CRP1, a LIM Domain Protein Implicated in Muscle Differentiation, Interacts with α-Actinin

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Abstract. Members of the cysteine-rich protein (CRP) family are LIM domain proteins that have been implicated in muscle differentiation. One strategy for defining the mechanism by which CRPs potentiate myogenesis is to characterize the repertoire of CRP binding partners. In order to identify proteins that interact with CRP1, a prominent protein in fibroblasts and smooth muscle cells, we subjected an avian smooth muscle extract to affinity chromatography on a CRP1 column. A 100-kD protein bound to the CRP1 column and could be eluted with a high salt buffer; Western immunoblot analysis confirmed that the 100-kD protein is α-actinin. We have shown that the CRP1–α-actinin interaction is direct, specific, and saturable in both solution and solid-phase binding assays. The K_d for the CRP1–α-actinin interaction is 1.8 ± 0.3 μM. The results of the in vitro protein binding studies are supported by double-label indirect immunofluorescence experiments that demonstrate a colocalization of CRP1 and α-actinin along the actin stress fibers of CEF and smooth muscle cells. Moreover, we have shown that α-actinin coimmunoprecipitates with CRP1 from a detergent extract of smooth muscle cells. By in vitro domain mapping studies, we have determined that CRP1 associates with the 27-kD actin–binding domain of α-actinin. In reciprocal mapping studies, we showed that α-actinin interacts with CRP1-LIM1, a deletion fragment that contains the NH_2-terminal 107 amino acids (aa) of CRP1. To determine whether the α-actinin binding domain of CRP1 would localize to the actin cytoskeleton in living cells, expression constructs encoding epitope-tagged full-length CRP1, CRP1-LIM1(aa 1-107), or CRP1-LIM2 (aa 108-192) were microinjected into cells. By indirect immunofluorescence, we have determined that full-length CRP1 and CRP1-LIM1 localize along the actin stress fibers whereas CRP1-LIM2 fails to associate with the cytoskeleton. Collectively these data demonstrate that the NH_2-terminal part of CRP1 that contains the α-actinin–binding site is sufficient to localize CRP1 to the actin cytoskeleton. The association of CRP1 with α-actinin may be critical for its role in muscle differentiation.

Myogenesis is a complex multistep process that involves the specification of muscle progenitor cells, the determination of a subset of these cells to become myoblasts, the proliferation of these determined cells, and ultimately the differentiation of these cells into fully functional muscle. A variety of growth factors and transcription factors, including members of the MyoD family of basic helix–loop–helix proteins and the MEF2 family, contribute to the coordinated control of muscle cell differentiation. These myogenic factors regulate both the exit of myoblasts from the cell cycle as well as the initiation of muscle-specific gene transcription (Cossu et al., 1996; Molkentin and Olson, 1996). The ultimate product of the muscle differentiation program is the ordered assembly of an actomyosin-rich contractile machinery.

Recently, members of a family of proteins called the cysteine-rich protein (CRP)[1] family have been shown to be involved in a late stage in muscle differentiation. In vertebrates, the CRP family is comprised of three closely related proteins, CRP1, CRP2, and the muscle LIM protein (MLP), also referred to as CRP3 (Weiskirchen et al., 1995). CRP1 expression is prominent in smooth muscle derivatives and is correlated with muscle development in avian embryos (Crawford et al., 1994). Furthermore, overexpression of either CRP1 or MLP/CRP3 potentiates the differentiation of myoblasts in culture (Arber et al., 1994). Perturbation of MLP/CRP3 expression by anti-sense RNA technology results in a failure of muscle differentiation (Arber et al., 1994). Similarly, elimination of MLP/CRP3 function in the mouse by targeted gene disruption results in dramatic disorganization of myofibrils (Arber et al., 1997). Recently,

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1. *Abbreviations used in this paper*: aa, amino acids; CEF, chicken embryo fibroblasts; CRP, cysteine-rich protein; HBB, Hepes binding buffer; GST, glutathione-S-transferase.

two *Drosophila* CRP family members, *Mlp60A* and *Mlp84B*, have also been described (Arber et al., 1994; Stronach et al., 1996). Both proteins exhibit muscle-specific expression in developing embryos and a cytoskeletal localization when expressed in vertebrate cells (Stronach et al., 1996). Collectively, although the specific mechanism of action of CRP family members is unknown, the available data suggest a role for CRPs as essential positive regulators of muscle differentiation.

Members of the CRP family exhibit a conserved molecular architecture (Weiskirchen et al., 1995). CRPs exhibit two tandemly arrayed LIM domains, each of which is flanked by a conserved glycine-rich repeat (Weiskirchen et al., 1995). The LIM domain is a cysteine-rich sequence ($CX_2CX_{16-23}HX_2CX_2CX_2CX_{16-21}CX_2$[C,H,D]) (Freyd et al., 1990; Sadler et al., 1992) that coordinates two zinc atoms (Michelsen et al., 1993) and mediates specific protein–protein interactions (Schmeichel and Beckerle, 1994). LIM domains are found in a number of proteins that are involved in control of gene expression and cell differentiation. The LIM motif was first identified in three developmentally regulated transcription factors, *Caenorhabditis elegans* Lin-11, rat Isl-1, and *C. elegans* Mec-3, from which the term LIM is derived (Freyd et al., 1990; Karlsson et al., 1990). LIM domains can be found in association with functional domains such as kinase domains, transcriptional activation domains, or DNA-binding homeodomains. Alternatively, LIM domains sometimes represent the primary sequence information in a protein.

In addition to their common structural features, CRP family members are functionally related as well. CRP1 was initially identified as a binding partner for zyxin, a low abundance phosphoprotein that is concentrated at adhesion plaques and in association with actin filament arrays (Sadler et al., 1992; Crawford et al., 1994). All three CRP family members have now been shown to bind directly to zyxin (Louis et al., 1997). Moreover, all three proteins are prominently associated with the actin cytoskeleton (this report; Louis et al., 1997).

To understand the mechanism by which CRP1 affects muscle differentiation, we have initiated an effort to identify CRP1-binding proteins in chicken smooth muscle, the source from which CRP1 was originally purified (Crawford et al., 1994). Here we report that CRP1 interacts directly with the actin-binding protein, α-actinin. Moreover, we demonstrate that the two proteins are substantially colocalized along the actin stress fibers. The findings reported here suggest that CRPs may function as regulators of myogenesis by virtue of their ability to interact directly with α-actinin, an essential structural element in the myofibril.

Materials and Methods

Isolation of Avian Smooth Muscle Proteins

Avian smooth muscle proteins were extracted from frozen chicken gizzards as described previously (Crawford and Beckerle, 1991; Crawford et al., 1994). The resulting extract was sequentially precipitated with 27–34, 34–43, and 43–61% saturated ammonium sulfate. These ammonium sulfate precipitates were dialyzed against the column buffer (20 mM Tris-acetate, pH 7.6, 0.1% 2-mercaptoethanol, 0.1 mM EDTA) before loading onto affinity columns. The 27–34% ammonium sulfate precipitate contains α-actinin whereas the 34–43% ammonium sulfate precipitate contains CRP1.

Purification and Radioiodination of α-Actinin from Avian Smooth Muscle

α-Actinin was purified from the 27–34% ammonium sulfate precipitate as described previously (Crawford et al., 1992). Cleavage of α-actinin by the proteolytic enzyme thermolysin (Sigma Chemical Co., St Louis, MO) was performed in 40 mM ammonium acetate, 1 mM $CaCl_2$ for 5 h at 20°C with an enzyme to substrate ratio of 1:25. The α-actinin concentration was 3.2 mg/ml.

Purified α-actinin was radioiodinated as described previously (Crawford et al., 1992), except that the incubation period of α-actinin with [^{125}I]Na was reduced to 2.5 min. The purity of the labeled α-actinin was ascertained by SDS-PAGE followed by autoradiography.

Purification of Bacterially Expressed CRP1, CRP1-LIM1, and CRP1-LIM2

CRP1-LIM1 corresponds to the NH_2-terminal part of chicken CRP1 (cCRP1) (amino acids [aa] 1–107) including the NH_2-terminal LIM domain followed by the first glycine-rich repeat of the protein. CRP1-LIM2 corresponds to the COOH-terminal portion of cCRP1 (aa 108–192) containing the COOH-terminal LIM domain and the second glycine-rich repeat of the protein. Techniques for the purification of the bacterially expressed full-length CRP1 and CRP1-LIM2 were described previously (Michelsen et al., 1993; Kosa et al., 1994). CRP1-LIM1 was purified in the same manner as CRP1-LIM2 except that the CM-52 cation-exchange column was equilibrated in 5 mM potassium phosphate and 0.01% 2-mercaptoethanol.

hCRP1 Expression, Isolation, and Radiolabeling

A plasmid engineered for the bacterial expression of human CRP1 (hCRP1) was generously provided by S.A. Liebhaber. The methods for expression, purification and radiolabeling of the glutathione-S-transferase (GST)-hCRP1 fusion protein were described previously (Feuerstein et al., 1994). The GST–hCRP1 fusion protein was used in blot overlay assays and in the solution binding assay whereas cCRP1 was used in all the other experiments. There is a high degree of sequence similarity between human and chicken forms of CRP1 (91% identity; Crawford et al., 1994), and the two proteins appear to be functionally interchangeable.

Affinity Chromatography

Bacterially expressed CRP1 or BSA was covalently coupled to Affi-gel 10 (Bio-Rad Laboratories, Hercules, CA) in coupling buffer (0.1 M Hepes, pH 7.8, 0.1% 2-mercaptoethanol, 0.1 mM EDTA) for 4 h at 4°C. The affinity resins were transferred to two different columns, washed with coupling buffer, and then equilibrated with the column buffer (20 mM Tris-acetate, pH 7.6, 0.1% 2-mercaptoethanol, 0.1 mM EDTA). A 27–34% ammonium sulfate precipitate from avian smooth muscle was loaded onto each column. The columns were washed with 0.1 M NaCl in column buffer. Proteins eluted with 1 M NaCl in column buffer were collected in 300-μl fractions and 15 μl of each fraction were resolved by electrophoresis on 12.5% SDS–polyacrylamide gels. α-Actinin was detected by Western immunoblot analysis using a polyclonal antibody raised against chicken α-actinin that was generously provided by K. Burridge.

Gel Electrophoresis and Western Immunoblot Analysis

SDS-PAGE was performed according to the method of Laemmli (1970) except with 0.13% bisacrylamide. 12.5% polyacrylamide gels were used routinely, however 17.5% gels were employed to resolve low mol wt proteins such as CRP1-LIM1 and CRP1-LIM2. Western immunoblot analysis was performed using horseradish peroxidase linked to protein A (Amersham Life Science Inc., Cleveland, OH) as a second reagent and enhanced chemiluminescent detection (Amersham Life Science Inc.).

Solution Binding Assay

GST-hCRP1 or GST agarose beads were incubated at 20°C with purified α-actinin or a 27–34% ammonium sulfate precipitate from avian smooth

muscle for 1.5 h on an orbital shaker. The agarose beads were washed three times with PBS and three times with buffer B10 (20 mM Tris-acetate, pH 7.6, 10 mM NaCl, 0.1 mM EDTA, 0.1% 2-mercaptoethanol). The beads were then mixed in 40 μl 2× Laemmli sample buffer (Laemmli, 1970), boiled, and the supernatants were analyzed by SDS-PAGE and Western immunoblot using a polyclonal antibody raised against chicken α-actinin.

In competition experiments, GST-hCRP1 agarose beads were incubated at 20°C with 100 μl of [^{125}I]α-actinin (500,000 cpm) for 1.5 h on an orbital shaker in the absence of competing protein or in the presence of unlabeled α-actinin or BSA. The agarose beads were washed three times with PBS, centrifuged, and the counts bound to the agarose beads were analyzed using a Packard Multi-Prias 1 γ counter (Packard Instrument Co., Inc., Meriden, CT).

Blot Overlay Assay

Blot overlay assays were performed as previously described (Crawford et al., 1992). Proteins were resolved by SDS-PAGE and transferred to nitrocellulose. The nitrocellulose strips were incubated in the presence of [^{32}P]GST or [^{32}P]GST-hCRP1 fusion protein probes (600,000 cpm/ml), or an [^{125}I] α-actinin probe (250,000 cpm/ml). For competition experiments, unlabeled competing proteins were added into the blot overlay buffer immediately before the introduction of the labeled probe. Autoradiography was performed at −80°C with an intensification screen.

Solid-phase Binding Assay

Removable microtiter wells (Dynatech Laboratories, Inc., Chantilly, VA) were coated overnight at 4°C with 120 μl of bacterially expressed CRP1 at 0.1 mg/ml. The wells were washed three times with Hepes binding buffer (HBB) (20 mM Hepes, pH 7.4, 10 mM NaCl, 0.1 mM EGTA, 0.1% 2-mercaptoethanol) and blocked with 300 μl 2% BSA in HBB. After a 120-min incubation at 37°C, the blocking solution was removed and the wells were washed with HBB plus 0.2% BSA. The wells were next incubated for 2.5 h at 37°C with [^{125}I]α-actinin, in the presence of competing proteins in HBB. The final volume was 120 μl. At the end of the incubation period, the radioactive material was removed from the wells and they were washed six times with HBB plus 0.2% BSA followed by a final rinse in HBB. The wells were air dried and bound counts were determined using a γ counter. For these solid-phase binding studies, the α-actinin was radioiodinated to a specific activity between 5.8×10^6 and 14.4×10^6 cpm/μg.

Confocal Immunofluorescence Microscopy

Chicken embryo fibroblasts (CEF) were cultured on glass coverslips in DME supplemented with 10% FBS. Smooth muscle cells were prepared from gizzards taken from 16-d-old chicken embryos as previously described, except that trypsin was used instead of collagenase (Gimona et al., 1990). Primary cultures derived from smooth muscle contained both fibroblasts and smooth muscle cells. We previously showed that differentiated smooth muscle cells, which express the smooth muscle marker calponin, also exhibit dramatically higher levels of CRP expression than fibroblasts and undifferentiated smooth muscle cells in the culture (Crawford et al., 1994). Based on these observations, one can unequivocally identify differentiated smooth muscle cells in the population based on their CRP levels. Double-label indirect immunofluorescence (Beckerle, 1986) was performed using an anti–α-actinin primary monoclonal antibody (ICN Biomedicals Inc., Irvine, CA) followed by an FITC-conjugated goat anti–mouse secondary antibody, and an anti–cCRP1 primary polyclonal antibody (B37) raised against the eleven carboxy-terminal amino acids GQGAGALIHSQ of cCRP1 followed by a Texas red–conjugated goat anti–rabbit secondary antibody. The B37 antibody was generated by K. Shepard and J.D. Pino in the Beckerle laboratory (University of Utah, Salt Lake City, UT). All fluorochrome-labeled secondary antibodies were obtained from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). Cells were viewed on a confocal laser scanning microscope (Bio-Rad Laboratories, Hercules, CA) with an optical section height of 1 μm.

Cell Labeling and Immunoprecipitation

CEF cells were radiolabeled with [^{35}S]methionine-cysteine (Tran^{35}S-label; ICN Biomedicals Inc., Irvine, CA). Metabolic labeling was carried out with adherent cells that were washed twice with PBS at 37°C and incubated in one part DME, plus nine parts DME without methionine and cysteine supplemented with 10% FBS in the presence of 200 μCi of [^{35}S]methionine-cysteine for 18 h. After three washes with PBS, the cells were lysed in Laemmli sample buffer with protease inhibitors (0.1 mM PMSF, 0.1 mM benzamidine, 1 μg/ml pepstatin A, 1 μg/ml phenantholine), and scraped off the dish. Cell lysates were boiled for 5 min. Immunoprecipitation was then performed as described below. In immunoprecipitation experiments using nonlabeled cells, smooth muscle cells from adult chicken gizzards were lysed in 10 mM Tris, pH 8, 140 mM NaCl, 1% Triton X-100, 0.2% deoxycholate, 0.02% SDS, 0.1 mM PMSF, 0.1 mM benzamidine, 1 μg/ml pepstatin A, 1 μg/ml phenantholine, and scraped off the dish. After incubation on ice for 30 min, the lysate was centrifuged at 10,000 rpm for 10 min, and the soluble material was recovered in the supernatant. The supernatant was then incubated with protein A–agarose beads (Sigma Chemical Co.) for 1 h at 4°C under gentle agitation. After a 2-min centrifugation at 2,000 rpm, the supernatant was incubated for 1 h at 4°C with either 3 μl of the polyclonal antibody B37 raised against CRP1 or 3 μl of the corresponding preimmune serum, followed by a 1.5-h incubation with protein A–agarose beads. At the end of the incubation period, the beads were washed twice with the lysis buffer to remove the unbound proteins; more extensive washing resulted in a loss of our ability to detect protein that coimmunoprecipitated with CRP1. 40 μl of 2× Laemmli sample buffer were then added to the pelleted beads and boiled for 5 min. The immunoprecipitated proteins were resolved by SDS-PAGE. Gels containing metabolically labeled, immunoprecipitated proteins were dried and subjected to autoradiography, while nonlabeled proteins were transferred to nitrocellulose for immunoblotting as described above. CRP1 was detected using the polyclonal antibody B37, while α-actinin was detected using the polyclonal antibody raised against chicken α-actinin provided by K. Burridge.

Heterologous Expression and Immunofluorescence

Expression vector construction involved amplifying coding regions from full-length cCRP1 cDNAs by PCR using Pfu Polymerase (Stratagene, La Jolla, CA). Primers encoded EcoRV (5′ end) or NotI (3′ end) restriction sites. Amplified fragments were digested and ligated into a pcDNA1/NEO vector (Invitrogen, Carlsbad, CA) that was modified by inserting sequences encoding the myc epitope (EQKLISEEDLL) downstream from the NotI site. Ligation at this site generated in-frame CRP1, CRP1-LIM1, and CRP1-LIM2 fusions with myc. Constructs were sequenced prior to use. Plasmid DNAs were isolated using a polyethyleneglycol precipitation procedure (Sambrook et al., 1989) and were ultimately resuspended in PBS for microinjection. Rat embryo fibroblast (REF52) cells were grown to 50–70% confluence on coverslips in a 1:3 mixture of Ham's F-12 and DME containing 10% FBS, and microinjected with plasmid DNA at 250 ng/μl by a previously described technique in Beckerle and Porter (1983), except an inverted microscope was used. Cells were fixed 24 h later and processed for fluorescence microscopy with rhodamine-phalloidin (Molecular Probes, Inc., Eugene, OR) and indirect immunofluorescence (Beckerle, 1986) with anti-myc primary monoclonal antibody and FITC-conjugated goat anti–mouse secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA).

Results

Recovery of α-Actinin from a CRP1-affinity Column

Affinity chromatography was used to identify CRP1-binding proteins in an avian smooth muscle extract. Briefly, proteins extracted from smooth muscle preparations were fractionated by precipitation with increasing amounts of ammonium sulfate (27–34, 34–43, and 43–61% saturation). Each of the ammonium sulfate precipitates was subjected to affinity chromatography on a CRP1 column. The CRP1 column was prepared from bacterially expressed avian smooth muscle CRP1. We have shown previously that bacterially expressed CRP1 exhibits a native structure (Michelsen et al., 1993, 1994) and retains the ability to bind zyxin (Schmeichel and Beckerle, 1994). When a 27–34% ammonium sulfate precipitate from the avian smooth muscle extract (Fig. 1 *A*, lane *2*) is loaded on a CRP1 column, four proteins of ~115, 100, 41, and 35 kD elute from the column

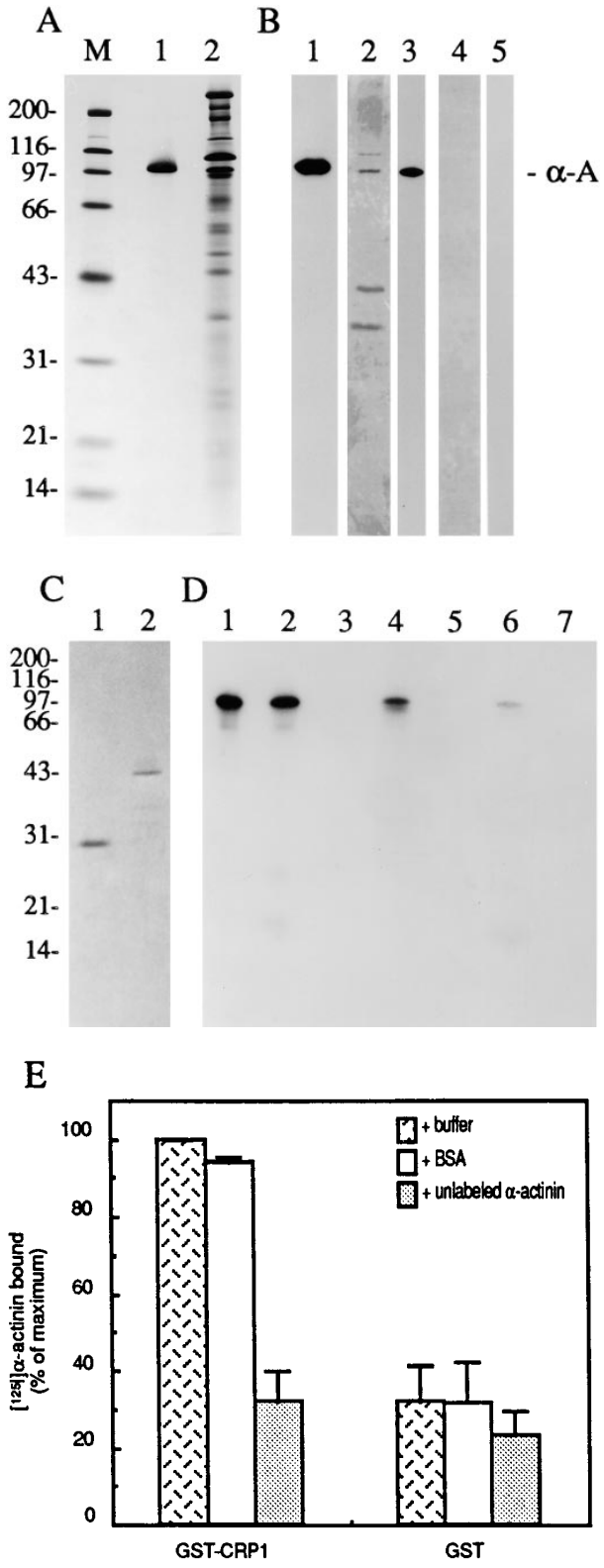

Figure 1. Specificity of the α-actinin–CRP1 interaction under nondenaturing conditions. (*A*) A Coomassie blue–stained gel showing molecular mass markers *M*, purified α-actinin (lane *1*), and the 27–34% ammonium sulfate precipitate from avian smooth muscle extract (lane *2*) that was loaded onto the affinity columns and used in the affinity resin binding assay. (*B*) Lane *1*, Western immunoblot analysis of the 27–34% ammonium sulfate precipitate that was loaded onto the affinity columns using a polyclonal antibody raised against chicken α-actinin; lane *2*, silver-stained gel showing the proteins eluted from the CRP1 column; lane *3*, Western immunoblot analysis of the proteins shown in lane *2* using a polyclonal antibody raised against α-actinin; lane *4*, silver-stained gel showing the material eluted from the BSA column; lane *5*, Western immunoblot revealed that no α-actinin bound to the BSA column (α-a, α-actinin). (*C*) Coomassie blue–stained gel showing the purified GST (lane *1*) and GST-CRP1 (lane *2*) proteins that were used to generate the affinity resins. (*D*) Western immunoblot analysis to detect chicken α-actinin. The gel was loaded with α-actinin (lane *1*) or a 27–34% ammonium sulfate precipitate from a smooth muscle cell extract (lane *2*). Purified α-actinin or proteins found in the 27–34% ammonium sulfate precipitate were incubated with GST agarose (lanes *3* and *5*) or GST-CRP1 agarose (lanes *4* and *6*). α-Actinin binds to the GST-CRP1 affinity resin. A mock affinity resin binding assay was performed with GST-CRP1 agarose beads in the absence of α-actinin; no immunoreactive product is observed (lane *7*). (*E*) [^{125}I]α-actinin was incubated with GST-CRP1 (*left*) or GST agarose beads (*right*) in the absence of competing proteins (+ buffer), in the presence of a 2,000-fold molar excess of unlabeled α-actinin (+ unlabeled α-actinin), or in the presence of an equivalent molar amount of BSA (+ BSA). The counts bound to the agarose beads were analyzed using a γ counter and expressed as a percentage of bound [^{125}I]α-actinin in absence of competing proteins. Mean and SEM from three experiments are shown.

with a high salt buffer as detected by silver staining (Fig. 1 *B*, lane *2*). By Western immunoblot analysis using specific antibodies, we determined that the 100-kD protein that binds to the CRP1 column is α-actinin (Fig. 1 *B*, lane *3*). No protein was detected using antibodies against the two cytoskeletal proteins, talin and vinculin (data not shown). Because zyxin has previously been shown to interact with both α-actinin and CRP1 (Crawford et al., 1992; Sadler et al., 1992), zyxin could theoretically have been responsible for linking α-actinin to CRP1 in this experiment. However, no zyxin is detected in the 27–34% ammonium sulfate precipitate (Crawford and Beckerle, 1991), and therefore the CRP1-α-actinin interaction can not be mediated by zyxin. In control experiments, the 27–34% ammonium sulfate precipitate was loaded on a BSA column. In this case, no α-actinin was recovered after a high salt buffer elution as monitored by silver staining and Western immunoblot (Fig. 1 *B*, lanes *4* and *5*). Collectively, the results of these experiments suggest that CRP1 can interact either directly or indirectly with the actin binding protein α-actinin.

A Direct Interaction between CRP1 and α-Actinin Is Detected under Nondenaturing Conditions

To examine whether CRP1 can interact directly with α-actinin, we used an affinity resin-binding assay. Purified α-actinin or a 27–34% ammonium sulfate precipitate containing α-actinin (Fig. 1 *A*) was incubated with GST-CRP1 or GST (Fig. 1 *C*) coupled to glutathione-agarose beads. After washing the beads, the proteins that remained bound to the GST or GST-CRP1 affinity resins were resolved by SDS-PAGE. Western immunoblot analysis revealed that GST-CRP1 (Fig. 1 *D*, lanes *4* and *6*), but not GST alone (Fig. 1 *D*, lanes *3* and *5*), extracts α-actinin from a solution of purified protein or from a complex mixture of proteins. No band corresponding to α-actinin is detected when GST-CRP1 agarose beads are incubated with

a solution that lacks α-actinin (Fig. 1 *D*, lane *7*); this control confirms that detection of the immunoreactive 100-kD protein is dependent on the addition of α-actinin and cannot be due to crossreactivity of the antibody with fusion protein dimers that migrate at a similar molecular mass. These experiments show a direct interaction between CRP1 and α-actinin under nondenaturing conditions.

To examine the specificity of the CRP1–α-actinin interaction observed by the affinity resin-binding assay, we performed a competition experiment (Fig. 1 *E*). [^{125}I]α-Actinin was incubated with GST-CRP1 or GST agarose beads in the absence of competing proteins or in the presence of a 2,000-fold molar excess of either unlabeled α-actinin or unlabeled BSA. The counts bound to the agarose beads were measured using a γ counter. The GST-CRP1 agarose beads bind the radioiodinated α-actinin in the absence of competing proteins or in the presence of BSA. In the presence of an excess of unlabeled α-actinin, binding of the radiolabeled α-actinin to GST-CRP1 is reduced to the level obtained with GST agarose beads. These results demonstrate that the interaction between CRP1 and α-actinin is direct, specific, and saturable under nondenaturing conditions.

A Direct Interaction between CRP1 and α-Actinin Is Detected by the Blot Overlay Assay

We have further characterized the CRP1–α-actinin interaction using a blot overlay assay that has been used previously to study many protein–protein interactions (Belkin and Koteliansky, 1987; Crawford et al., 1992; Sadler et al., 1992). We evaluated the ability of [^{125}I]α-actinin to bind directly to CRP1 present in fractions derived from an avian smooth muscle extract. Three different ammonium sulfate precipitates that include a diverse collection of smooth muscle-derived proteins were resolved by SDS-PAGE (Fig. 2 *A*) and transferred to nitrocellulose. CRP1 is found in the 34–43% ammonium sulfate (Fig. 2, lane *2*) but not in the 27–34 and the 43–61% ammonium sulfate precipitates. Purified α-actinin was radioiodinated and used as a probe to examine its ability to interact with CRP1 that was immobilized on nitrocellulose (Fig. 2 *B*). The purity of the [^{125}I]α-actinin used in this experiment is shown in Fig. 2 *C*. Among the proteins that are precipitated from the smooth muscle extract, [^{125}I]α-actinin recognizes a protein that exhibits an apparent molecular mass of 23 kD, corresponding to the molecular mass of CRP1. A number of other abundant proteins present on the nitrocellulose membrane fail to interact with the radioiodinated α-actinin showing the selectivity of the radiolabeled probe.

To analyze further the specificity of the CRP1–α-actinin interaction, a competition experiment was performed using the blot overlay assay. Purified CRP1 was resolved by SDS-PAGE (Fig. 3 *A*) and transferred to nitrocellulose. The immobilized CRP1 was probed with radioiodinated α-actinin in the absence or presence of a 2,000-fold molar excess of unlabeled α-actinin or BSA (Fig. 3, *B–D*). The radioiodinated probe interacts with the purified CRP1 confirming that, under the conditions of this experiment, [^{125}I]α-actinin interacts directly with CRP1. Moreover, in the presence of unlabeled α-actinin, but not in the presence of an equimolar amount of unlabeled BSA, the binding of the radioiodinated α-actinin to CRP1 was dramatically reduced. Collectively, these experiments demonstrate that in the blot overlay assay, the association between CRP1 and α-actinin is direct, specific and saturable.

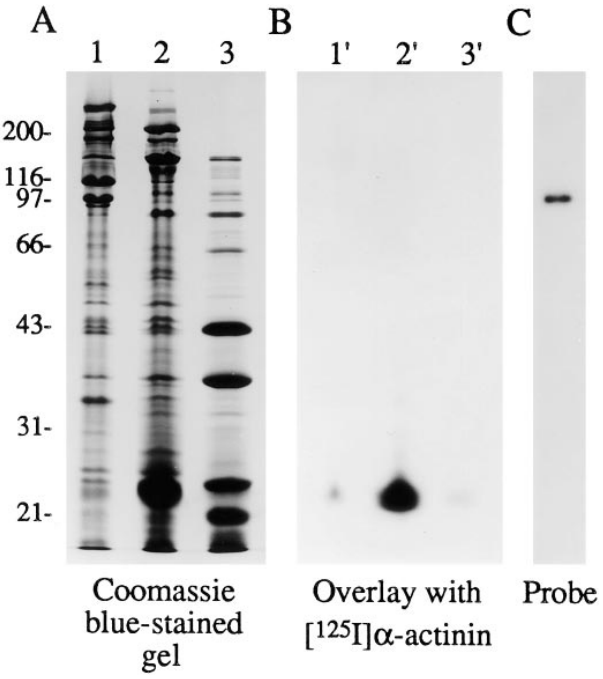

Figure 2. Demonstration of a direct interaction between CRP1 and [^{125}I]α-actinin using a blot overlay assay. (*A*) Coomassie blue–stained gel showing a 27–34 (lane *1*), a 34–43 (lane *2*), and a 43–61% (lane *3*) ammonium sulfate precipitates from an avian smooth muscle extract. Proteins from a parallel gel were transferred to nitrocellulose and the nitrocellulose strip was probed with [^{125}I]α-actinin. The resulting autoradiograph shown in *B* illustrates [^{125}I]α-actinin binding to CRP1. (*C*) Autoradiograph demonstrating the purity of the radioiodinated α-actinin probe. The position of the molecular mass markers is indicated on the left, in kD.

The α-Actinin–CRP1 Interaction Displays a Dissociation Constant in the Micromolar Range

The affinity of the association between α-actinin and CRP1 was characterized by a solid-phase binding assay. Purified bacterially expressed CRP1 (Fig. 3 *A*) was adsorbed to microtiter wells, unoccupied sites on the plastic wells were blocked with BSA, and the immobilized CRP1 was incubated with [^{125}I]α-actinin. The amount of bound [^{125}I]α-actinin was determined by γ counting. The specificity of the CRP1–α-actinin interaction in this solid-phase binding assay was evaluated by comparing the ability of unlabeled α-actinin or BSA to compete with radiolabeled α-actinin for binding to CRP1 (Fig. 4 *A*). A constant amount of [^{125}I]α-actinin was incubated in CRP1-coated wells in the presence of increasing concentrations of competing proteins. The interaction between CRP1 and [^{125}I]α-actinin is inhibited by the unlabeled α-actinin but not by an equivalent molar excess of BSA, demonstrating the specificity of the interaction between CRP1 and α-actinin in the solid-phase binding assay. A typical curve predicted by the simple binding reaction: CRP1 + α-A ↔ CRP1·α-A, was

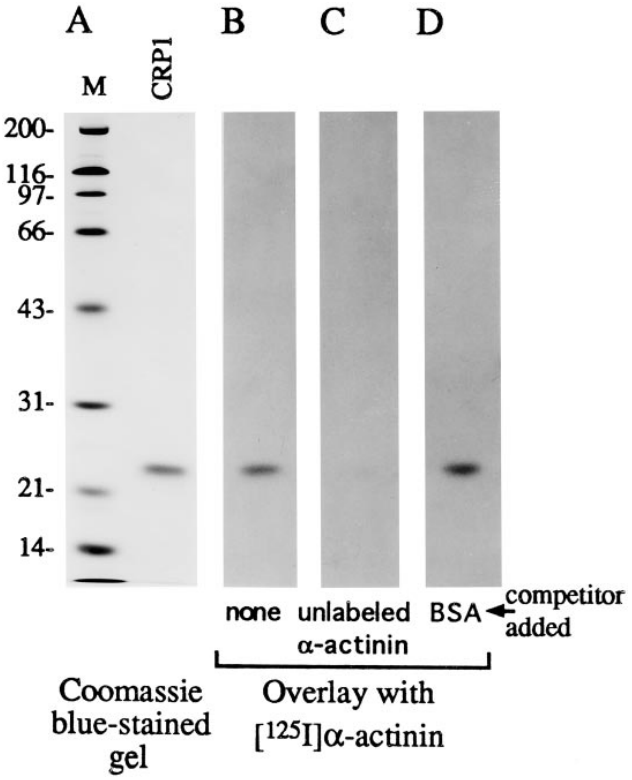

Figure 3. Specificity of the [^{125}I]α-actinin–CRP1 interaction. (*A*) Coomassie blue–stained gel showing molecular mass markers and the purified recombinant CRP1. Autoradiograph of parallel nitrocellulose strips probed with [^{125}I]α-actinin in the absence of competing protein (*B*), or in the presence of either a 2,000-fold molar excess of unlabeled α-actinin (*C*), or a 2,000-fold molar excess of unlabeled BSA (*D*).

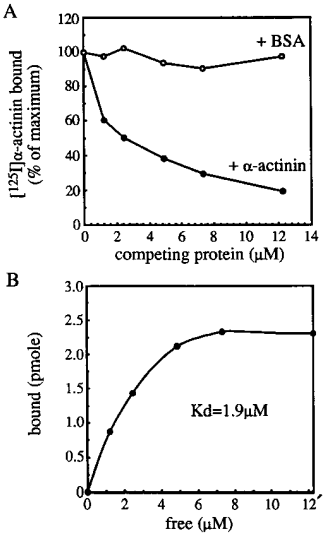

Figure 4. Binding of [^{125}I]α-actinin to CRP1 in a solid-phase binding assay. (*A*) The specificity of the association between CRP1 and [^{125}I]α-actinin in a solid-phase binding assay was analyzed in a competition experiment. A constant amount of [^{125}I]α-actinin (0.26 pmoles in 120 μl) was incubated in CRP1-coated wells with increasing concentrations of unlabeled α-actinin (+ α-actinin) or BSA (+ BSA). In this experiment, a total of 3,076 cpm were bound specifically to CRP1 when no competing unlabeled α-actinin was added. The data are expressed as a percentage of the maximum binding obtained when the [^{125}I]α-actinin is incubated with the CRP1-coated wells in the absence of competing protein. (*B*) From the competition experiment shown in *A*, the moles of α-actinin bound to CRP1 were plotted as a function of the free α-actinin concentration. In this particular experiment, the α-actinin was radioiodinated to a specific activity of 5.8×10^6 cpm/μg; assuming a mol wt of 200,000 g/mol for α-actinin. The calculated dissociation constant (K_d) was 1.9 μM. The mean dissociation constant determined from three different experiments using two different probes is 1.8 ± 0.3 μM (mean ± SEM).

obtained by plotting the moles of α-actinin bound to CRP1 against the concentration of free α-actinin (Fig. 4 *B*). Half maximum binding in this experiment occurs at 1.9 μM free ligand. From the average of three different experiments using two different probes we calculate an average K_d of 1.8 ± 0.3 μM (mean ± SEM) for the CRP1–α-actinin interaction.

CRP1 and α-Actinin Display Overlapping Subcellular Distributions in CEF and Smooth Muscle Cells

The work described above reports the ability of CRP1 and α-actinin to associate with each other in vitro. If this interaction also occurs in vivo, one might expect CRP1 and α-actinin to be colocalized in cells. To examine this possibility, we performed double-label immunofluorescence microscopy using an anti-peptide antibody (B37) raised against a sequence in cCRP1. By Western blot analysis of a CEF lysate, the B37 antibody recognizes a single band that exhibits an apparent molecular mass of 23 kD (Fig. 5 *B*) and comigrates with CRP1 (data not shown); no protein is detected using the preimmune serum (Fig. 5 *B*). Similarly, a single band that migrates at an apparent molecular mass of 23 kD is immunoprecipitated from a detergent extract of [^{35}S]methionine–labeled CEF under denaturing conditions, whereas no immunoprecipitated band is detected under the same conditions when the preimmune serum is used (Fig. 5 *C*). The B37 antibody was used to compare the subcellular distributions of CRP1 and α-actinin using double-label indirect immunofluorescence in CEF cells and in a primary culture of smooth muscle cells from chicken gizzard (Fig. 6). By this approach, we observe that CRP1 and α-actinin are extensively colocalized in cells along the actin stress fibers (Fig. 6, *C* and *F*), in accordance with the idea that they could interact in vivo. We also observed that both proteins are present at the leading edge of the cells, and in the adhesion plaques (Fig. 6 *F*). However, in some adhesion plaques, where α-actinin is present, CRP1 is not detected (data not shown). This observation is consistent with a previous report showing that CRP1 is present in some adhesion plaques of CEF cells but not in others (Crawford et al., 1994).

An In Vivo Interaction between CRP1 and α-Actinin in Smooth Muscle Cells

We performed a coimmunoprecipitation experiment to evaluate the ability of CRP1 to interact with α-actinin in vivo. CRP1 can be immunoprecipitated from a smooth muscle cell extract of smooth muscle cells under nondenaturing conditions using the B37 anti–CRP1 antibody (Fig. 7 *A*). Under these conditions, α-actinin is detected in the immunoprecipitate with CRP1 (Fig. 7 *B*), whereas another cytoskeletal protein, vinculin, is not detected (data not shown). Neither CRP1 nor α-actinin is detected when the preimmune serum is used in the immunoprecipitation assay (Fig. 7, *A* and *B*). These data provide evidence that α-actinin and CRP1 can be recovered as a complex from smooth muscle cells.

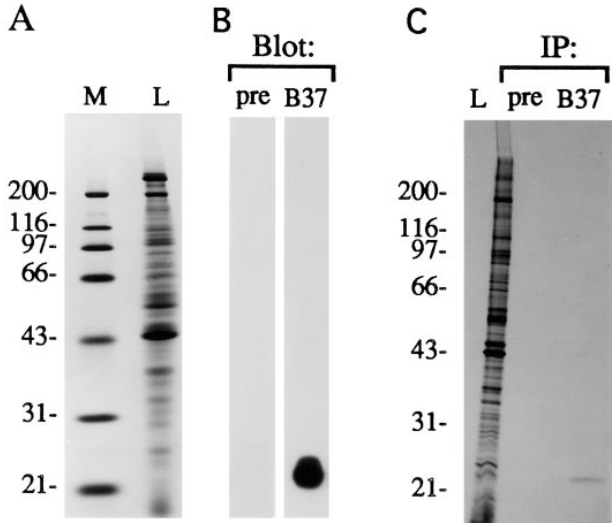

Figure 5. Characterization of an anti-peptide antibody (B37) directed against cCRP1. (*A*) A Coomassie blue–stained gel showing molecular mass markers *M* and total CEF proteins *L. (B*) A parallel gel was transferred to nitrocellulose and probed with the anti-CRP1 antibody *B37* or its corresponding preimmune serum *pre.* A single polypeptide of 23 kD is recognized by the antibody. (*C*) Autoradiograph of a gel loaded with a CEF lysate prepared from [^{35}S]methionine-cysteine–labeled cells *L*, the proteins immunoprecipitated from this lysate with the polyclonal antibody raised against CRP1 *B37*, and with its corresponding preimmune serum *pre.* A single protein of 23 kD is specifically immunoprecipitated with the antibody against CRP1.

Mapping the Domains of CRP1 and α-Actinin That Participate in the Interaction of the Two Proteins

To map the binding site for CRP1 on α-actinin, we performed a blot overlay experiment using labeled CRP1. α-Actinin can be separated into two well-characterized proteolytic products of 53 and 27 kD by cleavage with thermolysin. The 27-kD fragment has been shown to interact with zyxin, vinculin, and actin, whereas the 53-kD fragment is essential for dimerization of the protein and for interacting with the cytoplasmic domain of β_1 integrin receptors for extracellular matrix (Mimura and Asano, 1986; Otey et al., 1990; Pavalko and Burridge, 1991; Crawford et al., 1992). Fig. 8 *A* shows a Coomassie blue–stained gel of purified α-actinin and the products of partial proteolytic cleavage with thermolysin. Proteins from a parallel gel were transferred to nitrocellulose and the resulting blot was incubated with [^{32}P]GST-CRP1 (Fig. 8 *B*). By this blot overlay approach, [^{32}P]GST-CRP1 associates directly with α-actinin and also prominently with the 27-kD actin-binding domain of α-actinin; no interaction of CRP1 with the 53-kD fragment is observed. When [^{32}P]GST is used as a probe, no detectable protein binding is observed (Fig. 8 *C*). The purity of the ^{32}P-labeled probes used in this experiment is shown in Fig. 8 *D*. These results demonstrate that the binding site for CRP1 on α-actinin is in the 27-kD actin-binding domain of α-actinin.

CRP1 displays two LIM domains separated by 56 amino acids (Crawford et al., 1994). To characterize the binding site for α-actinin on CRP1, we compared the ability of α-actinin to interact with full-length CRP1 and two peptides, CRP1-LIM1 and CRP1-LIM2, derived from the intact protein. CRP1-LIM1 corresponds to the NH_2-terminal part of CRP1 (aa 1–107) containing the NH_2-terminal LIM domain followed by the first glycine-rich repeat of the protein, and CRP1-LIM2 corresponds to the COOH-terminal part of the protein (aa 108–192) containing the COOH-terminal LIM domain and the second glycine-rich repeat. CRP1, CRP1-LIM1, and CRP1-LIM2 were resolved by SDS-PAGE (Fig. 9 *A*), were transferred to nitrocellulose and were probed for their ability to interact with [^{125}I]α-actinin in a blot overlay assay (Fig. 9 *B*). The radioiodinated α-actinin interacts with the bacterially expressed purified CRP1 and with CRP1-LIM1. The molar amounts of the two single LIM peptides, CRP1-LIM1 and CRP1-LIM2, loaded on the gel was twice the amount loaded for the double LIM protein, CRP1. Although the [^{125}I]α-actinin bound only to intact CRP1 and the CRP1-LIM1 peptide, the binding to the deletion construct reached only about 50% of the binding observed with full-length CRP1, as measured by PhosphorImager analysis (data not shown). No interaction is detected between the [^{125}I]α-actinin and CRP1-LIM2. Thus it appears that the CRP1-LIM1 peptide contains sequence information that establishes a docking site for α-actinin; the generation of the CRP-LIM1 truncation may have rendered the α-actinin binding site suboptimal. We cannot rule out the possibility that other low affinity binding sites for α-actinin exist in CRP1, however, the only site we have been able to map is within the CRP1-LIM1 region.

Colocalization of CRP1 and CRP1-LIM1 with Actin Filaments

Given the fact that α-actinin interacts with CRP1-LIM1, the NH_2-terminal part of CRP1, we examined the possibility that the CRP1-LIM1 peptide contains sequence information involved in targeting the protein to the actin cytoskeleton. Eukaryotic expression constructs encoding epitope-tagged full-length CRP1, CRP1-LIM1, and CRP1-LIM2 were microinjected into cells. We used double-label indirect immunofluorescence to compare the subcellular distributions of the expressed portions of CRP1 and the actin stress fibers. The expressed CRP1 is associated with the actin cytoskeleton (Fig. 10, *A* and *B*); this localization corresponds to the typical distribution of endogenous CRP1 in fibroblasts (Sadler et al., 1992; Crawford et al., 1994). The CRP1-LIM1 peptide also localizes with F-actin (Fig. 10, *C* and *D*) whereas expressed CRP1-LIM2 fails to associate with the actin cytoskeleton (Fig. 10, *E* and *F*). We detected some nuclear localization of the two deletion constructs, CRP1-LIM1 and CRP1-LIM2; however, the significance of this finding is not clear. Some expressed protein is found in a punctate pattern that does not correspond to the distribution of filamentous actin; because we do not observe such a prominent punctate pattern when we visualize endogenous CRP1 by indirect immunofluorescence, the physiological relevance of this distribution is questionable. These heterologous expression studies in rat embryo fibroblasts reveal that deletion of aa 1–107 from CRP1 eliminates the protein's ability to localize to the actin cytoskeleton. The NH_2-terminal 107 aa of CRP1 retains the capacity to localize to the cytoskeleton illustrat-

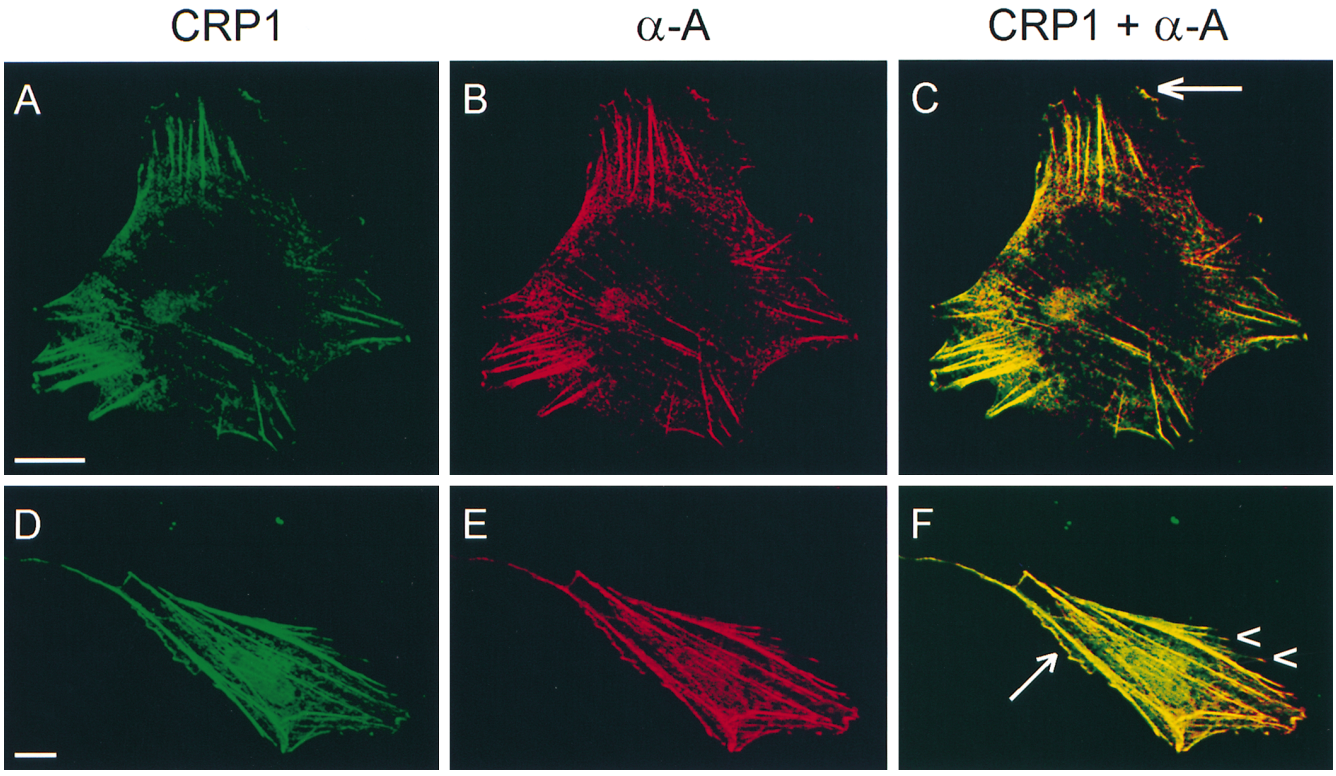

Figure 6. CRP1 and α-actinin are extensively codistributed in CEF and in smooth muscle cells. CEF cells (*A–C*) and smooth muscle cells (*D–F*), prepared for confocal indirect immunofluorescence microscopy, were double-labeled with a polyclonal antibody raised against CRP1 (*A* and *D*), and a monoclonal antibody raised against α-actinin (*B* and *E*). *C* and *F* are composite images of CRP1 (*green*) and α-actinin (*red*) staining; the overlapping regions appear in yellow. Confocal microscopy reveals that CRP1 and α-actinin are extensively colocalized along the actin stress fibers. Both α-actinin and CRP1 are detected at the leading edges of the cells (*arrows*) and in the adhesion plaques (*arrowheads* and data not shown). Bars, 30 μm.

ing that this region is both necessary and sufficient to support the cytoskeletal association of CRP1.

Discussion

In this study, we have identified the actin-binding protein, α-actinin, as a new binding partner for CRP1, a LIM domain protein that has been implicated in the process of muscle differentiation. We have used a variety of solution and solid-phase binding assays to demonstrate and characterize an association between α-actinin and CRP1. By these approaches we have shown a direct, specific, and saturable interaction between α-actinin and CRP1. Because both smooth muscle and bacterially expressed CRP1 interact with α-actinin, eukaryotic posttranslational modification of CRP1 is not required for binding of these two proteins. From our solid-phase binding studies, the interaction between α-actinin and CRP1 appears to occur at a single site with a ~1.8 μM K_d, corresponding to a moderate affinity interaction between the two proteins. The dissociation constant values calculated for the interactions between α-actinin and its other binding partners are in the same range (Fig. 11 *A*). The biochemical studies that provide evidence for an interaction between α-actinin and CRP1 are supported by immunocytochemical studies that demonstrate that the primary distributions of α-actinin and CRP1 in CEF and smooth muscle cells are very similar, with both proteins prominently concentrated along the actin cytoskeleton and to a more limited extent within adhesion plaques. Although some immunostaining of cell nuclei is evident with anti-CRP antibodies and some accumulation of the proteins within nuclei is observed in overexpression studies, the physiological relevance of the nuclear CRPs is not clear. CRPs are relatively small proteins that would not be excluded from nuclei based on size. If CRP did diffuse into the nucleus of a cell, it might be

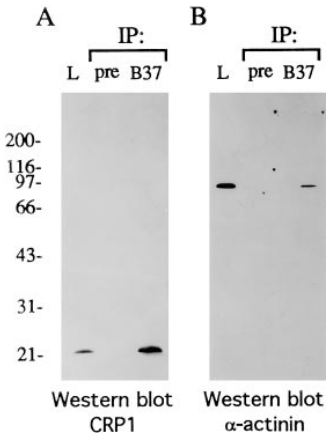

Figure 7. An in vivo interaction between CRP1 and α-actinin in smooth muscle cells. Proteins were immunoprecipitated from a chicken gizzard smooth muscle lysate *L* with the polyclonal antibody raised against CRP1 *B37* and with the corresponding preimmune serum *pre*. The immunoprecipitated proteins were resolved by SDS-PAGE and were transferred to nitrocellulose and probed with polyclonal antibodies raised against CRP1 (*A*) or α-actinin (*B*). α-actinin is immunoprecipitated under nondenaturing conditions with the anti-CRP1 antibody, but not with the preimmune serum. The position of the molecular mass markers is indicated on the left in kD.

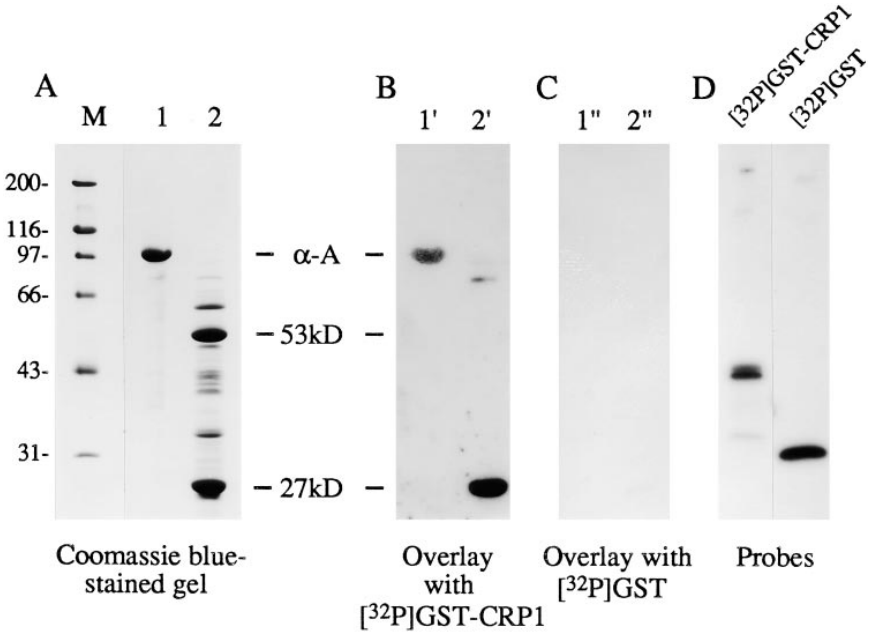

Figure 8. CRP1 interacts with the 27-kD actin-binding site of α-actinin. (*A*) A Coomassie blue–stained gel showing molecular mass markers *M*, purified α-actinin (lane *1*), and the 53- and 27-kD proteolytic products of α-actinin generated by thermolysin cleavage (lane *2*). Autoradiograph of overlay assay performed on parallel nitrocellulose strips containing purified α-actinin (lanes *1′* and *1″*) and the proteolytic fragments of α-actinin (lanes *2′* and *2″*) probed with [^{32}P]GST-CRP1 (*B*), or [^{32}P]GST (*C*). Note that in the experiment shown, thermolysin cleavage of α-actinin was not complete, therefore products other than the 53- and 27-kD fragments are also detected. (*D*) Autoradiograph illustrating the quality of the bacterially expressed, purified, ^{32}P-labeled probes, [^{32}P]GST-CRP1 and [^{32}P]GST.

passively trapped there as a result of its basic nature; the isoelectric point of CRP1 is 8.5 (Crawford et al., 1994). That said, it is not possible to rule out a nuclear function for CRP1; this possibility remains intriguing since the three-dimensional conformation of a LIM domain derived from CRP1 exhibits features that would be expected to be compatible with nucleic acid binding (Perez-Alvarado et al., 1994). Moreover, *Drosophila* CRPs are not excluded from cell nuclei in the developing musculature, in contrast with myosin which is clearly excluded from the nuclear compartment in the same cells (Stronach et al., 1996).

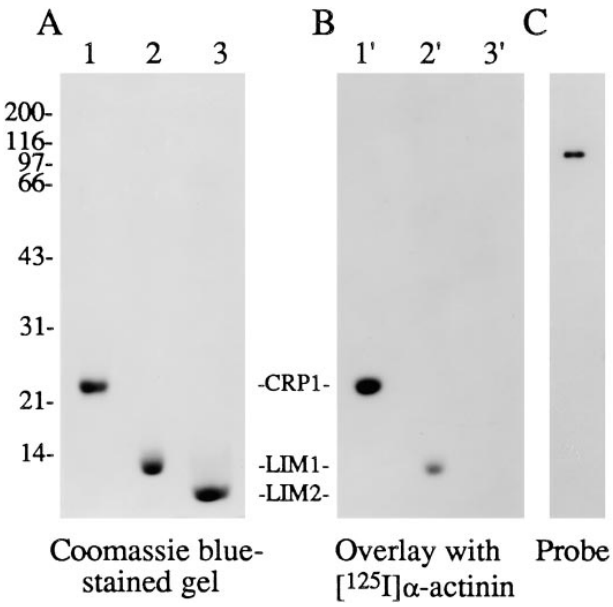

Figure 9. The binding site for α-actinin on CRP1 is contained within the CRP1-LIM1 fragment. (*A*) Coomassie blue–stained gel showing the purified CRP1 (lane *1*), the purified CRP1-LIM1 fragment (lane *2*) and the purified CRP1-LIM2 fragment (lane *3*). 100 pmoles of CRP1, 200 pmoles of CRP1-LIM1, and 200 pmoles of CRP1-LIM2 were loaded on the gel. The positions of CRP1, CRP1-LIM1, and CRP1-LIM2 are marked (*CRP1*, *LIM1*, and *LIM2*, respectively). The corresponding blot overlay assay probed with [^{125}I]α-actinin is shown in *B*. (*C*) Autoradiograph illustrating the purity of the radioiodinated α-actinin probe. The position of the molecular mass markers is indicated on the left in kD.

α-Actinin has been extensively studied and much is understood about its biochemical properties. α-Actinin forms antiparallel homodimers (Wallraff et al., 1986; Imamura et al., 1988) that cross-link actin filaments into parallel arrays (Maruyama and Ebashi, 1965; Podlubnaya et al., 1975). In nonmuscle cells such as cultured fibroblasts, α-actinin is found along the stress fibers and in the adhesion plaques where actin filament bundles associate with the plasma membrane (Lazarides and Burridge, 1975). In striated and smooth muscle, α-actinin is most prominently concentrated in the Z discs and dense bodies and plaques, respectively (Blanchard et al., 1989). Mutation of the gene encoding α-actinin in *Drosophila* results in disorganized myofibrillar arrays and reduced muscle function (Fyrberg et al., 1990; Roulier et al., 1992).

Prior to this report it was known that α-actinin has the capacity to interact with four different adhesion plaque and cytoskeletal proteins: integrin, vinculin, zyxin, and actin. A model for the associations among these proteins, based on what has been learned from protein binding studies, is shown in Fig. 11 *B*. Binding studies using the proteolytic fragments of α-actinin digested by thermolysin have shown that the β_1 integrin subunit interacts with the 53-kD rod-like domain of α-actinin (Otey et al., 1990), whereas vinculin, zyxin, and actin interact with the 27-kD globular head of α-actinin (Mimura and Asano, 1986; Pavalko and Burridge, 1991; Crawford et al., 1992). Here we have demonstrated an interaction between CRP1 and the 27-kD globular head domain of α-actinin. We have also shown that α-actinin interacts with the NH_2-terminal region of CRP1 (CRP1-LIM1) which contains one LIM domain followed by a glycine-rich repeat. Moreover, by heterologous protein expression experiments, we have

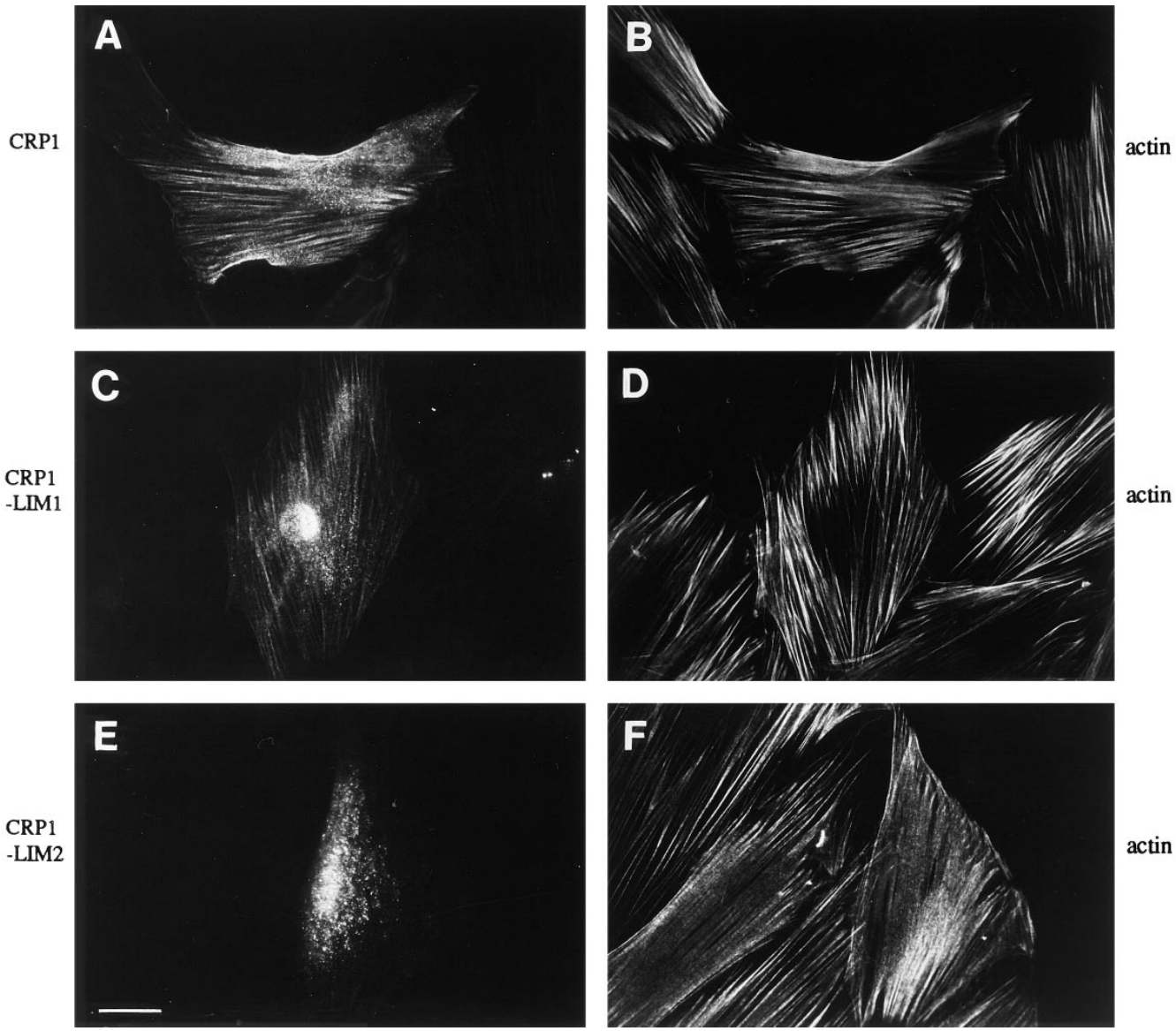

Figure 10. CRP1-LIM1 is targeted to actin stress fibers. Expression constructs encoding myc-tagged CRP1 (*A* and *B*), CRP1-LIM1 (*C* and *D*), or CRP1-LIM2 (*E* and *F*) were microinjected into rat embryo fibroblast (REF52) cells. Double-label immunofluorescence was used to compare the subcellular distributions of the expressed CRP1 polypeptides (*A*, *C*, and *E*) and the actin stress fibers (*B*, *D*, and *F*). The expressed proteins were visualized using an anti-myc monoclonal antibody whereas the actin stress fibers were visualized with phalloidin. Bar, 30 μm.

shown that the full-length CRP1 and the CRP1-LIM1 peptide have the capacity to localize along the actin cytoskeleton whereas the COOH-terminal part of the protein does not. These results illustrate that the sequence information required both for α-actinin binding and the cytoskeletal localization of CRP1 is contained within the NH_2-terminal 107 aa of the protein. Based on these observations, we speculate that it is CRP1's ability to bind to α-actinin that targets it to the actin cytoskeleton; however, additional work will be necessary to demonstrate whether this is indeed the case. The primary features of the NH_2-terminal 107 aa of CRP1 are the presence of a single LIM domain and a glycine-rich repeat. Since LIM domains have been shown to function in specific protein–protein interactions, it seems likely that the COOH-terminal LIM domain of CRP1 will also interact with a specific binding partner. CRP1 could thus serve as an adaptor protein that is targeted to the actin cytoskeleton by virtue of an interaction with α-actinin. The COOH-terminal LIM domain of CRP1 may function as a ligand for a factor whose function depends on a cytoskeletal localization (Fig. 11 *B*).

CRP family members appear to play a role in muscle differentiation; however, the mechanism by which they might act has not been clarified. The ability of CRP1 to interact in vivo with α-actinin in smooth muscle cells raises the possibility that α-actinin and CRP1 cooperate to perform an essential function in smooth muscle differentiation. One intriguing possibility is that the two proteins collaborate to localize protein machineries involved in actin assembly or dynamics (Fig. 11 *B*). It is interesting in this

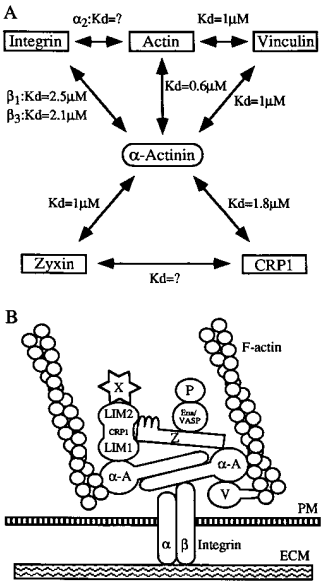

Figure 11. α-Actinin and its binding partners. (*A*) A summary of the interactions between α-actinin and its binding partners: integrin, zyxin, actin, vinculin, and CRP1. References for the dissociation constant values are as follows: α-actinin–integrin, (Otey et al., 1990); α-actinin–zyxin, (Crawford et al., 1992); α-actinin–vinculin, (Wachsstock et al., 1987); α-actinin–actin, (Wachsstock et al., 1993); α-actinin–CRP1, this report; vinculin–actin, (Menkel et al., 1994). (*B*) A schematic representation of an adhesion plaque showing a model for the association of α-actinin with its known binding partners within a cell. α-Actinin and CRP1 may cooperate to localize a complex of zyxin, Ena/VASP, and profilin and thus could participate in the regulation of actin assembly dynamics. (*Z*) zyxin; (*P*) profilin; (*V*) vinculin; (α-A) α-actinin; (*X*) other CRP1-binding partners; (*PM*) plasma membrane; (*ECM*) extracellular matrix.

regard that both α-actinin and CRPs bind zyxin, a protein that has been implicated in the spatial control of actin assembly by virtue of its ability to bind Ena/VASP family members that associate with profilin (Reinhard et al., 1995 *a*, *b*; Gertler et al., 1996). In future work, it will be very important to perform immunocytochemical studies to characterize the subcellular distributions of CRP1 and α-actinin within intact tissues. Likewise, functional studies will be essential in order to assess the physiological significance of the CRP1–α-actinin interaction in vivo.

In summary, using in vitro and in vivo biochemical studies and immunochemistry we have demonstrated a direct and specific interaction between the LIM domain protein CRP1 and the cytoskeletal protein, α-actinin. Our data are consistent with the possibility that the localization of CRP1 along the actin cytoskeleton is due to the interaction between the NH_2-terminal LIM domain of CRP1 and the actin-binding protein, α-actinin. CRP1 has been implicated as a key regulator in the control of muscle differentiation. The appropriate targeting of CRP1 to the actin cytoskeleton is likely to be important for the function of the protein during myogenesis. Given the finding that loss of one CRP family member, MLP/CRP3, results in dramatic disorganization of myofibrils (Arber et al., 1997), it is reasonable to speculate that the appropriate localization and function of CRPs at subcellular domains that are enriched in α-actinin may be required in order for a cell to build or maintain the semicrystalline arrays of actin and myosin filaments that constitute the contractile machinery.

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