

The Cytoskeletal Protein Talin Contains at Least Two Distinct Vinculin Binding Domains

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Abstract. We have mapped the vinculin-binding sites in the cytoskeletal protein talin as well as those sequences which target the talin molecule to focal contacts. Using a series of overlapping talin-fusion proteins expressed in *E. coli* and ¹²⁵I-vinculin in both gel-overlay and microtitre well binding assays, we present evidence for three separable binding sites for vinculin. All three are in the tail segment of talin (residues 434–2541) and are recognized by the same fragment of vinculin (residues 1–258). Two sites are adjacent to each other and span residues 498–950, and

the third site is more than 700 residues distant in the primary sequence. Scatchard analysis of ¹²⁵I-vinculin binding to talin also indicates three sites, each with a similar affinity ($K_d = 2-6 \times 10^{-7}$ M). We also detect a substoichiometric interaction of higher affinity ($K_d = 3 \times 10^{-8}$ M) which remains unexplained. By expressing regions of the chicken talin molecule in heterologous cells, we have shown that the sequences required to target talin to focal contacts overlap those which bind vinculin.

TALIN is one of a number of cytoskeletal proteins including vinculin and α -actinin which are localized in focal contacts, specialized junctions between cells and the extracellular matrix (ECM).¹ Here they are thought to link the cytoplasmic domains of integrins, cellular receptors for ECM proteins, to filamentous actin (Burrige et al., 1988; Hynes, 1992). The first indications that ECM proteins might be linked to the actin cytoskeleton stemmed from the observation that fibronectin fibrils on the surface of fibroblasts coaligned with F-actin stress fibers within the cell (Hynes and Destree, 1978). A molecular basis for this observation began to emerge when it was shown that integrins were able to bind to talin (Horwitz et al., 1986). Talin has in turn been shown to bind to vinculin (Burrige and Mangeat, 1984), and vinculin can bind to the actin-bundling protein α -actinin (Belkin and Kotliansky, 1987; Wachsstock et al., 1987) providing a possible route linking integrins to the actin cytoskeleton. However, talin can bind directly to

F-actin (Muguruma et al., 1990, 1992; Kaufmann et al., 1991), and the cytoplasmic domains of $\beta 1$ and $\beta 3$ integrins can bind to α -actinin (Otey et al., 1990) providing alternative mechanisms for linking integrins to F-actin. There is accumulating evidence that these links are important to integrin function. Mutant integrins containing deletions in the $\beta 1$ cytoplasmic domain fail to localize to focal contacts (Solowska et al., 1989) and are unable to support adhesion (Hayashi et al., 1990), and microinjection of antibodies to both vinculin (Westmeyer et al., 1990) and talin (Nuckolls et al., 1992) inhibit the formation of focal contacts. A more detailed description of the interactions among the various components in focal contacts is therefore likely to be fundamental to our understanding of the molecular basis of cell adhesion.

Towards this end, the primary sequences of talin (270 kD) (Rees et al., 1990), vinculin (117 kD) (Coutu and Craig, 1988; Price et al., 1987, 1989; Weller et al., 1990), and α -actinin (100 kD) (Baron et al., 1987; Noegel et al., 1987) have been determined, and some progress has been made in identifying the various binding domains within these proteins. Thus, we have shown that the talin-binding domain in vinculin is within the NH₂-terminal 258 residues of the molecule (Jones et al., 1989; Gilmore et al., 1992), and the binding site for paxillin, a 68-kD protein also localized in focal contacts, has been identified within the COOH-terminal region of the vinculin molecule (Turner et al., 1990). Similarly, we have defined an actin-binding site within the NH₂-terminal region of α -actinin (Hemmings et al., 1992; Kuhl-

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1. *Abbreviations used in this paper:* ECM, extracellular matrix; GST, glutathione-S-transferase.

man et al., 1992), a domain which also appears to bind zyxin (Crawford et al., 1992; Sadler et al., 1992), an 82-kD protein also localized in focal contacts. Furthermore, $\beta 1$ and $\beta 3$ integrins have been shown to bind to the spectrin-like repeats in α -actinin (Otey et al., 1990). The NH₂-terminal 47-kD fragment of talin liberated by calpain II cleavage before residue 434 (Rees et al., 1990) is important in targeting the molecule specifically to cell-matrix as apposed to cell-cell adherens-type junctions (Nuckolls et al., 1990). Interestingly, this fragment contains a region of homology (residues 164–373) with the NH₂-terminal region of three other cytoskeletal proteins, band 4.1, ezrin (Rees et al., 1990), and radixin (Funayama et al., 1991) as well as with the NH₂-terminal regions of moesin (Lankes and Furthmayr, 1991) and protein tyrosine phosphatase 1 (Yang and Tonks, 1991). The significance of these homologies remains to be determined. Relatively little progress has been made in identifying the binding sites for integrins (Horwitz et al., 1986), vinculin (O'Halloran and Burridge, 1986), and actin (Muguruma et al., 1990, 1992; Kaufmann et al., 1991) within talin. All are reportedly contained within the large COOH-terminal polypeptide (apparent *M_r* 190 kD) liberated by calpain II cleavage. In the present study we have used a series of overlapping talin fusion proteins to define the vinculin binding site(s) in talin. We have also expressed a series of chicken talin cDNAs in monkey Cos cells and mouse NIH 3T3 cells in an attempt to establish whether there is any correspondence between the sequences required for targeting talin to focal contacts and those involved in binding to vinculin.

Materials and Methods

Chicken Talin cDNAs

Chicken talin cDNAs were isolated from both oligo-dT and random primed chicken embryo cDNA libraries constructed in λ gt11 and λ ZAP (Stratagene Inc., La Jolla, CA) either by antibody screening or by screening with mouse talin cDNA probes (Rees et al., 1990). The complete sequence of chicken talin deduced from analysis of these clones shows a high degree of similarity with that of mouse talin (Rees et al., 1990) and will be reported in detail elsewhere.

Expression of Talin Polypeptides as Fusion Proteins

Chicken talin polypeptides were expressed as fusion proteins with glutathione-s-transferase (GST) using the prokaryotic expression vector pGEX (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). Inserts from the talin cDNA Bluescript constructs 54A, G3, G2, and GG4A were liberated by EcoRI digestion and were subcloned in frame into the EcoRI site of the appropriate pGEX vector. Because the talin cDNA 23B has an internal EcoRI site, the cDNA insert was isolated by PCR (see below). BamHI sites were incorporated into the primers and the PCR product was subcloned into the BamHI site in pGEX. The talin cDNA pGEX constructs were transformed into *E. coli* strain JM101. Overnight cultures were diluted and grown to an OD_{600nm} of 0.5. Fusion protein expression was induced by addition of 0.5 mM IPTG, and the cells harvested after an additional 3-h growth at 37°C.

Generation of Additional Talin cDNAs by PCR

Additional cDNAs encoding shorter talin polypeptides were generated by PCR using DNA from clones 23B, G3, and GG4A as templates (Fig. 1). The oligonucleotide primers used contained an adjacent EcoRI site followed by four additional nucleotides. PCR was carried out by standard procedures (Sambrook et al., 1989) with an annealing temperature of 55°C. PCR products were ethanol precipitated, cut with EcoRI, gel purified, and subcloned into pGEX. The 5' and 3' ends of all constructs were sequenced using pGEX

oligonucleotide primers. To generate a talin cDNA overlapping clones 54A and 23B, two short PCR products were synthesized, one from clone 54A and extending from the KpnI site to the 3'EcoRI site, and one from 23B extending from the 5' end of the clone to the internal EcoRI site. These two products, which have a 60-bp overlap, were mixed and used in a second PCR reaction containing the 5' 54A primer and the 3' 23B primer, both of which contained novel EcoRI sites. The product of the second PCR reaction was subcloned into pGEX and sequenced. The cDNA generated in this way encoded a talin polypeptide extending from residue 498–853.

Purification and Iodination of Vinculin and Talin

Vinculin was purified from chicken gizzard by the method of Evans et al. (1984) and talin by the method of Molony et al. (1987). Proteins were iodinated using 0.5 mCi of Bolton and Hunter reagent (Amersham Corp., Arlington Heights, IL) essentially according to the manufacturer's instructions. 100 μ l of vinculin or talin (1 mg/ml) in 50 mM sodium phosphate buffer, pH 8.5, was incubated with the reagent for 15 min on ice, and unincorporated label removed by gel filtration. The specific activity of the labeled proteins was $\sim 4\text{--}6 \times 10^6$ cpm per μ g.

Analysis of the Binding of ¹²⁵I-Vinculin to Talin Fusion Proteins Using a Gel-Overlay Assay

E. coli lysates containing the expressed chicken talin fusion proteins were separated in 9% SDS-polyacrylamide gels. Proteins were transferred electrophoretically to nitrocellulose and the position of the fusion proteins marked after visualization with Ponceau S stain (BDH, Poole, Dorset, UK). Filters were incubated in 25 mM Tris, pH 7.5/150 mM NaCl/0.2% Tween 20/1 mM EDTA/4% BSA for 2 h, and incubated with ¹²⁵I-vinculin (4 nM) in the same buffer overnight at room temperature, with gentle agitation. Filters were then washed three times with a large excess of buffer, dried and bound ¹²⁵I-vinculin detected by autoradiography. Where appropriate, filters were preincubated with unlabeled vinculin before the addition of the iodinated protein.

Binding of ¹²⁵I-Vinculin to Talin Fusion Proteins Adsorbed to Microtitre Wells

Fusion proteins were purified from bacterial extracts using glutathione-agarose beads after standard procedures (Smith and Johnson, 1988). The purity of the fusion proteins was assessed by SDS-PAGE, and the yields determined using a Bradford protein assay. Fusion proteins (10 μ g/ml) in 25 mM Tris, pH 7.5/150 mM NaCl (TBS) were adsorbed to microtitre wells for 4 h, and excess protein binding sites blocked by incubation for an additional 4 h with 4% BSA in TBS. ¹²⁵I-vinculin (4 nM) was added to the wells in 100 μ l of 4% BSA/TBS/0.1% Tween 20. After overnight incubation 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 determined in a 5500 gamma counter (Beckman Instrs., Inc., Fullerton, CA).

Expression of Chicken Talin Polypeptides in Monkey Cos Cells

Chicken talin cDNAs were subcloned into the eukaryotic plasmid expression vector pECE (Ellis et al., 1986), expression of the talin cDNAs being driven from the SV40 early promoter. The orientation of the inserts was established by restriction enzyme mapping. Covalently closed circular plasmid DNA was prepared by polyethylene glycol precipitation (Sambrook et al., 1989). pECE constructs were transiently expressed (48 h) in monkey Cos 1 cells using a DEAE transfection procedure (Cullen, 1987).

Isolation of Stable Mouse Cell Lines Expressing Chicken Talin Polypeptides

Mouse NIH 3T3 cell lines stably expressing chicken talin polypeptides were generated in one of two ways. Either cells were cotransfected with recombinant pECE vectors and a plasmid (pICR) encoding resistance to the antibiotic G418 (GIBCO BRL Laboratories, Gaithersburg, MD) or cells were transfected with talin cDNAs cloned into the pRc/CMV eukaryotic expression plasmid (Invitrogen, San Diego, CA) and expressed from the human cytomegalovirus promoter. This plasmid also contains the neomycin-resistance gene which is expressed from the SV40 early promoter. DNA was transfected into cells by the calcium phosphate method, essentially as described in Solowska et al. (1989). Clones of G418-resistant cells expressing

chicken talin polypeptides were identified using immune precipitation from [³⁵S]methionine-labeled cell extracts.

Rabbit Polyclonal Antibody to Chicken Talin

An antiserum against purified chicken gizzard talin was raised in rabbits. The antiserum immunoprecipitated a single protein from extracts of [³⁵S]methionine-labeled chicken embryo cells which comigrated with authentic chicken talin. The antiserum also recognized a protein of the same molecular weight in Western blots of chicken embryo cells, and stained focal contacts of chicken embryo cells in a manner indistinguishable from an antibody to chicken talin kindly provided by Dr. K. Burridge (University of North Carolina, Chapel Hill, NC). Immunofluorescence and immune precipitation experiments showed that the antiserum did not recognize either monkey Cos cell or mouse NIH 3T3 cell talin.

Immunofluorescence and Immune Precipitation

Coverslip cultures of cells were fixed and permeabilized for fluorescence microscopy as previously described (Jackson et al., 1989). For double staining of talin and vinculin, cells were first incubated with the chicken-specific rabbit antibody to talin (diluted 1:500) and a mouse vinculin monoclonal antibody (V284) (diluted 1:1,000), a generous gift from Dr. M. Wilkinson (Royal College of Surgeons, London). Bound antibodies were detected using a 1:50 dilution of Texas red-labeled donkey anti-rabbit and a 1:25 dilution of fluorescein-labeled sheep anti-mouse (Amersham Corp.). All antibodies were diluted in PBS/0.1% BSA. For double staining of talin and actin, cells were first incubated with the rabbit anti-talin followed by the Texas red-labeled donkey anti-rabbit. F-actin was then visualized by staining with NBD-phalloidin (Molecular Probes, Inc., Eugene, OR) 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. Talin was immunoprecipitated from cells labeled with [³⁵S]methionine exactly as previously described for vinculin (Jones et al., 1989).

Results

To define which regions of the talin molecule are able to bind to vinculin, we have expressed a number of overlapping chicken talin cDNAs in *E. coli* as fusion proteins with GST. The position of the amino acid sequences encoded by these cDNAs relative to the structure of talin is shown in Fig. 1. Analysis of the expressed fusion proteins by SDS-PAGE showed that all of the fusion proteins encoded by the talin cDNAs were of the expected size (Fig. 2). All reacted with a polyclonal antibody to chicken gizzard talin with the exception of GST/102-497 and GST/2269-2541 (data not shown). However, these latter fusion proteins did react with monoclonal antibodies to human platelet talin. All fusion proteins showed some evidence of degradation, and this was most apparent in the case of fusion proteins spanning residues 102-497 and 852-1328 (see Fig. 5).

Binding of ¹²⁵I-Vinculin to Talin Fusion Proteins Analyzed Using a Gel-Overlay Assay

To determine which talin fusion proteins bind to vinculin, cell lysates expressing the fusion proteins were fractionated by SDS-PAGE, transferred to nitrocellulose, and the filters incubated with ¹²⁵I-vinculin. The specificity of this assay has been documented previously (Burridge and Mangeat, 1984; O'Halloran and Burridge, 1986), and is demonstrated here by the finding that the binding of ¹²⁵I-vinculin to talin is markedly reduced by 50 nM vinculin (Fig. 3, A and B; lane I) and completely inhibited by 400 nM vinculin (Fig. 3 C;

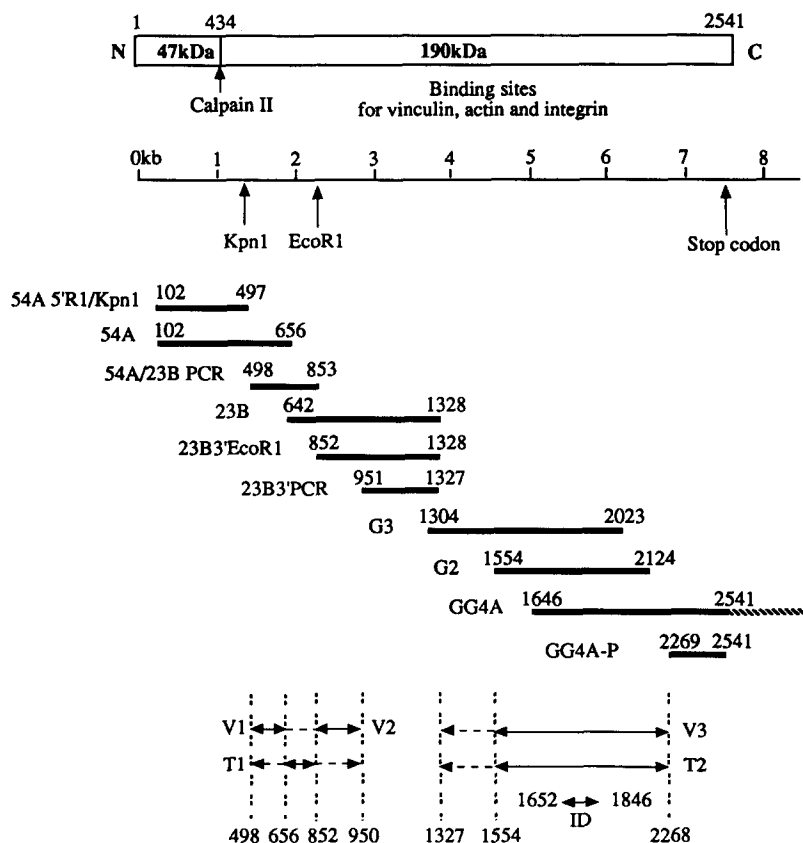


Figure 1. Alignment of a series of overlapping chicken talin cDNA clones with respect to the domain structure of the protein. The domain structure of talin deduced from the complete sequence of the mouse protein (Rees et al., 1990) is shown, and the size (as determined by SDS-PAGE) of the two proteolytic fragments generated by calpain II cleavage between residues 433-434 are indicated. The position of the talin cDNA clones (54A 5'EcoRI/KpnI, 54A, 54A/23B PCR, 23B, etc.) relative to the full length mRNA (~8 kb), and the amino acid residues encoded by these cDNAs are also shown. The position of the stop codon in the talin mRNA, as well as the KpnI and EcoRI restriction enzyme sites are indicated with arrows. Dashed lines show the extent of the 3' untranslated sequence contained within the clone GG4A. A summary diagram indicating the approximate locations of the three identified vinculin-binding sites (V1-V3) and focal contact targeting sequences (T1 and T2) are shown at the bottom of the figure. ID refers to the epitope recognized by the anti-idiotypic antibody (Lee et al., 1992).

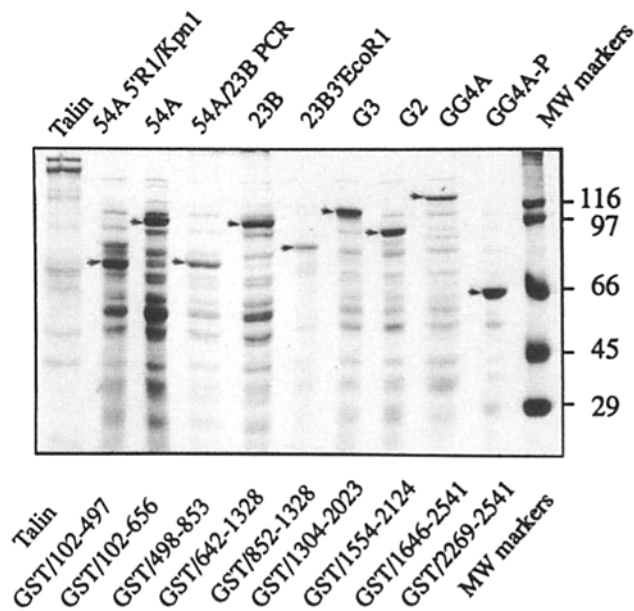


Figure 2. Expression of chicken talin cDNAs as fusion protein in *E. coli*. The chicken talin cDNAs shown in Fig. 1 were cloned into the plasmid vector pGEX and the talin polypeptides expressed as GST fusion proteins in *E. coli*. Proteins present in bacterial cell extracts were resolved by SDS-PAGE (9% gel). Partially purified chicken talin was included for reference. The sample was a mixture of intact talin and the 190-kD proteolytic fragment of talin. SDS-gel stained with Coomassie blue. The positions of intact fusion proteins are indicated (→); molecular weight standards (kD) are shown to the right of the figure. The names of the talin cDNA clones are indicated at the top of the figure. Fusion proteins are named according to the amino acid residues encoded by particular cDNAs at the bottom of the figure.

lane D). Analysis of the binding of ^{125}I -vinculin to the talin fusion proteins showed that all bound ^{125}I -vinculin to some degree, with the exception of GST/102-497, which was consistently negative in this assay. Binding to the COOH-terminal fusion protein GST/2269-2541 was also much lower than to other fusion proteins. Binding could be progressively inhibited by excess unlabeled vinculin, and there was no evidence that ^{125}I -vinculin bound either to GST alone (data not shown) or to any of the *E. coli* proteins present in the cell extracts. Very similar results were obtained using a series of overlapping mouse talin β -galactosidase fusion proteins, except that a fusion protein spanning residues 922-1379 did not bind ^{125}I -vinculin (data not shown). We therefore used PCR to synthesize a chicken cDNA (23B3'PCR) from the 3' end of the 23B cDNA clone which encoded chicken talin residues 951-1327. This fusion protein did not bind ^{125}I -vinculin in the gel-overlay assay (Fig. 4) in agreement with the results obtained using the mouse talin fusion proteins. The results of these experiments are summarized in Table I and indicate that there are several separable regions within the chicken talin molecule able to bind vinculin, i.e., residues 498-656, 852-950, and 1328-2268.

Binding of ^{125}I -Vinculin to Talin Fusion Proteins Adsorbed to Plastic

To establish that ^{125}I -vinculin was able to bind to talin fusion proteins which have not been subject to denaturing gel

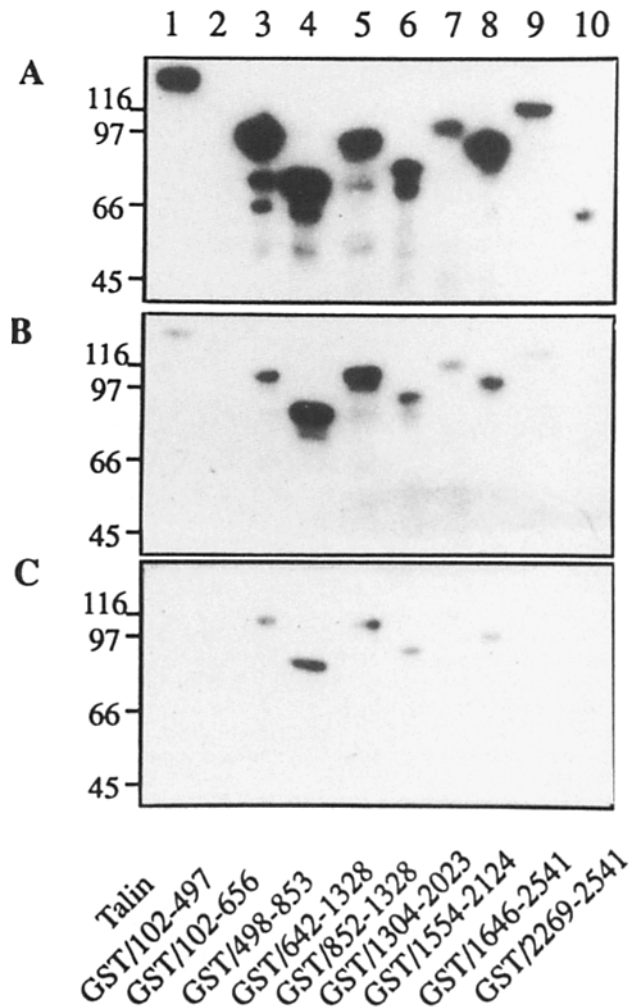


Figure 3. ^{125}I -vinculin binding to chicken talin fusion proteins analyzed using a gel-blot assay. Talin cDNAs were expressed as fusion proteins in *E. coli* and the proteins present in total bacterial cell extracts resolved by SDS-PAGE (9% gel) and blotted to nitrocellulose. The protein loadings were equivalent to those used in Fig. 2. Filters were incubated with ^{125}I -vinculin (4 nM) (A) and ^{125}I -vinculin in the presence of (B) 50 nM and (C) 400 nM unlabeled vinculin as described in Materials and Methods. Bound ^{125}I -vinculin was detected by autoradiography. Fusion proteins are named according to the amino acid residues encoded by particular cDNAs. The position of molecular weight standards (kD) is shown to the left of the figure.

electrophoresis, we purified the expressed chicken talin GST-fusion proteins from cell lysates using glutathione-agarose beads, and studied binding of ^{125}I -vinculin to the fusion proteins adsorbed to microtitre wells. All of the purified fusion proteins were relatively stable, although some showed evidence of limited proteolysis (Fig. 5). ^{125}I -vinculin bound to microtitre wells coated with talin and binding was inhibited progressively by 50 and 400 nM unlabeled vinculin (Fig. 6 A). In contrast, only low levels of ^{125}I -vinculin bound to wells coated with BSA or GST, and binding was not inhibited by unlabeled vinculin. Analysis of the binding of ^{125}I -vinculin to the various fusion proteins produced results which were similar but not identical to those obtained from the gel-overlay assay. The NH₂-terminal talin fusion protein GST/102-497 did not bind ^{125}I -

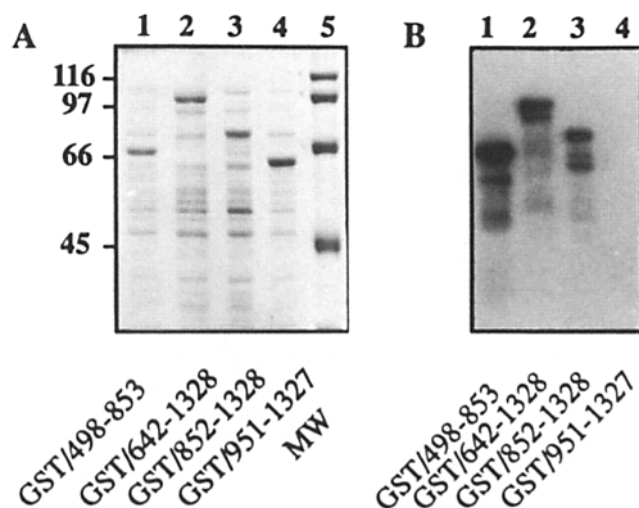


Figure 4. ^{125}I -vinculin binding to talin fusion proteins derived from the cDNA clone 23B. Talin cDNAs were expressed as fusion proteins in *E. coli* and total bacterial cell extracts were resolved by SDS-PAGE. Proteins were either stained with Coomassie blue (A) or were electroblotted to nitrocellulose (B) and the filters incubated with ^{125}I -vinculin (4 nM). Bound vinculin was detected by autoradiography. Fusion proteins are named according to the amino acid residues encoded by particular cDNAs. MW indicates molecular weight standards, the sizes of which are shown in kD on the left of A.

vinculin at levels significantly above background whereas the fusion proteins GST/102-656, GST/498-853, GST/642-1328, and GST/852-1328 each bound ^{125}I -vinculin, and binding was inhibited by unlabeled vinculin. However, ^{125}I -vinculin did not bind to a fusion protein spanning residues 951-1327 supporting the conclusion from the gel-overlay assay that residues 498-950 comprise a vinculin-binding region within the talin molecule. The fact that the fusion proteins GST/102-656 and GST/855-1328 both bind but do not overlap implies that there must be more than one vinculin-binding site within this region of talin. Analysis of the binding of ^{125}I -vinculin to the fusion proteins GST/1554-2124 and GST/1646-2541 revealed a further vinculin-binding site

Table I. Comparison of the Vinculin-binding Activity of Various Chicken Talin Polypeptides with Their Ability to Target to Focal Contacts when Expressed in Mammalian Cells.

Talin cDNA	Amino acid residues*	Vinculin binding		Targeting	
		Gel-blot assay	Microtitre well assay	Cos cells	NIH3T3 cells
54A5'RI/KpnI	102-497	-	-	nd	nd
54A	102-656	+++	+	-	-
54A/23B PCR	498-853	+++	+++	nd	++
23B	642-1328	+++	+++	+	+
23B3'EcoRI	852-1328	++	++	-	-
23B3'PCR	951-1327	-	-	nd	-
G3	1304-2023	++	+/-	+	-
G2	1554-2124	+++	+	+	+
GG4A	1646-2541	++	+	+	+
GG4A-P	2269-2541	+/-	-	nd	nd

*. Talin amino acid residues encoded by cDNA; nd, not determined.

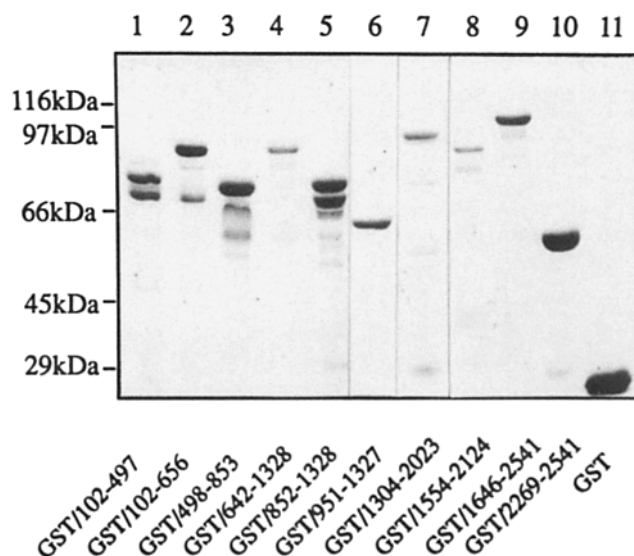


Figure 5. Purified talin fusion proteins. GST/talin fusion proteins were expressed in *E. coli* and purified from cell lysates by standard procedures (Smith and Johnson, 1988). Purified proteins were resolved by SDS-PAGE and visualized by staining with Coomassie blue. Fusion proteins are named according to the amino acid residues encoded by particular cDNAs. GST, glutathione-s-transferase alone. The position of molecular weight standards (kD) is shown to the left of the figure.

in talin. As ^{125}I -vinculin binding to fusion proteins GST/1304-2023 and GST/2269-2541 was close to background, the data suggest that the major determinants of vinculin binding to this region of the talin molecule are contained within residues 2024-2268.

We have previously provided evidence that the talin-binding site in vinculin is within residues 1-258 (Gilmore et al., 1992). To establish that this region of the vinculin molecule is able to bind to the same sites in talin recognized by intact vinculin, we examined the ability of a vinculin fusion protein containing residues 1-258 to compete with ^{125}I -vinculin for binding to talin fusion proteins adsorbed to microtitre wells (Fig. 6B). The results were similar to those obtained using whole vinculin in the competition binding assay (Fig. 6A) and clearly establish that the talin-binding site in vinculin can interact with several distinct regions of the talin molecule.

The results from gel-overlay and microtitre well binding assays are compared in Table I and Fig. 1. They are in reasonably good agreement and can be interpreted to define at least three vinculin-binding sites within talin located between residues 498-656, 852-950, and 2024-2268. Data from the gel-overlay assay suggest the possibility of a fourth site between residues 1304-2023.

Scatchard Analysis of ^{125}I -Vinculin Binding to Talin

To investigate the possibility of multiple vinculin binding sites within the talin molecule in more detail, we analyzed the binding of ^{125}I -vinculin to a fixed amount of talin adsorbed to plastic microtitre wells at concentrations of ^{125}I -vinculin between 10^{-9} M and 4×10^{-6} M. At concentrations of ^{125}I -vinculin above $0.1 \mu\text{M}$, it was not possible to achieve sufficiently high concentrations of unlabeled vinculin to abolish completely binding of the labeled ligand. The

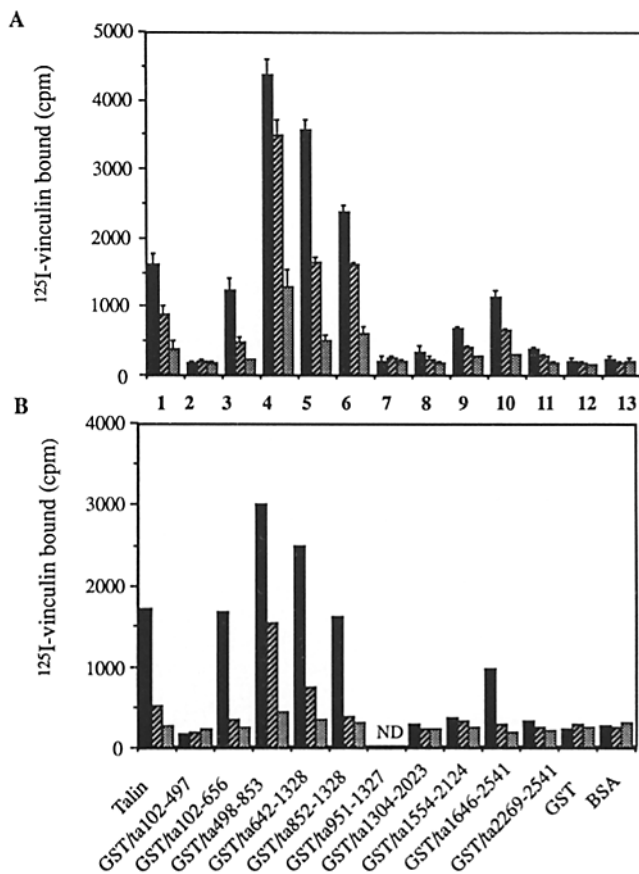


Figure 6. Binding of ^{125}I -vinculin to talin fusion proteins adsorbed to microtitre wells. (A) Microtitre wells were coated with solutions containing equivalent concentrations ($10\ \mu\text{g/ml}$) of the talin fusion proteins, and binding of ^{125}I -vinculin (\blacksquare) to these proteins was determined in quadruplicate as described in Materials and Methods. Binding was also determined in the presence of $50\ \text{nM}$ (▨) and $400\ \text{nM}$ (\blacksquare) unlabeled vinculin. Error bars indicate SD. The experiment was repeated twice. (B) Binding of ^{125}I -vinculin to talin fusion proteins (\blacksquare) was determined in the presence of the unlabeled vinculin fusion protein GST/1-258; $50\ \text{nM}$ (▨) and $400\ \text{nM}$ (\blacksquare). Fusion proteins are named according to the amino acids encoded by particular cDNAs. *GST*, glutathione-s-transferase alone; *BSA*, bovine serum albumin; *ND*, not determined.

amount of nonspecific binding was therefore estimated either by measuring ^{125}I -vinculin binding to wells coated with BSA, or by extrapolation from results obtained with concentrations of ^{125}I -vinculin below $0.1\ \mu\text{M}$ where a 100-fold molar excess of unlabeled ligand could be achieved. Both methods gave similar results. Binding of ^{125}I -vinculin to talin was biphasic with clear evidence for a high and lower affinity interaction (Fig. 7 A). Scatchard analysis of the data (Fig. 7 B) produced K_d values of $3 \times 10^{-8}\ \text{M}$ and $5.5 \times 10^{-7}\ \text{M}$ for the high and lower affinity interaction, respectively, with B_{max} values of 0.15 and 3.0 mol of vinculin bound per mol of talin. We have also analyzed the binding of ^{125}I -vinculin to talin adsorbed to microtitre wells in the presence of increasing concentrations of unlabeled vinculin (Fig. 7 C). Scatchard analysis of the binding data produced K_d values ($10^{-8}\ \text{M}$ and $2.4 \times 10^{-7}\ \text{M}$) close to the values determined from the saturation binding analysis. Binding of ^{125}I -vinculin to the 190-kD fragment of talin (Fig. 7 D) was similar to that of the intact protein (K_d values $1.6 \times 10^{-8}\ \text{M}$

and $2.2 \times 10^{-7}\ \text{M}$) suggesting that the binding sites for vinculin are indeed localized COOH-terminal to the calpain II cleavage site.

Transient Expression of Chicken Talin cDNAs in Monkey Cos Cells

To investigate the relationship between the ability of various talin polypeptides to bind to vinculin *in vitro* and to localize to focal contacts *in vivo*, we transiently expressed some of the overlapping chicken talin cDNAs in monkey Cos cells. Expression of talin polypeptides from these cDNAs relies on translation initiating at internal AUG codons close to the 5' end of the cDNAs. Cos cells transfected with the talin cDNAs cloned into the pECE eukaryotic plasmid expression vector were labeled with [^{35}S]methionine, and the expressed chicken talin polypeptides isolated by immune precipitation using an antibody specific for chicken talin. Labeled chicken talin polypeptides were specifically precipitated from cells transfected with all constructs, and the polypeptides were of the expected sizes deduced from the amino acid sequence (data not shown). To examine the distribution of the expressed chicken talin polypeptides in Cos cells, coverslip cultures of transfected cells were fixed and stained with the antibody to chicken talin. The chicken talin polypeptides encoded by the cDNAs 23B (Fig. 8 C), G3 (Fig. 8 G), G2 (Fig. 8 I), and GG4A (Fig. 8 K) all colocalized with vinculin in focal contacts. In contrast, the polypeptides encoded by 54A (Fig. 8 A) and the 3' EcoRI fragment of 23B (Fig. 8 E) were never found in focal contacts. Indeed, neither of these polypeptides could be detected in transfected cells by immunofluorescence even though it is clear from the immune precipitation experiments that the polypeptides are expressed. It is probable that these polypeptides remain in a soluble compartment and were extracted from cells during permeabilization before fixation. The fact that the chicken talin polypeptide encoded by the 23B cDNA localized to focal contacts, whereas that encoded by 23B 3'EcoRI fragment failed to do so, suggests that there is a targeting signal in the vicinity of talin residues 642-851. The finding that talin polypeptides encoded by the overlapping cDNAs G3, G2, and GG4A also target to focal contacts, suggests that there is at least one additional targeting signal COOH-terminal to residue 1329.

Stable Expression of Chicken Talin cDNAs in Mouse NIH 3T3 Cells

The levels of expression of proteins in Cos cells using plasmid vectors containing the SV40 origin of replication can be very high, and the localization of overexpressed proteins may therefore be subject to artifact. We therefore isolated a number of stable NIH 3T3 cell lines expressing similar amounts of the various chicken talin polypeptides (data not shown). In complete agreement with the results obtained from transient expression, the talin polypeptides encoded by the 54A cDNA (Fig. 9, A and B) and the 23B3'EcoRI (data not shown) failed to localize to focal contacts whereas that encoded by the 23B cDNA was able to do so (Fig. 9, E and F). The chicken talin polypeptide expressed from the cDNA 54A/23B PCR also localized to focal contacts (Fig. 9, C and D), and the staining was noticeably stronger than that obtained with the 23B cDNA. The talin polypeptide encoded

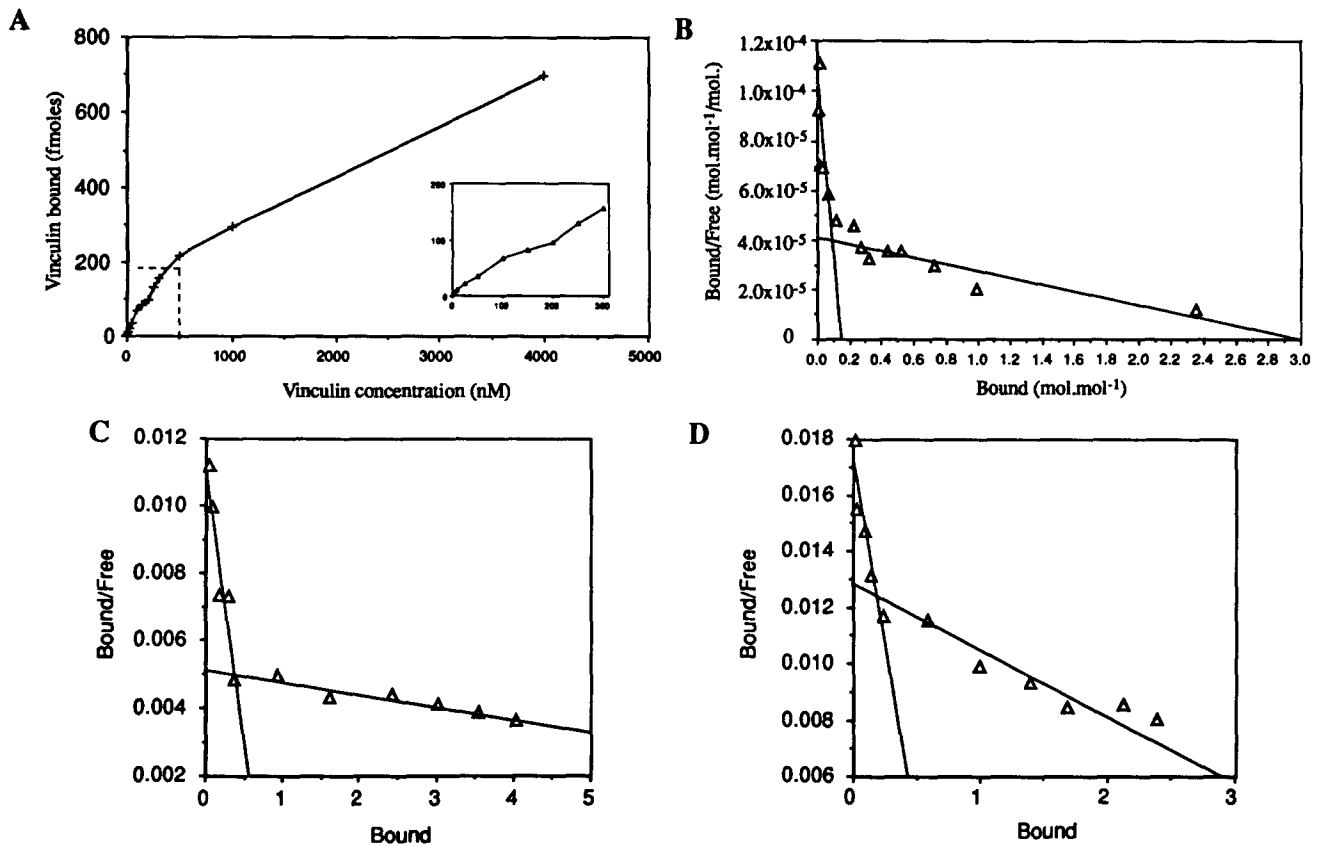


Figure 7. Binding of ¹²⁵I-vinculin to microtitre wells coated with purified chicken gizzard talin. (A) Microtitre wells were coated with purified chicken gizzard talin (10 μg/ml) and the amount bound (300 fmol) was determined using ¹²⁵I-talin. Talin-coated wells were incubated with increasing concentrations of ¹²⁵I-vinculin (400,000 cpm/μg; 1 nM–4 μM), and the amount bound determined as outlined in Materials and Methods. The results are the means of triplicates and have been corrected for nonspecific binding of ¹²⁵I-vinculin to wells coated with BSA. The experiment was repeated three times with essentially identical results. Inset shows the region of the curve from 1 nM to 300 nM ¹²⁵I-vinculin. (B) Scatchard analysis of the data shown in A. (C and D) Binding of ¹²⁵I-vinculin (0.4 nM; 4 × 10⁶ cpm/μg) to wells coated with talin (C) or the 190-kD proteolytic fragment of talin (D) was determined in the presence of increasing amounts of unlabeled vinculin (0–200 nM). The results were corrected for nonspecific binding to wells coated with BSA, and used in Scatchard analysis.

by the G3 cDNA did not localize to focal contacts in mouse stable cell lines (Fig. 9, *G* and *H*), a result at variance with that obtained from transient expression (Fig. 8, *G* and *H*). In contrast, the talin polypeptides encoded by the G2 and GG4A cDNAs both localized at the ends of actin filaments in focal contacts (Fig. 9, *I–L*), a result in agreement with those obtained from transient expression. Overall, the results of these studies indicate that there are at least two separate regions in the talin molecule which are responsible for the localization of talin to focal contacts (Table I and Fig. 1).

Discussion

Consideration of the results from the various experimental approaches used in this study along with the literature suggests that there are multiple sites within talin both for vinculin binding and for targeting to focal contacts. There is some overlap between these two classes of site, but also some clear distinctions. The Scatchard analyses indicate at least three vinculin-binding sites within talin ($K_d = 2\text{--}6 \times 10^{-7}$ M), all of which are located within the 190-kD COOH-terminal talin fragment. These data are in good agreement with the previous finding that vinculin binds exclusively to the 190-

kD fragment of talin (O'Halloran and Burridge, 1986). In addition, we have detected a higher affinity interaction with a K_d of 3×10^{-8} M and a $B_{max} = 0.15$ mol of vinculin bound per mol of talin. Similarly, Burridge and Mangeat (1984) have also reported high affinity ($K_d 10^{-8}$ M, $B_{max} 0.28$ mol mol⁻¹) and low affinity ($K_d 2 \times 10^{-8}$ M) binding of vinculin and talin. The fact that the stoichiometry of the high affinity interaction is <1 is puzzling. One possible explanation is that the preparation of talin or indeed vinculin contains molecules which are heterogeneous with respect to their affinity for the corresponding ligand. Such heterogeneity could arise via posttranslational modification. Both vinculin and talin are phosphoproteins (Turner et al., 1989) and ~6% of chicken gizzard talin is *O*-glycosylated (Hagmann et al., 1992), but there is no information on the effect of such modifications on the interaction between these two proteins. Alternatively, the presence of a small fraction of vinculin multimers (Milam, 1985) able to interact with two or more sites within talin may be responsible for the high affinity sub-stoichiometric binding.

The results from the gel-overlay and microtitre well binding assays with the talin fusion proteins indicate that there are two or more likely three, separable vinculin-binding sites

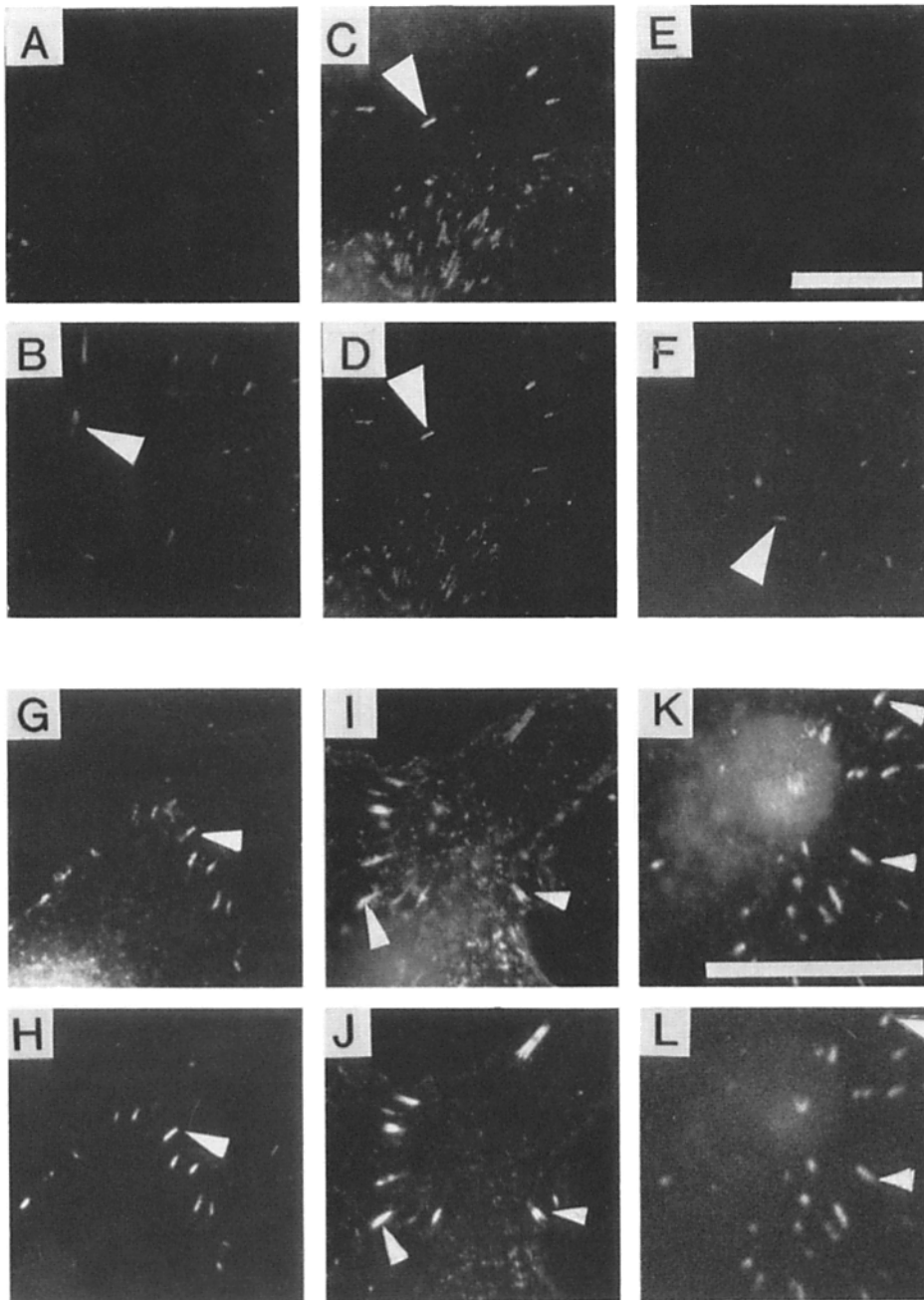


Figure 8. Immunofluorescence localization of chicken talin polypeptides expressed in monkey Cos cells. Cos cells expressing chicken talin cDNAs were double stained for the expressed chicken talin polypeptides (*A*, *C*, *E*, *G*, *I*, and *K*) and for endogenous monkey vinculin (*B*, *D*, *F*, *H*, *J*, and *L*) as described in Materials and Methods. (*A* and *B*) 54A cDNA; (*C* and *D*) 23B cDNA; (*E* and *F*) 23B 3'EcoRI cDNA; (*G* and *H*) G3 cDNA; (*I* and *J*) G2 cDNA; (*K* and *L*) GG4A cDNA. Bars: (*A*-*F*) 3 μ m; (*G*-*L*) 10 μ m.

within talin. Two regions of talin, residues 498-950 and 1554-2268 contain vinculin-binding activity in both assays and are clearly separated by a region which does not bind vinculin (residues 951-1327). The ends of the protein (1-497 and 2269-2541) also fail to interact with vinculin in good agreement with previous results (O'Halloran and Burridge, 1986; Lee et al., 1992). Less extensive analysis of the murine talin fusion proteins (our unpublished results) also conform well with this interpretation. The data for the NH₂-terminal vinculin-binding region (498-950) are best interpreted in terms of two separable binding sites (Fig. 1 and Table I). One site (V1) lies between residues 498-656 and accounts for the vinculin-binding activity of fusion protein 54A (102-656). A second site (V2) between residues 852 and 950 can account for the vinculin-binding activity of

fusion proteins 23B (642-1328) and 23B3'EcoRI (852-1328) since the fusion protein 23B3'PCR (951-1327) does not bind vinculin. These two vinculin-binding sites (498-656 and 852-950) are not clearly separated by a nonbinding segment and could be contiguous. However each site can function independently. The conclusion that this region of the talin molecule has vinculin-binding activity is consistent with the data of Lee et al. (1992) who identified a proteolytic fragment of talin spanning residues 482-1653 which bound vinculin in a gel-overlay assay similar to that used in this study.

The boundaries of the most COOH-terminal vinculin-binding region are less well defined and require further analysis. A fusion protein spanning residues 1304-2023 bound ¹²⁵I-vinculin in the gel-overlay assay, but binding was close to background in the microtitre well binding assay. ¹²⁵I-

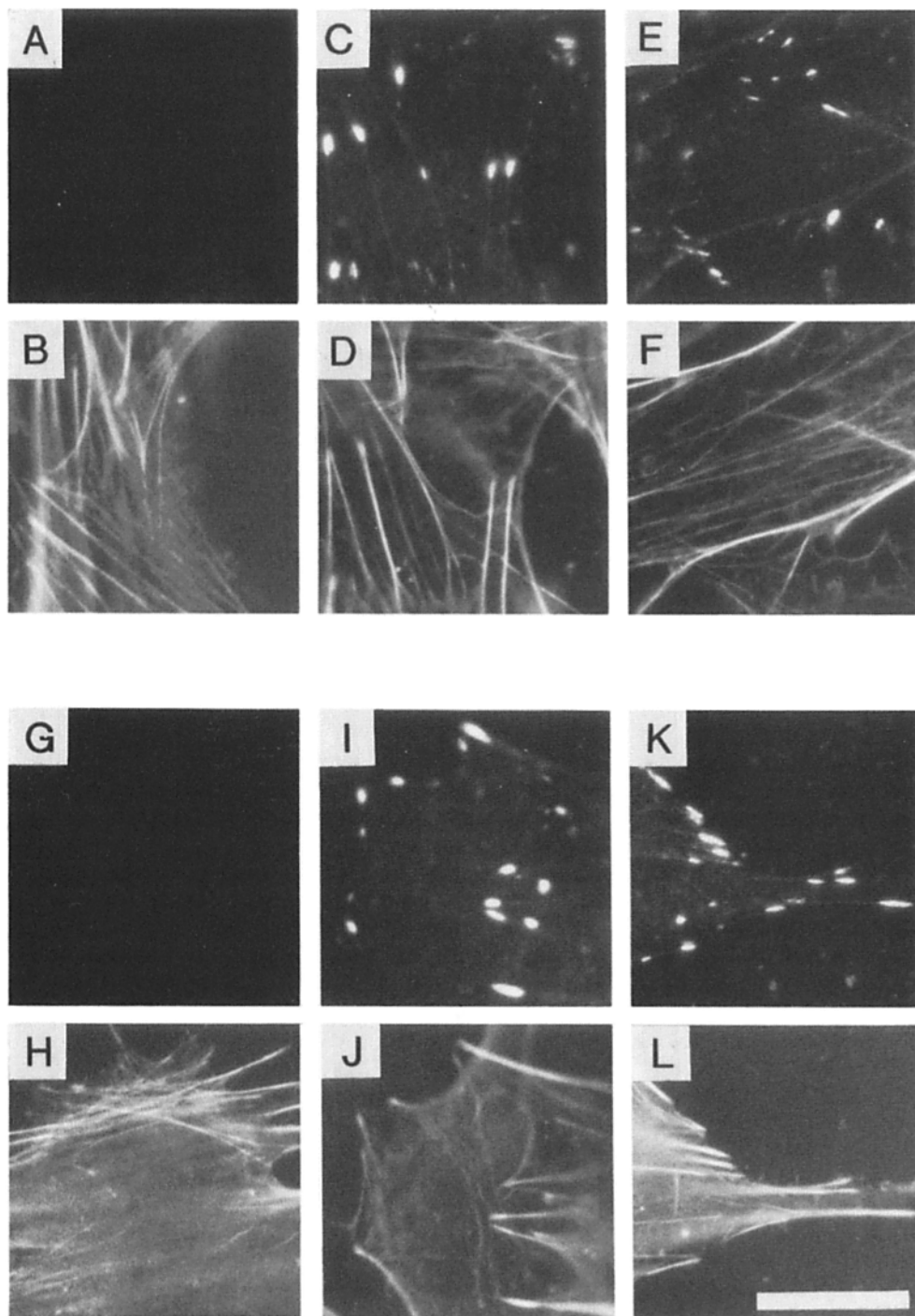


Figure 9. Immunofluorescence localization of chicken talin polypeptides expressed in stable mouse NIH 3T3 cell lines. Mouse NIH 3T3 cells stably expressing chicken talin cDNAs were double stained for either the expressed chicken talin polypeptides (*A*, *C*, *E*, *G*, *I*, and *K*) or for F-actin (*B*, *D*, *F*, *H*, *J*, and *L*) as described in Materials and Methods. (*A* and *B*) 54A cDNA; (*C* and *D*) 54A/23BPCR cDNA; (*E* and *F*) 23B cDNA; (*G* and *H*) G3 cDNA; (*I* and *J*) G2 cDNA; (*K* and *L*) GG4A cDNA. Bar, 5 μ m.

vinculin also bound quite strongly to a fusion protein spanning residues 1554–2124 in the gel-overlay assay whereas binding was relatively low in the microtitre well assay. It is possible that the larger amounts of fusion proteins present in the blot assay (~ 3 –11 pmol) versus the microtitre well assay (0.3 pmol) simply permit detection of lower levels of vinculin-binding activity. However, the talin fusion protein GST/1646–2541 was clearly positive for binding in both assays. The boundaries of the COOH-terminal vinculin binding segment identified using the microtitre well binding assay (residues 2024–2268) are therefore narrower than those identified using the gel-overlay assay (residues 1328–2268).

This latter region includes the epitope (residues 1653–1848) recognized by an anti-idiotypic antibody (Lee et al., 1992).

The simplest interpretation of all the available data suggests a model in which the NH₂-terminal 47 kD domain of talin is completely devoid of vinculin-binding activity whereas the COOH-terminal alanine-rich tail contains three vinculin-binding sites between residues 498–656, 852–950, and 1328–2268 all of which have similar affinities for vinculin ($K_d = 2$ – 6×10^{-7} M). It is conceivable that the two adjacent binding sites might account for the higher affinity vinculin-binding seen with whole talin.

How do these conclusions on vinculin-binding sites con-

form with the data on targeting of various regions of talin to focal contacts? Previous studies have shown that talin localizes specifically to focal contacts when microinjected into epithelial cells whereas the 190-kD fragment targets to focal contacts and cell-cell adherens-type junctions both of which contain vinculin (Nuckolls et al., 1990). These results indicate that there is a focal contact targeting sequence in the 47-kD head domain (1-433) of talin, although this region of the protein localizes only weakly to focal contacts on its own (Nuckolls et al., 1990). The 47-kD talin fragment does not bind vinculin, and targeting must therefore depend on interactions with other focal contact components. Talin residues 164-373 are homologous to band 4.1 (Rees et al., 1990), a membrane attachment protein in erythrocytes, suggesting one possible function for this region of the talin molecule. The results of expressing overlapping chicken talin polypeptides spanning the COOH-terminal alanine-rich region of the molecule in both Cos cells and in NIH 3T3 cells are summarized in Table I. They show that there is indeed considerable overlap between focal contact targeting sequences and vinculin-binding sites, although the match is not perfect. Thus, the talin polypeptide expressed from the 54A cDNA failed to form a stable association with focal contacts yet contains the vinculin-binding site V1 (498-656) as well as the region of homology with band 4.1. Either the V-1 site is insufficient, or it is occluded, or is otherwise inactive when expressed in cells. In contrast, the talin polypeptide expressed from the 54A/23BPCR cDNA, which contains the same vinculin-binding site plus an additional 197 residues of COOH-terminal sequence does target to focal contacts. Whether this additional flanking sequence is required for proper folding of the V1-site within the cell or contains a novel targeting sequence is unclear. The talin polypeptide expressed from the 23B cDNA, which contains the second vinculin-binding site V2 (852-950), targets to focal contacts whereas that expressed from the 23B3'EcoRI cDNA fails to do so even though this cDNA contains the coding sequence for residues 852-1328, and therefore the V2-site. This discrepancy may be due to the fact that translation of the talin polypeptide expressed in transfection experiments probably initiates at the AUG codon for Met-869 which may result in inactivation of the V2-site. Overall, the available results suggest that there is at least one focal contact targeting sequence in the vicinity of residues 498-853 whereas vinculin-binding maps to between residues 498-656 and 852-950. Because of the proximity of the targeting and vinculin-binding sites, it is tempting to speculate that they are one and the same.

The results on stable expression of the talin polypeptides encoded by the G3, G2, and GG4A cDNAs in NIH 3T3 cells can all be interpreted in terms of a site COOH-terminal to residue 1554 which is responsible for targeting to focal contacts and which overlaps with the vinculin-binding site V3. However, this interpretation is complicated by the fact that the polypeptide encoded by the G3 cDNA targets to focal contacts when transiently expressed in Cos cells. The G3 talin polypeptide does possess some vinculin-binding activity in the gel-overlay assay and overexpression of this polypeptide may allow it to bind to vinculin in focal contacts via a low affinity interaction. Alternatively, the G3 polypeptide may contain a low affinity binding site for another component of focal contacts such as actin or the $\beta 1$ integrin subunit. The COOH-terminal boundary of this targeting region is not

yet defined although one can argue that sequences beyond residue 2124 are not required since the G2 polypeptide targets as well as the GG4A polypeptide.

In summary, there are at least three regions of talin which are important for targeting of the molecule to focal contacts. The 47-kD NH₂-terminal segment (residues 1-433) restricts talin to focal contacts rather than cell-cell adherens-type junctions, both of which contain vinculin (Geiger et al., 1985; Nuckolls et al., 1990). The other two targeting sequences in talin overlap those regions shown to bind vinculin, one in the NH₂-terminal 450 amino acids of the alanine-rich tail segment of talin, and one in the COOH-terminal half of the tail segment (Fig. 1). Multivalent interactions of talin with vinculin and other constituents of focal contacts may facilitate assembly of the structure. Binding of talin to integrins and F-actin is likely to be important in this regard, and we are currently attempting to identify the binding sites for these proteins within the talin molecule.

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