

An Interaction between Zyxin and α -Actinin

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Abstract. Zyxin is an 82-kD protein first identified as a component of adhesion plaques and the termini of stress fibers near where they associate with the cytoplasmic face of the adhesive membrane. We report here that zyxin interacts with the actin cross-linking protein α -actinin. Zyxin cosediments with filamentous actin in an α -actinin-dependent manner and an association between zyxin and α -actinin is observed in solution by analytical gel filtration. The specificity of the interaction between zyxin and α -actinin was demonstrated by blot overlay experiments in which ^{125}I -zyxin recognizes most prominently α -actinin among a complex mixture of proteins extracted from avian smooth muscle. By these blot overlay binding studies, we determined that zyxin interacts with the NH_2 -terminal

27-kD domain of α -actinin, a region that also contains the actin binding site. Solid phase binding assays were performed to evaluate further the specificity of the binding and to determine the affinity of the zyxin- α -actinin interaction. By these approaches we have demonstrated a specific, saturable, moderate-affinity interaction between zyxin and α -actinin. Furthermore, double-label immunofluorescence reveals that zyxin and α -actinin exhibit extensive overlap in their subcellular distributions in both chicken embryo fibroblasts and pigmented retinal epithelial cells. The significant colocalization of the two proteins is consistent with the possibility that the interaction between zyxin and α -actinin has a biologically relevant role in coordinating membrane-cytoskeletal interactions.

CELLS can adhere to extracellular matrix and each other through specializations in the plasma membrane termed adherens junctions. These membrane-associated junctional complexes are associated with bundles of actin filaments that impinge on the cytoplasmic face of the plasma membrane (Singer, 1979). In cultured fibroblasts, these actin arrays are often organized into structures called stress fibers that terminate at adhesion plaques, areas of close membrane-substratum apposition that can be visualized directly by interference reflection microscopy (Heath and Dunn, 1978; Wehland et al., 1979). One of the principal proteins believed to be involved in organizing individual actin filaments into stress fibers is the actin-binding protein, α -actinin. α -Actinin forms antiparallel homodimers (Wallraff et al., 1986; Imamura et al., 1988) that have the capacity to cross-link actin filaments *in vitro* (Maruyama and Ebashi, 1965; Podlubnaya et al., 1975). By immunofluorescence, α -actinin is found periodically along stress fibers as they traverse the cytoplasm and is also located at the termini of these actin filament bundles in the adhesion plaque itself (Lazarides and Burridge, 1975). Because of its presence at sites where cells contact the extracellular matrix, α -actinin has been postulated to be involved in the attachment of the cytoskeletal framework to the plasma membrane (Lazarides and Burridge, 1975) and the ability of α -actinin to interact with other known adhesion plaque proteins has been investigated extensively.

In addition to binding actin, α -actinin has been shown to interact with other proteins involved in membrane-cyto-

skeleton interaction. An association between α -actinin and vinculin, a 116-kD cytoplasmic component of adhesion plaques (Geiger, 1979; Burridge and Feramisco, 1980), was demonstrated by blot overlay techniques using ^{125}I - α -actinin (Belkin and Koteliansky, 1987) and by solution binding assays (Wachsstock et al., 1987). Subsequently (Otey et al., 1990), it was shown by solid phase binding assays that α -actinin can also interact with the cytoplasmic domain of the β_1 subunit of integrin, a heterodimeric transmembrane receptor for extracellular matrix molecules found at sites of cell-substratum interaction (Damsky et al., 1985; Chen et al., 1985). Thus α -actinin may contribute to membrane-cytoskeletal interactions by more than one mechanism. In one scheme, stress fibers may be tethered directly to the membrane by α -actinin molecules bound to the cytoplasmic domain of β_1 -integrins. Alternatively, stress fibers may be attached to the membrane in a less-direct manner through a complex linkage of proteins assembled at adhesion plaques. Consistent with this multiprotein model, the adhesion plaque protein, talin (Burridge and Connell, 1983; Beckerle and Yeh, 1990), has been shown to interact with both integrin (Horwitz et al., 1986) and vinculin (Burridge and Mangeat, 1984). Based on a number of *in vitro* biochemical studies, it has been proposed that associations among α -actinin, vinculin, talin, and integrin could also contribute to anchoring actin filaments at sites of adhesion (for reviews see Burridge et al., 1988; Crawford and Beckerle, 1990). Our understanding of the molecular complexity of adhesion plaques continues to increase as more components and pro-

tein-protein interactions are discovered. It now appears that cells have several mechanisms for establishing and maintaining transmembrane connections between extracellular substratum and the cytoskeleton.

In addition to the above-mentioned proteins, there are a number of adherens junction components that have been identified immunochemically, but have not yet been well characterized. Studies of these proteins are likely to contribute to our understanding of the mechanism by which cells interact with their underlying substratum. One such protein is zyxin, an 82-kD protein first characterized as an adhesion plaque component of chicken embryo fibroblasts by analysis of a nonimmune rabbit serum (Beckerle, 1986). Zyxin is now known to reside at other types of adherens junctions as well, including the dense plaques of smooth muscle cells and the apical cell-cell contacts of pigmented retinal epithelial cells (Crawford and Beckerle, 1991). The protein has been purified from avian smooth muscle, and is present at stoichiometric levels relative to many adherens junction components, including vinculin, talin, and α -actinin. Many of the biochemical and biophysical properties of zyxin have been characterized, revealing that it is an asymmetric, monomeric protein that is phosphorylated on multiple sites *in vivo* (Crawford and Beckerle, 1991; and our unpublished results).

We have investigated the ability of zyxin to associate with other components of adherens junctions and report here that zyxin can interact *in vitro* with the actin-binding protein, α -actinin. The interaction between zyxin and α -actinin is specific and of moderate affinity. Moreover, the two proteins display overlapping subcellular distributions *in vivo*. Zyxin's association with α -actinin may thus provide a molecular mechanism for targeting zyxin to particular regions of the cell.

Materials and Methods

Protein Purification and Iodination

Actin was purified from rabbit skeletal muscle acetone powder using a modification (Pardee and Spudich, 1982) of the procedure of Spudich and Watt (1971). The resulting monomeric actin (G-actin) in buffer E (2 mM Tris-HCl, pH 8.0, at 25°C, 0.2 mM ATP, 0.2 mM CaCl₂, 0.1% 2-mercaptoethanol) was polymerized by the addition of 50 mM KCl, 1 mM ATP, and 1 mM MgCl₂ with stirring for 2 h at 4°C. The filamentous actin (F-actin) was stored at 4°C in the presence of 0.02% Na₃N before further purification. To maximize the purity of the actin used in these studies, F-actin was depolymerized by dialysis against buffer E at 4°C, clarified by centrifugation, and the resulting G-actin fraction was subjected to gel filtration chromatography (MacLean-Fletcher and Pollard, 1980) on a Sephadex G-150 column equilibrated in buffer E. Fractions containing G-actin were pooled and the actin was polymerized at 4°C as described above. Zyxin was purified from fresh avian smooth muscle as described previously (Crawford and Beckerle, 1991). For the sedimentation and gel filtration binding studies, it was necessary to use partially purified zyxin to have enough material to monitor the behavior of the protein in the assay; in these cases the zyxin isolation protocol was followed through the first anion exchange column step in our standard procedure and the resulting pool of zyxin-containing fractions is referred to as DE52-zyxin. A typical polypeptide profile of DE52-zyxin is shown in Fig. 1 B, lane 1 or can be found in our original report (Crawford and Beckerle, 1991); zyxin is detected in this fraction by Western immunoblot. DE52-zyxin fractions were dialyzed against buffer B (20 mM Tris-acetate, pH 7.6, 20 mM NaCl, 0.1 mM EDTA, 0.1% 2-mercaptoethanol) before use. α -Actinin and vinculin were also purified from avian smooth muscle using the procedure of Feramisco and Burridge (1980) with an additional anion exchange chromatography step on a Mono Q HR 5/5 column (Pharmacia Fine Chemicals, Piscataway, NJ) for α -actinin (Otey et al., 1990). Cleavage of α -actinin by the proteolytic enzyme, ther-

molysin (Sigma Chemical Co., St. Louis, MO) was performed in buffer B for 4 h at 22–24°C with an enzyme-to-substrate ratio of 1:20. The resulting 27- and 53-kD proteolytic products were purified by ion exchange chromatography as described previously (Otey et al., 1990). Except in the case of actin, protein concentrations were determined using the method of Bradford (1976) with BSA as a standard. BSA and ovalbumin used in these studies were obtained from Sigma Chemical Co. The concentration of actin was determined by measuring UV absorbance at 290 nm using an extinction coefficient of 0.62 cm² × mg⁻¹ (Tseng et al., 1984).

Purified protein to be iodinated was dialyzed into 150 mM NaCl, 50 mM Tris-HCl, pH 7.6, for 4–6 h (zyxin) or overnight (α -actinin). 40 μ l of Iodogen (Pierce Chemical Co., Rockford, IL; 1 mg/ml in chloroform) was dispensed into an Eppendorf tube (Brinkman Instruments Inc., Westbury, NY) and was dried down onto the wall of the tube by overnight evaporation in a fume hood. For radioiodination, 100–150 μ l of purified protein was placed in an Iodogen-coated tube, followed by the addition of 1 mCi Na-¹²⁵I (carrier free; ICN Biomedicals, Inc., Irvine, CA) and the mixture was incubated on ice for 5 (α -actinin) or 7 (zyxin) min. Labeled protein was separated from free iodine by chromatography on sephadex G-50 (0.7 × 15 cm) and samples were stored in the elution/equilibration buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.6, 0.1% sodium azide, 0.2% gelatin, 1 mM EDTA, and 0.1% 2-mercaptoethanol). The purity of the labeled proteins was ascertained by SDS-PAGE followed by autoradiography. For the binding studies, radioiodinated protein was used within 2–3 d of labeling.

Gel Electrophoresis and Immunoblot Analysis

Proteins were resolved on 10% SDS-polyacrylamide gels (Laemmli, 1970) using 0.13% bisacrylamide. Molecular mass markers were obtained from Bio-Rad Laboratories (Richmond, CA). Western immunoblot analysis using ¹²⁵I-protein A (ICN Biomedicals, Inc.) as a second reagent was performed according to a previously described modification (Beckerle, 1986) of the method developed by Towbin et al. (1979).

Antibodies and Immunocytochemistry

A monospecific, polyclonal anti-zyxin antibody (M2) was prepared by immunizing a mouse with electrophoretically isolated zyxin and has been characterized previously (Crawford and Beckerle, 1991). Antibody raised against chicken gizzard α -actinin was a generous gift of Dr. John Singer (University of California, San Diego, CA). This rabbit polyclonal antiserum recognized α -actinin specifically by Western immunoblot and has been described previously (Geiger and Singer, 1979). Indirect immunofluorescence was performed as described previously (Beckerle, 1984) except cells were prepermeabilized in 0.5% Triton X-100 in 100 mM Pipes, pH 7.3, 1 mM MgCl₂, 1 mM EGTA for 2–5 s before fixation. For chicken embryo fibroblasts, fluorescein-conjugated goat anti-mouse IgG and rhodamine-conjugated goat anti-rabbit IgG were used, and the cells were examined on a Zeiss axiophot microscope (Carl Zeiss, Inc., Thornwood, NY). For pigmented retinal epithelial cells, an affinity-purified Texas red-conjugated goat anti-rabbit IgG was used in lieu of the rhodamine probe and the labeled cells were viewed on a confocal laser scanning microscope (Bio-Rad Laboratories) with an optical section height of 2 μ m. All fluorochrome-labeled second antibodies were obtained from Cappel Laboratories (Malvern, PA) or from Jackson ImmunoResearch Laboratory (West Grove, PA).

Sedimentation Assay

Actin cosedimentation studies were performed according to the method described previously by Cooper and Pollard (1982). Specifically, 80 μ l F-actin (0.3 mg/ml) was mixed with 20 μ l 10 \times polymerization buffer (500 mM KCl, 100 mM Tris-HCl, pH 7.6), 90 μ l DE52-zyxin (described above) or buffer B, and 10 μ l purified α -actinin (1.7 mg/ml) or buffer B. The fractions containing zyxin and α -actinin were dialyzed into buffer B before use in the sedimentation assays. The protein mixtures (200 μ l total vol) were mixed for 5 s and were then placed atop a 60 μ l sucrose cushion (30% sucrose in a buffer equivalent to the sample) in an 8 × 20-mm ultraclear centrifuge tube (Beckman Instruments, Inc., Fullerton, CA). The samples were incubated for 30 min at 25°C and were then centrifuged for 10 min at 23 psi using an A-95 rotor in an airfuge (Beckman Instruments, Inc.). After centrifugation, 100 μ l of supernatant was carefully removed and mixed 1:1 with 2 \times Laemmli sample buffer (Laemmli, 1970). The pellet was resuspended in 200 μ l 1 \times Laemmli sample buffer. After boiling, the samples were analyzed by SDS-PAGE and Western immunoblot using anti-zyxin antibody.

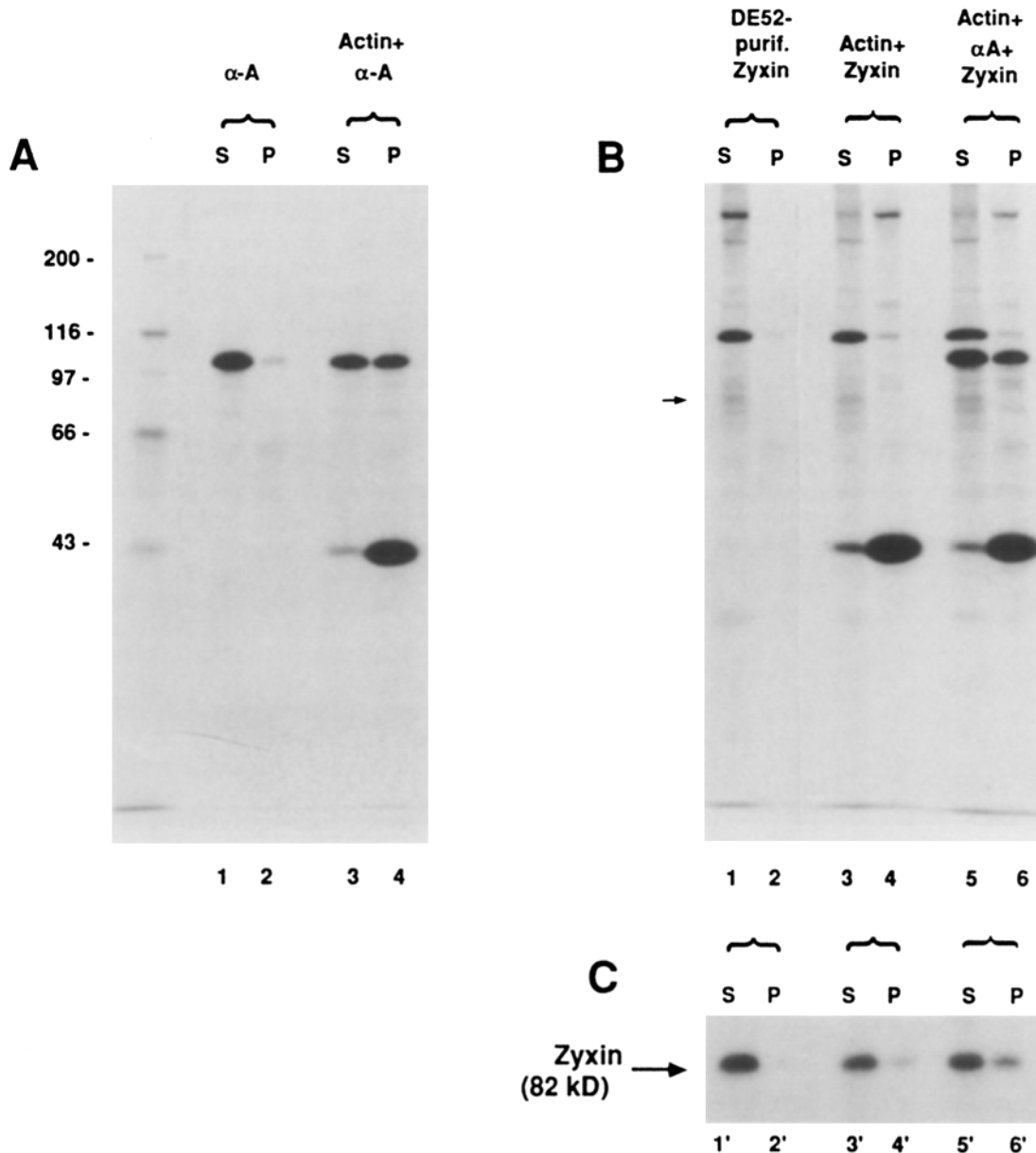


Figure 1. α -Actinin-dependent cosedimentation of zyxin with F-actin. (A) Coomassie blue-stained gel of sedimentation assays with α -actinin and F-actin. Upon centrifugation, purified α -actinin (lanes 1 and 2) remains primarily in the supernatant (S, lane 1); however, when centrifuged in the presence of F-actin (lanes 3 and 4), \sim 50% of the α -actinin is found in the pellet (P, lane 4) with the majority of the F-actin. (B and C) Coomassie blue-stained gel (B) and the 82-kD region of the corresponding Western immunoblot probed with anti-zyxin antibodies (C). The arrow marks the location of zyxin determined from examination of corresponding Western immunoblots; in the Coomassie blue-stained gel shown here, zyxin is not unambiguously detected because of its low abundance and the presence of numerous other polypeptides that migrate with similar apparent molecular mass. (Lanes 1 and 2) When partially purified zyxin is centrifuged, nearly all detectable zyxin is contained in the supernatant fraction (C, lane 1'). (Lanes 3 and 4) When zyxin is centrifuged in the presence of F-actin, the majority of the zyxin is retained in the supernatant fraction (C, lane 3'). (Lanes 5 and 6) Inclusion of purified α -actinin in the sedimentation assay results in a significant enhancement of zyxin accumulation in the pellet with F-actin (C, lane 6'). α -A, α -actinin.

Gel Filtration Binding Studies

Zyxin (225 μ l DE52-zyxin, 60 μ l buffer B, 15 μ l 20 \times KCl-ATP), α -actinin (225 μ l buffer B, 60 μ l α -actinin, 15 μ l 20 \times KCl-ATP), or a mixture of the two protein preparations (225 μ l DE52-zyxin, 60 μ l α -actinin, 15 μ l 20 \times KCl-ATP) was placed into a 8 \times 20 mm ultraclear centrifuge tube and incubated for 30 min at 25 $^{\circ}$ C. The proteins were dialyzed against buffer B before use; 20 \times KCl-ATP = 1 M KCl, 2 mM ATP, 0.4% Na₃. The sam-

ple was precleared by centrifugation in an airfuge (Beckman Instruments, Inc.) for 10 min at 23 psi in an A-95 rotor and a 200- μ l aliquot of the sample was then chromatographed on a Superose 6 HR 10/30 gel filtration column (Pharmacia Fine Chemicals). Gel filtration was performed at 4 $^{\circ}$ C on a Waters 600 HPLC (Millipore Corporation, Bedford, MA). (300- μ l) Fractions were collected and the elution profiles of the proteins were analyzed by SDS-PAGE and Western immunoblot using anti-zyxin antibody.

Blot Overlay Binding Studies

For detection of the binding of radioiodinated probe to proteins immobilized on nitrocellulose, a modification of the procedure developed previously by Otto (1983) was used. Briefly, protein to be overlaid with ^{125}I -labeled probes was separated by SDS-PAGE, transferred to nitrocellulose, and blocked overnight with 2% BSA in 50 mM Tris-HCl, 150 mM NaCl, 0.1% NaN_3 . ^{125}I -labeled protein was used at a concentration of 250,000 cpm/ml of overlay buffer (0.5% BSA, 0.25% gelatin, 1% NP-40, 1 mM EDTA, 0.1% 2-mercaptoethanol, 10 mM NaCl, 20 mM Hepes, pH 7.5). (The inclusion of NP-40 in the overlay buffer significantly reduces the overall background on the resulting autoradiogram but does not affect the specific binding observed.) The nitrocellulose strips were incubated with sufficient radiolabeled probe to cover the strip (~ 10 ml), were placed on an orbital shaker at room temperature for 4 h, and were washed 4×5 min to release nonspecifically bound counts. Autoradiography was performed at -80°C with film (X-OMAT; Eastman Kodak Co., Rochester, NY) with an intensification screen. For control experiments using heat-denatured probe, the radiolabeled protein was heated at $\sim 95^\circ\text{C}$ for 10 min before use. For the gel overlay experiment in which we examined the binding of radioiodinated zyxin to proteins eluting from a DEAE-cellulose column, we prepared a smooth muscle protein extract from chicken gizzard (Crawford and Beckerle, 1991), precipitated a subset of these proteins using 26–34% saturated ammonium sulfate, and fractionated the precipitated proteins on a DEAE-cellulose column eluted with a linear salt gradient (0–370 mM NaCl) in buffer B.

Solid Phase Binding Assay

Purified zyxin or 2% BSA (50 μl) was dispensed into removeable microtiter wells (Dynatech Laboratories, Inc., Chantilly, VA) and the protein was allowed to bind to the microtiter wells for 60 min at 37°C . At least 80% of the zyxin bound to the wells during this period as determined by quantitative, densitometric analysis of stained SDS-polyacrylamide gels. (Because of the low abundance of zyxin, it is not possible to estimate accurately the absolute concentration of zyxin in the purified protein preparation.) The wells were washed briefly ($3 \times$) with Hepes binding buffer (HBB) (20 mM Hepes, pH 7.4, 10 mM NaCl, 0.1 mM EGTA, and 0.1% 2-mercaptoethanol) and were subsequently blocked with 350 μl 2% BSA in HBB. After a 60-min incubation at 37°C , the blocking solution was removed and the wells were washed briefly with HBB containing 0.2% BSA. The wells were next incubated for 2.5 h at 37°C with 10 μl ^{125}I - α -actinin (50,000–100,000 cpm) plus 100 μl competing protein in HBB plus 0.2% BSA. The radiolabeled protein was mixed with the unlabeled protein samples before addition to the microtiter wells. At the end of the incubation period, the radioactive material was removed from the wells and the wells were washed thoroughly (six to seven times) with HBB plus 0.2% BSA with a final rinse in HBB. The wells were air dried and bound counts were determined using a Packard Multi-Prias 1 gamma counter (Packard Instruments Co., Inc., Meriden, CT). For controls, ^{125}I - α -actinin was heat denatured for 10 min at 65°C or 95°C ; these two heat treatment protocols yielded similar results. For these solid phase binding studies, the α -actinin was radioiodinated to a specific activity between 2.0×10^5 and 1.3×10^6 cpm/ μg . Four independent experiments were performed using three different preparations of ^{125}I - α -actinin.

Results

The distribution of zyxin in cultured chicken embryo fibroblasts was examined previously by indirect immunofluorescence, revealing that zyxin is concentrated at areas of membrane-substratum adhesion (Beckerle, 1986; Crawford and Beckerle, 1991). Interestingly, in contrast with many other adhesion plaque components, it was observed that zyxin is not strictly confined to the domain of the adhesion plaque defined by interference reflection microscopy, but is also present along actin filament bundles near where they terminate at sites of membrane-substratum interaction. Because zyxin is colocalized with membrane-associated actin fila-

ments, we examined the possibility that zyxin could interact directly with filamentous actin. We approached this possibility by using a cosedimentation assay that has been used by others to identify proteins that bind F-actin. This assay takes advantage of the fact that F-actin sediments upon centrifugation (Cooper and Pollard, 1982); the sedimentation of actin-binding proteins is dependent on the presence of F-actin. For example, the purified actin-binding protein, α -actinin, fails to sediment to a significant extent upon centrifugation (Fig. 1 A, lanes 1 and 2), but is found in the pellet fraction when incubated with F-actin before centrifugation (Fig. 1 A, lanes 3 and 4). To examine the possibility that zyxin behaves as an actin-binding protein in such a sedimentation assay, we prepared a smooth muscle extract from chicken gizzard and fractionated the extract by anion exchange chromatography to enrich for zyxin (DE52-zyxin; Fig. 1 B, lane 1). Because zyxin is a low abundance protein that is difficult to detect by SDS-PAGE (and consequently is not visible in the Coomassie blue-stained gel in Fig. 1 B), we used Western immunoblot analysis to monitor the behavior of the protein in the sedimentation assays. When DE52-zyxin is centrifuged alone, zyxin is found almost exclusively in the supernatant fraction (Fig. 1, B and C, lanes 1 and 1'). When centrifuged in the presence of filamentous actin, the majority of the zyxin also remains in the supernatant (Fig. 1, B and C, lanes 3 and 3') though a small amount cosediments with the filamentous actin and is recovered in the actin-rich pellet (Fig. 1, B and C, lanes 4 and 4'); the significance (if any) of this limited cosedimentation of zyxin with the actin filaments is not clear at this point. Interestingly, however, when the actin-binding protein, α -actinin, is added to the mixture of actin and DE52-zyxin, a significantly larger proportion of zyxin is contained within the pellet after centrifugation (Fig. 1, B and C, lanes 6 and 6'). The amount of actin in the pellets is not significantly affected by the inclusion of α -actinin in the assay. The increased sedimentation of zyxin with F-actin in the presence of the actin-binding protein, α -actinin, raises the possibility that zyxin is sedimenting with the actin via an association with α -actinin. In control experiments in which zyxin and α -actinin are mixed and centrifuged in the absence of F-actin, very little zyxin or α -actinin is contained in the pelleted material (data not shown). Therefore, F-actin, while not sufficient to support extensive sedimentation of zyxin, is required for the sedimentation of zyxin we observe in the presence of α -actinin.

We explored the possibility that zyxin can interact with α -actinin in the absence of actin by performing gel filtration binding studies as shown in Fig. 2. Zyxin is an asymmetric, monomeric protein having a Stokes radius of 5.6 nm (Crawford and Beckerle, 1991) and it consistently elutes from an HPLC gel filtration column with the profile shown in the Western immunoblot in Fig. 2 A. When preincubated with purified α -actinin before chromatography, the elution profile of zyxin shifts toward the void volume, and a portion of the zyxin coelutes with α -actinin (Fig. 2 B). By this technique, only a fraction of the total zyxin coelutes with α -actinin, although the entire zyxin profile is shifted toward the void volume in the presence of α -actinin. The elution profile of α -actinin alone is shown in the Coomassie blue-stained gel in Fig. 2 C. A variety of other proteins including vinculin, ovalbumin, and IgG (Crawford and Beckerle, 1991; and data not shown) have no effect on the apparent Stokes radius of zyxin.

1. Abbreviation used in this paper: HBB, Hepes binding buffer.

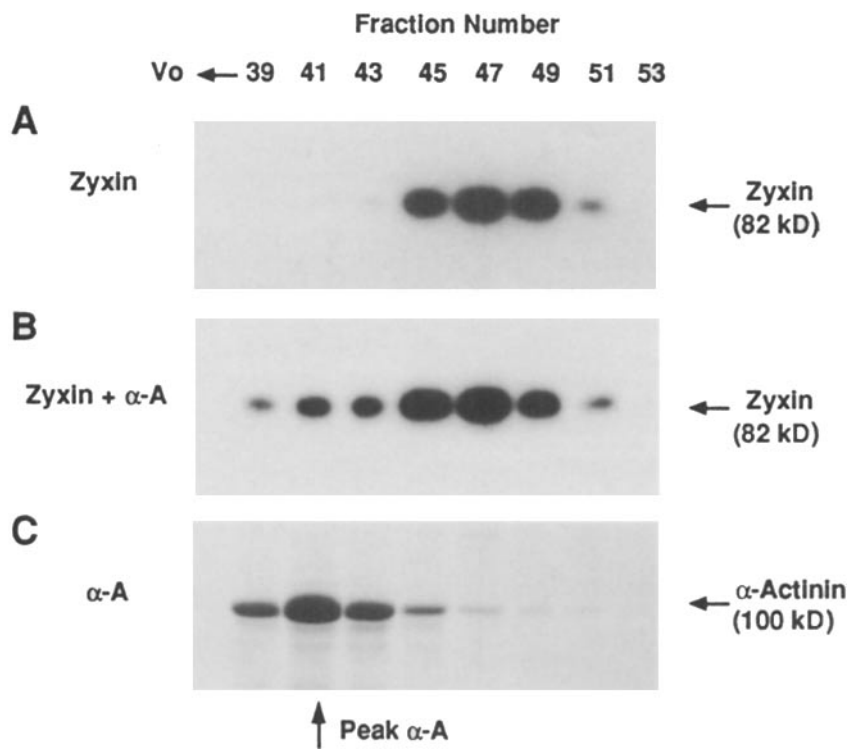


Figure 2. An interaction between zyxin and α -actinin detected by analytical gel filtration. Western immunoblot showing the elution profile of zyxin when chromatographed in the absence (A) and presence (B) of purified α -actinin. In the presence of α -actinin, the elution profile of zyxin is shifted toward the void volume of the column and some zyxin now coelutes from the column with α -actinin. A Coomassie blue-stained gel showing the elution profile of α -actinin is shown in C. α -A, α -actinin.

The result of this study suggests that zyxin can interact directly or indirectly with α -actinin; we were unable to demonstrate a direct interaction between α -actinin and zyxin by this method as it was necessary to use zyxin from an early

stage in its purification (DE52-zyxin) to have sufficient material to detect the protein after gel filtration.

In an effort to examine whether zyxin can interact directly with α -actinin, we used a modification of a sensitive gel-

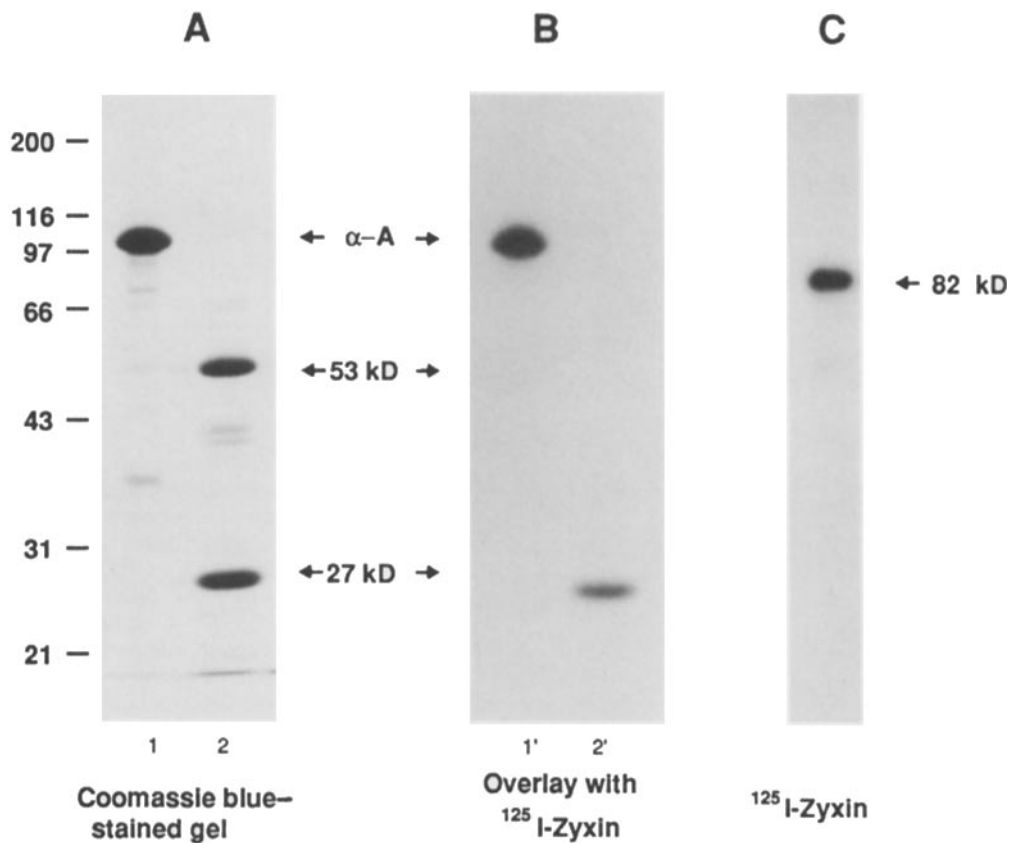


Figure 3. A direct interaction between 125 I-zyxin and α -actinin is observed by a blot overlay assay. (A) A Coomassie blue-stained gel showing purified α -actinin (lane 1) and the 53- and 27-kD proteolytic products of α -actinin generated by thermolysin cleavage (lane 2). Proteins from a parallel gel were transferred to nitrocellulose and the nitrocellulose strip was probed with 125 I-zyxin. The resulting autoradiograph is shown in B and illustrates 125 I-zyxin binding to α -actinin and its 27-kD proteolytic fragment. C is an autoradiogram demonstrating the purity of the radiiodinated zyxin; 750,000 cpm were loaded on the gel and the dried gel was exposed to film for 10 min before development. α -A, α -actinin.

overlay assay that has been described previously (Otto, 1983) and has been used successfully by a number of investigators to study protein-protein interactions (Belkin and Kotliansky, 1987; Burrige and Mangeat, 1984; O'Halloran et al., 1985; Fowler, 1987; Turner et al., 1990). Purified zyxin was radioiodinated and used as a probe to examine its ability to interact with purified α -actinin immobilized on nitrocellulose. As can be seen in Fig. 3 B, lane 1, radioiodinated zyxin interacts with α -actinin purified from avian smooth muscle. α -Actinin can be separated into two major fragments by cleavage with the proteolytic enzyme, thermolysin (Mimura and Asano, 1986): a 27-kD globular fragment which contains the actin-binding domain, and a 53-kD

fragment which is essential for dimerization of the intact molecule (Mimura and Asano, 1987). By this blot overlay approach, zyxin associates exclusively with the 27-kD actin-binding domain of α -actinin, with no detectable radiolabel bound to the 53-kD fragment (Fig. 3 B, lane 2). Heat-denatured, radioiodinated zyxin exhibits a significantly reduced ability to bind to α -actinin and its 27-kD thermolysin cleavage product (data not shown). The purity of the radioiodinated zyxin was determined by SDS-PAGE followed by autoradiography, by which ^{125}I -zyxin appeared to be a homogeneous preparation (Fig. 3 C); the faint signal present at 55 kD is derived from a breakdown product of zyxin.

To examine the specificity of the interaction between zyxin

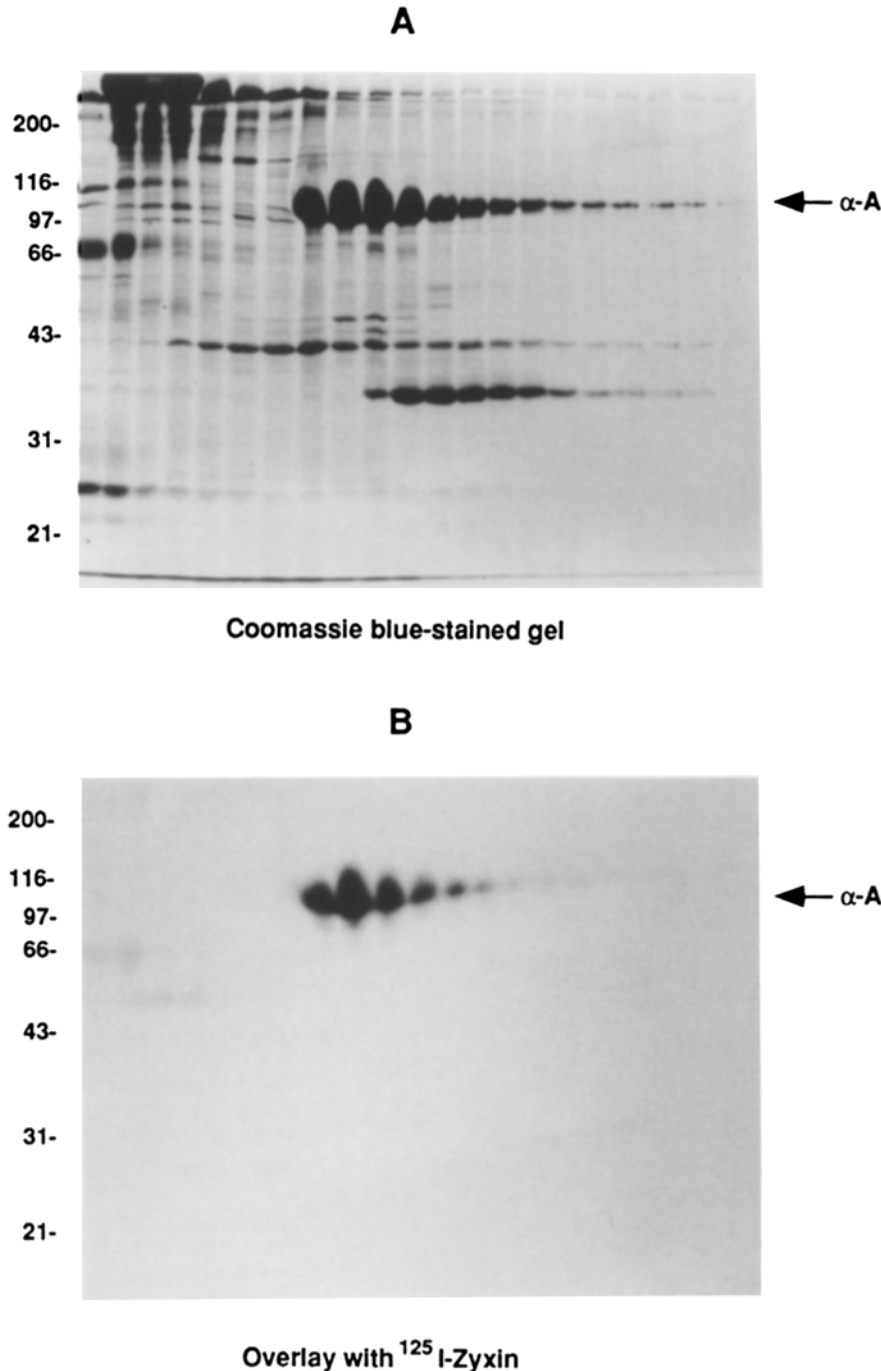


Figure 4. Characterization of the specificity of the interaction between ^{125}I -zyxin and α -actinin by blot overlay. *A* shows a Coomassie blue-stained gel of a subset of proteins extracted from avian smooth muscle and fractionated by chromatography on DEAE-cellulose. The position of α -actinin is marked by the arrow. The corresponding blot overlay probed with ^{125}I -zyxin is shown in *B*. The radioiodinated probe recognizes α -actinin most prominently among this complex mixture of proteins. Note that a number of other abundant proteins, including filamin (250,000 M_r), fail to bind radioiodinated zyxin. α -A, α -actinin.

and α -actinin, we used radioiodinated zyxin to probe a more complex mixture of proteins. Fig. 4 *A* shows a Coomassie blue-stained gel of proteins extracted from avian smooth muscle and fractionated by ammonium sulfate precipitation and chromatography on DEAE-cellulose. A large number of proteins associated with the actin cytoskeleton, including α -actinin, vinculin, filamin, talin, and caldesmon, are represented in the elution profile. Proteins from a parallel gel were transferred to nitrocellulose and incubated with radioiodinated zyxin. As can be seen in Fig. 4 *B*, the radiolabeled zyxin recognizes α -actinin most prominently among this complex mixture of proteins and is observed to bind α -actinin over its entire elution profile. The gel and corresponding blot shown in Fig. 4 were heavily loaded to enable detection of potential zyxin-binding proteins that are present at relatively low levels. Although the major zyxin-binding protein detected by this method is α -actinin, other proteins that associate with radiolabeled zyxin are also visible in the autoradiogram. For example, in the subset of smooth muscle proteins examined here, we observe some association of zyxin with polypeptides of \sim 50–55 and 65–70 kD. These proteins may be bonafide zyxin-binding proteins that are less readily detected because they are present at lower abundance in these cell extracts than is α -actinin.

We have also performed the reciprocal blot overlay experiment to examine the ability of radioiodinated α -actinin to in-

teract with purified zyxin. As shown in Fig. 5, purified zyxin is recognized by radioiodinated α -actinin (Fig. 5 *B*) but not by heat-denatured, radioiodinated α -actinin (Fig. 5 *C*). The purity of the radioiodinated α -actinin was confirmed by SDS-PAGE followed by autoradiography (Fig. 5 *D*). In previously published work, ^{125}I -labeled α -actinin has been used to probe human platelet extract by a similar blot-overlay method (Belkin and Koteliansky, 1987) and no interaction with an 82-kD cytoskeletal protein was detected by this analysis. This lack of interaction may be accounted for by the low abundance of zyxin relative to other proteins that interact with ^{125}I -labeled α -actinin or, alternatively, zyxin may not have been present in the cytoskeletal preparations used by these investigators. We have also failed to detect a significant signal at zyxin's molecular mass when we have probed total chicken embryo fibroblast protein with radiolabeled α -actinin (data not shown). We suspect that this is because of the relatively low levels of zyxin in cells since we do observe a specific interaction when more highly concentrated zyxin preparations are probed. The blot overlay assay, while a very powerful method for examining interactions between relatively abundant or purified proteins, may not be as useful for detecting specific interactions involving proteins present at low levels.

The affinity of the association between α -actinin and zyxin was characterized by solid phase binding assays. Purified

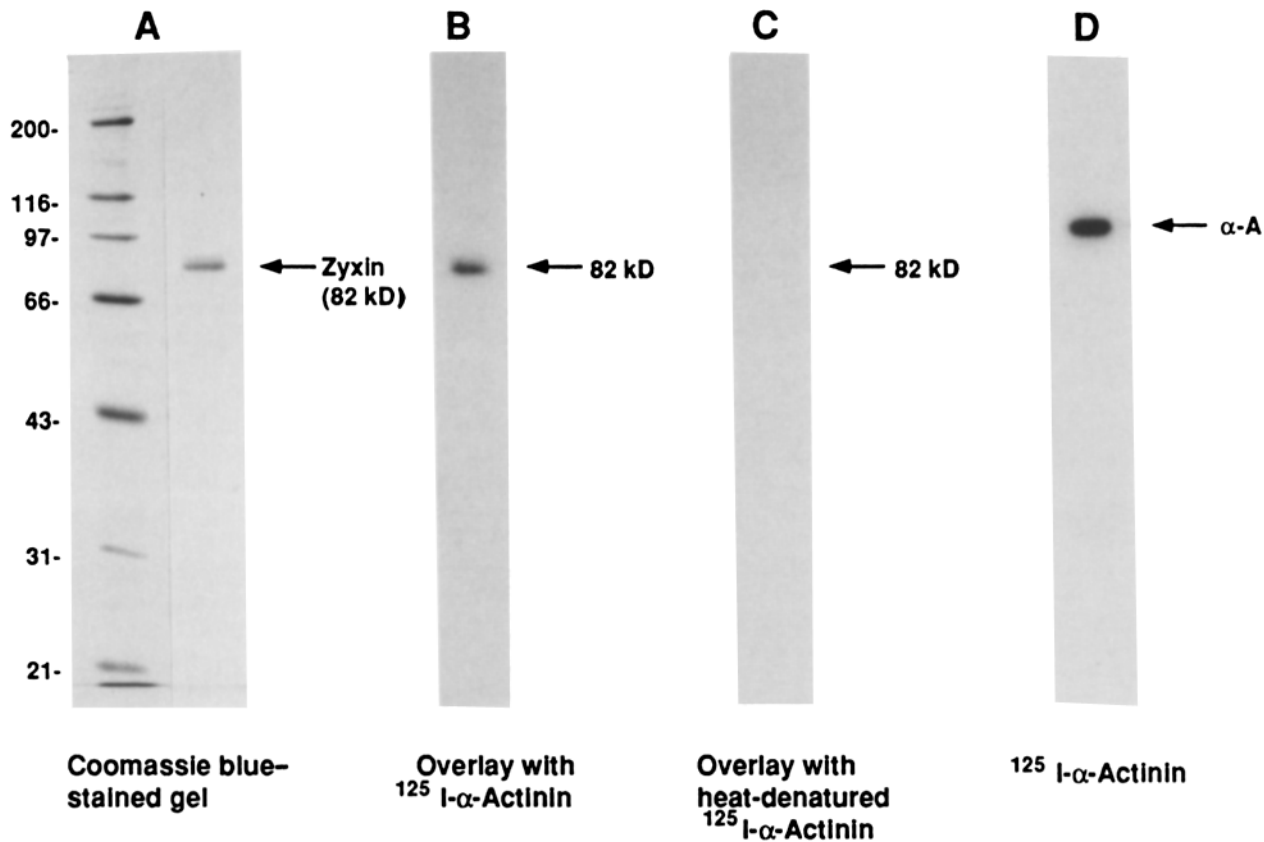


Figure 5. ^{125}I - α -actinin binds zyxin. (A) Coomassie blue-stained gel showing molecular mass markers and purified zyxin. (B) ^{125}I - α -actinin interacts with purified zyxin in a blot overlay assay. (C) Heat-denatured ^{125}I - α -actinin fails to bind zyxin in the blot overlay assay. (D) Autoradiogram illustrating the purity of the radioiodinated α -actinin; 250,000 cpm were loaded and the dried gel was exposed to film for 30 min before development. α -A, α -actinin.

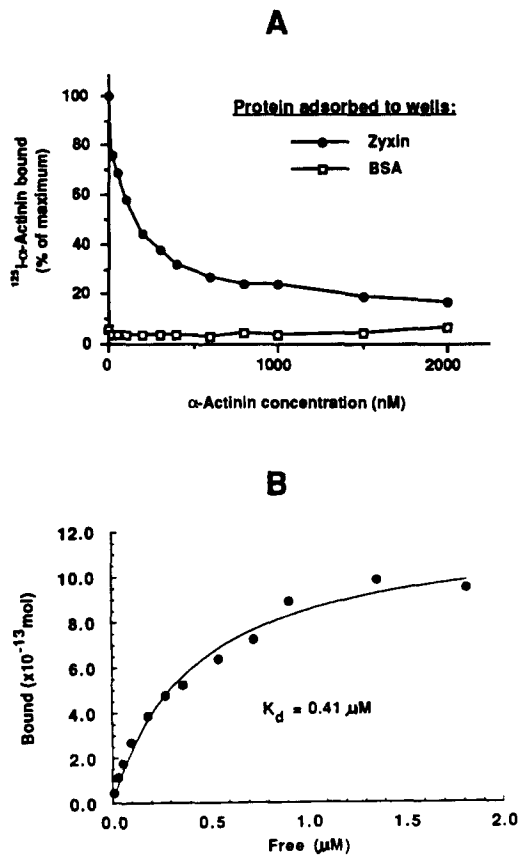


Figure 6. Binding of α -actinin to zyxin in a solid phase assay. (A) Binding of ^{125}I - α -actinin to microtiter wells coated with zyxin (\bullet) or BSA (\square) in the presence of increasing amounts of unlabeled α -actinin. The data are expressed as a percentage of the maximum counts bound. Each point represents the average value obtained from two duplicate trials within a single experiment. (B) The data from the experiment in Fig. 6 A were adjusted by subtracting the nonspecific counts bound to BSA from the total counts bound to zyxin and the moles of α -actinin bound to zyxin were plotted as a function of the free α -actinin concentration (μM). Our experimental data fit closely ($r^2 = 0.99$) the predicted curve (solid line) derived from the equation for a single site interaction between two proteins: $y = (y_{\text{max}} * x) / (K_d + x)$ where y = the moles of α -actinin bound, y_{max} = the maximum number of moles of α -actinin bound (total sites), x = the concentration of unbound (free) α -actinin (μM), and K_d = the dissociation constant (μM). In this particular experiment, the α -actinin was radioiodinated to a specific activity of 4.1×10^5 cpm/ μg and 3,569 cpm were bound specifically to zyxin when no competing unlabeled α -actinin was added. Assuming a molecular weight of 200,000 g/mol for α -actinin, the extrapolated value for $y_{\text{max}} = 12 \times 10^{-13}$ mol α -actinin and the resulting apparent $K_d = 0.41 \mu\text{M}$. As noted in the text, the mean K_d determined from four independent experiments is $1.07 \pm 0.32 \mu\text{M}$ (mean \pm SEM). The stoichiometry of binding cannot be estimated using this assay since it is not possible to determine accurately the number of functional zyxin molecules adsorbed to the wells.

zyxin was adsorbed to microtiter wells, unoccupied sites on the plastic dishes were blocked with BSA, and the immobilized protein was incubated with a constant amount of radioiodinated α -actinin in the presence of increasing amounts of unlabeled α -actinin. By this approach, we detected an association between zyxin and ^{125}I - α -actinin that was com-

petitively inhibited by unlabeled α -actinin (Fig. 6 A). Radioiodinated α -actinin failed to associate with control protein (BSA) under the same conditions (Fig. 6 A). Moreover, heat-denatured, radioiodinated α -actinin did not exhibit specific binding to zyxin in this assay (Fig. 7 A), an indication that an intact α -actinin tertiary structure is required for the zyxin- α -actinin interaction. Plotting the moles of α -actinin bound to zyxin vs. the concentration of free α -actinin (Fig. 6 B), we obtain the typical hyperbolic curve expected for a simple binding reaction: $Z + \alpha\text{-A} \leftrightarrow Z \cdot \alpha\text{-A}$. Our data for the zyxin- α -actinin interaction fit very closely the curve predicted from the binding equation that describes the interaction between two proteins at a single binding site (see legend, Fig. 6 B). Half-maximal binding in this particular experiment occurs at $0.41 \mu\text{M}$ free ligand. We obtained an average K_d of $1.07 \pm 0.32 \mu\text{M}$ (mean \pm SEM) from analysis of the results of four independent experiments. We are unable to estimate the number of binding sites per molecule because we do not know the fraction of zyxin molecules that adsorb to microtiter wells in a conformation that allows them to bind α -actinin.

The specificity of the interaction between zyxin and α -actinin was examined further by determining whether a number of other proteins were able to compete with radioiodinated α -actinin for binding to zyxin in solid phase binding assays (Fig. 7 B). Specifically, we compared the ability of equivalent molar amounts of vinculin, ovalbumin, α -actinin, and the 53- and 27-kD thermolysin cleavage products of α -actinin to compete with radioiodinated α -actinin for binding to zyxin. Only intact α -actinin successfully competed with radioiodinated α -actinin for zyxin binding. The fact that a number of other proteins fail to compete illustrates the specificity of this interaction. Somewhat surprisingly to us, the 27-kD thermolysin-generated cleavage product of α -actinin failed to compete with intact radioiodinated α -actinin for binding to zyxin, although we did observe an association between zyxin and the 27-kD fragment of α -actinin by blot overlay methods. It may be that the specific zyxin-binding site is masked upon proteolytic cleavage of native α -actinin and revealed once again when more denaturing procedures, such as the blot overlay methods, are used. Alternatively, intact zyxin may exhibit a significantly higher affinity for α -actinin than for its 27-kD fragment and consequently, the protein fragment may be ineffective at competing with intact α -actinin for zyxin binding. It is intriguing that in the presence of vinculin, the binding of α -actinin to zyxin appears to be slightly enhanced. We have not observed any direct interaction between zyxin and vinculin that might contribute to this result; however, α -actinin has been reported to associate with vinculin (Belkin and Kotliansky, 1987; Wachsstock et al., 1987), and it is therefore possible that vinculin binding to α -actinin might facilitate the α -actinin-zyxin interaction. Further experiments are required to resolve whether or not this is the case.

By a variety of biochemical approaches, we have demonstrated a specific, saturable, and moderate-affinity interaction between zyxin and α -actinin in vitro. If zyxin and α -actinin associate with each other in vivo as well, one might expect them to be colocalized in cells. To examine this possibility, we used double-label indirect immunofluorescence to compare the subcellular distributions of zyxin and α -actinin. As we reported previously, in chicken embryo fibroblasts

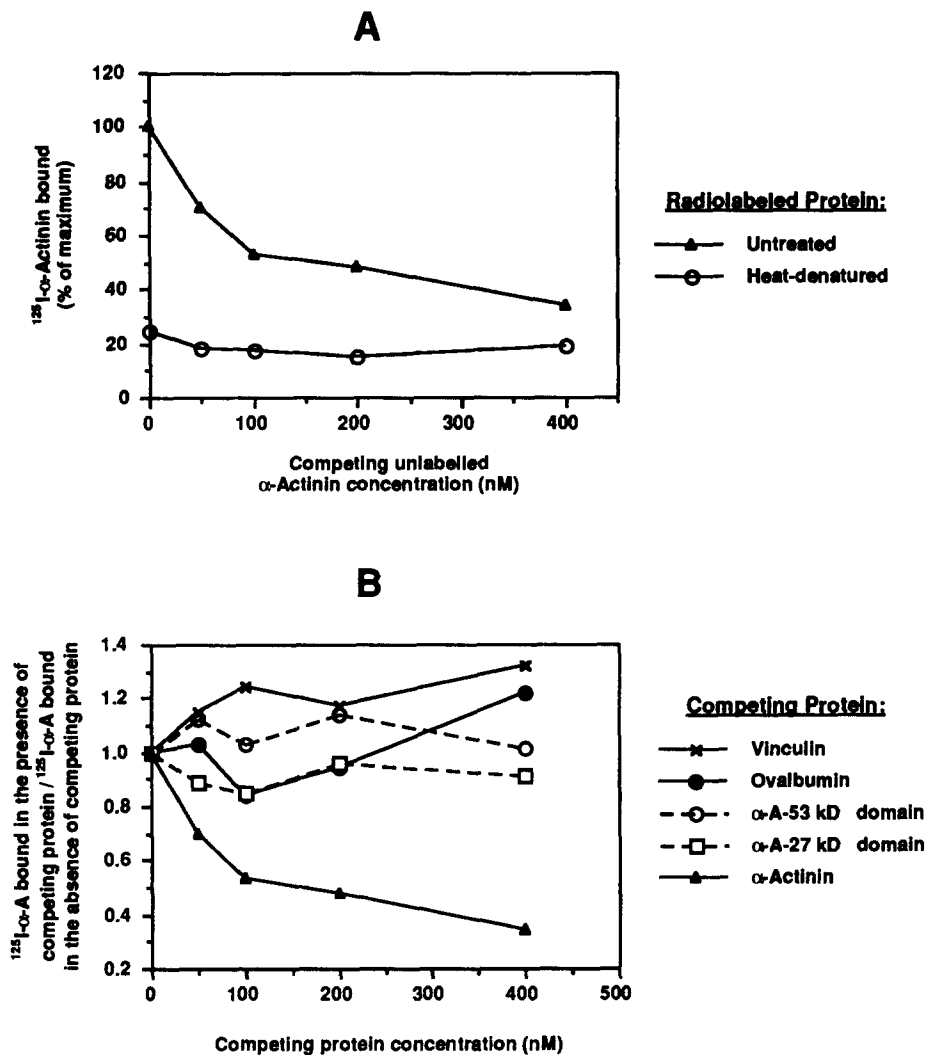


Figure 7. Specificity of the association between zyxin and α -actinin. (A) Comparison of the binding of untreated (\blacktriangle) and heat-denatured ^{125}I - α -actinin (\circ) to zyxin immobilized on microtiter wells. The data are expressed as a percentage of the maximal binding obtained. (B) Binding of ^{125}I - α -actinin to immobilized zyxin in the presence of increasing amounts of competing protein. Only intact α -actinin effectively competes with radioiodinated α -actinin for zyxin binding. The data are expressed as a ratio of the specific counts bound at each concentration of competing protein to the specific counts bound in the absence of competing protein. Each point represents the average value obtained from duplicate wells in multiple experiments. α -Actinin: $n = 4$; ovalbumin: $n = 3$; 55-kD domain: $n = 4$; 27-kD domain: $n = 5$; vinculin: $n = 4$. α -A, α -actinin.

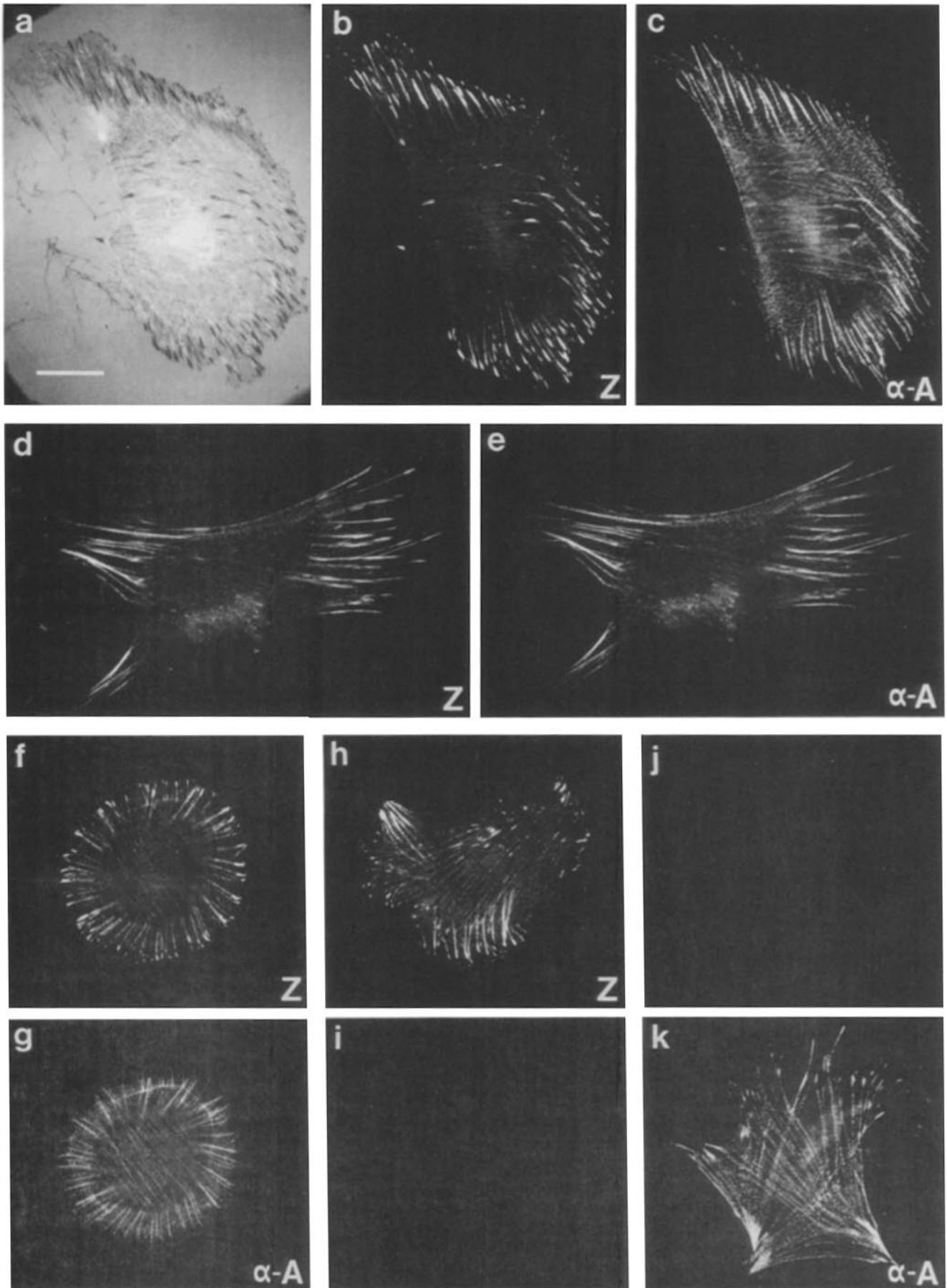
zyxin is typically localized at focal contacts as well as along actin filaments that associate with the membrane at sites of adhesion (Fig. 8 b); in some cells a faint periodic staining along stress fibers is also observed (see for example Fig. 8 h). α -Actinin is also found distinctly at some adhesion plaques (Fig. 8 c) but is more prominently concentrated at the ends of F-actin bundles where they attach at the membrane. In addition we observe α -actinin in a periodic distribution along actin filaments as has been reported previously (Lazarides and Burridge, 1975). There is a striking overlap in the subcellular distribution of zyxin and α -actinin in fibroblasts as can be seen in the representative selection of cells shown in Fig. 8. Interestingly, however, in many cases zyxin appears to be more prominent in the focal contact proper, whereas α -actinin staining is most striking along the actin filament bundles proximal to the adhesion plaques and extends further from these sites. From our examination of a large number of double-labeled chicken embryo fibroblasts, we conclude that the distributions of zyxin and α -actinin are substantially overlapping; however, they are not strictly identical by this technique. Control experiments illustrate that there is no transmission of the rhodamine signal in the fluorescein channel (Fig. 8, h and i) or vice versa (Fig. 8,

j and k). Furthermore our fluorochrome-labeled secondary antibodies exhibited no cross-species reactivity (data not shown).

We have also examined the extent of zyxin and α -actinin codistribution in pigmented retinal epithelial cells using confocal microscopy to compare precisely the relationship between the location of the two proteins. By this technique we find that zyxin and α -actinin are both present in a circumferential ring in an optical section that corresponds to the apical portion of the cell (Fig. 9). α -Actinin has previously been shown to be a component of the zonulae adherens of epithelial cells (Geiger et al., 1979). The demonstration of colocalization of zyxin with α -actinin at sites of cell-cell contact suggests that zyxin is a component of the zonulae adherens as well, though we have not yet demonstrated this conclusively by electron microscopic studies.

Discussion

In this paper, we report the results of solution and solid phase binding studies that demonstrate an interaction between zyxin and α -actinin *in vitro*. We have used four types of binding assays to demonstrate and characterize the association



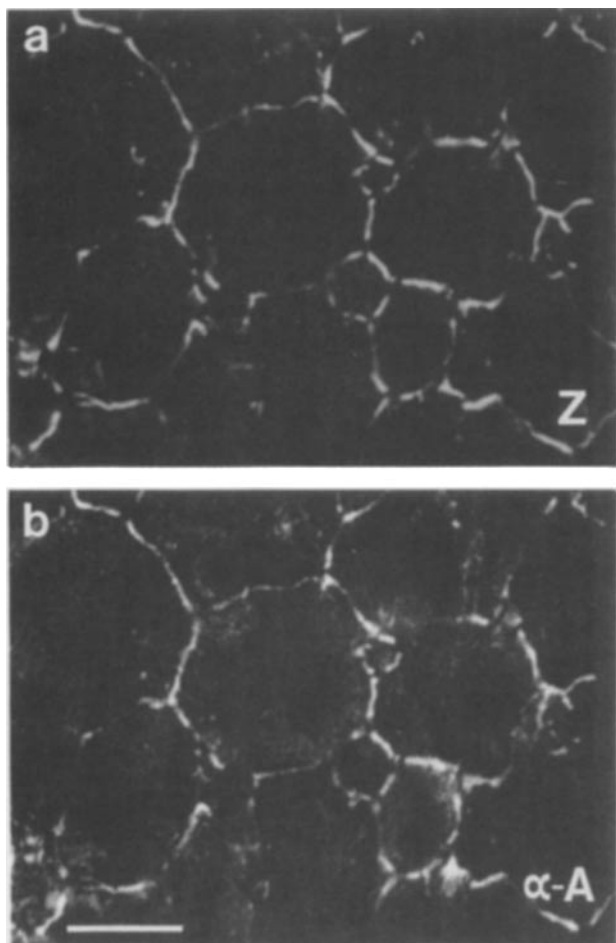


Figure 9. Zyxin and α -actinin are colocalized at sites of cell-cell adhesion in pigmented retinal epithelial cells. Confocal microscopy reveals that zyxin (a) and α -actinin (b) are colocalized at the apical junctional complexes of epithelial cells. Bar, 20 μ m.

between zyxin and α -actinin: an F-actin-based sedimentation assay, a gel filtration approach, blot overlay experiments, and a microtiter plate solid phase binding assay. By these approaches we have shown a direct and specific interaction between zyxin and α -actinin. The binding appears to occur at a single site with a K_d of 1.07 μ M. In addition, we have demonstrated that zyxin and α -actinin exhibit overlapping subcellular distributions *in vivo*: both proteins are found at cell-substratum adherens junctions and along stress fibers in fibroblasts and at cell-cell adherens junctions in retinal epithelial cells. Cumulatively, our findings from *in vitro* biochemical studies and immunocytochemistry suggest that the zyxin- α -actinin interaction at the membrane-cytoskeleton interface is biologically meaningful, although the physiological function of the zyxin- α -actinin complex remains to be determined.

The association between zyxin and α -actinin that we observe is specific based on a number of criteria. First, we have shown that unlabeled α -actinin competes effectively with radioiodinated α -actinin for zyxin binding whereas a variety of other proteins fail to prohibit the zyxin- α -actinin interaction. Second, if either zyxin or α -actinin is heat denatured before use in a binding study, we observe an extensive reduction in the interaction between the two proteins. Third, when radioiodinated zyxin is used to probe a blot containing a large number of smooth muscle proteins, α -actinin most prominently binds zyxin whereas a number of other abundant proteins present on the blot fail to interact with the radiolabeled probe.

We have demonstrated that the association between zyxin and α -actinin is direct and does not require any accessory proteins and that it is a saturable interaction. From our solid phase binding studies, we estimate a K_d of ~ 1 μ M for the zyxin- α -actinin interaction. The fraction of zyxin bound to α -actinin $\{[Z \cdot \alpha-A]/([Z] + [Z \cdot \alpha-A])\}$ would equal 1/2 when $[Z] = [Z \cdot \alpha-A]$. Based on our estimated K_d for the zyxin- α -actinin interaction $\{K_d = ([Z][\alpha-A])/[Z \cdot \alpha-A] = 1$ μ M $\}$, we would expect 50% of the available zyxin to be bound to α -actinin when the free α -actinin concentration ($[\alpha-A]$) equals 1 μ M. The actual concentration of α -actinin in fibroblasts has been estimated to be between 1.5 and 7.5 μ M (Pavalko and Burrige, 1991.) Consequently, although zyxin does not bind α -actinin with particularly high affinity, if we assume that the two proteins are available to interact freely with each other *in vivo*, a significant proportion of the cellular zyxin would be expected to be bound to α -actinin since α -actinin is present *in vivo* at concentrations equal to or greater than the K_d .

As pointed out previously (Crawford and Beckerle, 1991), all evidence gathered to date suggests that zyxin is present in cells at substoichiometric levels with respect to α -actinin. The relative abundance of the two proteins constrains how we can envision the interaction of the proteins *in vivo*. For example, it is clear that only a subset of the α -actinin molecules present in a cell will be associated with zyxin at any given point in time. Thus, if stoichiometric binding of zyxin to α -actinin affected the interaction between actin and α -actinin, for example, zyxin could simultaneously affect only a fraction of the α -actinin molecules in the cytoplasm. One could envision a more wide-reaching effect of zyxin on α -actinin function if zyxin were found to have some catalytic activity; however, to date we have not detected any enzymatic activity associated with zyxin. Nevertheless, the fact that the level of zyxin in cells appears significantly lower than the amount of a number of presumed structural components of adhesion plaques, including talin, vinculin, or α -actinin, raises the possibility that zyxin has a regulatory function.

We have shown here that α -actinin and zyxin are colocalized at both cell-cell and cell-substratum adherens junctional complexes. Although the distributions of zyxin and α -actinin in cells are significantly overlapping, at the light

Figure 8. Comparison of the distribution of zyxin and α -actinin in chicken embryo fibroblasts by double-label immunofluorescence. Interference reflection microscopy (a) reveals the location of focal contacts which appear black and are labeled with anti-zyxin (b) and anti- α -actinin (c) antibodies. There is also substantial overlap in the distribution of zyxin and α -actinin along the stress fibers (compare d with e and f with g). Control experiments illustrate that no fluorescein signal derived from the anti-zyxin-labeled cell in h is detected in the rhodamine channel (i), or vice versa (j and k). Bar, 20 μ m.

microscopic level at least, it appears that the two proteins also exhibit some differences in their relative local concentrations. For example, while both zyxin and α -actinin are present in focal contacts as well as along stress fibers, zyxin staining is relatively stronger at the adhesion plaques whereas α -actinin staining is more pronounced along the actin filaments than it is at focal contacts. By indirect immunofluorescence, zyxin staining at the adhesion plaque appears to extend beyond the region where α -actinin is detected. The differences in the staining of α -actinin and zyxin at adhesion plaques may simply reflect the degree of antigen accessibility at these sites. An alternative interpretation of this staining pattern is that zyxin is truly capable of associating with regions of adhesion plaques that lack detectable α -actinin, an observation that implies that zyxin is bound to another adhesion plaque protein. Indeed, we have identified other zyxin-binding proteins in smooth muscle cell extracts using the blot overlay approach. We are currently developing procedures for isolating these proteins and are particularly interested in whether these putative zyxin-binding proteins also reside at sites of actin-membrane interaction.

The recently deduced amino acid sequence of α -actinin (Noegel et al., 1987; Baron et al., 1987) reveals that three distinct domains exist within the molecule: an NH₂-terminal actin-binding domain, a central region containing four repeating units apparently involved in dimerization of the molecule (Mimura and Asano, 1987), and a COOH-terminal region composed of two EF-hand motifs which may coordinate Ca²⁺ binding by some α -actinin isoforms (Noegel et al., 1987; Baron et al., 1987). Thus far, α -actinin has been shown to interact with four proteins associated with adhesion plaques: integrin, vinculin, zyxin, and actin. Binding studies using the thermolysin cleavage products of α -actinin have aided in clarifying the gross domains of the α -actinin molecule with which these adhesion plaque proteins interact. For example, the 53-kD rodlike region containing the central repeats associates with integrin (Otey et al., 1990), while the 27-kD NH₂-terminal thermolysin fragment of α -actinin binds vinculin (Pavalko and Burridge, 1991), zyxin (shown here), and actin (Mimura and Asano, 1986). The 27-kD domain of α -actinin contains a short sequence (27 amino acids in length) that is conserved in a number of actin-binding proteins and has been shown to be essential for the interaction between the *Dictyostelium* actin cross-linking protein, ABP-120, and actin (Bresnick et al., 1990). Although more detailed analysis is required to define the zyxin-binding sequence on the 27-kD fragment of α -actinin, it is likely that zyxin recognizes some sequence other than the conserved actin-binding motif since we did not observe an association between zyxin and filamin, a protein that also exhibits this sequence motif. It is interesting to note that recent work (Pavalko and Burridge, 1991) has shown that microinjection of a molar excess of the 27-kD thermolysin cleavage product of α -actinin into fibroblasts causes disruption of stress fibers; this effect may be mediated, at least in part, by futile binding of endogenous zyxin to the 27-kD fragment of α -actinin, leading ultimately to a loss of zyxin or zyxin- α -actinin function.

α -Actinin is present in many nonmuscle cells and has been postulated to play an integral role in actin organization and membrane attachment. Interestingly, however, recent genetic and molecular genetic data have suggested that cells

can perform many actin-dependent functions in the absence of α -actinin. For example, it was recently demonstrated that *Dictyostelium* cells that express defective α -actinin gene products exhibit unimpaired chemotaxis and motility and exhibit normal changes in cell shape (Schleicher et al., 1988). In addition, although *Drosophila* α -actinin is essential late in development, it does not appear to be necessary during early embryogenesis (Fyrberg et al., 1990). These findings have prompted the suggestion that the function of α -actinin can be replaced by other α -actinin-like proteins (Schleicher et al., 1988; Fyrberg et al., 1990). α -Actinin has significant homology with spectrin (Wasenius et al., 1989) and dystrophin (Hammonds, 1987; Davison and Critchley, 1988; Koenig et al., 1988), proteins which are now considered to be members of the α -actinin superfamily. Given the significant sequence similarities between α -actinin, dystrophin, and spectrin, it will be important to address whether zyxin interacts with other members of the α -actinin superfamily.

In conclusion, we have demonstrated an interaction between the 82-kD adherens junction protein, zyxin, and the actin-binding protein, α -actinin. Adherens junctions are sites of membrane-cytoskeletal interaction and cell adhesion that are dynamic, physiologically responsive structures. The organization and elaboration of adherens junctions has been postulated to be important in morphogenesis, control of cell growth and differentiation, and signal transduction in response to extracellular ligand binding. Although the results of many experiments illustrate the physiological importance of these sites of transmembrane connection between the extracellular matrix and the cytoskeleton, the molecular organization and regulation of these assemblages is only now being clarified. The results presented here demonstrate a new protein-protein interaction at adherens junctions. The reported association between zyxin and α -actinin may be important for the localization of zyxin within cells or may contribute to the biological activities of the proteins in vivo.

The authors thank Dr. S. J. Singer for generously providing the anti- α -actinin antiserum, Ms. Sarah Locke for expert technical assistance with the immunocytochemistry, and members of our lab for critical reading of the manuscript. We are also grateful to David Goldenberg, Vann Bennett, Velia Fowler, and members of Keith Burridge's lab for many helpful discussions.

This work was supported by grants from the National Science Foundation and the American Heart Association. M. C. Beckerle is an Established Investigator of the American Heart Association.

Received for publication 28 August 1991 and in revised form 7 November 1991.

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