Zyxin and cCRP: Two Interactive LIM Domain Proteins Associated with the Cytoskeleton

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Abstract. Interaction with extracellular matrix can trigger a variety of responses by cells including changes in specific gene expression and cell differentiation. The mechanism by which cell surface events are coupled to the transcriptional machinery is not understood, however, proteins localized at sites of cell-substratum contact are likely to function as signal transducers. We have recently purified and characterized a low abundance adhesion plaque protein called zyxin (Crawford, A. W., and M. C. Beckerle. 1991. *J. Biol. Chem.* 266:5847–5853; Crawford, A. W., J. W. Michelsen, and M. C. Beckerle. 1992. *J. Cell Biol.* 116:1381–1393). We have now isolated and sequenced zyxin cDNA and we report here that zyxin exhibits an unusual proline-rich NH_2-terminus followed by three tandemly arrayed LIM domains. LIM domains have previously been identified in proteins that play important roles in transcriptional regulation and cellular differentiation. LIM domains have been proposed to coordinate metal ions and we have demonstrated by atomic absorption spectroscopy that purified zyxin binds zinc, a result consistent with the idea that zyxin has zinc fingers. In addition, we have discovered that zyxin interacts in vitro with a 23-kD protein that also exhibits LIM domains. Microsequence analysis has revealed that the 23-kD protein (or cCRP) is the chicken homologue of the human cysteine-rich protein (hCRP). By double-label indirect immunofluorescence, we found that zyxin and cCRP are extensively colocalized in chicken embryo fibroblasts, consistent with the idea that they interact in vivo. We conclude that LIM domains are zinc-binding sequences that may be involved in protein–protein interactions. The demonstration that two cytoskeletal proteins, zyxin and cCRP, share a sequence motif with proteins important for transcriptional regulation raises the possibility that zyxin and cCRP are components of a signal transduction pathway that mediates adhesion-stimulated changes in gene expression.

Cellular adhesion to extracellular ligands can induce profound changes in cell behavior ranging from alteration in cell morphology to induction of specific gene expression. The response of a cell to ligand binding depends on the generation of a transmembrane signal to couple the adhesion event to some intracellular machinery such as the cytoskeleton or the transcriptional apparatus. While dramatic responses of cells to extracellular matrix binding have been widely documented (reviewed in Hynes, 1992), little is known about the post-receptor events that mediate the profound and diverse effects on cell morphology, behavior, and developmental fate of cells stimulated by receptor-ligand binding.

One class of extracellular matrix receptors, the integrins, participates directly in ligand-stimulated transmembrane signaling events (reviewed in Hynes, 1992). Integrins are heterodimeric ($\alpha\beta$) receptors for extracellular matrix proteins that, depending on their subunit composition and cell-type specific environment, can mediate cell binding to a large number of different matrix constituents including collagen, fibronectin, fibrinogen, and von-Willebrand factor (Albelda and Buck, 1990; Hynes, 1992). In some cases, ligand binding to integrins has been shown to induce a conformational change in the receptor itself (Frelinger et al., 1988), an event that could enable the integrin receptor to act directly as a transmembrane signaling molecule. The specific binding of integrins to their respective ligands can trigger a variety of secondary responses including increased cytoplasmic tyrosine kinase activity (Ferrell and Martin, 1989; Golden et al., 1990; Shattil and Brugge, 1991; Guan et al., 1991; Kornberg et al., 1991), increased intracellular pH (Schwartz et al., 1991*a*,*b*), and changes in cellular differentiation or specific gene expression (Werb et al., 1989; Adams and Watt, 1989; Menko and Boettiger, 1987). Each of these responses to matrix binding has the potential to exert a profound influence on cellular physiology and function.

Although many of the responses of cells to integrin-matrix interactions have been characterized, the specific mechanism by which an integrin-mediated adhesive event participates in transmembrane signaling is not understood. However, it is likely that the signal transduction machinery that mediates the response of cells to changes in substratum adhesion is localized proximal to the adhesive membrane

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where integrins are concentrated (Damsky et al., 1985; Chen et al., 1985). Consequently, in order to understand the events that occur in response to matrix binding, it is first important to define the detailed molecular architecture of an adhesive membrane. One model system for studying the molecular organization of an adhesive membrane is the adhesion plaque or focal contact of cells in culture. At these specialized regions of the cell surface, the cell membrane is in very close contact with the underlying substratum and an integrin-mediated transmembrane connection between the extracellular matrix and the actin cytoskeleton is established (Singer, 1979; Horwitz et al., 1986). A number of proteins that are believed to perform structural functions at adhesion plaques have been identified and characterized (for reviews see Burridge et al., 1988; Crawford and Beckerle, 1990; Beckerle and Yeh, 1990; Otto, 1990). Furthermore, potential regulatory proteins, including a specific calcium-dependent protease (Beckerle et al., 1987), protein kinase C (Jaken et al., 1989), and certain tyrosine kinases (Rohrschneider, 1980; Rohrschneider and Najita, 1984; Nigg et al., 1982; Schaller et al., 1992) are localized on the cytoplasmic face of the adhesion plaque membrane where they could be involved in post-receptor signaling events or in the dynamic remodeling of the adhesive membranes that occurs in normal cells.

We have identified another low abundance, cytoplasmic constituent of focal contacts called zyxin (Crawford and Beckerle, 1991) that like the above-mentioned catalytic components of adhesion plaques, may be involved in some of the regulatory or signal transduction events that occur at adhesive membranes. Zyxin was first discovered as an adhesion plaque protein with an apparent molecular mass of 82 kD by analysis of a nonimmune rabbit serum that stained these specialized regions of the cell membrane by indirect immunofluorescence (Beckerle, 1986). Zyxin was subsequently purified from avian smooth muscle (Crawford and Beckerle, 1991) and was shown to be present in cells at low abundance relative to the major, well-characterized structural components of focal contacts: vinculin, talin, α-actinin and actin itself. Interestingly, zyxin interacts directly with the relatively abundant cytoskeletal protein, α-actinin, an association that may be important for localizing zyxin to particular subcellular domains, such as the ends of actin filaments (Crawford et al., 1992).

Here we report the results of molecular cloning studies that reveal that zyxin displays two very interesting structural features: an unusual proline-rich NH_2-terminal domain and a COOH-terminal region consisting of three tandemly arrayed LIM domains. LIM domains (Freyd et al., 1990) are putative zinc-binding structural motifs with the consensus amino acid sequence $CX_2CX_{16\text{-}23}HX_2CX_2CX_2CX_{16\text{-}21}CX_{2\text{-}3}$ (C,H,D). LIM domains have now been identified in a number of proteins that appear to be involved in control of gene expression and differentiation. For example, the LIM motif is found in five homeodomain proteins: *Caenorhabditis elegans* Lin-11 and Mec-3 (Freyd et al., 1990; Way and Chalfie, 1988), rat Isl-1 (Karlsson et al., 1990), *Xenopus* Xlim-1 (Taira et al., 1992) and *Drosophila* apterous (Cohen et al., 1992). The LIM-homeodomain proteins are presumed to function as transcription factors and most have clearly been shown to be required for cell lineage determination and/or pattern formation during embryogenesis. LIM proteins lacking homeodomains or other obvious functional domains have also been identified: CRIP (Birkenmeier and Gordon, 1986), ESP1 (Nalik et al., 1989), hCRP (Liebhaber et al., 1990), and rhombotin (Boehm et al., 1990*b*, 1991*a*; McGuire et al., 1989). Rhombotin, which is also referred to as Ttg-1, is a nuclear protein (McGuire et al., 1991) that is believed to be a proto-oncogene product (Boehm et al., 1988; Boehm et al., 1990*a*). Like many developmentally important *Hox* genes and their products (McGinnis and Krumlauf, 1992), rhombotin is expressed in a spatially restricted segmental pattern in the developing mouse central nervous system (Greenberg et al., 1990; Boehm et al., 1991*b*). While the function of the LIM domain has not been determined, these sequences are often associated with proteins that have roles in controlling gene expression or development.

Our demonstration that the adhesion plaque protein zyxin exhibits LIM repeats raises the intriguing possibility that zyxin may be one of the long sought proteins involved in coupling the mechanical events of cell adhesion to the cellular machinery required to affect the differentiation state or developmental fate of a cell. Furthermore, in a search for proteins that interact with zyxin, we have identified a highly conserved 23-kD zyxin-binding protein (cCRP) that also exhibits LIM repeats. We suggest that the LIM sequences may function as protein-binding interfaces that serve to regulate the subcellular localization or biological activity of the LIM domain proteins.

Materials and Methods

Protein Purification and Sequencing

Zyxin was purified from avian smooth muscle according to the previously published procedure of Crawford and Beckerle (1991) with two modifications: (*a*) fresh (unfrozen) chicken gizzards were routinely used as the starting material and (*b*) ammonium sulfate fractionation was performed with 16 *g* ammonium sulfate per 100 cc of extract. cCRP was also purified from avian smooth muscle; the procedure for isolating cCRP will be described elsewhere (Crawford, A. W., and M. C. Beckerle, manuscript in preparation). Vinculin was purified from avian smooth muscle by a previously described method (Feramisco and Burridge, 1980).

Purified zyxin in phosphate buffer (15 mM potassium phosphate [mono- and dibasic] pH 7.2, 10 mM NaCl, 0.1 mM EDTA, 0.1% 2-mercaptoethanol) or cCRP in Tris buffer (25 mM Tris-HCl pH 7.6, 1 mM EDTA) was subjected to proteolytic cleavage using sequencing grade endoproteinase Lys-C (Boehringer Mannheim Corp., Indianapolis, IN) at an enzyme to substrate ratio of 1:50. Proteolytic digestion was allowed to proceed for 7 h at 37°C for zyxin and 12.5 h at 37°C for cCRP. The resulting cleavage products were rapidly frozen in a dry ice-ethanol bath and lyophilized before suspension in 2% trifluoro acetic acid. The proteolytic peptides were resolved by HPLC using a narrow bore (2.1 × 25 mm) C-18 column (Waters Chromatography Division, Milford, MA). Intact cCRP or individual peptides derived from zyxin or cCRP were sequenced on a protein sequencer (model 477A, Applied Biosystems, Inc., Foster City, CA). To identify cysteine residues in the cCRP sequence, the intact protein and an isolated peptide were derivatized with 4-vinyl pyridine prior to sequence analysis.

Isolation and Characterization of Zyxin cDNA Clones

A mouse polyclonal antibody (M-2) (Crawford and Beckerle, 1991) that was raised against electrophoretically isolated zyxin was used to screen a chicken embryo cDNA library cloned into the EcoRI site of the expression vector λgt11 (Clontech Laboratories, Inc., Palo Alto, CA). Expression screening was performed by standard procedures (Young and Davis, 1983). From ~1.5×10^6 recombinants screened, eight immunoreactive plaques were identified and the phage were purified. These initial isolates were rescreened using a second, independently generated anti-zyxin antibody.

Six phage isolates were recognized by this antibody as well, and five of these were selected for further analysis. These λ isolates fell into two distinct categories based on their cDNA insert sizes of 2.6 kb (λcZyx-1) and 1.4 kb (λcZyx-2). The EcoRI inserts (cZyx-1 and cZyx-2) were purified, cloned into the vector, pBluescript II KS+ (pBS, Stratagene Inc., La Jolla) and restriction mapped. Partial sequencing of cZyx-1 and cZyx-2 revealed that neither clone contained the translation initiation codon. Therefore, we rescreened the original chicken embryo cDNA library with an EcoRI-KpnI restriction fragment derived from cZyx-1 that represented the 5'-most zyxin cDNA sequences identified thus far. Using this screen we were able to isolate λcZyx-3 which still did not contain a suitable start codon. We therefore used the 5'-EcoRI-HindIII fragment derived from cZyx-3 to screen a chicken embryo fibroblast cDNA library which was generously provided by J. Tamkun (Tamkun et al., 1986); this screen yielded λcZyx-4 which contained the start codon (Fig. 1 *A*). All zyxin cDNAs were purified and subcloned into pBS. A composite clone (pBScZyx-5) that contained both the start and stop codons was constructed by ligating pBScZyx-1 to pBScZyx-4 at the HindIII site.

DNA Sequencing

A series of nested deletions was generated from zyxin cDNAs in pBS using Exonuclease III and S1 digestion as described previously (Guo and Wu, 1983; Sambrook et al., 1989). The sequence of the zyxin cDNA was determined by the dideoxy chain termination method of Sanger (Sanger et al., 1977) using the modified T7 polymerase, Sequenase (U.S. Biochemical Corp., Cleveland, OH) and ^{35}S-dATP. We followed the procedure recommended by the manufacturer for double-stranded sequencing (U.S. Biochemical Corp.). To sequence regions not represented in the deletion series, primers were synthesized using a DNA synthesizer (model 380B, Applied Biosystems, Inc., Foster City, CA). Both strands corresponding to the composite zyxin cDNA, cZyx-5, were sequenced in entirety.

Sequence analysis was performed using the University of Wisconsin GCG software package (Devereux et al., 1984). The programs TFASTA and WordSearch were used to search the GenEMBL and Swissprot databases for protein sequences related to the derived zyxin amino acid sequence. Tfasta was also used to search for peptide sequences or implied peptide sequences with similarity to the peptide sequences determined from the purified 23-kD protein (cCRP). The program BESTFIT was used to identify regions of similarity between the derived zyxin amino acid sequence and other sequences of interest such as the radixin, MARCKS, and ezrin sequences. The multiple sequence alignment program PILEUP was utilized to compare all of the known LIM domain sequences to each other.

In vitro Transcription/Translation and Immunoprecipitation

In vitro transcription and translation was performed using Promega reagents (pGEM Express core system and nuclease-treated rabbit reticulocyte lysate) according to the procedures recommended by the manufacturer (Promega Corporation, Madison, WI). RNA was transcribed from the template plasmid DNA (1-5 μg of pBScZyx-5 or pBS without insert) using the T3 polymerase. The transcription reaction was precipitated with EtOH and resuspended in nuclease-free water. The resulting RNA from each reaction was translated in the presence of ^{35}S-methionine. After the translation reaction was complete, the reaction mixture was split in half. One half of the material was subjected to immunoprecipitation using anti-zyxin antibody or the corresponding preimmune serum. Immunoprecipitation was performed according to previously described techniques (Beckerle et al., 1986) except that Protein A-agarose (Sigma Chemical Co., St. Louis, MO) was used to collect immune complexes. The translation reaction mix as well as the translation products that were immunoprecipitated were analyzed by SDS-PAGE followed by autoradiography.

SDS-PAGE and Western Immunoblot Analysis

SDS-PAGE was performed according to the method of Laemmli (1970) except with 0.13% bisacrylamide. 10% polyacrylamide gels were used except when we were trying to resolve low molecular weight proteins such as cCRP when we used 12.5% gels. Western immunoblot analysis was performed using a modification of the procedure of Towbin and colleagues (1979) that has been described previously (Beckerle, 1986). ^{125}I-Protein A followed by autoradiography was used as the antibody detection method.

Metal Analysis

Purified zyxin was resolved on an HPLC hydroxylapatite column (BioRad Corp.). The relative zyxin concentration in each fraction was determined by densitometric scanning of a Coomassie blue-stained gel. The amount of zinc present in each fraction was determined using an atomic absorption spectrophotometer (model 305A, Perkin-Elmer Corp., Norwalk, CT). Atomic absorption spectroscopy was also utilized to evaluate whether zyxin is liganded to any detectable copper, iron, or cadmium; this was accomplished by comparing the levels of these metals with the level of zinc present in a number of independently prepared zyxin isolates ($n = 5$ for iron, $n = 4$ for copper and $n = 2$ for cadmium). All zyxin samples analyzed contained significant quantities of zinc ($n = 7$). To quantify the amount of zinc bound to zyxin, parallel zyxin protein samples were subjected to atomic absorption spectroscopy and amino acid analysis. Amino acid analysis was performed on an analyzer (model 6300, Beckman Instruments, Carlsbad, CA) after the protein was subjected to hydrolysis in 5.7N HCl/0.1% phenol at 110°C for 24 h. We used the amino acid composition of zyxin derived from the cDNA sequence to determine the zyxin concentration in the sample. In each experiment that was performed in an effort to define the stoichiometry of zinc binding to zyxin ($n = 2$), both the metal analysis and the amino acid analysis were performed in triplicate.

The Blot Overlay Binding Assay

For identification of zyxin-binding proteins, a variation of the gel overlay method described by Otto (1983) was used. Purified zyxin, vinculin, or bovine serum albumin was iodinated using Iodogen (Pierce Chemical Co., Rockford, IL) as described previously (Crawford et al., 1992). The blot overlay assays were performed as previously described (Crawford et al., 1992). In addition, we performed the assay using 150 mM NaCl and obtained the same results as with our normal low ionic strength overlay buffer. For the dot blots, equivalent molar amounts of cytochrome *c*, histone H1, ferredoxin (Sigma Chemical Co., St. Louis, MO) and the purified 23-kD protein (cCRP) were applied to nitrocellulose using a hybridot manifold and vacuum.

For examination of zyxin's ability to interact with hCRP, X. Wang, N. Cooke, and S. Liebhaber (University of Pennsylvania, Philadelphia, PA) generously provided us with hCRP cDNA cloned into the expression vector pGEMEX-1 (Promega Corp., Madison, WI). Expression of the hCRP fusion protein was performed in the bacterial strain, JM109(DE3) (Yanisch-Perron et al., 1985; Studier et al., 1990).

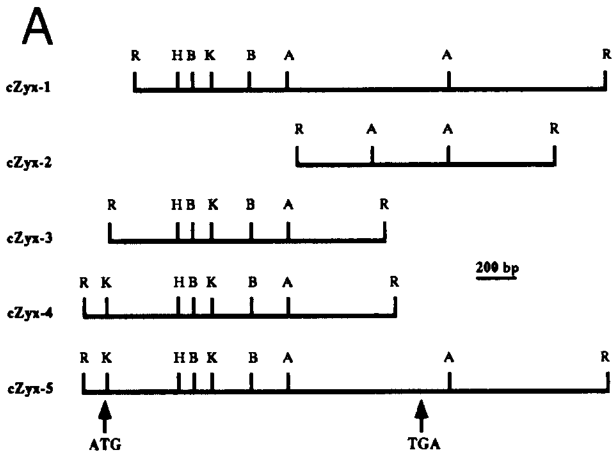
Antibody Preparation and Immunocytochemistry

The anti-zyxin antiserum, M2, which was used in expression screening has been described previously (Crawford and Beckerle, 1991). Antibodies directed against nonoverlapping protein sequences encoded by the zyxin cDNA clones were generated as follows. The cDNA inserts derived from pBScZyx-2 and the 5' EcoRI-BamHI fragment from pBScZyx-3 were cloned into the bacterial expression vector pWR590 (Guo et al., 1984). The β-galactosidase-zyxin fusion proteins were resolved by SDS-PAGE. The fusion proteins, isolated electrophoretically and immobilized on nitrocellulose, were used to immunize New Zealand white rabbits as previously described (Knudsen, 1985; Crawford and Beckerle, 1991). Antibodies against cCRP were prepared in rabbits (rabbits B31 and B32) as above. Western immunoblot characterization and indirect immunofluorescence using B31 is shown here, however both antibodies gave similar results. To probe further the specificity of the anti-cCRP antibodies, we examined the possibility that the antibodies would recognize other LIM domain sequences, specifically the Mec-3 and zyxin LIM domains expressed in bacteria. A plasmid expression clone encoding the LIM domains of Mec-3 was generously provided by Jeffrey Way, Rutgers University (New Brunswick, NJ). Anti-cCRP antibodies failed to recognize Mec-3 or zyxin LIM domains by Western immunoblot. Double label indirect immunofluorescence was performed on chicken embryo fibroblasts as described previously (Beckerle, 1984).

Results

Isolation of Zyxin cDNA Clones

The adhesion plaque protein, zyxin, has been purified from avian smooth muscle and many of its biochemical and bio-



B

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                                                                                                                     TCC       3
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                               M  A  S  P  G  T  P  G  T  R  M  T  T  T  V  S  I  N  I  S  T  P  S  F  Y  N  P  Q  K  K    30

TTTGCACCCGTGGTTGCCCCTAAACCCAAGGTGAATCCCTTCAAGACTGGGGGTACATCGGAGTCATCGCAGCCACAGCCTCCTGGAACTGGTGCCCAGCGTGCCCAGATAGGGAGAGTG   243
 F  A  P  V  V  A  P  K  P  K  V  N  P  F  K  T  G  G  T  S  E  S  S  Q  P  Q  P  P  G  T  G  A  Q  R  A  Q  I  G  R  V    70

GGAGAGATCCCCGTATCTGTGACAGCAGAAGAGCTGCCGCTGCCACCTCCTCCCCCACCTGGAGAGGAGCTAAGTTTCTCCTCAAACTGTGCTTTTCCTCCACCCCCACCACCCTTTGAA   363
 G  E  I  P  V  S  V  T  A  E  E  L  P  L  P  P  P  P  P  P  G  E  E  L  S  F  S  S  N  C  A  F  P  P  P  P  P  P  F  E   110

GAGCCTTTCCCACCAGCCCCAGATGAAGCTTTTCCTTCTCCTCCTCCACCTCCTCCACCAATGTTTGATGAAGGACCTGCCCTACAGATACCTCCAGGATCCACGGGTTCTGTGGAGAAA   483
 E  P  F  P  P  A  P  D  E  A  F  P  S  P  P  P  P  P  P  P  M  F  D  E  G  P  A  L  Q  I  P  P  G  S  T  G  S  V  E  K   150

CCGTTGGCCCCAAAAGCTCACGTGGAAATCTCATCTGCACCCAGAGATCCTACTCCTCCTTTTCCTTCCAAGTTCACTCCAAAGCCAAGTGGTACCTTATCTTCCAAGCCCCCTGGATTG   603
 P  L  A  P  K  A  H  V  E  I  S  S  A  P  R  D  P  T  P  P  F  P  S  K  F  T  P  K  P  S  G  T  L  S  S  K  P  P  G  L   190

GATTCAACTCCTGCCCCAGCTCCATGGGCAGCTCCACAGCAGCGCAAGGAGCCCCTAGCCTCAGTCCCTCCACCCCCCTCTCTCCCTTCTCAGCCTACTGCTAAATTCACACCACCCCCT   723
 D  S  T  P  A  P  A  P  W  A  A  P  Q  Q  R  K  E  P  L  A  S  V  P  P  P  P  S  L  P  S  Q  P  T  A  K  F  T  P  P  P   230

GTTGCCAGCTCTCCTGGATCCAAACCAGGTGCCACTGTTCCCATGGCTCCTTCAAACTCTACAAGATATCCTACATCCCTTCAGACTCAGTTCACTGCCCCTTCACCCTCCGGTCCCTTG   843
 V  A  S  S  P  G  S  K  P  G  A  T  V  P  M  A  P  S  N  S  T  R  Y  P  T  S  L  Q  T  Q  F  T  A  P  S  P  S  G  P  L   270

TCTCGACCTCAGCCTCCCAATTTCACCTATGCTCAGCAGTGGGAAAGACCTCAGGTGCAGGAGAAACCTGTTCCCACTGAAAAATCTGCTGCTGTAAAAGACATGCGTAGACCCACTGCA   963
 S  R  P  Q  P  P  N  F  T  Y  A  Q  Q  W  E  R  P  Q  V  Q  E  K  P  V  P  T  E  K  S  A  A  V  K  D  M  R  R  P  T  A   310

GATCCGCCTAAGGGAAACTCTCCTCTGACCATGAAGGAGGTAGAAGAGCTGGAGCTGTTGACCCAGAAACTAATGAAGGATATGGATCATCCACCTCCAGTAGAAGCTGCTACTTCTGAG  1083
 D  P  P  K  G  N  S  P  L  T  M  K  E  V  E  E  L  E  L  L  T  Q  K  L  M  K  D  M  D  H  P  P  P  V  E  A  A  T  S  E   350

CTCTGTGGCTTCTGTCGGAAGCCCCTGTCACGGACCCAGCCAGCTGTGAGAGCTCTGGACTGCCTTTTCCACGTGGAGTGCTTCACCTGCTTCAAGTGTGAGAAGCAGCTGCAGGGGCAG  1203
 L  C  G  F  C  R  K  P  L  S  R  T  Q  P  A  V  R  A  L  D  C  L  F  H  V  E  C  F  T  C  F  K  C  E  K  Q  L  Q  G  Q   390

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CGCTGCTCAGTATGTAGTGAACCTATCATGCCAGAGCCTGGGAAAGATGAGACAGTGCGTGTGGTGGCACTGGAGAAAAACTTCCACATGAAATGTTACAAGTGTGAGGACTGTGGGAGG  1563
 R  C  S  V  C  S  E  P  I  M  P  E  P  G  K  D  E  T  V  R  V  V  A  L  E  K  N  F  H  M  K  C  Y  K  C  E  D  C  G  R   510

CCCTTATCTATTGAGGCTGATGAAAATGGCTGCTTTCCACTGGATGGGCACGTACTATGTATGAAATGTCACACTGTTCGTGCTAAAACAGCGTGCTGAGGAGCTTGGAATGAGCATACA  1683
 P  L  S  I  E  A  D  E  N  G  C  F  P  L  D  G  H  V  L  C  M  K  C  H  T  V  R  A  K  T  A  C  *                         542

CTCAAGACCCCTGCACTCAGTACTCTTTCCTTCCCCACCATTGTCCTACCCCTCTGCCTGACAAGGCAGACTGCTACCAGTGAAGACTCTGCCAGTGCAGTTGGTTACCCAGATAGGATT  1803
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TTGAATAACTACGCTGTCCGACCACTGTTTGCATCCAATAAAATCGTGAGTTTTTTTCCATTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA                            2737
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Figure 1. Characterization of zyxin cDNA clones. (*A*) Partial restriction maps of the zyxin cDNA inserts. Clones λcZyx1-4 were identified by library screens. pBScZyx-5 represents a composite clone that encodes the complete zyxin protein. The positions of the translation start and stop codons are indicated by the arrows. Abbreviations for the restriction endonucleases are as follows: *R* = EcoRI; *H* = HindIII; *B* = BamHI; *K* = KpnI; *A* = AccI. (*B*) Nucleotide and corresponding amino acid sequence of zyxin cDNA. The amino acid and nucleotide

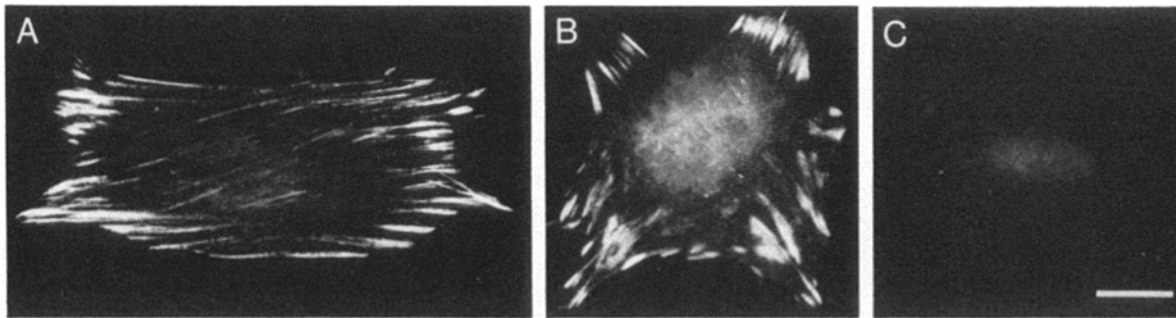


Figure 2. Anti-fusion protein antibodies stain adhesion plaques. Indirect immunofluorescence of chicken embryo fibroblasts stained with (*A*) anti-zyxin antibody; (*B*) anti-β-galactosidase/cZyx-2 fusion protein antibody (rabbit B28); or (*C*) pre-immune serum (rabbit B28). Bar = 20 μm.

physical properties have been characterized (Crawford and Beckerle, 1991). In an effort to learn more about the properties of zyxin and hopefully gain some insight into its function in cells, we set out to isolate and characterize zyxin cDNA clones. By screening of chicken embryo and chicken embryo fibroblast cDNA libraries, we identified four overlapping zyxin cDNA clones referred to as λcZyx 1-4 (see Materials and Methods for details). A composite clone (pBScZyx-5), which is full length with respect to coding capacity, was constructed by ligating pBScZyx-1 to pBScZyx-4 at the HindIII site. The relationships of the zyxin cDNA inserts to each other is shown in Fig. 1 *A*.

Features of the Zyxin cDNA Sequence

The 2737 nucleotide sequence corresponding to the composite zyxin cDNA (cZyx-5) is shown in Fig. 1 *B*. The ATG marked with the arrow is presumed to correspond to the initiation codon in zyxin mRNA because it is flanked by nucleotides that conform to the canonical eukaryotic translation initiation consensus sequence (Kozak, 1986) and the open reading frame is discontinued 15 nucleotides upstream of this position. The putative initiation codon is followed by a 1,623 base open reading frame that terminates with a TGA stop codon and an additional 3′ noncoding sequence of 1,075 nucleotides. A typical poly-adenylation signal (AATAAA) (Proudfoot and Brownlee, 1976) is found at nucleotides 2,680–2,685 preceding the poly-A tail by 21 nucleotides.

We confirmed that the sequenced cDNAs were bona fide zyxin cDNA clones by two independent strategies. First, antibodies were raised against bacterially produced β-galactosidase fusion proteins encoded by both the cZyx-2 and the 5′ EcoRI-BamHI fragment of cZyx-3 cDNAs cloned into the plasmid expression vector, pWR590 (Guo et al., 1984). The resulting antisera recognized zyxin by Western immunoblot analysis (data not shown) and stained adhesion plaques and the termini of stress fibers by indirect immunofluorescence mimicking the pattern obtained with anti-zyxin antibody. An example of the staining obtained with anti-β-gal/cZyx-2 antibody is shown in Fig. 2. In addition, we obtained the sequence of four peptides derived from purified smooth muscle zyxin by cleavage with Endo-Lys-C. The zyxin peptide sequences (1:FTPKPSGTL; 2:FTPPPV-ASSPG; 3:PGATV?M; and 4:ATGNSYHPQ?FT) matched exactly the amino acid sequence derived from the cDNA clones with the two undetermined residues corresponding to a proline or a cysteine in the deduced amino acid sequence. The underlined sequences in Fig. 1 *B* show the positions of the derived amino acid sequences that were verified by comparison to microsequenced zyxin peptides. By these immunological and biochemical approaches we have confirmed unequivocally that the cDNA sequence reported here encodes zyxin.

Predicted Amino Acid Sequence of Zyxin

The isolated zyxin cDNAs encode a 542 amino acid protein with an unmodified molecular weight of 58,537. Because the apparent molecular mass of zyxin based on its mobility on SDS-polyacrylamide gels is 82 kD (Beckerle, 1986), which is significantly higher than the molecular weight predicted from the analysis of zyxin cDNA, we performed in vitro transcription and translation experiments to examine the electrophoretic behavior of the protein encoded by the composite zyxin cDNA, cZyx-5. As can be seen in Fig. 3, the major protein produced by in vitro transcription and translation using the cDNA template, pBScZyx-5, migrates at an apparent molecular mass of 82 kD on SDS-polyacrylamide gels (lane *1*) and is specifically immunoprecipitated with anti-zyxin antibody (lane *2*). The unusually slow migration of zyxin on SDS-polyacrylamide gels appears to be due to the high proline content of the protein which will be discussed below.

Searches of both the GenEMBL and Swiss Protein data-

numbers are indicated in the right margin. Regions of the deduced amino acid sequence that correspond to the four microsequenced zyxin peptides are underlined in bold type. The proline residues are boxed; cysteine, histidine and aspartic acid residues that contribute to the LIM motif consensus are circled. The ATG start codon is marked with an arrow and the presumptive polyadenylation signal (AATAAA) is underlined in regular type. One sequence discrepancy was noted in the collected cDNA sequences. In cZyx-2, the nucleotide at position 1421 was a T instead of an A, creating a GTC codon; this alteration generated an AccI restriction site (*A*) and would result in the change of amino acid 463 from an Aspartic Acid (*D*) to a Valine (*V*). Three out of four cDNAs covering this region exhibited an A at position 1421, and therefore coded for an Aspartic Acid at residue 463. This variation in the cDNA sequences may be due to a reverse transcriptase error or it could represent a polymorphism.

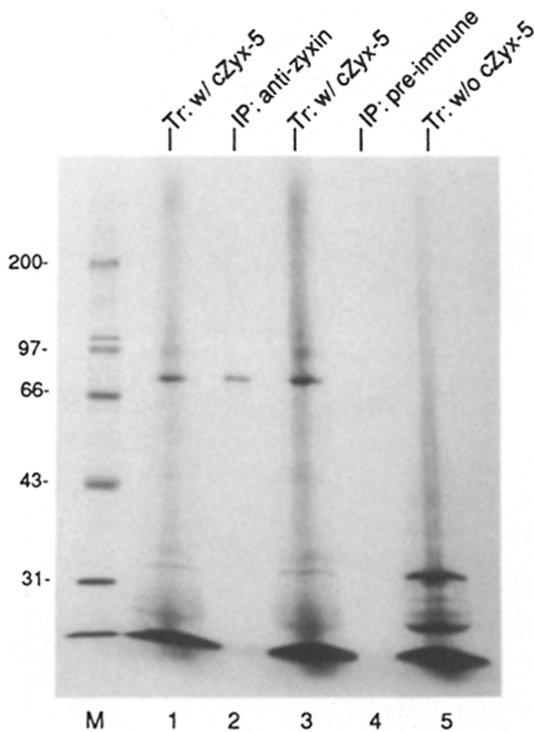


Figure 3. The composite zyxin cDNA, cZyx-5, encodes a protein that migrates with an apparent molecular mass of 82 kD on SDS-polyacrylamide gels. cZyx-5 DNA was transcribed and translated in vitro in the presence of ^{35}S-methionine and the products were analyzed by autoradiography. (Lane *1*) In vitro translation products of synthetic cZyx-5 RNA. (Lane *2*) Immunoprecipitation of the material shown in lane *1* with anti-zyxin antibody. A radio-labeled polypeptide that migrates at 82 kD is specifically immunoprecipitated. (Lane *3*) In vitro translation products of synthetic cZyx-5 RNA. (Lane *4*) Immunoprecipitation of the material shown in lane *3* with preimmune serum. (Lane *5*) In vitro translation products of synthetic plasmid-derived RNA.

bases with the zyxin protein sequence were performed and revealed that zyxin is not identical to any protein or derived amino acid sequence currently deposited in these databases. In addition, we specifically examined the possibility that zyxin might be related to cytoskeletal proteins such as ezrin (Gould et al., 1989; Turunen et al., 1989), radixin (Funayama et al., 1991), moesin (Lankes and Furthmayr, 1991), protein kinase C (Ono et al., 1986; Knopf et al., 1986), the calcium dependent protease (Aoki et al., 1986), plakoglobin (Franke et al., 1989), the MARCKS protein (Graff et al., 1989), and gelsolin (Kwiatkowski et al., 1986) that exhibit a similar molecular mass on SDS-polyacrylamide gels; we found no significant sequence relationship between zyxin and any of these proteins.

Based on analysis of the deduced amino acid sequence of zyxin, the predicted unmodified pI of the protein is 7.04, a value that is consistent with the observed pI of zyxin which ranges from 6.4–7.2 (Crawford and Beckerle, 1991). Interestingly, a plot of predicted pI as a function of amino acid sequence illustrates that zyxin exhibits substantial position-dependent fluctuations in pI; when viewed in 100 amino acid intervals, we observe alternating basic (aa 1–100, pI = 9.4), acidic (aa 101–200, pI = 4.4), and basic (aa 201–300, pI = 11.4) regions with the pI stabilizing around pI = 6.5 for the remainder of the protein. The areas exhibiting very divergent pI's may correspond to functional domains in the protein. Hydropathy analysis illustrates that the zyxin sequence is hydrophilic overall, with no stretches of hydrophobic amino acids that might represent a membrane spanning domain or a signal sequence. This analysis is consistent with the biochemical features of the protein which earlier prompted us to assert that zyxin is a cytoplasmic protein (Crawford and Beckerle, 1991).

Examination of the predicted amino acid sequence of zyxin (Fig. 1 *B*) reveals two striking features of the protein. First, zyxin is a proline-rich protein and, second, zyxin contains a COOH-terminal domain that is enriched in cysteine and histidine residues. With regard to proline content, zyxin exhibits 18% proline overall, a level which vastly exceeds the 4.5–5.5% proline typically found in proteins derived from eukaryotic cells (Doolittle, 1987). The prolines found in zyxin are not evenly distributed throughout the protein sequence, rather they are concentrated in the NH_2-terminal 2/3 of the protein. In this region, there is one 146 amino acid sequence (aa 85–230) in which the proline content is 35%. Numerous arrays of up to seven contiguous proline residues are apparent in this region of the protein. Regions of protein sequence exhibiting clusters of prolines would be predicted to form a type-II polyproline helix, an extended helical conformation (Sasisekharan, 1959), that would be expected to contribute to the observed asymmetric shape of zyxin (Crawford and Beckerle, 1991). The high proline content of zyxin could also be responsible for the aberrant mobility of the protein on SDS-polyacrylamide gels which has also been noted for other proline-rich proteins (see for example, Carroll and Scott, 1985; Ollo and Maniatis, 1987).

The cysteine and histidine residues that are concentrated in the COOH-terminal 1/3 of the zyxin sequence are organized into three copies of a sequence motif referred to as the LIM motif (Freyd et al., 1990). The acronym, LIM, is derived from the names of three homeodomain proteins, Lin-11, Isl-1, and Mec-3, that exhibit this sequence feature. The LIM motif has been identified in a number of proteins, many of which appear to have important developmental functions (Freyd et al., 1990; Cohen et al., 1992; Karlsson et al., 1990; Taira et al., 1992; Greenberg et al., 1990). The known LIM domain proteins are illustrated schematically in Fig. 4 *A* and the LIM sequences are compared with each other in Fig. 4 *B*. In addition to the absolutely conserved six cysteine and one histidine residues, there is always a cysteine (C), histidine (H) or aspartic acid residue (D) in the final position in the consensus motif. There are also two other highly conserved features of the LIM motif (*arrows*): one always finds an aromatic residue just before the conserved histidine in the LIM consensus and a leucine (L) is typically found in the fourth position following the central cluster of cysteine and histidine residues. While the remaining sequences in the LIM domains may be quite variable, some LIM domain proteins are similar to each other even in these more divergent regions.

Zyxin Is A Zinc-Binding Metalloprotein

Previous investigators have noted that the LIM motif is reminiscent of a metal liganding sequence (Freyd et al., 1990; Liebhaber et al., 1990; Karlsson et al., 1990). It has been proposed that each LIM domain might bind two zinc

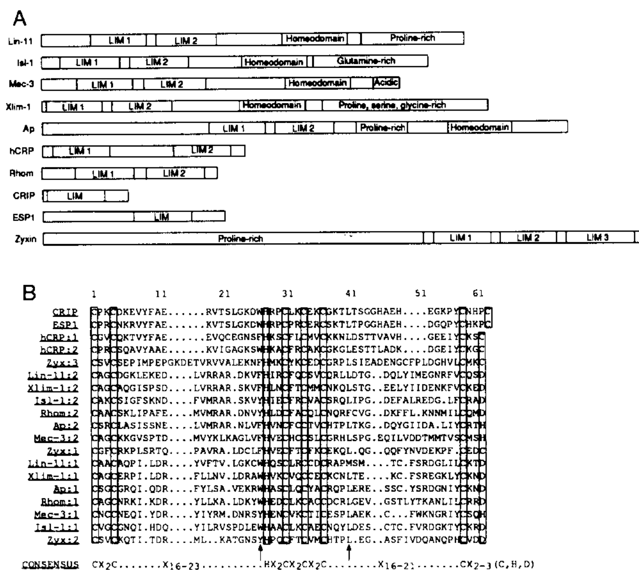
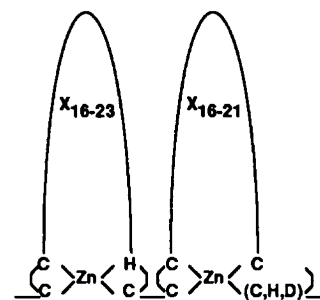
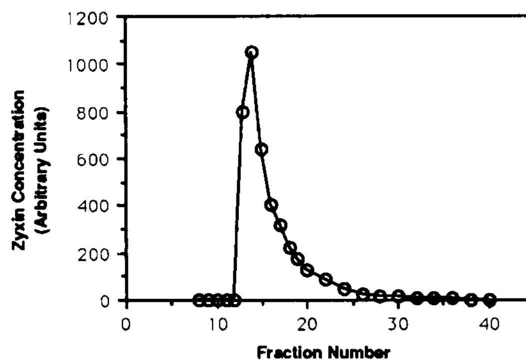


Figure 4. The LIM family of proteins. (*A*) Schematic representation of proteins exhibiting LIM domains. Five LIM-homeodomain proteins, Lin-11, Isl-1, Mec-3, Xlim-1, and apterous (*Ap*) have been described thus far. In addition, the non-homeodomain proteins, hCRP, Rhombotin, CRIP, ESP-1, and zyxin exhibit LIM domains. Proteins having from one to three LIM repeats have been identified. It should be noted that while the published ESP1 sequence reveals only a single LIM domain in that protein (Nalik et al., 1989), it has recently been suggested that ESP1 may, in fact, have two LIM domains (Wang et al., 1992.) Rhombotin (also referred to as Ttg-1) is used here to represent the rhombotin family which now also includes the related proteins Rhom-2 (or Ttg-2) and Rhom-3 (Royer-Pokora et al., 1991; Boehm et al., 1991*a*). (*B*) Comparison of the LIM sequences of the proteins listed above. Each LIM domain is designated 1, 2, or 3 based on proximity to the NH_2-terminus of the protein, with LIM 1 residing closest to the NH_2-terminus. Each LIM sequence analyzed begins with the first cysteine in the consensus and terminates with the final cysteine (*C*), histidine (*H*), or aspartic acid (*D*) in the consensus. The multiple sequence alignment program, PILEUP, from the UWGCG Software Package (Devereux et al., 1984) was used for the initial alignment of available LIM sequences and minor manual adjustment was performed in order to line up the residues that contribute to the LIM consensus sequence. In addition to the absolutely conserved cysteine, histidine and aspartic acid residues in the LIM consensus sequence (*boxed residues*), arrows mark the positions of a conserved aromatic residue (*W*, *F*, or *Y*) which is located adjacent to the first histidine in the LIM consensus and a leucine (*L*) which is found in 17 out of 19 LIM repeats.

atoms to generate two zinc fingers (Liebhaber et al., 1990). While it should be noted that it is not possible to predict the structure of metal liganding protein domains based on primary amino acid sequence alone (Vallee et al., 1991), one proposal for how a LIM domain could bind zinc is illustrated schematically in Fig. 5 *A*. As was suggested most recently by Wang and colleagues (Wang et al., 1992), the first zinc could be liganded by the first three cysteines and the first histidine highlighted in the consensus sequence to form a C_2HC finger; the second zinc could be liganded by the next three cysteines plus an aspartic acid, cysteine or histidine residue in the final position to yield a $C_3(C,H,D)$ finger. Aspartic acid, cysteine and histidine residues have all been shown to participate in metal coordination by zinc metalloproteins (Vallee and Auld, 1990*a*,*b*).





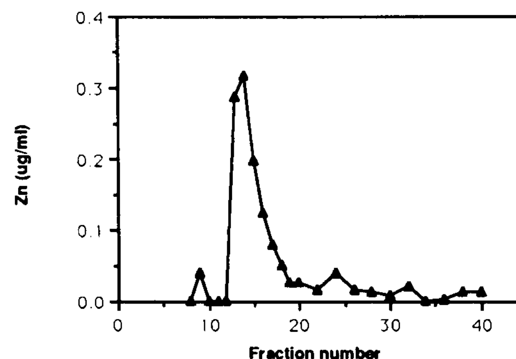


Figure 5. Zyxin is a zinc-binding metalloprotein. LIM domains have been proposed to bind zinc as illustrated schematically in *A*. To determine whether zyxin is a zinc-binding protein, zyxin purified from avian smooth muscle was fractionated on an HPLC hydroxylapatite column. Relative zyxin protein concentration determined by densitometry (*B*) and zinc concentration determined by atomic absorption spectroscopy (*C*) are plotted as a function of fraction number. The profile of zinc absorbance corresponds precisely to the zyxin elution profile.

To determine directly whether zyxin binds zinc, we used atomic absorption spectroscopy to measure the metal content of zyxin purified from avian smooth muscle (Fig. 5, *B* and *C*). By this approach we have demonstrated that zyxin does indeed bind zinc. Estimates of the stoichiometry of zinc binding reveal that purified zyxin displays between 2 and 6 zinc atoms per protein molecule. We observed no detectable iron, copper or cadmium associated with purified zyxin. On the basis of a number of independent experiments in which the metal content of zyxin was analyzed, we conclude that zyxin is a specific zinc-binding metalloprotein.

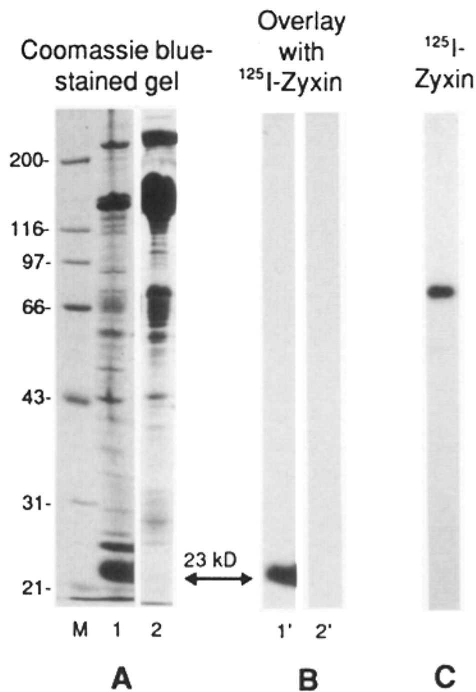


Figure 6. Identification of a 23-kD zyxin-binding protein by a blot overlay assay. (*A*) A Coomassie blue-stained gel showing molecular mass markers (lane *M*) and a collection of proteins extracted from avian smooth muscle and fractionated by anion exchange chromatography (lanes *1* and *2*). Proteins from a parallel gel were transferred to nitrocellulose and the nitrocellulose strip was probed with ^{125}I-zyxin. The resulting autoradiograph is shown in *B* and illustrates that radioiodinated zyxin interacts prominently with a polypeptide with an apparent molecular mass of 23-kD (*arrow*) found in lane *1'*. The purity of the radiolabeled zyxin is shown in *C*.

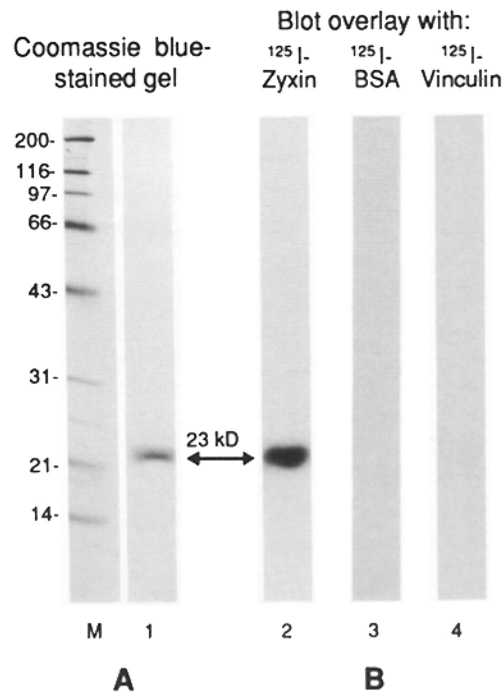


Figure 7. Specific interaction of the purified 23-kD protein with ^{125}I-zyxin. (*A*) Coomassie blue-stained gel showing the 23-kD protein purified from avian smooth muscle (lane *1*). (*B*) Using the blot overlay assay, we have examined the ability of a number of radioiodinated proteins to interact with the 23-kD protein. ^{125}I-zyxin interacts with the 23-kD protein, whereas neither ^{125}I-vinculin nor ^{125}I-BSA bind to the 23-kD protein. These results illustrate the specificity of the interaction between the 23-kD protein and ^{125}I-zyxin.

Identification Of P23, A Zyxin-Binding Protein That, Like Zyxin, Exhibits LIM Repeats

In spite of its presence in a group of biologically interesting proteins, the specific function of the LIM domain has not been defined. It has been postulated that LIM domains might interact with either nucleic acids or protein (see for example Freyd et al., 1990; Boehm et al., 1990*a*; Li et al., 1991). We suggest that LIM domains serve as protein-binding interfaces since, as discussed below, we have demonstrated a direct interaction between zyxin and a protein that is comprised primarily of LIM domains.

We set out to identify zyxin-binding proteins using a blot overlay assay which had been useful in demonstrating an interaction between zyxin and α-actinin (Crawford et al., 1992). In this approach, a complex mixture of proteins extracted from avian smooth muscle is resolved by SDS-PAGE and the proteins are electrophoretically transferred to nitrocellulose; the immobilized proteins are then screened for their ability to bind radioiodinated zyxin. Using this strategy, we identified a 23-kD protein that interacts with the radiolabeled zyxin (Fig. 6, lanes *1* and *1'*). A number of other proteins (e.g., Fig. 6, lanes *2* and *2'*) failed to interact with ^{125}I-zyxin in this assay. The purity of the ^{125}I-zyxin used in these experiments is shown in Fig. 6 *C*. The 23-kD protein was purified to homogeneity (Crawford, A. W., and M. C. Beckerle, manuscript in preparation) and we demonstrated that the purified 23-kD protein retained the ability to bind ^{125}I-zyxin (Fig. 7, lane *2*). Neither ^{125}I-BSA (Fig. 7, lane *3*) nor ^{125}I-vinculin (Fig. 7, lane *4*) bound to the purified 23-kD protein illustrating that the association was specific to zyxin and not simply a reflection of adventitious binding of radioiodinated proteins to the immobilized 23-kD protein.

We obtained the NH_2-terminal sequence and substantial internal sequence of the 23-kD protein by microsequencing the intact protein as well as five peptides obtained by cleavage of the purified protein with Endo-Lys-C. By a computer-assisted homology search using the UWGCG program, Tfasta (Devereux et al., 1984), we determined that the purified 23-kD protein is closely related to hCRP (Liebhaber et al., 1990), a 193-amino acid LIM domain protein illustrated in Fig. 4. hCRP displays two tandemly arrayed LIM domains separated by a short linker region that contains a potential nuclear targeting sequence (Liebhaber et al., 1990). The function of hCRP is not known, however, the human *crp* gene is an immediate-response gene whose serum-induced expression parallels that of the cellular proto-oncogene, *c-myc* (Wang et al., 1992). Comparison of the peptide sequences obtained from the purified 23-kD protein with the deduced amino acid sequence of hCRP reveals that of the 98 residues unequivocally identified in the 23-kD protein, 89 residues (or 91%) are identical to the hCRP sequence (Fig. 8). The striking sequence similarity between

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cCRP:     PNWGGGKKXGVXQKAVYFAEEVQCEGSSFHKSXFLXMVXKKNLDSTTVA
          |||||||| || || ||||||||||| ||||| || || ||||||||||
hCRP:  1 MPNWGGGKKCGVCQKTVYFAEEVQCEGNSFHKSCFLCMVCKKNLDSTTVA 50

         VHGDEIYXK          GYGYGMGAGTLSTDK
         ||| ||| |          ||||| |||||||||
      51 VHGEEIYCKSCYGKKYGPKGYGYGQGAGTLSTDKGESLGIKHEEAPGHRP 100

                     VGGSDGCPRCGQAVYAAEK
                      |||  |||| ||||||||
     101 TTNPNASKFAQKIGGSERCPRCSQAVYAAEKVIGAGKSWHKACFRCAKCG 150

                                     GFGFGQGAGALI
                                     |||||||||||
     151 KGLESTTLADKDGEIYCKGCYAKNFGPKGFGFGQGAGALVHSE        193
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Figure 8. **Identification of the 23-kD protein as the chicken homologue of hCRP. The NH_2-terminus of the purified 23-kD protein (cCRP) plus five endoproteolytic peptides generated by cleavage with Endo-Lys-C were microsequenced. The amino acid sequence derived from the purified 23-kD protein shows striking similarity to the deduced amino acid sequence of the human Cysteine-Rich Protein, hCRP, which exhibits two tandemly arrayed LIM repeats. We obtained sequence corresponding to amino acids 2–45, 16–24, 43–59, 70–84, 113–131, and 179–190 in the hCRP primary amino acid sequence. Residues in the cCRP sequence that could not be identified unequivocally appear as "X"s and always correspond to cysteine residues (*C*) in the hCRP sequence. By derivitization of the intact protein and peptide 113–131 with 4-vinyl pyridine, we were able to confirm that the amino acids corresponding to residues 26, 119, and 122 in the hCRP sequence are indeed cysteines. Vertical lines indicate identical amino acids. The initiator methionine is apparently removed post-translationally in the chicken protein. Because of the close relationship between the sequences of hCRP and the 23-kD protein (91% identical over the sequenced regions), we now refer to the 23-kD protein as chicken CRP (cCRP).**

the 23-kD protein and hCRP leads us to conclude that the 23-kD protein is the chicken smooth muscle homologue of the human Cysteine-Rich Protein, and consequently we refer to it as cCRP (chicken Cysteine-Rich Protein).

Because it remained a formal possibility that ^{125}I-zyxin was interacting with a protein that copurifies with cCRP but is not itself a homologue of hCRP, we examined the ability of ^{125}I-zyxin to bind to a T7 gene 10/hCRP fusion protein expressed in bacteria. As can be seen in Fig. 9, ^{125}I-zyxin fails to bind to gene 10 sequences (lanes *2* and *2'*), but specifically recognizes the gene 10/hCRP fusion protein (lanes *4* and *4'*) even in the context of a complex bacterial extract. This result demonstrates clearly that zyxin can interact with the LIM domain protein, hCRP, and strongly supports the conclusion that it is the cCRP sequences in our preparation of 23-kD protein that bind zyxin in the blot overlay assay.

Examination of the amino acid sequence of hCRP reveals that in addition to the relatively large number of cysteine and histidine residues that contribute to the LIM repeats in hCRP, the protein is also unusually basic with a predicted pI of 10.4 (Liebhaber et al., 1990). Because of these features, we explored further the specificity of zyxin's association with cCRP in the blot overlay assay by comparing the binding of ^{125}I-zyxin to purified cCRP, two very basic proteins, histone H1 (DeNooij and Westenbrink, 1962) and cytochrome *c* (Margoliash et al., 1961), and another cysteine-containing metalloprotein, ferredoxin (Matsubara et al., 1967). Equivalent molar amounts of these proteins were adsorbed onto nitrocellulose; staining of a parallel nitrocellulose strip confirmed that all four proteins were immobilized on the

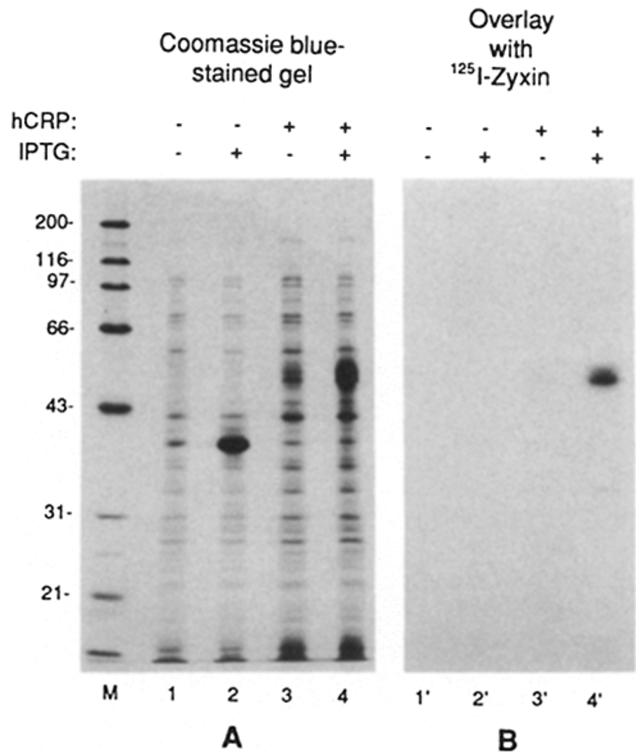


Figure 9. ^{125}I-zyxin interacts with bacterially-expressed hCRP sequences. (*A*) A Coomassie blue-stained gel showing proteins produced in E. coli strain, JM109(DE3), that carries either the plasmid pGEMEX-1 which encodes a T7 gene 10 leader peptide (lanes *1* and *2*) or pGEMEX-1/hCRP which encodes a gene 10/hCRP fusion protein (lanes *3* and *4*). Expression of plasmid-encoded sequences was induced with IPTG (lanes *2* and *4*). As expected, the gene 10/hCRP fusion protein (lane *4*) migrates with an apparent molecular mass ~20 kD greater than the gene 10 leader peptide (lane *2*). (*B*) The corresponding blot probed with ^{125}I-zyxin reveals that the radioiodinated zyxin interacts specifically with the gene 10/hCRP fusion protein (lane *4'*, and faintly in the uninduced cells, lane *3'*) but fails to recognize gene 10 sequences alone (lane *2'*) or a host of other bacterial proteins.

membrane. As can be seen in Fig. 10, radioiodinated zyxin fails to bind to cytochrome *c*, histone H1 or ferredoxin, but does bind in a concentration dependent manner to purified cCRP. Consequently, we conclude that the association we observe between zyxin and cCRP in the blot overlay assay is specific.

Colocalization of Zyxin and cCRP in Fibroblasts

If zyxin and cCRP interact with each other in living cells, we predicted that we should observe some colocalization of the two proteins in vivo. To examine this possibility, we prepared polyclonal antibodies against purified cCRP. By Western immunoblot analysis of total chicken embryo fibroblast protein, anti-cCRP antibody specifically recognizes a polypeptide that migrates at an apparent molecular mass of 23 kD by SDS-PAGE (Fig. 11). Anti-cCRP antibody also recognizes purified cCRP and gene 10/hCRP fusion protein expressed in bacteria (data not shown). Importantly, anti-cCRP antibody fails to recognize zyxin present in the chicken embryo fibroblast sample even though, as we have

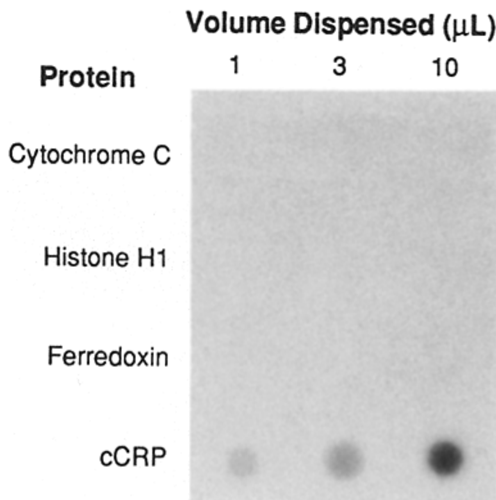
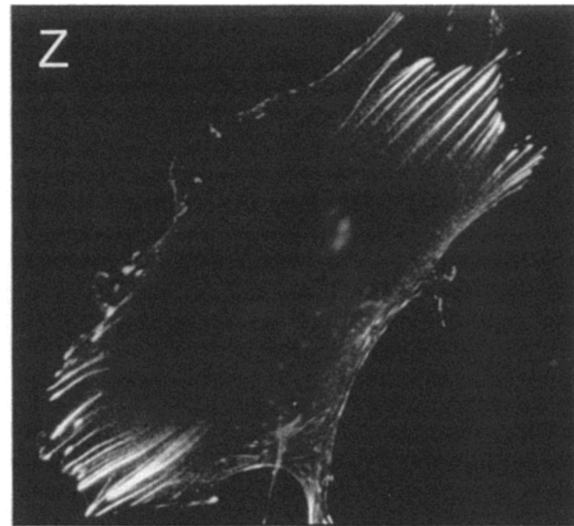


Figure 10. Radioiodinated zyxin fails to interact generally with basic or cysteine-rich proteins. Equivalent molar amounts of the basic proteins, cytochrome *c* and histone H1, or the cysteine-rich protein, ferredoxin, were adsorbed to nitrocellulose and compared with cCRP for their ability to bind to ^{125}I-zyxin. The resulting autoradiograph shows that zyxin interacts specifically with cCRP which illustrates that zyxin's association with cCRP cannot be explained on the basis of a nonspecific affinity for basic or cysteine-rich proteins.

now demonstrated, both proteins have LIM domains. The inability of anti-cCRP antibodies to recognize LIM domains of either zyxin or Mec-3 expressed in bacteria (data not shown) further supports the conclusion that the antibodies are specific for cCRP.

To compare the subcellular distributions of zyxin and cCRP, we performed double label indirect immunofluorescence (Fig. 12). As described previously (Beckerle, 1986), zyxin is found at adhesion plaques as well as along actin filament bundles, particularly near where they terminate at adhesion plaques (Fig. 12 *A*). Antibodies directed against cCRP also stain the stress fibers as they approach the adhesion plaques (Fig. 12 *B*). Though not as prominently labeled as with anti-zyxin antibodies, the adhesion plaques are occasionally recognized by anti-cCRP antibodies. In contrast to the rather discrete subcellular distribution of zyxin, we typically observe significant cytoplasmic staining with antibodies directed against cCRP, which may reflect the presence of a cytosolic pool of cCRP in addition to the cytoskeleton-associated protein. In addition, we occasionally detect faint nuclear staining with anti-cCRP antibodies. From this immunocytochemical analysis, it is clear that although cCRP has a broader subcellular distribution than zyxin, the two proteins are colocalized to some extent, a finding that is consistent with the idea that zyxin and cCRP interact in vivo.



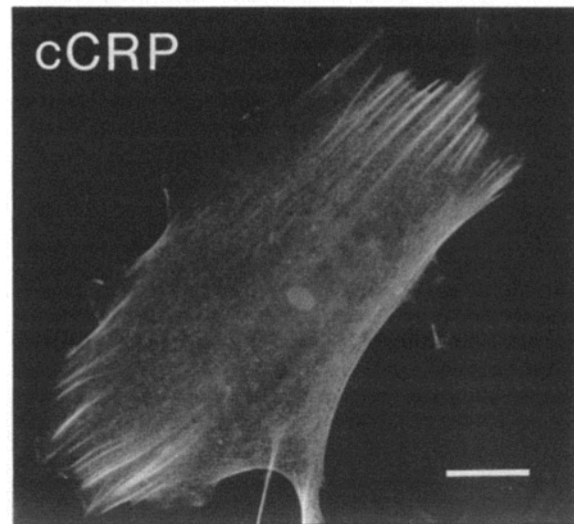


Figure 12. Zyxin and cCRP exhibit overlapping subcellular distributions in chicken embryo fibroblasts. By double label indirect immunofluorescence, zyxin and cCRP are both found along the stress fibers, most prominently at the ends of the stress fibers near where they terminate at the adhesion plaque. Antibodies directed against cCRP also stain the cytoplasm and the nucleus. Bar = 25 μm.

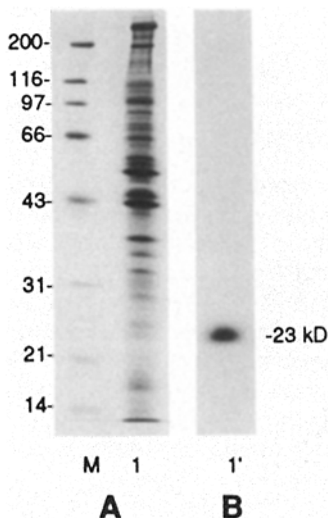


Figure 11. Specificity of anti-cCRP antibodies. (*A*) A Coomassie blue-stained gel showing molecular mass markers (*M*) and total chicken embryo fibroblast proteins (lane *1*). (*B*) Corresponding Western immunoblot probed with anti-cCRP antibody reveals that the antibody specifically interacts with a polypeptide of 23 kD that comigrates with purified cCRP. (Note: the material loaded in lane *1'* was twice what was loaded in lane *1*.)

Discussion

Zyxin is a low abundance α-actinin-binding protein that is localized at adhesion plaques and on the membrane-asso-

ciated termini of actin filament bundles in fibroblasts (Beckerle, 1986). In this report, we have demonstrated that zyxin is a novel, zinc-binding protein that exhibits three tandemly-arrayed LIM repeats as well as a distinctive proline-rich domain. Using an in vitro binding assay, we have identified a 23-kD zyxin-binding protein (cCRP) that, like zyxin, has LIM repeats, a result that raises the possibility that LIM domains function in protein–protein interaction. Because all LIM proteins for which a function has been defined appear to be involved in some aspect of regulation of gene expression or differentiation, it is possible that the cytoskeletal proteins, zyxin and cCRP, may have a related function, perhaps participating in some aspect of signal transduction that leads to altered gene expression in response to cell adhesion. Alternatively, our results are also consistent with the idea that the LIM domain is a protein-binding module found in functionally diverse proteins.

Zyxin Protein Sequence

The Proline-rich NH_2-Terminus. One of the striking features of the zyxin protein sequence is the presence of a relatively long proline-rich region beginning near the NH_2-terminus of the protein. Several other cytoskeletal proteins including vinculin (Coutu and Craig, 1988; Price et al., 1989), ezrin (Gould et al., 1989; Turunen et al., 1989), and radixin (Funayama et al., 1991), exhibit small clusters of contiguous proline residues, but the overall proline content of zyxin is much higher than in these proteins. Homology searches with the zyxin protein sequence have revealed that a number of DNA tumor virus-encoded proteins (for example, Cheung, 1989; Baer et al., 1984) and a presumed transcription factor, the hox 2.6 protein (Graham et al., 1988), display regions of proline-rich sequence reminiscent of the proline arrays found in zyxin; however, no functional relationship between zyxin and these proteins has yet been defined.

An abundance of prolines in a protein sequence would be expected to affect significantly the overall structure of the protein by disrupting α-helices and contributing to an extended or highly kinked protein conformation. Moreover, proline-rich sequences may be important for a variety of distinct functions: (*a*) For example, the introduction of a run of prolines into a protein sequence could serve to separate physically two functionally distinct domains of a protein (Guerini and Klee, 1989). One intriguing proposal is that polyproline stretches act as molecular verniers that could facilitate, because of their rigid and extended helical conformation, the displacement of two functional domains by a relatively fixed distance (Stryer and Haugland, 1967; Stryer, 1978). (*b*) In contrast with the idea that polyproline stretches serve more or less as biochemically inert spacers, it is also possible that the folding of proline-rich sequences provides a structural framework to generate a binding surface that mediates specific interactions with potential ligands. In this regard, it is interesting that a variety of transcription factors including CTF/NF1 (Mermod et al., 1989), AP-2 (Williams et al., 1988), Jun (Struhl, 1987), and Drosophila Krüppel (Rosenberg et al., 1986) exhibit regions of high proline content. In the case of CTF/NF1, a 100-amino acid domain that exhibits nearly 25% proline is required for transcriptional activation and it is believed that this domain mediates an essential interaction between CTF and a second key protein in the transcriptional apparatus (Mermod et al., 1989). (*c*) Alternatively, polyproline and proline-rich sequences could also represent binding sites for proteins that have a specific affinity for proline residues. Interestingly, profilin and profilin-actin complexes can be purified on the basis of their affinity for poly-L-proline (Tanaka and Shibata, 1985). It is intriguing to consider the possibility that profilin might interact with zyxin, an association that could serve to localize profilin near sites of actin-membrane interaction where the plus (+) or fast-growing ends of actin filaments are concentrated (Wang, 1985; Tilney et al., 1981). Further experiments are required to define the functional significance of zyxin's proline-rich sequence.

The COOH-Terminal LIM Domains. Perhaps the most interesting feature of zyxin's primary amino acid sequence is the presence of three tandemly arrayed LIM motifs. Zyxin shares the consensus amino acid sequence for LIM repeats, however it is unusual among LIM-motif proteins in a number of respects. First, zyxin is associated with adhesion plaques and the cytoskeleton whereas many other LIM domain proteins are nuclear or presumed to be nuclear (McGuire et al., 1991; Liebhaber et al., 1990; Freyd et al., 1990; Karlsson et al., 1990). Second, zyxin exhibits the largest molar ratio of LIM domains with a total of three repeats per zyxin monomer. And, finally, in contrast to the other LIM-proteins that are illustrated in Fig. 4 *A*, zyxin's LIM repeats are clustered at the COOH-terminus of the protein, a feature which carries unknown functional significance.

LIM Domains As Zinc-Binding Sequences

As discussed above, LIM domains have previously been postulated to bind metal. Metal coordination can serve to stabilize protein structures required for DNA, RNA or protein interactions (Berg, 1990). Interestingly, the single LIM domain protein, CRIP, has recently been shown to bind exogenous, radiolabeled zinc (Hempe and Cousins, 1991). We have shown here that zyxin isolated from avian smooth muscle binds zinc, but exhibits no detectable iron, cadmium or copper. In contrast with the metal profile of zyxin, bacterially expressed LIM sequences derived from Lin-11 have been reported to bind zinc as well as iron in a redox sensitive iron-sulfur cluster (Li et al., 1991). The reason for the apparent difference in metal liganding by zyxin and Lin-11 has not been resolved. It may be that the sequence differences between zyxin's and Lin-11's LIM domains are significant with respect to metal coordination and that LIM domains, though sharing a subset of highly conserved amino acids, may exhibit both quantitative and qualitative differences in metal binding. Alternatively, it is possible that bacterially expressed LIM domains may not faithfully reflect the metal liganding capacity and specificity of the protein as it exists in its normal eukaryotic context. We are concerned that this may indeed be the case because cCRP, another LIM domain protein that we have purified from its eukaryotic source, has a metal profile similar to zyxin, exhibiting zinc but no iron bound (our unpublished observations).

Demonstration of an Interaction Between Two LIM Domain Proteins

Using a blot overlay assay to search for zyxin binding proteins we have identified two proteins that interact promi-

nently with radioiodinated zyxin, a 100-kD protein which we subsequently identified as α-actinin (Crawford et al., 1992) and a 23-kD protein (cCRP) that is the chicken homologue of the human Cysteine-rich protein. As shown here, CRPs are highly conserved proteins. This sequence conservation suggests that the specific primary amino acid sequence of CRP is required for function, as opposed to more general specification of secondary structure. In addition, the identification of sequences related to the *crp* gene in a broad range of evolutionarily divergent groups including birds, mammals, invertebrates, fungi, and plants (Liebhaber et al., 1990; Wang et al., 1992) suggests that the *crp* gene is an ancient sequence that encodes a protein of fundamental importance.

The association between zyxin and cCRP that we detect in vitro using the blot overlay assay is direct and specific as illustrated by the fact that other radioiodinated proteins fail to bind to cCRP in this assay and zyxin fails to bind to proteins such as histone H1, cytochrome *c* and ferredoxin which share certain biochemical properties with cCRP. The observation that the two proteins exhibit overlapping subcellular distributions is consistent with the proposal that zyxin and cCRP do interact with each other in vivo. Experiments are in progress to develop solution binding assays with which to study further the interaction between zyxin and cCRP.

The subcellular location of cCRP, while overlapping with the distribution of zyxin, does not precisely mirror the pattern of zyxin in cells. Whereas anti-cCRP antibodies, like anti-zyxin antibodies, label sections of actin filaments proximal to the plasma membrane, anti-cCRP antibodies also label the cytoplasm and nuclei of chicken embryo fibroblasts. The nuclear staining is difficult to interpret at present because it is somewhat variable from cell to cell. However, the complete absence of nuclear staining with the preimmune serum suggests that the nuclear staining we observe is dependent on the presence of anti-cCRP antibodies. Interestingly, hCRP does exhibit a potential nuclear targeting sequence in the region between the first and second LIM domains (Liebhaber et al., 1990) and, by analysis of cCRP cDNAs, we have found that a closely related sequence is present in cCRP as well (Pino and Beckerle, unpublished observations).

The demonstration of an association between zyxin and cCRP represents the first report of a direct interaction between two LIM domain proteins. While our results do not explicitly exclude the possibility that LIM domains participate in protein-nucleic acid associations, as has been suggested previously (see for example Li et al., 1992), they cause us to look more closely at the idea that LIM domains are important for protein–protein interactions. Although domain mapping studies will be required to demonstrate unequivocally the involvement of LIM sequences in the cCRP-zyxin interaction, we suspect that the LIM domains displayed by cCRP are directly involved in its ability to interact with zyxin because the LIM motifs account for the major sequence content of cCRP.

Functional Implications

Our finding that two interactive cytoskeletal proteins exhibit LIM domains may have implications for the biological function of LIM domains present in other proteins. Genetic and molecular genetic studies have provided evidence that many of the LIM-homeodomain proteins play significant roles in development: Lin-11 in vulval cell lineage determination (Ferguson et al., 1987), Mec-3 in *C. elegans* touch receptor neuron differentiation (Way and Chalfie, 1988), Xlim-1 in *Xenopus* embryonic pattern formation (Taira et al., 1992) and apterous in wing and haltere imaginal disc development in *Drosophila* (Cohen et al., 1992). Our results raise the intriguing possibility that these LIM-homeodomain proteins share with zyxin and cCRP the capacity to associate with other LIM-domain proteins. Such interactions could serve to regulate the activity of a LIM-homeodomain transcription factor either by localizing it to a particular subcellular compartment where relevant incoming signals may be transmitted or by directly enhancing or depressing its transcriptional activity.

With regard to the possibility that LIM domains might participate in localizing a protein to a particular subcellular compartment, the fact that two LIM domain proteins, zyxin and cCRP, are associated with the actin cytoskeleton suggests a mechanism by which certain developmentally important asymmetries that require LIM domain proteins might be established. It has been suggested that differential cytoplasmic segregation of the LIM-homeodomain protein, Lin-11, could be important for controlling the developmental fates of vulval precursor cells (Horvitz and Herskowitz, 1992). A LIM-mediated cytoskeletal association of Lin-11 could contribute to an asymmetric partitioning of the Lin-11 protein at the time of the determinative division.

Protein–protein interactions have been shown to play a pivotal role in the regulation of a variety of transcription factors and this may also be true for the LIM-transcription factors such as Isl-1. The protein–protein interactions that contribute to control of transcription factor localization and function are mediated by a variety of different structural motifs including the leucine zipper as in the case of cFos and cJun (Landschulz et al., 1988; Turner and Tjian, 1989; Gentz et al., 1989), the helix-loop-helix motif in the case of MyoD and Id (Murre et al., 1989*a*,*b*; Benezra et al., 1990), and ankyrin repeats in the case of GABP α and β (Thompson et al., 1991; LaMarco et al., 1991) and NF-κB and IκB (Baeuerle and Baltimore, 1988; Hatada et al., 1992). The LIM motif may emerge as yet another protein-interactive domain that is utilized in transcriptional regulation.

Conclusions/Perspectives

We have demonstrated that the cytoskeletal protein, zyxin, exhibits an unusual proline-rich domain as well as three tandemly arrayed LIM domains. In addition we found that zyxin has the ability to interact with a highly conserved LIM domain protein called cCRP. LIM domains have previously been identified in a variety of proteins, many of which are known to be involved in the control of gene expression and differentiation. This report describes the first case of a LIM domain protein associated with the cytoskeleton and also provides the first evidence that two LIM domain proteins are capable of interacting with each other.

In conclusion, we consider the impact of these observations on our understanding of the functions of zyxin, cCRP and LIM domains. Often, as in the case of non-receptor tyrosine kinase domains which are present in a number of proteins that have been implicated in oncogenic transformation, one observes a rather strict conservation of both domain sequence and function. Such a convergence of functional

significance with sequence homology naturally leads to the conviction that other proteins exhibiting homologous sequences will likely share the more global functional similarities as well. Applying this logic to LIM domain proteins, it is both plausible and intriguing to postulate that zyxin and cCRP play a role in adhesion-stimulated changes in gene expression or differentiation, perhaps by interacting with and thus regulating LIM-transcription factors. However, unlike in the case of the tyrosine kinase domain, a specific, generalized function for the LIM domain has not yet been defined. Thus far, the presence of a LIM domain is correlated with, but not causally related to certain examples of regulated gene expression. Indeed, the LIM domain may represent a structural motif involved in protein-protein interaction that is present in functionally diverse proteins. In this view, the LIM domain may be more akin to ankyrin repeats that are found in a wide variety of proteins including a cytoskeletal component, transmembrane proteins, transcription factors, and proteins involved in cell cycle control, where they are thought to serve as versatile dimerization interfaces (Lux et al., 1990; Thompson et al., 1991; Haskill et al., 1991). Future work will be directed toward defining the structure and function of the LIM domain and the physiological roles of the cytoskeletal members of the LIM protein family.

The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X69190.

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