The Ca²⁺-binding Domains in Non-muscle Type α-Actinin: Biochemical and Genetic Analysis

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Abstract. Dictyostelium α-actinin is a Ca²⁺-regulated F-actin cross-linking protein. To test the inhibitory function of the two EF hands, point mutations were introduced into either one or both Ca²⁺-binding sites. After mutations, the two EF hands were distinguishable with respect to their regulatory activities. Inactivation of EF hand I abolished completely the F-actin cross-linking activity of Dictyostelium discoideum α-actinin but Ca²⁺ binding by EF hand II was still observed in a ⁴⁰Ca²⁺ overlay assay. In contrast, after mutation of EF hand II the molecule was still active and inhibited by Ca²⁺; however, ~500-fold more Ca²⁺ was necessary for inhibition and ⁴⁰Ca²⁺ binding could not be detected in the overlay assay. These data indicate that EF hand I has a low affinity for Ca²⁺ and EF hand II a high affinity, implying a regulatory function of EF hand I in the inhibition of F-actin cross-linking activity. Biochemical data is presented which allows us to distinguish two functions of the EF hand domains in D. discoideum α-actinin: (a) at the level of the EF-hands, the Ca²⁺-binding affinity of EF hand I was increased by EF hand II in a cooperative manner, and (b) at the level of the two subunits, the EF hands acted as an on/off switch for actin-binding in the neighboring subunit. To corroborate in vitro observations in an in vivo system we tried to rescue the abnormal phenotype of a mutant (Witke, W., M. Schleicher, A. A. Noegel. 1992. Cell. 68:53-62) by introducing the mutated α-actinin cDNAs. In agreement with the biochemical data, only the molecule modified in EF hand II could rescue the abnormal phenotype. Considering the fact that the active construct is "always on" because it requires nonphysiological, high Ca²⁺ concentrations for inactivation, it is interesting to note that an unregulated α-actinin was able to rescue the mutant phenotype.

α-Actinin is the prototype of a family of actin-binding proteins that share common structural and functional characteristics. Dictyostelium discoideum α-actinin, a typical non-muscle type α-actinin, is a homodimer and each subunit consists of three distinct domains (Witke et al., 1991a): (a) an NH₂-terminal actin-binding domain, that is highly conserved in different F-actin-binding proteins (Noegel et al., 1989); (b) a central rod domain formed by four spectrin like α-helical repeats (Baron et al., 1987); (c) two EF hands at the COOH-terminus thought to be responsible for the Ca²⁺ regulation (Noegel et al., 1987; Witke et al., 1991a). So far this domain structure was found in all α-actinins isolated from different organisms and tissues (for review see Blanchard et al., 1989). Skeletal muscle α-actinin, which is Ca²⁺ independent in its cross-linking activity, also contains two, but in most cases incomplete, EF hand structures (Burridge and Feramisco, 1981; Arimura et al., 1988; Waite et al., 1992). The actin-binding domain is conserved in all members of the α-actinin superfamily which includes dystrophin (Koenig et al., 1988), spectrin (Byers et al., 1989) and fimbrin (de Arruda et al., 1990). In the D. discoideum gelation factor (Noegel et al., 1989) and human filamin (Gorlin et al., 1990), representing a second family of F-actin cross-linking proteins, the α-helical repeats and EF hand regions are missing but similar actin-binding domains are responsible for binding to actin filaments (Noegel et al., 1989; Bresnick et al., 1990, 1991). In its native form the two subunits of D. discoideum α-actinin are organized in an antiparallel fashion (Wallraf et al., 1986) leading to a molecule with one actin-binding site at each end of the dimer. The investigation of proteolytic fragments supports an antiparallel dimerization of the α-actinin rod domains (Imamura et al., 1988).

At Ca²⁺-concentrations below 10⁻⁷ M and pH values of 6.6-6.8, D. discoideum α-actinin is highly active in cross-linking actin filaments into a three-dimensional network. Micromolar concentrations of Ca²⁺ and pH values above 7 inhibit the cross-linking activity (Condeelis and Vahey, 1982; Fechheimer et al., 1982). It has not been investigated so far how Ca²⁺ binding and regulation of the activity are exerted by the EF hands. In the case of macrophage α-actinin a stoichiometry of two Ca²⁺ ions per subunit has been found (Bennett et al., 1984), but differences in the Ca²⁺ affinity or the regulatory influence of the two EF hands have not been determined. Mutation of both EF hand domains of D. discoideum α-actinin allowed us to distinguish between the functional importance of the two Ca²⁺-binding sites and their impact on F-actin cross-linking. These results could be
correlated with data from transfection experiments using mutant cells which exhibit a defect in development when two cross-linking proteins are missing (Witke et al., 1992). By introducing selectively mutated α-actinin genes into the double mutant we screened for the minimal requirements that were adequate to rescue the abnormal phenotype.

**Materials and Methods**

**Point Mutation of the EF Hands**

Essentially the strategy described by Ho et al. (1989) was used to introduce into each EF-hand region separately the point mutations via PCR (Saiki et al., 1985). In a second PCR reaction the two mutated fragments were fused by overlap extension as described by Horton et al. (1989). Fig. 1A shows the methodological approach for the production of α-actinin with point mutations in EF hand I. In the first step two overlapping DNA fragments were amplified with internal 70-mer oligonucleotide primers encoding the overlapping region that carried either the four mutations introduced into EF hand I or the four mutations introduced into EF hand II. The amplified fragments were purified by Gene clean (Bio 101 Inc., La Jolla, CA), mixed in a 1:1 ratio and amplified in a second step using the two outer oligonucleotide primers in order to fuse the single fragments (Fig. 1A). The endogenous 1.1-kb Xhol/PvuII fragment encoding the EF hands in α-actinin was exchanged by the mutated 1.1-kb Xhol/PvuII fragment. Screening for clones carrying the mutations was facilitated because of the loss of restriction sites caused by the mutations. Mutation of EF hand I led to the loss of an EcoRI site, while mutation of EF hand II led to the loss of a Clal site. Mutation of both EF hands I + II was performed by using in the first amplification step the oligonucleotide primer for mutation of EF hand I and a clone containing the α-actinin mutated in EF hand I as the template. The second PCR step was done as described above. Clones having both EF hands mutated were selected by their loss of the EcoRI and Clal site. All mutations were verified by sequencing of the amplified fragments. The mutated α-actinin cDNA sequences were cloned into a D. discoideum transformation vector (Paix et al., 1992), which allowed transcription of sequences under the control of the actin 15 promoter, and transformed into the α-actinin-deficient strain HGi130 (Wallraff et al., 1986; Schleicher et al., 1988; Witke and Noegel, 1990) or the α-actinin/gelation factor double mutants (Witke et al., 1992) as described (Witke et al., 1987). The plasmids generated were designated pMI carrying mutations in EF hand I, pMII carrying mutations of EF hand II, and pMIII carrying mutations in both EF hands.

**Protein Purification**

Wild type and mutated α-actinins were prepared from AX2 cells or from transformants of strain HGi130 expressing the mutated α-actinin. 10 liters of stationary phase cells were washed with 17 mM Sorensen buffer, pH 6.0, and pulsed every 6 min with 20 nM cAMP for 6 h. Protein purification was performed as described by Condeelis and Vahey (1982) and modified by Schleicher et al. (1988). The purified proteins were >95% pure as judged from Coomassie blue stained SDS-polyacrylamide gels. The absence of gelation factor in our preparation was proven by immunoblotting and probing with Mabs directed against this protein (Brink et al., 1990). Wild type and mutated α-actinins exhibited identical properties during purification.

**4Ca2+ Overlay**

Equal amounts of purified protein (200 μg, 0.5 mg/ml) were bound onto nitrocellulose (BA85; Schleicher & Schuell, Dassel, Germany) which was equilibrated in buffer A (40 mM NaCl, 10 mM imidazol, pH 6.8) or B (5 mM MgCl2, 40 mM NaCl, 10 mM imidazol, pH 6.8). The filters were incubated for 15 min with 2 μCi/ml (1.2 μM final concentration of Ca2+) 45CaCl2 (0.3 mCi/mg; New England Nuclear, Dreieich, Germany) in the same buffer. After incubation the filters were washed twice for 2 min with 50% ethanol/water, dried, and autoradiographed (Maruyama et al., 1984). The bands were scanned in a CAMAG (Muttenz, Switzerland) electrophoresis scanner.

**Low Shear Viscosity**

Low shear viscosity was performed as described by MacLean-Fletcher and Pollard (1980). 0.5 mg/ml of G-actin (160 μl final volume) was polymerized for 30 min at 25°C with different amounts of normal and mutated α-actinins in the presence of 0.2 mM CaCl2 or 1 mM EGTA in a buffer containing 2 mM MgCl2, 1 mM ATP, 10 mM imidazol, pH 7.2. All values were normalized to control samples without α-actinin and expressed as percent relative viscosity (Pollard and Cooper, 1982).

**D. discoideum Strains**

Strain AX2-214 (Harleff et al., 1990) was used as wild-type strain. The α-actinin negative strain HG1130 (Wallraff et al., 1986) was transformed with the plasmids pMI, pMII, and pMIII giving rise to transformants HG1130(pMI), HG1130(pMII), and HG1130(pMIII) which expressed the corresponding mutated α-actinins. These plasmids were also introduced by cotransformation with pDel09 (Egelhoff et al., 1989) into the gelation factor and α-actinin-deficient strain GA1 (Witke et al., 1992) generating GAI(pMI), GAI(pMII), and GAI(pMIII).

**Dictyostelium Growth and Transformation**

D. discoideum strains were grown at 21°C, either on SM agar plates with Enterobacter aerogenes (Williams and Newell, 1986), or in liquid medium (Claviez et al., 1982). DNA was introduced using the method described by Nellen et al. (1984). Transformants were selected in medium containing 20 μg gentamicin (Sigma Chemicals, St. Louis, MO) per ml, and cloned by dilution on SM agar plates with E. aerogenes. When plasmids were introduced by cotransformation with plasmid pDel09 (Egelhoff et al., 1989) transformants were selected in HLP medium containing hygromycin (30 μg/ml) (Calbiochem-Novabiochem, Bad Soden, Germany). For screening of α-actinin expressing mutant strains, the colony blot technique was used (Wallraff et al., 1986). The blots were labeled with iodinated α-actinin specific mAb47-62 (Schleicher et al., 1988).

**Southern, Northern, and Western Blots**

DNA and RNA from wild type and transformant cells were prepared as described (Noegel et al., 1985). For hybridization analysis α-actinin cDNA fragments were used (Noegel et al., 1987). Hybridizations were done for 16 h at 37°C in a hybridization buffer containing 50% formamide and 2× SSC. SDS-PAGE was performed in 10% gels (Laemmli, 1971). Total proteins from 2 × 106 cells were separated per lane, blotted onto BA85 nitrocellulose (Schleicher & Schuell, Dassel, Germany) and incubated with 125I-labeled antibodies.

**Miscellaneous Methods**

For experiments performed in the presence of Ca2+-EGTA buffers, stock solutions containing variable molar ratios of Ca2+ and EGTA (2:1-2:1) were used. The final EGTA concentration was 1 mM in all experiments, the free Ca2+ concentration was calculated with a program kindly provided by Dr. A. Wegner (Ruhr-Universität Bochum, Bochum, Germany). Values were corrected for the Mg2+ and ATP concentrations present.

Protein concentrations were determined by the method of Bradford (1976). Immunoblots were performed as described by Towbin et al. (1979). Sequencing of plasmid DNA was carried out according to Sanger et al. (1977) as modified by Chen and Seeburg (1985).

**Results**

**Point Mutations in EF Hand I and II and Expression of Mutated α-Actinins in D. discoideum**

EF hands are defined as helix-loop-helix Ca2+-binding motifs (Kretsinger, 1980) where the loop usually consists of 12 contiguous residues from which the complexing oxygens are derived. In the original description of an octahedral geometry six residues (#1, 3, 5, 7, 9, and 12) occupy the vertices and the free Ca2+ concentration was calculated with a program kindly provided by Dr. A. Wegner (Ruhr-Universität Bochum, Bochum, Germany). Values were corrected for the Mg2+ and ATP concentrations present.

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The α-actinin cDNA was inserted into a D. discoideum vector to express the altered α-actinins under the control of the Dictyostelium actin 15 promoter (Pfax et al., 1992). The α-actinin defective D. discoideum strain HGI130 (Walruff et al., 1986; Schleicher et al., 1988; Witke and Noegel, 1990) was used for transformation. Clones expressing high amounts of mutated α-actinin were isolated. The amounts of α-actinin represented at least 2% of total cellular protein as judged from Coomassie blue-stained gels. In wild type AX2 cells, α-actinin amounts to ~1% of total cellular protein. The isolation procedures for wild type and mutated α-actinins were identical and no different behavior during the various purification steps was noted.

Ca2+ Binding of Normal and Mutated D. discoideum α-Actinin

The Ca2+-overlay assay for Ca2+-binding proteins (Murayama et al., 1984) gave highly reproducible results in all experiments. Purified normal or mutated α-actinin was bound to nitrocellulose and incubated with 45Ca2+ in the presence and absence of 5 mM Mg2+ (Fig. 2). In α-actinin with only EF hand II intact, Ca2+ binding was observed and the signal was reduced by ~55% compared to normal α-actinin. Inactivation of EF hand II and leaving EF hand I intact, led to a loss of Ca2+ binding in the overlay assay. The same result was obtained when both EF hands were mutated. We conclude from the 45Ca2+-overlay assays that binding of Ca2+ to EF hand II in α-actinin can be detected due to the high

Figure 2. 45Ca2+ overlay with α-actinin and α-actinin mutated in EF hand I or II. Purified proteins were bound to nitrocellulose and incubated with 45CaCl2 in the presence (+) or absence (-) of 5 mM Mg2+. The same amount of a 26-kD polypeptide representing the actin-binding domain of α-actinin was bound as a control for non-specific binding of Ca2+. The amount of radioactivity bound by intact α-actinin (-Mg2+) was set to 100% and Ca2+ binding by mutated α-actinins was quantified as relative to that of wild type α-actinin. The sum of Ca2+ binding by single EF hands in GAI (pM I) and GAI (pM II) is less than that in wild type α-actinin. This indicates cooperativity when both EF-hands are intact.

Figure 1. (A) Alignment of the EF hands of α-actinin. Only the mutation of EF hand I is shown; the mutation of EF hand II was carried out in similar manner. For EF hand I and II mutation an α-actinin cDNA carrying a mutation in EF hand II was the template in the PCR reaction. In the polymerase chain reaction #1 two DNA fragments were amplified in separate experiments which overlapped in the region carrying the mutations. The template was a XhoII/PvuII fragment encoding the two EF hands. The oligonucleotides used are indicated by arrows. An X indicates oligonucleotides carrying mutations of the EF hands (black bars). In the reaction #2 the products a and b from PCRs #1 after gel purification were amplified with oligonucleotides located at the ends of the XhoII/PvuII fragment. The resulting product was cloned into α-actinin cDNA thereby exchang

from different sources. The positions important for Ca2+ binding are indicated as well as the essential amino acids in EF hands I and II. To test the affinities and regulatory properties of EF hands I and II we substituted the Ca2+-liganding amino acids by alanine and expressed the mutated α-actinins in D. discoideum. In a first construct we mutated the amino acids at position X, Y, Z, and -Z in EF hand I. A second construct carried mutations at position X, Y, -X, -Z in EF hand II. In a third construct the mutations in EF hands I and II were combined to generate a molecule with two non-functional EF hands (Fig. 1). The mutated α-actinin cDNAs were cloned into a Dictyostelium transformation vector to express the altered α-actinins under the control of the Dictyostelium actin 15 promoter (Pfax et al., 1992). The α-actinin defective D. discoideum strain HGI130 (Walruff et al., 1986; Schleicher et al., 1988; Witke and Noegel, 1990) was used for transformation. Clones expressing high amounts of mutated α-actinin were isolated. The amounts of α-actinin represented at least 2% of total cellular protein as judged from Coomassie blue-stained gels. In wild type AX2 cells, α-actinin amounts to ~1% of total cellular protein. The isolation procedures for wild type and mutated α-actinins were identical and no different behavior during the various purification steps was noted.

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affinity for Ca\textsuperscript{2+}; second, the affinity of EF hand I is low compared to EF hand II and therefore Ca\textsuperscript{2+} binding is not detectable in the overlay assay; and third, relative to 100% compared to EF hand II and therefore Ca\textsuperscript{2+} binding is not detected in native α-actinin; this suggests in wild type α-actinin an intact single EF hand/actin ratio of 1:25 in a Ca\textsuperscript{2+}-EGTA buffer system. For normal α-actinin a transition from the active to the inactive state occurred in the range of 5 × 10\textsuperscript{-8} M to 1 × 10\textsuperscript{-5} M free Ca\textsuperscript{2+} (Fig. 4; see also Fechheimer et al., 1982). 50% inhibition was obtained at 10\textsuperscript{-7} M free Ca\textsuperscript{2+}, which is close to the intracellular Ca\textsuperscript{2+} concentration in a resting cell. The Ca\textsuperscript{2+} dependency of α-actinin mutated in EF hand II was also assayed (Fig. 4). Compared to native α-actinin the half-maximal inhibition for the protein mutated in EF hand II was shifted to 50 μM Ca\textsuperscript{2+}.

The results indicate that the two EF hands in α-actinin are distinct in their regulatory properties. EF hand I binds Ca\textsuperscript{2+} with low affinity and is necessary for Ca\textsuperscript{2+} inhibition of the cross-linking activity. Furthermore, for α-actinin with a mutated EF hand I no cross-linking activity was observed. Together these data indicate a crucial function of EF hand I for Ca\textsuperscript{2+} regulation and for the active state of α-actinin in the absence of Ca\textsuperscript{2+}. EF hand II binds Ca\textsuperscript{2+} with high affinity and most likely increases the sensitivity for Ca\textsuperscript{2+} inhibition. Comparison of the Ca\textsuperscript{2+} sensitivity of native α-actinin and α-actinins mutated in EF hand II (Fig. 4) suggests a cooperative Ca\textsuperscript{2+} binding of both EF hands in native α-actinin in such a manner that the affinity of EF hand I is increased if EF hand II binds Ca\textsuperscript{2+}.

Low-shear viscometry is a highly sensitive but not quantitative assay used to detect minute F-actin cross-linking activities. To obtain qualitative data and to test whether the α-actinin derivatives were still able to bind to F-actin without necessarily to exhibit F-actin cross-linking activity, we also used a spin-down assay using an airfuge. With this approach we found that all α-actinin modifications resulted in some cosedimentation with F-actin although clearly reduced in comparison to wild type α-actinin. Due to the qualitative nature of this assay we prefer not to calculate dissociation constants on this experimental basis. However, the ability of the α-actinin derivatives to interact with F-actin in a spin-down assay is in agreement with the residual binding properties of isolated actin-binding regions of α-actinin (unpublished data; Way et al., 1992).

Expression of Mutated α-Actinins in the D. discoideum Mutant GA1

Dictyostelium mutants deficient in the two F-actin cross-linking proteins α-actinin and gelation factor show an impaired morphogenesis and do not complete development under routine laboratory conditions. Development can be restored by expressing native α-actinin or gelation factor (Witke et al., 1992). To test the in vivo activity of the mutated α-actinins, the corresponding expression vectors were introduced into the double mutant GA1 by cotransformation with pDel09 (Egelhoff et al., 1989). In strain GA1 the gelation factor gene had been inactivated by nitrosoguanidine treatment and the α-actinin gene disrupted after homologous recombination. Several independent colonies expressing mutated α-actinin were identified by immunoblotting. Development was tested on SM agar plates containing E. aerogenes. Fig. 5 shows cell aggregates after 48 h of development on agar plates. In wild type strains this time period would be sufficient to allow formation of fruiting bodies with fully developed stalks and spore heads. The double mutant GA1 lacking the two F-actin cross-linking proteins is blocked.
Figure 4. Ca²⁺ inhibition of native α-actinin and α-actinin mutated in EF hand II. The cross-linking activity of native and the mutated α-actinin was measured at constant protein concentrations in a Ca²⁺-EGTA buffer. The protein concentration was kept at 18 μg/ml (0.1 μM) for native α-actinin and 120 μg/ml (0.65 μM) for the mutated α-actinin. The maximal cross-linking activity in the absence of Ca²⁺ was set to 100%. Whereas normal α-actinin shows a sigmoidal curve with the activity being regulated in a range of 1–2 orders of magnitude (○), the mutated α-actinin shows a wide range of Ca²⁺ inhibition (●), which could be explained by a cooperative influence of EF hand II on EF hand I in normal α-actinin. From the Ca²⁺ concentration needed for half maximal inhibition, dissociation constants of 10⁻⁷ M for native α-actinin and 5 × 10⁻⁵ for α-actinin mutated in EF hand II can be calculated. The error bars show the deviations of at least three measurements.

at the formation of tipped aggregates. The same blockage was observed after introducing pMI and pMIII into the double mutant. Only transformants expressing an α-actinin that carried mutations in EF hand II were able to complete development (Fig. 5).

Analysis of Rescued Mutant Strains on the DNA and Protein Level

Since homologous recombination is a frequent event in

Discussion

The distance between the actin-binding domain and the EF-hands of an α-actinin subunit is more than 20 nm as measured by EM (Meyer and Aebi, 1990) and a mutual regulatory influence between the two subunits has been suggested (Noegel et al., 1987). Since two α-actinin monomers form a dimer with antiparallel subunits, the actin-binding site and the EF hands probably form the ends of the molecule, which are often seen as globular structures in the electron microscope (Wallraff et al., 1986; Imamura et al., 1988). A direct interaction between the EF hands of one molecule and the actin-binding site of the second one has not been demonstrated so far. Such an interaction is not required for F-actin binding of α-actinin since amino terminal peptides are able to bind to and to cosediment with F-actin (Imamura et al., 1988; Tokuue et al., 1991; Witke et al., 1991b; Hemmings et al., 1992). However, as shown here, F-actin cross-linking activity is diminished to different extents as soon as the EF

Figure 5. Morphology of mutant GA1 and transformed mutant strains GA1(pMI), GA1(pMII), and GA1(pMIII). Dic- tyostelium strains were grown on SM agar plates containing E. aerogenes. GA1, GA1(pMI) and GA1(pMIII) formed tipped aggregates, GA1(pMII) formed fruiting bodies consisting of spores and stalks.
hand regions are genetically manipulated. In fact, any mutation we introduced in the EF hand regions (irrespective whether these were deletions, insertions, or point mutations) lowered the cross-linking activity of the mutated α-actinins as compared to wild type protein (unpublished data). This suggests that the actin-binding site of one strand requires an EF hand domain from the second subunit for high affinity binding to F-actin. Consistent with this observation is the finding that a peptide containing only the F-actin-binding domain (26 kD, used in Fig. 2 as a control) exhibited a reduced affinity for F-actin (unpublished). This stabilizing function might explain why all α-actinins whose sequences are known possess EF hands irrespective of exhibiting Ca²⁺-regulated or unregulated cross-linking activity.

Interactions between two chains and their impact on F-actin binding and cross-linking activity has also been reported for fodrin. Fodrin belongs to the α-actinin superfamily and consists of α- and β-subunits. The β-subunit has an actin-binding site at its amino terminus which is closely related to the α-actinin actin-binding site, the α-subunit carries two EF hand structures. Cleavage of the α-chain by the protease calpain rendered the fodrin molecule Ca²⁺ sensitive with regard to F-actin cross-linking which is indicative of an interaction between actin-binding site and EF hand structures (Harris and Morrow, 1990).

**Ca²⁺ Regulation of Cross-linking Activity**

A very important difference of our studies on α-actinin as compared to similar studies performed on the monomeric molecules calmodulin or troponin C is the complexity of the EF hand region of α-actinin in conjunction with the neighboring actin-binding site of the second subunit. In principle we have to consider the EF hands of α-actinin as being similar to the EF hands of calmodulin in a complex with a calmodulin-binding protein. This renders studies on isolated EF hands in α-actinin as artificial and therefore we were interested mainly in an overall analysis of the cross-talk between two EF hands of one subunit and the actin-binding region of the corresponding subunit.

In all known α-actinin sequences two EF-hands with different degrees of conservation were found in the COOH-terminal region despite the fact that only non-muscle type α-actinins including *Dictyostelium discoideum* α-actinin, but not skele-

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**Figure 6.** Northern and Southern blot analysis of mutant GAI expressing α-actinin with mutations in EF hand II. (A) 10 μg of total RNA from wild type AX2 cells, mutant GAI, and GAI(pMII) was separated under denaturing conditions in a 1.2% agarose gel, transferred to nitrocellulose and probed with the ³²P-labeled 1.2-kb EcoRI fragment derived from the α-actinin gene. The sizes of the mRNA species detected are indicated. In B a Southern blot analysis of nuclear DNA from strains AX2, GAI and GAI(pMII) is shown. 10 μg of DNA were digested with NdeI and the fragments separated in an 0.7% agarose gel in Tris-phosphate buffer, pH 7.8. The α-actinin–specific fragments were detected with the ³²P-labeled 0.7-kb EcoRI fragment derived from α-actinin cDNA. The location of HindIII-generated λDNA fragments is indicated. In C a schematic drawing of the gene disruption event in GAI is shown. The vector pDabx1.2 integrated via its α-actinin gene-derived 1.2-kb EcoRI fragment into the endogenous sequences thereby introducing an NdeI site into the α-actinin gene. The neomycin resistance gene of vector pDabx1.2 is hatched, the 1.2-kb EcoRI fragment is cross-hatched. The location of the 0.7-kb EcoRI fragment which was used as probe is indicated by a bar. It recognized an enlarged fragment of 9.0 kb in GAI and GAI(pMII).
tural muscle α-actinin are regulated by Ca²⁺ (Burridge and Feramisco, 1981). A comparison of the EF hand sequences present in α-actins (Fig. 1B) revealed that most of these regions are defective and probably do not bind Ca²⁺. An ideal EF hand structure is present in the first EF hand of Droso-phiila muscle α-actinin, and an almost complete set of liganding oxygens (excluding the -X position) can be found in EF hands I of chicken fibroblast α-actinin (Arimura et al., 1988) and brain α-actinin (Waites et al., 1992; Fig. 1B).

In contrast to these predictions, macrophage α-actinin was found to bind two Ca²⁺ ions per subunit (Bennett et al., 1984). The reason for this discrepancy is unknown. In the case of skeletal muscle α-actinin none of the EF hands fulfills the criteria of functional Ca²⁺-binding loops.

A comparison of the EF hands in D. discoideum α-actinin shows clearly that EF hand I is not ideal, since it lacks the obligatory Gly 758 (Asn will not substitute) and has unusually a hydrophobic residue in place of the usual acidic or basic amino acid next to the -Z glutamic acid (Fig. 1B, V and X, respectively). EF hand II of D. discoideum α-actinin has the ideal structure of a Ca²⁺-specific site. These predictions are fully consistent with the findings in this paper in showing a high-affinity Ca²⁺-specific site in EF hand II and a low-affinity regulatory site in EF hand I.

It is a matter of common sense that the use of genetically engineered proteins in biochemical studies on structure/function relationships carries some limitations. Structural data from NMR spectroscopy or crystallography would show whether a specific change in a protein sequence in fact accounts for the differences observed at the biochemical level. To overcome at least some of these limitations we chose D. discoideum for expression of the mutated proteins in their native environment and a genetic rescue experiment of mutants. From introducing point mutations into EF hands I and II of D. discoideum α-actinin the conclusion can be drawn that EF hand I confers as regulatory site the Ca²⁺-inhibition of the cross-linking activity. EF hand II supports the regulatory function of EF hand I by increasing the sensitivity to Ca²⁺. The sigmoid shape of the activity curve and the data from the ⁴⁰Ca²⁺ overlay an indication of such a mechanism. These findings might explain why fibroblast and brain α-actinin with almost ideal EF hands I are Ca²⁺-sensitive but because of the lack of an intact EF hand II the sensitivity towards Ca²⁺ is lowered. In the case of brain α-actinin half maximal inhibition was observed at 10⁻⁸ M Ca²⁺ (Duhaiman and Bamberg, 1984). Under these conditions D. discoideum wild type α-actinin is nearly inactive (see Fig. 4), whereas for half-maximal inhibition of α-actinin with mutated EF hand II 5 × 10⁻⁸ M Ca²⁺ are required. Furthermore, Waites et al. (1992) reported that they could not demonstrate ⁴⁰Ca²⁺ binding in a blot assay for brain α-actinin. These observations are very similar to the data obtained with mutated α-actinin carrying mutations in EF hand II. The idea that EF hand I is the key regulatory Ca²⁺-binding site in α-actinin is consistent with the observation that this is a region of alternative splicing in the chick α-actinin gene described by Waites et al. (1992). This results in expression of a smooth (Ca²⁺ insensitive) and non-muscle (Ca²⁺ sensitive) isoform of the protein from a single gene.

**In Vivo Role of α-Actinin**

Dictyostelium mutants lacking both α-actinin and the gela-

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