

# Comparison of Three Members of the Cysteine-rich Protein Family Reveals Functional Conservation and Divergent Patterns of Gene Expression\*

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Members of the cysteine-rich protein (CRP) family are evolutionarily conserved proteins that have been implicated in the processes of cell proliferation and differentiation. In particular, one CRP family member has been shown to be an essential regulator of cardiac and skeletal muscle development. Each of the three vertebrate CRP isoforms characterized to date is composed of two copies of the zinc-binding LIM domain with associated glycine-rich repeats. In this study, we have addressed the biological significance of the CRP multigene family by comparing the subcellular distributions, biochemical properties, and expression patterns of CRP1, CRP2, and CRP3/MLP. Our data reveal that all three CRP family members, when expressed in adherent fibroblasts, associate specifically with the actin cytoskeleton. Moreover, all three CRP isoforms are capable of interacting with the cytoskeletal proteins  $\alpha$ -actinin and zyxin. Together, these observations suggest that CRP family members may exhibit overlapping cellular functions. Differences between the three CRPs are evident in their protein expression patterns in chick embryos. CRP1 expression is detected in a variety of organs enriched in smooth muscle. CRP2 is restricted to arteries and fibroblasts. CRP3/MLP is dominant in organs enriched in striated muscle. CRP isoform expression is also developmentally regulated in the chick. Our findings suggest that the three CRP family members perform similar functions in different muscle derivatives. The demonstration that all members of the CRP family are associated with cytoskeletal components that have been implicated in the assembly and organization of filamentous actin suggests that CRPs contribute to muscle cell differentiation via effects on cytoarchitecture.

Members of the cysteine-rich protein (CRP)<sup>1</sup> family are evolutionarily conserved proteins that have been implicated in myogenesis. CRPs exhibit a common domain structure, being composed primarily of two tandemly arrayed LIM domains (1,

2). Each LIM domain, defined by the consensus sequence  $CX_2CX_{16-23}HX_2CX_2CX_2CX_{16-21}CX_{2-3}(C/H/D)$ , displays two closely associated zinc-binding modules or "fingers" (3–7). A number of recent investigations have indicated that LIM domains are capable of functioning as specific protein-binding interfaces and are found in a variety of proteins that are involved in cell differentiation (reviewed in Refs. 8–11). A typical LIM protein displays multiple copies of the LIM motif. By virtue of their protein binding capabilities, the LIM regions of proteins are thought to be important for targeting proteins to specific subcellular locations and for mediating the assembly of multimeric protein complexes (9, 12, 13). Because of the presence of two LIM domains in their structures, CRP family members have been proposed to serve as scaffolds that link protein partners in a productive fashion (9, 14).

Three members of the CRP family (CRP1, CRP2, and CRP3/MLP) have been characterized in vertebrates (1, 15–20). The first CRP family member to be identified was CRP1 (15, 16). CRP1 sequences are conserved within vertebrates, being 92% identical at the amino acid level in chick and human (15, 16, 18). In fibroblasts, CRP1 is localized at adhesion plaques and in association with filamentous actin (16, 18, 21). CRP1 has been purified to homogeneity from chicken gizzard, and much is known about its biochemical properties (18). Two protein-binding partners have been identified for CRP1; CRP1 interacts directly with the adhesion plaque protein, zyxin (9, 16, 22), and with the actin-cross-linking protein,  $\alpha$ -actinin (21). Both zyxin and  $\alpha$ -actinin are important regulators of actin cytoskeletal organization (23–25).

The transcript encoding CRP2 was identified in a subtractive hybridization screen for genes whose expression is significantly reduced in *myc*-transformed cells (1, 17). Subsequent studies revealed that CRP2 mRNA is undetectable in a variety of oncogenically and chemically transformed cells and that the dramatic loss of CRP2 expression correlates with the acquisition of the transformed phenotype (1, 17). As has been shown for CRP2, CRP1 expression is also reduced significantly upon fibroblast cell transformation (1). Thus, expression of both CRP1 and CRP2 is inversely correlated with cell proliferation.

The third CRP family member, referred to as MLP (muscle LIM protein) or CRP3, was originally identified in a subtractive hybridization screen designed to find genes whose expression was up-regulated in skeletal muscle after denervation (19). Products of genes that exhibit this property represent candidate myogenic factors. The possible involvement of CRP3/MLP in muscle differentiation was first tested using cultured C2 myoblasts. Consistent with the findings showing that CRP1 and CRP2 protein levels are highest in cells displaying a normal differentiated phenotype, CRP3/MLP expression was shown to be up-regulated dramatically when the C2 myoblasts were stimulated to exit the cell cycle and differentiate. Using

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<sup>1</sup> The abbreviations used are: CRP, cysteine-rich protein; PAGE, polyacrylamide gel electrophoresis.

the same culture system, it was demonstrated that elimination of CRP3/MLP by antisense oligonucleotide expression blocked muscle differentiation in these cells (19). Moreover, overexpression of CRP3/MLP in presumptive myoblasts enhanced the progression of the myogenic program (19). From the studies of myogenic cells in culture, it appeared that CRP3/MLP was essential for muscle differentiation. In fact, recent gene disruption studies in the mouse have confirmed the requirement for CRP3/MLP in both cardiac and skeletal muscle differentiation (26). The involvement of CRP3/MLP in the execution of the myogenic program comes late in the differentiation pathway, after the time that cells are determined in response to factors including the basic helix-loop-helix proteins MyoD and myogenin. In mice that lack CRP3/MLP expression, the muscle cell cytoarchitecture is disturbed, and the normal semicrystalline arrays of contractile proteins fail to organize (26).

Based on the fact that another CRP family member, CRP1, has been shown to interact directly with the cytoskeletal proteins zyxin and  $\alpha$ -actinin, it was postulated that failure of CRP3/MLP homozygous mutant mice to assemble myofibrillar arrays is due to defects in processes that depend on interactions between CRP3/MLP and cytoskeletal partners (21). However, direct support for this hypothesis was lacking since no protein-binding partners had been identified for CRP3/MLP. In this report, we compare the abilities of the three CRP family members to interact with zyxin and  $\alpha$ -actinin and to associate with the actin cytoskeleton. By monitoring transient expression of epitope-tagged versions of each CRP isoform and by performing protein binding assays, we have demonstrated that the three family members display similar subcellular localizations and biochemical properties. Our inability to distinguish between the three CRP isoforms based on functional criteria led us to speculate that the different CRP family members might be expressed in distinct locations within the organism. We have generated isoform-specific antibodies directed against each of the chick CRPs and have demonstrated that these three gene products exhibit distinct patterns of expression with only limited overlap. Also, for those organs examined, each CRP family member displays a characteristic expression profile during embryonic development. The results of our analysis are consistent with the idea that CRP family members have comparable functions that are executed in distinct locations within vertebrate organisms. Our findings make interesting predictions about the mechanism of action of CRP3/MLP in cardiac and skeletal muscle cell differentiation and about the roles of CRP1 and CRP2 in other contractile cells.

#### EXPERIMENTAL PROCEDURES

**Heterologous Expression of CRP1, CRP2, and CRP3/MLP in Cultured REF52 Cells**—Mammalian expression vectors were engineered to express Myc epitope-tagged chicken CRP1, CRP2, or CRP3/MLP in cultured fibroblasts using methods described previously (2, 21, 27). CRP coding regions were amplified in polymerase chain reactions using *Pfu* polymerase (Stratagene, La Jolla, CA) and CRP-specific primers supplemented with *EcoRV* and *NotI* restriction enzyme recognition sites. Once digested with *EcoRV* and *NotI*, the amplified fragments were ligated into the polylinker of the pcDNA1/NEO vector (Invitrogen, San Diego, CA). The pcDNA1/NEO vector used in these studies was supplemented with a sequence encoding an antigenic peptide from the c-Myc protein (EQKLISEEDLL) (21); this modification allowed for the expression of the CRPs with a C-terminal c-Myc epitope tag. All CRP expression vectors were sequenced prior to use via automated sequencing technology on a Model 373A DNA sequencer (Applied Biosystems, Inc., Foster City, CA). Large-scale purifications of plasmid DNAs were performed using a polyethylene glycol precipitation procedure (28). Purified plasmids, resuspended at a concentration of 250 ng/ $\mu$ l in phosphate-buffered saline, were microinjected into REF52 cells that had been plated onto glass coverslips. After a 24-h incubation at 37 °C, cells were fixed and processed for immunofluorescence microscopy.

**Cell Culture and Immunofluorescence**—REF52 cells were cultured in

a 1:3 mixture of Ham's F-12 medium and Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (29). For immunofluorescence analyses, cells were grown to 50–70% confluence on glass coverslips. Indirect immunofluorescence was performed exactly as described previously (30). A mouse monoclonal antibody directed against the c-Myc peptide epitope (American Type Culture Collection, Rockville, MD) was used as a primary antibody to detect heterologous expression and localization of Myc-tagged versions of CRP1, CRP2, or CRP3/MLP in cultured cells. Primary antibodies were detected using fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibodies (Cappel, Durham, NC). F-actin was monitored by incubating fixed cells with rhodamine-conjugated phalloidin (Molecular Probes, Inc., Eugene, OR).

**Expression, Purification, and Quantitation of Recombinant CRPs**—Chicken CRP1 and CRP2 were produced as recombinant proteins in bacteria. As described previously, CRP1 cDNA sequences were subcloned into a pET5 expression vector (Novagen, Madison, WI) such that CRP1 was translated from its endogenous start codon, without any additional leader sequences (4). Similar protocols were employed to generate a pET5-CRP2 expression vector. Cloned cDNA sequences were verified by double-stranded DNA sequence analysis (cycle sequencing kit, Life Technologies, Inc.). Both CRP1 and CRP2 were expressed and purified using protocols described previously for CRP1 (3, 4). Briefly, expression vectors were introduced into BL21(DE3) cells, and transformed cells were grown in LB medium supplemented with 200  $\mu$ g/ml ampicillin (Sigma) to  $A_{600} = 0.7$ – $0.9$ . Protein expression was induced for 3 h at 37 °C in the presence of 0.4 mM isopropyl- $\beta$ -D-thiogalactopyranoside (Sigma). To purify recombinant CRP1 and CRP2, 1 liter of induced cells was harvested by centrifugation, resuspended in 20 ml of lysis buffer (10 mM KCl, 10 mM dithiothreitol, and 10 mM potassium phosphate (pH 7.2)), and homogenized by macrotip sonication (Branson Ultrasonics Corp., Danbury, CT). The resulting lysates were fractionated by centrifugation, and the supernatant was dialyzed overnight against Buffer C2 (0.01% 2-mercaptoethanol and 10 mM potassium phosphate (pH 7.2)). The dialyzed sample was loaded onto a CM52 cation-exchange column pre-equilibrated in Buffer C2. After extensive washing with Buffer C2, purified CRP1 and CRP2 were eluted from the column in a 0–250 mM KCl gradient.

Rat CRP3/MLP was expressed in bacteria as a His-tagged fusion protein (QIAGEN Inc., Chatsworth, CA). Rat CRP3/MLP is nearly 90% identical to its chicken counterpart (19). The CRP3/MLP bacterial expression construct, pQEG-MLP, was a generous gift of S. Arber and P. Caroni (14). Purification of His-tagged CRP3/MLP was performed following the protocol suggested by the manufacturer (31).

Bradford (Bio-Rad) and Lowry (Pierce) protein assays were performed to estimate the concentrations of purified recombinant CRPs. In both assays, bovine serum albumin was used as a standard. These methods yielded comparable quantitative results.

**Blot Overlay Assays**—Blot overlay assays were performed as described previously (9, 21, 22, 32). For these experiments, 100 pmol of CRP1, CRP2, and CRP3/MLP were resolved by SDS-PAGE along with the chicken gizzard fractions described below. Coomassie Blue-stained gels were analyzed by densitometry to evaluate the relative levels of purified CRPs present on the gel. Parallel gels were transferred to nitrocellulose and probed with radioactively labeled  $\alpha$ -actinin or zyxin probes in blot overlay buffer (0.5% bovine serum albumin, 0.25% gelatin, 1.0% Nonidet P-40, 0.1 mM EDTA, 0.1% 2-mercaptoethanol, 10 mM NaCl, and 20 mM Hepes (pH 7.5)).  $\alpha$ -Actinin (purified from chicken gizzard) was radioiodinated using previously described methodologies (21, 32); 250,000 cpm/ml  $^{125}$ I-labeled chicken  $\alpha$ -actinin was included in each overlay assay. The zyxin probe used in this study was a recombinant  $^{32}$ P-labeled glutathione S-transferase fusion protein containing sequences from the zyxin LIM region (chicken zyxin amino acids 349–542); this probe was previously shown to be sufficient for mediating interactions with CRP1 (9). Glutathione S-transferase-zyxin probes were included in the blot overlay binding reaction at 600,000 cpm/ml. After a 2-h incubation with the probes, blots were subsequently washed and evaluated by both autoradiography and PhosphorImager analysis (Imagequant software, Version 3.5, Molecular Dynamics, Inc., Sunnyvale, CA). Relative CRP binding activities were normalized to protein levels determined by densitometry; these values are presented here as a percentage of the binding observed for CRP1  $\pm$  S.E.

**SDS-PAGE and Western Immunoblotting**—SDS-PAGE was performed according to the method of Laemmli (33) with a bisacrylamide concentration of 0.13%. 15% gels were utilized to resolve all CRP-containing samples examined in this study. Molecular mass markers (Bio-Rad) correspond to myosin (200 kDa),  $\beta$ -galactosidase (116 kDa), phosphorylase b (97 kDa), serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21 kDa), and

lysozyme (14 kDa). Western immunoblot analyses were performed following the protocols developed by Towbin *et al.* (34) using horseradish peroxidase-coupled protein A as a secondary agent. Detection was performed via chemiluminescent techniques (ECL, Amersham Life Science, Inc.).

**Antibody Production**—Isoform-specific antipeptide antibodies were generated for chicken CRP1, CRP2, and CRP3/MLP. Unique peptides from divergent regions within each CRP were identified according to the criteria of Doolittle (35). The exact peptide sequences were as follows: CRP1, KYEEGQSHRPTNPNASRM (amino acids 91–108); CRP2, KPESTPSPHRPTTNPNTSKF (amino acids 91–110); and CRP3/MLP, FGGLTQVEKKE (amino acids 184–194). Peptides were synthesized using an Applied Biosystems Model 431A peptide synthesizer. Each peptide was supplemented with an amino-terminal cysteine to allow for subsequent coupling to bovine serum albumin (Sigma) or to keyhole limpet hemocyanin (Sigma) carrier proteins (35). Chemical coupling to bovine serum albumin or keyhole limpet hemocyanin was performed using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Pierce) as described previously (36). Carrier-coupled peptides were injected into New Zealand White rabbits, and immune sera were collected following established protocols (37). CRP1-, CRP2-, and CRP3/MLP-specific antisera are referred to below as anti-CRP1, anti-CRP2, and anti-CRP3, respectively. A previously described anti-zyxin antibody, called B38 (38), was also used in Western immunoblot analyses.

**Embryonic Chicken Organ/Tissue Sample Preparation**—Selected embryonic chicken organs and tissues were prepared and homogenized as described previously (30). Briefly, 19-day chick embryos were dissected to recover protein samples from heart, stomach, brain, crop, liver, arteries, gizzard, lung, intestine/colon, leg skeletal muscle, and fibroblasts. For developmental time course studies, gizzards, arteries, and hearts were extracted from 11-, 13-, 15-, and 18-day embryos. Samples from each organ/tissue were homogenized rapidly in distilled H<sub>2</sub>O containing 1 mM phenylmethylsulfonyl fluoride (Sigma) at a ratio of 5 ml of homogenization buffer/g of organ or tissue (wet weight). Samples were prepared for SDS-PAGE in Laemmli sample buffer (33); DNA was sheared by passing samples through a 26-gauge syringe. Samples were boiled for 4 min prior to loading onto gels. 10  $\mu$ l of each sample were loaded per lane.

Protein extracts used in the blot overlay assays were obtained from adult chicken gizzards as described previously (18, 39). Whole gizzard extracts were sequentially precipitated with increasing concentrations of ammonium sulfate to give rise to two different complex protein fractions, one (from a 34–43% precipitation) that contained CRP and one (from a 43–61% precipitation) that did not. Precipitated protein fractions, once resuspended, were diluted 1:2 in 2  $\times$  Laemmli sample buffer and boiled for 3 min. 10  $\mu$ l of each fraction were loaded per lane onto denaturing gels.

## RESULTS

Recent studies have revealed the existence of three proteins, called CRP1, CRP2, and CRP3/MLP, that are members of a family of highly conserved gene products (1, 15–19). CRP family members are characterized by the presence of two tandemly arrayed zinc-binding LIM domains, each of which is followed by a conserved glycine-rich repeat (Fig. 1A) (1, 2, 18). Pairwise comparisons of the three CRPs show that these molecules are very similar to each other, exhibiting >65% amino acid sequence identity (Fig. 1, B and C). The similarity of these three proteins, in combination with data implicating CRPs in muscle function (18, 20, 26), raises a number of questions regarding the physiological significance of the existence of multiple CRP isoforms. We were interested in determining whether the three CRPs display distinct functions or expression patterns. We have analyzed the biochemical and cellular functions of each CRP family member by examining their subcellular localizations and binding-partner preferences. We have also examined CRP expression patterns in various embryonic chicken organs.

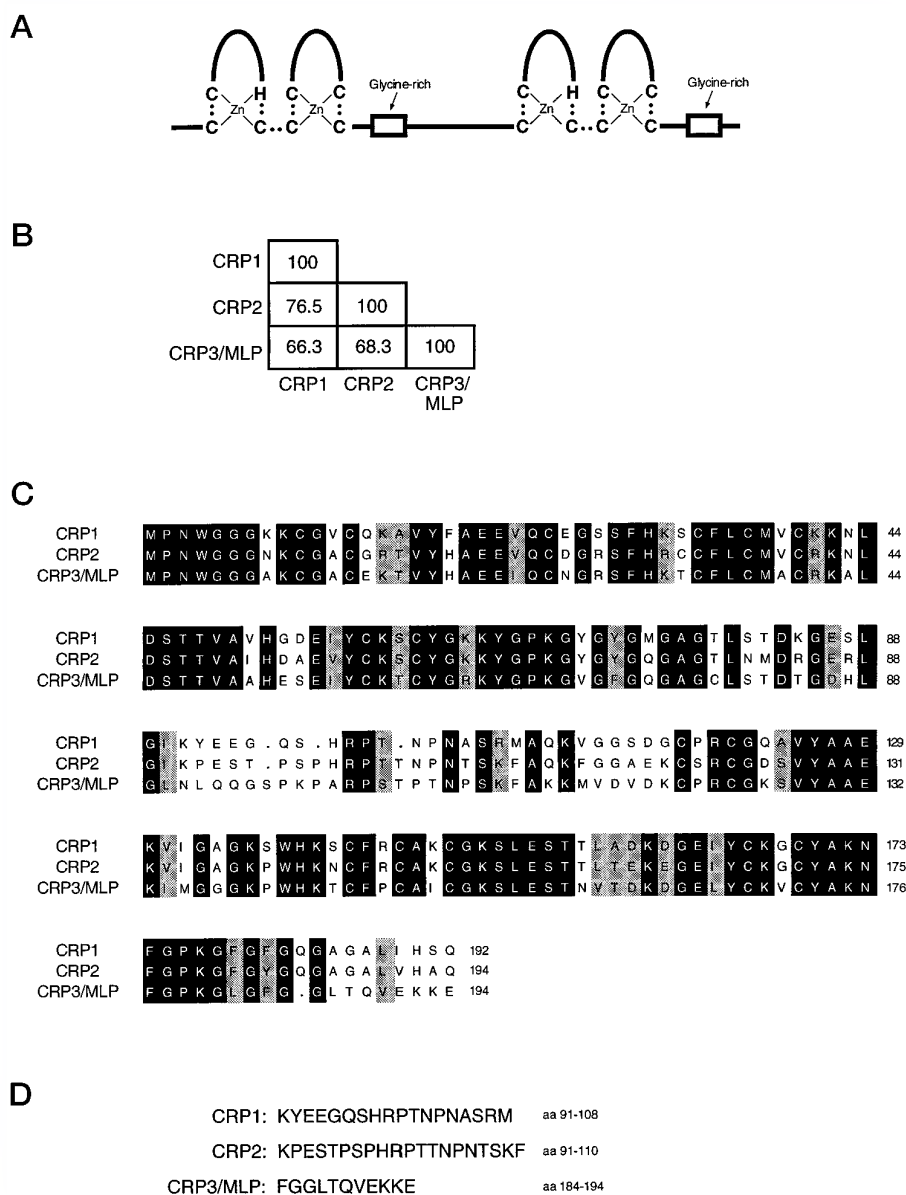
**Subcellular Distribution of CRP Family Members in Cultured Fibroblasts**—We first explored the possibility that the three CRP family members display distinct subcellular localizations. Classically, localization studies are performed by indirect immunofluorescence. However, because available CRP isoform-specific antibodies fail to recognize native CRPs in fixed and permeabilized cells, we monitored the subcellular

localization patterns of CRP1, CRP2, and CRP3/MLP by transiently expressing epitope-tagged versions of the proteins.

Three mammalian expression constructs were generated, each encoding one of the chicken CRPs. CRPs expressed from these vectors contained a C-terminal c-Myc epitope tag that allowed for the detection of heterologously expressed CRPs within the transfected cells. Purified expression constructs were injected into the nuclei of adherent REF52 cells, a fibroblastic cell line known for its well organized actin filament arrays (40, 41). After a 24-h incubation, CRP expression was visualized in the cells by indirect immunofluorescence using an anti-Myc antibody. The three different CRP isoforms exhibited similar subcellular localizations (Fig. 2). Each protein colocalized with filamentous actin; some punctate cytoplasmic staining was also observed. No staining was detected in cells injected with a control vector lacking CRP sequences (data not shown). Contrary to previous reports for CRP2 (20) and CRP3/MLP (14), we did not consistently observe accumulation of any of the three CRP isoforms in the nuclei of these cells. The cytoskeletal staining patterns observed here were comparable to those observed in previous experiments that used polyclonal antisera to detect endogenous CRP (18). Thus, the subcellular distributions observed in this study with ectopically expressed CRPs are likely to be physiologically relevant.

**Characterization of CRP Binding-partner Preferences**—Previous studies have demonstrated that the CRP1 protein interacts specifically with two cytoskeletal proteins,  $\alpha$ -actinin (21) and zyxin (9, 16). To determine whether each of the three CRP isoforms is capable of interacting with these protein partners, we performed *in vitro* binding assays. For these studies, equimolar amounts of purified recombinant CRP1, CRP2, and CRP3/MLP were resolved by SDS-PAGE (Fig. 3A, lanes 1–3). Two complex fractions derived from a chicken gizzard extract were also included on the gel (Fig. 3A, lanes 4 and 5); the fraction resolved in lane 4 contains CRP, whereas the fraction in lane 5 does not. Parallel gels were transferred to nitrocellulose and probed with one of two purified radiolabeled proteins: <sup>125</sup>I-labeled  $\alpha$ -actinin or <sup>32</sup>P-labeled zyxin. The  $\alpha$ -actinin probe was purified from avian smooth muscle; the zyxin probe, containing sequences from the zyxin C-terminal LIM region, was generated as a glutathione *S*-transferase fusion protein in bacteria (Fig. 3D). Autoradiographic analysis of the blot overlay assays revealed that all three CRP isoforms were capable of interacting with both the  $\alpha$ -actinin and zyxin protein probes (Fig. 3, B and C, respectively). <sup>32</sup>P-Labeled glutathione *S*-transferase did not interact with any proteins present on the blot (Refs. 9 and 22; data not shown). Moreover, the probes utilized in these studies failed to interact with other proteins present in the smooth muscle fractions (Fig. 3, A–C, lanes 4 and 5; data not shown), suggesting that the observed interactions are specific. Quantitation of these data revealed that the  $\alpha$ -actinin probe bound each CRP family member at comparable levels (Fig. 3E); similar results were obtained with the zyxin probe (Fig. 3E). These findings demonstrate that CRP1, CRP2, and CRP3/MLP have common biochemical activities.

**Antipeptide Antibody Design and Characterization**—As described above, CRP family members cannot be distinguished from each other based on their subcellular distributions or their binding-partner preferences. In the following studies, we examined the possibility that the CRPs exhibit unique patterns of protein expression. Due to the overall sequence conservation of CRP1, CRP2, and CRP3/MLP, we reasoned that previously generated polyclonal antibodies were likely to recognize epitopes common to multiple CRPs, thereby precluding their use in comparative Western immunoblot analyses (18, 21). Therefore, to characterize the expression profiles for each CRP,



**FIG. 1. The chicken CRP multigene family is composed of three highly conserved proteins.**

**A**, the molecular architecture of CRP family members is characterized by the presence of two zinc-binding LIM domains, each of which is followed by a conserved glycine-rich repeat. **B**, pairwise comparisons were made between chicken CRP sequences, and identities were determined according to the parameters set by Lipman and Pearson (54). Collectively, this analysis demonstrates that CRP1, CRP2, and CRP3/MLP share a high degree of sequence identity. **C**, sequence alignment of all three chicken CRP amino acid sequences illustrates the specific regions within these proteins that share the highest degree of similarity. *Black boxes* denote residues that are absolutely identical between the three molecules; *gray boxes* indicate those residues that, while not identical between all three proteins, display notable sequence similarity (similarity groups were assigned as described previously (1): Ala, Ser, Thr; Asp, Glu; Asn, Gln; Arg, Lys; Ile, Leu, Met, Val; Phe, Tyr, Trp). The chicken CRP3/MLP sequence shown here differs in four positions from the originally reported sequence (at amino acids 92, 93, 94, and 114) (19). The corrections to the CRP3/MLP sequence that are reported here result in slightly greater degrees of similarity between CRP isoforms than were described previously (1, 19). **D**, shown are the isoform-specific peptide sequences that were used to generate antibodies against each of the CRP family members. *aa*, amino acids.

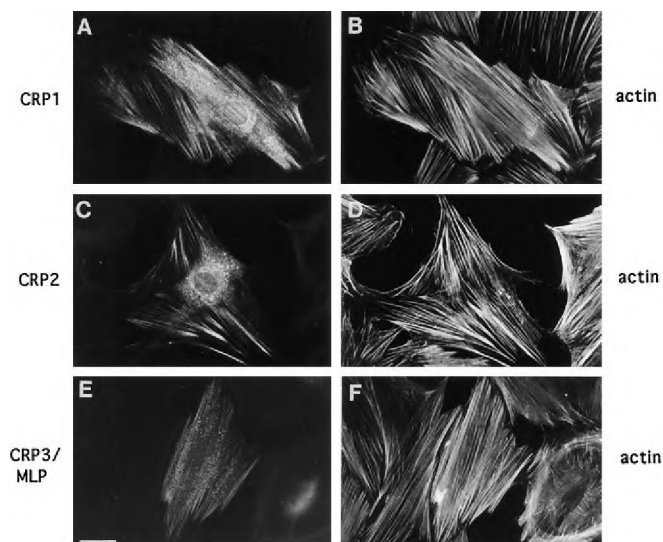
it was necessary to generate isoform-specific anti-CRP antibodies for use in this study.

To develop isoform-specific reagents, we identified divergent regions within each CRP family member and synthesized peptides corresponding to the distinguishing sequences (Fig. 1D). All peptides chosen were <55% identical to sequences occupying similar positions in the other two molecules. Specifically, the selected CRP1 and CRP2 peptides, derived from the same region in the molecule, are 55% identical (65% similar) to each other. While the CRP1 peptide is only 24% identical (33% similar) to corresponding CRP3/MLP sequences, the CRP2 peptide sequence is 43% identical (48% similar). Finally, the CRP3/MLP peptide displays 25% identity (33% similarity) and 17% identity (33% similarity) to corresponding CRP1 and CRP2 sequences, respectively. These peptides were coupled to carrier molecules and used as immunogens. CRP-specific antipeptide antibodies were raised in rabbits, and antisera were characterized by Western immunoblot analysis (Fig. 4).

To characterize the specificity of the antipeptide antibodies, equimolar amounts of purified recombinant CRP1, CRP2, and CRP3/MLP, as well as a sample of embryonic chicken gizzard extract, were resolved by SDS-PAGE and visualized by Coomassie Blue staining.

As shown in Fig. 4A, each of the three CRPs was loaded at comparable levels onto the gel. The faint 16-kDa band observed in the CRP2 preparation is a proteolytic product of the purified protein (Fig. 4A, lane 3). Parallel gels were transferred to nitrocellulose and probed with each of the anti-CRP antibodies and their corresponding preimmune sera (Fig. 4, B–D).

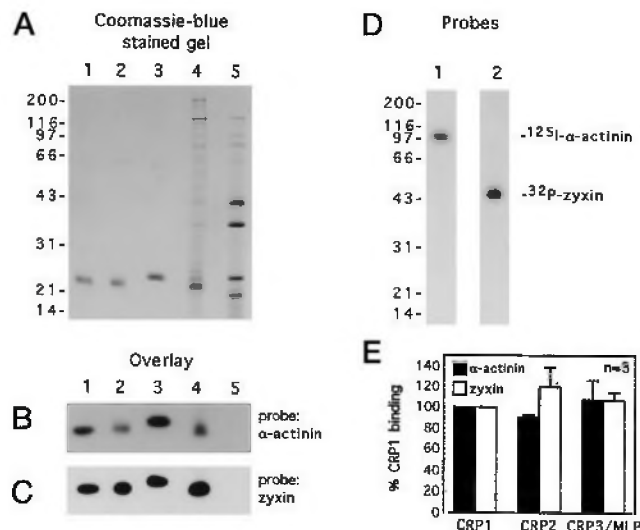
The Western immunoblots in Fig. 4 demonstrate that the isoform-specific antipeptide antibodies are in fact capable of distinguishing between each of the three CRP family members. The anti-CRP1 antibody bound to purified CRP1, but failed to interact with CRP2 or CRP3/MLP at the dilution used (Fig. 4B, lanes 2–4). (At a higher concentration, this antibody did show some affinity for CRP2 (data not shown).) The anti-CRP2 serum specifically recognized purified CRP2 (appearing on the blot as a doublet that is likely to be due to incomplete sample reduction) as well as the CRP2 degradation product visualized faintly on the Coomassie Blue-stained gel (Fig. 4, A and C, lanes 3). Finally, the anti-CRP3 antibody recognized CRP3/MLP, but not CRP1 or CRP2 (Fig. 4D, lane 4). In all cases, the preimmune sera failed to interact with any of the three purified proteins or with any of the proteins present in the complex



**FIG. 2. All three CRP family members associate with the actin cytoskeleton in adherent fibroblasts.** REF52 cells were microinjected with expression constructs encoding Myc-tagged CRP1 (A and B), CRP2 (C and D), and CRP3/MLP (E and F). After a 24-h incubation at 37 °C, cells were processed for immunofluorescence using anti-Myc antibodies to visualize Myc-tagged CRPs (A, C, and E) or using rhodamine-conjugated phalloidin to visualize filamentous actin (B, D, and F). Each of the three CRPs colocalizes with actin filaments in the REF52 cells. A punctate pattern that does not codistribute with the actin filaments is also observed for all three CRP family members. The significance of this punctate staining is not clear; it could represent an association of CRPs with some small organelle, or it may represent protein aggregates that result upon overexpression of CRPs. Since the cells were transiently transfected, we do not know the concentration of CRP protein expressed in the sample; therefore, it is not possible to assess whether we have saturated all CRP-binding sites. Bar = 32  $\mu$ m.

smooth muscle extract lanes (Fig. 4, B–D, lanes 1'–4'). Interestingly, of the three isoform-specific antibodies, only the anti-CRP1 antibody recognized a 23-kDa CRP in the complex chicken gizzard extract (Fig. 4, B–D, lanes 1). This result provided the first indication that CRP family members would exhibit distinct expression patterns.

**Organ/Tissue Distribution of CRP Family Members**—CRP isoform-specific antibodies were used in Western immunoblot analyses to screen protein extracts derived from 19-day chicken embryos. Proteins from chicken embryo brain, heart, arteries, crop, stomach, gizzard, intestine/colon, skeletal muscle, liver, lung, and fibroblasts were resolved by SDS-PAGE. The Coomassie Blue-stained gel in Fig. 5A illustrates the protein complexity of each organ or tissue. Parallel gels were analyzed by Western immunoblotting using each of the CRP isoform-specific antibodies described above (Fig. 5, B–D) as well as an antibody specific for the CRP binding partner zyxin (Fig. 5E) (38). This analysis revealed that each CRP family member exhibits a distinct expression pattern. CRP1 is found predominantly in organs enriched in smooth muscle, including arteries, stomach, gizzard, and intestine/colon, as well as in lung and fibroblasts (Fig. 5B, lanes 3, 5–7, 10, and 11). CRP2 expression is limited to arteries and fibroblasts (Fig. 5C, lanes 3 and 11), and CRP3/MLP is present exclusively in heart, crop, and skeletal muscle (Fig. 5D, lanes 2, 4, and 8). Using the anti-CRP3 antibody, we sometimes observed two immunoreactive polypeptides in heart and skeletal muscle: one migrated as expected at 23 kDa, and the other migrated at 33 kDa (Fig. 5D, lanes 2 and 8). The significance of this additional band is currently unclear, but it appears to occur only in very late stages of chick development. With the exception of the crop, the CRP binding partner zyxin is observed in all organs and tissues containing at least one CRP family member (Fig. 5E). Collec-



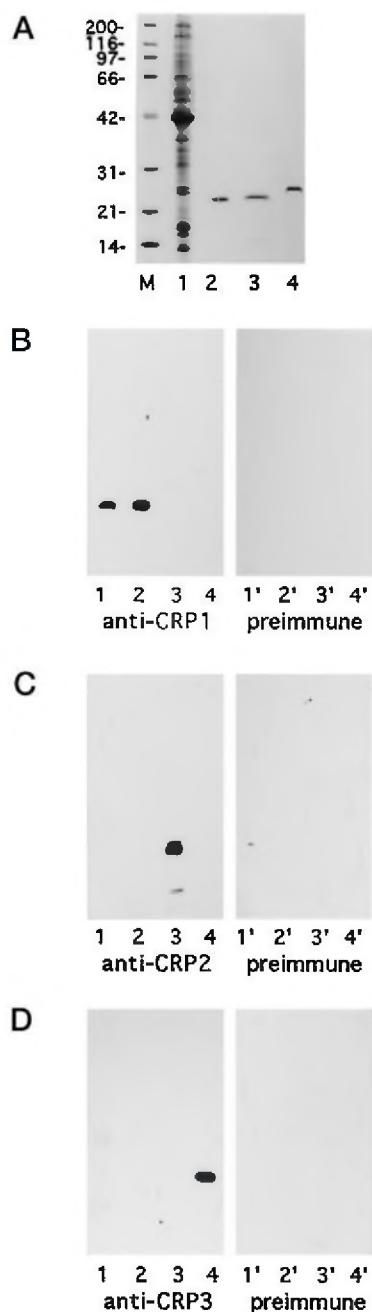
**FIG. 3. CRP1, CRP2, and CRP3/MLP exhibit similar binding-partner preferences.** A, Coomassie Blue-stained gel illustrating proteins used in the blot overlay assay. Lanes 1–3 were loaded with 100 pmol of purified recombinant chicken CRP1, chicken CRP2, and rat CRP3/MLP, respectively. Lanes 4 and 5 were loaded with two different ammonium sulfate precipitation fractions derived from a chicken gizzard lysate. The extract in lane 4 contains CRP, whereas the extract in lane 5 does not. B and C, autoradiographic analysis of parallel gels that were transferred to nitrocellulose and probed in blot overlay assays with  $^{125}$ I-labeled  $\alpha$ -actinin and  $^{32}$ P-labeled zyxin, respectively. D, autoradiograph demonstrating the homogeneity of the radiolabeled  $\alpha$ -actinin and zyxin probes. E, blot overlay assays quantitated by Phosphor Imager analysis. Quantitative data from three independent experiments were normalized relative to the amount of protein present on the blot, as determined by densitometric analysis of parallel Coomassie Blue-stained gels. These normalized values have been expressed here as a percentage of the maximal binding observed for the CRP1 protein  $\pm$  S.E. Collectively, these results demonstrate that  $\alpha$ -actinin and zyxin are capable of interacting at comparable levels with each of the CRPs.

tively, these results demonstrate that each of the three CRP family members has a distinct expression profile (Table I).

**Developmental Expression Patterns of CRP Family Members**—Having demonstrated that CRP family members display spatially distinct patterns of expression, we next explored the possibility that these proteins may also exhibit temporal variation in their expression patterns during embryogenesis. We utilized the isoform-specific antibodies to monitor CRP expression levels in organs extracted from 11-, 13-, 15-, and 18-day chick embryos. Based on the organ-specific expression patterns observed above, we chose to evaluate CRP1 in gizzard and arteries, CRP2 in arteries, and CRP3/MLP in heart. Proteins from the selected organs were visualized by Coomassie Blue staining of SDS-polyacrylamide gels (Fig. 6). Parallel gels were transferred to nitrocellulose and analyzed by Western immunoblotting. As observed in previous studies (18), CRP1 levels were shown to increase gradually as a function of developmental time in both chicken gizzard and arteries (Fig. 6, A and B). In contrast, the level of CRP2 in arteries increased markedly between days 11 and 13 of development; CRP2 expression remained constant between days 13 and 18 (Fig. 6B). CRP3/MLP expression levels were constant in embryonic heart at the developmental stages examined (Fig. 6C). Therefore, in addition to exhibiting spatially distinct patterns of expression in embryos, CRP family members also display differences in their temporal regulation during development.

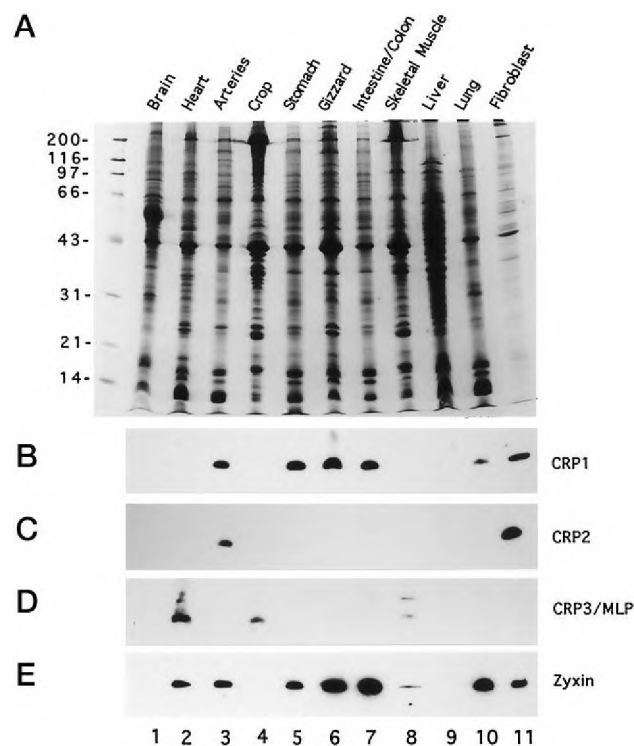
#### DISCUSSION

Recent studies revealing the sequence similarity of CRP1, CRP2, and CRP3/MLP (1) have raised interesting questions



**FIG. 4. Characterization of CRP isoform-specific antipeptide antibodies.** Gels for use in Western immunoblot analyses were loaded as follows: lane *M*, molecular mass markers; lane *1*, embryonic chicken smooth muscle extract; lane *2*, purified recombinant CRP1; lane *3*, purified recombinant CRP2; lane *4*, purified recombinant CRP3/MLP. Proteins were visualized in *A* by Coomassie Blue staining. Parallel gels were transferred to nitrocellulose and probed with anti-CRP1 (*B*), anti-CRP2 (*C*), and anti-CRP3 (*D*) antibodies or with their corresponding preimmune sera (*B–D*, lanes 1'–4'). These data illustrate that the antipeptide antibodies generated here are CRP isoform-specific. The anti-CRP1 antibody is the only antibody that recognizes a 23-kDa band in the embryonic smooth muscle extract.

regarding the physiological and biological significance of the CRP multigene family. In this report, we have compared the subcellular distributions, biochemical properties, and expression patterns of three vertebrate CRPs. Transient expression of epitope-tagged CRP1, CRP2, and CRP3/MLP in rat embryo fibroblasts revealed that all three CRP isoforms associate with the actin cytoskeleton. Moreover, all three proteins have the capacity to bind  $\alpha$ -actinin and zyxin. Together, these observa-



**FIG. 5. CRP family members exhibit distinct patterns of expression.** Shown is a Coomassie Blue-stained gel illustrating the proteins present in samples prepared from a 19-day chicken embryo (*A*). Western immunoblot analyses were performed on parallel blots using isoform-specific anti-CRP antibodies (*B–D*) or using an anti-zyxin antibody (*E*). These experiments demonstrate that CRP1 is expressed predominantly in arteries, stomach, gizzard, intestine/colon, lung, and fibroblasts (*B*); that CRP2 is observed primarily in arteries and fibroblasts (*C*); and that CRP3/MLP expression is limited to heart, crop, and skeletal muscle (*D*). With the exception of the crop, the CRP binding partner zyxin is coexpressed with at least one CRP family member in every organ/tissue examined (*E*).

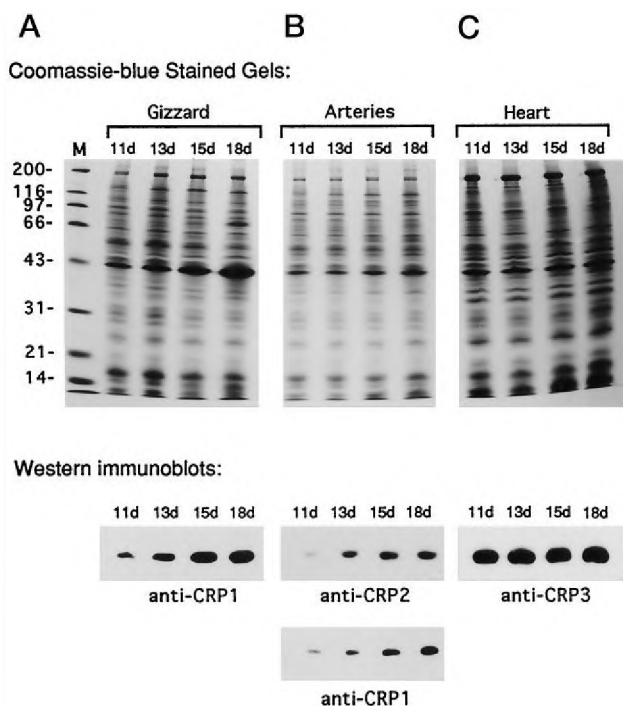
TABLE I  
Expression of CRPs in embryonic chicken organs and tissues

Organ/Tissue	CRP1	CRP2	CRP3/MLP	Zyxin
Brain	– <sup>a</sup>	–	–	–
Heart	–	–	+	+
Arteries	+	+	–	+
Crop	–	–	+	–
Stomach	+	–	–	+
Gizzard	+	–	–	+
Intestine/colon	+	–	–	+
Skeletal muscle	–	–	+	+
Liver	–	–	–	–
Lung	+	–	–	+
Fibroblast	+	+	–	+

<sup>a</sup> –, no protein expression detected; +, detectable levels of protein expression above background.

tions suggest that the three CRP family members display similar biochemical properties and subcellular distributions.

In contrast, significant differences among the CRPs are evident in their temporal and spatial patterns of expression. In chicken, the distribution of CRP isoforms appears to be largely restricted to organs containing muscle cell tissue, thereby implying a general role for the CRP multigene family in muscle function. We detected CRP3/MLP in avian heart and skeletal muscle, a finding consistent with the demonstration that CRP3/MLP is necessary for the differentiation of cardiac and skeletal muscle cells in the mouse (26). We also found CRP3/MLP in the crop, an organ unique to avian species that is enriched in smooth muscle (42–44). Our analysis shows that



**FIG. 6. CRP family members display different patterns of developmental regulation.** Gizzards, arteries, and hearts were extracted from chick embryos from four developmental stages: 11, 13, 15, and 18 days. The *upper panels* in A–C show Coomassie Blue-stained gels, illustrating the protein profiles of embryonic gizzard, arteries, and heart. Parallel gels were transferred to nitrocellulose and analyzed by Western immunoblotting using the isoform-specific anti-CRP antibodies (*lower panels* in A–C).

while CRP1 and CRP2 are not found in organs containing striated muscle, they are prominent in a variety of tissues rich in smooth muscle. For example, CRP1 is detected in gizzard, stomach, intestine/colon, arteries, and lung, all of which contain substantial amounts of smooth muscle tissue (45). CRP1 is also present in fibroblasts, which are smooth muscle-like in terms of their protein expression patterns. CRP2 is expressed in a subset of these organs, specifically in arteries and fibroblasts; while this manuscript was in preparation, Jain *et al.* (20) described the expression of CRP2 (also referred to as SmLIM) in rat aortic smooth muscle. We suspect that immunocytochemical analysis will reveal that CRP1 and CRP2 are generally present in the vasculature, but that we do not have the sensitivity to detect them in whole organ samples, except in isolated arteries. High resolution immunocytochemical or *in situ* hybridization studies will be essential to identify the cell types within arteries (for example) that express each CRP isoform. Such an analysis of early embryonic stages would also allow us to define the point at which tissue-restricted expression of CRP isoforms occurs. In general, CRP family members may serve as markers for different types of muscle. Consistent with findings for vertebrate CRPs, two CRP family members identified in *Drosophila* also exhibit muscle-specific expression (2, 19).

Based on the fact that CRP family members have many functions in common and no unique functions detected thus far, it is reasonable to speculate that these proteins may play similar roles in cells. One possibility is that CRP family members may play a cytoarchitectural role during the assembly of actin cytoskeletal networks and thereby may affect muscle cell differentiation. This idea is supported by our findings that all three CRPs are capable of associating with the actin cytoskeleton and that they all interact directly with two cytoskeletal proteins,  $\alpha$ -actinin and zyxin, both of which have been impli-

cated in the control of actin assembly and organization (21, 25). This view is also consistent with the phenotype of mice that lack CRP3/MLP; in these mice, the striated muscle cells display markedly disorganized myofibrillar arrays and thus fail to function properly (26).

Another possibility, which is not mutually exclusive, is that the CRP family members may function in the switching of master controls that direct a cell's decision to proliferate or differentiate. This idea is consistent with previous findings demonstrating that levels of transcripts encoding both CRP1 and CRP2 decline precipitously in a variety of transformed cells (1, 17). It has also been reported that CRP2 expression is down-regulated in rat arterial smooth muscle cells in response to vascular injuries that trigger cell proliferation (20). In the case of CRP3/MLP, it has been clearly demonstrated that protein expression is low in proliferating myoblasts and increases as myogenesis proceeds (19). The fact that all three CRP family members are associated with the actin cytoskeleton, perhaps via interactions with the cytoskeletal proteins  $\alpha$ -actinin and zyxin, suggests that the cytoskeleton represents one execution site for CRP activity.

Our results indicate that the different CRP family members are likely to perform their cellular functions in distinct locations within an organism. The observations that CRP3/MLP is expressed in organs containing striated muscle and that mice lacking CRP3/MLP exhibit severe defects in cardiac and skeletal musculature (26) indicate that, in the mouse, CRP3/MLP is likely to function predominantly during the development of striated muscle tissue. In the chick system, CRP3/MLP may also be essential for smooth muscle differentiation in the crop. By analogy to what is known about vertebrate CRP3/MLP function, one would predict that CRP1 and CRP2 could play essential roles during smooth muscle cell differentiation. Based on the patterns of expression of CRP1 and CRP2, CRP1 may play a significant role in the differentiation of nonvascular smooth muscle, such as occurs in the digestive and respiratory tracts, whereas CRP2 may function in the differentiation of vascular smooth muscle, either alone or in conjunction with CRP1. Higher resolution analysis of the expression patterns of CRP1 and CRP2 will be necessary to refine further our understanding of the roles of these two proteins in development. In particular, it will be of interest to learn whether the segregation of CRPs observed late in chick embryonic development (Fig. 5) also occurs at earlier stages.

The significance of isoform diversity has been probed in a number of other protein families, including groups of cytoskeletal proteins such as tropomyosins, actins, myosins, and tubulins (46–51). For example, a number of closely related actin isoforms that are expressed in temporally and spatially discrete patterns have been characterized (47, 48, 52). Mammals exhibit four muscle-specific actin isoforms that display a high degree of sequence identity (47, 48, 53). Nevertheless, the substitution of smooth muscle  $\gamma$ -actin for cardiac  $\alpha$ -actin in a mouse model results in abnormal cardiac muscle morphology and function (53). Studies on the actin family point out that even a high degree of sequence identity is not sufficient to ensure functional conservation. Although we have not yet detected any differences among the CRP family members in terms of binding-partner specificity or subcellular distribution, we cannot rule out the possibility that within a specific cellular context, these proteins perform unique functions. Experiments designed to test whether CRP isoforms can substitute for each other within a living organism will provide the ultimate test of their functional relationships.

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