

An Interaction between α -Actinin and the β_1 Integrin Subunit In Vitro

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Abstract. A number of cytoskeletal-associated proteins that are concentrated in focal contacts, namely α -actinin, vinculin, talin, and integrin, have been shown to interact in vitro such that they suggest a potential link between actin filaments and the membrane. Because some of these interactions are of low affinity, we suspect that additional linkages also exist. Therefore, we have used a synthetic peptide corresponding to the cytoplasmic domain of β_1 integrin and affinity chromatography to identify additional integrin-binding proteins. Here we report our finding of an interaction between the cytoplasmic domain of β_1 integrin and the actin-binding protein α -actinin. β_1 -integrin cytoplasmic domain peptide columns bound several proteins from Triton extracts of chicken embryo

fibroblasts. One protein at ~ 100 kD was identified by immunoblot analysis as α -actinin. Solid phase binding assays indicated that α -actinin bound specifically and directly to the β_1 peptide with relatively high affinity. Using purified heterodimeric chicken smooth muscle integrin (a β_1 integrin) or the platelet integrin glycoprotein IIb/IIIa complex (a β_3 integrin), binding of α -actinin was also observed in similar solid phase assays, albeit with a lower affinity than was seen using the β_1 peptide. α -Actinin also bound specifically to phospholipid vesicles into which glycoprotein IIb/IIIa had been incorporated. These results lead us to suggest that this integrin- α -actinin linkage may contribute to the attachment of actin filaments to the membrane in certain locations.

IN many cell types, the adhesion of the cell to the extracellular matrix (ECM)¹ and the attachment of the actin cytoskeleton to the cell membrane are both mediated through transmembrane proteins that act as receptors for the ECM. These proteins, known as integrins, are heterodimers composed of α and β subunits, many of which have been cloned and sequenced (Buck and Horwitz, 1987; Hynes, 1987; Ruoslahti and Pierschbacher, 1987). Based on integrin sequence data, the β subunits from diverse cell types can be divided into at least five groups (β_1 , β_2 , β_3 , β_4 , β_5). These β chains are very similar to one another as well as being highly conserved among different species. The integrin α chains are more variable, however, so that ECM-binding diversity can be generated through the association of different α subunits with a common β subunit.

One integrin that has been especially well-characterized is the fibronectin receptor ($\alpha_5\beta_1$). The β_1 subunit of this fibronectin receptor is shared by the receptors for laminin (Gehlsen et al., 1988; Sonnenberg et al., 1988; Ignatius and Reichart, 1988) and collagen (Kunicki et al., 1988; Wayner et al., 1988), but the α chain is unique to the fibronectin receptor. As with all integrins, each fibronectin receptor subunit possesses a large extracellular domain, a short trans-

membrane segment, and a short cytoplasmic domain. Much attention has been focused on the extracellular domains and on their binding to the ECM, which has been characterized in some detail. Although the cytoplasmic segments of the integrin subunits are known to be highly conserved, relatively little is known about the role of the integrin cytoplasmic domains in attaching actin to the cell membrane. One such site of attachment for actin filaments to the membrane occurs at focal contacts, which are sites of adhesion with the ECM.

Much of our current understanding of how microfilaments attach to focal contacts has been derived from in vitro binding studies of purified proteins. These studies have suggested a possible interaction between α -actinin, an actin-binding protein, and vinculin (Wachsstock et al., 1987). Vinculin has been shown to bind talin (Otto, 1983; Wilkins et al., 1983; Burridge and Mangeat, 1984) and an interaction between talin and integrin has also been demonstrated (Horwitz et al., 1986), forming a potential protein-protein chain from the microfilament bundles to the membrane. These interactions have provided the basis for a working model of focal contact structure; however, at least two of these associations are of low affinity, suggesting that other proteins may be involved in stabilizing this complex in vivo, or that other linkages between actin and the membrane may exist (Burridge et al., 1988).

We are interested in identifying cytoplasmic proteins that

1. *Abbreviations used in this paper:* CEF, chick embryo fibroblast; ECM, extracellular matrix; GP IIb/IIIa, glycoprotein IIb/IIIa; WGA, wheat germ agglutinin.

might be involved in attaching the actin cytoskeleton to the membrane. To this end, we have made use of published integrin sequences to produce a synthetic peptide corresponding to the cytoplasmic domain of the β_1 integrin subunit (Argaves et al., 1987; Tamkun et al., 1986). This peptide was used in affinity chromatography experiments to identify cytoplasmic fibroblast proteins that bind to integrin. Here, we report an *in vitro* interaction between the β_1 integrin cytoplasmic domain and α -actinin, a widely distributed actin-binding protein.

Materials and Methods

Cell Culture

Primary chick embryo fibroblast (CEF) cultures were prepared by trypsinization of skin from 8–10-d-old embryos. Cells were grown in DME (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS (Gibco Laboratories), 50 U/ml penicillin, and 50 U/ml streptomycin. For experiments using ^{35}S -labeled cells, the fibroblasts were grown overnight in methionine-free MEM (Gibco Laboratories) supplemented with 10% FCS and 10% DME containing 1 mCi ^{35}S -methionine (ICN Biomedicals Inc., Costa Mesa, CA).

Affinity Chromatography

A peptide corresponding to residues 752–798 of the β_1 integrin subunit (Tamkun et al., 1986; Argaves et al., 1987) was synthesized for us by the Protein Chemistry Facility of University of North Carolina at Chapel Hill–National Institute of Environmental Health and Safety (University of North Carolina, Chapel Hill, NC), with the sequence $\text{NH}_2\text{-Cys-Lys-Leu-Leu-Met-Ile-Ile-His-Asp-Arg-Arg-Glu-Phe-Ala-Lys-Phe-Glu-Lys-Lys-Lys-Met-Asn-Ala-Lys-Trp-Asp-Thr-Gly-Glu-Asn-Pro-Ile-Tyr-Lys-Ser-Ala-Val-Thr-Thr-Val-Val-Asn-Pro-Lys-Tyr-Glu-Gly-Lys-COOH}$. An additional NH_2 -terminal cysteine (not part of the sequence of β_1 integrin) was included in the synthetic peptide for use in coupling with an appropriate orientation. Although it has not been resolved whether the site of exit from the membrane is at the lysine (752) or the histidine (758), we chose to use this 47 amino acid peptide to represent the cytoplasmic domain of β_1 integrin. The β_1 cytoplasmic domain peptide was purified using reverse-phase HPLC, and subjected to amino acid analysis and partial sequence analysis to confirm that it contained the correct sequence. Purified peptide was coupled to thiopropyl sepharose 6B (Pharmacia Fine Chemicals, Piscataway, NJ) at a concentration of 6 mg peptide per ml of resin.

Cultured CEFs were extracted in 5 ml of 1% Triton X-100 in column wash buffer (50 mM Tris acetate, 50 mM NaCl, 10 mM EGTA, and 2 mM MgCl_2 , pH 7.6) containing 125 $\mu\text{g/ml}$ aprotinin and leupeptin, and the extract was centrifuged at 100,000 g at 4°C for 1 h. The CEF extract was preabsorbed on a sepharose 6B column, then mixed with the peptide affinity resin for 20 min at 4°C, washed batchwise five times with 10 vol of wash buffer, and eluted three times with 2 vol of 300 mM NaCl in wash buffer.

Immunoblot Analysis

Eluates from the peptide affinity column were dialyzed in 50 mM ammonium bicarbonate to remove salt, lyophilized, resuspended in sample buffer, and electrophoresed on 10% polyacrylamide gels according to the method of Laemmli (1970), except that the bisacrylamide concentration was 0.13%. Fractions from the integrin purification on wheat germ agglutinin-sepharose (WGA-sepharose) were electrophoresed without prior dialysis. Immunoblot analysis was performed as described by Towbin et al. (1979) with a few modifications. Proteins were transferred from the gel to nitrocellulose, the nitrocellulose was incubated for 1 h in blocking buffer (2.5% BSA, 0.2% gelatin, and 0.05% Tween 20 in TBS) and then cut into strips and incubated for 1 h in primary antibodies diluted 1:500 in blocking buffer. After washing for 1 h in washing buffer (0.2% gelatin, 0.05% Tween 20 in TBS), the strips were incubated for 1 h in second antibody (^{125}I -goat antirabbit for the peptide affinity column eluates and HRP-goat antirabbit for the WGA-sepharose column eluates), and washed again for 1 h. ^{125}I -labeled blots were mounted on cardboard and exposed to Kodak X-Omat film for 2–4 d, and peroxidase-labeled blots were reacted with 0.5 mg/ml 4-chloro-1-naphthol.

Antibodies to myosin, talin, vinculin, filamin, tropomyosin, and α -ac-

tinin were prepared in the lab and were characterized previously. Antivimentin was obtained from Sigma Chemical Co. (St. Louis, MO). Antitubulin was the generous gift of Dr. Ted Salmon (University of North Carolina, Chapel Hill, NC) and antifibulin was the generous gift of Dr. Scott Argaves (American Red Cross, Rockville, MD).

Protein Purification and Iodination

Purification of vinculin and α -actinin from chicken gizzard was performed as previously described (Feramisco and Burridge, 1980), with an additional purification by Mono Q column chromatography. Tropomyosin was purified from a 50–75% ammonium sulfate fraction of a gizzard extract prepared as in the vinculin and α -actinin purification. This fraction was dialyzed into buffer B (20 mM Tris acetate, 20 mM NaCl, 0.1 mM EDTA, 0.1% β -mercaptoethanol, pH 7.6) and subjected to anion exchange chromatography on DEAE-cellulose (DE52; Whatman Inc., Clifton, NJ). Tropomyosin-containing fractions were pooled and further purified on a hydroxylapatite column. Smooth muscle integrin was purified from chicken gizzard on WGA-sepharose as described previously (Kelly et al., 1987), except that the initial wash steps were performed with TBS (150 mM NaCl, 50 mM Tris-HCl, 0.1% NaN_3 , pH 7.6) instead of water. Platelet glycoprotein IIb/IIIa was purified from human platelets as described by Parise and Phillips (1985).

Purified α -actinin, vinculin, and tropomyosin were labeled with ^{125}I iodine using the iodogen method. Iodogen (30 μl of 5 mg/ml in CHCl_3 ; Pierce Chemical Co., Rockford, IL) was dried onto the inside of an eppendorf tube under a gentle nitrogen stream. Protein, in TBS, was added to the tube followed by 0.75 mCi of $\text{Na-}^{125}\text{I}$ (New England Nuclear, Wilmington, DE), and the reaction was carried out at 4°C for 5 min. Saturated tyrosine solution was added to terminate the reaction. Labeled protein was separated from free iodine and iodo-tyrosine by chromatography on sephadex G-50 in buffer B plus 0.2% gelatin as carrier. The proteins were labeled to a specific activity of 1×10^7 cpm/ μg for α -actinin, 5×10^6 cpm/ μg for vinculin, and 3×10^6 cpm/ μg for tropomyosin.

Solid Phase Binding Assay

Removable microtiter wells (Dynatech Laboratories, Inc., Alexandria, VA) were coated with 50 μl of β_1 cytoplasmic domain peptide (1 mg/ml in PBS) or with 50 μl of purified integrin for 1 h at 37°C. The wells were rinsed briefly with wash buffer (0.1% BSA, 0.1% NaN_3 , in PBS), blocked with 2% BSA for 30 min at 37°C, then washed again. Control wells were coated with 2% BSA alone. Iodinated protein and unlabeled protein were added to the wells and diluted to a final volume of 100 μl with PBS. The wells were incubated for 1 h at 37°C, then washed briefly four times with wash buffer, and allowed to dry. Individual wells were removed and counted in a tabletop gamma counter (LKB Instruments, Gaithersburg, MD).

To assess the ability of actin-binding proteins tropomyosin and α -actinin to bind to the β peptide in the presence of actin filaments, iodinated protein was preincubated with polymerized rabbit skeletal muscle actin in 0.2 mM ATP, 0.05 mM CaCl_2 , 2 mM Tris-HCl, 100 mM KCl, pH 8, for 90 min at 37°C before their use in peptide binding assays. In this case, the level of protein binding in the absence of F-actin was determined using the same buffer, except without F-actin. Background binding of ^{125}I - α -actinin and ^{125}I -tropomyosin to BSA-coated wells, also in the absence of F-actin, was subtracted from total counts bound and, in all cases, was <2% of total counts added.

Proteolytic Cleavage of α -Actinin

α -Actinin was dialyzed into 40 mM ammonium acetate (pH 8) containing 1 mM CaCl_2 , then digested with thermolysin (Sigma Chemical Co.) at a 1:25 enzyme/ α -actinin ratio for 2 h at 24°C with constant agitation. The cleavage products were fractionated on a Mono Q column equilibrated with buffer B (20 mM Tris acetate, 20 mM NaCl, 0.1 mM EDTA, 0.1% β -mercaptoethanol, pH 7.6) and eluted with a 0–400 mM NaCl gradient in buffer B.

Liposome Binding Assay

Purified glycoprotein IIb/IIIa (GP IIb/IIIa) was incorporated into synthetic liposomes by the procedure of Parise and Phillips (1985). Briefly, Triton X-100 was removed from the purified GP IIb/IIIa by adsorption of the GP IIb/IIIa onto a Con A affinity column. The column was washed with Tris buffer containing octyl glucoside (50 mM Tris-HCl, 2 mM CaCl_2 , 0.1 M NaCl, 60 mM octyl glucoside, and 0.02% NaN_3 , pH 7.3), and GP IIb/IIIa was eluted with α -methylmannoside (100 mM in the Tris wash buffer). The

GP IIb/IIIa was dialyzed in the Tris wash buffer to remove α -methylmannoside, and applied to a Sephadex G-75 column to remove contaminating Con A.

To make the liposomes, phosphatidylserine and phosphatidylcholine (Avanti Polar Lipids, Inc., Birmingham, AL) were mixed in a 70:30 ratio and dried under nitrogen to the sides of a glass test tube. GP IIb/IIIa (with a trace amount of ^{131}I -GP IIb/IIIa) was mixed with the phospholipids at a protein/phospholipid ratio of 1:2.9, wt/wt. Octyl glucoside was removed by dialysis against Tris buffer (50 mM Tris-HCl, 3 mM CaCl_2 , 0.1 M NaCl, 0.02% NaN_3 , pH 7.3), and the phospholipid vesicles were isolated by centrifugation.

For the binding assay, 50 μl of ^{125}I - α -actinin, 50 μl of vesicles (either with or without incorporated GP IIb/IIIa), 50 μl of competitor (either β_1 peptide, unlabeled α -actinin, or liposome buffer), and 50 μl of liposome buffer (50 mM Tris-HCl, 6 mM CaCl_2 , 0.1 M NaCl, 0.02% NaN_3 , 2% BSA, pH 7.3) were incubated in an eppendorf tube at room temperature for 1 h, with occasional mixing. The liposomes were collected on filters and washed two times with 1 ml of the Tris dialysis buffer, and the amount of bound ^{125}I - α -actinin was determined in a gamma counter.

Results

A synthetic peptide corresponding to the complete, 47 amino acid cytoplasmic domain of the integrin β_1 subunit was synthesized and purified by HPLC. NH_2 -terminal cysteine was included in the sequence to facilitate coupling to the column matrix, thiopropyl sepharose 6B, a resin which allows specific coupling through SH groups. Thus, the β_1 peptide was bound through the terminal cysteine and maintained an orientation similar to that of the native protein at the cytoplasmic face of the membrane. This affinity matrix was used to isolate integrin-binding proteins from Triton X-100 extracts of ^{35}S -methionine-labeled cultured CEFs. As shown in Fig. 1, elution with 300 mM salt revealed a number of proteins retained by the peptide affinity resin.

To determine if any of these bands represented known cytoskeletal proteins, samples of the column eluate were analyzed by immunoblotting with a panel of specific antibodies. Antibodies to fibulin, filamin, myosin, talin, vimentin, tropomyosin, and tubulin failed to react with proteins eluted from the peptide column (data not shown). As seen in Fig.

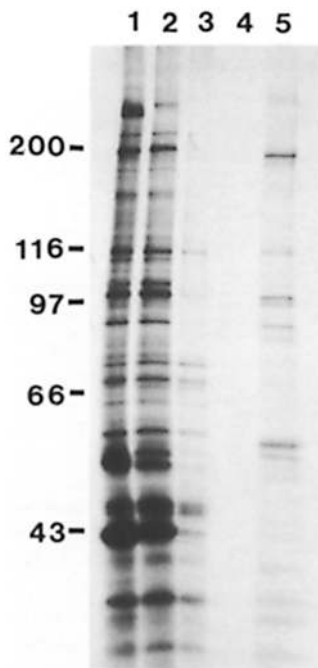


Figure 1. SDS-PAGE analysis of affinity chromatography with the integrin cytoplasmic domain peptide. The β_1 peptide was conjugated to thiopropyl sepharose 6B. An extract of ^{35}S -labeled CEFs (lane 1) was centrifuged to remove particulates, and the supernatant (lane 2) was applied to the peptide column. Flowthrough (lane 3) was collected and the column was washed extensively (wash, lane 4), then eluted with 300 mM salt (eluate, lane 5). The positions of molecular mass standards are shown at left in kilodaltons.

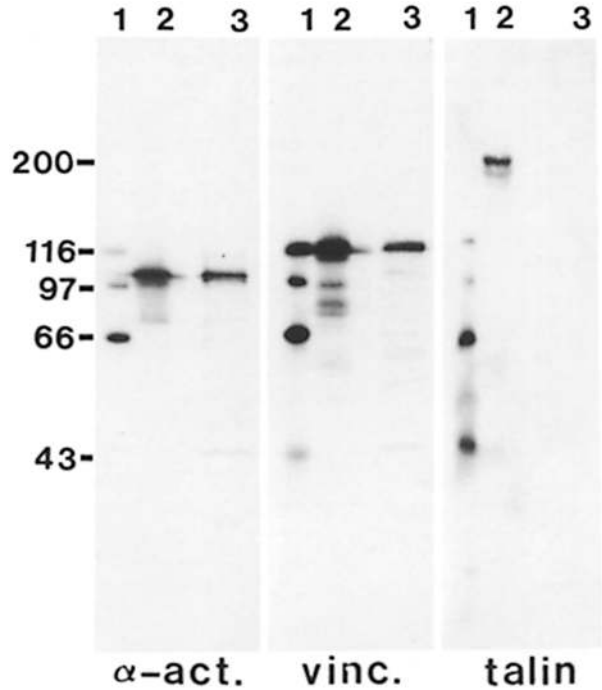


Figure 2. Immunoblot analysis of β_1 -column eluate. ^{125}I -labeled molecular weight standards were loaded in lane 1 of each set. Whole CEF extract was loaded in lane 2. β_1 -column eluate was loaded in lane 3. The gel was blotted onto nitrocellulose and stained with antibodies to α -actinin, vinculin, and talin, followed by ^{125}I -goat antirabbit. Note that talin is not detected in the column eluate, unlike vinculin and α -actinin.

2, only antibodies to α -actinin and vinculin reacted positively in the immunoblot assays.

We wanted to know if α -actinin, vinculin, or both were capable of binding directly to the β_1 cytoplasmic domain, and we addressed this question using a solid phase protein binding assay. For these experiments, microtiter wells were coated with the β_1 peptide, and the binding of ^{125}I -labeled protein was measured by competitive inhibition in the presence of increasing concentrations of unlabeled protein. In this assay, no significant binding of ^{125}I -vinculin to the integrin peptide was detected (Fig. 3 A), suggesting that vinculin may bind to the peptide affinity column through an association with some other integrin-binding protein. However, significant binding of ^{125}I - α -actinin to the β_1 cytoplasmic domain was observed, and this binding was inhibited by the addition of excess unlabeled α -actinin (Fig. 3 B). Scatchard analysis of these data demonstrated an apparent K_D of $1.6 \times 10^{-8}\text{M}$ (Fig. 3 C).

Using the solid phase binding assay, we characterized more fully the specificity of α -actinin binding to the β_1 cytoplasmic domain. In these experiments, we asked if unrelated proteins (both globular and rod-shaped) could compete with α -actinin for binding to the integrin peptide. As shown in Fig. 4, the binding of α -actinin to β_1 peptide was inhibited by the addition of increasing concentrations of unlabeled α -actinin or free β_1 peptide, but not by several other proteins, including BSA, ovalbumin, talin, or vinculin. Surprisingly, binding of α -actinin to β_1 peptide was also inhibited by tropomyosin (Fig. 4), and we found that ^{125}I -

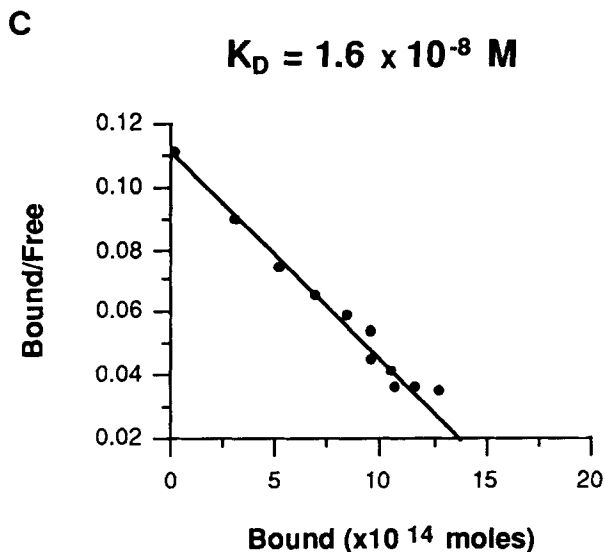
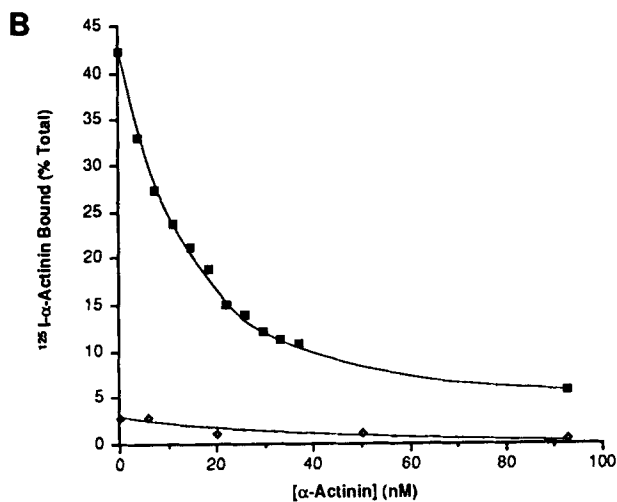
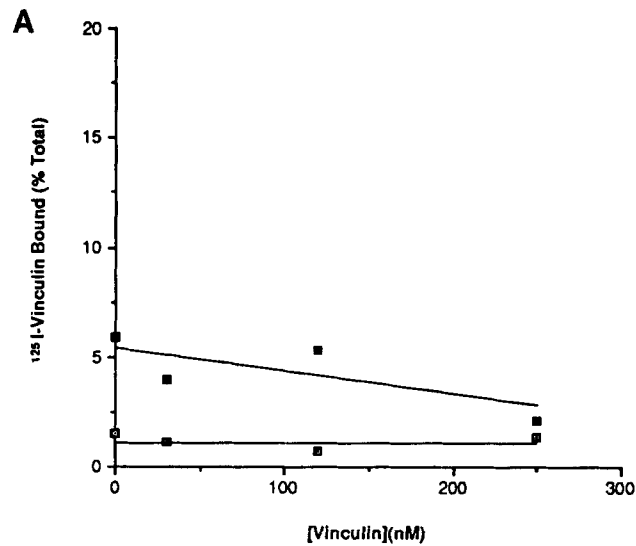


Figure 3. Binding of vinculin and α -actinin to β_1 integrin cytoplasmic domain peptide. (A) Binding of ^{125}I -vinculin to wells coated with β_1 cytoplasmic domain (closed squares) or BSA (open squares) in the presence of excess unlabeled vinculin. (B) Binding

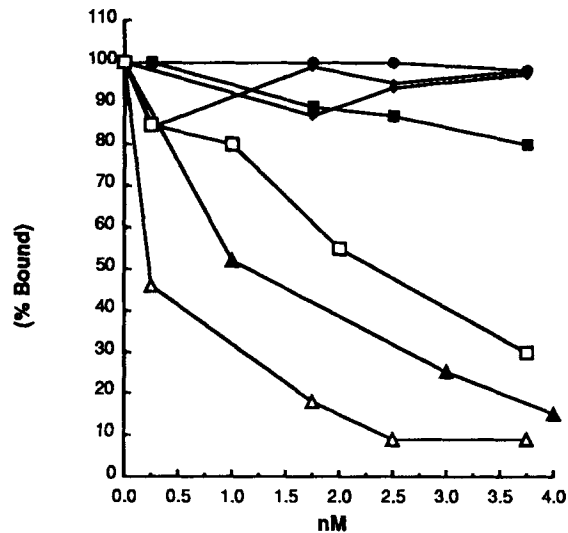


Figure 4. Specificity of α -actinin binding to the β_1 integrin cytoplasmic domain peptide. Binding of ^{125}I - α -actinin to β_1 peptide in the presence of increasing concentrations of free β peptide (open triangle); α -actinin (solid triangle); BSA (solid circle); ovalbumin (open diamond); talin (solid diamond); vinculin (solid square); or tropomyosin (open square). Specific activity of α -actinin was the same as in Fig. 4, and 6×10^4 cpm were added to each well.

tropomyosin bound to β_1 peptide in a solid phase assay (data not shown). Tropomyosin contains a large amount of α -helix arranged in a coiled coil structure (Cohen and Holmes, 1963). α -Actinin is a protein with a high α -helical content (Suzuki et al., 1976), some of which may also be arranged in a coiled coil structure. This raises the possibility that the β_1 peptide may be binding specifically to a coiled coil conformation. To test this, we isolated the light meromyosin fragment of myosin which is composed largely of coiled coil α -helices (Lowey and Cohen, 1962), and used this as a competitor in the solid phase binding assay. However, the solubility requirements of light meromyosin demand a high ionic strength buffer, and this buffer inhibited the binding of α -actinin to the β peptide in the solid phase assay, so that we were unable to obtain interpretable results for the binding of light meromyosin to the β peptide. Further experiments will be aimed at testing the affinity of the β_1 peptide for other proteins with a coiled coil structure.

Since tropomyosin binds to the isolated β_1 cytoplasmic domain peptide, we were surprised that it was not detected eluting from the peptide affinity column. One possibility is that the peptide-binding site on tropomyosin is obscured by binding to actin filaments. We found that the binding of tropomyosin to the peptide was inhibited by very low actin concentrations (Fig. 5). At equivalent concentrations of ac-

of ^{125}I - α -actinin to wells coated with β_1 cytoplasmic domain peptide (squares) or BSA (diamonds) in the presence of excess α -actinin. All points represent the average of duplicate experiments. ^{125}I - α -actinin did not bind to wells coated with a control, unrelated peptide (data not shown). (C) Scatchard plot analysis of α -actinin binding to β_1 peptide. The iodinated proteins were labeled with specific activities of 1×10^7 cpm/ μg for α -actinin and 5×10^6 cpm/ μg for vinculin. In each assay, 12 ng of ^{125}I -vinculin and 6 ng of ^{125}I - α -actinin were used, so that 6×10^4 cpm were added to each well.

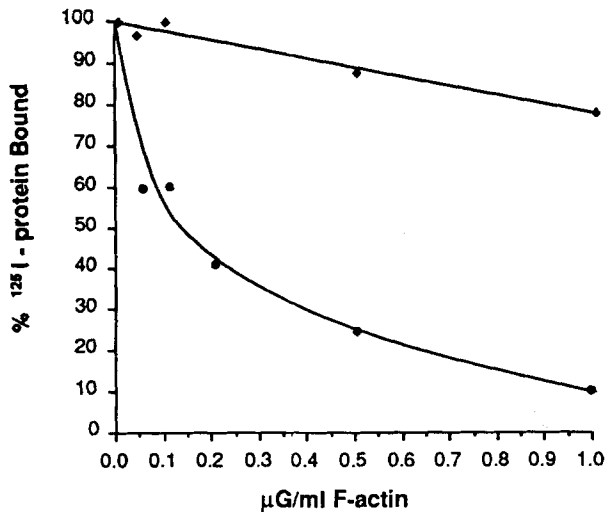


Figure 5. Effect of filamentous actin on the binding of tropomyosin and α -actinin to β integrin. ^{125}I -labeled protein was preincubated with varying concentrations of F-actin for 90 min, and this mixture was then added to wells coated with β_1 cytoplasmic domain peptide. ^{125}I - α -actinin (diamonds); ^{125}I -tropomyosin (circles). All points represent the average of duplicate experiments. Binding is expressed as a percentage of ^{125}I -protein bound to the β_1 -peptide in the absence of F-actin. 6 ng of α -actinin (at 1×10^4 cpm/ng) and 20 ng of tropomyosin (at 3×10^3 cpm/ng) were added in each assay. Maximal tropomyosin bound (100%) was 2.2×10^4 cpm, or 38% of total counts added. Maximal α -actinin bound was 2.9×10^4 cpm, or 48% of total counts added.

tin, α -actinin binding to the peptide was much less affected, although this too was inhibited at higher actin concentrations. This inhibition of α -actinin binding may be due to crosslinking of actin filaments preventing access of α -actinin to the peptide in this assay.

We next wanted to determine which domain of the α -actinin molecule was responsible for binding to integrin. To address this question, we generated the two major proteolytic fragments of α -actinin that can be obtained by cleavage with thermolysin, as has been described previously (Mimura and Asano, 1987). Thermolysin digestion yields a 27-kD globular fragment, which contains the actin-binding domain, and a 53-kD rod-like fragment, which contains four repeating units that may be involved in dimerization. These two α -actinin fragments were purified by FPLC (Fig. 6A). Solid phase binding assays were carried out to determine which fragment could compete with ^{125}I - α -actinin for binding to the β_1 cytoplasmic domain. As shown in Fig. 6B, binding of ^{125}I - α -actinin to the β_1 peptide was inhibited by the 53-kD fragment, but not by the 27-kD actin-binding fragment. These results indicate that the integrin-binding site is located within the domain of α -actinin that lacks the actin-binding site.

We extended these findings by asking if α -actinin could bind to intact heterodimeric integrin, as well as to the synthetic β_1 cytoplasmic domain peptide. Also, we wanted to know if integrins containing β chains other than β_1 were capable of binding to α -actinin. To address these questions, we used two different integrins: the chicken smooth muscle integrin and the platelet GP IIb/IIIa. Smooth muscle integrin has not been sequenced yet, but it has been shown to cross react with a polyclonal antibody specific for the β_1 cytoplasmic domain (Marcantonio and Hynes, 1988) and has therefore been classified as a β_1 integrin (C. Otey, unpublished observation). The platelet GP IIb/IIIa has been classified as a β_3 integrin based on sequence data. These two integrins were purified from chicken gizzard and human platelets, respectively, (Fig. 7A), adsorbed to microtiter wells, and allowed to bind to ^{125}I - α -actinin either alone or in the presence of an excess of unlabeled α -actinin. Very similar results were obtained with both purified integrins: ^{125}I - α -actinin

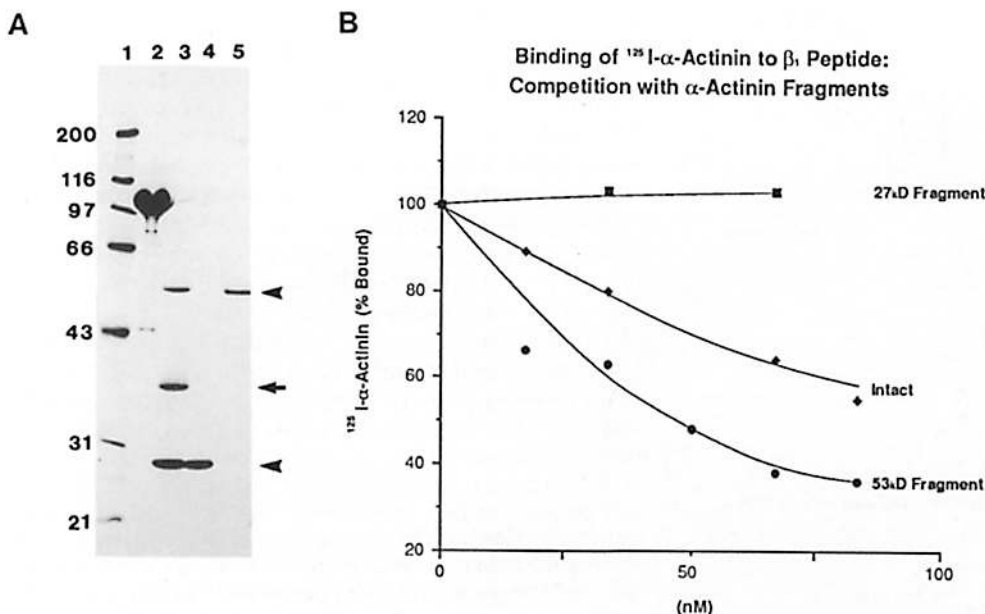


Figure 6. Binding of α -actinin fragments to the integrin β_1 cytoplasmic domain peptide. (A) Coomassie blue-stained SDS-polyacrylamide gel. (lane 1) Molecular weight standards; (lane 2) intact α -actinin, overloaded to demonstrate purity; (lane 3) products of thermolysin cleavage. Positions of thermolysin cleavage (arrow) and the two major α -actinin fragments (arrowheads) are labeled. (lane 4) Purified 27-kD α -actinin fragment; (lane 5) purified 53-kD α -actinin fragment. (B) Binding inhibition assay. Wells were coated with β_1 cytoplasmic domain peptide. Intact ^{125}I - α -actinin was added to the wells in the presence of increasing concentrations of intact α -actinin (diamonds), the 27-kD fragment (squares), or the

53-kD fragment (circles) of α -actinin. The concentrations of 27- and 53-kD fragments were estimated by comparison with BSA standards on SDS gels. The specific activity, total counts added, and concentration of ^{125}I - α -actinin was approximately the same as in Fig. 4. Binding is expressed as a percentage of maximal ^{125}I - α -actinin bound to the β_1 peptide in the absence of any inhibitor.

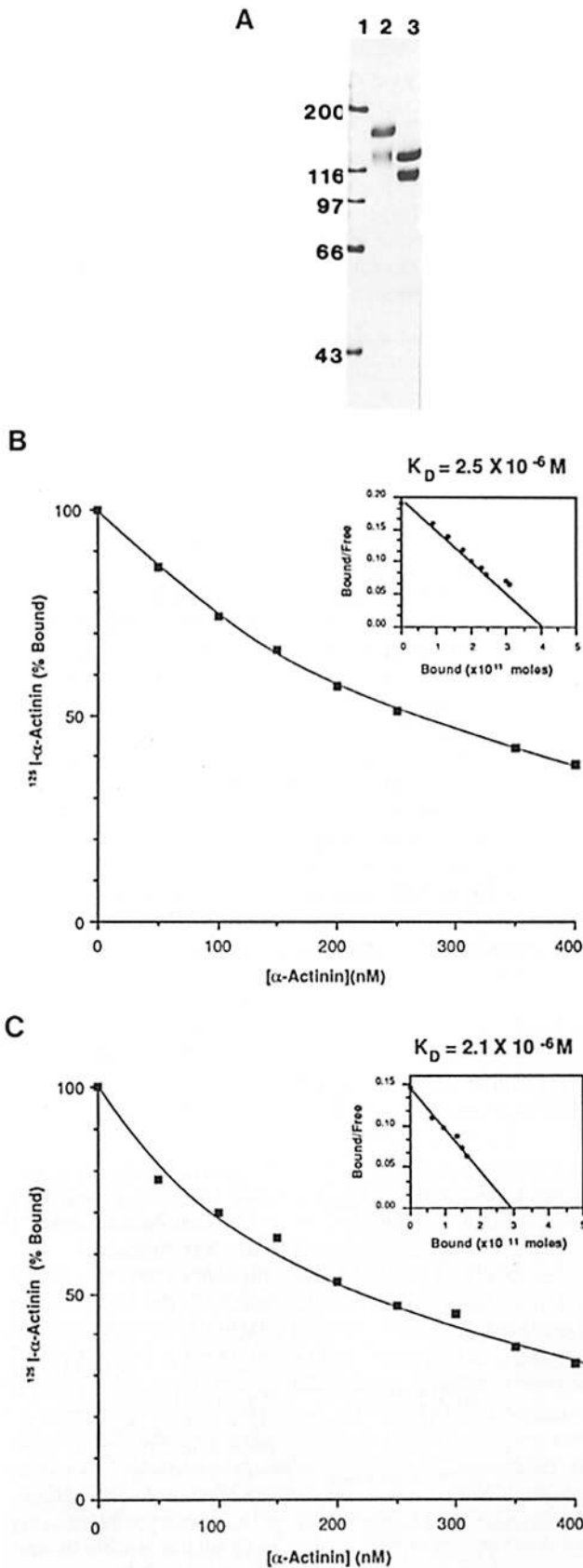


Figure 7. Binding of α -actinin to purified intact integrins. (A) Coomassie blue-stained SDS-polyacrylamide gel. (lane 1) Molecular weight standards; (lane 2) smooth muscle integrin (β_1) purified from chicken gizzard; (lane 3) glycoprotein IIb/IIIa (β_3) purified

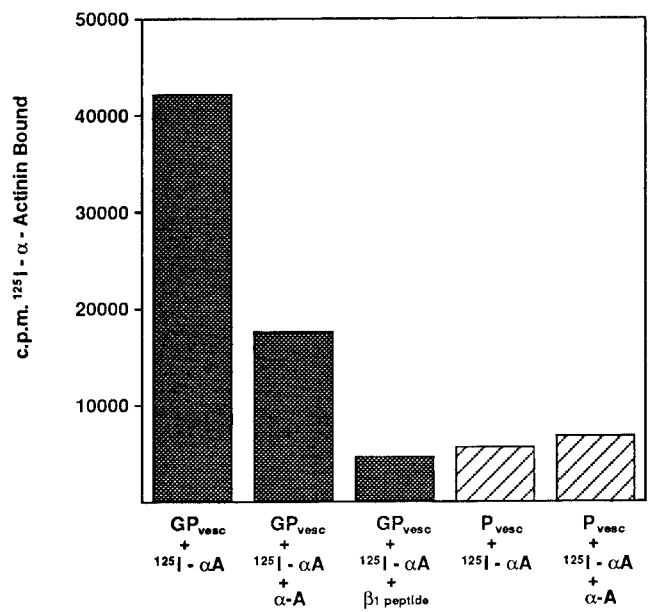


Figure 8. Binding of ^{125}I - α -actinin to synthetic phospholipid vesicles. Vesicles were prepared either with GP IIb/IIIa (solid bar) or without incorporated protein (hatched bar), and were incubated with ^{125}I - α -actinin alone or with excess unlabeled α -actinin (3 mg/ml) or cytoplasmic domain peptide (10 mg/ml) as competitors. Values were normalized with respect to ^{131}I -labeled GP IIb/IIIa, which was incorporated in trace amounts to monitor the relative number of vesicles used in each experiment. (*GP vesc*) vesicles containing GP IIb/IIIa; (*P vesc*) plain vesicles; (α -A) α -actinin.

bound to each integrin and was displaced by unlabeled α -actinin (Fig. 7, B and C). When these binding data were submitted to Scatchard plot analysis, an apparent K_D of 2.5×10^{-6} was obtained for binding of α -actinin to smooth muscle integrin and 2.1×10^{-6} for binding to GP IIb/IIIa.

These results indicate that α -actinin can bind to intact integrin adsorbed to the surface of a microtiter well, but because integrins in the cell exist in a lipid bilayer, we wanted to determine if α -actinin could interact with integrin in a membrane environment. Other labs have demonstrated the usefulness of incorporating purified integrin into phospholipid vesicles to measure binding of ligands to the extracellular domain of integrin, and this system has been used most successfully with GP IIb/IIIa (Parise and Phillips, 1985, 1986). It has been reported previously that with large phospholipid vesicles, 55% of the GP IIb/IIIa molecules are oriented with their extracellular domains on the outside of the vesicle (Parise and Phillips, 1985), suggesting that the remaining 45% are oriented "inside out," with their cytoplasmic domains exposed. Thus, these synthetic vesicles could also be used to measure binding of ligands to the cytoplasmic domain of integrin.

from human platelets. (B) Binding of ^{125}I - α -actinin to wells coated with smooth muscle integrin. Maximal binding represents 8×10^3 cpm, of the 6×10^4 cpm added per assay. (C) Binding of ^{125}I - α -actinin to wells coated with glycoprotein IIb/IIIa. Maximal binding represents 8×10^3 cpm, of the 6×10^4 cpm added per assay. (inset graphs) Scatchard plot analysis of α -actinin binding to smooth muscle integrin (B) or GP IIb/IIIa (C).

For the liposome binding assay, synthetic vesicles were prepared either with or without incorporated GP IIb/IIIa. To measure the specific binding of α -actinin to the GP IIb/IIIa, plain vesicles or GP IIb/IIIa vesicles were incubated with ^{125}I - α -actinin together with unlabeled α -actinin, free β peptide, or buffer alone. The vesicles were then collected on filters, washed, and counted in a gamma counter. As shown in Fig. 8, significant binding of ^{125}I - α -actinin to GP IIb/IIIa vesicles, but not plain vesicles, was detected. This binding was competitively displaced by the addition of either excess α -actinin or β peptide, suggesting that the binding of α -actinin to GP IIb/IIIa is specific for the cytoplasmic domain of the integrin.

If α -actinin does interact directly with integrin, as these *in vitro* experiments suggest, one might expect α -actinin to associate with the integrin heterodimer through the initial steps of its isolation. To address this question, smooth muscle integrin from chicken gizzard was eluted from a WGA-Sepharose column (Fig. 9 A), and integrin-containing fractions were then analyzed by immunoblot to determine if α -actinin was also present. As shown in Fig. 9 B, α -actinin was detected eluting from the column together with integrin. Although this is consistent with an *in vivo* interaction between α -actinin and integrin, a direct interaction between α -actinin and integrin is not conclusively demonstrated because the column eluate also contained actin, as well as several other proteins.

Discussion

In this report, we describe a specific, high-affinity interaction between α -actinin and the cytoplasmic domain of the β_1 integrin subunit. At the outset of this work, we did not anticipate finding an interaction between α -actinin and integrin. Since its discovery in nonmuscle cells, α -actinin has been suggested on several occasions to be a potential link between actin filaments and cell membranes. The popularity of this idea has vacillated. Originally, α -actinin was noted in focal contacts at the ends of stress fibers and this led to the idea that it might be involved in anchoring these structures (Lazarides and Burridge, 1975). Then it was shown that α -actinin

could be extracted from isolated plasma membranes leaving much of the actin still associated with the membranes (Burridge and McCullough, 1980). However, these membranes were prepared from cells grown in suspension which would have lacked both stress fibers and focal contacts. Subsequent work using immunoelectron microscopy suggested that α -actinin is located farther from the membrane than vinculin or talin (Chen and Singer, 1982). The role of α -actinin in anchoring skeletal muscle myofibrils is also unclear. Although immunoelectron microscopy of chicken skeletal muscle suggested that α -actinin may be absent from the myofibrillar termini (Tidball, 1987), genetic studies of insect flight muscle have shown that the sites of muscle insertion are disrupted in mutants lacking normal α -actinin (Fyrberg et al., 1990). In general, the view has evolved that α -actinin may be located some distance from the membrane, and that it may be just one link in a chain of proteins involved in attachment of actin to the membrane (Burridge et al., 1988). Our findings in this paper suggest a more direct role for α -actinin, and that α -actinin may form a bridge between actin and the cytoplasmic domain of integrin in some circumstances.

Our results differ from those of an earlier study which demonstrated that talin, and not α -actinin, bound to integrin in an equilibrium gel filtration assay (Horwitz et al., 1986). Our lack of detection of talin binding in the current assays is consistent with the low affinity for the talin-integrin interaction previously noted. That binding of α -actinin to integrin was not detected in the former work is perhaps surprising, but may have been due to the harsh conditions used to elute the integrin from the immunoaffinity column. Alternatively, the α -actinin-integrin complex may have not generated a sufficient shift in Stokes' radius to be detected in that assay. In our current study, we initially detected binding of α -actinin to a synthetic peptide that corresponds to the cytoplasmic domain of the integrin β_1 polypeptide, and then verified our results with two purified integrins. We found that α -actinin interacts with the integrin β -chain cytoplasmic domain under three distinct sets of conditions. First, α -actinin from a whole cell detergent extract bound to the synthetic peptide immobilized to an affinity matrix. Second, purified chicken gizzard α -actinin bound both to the peptide and to purified heterodimeric integrin from two sources, chicken gizzard and human platelets (GP IIb/IIIa). Third, purified α -actinin bound to glycoprotein IIb/IIIa which had been incorporated into phospholipid vesicles, indicating that binding does occur when integrin is in a membrane environment.

The results of our solid phase binding assays indicate that α -actinin binds with 100-fold higher affinity to the β_1 synthetic peptide than to purified heterodimeric integrin. It is possible that the presence of the α subunit in the integrin heterodimer exerts some regulatory effect on the binding of α -actinin to the β subunit, such that the affinity of α -actinin for intact integrin is reduced. Alternatively, the reduced affinity could result from the cytoplasmic domain of the intact integrin adopting a less favorable conformation than the free peptide adsorbed to microtiter wells. The experiments using purified integrins also demonstrated that α -actinin binds to integrins from both the β_1 and β_3 subfamilies. The cytoplasmic domains of these two β subunits are highly homologous, but not identical. We plan to investigate the possibility that α -actinin may interact with integrins from other subfamilies as well.

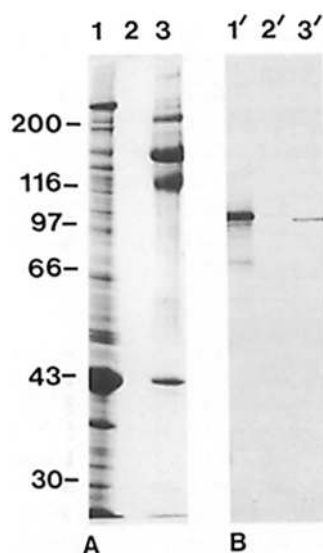


Figure 9. Detection of α -actinin with partially purified smooth muscle integrin. (A) Coomassie blue-stained SDS-polyacrylamide gel. A Triton X-100 extract of chicken gizzard (lane 1) was applied to a WGA-sepharose column, washed extensively (wash fraction, lane 2), and eluted with N-acetyl-glucosamine (eluate, lane 3). (B) Duplicate lanes were transferred to nitrocellulose and stained with a polyclonal antibody to avian α -actinin followed by peroxidase-labeled second antibody, and reacted with 4-chloro-1-naphthol.

Very recently, an approach similar to ours was utilized by Argraves et al. (1989), who also performed affinity chromatography experiments with a synthetic β_1 cytoplasmic domain peptide. Using extracts of human placenta, Argraves and coworkers identified a single major 100-kD band which could be eluted from the β_1 column with EDTA, and this protein was given the name fibulin. Sequence analysis and immunoblotting demonstrated that fibulin is clearly distinct from α -actinin. Argraves et al. (1989) did not detect α -actinin binding to their β_1 peptide columns, but the synthetic peptide used in their columns was 10 residues shorter than our peptide, and it is possible that these 10 residues may contribute to α -actinin binding. Also, the detergents and ionic conditions used by Argraves et al. (1989) differed significantly from ours. In particular, Argraves et al. used calcium-containing buffers and were able to elute fibulin with EDTA, while we used EGTA-containing buffers throughout, which may explain the absence of fibulin in our column eluates.

Our results may relate to a recent analysis of the sequence homology that α -actinin shares with dystrophin, the protein absent from patients with Duchenne muscular dystrophy (Hammond, 1987; Koenig et al., 1988; Davison et al., 1989). Dystrophin has been localized to the sarcolemma of normal skeletal muscle (Zubrzycka-Gaarn, et al., 1988; Arahata et al., 1988; Watkins et al., 1988), and there is evidence to suggest that dystrophin may associate with a membrane glycoprotein in muscle (Campbell and Kahl, 1989). Dystrophin shares homology with α -actinin in its NH₂-terminal actin-binding domain, in a COOH-terminal region, and in its multiple repeats of \sim 110 amino acids (Hammond, 1987; Koenig et al., 1988; Davison et al., 1989). Based on these observations, it will be interesting to determine whether the integrin-binding site in α -actinin is conserved in dystrophin and whether dystrophin interacts with integrin in the muscle sarcolemma. Possibly the lack of α -actinin in terminal Z-discs and myotendinous junctions (Tidball, 1987) reflects its replacement in these sites by dystrophin, which has been localized to the myotendinous junctions (Shimizu et al., 1989).

The eluted fractions from our peptide columns also contained a number of unidentified proteins which we are continuing to characterize. Some of these proteins may have bound to the affinity column through interactions with other integrin-binding proteins, as may be the case with vinculin. However, it is possible that some of these uncharacterized proteins may bind to integrin directly and with high affinity. Currently, we are working to purify these unknown proteins in order to investigate their interactions with other focal contact proteins.

Although our data suggest an interaction between integrin and α -actinin, it should be noted that most of these experiments were based on *in vitro* assays using purified proteins. We are now focusing on experiments designed to ask if α -actinin and integrin also interact *in vivo*. This was addressed in a preliminary manner by asking if α -actinin copurifies with the integrin heterodimer. Although α -actinin was detected in integrin-containing column fractions, these also contain actin and some other proteins. Thus, the presence of α -actinin in the integrin preparations cannot be taken as unambiguous evidence of a direct, physiological interaction between α -actinin and integrin. Further experiments are being aimed at resolving this issue.

The evidence to date suggests that more than one type of linkage attaches the actin cytoskeleton to the focal contact: one may involve an integrin-talin association and another, identified here, may involve an interaction between integrin and α -actinin. The discovery of a number of proteins, as yet uncharacterized, that are localized to the focal contact (Beckerle, 1986; Glenney and Zokas, 1989; Wilkins et al., 1986) suggests that additional linkages may also exist. These multiple linkages could reflect different stages in the maturity of a focal contact or different structural requirements. One of the challenges in this area of research will be to understand how these multiple linkages are regulated as well as their roles in such diverse integrin functions as embryogenesis, fibroblast motility, and platelet aggregation.

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Note Added in Proof. We have found that a synthetic peptide with a coiled coil conformation and two myosin fragments (long s-2 and short s-2) with high coiled coil content do not compete with α -actinin for binding to the β_1 integrin peptide.

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