Specificity of single LIM motifs in targeting and LIM/LIM interactions in situ

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The LIM motif defines a double zinc finger structure found in proteins involved in cell fate determination and growth control. LIM proteins, which include LIM homeo domain, LIM kinase, focal adhesion, and LIM-only proteins, usually contain two or more LIM motifs clustered at their amino- or carboxy-terminal end. At present, the mode of action of the LIM domain is not clear. In this study we have analyzed the binding properties of LIM motifs in the cellular environment. We show that MLP, CRP, and βCRP define a subclass of LIM-only proteins with unique dual subcellular localization in the nucleus and along actin-based filaments in the cytosol. A double MLP construct that accumulated nearly exclusively along actin filaments promoted myogenic differentiation efficiently, arguing for a functional role of cytoskeleton-associated MLP. Binding of MLP to the actin cytoskeleton is specifically attributable to its second LIM motif. An additional LIM motif potentiates binding. Potentiating LIM motifs can be interchanged, resulting in differential targeting of interacting proteins. To analyze LIM–LIM interactions in situ, this property was exploited to develop a hybrid interaction approach based on the relocalization of LIM-containing constructs to the actin cytoskeleton. These experiments revealed the existence of marked selectivity in the interactions of single LIM motifs, and among LIM domains from different LIM–homeo domain and LIM-only proteins. Furthermore, the analysis suggested that the LIM motif has two interacting interfaces. On the basis of these findings, we propose that LIM motifs function as specific adapter elements to promote the assembly and targeting of multiprotein complexes.

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The LIM motif defines a unique double zinc finger structure associated with a subclass of proteins involved in cell identity, differentiation, and growth control (Sanchez-Garcia and Rabbitts 1994). One LIM motif consists of the sequence (C-X2-C-X16–23-H-X2-C)–X2–([C-X2–C-X16–23–C-X2–C(D,H)]. The parentheses indicate the zinc fingers C2HC and C4(C3D, C3H), which coordinate one zinc ion each (Michelsen et al. 1993, 1994). In most cases, LIM proteins have two or more closely spaced LIM motifs at their amino- or carboxy-terminal end.

The designation LIM derives from the Caenorhabditis elegans cell fate genes lin-11 and mec-3, and from the insulin gene enhancer binding protein Isl-1. These proteins belong to the class of LIM–homeo domain proteins, a growing group of transcription factors involved in cell fate determination and differentiation (Sanchez-Garcia and Rabbitts 1994; Dawid et al. 1995). Like LIM–homeo domain proteins, LIM kinases consist of two amino-terminal LIM fingers, a large spacer region, and a carboxy-terminal kinase domain (Bernard et al. 1994; Mizuno et al. 1994). In addition to these proteins with well-defined functional domains, LIM motifs were also found at the carboxy-terminal end of zyxin (Sadler et al. 1992) and paxillin (Turner and Miller 1994), two proteins associated with focal adhesion points, and in a growing list of proteins of unknown function (Dawid et al. 1995). Finally, LIM motifs were found in proteins that appear to be devoid of any additional structural or functional motif. These LIM-only proteins mainly have two LIM motifs, but at least two consist of one LIM motif with very short flanking sequences (Sanchez-Garcia and Rabbitts 1994; Dawid et al. 1995).

Most LIM proteins have highly restricted expression patterns. In addition, their expression usually coincides with differentiation or growth regulation processes (Sanchez-Garcia and Rabbitts 1994). In many cases, the functional importance of LIM proteins has been demonstrated directly through loss- and gain-of-function studies in vivo. In addition to C. elegans and Drosophila mutants, analysis of mice depleted of particular LIM–homeo domain proteins revealed dramatic and specific defects in the formation of certain tissues or organs (e.g., Bourguin et al. 1992; Cohen et al. 1992; Shawlot and Behringer 1995). Significantly, LIM-only proteins also appear to play essential roles in differentiation and cell
growth. The LIM-only protein rhombotin-2 is required for the development of the hematopoietic system [Warren et al. 1994], and its ectopic expression induces T-cell tumors [Larson et al. 1994]. The related LIM-only protein rhombotin-1 (RBTN1) is mainly expressed in the nervous system and is also a T-cell oncogene [McGuire et al. 1989; Boehm et al. 1990]. In cultured cells, the LIM-only protein muscle LIM protein (MLP) is required for myogenic differentiation [Arber et al. 1994].

Although the specific function of LIM motifs is presently not clear, the available evidence suggests that they may mediate protein–protein interactions. Thus, LIM-only proteins can bind specifically to certain transcription factors [German et al. 1992; Valge-Archer et al. 1994; Wadman et al. 1994; Bach et al. 1995]. In addition, these proteins can bind one another directly through their LIM motifs. Such interactions have been demonstrated in vitro and in two-hybrid systems, and appear to involve some specificity [Feuerstein et al. 1994; Schmeichel and Beckerle 1994]. The LIM motifs of the homeo domain protein Lmx-1 interact with a basic helix–loop–helix (bHLH) protein that binds to an adjacent site on the insulin promoter. This allows formation of a mini-enhancer that is required for cell-type-specific expression of the insulin gene [German et al. 1992]. Finally, in an experiment that may suggest a role for LIM-based interactions in promoting transcription factor specificity, inactivation of the LIM motifs of the LIM–homeo domain protein Xlim-1 led to induction of neural and muscle structures in injected frog embryos, whereas the wild-type protein had no inductive activity [Taira et al. 1994].

The functional importance of LIM proteins raises the question of what may confer unique properties to the LIM motif. One major issue is whether and to what extent single LIM motifs are endowed with unique properties. In this study we demonstrate that MLP [Arber et al. 1994], cysteine-rich protein [CRP] [Liebhaber et al. 1990], and βCRP [Weiskirchen and Bister 1993] define a subclass of LIM-only proteins associated with cellular actin filaments. We exploited this association to define the specificity and versatility of LIM-based interactions in the cellular environment. The experiments document the existence of a substantial degree of specificity in LIM-based interactions and demonstrate that single LIM motifs define binding specificity. On the basis of the results we propose that LIM motifs function as molecular adapters to promote the assembly of specific protein complexes.

Results

MLP accumulates in the nucleus and along actin-based filaments in the cytosol

The myogenic LIM-only protein MLP accumulates in the nucleus and cytosol of differentiating skeletal muscle cells [Arber et al. 1994]. To learn more about where it may act in the cell, we analyzed its subcellular localization in transient transfection experiments with myogenic and nonmyogenic cells. As shown in Figure 1, when MLP was transfected into otherwise MLP-free C2 myoblasts, the transfected protein accumulated in the nucleus and along actin-based filaments. Strikingly, when MLP-expressing cells were compared in the same transfected culture, three types of distribution were observed: exclusively nuclear, exclusively f-actin-associated, or both nuclear and f-actin-associated (Fig. 1D, see also Fig. 1B,C). None of these subcellular localizations were detected in stably transfected C2 cells (Fig. 1D), suggesting that neither nuclear nor actin-associated localization were caused by overexpression of this protein. The same types of subcellular localization, although with different prevalences, were observed when MLP was expressed in nonmyogenic kidney epithelium-derived COS-1 cells or 3T3 fibroblasts (Fig. 1D). In primary heart cells, endogenous MLP was mainly detected along f-actin filaments (Fig. 1D, see also Fig. 2). In these cells, MLP did not associate with all f-actin-based structures. MLP-devoid structures included focal adhesion points [Fig. 2a] and peripheral ruffles during the initial phases of cell spreading [Fig. 2b,c].

In attempts to determine what may control the subcellular localizations of MLP, we carried out agonist and inhibitor experiments in transiently transfected cells. As shown in Figure 2f, depolymerization of actin filaments with cytochalasin B did not abolish the association of MLP with actin-containing structures. Significantly, perturbation of the actin-based cytoskeleton did not affect the relative proportion of nuclear and actin-associated MLP. Similarly, a variety of agents affecting protein phosphorylation, including staurosporine, vanadate, and phorbol esters, inhibition of protein synthesis with cycloheximide, and lowering serum concentrations to levels that prevent cell division all were ineffective in these experiments (data not shown). As discussed below, a possible interpretation of these findings is that the prevalence of f-actin-associated MLP in a particular cell may be governed by the protein composition of its actin-based filaments.

MLP, CRP, and βCRP define a subclass of related LIM-only proteins with shared subcellular localization properties

MLP is most homologous to the LIM-only proteins CRP [Liebhaber et al. 1990] and βCRP [Weiskirchen and Bister 1993]. Recently, it has been proposed that CRP and βCRP be renamed CRP1 and CRP2, respectively [Weiskirchen et al. 1995]. Shared properties include extensive details in the LIM double finger structures, the presence of Gly-rich sequences carboxy-terminal from each LIM motif, and the length of the spacer region between the LIM motifs [Fig. 3A]. To determine whether the relation between these proteins may extend to their subcellular localizations we carried out corresponding transfection experiments in COS-1 cells. As shown in Figure 3 (A,B), CRP and βCRP displayed nuclear, actin-associated, and nuclear plus actin-associated distribu-
Specificity of LINI-based interactions

MLP accumulates in the nucleus and along actin-based filaments in the cytosol. (A) Schematic representation of the antibodies used to detect MLP and LINI protein constructs. (M1, M2) First and second LINI motif of MLP; (GRR) Gly-rich region. (B) Distribution of MLP in transiently transfected C2 myogenic cells and 3T3 fibroblasts. Double-labeling immunocytochemistry was used; MLP, antiserum to "w/o C-term" [a,b, rhodamine-phalloidin to visualize actin-containing filaments [a',b']. (C) Subcellular localization of MLP in transiently transfected monkey kidney epithelial COS-1 (a-a') and myogenic C2 (b,b') cells. [a-a'] Triple-labeling experiment; [a] nuclear dye DAPI, [a'] MLP, carboxy-terminal antibody, [a''] rhodamine-phalloidin. (b,b'): Double-labeling experiment: (b) DAPI; [b'] MLP-tag (GLVVMNIT) immunocytochemistry. (D) Prevalences of subcellular localization patterns of MLP in transient transfections depend on the cellular background, but not on apparent expression levels. (Nucleus) antigen detected only in the nucleus; [f-actin] no nuclear localization detected; [both] nuclear and f-actin accumulation detected in the same transfected cell. Numbers refer to prevalences among cells from the same transfection and are estimated values from representative experiments [see Materials and methods]. (all) Values include all MLP-expressing cells, irrespective of apparent expression level; [low] only cells with lowest levels of MLP immunoreactivity included (about one-fifth of total). (C2) two separate batches of the mouse myogenic cell line C2C12. C2 [low] and C2 [stable] refer to the batch of C2 cells to the left. (heart cells) Subcellular localizations of endogenous MLP in cultured cells from rabbit heart. (B) Bar, 60 μm; (C) bar, 20 μm.

Actin filament association is attributable to the second LINI motif of MLP and requires one additional LINI motif for stabilization

To identify the structural elements responsible for the association of MLP with actin filaments we analyzed a series of deletion and chimeric constructs in transient transfection assays. In the following, the LINI motifs of MLP are designated M1 and M2 [MLP is M1–M2], and those of RBTN1 are designated R1 and R2. Like DMLP1, the single LINI motif constructs M1 and M2 accumulated mainly in the nucleus and did not associate with actin filaments [Fig. 4]. M1 was also detected frequently in the cytosol where it did not appear to associate with restricted to differentiating skeletal muscle and is highly homologous to MLP accumulated in the nucleus and displayed a diffuse cytosolic pattern in a subset of cells. In contrast to MLP, it did not colocalize with f-actin-containing structures [Fig. 3A,C]. Finally, and as expected, the LINI homeo domain proteins Apterous [Bourguoin et al. 1992; Cohen et al. 1992] and Isl-1 [Karlsson et al. 1990] almost exclusively accumulated in the nucleus [Fig. 3A,C]. Therefore, a unique subcellular distribution is shared between a subclass of structurally related LINI-only proteins. The characteristic association of these proteins with f-actin-containing structures provides a convenient visual assay to define the specific structural requirements of an LINI-based interaction in the cellular environment.

Figure 1. MLP accumulates in the nucleus and along actin-based filaments in the cytosol. (A) Schematic representation of the antibodies used to detect MLP and LINI protein constructs. (M1, M2) First and second LINI motif of MLP; (GRR) Gly-rich region. (B) Distribution of MLP in transiently transfected C2 myogenic cells and 3T3 fibroblasts. Double-labeling immunocytochemistry was used; MLP, antiserum to "w/o C-term" [a,b, rhodamine-phalloidin to visualize actin-containing filaments [a',b']. (C) Subcellular localization of MLP in transiently transfected monkey kidney epithelial COS-1 (a-a') and myogenic C2 (b,b') cells. [a-a'] Triple-labeling experiment; [a] nuclear dye DAPI, [a'] MLP, carboxy-terminal antibody, [a''] rhodamine-phalloidin. (b,b'): Double-labeling experiment: (b) DAPI; [b'] MLP-tag (GLVVMNIT) immunocytochemistry. (D) Prevalences of subcellular localization patterns of MLP in transient transfections depend on the cellular background, but not on apparent expression levels. (Nucleus) antigen detected only in the nucleus; [f-actin] no nuclear localization detected; [both] nuclear and f-actin accumulation detected in the same transfected cell. Numbers refer to prevalences among cells from the same transfection and are estimated values from representative experiments [see Materials and methods]. (all) Values include all MLP-expressing cells, irrespective of apparent expression level; [low] only cells with lowest levels of MLP immunoreactivity included (about one-fifth of total). (C2) two separate batches of the mouse myogenic cell line C2C12. C2 [low] and C2 [stable] refer to the batch of C2 cells to the left. (heart cells) Subcellular localizations of endogenous MLP in cultured cells from rabbit heart. (B) Bar, 60 μm; (C) bar, 20 μm.

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Figure 2. Preferential accumulation of MLP at a subset of f-actin-based structures. [a–e] Endogenous MLP in heart-derived primary cultures, [f] localization of transfected MLP in cytochalasin-B-treated COS-1 cells. Double-labeling experiments: [MLP] Carboxy-terminal antibody [a–f], antiphosphotyrosine [a’]; rhodamine-phalloidin [b–f’]. [a,d,e] Cultures 1 week after seeding. Note that MLP does not accumulate at phosphotyrosine-positive focal adhesion points [a, a’]. Cells in [b and c] show the distribution of MLP and f-actin during spreading on the substratum, [b,b’] 0.5 hr, [c, c’] 2 hr after replating. Note the absence of MLP at peripheral ruffles (b) and its preferential accumulation at vertices of actin-based structures (c,d). Also note that MLP still associated with actin structures in the presence of cytochalasin-B (20 min), and that f-actin depolymerization did not promote translocation of MLP to the nucleus. [a,f] Bar, 60 μm; [b–e] bar, 25 μm.

any particular structure [Fig. 4A]. Because of the possibility that the small one-LIM constructs may diffuse rapidly into the nucleus where they may be retained, thus precluding the detection of weak associations with the actin-based cytoskeleton, we analyzed the subcellular localization of chimeric constructs with a molecular mass comparable to that of MLP [M1–CAT, M2–CAT]. The passive targeting properties of the chloramphenicol acetyltransferase (CAT) moiety have been described in previous studies. As shown in Figure 4A, M1–CAT and M1 displayed undistinguishable subcellular localizations. In contrast, although predominant association of M2–CAT with actin filaments was not detected, a large proportion of transfected cells displayed a strong nuclear signal paired to a weaker but distinct actin-associated signal [Fig. 4B]. These findings suggested that M2 had actin-targeting properties. To explore this possibility further, we analyzed the subcellular localization of two-LIM constructs. Significantly, targeting of M1–M1 and M2–M2 were very different, and only the latter one co-distributed with f-actin [Fig. 4A,B]. In addition, when compared to MLP [i.e., M1–M2], the second M2 motif in M2–M2 greatly augmented the proportion of actin-associated transgene product [Fig. 4A]. These results indicated that M2 accounts for the association of MLP with actin-containing filaments and that two copies of M2 greatly potentiate binding. In addition, they suggested that the presence of a second LIM motif [M1 in MLP] may potentiate binding even if that particular motif cannot by itself direct targeting to the actin-based cytoskeleton. To test this possibility we analyzed the distribution of R1–M2, a construct where the amino-terminal end of MLP, including M1 and the adjacent Gly-rich sequence, was substituted with that of the nuclear protein RB7N1. As predicted, R1–M2 associated with actin-containing filaments to an extent comparable to that of MLP [Fig. 4A; see also Fig. 7, top row, see below].

To define further the structural requirements for M2-driven association with f-actin we analyzed a series of M2-containing constructs [Fig. 4]. The results revealed that [1] an M1–M2–M1 three LIM construct had a subcellular distribution comparable to that of M2–M2, further supporting the notion that additional LIM motifs potentiate binding [Fig. 4A], and [2] the association of MLP with actin-based filaments did not depend on the length of the spacer between M1 and M2 [Fig. 4A] and was not prevented by the inclusion of a nuclear localization sequence in MLP [Fig. 4C]. The specificity of the M2-based interaction with cellular actin-containing fil-
Specificity of LIM-based interactions

A

| LIM Protein | Nucleus (%) | Cytosol (%) | Nuclear (% | Actin (%) | Other |
|-------------|-------------|-------------|------------|-----------|-------|
| MLP         | 15%         | 10%         | 75%        |           |       |
| CRP         | 20%         | 10%         | 70%        |           |       |
| δ-CRP       | 15%         | 5%          | 80%        |           |       |
| DMLP1       | 70%         | —           | (30%) cortex | 40%       |
| RbM1        | 60%         | (10%) spots | (30%) spots |           |       |
| Apertin     | 95%         | —           | (5%) cytoplasm |           |
| Is1-1       | 95%         | —           | (5%) cytoplasm |           |

Figure 3. MLP and the structurally related proteins CRP and δCRP define a subclass of LIM-only proteins accumulating at actin-based filaments in the cytosol and in the nucleus. (A) Prevalences of subcellular localizations for indicated LIM proteins. Data from transient transfection experiments in COS-1 cells, as described in Fig. 1D. Values in parentheses indicate that the antigen accumulated at a cytosolic location distinct from f-actin. (Cortex) Peripheral staining along the plasma membrane; (spots) predominantly perinuclear spotted structures, most evident in Fig. 4D (RbM1–RbM1 construct). The solid circles at the carboxy-terminal end of constructs indicate that these contained an epitope tag, which was used for immunocytochemical visualization. (DMLP1) Drosophila one-LIM protein with homology to MLP; (HD) homeo domain. (B, top) Double transfection with MLP and CRP construct, and independent detection of the transgenes in double-labeling experiment. (bottom) δCRP transfection, double labeling for δCRP and rhodamine-phalloidin. (C) Single transfection experiments. Labeled structures are nuclei. Bar, 50 μm.

ments was demonstrated further by the subcellular localization of MLP–MLP and RBTN1–RBTN1 double constructs. In C2 cells, the distribution of the MLP–MLP construct was undistinguishable from that of f-actin, and no nuclear signal was detected [Fig. 4C]. In contrast, although exclusively cytosolic, the RBTN1–RBTN1 construct accumulated at large perinuclear structures, but not along actin-containing filaments [Fig. 4C]. Therefore, we conclude that the combination of one M2 LIM motif and one additional LIM motif is sufficient to efficiently and specifically direct LIM-only proteins to actin-containing filaments.

Functional significance of actin-associated MLP

Overexpression of MLP in differentiating C2 myoblasts promotes myotube formation [Arber et al. 1994]. The exclusive association of the MLP–MLP construct with the actin cytoskeleton allowed us to ask whether actin-associated MLP may be active in this assay. C2 myoblasts growing at low density were transfected with insert-free vector [NEO], MLP, or MLP–MLP, and cultures were then switched to differentiation-promoting conditions. In these experiments, transfection efficiencies ranged between 20% and 35%. As shown in Figure 4D, the MLP–MLP construct was at least as effective as MLP in promoting the formation of myosin heavy chain-positive myotubes. The apparently higher efficiency of the double MLP construct may be attributable to the higher metabolic stability of this construct in transfected cells [data not shown]. Therefore, although overexpression of LIM-only proteins in the nucleus of differentiating myoblasts may also promote myogenesis [Arber et al. 1994], these findings support the notion that actin-associated MLP plays a functional role in promoting myogenic processes. As discussed below, one possibility consistent with the phenotype of MLP-deficient mice [S. Arber and P. Caroni, in prep.] is that actin-associated MLP may promote myofibril assembly.

Relocalization of one- and two-LIM proteins through specific LIM–LIM interactions

The observation that a particular LIM motif can have
Figure 4. The second LIM motif of MLP binds specifically to a component localized along actin-containing filaments in the cytosol. Efficient localization to f-actin is promoted by the presence of an additional LIM motif. [A] Prevalences of subcellular localizations as described in Fig. 1D (COS-1 cells). [M1, M2] First and second LIM motif in MLP; [R1] first LIM motif in RBTN1; [M1, M2-CAT] fusion proteins of single LIM motifs with CAT (see Materials and methods). Note that M2-CAT localized to f-actin in a large proportion of transfected cells but that these always also contained transgene in the nucleus. In contrast, M2-M2, a construct of comparable molecular size, efficiently accumulated along actin filaments. [B] Immunocytochemical detection of one-LIM and two-LIM constructs in single transfection experiments (COS-1 cells). [C] Specificity of the association of MLP-based constructs with the actin cytoskeleton. Data for single transfection experiments in C2 cells. The MLP-MLP photograph shows transfected C2 cells, whereas the Rbtn–Rbtn photograph shows a transfected COS-1 cell. [D] The actin-associated double-MLP construct promotes myogenic differentiation. C2 cells were transfected at 20% confluency, switched to differentiation medium, and analyzed 3 days later. Labeled cells [alkaline phosphatase reaction] are myosin heavy chain (MHC)-positive myotubes. The photographs are representative for five independent experiments, numbers of labeled cells in the photographs are 18 [NEO], 34 [MLP], and 48 [MLP-MLP]. [NEO] Cells transfected with insert-free vector. The histogram is based on the data from five independent experiments. Transfection efficiencies are from parallel cultures assayed 1 day after transfection for β-galactosidase activity. [MLP–MLP] Bar, 25 μm; [B, Rbtn–Rbtn] bar, 50 μm; [D] bar, 225 μm.
specific targeting properties raises the question of what may be its cellular receptors. One possibility is that binding may be mediated by specific LIM–LIM interactions. To address this question we carried out in vitro and in situ interaction experiments.

In gel overlay experiments recombinant MLP bound to itself, RBTN1, and the LIM–homeo domain protein Apterous [Fig. 5A]. It did not bind detectably to BSA, the myogenic bHLH protein myogenin, the tumor suppressor protein p53, or a mixture of commercially available molecular weight markers [Fig. 5A]. These results suggest that under these experimental conditions LIM proteins bind to one another, although with little selectivity. Binding of MLP to RBTN1 was saturable and could be competed by excess unlabeled ligand (Fig. 5B), suggesting that it was specific. In a different type of in vitro experiment, recombinant MLP was bound to a glutathione S-transferase (GST)–MLP column. This binding was reduced by ~50% in the presence of 5 mM EDTA [data not shown] and was suppressed in the presence of 50 mM of this specific divalent metal chelator [Fig. 5B]. In contrast to these binding experiments in cell-free systems, double transfection experiments revealed a substantial degree of selectivity in LIM–LIM interactions in the cellular environment. When MLP and RBTN1 were expressed in the same transfected cells, the two LIM-only proteins did not influence detectably their respective subcellular distributions [Fig. 6A,B]. A similar outcome was observed in Apterous plus MLP cotransfections [Fig. 6B]. In contrast, cotransfection of RBTN1 with the R1-M2 construct produced a dramatic redistribution of RBTN1, which now mainly colocalized with R1-M2 along actin-containing filaments [Fig. 6B,C]. In control experiments, an M2–M2 construct failed to affect the distribution of RBTN1, indicating that it was R1 in R1-M2 that interacted with RBTN1 [i.e., R1–R2]. To identify more directly interacting LIM motifs we carried out double transfection experiments with two LIM and one LIM constructs. As shown in Figure 6C, R1–M2 induced redistribution of R2, but not R1. In control experiments, minimal redistribution of R1 or R2 by MLP or M2–M2 was detected [Fig. 6B]. These findings indicate that the LIM motifs of RBTN1 interact specifically to form heterodimers of R1–R2. Similar experiments revealed that the LIM motifs of MLP preferentially form M1–M2 heterodimers [Fig. 6B].

In the double transfections with R1–M2 a substantial fraction of R2 colocalized with R1–M2 along actin filaments. Significantly, cotransfection with R2 markedly affected the distribution of R1–M2. Thus, although R1–M2 codistributed with and did not perturb actin filaments in single transfection experiments [Fig. 7, top row], double transfection with R2 [but not with R1] led to colocalization of R1–M2 and R2 at the spotted perinuclear structures that are characteristic of the subcellular distribution of R2, and at abnormally condensed actin-containing structures [Fig. 7, bottom; Fig. 6C]. By demonstrating that the single-LIM motif R2 can affect the localization of R1–M2, these observations suggest that single-LIM motifs can interact at the same time with a LIM motif and with an additional binding component.

To determine whether LIM motifs may mediate specific interactions between different LIM proteins we carried out corresponding f-actin targeting assays. As shown in Figure 8, an II–I2–CAT construct consisting of the LIM motifs of the LIM–homeo domain protein Isl-1 fused to CAT accumulated in the nucleus of transfected cells. Cotransfection with a RBTN1–MLP construct, but not with a MLP–MLP construct, induced targeting of II–I2–CAT to f-actin efficiently [Fig. 8]. In similar experiments, II–I2–M2 induced targeting of RBTN1–CAT. Finally, in preliminary experiments II–I2–M2 selectively induced targeting of certain LIM–LIM–CAT constructs derived from LIM–homeo domain proteins [data not shown]. These results demonstrate the existence of efficient and selective interactions among the LIM motifs of LIM–homeo domain and LIM-only proteins.

Discussion

In this study we have defined principles underlying LIM
motif-based interactions in the cellular environment. First, we showed that the myogenic LIM-only protein MLP has a unique subcellular distribution in that it accumulates both in the nucleus and along actin-containing filaments in the cytosol. This characteristic property defines a subclass of structurally related LIM-only proteins, including MLP, CRP, and BCRP. Further analysis revealed that the second LIM motif of MLP binds specifically to a site associated with f-actin and that an additional LIM motif appears to have a cooperative effect in binding. This was exploited in a manner analogous to that of the two-hybrid approach to analyze the properties of LIM–LIM interactions in double transfection experiments. These experiments revealed that although LIM proteins were capable of interacting with each other with little apparent specificity in cell-free systems, LIM–LIM interactions in the cellular environment displayed a high degree of specificity. In addition, by showing that single LIM constructs can interact with a LIM motif and an additional binding site, the results suggest that the LIM motif has two binding interfaces.

Specificity and versatility of single-LIM motif interactions

The results of this study are in agreement with recent reports indicating that the LIM motif can function as a protein dimerization domain. In one study CRP (i.e., C1–C2 according to the LIM motif nomenclature used in this study) dimerization was shown to be attributable to...
Specificity of LIM-based interactions

LIM–LIM interaction in a yeast two-hybrid system and in gel overlay experiments (Feuerstein et al. 1994). Interaction appeared to be stronger in the presence of two LIM motifs, and was affected by mutagenesis of Cys residues in either zinc finger, suggesting that the double finger structure is involved in binding (Feuerstein et al. 1994). In the gel overlay assay, homo- and heterodimers of C1 and C2 where detected. This is in agreement with our findings indicating little selectivity of LIM–LIM interactions in this cell-free assay. An important recent study demonstrated specific LIM-dependent interaction between CRP and zyxin in affinity chromatography and gel overlay assays (Schmeichel and Beckerle 1994). Significantly, the first LIM motif of zyxin was necessary and sufficient for interaction with CRP. These findings led to the proposal that LIM motifs may be versatile, modular protein-binding interfaces (Schmeichel and Beckerle 1994). Our results now fully support and significantly extend this conclusion. Thus, we find a significant degree of specificity in LIM–LIM interactions in the cellular environment. We also find that single LIM motifs of LIM-only proteins interact with distinct and specific cellular-binding sites, thus functioning as targeting determinants. Finally, we find that single LIM motifs can function to target interacting LIM proteins, suggesting that they can engage in two binding interactions. These findings led us to propose that the LIM domain may be a specific adapter element. According to this hypothesis, LIM-only proteins of the RBTN or MLP type could function as linkage elements [adapters] to promote the assembly of specific multicomponent complexes. A further implication is that LIM/LIM interactions may promote the assembly of specific components at defined cellular sites. Possible examples include assembly at focal adhesion points (zyxin, paxillin), or at transcription complexes (LIM–homeo domain proteins). How could a single LIM motif function as a molecular adapter? One possibility is revealed by a comparison with the highly related zinc finger motifs of GATA-type proteins. These cell type-specific transcription factors share one conserved motif of the type \([C-X_2-C-X_{17}-C-X_2-C]-X_3o-[C-X_2-C-X_{17}-C-X_2-C]\). This is nearly identical to the LIM motif,

Figure 7. The one-LIM construct R2 alters the localization of an R1–M2 construct. Double-labeling of transfected C2C12 cells is shown. In single transfected cells (top row), R1–M2 accumulates at stress fibers. In double-transfected cells (bottom three rows) R2 alters the distribution of R1–M2 and the appearance of the actin cytoskeleton. Double-labeling experiments are shown (R1–M2, left). Note accumulation of R1–M2 at perinuclear spots (arrow in third row, long arrows in fourth row), and clumping of actin-based cytoskeleton [e.g., short arrow in fourth row]. [DAPI] Nuclear stain. (Top row) Bar, 10 μm; (bottom rows) bar, 25 μm.

Figure 8. Specific interaction between the LIM motifs I1–I2 from Isl-1 and R1–R2 from RBTN1 in transfected C2 cells. The single-transfection experiment (left) was double labeled for I1–I2–CAT [antibody to CAT] and f-actin. The double transfection experiments (right) were labeled for I1–I2–CAT and either RBTN1–MLP or MLP–MLP. Note that RBTN1–MLP, but not MLP–MLP targeted I1–I2–CAT to f-actin efficiently. Bar, 20 mm.
except for the spacer region between the two zinc fingers, which consists of only two residues in the LIM motif. Solution nuclear magnetic resonance (NMR) analysis revealed that the second finger of GATA-1 folds into a structure that is essentially identical to that of the second finger in C2 of CRP (Perez-Alvarado et al. 1994). In GATA-1, the second zinc finger is necessary and sufficient for DNA binding, whereas the first finger functions as a specific dimerization motif interacting with the corresponding finger of GATA-1 or GATA-2, but not GATA-5 (Yang and Evans 1995). This raises the attractive possibility that single zinc fingers of LIM motifs may represent distinct interactive entities. In further analogy to LIM proteins, it has been suggested that in GATA-1 the protein interacting motif may be involved in bridging of distant GATA elements to promote cell-specific gene expression (Yang and Evans 1995). This is highly reminiscent of the mini-enhancer promoting function that was demonstrated for the LIM motifs of Lmx-1 (German et al. 1992). In a similar vein, specific LIM–LIM interactions in LIM–homeo domain proteins may mediate the activation of cell type-specific genes (see e.g., Tsuchida et al. 1994).

Cellular LIM-binding sites

Besides binding to LIM elements, LIM motifs are involved in binding to other types of proteins. LIM-binding proteins identified so far are transcription factors of the POU [Bach et al. 1995] and bHLH (German et al. 1992; Valge-Archer et al. 1994, Wadman et al. 1994) types, but further interacting species are likely to exist (see, e.g., Wu and Gill 1994). The nature of the site responsible for association of MLP to actin-based filaments is not known. Zyxin and paxillin (i.e., the two LIM-containing proteins known to associate with actin-based structures at focal adhesion points) are unlikely to be involved. Thus, in double transfection experiments zyxin and MLP only colocalized to a minor extent along actin filaments [data not shown]. In addition, MLP was excluded from focal adhesion points and peripheral ruffle structures in heart-derived myoblasts [Fig. 2]. The data shown in Figure 2 also appear to argue against a direct association between MLP and actin filaments. Therefore, we assume that the LIM motif M2 of MLP associates with one or several actin-binding proteins of widespread distribution.

What may be the function of the association of MLP, CRP, and βCRP with actin-containing filaments? We found no evidence suggesting that these LIM proteins may affect the overall appearance of the actin-based cytoskeleton in transfected cells. Thus, although association with f-actin tended to be more frequent and pronounced in cells with prominent microfilaments, we noticed no difference in the distribution of f-actin patterns between expressing and nonexpressing cells of the same transient transfection experiment. We therefore find it more likely that, in agreement with the adapter model proposed above, these LIM-only proteins may promote the assembly of interacting components along the actin-based cytoskeleton. The differentiation experiments with C2 cells expressing an MLP–MLP construct suggest that actin filament-bound MLP promotes myogenic processes. A potential mechanism is suggested by the dramatic disruption of myofibrils in MLP-deficient mice (S. Arber and P. Caroni, in prep.). Preliminary analysis suggests that the primary defect in these mice may be linked specifically to myofibril assembly (i.e., a process that may use actin filaments as a scaffold). Conceivably, binding of the LIM-only myogenic protein MLP to actin-containing filaments would be a prerequisite for it to function as a regulator of myofibril assembly. The availability of myofibril assembly and MLP-deficient muscle cells should now allow critical testing of these concepts.

Materials and methods

Reagents, cell culture, and transfections

Cell lines were obtained from American Type Cell Culture Collection (mouse myogenic cell line C2C12, Swiss mouse embryo fibroblasts 3T3, monkey kidney epithelial cells COS-1) and cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% FCS. The C2–MLP stable transfectants have been described (Arber et al. 1994). Primary cultures of cardiac fibroblasts from adult rabbit heart were prepared following the protocol of Eghbali et al. [1991] with slight modifications, and cultured in DMEM–10% FCS. For transfection experiments, cDNA constructs [see below] were cloned into the cytomegalo-virus (CMV)-promoter based eukaryotic expression vector pcDNA3 [Invitrogen]. Cells were transfected using the lipo-tetamine reagent (GIBCO) as described by the manufacturer. Twenty-four hours after transfection, cells were fixed and stained as described (Arber et al. 1994). For differentiation experiments, low-density [20% confluency] C2C12 cells were transfected with appropriate constructs and pcDNA3–LacZ [CMV-driven expression of lacZ] and then maintained in a 1:1 mixture of Opti-Mem (GIBCO) and DMEM–2% FCS for 12 hr. The medium was then changed to DMEM–2% FCS. Three days after transfection, cells were stained for myotube markers, as described [Arber et al. 1994]. In control experiments, parallel cultures were assayed for β-gal activity [transfection efficiency] 1 day after transfection. RTC–phalloidin, DAPI, biotinylated, or RTC-labeled secondary antibodies, and streptavidin–lucifer yellow were from Molecular Probes Inc.

cDNA constructs

The following cDNAs were used for transfection experiments [the sources of the cDNAs, which included complete coding sequences, are mentioned in parentheses]: rat MLP [Arber et al. 1994], DMLP1 [Arber et al. 1994], human CRP [cloned by PCR from a human fibroblast cDNA library, Stratagene], chick βCRP [M.C. Beckerle, University of Utah, Salt Lake City; this clone was originally published as the avian homolog of CRP [Weiskirchen and Bister 1993] and was then renamed βCRP to indicate that it corresponds to a holomeric but distinct gene [Weiskirchen et al. 1995; K. Bister, pers. comm.]], mouse RTBN1 [clone pN2A; T.H. Rabbitts, Medical Research Council, LMB, Cambridge, England], Drosophila Apterus B8 [S.M. Cohen, European Molecular Biology Laboratory, Heidelberg, Germany], and rat Isl-1 [T. Edlund, Department of Microbiology, University of Umeå, Umeå, Sweden]. The corresponding proteins were detected using the following antibodies. Rat MLP was recognized specifically by rabbit sera produced against the carboxy-terminal peptide of MLP [GGLTHQVEKK; Arber et
al. 1994), the internal peptide FOQSSPKPARAAATTS, or recombinantly produced MLP (see below). The following proteins were produced and detected as carboxy-terminally tagged proteins (the sequence coding for GLVVMNIT was added by PCR; Arber et al. 1994): MLP, CRP, bCRP, DMLP1, RBTN1, Apterous, Isl-1, M1, M2, RBTN–RBTN. Some constructs were also tagged using the human Myc sequence EQKLISEEDLN (RBTN, R1, R2, M2, MLP, Apterous, Isl-1, R2–M2), which is recognized specifically by a commercially available monoclonal antibody [line MYCL–GE10 from European Collection of Cell Culture]. Constructs containing the CAT sequence were recognized by a monoclonal antibody [Davies et al. 1986; kind gift from J. Gannon and D. Lane, Imperial Cancer Research Foundation, UK]. Chimeric protein constructs were produced by ligation of PCR fragments using NotI as a restriction site. Initial and final amino acids for all fragments in the constructs are listed below; amino acids in the parentheses refer to the rat MLP sequence (Arber et al. 1994) unless indicated otherwise. CAT was amplified by PCR from pcDNA3–CAT [Invitrogen]. M1, (M1–G835); M2, [MPC120–E164] or [MA117–E194] M1–CAT, [M1–G835]–CAT–CAT; M2–CAT, [MA102–E194]–CAT–CAT; M1–M1, [M1–A102]–[M1–T64]; M2–M2, [MA102–E194]–[Leu85–E194]; R1–M2, [M1–R90]–[RBTNI]–[C78–E194] long spacer, [M1–K212]–[Y62–E194] short spacer, [M1–C90]–[E184–E194] M1–M2–M1, [M1–E194]–[M1–T64]; MLP–NLS (SV40-NLS), [M1–L40]–[PKKKRRQRRR]22–E194, MLP–MLP, [M1–E194]–[M1–E194], RBTN–RBTN, [M1–Q165]–[RBTNI]–[M1–Q155]–[RBTNI]–[C85–E194] R1, [M1–N66]–[RBTNI]–R2, [MR86–Q155]–[RBTNI]–[C85–E194] R1, [M1–G835]–1–2–CAT, [M1–G835]–1–2–CAT.

Bacterial expression of recombinant proteins

Unless specifically mentioned, the following proteins were expressed in bacteria from pQE9 plasmids [Qiagen Inc.] as aminoterminal Hexa–His-tagged proteins. The initial and final amino acids of the recombinant proteins are indicated in the parentheses: rat MLP [M1–E194], Drosophila Apterous B8 [M1–D190], mouse RBTN1 [M1–Q155], chick myogenin [pRSETB [Invitrogen]]; B.M. Paterson, National Institutes of Health, Bethesda, MD). Proteins were purified under native [MLP; during the isolation procedure the pH ranged between 7.0 and 7.6] or denaturing (Apterous; RBTN1 and myogenin) conditions, as recommended by the manufacturer (Qiagen Inc.), with minor modifications. ZnCl2 was added to a 10-equivolal ratio to isolated LIM proteins [Michelsen et al. 1993]. Glutathione fusion proteins [MLP–GST or GST; Smith and Johnson 1988] were expressed from pGEX–2T plasmids [Pharmacia], and purified under native conditions. For binding studies, MLP–GST or GST were immobilized to glutathione–Sepharose 4B [Pharmacia] and binding of Hexa–His-tagged MLP was performed at 4°C in PBS–0.1% BSA, in the presence of 40 μM ZnCl2 or 50 mM (or 5 mM) EDTA. After extensive washing with PBS, bound proteins were eluted with 5 mM reduced glutathione [Sigma] in 50 mM Tris–HCl (pH 8.0), and analyzed by SDS–PAGE and Western blotting. Untagged recombinant human tumor suppressor protein p53 was obtained from P. Chene, Ciba Geigy, Basel, Switzerland.

Gel overlay experiments

For overlay experiments, protein gels were blotted onto PVDF membranes [Millipore]. Membranes were then blocked overnight at room temperature in PBS, 0.05% Tween, 1% skim milk powder [Fluka]. Subsequent incubations with biotinylated His-tagged MLP [100 ng/ml] or as indicated, biotinylation with N-biotinyl-6-amino capric acid-N-Succinidylester [Fluka] were performed for 4 hr at room temperature in PBS, 0.05% Tween 20, 0.5% skim milk powder. Washes were performed in PBS, 0.05% Tween 20, 0.5% skim milk powder, and bound biotinylated proteins were detected with Extravidin–peroxidase [Sigma] and the enhancer chemiluminescence (ECL) system [Amersham]. In control experiments, inclusion of 1 mM DTT during the binding procedure did not affect binding of biotinylated MLP to blot-bound LIM proteins, indicating that it was not attributable to formation of dithiobridges.

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