Isolation of peptides from phage-displayed random peptide libraries that interact with the talin-binding domain of vinculin

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Peptides isolated from combinatorial libraries typically interact with, and thus help to characterize, biologically relevant binding domains of target proteins. To characterize the binding domains of the focal adhesion protein vinculin, vinculin-binding peptides were isolated from two phage-displayed random peptide libraries. Altogether, five non-similar vinculin-binding peptides were identified. Despite the lack of obvious sequence similarity between the peptides, binding and competition studies indicated that all five interact with the talin-binding domain of vinculin and do not disrupt the binding of α-actinin or paxillin to vinculin. The identified peptides and talin bind to vinculin in a comparable manner; both bind to immobilized vinculin, but neither binds to soluble vinculin unless the C-terminus of vinculin has been deleted. An analysis of amino acid variants of one of the peptides has revealed three non-contiguous motifs that also occur in the region of talin previously demonstrated to bind vinculin. Amino acid substitutions within a 127-residue segment of talin capable of binding vinculin confirmed the importance of two of the motifs and suggest that residues critical for binding are within a 16-residue region. This study demonstrates that the vinculin-binding peptides interact with vinculin in a biologically relevant manner and represent an excellent tool for further study of the biochemistry of vinculin.

INTRODUCTION

At the cytoplasmic face of the cell membrane, focal adhesions anchor integrins to the actin cytoskeleton. A number of focal adhesion-associated proteins have been identified, including α-actinin, focal adhesion kinase, paxillin, talin, tensin, vinculin and zyxin. Molecular interactions between these proteins have been proposed to regulate cell adhesion and modulate such processes as cell growth, differentiation, wound healing and tumour metastasis [1–5]. In this study we have used peptides isolated from phage-displayed random peptide libraries to investigate the protein–protein interactions of the focal adhesion protein vinculin. Central to this strategy is the observation that peptides isolated by affinity selection from such libraries typically interact with biologically relevant domains of the target proteins [6,7]. For example, peptides selected with antibodies [8–11], streptavidin [12], Src [13–16], integrins [17], calmodulin [18,19] or receptors [20] have been shown to be either antagonists or agonists of these proteins.

We have identified five different peptide sequences from phage-displayed random peptide libraries that bind to the cytoskeletal protein vinculin. Gel overlay experiments of proteins present in cell lysates revealed that the peptides are highly specific for vinculin. To dissect the binding of the peptides to vinculin we performed saturation mutagenesis on one sequence and identified three motifs important for vinculin binding. Binding studies showed that the peptides interact with the talin-binding domain of vinculin in a manner comparable to talin. Use of these peptides in intact cells, either as blocking reagents or as fusions to other proteins to impart novel functions, should provide a wealth of additional information.

MATERIALS AND METHODS

Materials

General biochemical reagents were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Vinculin, talin, paxillin and α-actinin were purified to various degrees by DEAE-cellulose column chromatography of chicken gizzard extracts, as previously described [21]. Peptides (pC, pV, pVR and pT) were synthesized by standard fluoren-9-ylmethoxycarbonyl chemistry by Dr. J. Mark Carter (Cytogen Corporation, Princeton, NJ, U.S.A.), with biotin added to their N-termini.

Screening of phage-displayed random peptide libraries

Vinculin (1 μg) was adsorbed on non-adjacent wells of a 96-well polystyrene mictotitre plate, non-specific binding sites were blocked with 1.0% (w/v) BSA, and then 10^{11} phage particles in wash buffer [PBS (pH 7.5)/0.1% Tween-20/0.1% BSA] were incubated for 4 h at room temperature. Two bacteriophage M13 libraries, displaying either 22 [15] or 26 [19] random amino acid residues at the N-terminus of protein III, were screened for vinculin-binding phages. Non-bound phages were removed from the wells by repeated washes and the bound phages were eluted with 50 mM glycine/HCl, pH 2.0. After neutralization of the pH, the recovered phages were amplified in DH5αF′ *Escherichia coli* cells for 6 h; the resulting phages were screened two additional times. The recovered phages were finally plated at a limiting concentration to yield individual plaques for clonal analysis [22].

Quantification of phage–vinculin and peptide–vinculin interactions

Plaques, representing single clones, were amplified in DH5αF′ cells and the resulting phage particles were evaluated for binding to vinculin-coated microtitre plate wells by ELISA, using a horseradish peroxidase-coupled anti-M13 antibody in accordance with the manufacturer's instructions (Pharmacia, Piscataway, NJ, U.S.A.). The sequences of the phage-displayed peptides

Abbreviations used: GST, glutathione S-transferase; SaAP, streptavidin-conjugated alkaline phosphatase.

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were deduced by sequencing both DNA strands of the appropriate region in the viral genome.

For the detection of peptide–vinculin interactions on membranes, protein samples were resolved by SDS/PAGE, transferred to PVDF membranes (Millipore, Bedford, MA, U.S.A.), blocked with 0.1 % poly(vinyl alcohol) and then incubated with individual peptides precomplexed with streptavidin-conjugated alkaline phosphatase (SaAP) in wash buffer. The membranes were washed four times (15 min each at room temperature); bound SaAP–peptide complexes were detected with Nitroblue Tetrazolium chloride and 5-bromo-4-chloro-3-indoyl phosphate *p*-toluidine salt in 0.1 M Tris/HCl (pH 9.4)/0.1 M NaCl/50 mM $MgCl_2$. A similar protocol was used to monitor peptide–vinculin interactions in microtitre plate wells, except that alkaline phosphatase activity was detected with *p*-nitrophenyl phosphate and measured spectrophotometrically.

Expression and purification of glutathione S-transferase (GST)–vinculin fusion proteins

The plasmids V1-252, V1-430, V811-1066 and V1-1066 (gifts from Dr. Susan Craig, Johns Hopkins University, Baltimore, MD, U.S.A.) and V431-850 (constructed in the same manner) were transformed into *E. coli* LE392 cells, grown to mid-exponential growth phase, and expression of the fusion protein was induced with 0.05 % isopropyl β-D-thiogalactoside for 4 h. The GST fusion proteins were purified by affinity chromatography with glutathione–agarose and recovered by either thrombin cleavage or glutathione elution [23].

Phage binding and competition experiments

Interactions between isolated phages and vinculin were monitored by coating microtitre plates wells with vinculin (see above) and following the binding of phage particles by ELISA (see above). For competition studies on the interaction, wells were preincubated in wash buffer containing the appropriate peptide (10 μM) for 10 min before the addition of phage particles. The binding of various cytoskeletal proteins to immobilized vinculin was determined in a similar manner except that protein fractions from an anion-exchange column (see above) enriched for individual chicken gizzard proteins (approx. 2 μg per well) were added instead of phage particles. The binding of these proteins was followed immunologically with a polyclonal anti-talin antibody (gift from Dr. Keith Burridge, University of North Carolina at Chapel Hill, Chapel Hill, NC, U.S.A.), a monoclonal anti-paxillin antibody (Transduction Labs, Lexington, KY, U.S.A.) or a polyclonal anti-α-actinin antibody (Sigma) and detected with the appropriate alkaline phosphatase-linked secondary antibodies.

Detection of peptide–vinculin interactions in solution

Biotinylated peptide (0.3 μg) was incubated with approx. 2 μg of purified protein in 0.5 ml of wash buffer for 1 h at room temperature. Peptide–protein complexes were recovered by incubating the mixtures for 30 min with 10 μl of streptavidin-coated magnetic beads (PerSeptive Diagnostics, Cambridge, MA, U.S.A.). The beads were washed once in wash buffer and once in PBS/0.1 % Tween-20; the bound proteins were then eluted at 100 °C in 20 μl of gel-loading dye [24]. The beads were removed by centrifugation and the proteins in the supernatant were resolved by SDS/PAGE [8 % (w/v) gel] and detected by Coomassie Blue staining.

Binding studies with wild-type and mutant segments of mouse talin

Segments of mouse talin were generated by PCR from a mouse fibroblast cDNA library (gift from Dr. Terry Van Dyke, University of North Carolina at Chapel Hill, Chapel Hill, NC, U.S.A.) and cloned into the SP35T vector, which contains the 5′ and 3′ untranslated sequences of rabbit β-globin mRNA and an SP6 RNA polymerase promoter. [^{35}S]Methionine-labelled talin fragments were expressed *in vitro* in a transcription-coupled translation expression system in accordance with the manufacturer's instructions (Promega, Madison, WI, U.S.A.). Site-directed mutagenesis was performed as described [25]. To microtitre plate wells coated with vinculin, wild-type and mutant talin fragments translated *in vitro* were added in wash buffer. After 4 h of incubation the wells were washed and the contents removed with gel-loading buffer for evaluation by SDS/PAGE and quantification with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.). A more detailed description is available via the Internet (http://www.unc.edu/depts/biology/kayref.html).

RESULTS

Isolation of vinculin-binding phages from random peptide libraries

To identify peptides that bound to the focal adhesion protein vinculin, bacteriophage M13 libraries displaying 22- and 26-residue random peptides were screened by affinity selection. After three rounds of screening with microtitre plate wells coated with chicken gizzard vinculin, recovered phages were isolated, amplified and their binding properties evaluated. The phage isolates seemed to bind specifically to vinculin and not to a variety of other proteins (results not shown). Sequence analysis of the isolates showed that they displayed one of five different peptides, without obvious sequence similarity (Figure 1).

Competition of peptide with talin for binding to vinculin

Vinculin interacts with a number of focal adhesion-associated proteins including α-actinin [26,27], actin [28,29], paxillin [30,31], talin [32,33], tensin [34] and itself [32]. To evaluate whether the peptides interacted with vinculin in a biologically relevant manner, a competition experiment was performed to determine whether a vinculin-binding peptide could disrupt the interaction

Vin.12-1	ST GGFD **DVY** DWAR G VSSA **LTTTL** VA TR
Vin.12-1R	ST GGFD **DVY** DW **ARRVS** SA **LTTTL** VA TR
Vin.26b-2	SR GVNFSEWLYDMS A AMKEASNVFPSRR SR
Vin.26d-1	SS QNWDMEAG **VEDLT** A AMLGLLSTIHSS SR
Vin.26d-2	SS PSLYTQFLVNYES A ATRIQDLLIASRP SR
Vin.26d-3	SS TGWVDLLGALQRA A DATRTSIPPSLQN SR

Figure 1 Amino acid sequences of vinculin-binding peptides

Vinculin-binding phages were isolated from the T12 (Vin.12-1) and the R26 (Vin.26b-2, Vin.26d-1, Vin.26d-2 and Vin.26d-3) random peptide libraries displayed at the N-terminus of protein III of bacteriophage M13. Single underlined positions indicate residues that were fixed in each library. Residues shown in bold indicate sequence motifs shared with talin (see Figure 6). Phages Vin.26b-2 and Vin.26d-1 are shorter than Vin.26d-2 and Vin.26d-3 owing to single-residue deletions, which most probably occurred during assembly and cloning of the oligonucleotides. The sequence labelled Vin.12-1R corresponds to an amino acid variant of Vin.12-1 with improved binding properties, where the central glycine residue has been replaced by arginine (double underline).

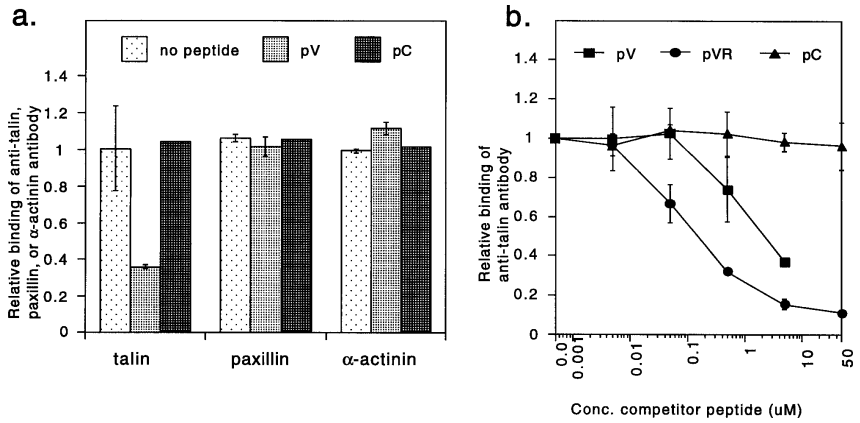

Figure 2 pV, the peptide displayed by Vin.12-1, competes with talin, but not paxillin or α-actinin, in binding vinculin

Chicken vinculin was immobilized on microtitre plate wells, preincubated with competitor peptide and then incubated with chromatographic fractions containing talin, paxillin or α-actinin. After repeated washes, the amount of protein bound to the wells was determined with the appropriate primary and enzyme-linked secondary antibodies. (**a**) peptide pV (biotin-STGGFDDVYDWARGVSSALTTTLVATR) (5 μM) disrupts the talin–vinculin interaction but not the paxillin–vinculin or the α-actinin–vinculin interactions. The same concentration of an unrelated competitor peptide, pC (biotin-STVPRWIEDSLRGGAARAQTRLASATR), had no effect. (**b**) pV and pVR (biotin-STGGFDDVYDWARRVSSALTTTLVATR) compete with talin for binding to immobilized vinculin in a concentration-dependent manner. The average of two independent determinations is plotted; the range is indicated by error bars.

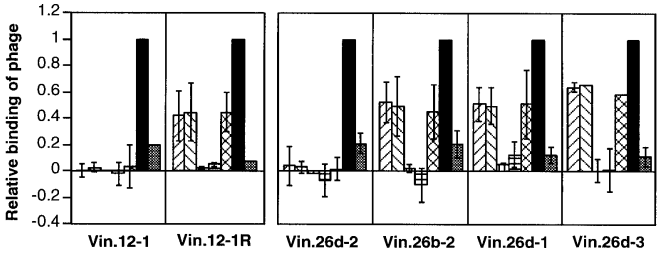

Figure 3 All five peptides recognize the same site on vinculin

The vinculin-binding phages were examined for binding to various segments of vinculin by ELISA. Segments of vinculin [residues 1–252 (▨), 1–430 (▧), 431–850 (▥), 811–1066 (▤) and 1–1066 (▩)] were expressed in *E. coli* as GST fusion proteins [49]. ■, Full-length vinculin isolated from chicken gizzard. ▨, Wells preincubated with 10 μM pVR before the addition of phages. (Binding was not affected by preincubation with a similar amount of the unrelated peptide pC.) Column heights represent the average relative spectrophotometric signal of three separate determinations, with the error bars representing the range. The values were adjusted by subtracting the background signal from BSA-coated wells and scaling the values to that of the chicken-gizzard vinculin samples.

of vinculin with any of these cytoskeletal proteins. Protein preparations enriched for talin, paxillin or α-actinin were added to microtitre plate wells containing immobilized vinculin and the amount of bound talin, paxillin or α-actinin was determined by ELISA. Figure 2(a) shows that a peptide (pV), corresponding to the insert of phage Vin.12-1, disrupts the interaction of vinculin with talin but not with paxillin or α-actinin. We interpret this result to suggest that the peptide binds at or near the talin-binding domain of vinculin and not to the sites where paxillin and α-actinin bind.

Localization of peptide-binding sites on vinculin

To map the binding site(s) of the five phages, we evaluated their binding to different segments of vinculin. As seen in Figure 3, three of the phages bound to every fragment that contained the N-terminal 252 amino acid residues, corresponding to the talin-binding domain of vinculin [3,35]. Whereas the two other phages bound immobilized vinculin, they did not bind to any of the *E. coli*-expressed fragments (Figure 3). A single amino acid variant (see below) of the peptide sequence displayed by phage Vin.12-1 was found to promote binding to vinculin fragments. When the soluble form of this peptide (pVR) was added to the microtitre wells, it was observed to be an effective inhibitor of binding of all five phages to full-length vinculin. In contrast, pVR did not disrupt the paxillin–vinculin or the α-actinin–vinculin interactions (results not shown). We conclude that all five vinculin-binding phages bind to the same general site in vinculin.

Identification of amino acid substitutions in pV that affect binding to vinculin

To determine which amino acid residues in the pV sequence contribute to vinculin-binding, a library displaying 10^6 variants of the Vin.12-1 peptide was constructed [36]. The phage-display system is a useful one for generating and evaluating the binding properties of amino acid variants [37,38]. Phage isolates were selected at random from the library, tested for binding to immobilized vinculin and sequenced. Single amino acid substitutions that disrupted binding tended to occur throughout the length of the peptide, whereas most non-disruptive substitutions were clustered at the N-terminus (Figure 4). These results indicate that a significant portion of the peptide sequence is involved in vinculin binding.

To identify improved binding peptides from the library of variants, the evolved library was subjected to one round of screening with a decreased amount of vinculin immobilized on microtitre plates. A number of recovered phages were confirmed to have improved binding strength to vinculin. Sequence analysis of those isolates revealed that amino acid substitutions occurred at a small number of positions, of which the most common was the replacement of the central fixed glycine residue (Figure 1) by an arginine (e.g. Vin.12-1R, pVR).

The concentration dependence of the peptides pV and pVR on the disruption of the talin–vinculin interaction was determined. Figure 2(b) shows that pVR is a more potent inhibitor than pV,

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Non-binders
....C......................
........CE.................
..........C...*............
.......E................*..
.......A...........*.......
..D.....Q..................
......YE....K..............
....LY........I.......*....
...........S..I............
...EV........R...P...*.....
..................H........
.................P.P.*.....
........D...........I.-....
..................*.P......
.....*..............S.V....
........*.............V....
......................V....
.......*................V..
...*....................T..
........................P..
STGGFDDVYDWARGVSSALTTTLVATR
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     Binders
     ...*L..*...................
     ....L...F..................
     .....E.....................
     .....H.....................
     ..R......A.................
     .....VN.*Y.................
     ..........L.....*....*.....
     ................A......*...
     ..S...Y......*.*....A......
     Improved binders
     ....................A......
     ....................A......
     .......................L...
     .............A.........L...
     ...*.A.......R.T...........
     ..**.VL......E.............
pVR= .............R.............
     ........F....R.............
     ...E....FH..........*......
     .........Y......*......*...
     STGGFDDVYDWARGVSSALTTTLVATR
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Figure 4 Saturation mutagenesis of the pV sequence

A library of phages displaying amino acid variants of the pV peptide was constructed according to published procedures [36]. Phage isolates were picked at random, sequenced and tested for binding to vinculin in microtitre plate wells by ELISA. Isolates classified as 'binders' produced a signal at least half as strong as the original Vin.12-1 phage, whereas 'non-binders' produced a signal one-tenth as strong as the parent clone. To identify improved binding peptides, the library was subjected to one round of affinity selection with a small of amount of vinculin (0.05 μg per well) and output clones were tested as described above. Most isolates from this screen produced signals up to 5-fold stronger than that of the parent phage. The peptide displayed by improved binding phage, Vin.12-1R (pVR), is used in several experiments in this paper. Primary structures of the various peptides are shown relative to pV (bottom): full points indicate identity, amino acid differences are indicated with capital letters, asterisks indicate silent nucleic acid substitutions and underlined positions indicate residues fixed in the original T12 library.

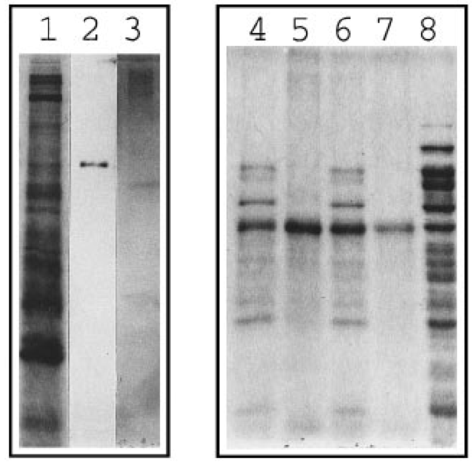

Figure 5 pVR peptide recognizes vinculin in gel overlays and in solution

A whole-cell lysate from chicken embryo fibroblast cells was resolved by SDS/PAGE and revealed by Coomassie Blue staining (lane 1). The same lysate was also transferred to a PVDF membrane by standard blotting techniques. The immobilized proteins were detected with pVR precomplexed to SaAP and then revealed with the chromogenic reagents Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (lane 2). In lane 3 the blot was pretreated with a 100-fold excess of pVR peptide precomplexed to streptavidin; this treatment blocks the interaction of pVR precomplexed to SaAP, as detected with Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Lanes 4–8 represent samples resolved by SDS/PAGE and stained with Coomassie Blue. Lane 8 contains bacterially expressed vinculin fragments from the full-length vinculin GST fusion construct, V1-1066. These proteolytically cleaved fragments were truncated at their C-termini because only fragments fused to the N-terminal GST protein would be recovered by glutathione–agarose chromatography. Lanes 4–7 are the subset of those fragments recovered with streptavidin-coated magnetic beads after incubation in solution with biotinylated pV peptide and (lane 4) no additional peptide, (lane 5) a 100-fold excess of non-biotinylated pV, (lane 6) a 100-fold excess of an unrelated non-biotinylated peptide pC, or (lane 7) an unrelated biotinylated peptide pC. The band common to lanes 4–8 is BSA, as it was present in the wash solution.

with an IC_{50} of approx. 100 nM. This result supports the observation that the glycine–arginine substitution in Vin.12-1 improves the apparent binding strength of the peptide to vinculin.

Detection of vinculin in a cell lysate with a vinculin-binding peptide

To examine the specificity of the peptide–vinculin interaction, whole-cell lysates from chicken embryo fibroblast cells were resolved by SDS/PAGE, transferred to a PVDF membrane and incubated with SaAP complexed to pVR (Figure 5). The complexed peptide reacted strongly with a 130 kDa species, with no observable cross-reactivity with any other protein (lane 2). This species is likely to be vinculin, as demonstrated by positive reaction with purified vinculin in other blots (results not shown). The binding of the pVR peptide to the 130 kDa is saturable, as its detection can be competed by excess pVR. These results demonstrate that peptides can be used as detection reagents in a gel overlay.

Interaction of the vinculin-binding peptide with soluble vinculin

Although a strong interaction between the vinculin-binding peptides and immobilized vinculin is readily observed, we were unable to detect an interaction between the peptides and soluble vinculin (results not shown). To investigate this paradox, we tested whether the peptides could bind soluble fragments of vinculin expressed in *E. coli*. Figure 5 demonstrates that only proteolytically truncated fragments of vinculin bind to the pVR peptide. Because vinculin is fused at its N-terminus to GST, the proteolytic fragments that are purified by glutathione–agarose chromatography are probably truncated at the C-terminus. These results, consistent with previous studies [39], indicate that the C-terminus of vinculin interacts with the talin-binding domain in the N-terminus and excludes binding of the pVR peptide.

Sequence comparison of the vinculin-binding peptides with known vinculin-binding proteins

Although there is little sequence similarity between the five vinculin-binding peptides, sequence comparisons between the peptides and talin revealed two segments of similarity (Figure 6). The first segment is composed of two five-residue peptide motifs: LTTTL from Vin.12-1 and VEDLT from Vin.26d-1 matched residues 1807–1814 (VEDLTTTL) from talin [40]. The second segment is composed of five- and three-residue peptide motifs (DVY and ARRVS, both in Vin.12-1R, termed the DVY/ARRVS motif), which occurred within a 16-residue segment of talin.

To determine whether the motifs shared by talin and the phages are involved in binding vinculin, a number of experiments were performed. First, a short peptide of talin (pT), containing the sequence VEDLTTTL, was synthesized and found not to bind either immobilized or soluble vinculin (results not shown). Secondly, segments of the mouse talin cDNA were amplified by PCR, cloned and expressed *in vitro*. The resulting 378-residue (1702–2079) and 127-residue (1891–2017) fragments of talin, termed Tal378 and Tal127 respectively, had the capacity to bind

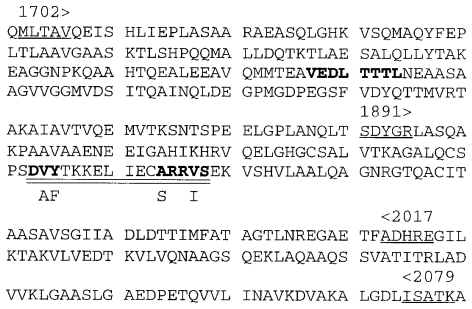

Figure 6 Mouse talin, sites of oligonucleotides for PCR amplification, sequence motifs and sites of mutations

Segments of the mouse talin coding [40] were amplified by PCR from a mouse fibroblast cDNA library by using the oligonucleotides 1702 and 2079, or 1891 and 2017 (numbers correspond to the positions of the amino acid residues encoded by the oligonucleotides, underlined). These segments, encoding 378- and 127-residue fragments of mouse talin respectively, were cloned and expressed *in vitro* in a transcription-coupled translation system to generate the ^{35}S-labelled Tal378 and Tal127 polypeptides (see Table 1). Residues in bold type correspond to the three motifs described in the text present in several of the vinculin-binding phages. Amino acid substitutions incorporated into the Tal127 fragments are shown within a 16-residue region (double underline), generating the ΔAFSI polypeptide.

Table 1 Relative binding of [^{35}S]Met-labelled talin fragments translated *in vitro* to immobilized vinculin

Bound products were eluted with SDS/dithiothreitol, resolved by electrophoresis and quantified with a Phosphorimager. Abbreviation: n.d., not done.

	Relative binding (%)			
	Competitor peptide (5 μM)			
Talin fragment	None	pVR	pT	Mutation ΔAFSI
Tal378	100	14	101	n.d.
Tal127	100	9	118	32*

* Average of three independent determinations.

immobilized vinculin. Their binding, however, could be blocked with the pVR, but not the pT (biotin-SGSGRTEAVEDLTTTL-NEAASK), peptide (Table 1). Thirdly, to assess the significance of the DVY/ARRVS motif, site-directed mutagenesis was used to create four amino acid substitutions within the motif. This mutant form of the 127-residue segment, termed ΔAFSI, showed decreased binding, suggesting that the DVY/ARRVS motif of talin is involved in binding to vinculin.

DISCUSSION

In this study, five peptides were isolated from phage-displayed random peptide libraries that bound to the focal adhesion protein vinculin. Binding and competition experiments showed that all five peptides interacted with the talin-binding domain of vinculin. Curiously, the peptides lacked sequence similarity. This finding is reminiscent of the discovery of mimotopes [41], where short peptides bind to the antigen-binding site of antibodies even though they differ in sequence from the antigen. Presumably either the tertiary structures of the five peptides are similar, even though their primary structures differ, or the five peptides bind at neighbouring sites on vinculin.

The inability of the vinculin-binding peptides to disrupt the interaction of vinculin with α-actinin or paxillin suggests that these interactions occur at distinct sites. This result is consistent with previous studies that mapped vinculin's α-actinin-, talin- and paxillin-binding domains to the N-terminal 107 [27] and 252 amino acid residues [35,42] and to the C-terminus [43] respectively. Our work extends these results by demonstrating that talin and α-actinin bind vinculin at distinct sites.

A particularly interesting finding of this study was that although the peptides could bind immobilized vinculin they did not seem to bind full-length vinculin in solution. However, if the C-terminus was truncated from soluble vinculin, their binding was restored. These results are consistent with previous studies showing that both the talin-and actin-binding domains of vinculin are masked by an intramolecular interaction between the N- and C-termini [39,44] and that the conformation of vinculin is regulated biologically between the 'open' and 'closed' states by PtdIns(4,5)P_2 [45]. We hypothesize that when vinculin is adsorbed on surfaces (microtitre dish or PVDF membranes) it adopts an open state that is competent to bind talin or our peptides.

A detailed analysis of one of the vinculin-binding peptides first drew our attention to two sequence motifs (DVY/ARRVS and VEDLTTTL), present in the peptide and talin, as being potentially important for binding. However, we later found that a 127-residue fragment of talin (1891–2017) that contained the DVY/ARRVS, but not the VEDLTTTL, motif was still capable of binding to vinculin and that a peptide corresponding to the VEDLTTTL motif region of talin was not an effective competitor of the talin–vinculin interaction. Mutation of the DVY/ARRVS motif in talin, in contrast, diminished binding to vinculin. Thus it seems that a 16-residue region of talin (1948–1963) is critical in binding vinculin. This result is consistent with previous work [46] that mapped a vinculin-binding domain of talin to residues 1328–2268 and recent work [46a] that narrowed this domain to residues 1929–2029. Additional work will be needed to delineate the details of the molecular interaction between talin and vinculin, as this general region is not well conserved in sequence between mouse and nematode talin [47], and other experiments [48] with an anti-idiotype antibody mapped the vinculin-binding domain of talin to residues 1653–1848, which contains the VEDLTTTL motif, but not to the DVY/ARRVS motif.

The vinculin-binding peptides represent an excellent tool for further study of the biochemistry of vinculin. The peptides could be used as detection reagents, in both ELISA and in gel overlay formats. In addition, the peptides can be used to differentiate between open and closed conformations of vinculin. It will be interesting in the future to express such sequences *in vivo* as a potential means of disrupting the talin–vinculin interaction or to target fusion proteins to the talin-binding domain of vinculin.

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