Talin contains three similar vinculin-binding sites predicted to form an amphipathic helix

Mark D. BASS, Beverley J. SMITH, Sally A. PRIGENT and David R. CRITCHLEY¹

Department of Biochemistry, University of Leicester, University Road, Leicester LE1 7RH, U.K.

Using recombinant talin polypeptides and an SDS/PAGE–blot overlay assay, we have previously identified three regions of talin that are involved in binding to vinculin [Gilmore, Wood, Ohanian, Jackson, Patel, Rees, Hynes and Critchley (1993) *J. Cell Biol.* **122**, 337–347]. We have confirmed these observations by using a yeast two-hybrid assay and shown that talin residues 498–656, 852–950 and 1929–2029 are each capable of binding to vinculin residues 1–258. We have further defined the three vinculin-binding sites in talin to residues 607–636, 852–876 and 1944–1969; alignment of these sequences shows 59% similarity, although there are only two identical residues. Predictions of secondary structure indicate that this vinculin-binding motif forms an amphipathic α -helix. The hydrophobic face of helix 607–636 contains three aligned leucines (residues 608, 615 and 622), which show conservative substitutions in the other two

sites. To test the possibility that this might constitute a leucine zipper involved in vinculin binding, we mutated each leucine residue to an alanine. The results showed that this leucine repeat is not essential to the interaction between talin and vinculin. We also used the yeast two-hybrid system to define further the talin-binding site within vinculin residues 1–258. C-terminal deletions made in accordance with exon boundaries showed that vinculin residues 1–167 are capable of interacting with each of the three vinculin-binding sites in talin. However, all N-terminal deletions abolished binding. The results suggest that the talin-binding site in vinculin has a relatively complex fold, whereas the vinculin-binding motif in talin is contained within a short linear peptide sequence that is repeated three times in the talin rod domain.

Key words: focal adhesions, integrins, yeast two-hybrid assay.

INTRODUCTION

Many if not all members of the integrin family of cell adhesion molecules are linked to the actin cytoskeleton via a series of interacting cytoskeletal proteins including talin, vinculin and α -actinin [1]. Such a link provides a mechanism for coupling adhesion to the actomyosin contractile machinery and is therefore likely to be important in cell motility. In addition, there is evidence that the connection between integrins and the actin cytoskeleton is important in integrin signalling [2], and several signalling molecules have been shown to bind to cytoskeletal proteins including pp125 focal adhesion kinase, which binds to talin [3], and phosphatidylinositol 3' kinase [4] and novel protein kinase PKN [5], which bind to α -actinin, at least *in vitro*.

Current evidence suggests that talin has a pivotal role in the early stages of cell adhesion, including the formation of filopodia and lamellipodia. Talin accumulates at the tips of filopodia in *Dictyostelium discoideum* stimulated with cAMP [6] and inactivation of talin in neuronal growth cones inhibits filopodial extension [7]. In fibroblasts, talin is found in membrane ruffles as well as in focal adhesions [8,9], and talin is recruited to nascent focal adhesions before vinculin [10]. Down-regulation of talin expression in HeLa cells slowed down the rate of cell spreading and caused a decrease in the size of focal adhesions [11]; microinjection of antibodies against talin into fibroblasts led to the disruption of focal adhesions and actin stress fibres [9,12], as did certain talin polypeptides [13]. Finally, mouse embryonic stem cells in which both copies of the talin gene were disrupted showed extensive membrane blebbing, a decrease in cell polarity and an inability to form focal adhesions [14].

The biochemical properties of talin are consistent with its proposed role in linking integrins to the actin cytoskeleton. Talin is a dimeric elongated flexible protein [15,16] that can bind directly to β 1 [17,18] and β 3 [19] integrins; a putative talin-binding site in the β 1-integrin cytoplasmic domain has been identified [20]. These observations are consistent with experiments *in vivo* showing that integrins are key determinants of the subcellular localization of talin [21,22]. Talin is also an F-actin-binding protein [13,23,24] and is reported to possess activity in both actin nucleation [25] and actin bundling [26]. Therefore talin has the potential to link integrins directly to the actin cytoskeleton. However, talin also binds to the cytoskeletal protein vinculin [27–29], an actin-binding protein [30] that also contains binding sites for several other focal adhesion proteins including paxillin [31] and vasodilator-stimulated phosphoprotein (VASP) [32]. Vinculin can also bind to the actin-bundling protein α -actinin [33], which has been shown to bind to integrin cytoplasmic domain peptides *in vitro* [34]. These observations suggest that integrins might be linked to the actin cytoskeleton by more than one pathway.

As part of an effort to define further the role of talin in integrin-mediated cell adhesion, we have begun to map the binding sites in talin for its various binding partners, including actin and vinculin. We have previously provided evidence that there is more than one vinculin-binding site in talin [29]; in the present study we have used a yeast two-hybrid approach to define these sites further. The results show that the talin rod domain contains three similar vinculin-binding sites, each of which contains approx. 22 residues, and is predicted to form an amphipathic helix. In contrast, the talin-binding site in vinculin

Abbreviation used: VASP, vasodilator-stimulated phosphoprotein.

¹ To whom correspondence should be addressed (e-mail drc@le.ac.uk).

seems to be more complex and is contained within residues 1–167.

MATERIALS AND METHODS

Generation of talin and vinculin cDNA constructs in yeast two-hybrid expression vectors

Chicken talin and vinculin cDNA species were generated by PCR by using primers that contained unique restriction sites, allowing the PCR products to be cloned directionally into the appropriate yeast expression vector. Chicken talin cDNA species were amplified by using DNA from pGEX plasmids 54A, 23B and GG4A [29] (which encode residues 102–656, 950–1327 and 1646–2541) as template; PCR products were cloned into the yeast GAL4 transactivation domain expression vector pGAD GH (Clontech, Basingstoke, Hants., U.K.). A cDNA encoding talin residues 498–656 was cloned into the unique *EcoRI*/*Sall* sites in pGAD GH, whereas cDNA fragments encoding talin residues 852–950 and 1881–2042 were cloned into the *BamHI*/*EcoRI* sites. Chicken vinculin cDNA species were amplified from a cDNA encoding residues 1–398 in pGEX-1 [28] and were cloned into the *EcoRI*/*BamHI* sites of the yeast LexA DNA-binding domain expression vector pBTM116 [35]. The sequence of each construct was confirmed by automated DNA sequencing.

Yeast two-hybrid system

Yeast plasmids were transformed into the L40 strain of *Saccharomyces cerevisiae*. The genotype of the L40 reporter strain was *MATa ura3 his3 ade2 lys2 trp1 gal80 LYS2::GAL1-GAL1-HIS3-URA3::GAL4-CYC1-lacZ* [36]. Yeast transformed with both pBTM116 and pGAD GH plasmids were maintained on selective medium lacking leucine and tryptophan supplements. Positive interactions were identified by the ability of yeast to grow in the absence of a histidine supplement in the presence of 2 mM 3-amino-1,2,4-triazole, and by assaying for β -galactosidase expression. The level of β -galactosidase activity expressed in yeast grown on filter papers was assayed qualitatively by using 5-bromo-4-chloroindol-3-yl β -D-galactopyranoside as substrate [37]. Quantitative values for β -galactosidase activity were measured by using *O*-nitrophenyl β -D-galactopyranoside as substrate and yeast lysates made from liquid cultures [38]. For each interaction, lysates were made from 12 separate colonies and assayed for β -galactosidase activity. The activity (arbitrary units) was normalized within each experiment for the amount of yeast used, as estimated from a D_{600} reading. An [Arg¹⁸⁶]Ha-ras–LexA DNA-binding domain construct, and a byr2 (Ras-binding protein)–GAL4 transactivation domain construct (kindly provided by Dr M. H. Wigler, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, U.S.A.) [39], were used as a positive control. A human lamin C (residues 66–230)–LexA DNA-binding domain construct [36] was used to test for non-specific interactions with talin–GAL4 transactivation domain constructs. The byr2–GAL4 transactivation domain fusion construct was used to test for non-specific interactions with vinculin–LexA DNA-binding domain constructs.

Site-directed mutagenesis

Single amino acid substitutions were generated within talin residues 498–636 by using PCR-based site-directed mutagenesis and a talin cDNA in the pGAD GH vector as template. Each pair of primers was designed to introduce a single amino acid substitution and to introduce or destroy a restriction site at the point of mutation. Primer pairs contained a 20 nt overlap

covering the site of mutation, and 20 nt 3' overhangs to facilitate efficient priming of the PCR reactions. Linear copies of the complete plasmid were made with the Expand[™] Long Template PCR System (Boehringer Mannheim, Lewes, East Sussex, U.K.). Template DNA was destroyed by digesting PCR products with the methylation-specific restriction enzyme *DpnI*; the digest was transformed directly into the *Escherichia coli* strain DH5 α . Plasmids containing the required mutation were identified with the appropriate restriction endonuclease. To avoid the risk of introducing PCR errors into the plasmid, mutant talin cDNA species were excised and recloned into the pGAD GH vector. The sequence of each mutant was confirmed by automated DNA sequencing.

RESULTS

Identification of three separate vinculin-binding sites in talin

Using ¹²⁵I-vinculin, solid-phase binding assays and a series of overlapping talin polypeptides expressed in *E. coli*, we have previously provided evidence that there are at least three separate vinculin-binding sites in talin contained within residues 498–656, 852–950 and 1929–2029 [13,29]. To establish that each of these regions is indeed capable of binding vinculin, we used the yeast two-hybrid system. The talin-binding region of vinculin (residues 1–258) [28] was expressed as a LexA DNA-binding domain fusion protein; each of the proposed vinculin-binding sites in talin was expressed as a GAL4 transactivation domain fusion protein. Growth tests in the absence of a histidine supplement (Figure 1A) and β -galactosidase filter assays (results not shown) showed that all three talin polypeptides were able to interact with vinculin, activating the *HIS3* and *lacZ* reporter genes. These results were confirmed with the use of a β -galactosidase solution assay (Figure 1B); none of the talin or vinculin constructs gave a signal with control proteins. Interestingly, talin residues 1929–2029 consistently gave a stronger signal than talin residues 498–656 or 852–950.

Further definition of the three vinculin-binding sites in talin

To define further each of the three vinculin-binding sites in talin, we made a series of C-terminal deletion constructs that were tested for their ability to interact with vinculin residues 1–258 in the yeast two-hybrid system. Deletions were selected on the basis of conservation between chicken, mouse and *Caenorhabditis elegans* protein sequences, the predicted boundaries of the alanine-rich repeats found throughout the talin rod region [40] and the similarity of talin residues 1942–1957 to a vinculin-binding polypeptide identified by phage display [41]. The polypeptide encompassing talin residues 498–656 could be truncated to residue 636 without compromising binding to vinculin (Figure 2A). However, deletion to residue 607 completely abolished the interaction, suggesting that the vinculin-binding site lies within talin residues 607–636. To test this hypothesis, residues 607–636 were expressed as a GAL4 transactivation domain fusion protein and were found to interact with vinculin residues 1–258 expressed as a LexA DNA-binding-domain fusion protein. These results confirm that talin residues 607–636 contain a vinculin-binding site. Similar deletion analysis on the second vinculin-binding site in talin (residues 852–950) showed that it could be truncated to residue 876 without abolishing the interaction with the vinculin (Figure 2B). This defines a second short sequence in talin (residues 852–876) that is capable of binding vinculin. Several of the C-terminal deletions introduced within talin residues 498–656 and

Figure 1 Identification of three separate vinculin-binding sites in talin with the use of the yeast two-hybrid system

Talin polypeptides spanning residues 498–656, 852–950 and 1929–2029 were expressed as GAL4 transactivation domain fusion proteins and tested for binding to vinculin residues 1–258 (Vinc 1–258) expressed as a LexA DNA-binding domain fusion protein. Interactions were detected by the activation of *HIS3* and *lacZ* reporter genes. **(A)** The activity of the *HIS3* reporter was assayed by growing yeast on histidine-deficient agar. **(B)** Expression of the *lacZ* reporter gene was analysed by assaying relative β -galactosidase activity in yeast cell lysates. For each interaction, 12 lysates were tested; results are means $\pm$ S.E.M. In both assays the interactions between talin–GAL4 and vinculin–LexA fusion proteins (left-hand columns) were compared with the interactions between the talin–GAL4 and lamin–LexA fusion proteins used as negative controls (right-hand columns). The interaction between Ras–LexA and byr2–GAL4 fusion proteins was used as a positive control; the interaction between the vinculin–LexA and byr2–GAL4 fusion proteins was used as a further negative control.

852–950 resulted in increased binding to vinculin residues 1–258, most notably the truncations to residues 636 and 883 respectively. This might be due to the expression of more stable fusion proteins, although this remains to be established. A larger talin polypeptide spanning residues 1881–2042 was used for deletion analysis of the most C-terminal vinculin-binding site. Deletion to residue 1969 had no effect on the interaction with vinculin, but further deletion to residue 1950 completely abolished binding (Figure 2C). On the basis of these observations, a polypeptide spanning talin residues 1951–1969 was expressed in the yeast two-hybrid system but it failed to show an interaction with

Figure 2 Further definition of the three vinculin-binding sites in talin by deletion mutagenesis

Talin polypeptides spanning residues 498–656 **(A)**, 852–950 **(B)** and 1881–2042 **(C)** and their C-terminal deletion mutants, expressed in yeast as GAL4 transactivation domain fusion proteins, were tested for binding to vinculin residues 1–258 expressed as a LexA DNA-binding domain fusion protein. Interaction between the talin and vinculin polypeptides was monitored by assaying relative β -galactosidase activity in yeast cell lysates (left-hand columns). In this way the vinculin-binding sites were further localized to talin residues 607–636, 852–876 and 1944–1969. In addition, each of these short sequences was shown to be able to support an interaction with vinculin residues 1–258. Lamin–LexA (right-hand columns) and byr2–GAL4 fusion proteins were used as negative controls. For each interaction, 12 lysates were tested; results are means $\pm$ S.E.M.

vinculin. However, a slightly larger polypeptide spanning talin residues 1944–1969 was able to interact specifically with vinculin. The results of the above analysis define three short sequences in talin containing 25–30 residues that are able to interact with vinculin residues 1–258.

Figure 3 Sequence analysis of the three vinculin-binding sites in talin

(A) The three short vinculin-binding polypeptides (residues 607–636, 852–876 and 1944–1969) identified in chicken talin with the use of the yeast two-hybrid system, plus flanking sequences, were aligned by using CLUSTAL protein alignment software. Talin residues that have been shown to support vinculin binding are shaded. Identical (●) and similar (◐) residues are indicated above the alignment. The relationship between the vinculin-binding sites and the hydrophobic core of the alanine-rich repeat predicted by McLachlan et al. [40] is indicated, as is the proposed 22-residue putative vinculin-binding motif. (B) Each of the three short vinculin-binding polypeptides was arranged around a helical wheel to reveal an amphipathic helix with a basic face, and a hydrophobic face containing a seven-residue repeat that might form a putative leucine zipper (broken outline).

Structural predictions on the vinculin-binding sites in talin

The three vinculin-binding sites in talin were aligned by using the CLUSTAL protein alignment software; the results show that the sites are similar (Figure 3A). There is a 58% similarity between the 22 residues comprising the site, with two residues completely conserved. In contrast, a comparison of pairs of the alanine-rich repeats identified by McLachlan et al. [40] with each of the repeats containing vinculin-binding activity shows an average similarity of 25%, with a maximum similarity of 41%. This suggests that there is a vinculin-binding motif in talin that is repeated three times along the talin rod. The complete chicken talin amino acid sequence was analysed with the HeiDelberg PHD secondary-structure program, which predicted the protein to contain 69% α -helix. Within this sequence, the three vinculin-binding sites were predicted to have a 90% probability of forming an α -helix, with the exception of residues 627–636 within the N-terminal site. These residues extend beyond the aligned sites and do not seem to be part of the consensus vinculin-binding motif.

On the basis of these predictions, talin residues 607–628, 855–876 and 1948–1969 were arranged around helical wheels to establish the alignment of charged or hydrophobic residues along

Figure 4 Role of the leucine repeat within talin residues 498–636 in vinculin binding; the effect of Leu → Ala mutations

Helical wheel alignment of the N-terminal vinculin-binding site in talin (residues 607–629) suggests that Leu-608, Leu-615 and Leu-622 might be involved in a leucine zipper-like interaction with vinculin. To test this hypothesis, each leucine residue was mutated, both singly and in combination, to an alanine residue. Leu-623, which does not form part of the seven-residue repeat, was mutated to arginine as a control. The effects of the mutation on the interaction of this site with vinculin residues 1–258 were assessed by using the yeast two-hybrid system and a β -galactosidase solution assay (left-hand columns). Lamin–LexA (right-hand columns) and byr2–GAL4 fusion proteins were used as negative controls. For each interaction, 12 lysates were tested; results are means $\pm$ S.E.M.

the predicted α -helices (Figure 3B). Each of the core repeats in talin is predicted to form an amphipathic helix [40], a structure that is particularly evident in the vinculin-binding helices, which have a hydrophilic face containing predominantly basic residues. The helix predicted at residues 607–628 contains three leucine residues spaced at seven residue intervals that align down one side of the helix. Residues 852–876 contain two of the three leucine residues, with methionine replacing leucine at the third position. Residues 1944–1969 contain only one of the leucine residues, with valine at the other two positions.

Secondary-structure analysis of the talin-binding region in vinculin (residues 1–258) predicts a number of α -helices within this region. Helical wheel presentation reveals a number of aligned hydrophobic residues, mainly leucines, although substitutions to valine are again common (results not shown). The analysis raises the possibility that talin and vinculin contain potential leucine zipper motifs [42], which might be important in the interaction between the two proteins.

Site-directed mutagenesis of talin residues 498–636

To investigate the role of Leu-608, Leu-615 and Leu-622 within the N-terminal vinculin-binding site in talin, each was mutated individually and in combination to an alanine residue (Figure 4). Nucleotide substitutions were introduced into the talin cDNA encoding residues 498–636, and the effect of these mutations was analysed in the yeast two-hybrid system. Each of the individual amino acid substitutions L608A, L615A and L622A caused no loss of reporter gene activation when compared with the wild-type; the replacement of a fourth leucine residue (not part of the predicted leucine repeat) with an arginine residue (L623R) similarly caused no loss in reporter gene activity. The L608A, L622A double mutant led to a decrease in reporter gene activity but still showed a positive interaction with vinculin

Figure 5 Further definition of the talin-binding site in vinculin by deletion mutagenesis

The effects of both N-terminal and C-terminal deletions of the talin-binding site in vinculin were assessed by using the yeast two-hybrid assay. Vinculin polypeptides expressed as Lex A DNA-binding domain fusion proteins were co-expressed with GAL4 fusion proteins containing each of the three vinculin-binding sites in talin. The effects of the deletions on binding were assayed by using a β -galactosidase solution assay. **(A)** C-terminal deletions of vinculin residues 1–258 localize the talin-binding site to residues 1–167. **(B)** N-terminal deletions of vinculin residues 1–258 suggest that residues 57–258 might retain some capacity to bind to talin but that further deletion mutants showed no specific interaction with talin. For each interaction, 12 lysates were tested; results are means $\pm$ S.E.M. A byr2–GAL4 fusion protein was used as a negative control.

residues 1–258. The results suggest that Leu-608, Leu-615 and Leu-622 contained within the predicted amphipathic helix spanning talin residues 607–629 are not essential to vinculin binding.

Further definition of the talin-binding site in vinculin

By using ^{125}I -talin, a series of overlapping vinculin polypeptides expressed in *E. coli*, and blot overlay assays, we have previously shown that vinculin residues 1–258 contain the talin-binding site [28]. To define this site further, we used the yeast two-hybrid system to analyse the effect of both N-terminal and C-terminal deletions on binding to each of the three vinculin-binding sites in talin. Deletions were made in accordance with exon boundaries [43]. C-terminal deletions to vinculin residues 208 and 167 failed to compromise binding to any of the three vinculin-binding sites in talin (Figure 5A), indicating that vinculin residues 1–167 contain a complete talin-binding site. N-terminal deletions to residues 57, 131 and 167 all caused the loss of a specific interaction with each of the vinculin-binding sites in talin (Figure 5B).

Although there was some residual binding to talin, it seemed to be non-specific because β -galactosidase filter assays showed that these constructs also bound weakly to byr2, which was used as a control.

DISCUSSION

In a previous study we provided evidence for three vinculin-binding sites in talin; they were mapped by deletion mutagenesis to residues 498–656, 852–950 and 1929–2029 [13,29]. The analysis was based on the use of recombinant talin polypeptides expressed in *E. coli*, and an SDS/PAGE–blot overlay assay. Here we have used yeast two-hybrid technology to confirm that the above sequences contain vinculin-binding sites; we have further defined these sites to residues 607–636, 852–876 and 1944–1969. The sites are similar and are predicted to form an amphipathic helix. We have taken a similar approach in an attempt to define further the talin-binding site(s) in vinculin, previously mapped to residues 1–258 with the use of recombinant vinculin polypeptides and solid-phase binding assays [28]. Although we have established that vinculin residues 1–167 can interact with all three sites in talin, further deletions led to a loss of activity, suggesting that the talin-binding site in vinculin is not a simple linear peptide sequence.

Alignment of the above talin sequences suggests that the vinculin-binding motif can be further localized to residues 607–628, 855–876 and 1948–1969 (Figure 3A). The three sites, which contain 22 amino acids, show 58% similarity; however, they have only two identical residues, leucine and alanine. Cross-species comparisons indicate that the vinculin-binding sites identified in chicken talin show no greater degree of sequence conservation than the whole protein. Of the residues that are conserved between species, most are hydrophobic. The leucine residue found in all three vinculin-binding sites in chicken talin is totally conserved in mouse and *C. elegans* talin. The alanine residue is conserved in two of the three sites but is replaced by threonine in site 1 in *C. elegans*. In addition, it is noteworthy that the sequence VLAAL in site 3 is identical in all three species (Figure 6). We have considered the possibility that the conserved hydrophobic residues are important determinants of the interaction between talin and vinculin. More specifically, we have investigated the idea that the leucine repeat in site 1 might be involved in a leucine-zipper-type interaction with vinculin, which is also predicted to contain several amphipathic helices with candidate leucine repeats. However, we have demonstrated that the leucine repeat in site 1 in chicken talin is not essential for binding to vinculin residues 1–258. We conclude that these conserved leucine residues are probably important in defining the overall secondary and tertiary structure of the vinculin-binding sites, and that the less conserved hydrophilic residues are more likely to be responsible for the interaction with vinculin.

McLachlan et al. [40] proposed that the talin rod is composed of 50–60 alanine-rich repeats, each containing approx. 34 residues. The repeat is predicted to contain a short leader sequence rich in prolines and acidic residues, a conserved hydrophobic core of approx. 21 residues predicted to form an amphipathic helix, and a highly variable linker that joins to the next repeat. The hydrophobic face of the core has a characteristic pattern of large and small hydrophobic residues (mainly leucine and alanine) repeated every seven residues and is envisaged to participate in the packing of the helices along the talin rod. The conserved leucine residue at the N-terminus of all three vinculin-binding sites forms the start of the core of repeats 5, 13 and 47 predicted by McLachlan et al. [40]; the C-terminus of each of the binding sites coincides with the boundary between the core and

Figure 6 Alignment of the vinculin-binding sites in chicken talin

Sequence conservation of the vinculin-binding sites in talin, for chicken (G.g.) [13], mouse (M.m.) [58], nematode (C.e.) [22] and *D. discoideum* (D.d.) [6]. The sequences were aligned by using the CLUSTAL program; conserved residues were highlighted by using the BOXSHADE program. Site 1 is compared with α -catenin [47] and a sequence towards the C-terminal region of chicken talin. The relationship between site 3 and a vinculin-binding peptide identified by phage display [41] is shown.

Figure 7 Domain structure of talin

(A) The domain structure of talin, showing the positions of the binding sites for pp125 focal adhesion kinase, layilin [3], vinculin [27-29] and F-actin [13,23,24]. (B) The possible relationship between the three vinculin-binding sites and the C-terminal actin-binding site in a talin homodimer. Vinculin is shown as a trimer cross-linking the talin subunits to each other and to F-actin.

the linker region (Figure 3A). The predicted amphipathic helices formed by these repeats in chicken talin have a predominantly basic face. Interestingly, vinculin residues 1-167 have a pI of 4.9, suggesting that the interaction between vinculin and talin might

involve electrostatic interactions, although we do not exclude a contribution from hydrophobic residues.

Apart from talin, other proteins have been reported to bind to the 90 kDa vinculin head, including the vinculin C-terminal tail [44], α -actinin [45] and α -catenin [46]. Interestingly, the most N-terminal vinculin-binding site in chicken talin showed 48% identity (68% similarity) with residues 818-842 in chicken α -catenin (Figure 6) [47]. Two regions of α -catenin have been shown to bind vinculin, one of which (residues 697-906) contains the above region of similarity [46]. The α -catenin sequence contains the conserved leucine and alanine residues found in all three vinculin-binding sites in talin. This suggests that α -catenin might recognize the same site in vinculin as talin; it will be interesting to see whether these two proteins compete for binding to vinculin. Although vinculin is found in both cell-cell and cell-matrix adherens-type junctions [48], α -catenin is found only in the former [47] and talin in the latter [48], so these proteins are unlikely to compete for binding to vinculin *in vivo*. BLAST searches for other proteins containing sequences similar to the vinculin-binding sites in talin resulted in no hits, although a match was revealed with talin residues 2344-2369 (50% identity, 73% similarity to site 1). Interestingly, a glutathione S-transferase fusion protein containing talin residues 2269-2541 showed weak vinculin-binding activity in an SDS/PAGE-blot overlay assay but failed to bind in a microtitre plate binding assay [29]. The vinculin-binding peptide identified by phage display [41] shows some similarity to the third vinculin-binding site in talin. Experiments with mutant peptides indicate that the residues ARRVS might have an important role in the binding of vinculin to this site, although this sequence is not particularly highly conserved across species (Figure 6).

Previous studies with recombinant vinculin polypeptides and a number of different solid-phase binding assays have shown that the talin-binding site in vinculin is located within residues 1-258 [28]. This is a highly conserved region of the protein encoded by exons 1-6. However, attempts to define this site further by using the above assays proved unsuccessful; both N-terminal deletions and internal deletions made in accordance with exon boundaries abolished binding to talin [28]. Similarly, we have found that the deletion of N-terminal sequences in vinculin abolished binding to talin by using the yeast two-hybrid assay, although C-terminal deletions allowed us to localize the site further to residues 1-167. The results suggest that the talin-binding site in vinculin is not defined by a short peptide sequence.

The positions of the three vinculin-binding sites in talin relative to those for other vinculin-binding proteins are shown in Figure 7(A). Whether the three sites are equivalent and serve precisely the same function is unknown. We have determined the K_d for vinculin binding to glutathione S-transferase-talin fusion proteins containing residues 565-1328 and 1646-2541: the values were similar ($K_d \approx 100$ nM) [29]. Two of the sites (residues 607-629 and 852-876) are close together in the N-terminal half of talin, whereas the other (residues 1944-1969) is towards the C-terminal region of the molecule. Bearing in mind that talin can exist as an anti-parallel dimer [15,49], sites 1 and 2 might be close to site 3 in the adjacent subunit (Figure 7B). Vinculin has been proposed to form homodimers and trimers that might be stabilized by the binding of VASP to the Phe-(Pro)₄ motif within the proline-rich region of vinculin [50]. Such vinculin-VASP complexes might be able to bind to sites in both talin subunits, cross-linking the talin dimer. Our previous results provided evidence that talin also has more than one actin-binding site [13], although the best characterized of these is that towards the C-terminus of the protein [9,13,24]. In an anti-parallel dimer the C-terminal actin-binding site in talin would be close to the vinculin-

binding sites (Figure 7B). Vinculin is also an actin-binding protein [51] and it is possible that vinculin might serve to cross-link, and thereby stabilize, the interaction between talin and actin.

Such a role for vinculin in integrin-mediated cell adhesion is consistent with a number of observations. Microinjection of a monoclonal antibody against vinculin led to the disruption of actin stress fibres and focal adhesions in fibroblasts [52]. Similar effects were seen when a talin polypeptide containing the C-terminal vinculin-binding site was microinjected into fibroblasts, whereas a talin polypeptide containing just the C-terminal actin-binding site lacked such effects [13]. Moreover, the down-regulation of vinculin in Balb/c 3T3 cells with the use of anti-sense technology resulted in cells that were less well spread, assembled smaller focal adhesions and were more motile [53], whereas over-expression of vinculin in the same cell line led to the assembly of larger focal adhesions and a lower motility of cells [54]. However, gene disruption studies have shown that, whereas talin is essential to the assembly of focal adhesions in mouse embryonic stem cells, vinculin is not [14]. Neither is vinculin essential for focal adhesion assembly in mouse F9 embryonic carcinoma cells, although the F9 cells were less well spread and showed decreased adhesion to laminin and fibronectin [55]. The results support a hypothesis in which talin acts as the key link between integrins and the actin cytoskeleton, whereas vinculin stabilizes focal adhesions possibly by cross-linking talin to F-actin. The fact that the ability of vinculin to bind talin and actin can be regulated by $\text{PtdIns}(4,5)\text{P}_2$ [56,57] suggests that vinculin might have a key role in modulating cell adhesion and cell motility.

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