Further characterisation of the talin-binding site in the cytoskeletal protein vinculin

A.P. GILMORE, P. JACKSON*, G.T. WAITES and D.R. CRITCHLEY†

Department of Biochemistry, University of Leicester, Leicester LE1 7RH, UK

*Present address: Division of Cell Biology, John Curtin School of Medical Research, Australian National University, Canberra ACT 2601, Australia
†Author for correspondence

Summary

The cytoskeletal protein vinculin is a component of adherens-type junctions where it is one of a number of interacting proteins thought to link the cytoplasmic domain of adhesion receptors to F-actin. Vinculin has been shown to bind to at least three other cytoskeletal proteins, talin, paxillin and α-actinin. In this study, we further characterise the talin-binding domain in vinculin using a series of chick vinculin polypeptides expressed as glutathione-S-transferase fusion proteins in Escherichia coli. Thus 125I-talin bound to a fusion protein spanning residues 1-398, but not to those spanning residues 399-881 or 881-1066 in an SDS-PAGE gel-blot assay. We have previously characterised two chick vinculin cDNAs (2.89 kb cDNA and cVIn5) which are identical in the region of overlap except that cVIn5 lacks coding sequence for residues 167-207. Interestingly, a fusion protein spanning residues 1-398, but lacking residues 167-207, was unable to bind talin. However, further analysis showed that residues 167-207 are insufficient to support binding, and deletion of as few as 31 N-terminal residues abolished binding activity. The results of the gel-blot assay were essentially confirmed using purified fusion proteins adsorbed to glutathione-agarose beads. The smallest vinculin fusion protein able to bind talin contained residues 1-258. This fusion protein was as effective as whole vinculin in inhibiting the binding of 125I-vinculin to talin-coated microtitre wells. Interestingly, mutations which altered the charge characteristics of the highly conserved residues 178 and 181 abolished binding, whereas conservative substitutions were without effect. However, such mutations did not abolish the ability of mutant polypeptides spanning residues 1-398 to target to cell-matrix junctions in Cos cells. We have investigated the possible origin of the cDNA clone cVIn5 by defining the structure of a 5′ portion of the chicken vinculin gene, and by analysing vinculin transcripts in a variety of adult tissues and embryonic fibroblasts using reverse transcriptase and polymerase chain reaction. Although residues 167-207 are encoded on a separate exon, we have been unable to identify a tissue where this exon is alternatively spliced.

Key words: vinculin, talin, integrins, cell adhesion, extracellular matrix.

Introduction

The interaction between cells and adhesive glycoproteins of the extracellular matrix is frequently mediated by members of the integrin super-family of transmembrane heterodimeric glycoproteins (Humphries, 1990; Hynes, 1992). The highly conserved cytoplasmic domains of the β1 and β3 integrins (DeSimone and Hynes, 1988) are thought to associate indirectly with actin microfilaments via a number of interacting cytoskeletal proteins, which include talin, vinculin and α-actinin (Burridge and Mangeat, 1984). Evidence that integrins might be linked to cytoskeletal proteins initially stemmed from the observation that certain members of the integrin super-family co-distributed with cytoskeletal proteins in cell-matrix junctions (Damsky et al., 1985; Singer et al., 1988). Subsequently, biochemical evidence that the cytoplasmic domain of β1 integrins can bind directly to the cytoskeletal protein talin was obtained (Horwitz et al., 1986; Tapley et al., 1989), although the interaction is apparently of low affinity. Talin has been shown to bind to vinculin with relatively high affinity (Kd 10⁻⁸ M) (Burridge and Mangeat, 1984), and vinculin binds with low affinity (Kd 10⁻⁶ M) to α-actinin (Belkin and Koteliensky, 1987; Wachstock et al., 1987), an F-actin binding protein (Blanchard et al., 1989). However, other interactions are likely. Firstly, the cytoplasmic domains of β1 and β3 integrins have been shown to bind to α-actinin (Otey et al., 1990), and talin can bind directly to F-actin (Muguruma et al., 1990; Kaufmann et al., 1991). Secondly, a number of other cytoskeletal proteins have been localised to cell-matrix junctions (Burridge et al., 1988) including paxillin, which can bind to vinculin (Turner et al., 1990), and zyxin, which binds to α-actinin (Crawford et al., 1992). The finding that the association of integrins with the
The cytoskeleton is important to integrin function is supported by two lines of evidence. Firstly, deletions within the cytoplasmic domain of the βι subunit of the so-called high affinity fibronectin receptor (αβι), abolish the ability of this integrin to support adhesion of cells to fibronectin (Hayashi et al., 1990). Interestingly, mutant βι subunits were still able to form heterodimers with αζ subunits, and mutant heterodimers were expressed at the cell surface although they remained diffusely distributed and failed to localise to cell-matrix junctions. Given that the cytoplasmic domain of the βι integrin subunit is thought to contain a binding site for talin (Horwitz et al., 1986), the above results strongly suggest that the link between the αζβι integrin and the cytoskeleton is essential to integrin function. Similar experiments with the cadherin family of cell-cell adhesion molecules also suggest that cadherin function is dependent on the link with the cytoskeleton (Nagafuchi and Takeichi, 1988). Secondly, micro-injection of monoclonal antibodies to talin (Nuckolls and Burridge, 1990) or vinculin (Westmeyer et al., 1990) into fibroblasts have been shown to disrupt cell adhesion to the extracellular matrix.

The fact that the cytoplasmic domain of βι integrin (Hirst et al., 1986; Tapley et al., 1989), as well as the cytoskeletal proteins talin (Pasquale et al., 1986; Turner et al., 1989), vinculin (Werth and Pastan, 1984; Kellie et al., 1986; Nigg et al., 1986) and paxillin (Turner et al., 1991), are all substrates for a variety of protein kinases suggests that cell adhesion might be regulated by modulating the interaction between integrins and the cytoskeleton (Burridge et al., 1988; Hynes, 1992; Woods and Couchman, 1992). However, initial attempts to correlate phosphorylation of cytoskeletal proteins with altered cell adhesion have met with only limited success (Kellie et al., 1986; Nigg et al., 1986; Turner et al., 1989). This has been largely due to the lack of information concerning the structure of the proteins involved and the mechanisms by which they interact. The primary sequences of talin (Rees et al., 1990), vinculin (Price et al., 1987, 1989; Coutou and Craig, 1988; Weller et al., 1990) and α-actinin (Baron et al., 1987; Noegel et al., 1987; Arimura et al., 1988) have now been determined, providing some insight into the structural domains within these proteins which contribute to the various binding interactions (reviewed by Critchley et al., 1991). For example, both the βι integrin (Horwitz et al., 1986) and vinculin binding sites (O’Halloran and Burridge, 1986) in talin are thought to be contained within a 190 kDa talin polypeptide (residues 435-2541), which can be liberated by cleavage of talin with calpain II (Beckerle et al., 1987). Interestingly, the N-terminal 47 kDa talin polypeptide liberated by calpain II contains a region of sequence homology with the band 4.1 family of cytoskeletal proteins (Rees et al., 1990), and seems to be important for the specific targeting of talin to cell-matrix as opposed to cell-cell junctions (Nuckolls et al., 1990). Similarly, an actin-binding site in α-actinin has been localised to between residues 120 and 140 (Kuhlman et al., 1992; Hemmings et al., 1992), and evidence has been presented that there is a βι integrin binding site within the four spectrin-like repeats in α-actinin (Otey et al., 1990).

We have previously shown that there is a talin binding site within the first 398 residues of the globular head of the vinculin molecule, and that residues 167-207 play an important role in this interaction (Jones et al., 1989). In the present study we have carried out a detailed analysis of the talin binding site in vinculin using a variety of molecular and biochemical approaches. The results of these studies show that vinculin residues 167-207 on their own are insufficient to support talin binding, and suggest that the N-terminal 258 amino acids of the vinculin molecule are required to form a functional talin binding domain.

Materials and methods

Chick vinculin cDNAs

We have previously isolated two chick vinculin cDNAs referred to as the 2.89 kb cDNA and cVin5 (Price et al., 1987, 1989). The 2.89 kb cDNA is equivalent to the cDNA clone termed cVin1 isolated from the same library by Bendori et al. (1989). The 2.89 kb cDNA contains 247 bp of 5’ untranslated sequence and encodes 881 of the 1066 amino acids which comprise the vinculin molecule. The clone cVin5 (approximately 5 kb) contains 36 bp of 5’ untranslated sequence, the entire coding sequence except for 123 bp encoding residues 167-207, and 2.5 kb of 3’ untranslated sequence.

Expression of vinculin polypeptides as fusion proteins with glutathione-S-transferase

DNA fragments encoding various regions of the vinculin molecule were generated either by using convenient restriction enzyme sites within the above cDNAs, or by polymerase chain reaction (PCR) using the cDNAs as templates. DNA fragments were subcloned in frame into the glutathione-S-transferase (GST) gene of the plasmid expression vector pGEX (Smith and Johnson, 1988), and the GST/vinculin fusion proteins expressed in *Escherichia coli* by standard methods. Details of the various constructions are as follows. To generate a stable fusion protein containing vinculin residues 1-398 (GST/1-398), the 5’ EcoRI/BamHI fragment of the 2.89 kb cDNA was first subcloned into phase M13mp18, and the 247 bp of 5’ untranslated sequence deleted using mutagenesis. The resultant EcoRI/BamHI fragment was then blunt end ligated into the *Smal* site of pGEX-1. The fusion protein GST/1-398 (Δ167-207) was generated by blunt end ligation of the 5’ EcoRI/BamHI fragment from cVin5 into the *Smal* site of pGEX-2. The 36 bp of 5’ untranslated sequence between the GST gene and the vinculin coding sequence did not affect the stability of the resultant fusion protein. The fusion protein GST/399-881 was generated by subcloning the 5’ BamHI/EcoRI fragment of the 2.89 kb cDNA into the equivalent sites in pGEX-1. The fusion protein GST/881-1066; a DNA fragment encoding vinculin residues 881-1066 was generated by PCR using cVin5 as template. The 5’ primer incorporated a *BamHI* site and the 3’ primer (30 bp 3’ to the stop codon) contained an EcoRI site. The resultant PCR product was force cloned into pGEX-2. The fusion protein GST/32-398 was generated by blunt end ligation of the AccI/BamHI fragment of the 2.89 kb cDNA into pGEX-1. The fusion protein GST/67-207 was generated by blunt end ligation of a BglII/BglII fragment of the 2.89 kb cDNA into pGEX-2. The fusion proteins GST/118-398, GST/161-398, GST/1-258 were all generated by deletion mutagenesis using an M13mp18 construct containing the 5’ EcoRI/BamHI fragment of the 2.89 kb cDNA. Single-stranded template was prepared in the *dut* + *ung* strain of *E. coli* (Kunkel, 1985). The authentic initiation codon was maintained in all mutants. The mutant EcoRI/BamHI fragments were excised from M13 and blunt end ligated into the *Smal* site in pGEX. Fusion proteins spanning residues 1-398 but containing point mutations were made in the same way. Mutants spanning...
residues 1-398 but containing deletions designed according to exon boundaries (Δ57-80; Δ81-130; and Δ131-166) were engineered using the Promega pSelect system. All pGEX constructs were verified by double-strand sequencing using primers 5' and 3' to the cloning site.

**Purification and iodination of vinculin and talin**

Vinculin and talin were purified from chicken gizzard as described by Evans et al. (1984) and Molony et al. (1986), respectively. Both proteins (20 µg) were iodinated using 500 µCi of [125I] (Amerham, UK) and iodobeads (Pierce) according to the manufacturer’s instructions. Specific activities of the labelled proteins were of the order of 3 µCi/µg.

**Binding of [125I]talin to vinculin fusion proteins analysed using a gel-blot assay**

*E. coli* transformed with pGEX constructs containing various vinculin cDNAs were grown to an A600 of 0.5, and fusion protein expression induced by the addition of 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Cells were grown for a further 3 h, harvested by centrifugation and lysed in SDS-PAGE sample buffer. Solubilised proteins were resolved in 10% SDS-polyacrylamide gels and electro-blotted to nitrocellulose filters (Towbin et al., 1979). Filters were incubated in 25 mM Tris-HCl, pH 7.5, 150 mM NaCl (TBS), 0.2% Tween 20, 1 mM EDTA, 4% BSA for 2 h and overnight with [125I]-talin (0.37 nM; 5x10^5 cts/min per ml) in the presence or absence of a 100-fold molar excess of unlabelled talin. Filters were washed twice with buffer alone (5 min each wash), and bound [125I]-talin detected by autoradiography.

**Binding of [125I]talin to purified vinculin fusion proteins adsorbed to glutathione-agarose beads**

GST/vinculin fusion proteins expressed in *E. coli* were purified from 50 ml cultures by standard methods (Smith and Johnson, 1988). Briefly, cells were harvested by centrifugation, resuspended in 0.5 ml of PBS/1% Triton X-100, lysed by sonication (two 30 second bursts on ice) and the solubilised fusion proteins were purified by adsorption to glutathione-agarose beads (Sigma). Beads were washed with phosphate buffered saline (PBS) to remove contaminating *E. coli* proteins. The purity of the fusion protein was analysed by boiling a small sample of the beads in SDS-PAGE sample buffer followed by analysis on 10% gels. The amount of fusion protein bound was estimated by Coomassie blue staining and overnight with [125I]-talin (0.37 nM; 5x10^5 cts/min per ml) in the same buffer. Beads were washed twice with buffer alone (5 min each wash), and bound [125I]-talin detected by autoradiography.

**Binding of [125I]-vinculin to talin adsorbed to microtitre wells**

Talin (100 ng in 5 µl of TBS) was adsorbed to wells for 4 h, and excess protein binding sites were blocked by incubation for a further 4 h with 4% BSA in TBS (200 µl). [125I]-vinculin was added in 100 µl of 4% BSA/TBS/0.1% Tween 20 at a final concentration of 0.37 nM, and incubated overnight at room temperature. Unbound ligand was removed, the wells washed three times in TBS/0.1% Tween 20, and the amount of [125I]-vinculin bound was determined in a Beckman 5500 gamma counter.

**Expression of vinculin cDNAs in Cos cells using the pECE eukaryotic expression vector**

Restriction enzyme fragments derived from the two chick vinculin cDNAs were subcloned into the polylinker sequence of the pECE expression vector which contains the SV40 early promoter, poly(A) addition signal and poly(A) tract (Ellis et al., 1986). Plasmids containing inserts in the correct orientation were identified by restriction enzyme mapping. Covalently closed circular plasmid DNA for transfection was prepared by polyethylene glycol precipitation (Sambrook et al., 1989). Plasmid DNA was transfected into Cos cells using the DEAE-dextran method of Cullen (1988).

**Localisation of chick vinculin polypeptides expressed in Cos cells by fluorescence microscopy**

Coverslip cultures of cells were fixed and permeabilized for fluorescence microscopy 48 h post-transfection as previously described (Jackson et al., 1989). For double labelling of vinculin and F-actin, cells were first stained with a rabbit antiserum to chick vinculin (Kellie et al., 1986), followed by a Texas red-labelled donkey anti-rabbit serum (Amersham). Before mounting, cells were stained for F-actin with NBD-phallacidin (Molecular Probes, Eugene, OR USA), exactly as described by the manufacturers. Photographs were taken with a Zeiss Axiophot photomicroscope equipped with epifluorescence using Ilford HPS film (ASA 400) uprated to 1600 ASA.

**Radiolabelling and immune precipitation of chick vinculin polypeptides expressed in Cos cells**

Cells grown for 24 h post-transfection were cultured for a further 18 h in the presence of [125I]methionine (Amersham), and the labelled vinculin polypeptides were isolated by immune precipitation using an antibody specific to chick vinculin and Protein A-agarose, exactly as described previously (Jackson et al., 1989). Proteins isolated in this way were analysed by SDS-PAGE and fluorography.

**Analysis of vinculin mRNA using PCR**

Poly(A)+ RNA was isolated from growing chick embryo cells in culture and from a variety of adult chicken tissues using the guanidine isothiocyanate/hot phenol procedure and oligo(dT)-cellulose (Collaborative Research, Levington, USA) (Sambrook et al., 1989). The buffer used for reverse transcription and PCR amplification was 50 mM KCl, 10 mM Tris-HCl (pH 8.0), 1.5 mM MgCl2, 200 µM dNTPs, with 1 µg of mRNA in each reaction. Reverse transcription was carried out in 10 µl of buffer using 10 units of Moloney murine reverse transcriptase and the following primer 5′-CTTTCATGGCCTACGTGCCAACG-3′. The second primer in the reaction was then increased to 50 µl by addition of the remaining components for amplification including an upstream primer (5′-TTGGGAAAGAACTGTGTCCAGACA-3′). Amplification was for 20 cycles (94°C for 1 min, 65°C for 1 min, and 72°C for 2 min). 1 µl of this reaction was used as a template for a second round of PCR, using a pair of nested primers. The upstream primer was 5′-TTTAAAGCTTGAGAAGCA-GATCGT-3′; the second primer was 5′-TCCGGAATTC-CCAGGATCTGCATCTTCATC-3′ corresponding to nucleotides 440-930 in the sequence of the 2.89 kb chick vinculin cDNA (Price et al., 1987). Amplification was for 15 cycles with the annealing temperature set at 55°C. The upstream primer was labelled at the 5′ end with [γ-32P]dATP using polynucleotide kinase (Pharmacia). The second round reactions were analysed on a 7% polyacrylamide gel containing 7 M urea and 20% formamide, and the products were detected by autoradiography.

**Isolation of chick vinculin genomic clones and determination of intron/exon boundaries**

A chick genomic library in the EMBL 3 replacement vector
(kindly provided by Professor D. Engel, Northwestern University, Evanston, IL 60208, USA) was screened with a 5′ EcoRI/BamHI restriction enzyme fragment (1.4 kb) purified from the chick 2.89 kb vinculin cDNA (Price et al., 1987). Positive clones were tested for cross-hybridisation to a series of smaller probes spanning nucleotides 442-1030 and generated by PCR. These were SW1-2 (nt 442-810), SW1-3 (nt 442-1021), SW3-5 (nt 772-1021). The probes were labelled with [α-32P]dCTP by the random priming method (Feinberg and Vogelstein, 1984) and plaque-screening by DNA-DNA hybridisation was by standard methods (Sambrook et al., 1989) with a final wash in 15 mM NaCl/1.5 mM sodium citrate, pH 7.0, 0.1% (w/v) SDS at 65°C. Filters were stripped between hybridisation and the efficiency of probe removal checked by autoradiography. Selected positive plaques were purified to homogeneity and recombinant phage was purified by CsCl gradient centrifugation (Sambrook et al., 1989). The sizes of the inserts were determined by gel electrophoresis of SalI-digested DNA, and the relationships between clones were established by mapping with combinations of SalI, EcoRI and BamHI restriction endonucleases. Restriction enzyme fragments were transferred to Hybond-N membranes (Amersham) and hybridized with the [α-32P]dCTP-labelled probes SW1-2, SW1-3, SW3-5 in succession. Hybridization was carried out at temperatures calculated for each oligonucleotide. Selected hybridizing fragments were gel purified and subcloned into the Bluescript SK+ vector (Stratagene) for sequencing. Intron/exon boundaries were determined using oligonucleotides based on the genomic sequence of human vinculin (Weller and Critchley, unpublished data).

**Results**

**Binding of 125I-talin to vinculin fusion proteins analysed using a gel-blot assay**

We have previously shown that a vinculin polypeptide containing residues 1-398 synthesised *in vitro* from the 2.89 kb vinculin cDNA (Price et al., 1987) was able to bind to microtitre wells coated with talin (Jones et al., 1989). In contrast, a chick vinculin polypeptide synthesised from the equivalent region of a second vinculin cDNA (cVin5), which lacks coding sequence for residues 167-207, was unable to bind talin. These results suggest that there is a talin-binding site within residues 1-398 of the vinculin molecule, with residues 167-207 playing an important role in the binding interaction.

To investigate the possibility that other regions of the vinculin molecule might also be involved in talin binding, we expressed different regions of the vinculin molecule as glutathione-S-transferase (GST) fusion proteins in *E. coli* (Fig. 1A). The ability of the vinculin fusion proteins to bind talin was assessed using a gel-blot assay. Thus, cells expressing the fusion proteins were lysed, total cell proteins were resolved by SDS-PAGE and the proteins electroblotted to nitrocellulose filters. Filters were then incubated with 125I-talin, and binding to the fusion proteins was detected by autoradiography. In agreement with earlier experiments, 125I-talin was able to bind to a vinculin fusion protein containing residues 1-398, but did not bind to a protein spanning residues 1-398, which lacked residues 167-207. 125I-talin did not bind to fusion proteins spanning vinculin residues 399-881 or 881-1066 (Fig. 1B,C). Binding of the 125I-talin was specific in that it was markedly reduced in the presence of excess unlabelled talin (Fig. 1D). The results are consistent with the conclusion that talin binding to vinculin is limited to the N-terminal 398 residues of the vinculin molecule, with residues 167-207 playing an important role in the interaction.

To define further the talin-binding site within the N-terminal 398 residues of the vinculin molecule, we expressed a series of vinculin fusion proteins containing deletions both N- and C-terminal to residues 167-207 (Fig. 2A). These were then tested for the ability to bind talin in the gel-blot assay. Interestingly, the only truncated fusion protein which retained talin-binding activity was that containing residues

![Fig. 1. 125I-talin binding to vinculin fusion proteins expressed in E. coli detected using a gel-blot assay.](image-url)

(A) The regions of the vinculin molecule expressed as GST/fusion proteins were: (1) GST/vinc1-398; (2) GST/vinc1-398(A167-207); (3) GST/vinc399-881; (4) GST/vinc881-1066. Numbering in all cases includes the initiating methionine. aa, amino acid. (B) Coomassie blue-stained SDS-polyacrylamide gel (10%) of *E. coli* cell extracts expressing the vinculin fusion proteins shown in A. The fusion protein is the major band in all extracts. Molecular mass markers (kDa) are shown to the left of the figure. (C) Autoradiograph showing 125I-talin binding to the same vinculin fusion proteins electroblotted to nitrocellulose. (D) 125I-talin binding to vinculin fusion proteins in the presence of a 100-fold molar excess of unlabelled talin.
The talin-binding domain in vinculin

1-258 (Fig. 2B-D, lane 5). All N-terminal deletions tested including removal of just 31 residues of the vinculin molecule abolished the ability of the fusion protein to bind talin. The results suggest that the talin-binding site in vinculin is located within residues 1-258 and demonstrate that residues 167-207 are not in themselves sufficient for talin binding.

In an attempt to identify vinculin sequences which might be required in addition to residues 167-207 to form a functional talin-binding site, we made a series of internal deletions (Fig. 3A) the boundaries of which corresponded to individual exons (see Fig. 10). However, all such deletion mutants lacked talin binding activity as determined using the gel-blot assay (Fig. 3B,C). This result suggests that residues 1-258 comprise the talin-binding domain in vinculin and that deletions within this domain inactivate binding.

Binding of 125I-talin to purified vinculin fusion proteins adsorbed to glutathione-agarose

In the course of the gel-blot assay, vinculin fusion proteins are denatured by boiling in SDS-PAGE sample buffer. In order to establish that the results described above were not an artifact of the assay procedure, vinculin fusion proteins were purified under non-denaturing conditions from E. coli cell extracts using glutathione-agarose beads. The ability of the fusion proteins adsorbed onto the surface of the beads to bind 125I-talin was then determined using a centrifugation assay. The results of these experiments were essentially similar to those obtained with the gel-blot assay (Fig. 4). Thus, 125I-talin bound to GST fusion proteins spanning residues 1-258 and 1-398 whereas binding to the fusion protein GST/1-398(Δ167-207) was similar to that observed with GST alone. However, fusion proteins GST/67-208 and GST/32-398 displayed some low-level residual binding activity not seen using the gel-blot assay. Unfortunately, vinculin fusion proteins spanning residues 118-398 and 161-398 were insoluble and could not be tested in this assay.

Inhibition of 125I-vinculin-binding to talin-coated microtitre wells by vinculin and vinculin fusion proteins

In order to establish the relative binding affinities of whole vinculin and vinculin fusion proteins for talin, we compared their abilities to inhibit binding of 125I-vinculin to talin immobilised in microtitre wells. Vinculin fusion protein containing residues 1-258 and 1-398 were as effective as whole vinculin in inhibiting the binding of 125I-vinculin to talin (Fig. 5). The IC50 values (concentration of inhibitor bringing about a 50% reduction in binding) were between 5 nM and 10 nM, respectively. As expected, a fusion protein spanning residues 1-398 but lacking residues 167-207...
was inactive in this assay (data not shown) as was GST alone (Fig. 5).

The effect of mutations in vinculin residues 178 and 181 on talin binding in vitro and localisation of expressed vinculin polypeptides in Cos cells

Comparison of the sequence of vinculin residues 167-207 between human and chick shows that the two are identical. However, nematode vinculin (Barstead and Waterston, 1989) is somewhat divergent in this region except for a block of eight amino acids which are totally conserved (residues 178-185) (Weller et al., 1990). To investigate the importance of these residues in talin binding, we made a number of point mutations introducing conservative and

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**Fig. 3.** $^{125}$I-talin binding to vinculin fusion proteins containing internal deletions within residues 1-398 detected using a gel-blot assay. (A) The regions of vinculin molecule expressed as fusion proteins were: (1) GST/vinc1-398; (2) GST/vinc1-398Δ131-166; (3) GST/vinc1-398Δ81-130; (4) GST/vinc1-398Δ57-80. (B) Coomassie blue-stained SDS-polyacrylamide gel (10%) of *E. coli* cell extracts expressing the vinculin fusion proteins shown in A. Molecular mass markers (kDa) are shown to the left of the figure. (C) Autoradiograph showing $^{125}$I-talin binding to the same vinculin fusion proteins electroblotted to nitrocellulose.

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**Fig. 4.** Binding of $^{125}$I-talin to vinculin fusion proteins adsorbed to glutathione-agarose beads. Binding to each fusion protein was analysed as described in Materials and methods and was carried out in triplicate. Error bars show standard deviation.

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**Fig. 5.** Inhibition of $^{125}$I-vinculin binding to talin-coated microtiter wells by unlabelled vinculin and vinculin fusion proteins. $^{125}$I-vinculin binding was assayed in the presence of increasing concentrations of vinculin (■—■), vinculin fusion proteins GST/vinc1-398 (▲—▲) and GST/vinc1-258 (△—△), and GST alone (■—■). Binding is expressed as a percentage of that observed minus inhibitor. The points are means of triplicates with <10% variation about the mean.
non-conservative amino acid substitutions. Thus a Glu181 to Asp mutant still retained talin binding activity in the gel-blot assay (Fig. 6A,B, lane 2). However, substitutions which changed the charge characteristics of residue 178 (Arg to Glu) or residue 181 (Glu to Lys) resulted in loss of activity (Fig. 6A,B, lanes 3,4, respectively).

To establish whether such mutations also affected the ability of this region of the vinculin molecule to target to cell-matrix junctions, a number of mutant chick vinculin cDNAs encoding residues 1-398 were expressed in monkey COS cells. Both wild-type and mutant vinculin polypeptides were expressed to about the same level as judged by autoradiography. As a control the analysis was also carried out using the 2.89 kb vinculin cDNA and cVin5 as templates. Using this method we could find no evidence for a transcript equivalent to the 123 bp encoding residues 167-207 (Price et al., 1989). To investigate the possibility that this difference arose through alternative splicing, we initially decided to characterise the exon structure of the chick vinculin gene in the region encoding the talin-binding domain. A chick genomic library in the lambda vector EMBL3 was screened with a 5′ EcoRI-BamHI restriction enzyme fragment isolated from the 2.89 kb vinculin cDNA. The restriction enzyme map of one of the genomic clones isolated in this way is shown in Fig. 9A. Sequencing subfragments derived from this genomic clone permitted the identification of several intron/exon boundaries, and showed that residues 167-207 are indeed encoded by a single exon (Fig. 9B). The structure of this region of the chick vinculin gene is identical to the human gene (Weller and Critchley, unpublished data). Residues 167-207 are encoded by exon 5.

To explore the possibility that this exon is alternatively spliced, we isolated poly(A)+ RNA from a variety of chicken tissues and analysed the vinculin transcripts using an assay based on reverse transcriptase and the polymerase chain reaction (RT/PCR). Initially, the reverse transcriptase step was followed by a 20-cycle PCR amplification using oligonucleotide primers within exons 2 and 6. The products of the first round amplification were then subjected to a further 15 cycles of PCR amplification using primers nested with respect to those used in the first round amplification. One of the primers was end labelled with γ-32P-ATP and the PCR products were analysed by electrophoresis using denaturing polyacrylamide gels and autoradiography. As a control the analysis was also carried out using the 2.89 kb vinculin cDNA and cVin5 as templates. Using this method we could find no evidence for alternative splicing of residues 167-207 in adult gizzard, kidney, liver, heart, lung, stomach or spleen (Fig. 10A). The two vinculin cDNAs were isolated from a chick embryo cell cDNA library (Tamkun et al., 1986) and we therefore analysed mRNA isolated from tertiary cultures of chick embryo cells derived from 10-day-old embryos. Again we could find no evidence for a transcript equivalent to the

Fig. 6. 125I-talin binding to vinculin fusion proteins spanning residues 1-398 detected using a gel-blot assay. The effect of point mutations at residues 178 and 181. (A) Coomassie blue-stained SDS-polyacrylamide (10%) gel of E. coli cell extracts showing expressed vinculin fusion proteins. Lanes: (1) GST/vinc1-398; (2) GST/vinc1-398(E181-D); (3) GST/vinc1-398(R178-E); (4) GST/vinc1-398(E181-K). The major band is the fusion protein in all cases. Molecular mass markers (kDa) are shown to the left of the figure. (B) Autoradiograph showing 125I-talin binding to the expressed vinculin fusion proteins electroblotted to nitrocellulose. The relative mobilities of the fusion proteins in lanes 1 and 2 of A and B was variable and is probably the result of different amounts of proteins loaded.

Fig. 7. Immune precipitation of chick vinculin polypeptides expressed in monkey COS cells. [35S]methionine-labelled extracts of cells transiently expressing cDNAs encoding vinculin residues 1-398 (lanes A,E) and the point mutants E181-D (lanes B,F), R178-E (lanes C,G) and E181-K (lanes D,H) were immune-precipitated with either rabbit antiserum specific to chick vinculin (lanes A, B, C, D) or preimmune rabbit serum (lanes E, F, G, H) as described in Materials and methods. Immune precipitates were analysed by SDS-PAGE and autoradiography.
Fig. 8. Immunofluorescence localisation of chick vinculin polypeptides expressed in monkey COS cells. COS cells transiently expressing: (A,B) chick vinculin residues 1-398; (C,D) point mutant E181-D; (E,F) point mutant R178-E; (G,H) point mutant E181-K were stained for chick vinculin (A, C, E, G) or F-actin (B, D, F, H) as described in Materials and methods. Both the wild-type and mutant vinculin polypeptides localise to the ends of actin filaments in cell-matrix junctions. Bar, 5 µm.
The talin-binding domain in vinculin cDNA clone cVin5 (Fig. 10B). Neither could we find such a transcript in mRNA isolated from whole 2-day-old chick embryos (Dr. P. Jones, unpublished data). However, the cDNA clone cVin5 was readily detected in the original λgt11 cDNA library using PCR (data not shown), but we could find no equivalent clones in other chick cDNA libraries, including embryonic gizzard (Clontech), small intestinal epithelial cell (deArruda et al., 1990), and skeletal muscle libraries (Reinach and Fischman, 1985). The origin of the cDNA clone cVin5 within the chick embryo cell cDNA library is therefore unresolved.

Discussion

Analysis of the deduced sequence of chick (Price et al., 1987, 1989; Coutu and Craig, 1988) and human (Weller et al., 1990) vinculin shows it to be a highly conserved protein (>95% sequence identity) containing 1066 amino acids.
with a deduced molecular mass of 116.5 kDa. The higher molecular mass isoform of vinculin termed metavinculin arises via alternative splicing of a single exon. In humans this exon encodes 68 amino acids which are inserted between vinculin residues 915 and 916 (Koteliansky et al., 1992). In the electron microscope, vinculin appears to possess a globular head and an extended tail (Milam, 1985). The globular head can be liberated from the tail by V8 protease, which cleaves the protein at two sites (residues 851 and 858) contained within a proline-rich region of the molecule (Price et al., 1989). The 90 kDa globular head contains the N terminus of vinculin, three 112 amino acid repeats, and one or more binding sites for the cytoskeletal protein talin. This site may account for the ability of this region of the protein to localise to cell-matrix junctions when expressed in Cos cells (Bendori et al., 1989; Jones et al., 1989). The 32 kDa vinculin tail, which is relatively basic, contains a second independent site capable of targeting vinculin to cell-matrix junctions (Bendori et al., 1989). This site may be equivalent to the binding site for the cytoskeletal protein paxillin, which has recently been localised to this region of the vinculin molecule (Turner et al., 1990).

In an attempt to define further the talin-binding site(s) in vinculin, we have expressed contiguous regions of the vinculin molecule as fusion proteins in E. coli and monitored the ability of these proteins to bind $^{125}$I-talin using a gel-blot assay. Talin binding was restricted to a fusion protein spanning vinculin residues 1-398, and there was no binding to other regions of the molecule. We have previously isolated two chick vinculin cDNAs (a 2.89 kb cDNA, and a 5 kb cDNA called cVin5) which are essentially identical in the region of overlap except that cVin5 lacks the coding sequence for residues 167-207 (Price et al., 1987, 1989). Interestingly, residues 1-261 are encoded by a single exon raises the possibility that these residues might represent a distinct structural domain which might therefore represent a distinct structural domain containing the talin-binding site.

However, further experiments clearly demonstrate that vinculin residues 167-207 are not in themselves sufficient to support talin binding. For example, a fusion protein spanning residues 32-398 lacked talin binding activity as detected by the gel-blot assay, and expressed a very low level of activity in the solid-phase binding assay. A number of other fusion proteins with more extensive deletions N-terminal to residues 167-207 were also without activity. Reference to the literature on the central cell binding domain in the adhesive glycoprotein fibronectin is of interest in this regard. Whilst it is apparent that the RGD sequence in the fibronectin type III repeat number 10 is absolutely required for the activity of this domain, small deletions in repeats 8 and 9 also dramatically reduce activity (Aota et al., 1991). However, we have found no evidence for short sequences within the N-terminal region of the vinculin molecule which co-operate with residues 167-207 in binding talin. Thus, fusion proteins spanning residues 1-398 but containing internal deletions designed according to determined exon boundaries were all without activity in the gel-blot assay.

The smallest vinculin fusion protein we have generated which retains talin-binding activity in vitro and targets to cell-matrix junctions in vivo (data not shown) spans residues 1-258 (Table 1). Sequence alignments suggest that residue 259 is the start of the three 112-residue repeats, which might therefore represent a distinct structural domain (Price et al., 1987, 1989). Interestingly, residues 1-261 are encoded by the first six exons of the vinculin gene, the pre-

### Table 1. Comparison of the talin-binding activity of various vinculin polypeptides with their ability to target to cell-matrix junctions when expressed in Cos cells

<table>
<thead>
<tr>
<th>Construct number</th>
<th>Vinculin polypeptide expressed</th>
<th>Talin binding in vitro*</th>
<th>Localisation to cell-matrix junctions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1-398</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>1-398 (Δ167-207)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>399-881</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>881-1066</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>1-258</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>67-208</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td>32-398</td>
<td>–</td>
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<td>9</td>
<td>161-398</td>
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<tr>
<td>11</td>
<td>1-398 (Δ81-130)</td>
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<td>ND</td>
</tr>
<tr>
<td>12</td>
<td>1-398 (Δ153-166)</td>
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<td>ND</td>
</tr>
<tr>
<td>13</td>
<td>1-398 (R178→E)</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>1-398 (E181→D)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>1-398 (E181→K)</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>16</td>
<td>1-398 (Δ167-173)</td>
<td>–</td>
<td>+</td>
</tr>
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<td>1-398 (Δ174-180)</td>
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<td>–</td>
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<tr>
<td>18</td>
<td>1-398 (Δ181-187)</td>
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<td>19</td>
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<td>20</td>
<td>1-398 (Δ195-201)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>21</td>
<td>1-398 (Δ202-208)</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Vinculin polypeptides encoded by constructs 1-15 were expressed in vitro as glutathione-S-transferase fusion proteins (this study). Those encoded by constructs 16-21 were expressed using an in vitro transcription/translation system (Jones et al., 1989). The ability of the vinculin polypeptides either to bind talin in vitro or to localise to cell-matrix junctions when expressed in Cos cells is indicated by a +. –, No activity. ND, not done. The data on constructs 16-21 are from Jones et al. (1989).
dicted start of the repeats being close to the 5′ boundary of exon 7. We therefore conclude that vinculin residues 1-258 are required to form a fully functional talin-binding domain. This view is consistent with the observation that a vinculin fusion protein spanning residues 1-258 is as effective as residues 1-398 or intact vinculin in competing with

\( ^{125}I \)-vinculin for binding to talin. Interestingly, Scatchard analysis of the binding data suggests that there may be high- and low-affinity interactions between vinculin and talin (not shown), a conclusion reached previously by Burridge and Mangeat (1984).

The concept that residues 167-207 are important to the activity of the talin binding domain remains. We have previously shown (Jones et al., 1989) that deletions of groups of seven residues spanning this region abolish talin binding in vitro with the exception of the deletion of residues 202-208, which was without any marked effect (data summarised in Table 1). Comparison of the sequence of residues 167-207 between chick and human vinculin shows it to be identical, and there are only five sequence differences in the first 258 residues of the protein (Weller et al., 1990). Nematode vinculin shows a much greater sequence divergence in this region (Barstead and Waterston, 1989), and residues 167-207 show only a 39% identity to the chick sequence (Weller et al., 1990). However, within this region there is a block of eight residues (residues 178-185; RQQELTHQ) which are identical in all three vinculins sequenced to date. Interestingly, the sequence RQQEL is found in the N-terminal domain of the cadherin-associated protein α-catenin (Nagafuchi et al., 1991). This domain shows a 25.6% sequence identity to vinculin residues 6-208. Point mutations in this sequence which alter the charge characteristics of residues 178 or 181 (R178 to E; E181 to K) abolished vinculin binding to talin in vitro. However, these mutations did not affect the ability of vinculin polypeptides spanning residues 1-398 to localise to cell-matrix junctions when expressed in Cos cells. We have previously noted that a number of small deletions within residues 167-207 which inhibit talin-binding in vitro do not affect targeting of the expressed protein in Cos cells (Jones et al., 1989). One possible explanation for these observations is that such vinculin mutants retain some capacity to interact with talin, but with an affinity which is too low to be detected in the gel-blot assay. A low affinity interaction might be sufficient for normal targeting of the mutant vinculin polypeptides when overexpressed in Cos cells. Alternatively, it is conceivable that there is a binding site for another cytoskeletal protein within vinculin residues 1-398, and that this site is responsible for the successful targeting of vinculin polypeptides unable to bind to talin. For this explanation to be viable, one must suppose that the activity of this second site is also dependent on residues 167-207, as the mutant 1-398Δ167-207 is unable to target to cell-matrix junctions. Point mutations and small deletions within residues 167-207 which abolish talin-binding might have little or no effect on the second site.

The origin of the chick vinculin cDNA clone cVin5, which lacks the 123 bp coding for amino acid residues 167-207 remains unresolved. The fact that the coding sequence for these residues is contained entirely on exon 5 in both chick (this study) and human (P.A. Weller and D.R. Critchley, unpublished data) supports the view that this might be a region of alternative splicing resulting in expression of a novel vinculin isoform unable to bind talin. This concept has some attraction because vinculin is not only found in cell-matrix junctions, but also at sites of cell-cell adhesion mediated by members of the cadherin family. Such junctions lack talin (Geiger et al., 1985). However, using a sensitive RT/PCR-based method and poly(A)+ RNA isolated from a variety of adult chick tissues, we have been unable to find evidence for this splice variant. We have previously used the RT/PCR method to map alternatively spliced α-actinin transcripts and have shown that it will detect mRNAs which comprise <1-3% of the total population of α-actinin mRNAs (Waites et al., 1992). The cDNA clone cVin5 was originally isolated from a chick embryo fibroblast λgt11 library (Tamkun et al., 1986). We have had no difficulty in detecting both the 2.89 kb cDNA and cVin5 in this library by PCR, but we have been unable to detect an mRNA equivalent to cVin5 in poly(A)+ RNA isolated from cells cultured from 10-day-old chick embryos using either RT/PCR or S1 nuclease mapping. Neither have we been able to find the equivalent of cVin5 in other chick cDNA libraries including those derived from embryonic brain, small intestinal epithelial cells, or smooth muscle. Similarly, we have screened human liver, brain, retina, colon, lung and placenta cDNA libraries using PCR and found no evidence for such a variant (Dr E.P. Moiseyeva and Dr. D.R. Critchley, unpublished data). Whilst we do not exclude the possibility that an mRNA equivalent to cVin5 might be expressed in some tissue or at some developmental stage which we have not yet analysed, neither can we exclude the possibility that it was derived from a rare aberrantly spliced vinculin mRNA. The 5′ splice site of exon 5, which encodes residues 167-207, is very similar to one shown to be weak when compared with the consensus sequence (Lear et al., 1990). In contrast the 5′ splice site of exon 4 would be predicted to be quite strong, and it is conceivable that this might result in splicing of exon 4 to exon 6 in a small proportion of vinculin mRNAs.

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