactions of talin VBSs within the hydrophobic cleft in Vh created by helical bundle conversion. Talin VBS3 interacts most intimately within this cleft ($K_d$ of 3.1 nM), whereas the VBS2 interaction is significantly weaker ($K_d$ of 32.8 nM). Vinculin binds more efficiently to Vh than to the full-length protein (45), suggesting that talin activation is a necessary step toward linking its VBSs with vinculin. Talin appears rapidly activated by guest on July 25, 2018http://www.jbc.org/Downloaded from

Each of the talin VBSs is individually capable of binding to the same site in Vh. When bound by lipid and/or actin talin can exist as an antiparallel homodimer (46), whereas vinculin has been proposed to form homodimers or trimers after its interaction with vasodilator stimulatory protein (47). In this scenario a vinculin trimer has been proposed to bind to the antiparallel talin homodimer to provoke changes in the actin cytoskeleton (23). However, gel filtration and electron microscopy have revealed that under physiological conditions talin and vinculin are both monomers (48–50). These findings, together with our
structures and biochemical studies, suggest a simpler model whereby binding of one talin molecule could serve to activate three molecules of vinculin (Fig. 8). This scenario might then explain how outside-in signaling provoked by focal adhesions induces such rapid changes in the actin cytoskeleton. The precarious nature of talin allows the protein to bind simultaneously to integrin receptors through its head domain and to multiple vinculin molecules through its three VBS motifs, and the multiplicity of these interactions effectively amplifies the signaling response. Thus, talin can no longer be viewed simply as a scaffold protein in focal adhesions, but rather by altering the structures of its binding partners, as a signaling effector that directs and amplifies both inside-out and outside-in integrin signaling.

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REFERENCES

1. Critchley, D. R. (2000) Curr. Opin. Cell Biol. 12, 133–139
2. Xu, W., Baribault, H., and Adamson, E. D. (1998) Development 125, 327–337
3. Friddle, H., Hemmings, L., Monkley, S., Woods, A., Patel, B., Sutton, D., Dunn, G. A., Zicha, D., and Critchley, D. R. (1998) J. Cell Biol. 142, 1121–1133
4. Steenbergen, C. H., and Jennings, R. B. (1987) Circ. Res. 60, 478–486
5. Lesay, A., Hickman, J. A., and Gibson, R. M. (2001) Neuronreport 12, 2111–2115
6. Propato, A., Cutrera, G., Franzavilla, V., Ulivi, M., Schiaffella, E., Landi, O., Danhar, R., Cerundolo, V., Ferrarini, M., and Barnaba, V. (2001) Nat. Med. 7, 807–813
7. Bailly, M. (2003) Trends Cell Biol. 13, 163–165
8. Schwartz, M. A., and Ginsberg, M. H. (2002) Nat. Cell Biol. 4, 65–68
9. Kim, M., Carman, C. V., and Springer, T. A. (2003) Science 301, 1720–1725
10. Ridley, A. J., Schwartz, M. A., Burridge, K., Firtel, R. A., Ginsberg, M. H., Boras, G., Parsons, J., and Horwitz, A. R. (2003) Science 302, 1704–1709
11. Turner, C. E. (2000) Nat. Cell Biol. 2, 231–236
12. Parsons, J. T. (2003) J. Cell Sci. 116, 1409–1416
13. Calderwood, D. A., Yan, B., de Pereda, J. M., Alvarez, B. G., Fujikita, Y., Liddington, R. C., and Ginsberg, M. H. (2002) J. Biol. Chem. 277, 21749–21758
14. Garcia-Alvarez, B., de Pereda, J. M., Calderwood, D. A., Ulmer, T. S., Critchley, D., Campbell, I. D., Ginsberg, M. H., and Liddington, R. C. (2003) Mol. Biol. Cell 14, 49–58
15. Gilmore, A. P., Wood, C., Ohanian, V., Jackson, P., Patel, B., Rees, D. J. G., Hynes, R. O., and Critchley, D. R. (1993) J. Cell Biol. 122, 337–347
16. Hemmings, L., Rees, D. J., Ohanian, V., Bolton, S. J., Gilmore, A. P., Patel, B., Priddle, H., Trevithick, J. E., Hynes, R. O., and Critchley, D. R. (1996) J. Cell Sci. 109, 2715–2726
17. Bass, M. D., Smith, B. J., Prigent, S. A., and Critchley, D. R. (1999) Biochem. J. 341, 257–263
18. Critchley, D. R., Holt, M. R., Barry, S. T., Priddle, H., Hemmings, L., and Norman, J. (1999) Biochem. Soc. Symp. 65, 79–99
19. Tadokoro, S., Shattil, S. J., Rho, K., Tal, V., Liddington, R. C., de Pereda, J. M., Ginsberg, M. H., and Calderwood, D. A. (2003) Science 302, 103–106
20. Di Paolo, G., Pellegrini, L., Letinic, K., Cecca, G., Zucconi, R., Voronov, S., Chang, S., Guo, J., Wenk, M. R., and De Camilli, P. (2002) Nature 414, 85–89
21. Ling, K., Doughman, R. L., Firestone, A. J., Bunc, M. W., and Anderson, R. A. (2002) Nature 420, 89–93
22. Barsukov, I. L., Prescott, A., Bate, N., Patel, B., Floyd, D. N., Bhanji, N., Bagshaw, C. R., Letinic, K., Di Paolo, G., De Camilli, P., Roberts, G. C., and Critchley, D. R. (2003) J. Biol. Chem. 278, 31202–31209
23. Bass, M. D., Patel, B., Barsukov, I. G., Fillingham, I. J., Mason, R., Smith, B. J., Bagshaw, C. R., and Critchley, D. R. (2002) Biochem. J. 362, 761–768
24. Winkler, J., Lundsford, H., and Jockusch, B. M. (1996) J. Struct. Biol. 116, 270–277
25. Gilmore, A. P., and Burridge, K. (1996) Nature 381, 531–535
26. Miller, G. J., Dunn, S. D., and Ball, E. H. (2001) J. Biol. Chem. 276, 11729–11734
27. McGregor, A., Blanchard, A. D., Rowe, A. J., and Critchley, D. R. (1994) Biochem. J. 301, 225–233
28. Johnson, R. P., and Craig, S. W. (1994) J. Biol. Chem. 269, 12611–12619
29. Johnson, R. P., and Craig, S. W. (1995) Nature 373, 261–264
30. Johnson, R. P., Niggli, V., Durrer, P., and Craig, S. W. (1998) Biochemistry 37, 10211–10222
31. Bakkevold, C., de Pereda, J. M., Bhanji, N., Bagshaw, C. R., Letinic, K., Di Paolo, G., De Camilli, P., Roberts, G. C., and Critchley, D. R. (1999) Cell 99, 403–413
32. Izard, T., Evans, G., Borgen, R. A., Rush, C. L., Bricogne, G., and Bois, P. R. J. (2004) Nature 427, 171–177
33. Rush, C. L., and Izard, T. (2004) Acta Crystallogr. Sect. D Biol. Crystallogr. 60, 945–947
34. Navaza, J. (1994) Acta Crystallogr. Sect. A 46, 619–620
35. Jones, T. A., Zou, J.-Y., Cowan, S. W., and Kjeldgaard, M. (1991) Acta Crystallogr. Sect. A 47, 110–119
36. Bricogne, G. (1997) Methods Enzymol. 276, 361–423
37. Trounson, D. E., Eyck, L. F. T., and Matthews, B. W. (1987) Acta Crystallogr. Sect. A 43, 489–501
38. Collaborative Computational Project 4 (1994) Acta Crystallogr. Sect. D Biol. Crystallogr. 50, 760–763
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39. Pokutta, S., and Weiss, W. I. (2000) Mol. Cell 5, 533–543
40. Isenberg, G., and Goldmann, W. H. (1998) FEBS Lett. 426, 165–170
41. Martel, V., Racaud-Sultan, C., Dupe, S., Marie, C., Paulhe, F., Galmiche, A.,
   Block, M. R., and Albiges-Rizo, C. (2001) J. Biol. Chem. 276, 21217–21227
42. Calderwood, D. A., Zent, R., Grant, R., Rees, D. J., Hynes, R. O., and Ginsberg,
   M. H. (1999) J. Biol. Chem. 274, 28071–28074
43. Jiang, G., Giannone, G., Critchley, D. R., Fukumoto, E., and Sheetz, M. P.
   (2003) Nature 424, 334–337
44. Vinogradova, O., Velyvis, A., Velyviene, A., Hu, B., Haas, T., Plow, E., and Qin,
   J. (2002) Cell 110, 587–597
45. Burridge, K., and Mangeat, P. (1984) Nature 308, 744–746
46. Goldmann, W. H., Bremer, A., Haner, M., Aebi, U., and Isenberg, G. (1994) J.
   Struct. Biol. 112, 3–10
47. Huttelmaier, S., Mayboroda, O., Harbeck, B., Jarchau, T., Jockusch, B. M., and
   Rudiger, M. (1998) Curr. Biol. 8, 479–488
48. Molony, L., McCaslin, D., Abernethy, J., Paschal, B., and Burridge, K. (1987)
   J. Biol. Chem. 262, 7790–7795
49. Winkler, J., Lunsdorf, H., and Jockusch, B. M. (1997) Eur. J. Biochem. 243,
   430–436
50. Eimer, W., Niermann, M., Eppe, M. A., and Jockusch, B. M. (1993) J. Mol. Biol.
   229, 146–152
51. Nicholls, A., Sharp, K. A., and Honig, B. (1991) Proteins 11, 281–296
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