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Molecular Dissection of a LIM Domain

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LIM domains are novel sequence elements that are found in more than 60 gene products, many of which function as key regulators of developmental pathways. The LIM domain, characterized by the cysteine-rich consensus $CX_2CX_{16-23}HX_2CX_2CX_2CX_{16-21}CX_{2-3}$(C/H/D), is a specific metal-binding structure that consists of two distinct zinc-binding subdomains. We and others have recently demonstrated that the LIM domain mediates protein–protein interactions. However, the sequences that define the protein-binding specificity of the LIM domain had not yet been identified. Because structural studies have revealed that the C-terminal zinc-binding module of a LIM domain displays a tertiary fold compatible with nucleic acid binding, it was of interest to determine whether the specific protein-binding activity of a LIM domain could be ascribed to one of its two zinc-binding subdomains. To address this question, we have analyzed the protein-binding capacity of a model LIM peptide, called zLIM1, that is derived from the cytoskeletal protein zyxin. These studies demonstrate that the protein-binding function of zLIM1 can be mapped to sequences contained within its N-terminal zinc-binding module. The C-terminal zinc-binding module of zLIM1 may thus remain accessible to additional interactive partners. Our results raise the possibility that the two structural subdomains of a LIM domain are capable of performing distinct biochemical functions.

INTRODUCTION

The LIM motif is a modular sequence element that typically conforms to the consensus $CX_2CX_{16-23}HX_2CX_2CX_2CX_{16-21}CX_{2-3}$(C/H/D) (Sadler *et al.*, 1992; Sanchez-Garcia and Rabbitts, 1994; Dawid *et al.*, 1995; Gill, 1995). More than 60 gene products have been identified that display from one to five copies of the LIM domain (reviewed in Sanchez-Garcia and Rabbitts, 1994; Dawid *et al.*, 1995; Gill, 1995). Within these gene products, LIM domains may be coupled to specific functional domains, such as DNA-binding homeodomains, or alternatively they may represent the primary sequence information in a protein; thus, the LIM domain is capable of functioning in a variety of molecular contexts.

LIM domain-containing proteins are key players in a number of fundamental pathways controlling development. For example, the Lhx1 gene product (formerly referred to as Lim1), is a LIM homeodomain protein that is prominently expressed in the organizer region in mouse embryos in a subset of cells that is required for the proper induction of adult head and forebrain structures (Barnes *et al.*, 1994; Shawlot and Behringer, 1995). Transgenic mice that lack the *lhx1* gene fail to form anterior head structures, suggesting that *lhx1* is an essential regulator of vertebrate organizer function (Shawlot and Behringer, 1995). LIM domain proteins that lack DNA-binding homeodomains also contribute to developmental pathways. LMO2 (previously called Rhombotin2, or RBTN2), which is comprised primarily of two LIM domains, is one of several master regulators of erythroid precursor cell differentiation (Warren *et al.*, 1994). The fact that LIM domain proteins apparently participate in many developmental programs has inspired investigations of the structure and function of the LIM domain.

Biochemical and biophysical data have clearly demonstrated that LIM domains are specific zinc-binding structures. Each LIM domain binds two atoms of zinc via two tetrahedral metal-coordinating centers established by the conserved cysteine, histidine, and aspartic acid residues of the consensus (Michelsen *et al.*, 1992, 1994; Khoo and Cousins, 1993; Archer *et al.*, 1994; Kosa *et al.*, 1994; Pérez-Alvarado *et al.*, 1994, 1996). The

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first four conserved metal-liganding residues of the motif cooperate to form the N-terminal zinc-binding module of the domain, whereas the last four conserved residues comprise the second, C-terminal zinc-binding module. Zinc coordination is required to stabilize the secondary and tertiary folds of the LIM domain (Michelsen *et al.*, 1994).

The three-dimensional structure of the LIM domain has been defined by two-dimensional nuclear magnetic resonance (NMR) spectroscopy (Pérez-Alvarado *et al.*, 1994, 1996; Hammarström *et al.*, 1996; Summers *et al.*, unpublished results). The three intact LIM domains that have been analyzed to date display very similar tertiary folds, indicating that the structure of the LIM domain may be generally conserved. Each LIM domain displays two spatially discrete subdomains that are centered around the metal coordination sites. Although the overall structure of the LIM domain is unique, the tertiary fold of its C-terminal zinc-binding module bears a striking similarity to that of the DNA-binding zinc fingers of the glucocorticoid receptor (GR) and GATA-1 transcription factors (Pérez-Alvarado *et al.*, 1994, 1996; Sanchez-Garcia and Rabbitts, 1994). Based on this structural concordance, it appears that the C-terminal zinc-binding module of the LIM domain could be compatible with a nucleic acid-binding function. However, as yet there is no published experimental evidence demonstrating a direct interaction between a LIM domain and either DNA or RNA.

Interestingly in a number of recent reports, the LIM domain has been described as a specific protein-binding interface (Feuerstein *et al.*, 1994; Schmeichel and Beckerle, 1994; Valge-Archer *et al.*, 1994; Wadman *et al.*, 1994; Wu and Gill, 1994; Bach *et al.*, 1995; Osada *et al.*, 1995; Jurata *et al.*, 1996). We previously demonstrated the protein-binding function of a LIM domain while exploring the molecular basis of the interaction between the LIM protein, zyxin, and its binding partner, the cysteine-rich protein (CRP1) (Sadler *et al.*, 1992; Crawford *et al.*, 1994; Schmeichel and Beckerle, 1994). A specific association between these two proteins has been detected in vitro by both solid-phase and solution-binding assays. In addition, by indirect immunofluorescence analyses, zyxin and CRP1 were found to colocalize along filamentous actin structures and at sites of cell adhesion in adherent fibroblasts. By performing a deletion mapping analysis of zyxin, which contains three tandemly arrayed LIM domains, we demonstrated that a single LIM domain, called zLIM1, is necessary and sufficient to mediate zyxin's specific association with CRP1 (Schmeichel and Beckerle, 1994). These findings, along with those of others (Feuerstein *et al.*, 1994; Valge-Archer *et al.*, 1994; Wadman *et al.*, 1994; Wu and Gill, 1994; Bach *et al.*, 1995; Osada *et al.*, 1995; Jurata *et al.*, 1996), demonstrate that the LIM domain functions as a protein-binding interface.

As described above, each LIM domain is comprised of two zinc-binding modules. However, the sequences that define the protein-binding specificity of the LIM domain had not yet been identified. Because structural analyses have indicated that one zinc-binding module of the LIM domain exhibits a conformation that is reminiscent of known DNA-binding zinc fingers, it was of interest to determine whether the protein-binding capacity of a LIM domain mapped to a single zinc-binding subdomain and, if so, to which one. We have performed a molecular dissection of zyxin's LIM domain, zLIM1, to define the regions that are required for its protein-binding function. Here, we report that the sequences necessary to specify the interaction between zyxin and its binding partner, CRP1, are contained within the N-terminal zinc-binding module of zLIM1. Thus, the C-terminal module of zLIM1 which appears from structural studies to be compatible with nucleic acid binding may remain accessible to additional interactive partners.

MATERIALS AND METHODS

zLIM1 Truncation Constructs

Truncations of zyxin's N-terminal-most LIM domain, zLIM1 (avian zyxin AA349-406), were generated via a polymerase chain reaction-based approach. zLIM1 is comprised of two tetrahedral metal-coordinating centers, an N-terminal site that consists of three cysteines and a histidine (referred to as C_3H) and a C-terminal site that is comprised of four cysteine residues (referred to as C_4). Oligonucleotide primers were generated to amplify zLIM1 sequences corresponding to either the C_3H or the C_4 zinc-binding module (zyxin AA349-379 or AA378-406, respectively). The primers were designed such that each amplified fragment was supplemented with an in-frame stop codon as well as flanking *Eco*RI restriction sites to facilitate subcloning. The resulting DNA fragments were inserted into the pGEX-2T$^{128}/_{129}$ vector and were expressed as glutathione *S*-transferase (GST)-zyxin fusion proteins (Blanar and Rutter, 1992; Schmeichel and Beckerle, 1994). These fusion protein probes are referred to in the text as GST-zLIM1A and GST-zLIM1B. In this nomenclature, the letter "A" corresponds to the sequences of the N-terminal C_3H zinc-binding module of zLIM1 and "B" corresponds to the sequences of the C-terminal C_4 zinc-binding module of zLIM1. The fidelity of all expression constructs was verified by sequence analysis (dsDNA Cycle Sequencing System, Life Technologies, Inc., New York).

Chimeric LIM Domain Constructs

The polymerase chain reaction was used to amplify four DNA fragments corresponding to the four independent zinc-binding modules of zLIM1 and zLIM2 (zLIM1A = zyxin AA349-377, zLIM1B = zyxin AA380-406, zLIM2A = zyxin AA407-435, and zLIM2B = zyxin AA438-467). The oligonucleotide primers used in these reactions were designed such that each of the four resulting DNA fragments was flanked on one end by a vector-compatible *Eco*RI restriction site and on the other end with a *Dra*I recognition sequence. In the zLIM1A and zLIM2A fragments, the *Eco*RI sites were positioned at the 5′ end of the coding strand and the *Dra*I sites were positioned at the 3′ end; in zLIM1B and zLIM2B, the restrictions sites were placed in the opposite orientation with the *Dra*I site

at the 5′ end of the coding strand and the *Eco*RI site at the 3′ end. Hybrid LIM domains were subcloned into the bacterial expression vector pGEX-2T$^{128}/_{129}$ by ligating the appropriate reciprocal zLIM1- and zLIM2-derived DNA fragments to each other at the internal *Dra*I recognition sites and to the vector at the *Eco*RI sites. Both of the resulting chimeric LIM domains exhibit a heterologous dipeptide linker (phenylalanine-lysine, or FK) that is encoded by the engineered *Dra*I sequence; note that these dipeptide linkers contain lysine residues in positions that, in wild-type zLIM1 and zLIM2, are occupied by a threonine residue. These constructs allowed for the expression of two chimeric LIM domain probes that are referred to in the text as GST-zLIM1A2B"FK" and GST-zLIM2A1B"FK."

Site-directed Mutagenesis of Intact zLIM1 and zLIM1/zLIM2 Chimeras

Promega's Altered Sites II in vitro Mutagenesis System (Madison, WI) was used to alter specific DNA sequences encoding the linker dipeptide and metal-liganding residues of the zLIM1 sequences indicated in the text. The efficacy of the mutagenesis reactions was evaluated and verified by sequence analysis. The mutagenized zyxin sequences were subsequently subcloned into pGEX-2T$^{128}/_{129}$ and were expressed as bacterial GST fusion proteins. These mutant protein probes are referred to in the text as GST-zLIM1"FK", GST-zLIM1A2B"FT", GST-zLIM2A1B"FT", and GST-zLIM1(C5A).

Purification of Recombinant CRP1

Recombinant CRP1 was expressed and purified as described previously (Michelsen *et al.*, 1992; Kosa *et al.*, 1994). The CRP1 used in these studies was estimated to be >98% pure by analysis of Coomassie blue-stained gels. CRP1 concentrations were estimated using a M extinction coefficient of $2.66 \times 10^4\ M^{-1} \cdot cm^{-1}$ (Michelsen *et al.*, 1992). The recombinant CRP1 used in this study has previously been referred to as CRP (Michelsen *et al.*, 1992; Sadler *et al.*, 1992; Crawford *et al.*, 1994; Weiskirchen *et al.*, 1995).

Expression, Purification, and Radiolabeling of GST-Zyxin Fusion Protein Probes

Bacterially produced GST-zyxin fusion proteins were expressed and purified according to published methods (Ausubel *et al.*, 1995). GST-zyxin fusion proteins or GST leader peptides, once immobilized on glutathione agarose affinity beads, were labeled with ^{32}P as described previously (Blanar and Rutter, 1992; Schmeichel and Beckerle, 1994). Relative levels of ^{32}P incorporation by each fusion probe was estimated by Cherenkov counting. Parallel mock-treated fusion proteins were prepared in the absence of radioactivity; these unlabeled samples were evaluated for protein concentration with Bradford protein assays (Bio-Rad, Richmond, CA) using bovine serum albumin (BSA) as a standard. Based on these analyses, the radiolabeled GST-zyxin probes exhibited comparable specific activities of approximately 36,000 cpm/pmol. Relative zinc contents of the unlabeled GST-zyxin fusion proteins were determined by atomic absorption spectroscopy. Levels of zinc binding are reported in the text as a percentage of wild-type GST-zLIM1 ± SE of the mean (SEM).

Blot Overlay Assays

Blot overlay protein-binding assays were performed as described previously (Crawford *et al.*, 1992, 1994; Sadler *et al.*, 1992; Schmeichel and Beckerle, 1994). Briefly, purified recombinant CRP1 (100–200 pmol/lane) or complex bacterial cell extracts were resolved by SDS-PAGE. Proteins were subsequently transferred to nitrocellulose and renatured for 3 to 4 h in a physiological blocking buffer (2.5% BSA in 50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 0.1% NaN_3, 0.05% Tween 20, and 0.2% gelatin). Protein blots were then probed in triplicate (to control for accuracy of sample loading) at room temperature for 2 to 4 h with 600,000 cpm/ml of the appropriate ^{32}P-labeled GST-zyxin fusion proteins in overlay buffer (0.5% BSA, 0.25% gelatin, 1.0% Nonidet P-40, 0.1 mM EDTA, 0.1% 2-mercaptoethanol, 10 mM NaCl, 20 mM HEPES, pH 7.5). The resulting blots, once washed, were evaluated by autoradiography and by PhosphorImager analysis (Imagequant software, Version 3.5, Molecular Dynamics, Sunnyvale, CA). The quantitations reported here reflect those experiments in which triplicate values displayed a SD of less than 25%. CRP1-binding activities for each of the zyxin-derived probes were normalized relative to wild-type zLIM1 levels and are expressed here as a percentage of GST-zLIM1 binding ± SE of the mean (SEM). For each ^{32}P-labeled probe used in the blot overlay assays, the SEM values reported are proportional to the mean CRP1-binding level observed with that probe. The significance of the difference between the mean binding activities of the zyxin-derived probes was evaluated using Student's *t* test (Statworks, Version 1.2). Data sets for which p values were calculated to be > 0.05 were not considered to be significantly different.

SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli (1970) which was modified to incorporate the use of 0.13% bisacrylamide as a cross-linker. Gels that were 12.5% or 15% were used to resolve CRP1 in all experiments.

RESULTS

The LIM domain displays a striking structural similarity to the DNA-binding domains of the GR and GATA-1 transcription factors (Pérez-Alvarado *et al.*, 1994, 1996). As can be seen in Figure 1, the LIM domain, like both GR and GATA-1, exhibits two discrete zinc-binding subdomains or fingers (Härd *et al.*, 1990; Luisi *et al.*, 1991; Omichinski *et al.*, 1993; Pérez-Alvarado *et al.*, 1994, 1996). In the case of both GR and GATA-1, the two zinc fingers are functionally distinct. The N-terminal zinc finger of GR functions in site-specific nucleic acid-binding, whereas its C-terminal zinc finger mediates receptor dimerization (Dahlman-Wright *et al.*, 1991, 1992). Likewise, recent studies have demonstrated that the N-terminal zinc finger of GATA-1, in addition to stabilizing the nucleic acid interactions established by the C-terminal DNA-binding finger, mediates GATA-1 self-association (Yang and Evans, 1992; Crossley *et al.*, 1995). It has not yet been determined whether the two zinc-binding modules of a LIM domain are capable of performing independent biochemical functions. However, given the evidence that an intact LIM domain can function in protein–protein interactions and the demonstration that its C-terminal subdomain has a conformation that is compatible with nucleic acid binding, it is plausible that the LIM domain's two zinc-binding modules may have the capacity to associate with distinct partners.

Because LIM domain function is best understood in the context of protein–protein interactions, one step toward understanding the molecular basis of LIM domain function is to determine whether its protein-binding activity can be ascribed to the sequences contained within one of the LIM domain's two zinc-

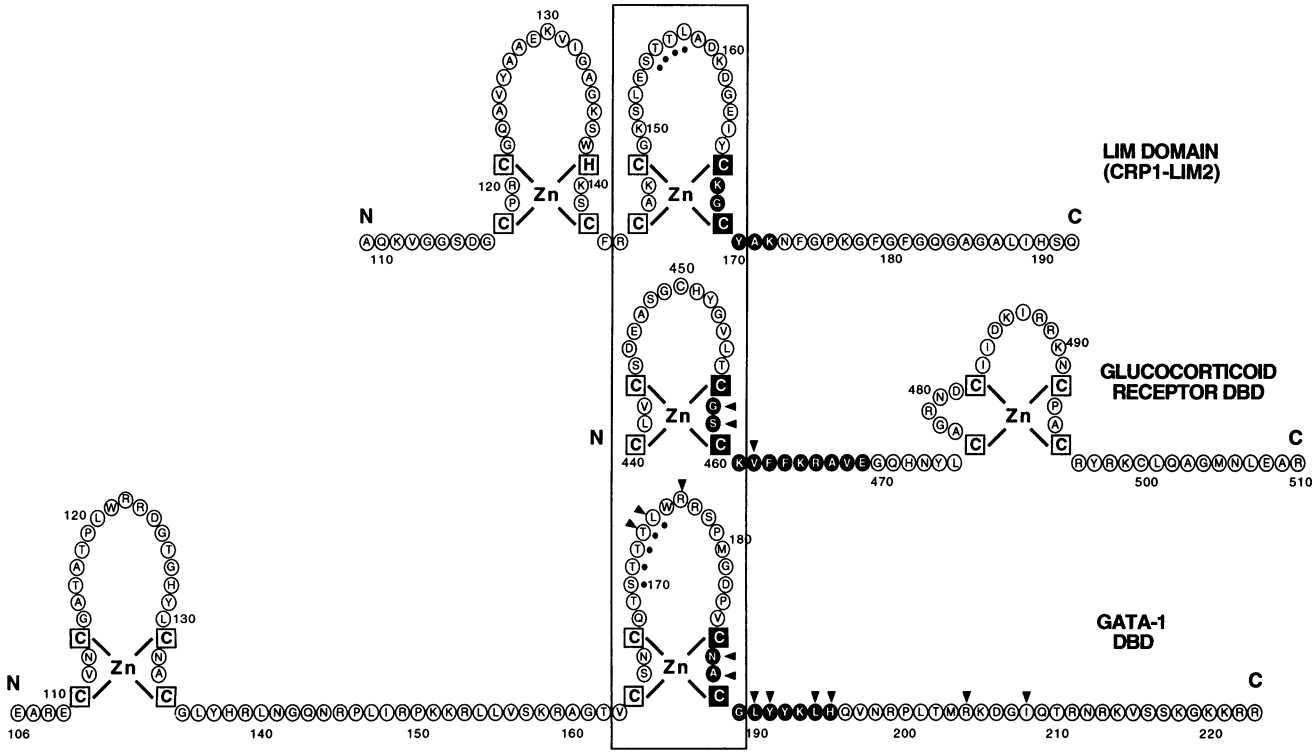

Figure 1. Comparison of the double zinc finger domains of CRP1-LIM2, the GR and GATA-1. The LIM domain, represented here by CRP1-LIM2 (avian CRP1, AA109-192), displays striking similarity to the DNA-binding domains (DBDs) of the rat GR (AA 440–510) and avian GATA-1 (AA 106–224) transcription factors (Härd *et al.*, 1990; Luisi *et al.*, 1991; Omichinski *et al.*, 1993; Pérez-Alvarado *et al.*, 1994, 1996). Each domain consists of two zinc-binding substructures. The zinc-binding modules indicated in the boxed region all display comparable tertiary folds (Härd *et al.*, 1990; Luisi *et al.*, 1991; Omichinski *et al.*, 1993; Pérez-Alvarado *et al.*, 1994, 1996). In both GR and GATA-1, this structurally conserved finger functions to mediate specific protein-DNA interactions. The other zinc finger of both GR and GATA-1 mediates specific protein–protein interactions (Dahlman-Wright *et al.*, 1991; Crossley *et al.*, 1995). Although the LIM domain has been generally defined as a protein-binding domain (Feuerstein *et al.*, 1994; Schmeichel and Beckerle, 1994; Valge-Archer *et al.*, 1994; Wadman *et al.*, 1994; Wu and Gill, 1994; Bach *et al.*, 1995; Osada *et al.*, 1995; Jurata *et al.*, 1996), the specific functional contributions made by each of the LIM domain's two zinc-binding modules has not been established. Arrowheads indicate residues of GR and GATA-1 that engage in direct interactions with DNA. Notably, a short sequence of amino acids in the C-terminal zinc-binding module of CRP1-LIM2 (STTL, ●) is similar in sequence and position to a region in GATA-1 (STTTL) that is known to make specific contacts with target DNAs. The blackened residues correspond to the α-helical regions in each molecule that, in the case of GR and GATA-1, are known to mediate contacts in the major groove of DNA. In CRP1-LIM2, residues beyond K172 fail to display ordered structure by NMR analysis.

binding substructures. Here, we have performed a molecular dissection of a model LIM domain peptide to determine whether either of its two zinc-binding modules is sufficient to mediate its protein-binding function.

Analysis of the Zinc-binding Subdomains of zLIM1

Avian zyxin displays a C-terminal cluster of three tandemly arrayed LIM domains, one of which (zLIM1) is necessary and sufficient to mediate zyxin's association with the 23-kDa cysteine-rich protein, CRP1 (Figure 2; Sadler *et al.*, 1992; Schmeichel and Beckerle, 1994). The interaction between zLIM1 and CRP1 has been extensively characterized in previous studies and can be readily monitored using in vitro blot overlay binding assays (Sadler *et al.*, 1992; Crawford *et al.*, 1994; Schmeichel and Beckerle, 1994). In blot overlay assays, target-binding proteins are immobilized on nitrocellulose and probed with radiolabeled zyxin. As can be seen in Figure 2, a ^{32}P-labeled GST-zyxin fusion protein containing sequences from zyxin's N-terminal LIM domain, zLIM1, clearly binds specifically to both purified CRP1 (Figure 2A, lane 6) and CRP1 contained within a complex bacterial cell extract (Figure 2A, lane 5), but does not interact substantially with other abundant proteins displayed on the blot (Figure 2A, lanes 4 and 5). The radiolabeled GST leader peptide alone fails to bind appreciably to CRP1 (Figure 2A, lanes 7–9). We have used this binding assay to explore further zLIM1's protein-binding function.

In an effort to evaluate the roles of the two structural subdomains of zLIM1 in mediating protein–protein

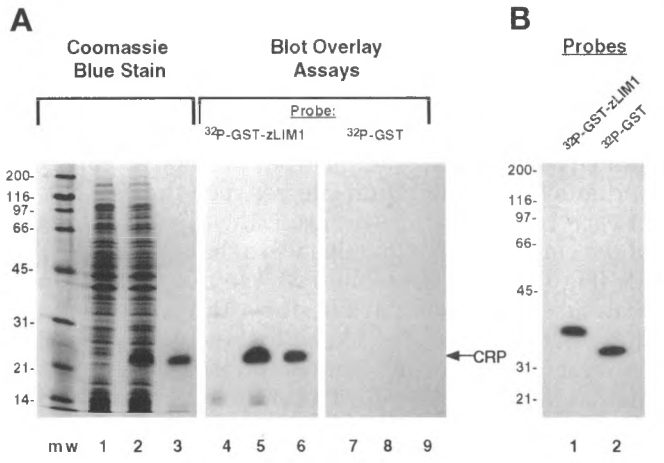

Figure 2. ^{32}P-Labeled GST-zLIM1 interacts specifically with CRP1. (A) Control bacterial extracts, extracts from bacterial cells expressing CRP1, and purified recombinant CRP1 were resolved by SDS-PAGE and visualized by Coomassie blue staining (lanes 1, 2, and 3, respectively). Parallel nitrocellulose blots were subsequently probed with either ^{32}P-labeled GST-zLIM1 (containing zyxin's N-terminal-most LIM sequences, AA349-406; lanes 3–6) or ^{32}P-labeled GST leader peptide (lanes 7–9). Autoradiographic analysis revealed that the ^{32}P-labeled GST-zLIM1 probe interacts most prominently with CRP1, whereas the ^{32}P-labeled leader peptide fails to bind appreciably to proteins present on the blot. (B) Equivalent cpm/ml of the ^{32}P-labeled GST-zLIM1 or ^{32}P-labeled GST probes used in the blot overlay assay were resolved by SDS-PAGE and analyzed by autoradiography. The quality of the probes illustrated here is characteristic of those used throughout this study.

interactions, we generated two GST-zyxin fusion proteins: 1) GST-zLIM1A, which included a 31-amino acid peptide corresponding to the N-terminal C_3H zinc-binding module of zLIM1; and 2) GST-zLIM1B, which contained the 26-amino acid C-terminal C_4 module of zLIM1 (Figure 3A). (Throughout the text, N-terminal zinc-binding subdomains will be generally referred to with the letter A and C-terminal zinc-binding subdomains will be referred to with the letter B.) Both proteins were stably expressed and readily purified as soluble proteins from bacterial extracts. As would be expected if these fusion proteins were properly folded, GST-zLIM1A and GST-zLIM1B bound approximately one-half the amount of zinc as the intact GST-zLIM1 fusion, as estimated by atomic absorption spectroscopy. When compared with GST-zLIM1 (zinc binding, 100%), GST-zLIM1A and GST-zLIM1B bound an average of 37.6 ± 2.7% and 49.5 ± 3.0% zinc, respectively, in three independent experiments. These results suggest that the individual subdomains of zLIM1, although physically separated, coordinate zinc and thus appear to be properly folded in the context of the fusion protein.

Purified GST-zLIM1A and GST-zLIM1B fusion proteins were radiolabeled and used as probes in blot overlay assays to evaluate their ability to interact with CRP1. As can be seen in Figure 3, although intact

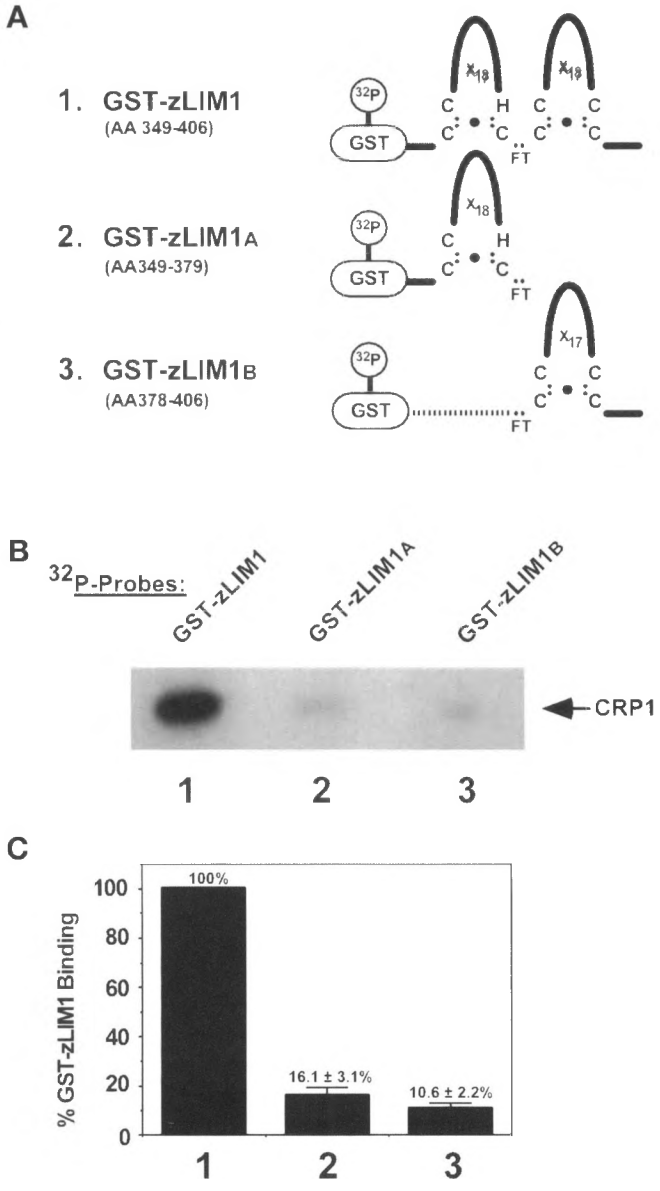

Figure 3. zLIM1 half molecules do not bind CRP1. (A) Two truncated zLIM1 peptides were expressed as GST-labeled fusions: GST-zLIM1A contains sequences from zLIM1's N-terminal zinc-binding module (AA406-379), and GST-zLIM1B contains sequences from zLIM1's C-terminal zinc-binding module (AA378-406). Triplicate nitrocellulose strips embedded with purified CRP1 were probed in blot overlay assays with equivalent cpm/ml of each of the ^{32}P-labeled probes indicated in A. The resulting blots were evaluated by autoradiography (B) and were quantitated by PhosphorImager analysis (C). The data presented in C have been normalized to wild-type GST-zLIM1 binding levels and are expressed here as a percentage of GST-zLIM1 binding ± SEM. These data are representative of six independent experiments. Collectively, these analyses reveal that neither of the zinc-binding modules of zLIM1, when independently expressed, binds CRP1.

zLIM1 exhibits robust binding to CRP1, neither GST-zLIM1A nor GST-zLIM1B was capable of binding to

CRP1 at wild-type levels. (The amounts of GST-zLIM1A and GST-zLIM1B that were bound to CRP1 were not significantly different [$p > 0.05$]). The slight CRP1-binding activity retained by these half molecules is reminiscent of that observed previously with other LIM domain-containing probes (Schmeichel and Beckerle, 1994). It is possible that this low level of residual binding may be due to an overall compatibility of the LIM domain fold with a binding site on CRP1, since the GST leader peptide reproducibly fails to exhibit detectable CRP1 binding. Alternatively, the level of CRP1 binding observed with GST-zLIM1A and GST-zLIM1B probes may be due to a nonspecific association, perhaps via promiscuous disulfide bond formation, between the cysteine-rich zyxin probes and a small percentage of CRP1 that has failed to refold appropriately on the blot.

Assuming that the zLIM1A and zLIM1B half-LIM domains are properly folded and are not obscured by the GST leader peptide, we have considered two possible explanations for their failure to associate with CRP1 at wild-type levels: 1) the CRP1-binding site could be specified by a complex surface derived from both halves of zLIM1; or 2) the sequences essential for binding to CRP1 could be contained within one of the two halves of zLIM1 but presentation in the context of an intact LIM domain could be required for function. We have distinguished between these two possibilities by performing an analysis of the CRP1-binding function of zyxin-derived, chimeric LIM polypeptides.

Probing the Protein-binding Function of zLIM1's Zinc-binding Modules by Analysis of Chimeric LIM domains

To explore the CRP1-binding capacity of the two zinc-binding modules of zLIM1 in the context of an intact LIM structure, we generated a collection of hybrid LIM domains. Our strategy took advantage of the fact that while the most N-terminal LIM domain of zyxin (zLIM1) binds specifically to CRP1, the second and third LIM domains of zyxin (zLIM2 and zLIM3) exhibit only a low level of binding (approximately 10% of that observed with zLIM1; Schmeichel and Beckerle, 1994). Although all three of zyxin's LIM domains differ significantly in terms of sequence composition outside of the LIM consensus, zLIM1 and zLIM2 are related with respect to overall size and linker dipeptide composition (Figure 4A). Thus, we chose to generate fusions of zLIM1 and zLIM2. Within these chimeric probes, the sequences derived from zLIM1 should convey CRP1-specific recognition elements, whereas zLIM2 sequences should furnish the balance of the LIM structure.

The chimeric LIM domain fusion proteins used in this study were designed such that one contained the N-terminal zinc-binding module of zLIM1 and the C-terminal zinc-binding module of zLIM2 (GST-zLIM1A2B"FK"), and a reciprocal molecule consisted of the N-terminal zinc-binding module of zLIM2 linked to the C-terminal subdomain of zLIM1 (GST-zLIM2A1B"FK"; see Figure 4B). Our initial cloning strategy resulted in the incorporation of a 6-bp *Dra*I endonuclease recognition site between the two halves of each LIM domain; as a result, the dipeptide linkers of the chimeric LIM domains were changed from FT to FK (Figure 4B, probes 2 and 3). We initially reasoned that substitution of a lysine for a threonine in these dipeptide linkers was likely to be tolerated in the chimeric molecules since only a single amino acid was altered and since the third LIM domain of zyxin displays a lysine residue at this C-terminal position (Figure 4A). Moreover, FK dipeptide linkers, although not present in any of zyxin's LIM domains, are observed in LIM domains derived from other proteins (Boehm *et al.*, 1991; Baltz *et al.*, 1992; Stronach *et al.*, 1996).

The blot overlay assay was used to assess the CRP1-binding activity of the zyxin-derived chimeric LIM domains. Neither the GST-zLIM1A2B"FK" nor the GST-zLIM2A1B"FK" chimera supported an interaction with CRP1 that was comparable to the wild-type zLIM1 peptide (Figure 4, C and D, lanes 1–3). Although the GST-zLIM1A2B"FK" was found in some experiments to exhibit slightly higher binding to CRP1 than observed with the GST-zLIM2A1B"FK" chimera, statistical analyses of these binding data revealed no significant difference in the CRP1-binding capacities of the two probes ($p > 0.05$). The failure of both of the chimeric proteins to restore wild-type levels of CRP1 binding led us to investigate further the impact of the single amino acid alterations in the dipeptide linker.

We explored the functional significance of the dipeptide linker sequence composition by analyzing the protein-binding activity of a mutant peptide referred to in Figure 4B as zLIM1"FK" (see probe 4). zLIM1"FK" differs from wild-type zLIM1 by only a single amino acid; to mimic the situation created in the LIM chimeras described above, the threonine residue in the wild-type dipeptide linker was changed to a lysine residue. The zLIM1"FK" peptide was stably expressed as a GST fusion protein and was found to bind wild-type levels of zinc (if GST-zLIM1 = 100% zinc binding, then on average GST-zLIM1"FK" = 109.8 ± 10.3%; $n = 4$). However, compared with wild-type GST-zLIM1, GST-zLIM1"FK" binds CRP1 at a significantly reduced level that is not statistically different from the binding observed with the nonspecific GST-zLIM2 probe ($p > 0.05$; Figure 4, C and D). These results suggest that this dipeptide sequence is in fact a key element required to maintain the optimal protein-binding function of zLIM1.

Because the sequence composition of the dipeptide linker is critical to the CRP1-binding function of zLIM1, we sought to reevaluate the protein-binding

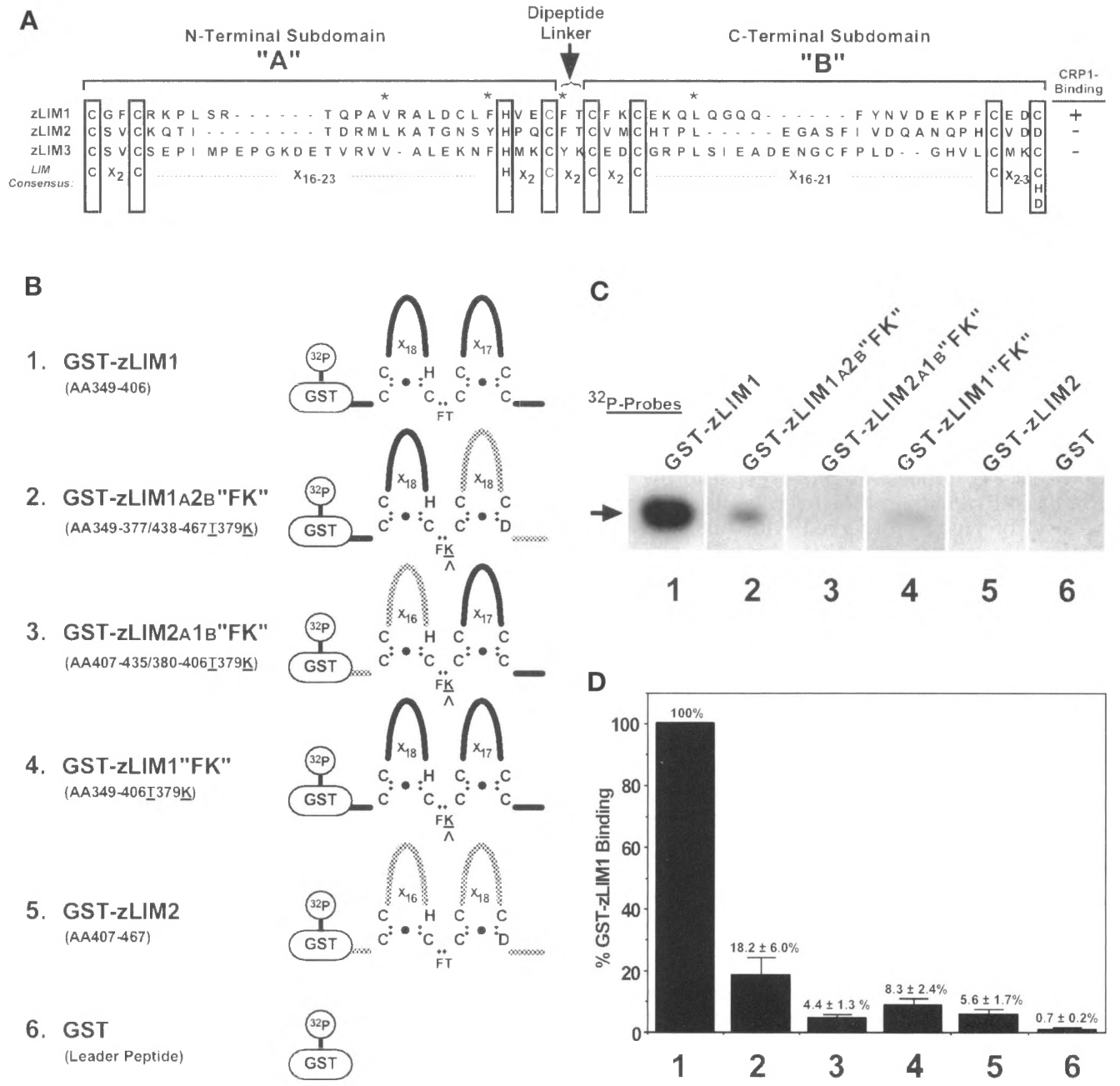

Figure 4. zLIM1/zLIM2 chimeras with heterologous dipeptide linkers do not reconstitute CRP1-binding activity. (A) Apart from the residues defining the LIM consensus and a core of loosely conserved hydrophobic residues that are thought to stabilize LIM domain structure (*), the amino acid compositions of the three LIM domains of zyxin are notably different. Of these three LIM domains, only zLIM1 exhibits a robust binding to CRP1 (Schmeichel and Beckerle, 1994). (B) The two zinc-binding subdomains of zLIM1 (black lines) were independently fused to reciprocal subdomains from zLIM2 (gray lines) to generate two zyxin LIM domain chimeras (probes 2 and 3). (Note that due to the cloning strategy used, these chimeras display FK instead of FT dipeptide linkers (B, arrowheads). For probe 4, site-directed mutagenesis was used to convert codon 379 of wild-type zLIM1 from a threonine to a lysine encoding sequence. (C) Equimolar amounts of purified CRP1 (arrow) were probed in blot overlay assays with equivalent cpm/ml of each of the ^{32}P-labeled fusions indicated in B. CRP1 binding was evaluated by autoradiography. (D) PhosphorImager analysis was performed on triplicate blots to determine relative levels of CRP1-binding activity. CRP1 binding is expressed in D as a percentage of GST-zLIM1 binding ± SEM; the data are representative of four independent experiments. These data indicate that none of the zyxin-derived molecules displaying an FK dipeptide linker is capable of reconstituting wild-type CRP1-binding activity.

function of the zLIM1/zLIM2 chimeras by engineering hybrid molecules that retain wild-type FT dipeptide linker sequences. The resulting chimeric peptides, zLIM1A2B"FT" and zLIM2A1B"FT" (Figure 5A), were expressed as GST fusion proteins and used as probes in the blot overlay assay with purified CRP1. As can be seen in Figure 5, B and C, CRP1-binding activity is effectively restored by the zLIM1A2B"FT" probe, whereas the zLIM2A1B"FT" probe binds CRP1 only at background levels. The inability of the zLIM2A1B"FT" probe to bind CRP1 is likely not due to structural instability of this probe since this molecule is capable of coordinating zinc at levels that are comparable to the wild-type zLIM1 polypeptide (if GST-zLIM1 = 100% zinc binding, then GST-zLIM2A1B"FT" = 103.5 ± 9.0%; n = 3). Some variability was observed in the binding of the zLIM1A2B"FT" probe to CRP1. However, as with other probes used in these blot overlay assays, the variability observed with the zLIM1A2B"FT" probe was proportional to the mean level of CRP1 binding. Moreover, in all experiments the level of binding observed with this probe was significantly greater than that observed with other chimeric LIM probes described in this study. Thus, a LIM domain peptide containing sequences derived from the first zinc-binding module of zLIM1 displays an ability to bind to CRP1 that is, on average, at least as good as intact zLIM1. Collectively, these experiments reveal the importance of sequences contained within the N-terminal zinc-binding module of zLIM1 in specifying zyxin's association with CRP1.

Modification of a Metal-coordinating Residue in the C_4 Subdomain of zLIM1 Diminishes Its Protein-Binding Function

Our analysis of the zLIM1/zLIM2 chimeras indicates that although the C-terminal C_4 subdomain makes no sequence-specific contributions in the CRP1 interaction, this region is required for optimal reconstitution of the protein-binding function of the LIM domain probes. To evaluate further the functional requirement for a C-terminal C_4 subdomain, we have examined whether disruption of the metal-binding capacity of the C_4 subdomain limits its ability to complement the C_3H zinc-binding module of zLIM1 in its protein-binding function.

Previous work has demonstrated that the modification of a single metal-coordinating residue in a LIM domain can disrupt the fold of the mutant zinc-binding module without perturbing the adjacent zinc-binding subdomain (Michelsen *et al.*, 1994). Thus, we reasoned that, by diminishing the zinc-binding potential of the C-terminal C_4 module of zLIM1, we could effectively target the disruption of the tertiary structure in this region, as is illustrated schematically in Figure 6A. Such a metal-compromised probe could be used to determine whether the C-terminal zinc-binding subdomain must achieve a native fold to support the protein-binding function of the LIM domain.

To perturb zinc coordination in the C_4 module of zLIM1, a mutant probe was generated [referred to as GST-zLIM1(C5A)] in which the fifth conserved residue of zLIM1's zinc-binding consensus was changed from a cysteine to an alanine, an amino acid whose side chain is not compatible with metal binding (Figure 6A). We determined by atomic absorption spectroscopy that the mutant peptide exhibited a reduced metal-binding capacity in comparison to wild-type GST-zLIM1 (Figure 6B). Although it was somewhat surprising that the zinc content in the zLIM1(C5A) fusion protein was not reduced to 50% of wild type, it is possible that the altered metal-binding center partially retains its zinc-binding potential (O'Connor *et al.*, 1993; Giraud-Panis *et al.*, 1995). The CRP1-binding capacity of the GST-zLIM1(C5A) was evaluated by the blot overlay assay and was determined to be significantly and reproducibly less than that observed with the wild-type probe (Figure 6, C and D). Further analysis of these data revealed that the CRP1-binding activity of GST-zLIM1(C5A) is significantly lower than that observed with the GST-zLIM1A2B"FT" probe that was shown in Figure 5 to restore CRP1-binding function ($p < 0.05$). This finding is consistent with the interpretation that the GST-zLIM1(C5A) probe displays a reduction in its capacity to bind CRP1. Taken together with the results described above, this mutagenesis study supports the conclusion that a structurally intact LIM domain is required for zLIM1's ability to function as a protein-binding interface.

DISCUSSION

The LIM domain is a cysteine-rich sequence element that exhibits the consensus $CX_2CX_{16-23}HX_2CX_2CX_2CX_{16-21}CX_{2-3}$(C/H/D) (Sadler *et al.*, 1992; Sanchez-Garcia and Rabbitts, 1994; Dawid *et al.*, 1995; Gill, 1995). NMR structural analyses of four LIM domains with varying sequence compositions have demonstrated a common fold for the LIM domain: the conserved residues within a single LIM domain give rise to two consecutive zinc-binding modules that display characteristic anti-parallel β-sheets and are connected by a short dipeptide linker (Pérez-Alvarado *et al.*, 1994, 1996; Hammarström *et al.*, 1996; Summers *et al.*, unpublished results). LIM motifs have been observed in a variety of gene products, including transcription factors, a proto-oncogene product, and a number of proteins associated with the actin cytoskeleton (reviewed in Sadler *et al.*, 1992; Sanchez-Garcia and Rabbitts, 1994; Dawid *et al.*, 1995; Gill, 1995). Because LIM domain-containing proteins, in general, appear to be critical determinants of cell differentiation events that occur during development, many studies

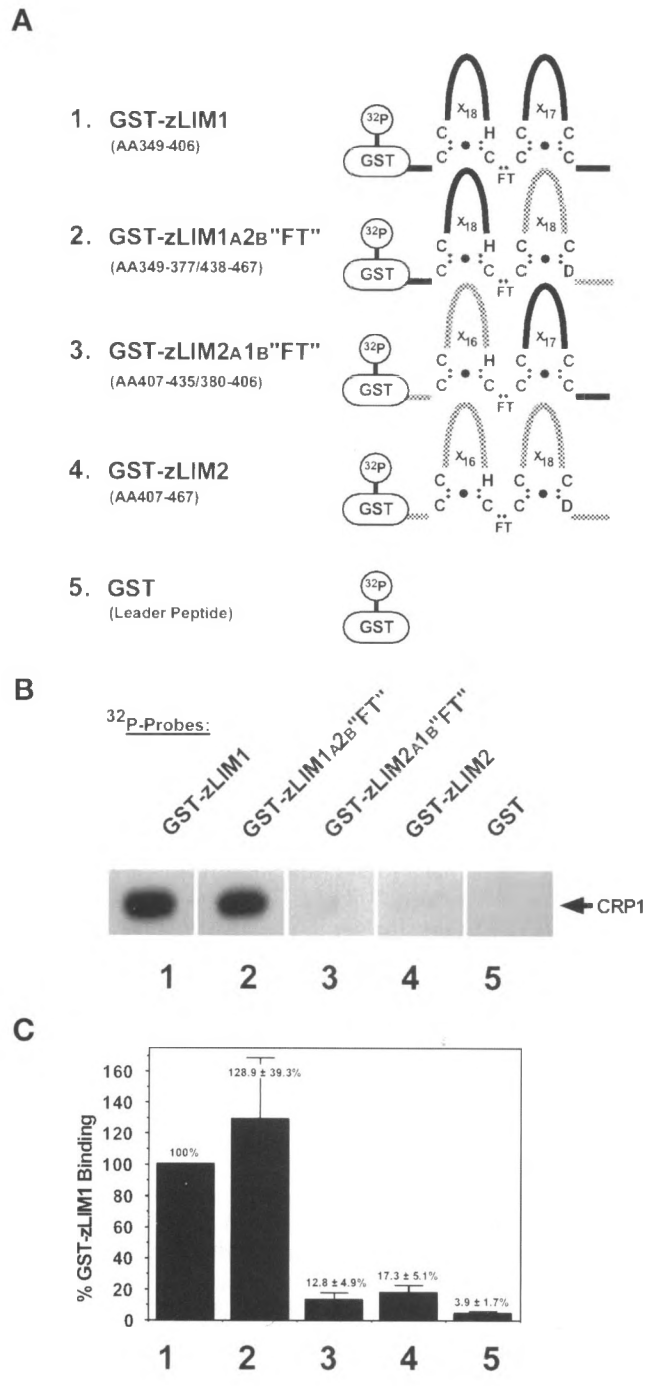

Figure 5. The N-terminal zinc-binding subdomain of zLIM1 specifies CRP1 binding in an intact LIM domain. (A) Site-directed mutagenesis was used to reengineer the zyxin LIM chimeras described in Figure 4 with wild-type FT dipeptide linkers. The CRP1-binding activity of this second set of chimeric probes was subsequently evaluated in triplicate blot overlay assays. The autoradiographs in B and the quantitations presented in C demonstrate that the reengineered LIM chimera containing the N-terminal zinc-binding module of zLIM1 binds to CRP1. Data from four independent experiments are presented in C as the mean percentage of GST-zLIM1 binding ± SEM.

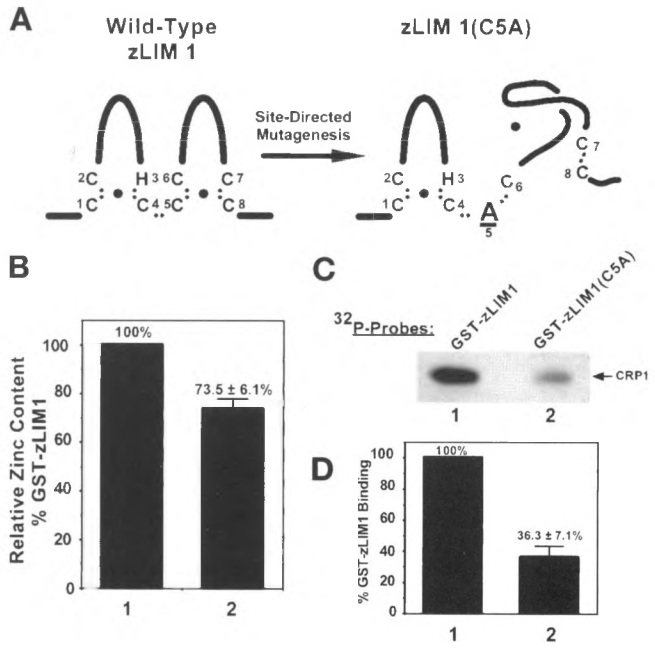

Figure 6. The CRP1-binding activity of zLIM1 requires a structurally intact C-terminal zinc-binding module. (A) To target disruption of the zLIM1's C-terminal zinc-binding module, site-directed mutagenesis was used to change the codon for zLIM1's fifth position cysteine (Cys 377) to an alanine codon. (B) Zinc coordination by the wild-type GST-zLIM1 and the mutant GST-zLIM1(C5A) polypeptides was evaluated by atomic absorption spectroscopy. The zinc-binding capacity of the mutant peptide was reproducibly lower than that observed with the wild-type peptide. Zinc-binding levels are reported here as a percentage of wild-type GST-zLIM1 ± SEM and are reflective of eight independent trials. (C–D) The protein-binding function of the mutant GST-zLIM1(C5A) probe was evaluated in blot overlays with purified CRP1. Representative autoradiographs illustrated in C and the quantitations in D demonstrate that the CRP1-binding activity of the metal-compromised GST-zLIM1(C5A) probe is reduced in comparison to wild-type zLIM1 peptides. The quantitative results presented here were calculated as a percentage of wild-type zLIM1 ± SEM and were derived from seven independent experiments.

have explored the specific functional attributes conveyed to a protein by the LIM domain.

It has been previously demonstrated that the LIM domain is capable of functioning as a protein-binding interface (Feuerstein *et al.*, 1994; Schmeichel and Beckerle, 1994; Valge-Archer *et al.*, 1994; Wadman *et al.*, 1994; Wu and Gill, 1994; Bach *et al.*, 1995; Osada *et al.*, 1995; Jurata *et al.*, 1996). For example, we have shown that the cytoskeletal protein zyxin, which harbors a C-terminal cluster of three LIM domains, binds to CRP1 via a single LIM domain that is referred to here as zLIM1 (Schmeichel and Beckerle, 1994). Because a single LIM domain is comprised of two distinct zinc-binding substructures, we were interested in determining the functional contributions of the two subdomains of zLIM1 in the zyxin-CRP1 interaction. In this study, we have examined the CRP1-binding proper-

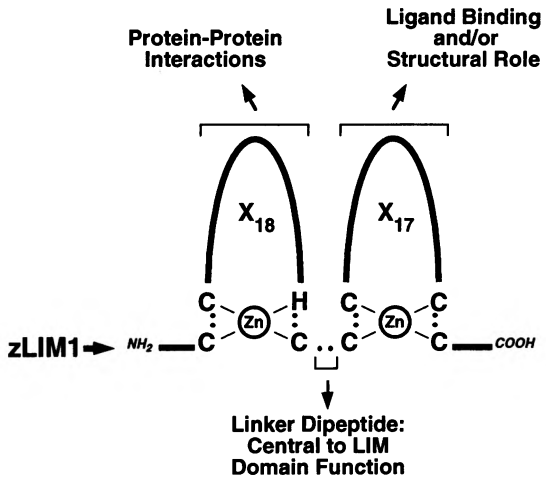

Figure 7. A dual function for a LIM domain? Structural studies have established that the LIM domain consists of two zinc-binding subdomains. Our data demonstrate that sequences contained within the N-terminal zinc-binding module of zLIM1 can specify its association with the protein CRP1. zLIM1's C-terminal zinc-binding module, which is likely to exhibit a tertiary fold that is similar to known DNA-binding zinc fingers, may function as a distinct molecular interface in mediating protein–nucleic acid or protein–protein interactions. The C-terminal zinc-binding module also plays a structural role in optimizing zLIM1's CRP1-binding function. The dipeptide linker that joins the two zinc-binding modules of zLIM1 is also a key element involved in mediating the zyxin-CRP1 interaction.

ties of zyxin-derived chimeric LIM domains and have shown that the N-terminal zinc-binding module of zLIM1 is a key functional element which is required to specify the zyxin-CRP1 interaction.

Because the protein-binding determinants of zLIM1 can be mapped to its N-terminal zinc-binding subdomain, it is possible that zLIM1's C-terminal zinc-binding module could exist as a functionally distinct molecular docking site (Figure 7). Interestingly, high-resolution structural studies have revealed that the C-terminal zinc-binding module of a LIM domain exhibits a conformation that is likely to be compatible with a DNA-binding function (Pérez-Alvarado *et al.*, 1994, 1996; Hammarström *et al.*, 1996; Summers *et al.*, unpublished results). Because in many proteins there is a well-established link between protein conformation and function, regardless of the level of amino acid identity (Branden and Tooze, 1991; Chothia, 1992), it is plausible that while the N-terminal zinc-binding module of zLIM1 engages in protein–protein interactions, zLIM1's second zinc-binding module mediates specific interactions with nucleic acids. This type of arrangement is exemplified by the DNA-binding domain of the GR, which also consists of two tandemly arrayed zinc fingers (Figure 1; Härd *et al.*, 1990; Luisi *et al.*, 1991; Omichinski *et al.*, 1993). The N-terminal zinc finger of GR mediates specific contacts with target DNA sequences, whereas its C-terminal zinc finger can be simultaneously engaged in receptor dimerization (Dahlman-Wright *et al.*, 1991, 1992; Luisi *et al.*, 1991). This type of functional subdivision has also been reported for the two zinc fingers of the GATA-1 transcription factor (Yang and Evans, 1992; Crossley *et al.*, 1995).

Nevertheless, because there are examples demonstrating that structural similarities are not necessarily sufficient to predict a protein's function (Branden and Tooze, 1991), one must be cautious in inferring function based on structural considerations alone. In the case of the LIM domain, the polypeptide fold appears to be compatible with a nucleic acid-binding function, however a direct association between a LIM domain and DNA has not been reported. Therefore, it remains possible that both of the zinc-binding modules of zLIM1 function in protein binding. If this were the case, the C-terminal zinc-binding module of zLIM1, which is likely to display a tertiary fold similar to that of known DNA-binding zinc fingers, would instead participate in protein–protein interactions. Protein binding by zinc fingers, including those known to function in DNA binding, has been described previously (Lee *et al.*, 1993; Seto *et al.*, 1993; Geisberg *et al.*, 1994; Crossley *et al.*, 1995; Merika and Orkin, 1995).

An alternative view, that is not necessarily mutually exclusive, is that the C-terminal zinc-binding module within zLIM1 plays a structural role in optimizing the CRP1-binding function of the N-terminal zinc-binding site. Support for this idea comes from our finding that when the N-terminal C_3H subdomain of zLIM1 (zLIM1A) is physically separated from its C-terminal C_4 counterpart, it fails to exhibit robust CRP1 binding. In this case, the inability of the zLIM1A probe to bind CRP1 was not due to an aberrant fold in the molecule since this fusion protein was competent to bind appropriate levels of zinc. Moreover, a recent report describing the solution structure of the N-terminal zinc-binding module of a LIM domain from the protein Lasp-1 illustrates that in the case of the Lasp-1 LIM domain, the N-terminal subdomain is capable of assuming a characteristic LIM fold independently of its C-terminal counterpart (Hammarström *et al.*, 1996). Interestingly, when zLIM1A peptides are supplemented with a C-terminal zinc-binding module from an irrelevant LIM domain, the protein-binding activity of the LIM domain is restored. The functional interplay that is observed between the two zinc-binding modules of zLIM1 is not altogether surprising since structural studies have demonstrated that within a single LIM domain, the two individual zinc-binding modules are found to be closely packed together via a collection hydrophobic amino acids that are loosely conserved within the LIM consensus (Pérez-Alvarado *et al.*, 1994, 1996).

The importance of the structural contributions of zLIM1's C-terminal zinc-binding subdomain in CRP1

binding was also revealed in our studies of the CRP1-binding activity of GST-zLIM1(C5A). This molecule, which was engineered specifically to disrupt the fold of zLIM1's C-terminal metal-coordinating pocket, displays a significant reduction in CRP1-binding potential. Moreover, had the zinc content of the GST-zLIM1(C5A) been more extensively depleted in the GST-zLIM1(C5A) probe, it is reasonable to speculate that the protein-binding activity may have been reduced even more dramatically. Combined with the studies described above, this finding emphasizes the fact that the two structurally intact zinc-binding modules of zLIM1 act in concert to facilitate CRP1 binding. Although the C-terminal C_4 zinc-binding module of zLIM1 makes no sequence-specific contacts with CRP1, this subdomain appears to be required for proper presentation of the protein-binding sequences displayed within the N-terminal C_3H module.

In this study, we also present results that illustrate the functional importance of the dipeptide that couples the two zinc-binding subdomains of zLIM1. Substitution of a lysine for a threonine at the second amino acid of zLIM1's dipeptide linker resulted in a dramatic reduction in CRP1-binding activity. One interpretation of this observation is that the amino acid substitution in the dipeptide linker may have adversely affected the overall conformation of zLIM1. Consistent with this possibility, recent NMR studies have suggested a role for the dipeptide linker in the establishment of the relative orientations of the two zinc-binding modules within a LIM domain (Pérez-Alvarado *et al.*, 1994, 1996). The spatial positioning of the two zinc-binding modules, in turn, may be a general determinant defining the ligand-binding specificities of a LIM domain.

We have performed a molecular dissection of the LIM peptide, zLIM1, to determine whether the protein-binding function of a LIM domain can be attributed to one of its two zinc-binding subdomains. Our data indicate that sequences contained within the N-terminal C_3H zinc-binding module of zLIM1 are required to mediate specific protein–protein interactions. The protein-binding function of this N-terminal zinc-binding module is optimized in the context of an intact LIM structure and can be influenced by alterations in zLIM1's dipeptide linker. Our demonstration of a role for zLIM1's N-terminal zinc-binding module in protein–protein interactions in combination with structural data suggesting that the fold of the C-terminal zinc-binding subdomain is likely to be compatible with nucleic acid binding is consistent with the possibility that zLIM1 is both structurally and functionally bipartite (Figure 7). Additional research will be required to evaluate whether such a functional subdivision is a general characteristic of all LIM domains.

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