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Biochemical and Molecular Characterization of the Chicken Cysteine-rich Protein, a Developmentally Regulated LIM-Domain Protein That Is Associated with the Actin Cytoskeleton

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Abstract. LIM domains are present in a number of proteins including transcription factors, a proto-oncogene product, and the adhesion plaque protein zyxin. The LIM domain exhibits a characteristic arrangement of cysteine and histidine residues and represents a novel zinc binding sequence (Michelsen et al., 1993). Previously, we reported the identification of a 23-kD protein that interacts with zyxin in vitro (Sadler et al., 1992). In this report, we describe the purification and characterization of this 23-kD zyxin-binding protein from avian smooth muscle. Isolation of a cDNA encoding the 23-kD protein has revealed that it consists of 192 amino acids and exhibits two copies of the LIM motif. The 23-kD protein is 91% identical to the human cysteine-rich protein (hCRP); therefore we refer to it as the chicken cysteine-rich protein (cCRP). Examination of a number of chick embryonic tissues by Western immunoblot analysis reveals that cCRP exhibits tissue-specific expression. cCRP is most prominent in tissues that are enriched in smooth muscle cells, such as gizzard, stomach, and intestine. In primary cell cultures derived from embryonic gizzard, differentiated smooth muscle cells exhibit the most striking staining with anti-cCRP antibodies. We have performed quantitative Western immunoblot analysis of cCRP, zyxin, and α-actinin levels during embryogenesis. By this approach, we have demonstrated that the expression of cCRP is developmentally regulated.

ADHESION plaques are specialized structures of the plasma membrane present at discrete locations along the ventral surface of the cell (Wehland et al., 1979; Heath and Dunn, 1978), which act as a physical, molecular link between the actin cytoskeleton and the extracellular matrix (Singer, 1979; for reviews see Burridge et al., 1988; Crawford and Beckerle, 1990). These regions of the membrane represent the closest approach of the cell to its underlying substratum (Curtis, 1964) and contain a number of proteins that are believed to play a structural role in cell substratum adhesion, such as vinculin (Geiger, 1979; Otto, 1990), talin (Burridge and Connell, 1983; Beckerle and Yeh, 1990) and α-actinin (Lazarides and Burridge, 1975; Blanchard et al., 1989). In addition, a number of low abundance proteins that are predicted to perform regulatory or signaling functions at adhesive membranes have been identified. One such protein is zyxin, which was first identified through analysis of a nonimmune rabbit serum (Beckerle, 1986). Molecular genetic characterization of zyxin revealed that the protein has an unusual primary amino acid sequence (Sadler et al., 1992). For instance, the NH_2-terminal region of zyxin is proline rich, containing one consecutive stretch of 146 amino acids that is comprised of ~35% proline residues. The COOH terminus of zyxin contains a number of cysteine and histidine residues that are organized into three copies of a zinc binding sequence, termed the LIM motif (Sadler et al., 1992; Michelson et al., 1993).

The LIM motif (Freyd et al., 1990), which exhibits the consensus amino acid sequence $CX_2CX_{16-23}HX_2CX_2CX_2$-$CX_{16-21}CX_{2-3}$(C,H,D) (Sadler et al., 1992), has been identified in a number of gene products (Table I), several of which have been proposed to function as transcription factors. The LIM motif was first defined in proteins that also contain homeodomain sequences, including Lin-11 (Freyd et al. 1990), Isl-1 (Karlsson et al., 1990), and Mec-3 (Way and Chalfie, 1988). Although the exact function of the LIM domain has not been determined, many proteins that display these domains are involved in developmental regulation or cellular differentiation. For instance, *lin-11* and *mec-3* are *C. elegans* genes required for specification of cell fate (Ferguson et al., 1987; Way and Chalfie, 1988). The mRNA encoding another LIM homeodomain protein, Xlim-1, is locally expressed at the dorsal lip of the blastopore and dorsal mesoderm of *Xenopus* embryos at the onset of gastrulation, suggesting that Xlim-1 plays a role in determining body pattern in the developing embryo (Taira et al., 1992). Finally, the *Drosophila* LIM

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Table I. LIM-Domain Proteins

Protein (source)	No. of LIM domains	Homeo-domain	Reference
Lin-11 (*C. elegans*)	2	+	Freyd et al., 1990
Mec-3 (*C. elegans*)	2	+	Way and Chalfie, 1988
Isl-1 (rat)	2	+	Karlsson et al., 1988
Apterous (*Drosophila*)	2	+	Cohen et al., 1992; Bourgouin et al., 1992
X-lim1 (*Xenopus*)	2	+	Taira et al., 1992
lmx-1 (hamster)	2	+	German et al., 1992
LH-2 (mouse, rat)	2	+	Xu et al., 1993
Zyxin (chicken)	3	−	Sadler et al., 1992
cCRP (chicken)	2	−	This report
hCRP (human)	2	−	Leibhaber et al., 1990
Rhombotin (Ttg-1) (mouse)	2	−	Boehm et al., 1990; McGuire et al., 1989
SF3 (sunflower)	2	−	Baltz et al., 1992
CRIP (rat)	1	−	Birkenmeier and Gordon, 1986
ESP-1 (rat)	1*	−	Nalik et al., 1989

* It should be noted that while the published sequence of ESP-1 has one LIM domain, it has been suggested that ESP-1 may actually have two LIM domains (Wang et al., 1992).

homeobox gene, *apterous*, is required during embryogenesis for proper development of wing and haltere imaginal discs (Wilson, 1981; Cohen et al., 1992; Bourgouin et al., 1992).

In addition to the LIM-homeodomain proteins, a number of small (10–30 kD) proteins that are comprised primarily of LIM domains have been identified. These include rhombotin (or Ttg-1) (McGuire et al., 1989; Boehm et al., 1990, 1991), the cysteine-rich intestinal protein (CRIP)[1] (Birkenmeier and Gordon, 1986), and the human cysteine-rich protein (hCRP) (Liebhaber et al., 1990). Interestingly, several of these non-homeodomain LIM proteins exhibit developmentally regulated expression. For instance, the level of mRNA encoding CRIP, an intestinal protein which contains a single LIM domain, is dramatically increased at the transition from suckling to weaning behavior in rats (Birkenmeier and Gordon, 1986). Rhombotin is a proto-oncogene product (McGuire et al., 1992) that is postulated to function in nervous system development (Greenberg et al., 1990). Thus, although a number of LIM domain proteins lack any well-characterized DNA-binding domain, many of these proteins have also been implicated in processes such as cell differentiation and embryonic development.

Our interest has been focussed on the LIM domain protein, zyxin, that is localized at sites of membrane–substratum contact. In an effort to define the cellular function of zyxin, we initiated a search for zyxin binding partners. We hoped to identify proteins that might target zyxin to its subcellular location or might be components of a common molecular pathway with zyxin. By use of an in vitro biochemical screen, we identified two proteins that interact prominently with zyxin. One protein migrated at an apparent molecular mass of 100 kD and was subsequently determined to be α-actinin, a well known constituent of adhesion plaques that could be involved in localizing zyxin to those sites (Crawford et al., 1992). A second zyxin-binding protein that migrated at 23 kD was also identified in our screen (Sadler et al., 1992). Here we describe the purification, biochemical properties, and molecular characterization of this 23-kD zyxin-binding protein. The 23-kD protein exhibits two copies of the LIM motif in its primary amino acid sequence and appears to be the chicken homologue of the human cysteine-rich protein (hCRP) mentioned above. Thus, our biochemical screen revealed an association between two proteins that display LIM domains. Like many other LIM domain proteins, the chicken cysteine-rich protein (cCRP) exhibits both tissue-specific and developmentally regulated expression during embryogenesis. The development of a procedure that allows the isolation of milligram quantities of a LIM domain protein from its endogenous source represents an important step toward characterizing the specialized function and biochemical properties of a LIM family member.

Materials and Methods

Purification of the 23-kD Protein from Avian Smooth Muscle

Protein was extracted from either fresh or frozen chicken gizzards as described previously (Crawford and Beckerle, 1991). Protein present in the extract was precipitated in the presence of 16 *g* ammonium sulfate/100 ml for 45 min at 4°C, collected by centrifugation (10′ at 16,000 *g*) and discarded. An additional 10 *g* ammonium sulfate/100 ml was added and the mixture was stirred at 4°C for 45–60 min. The resulting precipitate was collected by centrifugation (10′ at 16,000 *g*), resuspended in buffer B-10 (20 mM Tris-acetate, pH 7.6, 10 mM NaCl, 0.1% 2-mercaptoethanol, 0.1 mM EDTA) and loaded onto a 2.5 × 10 cm DEAE-cellulose column (Whatman Biosystems Ltd., Kent, UK). The column was washed with ~100 ml of buffer B-10; the 23-kD protein was collected in the non-adsorbed column fractions. Ammonium sulfate was added to the non-adsorbed fractions enriched in the 23-kD protein to a final concentration of 20% saturation and mixed briefly. This sample was loaded onto a 1.0 × 10 cm phenyl Sepharose CL-4B column (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) previously equilibrated with 20% saturated ammonium sulfate in buffer B-10. The phenyl Sepharose column was eluted first with a decreasing 30-ml gradient of 0–20% ammonium sulfate prepared in buffer B-10 followed by an increasing 30 ml gradient of 0–50% ethylene glycol also prepared in buffer B-10. The 23-kD protein elutes from this column at a concentration between 20 and 40% ethylene glycol. Fractions containing the 23-kD protein were identified by SDS-PAGE and were pooled. Proteins contained within the pooled fractions were precipitated with 50% saturated ammonium sulfate, collected by centrifugation (10′ at 16,000 *g*), carefully resuspended in 1–2 ml buffer B-10, and loaded onto a Sepharose CL-6B (Pharmacia LKB Biotechnology Inc.) gel filtration column (1 m × 1.2 cm). The column was eluted with buffer B-10; fraction volumes of 2.5–3 ml were collected. The 23-kD protein elutes from the gel filtration column with an effective Stokes radius of 2.0 nm. By this protocol, one can obtain this protein purified to apparent homogeneity, as assayed by SDS-PAGE. Fractions collected from the gel filtration column were stored at 4°C and the 23-kD protein contained in these fractions was found to be stable for approximately one month. The blot overlay assay used to identify the 23-kD protein as a zyxin-binding protein and to track the 23-kD protein through the purification procedure has been described in detail previously (Crawford et al., 1992; Sadler et al., 1992).

Isolation of cCRP cDNA

A chicken embryo fibroblast cDNA library cloned into the EcoRI site of lambda gt11 (Tamkun et al., 1986) was screened by hybridization to hCRP cDNA (Liebhaber et al., 1990) following standard protocols (Maniatis, 1989). Specifically, the 1.8-kb hCRP cDNA was labeled with ^{32}P by the random primer method (Pharmacia, LKB Biotechnology Inc.). Hybridizations were conducted overnight at 65°C in 5× SSC, 5× Denhardt's solution,

1. *Abbreviations used in this paper*: cCRP, chicken cysteine-rich protein; CRIP, cysteine rich intestinal protein; hCRP, human cysteine-rich protein.

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100 mg/ml single-stranded salmon sperm DNA, 0.2% SDS, and 10 mM Tris, pH 7.2. Two 5-min washes in 2× SSC/0.1% SDS were conducted at 25°C. These were followed by two 30-min washes in 0.2× SSC/0.1% SDS at 65°C. 1.5 × 10^6 recombinant phage were screened. Of these, 20 positive plaques were identified, collected, and purified by four rounds of plaque purification, using the hCRP cDNA as the probe. 10 of the cDNA inserts from these recombinant phage were analyzed further by Southern hybridization. One of these, a 1.3-kb insert, was further characterized as described below.

DNA Sequencing and Analysis

The 1.3-kb insert was cloned into the EcoRI restriction site of the vector, pBluescript II KS+ (pBS; Stratagene, La Jolla, CA) to generate pBS-cCRP. The majority of the nucleotide sequence of cCRP cDNA was determined by double-stranded DNA sequencing using the dideoxy chain termination method of Sanger (Sanger et al., 1977) and Sequenase II (United States Biochemical Corp., Cleveland, OH). Ambiguities were resolved by dideoxy chain termination sequencing of polymerase chain reaction products (GIBCO BRL Life Technologies Inc., Gaithersburg, MD), dideoxy chain termination sequencing with Taq polymerase (United States Biochemical Corp.), or a modification of the dideoxy chain termination method (Schuurman and Keulen, 1991). Sequencing was performed using a combination of specific primer directed DNA sequencing (Strauss et al., 1986) and sequence determination of deletion subclones. Primer synthesis was conducted using an Applied Biosystems DNA synthesizer, Model 380 B. The entire sequences of both strands were determined. Analysis of the sequence was performed using the University of Wisconsin GCG software (Devereux et al., 1984). The program "Bestfit" was used to compare the derived amino acid sequences of hCRP and cCRP.

Stoke Radius, Sedimentation Coefficient, Blot Overlay Assays, SDS-PAGE, and Western Immunoblot Analysis

The Stokes radius of cCRP was determined by calibrated gel filtration and the sedimentation coefficient was estimated by sucrose density gradients as described previously (Crawford and Beckerle, 1991). Purification of zyxin, protein iodination and blot overlay assays were performed as described by Crawford et al. (1992). ^{125}NaI was obtained from ICN Radiochemicals (Costa Mesa, CA). ^{125}I-zyxin was used at a concentration of 250,000 cpm/ml in blot overlay assays. SDS-PAGE was performed using 12.5% acrylamide (Bio Rad Laboratories, Richmond, CA) with a bis:acrylamide ratio of 1:120 according to the method of Laemmli (1970). Western immunoblot analysis was performed using a modification (Beckerle, 1986) of the technique described previously (Towbin et al., 1979). ^{125}I-Protein A was employed as a secondary reagent in immunoblot analysis.

Heat Stability Assay

Protein samples from non-adsorbed DEAE-cellulose fractions which contained cCRP were placed in a post-boiling (~95°C) water bath for 6 min. The sample was then centrifuged at 16,000 *g* in an Eppendorf microfuge for 10 min. Pelleted material was resuspended directly in Laemmli sample buffer for SDS-PAGE.

Antibody Production

New Zealand white rabbits were immunized twice by subcutaneous injection at 3-wk intervals. For these initial immunizations, electrophoretically purified cCRP that had been transferred to nitrocellulose and dissolved in DMSO was used as the immunogen, as described previously (Knudsen, 1985; Crawford and Beckerle, 1991). Two subsequent intravenous immunizations using biochemically purified cCRP followed at 3-wk intervals. Polyclonal antibody B31 was employed for the experiments described in this paper.

Preparation of Primary Smooth Muscle Cell Cultures and Immunofluorescence

Chicken gizzards removed from 14–16-d embryos were rinsed in HBSS, dissected into small pieces, and digested with 3 mg/ml collagenase (Boehringer Mannheim Corp., Indianapolis, IN) prepared in DME for 0.5–1.0 h at 37°C as described by Gimona and colleagues (1992). Treated cells were rinsed in DME, pelleted, resuspended in DME and grown on glass coverslips in preparation for immunofluorescence. Indirect immunofluorescence was performed exactly as described previously (Beckerle, 1986). Briefly, cells were fixed with 3.7% formaldehyde and were subsequently detergent permeabilized and processed for immuno-labeling. A rabbit polyclonal antibody (B31) was used to localize cCRP. The mouse polyclonal anti-zyxin antibody, M2, was described previously (Crawford and Beckerle, 1991). Monoclonal anti-calponin antibodies were a generous gift of Dr. M. Gimona (Austrian Academy of Sciences, Salzburg, Austria). Fluorescein-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-mouse IgG (Cappell Laboratories, Malvern, PA) were used as secondary reagents. No fluorescein signal was observed in the rhodamine channel or vice versa using these reagents. Cells prepared for immunofluorescence were examined on a Zeiss Axiophot microscope (Carl Zeiss Inc., Thornwood, NY).

Tissue Sample Preparation

Organs were isolated from appropriately aged embryonic chickens, rinsed in HBSS and homogenized in 10 volumes of homogenization buffer (0.1% Triton X-100, 0.1 mM benzamidine HCl, 1 ng/ml 1-10-phenanthroline, 1 ng/ml pepstatin A, and 0.1 mM phenylmethylsulfonyl fluoride) (Sigma Chemical Co., St. Louis, MO) and mixed 1:1 with Laemmli sample buffer. Samples were loaded onto SDS-polyacrylamide gels so that each lane contained the same total wet weight of material (typically 250–500 μg wet tissue weight). Bradford analysis (Bradford, 1976) using bovine serum albumin (Sigma Chemical Co.) as a standard was used to determine the total protein concentration of samples obtained from different staged gizzards. These values were used to normalize the values obtained by phosphoimage analysis when determining relative protein levels during embryonic development. Relative protein levels were determined by Western immunoblot techniques, and quantitative analysis was performed on a model 400 B phosphoimager (Molecular Dynamics, Inc., Sunnyvale, CA). Values obtained from phosphoimaging analysis were subjected to the Mann-Whitney U test (Mann and Whitney, 1947), a non-parametric statistical analysis appropriate for the small sample size used in our study. Rabbit serum B31 was used to identify cCRP, and polyclonal rabbit antiserum B23 was used to detect zyxin in Western immunoblots. Anti-α-actinin antiserum used in these studies was a generous gift of Dr. S. J. Singer (University of California, San Diego).

Results

Purification of a 23-kD Zyxin-binding Protein from Avian Smooth Muscle

We previously reported the identification of a 23-kD zyxin-binding protein in avian smooth muscle (Sadler et al., 1992). We detected the 23-kD zyxin-binding protein by use of a blot overlay assay in which electrophoretically resolved smooth muscle proteins were transferred to nitrocellulose and probed for their ability to interact with radio-iodinated zyxin (see Fig. 1 for example). We were able to take advantage of this assay system to develop a purification protocol for the 23-kD protein. We used ^{125}I-zyxin to detect the protein in avian smooth muscle extracts that were fractionated by ammonium sulfate precipitation. Briefly, proteins extracted from low ionic strength smooth muscle preparations were fractionated by precipitation with increasing amounts of ammonium sulfate (0–27, 27–34, 34–43, and 43–61% saturation). Samples from each of these precipitates were resolved by SDS-PAGE, transferred to nitrocellulose and prepared for blot overlay experiments. In the 34–43% precipitate, ^{125}I-zyxin prominently recognizes a protein that exhibits an apparent molecular mass of 23 kD on SDS-polyacrylamide gels (Fig. 1, lane *3'*). As expected based on our previous work (Crawford et al., 1992), we also observed an interaction between ^{125}I-zyxin and α-actinin, a 100-kD protein present in both the 0–27% and 27–34% ammonium sulfate precipitates (Fig. 1 *B*, lanes *1'* and *2'*). A small number of other poly-

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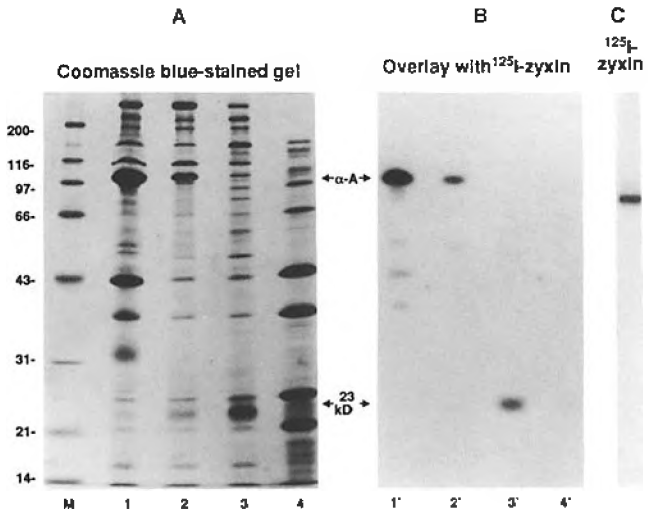

Figure 1. Identification of a 23-kD zyxin-binding protein by ^{125}I-zyxin blot overlay assays. Protein samples were extracted from gizzard and were fractionated by precipitation with increasing amounts of ammonium sulfate. The polypeptide content of each fraction was analyzed by SDS-PAGE. (*A*) Coomassie blue–stained gel showing the protein composition of samples precipitated with 0-27% (lanes *1* and *1'*), 27–34% (lanes *2* and *2'*), 34–43% (lanes *3* and *3'*) and 43-61% (lanes *4* and *4'*) saturated ammonium sulfate. (*B*) Corresponding ^{125}I-zyxin blot overlay of an equivalent gel transferred to nitrocellulose. A 23-kD protein is prominently recognized in the 34–43% saturated ammonium sulfate precipitate using ^{125}I-zyxin (lane *3'*). Other polypeptides are also recognized by ^{125}I-zyxin, including α-actinin (~100 kD, lanes *1'* and *2'*). (*C*) Autoradiogram showing the purity of radiolabeled zyxin used in this assay.

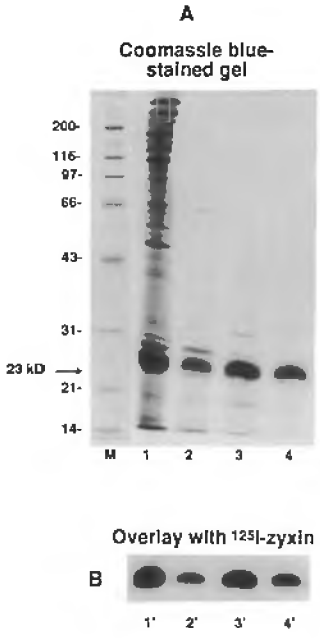

Figure 2. Purification of the 23-kD zyxin-binding protein from avian smooth muscle. (*A*) Successive chromatographic steps involved in the purification of the 23-kD protein from avian smooth muscle. Fractions enriched in the 23-kD protein were pooled from each of the chromatographic steps in the protocol and resolved on 12.5% polyacrylamide gels; 34–43% ammonium sulfate column load (lane *1*); proteins that fail to bind DEAE-cellulose (lane *2*); pooled fraction from phenyl Sepharose CL-4B column (lane *3*); purified cCRP after gel filtration on Sepharose CL-6B (lane *4*). (*B*) The 23-kD region of a corresponding blot overlay probed with ^{125}I-zyxin demonstrates that the 23-kD protein present in each fraction is recognized by the radiolabeled probe, confirming that the purified protein has the capacity to bind zyxin.

peptides that interact with ^{125}I-zyxin in this assay remain to be characterized.

The 23-kD zyxin-binding protein was effectively purified from the 34–43% ammonium sulfate precipitate of the avian smooth muscle extract by conventional chromatographic techniques (Fig. 2). Proteins precipitated with 34–43% ammonium sulfate (Fig. 2, lane *1*) were subjected to column chromatography on DEAE-cellulose (Fig. 2 *A*, lane *2*), phenyl Sepharose (Fig. 2 *A*, lane *3*), and Sepharose CL-6B (Fig. 2 *A*, lane *4*), a procedure that resulted in the purification of the 23-kD protein to apparent homogeneity. To verify that the purified protein obtained after chromatographic separation was the protein of interest, we performed ^{125}I-zyxin blot overlay assays on fractions eluted from each of the columns used in the purification protocol (Fig. 2 *B*); the purified 23-kD protein was recognized by ^{125}I-zyxin in the blot overlay assay (Fig. 2 *B*, lane *4'*). Approximately 20 mg of purified 23-kD protein is obtained from 300 g of starting material, indicating that this protein is substantially more abundant than zyxin in adult chicken gizzard.

Isolation and Molecular Characterization of cDNA Clones Encoding the 23-kD Protein

By microsequence analysis of peptides derived from the 23-kD protein, we determined that the purified protein was closely related to a previously described LIM domain protein, hCRP. Because of the sequence similarity between the 23-kD protein and hCRP, we used an hCRP cDNA clone (Liebhaber et al., 1990) to probe a chicken embryo fibroblast library in an effort to isolate the cDNA encoding the avian 23-kD protein. We isolated and sequenced a 1,327-bp cDNA which is complete with respect to coding capacity (Fig. 3). The translational start codon, located at position 70, was identified as such by direct comparison of the derived amino acid sequence with the NH_2-terminal amino acid sequence of the 23-kD protein. The NH_2-terminal sequence revealed that the initiator methionine is absent in the mature protein.

The single open reading frame encodes a 192–amino acid protein with a calculated molecular mass of 20.3 kD (Fig. 3). The derived amino acid sequence of this protein is 91% identical to the amino acid sequence of hCRP, with 6 of the 19 mismatches characterized as conservative amino acid substitutions (Fig. 4). Therefore, we conclude that the isolated cDNA encodes an avian homologue of hCRP, referred to hereafter as chicken CRP (cCRP). Furthermore, the deduced amino acid sequence of cCRP exhibits complete identity with sequence information obtained previously by direct microsequencing of peptides derived from the purified 23-kD protein (Sadler et al., 1992). Therefore, the isolated cDNA clone clearly encodes the 23-kD zyxin-binding protein.

As is the case for hCRP, the amino acid sequence of cCRP contains a number of cysteine and histidine residues organized into two tandemly arrayed LIM domains of the sequence $CX_2CX_{17}HX_2CX_2CX_2CX_{17}CX_2C$ (Fig. 3). In addition, the protein exhibits a glycine-rich repeat, GPKG(Y/F)G(Y/F)-G(M/Q)GAG, following each LIM domain (Fig. 3). A potential nuclear targeting signal (KKYGPK) noted in the sequence of hCRP (Liebhaber et al., 1990) is conserved in the chicken protein as well. The sequence of cCRP is rich in ba-

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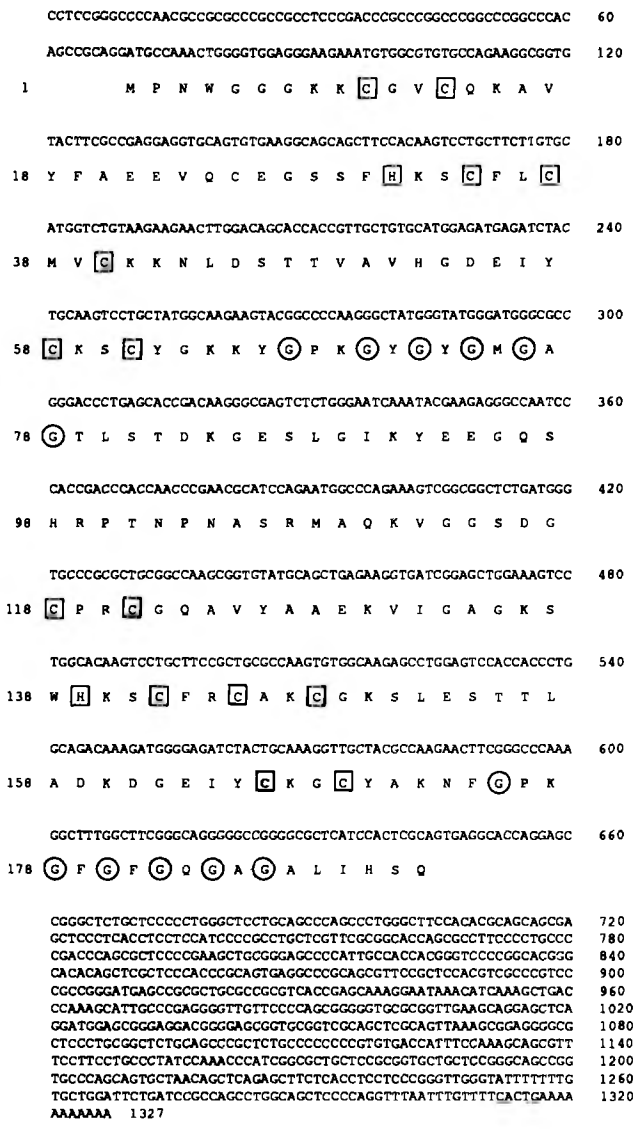

Figure 3. Nucleotide and derived amino acid sequence of cCRP cDNA. The nucleotide (numbered on the right) and derived amino acid sequences (numbered on the left) of the cCRP cDNA are shown. The cysteine and histidine residues that define the LIM consensus sequence are boxed. Glycine residues that occur in the glycine-rich repeat following each LIM domain are circled. A polyadenylation recognition element, CACTG (*underlined*), occurs just upstream of the poly A-tail (Berget, 1984). These sequence data are available from EMBL/GenBank/DDBJ under accession number X73831.

sic amino acids (13.5% Lys and Arg) and the protein exhibits a predicted, unmodified isoelectric point of 8.53.

Properties of Purified cCRP

The biophysical characteristics of cCRP isolated from avian smooth muscle are summarized in Table II. The Stokes radius of 2.0 ± 0.1 nm for purified cCRP in 110 mM NaCl was estimated by calibrated gel filtration chromatography (Nozaki et al., 1976). From this value, we calculated a frictional ratio (f/f_o) of 1.1 ± 0.1 for cCRP, which indicates that the protein is essentially spherical in shape. The relative

Figure 4. Comparison of the predicted amino acid sequence of cCRP to hCRP. The predicted amino acid sequence of cCRP (*bottom line*) is 91% identical to the amino acid sequence of hCRP (*top line*) with one single amino acid gap.

sedimentation coefficient of cCRP was determined to be 2.2 S ± 0.1 S by sucrose density gradient centrifugation (Martin and Ames, 1961). The method of Cohn and Edsall (1943) was used to calculate a partial specific volume of 0.72 g/ml for cCRP from its derived amino acid composition. We have used these hydrodynamic properties to estimate a native molecular mass of 18 kD for cCRP according to the method of Siegel and Monty (1965); this value suggests that purified cCRP is present predominantly in the form of a monomer.

We have also determined that cCRP is a heat stable protein. When a sample of partially purified cCRP is incubated at 95°C for 6′ and subsequently subjected to centrifugation, the majority of cCRP remains soluble and is retained in the supernatant, whereas other proteins precipitate and are found in the pelleted material (Fig. 5). We suspect that the small amount of cCRP present in the pellet results from nonspecific trapping of the protein in the heat-denatured protein aggregate. The heat stable 23-kD protein is recognized by ^{125}I-zyxin in blot overlay assays (data not shown) and by specific antibodies raised against purified cCRP (Fig. 5 *B*), confirming its identity as cCRP.

cCRP Exhibits Tissue Specific Protein Expression

To examine the tissue distribution of cCRP, we performed Western immunoblot analysis of proteins derived from several embryonic avian tissues. Tissues from day-20 embryos were homogenized in 10 volumes of homogenization buffer, and an equivalent volume of each sample was loaded onto SDS-polyacrylamide gels. A Coomassie blue-stained gel revealing the protein complexity of each tissue sample is shown in Fig. 6 *A*. Western immunoblot analysis using anti-cCRP antibodies reveals that cCRP is most prominent in intestine, stomach, and particularly gizzard (Fig. 6 *B*). A

Table II. Properties of Purified cCRP

Relative sedimentation coefficient	2.2 S ± 0.1 S
Stokes radius	2.0 nm ± 0.1 nm
Frictional ratio (f/f_0)	1.1 ± 0.1
Partial specific volume	0.72 g/ml
Calculated native molecular weight	18,000

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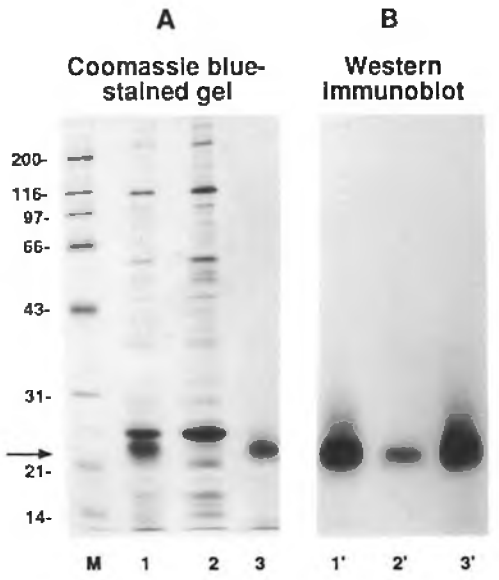

Figure 5. cCRP is heat stable. Samples enriched in cCRP were prepared by extraction of smooth muscle and chromatography on DEAE-cellulose (for example see Fig. 2, lane *2*). The partially purified cCRP was incubated at 95°C for 6′ and then centrifuged. *A* shows a 12.5% Coomassie blue–stained gel. The corresponding Western immunoblot using anti-cCRP antibodies to detect cCRP (*arrow*) is shown in *B*. (Lanes *1* and *1′*) Untreated protein sample; (lanes *2* and *2′*) material recovered in the pellet after heat treatment; (lanes *3* and *3′*) material that remains soluble after heat treatment; the majority of cCRP is found in this supernatant fraction. Twice as much material was loaded in lanes *2* and *2′* to detect residual cCRP in the pelleted material.

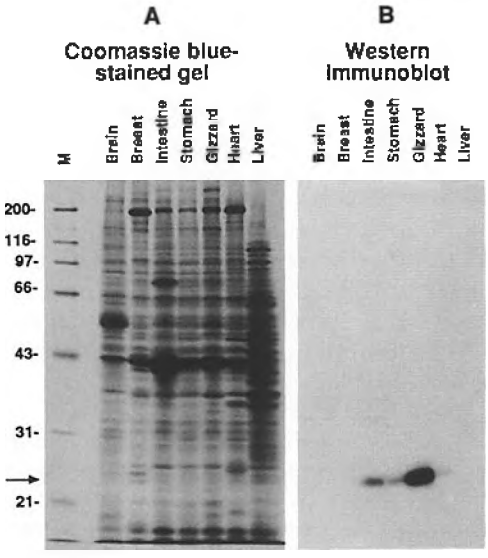

Figure 6. Tissue-specific expression of cCRP. Tissues from day-20 chicken embryos were homogenized and equivalent samples, based on wet tissue weight, were prepared for SDS-PAGE. *A* shows a Coomassie blue–stained gel of the tissues examined. *B* shows the corresponding immunoblot using anti-cCRP antibodies, which reveals that cCRP (*arrow*) is specifically recognized from these complex mixtures of proteins. cCRP is most prominent in intestine, gizzard and stomach, while less abundant in heart. No signal is observed in skeletal muscle (breast), brain, or liver.

lower amount of cCRP is observed in heart, whereas no cCRP is detected in liver, skeletal muscle, or brain. From this analysis of day-20 chicken embryo tissues, cCRP appears to be most abundant in smooth muscle sources. Comparable results were obtained with a second, independently generated antiserum.

We have investigated whether cCRP is present in the tissues shown in Fig. 5 *A* at earlier stages of development as well, including samples obtained from day-12 and day-16 embryos (data not shown). As in the case of day-20 embryos, we fail to detect cCRP in brain, skeletal muscle or liver in these younger embryos. However, as will be discussed below, we did observe that cCRP levels are modulated as a function of developmental time in smooth muscle sources, including intestine, stomach and gizzard.

Developmental Regulation of cCRP Expression

A number of LIM-containing proteins, such as Xlim-1 and rhombotin, are developmentally regulated during embryogenesis (Taira et al., 1992; Greenberg et al., 1990). Therefore, we performed Western immunoblot analysis to determine the extent to which cCRP levels are modulated during gizzard differentiation. We compared the amount of cCRP in gizzard derived from adult chickens with the amount of cCRP detected in gizzard obtained from 11-, 14-, 17-, and 20-day chick embryos (Fig. 7). Immunoblots of samples loaded equivalently by wet tissue weight were analyzed using a phosphoimager. The raw values obtained were adjusted to normalize for the protein concentration in each sample, as measured by Bradford analysis. Quantitative analysis of several independent experiments reveals that cCRP steadily increases in abundance as a function of developmental time (Table III). Statistical analysis revealed that there is a significant increase in the amount of cCRP detected in gizzard isolated from day-20 embryos when compared to either day-11 ($P < 0.001$) or day-14 ($P < 0.01$) embryos. The most dramatic increase in cCRP levels that we have detected prior to hatching occurs between day 14 and day 17 of embryonic development. In addition, we have observed that maximal expression of cCRP occurs in the adult tissue (Fig. 7); the

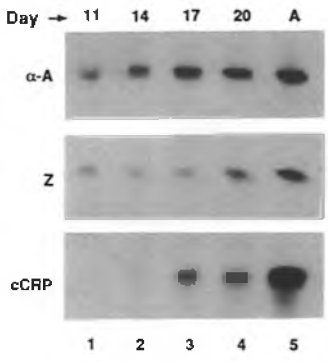

Figure 7. cCRP protein level is developmentally regulated. Proteins were extracted from chicken gizzards at the following developmental stages: embryonic days 11 (lane *1*), 14 (lane 2), 17 (lane *3*), 20 (lane *4*), and adult (lane *5*). These samples were analyzed by Western immunoblot for the presence of α-actinin (α-A), zyxin (Z), and cCRP (*cCRP*). α-Actinin and zyxin are detected in samples corresponding to all stages of development examined. Conversely, cCRP is significantly more abundant in older embryonic gizzard tissue. Half as much material was loaded in adult samples (lane *5*) in the immunoblot analyses shown here in an attempt to visualize less intense signals observed in samples from younger embryos. Quantitative analysis of the relative protein abundance of α-actinin, zyxin and cCRP is presented in Table III.

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*Table III. Protein Levels of α-Actinin, Zyxin, and cCRP in Gizzard at Different Developmental Stages**

Protein	Day 11	Day 14	Day 17	Day 20	Adult‡
α-Actinin	0.36 ± .05§	0.40 ± .09	0.44 ± .06	0.49 ± .05	1.0
Zyxin	0.33 ± .03	0.28 ± .06	0.26 ± .01	0.30 ± .05	1.0
cCRP	0.06 ± .01	0.09 ± .02	0.17 ± .02	0.24 ± .04	1.0

* Protein levels are expressed as a ratio (embryonic level/adult level).
‡ Adult levels have been given an arbitrary value of 1.0.
§ ±SEM.

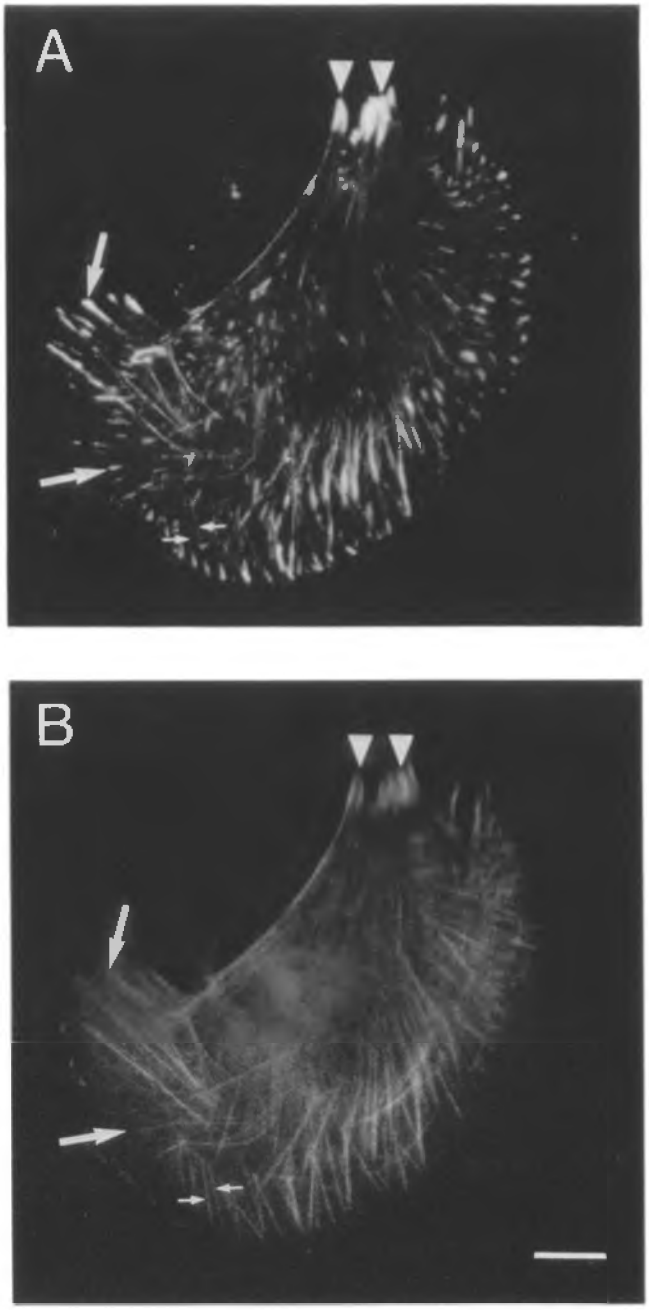

Figure 8. Comparison of the distributions of cCRP and zyxin in chicken embryo fibroblasts. Cells were double labeled by indirect immunofluorescence using a mouse polyclonal anti-zyxin antibody and a rabbit polyclonal anti-cCRP antibody. Both zyxin (*A*) and cCRP (*B*) are found along the stress fibers near where they terminate at adhesion plaques (*small arrows*). Although both zyxin and cCRP are found together in some adhesion plaques (*arrowheads*), in many cases cCRP is not detected at zyxin-rich focal contacts (*large arrows*). Cytoplasmic staining is more pronounced with anti-cCRP antibodies. Bar, 15 mm.

amount of cCRP detected in adult gizzard is ~15-fold greater than the amount found in gizzard isolated from day-11 embryos.

We have compared the relative level of two other cytoskeletal proteins, zyxin, and α-actinin, to that of cCRP at different stages during gizzard development (Fig. 7). In contrast to cCRP, levels of both zyxin and α-actinin remain fairly constant during embryogenesis, exhibiting little developmental regulation over the time period we have monitored (Table III). In addition, unlike cCRP, zyxin and α-actinin are clearly detectable at all stages investigated. Interestingly, there is a significant increase in the amount of all of these proteins (cCRP, zyxin, and α-actinin) in adult tissue as compared with embryonic samples. The elevated protein levels observed for these proteins in adult samples parallels the pattern observed for another adhesion plaque protein, vinculin (Volberg et al., 1986). Consistent with the finding of others (Hirai and Hirabayashi, 1983), we also observed that the levels of actin and myosin increase with developmental age (data not shown).

Subcellular Distribution of cCRP

The distribution of cCRP relative to its binding partner, zyxin, is complex. By double-label indirect immunofluorescence, we detect an extensive, but not absolute, overlap in the distributions of zyxin and cCRP. Both zyxin (Fig. 8 *A*) and cCRP (Fig. 8 *B*) are detected on actin filament bundles near where they terminate at the adhesion plaques. The prominent non-uniform staining of stress fibers with anti-zyxin and anti-cCRP antibodies provides support for the notion that cytoplasmic actin bundles display molecularly distinct, spatially restricted subdomains. As reported previously, zyxin is consistently observed at adhesion plaques. In contrast, cCRP is detected in some adhesion plaques but not in others. The lack of cCRP in certain adhesion plaques may reflect an underlying biochemical heterogeneity in cellular junctional complexes. Alternatively, the failure to detect cCRP consistently at adhesion plaques may be attributable to a problem of antigen presentation or antibody accessibility at adhesion plaques as has been reported previously for α-actinin (Pavalko and Burridge, 1991).

Because our biochemical analysis revealed that cCRP is most abundant in tissues that are rich in smooth muscle, such as gizzard, we have used primary cell cultures isolated from

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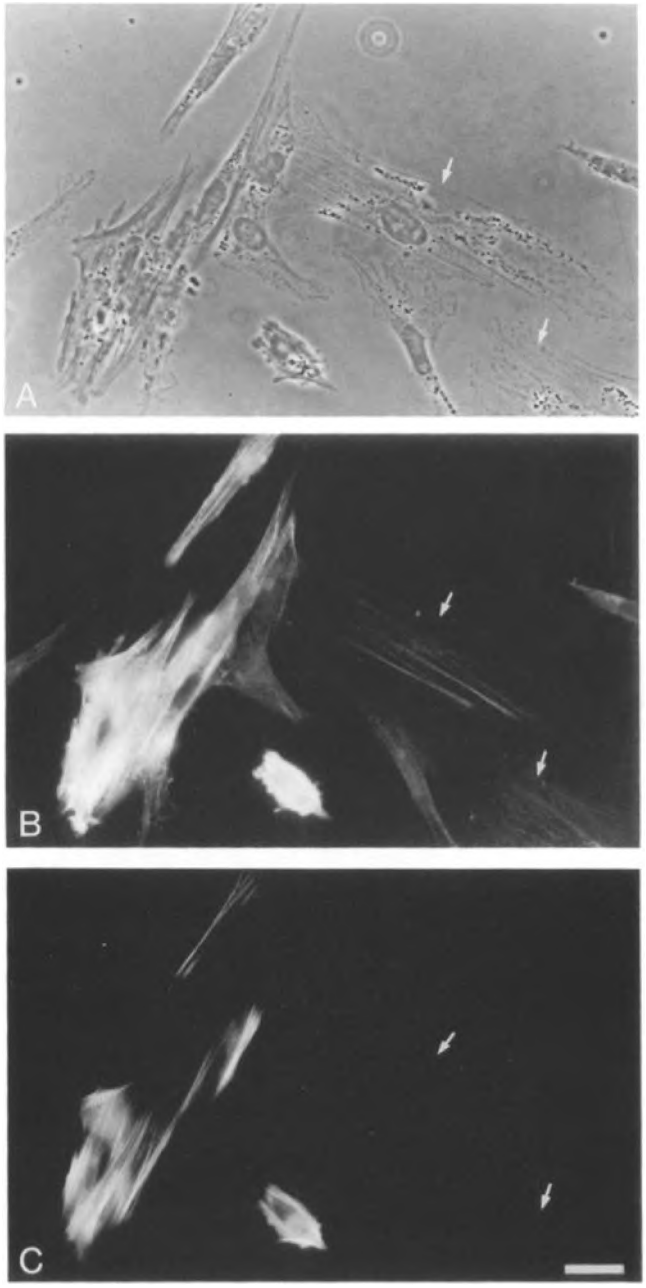

Figure 9. cCRP is enriched in smooth muscle cells. The distributions of cCRP and calponin were examined by double label indirect immunofluorescence in a heterogeneous population of cells isolated from embryonic day-16 gizzard. (*A*) Phase-contrast image. (*B*) Immunofluorescence image using anti-cCRP antibodies to determine the cellular distribution of cCRP in a heterogeneous cell population. (*C*) Immunofluorescence image using anti-calponin antibodies to identify smooth muscle cells. Note that smooth muscle cells defined by anti-calponin antibodies are more intensely labeled with anti-cCRP antibodies than are neighboring presumptive fibroblasts. Arrows in each panel correspond to cells that are labeled with anti-cCRP antibodies, but which do not stain with anti-calponin antibodies. Bar, 20 mm.

embryonic day-16 gizzard to investigate the cell-type distribution of cCRP. Primary cell cultures prepared from gizzard are comprised of a heterogeneous population of cells which includes fibroblasts as well as smooth muscle cells at various stages of differentiation. Indirect immunofluorescence using antibodies that specifically and exclusively recognize cCRP by Western immunoblot reveals that a subpopulation of cells are preferentially stained in these heterogeneous cultures (Fig. 9). We have used a smooth muscle cell-specific probe, anti-calponin antibody, to identify differentiated smooth muscle cells in the primary culture (Gimona et al., 1990). Cells that are calponin-positive (Fig. 9 *C*) correspond to cells that exhibit the most intense staining with anti-cCRP antibody (Fig. 9 *B*).

The intense staining of smooth muscle cells with anti-cCRP antibodies made it difficult to characterize the subcellular distribution of cCRP in these cells. However, we have observed that cCRP is present along stress fibers in less intensely stained calponin-positive cells (data not shown). In addition, cCRP is also present along actin-rich stress fibers in neighboring cells, presumably fibroblasts or de-differentiated smooth muscle cells, that are not recognized by anti-calponin antibodies (Fig. 9, *arrows*). These results are consistent with the staining observed in isolated chicken embryo fibroblasts (Fig. 8 *B*). Our results demonstrate that cCRP, unlike calponin, is not absolutely restricted to differentiated smooth muscle cells; however, cCRP is significantly more abundant in calponin-positive cells.

Discussion

We have used an in vitro binding assay to identify proteins that interact with zyxin, a LIM domain protein found at adhesion plaques. Using this approach, we have identified a 23-kD zyxin-binding protein in avian smooth muscle extracts. By peptide microsequence analysis and by isolation of a cDNA clone encoding the 23-kD protein, we determined that this zyxin-binding protein is the avian homologue of the human cysteine-rich protein (hCRP). We refer to the 23-kD protein as the chicken cysteine-rich protein (cCRP).

The primary amino acid sequence of cCRP is strikingly similar to that of its human homologue. The high degree of conservation between two species which have diverged at least 320 million years ago (Lombard and Sumida, 1992) highlights the importance of the primary amino acid sequence for specifying the function of the protein. cCRP is a basic protein with a calculated pI of 8.53 and like hCRP, it displays two copies of the LIM motif, each followed by the glycine-rich sequence: GPKG(Y/F)G(Y/F)G(M/Q)GAG. Although the biological significance, if any, remains to be determined, it is intriguing that this glycine-rich repeat is similar to the sequence, KG(Y/F)G(Y/F)VX(Y/F), found in RNA-binding proteins such as nucleolin (Dreyfuss et al., 1988).

The identification of cCRP in smooth muscle presented us with the opportunity to purify a LIM domain protein from its endogenous source. Although the LIM domain is present in a number of biologically significant proteins, thus far only one LIM domain protein has been biochemically isolated. The previously purified LIM protein is the adhesion plaque constituent zyxin, which is present only in very low abundance in cells (Sadler et al., 1992). As described here, we have developed a method for purifying cCRP from chicken gizzard. Isolated cCRP behaves as a globular, heat-stable, monomeric protein. cCRP is abundant in adult chicken gizzard, and interestingly, was noted previously in a study of

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particularly abundant smooth muscle proteins (Lees-Miller, 1987). The extensive cytoskeletal association of cCRP is particularly interesting since, with the notable exception of zyxin (Sadler et al., 1992), the majority of LIM domain proteins described to date are nuclear or presumed nuclear proteins (McGuire et al., 1991; Freyd et al., 1990).

Like many other LIM domain proteins, cCRP exhibits tissue-specific expression and is developmentally regulated. We have used Western immunoblot analysis to monitor the level of cCRP in a variety of embryonic avian tissues and have found that cCRP is most abundant in smooth muscle sources. This finding is consistent with our immunofluorescence studies which demonstrate that the fluorescent signal associated with cCRP in primary gizzard cell cultures is more prominent in smooth muscle cells than in presumptive fibroblasts. The level of cCRP in chicken gizzard is subject to developmental regulation. cCRP levels steadily increase in gizzard during embryogenesis, displaying a fourfold increase in abundance from day 11 to day 20 of embryonic development. The most dramatic increase in cCRP level during embryogenesis occurs from day 14 to day 17, a time that corresponds to a number of key events in the maturation of smooth muscle cells (Chou et al., 1992; Volberg et al., 1986).

Although CRP is an evolutionarily conserved protein, its biological function is not yet understood. In an effort to characterize the protein further and hopefully gain some insight into its role in cells, we have characterized the primary amino acid sequence, biophysical properties, tissue distribution, subcellular location, and expression pattern of cCRP during development. We have also determined that CRP is a binding partner for zyxin and is colocalized with zyxin in association with the cytoskeleton. Recently, it has been suggested that CRP may participate in some aspect of cell growth control. The *crp* gene is a primary response gene that is induced in quiescent cells in response to serum stimulation in a manner that parallels the induction of c-*myc* (Wang et al., 1992). Moreover, *crp* expression is suppressed in a variety of transformed avian cell lines that display unregulated cell growth properties (Weiskirchen and Bister, 1993). This finding is particularly interesting in light of recent work showing that merlin, a protein related to the cytoskeletal proteins moesin, ezrin, and radixin, may function as a tumor suppressor in humans (Rouleau et al., 1993; Trofatter et al., 1993).

Since CRP is comprised primarily of LIM domains, the structure and function of the LIM domain will clearly have significant influence on the biochemical role of CRP in cells. With regard to structure, we have demonstrated that the LIM motif defines a specific zinc-binding domain (Michelsen et al., 1993). LIM domains have been postulated to have a variety of functions. Initially, because the structure of the LIM domain is reminiscent of the zinc fingers found in certain transcription factors, it was suggested that the LIM domain might bind to nucleic acids (Freyd et al., 1990; Li et al., 1991). Alternatively, others have speculated that the LIM domain may function in protein–protein interactions (Rabbitts and Boehm, 1990). The demonstration of a direct interaction between two LIM domain proteins, zyxin and CRP, suggests that the LIM domain may indeed serve as a protein binding interface (Sadler et al., 1992). Further support for this proposal comes from recent studies showing that synergism between two transcription factors, one of which is a LIM-homeodomain protein, is eliminated if the LIM region is deleted (German et al., 1992).

Many LIM proteins for which functions have been established are believed to exert influence over developmental events by affecting gene expression (Freyd et al., 1990; Karlsson et al., 1990; Way and Chalfie, 1988). Even LIM domain proteins that lack a DNA-binding homeodomain have been postulated to function in regulating cellular differentiation. For example, rhombotin is thought to play a role in transcriptional regulation during development (McGuire et al., 1991; Boehm et al., 1990; Greenberg et al., 1990). Recent studies indicate that ectopic overexpression of rhombotin in transgenic mice leads to T-cell tumors (McGuire et al., 1992). Thus rhombotin appears to be a proto-oncogene product involved in differentiation during development that, when ectopically expressed, gives rise to the transformed phenotype. The structural similarities between rhombotin and cCRP raise the possibility that cCRP, like rhombotin, could be involved in specification or maintenance of cell phenotype or growth properties. However, this is clearly speculative at present.

It is also possible that cCRP, which is comprised primarily of LIM domains, has a more general role in cells. Whereas a number of LIM domain proteins exhibit additional functional domains, such as homeodomains, others such as the cysteine-rich intestinal protein (CRIP) and cCRP display LIM domains in the absence of other identifiable sequence motifs. A parallel relationship occurs in certain proteins that exhibit the Src homology regions 2 and 3 (SH2 and SH3). Typically, proteins that contain the SH2 and SH3 sequence motifs are involved in cytoplasmic signal transduction pathways. Although the SH2/SH3 domains are frequently associated with enzymatic activities such as tyrosine kinases (e.g., $pp60^{src}$), phospholipases (e.g., PLC-γ), and GTPase activators (e.g., GAP-ras) (for review see Schlessinger and Ullrich, 1992), the SH2 and SH3 domains are also found in a collection of proteins that fail to exhibit any other major sequence features (e.g., GRB2/sem 5, nck, and c-crk) (for review see Schlessinger and Ullrich, 1992). Both SH2 and SH3 domains appear to mediate specific protein-protein interactions that are critical for the assembly of signaling complexes. Proteins that display SH2 and SH3 domains as their primary or sole sequence feature appear to function as adaptor molecules that serve to juxtapose two participants in a signal transduction cascade (Koch et al., 1991). By analogy, LIM-only proteins such as cCRP may serve to link proteins together to generate functional complexes. Alternatively, the LIM-only proteins may be important regulatory proteins that compete, for example with LIM-homeodomain proteins, for binding to key co-factors.

In summary, we have described here the purification and molecular characterization of an abundant 23-kD LIM domain protein, cCRP, that is evolutionarily conserved (Liebhaber et al., 1990; Wang et al., 1992; Sadler et al., 1992). cCRP interacts directly with zyxin and is prominently associated with the actin cytoskeleton. cCRP is enriched in smooth muscle sources and displays temporally regulated expression during embryogenesis. The presence of LIM domains in a developmentally regulated protein which is associated with the cytoskeleton is intriguing, as this zinc-binding sequence motif has also been found in a variety of

proteins involved in the regulation of gene expression. The availability of a LIM domain protein isolated from its normal eukaryotic source in quantities sufficient for biochemical analysis, such as we have demonstrated for cCRP, should facilitate future investigations into the structure and function of the LIM domain.

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