Fine mapping of the α-actinin binding site within cysteine-rich protein

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The cysteine-rich proteins (CRPs) are a family of highly conserved LIM (an acronym derived from the three gene products lin-11, isl-1 and mec-3) domain proteins that have been implicated in muscle differentiation. All CRP family members characterized so far have been shown to interact with the filamentous actin cross-linker α-actinin. The region of CRP required for this interaction has previously been broadly mapped to the molecule's N-terminal half. Here we report that the α-actinin-binding region of CRP, which we have mapped by using a combination of blot overlay and Western immunoblot techniques, is confined to an 18-residue sequence occurring within the protein's N-terminal glycine-rich repeat. A site-directed mutagenesis analysis of the binding region has revealed the critical importance of a single lysine residue (lysine 65 in human CRP1). Alterations at this site lead to a 10-fold decrease in α-actinin binding in comparison with wild-type CRP. The critical lysine residue localizes within a short α-helix, raising the possibility that mutagenesis-induced alterations in α-actinin-binding capacity might be attributed to the disruption of a key structural element.

Key words: actin cytoskeleton, glycine-rich repeat, muscle, myogenesis.

INTRODUCTION

The cysteine-rich proteins (CRPs) are highly conserved proteins that have been implicated in muscle differentiation [1–7]. In vertebrate systems, three CRP family members have been identified: CRP1, which is expressed in vascular and visceral smooth-muscle cells [5,7]; CRP2, which is prominent in vascular smooth muscle [6,7]; and CRP3, also referred to as the muscle LIM protein (MLP; LIM is itself an acronym derived from the three gene products lin-11, isl-1 and mec-3), which is found exclusively in striated muscle [3]. The function of MLP/CRP3 has been probed extensively both *in vitro* and *in vivo* [3,4]. MLP/CRP3 overexpression in cell culture has been shown to potentiate myogenesis [3]. Conversely, the absence of the *MLP* gene product from mice causes early postnatal cardiomyopathy, hypertrophy and heart failure. Analysis of the cardiac muscle cells derived from these MLP/CRP3$^{-/-}$ mice reveals extensive disorganization of the cytoarchitecture, suggesting an important role for the protein in establishing and maintaining proper ultrastructure in these highly organized cells [4]. Although fewer functional studies have been performed with CRP1 and CRP2, they are postulated to have similar roles in tissues in which they are expressed, such as smooth-muscle derivatives [7].

Subcellular localization patterns of the CRPs offer additional evidence for a role in cytoarchitectural organization. Immunofluorescence studies of endogenous and ectopically expressed CRP family members in fibroblastic cell lines reveal extensive co-localization with actin stress fibres [7,8]. Moreover, MLP/CRP3 localizes to costameres, as well as to distinct bands flanking the Z disc, in cardiac muscle [4]. Interestingly, two *Drosophila* proteins, Mlp84B and Mlp60A, which are related to the vertebrate CRPs, localize to specific regions adjacent to the Z bands of larval midgut visceral muscles, further suggesting that the functional importance of the CRPs lies in their association with the actin cytoskeleton [2].

The predominant structural component of CRP family members is the LIM domain, which acts as a discrete protein-binding unit [9,10]. The association of LIM domains with specific protein partners is now known to have a critical role in the control of its subcellular distribution and activity [11–13]. Moreover, because of the capacity of individual LIM domains to dock with unique protein partners, it has been proposed that proteins with multiple LIM domains might act as molecular scaffolds, facilitating the assembly of biologically active protein complexes within the cell [14,15].

The solution dynamics of CRPs are compatible with their postulated scaffolding activity. Structural determinations have so far been performed on two family members (avian CRP1 and CRP2) by NMR spectroscopy, and the three-dimensional structures reveal that a flexible linker region separates the LIM domains in both molecules [16,17]. This characteristic confers both lateral and rotational freedom on the LIM domains, thereby potentially enabling CRP molecules within cells to present a wide variety of orientations for recruitment of protein partners.

In addition to the LIM domains, CRP family members display structurally interesting glycine-rich regions that are found C-terminal to each of the two LIM domains. These glycine-rich regions display substantial intramolecular similarity, with the N-terminal and C-terminal repeats of hCRP1 being 78 % similar [18]. These regions also exhibit a striking degree of evolutionary conservation: the N-terminal glycine-rich regions of human and avian CRP1 are 94 % identical [18,19]. The extensive sequence conservation in these regions suggests that the precise sequence is important for defining some aspect of the protein's function, such as providing a surface for association with a binding partner.

Although members of the CRP family are known to be essential for normal muscle function *in vivo*, little is known about the precise molecular mechanism by which they contribute to the normal physiology and contractile capacity of muscle cells. One strategy for developing insight into the mechanism of CRP function *in vivo* is to define the partners that associate with members of the family. Biochemical studies with purified CRP1 have led to the identification of two binding partners, both of

Abbreviations used: CRP, cysteine-rich protein; GST, glutathione S-transferase; LIM, acronym derived from the three gene products lin-11, isl-1 and mec-3; MLP, muscle LIM protein.

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which are associated with the cytoskeleton. The first binding partner for CRP to be identified was zyxin [20], a focal adhesion protein implicated in the regulation of actin organization and signalling between the cell surface and the nucleus [14,21]. Subsequently, α-actinin, the ubiquitously expressed cross-linker of filamentous actin, was identified as a binding partner for CRP1 [8]. Not surprisingly, given the high degree of sequence conservation, all three vertebrate CRPs share the ability to associate with both zyxin and α-actinin [7]. The fact that these two partners are proteins associated with the organization of the actin cytoskeleton is consistent with the postulated cyto-architectural role of CRP family members.

We have concentrated our efforts here on studying the association between CRP1 and α-actinin. We show that the ability of CRP1 to interact with α-actinin depends on an 18-residue sequence outside the LIM domains that is both necessary and sufficient to support the association. Moreover, by mutagenesis analysis we have identified a single lysine residue in CRP1 that is required for its partnership with α-actinin.

MATERIALS AND METHODS

Generation of constructs

Plasmids engineered for expression of the glutathione S-transferase (GST) fusion proteins hCRP1 (2–193), hCRP1 (62–118) and hCRP1 (80–118) were generously provided by Stephen Leibhaber [22]. Fusion constructs that linked the N-terminal or C-terminal glycine-rich repeat of hCRP1 (residues 62–79 or 171–188 respectively) to GST were generated as follows. Complementary oligonucleotides encoding each of these 18-residue regions were synthesized to include *Eco*RI overhangs. The oligonucleotides were annealed and the resultant cDNA species were subcloned into the *Eco*RI site of the pGEX2T (128/129) vector (a gift from Michael Blanar, Department of Cardiovascular Drug Discovery, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ, U.S.A.).

The generation of glycine-rich repeat constructs that carried single point mutations (Y66F, Y66A, K65N and N174K) was accomplished by PCR. Sense oligonucleotides that spanned an *Eco*RI site and harboured the appropriate point mutation were used for PCR amplification from a wild-type hCRP1 glycine-rich repeat template. Anti-sense oligonucleotides included a second *Eco*RI cloning site. PCR products were digested with *Eco*RI and subcloned into pGEX2T (128/129). We used the same strategy, with a single modification, to generate a construct in which the N-terminal glycine-rich repeat carried two point mutations (K65N/Y66F). In this instance the sense oligonucleotide was complementary to a Y66F template and harboured the K65N point mutation.

Sequence analysis was performed on all constructs to verify the integrity of the polymerase reaction and/or mutagenesis.

The expression construct encoding chicken CRP1 lacking glycine-rich repeat residues 63–89 (cCRP1 Δ63–89) was provided by Karen Schmeichel (Lawrence Berkeley National Laboratory, Life Sciences Division, Berkeley, CA, U.S.A.) [23].

Fusion protein expression and purification

The methods for the expression and purification of all GST fusion proteins used have been slightly modified from those previously described [22]. In brief, cleared bacterial lysates were incubated with 0.03 vol. of 50% (w/v) glutathione–agarose (Sigma-Aldrich Chemicals, St Louis, MO, U.S.A.) slurry for 10 min at room temperature. The protein-bound beads were washed four times with PBS/0.1% protease inhibitor cocktail (100 mM PMSF/100 mM benzamidine/HCl/1.5 mM pepstatin A/5 mM *o*-phenanthroline) and eluted with 8 bed vol. of 15 mM glutathione (Sigma-Aldrich Chemicals)/50 mM Tris (pH 8.0).

SDS/PAGE and Western immunoblot analysis

Gel electrophoresis was performed as described by Laemmli [24], except for use of 0.13% bisacrylamide as the cross-linking reagent; 15% (w/v) gels were used for the resolution of fusion proteins in all experiments.

Western immunoblots were performed as outlined by Towbin et al. [25]. A mouse monoclonal antibody (ICN Biomedicals, Aurora, OH, U.S.A.) was used for primary recognition of the α-actinin antigen, followed by an anti-mouse IgG secondary reagent coupled to horseradish peroxidase (Amersham Life Science, Cleveland, OH, U.S.A.). The membranes were subsequently treated with enhanced chemiluminescence reagents and analysed by autoradiography.

Blot overlay assay

hCRP1 truncation and point mutation constructs were resolved by SDS/PAGE [15% (w/v) gel] and were transferred electrophoretically at 0.6 A to nitrocellulose membranes. Additional protein-binding sites on the nitrocellulose were blocked overnight in 50 mM Tris/HCl (pH 7.6)/150 mM NaCl/1% (w/v) NaN_3/2.5% (w/v) BSA. The nitrocellulose membranes were then incubated with 20 nM α-actinin in overlay buffer [20 mM Hepes (pH 7.5)/0.5% BSA/0.25% gelatin/1% (v/v) Nonidet P40/10 mM NaCl/1 mM EGTA/0.1% (v/v) 2-mercaptoethanol] for 3.5 h. α-Actinin was purified from an avian smooth-muscle extract as described previously [26]. After washing four times for 5 min in approx. 50 ml of PBS, any α-actinin specifically bound was fixed to the membrane with 0.5% (v/v) formaldehyde/PBS for 20 min and washed with 2% (w/v) glycine/PBS, also for 20 min. Membranes were washed again in PBS, as described above, before the presence of α-actinin was detected with a Western immunoblot technique.

Quantification of binding assays

To determine precisely the effects of altering single residues within the glycine-rich repeats of hCRP1, densitometry analysis was performed on the autoradiographs of blot overlay assays performed with α-actinin and wild-type or mutated glycine-rich repeats. The binding of α-actinin to each construct was quantified with Gel Doc 2000 Volume Analysis Tools (Bio-Rad, Hercules, CA, U.S.A.). Some variation in the amount of each construct present on a given membrane occurred owing to differences in protein loading and was accounted for by performing densitometry analysis on Coomassie Blue-stained gels run in parallel with those transferred to nitrocellulose. α-Actinin binding to all constructs is expressed as a percentage of binding (mean ± S.D.) to the wild-type N-terminal glycine-rich repeat of hCRP1.

RESULTS

N-terminal glycine-rich repeat of hCRP1 is necessary for α-actinin binding

Because genetic studies in which CRP function is compromised suggest a role for the protein in maintenance of the stability of the contractile machinery, we have focused our attention on the association between CRP1 and its cytoskeletal partner, α-actinin.

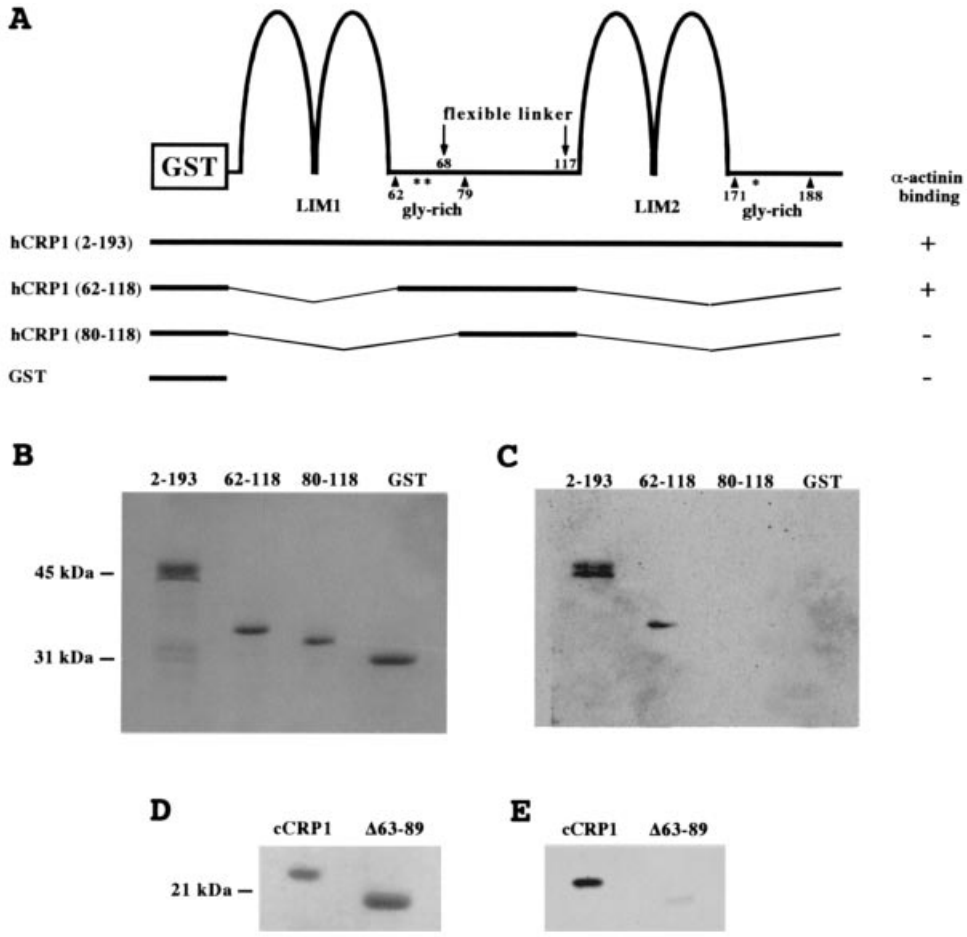

Figure 1 N-terminal glycine-rich repeat is necessary for α-actinin binding

(**A**) Molecular organization of hCRP1. Residues 62–79 and 171–188 are included in the N-terminal and C-terminal glycine-rich repeats respectively (arrowheads). Residues defining boundaries of the flexible linker are indicated by arrows. Point mutations at positions 65, 66 and 174 are indicated with an asterisk. Regions of the hCRP1 molecule included in GST fusion constructs used in experiments shown in (**B**) and (**C**) are indicated by thick lines. The ability of a given construct to bind α-actinin is indicated by + ; inability is indicated by −. (**B**) Purified recombinant protein revealed by staining with Coomassie Blue. (**C**) Autoradiograph of a blot overlay assay displaying the inability of α-actinin to interact with a recombinant hCRP1 molecule lacking the N-terminal glycine-rich repeat. Recombinant proteins were separated electrophoretically and transferred to nitrocellulose. Membranes were incubated with native α-actinin, then washed and treated with a fixative to capture α-actinin bound specifically. The interaction was subsequently detected by means of Western immunoblot techniques. (**D**) Purified bacterially expressed chicken CRP1 and CRP1 lacking glycine-rich repeat residues 63–89 (Δ63–89) as revealed with Coomassie Blue stain. (**E**) Representative autoradiograph of a blot overlay assay showing the inability of α-actinin to interact with CRP1 molecules lacking residues 63–89. α-Actinin binding activity was assayed as described for (**C**).

It has been previously reported that the α-actinin binding activity of CRP1 is contained within its N-terminal half [8], which includes a single LIM domain, the N-terminal glycine-rich repeat and a portion of the sequence intervening between the two LIM domains. Because structural studies performed so far indicate that this intervening region (hCRP1 residues 68–117) is likely to be highly mobile in solution [16,17], we refer to it as a flexible linker (Figure 1A). The N-terminal portion of CRP1 is also sufficient to target the protein to elements of the actin cytoskeleton, thus raising the possibility that the ability of CRP1 to bind α-actinin might contribute to its subcellular distribution and therefore be critical for its normal function in muscle cells. As we were interested in a high-resolution view of the region within CRP1 that is critical for the support of an α-actinin binding interaction, we tested the ability of α-actinin to interact with constructs representing discrete domains within this N-terminal segment. Using a blot overlay assay that has previously been demonstrated to be effective for the observation of the CRP1–α-actinin interaction [7,8,23], we performed a deletion analysis to identify regions in CRP1 that are necessary to support an association with α-actinin. As can be seen in Figure 1(C), a GST fusion protein harbouring full-length CRP1 sequences, residues 2–193, exhibited α-actinin binding. We used CRP1 sequences beginning at residue 2 for the full-length fusion protein because we have previously shown that the N-terminal methionine is cleaved post-translationally [19]. A GST fusion protein GST–CRP1 (62–118), from which both the N-terminal LIM domain and the C-terminal half of the protein had been deleted, retained the capacity to bind α-actinin. However, a further deletion mutant from which 18 additional residues (62–79), comprising the glycine-rich region, had been eliminated from CRP1 failed to interact with α-actinin. Bacterially expressed full-length CRP1 molecules lacking residues 63–89 also lacked the capacity to bind α-actinin (Figures 1D and 1E). Thus it seemed that residues 62–79 within the N-terminal glycine-rich region are essential for CRP1's ability to interact with α-actinin.

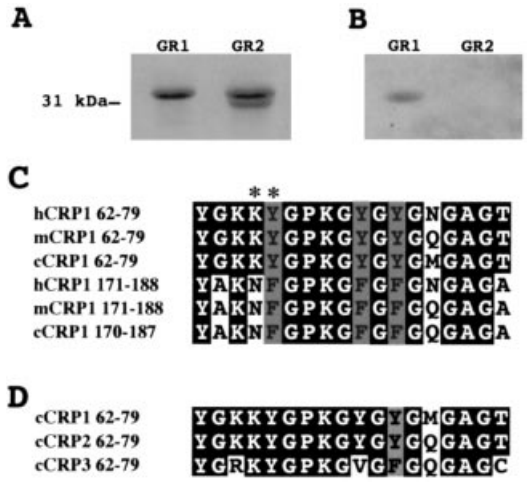

Figure 2 N-terminal glycine-rich repeat of hCRP1 is sufficient for α-actinin binding

(**A**) Purified recombinant GR1 and GR2 as revealed by staining with Coomassie Blue. (**B**) Autoradiograph of a representative blot overlay assay, showing the specificity of α-actinin's interaction with the N-terminal glycine-rich repeat. (**C**) Interspecies alignments reveal high levels of sequence identity between N-terminal and C-terminal glycine-rich repeats. CRP1 residues 62–79 and 170/171–187/188 were N-terminally linked to GST to form GR1 and GR2 respectively. Residues altered via site-directed mutagenesis are indicated with an asterisk. (**D**) Alignment of the N-terminal glycine-rich repeat from avian CRP family members.

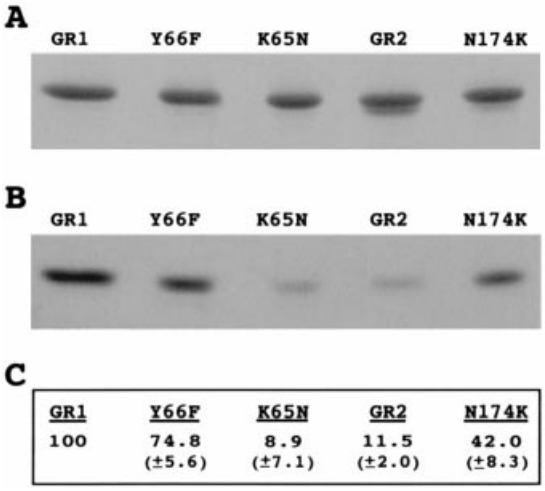

Figure 3 Single-residue changes within the N-terminal glycine-rich repeat affect α-actinin binding activity

(**A**) Purified hCRP1 glycine-rich repeat point mutants, resolved electrophoretically and revealed with Coomassie Blue stain. (**B**) Autoradiograph of a representative blot overlay assay, displaying the differential effects of point mutations within the glycine-rich repeats on binding of α-actinin. (**C**) α-Actinin binding activities were quantified by densitometric analysis of autoradiographs from four independent experiments and are shown here as percentages of wild-type (N-terminal glycine-rich repeat) binding; S.D. values are shown in parentheses. Measurements of optometric density from autoradiographs were adjusted to account for differences in protein loading (see the Materials and methods section).

N-terminal glycine-rich repeat of hCRP1 is sufficient for α-actinin binding

To characterize further the importance of this glycine-rich sequence for α-actinin binding, we sought to determine whether the 18-residue sequence present in hCRP1 (62–118) but absent from hCRP1 (80–118) was sufficient to support the binding interaction. We therefore expressed and purified a recombinant protein (GR1) in which hCRP1 residues 62–79 were N-terminally linked to GST for the detection of α-actinin binding by blot overlay (Figure 2A). To address the possibility that α-actinin could interact with any glycine-rich sequence, we expressed a second construct (GR2) in which the highly similar C-terminal glycine-rich repeat of hCRP1 (residues 171–188) was coupled to GST (Figure 2A). Testing these recombinant proteins for binding activity revealed that α-actinin interacted specifically with the N-terminal glycine-rich repeat of hCRP1 (Figure 2B), leading us to conclude that this 18-residue region was both necessary and sufficient for α-actinin binding. Although the binding to the GR1 sequence was not as high as binding to full-length CRP1 (results not shown), it was highly reproducible.

Identification of key residues within the α-actinin-binding region

Interspecies sequence comparisons of the N-terminal and C-terminal glycine-rich repeats of CRP1 revealed a high degree of sequence identity (Figure 2C). The similarity of these amino acid sequences led us to hypothesize that only a small number of amino acid residues(s) were likely to be present in the N-terminal glycine-rich repeat of hCRP1 but absent from the C-terminal repeat that were essential for supporting CRP's interaction with α-actinin. We used our knowledge that all avian CRP family members characterized so far interact with α-actinin [7] to eliminate as potentially essential any residue that was not conserved among the N-terminal glycine-rich repeats of cCRP1, cCRP2 and cCRP3. We concluded, for example, that the residue at position 71 was unlikely to be important for α-actinin binding because it was not conserved in the N-terminal glycine-rich region of cCRP3 (Figure 2D). Inspection of the sequences of all the available N-terminal glycine repeats, which have α-actinin binding capacity, and the C-terminal repeats, which do not, led us to focus on lysine 65 and tyrosine 66. These two positions are absolutely conserved in the N-terminal glycine-rich repeats of CRP1-3 but are altered in the C-terminal glycine-rich repeats.

To test the hypothesis that lysine 65 or tyrosine 66, or both, were essential within the α-actinin-binding site, we performed site-directed mutagenesis on the GR1 (hCRP1 62–79) coding sequence to create two mutants, Y66F and K65N. In both of these cases, the amino acids in GR1 were changed to their C-terminal counterparts (phenylalanine 175 and asparagine 174 respectively). Analysis of the α-actinin binding activity of the Y66F mutant by blot overlay assay revealed a 25% decrease in α-actinin binding relative to wild-type (GR1), suggesting a moderately important, although not essential, role for tyrosine 66. As can be seen in Figure 3, the effect of the K65N mutation on α-actinin binding was more marked. We observed this recombinant protein to be capable of only approx. 10% of wild-type binding to α-actinin. Interestingly, this single mutation seemed sufficient to decrease binding activity to a level comparable with that observed for GR2 (Figure 3C). It should be noted that Western immunoblot techniques with chemiluminescence reagents provide a temporal resolution gradient, thus explaining the slight discrepancy between levels of α-actinin binding to GR2 visible in Figures 2(B) and 3(B). We did not observe any additional decrease in α-actinin binding with a recombinant protein carrying the Y66F and K65N mutations in tandem (results not shown).

Once we had observed that changing lysine 65 in the N-terminal glycine-rich repeat resulted in a strong decrease in α-

actinin binding, we examined whether replacing the appropriate residue within the C-terminal repeat with lysine would enhance this region's ability to interact with α-actinin. We therefore created the N174K mutant, in which the residue within the C-terminal glycine-rich repeat analogous to lysine 65 was replaced with lysine. Analysing this mutant by blot overlay revealed a substantial increase in α-actinin binding activity (Figure 3C). These results, along with our observations on the K65N mutant, suggested that lysine 65 is critical for the interaction between α-actinin and the N-terminal glycine-rich repeat. Moreover, the absence of lysine from the analogous position within the C-terminal glycine-rich repeat might explain the absence of a robust interaction between this domain and α-actinin.

DISCUSSION

Our results define the critical role of CRP's N-terminal glycine-rich repeat in α-actinin binding. Using a series of blot overlay assays, we have demonstrated that recombinant proteins containing this region are competent to bind α-actinin, whereas proteins lacking this sequence are not. Because a highly similar glycine-rich region following CRP's second LIM domain failed to interact with α-actinin, we performed site-directed mutagenesis to identify residues critical for α-actinin binding within this N-terminal glycine-rich repeat. We found that an alteration at position 65, from lysine to asparagine, was sufficient to cause a 10-fold decrease in α-actinin binding. Moreover, we were able to establish α-actinin binding activity for the C-terminal glycine-rich repeat by the introduction of a lysine residue at the appropriate position.

One explanation for the apparent importance of lysine 65 is that it might participate in the formation of a key structural motif recognized by α-actinin. On the basis of the three-dimensional structure of CRPs ascertained by NMR spectroscopy, two short α-helices, whose locations correspond to portions of the first and second glycine-rich repeats, occur within the cCRP1 and qCRP2 molecules. Although structural analysis has not been performed on hCRP1, hCRP1 residues 59–67 and 167–173 are 100% identical with regions of cCRP1 and qCRP2 that have been reported to be helical [18,19,27]. It is interesting that the helix involving cCRP1's C-terminal glycine-rich repeat is shorter by three amino acid residues than the corresponding N-terminal helix (Figure 4). Under the Chou and Fasman scheme, which classifies amino acids on the basis of the frequency with which they appear in α-helices, asparagine is designated as a helix breaker. As the presence of asparagine at position 174 corresponds both to putative helix truncation and to a prohibition of α-actinin binding in the C-terminal glycine-rich repeat of hCRP1, it is reasonable to speculate that premature truncation of a critical α-helical binding site within the N-terminal repeat via the insertion of asparagine results in diminished partner-binding capacity.

The importance of α-actinin and MLP/CRP3 for the establishment of proper cytoarchitectural organization in muscle has been well documented in genetic studies performed in *Drosophila* and the mouse. *Drosophila fliA* mutants carry various lesions at the α-actinin locus that perturb muscle function. Homozygous *fliA* animals are flightless; subcellular phenotypes include the disruption of sarcomeres and myofibrillar insertions, as well as a lack of Z-disk continuity [28,29]. Interestingly, MLP/CRP3$^{-/-}$ mice experience early postnatal heart failure. Like the *fliA* mutants, these animals display defective cellular ultrastructure, including disruption of the cardiac myofibrillar array and of sites of actin cytoskeletal–membrane linkage. It will be interesting to ascertain what part of these phenotypes is due to a disruption of the CRP/α-actinin interaction, a question that could be addressed with the use of CRP constructs that would act as dominant-negatives with respect to α-actinin binding. Full-length molecules with amino acid substitutions in the α-actinin-binding region, particularly with residues that disrupt the short N-terminal α-helix, might be useful in this regard.

Lastly, the emergent functional importance of apparently minor structural elements within 'LIM-only' proteins should be noted. Although the LIM domains are the most prominent structural element of CRP family members, we have shown here that an inconspicuous glycine-rich/α-helical region is responsible for the α-actinin binding activity of these molecules.

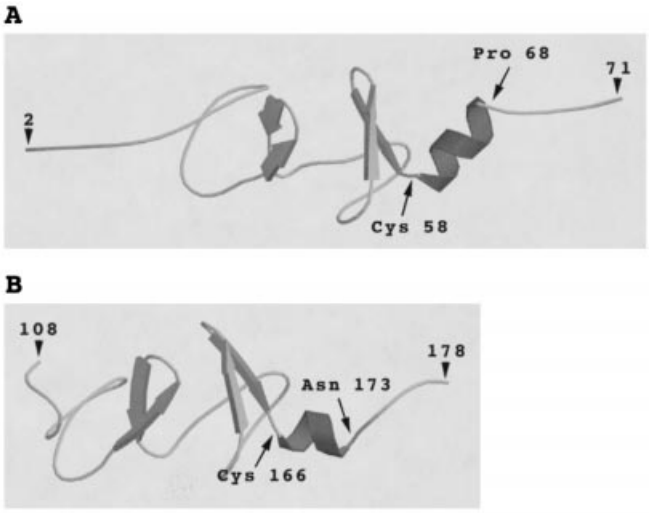

Figure 4 N-terminal and C-terminal glycine-rich repeats of hCRP1 differ in secondary structure

Ribbon diagrams of cCRP1's N-terminal (**A**) and C-terminal (**B**) LIM domains and α-helices. Note the differing lengths of the two helices: the N-terminal helix includes nine residues, three residues beyond the number included in the C-terminal helix, allowing it to complete approximately two full turns. Arrowheads indicate the amino acids at the terminus in each diagram; arrows indicate residues flanking the α-helices. Images were created in MOLSCRIPT [30].

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