The Activity of the Vinculin Binding Sites in Talin Is Influenced by the Stability of the Helical Bundles That Make Up The Talin Rod*§

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The talin rod contains ~11 vinculin binding sites (VBSs), each defined by hydrophobic residues in a series of amphipathic helices that are normally buried within the helical bundles that make up the rod. Consistent with this, talin failed to compete for binding of the vinculin Vd1 domain to an immobilized talin polypeptide containing a constitutively active VBS. However, talin did bind to GST-Vd1 in pull-down assays, and isothermal titration calorimetry measurements indicate a Kd of ~9 μM. Interestingly, Vd1 binding exposed a trypsin cleavage site in the talin rod between residues 898 and 899, indicating that there are one or more active VBSs in the N-terminal part of the talin rod. This region comprises a five helix bundle (residues 482–655) followed by a seven-helix bundle (656–889) and contains five VBSs (helices 4, 6, 9, 11, and 12). The single VBS within 482–655 is cryptic at room temperature. In contrast, talin 482–889 binds Vd1 with high affinity (Kd ~ 0.14 μM), indicating that one or more of the four VBSs within 656–889 are active, and this likely represents the vinculin binding region in intact talin. In support of this, hemagglutinin-tagged talin 482–889 localized efficiently to focal adhesions, whereas 482–655 did not. Differential scanning calorimetry showed a strong negative correlation between Vd1 binding and helical bundle stability, and a 755–889 mutant with a Q755H substitution in the talin rod failed to bind vinculin, indicating that this mutant is not accessible to vinculin binding. These data suggest that the stability of the helical bundles that make up the talin rod is an important factor determining the activity of the individual VBSs.

The cytoskeletal protein talin is of a number of proteins including filamin (1), α-actinin (2), tensin (3, 4), and ILK (5) implicated in coupling focal adhesions to actin filament bundles. The globular N-terminal talin head contains a FERM domain (residues 86–400) with binding sites for the cytoplasmic domains of the integrin β-subunit (21, 22) and also latulin (a hyaluronan receptor) (23), F-actin (24), and two signaling proteins, focal adhesion kinase (25) and the type 1 ρ-kinase (26–29), both of which are implicated in regulating FA dynamics. The talin rod, which is responsible for dimer formation (30), contains a conserved C-terminal actin-binding site (31), a second integrin-binding site (32), and several binding sites for the cytoskeletal protein vinculin (33), which itself has multiple binding partners including F-actin (34). Integrin signaling via focal adhesion kinase/Src promotes binding of talin to F-actin kinase (26). This activates PIP kinase, and results in translocation of the talin-PIP kinase complex to the plasma membrane (25). PIP2 has been shown to activate the integrin binding site(s) in talin (35), and the talin head has in turn been shown to activate integrins (36, 37), suggesting a model in which the localized production of PIP2 drives the assembly of integrin-talin-F-actin complexes (38) competent to engage the extracellular matrix. Recruitment of additional components might then facilitate the stabilization and maturation of these complexes into FAs.

One protein that has been implicated in stabilizing FAs is vinculin. Down-regulation of vinculin leads to smaller FAs and enhances cell motility (39), whereas overexpression of vinculin leads to larger FAs and suppresses motility (40). Studies on vinculin knock-out cells are consistent with these data (41–44). Moreover, vinculin is recruited to FAs in response to applied mechanical force, supporting the view that it plays a role in stabilizing these structures (45). Using SDS-PAGE blot assays (46) and yeast two hybrid deletion mutants analysis (33, 47), we previously identified three vinculin binding sites (VBSs) in the talin rod, each defined by a single amphipathic helix. More recent data using synthetic peptides equivalent to each of the predicted 62 helices that make up the talin rod (48) indicate that there are at least eight additional VBSs (49). Crystal structures show that the hydrophobic groups on one face of a talin VBS helix become embedded in a hydrophobic groove in the vinculin head (50–52), but our recent structural studies (52, 53) show that these vinculin binding determinants in talin are normally buried in the...
core of a series of amphipathic helical bundles that make up the talin rod (48). In the present study we have sought to establish whether some or all of the VBSs in the talin rod are cryptic or constitutively active.

MATERIALS AND METHODS

**Protein Expression and Purification**—cDNAs encoding mouse talin 482–636, 482–655, 482–789, and 755–889 and the chicken vinculin Vd1 domain (residues 1–258) were cloned into the expression vector pET-15b (Novagen, Cambridge Biosciences, Cambridge), whereas a cDNA encoding mouse talin 482–889 was cloned into pET-151/DTOPO (Invitrogen). Recombinant proteins were purified as described previously (52, 53), and proteins concentrations were determined using the CB Protein Assay (Calbiochem).

cDNAs encoding human talin 1 (residues 1–2541) and the talin 1 rod domain (residues 453–2541) were generated by PCR using pET30a constructs encoding the N-terminal talin head and the talin rod as templates (kindly provided by Dr. Stephen C.-T. Lam, University of Illinois, Chicago, IL). PCR products were cloned into pET30a (Novagen) between the Ndel and EagI sites such that the encoded proteins were expressed with a C-terminal His tag. Constructs were authenticated by DNA sequencing and shown to match the human talin 1 cDNA sequence in GenBank™ (accession number BC042923). Human talin 1 and the talin 1 rod were expressed in Escherichia coli BL21(DE3). In brief, cells (1 liter) were grown at 37 °C to an A 600 nm of 0.6 and induced with 0.2 mM isopropyl-β-D-galactopyranoside at 15 °C overnight. The cell pellet was resuspended into 35 ml of lysis buffer (20 mM Tris (pH 7.4), 0.4 mM NaCl) with 1 mM β-mercaptoethanol and lysed by passing through a French press (Thermo Electron). The soluble cell lysate was then recovered and passed through a 5-ml HiTrap affinity column (Amersham Biosciences) charged with Ni 2+. Fractions containing the required protein were combined, concentrated, and further purified by size exclusion chromatography (Superdex 200 10/30, Amersham Biosciences) in buffer containing 20 mM sodium phosphate (pH 7.5), 150 mM NaCl, and 0.1 mM EDTA. The relationship of the various talin polypeptides used in this study to full-length talin is illustrated in Fig. 1.

**Binding of the Vinculin Vd1 Domain to Talin**—The relative affinities of talin and talin polypeptides for the vinculin Vd1 domain was measured by competitive ELISA as described previously (52). In brief, NUNC immunoplates (F96-maxisorp) coated with a talin 482–636 polypeptide were incubated with the GST–vinculin Vd1 domain at a concentration of 5 μM (54) in the presence of increasing amounts of talin or recombinant talin polypeptides. Binding of GST–vinculin or GST alone was determined using a rabbit polyclonal anti-GST coupled to horseradish peroxidase (Santa Cruz). Binding of talin to GST–Vd1 was also measured using a pull-down assay as described previously (47). Analytical gel filtration chromatography of recombinant talin polypeptides and vinculin Vd1 (1–258) was performed using Superdex–75 (10/30) (Amersham Biosciences) at room temperature. The column was pre-equilibrated and run in 20 mM Tris (pH 8.0), 200 mM NaCl, and 2 mM dithiothreitol at a flow rate of 0.8 ml/min. In each case, 0.5-ml fractions were collected and analyzed using a 15% SDS-PAGE gel and stained using the GelCode blue reagent (Pierce).

**Cytometry**—Differential scanning calorimetry (DSC) experiments were performed on a N-DSC II differential scanning calorimeter (Calyomtry Sciences Corp, Provo, UT) at the scanning rate of 1000/min under 3.0 atm of pressure. Before measurement, protein samples were dialyzed against PBS. The dialysis buffer was used as the reference solution. Vd1 and talin polypeptides were used at concentrations between 18.0 and 25 μM. Isothermal titration calorimetry (ITC) was performed on a VP-ITC calorimeter from Microcal (Northampton, MA). 8-μl aliquots of solution containing 0.8–1.0 mM vinculin Vd1 (residues 1–258) were injected into the cell containing 40–100 μM talin or the talin polypeptide. In each experiment 37 injections were made. The experiments were performed at 23 °C. Before ITC titrations, all protein samples were dialyzed against PBS buffer. Experimental data were analyzed using Microcal Origin software provided by the ITC manufacturer (Microcal, Northampton, MA).

**Circular Dichroism Spectroscopy**—CD spectra were recorded using a Jasco J-715 spectropolarimeter equipped with a Jasco PTC-348W temperature control unit. Far-UV CD spectra were recorded at 20 °C over the wavelength range 200–250 nm in a quartz cell of 0.1-cm path length (scan rate 50 nm/min−1). Proteins were dissolved in 20 mM sodium phosphate (pH 6.5), 50 mM NaCl at concentrations of 25 μM. The mean residue molar ellipticity [θ] (deg × cm2 × dmol−1) was calculated according to the formula [θ] = θ/(n × 10 × C M × l), where θ is the measured ellipticity in degrees, n is the number of peptide bonds (residue), l is the path length in centimeters, and C M is the molar protein concentration. The factor 10 originates from the conversion of the molar concentration to the dmol cm−1 concentration unit. For urea denaturation studies, proteins were dissolved in 20 mM sodium phosphate, pH 6.5, 50 mM NaCl containing 0, 0.8, 1.6, 4.0, or 5.6 M urea.

**Expression of HA-tagged Talin Polypeptides in NIH3T3 Cells**—cDNAs encoding C-terminal HA-tagged talin polypeptides 482–636, 482–655, 482–789, and 482–889 were synthesized by PCR using the mouse talin-1 cDNA (55) as template and the following primers from Invitrogen: 482, 5'-CGGGATCCATGCAGGATGCACATGGCCTTT-3'; 636, 5'-GGATATACAAAGACGTAATCTGGAACATCGT-3'; 789, 5'-GGATATTCACAAGACGCTAATCTGGAACATCGT-3'; 889, 5'-GGATATTCACAAGACGCTAATCTGGAACATCGT-3'. PCR products were first cloned into pPCR-Script Amp SK(+) (Stratagene) and then subcloned into the expression vector pcDNA3 (Invitrogen) using BamHI and EcoRI sites. The authenticity of the cloned cDNAs was confirmed by sequencing. The constructs were transfected −2 μg of cDNA/6 μl of FuGENE 6 (Roche Applied Science) into 3 × 104 NIH3T3 cells grown on glass coverslips and cultured in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum. After 16 h at 37 °C, cells were fixed for 10 min in 4% paraformaldehyde/PBS, permeabilized for 5 min in 0.2% (v/v) Triton X-100/PBS, and quenched for 10 min in 50 mM NH4Cl/PBS. After 1 h of incubation in 1% bovine serum albumin/PBS, cells were incubated for 20 min with the anti-HA antibody (Santa Cruz Biotechnologies), washed 3 times with 0.02% bovine serum albumin/PBS, and then incubated for 20 min with fluorescein isothiocyanate-conjugated secondary antibody (Southern Biotechnology Associates, Inc.). F-actin was visualized by staining with phalloidin–Texas Red (Molecular Probes) and inspected using a Nikon TE300 inverted microscope and Openlab 4.0.2 software.

**RESULTS**

The VBSs in Talin Are in a Low Affinity State—Our recent biochemical (33, 47) and structural studies (52, 53) show that the VBSs in the talin rod (49) (Fig. 1) are each contained within a single amphipathic α-helix. Binding is determined by a series of hydrophobic residues on one face of the helix, but these are normally buried in the core of the helical bundles that make up the talin rod (52, 53). To investigate whether any
of the VBSs in intact talin are active, we set up a competitive ELISA-type assay. Because the talin-binding site in the N-terminal region of vinculin is normally masked by an intramolecular interaction with the C-terminal vinculin tail (54, 56), we used a GST–vinculin fusion protein containing just residues 1–258 (GST-Vd1) to assay talin binding. Similarly, because the single VBS contained within the N-terminal region the talin rod (residues 482–655, a 5-helix bundle) is cryptic (52), we used a construct (residues 482–636) in which the VBS has been activated by deletion of the C-terminal helix. We then measured the ability of GST-Vd1 to bind to talin 482–636 deposited on plastic in the presence of increasing concentrations of talin or talin fragments. As shown previously (52), preincubation of GST-Vd1 with increasing concentrations of talin 482–636 in solution progressively inhibited binding to talin 482–636 on plastic, with an IC_{50} of ~1 nM, whereas talin 482–655 was completely without effect (Fig. 2A). Interestingly, talin purified from turkey gizzard was also unable to inhibit GST-Vd1 binding, indicating that the VBSs in intact talin are cryptic or in a low affinity state (Fig. 2A). The integrin-binding sites in talin are also reportedly cryptic and can be activated by PIP2 (35) or by cleavage of the talin head from the rod (57). Preincubation of talin with PIP2 did not activate the VBSs in talin (Fig. 2A), although the purified talin rod (but not the head) did show significantly more Vd1 binding activity than intact talin (Fig. 2A).

The above assay provides a measure of the relative affinities of Vd1 for talin and talin fragments and does not necessarily indicate that intact talin cannot bind vinculin. To investigate this, we incubated purified talin with GST or GST-Vd1 and carried out a pull down assay. Talin binding was analyzed by SDS-PAGE followed by Western blotting using the TD77 monoclonal antibody that recognizes the extreme C terminus of talin (8). The results clearly establish that talin and talin rod fragments are able to bind to GST-Vd1 (Fig. 2B). To obtain a direct measure of the binding affinities, we used ITC (Table 1). At 23 °C, Vd1 bound talin with a K_{d} of ~9 μM, and PIP2 had relatively little effect on binding (K_{d} ~5 μM). The talin rod bound Vd1 with a slightly higher affinity (K_{d} = 2.4 μM), whereas Vd1 bound the short talin 482–636 polypeptide with a 30-fold higher affinity (K_{d} = 0.3 μM) than intact talin. As expected, no binding could be detected to talin 482–655. The results are in good agreement with the semiquantitative ELISA data and confirm that although intact talin can bind to vinculin, the interaction is of low affinity. Thus, talin is unable inhibit binding of Vd1 to talin 482–636, because the latter polypeptide binds Vd1 with much higher affinity.

**Binding of Vinculin Vd1 to Intact Talin Exposes Protease Cleavage Sites in the Talin Rod**—The affinity of Vd1 for talin suggests that the majority of the ~11 VBSs in talin are cryptic and must be regulated in some way. We, therefore, sought an assay that might identify the approximate location of any active VBSs in the talin rod. Binding of Vd1 to a recombinant talin 755–889 (which contains 3 VBSs) induces a marked conformational change in the talin polypeptide that can be readily demonstrated by NMR and also by an increase in sensitivity to proteolytic cleavage (53). We, therefore, explored the possibility that Vd1 binding to intact talin might expose previously buried trypsin cleavage sites that would indicate where Vd1 binding had occurred. Incubation of talin alone with trypsin liberated a protease-resistant 170-kDa fragment (Fig. 3A), detected by monoclonal antibody 8D4 (epitope residues 482–655) (Fig. 3B) but lacking the epitope for monoclonal antibody TD77 (residues 2494–2541) (Fig. 3C). Consideration of the size of the fragment and the antibody data indicates that trypsin cleaves off the talin head, liberating a rod fragment starting at about residue 482 that lacks the extreme C-terminal region of the rod. When intact talin was preincubated with Vd1, the ~170-kDa fragment was further cleaved to an ~140-kDa protease-resistant fragment (Fig. 2A) that lacked the epitope for 8d4 (Fig. 3B) as well as that for TD77 (Fig. 3C). N-terminal sequencing showed that the ~140-kDa fragment starts at residue 899. Interestingly, the N-terminal region cleaved from the talin rod does not accumulate as a Coomassie Blue-positive band, indicating that it is unstable. However, a series of 8d4 positive bands can be detected by Western blotting (Fig. 3B). The results show that Vd1 binding to intact talin markedly affects the conformation of the N-terminal region of the talin rod (residues 482–898), rendering it protease-sensitive and indicating that this region must contain at least one active VBS.
Talin Residues 656–889 Contain One or More Active VBSs
—We have recently determined the structures of several polypeptides spanning the N-terminal part of the talin rod (52, 53), and this has allowed us to propose a model (53) for the structure of residues 482–889 comprising a 5-helix bundle (residues 482–655; domain 1) followed by a 7-helix bundle (residues 656–889; domain 2) (53). Moreover, using synthetic peptides corresponding to each of the /H9251-H9261-helices, we have shown that helices 4, 6, 9, 11, and 12 bind Vd1 (49) (Fig. 1). To establish which if any of these VBSs is active, we analyzed the ability of various talin polypeptides spanning this region to bind Vd1 in both competitive ELISA and gel filtration assays. In the ELISA, talin 482–889 showed the highest affinity for GST-Vd1 and was even more effective at inhibiting binding of GST-Vd1 to immobilized talin 482–636 than soluble 482–636 itself (Fig. 4). These results are consistent with ITC data, which show that at 23 °C talin 482–889 binds Vd1 with a \( K_d \) of 0.14 μM compared with a \( K_d \) of 0.3 μM for talin 482–636 (Table 1). Because the single VBS (helix 4) in the 5 helix bundle is cryptic, we conclude that one or more of the four VBSs in the 7 helix bundle (domain 2) are able to bind Vd1 with high affinity. The talin 482–889 polypeptide also showed high affinity binding to Vd1 in gel filtration experiments and at a molar ratio of ~1:1 the proteins formed a complex with no unbound Vd1 remaining (Fig. 5A). Progressive increases in the molar ratio of talin 482–889/Vd1 suggests that this talin polypeptide can bind up to 3 molecules of Vd1 (at a molar ratio of 1:3 some free Vd1 was again detected).

Binding of Vd1 to Talin 482–655 Is Inhibited by the Stability of the 5-Helix Bundle
—One of the factors that might influence the activity of a VBS is the inherent stability of the talin helical bundle in which it is embedded. To explore this possibility, we expressed various talin rod constructs containing VBSs and used DSC to compare their stability with their ability to bind Vd1. As observed previously (54), a DSC scan shows that Vd1 has a rather broad melting profile and relatively low melting temperature (\( T_m^1 \sim 49 ^\circ C \) and \( T_m^2 \sim 58 ^\circ C \)) but becomes more ordered upon binding to the talin rod, resulting in the appearance of a sharp peak with a \( T_m \) of 66.0 °C (Fig. 6A). A similar sharp peak, albeit with a somewhat higher \( T_m \) (67.5 °C), is present in the DSC scan of the Vd1 complex with talin 482–636 (Fig. 6B), indicating that Vd1 binds to talin 482–636 in a similar manner to the talin rod. The low \( T_m \) (53.9 °C) and low unfolding enthalpy of the talin 482–636 4-helix bundle demonstrates that it is loosely folded, whereas the much higher \( T_m \) (60.2 °C) and higher unfolding enthalpy of the talin 482–655 5-helix bundle (Fig. 6C) shows that the additional helix stabilizes the bundle. These observations are in accord with the NMR data (52).

Incubation of the 482–636 polypeptide with Vd1 resulted in the formation of a 1:1 complex, and no free Vd1 remained (Fig. 6B). This is consistent with ELISA data (Fig. 2A and 4A) and also gel filtration data, which showed a 1:1 high affinity binding between talin 482–636 and affinity.

![FIGURE 2. The VBSs in talin are in a low affinity state.](image)

**TABLE 1**

| Construct          | \( K_d \) (μM) |
|--------------------|---------------|
| Talin              | 8.9 ± 3.4     |
| Talin + PIP2       | 4.7 ± 1.6     |
| Talin rod          | 2.4 ± 1.2     |
| 482–636            | 0.3 ± 0.1     |
| 482–655            | No binding    |
| 482–889            | 0.14 ± 0.01   |

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Interestingly, binding of the talin 482–655 polypeptide to Vd1 was also readily detected by DSC (Fig. 6C), whereas no binding was observed in ELISA, gel filtration, or ITC experiments conducted at room temperature. During DSC experiments, the sample temperature was raised, and one can speculate that at the elevated temperatures, the talin 482–655-fold became destabilized, permitting Vd1 binding. Interestingly, Vd1 seems to promote the destabilization of the talin 482–655-fold; there is a new peak with $T_m = 49.0 \, ^\circ C$ present in the DSC scan of the talin 482–655-Vd1 complex but not in the scan of 482–655 alone (Fig. 6C). This peak could represent melting of a fraction of unbound Vd1 or, alternatively, melting of talin 482–655 destabilized by Vd1 binding. If the first scenario were correct, then increasing 482–655 concentration in the sample should decrease the intensity of this peak, since less unbound Vd1 would be present. In fact, raising the talin 482–655 concentration 1.5-fold (Fig. 6C) caused a slight increase in the intensity of the 49.0 °C peak. Thus, this peak appears to represent melting of talin 482–655 destabilized by Vd1 binding. Together, these results dem-

**FIGURE 3.** Vinculin binding to talin exposes a cryptic trypsin cleavage site in the talin rod. Purified turkey gizzard talin was incubated with trypsin (lanes 2 and 4) in the absence (lane 2) or presence (lane 4) of vinculin Vd1. Samples were analyzed by SDS-PAGE, and talin was detected by staining with Coomassie Blue (A), monoclonal antibody (Mob) 8d4 (epitope residues 482–655) (B), monoclonal antibody TD77 (epitope residues 2494–2541) (C). The N-terminal sequence of the talin polypeptide (arrow) in panel A starts at residue 899. Right, molecular mass markers (kDa). Note the Vd1 (~28 kDa) has run off the bottom of the gel.

**FIGURE 4.** Talin residues 482–889 contain one or more active VBSs. Binding of GST-vinculin Vd1 to microtiter wells coated with talin polypeptide 482–636 was determined by ELISA (490 nm) (52) in the presence of increasing concentrations (nM) of talin polypeptides. A, 482–636 and 482–655; B, 755–889, 482–789, and 482–889.

Vd1 (52). Interestingly, binding of the talin 482–655 polypeptide to Vd1 was also readily detected by DSC (Fig. 6C), whereas no binding was observed in ELISA, gel filtration, or ITC experiments conducted at room temperature. During DSC experiments, the sample temperature was raised, and one can speculate that at the elevated temperatures, the talin 482–655-fold became destabilized, permitting Vd1 binding. Interestingly, Vd1 seems to promote the destabilization of the talin 482–655-fold; there is a new peak with $T_m = 49.0 \, ^\circ C$ present in the DSC scan of the talin 482–655-Vd1 complex but not in the scan of 482–655 alone (Fig. 6C). This peak could represent melting of a fraction of unbound Vd1 or, alternatively, melting of talin 482–655 destabilized by Vd1 binding. If the first scenario were correct, then increasing 482–655 concentration in the sample should decrease the intensity of this peak, since less unbound Vd1 would be present. In fact, raising the talin 482–655 concentration 1.5-fold (Fig. 6C) caused a slight increase in the intensity of the 49.0 °C peak. Thus, this peak appears to represent melting of talin 482–655 destabilized by Vd1 binding. Together, these results dem-

**FIGURE 5.** Analysis of vinculin Vd1 binding to talin 482–889 by gel filtration. A, Superdex-75 (10/30) gel filtration chromatography of talin 482–889 (0.05 mM) in the presence or absence of the vinculin Vd1 domain. Fractions (0.5 ml) were collected and analyzed using 15% SDS-PAGE. At a 1:1 molar ratio of talin 482–889/Vd1 (solid line), all the Vd1 is in complex with the talin polypeptide, indicative of a high affinity interaction (the complex elutes at approximately the same position as free talin polypeptide). At a 1:3 molar ratio (dotted line), the complex elutes slightly ahead of the free talin polypeptide, and some free Vd1 is detected both by gel filtration (arrow) and SDS-PAGE. At a molar ratio of 1:7 (solid line), an extra shift is observed in the elution of the complex (about 8 ml), but much of the Vd1 remains unbound.
onstrate that the greater stability of the talin 482–655 5-helix bundle (domain 1) renders the VBS in helix 4 cryptic at room temperature. This conclusion was substantiated by analyzing the ability of talin 482–655 to bind Vd1 at different temperatures using gel filtration (Fig. 7). At 20 °C, there was no evidence of binding, although some complex formation could be detected at 37 °C. Only when the temperature was increased to 45 °C was stoichiometric binding of Vd1 to talin 482–655 observed.

Analysis of Vd1 Binding to Talin Polypeptides 482–789 and 482–889 by DSC—To further investigate the relationship between helical bundle stability and vinculin binding, we next used DSC to analyze Vd1 binding to talin polypeptides containing domain 1 and either part of or the whole of domain 2 (residues 656–889), which contains four VBSs (Fig. 1), one or more of which is active as indicated by ELISA, gel filtration, and ITC experiments. The crystal structure of 482–789 reveals that the domain 1 5-helix bundle is followed by a 4-helix bundle that packs against domain 1 in a staggered manner (52). Interestingly, the melting temperature of talin 482–789 (70.4 °C) (Fig. 8A) was substantially higher than that of talin 482–655 (60.2 °C) (Fig. 6C), indicating that domain 1 is further stabilized by the adjacent 4-helix bundle. Binding of the 482–789 polypeptide to Vd1 (Fig. 8A) was greatly inhibited in DSC experiments, in agreement with both the ELISA (Fig. 4B) and gel filtration data (Fig. 5B). We conclude that the 482–789 polypeptide adopts a very stable fold and the additional VBSs in helices 6 and 9 are only partially active even at elevated temperatures.

We have developed a model of talin 482–889 (53) from the crystal structure of 482–789 (52) and the NMR structure of talin 755–889 (53), which overlap by one helix. The model predicts that domain 1 is actually followed by a 7-helix bundle. When 482–889 was analyzed by DSC (Fig. 8C), the main peak was at 69.2 °C with a shoulder at 75 °C. The fact that the Tm of the main peak (69.2 °C) is 1.2 °C less than that of the 482–789 polypeptide (70.4 °C) suggests that in this case, the incorporation of an additional three helices has a destabilizing effect on the overall fold adopted by the 7-helix bundle. Strong binding of Vd1 to 482–889 (Fig. 8C) was detected by DSC, in agreement with the data from ELISA (Fig. 4B), gel filtration (Fig. 5A), and ITC (Kd ~ 0.14 μM; Table 1). The above results reinforce the idea that the activity of VBSs within the talin
Talin Helical Bundle Stability Affects Vinculin Binding

This fold is characterized by two threonine pairs (Thr-775/Thr-809 and Thr-833/Thr-867) that are accommodated within the hydrophobic core of the bundle (53). Mutation of these threonines to more hydrophobic residues would be expected to stabilize the fold and to reduce the availability of the VBSs contained therein. We, therefore, expressed a talin 755–889 mutant polypeptide in which these threonine pairs were replaced by isoleucine/valine pairs T775V/T809I/T833I/T867V, referred to as the VIIV mutant. We confirmed that these mutations do not affect the capacity of GST-Vd1 to bind the VBSs contained within talin 755–889 using a SPOT-peptide assay (Supplemental Fig S1). The CD spectra of both the wild-type and VIIV mutant polypeptides were very similar and indicated, as expected, a predominantly helical fold (data not shown). Analysis of the change in the mean molar ellipticity at 222 nm with increasing temperature showed a cooperative unfolding of the wild-type 755–889 polypeptide with a T_m of 72.6 °C (Fig. 9A), in good agreement with the DSC data. By contrast, the VIIV mutant was much more stable and was less than 50% unfolded even at 95 °C. The contrast in stabilities of the two proteins was further illustrated by experiments in 5.6 M urea, where the T_m of the mutant was 78.4 °C, whereas the wild-type protein was completely unfolded even at 20 °C under these conditions (data not shown). Gel filtration experiments showed that, whereas wild-type 755–889 binds strongly to Vd1 (Fig. 9B), the VIIV mutant binds much more weakly (Fig. 9C); even with a 3-fold excess of vinculin Vd1, less than half the mutant is in the form of the complex. The results clearly establish that the inherent stability of the individual amphipathic helical bundles making up the talin rod is likely to play a significant role in determining the activity of any VBS contained within the bundle.

**Focal Adhesion Targeting of Talin Polypeptides Containing VBSs—**
Talin polypeptides containing active VBSs would be expected to localize in vinculin-containing FAs when expressed in mammalian cells. To test this prediction, NIH3T3 cells were transfected with HA-tagged talin 482–636 or 482–655, in which VBS1 is constitutively active or cryptic, respectively. As predicted, the 482–636 polypeptide localized to FAs, whereas the 482–655 did not (Fig 10). In contrast, a talin 482–636 polypeptide containing a double mutation in the VBS, which abolishes Vd1 binding in vitro (52), failed to localize to FAs. These results support the conclusion that localization of these talin polypeptides to FAs reflects the activity of the VBS. We then transfected cells with HA-tagged talin 482–789 and 482–889 constructs. Interestingly, only talin 482–889 localized to FAs. Collectively, the foregoing results indicate that although there are 3 VBSs within residues 482–789, they are not fully available for vinculin binding, whereas extension of the polypeptide by an additional 3 helices (two of which contains VBSs) results in a polypeptide (482–889) that can support vinculin binding in vitro and in vivo.

**DISCUSSION**

The VBSs in the talin rod are defined by hydrophobic residues on one face of a series of amphipathic helices. However, crystallographic (52) and NMR (53) structures of domains of the talin rod show that the key residues are normally buried within the hydrophobic cores of α-helical bundles. Indeed, mutagenesis shows that the VBS residues contribute to interhelical contacts and the stability of the bundle (52). In agreement with these observations, the competitive ELISA and ITC data shown here clearly indicate that most of the VBSs in either gizzard talin or recombinant talin are in a low affinity state, although the fact that Vd1 binding could be detected in pull down and ITC assays demonstrates that one or more VBSs in talin are available for binding. The finding that Vd1 exposes a cryptic trypsin cleavage site toward the N-terminal...
FIGURE 9. The talin 755–889 VIIIV mutant is more stable and shows reduced vinculin Vd1 binding. A, denaturation profiles of wild-type talin 755–889 (black circles) and the quadruple VIIIV mutant (white triangles), measured by monitoring the change in mean residue molar ellipticity at 222 nm ([θ]$_{222}$) with increasing temperature. B and C, binding of wild-type talin 755–889 (B) and the quadruple VIIIV mutant (C) to vinculin Vd1 determined by Superdex-75 (10/30) gel filtration chromatography. B, at a 1:1 molar ratio of talin 755–889/Vd1 (dot trace), all the Vd1 is in complex with the talin polypeptide. At a 1:3 molar ratio (line-dot trace), free Vd1 is detected, but all the talin 755–889 is in complex. C, in contrast, at a 1:1 molar ratio of the talin 755–789 VIIIV mutant/Vd1 (dot trace) most of the talin mutant and Vd1 are in their free form, although some of the Vd1 is in complex with the talin polypeptide (complex elutes at ~8 ml). At a molar ratio of 1:3 (line-dot trace), there is an increase in complex assembly, but still, most of the two proteins remain in the free form.
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region of the talin rod (between residues 898/899) strongly suggests that there is at least one active VBS in this vicinity. Because the remainder of the talin rod remains resistant to proteolysis in the presence of Vd1, we surmise that the VBSs contained therein must be in a low affinity state.

It is notable that the N-terminal region of the talin rod contains 5 of the ~11 VBSs in talin (Fig. 1) (49). Structures of this region show that it is composed of two domains (52, 53). Domain 1 (residues 482–655) is a five-helix bundle containing one VBS (helix 4), but this is cryptic at room temperature, as shown by competitive ELISA, gel filtration, and ITC experiments. Moreover, gel filtration experiments conducted at higher temperatures, presumably because the five-helix bundle is less stable under these conditions. Similarly, mutations to the hydrophobic core of the bundle that would be predicted to destabilize the bundle also activate Vd1 binding (52). In the slightly larger talin 482–789 polypeptide, the five helix bundle is followed by a four-helix bundle, and this polypeptide contains three VBSs (helices 4, 6, and 9). Although it does bind Vd1 in both gel filtration and competitive ELISA, the affinity is much lower than for the 482–636 four-helix bundle. Interestingly, the 482–789 polypeptide melts at a higher temperature (70.4 °C) than the talin 482–655 polypeptide (60.2 °C), indicating that domain 1 is stabilized by the adjacent four-helix bundle, and even in DSC, binding to Vd1 is greatly inhibited. The picture is quite different with the talin 482–889 polypeptide that contains an additional three helices, two of which (helices 11 and 12) are VBSs. This polypeptide binds Vd1 with high affinity and melts at a slightly lower temperature (69.2 °C) than the 482–789 polypeptide (70.4 °C), indicating that inclusion of the additional three helices has a slight destabilizing effect on the domain 2 fold. Moreover, the 482–889 polypeptide is cleaved by trypsin at residue 790, removing the last three helices (data not shown), suggesting that these are somewhat less stable and/or less tightly packed against the other helices. Therefore, we tentatively conclude that it is this region in intact talin that contains the active VBSs since Vd1 induces trypsin cleavage nearby (i.e. between residues 898/899).

A direct test of the relationship between fold stability and Vd1 binding was carried out by constructing a mutant of the 755–889 four-helix bundle (TT775V/T809I/T833I/T867V) predicted to have a more stable fold. In line with prediction, this mutant was substantially more stable to thermal and urea denaturation and was also markedly less effective at binding Vd1. Taken together, our results clearly establish that the inherent stability of the amphipathic helical bundles that make up the talin rod is an important determinant of vinculin binding. This is borne out by studies on the localization of HA-tagged talin polypeptides expressed in NIH3T3 cells. Thus, those that bind Vd1 with high affinity in vitro (482–636, 482–889) target to FAs, whereas those that do not bind (482–655) or bind with low affinity (482–789) fail to do so. A simple general correlation between overall thermal stability and Vd1 binding would only be expected if the talin bundle unfolded before interaction with Vd1; it is perhaps more likely that an initial interaction with Vd1 is required to initiate the structural changes in the talin bundle to expose the VBS so that the stability of the fold will not be the only factor.

Talin is ubiquitously expressed and is found in structures ranging from the myotendinous junction (58) and costameres in skeletal muscle (59), the intercalated discs in cardiac muscle (59), dense plaques in smooth muscle (60), the neuromuscular junction (61), the immunological synapse (62), neuronal growth cones, the synapse (63), phagocytic cups (64), and the mid-body in cytokinesis (65). Talin typically colocalizes with vinculin, and it may be significant that in most of these structures the integrin-talin complex is subject to force induced by actomyosin contraction. This raises the possibility that force-induced unfolding of the helical bundles in talin may be a significant factor in the exposing VBSs in the talin rod. Because vinculin is also an F-actin-binding protein (66, 67), it may be recruited to strengthen the link between talin and F-actin (45). Interestingly, in the immunological synapse the integrin LFA-1/talin complex in T-cells does not colocalize

FIGURE 10. Talin polypeptides which bind vinculin Vd1 target to focal adhesions. NIH3T3 cells were grown on glass coverslips and transiently transfected with cDNAs encoding the HA-tagged talin polypeptides indicated. After 16 h, the cells were stained with anti-HA followed by incubation with fluorescein isothiocyanate-conjugated secondary antibody. F-actin was visualized with phalloidin-Texas Red (Molecular Probes). Actin staining and merged images are shown just for the HA-tagged talin polypeptides that localized to FAs. Results are representative of 3–6 separate experiments. Scale bar, 10 μm. The expression of the various talin polypeptides was confirmed by Western blotting using an anti-HA antibody. Talin polypeptides (482–636, 482–789, and 482–636 M (52)) that have a low affinity for vinculin remain diffusely distributed within the cell, whereas those that bind vinculin with high affinity (482–636 and 482–889) localize to the end of actin filaments in FAs.

The expression of the various talin polypeptides was confirmed by Western blotting using an anti-HA antibody. Talin polypeptides (482–636, 482–789, and 482–636 M (52)) that have a low affinity for vinculin remain diffusely distributed within the cell, whereas those that bind vinculin with high affinity (482–636 and 482–889) localize to the end of actin filaments in FAs.
with vinculin (62). Perhaps the junction between T-cells and antigen-presenting cells is not subject to actomyosin contractile forces.

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