

# Identification of a Talin Binding Site in the Cytoskeletal Protein Vinculin

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**Abstract.** Binding of the cytoskeletal protein vinculin to talin is one of a number of interactions involved in linking F-actin to cell-matrix junctions. To identify the talin binding domain in vinculin, we expressed the NH<sub>2</sub>-terminal region of the molecule encoded by two closely similar, but distinct vinculin cDNAs, using an *in vitro* transcription translation system. The 5' Eco RI-Bam HI fragment of a partial 2.89-kb vinculin cDNA encodes a 45-kD polypeptide containing the first 398 amino acids of the molecule. The equivalent restriction enzyme fragment of a second vinculin cDNA (cVin5) lacks nucleotides 746–867, and encodes a 41-kD polypeptide missing amino acids 167–207. The radiolabeled 45-kD vinculin polypeptide bound to microtiter wells coated with talin, but not BSA, and binding was inhibited by unlabeled vinculin. In con-

trast, the 41-kD vinculin polypeptide was devoid of talin binding activity. The role of residues 167–207 in talin binding was further analyzed by making a series of deletions spanning this region, each deletion of seven amino acids contiguous with the next. Loss of residues 167–173, 174–180, 181–187, 188–194, or 195–201 resulted in a marked reduction in talin binding activity, although loss of residues 202–208 had much less effect. When the 45-kD vinculin polypeptide was expressed in Cos cells, it localized to cell matrix junctions, whereas the 41-kD polypeptide, lacking residues 167–207, was unable to do so. Interestingly, some deletion mutants with reduced ability to bind talin *in vitro*, were still able to localize to cell matrix junctions.

CELL adhesion is fundamental to such processes as cell growth (Folkman and Moscona, 1978), differentiation (Bissell and Barcellos-Hoff, 1987; Watt, 1987; Pignatelli and Bodmer, 1988), embryogenesis (Edelman, 1986; Ekblom et al., 1986), and wound healing (Grinnell et al., 1987), and as a consequence, much effort has been spent in determining the structure of the molecules involved. The membrane receptors implicated in such cellular interactions are typically transmembrane proteins (Buck and Horwitz, 1987a; Cunningham, 1986; Edelman, 1986), and in many cases, the cytoplasmic tail of these receptors are thought to interact with proteins of the cytoskeleton. In adherens-type cell-cell and cell-matrix junctions, membrane receptors are thought to be linked indirectly to F-actin by a number of additional interacting proteins, the most ubiquitous of which are the cytoskeletal proteins  $\alpha$ -actinin and vinculin (Geiger et al., 1987). Studies on cell-matrix junctions (adhesion plaques) formed when cultured fibroblasts attach to adhesive glycoproteins such as fibronectin have begun to shed some light on the mechanisms involved in assembly of these structures. Cells interact with fibronectin through at least one receptor,

an  $\alpha 5\beta 1$ -heterodimer which is a member of a large family of membrane receptors, the integrins (Hynes, 1987; Buck and Horwitz, 1987a). The cytoplasmic COOH-terminus of the  $\beta 1$ -subunit of the fibronectin receptor is very highly conserved (DeSimone and Hynes, 1988), and is essential to the assembly process. Interestingly, a B1-subunit with this COOH-terminal region deleted is still able to form a heterodimer with the appropriate  $\alpha$ -subunit, which can still bind to fibronectin, but the receptor fails to localize to adhesion plaques (Solowska et al., 1989). Evidence that this region of the  $\beta 1$ -subunit of the receptor binds to the cytoskeletal protein talin has been presented, although the interaction *in vitro* is apparently of low affinity (Horwitz et al., 1986; Buck and Horwitz, 1987b). Talin binds to vinculin with relatively high affinity ( $K_d$   $10^{-8}$  M) (Burrige and Mangeat, 1984), and vinculin has been reported to bind to  $\alpha$ -actinin (Belkin and Koteliansky, 1987; Wachsstock et al., 1987), although with low affinity ( $K_d$   $10^{-6}$  M).  $\alpha$ -Actinin in turn is well known to both bind to and cross-link, actin filaments (Maruyama and Ebashi, 1965; Bennett et al., 1984; Blanchard et al., 1989). Additional molecules have been localized to adhesion plaques, and may play either structural or regulatory roles (reviewed in Burrige et al., 1988).

It would appear that one of the signals that triggers the assembly of adhesion plaques is the interaction between the RGD sequence in fibronectin and the fibronectin receptor, as

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addition of the RGD peptide to cultured fibroblasts leads to the rapid disruption of this structure (Stickel and Wang, 1988). However, the RGD-containing cell binding domain in fibronectin alone is insufficient to support the formation of adhesion plaques, and the heparin binding domain of fibronectin is also required (Woods et al., 1986). The nature of the receptor for this domain is under investigation. Regulation of the stability of adhesion plaques is likely to be controlled, at least in part, by phosphorylation of one or more of the components (reviewed in BurrIDGE et al., 1988). Adhesion plaques are disrupted by agents that activate protein kinase C (Meigs and Wang, 1986), and by growth factors that bind to receptors with tyrosine kinase activity (Hermann and Pledger, 1985). Also, the protein tyrosine kinase encoded by the *src* oncogene is localized in adhesion plaques (Rohrschneider, 1980), and expression of kinase activity is frequently, but not always associated with disruption of these structures (Kellie et al., 1986). It is also noteworthy that a calcium-activated protease is localized in adhesion plaques, and that talin is a substrate for this enzyme (Beckerle et al., 1987).

To understand the biochemical mechanisms that control the interactions between these various cytoskeletal proteins and the membrane, we and others have begun to determine the sequence of the proteins involved. The complete sequences of  $\alpha$ -actinin (Baron et al., 1987; Noegel et al., 1987; Arimura et al., 1988) and vinculin (Price et al., 1987, 1989; Coutu and Craig, 1988) have been determined, and the sequence of talin is nearing completion. In this study, we report experiments that define a region in chick vinculin that is required for talin binding and for the localization of vinculin to adhesion plaques.

## Materials and Methods

### Vinculin cDNAs

The two chick vinculin cDNAs used in this study (referred to as the 2.89-kb cDNA and *cVin5*) were both isolated from a chick embryo fibroblast Lgt11 cDNA library, and have been described elsewhere (Price et al., 1987, 1989; Bendori et al., 1987).

### Identification of a Talin Binding Domain in Vinculin Using an In Vitro Transcription Translation System

5' Eco RI-Bam HI restriction enzyme fragments of the two vinculin cDNAs (Fig. 1) were cloned into the Bluescript vector SK<sup>+</sup> (Stratagene Corp., La Jolla, CA). RNA transcripts were synthesized in vitro using T7 RNA polymerase, and the transcripts were capped and translated using a rabbit reticulocyte lysate (Amersham International, Amersham, UK) in the presence of [<sup>35</sup>S]methionine (Amersham International). Total translation mixes diluted in NET<sup>1</sup> buffer/ (150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.0)/1 mM PMSF were added to microtiter wells coated with chick gizzard talin or BSA (100  $\mu$ g/ml each), and incubated for 1-2 h at 37°C. The wells were washed with three changes of NET buffer containing 0.05% NP-40, and bound, radiolabeled polypeptides were solubilized in SDS sample buffer and analyzed by SDS-PAGE and fluorography. In experiments using the vinculin deletion mutants, similar amounts of translation product of each mutant were added to each talin-coated microtiter well. These methods have been described in more detail elsewhere (Price et al., 1989).

### Mutagenesis

The 5' Eco RI-Bam HI fragment of the 2.89-kb vinculin cDNA was cloned

1. *Abbreviations used in this paper:* NET buffer, 150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.0.

into the M13-based mICE vector (Eperon, 1986). A series of six contiguous 21-bp deletions were introduced between nucleotides 745 and 870 by annealing mismatched synthetic oligonucleotides to single-stranded template DNA. The closed circular mutant strand was synthesized by 3' extension followed by ligation, using the Klenow fragment of DNA polymerase I, and T4 DNA ligase. Double-stranded DNA was gel purified, and transfected into *Escherichia coli* strain JM101. The efficiency of isolating the mutant strand was increased by preparing single-stranded template DNA in the *dut*, *ung* host strain RZ1032 (Kunkel, 1985). To ensure that the correct deletions had been correctly introduced, mutants were sequenced in the mICE vector using the dideoxy chain termination method of Sanger et al. (1977). Because the mICE vector contains the T7 promoter, RNA transcripts could be generated from the deletion mutants without further subcloning.

### Cell Culture

Monkey Cos cells (Gluzman, 1981) kindly provided by Dr. Paul Stevens (Celltech, Ltd., Slough, Bucks., UK), were cultured in Dulbecco's MEM supplemented with 10% newborn calf serum (NCS). Chick embryo fibroblasts were maintained in Dulbecco's MEM supplemented with 10% tryptic phosphate broth, 5% NCS, and 1% chick serum.

### Expression of Vinculin cDNAs in Cos Cells Using the pECE Eukaryotic Expression Vector

Vinculin cDNA fragments were subcloned into the polylinker sequence of the pECE eukaryotic expression vector, which contains the SV40 early promoter, poly(A) addition signal, and poly(A) tract (Ellis et al., 1986). Vinculin cDNA fragments were liberated from mICE vector constructs as Eco RI-Xba I fragments, and cloned into Eco RI-Xba I-cut pECE (there is a Bam HI site within the plasmid). Covalently closed circular plasmid DNA was prepared by cesium chloride/ethidium bromide equilibrium gradient centrifugation (Maniatis et al., 1982). Plasmids containing vinculin cDNA inserts were sequenced (Mierendorf and Pfeffer, 1987) to authenticate the constructs.

### Antisera

To obtain a vinculin antiserum that recognized the chick but not the monkey protein, a rabbit antiserum to chick gizzard vinculin (Kellie et al., 1986) was extensively adsorbed against monolayer cultures of monkey Cos cells fixed in 4% formaldehyde in PBS (15 min at room temperature), and permeabilized with 0.5% Triton-X100. A mouse mAb to human platelet vinculin was a generous gift from Dr. M. Wilkinson (Royal College of Surgeons, Lincoln's Inn Fields, London).

### Localization of Vinculin and Actin in Cells by Fluorescence Microscopy

Coverslip cultures were fixed and permeabilized for fluorescence microscopy as previously described (Kellie et al., 1986). For double labeling of vinculin and actin, cells were first stained with either a rabbit polyclonal vinculin antiserum or a mouse monoclonal vinculin antiserum, followed by Texas red-labeled donkey anti-rabbit or anti-mouse, respectively (Amersham International). F-actin was then stained with NBD-phalloidin (Molecular Probes, Inc., Eugene, OR) as described in the manufacturer's instructions. Photographs were taken with a photomicroscope (Axiophot; Carl Zeiss, Inc., Thornwood, NY) equipped with epifluorescence using Ilford HP5 film (ASA 400) uprated to 1,600 ASA.

### Transfection of Cos Cells

Cos cells grown on coverslips or culture dishes were transfected with 20  $\mu$ g of pECE DNA, plus or minus insert sequences, by the DEAE-dextran method (Cullen, 1988). After 48 h, cells were either fixed for fluorescence microscopy or harvested to make cell lysates.

### Radiolabeling and Immunoprecipitation

Cells grown for 24 h after transfection were rinsed twice in methionine-free Dulbecco's MEM plus 10% NCS and grown for a further 18 h in the same medium containing 25  $\mu$ Ci/ml [<sup>35</sup>S]methionine (>800 mCi/mmol; Amersham International). Cells were then washed twice in ice-cold PBS/1 mM PMSF, and harvested into 700  $\mu$ l of ice-cold NET lysis buffer containing 0.1% SDS, 1% NP-40, 1% sodium deoxycholate, and 2 mM PMSF. Cell ly-

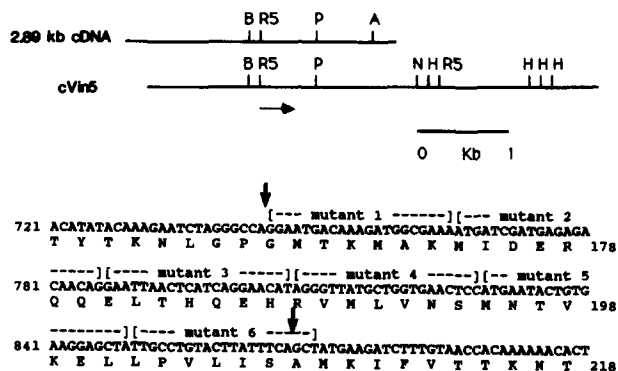
sates were incubated on ice for 10 min and then centrifuged at 12,000 *g* for 15 min at 4°C. Supernatants, containing equal amounts of TCA-precipitable radioactivity, were preadsorbed at 4°C with 5 µl of preimmune rabbit serum (1 h) followed by 30 µl of a 50% (vol/vol) suspension of protein A-Sepharose (Sigma Chemical Co., St. Louis, MO) in NET buffer containing 0.1% BSA, 0.1% SDS, 0.5% NP-40, 0.1% sodium deoxycholate (1 h). Samples were centrifuged at 6,000 *g* for 15 s and radiolabeled vinculin was precipitated from the supernatant by addition of either the unadsorbed (5 µl) or adsorbed (10 µl) rabbit antiserum, or the mouse mAb (1 µl) to vinculin, followed by protein A-Sepharose. Immune complexes bound to the protein A-Sepharose were washed three times with buffer and extracted with 100 µl of SDS sample buffer at 100°C for 2 min; the solubilized proteins were separated by SDS-PAGE. Gels were incubated in 1 M sodium salicylate for 1 h, dried, and exposed to Fuji NR x-ray film for 1–4 d.

## Results

We have previously analyzed a 2.89-kb chick vinculin cDNA which contained 246 bp of 5' untranslated sequence, the initiation codon, and 83% of the total coding sequence, but lacked 3' coding and noncoding sequence (Price et al., 1987). Comparison of the restriction map of this clone with that of a 5-kb chick vinculin cDNA isolated by Bendori et al. (1987) showed the latter to contain the additional 3' sequence missing from the 2.89-kb cDNA (Fig. 1), allowing us to determine the complete sequence of chick vinculin (Price et al., 1989). Sequencing of cVin5 from the 5' Eco RV site to the 3' end showed that the two clones were identical in the region of overlap, except for five third-base substitutions. None of these affected the deduced amino acid sequence, but one accounted for the unique Ava I site in the 2.89-kb cDNA (Price et al., 1989). Sequencing of cVin5 in a 5' direction from this Eco RV site has subsequently revealed a further four third-base substitutions, all of which were silent. In addition, cVin5 lacks 123 bp of coding sequence corresponding to amino acid residues 167–207 in the vinculin sequence deduced from the 2.89-kb cDNA (Fig. 1).

### Identification of the Talin Binding Site in Vinculin

We (Price et al., 1989), and others (Burrige and Mangeat, 1984; Milam, 1985) have previously shown that the talin binding site in vinculin is contained within the NH<sub>2</sub>-terminal 90-kD globular head region of the molecule, which is liberated when vinculin is cleaved by V8-proteinase. Furthermore, we have shown that the NH<sub>2</sub>-terminal 398 amino acids encoded by the 5' Eco RI-Bam HI fragment derived from the 2.89-kb vinculin cDNA (Fig. 1) is also able to bind talin (Price et al., 1989). Because the vinculin cDNA cVin5 lacks 123 bp encoding amino acids 167–207, it was of interest to establish whether the polypeptide encoded by the equivalent restriction enzyme fragment derived from this cDNA was also able to bind talin. Thus, transcripts were synthesized from the 5' Eco RI-Bam HI fragments derived from the two vinculin cDNAs cloned into the Bluescript vector and the transcripts were capped and then translated in the presence of [<sup>35</sup>S]methionine using a rabbit reticulocyte lysate system. Analysis of the total translation mix on SDS-gels showed that a polypeptide of apparent molecular weight 45,000 was synthesized in the presence, but not the absence, of the transcript generated from the Eco RI-Bam HI fragment of the 2.89-kb vinculin cDNA (Fig. 2, *a* and *b*). The observed molecular weight of this polypeptide is in close agreement with that predicted from the cDNA sequence. As expected, the molecular weight of the polypeptide encoded



**Figure 1.** Sequence divergence between two chick vinculin cDNAs. A restriction map of two vinculin cDNAs: (a) a 2.89-kb cDNA (Price et al., 1987); (b) a 5-kb cDNA referred to as cVin5 (Bendori et al., 1987). Each cDNA is contained on a single Eco RI fragment. The nucleotide sequence of cVin5 from the 5' Eco RV site (*arrow*) has been published previously (Price et al., 1989). A, Aval; B, Bam HI; E, Eco RI; H, Hind III; N, Nco I; P, Pst I; R5, Eco RV. The sequence of the 2.89-kb vinculin cDNA is shown between nucleotides 721–900 encoding amino acids 159–218. The vinculin cDNA cVin5, lacks nucleotides 746–867 encoding amino acids 167–207 (*arrows*). The positions of six 21-bp deletions introduced into the 5' Eco RI-Bam HI fragment of the 2.89-kb cDNA by site-directed mutagenesis are also shown.

by the equivalent restriction fragment of cVin5 was ~41,000 (Fig. 2 *c*). Both polypeptides were specifically precipitated from the total translation mix by an antibody to vinculin, but not by preimmune serum (Fig. 2, *d-f*). To test the ability of these vinculin polypeptides to bind to talin, total translation mixes containing similar amounts of each translation product were added to microtiter wells coated with talin or BSA, and



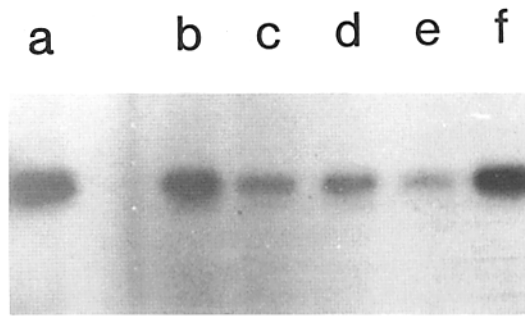
**Figure 2.** In vitro synthesis of NH<sub>2</sub>-terminal vinculin polypeptides, and analysis of their talin binding properties. RNA transcripts from the 5' Eco RI-Bam HI fragments of the 2.89-kb vinculin cDNA and cVin5 were translated in vitro in the presence of [<sup>35</sup>S]methionine, and labeled polypeptides were resolved by SDS-PAGE as described in Materials and Methods. Proteins synthesized from endogenous reticulocyte mRNA (*a*), and added transcript from the 5' Eco RI-Bam HI fragment of the 2.89-kb cDNA (*b*) or cVin5 (*c*). The positions of the 45- and 41-kD proteins synthesized in response to added transcript are shown on the left. That these were vinculin polypeptides was shown by immunoprecipitating aliquots of the total translation mixes (*b* and *c*) with rabbit anti-vinculin (*e* and *f*). Addition of preimmune rabbit serum to an aliquot of the total translation mix (*b*) did not precipitate the 45-kD vinculin polypeptide (*d*). The talin binding activity of the vinculin polypeptides was assayed by adding aliquots of the total translation mix to microtiter wells coated with talin (*g* and *i*) or BSA (*h* and *j*), as described in Materials and Methods. Translation mix (*b*) was added to wells (*g* and *h*); translation mix (*c*) to wells (*i* and *j*).

the bound protein was analyzed on SDS-gels. The 45-kD vinculin polypeptide encoded by the 5' Eco RI-Bam HI fragment of the 2.89-kb cDNA bound to wells coated with talin but not BSA (Fig. 2, *g* and *h*). Binding was specific in that none of the polypeptides synthesized from endogenous reticulocyte mRNA bound to talin, and binding of the 45-kD vinculin polypeptide was progressively inhibited by addition of increasing amounts of unlabeled vinculin, but not by BSA (Fig. 3). In contrast, the 41-kD vinculin polypeptide encoded by the 5' Eco RI-Bam HI fragment of *cVin5* was unable to bind talin. The result suggests that residues 167–207 in the vinculin molecule are important determinants of talin binding.

To further define the role of residues 167–207 of the vinculin molecule in the interaction with talin, we took the 5' Eco RI-Bam HI fragment from the 2.89-kb vinculin cDNA, and constructed a series of six deletion mutants covering this region. Each mutant contained a 21-bp deletion, each deletion being contiguous with the next (Fig. 1). The deletion mutants, each lacking a different stretch of seven amino acids within the region of residues 167–208, were then expressed using the *in vitro* transcription translation system. The polypeptides encoded by the deletion mutants had a similar mobility on SDS-gels to the 45-kD polypeptide encoded by the 5' Eco RI-Bam HI fragment from the 2.89-kb cDNA (Fig. 4, *a-h*). Similar amounts of translation product of each of the mutants were then added to talin-coated microtiter wells and assayed for their ability to bind talin. All but one of the deletions resulted in a dramatic reduction in the capacity of the expressed protein to bind to the talin-coated microtiter wells (Fig. 4, *i-n*). Thus, mutant 6, which lacked amino acid residues 202–208, retained a much higher capacity to bind talin than the other mutants. Because the assay is not strictly quantitative, it is not possible to say whether mutant six showed a significant reduction in talin binding activity compared with the wild-type 45-kD vinculin polypeptide. The results further define residues 167–201 within the vinculin sequence as important determinants of talin binding.

#### Expression of Vinculin Polypeptides in Cos Cells

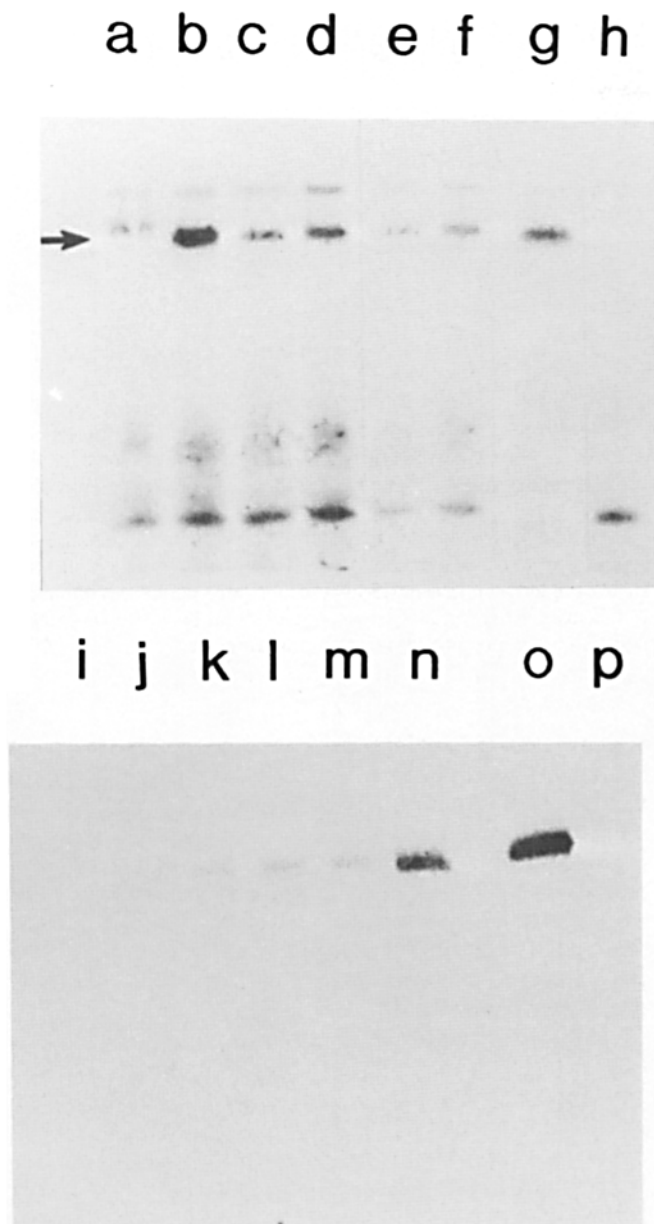
To establish whether residues within the talin binding site are also important determinants of the ability of vinculin to localize to adhesion plaques, we expressed the 5' Eco RI-Bam HI fragments from the 2.89-kb vinculin cDNA, as well as from *cVin5* and the deletion mutants in monkey Cos cells. To determine whether the chick vinculin polypeptides were indeed stably expressed in Cos cells, and to determine the localization of the expressed proteins within these cells, it was essential to use a chick-specific antivinculin antiserum. We have previously characterized a rabbit antiserum raised against chick gizzard vinculin (Kellie et al., 1986). In chick embryo fibroblasts, this antiserum clearly stained vinculin in adhesion plaques at the ends of actin filaments (Fig. 5, *a* and *b*, *arrowheads*). In monkey Cos cells, very faint specific vinculin staining was observed in only a few cells (data not shown). Adsorption of the antiserum against fixed and permeabilized Cos cells removed this unwanted cross-reactivity with Cos cell vinculin, although the adsorbed antiserum still retained the capacity to stain vinculin in chick cells (Fig. 5, *c* and *d*). That Cos cells do indeed possess vinculin containing adhesion plaques was demonstrated with an mAb raised against human platelet vinculin (Fig. 5, *e* and *f*, *arrowheads*).



**Figure 3.** Binding of the [<sup>35</sup>S]methionine-labeled 45-kD vinculin polypeptide to talin in the presence of unlabeled vinculin. Transcripts from the 5' Eco RI-Bam HI fragment derived from the 2.89-kb vinculin cDNA were translated *in vitro*, and the total translation mix added to talin-coated microtiter wells (*a*) or talin-coated wells preincubated with increasing amounts of unlabeled vinculin: (*b*) 250 ng; (*c*) 500 ng; (*d*) 1  $\mu$ g; (*e*) 5  $\mu$ g. In *f*, wells were analyzed by SDS-PAGE as described in Materials and Methods. Only the region of the gel containing the 45-kD vinculin polypeptide is shown. Binding of the radiolabeled 45-kD vinculin polypeptide was inhibited by 0% (*a*), 28.4% (*b*), 80.4% (*c*), 75.1% (*d*), or 86.4% (*e*) as judged by scanning densitometry of the autoradiogram.

The 5' Eco RI-Bam HI fragments of the various chick vinculin cDNAs were ligated into the pECE eukaryotic expression vector and transfected into Cos cells. To establish that the chick vinculin polypeptides were indeed expressed, transfected cells were labeled with [<sup>35</sup>S]methionine, and expressed polypeptides were precipitated from cell extracts using the chick-specific vinculin antiserum. As expected, 45- and 41-kD vinculin polypeptides were specifically precipitated from cells transfected with the 5' Eco RI-Bam HI fragment from the 2.89-kb cDNA and *cVin5*, respectively, and both polypeptides were expressed to a similar extent (Fig. 6, lanes 4 and 6). No such polypeptides were precipitated with preimmune serum, or from cells transfected with the pECE vector alone (Fig. 6, lanes 1, 2, 3, and 5). Neither did the chick-specific antibody precipitate Cos cell vinculin. To examine the distribution of these expressed chick vinculin polypeptides in Cos cells, coverslip cultures transfected with appropriate constructs were fixed and stained with the chick-specific vinculin antiserum. By double-fluorescence staining, the 45-kD vinculin polypeptide was clearly visible in adhesion plaques at the ends of actin filaments (Fig. 7, *a* and *b*, *arrows*). In contrast, the 41-kD polypeptide was never detected in adhesion plaques despite extensive analysis of many different transfected cell populations (Fig. 7, *c* and *d*). Neither was the protein detected in the cytosol, presumably because it was extracted during permeabilization of the cells. The results clearly demonstrate that residues 167–207, which are absent from the 41-kD vinculin polypeptide, are not only important determinants of talin binding, but are also required for the targeting of the vinculin to adhesion plaques.

In an attempt to further extend these studies, we also expressed the series of Eco RI-Bam HI fragments containing deletions of seven amino acids (spanning residues 167–208) in Cos cells. The polypeptides encoded by these deletion mutants were all expressed at a similar level, as determined by immunoprecipitation of radiolabeled cell extracts (data not shown). However, analysis of the ability of each of the mutant polypeptides to localize to adhesion plaques yielded un-



**Figure 4.** Talin binding activity of vinculin polypeptides containing deletions spanning residues 167–208. RNA transcripts from the 5' Eco RI-Bam HI restriction enzyme fragments of the six deletion mutants (*a–f*), and the 2.89-kb vinculin cDNA (*g*), were translated *in vitro* in the presence of [<sup>35</sup>S]methionine, and the translation products were analyzed by SDS-PAGE. (*a*) Deletion of residues 167–173; (*b*) 174–180; (*c*) 181–187; (*d*) 188–194; (*e*) 195–201; (*f*) 202–208; (*h*) total translation mix from endogenous reticulocyte mRNA. The talin binding activity of the vinculin polypeptides containing deletions (*a–f*) was assayed by adding the total translation mixes (*a–f*) to microtiter wells coated with talin. Bound proteins were solubilized, and analyzed by SDS-PAGE (*i–n*). The specificity of the talin binding assay was shown by adding the wild-type 45-kD vinculin polypeptide synthesized in *g* to wells coated with talin (*o*) or BSA (*p*). The experiment was repeated on three separate occasions with identical results.

expectedly complex results (Fig. 7, *e–p*, summarized in Table I). Mutant polypeptides lacking residues 167–173 (Fig. 7, *e* and *f*), 181–187 (Fig. 7, *i* and *j*), 188–194 (Fig. 7, *k* and *l*), and 202–208 (Fig. 7, *o* and *p*) still retained the ability to

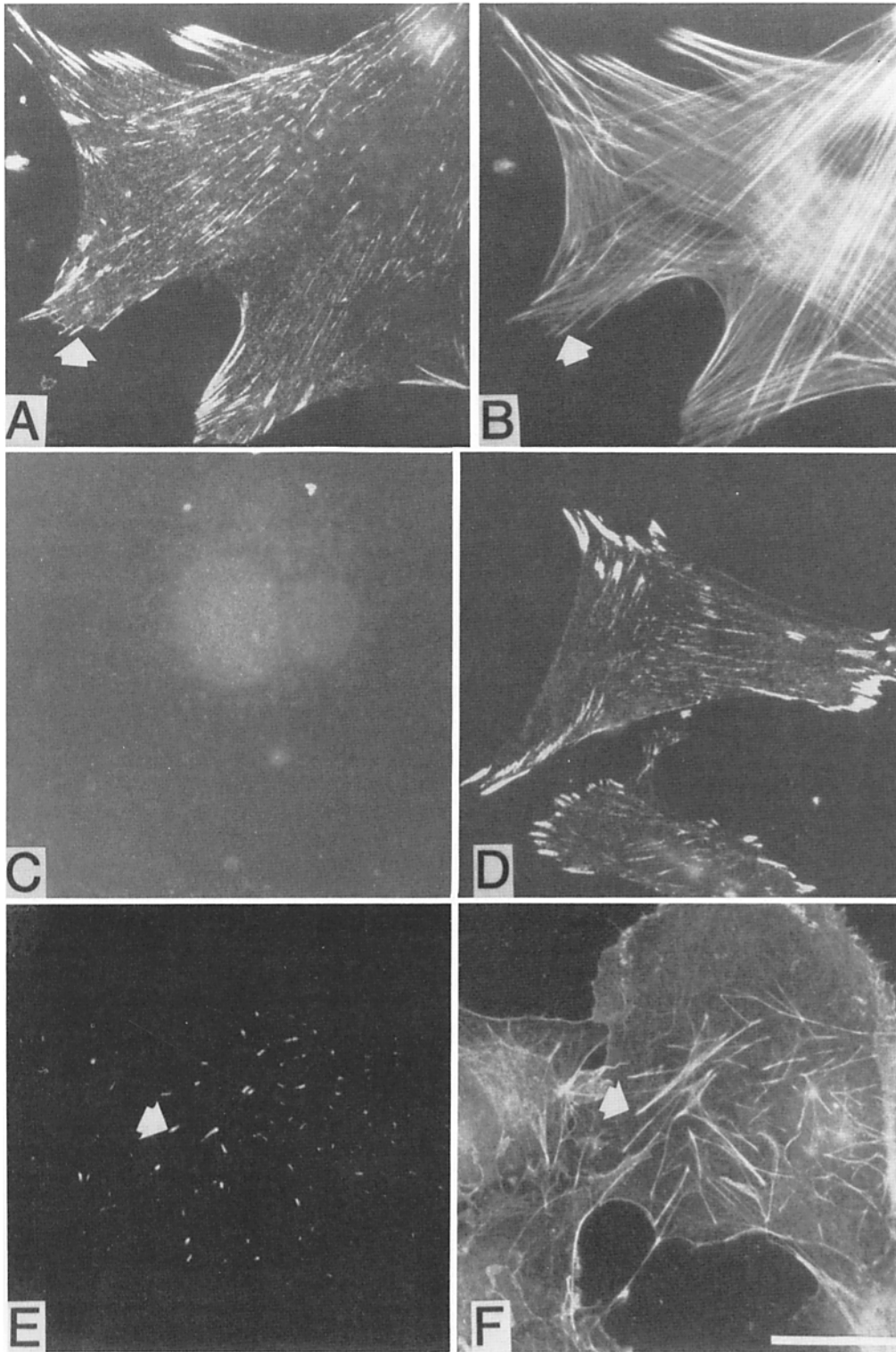
localize to adhesion plaques, whereas those lacking residues 173–179 (Fig. 7, *g* and *h*) and 195–201 (Fig. 7, *m* and *n*) failed to do so.

## Discussion

An analysis of the complete sequence of chick vinculin (Price et al., 1987, 1989; Coutu and Craig, 1988) shows that the molecule contains 1,066 amino acids, with a deduced molecular mass of ~117 kD. The most notable feature of the molecule is that it contains a proline-rich region spanning residues 837–879, with two V8-proteinase cleavage sites (Price et al., 1989). Cleavage at these sites liberates the globular head and extended tail regions of vinculin visualized by electron microscopy, as 90- and 32-kD fragments, respectively (Milam, 1985). The 90-kD fragment contains the NH<sub>2</sub> terminus of the protein, three 112 residue repeats (residues 259–589) of unknown function, and extends to ~residue 850. Gel overlay experiments show that the 90-kD fragment, but not the 32-kD fragment, retains the ability to bind talin (Burrige and Mangeat, 1984; Milam, 1985; Price et al., 1989). However, it has not proved possible to demonstrate talin binding to substantially smaller fragments derived from this region (Price et al., 1989).

To overcome this problem, we have used an *in vitro* transcription translation system to synthesize the NH<sub>2</sub>-terminal region of vinculin. Furthermore, we have synthesized vinculin polypeptides encoded by two closely related, but distinct vinculin cDNAs, the 2.89-kb cDNA and cVin5 (Price et al., 1989). The 5' Eco RI-Bam HI fragment of the 2.89-kb cDNA encodes a protein containing the NH<sub>2</sub>-terminal 398 amino acids of vinculin, and with a molecular mass of 45 kD. The equivalent restriction enzyme fragment of cVin5 encodes a vinculin polypeptide of only 41 kD, because cVin5 lacks the 123 bp that code for residues 167–207 in the 2.89-kb cDNA. Otherwise the 41- and 45-kD polypeptides derived from these two cDNAs are identical. The radiolabeled 45-kD vinculin polypeptide was able to bind to talin-coated microtiter wells, in agreement with our previous results (Price et al., 1989), and binding was specific in that it was substantially reduced, though not completely inhibited, by the addition of intact unlabeled vinculin. The inability of unlabeled vinculin to completely inhibit binding of labeled vinculin to talin, is similar to an observation reported by Avnur et al. (1983) in studies of vinculin binding to focal contacts. They were able to show binding of rhodamine-labeled vinculin to focal contacts but the binding was not saturable or efficiently inhibited by unlabeled vinculin. They suggested that vinculin might self-associate in focal contacts. Evidence for self-association of vinculin molecules has now been obtained from both direct binding assays (Otto, 1983; Belkin and Kotliansky, 1987) and EM (Milam, 1985; Molony and Burrige, 1985). We propose that self-association of vinculin molecules is responsible for the nonproportional inhibition of talin binding by unlabeled vinculin in our own experiments.

In contrast to the results obtained for the 45-kD peptide, the 41-kD vinculin polypeptide displayed little or no talin binding activity. We have further analyzed this region of 41 amino acids within vinculin by making smaller deletions spanning residues 167–208. The results clearly demonstrate that deletions of just seven amino acids covering residues 167–201 lead to a marked reduction in talin binding activity,

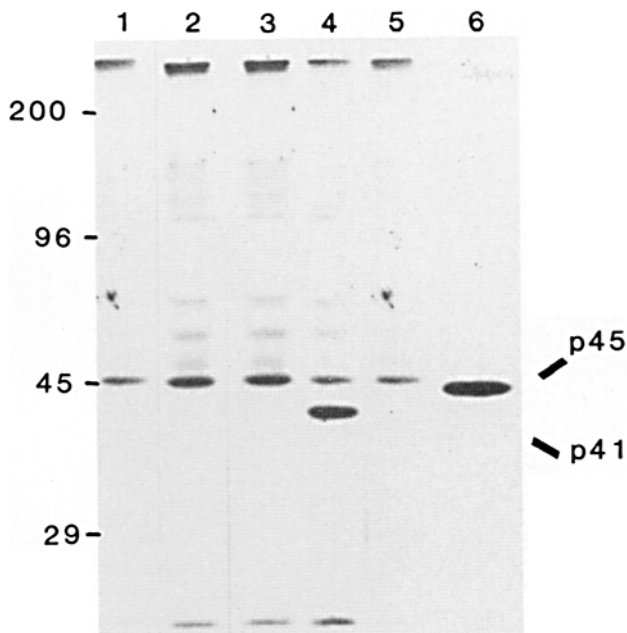


**Figure 5.** Characterization of a chick-specific antibody to vinculin by immunofluorescence. Distribution of vinculin in chick embryo fibroblasts (CEF) as visualized with an unadsorbed rabbit antibody to chick gizzard vinculin (*a*). F-actin visualized in the same cell with NBD-phalloidin (*b*). Cos cells stained with the vinculin antiserum adsorbed against fixed and permeabilized Cos cells (*c*). CEF stained with the adsorbed antibody to vinculin (*d*). Cos cell stained with a mouse mAb to human platelet vinculin (*e*); the same Cos cells stained with NBD-phalloidin (*f*). Arrows in *a*, *b*, and *e*, *f* indicate colocalization of vinculin with the termini of actin filaments. Bar, 10  $\mu$ m.

although deletion of residues 202–208 had a much smaller effect on binding. These observations are consistent with the view that residues 167–201 define a talin binding site in vinculin, although we cannot exclude the possibility that dele-

tions in this region alter the conformation of the NH<sub>2</sub>-terminal region of vinculin, inactivating talin binding activity, the actual binding site lying elsewhere in the molecule.

In an attempt to differentiate between these possibilities,



**Figure 6.** Expression of 45- and 41-kD vinculin polypeptides in Cos cells. Extracts of [<sup>35</sup>S]methionine-labeled Cos cells (lanes 1 and 2), and Cos cells transfected with the pECE vector containing the 5' Eco RI-Bam HI fragments of either *cVin5* (lanes 3 and 4) or the 2.89-kb vinculin cDNAs (lanes 5 and 6) were immunoprecipitated with either preimmune rabbit serum (lanes 1, 3, and 5), or a rabbit antibody to chick vinculin adsorbed against fixed and permeabilized Cos cells (lanes 2, 4, and 6). The positions of the molecular weight standards ( $\times 10^{-3}$ ) is shown to the left of the figure. Arrows indicate the position of the expressed 41- and 45-kD vinculin polypeptides.

we have cloned oligonucleotides encoding the putative talin binding site into the chloramphenicol acetyl transferase gene. We have shown that the fusion protein is now capable of binding talin whereas the wild-type enzyme is not, but it has proved difficult to establish the specificity of the interaction (Jones, P., unpublished data). It will also be interesting to see whether synthetic peptides covering this region are able to inhibit binding of vinculin to talin, and whether they can directly support talin binding when coupled to an inert matrix.

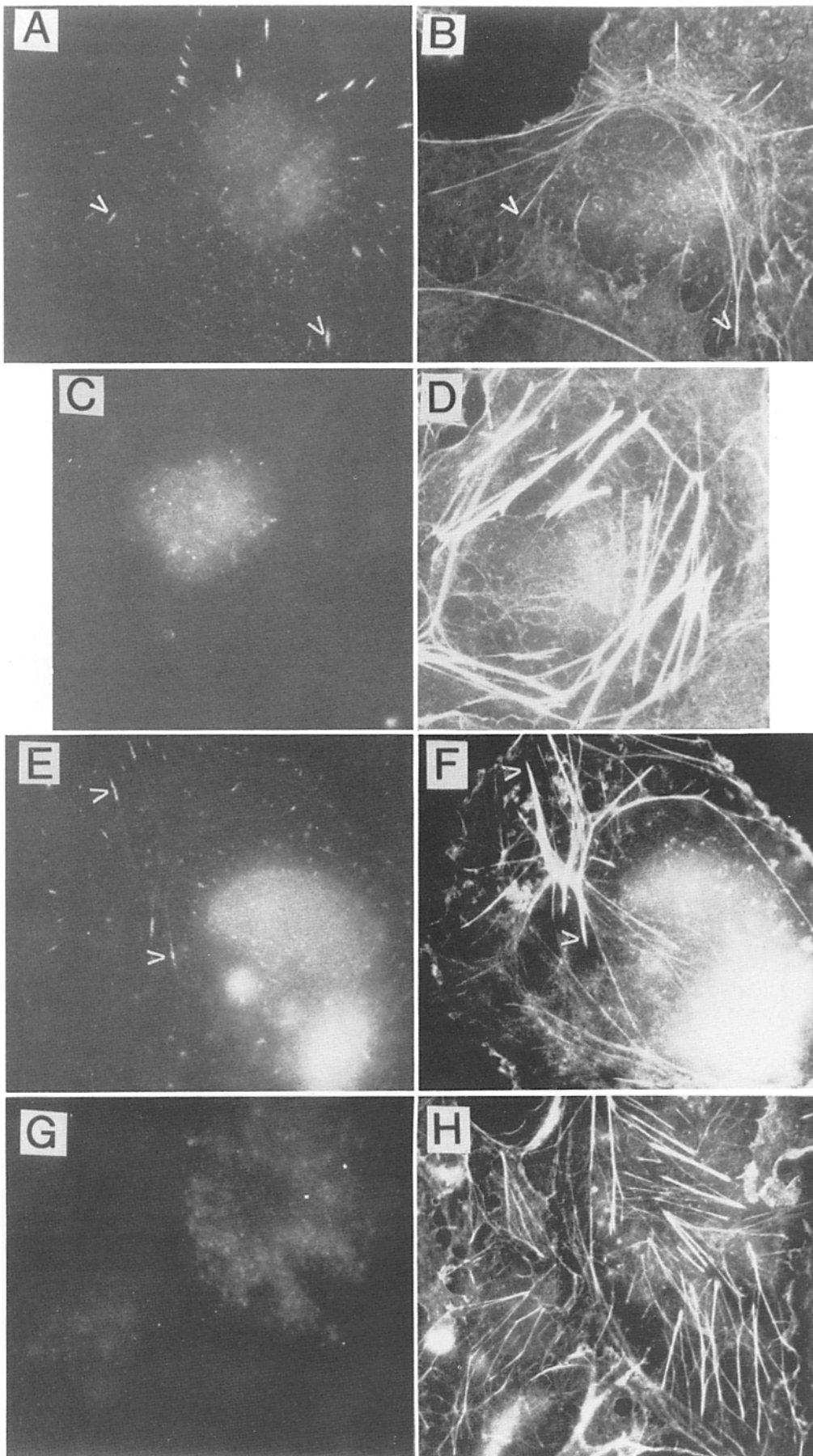
We have also examined the relationship between the ability of vinculin to bind to talin, and the localization of the molecule within the cell. Thus, when the NH<sub>2</sub>-terminal 45-kD vinculin polypeptide, which contains the talin binding site, was expressed in Cos cells, the polypeptide localized to adhesion plaques. In contrast, the NH<sub>2</sub>-terminal 41-kD vinculin polypeptide, which lacks the talin binding site, failed to do so. These observations are consistent with similar experiments described by Bendori et al. (1989). Together, these results clearly establish that the talin binding site in vinculin plays an important role in determining the distribution of vinculin within the cell. However, experiments with vinculin polypeptides containing deletions of just seven amino acids within residues 167–208 have revealed some discrepancies between the talin binding activity of vinculin assayed *in vitro*, and the ability of the expressed polypeptides to localize to adhesion plaques (Table I). Thus, deletions of residues 167–173, 181–187, or 188–194 in the NH<sub>2</sub>-terminal region of

vinculin did not have any detectable effect on the ability of the expressed polypeptides to localize to adhesion plaques, but did dramatically reduce their ability to bind to talin *in vitro*. At present, we are unable to offer an explanation for this discrepancy. It may simply be a reflection of the different assays used, immunofluorescence localization of vinculin polypeptides within a cell possibly being more sensitive, though less quantitative, than *in vitro* binding assays. Whatever the explanation, the result raises the possibility that the activities associated with this region of the vinculin molecule may not be defined by a simple linear stretch of amino acids.

It is also clear from other studies, that the talin binding site in vinculin is not the only factor governing the localization of the molecule to adhesion plaques. Thus, when a partial cDNA encoding just the COOH-terminal tail of vinculin is expressed in Cos cells, the polypeptide localizes to adhesion plaques, as does the vinculin encoded by *cVin5* (Bendori et al., 1989). The tail region of vinculin is known to be a self-association site (Molony and Burridge, 1985; Milam, 1985), and it therefore seems likely that the expressed tail polypeptides are simply associating with endogenous monkey vinculin. The COOH-terminal tail region of vinculin is predicted to be basic relative to the globular head, which is predicted to be acidic (Coutu and Craig, 1988). It is therefore also possible that the tail region of vinculin allows a direct association of the molecule with the polar head groups of phospholipids on the cytoplasmic face of the membrane. Vinculin is known to bind to acidic phospholipids (Ito et al., 1983), and it has been suggested that a part of the molecule penetrates the lipid bilayer (Niggli et al., 1986). However, it is difficult to see how such an association would allow the COOH-terminal tail of vinculin to localize specifically to adhesion plaques. Vinculin is also found in cell-cell adherens-type junctions that lack talin (Geiger et al., 1985), and it will be interesting to see which regions of vinculin determine localization of the protein to this site.

The results described here strongly suggest that there are distinct isoforms of vinculin with differing abilities to bind to talin. As mentioned previously, vinculin and talin do not always co-distribute within the same cell (Geiger et al., 1985). A study of vinculin and talin distribution in embryonic chick smooth muscle showed that both proteins are predominantly cytosolic during the first 14 d of development (Volberg et al., 1986). After 16–18 d, membrane-associated dense plaques appear, and talin is recruited to these structures. Vinculin, on the other hand remains cytosolic, only localizing to dense plaques 1–3 d after hatching. Interestingly, the level of vinculin in smooth muscle increases dramatically within 3 d of hatching. It is tempting to speculate that the vinculin synthesized by the embryo is an isoform unable to bind talin and therefore remains cytosolic, whereas the isoform synthesized after hatching is able to bind talin, and is therefore able to localize dense plaques. Similarly, talin, but not vinculin, is localized at the points of contact between cytotoxic T cells and their target cells, and between helper T cells and antigen-presenting B cells (Kupfer et al., 1986, 1987). Analysis of vinculin transcripts in these, and other tissues, should help to establish whether different vinculin isoforms are expressed in different tissues.

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**Figure 7.** Double-fluorescence staining of Cos cells transfected with the pECE vector containing the 5' Eco RI-Bam HI fragments of the 2.89-kb vinculin cDNA (*a* and *b*), cVin5 (*c* and *d*) or mutants with deletions of amino acid residues 167–173 (*e* and *f*), 174–180 (*g* and *h*), 181–187 (*i* and *j*), 188–194 (*k* and *l*), 195–201 (*m* and *n*) and 202–208 (*o* and *p*). Cells were stained with the chick-specific vinculin antibody (*a*, *c*, *e*, *g*, *i*, *k*, *m*, and *o*) and with NBD-phalloidin to label F actin filaments (*b*, *d*, *f*, *h*, *j*, *l*, *n*, and *p*). Arrows indicate colocalization of vinculin with the termini of actin filaments. Bar, 10  $\mu\text{m}$ .



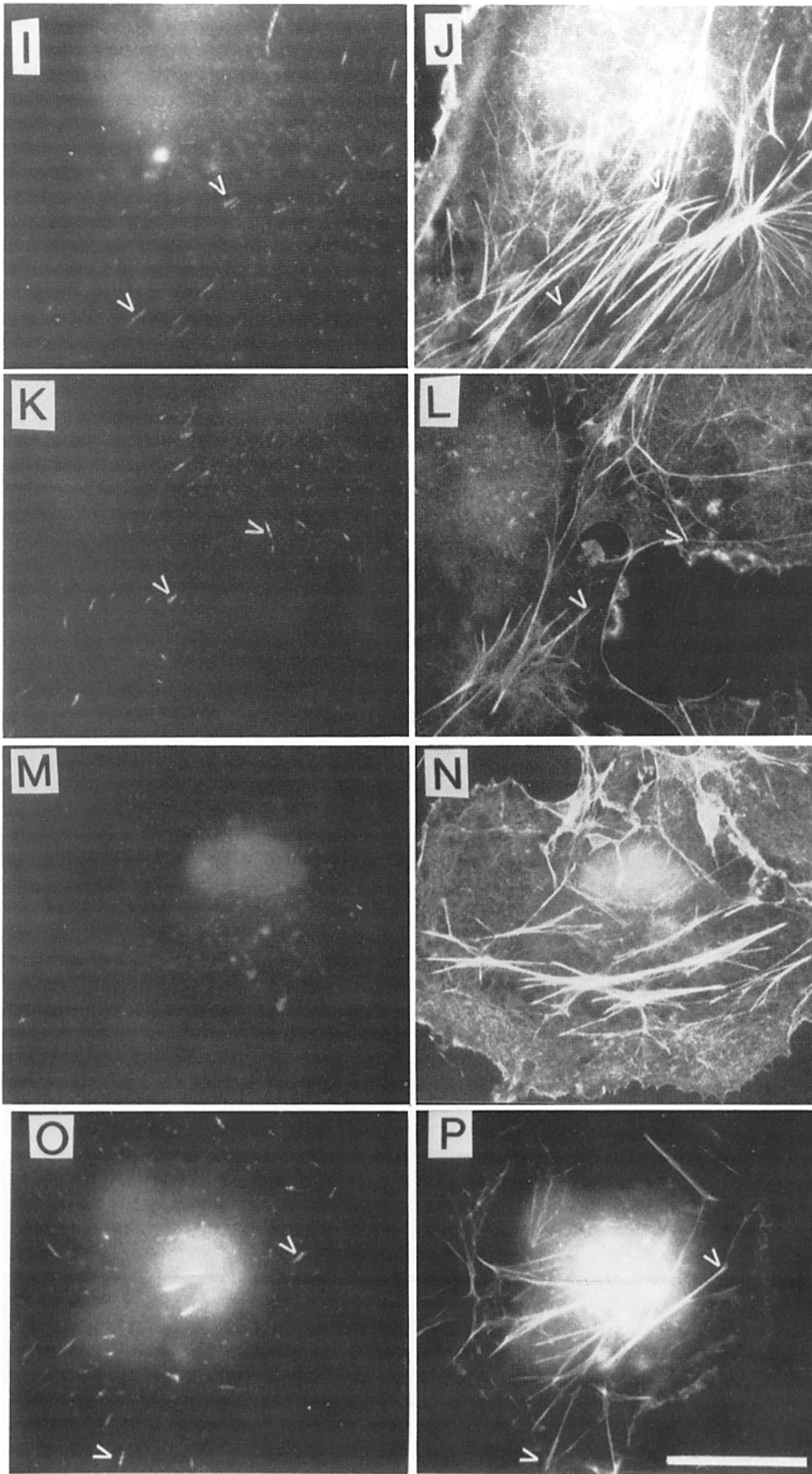


Figure 7.

**Table I. Expression of 5' Eco RI-Bam HI Restriction Enzyme Fragments of Chick Vinculin cDNAs in Cos Cells: Comparison of the Cellular Distribution of the Expressed Vinculin Polypeptides with Talin Binding Activity In Vitro**

cDNAs	Amino acid deletion	Size of deleted protein in Cos cells	Localization to adhesion plaques in Cos cells	Talin binding in vitro
		<i>kD</i>		
2.89-kb cVin5	None	45	Yes	Yes
Mutant 1	167-207	41	No	No
Mutant 2	167-173	~44	Yes	Severely reduced
Mutant 3	174-180	~44	No	Severely reduced
Mutant 4	181-187	~44	Yes	Severely reduced
Mutant 5	188-194	~44	Yes	Severely reduced
Mutant 6	195-201	~44	No	Severely reduced
	202-208	~44	Yes	Yes

The 5' Eco RI-Bam HI fragments of a 2.89-kb chick vinculin cDNA, six deletion mutants derived from this cDNA, and a 5-kb vinculin cDNA, cVin5 (see Fig. 1), were expressed in Cos cells, and the expressed NH<sub>2</sub>-terminal vinculin polypeptides were detected by either immunoprecipitation or by indirect immunofluorescence as described in Materials and Methods. All vinculin polypeptides were expressed in Cos cells at similar levels. Talin binding data were derived from Fig. 4.

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