Paxillin: A New Vinculin-binding Protein Present in Focal Adhesions

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Abstract. The 68-kD protein (paxillin) is a cytoskeletal component that localizes to the focal adhesions at the ends of actin stress fibers in chicken embryo fibroblasts. It is also present in the focal adhesions of Madin-Darby bovine kidney (MDBK) epithelial cells but is absent, like talin, from the cell-cell adherens junctions of these cells. Paxillin purified from chicken gizzard smooth muscle migrates as a diffuse band on SDS-PAGE gels with a molecular mass of 65-70 kD. It is a protein of multiple isoforms with pIs ranging from 6.31 to 6.85. Using purified paxillin, we have demonstrated a specific interaction in vitro with another focal adhesion protein, vinculin. Cleavage of vinculin with *Staphylococcus aureus* V8 protease results in the generation of two fragments of ~85 and 27 kD. Unlike talin, which binds to the large vinculin fragment, paxillin was found to bind to the small vinculin fragment, which represents the rod domain of the molecule. Together with the previous observation that paxillin is a major substrate of $pp60^{src}$ in Rous sarcoma virus-transformed cells (Glenney, J. R., and L. Zokas. 1989. *J. Cell Biol.* 108:2401-2408), this interaction with vinculin suggests paxillin may be a key component in the control of focal adhesion organization.

THE linkage between actin filaments and the plasma membrane of cells spread on plastic or glass in tissue culture has been demonstrated previously to involve the recruitment of a variety of cytoskeletal proteins (for review see Burridge et al., 1988), including talin (Burridge and Connell, 1983), vinculin (Geiger, 1979; Burridge and Feramisco, 1980) and α-actinin (Lazarides and Burridge, 1975). These specializations, where the cells come in closest proximity to the underlying substratum, have been variously referred to as focal adhesions, focal contacts or adhesion plaques. In vitro assays have enabled an apparent link between actin through these proteins to a family of glycoproteins, the integrins (for review see Hynes, 1987), to be suggested. In turn, the integrins span the plasma membrane and interact with a variety of extracellular matrix proteins, such as fibronectin and vitronectin (Buck and Horwitz, 1987; Ruoslahti and Pierschbacher, 1987).

Undoubtedly, other links to the membrane exist and a number of additional proteins, which localize to focal adhesions, have been described. These include fimbrin (Bretscher and Weber, 1980), an actin-bundling protein (Bretscher, 1981; Glenney et al., 1981), also present in large quantities in intestinal microvilli (Bretscher, 1981; Glenney et al., 1981), although its absolute requirement for focal adhesion stability has recently been questioned (De Pasquale, J. A., and C. S. Izzard, 1989. *J. Cell Biol.* 109:267a). An 82-kD protein (Beckerle, 1986) is also present in focal adhesions in low abundance, as is tensin (Risinger, M. A., J. A. Wilkins, and S. Lin. 1987. *J. Cell Biol.* 105:130a), which has been proposed to be responsible for the actin-capping activity present in partially purified vinculin preparations (Wilkins and Lin, 1986; Wilkins et al., 1986). Unfortunately, little is known with respect to the functional contribution of these and other as yet uncharacterized proteins present at these sites. Similarly, the regulation of focal adhesion integrity is poorly understood.

Much interest has been directed towards a variety of regulatory proteins which localize to focal adhesions. Potential regulatory proteins include the calcium-dependent protease, calpain II, which has been shown to be concentrated in the focal adhesions of epithelial cells (Beckerle et al., 1987), and a number of kinases. The loss of focal adhesion organization after the activation of protein kinase C by tumor promoters (Schliwa el al., 1984; Kellie et al., 1985; Meigs and Wang, 1986) and the presence of protein kinase C at focal adhesions (Jaken et al., 1989) have led to a number of studies focusing on the possible role played by serine and threonine phosphorylation of focal adhesion proteins, via the activation of protein kinase C, in the reorganization of these structures (Werth et al., 1983; Werth and Pastan, 1984; Litchfield and Ball, 1986; Turner et al., 1989; Beckerle, 1990). However, the stoichiometry of phosphorylation observed on many of these proteins is very low (Turner et al., 1989), and it is difficult to invoke a model for their involvement in the control of focal adhesion integrity without envisaging rapid phosphorylation/dephosphorylation events or phosphorylation of specific subpopulations of the proteins in question. Similarly, when chick embryo fibroblasts (CEFs)[1] are transformed by the Rous sar-

1. *Abbreviations used in this paper*: CEF, chick embryo fibroblast; HAP, hydroxylapatite; ZA, zonula adherens.

coma viral tyrosine kinase pp60^{src}, the proteins talin, vinculin, and integrin are substrates for phosphorylation (Sefton et al., 1981; Hirst et al., 1986; Pasquale et al., 1986; DeClue and Martin, 1987). A direct correlation between these post-translational modifications and altered cell morphology has been questioned, since comparable levels of phosphorylation of vinculin, for example, have been observed in cells containing a mutant variant of pp60^{src} lacking the membrane binding domain which does not give rise to a transformed phenotype (Kamps et al., 1986).

Recently, a number of mAbs have been generated against phosphotyrosine-containing proteins from RSV-transformed CEFs (Glenney and Zokas, 1989). One of these proteins, with a molecular mass of ~65–76 kD, localizes to focal adhesions in nontransformed cells. Upon transformation by RSV, 20–30% of this protein is phosphorylated on tyrosine and its cellular distribution becomes more diffuse (Glenney and Zokas, 1989), suggesting that phosphorylation of this protein may have a role in the disassembly of focal adhesions and stress fibers during transformation. In an effort to determine the contribution of the 68-kD protein in focal adhesion organization we describe herein the purification and initial biochemical characterization of this protein from chicken gizzard smooth muscle. In addition, its interactions with other known focal adhesion proteins were investigated. In view of its localization to focal adhesions in nontransformed CEFs we propose the name paxillin after the Latin "paxillus," meaning a small stake or peg, to fit in with the idea of proteins being tethered to the membrane at focal adhesions.

Materials and Methods

Cell Culture

Chick embryo dermal fibroblasts taken from 10-d embryos were grown in DME (Gibco Laboratories, Grand Island, NY) supplemented with 10% FBS (Gibco Laboratories), 50 μl/ml streptomycin and 50 U/ml penicillin and grown at 37°C in 5% CO_2. Madin–Darby bovine kidney (MDBK) epithelial cells were grown at 37°C with 5% CO_2 in Eagle's MEM with Earles's salts, 15 mM Hepes pH 7.3, 10% FBS, and penicillin and streptomycin.

Fluorescence Microscopy

CEFs were allowed to spread overnight and MDBK cells grown in culture until colonies of well spread cells were observed, usually after 2–3 d. Cells were fixed in 3.7% formaldehyde in PBS, pH 7.4 for 8 min and subsequently washed in Tris-buffered saline (TBS), pH 7.6 for 5 min. The cells were permeabilized for 2 min in 0.2% (vol/vol) Triton X-100 in TBS, and then washed as above in TBS. For double labeling, cells were incubated first with antipaxillin antibody (either culture supernatant used undiluted or purified Ig from ascites fluid at 1:1,000) for 30 min at 37°C in a humid chamber. The cells were then washed and incubated with rhodamine-conjugated goat anti-mouse Ig (Cappel Laboratories, West Chester, PA) diluted 1:50 for 30 min at 37°C. For double labeling of actin, fluorescein-conjugated phalloidin was included with the anti-mouse antibody at 1:500. Colocalization of vinculin or talin was achieved after the paxillin labeling by incubating the cells in either rabbit antivinculin (1:100) or rabbit antitalin antibody (1:500) followed by affinity-purified, species cross-adsorbed fluorescein goat anti-rabbit Ig (Cappel Laboratories) diluted 1:50. Labeling of the zonula adherens junctions in MDBK cells was achieved using a rabbit antiuvomorulin antibody at 1:100 (a generous gift of Dr. B. Gumbiner, University of California, San Francisco) followed by fluorescein-goat anti-rabbit Ig.

Paxillin Purification

150 g of finely chopped chicken gizzard smooth muscle was homogenized in 800 ml ice-cold 10 mM Tris-HCl pH 8.0, 2 mM EDTA, 0.1% β-mercaptoethanol, 0.5 mM PMSF and 5 μM leupeptin with 5 10-s bursts at top speed in a Waring blender before centrifugation at 16,000 *g* for 10 min in a GSA rotor (DuPont Corp., Wilmington, DE). The supernatant was saved and the pellet resuspended at medium speed three times for 5 s each in a further 800 ml of the above buffer. The extract was centrifuged as above and the supernatants from the two washes were combined and filtered through six layers of fine cheesecloth. The volume was measured and paxillin was precipitated by adding solid $(NH_4)_2SO_4$ (13.4 g/100 ml), with stirring for 60 min. The precipitate was collected by centrifugation at 12,000 *g* for 10 min and resuspended in buffer B (20 mM Tris/acetate pH 7.6, 20 mM NaCl, 0.1 mM EDTA, 0.1% β-mercaptoethanol) plus 0.5 mM PMSF and 5 μM leupeptin and dialyzed overnight against buffer B. Precipitated protein (mostly myosin) was removed by centrifugation at 100,000 *g* for 30 min (45 Ti rotor; Beckman Instruments, Inc., Fullerton, CA) and the clarified supernatant was loaded onto a 100 ml DEAE-cellulose (DE 52; Whatman Biosystems Ltd., Kent, UK) anion exchange column preequilibrated in buffer B. After loading, the column was washed with 300 ml of buffer B and proteins were eluted with a 650-ml salt gradient going from buffer B to buffer B containing 325 mM NaCl. Fractions containing protein were assayed by SDS-PAGE and Western blotting with the antipaxillin antibody. Relevant fractions were pooled and loaded directly onto a 25-ml hydroxylapatite (HAP) column equilibrated in buffer B. The proteins were eluted from the column with a 15–240 mM potassium phosphate, pH 7.5 gradient (120 ml) containing 0.1% β-mercaptoethanol. Paxillin enriched fractions were pooled and dialyzed against TBS and then loaded onto an antipaxillin antibody affinity column. Paxillin antibody was coupled to Affi-Gel 10 (Bio-Rad Laboratories, Cambridge, MA) according to the manufacturers' instructions. The column was washed extensively with PBS and then prewashed with 10 mM sodium phosphate pH 6.8 before elution of the paxillin with 100 mM glycine, HCl, pH 2.5 into tubes containing sufficient Tris/HCl pH 10 to neutralize the acid and 0.2% β-mercaptoethanol.

Iodination

60 μl of a 1 mg/ml stock of Iodogen (Pierce Chemical Co., Rockford, IL) in chloroform was dried onto the walls of an Eppendorf tube using a stream of nitrogen gas. 100-μl aliquots of paxillin or vinculin dialyzed into TBS were added to the tube in addition to 1 mCi carrier-free ^{125}I (DuPont Corp.) in 0.1 M potassium phosphate pH 7.2. The reaction was allowed to proceed for 7 min on ice before excess free iodine was quenched by the addition of 20 μl saturated tyrosine solution and incubation for a further 5 min on ice. Labeled protein was separated from free iodine by passage over a 0.7 × 15 cm G 50 column (Pharmacia Fine Chemicals, Piscataway, NJ), preblocked with buffer B containing 0.3% BSA, and eluted with the same buffer.

Tissue Extract Preparation

Tissues from 10-d chicken embryos were weighed and homogenized using a Dounce homogenizer in 10 vol of 1:1 H_2O and 2× Laemmli sample buffer (boiling). The samples were heated for a further 5 min and DNA was sheared by passage through a 27-gauge needle.

SDS-PAGE and Western Blotting

Protein samples were electrophoresed in one dimension on 10% polyacrylamide gels according to Laemmli (1970) with a bisacrylamide concentration of 0.13%. In the case of the vinculin fragments, 12.5% acrylamide gels were used with a bisacrylamide concentration of 0.1%. For protein overlay or Western blotting the electrophoresed proteins were transferred to nitrocellulose, blocked, and then probed with either iodinated paxillin (7.5 × 10^5 cpm/ml) for 90 min, before extensive washing in buffer B plus 0.2% gelatin and 0.05% Tween 20, or antipaxillin antibody for 60 min followed by ^{125}I-conjugated rabbit anti-mouse Ig or unlabeled rabbit anti-mouse followed by ^{125}I-labeled goat anti-rabbit Ig. For antibody blots the nitrocellulose was washed with TBS plus 0.2% gelatin and 0.05% Tween 20 between each incubation.

Two-dimensional PAGE

Two-dimensional electrophoresis was performed according to O'Farrell (1975) on a Minigel apparatus (Hoeffer Scientific Instruments, San Francisco, CA) with the following modifications. IEF gels for the first dimension consisted of 9.2 M urea, 4% acrylamide, 2.0% TX-100, 1.6% Bio-lyte 5/7 ampholyte, and 0.4% Bio-lyte 3/10 ampholyte. They were prerun at 200, 300, and 400 V for 10, 15, and 15 min respectively, after which the samples were loaded and focused at 500 V for 10 min followed by 4 h at 950 V. Proteins were separated in the second dimension on 8% mini gels. Carba-

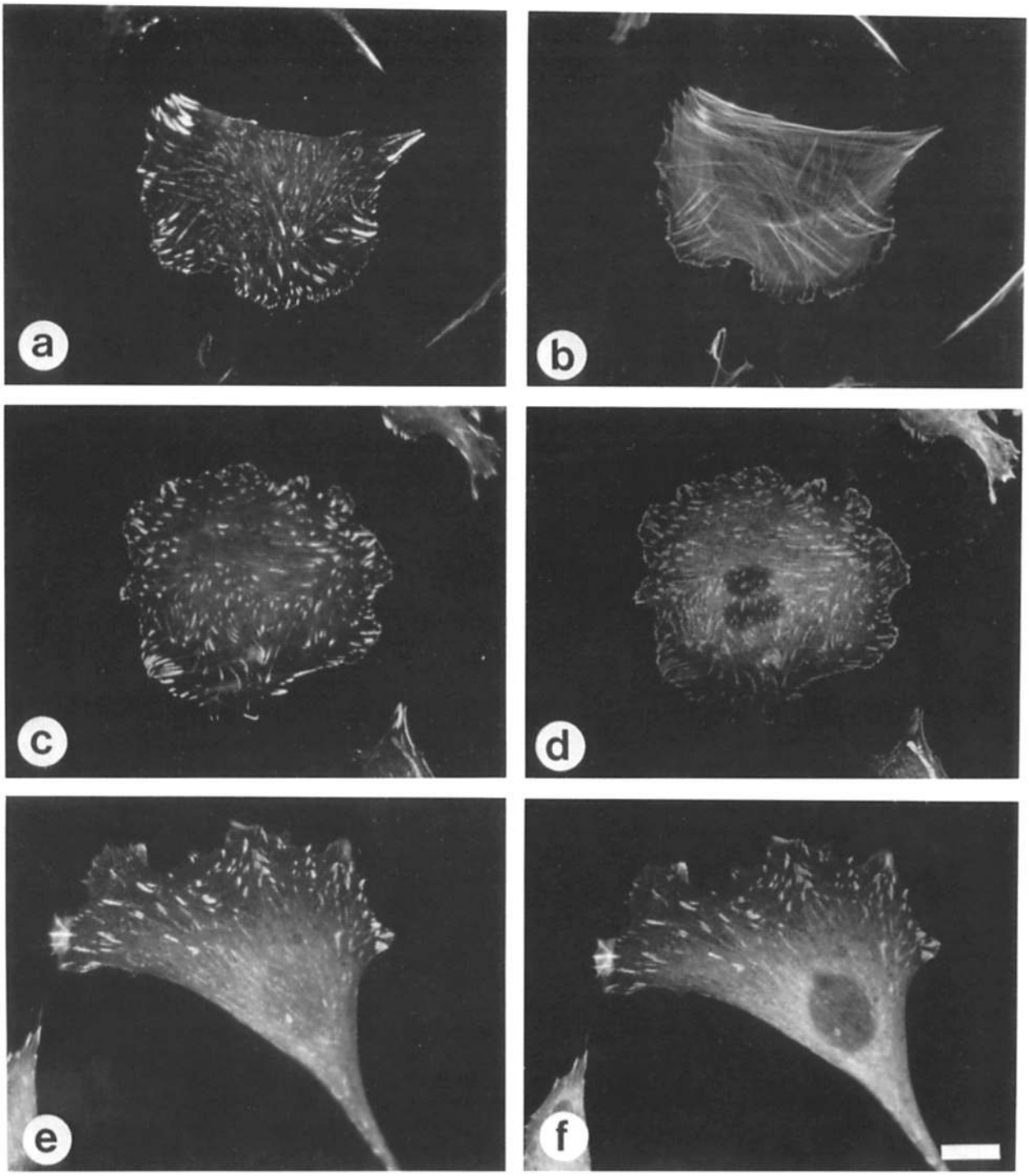


Figure 1. Paxillin colocalizes with talin and vinculin at the ends of actin stress fibers. CEFs allowed to spread overnight were fixed and permeabilized before labeling with an mAb to paxillin and a corresponding rhodamine-labeled second antibody (*a*, *c*, and *e*). The same cells were double-labeled for F-actin using fluorescein-conjugated phalloidin (*b*), for talin (*d*), or for vinculin (*f*) using rabbit antisera followed by fluorescein-labeled second antibody. Bar, 10 μm.

mylated creatine kinase was used to calibrate the isoelectric focusing, and prestained markers were included in the second dimension. Paxillin was localized by transferring proteins from the slab gel onto nitrocellulose and then probing with antipaxillin antibody.

Plate Binding Assay

50 μl of protein solution was pipetted into microtiter wells and allowed to adsorb to the surface for 60 min at 37°C. The wells were washed with buffer B containing 0.2% BSA and then blocked with 200 μl of buffer B plus 2% BSA for 60 min at 37°C. 50–100,000 cpm of iodinated protein was added to each well in 10 μl in addition to 100 μl of increasing concentrations of competing protein. After a further 60 min incubation at 37°C, the wells were washed extensively with buffer B plus 0.2% BSA before counting in a gamma counter.

Miscellaneous Procedures

For protein overlays of gizzard protein fractions, homogenized gizzard was extracted under low salt conditions as previously described (O'Halloran et

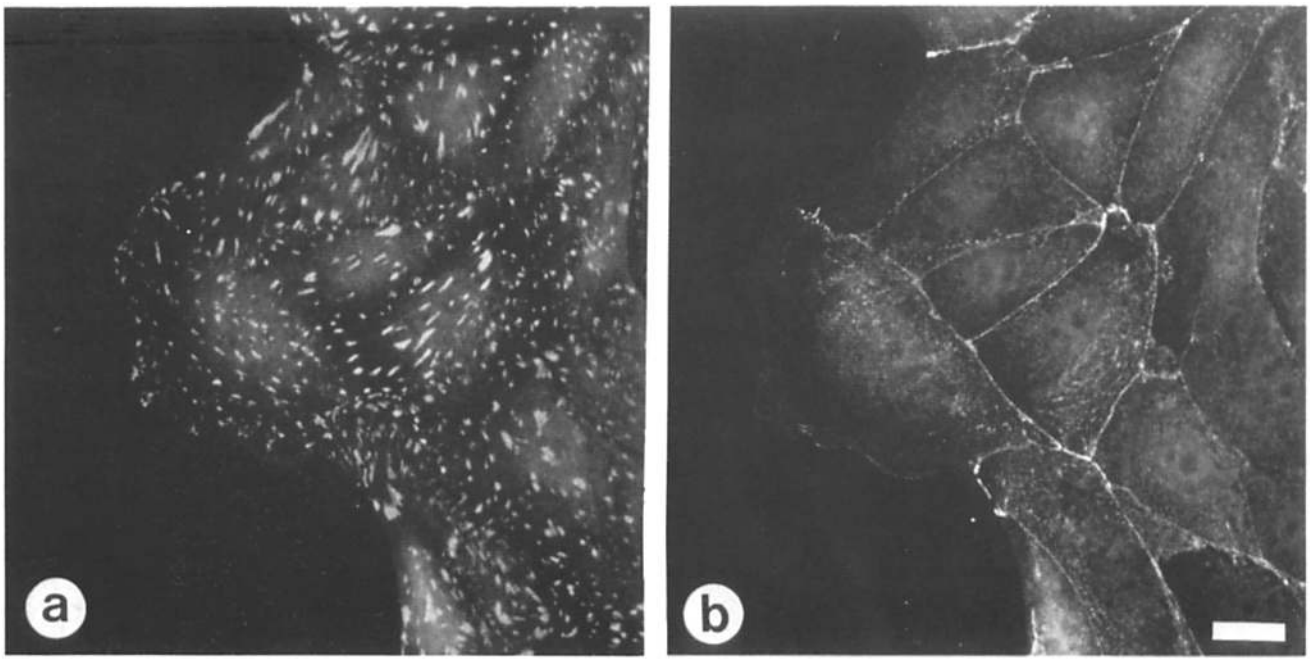


Figure 2. Paxillin is absent from the ZA cell–cell junctions of Madin–Darby bovine kidney (MDBK) cells. MDBK cells were allowed to adhere and spread on glass coverslips overnight before being fixed and permeabilized. The distribution of paxillin, visualized using a specific mAb, was restricted to the focal adhesions formed on the ventral surface of the cells (*a*). The cell–cell adherens junctions (ZAs) of the same cells were visualized using an antibody to uvomorulin (*b*). Both images were taken at the same focal plane. Bar, 10 μm.

al., 1986), dialyzed against buffer B_{10} (20 mM Tris-acetate, pH 7.6, 10 mM NaCl, 0.1 mM EDTA, 0.1% β-mercaptoethanol), and clarified at 100,000 *g* for 60 min before loading onto DEAE cellulose (DE 52; Whatman Biosystems). Proteins were eluted with a salt gradient going from buffer B_{10} to buffer B_{10} plus 400 mM NaCl.

Proteolytic cleavage of vinculin using *Staphylococcus aureus* V8 protease (Sigma Chemical Co., St Louis, MO) was performed as described previously (Groesch and Otto, 1990). The digestion was halted by heating in gel sample buffer.

Results

Immunofluorescence

Double-label immunofluorescence of chick embryo fibroblasts after they were plated for 18 h on glass coverslips, in the presence of serum, revealed that the 68-kD protein (hereafter referred to as paxillin) was localized to the ends of the actin stress fibers (Fig. 1 *a*) labeled with fluorescein-phalloidin (Fig. 1 *b*) as previously described (Glenney and Zokas, 1989). That paxillin was localizing to the focal adhesions was confirmed by double-labeling with antibodies against paxillin (Fig. 1, *c* and *e*) and either talin (Fig. 1 *d*) or vinculin (Fig. 1 *f*). The images were striking for the degree of colocalization between these two proteins and paxillin. Even the smallest contacts at the periphery of the cells, which are presumably newly forming structures, contained paxillin in conjunction with talin or vinculin. It is of note that when costaining with antibodies against either talin or vinculin the degree of labeling of paxillin was significantly attenuated compared with double labeling for actin. Only if the cells were incubated with both the antipaxillin antibody and its corresponding fluorescent secondary antibody before any talin or vinculin staining was the paxillin staining not reduced. This observation suggests that paxillin may be closer to the membrane than talin or vinculin, with the result that antibodies to these two proteins block sterically the binding of the paxillin antibodies. Paxillin could not be labeled on nonpermeabilized cells indicating the epitope for the antibody is intracellular (data not shown).

Epithelial cells grown in tissue culture demonstrate two forms of adherens junctions. They form focal adhesions on their ventral surface analogous to the focal adhesions found in chick embryo fibroblasts described above, and they also form cell–cell junctions of the zonula adherens (ZA) type (Farquhar and Palade, 1963). Whereas talin localizes exclusively to the focal adhesions (Geiger et al., 1985), vinculin localizes to both types of adherens junction (Geiger et al., 1981). In view of the localization of paxillin to focal adhesions in CEFs we were interested in determining if paxillin, like vinculin, was also located in the cell–cell junctions of epithelial cells. Fig. 2 *a* demonstrates that while paxillin localizes to the cell–substrate junctions of the focal adhesions, it was found to be absent from the cell–cell junctions that have been delineated in the same cells in Fig. 2 *b* using antisera to uvomorulin (L-CAM, E-cadherin). In this respect, the paxillin distribution in adherens junctions is similar to that of talin, which is restricted to the focal adhesions on the ventral surface (Geiger et al., 1985).

Tissue Localization of Paxillin

As a preliminary screening before the selection of a suitable tissue from which paxillin may be subsequently purified, a number of chicken tissues were taken from a day 10 embryo. The samples were electrophoresed on 10% gels and then either stained with Coomassie blue (Fig. 3 *A*) or transferred onto nitrocellulose and probed with the antipaxillin antibody (Fig. 3 *B*). The smooth muscle of the gizzard and that of the

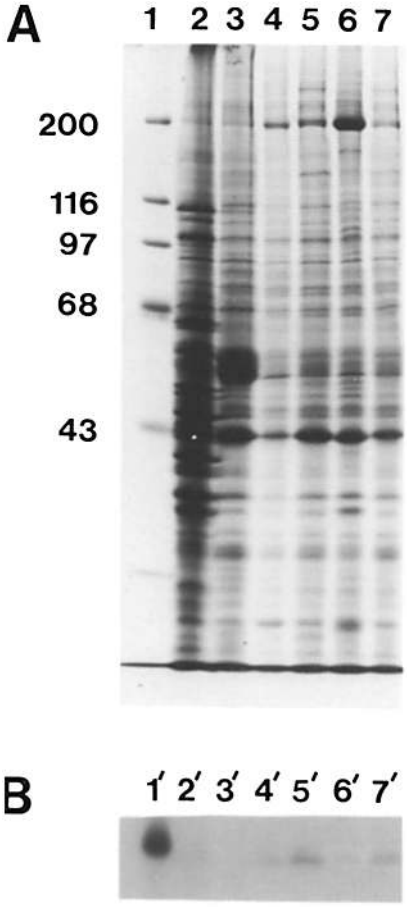


Figure 3. Tissue distribution of paxillin. Tissues from day 10 chick embryos were homogenized directly into 10 vol of boiling SDS-PAGE sample buffer. Equal volumes were loaded onto 10% gels and either stained with Coomassie brilliant blue (*A*) or transferred to nitrocellulose and probed with antipaxillin antibody (*B*). Lanes *1* and *1'*, molecular weight standards (^{125}I-labeled in *B*); lanes *2* and *2'*, liver; lanes *3* and *3'*, brain; lanes *4* and *4'*, skeletal muscle; lanes *5* and *5'*, smooth muscle (gizzard); lanes *6* and *6'*, cardiac muscle; lanes *7* and *7'*, intestine. The immunoblot of the appropriate region of the gel shows that paxillin is most abundant in gizzard and intestinal smooth muscle (lanes *5'* and *7'*). Skeletal and cardiac muscle contain intermediate levels (lanes *4'* and *6'*) while liver (lane *2'*) expresses only very low levels of paxillin and it is not detectable in brain (lane *3'*).

intestine (Fig. 3 *B*, lanes *5'* and *7'*, respectively) contained the largest amounts of paxillin. Skeletal and cardiac muscle exhibited intermediate levels (Fig. 3 *B*, lanes *4'* and *6'*). Low levels of paxillin were detected in liver (Fig. 3 *B*, lane *2'*). No paxillin was observed in brain at this level of sensitivity (Fig. 3 *B*, lane *3'*).

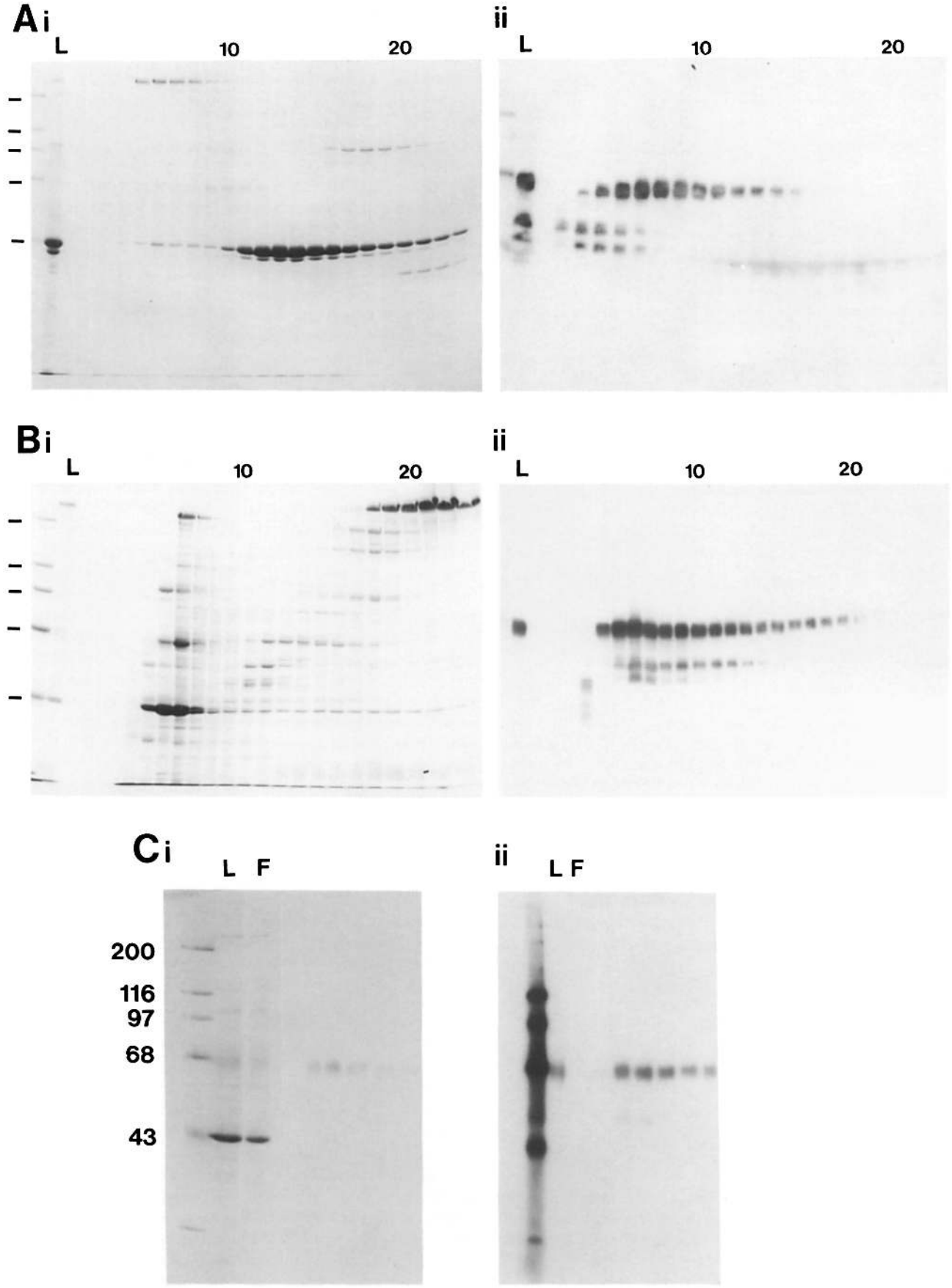
Purification of Paxillin from Chicken Gizzard Smooth Muscle

Many other focal adhesion proteins have been purified from chicken gizzard smooth muscle in milligram quantities (Molony et al., 1987; Feramisco and Burridge, 1980). Using the procedures for the purification of talin and vinculin (O'Halloran et al., 1986), we were able to determine by Western blotting with the paxillin antibody that the majority of the 68-kD protein was readily extracted and was highly enriched in the first two washes of the homogenized tissue, which are normally discarded for the other preparations (data not shown). The 68-kD protein could be precipitated from this extract with a 25% ammonium sulfate fractionation, leaving many of the contaminating proteins in solution. Note that fimbrin, another 68-kD focal adhesion protein, is precipitated in 55% ammonium sulfate (Glenney et al., 1981). Furthermore, antibodies to paxillin do not cross-react with fimbrin nor vice versa (Turner, C. E., unpublished observations).

After overnight dialysis against low salt to precipitate any myosin, the clarified supernatant was fractionated on a DEAE-cellulose (DE-52) anion exchange column by developing with a salt gradient from 20 to 325 mM NaCl. Fractions were electrophoresed on 10% SDS gels and either stained with Coomassie blue (Fig. 4 *A* [i]) or transferred to nitrocellulose and probed with the anti paxillin antibody (Fig. 4 *A* [*ii*]). It was apparent at this stage that paxillin migrated as a diffuse band with a molecular mass range of between 65 and 70 kD and was poorly stained with Coomassie blue. Two other immunoreactive bands of 46 and 44 kD were also observed. (It has not yet been determined if these represent breakdown products of paxillin or separate proteins, but the relative amounts of these contaminants compared with paxillin are variable, and often increase during chromatography on HAP, suggesting proteolysis may be occurring.) The fractions enriched in paxillin were pooled and loaded directly onto the HAP column without lowering the salt concentration. This was found to prevent the association of filamin and actin at this stage allowing for a good separation of these two proteins, with filamin eluting towards the end of the phosphate gradient. The paxillin eluted early in the gradient at ~30 mM phosphate (Fig. 4 *B*). Even after purification over two columns, paxillin was barely detectable with Coomassie blue (or silver stain) despite a strong signal when blotted with antipaxillin antibody (Fig. 4 *B* [*ii*]). We have attempted to purify paxillin further by conventional chromatography but the protein focuses very poorly and consequently the yield drops at each step. In view of these restrictions, an antipaxillin antibody affinity column was constructed and used to purify paxillin from pooled fractions from the HAP column enriched in paxillin. Coomassie blue analysis of the eluted fractions revealed a single diffuse band of ~68 kD (Fig. 4 *C* [*i*]). This was confirmed to be paxillin by performing a complementary Western blot (Fig. 4 *C* [*ii*]). The apparent molecular weight of paxillin was not affected by running the protein under nonreduced conditions (data not shown).

One possible explanation for the protein running as a diffuse band on reduced SDS gels was the presence of a carbohydrate moiety. However, incubation with neuraminidase or endoglycosidase H under appropriate conditions failed to produce a shift in mobility on SDS gels. Furthermore, paxillin was neither retained on Con A nor wheat germ agglutinin affinity columns (data not shown).

Since there is evidence to suggest that paxillin is a phosphoprotein, at least in transformed cells (Glenney and Zokas, 1989), it was possible that the protein purified from gizzard was present in a number of multiply phosphorylated forms. Two-dimensional electrophoresis analysis of paxillin-containing material from the DE 52 column demonstrated at least four isoforms of paxillin with pIs ranging from pH 6.31 to 6.86 (Fig. 5). There was a slight increase in molecular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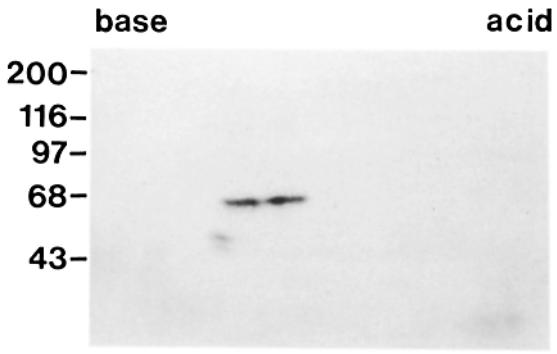

Figure 5. Two-dimensional gel analysis of paxillin. A sample of the paxillin-containing fractions from the HAP column was resolved by isoelectric focusing (*left* to *right*) followed by SDS-PAGE (*top* to *bottom*). Paxillin was visualized by transferring the proteins to nitrocellulose and probing the filter with antibody to paxillin. Paxillin exhibited a number of isoforms with pIs ranging from pH 6.31 to 6.86, with a slight increase in molecular weight towards the acidic end. The two lower molecular weight immunoreactive forms of 46 and 44 kD demonstrated pIs of pH 6.9. pIs were determined by comparison with carbamylated standards resolved under identical conditions.

weight in the more acidic isoforms consistent with there being multiple phosphorylation sites. The two lower molecular mass peptides (46 and 44 kD) also recognized by the paxillin antibody both exhibited pIs of pH 6.9.

Vinculin Binding

Because of the localization of paxillin to focal adhesions in spread fibroblasts, we were interested in determining if paxillin interacts with other known focal adhesion proteins in vitro. To this end, purified paxillin was radioiodinated, and used to overlay proteins extracted from chicken gizzard and fractionated on DEAE cellulose (DE 52 column) (Fig. 6 *A*, lanes *2–8*). After transfer of identical proteins as in *A* onto nitrocellulose and overlaying with iodinated paxillin, a single polypeptide of ~116 kD reacted strongly with the iodinated paxillin (Fig. 6 *B*, lanes *2′–4′*). This protein was confirmed to be vinculin by overlaying paxillin on a sample of purified vinculin (Fig. 6, *A* and *B*, lanes *9* and *9′*). No interaction with talin, filamin, α-actinin, or actin was observed in this assay despite their presence, in abundance, in the fractions probed. The purity of the radioiodinated paxillin was confirmed by autoradiographic analysis of an electrophoresed sample of radioiodinated paxillin (Fig. 6 *C*).

To characterize the specificity of the interaction between paxillin and vinculin further, a solid-phase binding assay was performed in which vinculin was bound to wells of microtiter plates. After blocking, the ^{125}I-paxillin was incubated with this vinculin in the presence of increasing amounts of soluble vinculin. Fig. 7 *A* demonstrates that the binding of the paxillin could be effectively competed with the soluble vinculin. Only background levels of counts were observed when BSA, instead of vinculin, was bound to the plates.

To determine an apparent dissociation constant for the interaction between paxillin and vinculin, purified paxillin was adsorbed onto the wells of the microtiter plates. By addition of radioiodinated vinculin and increasing amounts of cold vinculin, a similar inhibition curve to that using iodinated paxillin was obtained (Fig. 7 *B*). When these data were subjected to Scatchard analysis (Fig. 7 *B*, *inset*) a K_d of ~6 × 10^{-8} M was obtained in this particular experiment. An average value of 4.5 × 10^{-8} M was obtained from two experiments.

Paxillin Binds to the Rod Domain of Vinculin

Electron micrographs of rotary-shadowed vinculin monomers have previously shown that vinculin consists of a globular head domain attached to a rod-like tail domain (Milam, 1985; Molony and Burridge, 1985). These two domains, of ~85 and 27 kD, respectively, can be cleaved by digestion with *Staphylococcus aureus* V8 protease (Milam, 1985; Groesch and Otto, 1990) which is thought to cleave within the proline-rich region of the vinculin molecule (Coutu and Craig, 1988). To determine which region of the molecule contains the paxillin binding site, a sample of V8-digested vinculin was electrophoresed in parallel with a sample of intact vinculin (Fig. 8). The peptides were either stained with Coomassie blue (Fig. 8 *A*) or transferred to nitrocellulose and overlayed with radioiodinated paxillin (Fig. 8 *B*). The ^{125}I-paxillin bound to the intact vinculin (Fig. 8 *B*, lane *2′*) and to the 27-kD fragment but not to the 85-kD fragment (Fig. 8 *B*, lane *3′*). This indicates that the paxillin binding site is within the carboxy-terminal rod domain of the vinculin molecule.

Discussion

The current models of focal adhesion organization (see for example, Burridge et al., 1988) are clearly incomplete in view of the continuing identification of new proteins at these regions of actin–membrane attachment. The structure and regulation of these specializations will be better understood once a function has been assigned to each component. In this paper we have initiated the characterization of a novel focal adhesion protein with a molecular mass of 68 kD, for which we propose the name paxillin.

Like many other focal adhesion proteins, paxillin is most abundant in chicken gizzard smooth muscle and other mus-

Figure 4. Column purification of paxillin. Ammonium sulfate precipitated proteins were dialyzed against buffer B, loaded onto a DEAE-cellulose anion-exchange column and eluted with a 20–325 mM salt gradient. Fractions (every third) were electrophoresed and either stained with Coomassie brilliant blue (*A*[*i*]) or blotted with antipaxillin antibody (*A*[*ii*]). (*B*) HAP chromatography of paxillin-containing fractions pooled from the DEAE column, (*B*[*i*]) Coomassie brilliant blue staining. (*B*[*ii*]) Corresponding anti-paxillin blot. (*C*) immunoaffinity purification of paxillin from material eluted from the HAP column. (*i*) Coomassie brilliant blue staining. (*ii*) Antipaxillin blot. *L*, column load; *F*, flow through. Radioiodinated standards were coelectrophoresed in the Western blot samples. (M_r × 10^{-3}). Equal quantities of protein were loaded in (*A*[*i* and *ii*]), whereas one-third as much was loaded in (*B*[*ii*] and *C*[*ii*]) compared with their corresponding Coomassie brilliant blue–stained gels.

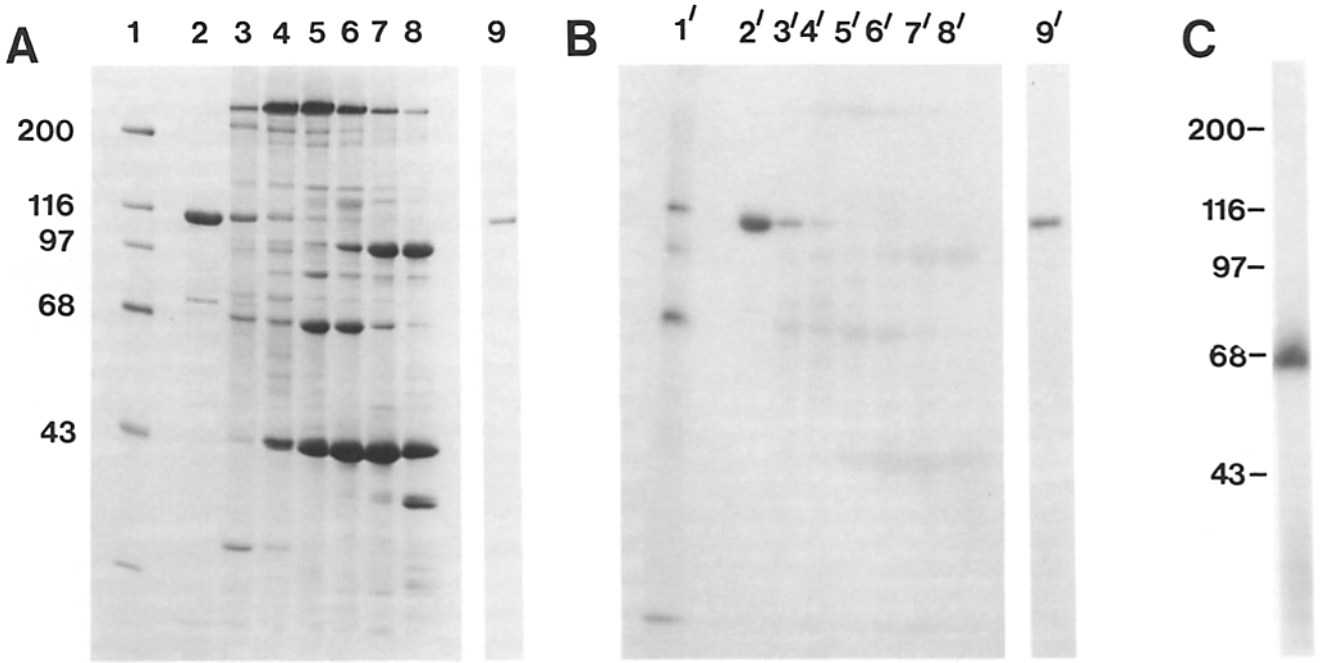

Figure 6. Paxillin binds vinculin in protein overlay assays. Proteins extracted by low salt from chicken gizzards were fractionated on an anion-exchange column, electrophoresed, and then either stained with Coomassie brilliant blue (*A*) or transferred to nitrocellulose and probed with radioiodinated paxillin (*B*). Lanes *1* and *1′*, molecular weight standards (iodinated in lanes *1′*); lanes *2–8* and *2′–8′*, column fractions. The paxillin interacted with a polypeptide of 116 kD in the column fractions, which was confirmed to be vinculin by overlaying purified vinculin with the ^{125}I-labeled paxillin (lanes *9* and *9′*). (*C*) autoradiograph of radioiodinated paxillin.

cle tissues. It is absent from neuronal tissue where a fodrin-based cytoskeleton is more prominent (Bennett et al., 1982). It remains unclear as to why the protein migrates as a diffuse band, but its solubility in the absence of detergents, its lack of binding to lectins and its insensitivity to glycosidases appears to rule out it being a glycoprotein as was first suggested (Glenney and Zokas, 1989). The numerous isoforms of paxillin visible after two-dimensional electrophoresis probably reflect multiple posttranslational covalent modifications of the protein, such as phosphorylation. In addition to being heavily phosphorylated on tyrosine after transformation, paxillin also contains high levels of phosphoserine and phosphothreonine when isolated from normal ^{32}P-labeled CEFs (Glenney, J. R., unpublished observations).

Using protein overlay techniques, a number of vinculin-binding proteins have been previously described (Otto, 1983; Wilkins et al., 1983; Burridge and Mangeat, 1984; Belkin and Koteliansky, 1987). Of these, only talin and α-actinin have been further characterized (Burridge and Mangeat, 1984; Wachsstock et al., 1987). The affinity of talin for vinculin in vitro is in the same range as that of paxillin for vinculin (~10^{-8} M), while α-actinin binds with a lower affinity of ~10^{-6} M (Wachsstock et al., 1987).

The association between talin and vinculin has been shown to occur via the NH_2-terminal globular head domain of vinculin by blot-overlay assays (Price et al., 1989; Turner and Burridge, 1989; Groesch and Otto, 1990) and by analyses of cDNA clones of vinculin, which have localized the talin binding site to a 41-amino acid region within the head domain (Price et al., 1989; Jones et al., 1989). In contrast, the rod-shaped portion of the vinculin molecule composing the carboxy terminus (Coutu and Craig, 1988) contains one or more self-association sites (Milam, 1985; Molony and Burridge, 1985). The finding that transfected vinculin peptides lacking the talin binding site still had the ability to localize to focal adhesions led Bendori et al. (1989) to propose that vinculin–vinculin interactions were responsible for their observations. Our findings that paxillin binds to the tail region of vinculin suggests that it may be an interaction between paxillin and vinculin that was responsible for the localization of such vinculin peptides to focal adhesions in these experiments. However, the two mechanisms are not mutually exclusive. Indeed, in the absence of the identification of other paxillin-binding proteins the accumulation of paxillin in focal adhesions at present relies upon the presence of vinculin molecules. It will be important to determine if paxillin interacts with other focal adhesion proteins, for example integrin, which was not present in the fractions probed with ^{125}I-paxillin, and how paxillin may affect the self-association of vinculin molecules.

The lack of colocalization of paxillin with vinculin in cell–cell junctions of epithelial cells is intriguing, especially in view of its ability to interact with vinculin in the in vitro assays. This situation is similar to that of talin (Geiger et al., 1985), the only other cytoskeletal protein known to be present in cell–substrate but not epithelial cell–cell junctions. The explanation for this result remains unclear, although the finding that a proteolytic fragment of talin can bind to the ZA of epithelial cells, possibly via an association with vinculin (Nuckolls et al., 1990) suggests that segregation is not a function of there being different isoforms of vinculin at these two locations. Rather, there are probably other proteins pres-

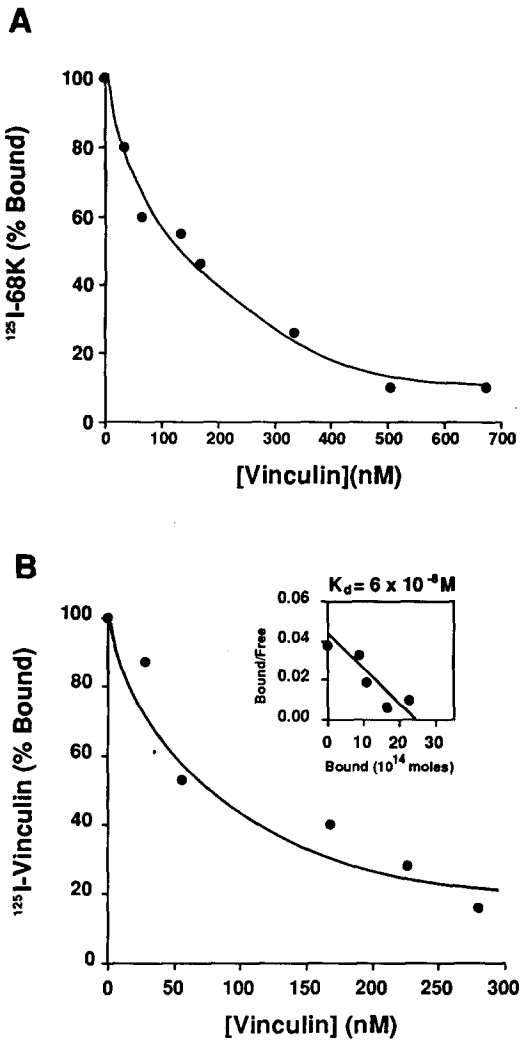

Figure 7. Quantitation of the binding between paxillin and vinculin. (*A*) Purified vinculin was adsorbed to microtiter wells. After blocking with BSA, radiolabeled paxillin was added in conjunction with increasing amounts of unlabeled vinculin, which reduced the binding of the paxillin to the adsorbed vinculin down to background levels. (*B*) purified paxillin was adsorbed to the microtiter wells. Radioiodinated vinculin was added in conjunction with increasing amounts of unlabeled vinculin. These data were subjected to Scatchard analysis (*Inset, B*) and an apparent K_d of 6×10^{-8} M was calculated.

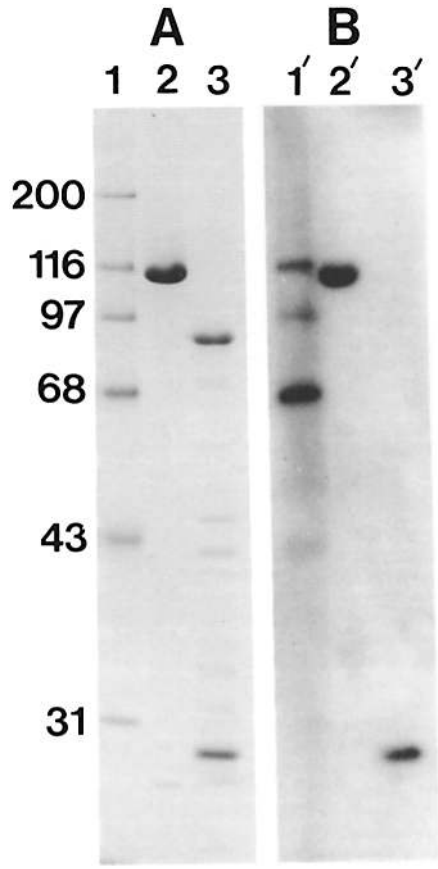

Figure 8. Paxillin binds to the rod domain of vinculin. Purified vinculin was digested with *Staphylococcus aureus* V8 protease and coelectrophoresed with intact vinculin on 12.5% gels. (*A*) Coomassie brilliant blue–stained gel. Lane *1*, molecular weight standards; lane *2*, intact vinculin; lane *3*; digested vinculin showing major fragments of 85 and 27 kD. Identical samples were transferred to nitrocellulose and overlayed with radioiodinated paxillin (*B*). The ^{125}I-paxillin bound to the intact vinculin (lane *2′*) and to the 27-kD vinculin fragment but not the 85-kD fragment (lane *3′*). Lane *1′*, ^{125}I-labeled molecular weight standards.

ent in the ZA that distinguish it from focal adhesions. These other components may prevent the access of paxillin and talin to vinculin in these regions.

The apparent low abundance of paxillin in comparison to other focal adhesion proteins like talin and vinculin suggests that paxillin may play a regulatory, rather than a structural, role within the focal adhesion. In RSV-transformed cells, where normal focal adhesion organization is disrupted, 20–30% of the paxillin in these cells has been shown to be phosphorylated on tyrosine residues (Glenney and Zokas, 1989). This is a significantly higher stoichiometry than the phosphorylation of other focal adhesion proteins previously examined, for example, ~1% of vinculin (Sefton et al., 1981) and talin (Pasquale et al., 1986; DeClue and Martin, 1987) molecules become phosphorylated in this situation. The paxillin purified from chicken gizzard as described in this paper is unlikely to contain much, if any, phosphotyrosine since this appears to be a feature of more dynamic tissues as demonstrated by the decrease in the detectable level of phosphotyrosine-containing proteins during chick embryonic development (Maher and Pasquale, 1988). In future experiments, we hope to determine if the presence of phosphotyrosine residues on paxillin perturbs its interactions with vinculin. This would represent a possible mechanism for the disruption of focal adhesion organization during RSV transformation.

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