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Characterisation of the paxillin-binding site and the C-terminal focal adhesion targeting sequence in vinculin

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SUMMARY

Paxillin and vinculin are cytoskeletal proteins that co-localise to focal adhesions, specialised regions of the cell involved in attachment to the extracellular matrix. These two molecules form part of a complex of proteins that link the actin network to the plasma membrane. Paxillin has been shown to bind directly in vitro to the C-terminal region of vinculin (Turner et al. (1990). *J. Cell Biol.* 111, 1059-1068), which also contains a focal adhesion targeting sequence (Bendori et al. (1989). *J. Cell Biol.* 108, 2383-2393). In the present study, we have used a series of vinculin deletion mutants to map more precisely the sites in vinculin responsible for paxillin binding and focal adhesion localisation. A glutathione-S-transferase fusion protein spanning vinculin residues 881-1000 was sufficient to support ^{125}I-paxillin binding in a gel-blot assay while no detectable binding was observed to a fusion protein spanning residues 881-978. Transfection experiments using cDNAs encoding chick vinculin residues 398-1066 and 398-1028 demonstrated that amino acids C-terminal to residue 1028 were not necessary for targeting to focal adhesions. In contrast, a vinculin polypeptide expressed from a cDNA encoding residues 398-1000 failed to localise to focal adhesions in stably transfected NIH3T3 cells. We have therefore identified a region of 50 amino acids (residues 979-1028) within the C-terminal region of vinculin that contains both the paxillin-binding site and the focal adhesion targeting sequence. This region is highly conserved in human and chicken vinculin and is likely to be important in regulation of the assembly of focal adhesions.

Key words: paxillin, vinculin, focal adhesion

INTRODUCTION

The attachment of cultured cells to extracellular matrix (ECM) proteins is often limited to discrete regions of the ventral plasma membrane. These structures known as focal adhesions or focal contacts are composed of a complex of proteins that provide a link between the ECM and the ends of actin bundles, or stress fibers within the cell (Burridge et al., 1988). Cell attachment is mediated most frequently through integrin molecules, a family of heterodimeric glycoproteins that traverse the plasma membrane (Hynes, 1992). Integrins have been shown to interact in vitro via their short cytoplasmic tails with the focal adhesion/cytoskeletal proteins talin (Horwitz et al., 1986) and α-actinin (Otey et al., 1992). Although talin and α-actinin have both been demonstrated to bind actin filaments (Muguruma et al., 1992) and could therefore provide the necessary linkage between the integrin molecules and stress fibers in vivo, the presence of additional proteins at the cytoplasmic face of focal adhesions suggests that the organization of these structures may be considerably more complex (for reviews see Turner and Burridge, 1991; Luna and Hitt, 1993). In fact vinculin, one of the first focal adhesion proteins to be identified (Geiger et al., 1980) has been shown to interact with both talin (Burridge and Mangeat, 1984) and α-actinin (Belkin and Koteliansky 1987; Wachsstock et al., 1987) in vitro.

Vinculin is a highly conserved 116 kDa protein containing 1066 amino acids in chickens and humans (Weller et al., 1990). Rotary shadowing and electron microscopy reveals a two domain structure consisting of a globular head and rod-like tail (Molony and Burridge, 1985). The globular head contains the N terminus of the protein, a talin-binding domain and three 112 residue repeats of unknown function (Price et al., 1987, 1989). It can be liberated from the C-terminal rod domain by V8-protease which cleaves vinculin at two sites within a proline-rich region spanning residues 837-878 (Price et al., 1989). Transfection experiments using cDNAs encoding the N- or C-terminal regions of vinculin have indicated that both contain sufficient information for targeting to focal adhesions (Bendori et al., 1989). Biochemical analysis of the interaction between vinculin and talin have localized the talin-binding site to the amino-terminal 258 amino acids within the globular head of vinculin (Jones et al., 1989; Gilmore et al., 1992), and this probably accounts for the ability of this region of the protein to target to focal adhesions. The targeting of the carboxyl terminal region of vinculin to focal adhesions could be attrib-

uted to the ability of this region of the molecule to self-associate. Such an interaction has been observed in rotary shadowed images (Molony and Burridge, 1985) and in some vinculin blot overlay assays. An alternative mechanism for the localization of the vinculin tail to focal adhesions involves binding to paxillin.

Paxillin is a 68 kDa focal adhesion protein that binds selectively to the rod domain of vinculin (residues 851-1066) with an apparent K_d of 6×10^{-8} M (Turner et al., 1990; Turner, 1993). Paxillin exhibits increased tyrosine phosphorylation following cell adhesion to ECM components (Burridge et al., 1992) and following transformation by Rous sarcoma virus (Glenney and Zokas, 1989). It is also heavily phosphorylated on tyrosine during chick embryonic development (Turner, 1991). The tyrosine phosphorylation of paxillin accompanies major cytoskeletal and focal adhesion reorganizations suggesting that the regulated phosphorylation of paxillin may be an important factor in controlling the integrity of these structures. One possible role for paxillin is the recruitment of vinculin molecules to precursors of focal adhesions through an interaction with the vinculin rod domain (Turner et al., 1990). Therefore in the present study, we have undertaken to define further the region within the vinculin molecule that is necessary for focal adhesion localization and to determine if the paxillin-binding site is also contained within this region of the molecule.

MATERIALS AND METHODS

Expression of chick vinculin polypeptides as fusion proteins

Chick vinculin polypeptides starting at residue 881 and extending either to the C terminus of the protein (residue 1066) or to residues 1028, 1021, 1012, 1000 and 978 were expressed in *E. coli* as fusion proteins with glutathione-S-transferase (GST) using the prokaryotic expression vector pGEX (Pharmacia). cDNAs encoding these polypeptides were generated by PCR using the chick vinculin cDNA cVin5 (Price et al., 1989) as template. The oligonucleotide primers contained 20 nt complimentary to the template sequence, plus an adjacent restriction endonuclease recognition site (*Bam*HI in the 5′ primer, *Eco*RI in the 3′ primer) followed by three additional nucleotides. PCR was performed using standard procedures (Sambrook et al., 1989) with an annealing temperature of 63°C. PCR products were ethanol precipitated, cut with *Bam*HI and *Eco*RI, gel purified and force-cloned into the appropriate pGEX vector cut with the same enzymes. The 5′ and 3′ ends of all constructs were sequenced using pGEX oligonucleotide primers flanking the cloning site. pGEX constructs were transformed into the *E. coli* strain JM101. Overnight cultures were diluted 1:100 and grown to an A_{600} of 0.4. Expression of the fusion proteins was induced by the addition of 0.5 mM isopropylthiogalactoside and the cells harvested after a further 1.5 hours growth at 37°C. The vinculin fusion proteins were purified from bacterial lysates using glutathione-agarose beads as described by Smith and Johnson (1988).

Iodination of purified chick vinculin fusion protein

Purified vinculin fusion proteins were iodinated using Iodobeads (Amersham) essentially as described by the manufacturer. A 40 μg sample of fusion protein in 500 μl of 100 mM potassium phosphate buffer, pH 7.2, was incubated with 0.5 mCi of ^{125}I-iodide in the presence of an Iodobead for 30 minutes on ice. The iodinated fusion protein was separated from unincorporated ^{125}I-iodide using a PD-10 gel filtration column (Pharmacia) equilibrated and eluted with 50 mM potassium phosphate buffer, pH 7.2. The specific activity of the ^{125}I-vinculin fusion proteins was typically $3\text{-}5\times10^6$ cpm/μg.

Partial purification of paxillin

Paxillin was partially purified according to the procedure described by Turner et al. (1990). Chicken gizzard was homogenized in 10 mM Tris-HCl buffer, pH 8.0, containing 2 mM EDTA, 0.1% β-mercaptoethanol, 0.5 mM PMSF, 5 mM leupeptin. The homogenate was centrifuged at 16,000 ***g*** for 10 minutes and the supernatant retained. Ammonium sulphate (13.4 g/100 ml) was added and the suspension stirred for 1 hour. The precipitated protein was recovered by centrifugation (12,000 ***g*** for 10 minutes), resuspended in 20 mM Tris-acetate buffer, pH 7.6, containing 20 mM NaCl, 0.1 mM EDTA and 0.1% β-mercaptoethanol and dialysed overnight against the same buffer. The sample was then loaded onto a DE-52 (Whatman) column (2×7 cm) and proteins eluted with a 0-325 mM NaCl gradient (650 ml). Fractions were analysed for the presence of paxillin by western blotting using a monoclonal antibody to paxillin as described previously (Turner et al., 1990).

Binding of ^{125}I-vinculin fusion proteins to partially purified paxillin

The proteins present in the partially purified paxillin preparation were resolved in 10% SDS-polyacrylamide gels and transferred to nitrocellulose filters (Sartorius). The filters were cut into strips and incubated in 3% BSA (w/v) in 20 mM Tris, pH 7.4, 0.15 M NaCl for 1 hour. Filters were incubated with the appropriate ^{125}I-vinculin fusion protein (12 nM) in the same buffer overnight with gentle agitation. Filters were subsequently washed three times with a large excess of buffer, dried and bound ^{125}I-vinculin fusion protein detected by autoradiography. Where appropriate, filters were pre-incubated with either unlabelled fusion protein or unlabelled vinculin prior to the addition of the iodinated fusion protein.

Binding of ^{125}I-paxillin to vinculin fusion proteins

Paxillin was purified to homogeneity from chicken gizzard smooth muscle and iodinated using iodogen, exactly as described previously (Turner et al., 1990). Purified vinculin fusion proteins were separated in 10% SDS-polyacrylamide gels, the proteins electroblotted to nitrocellulose and the blocked filters incubated with ^{125}I-paxillin (7.5×10^5 cpm/ml) in 20 mM Tris/acetate buffer, pH 7.6, 20 mM NaCl, 0.1 mM EDTA, 0.1% β-mercaptoethanol containing 3% BSA for 90 minutes at room temperature. Filters were then washed in buffer containing 0.2% gelatin and 0.05% Tween-20, and bound ^{125}I-paxillin detected by autoradiography.

Expression of chick vinculin polypeptides in monkey Cos cells

Chick vinculin cDNAs encoding residues 398-1066, 398-1028, 398-978, 398-928 and 398-881 were generated by PCR using the cVin5 cDNA as template. Both the 5′ and 3′ primers contained *Eco*RI sites, and products were subcloned into the *Eco*RI site in the eukaryotic expression vector pECE, which contains the SV40 early promoter (Ellis et al., 1986). Plasmids containing inserts in the correct orientation were identified by restriction enzyme mapping and sequencing. Covalently closed circular DNA was prepared by polyethylene glycol precipitation (Sambrook et al., 1989), and the pECE constructs transfected into monkey Cos cells using a DEAE-dextran method (Cullen, 1987).

Isolation of stable mouse cell lines expressing chick vinculin polypeptides

NIH3T3 cell lines stably expressing chick vinculin polypeptides were generated by sub-cloning the blunt ended vinculin cDNAs into the filled *Hin*dIII site in the eukaryotic expression vector pRc/CMV (Invitrogen). The inserted cDNAs are expressed from the human cytomegalovirus promoter. This plasmid vector also contains the

neomycin resistance gene, which is expressed from the SV40 promoter. DNA was transfected into cells by the calcium phosphate method (Solowska et al., 1989). G418 resistant clones were screened for those expressing the appropriate chick vinculin polypeptide by immune precipitation from cells grown in the presence of [^{35}S]methionine as described previously (Jackson et al., 1989; Jones et al., 1989).

Immunofluorescence

Coverslip cultures of cells were fixed and permeabilized for fluorescence microscopy as previously described (Jackson et al., 1989). Double staining of vinculin and actin was performed by first incubating with a chick-specific rabbit anti-vinculin (diluted 1:1000 with 0.1% BSA in phosphate buffered saline) and then with Texas Red-labelled donkey anti-rabbit (diluted 1:50) (Amersham). F-actin was then stained with NBD-phallocidin (Molecular Probes, Eugene, Oregon, USA) exactly as described by the manufacturers. Photographs were taken with a Zeiss Axiophot photomicroscope equipped with epifluorescence using Ilford HP5 film (ASA 400) uprated to 1600 ASA.

Cell culture

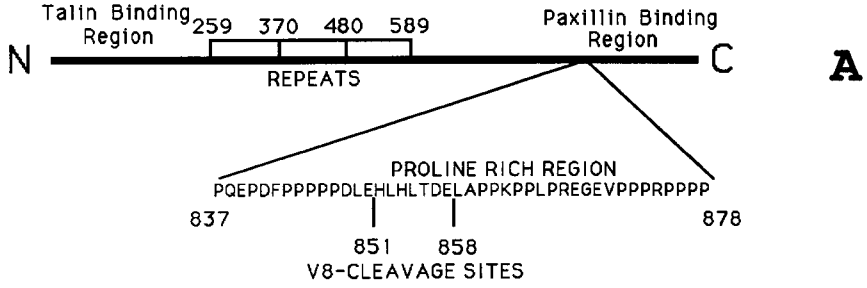
Monkey Cos cells and mouse NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum.

RESULTS

In order to define further the paxillin-binding site within the C-terminal region of the vinculin molecule, we expressed a series of overlapping vinculin cDNAs as glutathione-S-transferase (GST) fusion proteins in *E. coli* using the pGEX-2 prokaryotic expression plasmid. Initially we attempted to express a C-terminal fusion protein spanning residues 398-1066. However, it was unstable probably due to cleavage within the proline-rich region of the molecule (residues 837-878), which we have previously shown contains two V8-proteolytic cleavage sites (Price et al., 1989; and Fig. 1A). We therefore expressed fusion proteins spanning residues 881-1066, 881-1028 and 881-978. The molecular mass of the first two fusion proteins determined from SDS-PAGE was close to that predicted from the deduced amino acid sequence whereas that of the fusion protein spanning residues 881-978 was slightly less than that predicted (Table 1). All three fusion proteins were soluble, and were readily purified from *E. coli* cell lysates using glutathione-agarose beads. The fusion proteins were stable although GST/881-1028 displayed some evidence of degradation (Fig. 2).

Binding of ^{125}I-vinculin fusion proteins to paxillin using a gel-blot assay

To establish which if any of the above C-terminal vinculin fusion proteins contained paxillin-binding activity, paxillin was partially purified from chicken gizzard using a DE52 anion exchange column and the paxillin containing fractions resolved by SDS-PAGE and electroblotted to nitrocellulose. Filters were then incubated with each of the iodinated vinculin fusion



B

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                870          881                                        913
                 +            +                                          +
chick            VPPPRPPPPE   EKDEEFPEQK  AGE------A  INQPMMMAAR  QLHDEARKWS
human            VPPPRPPPPE   EKDEEFPEQK  AGE------V  INQPMNMAAR  QLHDEARKWS
nematode         SPPPRPPPPP   EYDEEEETRA  FWERYPLPQA  SHQPMLAAAH  NLHNELHQWS

                914          928                                        963
                 +            +                                          +
chick            SKGNDIIAAA   KRMALLMAEM  SRLVRGGSGN  KRALIQCAKD  IAKASDEVTR
human            SKGNDIIAAA   KRMALLMAEM  SRLVRGGSGT  KRALIQCAKD  IAKASDEVTR
nematode         SQENDIIAAA   KRMAILMARL  SQLVRGEGGT  KKDLINCSKA  IADSSEEVTR

                964          978  Putative paxillin b.s.  1000
                 +            +                           +
chick            LAKEVAKQCT   DKRIRTNLLQ  VCERIPTISR  QLKILSTVKA  TML-------
human            LAKEVAKQCT   DKRIRTNLLQ  VCESIPTIST  QLKILSTVKA  TML-------
nematode         LAVQLARLCT   DIKMRTALLQ  VSERIPTIAT  QLKVLSTVKA  TMLGSADVIG

                1007              1028                              1054
                  +    **  * *  *    +                                +
chick            --GRTNISDE   ESEQATEMLV  HNAQNLMQSV  KETVREAEAA  SIKIRTDAGF
human            --GRTNISDE   ESEQATEMLV  HNAQNLMQSV  KETVREAEAA  SIKIRTDAGF
nematode         PYGQPVEGSE   EDDEAMQQLV  HNAQNLMQSV  KDVVRAAEAA  SIKIRTNSGL

                1055         1066
                 +            +
chick            TLRWVRKTPW   YQ
human            TLRWVRKTPW   YQ
nematode         RLRWLRKPMW   SNF
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Fig. 1. Alignment of a series of PCR-generated chick vinculin cDNAs with respect to the structure of the vinculin molecule. (A) The domain structure of vinculin (1066 amino acids) is represented schematically to show the positions of the talin-binding domain, the three 112 amino acid repeats, the proline-rich region, the V8-protease cleavage sites and the paxillin-binding region. (B) Alignment of the C-terminal region of chicken (Coutu and Craig, 1988; Price et al., 1989); human (Weller et al., 1990); and nematode vinculin (Barstead and Waterston, 1989). Amino acid residue numbers based on the chick and human vinculin sequences are shown above the alignments. The four glutamate residues and one aspartate residue that form an acidic patch in the middle of a region that is otherwise predominantly basic are indicated (*). The paxillin-binding site (residues 978-1000) is boxed. Pad characters are indicated by a dashed line.

Table 1. Paxillin-binding activity and localization of chick vinculin polypeptides

(A) Paxillin-binding activity of various C-terminal chick vinculin polypeptides

Amino acids encoded by cDNA	Mol. mass (kDa)		Binding	
	Expected	Actual	^{125}I-fusion protein to paxillin	^{125}I-paxillin to fusion protein
881-1066	46.9	45	+	+
881-1028	42.5	41	+	+
881-1021	41.6	38	nd	+
881-1012	40.6	38	nd	+
881-1000	39.4	36	nd	+
881-978	36.9	34	–	–

(B) Localization of expressed chick vinculin polypeptides to focal adhesions

Amino acids encoded by cDNA	Mol. mass (kDa)		Localization	
	Expected*	Actual	Cos cells	NIH 3T3 cells
398-1066	71.8	73	+++	++
398-1028	67.3	70	++	++
398-1000	64.2	70	nd	–
398-978	61.8	66	+	–
398-928	56.3	65	–	nd
398-881	51.0	54	–	nd

* Predicted initiating methionine residue 412.

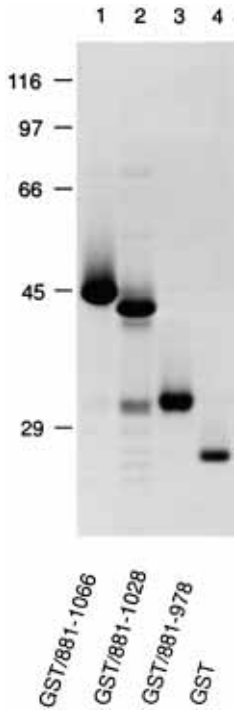

Fig. 2. Expression of chick vinculin cDNAs as fusion proteins in *E. coli*. Chick vinculin cDNAs encoding residues: lane 1, 881-1066; lane 2, 881-1028; and lane 3, 881-978; were cloned into the plasmid expression vector pGEX and the polypeptides expressed as GST-fusion proteins in *E.coli.* Fusion proteins were purified using glutathione-agarose beads and analysed by SDS-PAGE (10% gel). Lane 4, control, GST only. The gel was stained with Coomassie Blue. Molecular mass standards (kDa) are shown on the left.

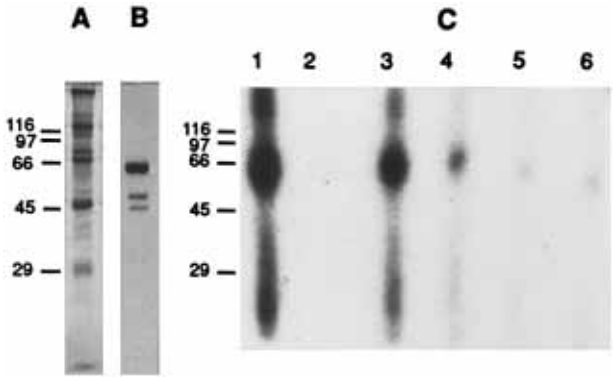

Fig. 3. Analysis of the binding of ^{125}I-labelled vinculin fusion proteins to paxillin using a gel-blot assay. Paxillin was partially purified from chick gizzard by chromatography on a DE-52 anion exchange column (Turner et al., 1990). The fractions containing paxillin were resolved by SDS-PAGE (10% gel) and either stained with Coomassie Blue (A) or electroblotted to nitrocellulose (B,C) and the blot stained with a monoclonal antibody to paxillin (B) or incubated with the ^{125}I-labelled vinculin fusion proteins (C); lanes 1,2, GST/881-1066; lanes 3,4, GST/881-1028; lanes 5,6, GST/881-978. Lanes 2,4 and 6 were pre-incubated with a 100-fold excess of the appropriate unlabelled fusion protein. The bound ^{125}I-labelled fusion protein was detected by autoradiography. Molecular mass markers (kDa) are shown to the left of the figure.

proteins, and binding detected by autoradiography. Coomassie Blue staining of the partially purified paxillin revealed the presence of several proteins although paxillin (68 kDa) itself is poorly stained by Coomassie Blue (Fig. 3A). In order to confirm that paxillin was present in the extract, a similar blot was incubated with a mouse monoclonal antibody to paxillin and bound antibody visualised with an anti-mouse alkaline phosphatase conjugate (Fig. 3B). A major immunoreactive band at 68 kDa was observed along with two minor immunoreactive bands at 48 kDa and 44 kDa. These two bands are believed to be breakdown products of paxillin (Turner et al., 1990). The iodinated fusion proteins GST/881-1066 and GST/881-1028 both bound to a protein of similar electrophoretic mobility to that of paxillin (Fig. 3C, lanes 1 and 3), whereas the iodinated fusion protein GST/881-978 bound only very weakly to this protein (Fig. 3C, lane 5). Binding of the two former fusion proteins to paxillin was specific in that it was inhibited by a 100-fold molar excess of the appropriate unlabelled fusion protein (Fig. 3C, lanes 2 and 4) and unlabelled vinculin (data not shown). In contrast, the low level binding of GST/881-978 was not inhibited by unlabelled fusion protein (Fig. 3C, lane 6). The results suggest that

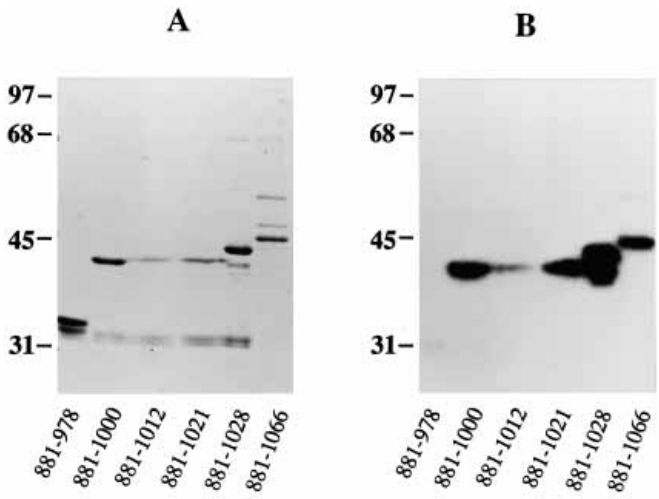

Fig. 4. Analysis of ^{125}I-paxillin binding to purified vinculin fusion proteins using a gel-blot assay. GST/vinculin fusion proteins were purified from *E. coli*, separated by SDS-PAGE (10% gel) and either stained with (A) Coomassie Blue or (B) electroblotted to nitrocellulose filters. The filters were incubated with ^{125}I-paxillin and bound protein detected by autoradiography. Molecular mass markers (kDa) are shown to the left of the figure. The vinculin residues encoded by each of the proteins are shown at the bottom of the figure. It is important to note that the amounts of the various fusions proteins have not been normalised (A).

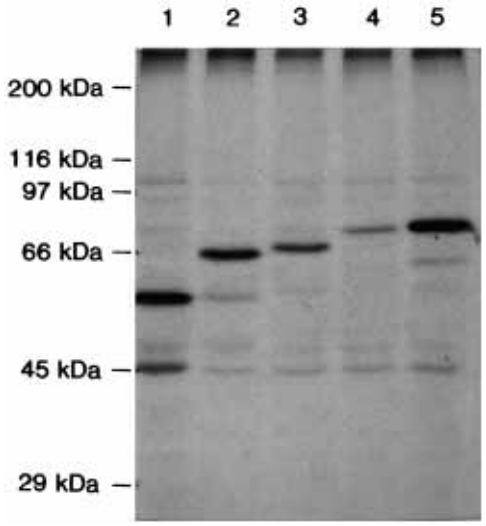

Fig. 5. Immune precipitation of chick vinculin polypeptides expressed in monkey Cos cells. Monkey Cos cells were transfected with chick vinculin cDNAs cloned into the eukaryotic expression vector pECE (Ellis et al., 1986). Cells were cultured in the presence of [^{35}S]methionine and expressed polypeptides isolated by immune precipitation using a rabbit polyclonal antibody to chick gizzard vinculin. Labelled polypeptides were analysed in 10% SDS-PAGE gels, and the labelled proteins detected by fluorography. The vinculin cDNAs expressed encoded amino acid residues: lane 1, 398-881; lane 2, 398-928; lane 3, 398-978; lane 4, 398-1028; and lane 5, 398-1066. Molecular mass markers (kDa) are shown to the left of the figure.

vinculin residues 978-1028 contain a paxillin-binding site, although we have not been able to prove formally that the protein recognised by the iodinated vinculin fusion proteins in the crude paxillin extract is indeed paxillin.

Binding of ^{125}I-paxillin to vinculin fusion proteins

Because of these difficulties, the purified vinculin fusion proteins were resolved by SDS-PAGE, electroblotted to nitrocellulose and the filters incubated with ^{125}I-paxillin. The ^{125}I-paxillin bound to fusion proteins spanning residues 881-1066 and 881-1028, but did not bind to the fusion protein spanning residues 881-978 (Fig. 4). GST alone showed no binding activity (data not shown). The results are in complete agreement with those obtained using the iodinated vinculin fusion proteins, and again suggest that paxillin binds to a site contained within vinculin residues 978-1028. Inspection of the amino acid sequence in this region of the vinculin molecule shows that residues 978-1000 have a predicted pI of 9.77 whereas residues 1000-1028 have a predicted pI of 4.41 due to a cluster of five acidic amino acids spanning residues 1013-1021 (Fig. 1B). To assess the relative contributions of these regions to paxillin binding, we expressed and purified three additional vinculin fusion proteins spanning residues 881-1021, 881-1012 and 881-1000. All of these fusion proteins bound ^{125}I-paxillin in the gel-overlay assay (Fig. 4). Whilst it is important to note that the paxillin-binding assay is not quantitative, there is no obvious indication that the fusion protein spanning residues 881-1000 is any less able to bind paxillin than one containing residues 881-1028. The result suggests that the paxillin-binding site in vinculin is contained within residues 978-1000 and establishes that the acidic patch within residues 1013-1021 is not essential for paxillin binding.

Transient expression of chick vinculin cDNAs in monkey Cos cells

Vinculin has previously been shown to contain a domain C-terminal to residue 881 that can support targeting of vinculin to cell-matrix junctions (Bendori et al., 1989). This domain functions quite independently of the N-terminal targeting domain (residues 1-258), which contains the talin-binding site (Jones et al., 1989; Gilmore et al., 1992). To establish whether the C-terminal targeting sequence in vinculin is the same as the paxillin-binding site, a chick vinculin cDNA encoding residues 398-1066 plus a number of C-terminal deletion mutants (residues 398-1028, -978, -928, -881) were transiently expressed in monkey Cos cells, and their ability to target to focal adhesions monitored by immunofluorescence. The expression of these polypeptides relies upon translation initiating at internal AUG codons close to the 5′ end of the cDNA. In order to establish that the expressed polypeptides were of the predicted size, Cos cells were labelled with [^{35}S]methionine and the expressed vinculin polypeptides immunoprecipitated with a polyclonal antibody specific for chick vinculin (Fig. 5). All of the vinculin polypeptides were expressed at similar levels, and all were of a similar size to that deduced from the amino acid sequence with the exception of that synthesised from the cDNA encoding residues 398-928, which was substantially larger than predicted (Table 1). However, the authenticity of this construct was confirmed by DNA sequencing, and the anomalous behaviour of the polypeptide encoded by this construct remains unexplained. The vinculin polypeptide synthesised from a cDNA encoding residues 398-881 gave

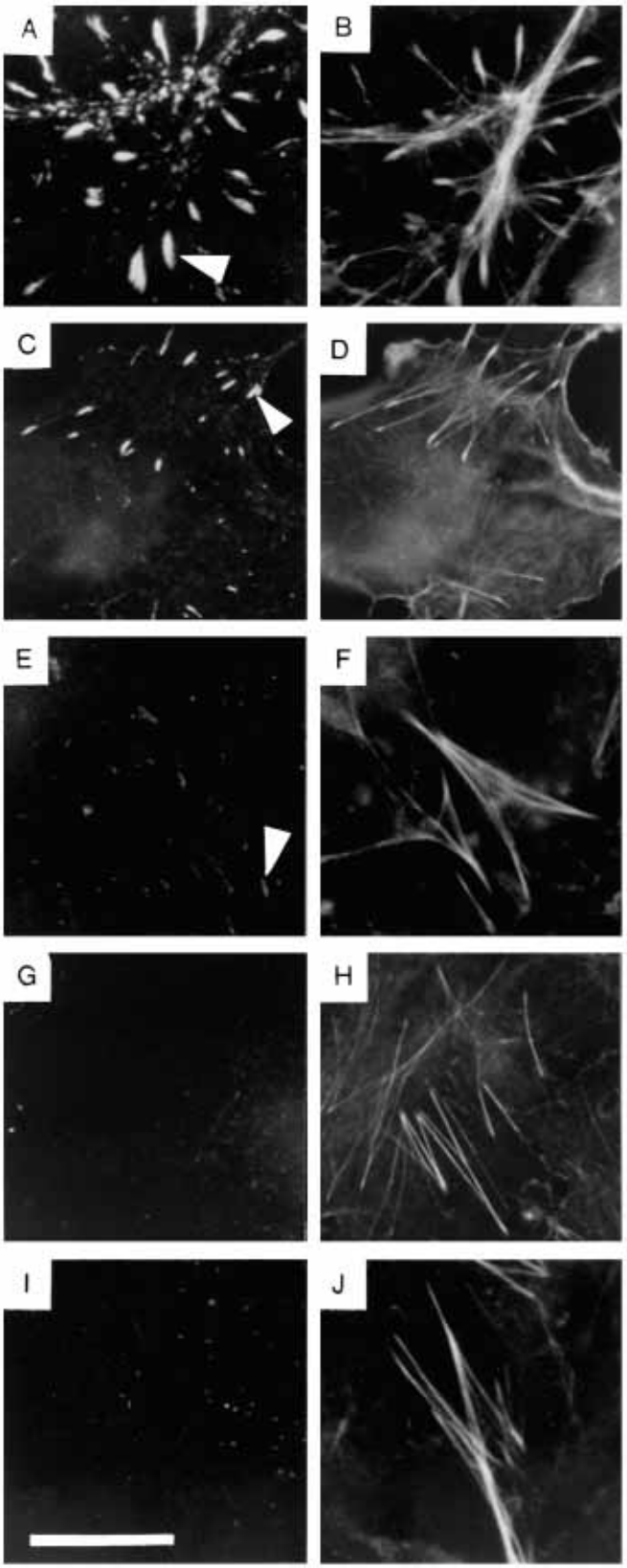

Fig. 6. Immunofluorescence localisation of chick vinculin polypeptides expressed in monkey Cos cells. Cos cells expressing chick vinculin cDNAs were double stained for the expressed chick vinculin polypeptides (A,C,E,G,I) or for F-actin (B,D,F,H,J). The vinculin cDNAs expressed encoded amino acid residues (A,B) 398-1066; (C,D) 398-1028; (E,F) 398-978; (G,H) 398-928 (I,J) 398-881. Bar, 3 μm.

rise to two major products. One of these polypeptides with a molecular mass of approximately 54 kDa was of the predicted size, but a smaller polypeptide of approximately 45 kDa was also expressed. This is likely to be a degradation product of the vinculin polypeptide, and indeed a similar size polypeptide was immunoprecipitated from cells expressing each of the vinculin cDNAs (Fig. 5).

In order to examine the cellular distribution of the expressed chick vinculin polypeptides in Cos cells, coverslip cultures of transfected cells were fixed and immunostained with a chick-specific polyclonal vinculin antibody. The vinculin polypeptide synthesised from the cDNA encoding residues 398-1066 localised very strongly to focal adhesions (Fig. 6A,B). Similarly, the polypeptide synthesised from the cDNA encoding residues 398-1028 also clearly localised to focal adhesions (Fig. 6C,D) although staining was somewhat less intense than the polypeptide with an intact C-terminal domain. In contrast, the polypeptide synthesised from the cDNA encoding residues 398-978 targeted only weakly to focal adhesions (Fig. 6E,F), and the polypeptides synthesised from cDNAs encoding residues 398-928 and 398-881 both failed to target to focal adhesions (Fig. 6G-J) even though it is clear from the immunoprecipitation experiments that these polypeptides are expressed in the Cos cells. The lack of any staining suggests that the polypeptides remain in a soluble cytoplasmic pool and are extracted by permeabilisation of cells prior to fixation.

Stable expression of chick vinculin cDNAs in mouse NIH3T3 cells

The levels of expression of proteins from plasmid vectors possessing the SV40 origin of replication in Cos cells is often very high. To verify the results of transient expression experiments, we isolated a number of mouse NIH3T3 cell lines stably expressing cDNAs encoding one of the following chick vinculin polypeptides; residues 398-1066, 398-1028 and 398-978. Clones expressing similar amounts of the various vinculin polypeptides were identified by immune precipitation from [^{35}S]methionine-labelled cells (Fig. 7). The vinculin polypeptides synthesised from the cDNAs encoding residues 398-1066 and 398-1028 both localised to focal adhesions in stable cell lines (Fig. 8A-D). These results are entirely consistent with those obtained using the transient expression system. Interestingly, the vinculin polypeptide synthesised from the cDNA encoding residues 398-978 failed to target to focal adhesions when stably expressed in NIH3T3 cells, although it did localise to focal adhesions, albeit weakly, when transiently expressed in Cos cells (Fig. 6E,F). The results of the transient and stable expression experiments indicate that the major determinants of targeting of the C-terminal region of vinculin to focal adhesions are localised between residues 978 and 1028. In an attempt to further define the focal adhesion targeting sequence in this region, we isolated a stable cell line expressing a vinculin cDNA encoding residues 398-1000. Although the protein was expressed at about the same level as the other vinculin polypeptides (Fig. 7), it failed to target to focal adhesions (Fig. 8). The results suggest that the C-terminal

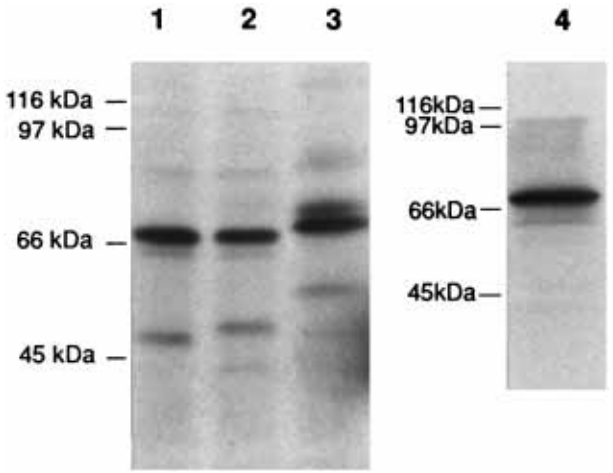

Fig. 7. Immune precipitation of chick vinculin polypeptides expressed in stable mouse NIH3T3 cell lines. Mouse NIH3T3 cells were transfected with chick vinculin cDNA's cloned into the eukaryotic expression vector pRc/CMV, and selected for G418 resistance as described in Materials and Methods. Cloned cell populations were cultured in the presence of [^{35}S]methionine and expressed polypeptides isolated by immune precipitation using a chick-specific polyclonal vinculin antibody. Labelled polypeptides were analysed by SDS-PAGE (10% gel) and detected by autoradiography. The vinculin cDNAs expressed encoded amino acid residues: lane 1, 398-978; lane 2, 398-1028; lane 3, 398-1066; lane 4, 398-1000. Molecular mass markers (kDa) are shown to the left of each autoradiograph.

targeting sequence in vinculin lies between residues 1000 and 1028.

DISCUSSION

The paxillin-binding region in vinculin was initially identified by studying binding of ^{125}I-paxillin to proteolytic fragments of vinculin using a gel-overlay assay identical to that used in the present study (Turner et al., 1990). ^{125}I-paxillin was found to bind to a 27 kDa fragment of vinculin liberated by V8-protease that we have previously shown to be derived from the C-terminal region of the protein by cleavage between residues 857 and 858 (Price et al., 1989). The paxillin-binding site thus defined is contained within residues 858-1066. In the present study, we have used a series of vinculin polypeptides expressed as fusion proteins in *E. coli* to further define the paxillin-binding site. Fusion proteins starting at residues 881 and extending to residues 1066 and 1028 were both shown to bind paxillin in the gel-blot assay, using either ^{125}I-labelled vinculin fusion proteins and unlabelled paxillin or ^{125}I-paxillin and unlabelled fusion proteins. In contrast, a fusion protein spanning vinculin residues 881-978 was negative in both assays suggesting that the paxillin-binding site is contained within residues 978-1028 (Table 1). The pI of vinculin C-terminal to residue 858 (as deduced from the amino acid sequence), is 9.7 whereas the amino terminal domain has a pI of 5.4 (Coutu and Craig, 1988). Within the C-terminal basic region there are two clusters of acidic residues, one (residues 883-892) immediately C-terminal to the proline-rich region,

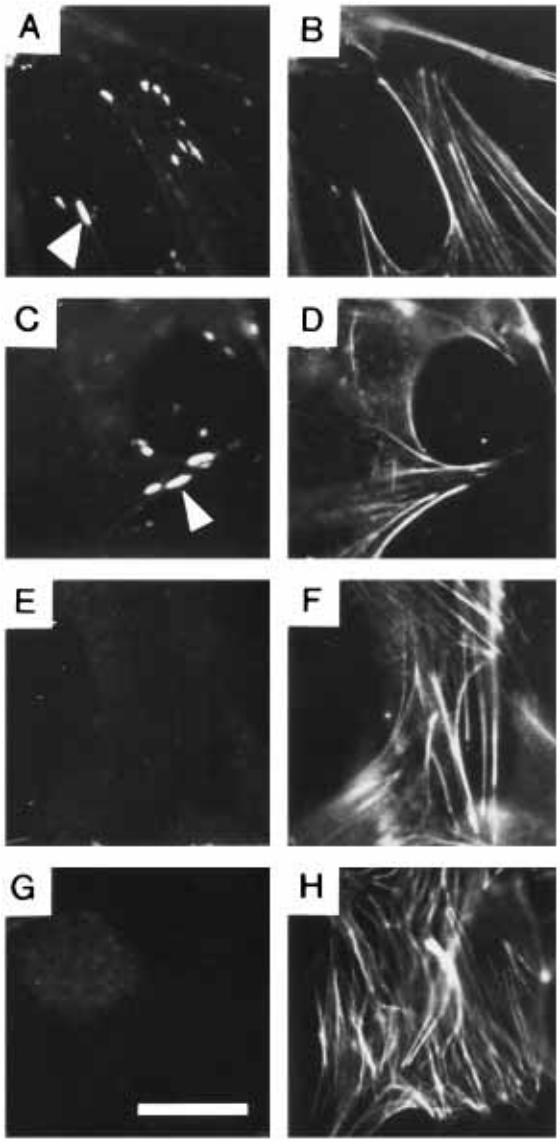

Fig. 8. Immunofluorescence localisation of chick vinculin polypeptides expressed in stable mouse NIH3T3 cell lines. Mouse NIH3T3 cells stably expressing chick vinculin cDNAs were double stained for either the expressed chick vinculin polypeptides (A,C,E,G) or for F-actin (B,D,F,H) as described in Materials and Methods. The vinculin cDNAs expressed encoded amino acid residues (A,B) 398-1066; (C,D) 398-1028; (E,F) 398-978; (G,H) 398-1000. Bar, 3 μm.

and the other (residues 1013-1021) within the paxillin-binding region identified above (Fig. 1B). Indeed, although the overall pI of residues 978-1028, which contain the paxillin-binding site is 5.78, the sequence can be sub-divided into residues 978-1000 with a pI of 9.77, and residues 1000-1028 with a pI of 4.41. However, analysis of the binding of ^{125}I-paxillin to fusion proteins lacking this acidic patch has clearly established that it is not required for paxillin binding, and these studies have further defined the paxillin-binding site to within residues 978-1000. This is a highly conserved region of vinculin with only five amino acid differences between chick and nematode

vinculin (Fig. 1B), although it is not known whether nematodes also contain paxillin.

The results of experiments designed to identify the C-terminal focal adhesion targeting sequence in vinculin have again focussed attention on residues 978-1028 (Table 1). Chick vinculin polypeptides expressed from cDNAs encoding residues 398-1066 and 398-1028 both targeted to focal adhesions when transiently expressed in monkey Cos cells or stably expressed in mouse NIH3T3 cells. In contrast, a vinculin polypeptide spanning residues 398-978 failed to localise to focal adhesions when stably expressed in NIH3T3 cells, although it did target weakly in Cos cells. Deletion of residues C-terminal to 978 may only partially inactivate the C-terminal targeting sequence reducing its affinity for the focal adhesion component(s) recognised by this region of the vinculin molecule. The level of expression of the vinculin polypeptide spanning residues 398-978 may be sufficiently high in Cos cells to support low affinity binding. To further define the boundaries of the targeting sequence, we stably expressed a vinculin cDNA encoding a polypeptide spanning residues 398-1000 in NIH3T3 cells. The fact that the polypeptide failed to target to focal adhesions suggests that the C-terminal residues important in localisation of vinculin to focal adhesions are contained within residues 1000-1028.

Because of the close proximity of the paxillin-binding region in vinculin (residues 978-1000) to the focal adhesion targeting sequence (residues 1000-1028), it is tempting to speculate that the sites are one and the same. However, we cannot and do not exclude the possibility that the paxillin-binding site and focal adhesion targeting sequence in vinculin are distinct sites. In evaluating the data, it is important to note that the paxillin-binding site was mapped using vinculin polypeptides resolved in denaturing SDS-gels. Although the polypeptides presumably refold following blotting to nitrocellulose filters, the resulting conformation may be sufficiently different from that of the polypeptides expressed in Cos cells or NIH3T3 cells to exhibit slightly different binding characteristics. We have noted similar slight discrepancies between the boundaries of the vinculin-binding regions in talin as defined by in vitro biochemical analysis, and those sequences that are required for targeting talin to focal adhesions (Gilmore et al., 1993).

The functional significance of the interaction between vinculin and paxillin remains to be established. It is interesting to note that although vinculin and paxillin co-localise in focal adhesions, vinculin but not paxillin is also found in cell-cell junctions (Turner et al., 1990). The two proteins can therefore function independently of each other. Several lines of evidence suggest that vinculin is an essential structural component of focal adhesions. Microinjection of antibodies to vinculin disrupts focal adhesions (Westmeyer et al., 1990), and an F9 teratocarcinoma cell line lacking vinculin has recently been shown to be adhesion defective (Samuels et al., 1993). Expression of the full length vinculin cDNA rescues this phenotype. The fact that vinculin binds to talin (Burridge and Mangeat, 1984) and α-actinin (Belkin et al., 1987; Wacchstock et al., 1987) as well as paxillin is also consistent with the view that vinculin plays a structural role in focal adhesions. The lower abundance of paxillin may point more toward a regulatory rather than a structural role for this protein in focal adhesions. Paxillin is heavily phosphorylated on tyrosine residues at an early stage during the assembly of focal adhesions on fibronectin (Burridge et al., 1992) and it may be a substrate for the focal adhesion kinase pp125FAK (Turner et al., 1993). Whether paxillin phosphorylation increases binding to vinculin and acts as a trigger for the assembly of focal adhesions is unknown, although tyrosine kinase inhibitors do inhibit assembly of focal adhesions (Burridge et al., 1992). Vinculin is itself a phosphoprotein, but it is not heavily phosphorylated on tyrosine residues during focal adhesion assembly on fibronectin (Burridge et al., 1992). However, vinculin has been reported to be phosphorylated by protein kinase C both in vivo and in vitro (Werth and Pastan, 1984) and inhibitors of protein kinase C can also block focal adhesion assembly (Woods and Couchman, 1992). Further insight into the role of the vinculin-paxillin interaction may come from experiments in which vinculin cDNAs lacking the paxillin-binding site are expressed in the vinculin-minus adhesion-defective F9 teratocarcinoma cells (Samuels et al., 1993). The recent isolation of paxillin cDNAs by one of us (C.E.T.) should also help in this regard.

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