Disruption of the Actin Cytoskeleton after Microinjection of Proteolytic Fragments of α-Actinin

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Abstract. α-Actinin can be proteolytically cleaved into major fragments of 27 and 53 kD using the enzyme thermolysin. The 27-kD fragment contains an actin-binding site and we have recently shown that the 53-kD fragment binds to the cytoplasmic domain of β1 integrin in vitro (Otey, C. A., F. M. Pavalko, and K. Burridge. 1990. J. Cell Biol. 111:721-729). We have explored the behavior of the isolated 27- and 53-kD fragments of α-actinin after their microinjection into living cells. Consistent with its containing a binding site for actin, the 27-kD fragment was detected along stress fibers within 10-20 min after injection into rat embryo fibroblasts (REF-52). The 53-kD fragment of α-actinin, however, concentrated in focal adhesions of REF-52 cells 10-20 min after injection. The association of this fragment with focal adhesions in vivo is consistent with its interaction in vitro with the cytoplasmic domain of the β1 subunit of integrin, which was also localized at these sites. When cells were injected with >5 μM final concentration of either α-actinin fragment and cultured for 30-60 min, most stress fibers were disassembled. At this time, however, many of the focal adhesions, particularly those around the cell periphery, remained after most stress fibers had gone. By 2 h after injection only a few small focal adhesions persisted, yet the cells remained spread. Identical results were obtained with other cell types including primary chick fibroblasts, BSC-1, MDCK, and gerbil fibroba cells. Stress fibers and focal adhesions reformed if cells were allowed to recover for 18 h after injection. These data suggest that introduction of the monomeric 27-kD fragment of α-actinin into cells may disrupt the actin cytoskeleton by interfering with the function of endogenous, intact α-actinin molecules along stress fibers. The 53-kD fragment may interfere with endogenous α-actinin function at focal adhesions or by displacing some other component that binds to the rod domain of α-actinin and that is needed to maintain stress fiber organization.

α-actinin is an actin binding protein that cross-links actin filaments in vitro (Maruyama and Ebashi, 1965; Burridge and Feramisco, 1981). Its native structure is a homodimer with a subunit molecular weight of 103 kD (Singh et al., 1977; Suzuki et al., 1979; Baron et al., 1987) and is visualized in the electron microscope as a dumbbell-shaped molecule, 30-40 nm long and 3-4 nm wide with an enlarged head domain at each end (Podlubnaya et al., 1975; Breitsch et al., 1979; Pollard, 1981). Distinct α-actinin isoforms have been identified in muscle (Endo and Masaki, 1982) and non-muscle cells (Burridge and Feramisco, 1981; Duhaian and Bamburg, 1984; Bennett et al., 1984; Landon et al., 1985); however, the only known functional difference between the isoforms is that binding of muscle α-actinin to actin is calcium insensitive, whereas the binding of nonmuscle α-actinins to actin is inhibited by calcium (Mimura and Asano, 1979; Burridge and Feramisco, 1981; Duhaian and Bamburg, 1984; Bennett et al., 1984; Landon et al., 1985).

Based on the complete amino acid sequence of α-actinin from various sources (Baron et al., 1987; Noegel et al., 1987) it has become clear that the α-actinin monomer is organized into three domains: a globular NH2-terminal domain which contains the binding site for actin (Mimura and Asano, 1986; Imamura et al., 1988; Baron et al., 1987), an extended rodlike domain which is responsible for dimer formation and consists of four internal repeating units of 122 amino acids each (Mimura and Asano, 1987; Imamura et al., 1988), and a COOH-terminal domain containing two EF-hand calcium-binding regions (Baron et al., 1987; Noegel et al., 1987). Comparison of α-actinin sequences with those of spectrins and dystrophin has revealed extensive homologies, leading to the suggestion that these three proteins are members of a single family of cytoskeletal proteins (Hammond, 1987; Koenig et al., 1988; Davison and Critchley, 1988; Davison et al., 1989).

In striated and smooth muscles, α-actinin is localized at Z-lines (Masaki et al., 1967; Goll et al., 1969) and dense bodies (Geiger et al., 1981), respectively, where it seems to be involved in anchoring actin thin filaments. In cultured nonmuscle cells α-actinin is localized along actin stress fibers with a periodic distribution reminiscent of muscle sarcomeres (Lazarides and Burridge, 1975). Here it may also be involved in anchoring actin filaments as well as bundling...
them into stress fibers. α-Actinin is also present at sites where actin filaments attach to the cytoplasmic face of the plasma membrane which are known as focal adhesions (Wehland et al., 1979). Several other proteins including vinculin (Geiger, 1979; Burridge and Faramisco, 1980), talin (Burridge and Connell, 1983), and integrin (Damsky et al., 1985; Chen et al., 1985; Kelly et al., 1987) are also present in focal adhesions and together with α-actinin these proteins have been proposed to link actin to integrin in the membrane via a series of protein interactions (reviewed in Burridge et al., 1988).

The role of α-actinin in mediating the attachment of actin to the membrane in nonmuscle cells is not entirely clear. Some work has suggested that actin can attach to the membrane via mechanisms that do not involve α-actinin. For example, it was possible to extract α-actinin from isolated plasma membranes obtained from cells grown in suspension without dissociating most of the actin (Burridge and McCulloch, 1980). Immunoelectron microscopy of cells with stress fibers and focal adhesions indicated that much of the α-actinin in the focal adhesions of cultured cells was located farther from the membrane than was vinculin (Chen and Singer, 1982). Also, the intensity of staining for α-actinin in focal adhesions varies among cell types and even among adhesions in the same cell (our unpublished observations) suggesting that the role of α-actinin at these sites may also vary. Based on these findings, together with the discovery that α-actinin can bind directly to vinculin (Wachstock et al., 1987), α-actinin in focal adhesions has generally been thought either to be involved in bundling actin filaments or to be one of several links in a chain of protein interactions between actin and the membrane.

Recently, however, we found that α-actinin purified from chicken gizzard smooth muscle was able to interact directly with the cytoplasmic domain of the β, subunit of integrin in vitro (Otey et al., 1990). The binding site on α-actinin for integrin was localized to a 53-kD proteolytic fragment of α-actinin that dimerizes in solution and corresponds to the rod domain (Otey et al., 1990). This study suggested a more direct role for α-actinin in linking actin to the membrane.

One prediction of this earlier work is that the 53-kD fragment of α-actinin would target to focal adhesions that contain integrin after its microinjection into live cells. We tested this prediction and found that microinjected 53-kD fragment of α-actinin concentrated in focal adhesions shortly after injection into cells, whereas the microinjected 27-kD fragment localized along stress fibers. When cells were cultured for longer periods of time following injection of either fragment of α-actinin, actin stress fibers and focal adhesions were disassembled. Possible mechanisms whereby α-actinin fragments may disrupt the actin cytoskeleton are discussed.

Materials and Methods

Cell Culture and Microinjection

Rat embryo fibroblasts (REF-52), primary chick embryo fibroblasts, African green monkey kidney cells (BSC-1), MDCK cells, and gerbil fibroma cells (IMR-33) were cultured in DMEM-H supplemented with 10% fetal calf serum (FCS), 50 U/ml penicillin, and 50 μl streptomycin in a humidified incubator with 7% CO2 at 37°C. Cells for microinjection were passaged by brief trypsinization and subcultured to 50% confluency on glass coverslips in a diaverter microscope using a 32× phase objective and microinjections were performed using a micromanipulator (Leitz, Inc., Rockleigh, NJ).

Protein Purification

α-Actinin and vinculin were purified from frozen chicken gizzards as described previously (Faramisco and Burridge, 1980), with an additional, final, purification by chromatography on an FPLC Mono Q column (Pharmacia Fine Chemicals; Piscataway, NJ). Actin was prepared from rabbit skeletal muscle acetone powder by the method of Spudich and Watt (1971) and further purified by gel filtration on a Sephadex G-150 column.

Thermolysin Cleavage of α-Actinin and Isolation of Proteolytic Fragments

8 ml of purified α-actinin (2 mg/ml) was treated with the enzyme thermolysin (Sigma Chemical Co., St. Louis, MO) at a 1:2.5 enzyme/protein ratio for 2 h at 37°C with frequent mixing. The fragments were separated by FPLC using 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. The 53-kD fragment of α-actinin was further purified on a Mono S column equilibrated in MES buffer (20 mM MES, 0.1 mM EDTA, 0.1% β-mercaptoethanol, pH 6.2). Under these conditions the 53-kD fragment flowed through the column and several trace contaminating proteins were retained by the column.

Fluorescent Labeling of α-Actinin and the 27- and 53-kD Fragment

Purified intact α-actinin and the 53-kD fragment were labeled with either fluorescent isothiocyanate (FITC), tetramethylrhodamine-isothiocyanate (TRITC; Research Organics, Inc., Cleveland, OH), or iodocacetamitetra-methyl rhodamine (IATR; Molecular Probes, Inc., Junction City, OR). The 27-kD fragment was labeled with either FITC or TRITC. For the IATR labeling, ~5 ml of purified protein at 1.5-2 mg/ml was dialyzed against 200 mM sodium borate buffer, pH 7.5. 22 μg of IATR (dissolved in DMSO) was added to the protein solution and incubated at 4°C for 6 h. The reaction was stopped by addition of DTT to a final concentration of 0.1%. For FITC and TRITC labeling, protein at approximately the same concentration as for IATR labeling was dialyzed against 200 mM sodium carbonate/bicarbonate (C/B) buffer at pH 9.5. The dialysis bag was then transferred to 600 ml of the C/B buffer and 18 mg of FITC or TRITC was added with stirring. The reaction was continued for 6 h at 4°C and then terminated by dialyzing against buffer B at pH 7.6. Free dye was removed from labeled proteins by extensive dialysis against microinjection buffer (75 mM KCl, 0.1% β-mercaptoethanol, 10 mM potassium phosphate, pH 7.5). Intact α-actinin and both of the labeled fragments were concentrated by ultrafiltration to a final concentration of ~200 μM. Dye/protein molar ratios for the labeled proteins were between 0.1 and 0.5 as determined by absorbance at 575/280 nm. Estimates of the α-actinin concentration in fibroblasts were made by RIA using rabbit polyclonal anti-α-actinin antiserum with purified α-actinin as a standard.

Fluorescence Microscopy

Cells (REF-52, BSC-1, IMR-33, MDCK, and CEF) were grown on 12-mm round glass coverslips and prepared for fluorescence microscopy and photographed as previously described (Pavalko et al., 1989). Antibodies specific for the cytoplasmic domain of the β, subunit of integrin were generously provided by Dr. Richard Hynes (Massachusetts Institute of Technology, Cambridge, MA). Polyclonal antibodies against α-actinin, talin and vinculin were raised against purified chicken gizzard proteins.

SDS-PAGE, Vinculin Iodination, and Blot Overlays

Electrophoresis of proteins was performed on 15% SDS-polyacrylamide gels according to Laemmli (1970) except that the bisacrylamide concentration was 0.89%. Vinculin was iodinated using Iodogen (Pierce Chemical

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Co., Rockford, IL) as has been described previously for talin (Turner and Burridge, 1989). For blot overlays, samples of digested a-actinin were transferred to nitrocellulose after electrophoresis. After incubating in buffer B containing 0.2% gelatin, 0.05% Tween-20 and 5% BSA (blocking buffer), the nitrocellulose was overlayed with 1.2 x 10^6 cpm/ml 125I-vinculin in blocking buffer for 90 min and subsequently washed in this same buffer minus BSA with several changes over a 2-h period. Protein bands binding to the 125I-vinculin were visualized by exposing the dried nitrocellulose sheet to x-ray film with an intensifying screen at -70°C for 5 d.

**Actin Sedimentation Assays**

To examine the binding of a-actinin, and the 27- and 53-kD fragments of a-actinin to F-actin, ~20 μg of each protein was added to 120 μg of G-actin and the actin was allowed to polymerize for 1 h by addition of KCl to a final concentration of 50 mM and MgCl₂ to 1 mM. F-actin and bound proteins were sedimented at 100000 g for 45 min in a Beckman TL-100 ultracentrifuge (Beckman Instrument Co., Fullerton, CA) and equivalent amounts of the pellets and supernatants were analyzed by SDS-PAGE.

**Results**

**Preparation of a-Actinin Fragments**

a-Actinin was purified from chicken gizzard smooth muscle and treated with the enzyme thermolysin to generate two major proteolytic fragments of 27- and 53-kD that were separated by FPLC (Fig. 1). Fluorescent probes were conjugated to the two fragments of a-actinin or to intact a-actinin (Fig. 1) for microinjection into non-muscle cells.

**Localization of Microinjected a-Actinin and the 27- and 53-kD Fragments in Cells**

Intact a-actinin that was microinjected into rat fibroblasts localized periodically along stress fibers (Fig. 2 b) and also at their ends in focal adhesions, as identified by staining with antibodies against talin (Fig. 2 a), consistent with previous observations (Feramisco, 1979; Kreis and Birchmeier, 1980; Sanger et al., 1984, 1986, 1987a; Meigs and Wang, 1986). The intensity of the fluorescent a-actinin incorporated into focal adhesions was variable. We have also found similar variability in the intensity of staining for a-actinin in focal adhesions when cells are stained with antiserum against a-actinin (our unpublished observations). A similar distribution of injected a-actinin was obtained when the protein was labeled with IATR, FITC, or TRITC (data not shown). When the 53-kD fragment was injected into fibroblasts and the cells were fixed ~10 min after being returned to 37°C, the fragment localized predominantly in focal adhesions but not along stress fibers (Fig. 2 d). The β₁ subunit of integrin co-localized with the 53-kD fragment in focal adhesions (Fig. 2 c). The fluorescence intensity of the 53-kD fragment in focal adhesions was considerably weaker than what we have generally observed with injected talin or vinculin. The 27-kD fragment of a-actinin localized along the entire length of stress fibers including the focal adhesions when cells were fixed ~10 min after being injected (Fig. 2, e and f). This is consistent with the 27-kD fragment containing an actin-binding site. We noted that the injected 27-kD fragment stained stress fibers more uniformly than did intact a-actinin which is periodically distributed. The reason for this difference is not clear. Nuclear staining was also observed in cells that were injected with either the fluorescently labeled 27- or 53-kD fragment. Although it is not known why these labeled fragments concentrate in and around the nucleus, we have no reason to believe that it is due to the recognition of a physiologic nuclear binding site. Indeed, FITC-labeled vinculin and the 47-kD talin fragment have also been found to concentrate in the nucleus (Burridge and Feramisco, 1980; Nuckolls et al., 1990).

**Disruption of the Actin Cytoskeleton by Microinjection of a-Actinin Fragments**

Changes in the organization of the actin cytoskeleton were first observed when cells were microinjected with either the 27- or 53-kD fragment of a-actinin and returned to 37°C for ~30 min before fixation. In the examples shown in Fig. 3, the 53-kD fragment which had been microinjected 30 min before fixation was concentrated in most focal adhesions at the ends of actin bundles that were much thinner than normal (Fig. 3, a and b). 30 min after injection of the 53-kD fragment the focal adhesions of injected cells still contained talin (Fig. 3, c and d), vinculin, a-actinin, and β₁ integrin (not shown). 30 min after microinjection of the 27-kD fragment similar changes in stress fibers organization were seen and the 27-kD fragment continued to be detected along thin stress fibers (not shown) although with less fluorescence intensity.

When viewed by IRM, cells that had undergone more extensive disruption of the actin cytoskeleton after injection with either fragment for ~60 min before fixation still retained numerous focal adhesions, predominantly at the cell periphery (Fig. 4, a and b). These adhesions still contained talin (Fig. 4, c and d), vinculin and integrin (not shown), but did not appear to stain for endogenous a-actinin (Fig. 4, e and f). It should be noted, however, that detecting a-actinin in focal adhesions is more problematic than the detection of either talin or vinculin at these sites. In normal cells the level of a-actinin in focal adhesions appears to vary and in some adhesions it is difficult to detect. In case there was a variation with cell type, we have injected both fragments into multiple cell types, including rat embryo fibroblasts, chick embryo.
Figure 2. Microinjection of intact α-actinin and the 53- and 27-kD fragments of α-actinin into rat embryo fibroblasts (REF-52). IATR-α-actinin was microinjected into REF-52 cells and incubated for 2 h before fixation (B). The location of focal adhesions in the same field of cells is shown by immunostaining with antisera against talin, followed by FITC-anti-rabbit Ig (A). FITC-53-kD fragment was microinjected into cells and incubated for 10 min before fixation (D). The distribution of β1 integrin in these cells is shown using β1 antisera followed by RITC-anti-rabbit Ig (C). FITC-27-kD fragment was microinjected into cells and incubated for 10 min before fixation (F). Inset, higher magnification of the cell in F. The distribution of actin is shown by staining with rhodamine-phalloidin (E). Bar, 20 μm.
fibroblasts, epithelial cells (BSC-1 and MDCK) and gerbil fibroma cells (IMR-33), and found that they all respond by disassembling their stress fibers beginning \(\sim 30\) min after injection of these fragments. We generally were unable to detect \(\alpha\)-actinin associated with focal adhesions beyond \(30\) min after injection of either \(\alpha\)-actinin fragment, even though talin and vinculin were still present until \(\sim 60\) min post-injection. Disruption of the cytoskeleton was not observed when cells were injected with fluorescently labeled intact \(\alpha\)-actinin, vinculin or with a control protein, TRITC-labeled ovalbumin.

At \(2\) h after injection, the injected cells had lost essentially all of their stress fibers (Fig. 5). Microtubule organization, however, appeared unaltered by injection of cells with either fragment (not shown). At this advanced stage of stress fiber disruption, cells injected with either fragment had few focal adhesions as determined by IRM (Fig. 6, \(b\) and \(d\)) or by immunofluorescence with antibodies against vinculin (Fig. 6 \(c\)), talin, or \(\beta_1\) integrin (not shown). Those focal adhesions that were present tended to be small and confined to the periphery of the cell.

We estimated that the maximum final concentration of labeled fragments injected into cells was \(\sim 20\) \(\mu\)M based on a needle concentration of 200 \(\mu\)M and assuming the volume of injected protein to be \(\sim 10\%\) of the total cell volume. Needle concentrations of \(\alpha\)-actinin fragments that ranged from \(\sim 50-200\) \(\mu\)M (final concentrations \(\sim 5-20\) \(\mu\)M in cells) resulted in stress fiber and focal adhesion disruption in all cells. We estimated an \(\alpha\)-actinin concentration in cells of 1.5-7.5 \(\mu\)M based on our measurement by RIA of \(\sim 1.5\) pg \(\alpha\)-actinin/fibroblast and a cell volume of 1-5 pl (Pavalko, unpublished results). When needle concentrations of either \(\alpha\)-actinin fragment were reduced below \(\sim 50\) \(\mu\)M (\(\sim 5\) \(\mu\)M final concentration in cells), stress fiber and focal adhesion disassembly were no longer observed suggesting that a molar concentration of \(\alpha\)-actinin fragment approximately equal to or greater than the endogenous \(\alpha\)-actinin concentration was required for cytoskeletal disassembly.

**Cytoskeletal Disruption after Microinjection of \(\alpha\)-Actinin Fragments Was Reversible**

When REF-52 cells were injected with \(\sim 200\) \(\mu\)M concentrations of either fragment of \(\alpha\)-actinin and then returned to
Figure 4. Focal adhesions, detected by IRM and antibody staining, persist in cells 60 min after injection with the 53-kD fragment of α-actinin although most stress fibers have been disrupted. Cells injected with the TRITC-53-kD fragment (indicated by *) were fixed after 60 min and stained with fluorescein-phalloidin (A), talin antisera (C), or α-actinin antisera (E) followed by FITC-anti-rabbit Ig antisera. (The α-actinin antisera was raised against mammalian α-actinin and cross-reacts poorly with chicken α-actinin 53-kD fragment.) (B, D, and F) The corresponding IRM images. Note that numerous small focal adhesions (arrows) are still present in the injected cells, especially around the cell margin. Note also that the focal adhesions remaining in the injected cell in C and D also stain for talin. These adhesions are generally smaller than their counterparts in uninjected cells such as the cell at the bottom of C and D. The injected cell in E and F has no detectable α-actinin in its remaining focal adhesions (arrows) although the uninjected cell at the left of the field stains normally for α-actinin. Bar, 20 μm.
37°C for 18 h before fixation and staining with phalloidin, ~70% of the cells had redeveloped stress fibers (35 of 49 cells injected with 53 kD; 26 of 38 cells injected with 27 kD). The remaining 30% of cells showed little or no stress fiber reformation although the cells remained spread. Since injection with these fragments resulted in a complete loss of stress fibers in all cells after 2 h, the ability of a majority of cells to recover indicates that in most cases the disruption was reversible and was not the result of cell death.

**Analysis of the Actin-binding Properties of α-Actinin Fragments**

The 27-kD fragment has previously been shown to contain the binding site for actin (whereas the 53-kD fragment does not bind to actin) (Mimura and Asano, 1986). This was confirmed in actin sedimentation assays (Fig. 7). Additionally, we found no evidence that 53-kD dimers could undergo an exchange of subunits with intact α-actinin (Fig. 7, lane 19). If such an exchange of subunits had occurred we would have expected to find a proportion of the 53-kD fragment sedimenting with actin/α-actinin complexes. Thus, it seemed unlikely that the 53-kD fragment could directly affect cross-linking of actin filaments by α-actinin or associate with soluble α-actinin to alter the exchange of α-actinin between stress fibers and the cytoplasm.

**Binding of Vinculin to Fragments of α-Actinin**

The binding domain on α-actinin for vinculin was determined by using 125I-labeled vinculin in a protein overlay assay of α-actinin fragments that had been separated by SDS-gel electrophoresis and transferred to nitrocellulose. Fig. 8 shows a Coomassie blue-stained gel of α-actinin digested with thermolysin for increasing lengths of time (A). 125I-vinculin binds to intact α-actinin, to a band just below intact α-actinin of ~90 kD, and to the 27-kD α-actinin fragment. Vinculin also bound to higher molecular weight bands from the crude gizzard extract which probably correspond to talin and to its 190-kD proteolytic fragment, which are both present in this extract and are known to bind vinculin (Burdige and Mangeat, 1984). The band at ~38 kD in lanes 5–9 of Fig. 8 is the enzyme thermolysin, which may bind vinculin since vinculin is a substrate for thermolysin (data not shown). Neither the 53-kD fragment nor its apparent precursors were recognized by vinculin.

**Discussion**

An interaction between purified α-actinin and the cytoskeletal domain of integrin was recently reported (Otey et al., 1990). The binding site on α-actinin for integrin was found...
Disruption of Actin Organization by the 53-kD Fragment

The initial localization of the microinjected 53-kD fragment of α-actinin in focal adhesions but not along stress fibers is consistent with the previously described interaction of this fragment with integrin cytoplasmic domains, but it could also result from other interactions. For example, it has been reported that α-actinin binds to vinculin, another protein concentrated in focal adhesions (Wachsstock et al., 1987). Using a blot assay we have failed to detect an interaction between the 53-kD fragment and vinculin but have shown that this apparent interaction occurs in the 27-kD domain of α-actinin. Alternatively, the 53-kD fragment may localize to fo-
Figure 7. Actin sedimentation assays with α-actinin and the 27- and
53-kD fragments of α-actinin. Supernatants are shown in all even
to 53-kD fragment are mixed together, only intact α-actinin sediments
in lanes (2 and 3) but not alone (lanes 4 and 5). the 27-kD fragment
sediments with actin (lanes 6 and 7) but not alone (lanes 8 and 9).
The 53-kD fragment does not sediment either with actin (lanes 10
and 11) or alone (lanes 12 and 13). When mixed together, intact
α-actinin and the 27-kD fragment sediment with actin (lanes 14 and
15) but not alone (lanes 16 and 17). When intact α-actinin and the
53-kD fragment are mixed together, only intact α-actinin sediments
with actin (lanes 18 and 19) demonstrating that the 53-kD fragment
do not undergo significant subunit exchange with intact α-actinin
dimers. Intact α-actinin does not sediment when mixed with the 53-
kD fragment in the absence of actin (lanes 20 and 21).

Figure 8. 125I-vinculin overlay of α-actinin and α-actinin frag-
ments generated by thermolysin cleavage. Lanes 1-10 show a poly-
acrylamide gel stained with Coomassie blue. Lane 1, molecular
mass standards. Lane 2, fraction of chicken gizzard proteins
enriched for α-actinin after DE52 anion exchange chromatography.
Lane 3, fraction of chicken gizzard proteins after an additional
purification step on a CL-6B gel filtration column. Lane 4, purified
α-actinin after Mono Q anion exchange chromatography. Lanes
5-9, time course of digestion of purified α-actinin with thermolysin
(lane 5 = 10 min, 6 = 30 min, 7 = 45 min, 8 = 90 min, and 9
= 120 min) at 37°C. Lane 10, FPLC-purified 27- and 53-kD frag-
ments mixed back together, i.e., no thermolysin is present in this
lane. Lanes 2'-10', samples identical to those in lanes 2-10 trans-
ferred to nitrocellulose and overlayed with 125I-vinculin. Note that
the vinculin binds to intact α-actinin and to the 27-kD fragment,
but not to the 53-kD fragment. In lane 2', vinculin binds to several
proteins of ~190-220 kD (probably talin and its fragments which
are known to be present in this DE52 fraction) and to unidentified
proteins ~80 kD. Vinculin also binds to thermolysin (TL) at 38 kD.
Molecular masses are shown at left in kilodaltons.

Disruption of stress fibers was complete 2 h after injection
of the 53-kD fragment. The number of focal adhesions was
also reduced although the time course of focal adhesion loss
lagged slightly behind the disassembly of stress fibers. By 2 h
after injection only a few small adhesions remained around
the cell periphery. Stress fiber and focal adhesion disassem-
bly occurred when the concentration of injected 53-kD fragment
exceeded ~5 μM. We had not anticipated this result since
microinjection of vinculin (Burdidge and Feramisco,
1980), talin or talin fragments (Hock et al., 1989; Nuckolls
et al., 1990) does not noticeably disrupt focal adhesions or
stress fibers. After their injection into cells, vinculin, talin,
and talin fragments accumulate in focal adhesions, either by
direct addition to these structures or by exchange with the
proteins already present. In contrast, reversible disruption of
stress fibers in nonmuscle cells, and in some cases of focal
adhesions as well, has been observed following microinjec-
tion of several actin-binding proteins that sever or cap actin
filaments (Fuchtbauer et al., 1983; Jockusch et al., 1985;
Weeds et al., 1985; Cooper et al., 1987; Huckriede et al.,
1990; Franck et al., 1990). Myofibrils in cardiac myocytes,
however, were resistant to disassembly by the actin cap-
ning/severing protein gelsolin (Sanger et al., 1987b) and gel-
solin from some sources had no effect on stress fibers in non-
muscle cells (Cooper et al., 1987; Huckriede et al., 1990).
Stress fibers have also been disrupted by microinjection of
vitamin D binding protein and DNase I, two other proteins
that bind monomeric actin (Sanger et al., 1990).

Our results differ from these previous studies in that the
53-kD fragment of α-actinin does not bind actin directly
(Mimura and Asano, 1986). We have confirmed this lack of
interaction with actin and have presented evidence indicating
that there is no detectable subunit exchange between the
53-kD fragment and intact α-actinin dimers, which would
result in defective molecules unable to cross-link actin
filaments. We can envisage at least three explanations for
why the microinjected 53-kD fragment disrupts stress fibers.
In the first case, the fragment may compete with endogenous
intact α-actinin for binding to integrin cytoplasmic domains
and thereby dislocate one of the attachments of stress fibers
to focal adhesions. The detachment of the stress fiber may
result in its disassembly. A second possible explanation is
that the 53-kD fragment may recognize and bind to a
presently unidentified focal adhesion component that is competed from focal adhesions and is necessary for maintaining actin attachments. Despite the lack of binding of the 53-kD fragment to stress fibers, a third possible explanation is that the 53-kD fragment competes with intact α-actinin for binding to some other protein along the length of stress fibers whose displacement results in an unbundling of actin filaments. Although our data do not yet allow us to distinguish among these possibilities, they suggest a role for protein binding sites within the rod domain of α-actinin in maintaining actin-integrin interactions and stress fiber organization in cells.

**Disruption of Actin Organization by the 27-kD Fragment**

The 27-kD fragment of α-actinin contains the binding site for actin and initially localizes along actin stress fibers and focal adhesions after its microinjection into nonmuscle cells. The actin-binding activity of this fragment presumably accounts for its ability to bind along stress fibers including at their ends where they terminate in focal adhesions. One possible explanation for the subsequent disassembly of stress fibers after microinjection of the 27-kD fragment may lie in the ability of this fragment to bind to vinculin or another, unidentified, focal adhesion component. Interference of a link between vinculin or some other protein and α-actinin at focal adhesions could potentially lead to a weakening of stress fiber-membrane attachments sufficient to disassemble stress fibers. The time course and concentration dependence of stress fiber and focal adhesion disruption was indistinguishable from that seen after injection of the 53-kD fragment. Neither fragment of α-actinin resulted in detachment of cells from the substrate despite the disruption of stress fibers and focal adhesions.

**Stress Fiber Disassembly Precedes Loss of Focal Adhesions**

At intermediate time points (∼60 min) after microinjection of α-actinin fragments, cells that lacked stress fibers often revealed small focal adhesions, particularly at their peripheries. These residual focal adhesions continued to stain for vinculin, talin, and integrin but not α-actinin. Evidence consistent with the loss of focal adhesions being secondary to the loss of stress fibers comes from the studies of Meigs and Wang (1986) who noted that loss of α-actinin from stress fibers and focal adhesions preceded the loss of vinculin from focal adhesions in BSC-1 cells treated with phorbol esters. Stress fibers can also be disrupted by microinjection into cells of a number of agents which appear to decrease or inhibit the interaction of myosin with actin, such as the catalytic subunit of the cAMP-dependent protein kinase, or antibodies to the myosin light chain kinase (Lamb et al., 1988) or type 1 protein phosphatase (Fernandez et al., 1990). Under these conditions it would be interesting to determine whether focal adhesions subsequently disassemble.

**What Is the Role of α-Actinin in Focal Adhesions?**

α-Actinin is normally included in lists of focal adhesion proteins (e.g., Burridge et al., 1988). However, we have frequently observed that the intensity of staining for α-actinin varies among different cell types. Even within a single cell, adhesions may vary in their intensity of staining with α-actinin antibodies. This raises the critical question of the relative role of α-actinin in focal adhesions. We suspect that multiple modes of attachment between actin filaments and the plasma membrane occur at focal adhesions, one of which may involve talin (Burridge et al., 1990) and another of which may involve α-actinin bridging directly between actin and integrin (Otey et al., 1990). Attachments involving different proteins may reflect different stages in the formation of focal adhesions. It could be, for example, that α-actinin is not important in the initial formation of a focal adhesion but is involved in the attachment of mature stress fibers to the focal adhesion. Consistent with this idea, in response to microinjection of the 53-kD fragment, we have observed focal adhesions lacking detectable α-actinin that stain for both vinculin and talin. In general these structures did not support prominent stress fibers. Future experiments will be aimed at testing this idea that the level of α-actinin in a focal adhesion varies with its maturity.

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