Molecular Interaction between Limb Deformity Proteins (Formins) and Src Family Kinases*

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Ld proteins (formins) are encoded by the limb deformity (ld) gene and define a family of related gene products regulating establishment of embryonic polarity. In this study we establish that chicken and murine Ld proteins interact directly with Src family kinases (c-Src and c-Fyn). Specific binding is mediated by the proline-rich domain present in Ld proteins and the ligand binding surface of the Src SH3 domain. Co-immunoprecipitation of Ld and c-Src proteins from transfected cells shows that these proteins associate in vivo. Immunolocalization and biochemical fractionation of fibroblasts confirms the predominant nuclear localization of Ld proteins, but unexpectedly identifies a population of Ld proteins associated to cellular membranes. This population of Ld proteins co-localizes with membrane-associated c-Src proteins at both plasma and perinuclear membranes. These studies indicate that the morphoregulatory Ld proteins interact with signal transduction cascades by association to membrane-bound Src family kinases.

Mutations of the limb deformity (ld) locus affect patterning of distal limb structures (1) and disrupt induction of metanephric kidneys in mice (2). The ld transcripts (3) were shown to encode predominantly nuclear proteins expressed in various cell types of vertebrate embryos and adults (4, 5). Several related genes have been identified from invertebrates and other phyla including Drosophila melanogaster (D. melanogaster diaphanous (dia); 6) and cappuccino (capu; 7)), yeast (BNI1, fus1, YIP9, for details, see Ref. 7) and Aspergillus (FigA; 8). Genetic analysis shows that the vertebrate Ld gene products and most of its relatives (capu, BNI1, FigA) participate in the establishment of embryonic and/or cellular polarity (1, 7). In particular, the D. melanogaster capu gene products regulate cytoskeletal architecture and the establishment of primary egg polarity (7, 9). Capu genetically interacts with profilin, an actin-associated protein (9), whereas diaphanous functions primarily during cytokinesis (6). In contrast, genetic analysis of the murine ld phenotype showed that the vertebrate Ld gene products regulate signals that control distal limb outgrowth and patterning (10, 11). Littie is known about their molecular functions, but comparison of different Ld family members shows they share two structurally and probably functionally conserved domains.

EXPERIMENTAL PROCEDURES

Production of Ld Protein by in Vitro Translation—The Ld proteins (murine isofrom I and chicken isofrom IV, 3, 5) were labeled by in vitro translation using the TNT reticulocyte lysate system (Promega) using (35)S)methionine (Amersham).

Production of GST-SH3 Fusion Proteins—The Abi-SH3 construct was made by polymerase chain reaction amplifying its SH3 domain from a mouse Abi-SH3 construct (oligos and cDNA provided by A. Musacchio). This polymerase chain reaction product was ligated in frame into pGEX2T (Pharmacia). All other GST-SH3 fusions are described elsewhere (19 to 22). The recombinant fusion proteins were produced in bacteria and purified as described (23).

Antisera against Src and Ld Proteins—Antiser against different Src domains were used as described (22). One monoclonal antibody recognizes amino acids 2–17 of the Src protein (α2–17; 22), whereas the other

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recognizes its SH3 domain (aSH3, mAb3 327; 24). A polyclonal anti-
serrre proteins were isolated using monoclonal aSH3
mAb3 327; 24). Ld antibodies were affinity-purified using bacte-
nuclear fusion proteins encoding either the chicken or murine
terminus of the chicken Ld isoform IV and several deletions were
generated using convenient restriction enzymes (see Fig. 2A). Protein
A-tagged proteins were detected by rabbit IgG-horseradish peroxidase
were incubated overnight and fixed in 1% (or 4%)
chicken embryonic fibroblasts, NIH3T3 fibroblasts, and NIH-3T3 cells
were harvested 2–3 days following transfection. Native cell
extracts were prepared as described (29).
Immunoprecipitation—Src antibodies were coupled to protein
A-Sepharose beads (Pharmacia) in IPP 150 buffer following standard
protocols (30). Following two washes in IPP buffer, beads were resus-
pended in 1 ml of IPP buffer and normalized amounts (about 600 µg) of
native protein extract were added. Immunocomplexes were allowed to
form for 3–4 h at 4°C. Complexes were washed three times with IPP 150
and analyzed on 7.5% SDS-polyacrylamide gels. Immunoblotting
was performed as described (5) using ECL detection.

RESULTS
Alignment of the chicken and murine proline-rich Ld
do mains (3, 5) (Fig. 1A) reveals that the non-proline residues
important for mediating interaction with the c-Abl SH3 domain (13; underti
ed in Fig. 1A) are not well conserved. Therefore, possible interactions of
both chicken and murine Ld proteins with different types of SH3 domains were compared using an in
vitro interaction assay (Fig. 1B). Interestingly, Ld proteins of both species bind equally well to SH3 domains of c-Fyn (Fig. 1B, lane 3) and c-Src (Fig. 1B, lane 8). Both Ld proteins also
interact with the SH3 domain of c-Abl (Fig. 1B, lane 1). However,
interaction and binding to the SH3 domains of PLCγ (Fig. 1B, lane 5) and p85 (regulatory subunit of the PI-3 kinase; Fig. 1B, lane 6) is much weaker than binding to Src family
kinases. Furthermore, no binding to the SH3 domains of n-Src
(Fig. 1B, lane 7; containing a 6-amino acid insert in comparison to c-Src; 32), Csk (Fig. 1B, lane 2), and GAP (GTPase activating
protein; Fig. 1B, lane 4) is observed. These results show that Ld
proteins interact preferentially with SH3 domains of Src family
kinases (c-Fyn and c-Src). The high affinity of the Ld-Src SH3
domain interaction is supported by the stability of the protein
complexes under high stringency conditions (see “Experimental
Procedures,” data not shown). Fig. 1C shows that this inter-
action depends on amino acids of the hydrophobic patch of
the SH3 domain, which are essential for binding to specific
ligands (20). Mutating two of these essential amino acids indi-
vidually (W118A, tryptophane at position 118 changed to ala-
inine; P133L, proline at position 133 changed to leucine; con-
ducted by Erpel et al. (20)) results in an almost complete loss of binding to Ld proteins (Fig. 1C, lanes 2 and 3).
Taken together, the results shown in Fig. 1 establish that
murine and chicken Ld proteins possess an apparently identi-
Calbind specificity for SH3 domains and bind best to SH3
domains of Src family kinases. Furthermore, comparative in
vitro binding studies showed that Ld proteins bind c-Src SH3
domains with higher affinity than a WW module (derived from human
YAP65 (33), data not shown).

Several proline-rich consensus binding sites that mediate in
vitro interactions with Src family SH3 domains have been
identified (reviewed in Ref. 34), but none of them is present in
the proline-rich Ld domains (data not shown). Furthermore, Ld
proteins of both species contain two additional short proline-
rich peptides located outside their proline-rich domains (3, 5).
Therefore, a series of deletions of the chicken Ld protein iso-
form IV were generated (Fig. 2A) to establish the importance of
the proline-rich domain in interactions with SH3 domains. The
recombinant Ld proteins were expressed in cultured cells (Fig.
2B, panel input) and assayed in vitro for interaction with the
c-Src SH3 domain (Fig. 2B, panels GST-Src SH3). Deletion of
the carboxyl-terminal domain (Fig. 2A, construct 2) does not
affect interaction with SH3 domains (Fig. 2B, compare lanes 1
and 2). However, deletion of the proline-rich domain results in
complete loss of binding (Fig. 2B, lanes 3). These results show that
the proline-rich Ld domain is essential for binding to SH3
domains.

Transfected COS cells expressing chicken Ld and/or c-Src
proteins were used to study the formation of Ld-Src complexes in
vitro (Fig. 3). Native protein extracts were prepared 2 days
after transfection and normalized for their protein content. c-Src proteins were immunoprecipitated using antibodies

1 The abbreviations used are: mAb, monoclonal antibody; CHAPS, 3-(cyclohexylamino)propanesulfonic acid.
2 C. Dickson, personal communication.
raised against different domains and associated Ld proteins were detected by immunoblotting using Ld antibodies (for details, see "Experimental Procedures"). The specificity of the c-Src-immunoprecipitation was controlled by competition with the peptide used to raise the α2–17 antisera (Fig. 3, compare lanes 1–3 and 4–6). It is important to note that COS cells already express c-Src proteins, whereas they do not express detectable levels of Ld proteins (Ref. 35, and data not shown). Indeed, antibodies raised against the amino- and carboxyl-terminal domains of c-Src proteins both immunoprecipitate Ld-Src protein complexes from transfected cells (Fig. 3, lanes 1–3 and 10–12). Note that significantly more Ld-Src protein complexes are immunoprecipitated after increasing c-Src protein levels by co-transfection (compare Fig. 3, lanes 1 and 10 to lanes 2 and 11). Most importantly, antibodies recognizing an epitope of the c-Src SH3 domain (αSH3, mAb 327) fail to co-precipitate Ld proteins (Fig. 3, lanes 7–9), despite the fact that native Src proteins are well precipitated (e.g. Ref. 22 and data not shown). These results indicate that Ld proteins and anti-SH3 antibodies compete for binding to the SH3 domain. These results independently suggest that the SH3 domain mediates complexing of Ld and Src proteins in intact cells.

Overexpression of Ld proteins in cultured cells causes abundant accumulation in both nucleus and cytoplasm, indicating that c-Src proteins could complex with non-nuclear Ld proteins in transfected COS cells (Fig. 3). Therefore, it was important to determine if the subcellular distributions of the predominantly nucleoplasmic Ld (5) and c-Src proteins show any overlap in fibroblasts. It is well established that c-Src proteins localize predominantly to plasma and perinuclear membranes (endosomes) in cultured fibroblasts (36–38). In an attempt to identify cellular compartments containing both Ld and Src proteins, both immunolocalization and biochemical fractionation studies were performed (Fig. 4). Analysis of primary chicken embryonic fibroblasts by immunofluorescence (using affinity-purified Ld antibodies) revealed the presence of non-nuclear Ld antigens in addition to the previously documented nucleoplasmic localization (Fig. 4A). Non-nuclear Ld proteins were also observed in NIH3T3 fibroblasts (Fig. 4B), where they seem mostly to be associated to cell membranes (arrowheads, Fig. 4B). The presence of membrane-associated Ld proteins was confirmed by biochemical fractionation of cultured cells (Fig. 4C). These results indicated that such non-nuclear Ld proteins could associate with membrane-bound c-Src proteins. Because

Fig. 1. High affinity interaction of Ld proteins (formins) with Src family SH3 domains. A, schematic representation of the two major Ld protein isoforms I (murine) and IV (chicken) and alignment of their proline-rich domains. The peptide used by Ren et al. (13) to study interaction with the c-Abl SH3 domain is underlined. Note that most of the amino-terminal domain of isoforms I and IV differ due to alternative splicing (49), whereas the other domains of chicken and murine Ld proteins are conserved. B, the affinity of in vitro translated (IVT) chicken and murine Ld proteins (isoforms IV (5) and I (3); [35S]methionine labeled) to different types of SH3 domains (expressed in bacteria as GST fusion proteins) was assayed by an in vitro interaction assay (see "Experimental Procedures"). Equal amounts of in vitro translated Ld proteins (lane 10) were incubated with different GST-SH3 fusion proteins bound to glutathione-agarose beads and specifically retained proteins detected by autoradiography. Lanes 1–8 correspond to the GST-SH3 fusions indicated on top. Lane 9, binding to the GST part of the fusion protein to assess non-specific interactions. C, the hydrophobic patch of the c-Src SH3 domain is essential for binding of Ld proteins. Lane 1, Ld binding to wild-type c-Src SH3 domain fusion proteins. Lanes 2 and 3, the SH3 domain mutations W118A and P133L (20) abolish association to Ld proteins.
endogenous c-Src proteins are not easily detected in fibroblasts, c-Src expressing NIH3T3 cells (Ref. 31 and Fig. 4C; see also Refs. 36–38) were used to co-localize c-Src and Ld proteins in optical sections by confocal laser microscopy (Fig. 4, D–F). Ld proteins were detected using affinity-purified antibodies recognizing all known Ld protein isoforms (αFP1; 5) and c-Src proteins were detected using monoclonal antibodies (mAb 327; 24). These studies show that a fraction of Ld (Fig. 4D) and c-Src proteins (Fig. 4E) co-localize to perinuclear (Fig. 4F, black arrowheads) and plasma membranes (Fig. 4F, white arrowheads). The overlap is partial, because c-Src proteins are more widely distributed in plasma and perinuclear membranes than Ld proteins (compare Fig. 4, D–E, and overlap in F). The previously unnoted association (4, 5) of a fraction of Ld proteins with membranes was confirmed by biochemical fractionation of NIH3T3 cells (Fig. 4G; for details see “Experimental Procedures”). Fractionation showed that Ld proteins (180 kDa, 5) are most abundant in the nuclear fraction, but are also detected in membrane fractions. This was never observed for other nuclear proteins such as the c-Jun transcription factor (Fig. 4G). As expected, c-Src proteins are most abundant in the membrane fraction, whereas the nuclear signal is most likely due to residual contamination by perinuclear proteins (see “Experimental Procedures” and data not shown). These studies establish that a fraction of non-nuclear Ld proteins is associated to plasma and perinuclear membranes and co-localizes with c-Src proteins in fibroblasts.

Most proteins interacting with Src family kinases are substrates for phosphorylation at tyrosine residues (reviewed in Refs. 17 and 39). Possible tyrosine phosphorylation of Ld proteins bound to Src kinases was assayed in vivo by kinase assays and antiphosphotyrosine immunoblotting using either Src-Ld complexes from transfected cells (see above) or purified Ld and Src proteins in vitro (data not shown). However, no evidence for phosphorylation of Ld proteins at tyrosine residues has been obtained (in agreement with Ref. 35, and data not shown), indicating that Ld proteins are most likely not substrates for Src tyrosine kinases (see also “Discussion”).

**DISCUSSION**

Genetic analysis of several Ld gene family members in different species has led to the proposal that the encoded proteins function in the establishment of cell and embryonic polarity by regulating either cytoskeletal architecture and/or cell signaling during morphogenesis (for details see Introduction). The vertebrate Ld proteins are predominantly nuclear proteins expressed in a variety of different cell types during embryogenesis (4, 5). However, these studies provided no insights into their molecular function and possible interactions with other proteins. The first evidence for interactions with other proteins was provided by Ren et al. (13). These authors established that a 33-amino acid peptide derived from the proline-rich domain of Ld proteins binds to the c-Abl SH3 domain in vitro. Recently, Chan et al. (16) isolated several SH3 and WW domains which bind in vitro to the proline-rich domain of murine formins. These studies suggested that the proline-rich domain of vertebrate Ld proteins acted as a protein-protein interaction domain, but did not provide evidence for these interactions occurring in vivo. Our studies establish that vertebrate Ld proteins possess high affinity to SH3 domains of Src family tyrosine kinases and show that Ld and c-Src proteins interact in cultured cells. Most intriguingly, this interaction seems to occur between a fraction of non-nuclear Ld proteins co-localizing with membrane-associated c-Src proteins in fibroblasts. These results indicate that subcellular localization of Ld proteins determines interaction with Src family kinases in vivo. Previous studies of the murine Ld gene products identified several form isoforms created by alternative splicing of their amino-terminal domains (3). It is possible that only particular Ld isoforms localize to cell membranes and interact with Src family tyrosine kinases in fibroblasts. The studies by Chan et al. (16) led to the proposal that SH3 domains and WW modules could compete for binding to the same proline-rich Ld domain.
Such competitive binding could mediate different functions of Ld proteins during developmental processes (reviewed by Ref. 15). Alternatively, our studies suggest that differential subcellular localization of Ld proteins (or of particular protein isoforms) could determine or be a consequence of interactions with alternative protein partners.

C-Src proteins are inserted into membranes by myristoylation and localize predominantly to plasma and perinuclear membranes (endosomes and secretory vesicles) (Refs. 36–38 and 40, reviewed by Ref. 17). Most membrane-associated c-Src proteins are inactive, whereas activated Src tyrosine kinases translocate to focal adhesions and phosphorylate adhesion plaque proteins (41, 42). Interestingly, Ld proteins associate with c-Src proteins at plasma and perinuclear membranes, but no co-localization at focal adhesions has been observed. Furthermore, association with c-Src proteins does not result in detectable tyrosine phosphorylation of Ld proteins (see also Ref. 35). Interestingly, YAP65, a proline-rich protein binding to the Yes tyrosine kinase via its SH3 domain, is also not a tyrosine kinase substrate (43). These results show that not all proteins interacting with Src family kinases are subject to tyrosine phosphorylation and suggest a different functional relevance of their interaction with Src tyrosine kinases. Experimental evidence suggests that proteins binding to kinases via SH3 domains can also regulate their subcellular localization, activate or repress kinase activity (as shown for the Sin protein; 44), or affect phosphorylation of substrates by competitive binding (reviewed in Refs. 17, 18, 39, and 45). Therefore, it is possible that Ld proteins exert some of their functions by altering subcellular localization and/or activity of Src family kinases and possibly other components of signal transduction cascades.

Taken together, our studies provide strong evidence for direct molecular interactions of Ld proteins with membrane-associated Src tyrosine kinases in fibroblasts, but the functional relevance of this interaction remains unclear. A combination of genetic and biochemical studies will be necessary to identify the proteins relevant during embryonic pattern formation. However, recent genetic and embryological analysis of Ld mutant mouse embryos provides independent evidence for direct interactions of Ld proteins with signaling cascades (10, 11). These studies show that establishment of the fibroblast growth factor-4/SHH signaling feedback loop is disrupted in limb buds of Ld mutant embryos. Interestingly, it has been shown that the c-Src kinase associates with fibroblast growth factor receptor-1 and that this association triggers tyrosine phosphorylation of Src substrates (46). Furthermore, constitutive activation of Src family kinases in embryos lacking a
functional Csk gene causes severe alterations of embryogenesis
(47, 48). These studies show that tight regulation of Src family
kinases is essential for normal progression of development.
Therefore, it is possible that molecular interaction of Ld pro-
tein with Src family kinases directly links Ld gene products to
the embryonic signaling cascades disrupted in ld mutant limb
buds (10, 11).

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