LIM domains of cysteine-rich protein 1 (CRP1) are essential for its zyxin-binding function

Karen L. SCHMEICHEL*†[1] and Mary C. BECKERLE†[2]

*Ernest Orlando Lawrence Berkeley National Laboratory, One Cyclotron Road, MS 83-101, Berkeley, CA 94720, U.S.A., and †University of Utah, Biology Department, 204 So. Biology, Salt Lake City, UT 84112-0840, U.S.A.

Previous studies have demonstrated that the adhesion-plaque protein, zyxin, interacts specifically with a 23 kDa protein, called the cysteine-rich protein 1 (CRP1), which has been implicated in myogenesis. Primary sequence analyses have revealed that both zyxin and CRP1 exhibit multiple copies of a structural motif called the LIM domain. LIM domains, which are defined by the consensus $CX_2CX_{16-23}HX_2CX_2CX_2CX_{16-23}CX_{2-3}$(C,H,D), are found in a variety of proteins that are involved in cell growth and differentiation. Recent studies have established that LIM domains are zinc-binding structures that mediate specific protein–protein interactions. For example, in the case of the zyxin–CRP1 interaction, one of zyxin's three LIM domains is necessary and sufficient for binding to CRP1. Because the CRP1 molecule is comprised primarily of two LIM domains, we were interested in the possibility that the binding site for zyxin on CRP1 might also be contained within a single LIM domain. Consistent with the hypothesis that the LIM domains of CRP1 are critical for the protein's zyxin-binding function, zinc-depleted CRP1 displays a reduced zyxin-binding activity. However, domain mapping analyses have revealed that neither of the two individual LIM domains of CRP1 can support a wild-type interaction with zyxin. Collectively, our results suggest that the binding site for zyxin on CRP1 is not contained within a single contiguous sequence of amino acids. Instead, the interaction appears to rely on the co-ordinate action of a number of residues that are displayed in both of CRP1's LIM domains.

INTRODUCTION

Many cellular proteins are comprised of a collection of modular domains that perform distinct molecular functions. One such protein domain is the LIM (an acronym derived from the three gene products lin-11, isl-1 and mec-3) domain, a 50–60 amino acid sequence that exhibits the consensus $CX_2CX_{16-23}HX_2CX_2CX_2CX_{16-23}CX_{2-3}$(C,H,D) [1–4]. The conserved cysteine, histidine and aspartate residues found in each LIM domain co-ordinate two atoms of zinc, giving rise to a structure that is comprised of two closely associated zinc-binding subdomains [5–11]. Biophysical studies have revealed that the structural integrity of the LIM domain is dependent on zinc co-ordination [6,7].

LIM domains are found in a variety of proteins, many of which participate in pathways controlling cell proliferation and differentiation [12–15]. Within some proteins, LIM domains are coupled to other functional modules, such as kinase domains or DNA-binding homeodomains [2,4]. Alternatively, LIM domains can serve as the primary sequence elements of proteins. As with Src-homology-2 and -3 domains, LIM domains function as protein-binding interfaces and, in this capacity, are thought to influence subcellular protein localization and regulate protein function [16].

The protein-binding activity of the LIM domain has been well documented [16–25]. Our studies of the LIM domain's protein-binding function have focused primarily on the observation that two avian LIM domain proteins, zyxin and CRP1, are capable of interacting *in vitro* [1,26,27]. By performing a domain-mapping analysis of zyxin, which exhibits a C-terminal cluster of three LIM repeats [1,28], we demonstrated previously that a single LIM domain from zyxin (zLIM1) is both necessary and sufficient to mediate a specific association with CRP1, thereby establishing the LIM domain as a modular protein-binding interface [16]. Additional studies have revealed that the zLIM1–CRP1 interaction is specified by sequences contained within zLIM1's N-terminal zinc-binding subdomain [29].

Zyxin's binding partner, CRP1, also exhibits multiple LIM domains [26,30,31]. The 23 kDa CRP1 protein is comprised primarily of two LIM domains, each of which is followed by a short glycine-rich region of unknown function. The LIM–glycine repeats of CRP1 are linked via a short 39 amino acid intervening region that, by itself, fails to conform to any known structural motif. The molecular organization displayed by CRP1 is characteristic of CRP proteins in general [31]. The three CRP proteins identified to date display high levels of sequence similarity, and all are capable of interacting with the LIM protein, zyxin [32]. However, the binding site for zyxin on CRP proteins had not been defined.

The fact that the LIM domains of CRP1 contribute significantly to the overall composition of the molecule, combined with the fact that LIM domains function as protein-binding interfaces, raised the possibility that the binding site for zyxin on CRP1 may involve one, or both, of CRP1's component LIM domains. Because a number of modular protein-binding domains, including leucine zippers and helix-loop-helix domains, mediate homotypic dimerization [33–35], we were particularly intrigued by the possibility that the interaction between zyxin and CRP1 could be achieved via a homotypic LIM–LIM interaction. Characterization of LIM-dependent protein–protein interactions

Abbreviations used: CRP1, cysteine-rich protein 1; LIM, an acronym derived from the three gene products lin-11, isl-1 and mec-3; zLIM1, a single LIM domain from zyxin; GST, glutathione S-transferase.

[1]Current address: Ernest Orlando Lawerence Berkeley Natonal Laboratory.

[2] To whom correspondence should be addressed (e-mail beckerle@bioscience.utah.edu).

thus far have suggested that LIM-dependent associations are generally heterotypic in nature [4].

To determine whether CRP1 and zyxin associate with each other via a LIM–LIM interaction, we have performed studies to define the binding site for zyxin on CRP1. Consistent with the hypothesis that the LIM domains of CRP1 play a critical role in the protein's zyxin-binding function, zinc-depleted CRP1 displays a reduced zyxin-binding activity. Moreover, deletion of sequences outside the LIM domains fails to affect the zyxin–CRP1 interaction. Nevertheless, deletion-mapping analyses have revealed that neither of the two individual LIM domains of CRP1 alone is sufficient to support wild-type levels of zyxin binding. Our results suggest that the binding site for zyxin on CRP1 is not contained within a short contiguous array of amino acids, but rather that zyxin's docking site is dependent upon the coordinate action of sequences found in both of CRP1's component LIM domains.

EXPERIMENTAL

CRP1 deletion constructs

N- and C-terminal CRP1 deletions

Terminally deleted CRP1 polypeptides were generated by subcloning chicken CRP1 cDNA fragments into the pET5 vector (Novagen; Madison, WI, U.S.A.); in the case of CRP1(108–192), the pAED4 vector was used. The cloning strategy employed to generate avian pET5-CRP1 and -CRP1(108–192) expression constructs has been described previously [5–7]; CRP1(108–192) has been referred to previously in the literature as CRP1-LIM2. The pET5-CRP1(1–107) expression vector was made by using PCR to amplify a DNA fragment corresponding to amino acids 1–107 in avian CRP1. The amplified product, which was engineered with an *Nde*I site at the 5′ end of the coding region and with an *Eco*RI site on the 3′ end, was ligated between the *Nde*I and *Eco*RI sites of the pET5 vector polylinker to allow for the expression of CRP1(1–107) from its endogenous start codon. The sequence composition of the pET5-CRP1(1–107) expression construct was confirmed by direct sequence analysis using the dsDNA cycle-sequencing system (Gibco–BRL; Grand Island, NY, U.S.A.). CRP1(1–107) has been referred to previously in the literature as CRP1-LIM1 [36].

CRP1(1–117) and CRP1(1–144) were expressed from pET5-CRP1 vectors that had been mutagenized using the Altered Sites II Mutagenesis System (Promega; Madison, WI, U.S.A.). For CRP1(1–117) and CRP1(1–144), mutagenic primers were generated to create premature stop codons in the CRP1 cDNA sequence at the desired sites (i.e. at codons 118 and 145, respectively). CRP1(33–192) was engineered into the pET5 expression vector using a PCR-based strategy that allowed for the incorporation of a novel N-terminal start codon adjacent to the codon encoding amino acid 33; proteins expressed from this vector lacked the most N-terminal zinc-binding module of CRP1. Finally, the CRP1(33–144) expression construct was generated by combining the truncated regions displayed by pET5-CRP1(1–144) and pET5-CRP1(33–192) vectors; the two CRP1 end deletions were incorporated into a unique construct by classical restriction digests of the parent molecules followed by ligation of the reciprocal fragments. In all cases, mutagenized and/or truncated DNA products were evaluated by dsDNA cycle-sequencing (Gibco-BRL) or automated-sequencer technologies to confirm the engineered alterations. The nomenclature used to describe the CRP1 expression constructs used in these studies refers to the CRP1 residues encoded by the vector. It should be noted that the initiator methionine is likely to be absent from the mature protein product.

Internally deleted CRP1 constructs

To generate each internally deleted CRP1 molecule, including CRP1(Δ63–89), CRP1(Δ90–111) and CRP1(Δ63–111), site-directed mutagenesis was used to introduce a pair of unique blunt-end-cutting restriction enzyme sites into the chicken CRP1 cDNA. For example, to generate the expression construct encoding the CRP1(Δ63–89) protein, an *Sna*BI cleavage site was introduced between codons 62 and 63 and an *Eco*RV site was placed between codons 89 and 90. Simultaneous digestion of this doubly mutated construct with *Sna*BI and *Eco*RV allowed for the removal of the DNA sequences residing between these two sites. Subsequent religation of the newly created vector ends resulted in the production of an internally truncated molecule that, outside of the introduced disruption, displayed a normal, in-frame array of amino acids. The CRP1(Δ90–111) and the CRP1(Δ63–111) expression vectors were generated using a similar strategy. For CRP1(Δ90–111), *Eco*RV and *Pml*I sites were introduced between codons 89 and 90, and 110 and 111, respectively; for CRP1(Δ63–111), *Sna*BI and *Pml*I sites were introduced between codons 62 and 63, and 110 and 111, respectively. The strategy used to create the CRP1(Δ90–111), in addition to giving rise to an extensive internal deletion, also resulted in the incorporation of a glycine-to-aspartic acid substitution at amino acid 89. All CRP1 expression constructs were verified by sequence analysis prior to protein expression and purification.

Expression and purification of CRP1 and CRP1-derived deletion fragments

All of the pET5-CRP1 constructs described above were expressed and purified by previously described methods [5–8]. Briefly, cultures of BL21(DE3) cells, containing the desired expression plasmid, were grown to an A_{600} of 0.7–0.8 and induced for 3 h with 0.3–0.4 mM isopropyl β-D-thiogalactopyranoside. Cells, collected by centrifugation, were resuspended in 20 ml of buffer C-2 [10 mM potassium phosphate (pH 7.2)/0.01 % β-mercaptoethanol] per litre of culture. Cells were lysed by sonication and supernatants were recovered after centrifuging the lysates at approximately 12000 ***g*** for 20 min. Supernatants were equilibrated in buffer C-2 by dialysis at 4 °C and were loaded directly on to 20 ml CM-52 cation-exchange columns (Whatman; Fairfield, NJ, U.S.A.). Proteins were eluted with a 0–250 mM KCl gradient; protein elution profiles varied depending on the CRP1 fragment. An alternative procedure, in which the above manipulations were performed in rapid succession using batch-preparation techniques, greatly improved the protein yield of several CRP1-derived polypeptides, including CRP1(1–117) and CRP1(1–144). Although abundantly expressed upon bacterial induction, the CRP1-derived proteins displayed variable proteolytic sensitivity during purification; the internally deleted CRP1 molecules proved to be extremely labile and could not be purified efficiently by these methods.

Preparation of zinc-depleted CRP1 and determination of zinc stoichiometries

Zinc-depleted CRP1 was prepared as described previously [5,6]. Briefly, purified CRP1 was denatured in 6 M guanidinium/HCl and was resolved from contaminating metals by gel-filtration chromatography; CRP1 was collected from the gel-filtration column under stringent reducing conditions (10 mM dithio-

threitol). The denatured CRP1 samples were then reconstituted by diluting them in Buffer C-1 [10 mM potassium phosphate (pH 7.2)/10 mM KCl/0.01 % β-mercaptoethanol] that contained either a 10-fold molar excess of zinc [to generate CRP1(+Zn)] or specific zinc chelators [to generate CRP1(−Zn)]. After extensive dialysis, the zinc content of known amounts of the reconstituted proteins was determined by atomic absorption spectroscopy using a 3100 impact bead spectrometer (Perkin–Elmer; Norwalk, CT, U.S.A.). This procedure resulted in the production of CRP1(+Zn) and CRP1(−Zn) that on average bound 4.3 and 0.3 zinc atoms per molecule, respectively [5].

Determination of CRP1 protein concentration

The concentrations of purified CRP1 and its related polypeptides were estimated according to experimentally determined extinction coefficients: an extinction coefficient of 0.95×10^4 M·cm^{-1} was used to estimate CRP1(1–107) and CRP1(108–192) peptide concentrations [6,7], whereas an extinction coefficient of 2.66×10^4 M·cm^{-1} was used to estimate levels of all other CRP1-derived polypeptides [5]. Relative levels of CRP1 proteins present in each zyxin-binding assay were quantitated via video densitometric analysis of Coomassie Blue-stained gels using NIH Image software (version 1.52; National Institutes of Health, U.S.A.) or by Phosphorimager analysis of Western immunoblots (Imagequant software, version 3.5; Molecular Dynamics; Sunnyvale, CA, U.S.A.). All quantitative Western-immunoblot analyses involved the use of a previously described anti-CRP1 antibody, B37 [36]. B37, which recognizes an avian CRP1 C-terminal peptide epitope (CRP1, amino acids 182–192), proved useful in establishing relative levels of CRP1 proteins displaying intact C-termini.

Zyxin-binding assays

^{32}P-Labelled probes

Radiolabelled GST (glutathione S-transferase)–zyxin fusion proteins were prepared for use in conjunction with *in vitro* blot-overlay assays as described previously [16,29,37]. For the experiments presented here, three zyxin-derived molecules were used routinely as probes. The probe referred to in the text as GST–zyxin displays sequences from zyxin's entire C-terminal LIM region (avian zyxin amino acids 349–542) [16]; the GST leader peptide was used alone as a control for specificity in all experiments. Interactions were also confirmed using GST–zLIM1 (containing avian zyxin amino acids 349–406), which was found previously to be necessary and sufficient to interact with CRP1 [16]. To prepare the GST fusions for use in binding assays, each protein was purified by affinity chromatography on glutathione–Sepharose (Pharmacia; Piscataway, NJ, U.S.A.) and was subsequently labelled *in vitro* with heart muscle kinase (Sigma; St. Louis, MO) in the presence of ^{32}P-γ-ATP (> 6000 Ci/mmol; DuPont-NEN; Wilmington, DE, U.S.A.). The radioactivity incorporated into each of these protein probes was estimated by Cerenkov counting. The protein probes displayed comparable specific activities after the labelling reaction [16,29].

Blot-overlay assays

Blot-overlay assays were performed as described elsewhere [1,16,26,29,37]. Briefly, CRP1 proteins were immobilized on nitrocellulose (pore size, 0.4 μm). For the majority of the binding assays performed in this study, CRP1 proteins were resolved by SDS/PAGE and electrophoretically transferred to nitrocellulose. However, for analysis of CRP1(+Zn) and CRP1(−Zn), protein samples were directly applied to nitrocellulose using a Hybridot manifold (Gibco–BRL); this procedure was employed to avoid the possibility that trace levels of zinc present during the routine SDS/PAGE and subsequent electrophoretic transfer might repopulate the CRP(−Zn) sample. The protein-embedded blots were subsequently blocked and incubated in blot-overlay buffer [20 mM Hepes (pH 7.5)/0.5 % BSA/0.25 % gelatin/1 % Nonidet P-40/10 mM NaCl/1 mM EGTA/0.1 % β-mercaptoethanol) containing 600000 cpm/ml of the appropriate ^{32}P-labelled GST-fusion-protein probe. After washing, the blots were evaluated by autoradiography.

Quantification of binding assays

In all blot-overlay experiments, zyxin binding was quantified using Phosphorimager analysis. Duplicate blots were evaluated in each zyxin-binding assay to control for variability in gel loading. The relative binding intensities calculated by this method were then normalized for the amount of protein loaded into each lane using one of two strategies. In the case of CRP1(+Zn) and CRP1(−Zn), as well as the internally deleted CRP1 fragments, relative protein levels were determined via Western-immunoblot analysis using the anti-peptide antibody, B37; ^{125}I-Protein-A was used as a secondary detection agent. Relative protein levels were estimated from the immunoblots by Phosphorimager analysis of duplicate blots. In the case of the terminally deleted CRP1 fragments, relative protein levels were estimated by video densitometric analysis of parallel Coomassie Blue-stained gels using NIH Image software; duplicate gels were analysed to control for variability in gel loading. Zyxin-binding activity was ultimately calculated as units of signal from the blot-overlay assay per unit of protein loaded on to the blot. These values, obtained from multiple independent experiments, are reported here as a percentage of the binding observed with intact wild-type CRP1 (±S.E.M.), with wild-type CRP1 displaying 100 % zyxin binding.

SDS/PAGE and Western immunoblots

CRP1 proteins examined in these studies were resolved using 17.5 % SDS/polyacrylamide gels, according to the method of Laemmli [38], which was modified to incorporate the use of 0.13 % bisacrylamide as a cross-linker. Western immunoblots were performed following protocols established by Towbin and co-workers [39]. To facilitate quantification of Western immunoblots by Phosphorimager analysis, primary antibodies were detected using ^{125}I-coupled Protein A.

RESULTS

The cytoskeletal proteins, zyxin and CRP1, display multiple copies of the zinc-binding motif known as the LIM domain (Figure 1). In previous studies, we used both solution and solid-phase binding assays to show that zyxin and CRP1 associate *in vitro* [1,16,26,29]. The binding site for CRP1 on zyxin has been mapped specifically to zyxin's most N-terminal LIM domain, zLIM1 [16,29]; the binding site for zyxin on CRP1 had not been defined. Because LIM domains are the most prominent sequence elements in CRP1 and have been shown to function as protein-binding interfaces, we reasoned that the binding site for zyxin on CRP1 might reside within one of CRP1's LIM domains. In this study, we have performed a series of experiments to probe the significance of the LIM domains of CRP1 in zyxin binding.

Zinc-depleted CRP1 exhibits a decrease in zyxin-binding potential

The metal-binding features of LIM domains derived from CRP1 have been studied extensively [6,7]. Conditions that result in the quantitative removal of zinc from the LIM domains of CRP1

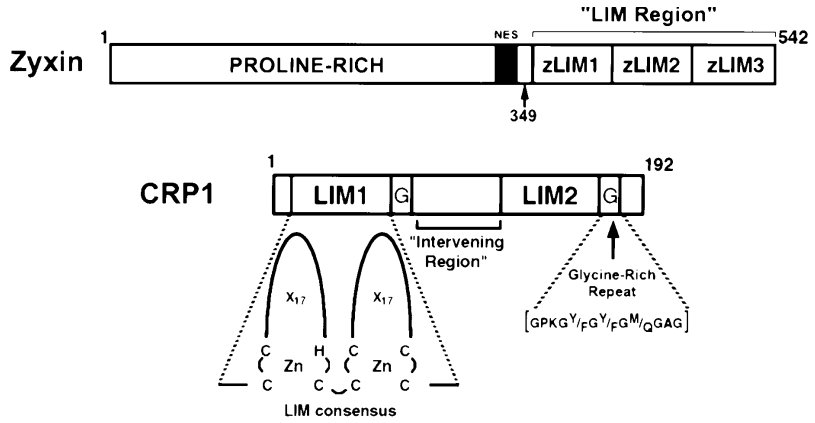

Figure 1 Molecular organization of the interacting LIM proteins, zyxin and CRP1

It has been shown previously that the cytoskeletal proteins, zyxin and CRP1, associate *in vitro* [1,16,26]. Zyxin is comprised of an N-terminal proline-rich domain, a short nuclear export sequence (NES) and a C-terminal cluster of LIM domains (LIM region; amino acids 349–542). CRP1 is a 23 kDa protein that displays two cysteine-rich LIM domains (within amino acids 10–61 and 118–169), each of which is followed by a short glycine-rich repeat (see consensus and boxes marked G). The two LIM-glycine repeats of CRP1 are physically linked by a 39 amino acid intervening region (amino acids 79–117) that does not conform to any known structural motif. A domain-mapping analysis of zyxin revealed that sequences contained within its most N-terminal LIM domain, zLIM1, are necessary and sufficient to interact with CRP1. The binding site for zyxin on CRP1 remained to be determined.

have been described and the consequences for protein conformation have been characterized [5–7]. Using a variety of biophysical approaches, including intrinsic fluorescence measurements, Stokes radius determination, CD spectroscopy, and ^{113}Cd-NMR, it has been shown that removal of zinc from CRP1 destabilizes both secondary and tertiary structures [5–7].

Assuming that such a structural destabilization is likely to impair LIM domain function, we explored the role of CRP1's LIM domains in zyxin binding by comparing the zyxin-binding activities of wild-type and metal-depleted CRP1. Using established procedures [5–7], we purified recombinant avian CRP1 and generated protein samples that were either fully metallated [CRP1(+Zn)] or metal-depleted [CRP1(−Zn)] (see Experimental for details). CRP1(+Zn) and CRP1(−Zn) were analysed for zyxin-binding activity using the blot-overlay binding assay. This method was one of several assays used previously to define and characterize the interaction between zyxin and CRP1 and has been proved to be amenable to quantitative analyses [1,16,26,29]. In these experiments, an equivalent amount of each CRP1 protein was dotted directly on to nitrocellulose. Parallel blots were analysed by Coomassie Blue staining (Figure 2A) and by Western-immunoblot analysis (Figure 2B), to confirm that CRP1(+Zn) and CRP1(−Zn) were present at comparable levels. For the binding assay, CRP1-embedded blots were incubated in a physiological blocking buffer and then probed with either ^{32}P-GST–zyxin, containing sequences from avian zyxin's LIM region (avian zyxin amino acids 349–542) [16], or ^{32}P-GST, the isolated leader peptide (see autoradiograph of ^{32}P-probes in Figure 2E).

Analysis of the blot-overlay assay results revealed that the zinc-depleted CRP1 protein, CRP1(−Zn), exhibited a significant decrease in its zyxin-binding capacity in comparison with its zinc-reconstituted counterpart, CRP1(+Zn) (Figure 2C). Moreover, the ^{32}P-GST probe failed to bind either CRP1 protein (Figure 2D), thereby demonstrating that the observed interaction is dependent upon the presence of zyxin LIM-region sequences. Quantitative analyses of five independent experiments revealed that CRP1(−Zn) bound to zyxin at levels that were, on average, only $30.8 \pm 5.2\%$ of that observed for metallated CRP1. These findings demonstrate that structural destabilization of CRP1's

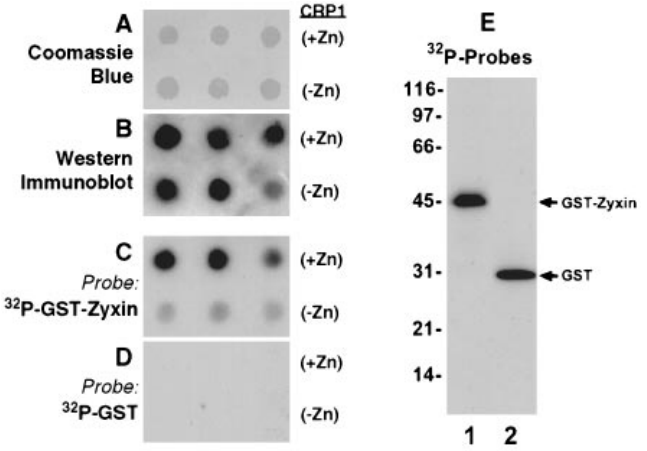

Figure 2 Zinc-depleted CRP1 exhibits a decrease in zyxin-binding potential

Purified recombinant CRP1 was biochemically manipulated to give rise to two CRP1 samples: CRP1(+Zn) bound a full complement of four zinc atoms, whereas CRP1(−Zn) was fully zinc-depleted. CRP1(+Zn) and CRP1(−Zn) (50 pmol) were dotted in triplicate on to nitrocellulose. Parallel blots were analysed by Coomassie Blue staining (**A**) and by Western immunoblot with the B37 antibody (**B**) to evaluate CRP1 protein levels on the blot. Zyxin-binding was evaluated on parallel nitrocellulose strips in blot-overlay assays with 600 000 cpm/ml ^{32}P-GST–zyxin (**C**) or ^{32}P-GST leader peptide (**D**). (**E**) Autoradiograph of the ^{32}P-labelled GST–zyxin (containing zyxin's three C-terminal LIM domains, amino acids 349–542) and GST leader-peptide probes used in the zyxin-binding assays.

LIM domains by zinc depletion results in a significant and reproducible reduction of zyxin-binding activity.

Neither LIM domain from CRP1 is sufficient to support maximal zyxin binding

To address the possibility that the association between zyxin and CRP1 is mediated by one of the two LIM domains of CRP1, we evaluated the zyxin-binding activity of two truncated CRP1

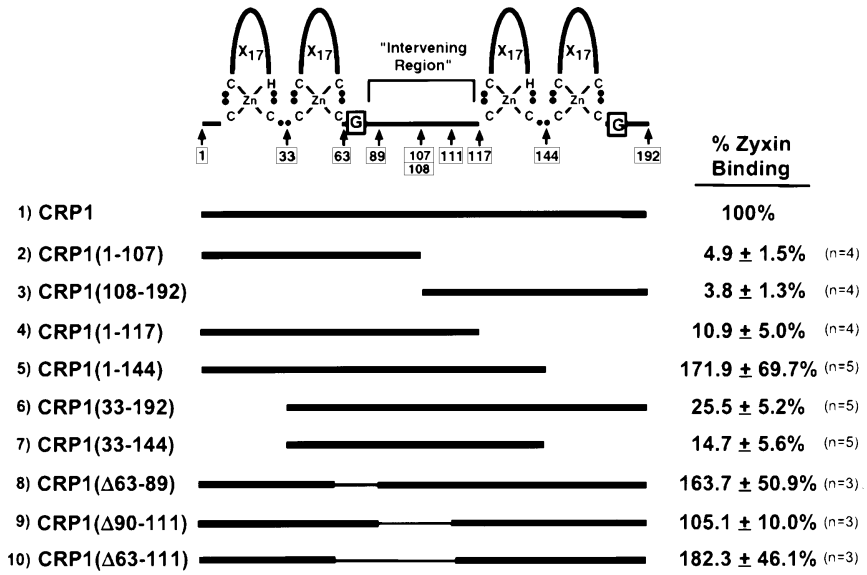

Figure 3 Diagram of recombinant CRP1-derived proteins used to map zyxin's binding site

pET5 bacterial expression vectors were engineered to produce a variety of end-truncated (2–7) or internally truncated (8–10) CRP1 proteins. The nomenclature of each N- or C-terminally truncated CRP1 molecule denotes the amino acid residues included in each protein; the names of the internally deleted CRP1 molecules indicate the regions removed from the protein. The composition of each CRP1 molecule is indicated by straight lines; each line corresponds to the regions of CRP1 as depicted at the top of the figure. Thick lines represent those CRP1 sequences present in a given protein and thin lines are indicative of internally deleted sequences. The zyxin-binding activity of each CRP1-derived protein was determined by performing blot-overlay binding assays and the results are summarized here. The levels of zyxin binding were quantificated by Phosphorimager analysis of the binding assay blots; the binding activities of each CRP1 polypeptide are reported here as a percentage of the binding observed with wild-type recombinant CRP1 (±S.E.M.).

proteins, referred to as CRP1(1–107) and CRP1(108–192) (depicted in Figure 3). Each of these molecules includes one of CRP1's two LIM domain structures as well as its accompanying glycine-rich region. The CRP1(1–107) and CRP1(108–192) polypeptides have been shown to adopt native conformations [7,8]. Moreover, the CRP1(1–107) peptide has been shown to retain the ability to interact with CRP1's binding partner, α-actinin [36].

The blot-overlay assay was used to determine whether either one of the CRP1-derived half molecules was sufficient to associate with zyxin. For these experiments, equimolar amounts of purified CRP1, CRP1(1–107) and CRP1(108–192) polypeptides were resolved by SDS/PAGE and were analysed by Coomassie Blue staining (Figure 4A, lanes 1–3) and by blot-overlay binding assays, using ^{32}P-labelled GST–zyxin (Figure 4B, lanes 1–3) as a probe. Autoradiographic analysis revealed that, although the zyxin probe interacts prominently with the full-length CRP1 protein (Figure 4B, lane 1), equimolar amounts of CRP1(1–107) (lane 2) and CRP1(108–192) (lane 3) display only very low levels of zyxin binding (see quantification in Figure 3). Lanes loaded with twice the amount of CRP1(1–107) and CRP1(108–192) (i.e. protein quantities that were comparable with CRP1 with respect to number of LIM domains loaded per lane) also failed to give rise to an interaction comparable with wild-type CRP1 (results not shown). Similar results were observed in blot-overlay assays in which purified CRP1 and its component half-molecules had been dotted directly on to nitrocellulose, and thus had not been subjected to denaturation during SDS/PAGE (results not shown). The ^{32}P-GST leader peptide consistently failed to interact with any CRP1 proteins in the blot-overlay assay ([16,29], and results not shown). Collectively, these data indicate that neither LIM domain of CRP1 alone is sufficient to display a wild-type zyxin-binding site, thereby suggesting that the association between zyxin and CRP1 is not mediated via simple homotypic LIM–LIM dimerization.

Characterization of the minimal CRP1 sequences required for zyxin binding

Given that the binding site for zyxin on CRP1 was not contained solely within either of CRP1's component LIM domains, we reasoned that a different subregion of CRP1 might be responsible for mediating an association with zyxin. To characterize the minimal CRP1 sequences required for zyxin binding, we examined the zyxin-binding activity of a collection of molecularly engineered N- and C-terminally truncated forms of CRP1 (summarized in Figure 3). Recombinant CRP1 proteins displaying terminal deletions all exhibited the capacity to bind significant amounts of zinc, suggesting that these proteins have the capacity to adopt a zinc-dependent fold. To assay for zyxin-binding activity, comparable amounts of each purified CRP1 polypeptide were resolved by SDS/PAGE and evaluated by Coomassie Blue staining (Figure 4A) or by blot-overlay assay with a ^{32}P-GST–zyxin probe (Figure 4B). The C-terminally truncated CRP1(1–144), lacking the second zinc-binding module of CRP1's second LIM domain, displays the capacity to bind zyxin (see Figures 4A and 4B, lanes 5). Another C-terminally-deleted CRP1 molecule, CRP1(1–117), which displays an intact intervening region, interacted with the zyxin probe at low levels that were reminiscent of those observed with CRP1(1–107) (Figure 4A and 4B, lanes 4). Finally, the CRP1(33–192) and CRP1(33–144) molecules, both of which harbour N-terminal deletions, exhibited only partial affinity for the zyxin probe (Figure 4A and 4B, lanes 6 and 7, respectively). An identical zyxin-binding profile was observed when GST–zLIM1 was used as a probe (results not shown), thereby confirming that the effects

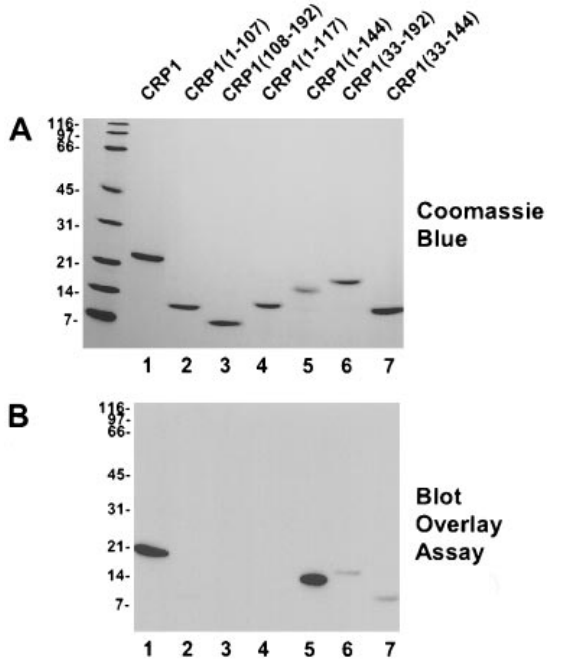

Figure 4 Zyxin-binding activity of the CRP1 half-molecules and the terminally deleted CRP1 fragments

(**A**) Coomassie Blue-stained gel of purified CRP1 protein derivatives. Approx. 100 pmol of each purified protein was loaded in each lane. (**B**) Parallel gels were transferred to nitrocellulose and analysed in blot-overlay assays with 600000 cpm/ml of the ^{32}P-labelled GST–zyxin probe. Positive interactions were visualized by autoradiography. Collectively this analysis indicates that, although neither of the LIM domains of CRP1 is sufficient to support an association with zyxin, the binding site for zyxin on CRP1 requires sequences that are found in both of CRP1's component LIM domains.

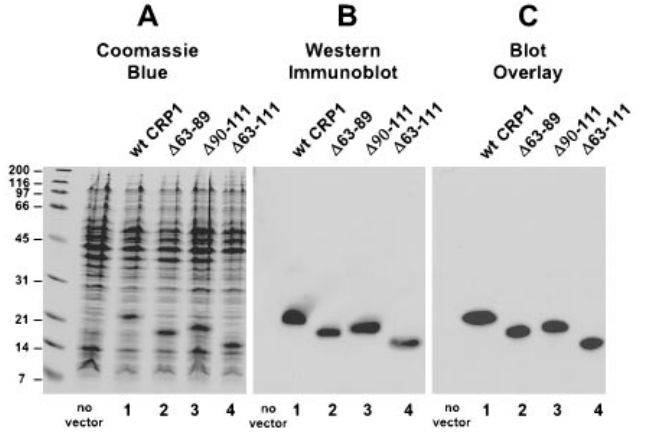

Figure 5 Removal of CRP1's central linker region does not affect zyxin binding

(**A**) Coomassie Blue-stained gel of BL21(DE3) bacterial cell lysates. Extracts were derived from naive cells (no vector) or from cell expressing CRP1 (lane 1), CRP1(Δ63-89) (lane 2), CRP1(Δ90–111) (lane 3) or CRP1(Δ63–111) (lane 4). (**B**) Parallel Western immunoblot of bacterial extracts using the B37 antibody demonstrate that comparable amounts of each protein were present after transfer to nitrocellulose. (**C**) Blot-overlay assay of a parallel blot probed with 600000 cpm/ml of the ^{32}P-GST-Zyxin probe. The results illustrate that amino acids 63–111 of CRP1 are not required for the zyxin-binding function of CRP1.

observed here were due to the presence of sequences contained in the N-terminal LIM domain of zyxin's LIM region. As in previous experiments, the ^{32}P-GST leader peptide probe did not bind any of the terminally deleted CRP1 proteins (results not shown). These results, which are summarized in Figure 3, indicate that zyxin's binding site on CRP1 does not appear to be contained within an array of contiguous amino acids.

Although the deletion-mapping studies described above revealed that sequences in CRP1's intervening region were not sufficient to support a wild-type zyxin-binding function, at this point we were not able to rule out the possibility that this region of CRP1 was important to optimize the association with zyxin. Therefore, to probe the significance of CRP1's intervening region in greater detail, we engineered three internal deletions in the CRP1 molecule that gave rise to proteins referred to as CRP1(Δ63–89), CRP1(Δ90–111) and CRP1(Δ63–111) (Figure 3). These internally deleted peptide derivatives of CRP1 were very unstable, making their purification technically difficult. Therefore, to analyse the zyxin-binding activity of these molecules, we performed blot-overlay assays on proteins that were presented in the context of complex bacterial lysates. As can be seen in the Coomassie Blue-stained gel in Figure 5(A), CRP1(Δ63–89), CRP1(Δ90–111) and CRP1(Δ63–111) proteins of expected sizes were effectively expressed in bacteria. Parallel gels were examined by Western immunoblot to assess relative CRP1 protein levels present on the blot (Figure 5B), and by blot overlay assay with the GST–zyxin probe to assay zyxin-binding activity (Figure 5C). Evaluation of results, such as those shown in Figure 5(C), revealed that each of the internally deleted CRP1 proteins retains significant zyxin-binding potential. Proteins present in the naive bacterial-cell extracts (no vector) do not account for this observed activity. The ^{32}P-GST probe did not exhibit any detectable interactions (results not shown). Quantitative analysis of these experiments showed that removal of the sequences that physically link the two LIM domains of CRP1 did not diminish the zyxin-binding capacity of the molecule (summarized in Figure 3). Binding studies performed using a ^{32}P-GST-zLIM1 probe showed similar binding preferences (results not shown).

DISCUSSION

LIM domains have been identified in over 60 gene products to date [2–4]. The importance of the LIM domain has become increasingly apparent as more studies reveal a prominent role for LIM proteins in processes such as embryogenesis, neural patterning and pathfinding, tissue differentiation and the control of cell proliferation [12–15,40–42]. The interaction between the two LIM domain proteins, zyxin and CRP1, has proved to be a useful model system for studying structure–function relationships within the LIM domain [1,26,27]. A domain-mapping analysis of zyxin revealed that zyxin's binding site for CRP1 is contained within one of its three LIM domains (zLIM1) [16,29]. This finding, as well as those of others [17–25], established the LIM domain as a specific protein-binding interface that is likely to function like other modular protein-binding domains in facilitating protein localization and function.

Here we have examined the zyxin–CRP1 interaction further to characterize the role of CRP1's LIM domains in zyxin binding. Our results show that zinc co-ordination by CRP1 is required for maximal zyxin-binding activity. Because zinc depletion results in the structural destabilization of the LIM domain [5–7], these findings indicate that properly folded LIM domains are essential for optimal binding of zyxin by CRP1. Interestingly, some residual zyxin binding is observed in CRP1 samples in which

metal had been quantitatively removed prior to the binding assay (Figure 2). It is not possible to determine whether this binding involves apo-CRP1 or if the apo-CRP1 may have been able to scavenge trace zinc ions during the course of the binding studies. Given the possibility that some metal co-ordination and refolding of CRP1 could occur during the binding assay, our analysis may represent an underestimation of the functional consequences of zinc removal from CRP1. It is also possible that the cysteine residues of a subpopulation of apo-CRP1 molecules may have formed disulphide bridges that effectively restore the structure of the CRP1 protein and allow for partial restoration of protein function in the absence of bound zinc [43]. In any case, the significant reduction of zyxin binding when metal is depleted from CRP1 is consistent with a role for the LIM domains in presentation of the zyxin-docking site.

Collectively, the results from our domain-mapping analysis indicate that the binding site for zyxin is not present in a linear amino acid array in either of CRP1's individual LIM domains, or in their associated glycine-rich repeats, since neither CRP1 half-molecule binds zyxin at wild-type levels. Instead, additional deletion-mapping studies suggest a more complicated interactive mechanism.

Our analyses clearly pinpoint several regions in CRP1 that are not essential for zyxin binding. For example, analysis of the CRP1(1–144) protein demonstrates that zyxin-binding activity does not require the second zinc-binding module of CRP1's second LIM domain and its adjacent glycine-rich sequences. Our results also reveal that sequences from CRP1's intervening region (amino acids 63–111) can be entirely removed without diminishing CRP1's zyxin-binding potential. This observation, combined with the fact that the CRP1(1–117) protein, which displays an intact intervening region, fails to associate with zyxin in our binding assay, suggests that CRP1's linker region does not participate in sequence-specific recognition of the zyxin protein. Although we cannot exclude the possibility that our solid-phase-binding analysis underestimates the structural contributions of the intervening region (e.g. with respect to presentation of LIM sequences), we conclude that these regions do not themselves constitute the zyxin-docking site. Minimally, the zyxin–CRP1 interaction requires sequences from CRP1's entire N-terminal LIM domain as well as sequences contained within the N-terminal zinc-binding module of LIM2; residues 112–116 of CRP1 may also be necessary for zyxin binding, but they are clearly not sufficient. Our results, demonstrating that both of CRP1's LIM domains contribute to zyxin's binding site, are consistent with our finding that zinc-depleted CRP1 displays a diminished zyxin-binding activity. The specific amino acid residues within CRP1's LIM domains that are required to specify zyxin binding remain to be determined.

Our findings do not rule out the possibility that interactions between CRP1 and other partners may be attributable specifically to one of CRP1's component LIM domains. Indeed, the interaction between CRP1 and the actin cross-linking protein, α-actinin, has been recently mapped to sequences contained within CRP1(1–107), which contains a single LIM domain [36]. Interestingly, however, because both the LIM domains of CRP1 appear to be required to mediate a stable association with zyxin, this study provides an example of a situation in which multiple LIM domains within a protein contribute to the ability of the protein to bind a single partner. This alternative interactive mechanism may be employed by other LIM proteins, some of which display up to five LIM repeats [3,4,44].

In conclusion, by characterizing the binding site for zyxin on CRP1, we have demonstrated that the interaction between zyxin and CRP1 requires the participation of LIM domain sequences from both proteins. Because zyxin's binding site is not reconstituted in a short linear peptide array within CRP1, we suggest that the zyxin–CRP1 interaction requires a precise structural alignment of a number of sequences found in both of CRP1's component LIM domains. The apparent complexity of zyxin's binding site on CRP1 underscores the fact that, unlike other protein-binding domains, no short peptide-binding-site consensus has yet emerged for the LIM domain.

We would like to thank all the members of the Beckerle laboratory for helpful discussions of these data, and P. Pomiès and D. Nix in particular for their assistance in preparing this manuscript. We would also like to thank D. Winge for his advice on the metal-binding assays, for assistance with the metal analyses performed in these studies and for many helpful discussions. J. Michelsen's help in generating the zinc-depleted CRP1 proteins is also greatly appreciated. Finally, we would like to thank B. Schackmann and the University of Utah Biotechnology Core Facility (CA-42014) for the synthesis of all primers used in the generation of the CRP1 mutant proteins and for peptide microsequencing. This work was supported by a National Institutes of Health predoctoral training grant (CA-09602) to K.L.S. and by a National Institutes of Health grant (GM-50877) to M.C.B., who is also the recipient of a Faculty Research Award from the American Cancer Society.

REFERENCES

1 Sadler, I., Crawford, A. W., Michelsen, J. W. and Beckerle, M. C. (1992) J. Cell Biol. **119**, 1573–1587
2 Sanchez-Garcia, I. and Rabbitts, T. H. (1994) Trends Genet. **10**, 315–320
3 Dawid, I. B., Toyama, R. and Taira, M. (1995) C. R. Acad. Sci. Ser. III **318**, 295–306
4 Gill, G. N. (1995) Structure **3**, 1285–1289
5 Michelsen, J. W., Schmeichel, K. L., Beckerle, M. C. and Winge, D. W. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 4404–4408
6 Kosa, J. L., Michelsen, J. W., Louis, H. A., Olsen, J. I., Davis, D. R., Beckerle, M. C. and Winge, D. R. (1993) Biochemistry **33**, 468–477
7 Michelsen, J. W., Sewell, A. K., Louis, H. A., Olsen, J. I., Davis, D. R., Winge, D. R. and Beckerle, M. C. (1994) J. Biol. Chem. **269**, 11108–11113
8 Pérez-Alvarado, G. C., Miles, C., Michelsen, J. W., Louis, H. A., Winge, D. R., Beckerle, M. C. and Summers, M. F. (1994) Nat. Struct. Biol. **1**, 388–398
9 Archer, V. E. V., Breton, J., Sanchez-Garcia, I., Osada, H., Forster, A., Thomson, A. J. and Rabbitts, T. H. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 316–320
10 Pérez-Alvarado, G. C., Kosa, J. L., Louis, H. A., Beckerle, M. C., Winge, D. R. and Summers, M. F. (1996) J. Mol. Biol. **257**, 153–174
11 Konrat, R., Weiskirchen, R., Krautler, B. and Bister, K. (1997) J. Biol. Chem. **272**, 12001–12007
12 Weiskirchen, R. and Bister, K. (1993) Oncogene **8**, 2317–2324
13 Warren, A. J., Colledge, W. H., Carlton, M. B. L., Evans, M. J., Smith, A. J. H. and Rabbitts, T. H. (1994) Cell **78**, 45–57
14 Shawlot, W. and Behringer, R. R. (1995) Nature (London) **374**, 425–430
15 Arber, S., Hunter, J. J., Ross, J., Hongo, M., Sansig, G., Borg, J., Perriard, J.-T., Chien, K. R. and Caroni, P. (1997) Cell **88**, 393–403
16 Schmeichel, K. L. and Beckerle, M. C. (1994) Cell **79**, 211–219
17 Valge-Archer, V. E., Osada, H., Warren, A. J., Forster, A., Li, J., Baer, R. and Rabbitts, T. H. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 8617–8621
18 Wadman, I., Li, J., Bash, R. O., Forster, A., Osada, H., Rabbitts, T. H. and Baer, R. (1994) EMBO J. **13**, 4831–4839
19 Wu, R.-Y. and Gill, G. N. (1994) J. Biol. Chem. **269**, 25085–25090
20 Bach, I., Rhodes, S. J., Pearse, R. V., Heinzel, T., Gloss, B., Scully, K. M., Sawchenko, P. E. and Rosenfeld, M. G. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 2720–2724
21 Osada, H., Grutz, G., Axelson, H., Forster, A. and Rabbitts, T. H. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 9585–9589
22 Durick, K., Wu, R., Gill, G. and Taylor, S. (1996) J. Biol. Chem. **271**, 12691–12694
23 Jurata, L. W., Kenny, D. A. and Gill, G. N. (1996) Proc. Natl. Acad. Sci., U.S.A. **93**, 11693–11698
24 Agulnick, A. D., Taira, M., Breen, J. J., Tanaka, T., Dawid, I. B. and Westphal, H. (1996) Nature (London) **384**, 270–272
25 Johnson, J. D., Zhang, W., Rudnick, A., Rutter, W. J. and German, M. S. (1997) Mol. Cell. Biol. **17**, 3488–3496
26 Crawford, A. W., Pino, J. D. and Beckerle, M. C. (1994) J. Cell Biol. **124**, 117–127
27 Beckerle, M. C. (1997) BioEssays **19**, 949–957
28 Macalma, T., Otte, J., Hensler, M. E., Bockholt, S. M., Louis, H. A., Kalff-Suske, M., Grzeschik, K.-H., von der Ahe, D. and Beckerle, M. C. (1996) J. Biol. Chem. **271**, 31470–31478
29 Schmeichel, K. L. and Beckerle, M. C. (1997) Mol. Biol. Cell **8**, 219–230

30 Liebhaber, S. A., Every, J. G., Urbanek, M., Wang, X. and Cooke, N. E. (1990) Nucleic Acids Res. **18**, 3871–3879
31 Weiskirchen, R., Pino, J. D., Macalma, T., Bister, K. and Beckerle, M. C. (1995) J. Biol. Chem. **270**, 28946–28954
32 Louis, H. A., Pino, J. D., Schmeichel, K. L., Pomiès, P. and Beckerle, M. C. (1997) J. Biol. Chem. **272**, 27484–27419
33 Abel, T. and Maniatis, T. (1989) Nature (London) **341**, 24–25
34 Kerrpola, T. K. and Curran, T. (1991) Curr. Opin. Struct. Biol. **1**, 71–79
35 Edmondson, D. G. and Olson, E. N. (1993) J. Biol. Chem. **268**, 755–758
36 Pomiès, P., Louis, H. A. and Beckerle, M. C. (1997) J. Cell Biol. **139**, 1139–1147
37 Schmeichel, K. L., Beckerle, M. C. and Crawford, A. W. (1997) Cell Biology: A Laboratory Handbook (J. E. Celis, ed.), pp. 441–449, Academic Press, San Diego
38 Laemmli, U.K. (1970) Nature (London) **227**, 680–685
39 Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
40 Arber, S., Halder, G. and Caroni, P. (1994) Cell **79**, 221–231
41 Blair, S. S., Brower, D. L., Thomas, J. B. and Zavortink, M. (1994) Development **120**, 1805–1815
42 Pfaff, S. L., Mendelsohn, M., Stewart, C. L., Edlund, T. and Jessell, T. M. (1996) Cell **84**, 309–320
43 Banecki, B., Leberek, K., Wall, D., Wawrzynow, A., Georgeopoulos, B., E., Tanfani, F. and Zylicz, M. (1996) J. Biol. Chem. **271**, 14840–14848
44 Stronach, B. E., Siegrist, S. E. and Beckerle, M. C. (1996) J. Cell Biol. **134**, 1179–1195

Received 5 January 1998/2 February 1998; accepted 3 February 1998