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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). Little is known about their molecular functions, but comparison of different Ld family members shows they share two structurally and probably functionally conserved domains. First, a part of their carboxyl-terminal domains is highly conserved (6, 7) and disruption of this domain in several of the murine *ld* and *D. melanogaster capu* alleles causes the phenotypic alterations observed in mutant embryos (7, 12). Second, all family members contain a proline-rich domain separating the conserved carboxyl- from the amino-terminal domain. Proline-rich domains function either as molecular hinges or interact with proteins encoding SH3 or WW domains (13–15). Indeed, *in vitro* binding studies using the proline-rich domain of murine Ld proteins (formins; 3) revealed specific binding to the c-Abl SH3 domain (13) and several novel WW domains (16). Furthermore, the genetic interaction of *Capu* and profilin seems to be mediated by the direct binding of profilin to the proline-rich domain of the Capu protein (9).

Our study focuses on the interactions of vertebrate Ld proteins with SH3 domain containing proteins, the identification of possible *in vivo* partners and the cellular compartments where interactions occur. SH3 domain containing proteins are of particular interest, because they are known to participate in signal transduction pathways and/or associate with the cytoskeleton (reviewed in Refs. 17 and 18). These pathways and structures are impaired by mutations affecting Ld family members (6, 7, 10, 11). Our initial *in vitro* binding assays establish that the chicken and murine Ld proteins interact best with SH3 domains of Src family tyrosine kinases (c-Src and c-Fyn) and they possess a much lower affinity to other types of SH3 domains tested. We show that interaction of Ld with c-Src proteins is mediated by the proline-rich domain of Ld and the SH3 domain of Src. Studies using transfected cells show that Ld-c-Src protein complexes form in intact cells. Immunolocalization and biochemical fractionation studies were performed to establish in which cellular compartment the two proteins interact. These studies show that chicken embryonic and mouse NIH3T3 fibroblasts contain non-nuclear Ld proteins, a fraction of which co-localizes with c-Src proteins at plasma and perinuclear membranes. Our studies establish that Ld proteins can interact with membrane-associated Src family kinases *in vivo* and propose direct molecular links of Ld proteins with signal transduction pathways.

EXPERIMENTAL PROCEDURES

Production of Ld Protein by in Vitro Translation—The Ld proteins (murine isoform I and chicken isoform 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 Abl-SH3 construct was made by polymerase chain reaction amplifying its SH3 domain from a mouse Abl-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—Antisera 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 (αSH3, mAb[1] 327; 24). A polyclonal antiserum recognizing the carboxyl-terminal tail was also used (αKin, antiserum cst.1; 25). The Ld proteins were detected using affinity-purified polyclonal antibodies directed against its highly conserved carboxyl-terminal domain (5). Ld antibodies were affinity-purified using bacterially expressed fusion proteins encoding either the chicken or murine carboxyl-terminal domains.

In Vitro Interaction Assay—A standard *in vitro* binding assay was used (*e.g.* Ref. 22) to study the interactions of Ld proteins with different GST-SH3 fusion proteins: 5–10 μg of GST-SH3 fusion protein was bound to 20 μl of glutathione-agarose beads (packed volume, Sigma). After washing in IPP 150 (150 mM NaCl, 10 mM Tris, pH 8, 0.1% Nonidet P-40, 0.1% sodium azide), the complexes were resuspended in 1 ml of IPP buffer and equal amounts of *in vitro* translated Ld proteins or native protein extracts were added. Binding was performed at 4 °C for 2–3 h. Complexes were washed 3 times with IPP 150 buffer and then analyzed on 7.5% SDS-polyacrylamide gels. RIPA buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton, 1% deoxycholate, 0.1% SDS) was used for washing under high stringency conditions.

Expression of Protein A-tagged Ld Proteins in Cultured Cells—A protein A tag containing four z-domains (26) was inserted close to the amino terminus of the chicken Ld isoform IV and several deletions were generated using convenient restriction enzymes (see Fig. 2*A*). Protein A-tagged proteins were detected by rabbit IgG-horseradish peroxidase conjugates (27) in combination with enhanced chemiluminescense (ECL, Amersham). The constructs were expressed in quail QT6 cells and native protein extracts were used for *in vitro* interaction assays.

Transfection of Cultured Cells and Preparation of Native Protein Extracts—Chicken c-Src proteins were expressed using a pSG5-derived vector (20), whereas Ld proteins were expressed using the Rc/CMV vector (Invitrogen). Cells were transfected after reaching about 80% confluency. The Ld and/or Src expression constructs (or vector for controls; 10 μg of DNA per 10-cm dish) were transfected using the calcium phosphate technique (28). The medium was changed 16 h later and 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 resuspended 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.

Biochemical Fractionation of Cultured Cells—Cells were harvested by scraping them into phosphate-buffered saline. All solutions contained protease inhibitors. Following centrifugation, cell pellets were equilibrated in hypotonic buffer (1 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl) and incubated on ice for 10 min. Subsequently cells were homogenized using a Dounce homogenizer (pestle B) and nuclei pelleted by centrifugation. The initial supernatant was recentrifuged at 100,000 × *g* for 1 h to separate membranes (pellet) and cytosolic fractions (supernatant). In parallel, the initial nuclear pellet was resuspended in 1 ml of Cu1 buffer (20 mM HEPES 7.9, 0.3 M sucrose, 1.5 mM $MgCl_2$, 0.2 mM EDTA) and nuclei were enriched further by pelleting through a cushion of Cu2 buffer (as Cu1 buffer, but containing 0.9 M sucrose, 5,000 × *g* for 15 min). The second nuclear pellet was washed twice in TESM-CHAPS (10 mM Tris-HCl, pH 7.5, 1 mM EGTA, 0.25 M sucrose, 2 mM $MgCl_2$, 2% CHAPS) to remove associated perinuclear material and nuclear envelopes.[2] Nuclear proteins were extracted by incubation in 5 volumes of NE buffer (10 mM HEPES, pH 7.9, 400 mM NaCl, 100 μM EGTA, 5% glycerol, 0.5 mM dithiothreitol, and proteinase inhibitors) for 10 min. Protein extracts were normalized by silver staining and equal amounts analyzed by immunoblotting and ECL. The quality of fractionation was assessed using tubulin (α-tubulin; Sigma) and c-Jun (α-Jun; Santa Cruz Biotechnology) as marker proteins for cytosolic and nuclear fractions, respectively.

Co-localization of c-Src and Ld Proteins by Immunofluorescence—Chicken embryonic fibroblasts, NIH3T3 fibroblasts, and NIH-3T3 cells expressing the wild-type chicken c-Src protein (31) were plated on gelatinized coverslips, grown overnight, and fixed in 1% (or 4%) paraformaldehyde for 30 min. Ld proteins were detected using affinity-purified polyclonal Ld antibodies (FP1; recognizing all known Ld protein isoforms, 5) and rhodamine-coupled secondary antibodies (goat α-rabbit; Cappel). c-Src proteins were detected using monoclonal αSH3 (mAb 327, Ref. 24) antibodies and fluorescein-coupled secondary antibodies (goat α-mouse, Jackson Immuno Research). Antibody incubations, washes, and detection were performed as described previously by Trumpp *et al.* (5). Results were analyzed either by conventional immunofluorescence or optical sections taken from a confocal laser microscope.

RESULTS

Alignment of the chicken and murine proline-rich Ld domains (3, 5) (Fig. 1*A*) reveals that the non-proline residues important for mediating interaction with the c-Abl SH3 domain (13; *underlined* in Fig. 1*A*) 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. 1*B*). Interestingly, Ld proteins of both species bind equally well to SH3 domains of c-Fyn (Fig. 1*B, lane 3*) and c-Src (Fig. 1*B, lane 8*). Both Ld proteins also interact with the SH3 domain of c-Abl (Fig. 1*B, lane 1*). However, this interaction and binding to the SH3 domains of PLCγ (Fig. 1*B, lane 5*) and p85 (regulatory subunit of the PI-3 kinase; Fig. 1*B, lane 6*) is much weaker than binding to Src family kinases. Furthermore, no binding to the SH3 domains of n-Src (Fig. 1*B, lane 7*; containing a 6-amino acid insert in comparison to c-Src; 32), Csk (Fig. 1*B, lane 2*), and GAP (GTPase activating protein; Fig. 1*B, 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. 1*C* shows that this interaction 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 individually (W118A, tryptophane at position 118 changed to alanine; P133L, proline at position 133 changed to leucine; constructs generated by Erpel *et al.* (20)) results in an almost complete loss of binding to Ld proteins (Fig. 1*C, lanes 2* and *3*). Taken together, the results shown in Fig. 1 establish that murine and chicken Ld proteins possess an apparently identical binding 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 isoform IV were generated (Fig. 2*A*) to establish the importance of the proline-rich domain in interactions with SH3 domains. The recombinant Ld proteins were expressed in cultured cells (Fig. 2*B, panel input*) and assayed *in vitro* for interaction with the c-Src SH3 domain (Fig. 2*B, panels GST-Src SH3*). Deletion of the carboxyl-terminal domain (Fig. 2*A*, construct *2*) does not affect interaction with SH3 domains (Fig. 2*B*, compare *lanes 1* and *2*). However, deletion of the proline-rich domain results in complete loss of binding (Fig. 2*B, 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 vivo* (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.

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); [^{35}S]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 nonspecific 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.

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,[3] 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 immunofluorescense (using affinity purified Ld antibodies) revealed the presence of non-nuclear Ld antigens in addition to the previously documented nucleoplasmic localization (Fig. 4*A*). Non-nuclear Ld proteins were also observed in NIH3T3 fibroblasts (Fig. 4*B*), where they seem mostly to be associated to cell membranes (*arrowheads*, Fig. 4*B*). The presence of membrane-associated Ld proteins was confirmed by biochemical fractionation of cultured cells (Fig. 4*G*). These results indicated that such non-nuclear Ld proteins could associate with membrane-bound c-Src proteins. Because

[3] J. L. de la Pompa and R. Zeller, unpublished observations.

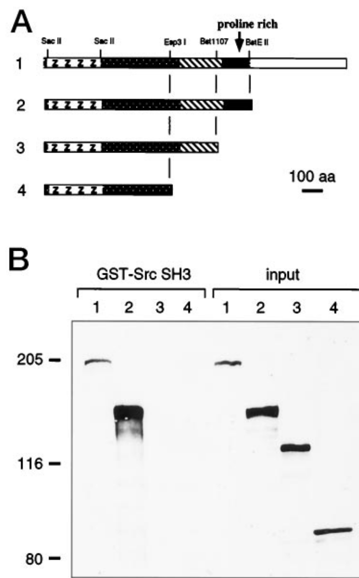

FIG. 2. **The proline-rich Ld domain is essential for interaction with the c-Src SH3 domain.** *A,* schematic representation of the deletion constructs used. The chicken isoform IV was tagged with four protein-A Z domains to enable immunoblot detection of the truncated proteins by rabbit IgG-horseradish peroxidase conjugates. *B, in vitro* binding of the full-length and truncated Ld proteins to the c-Src GST-SH3 fusion protein. The full-length and truncated Ld proteins were expressed in transiently transfected QT6 cells and native extracts containing the proteins (*panel input, lanes 1–4*) used for the *in vitro* interaction assay (*panel GST-SH3 fusion, lanes 1–4*). The protein A-tagged Ld proteins were detected by ECL immunoblot analysis. The Ld proteins present in *lanes 1–4* correspond to the constructs 1 to 4 shown in *A*.

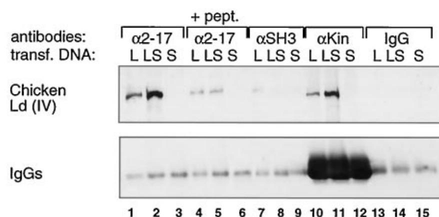

FIG. 3. **Ld proteins associate with c-Src tyrosine kinases in transfected cells.** Full-length chicken Ld protein alone (*L; lanes 1, 4, 7, 10,* and *13*), together with c-Src (*LS; lanes 2, 5, 8, 11,* and *14*), or c-Src protein alone (*S; lanes 3, 6, 9, 12,* and *15*) were expressed in COS cells. Non-transfected COS cells already express the c-Src tyrosine kinase, but Ld proteins are not detected. Two days after transfection normalized native protein extracts were prepared and immunoprecipitated using c-Src antibodies. Associated Ld proteins were detected by ECL immunoblotting using specific antibodies. *Lanes 1–6*, immunoprecipitation of c-Src protein complexes using antibodies (α2–17) raised against a Src peptide containing amino acids 2–17 (peptide 2–17; Ref. 22). Immunoprecipitation of c-Src-Ld complexes is significantly reduced by adding an excess of peptide 2–17 to the reaction (competition experiments shown in *lanes 4–6*; +peptide). *Lanes 7–9*, immunoprecipitation using antibodies raised against the SH3 domain of c-Src (αSH3, mAb 327) (24). Note that no c-Src-Ld complexes are detected. *Lanes 10–12*, immunoprecipitation using antibodies raised against the c-Src kinase domain (αKin, Cst.1) (25). *Lanes 13–15*, control immunoprecipitation using preimmune IgGs. The panel "*IgGs*" shows the IgGs recovered after immunoprecipitation and controls for quantitative recovery of immune complexes. Note that the αKin antibodies were used as crude serum (*lanes 10–12*), whereas all other antibodies were affinity-purified.

endogenous c-Src proteins are not easily detected in fibroblasts, c-Src expressing NIH3T3 cells (Ref. 31 and Fig. 4*C*; 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 monclonal antibodies (mAb 327; 24). These studies show that a fraction of Ld (Fig. 4*D*) and c-Src proteins (Fig. 4*E*) co-localize to perinuclear (Fig. 4*F, black arrowhead*) and plasma membranes (Fig. 4*F, 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. 4*G*; 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. 4*G*). 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 vitro* 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 to 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 formin 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.

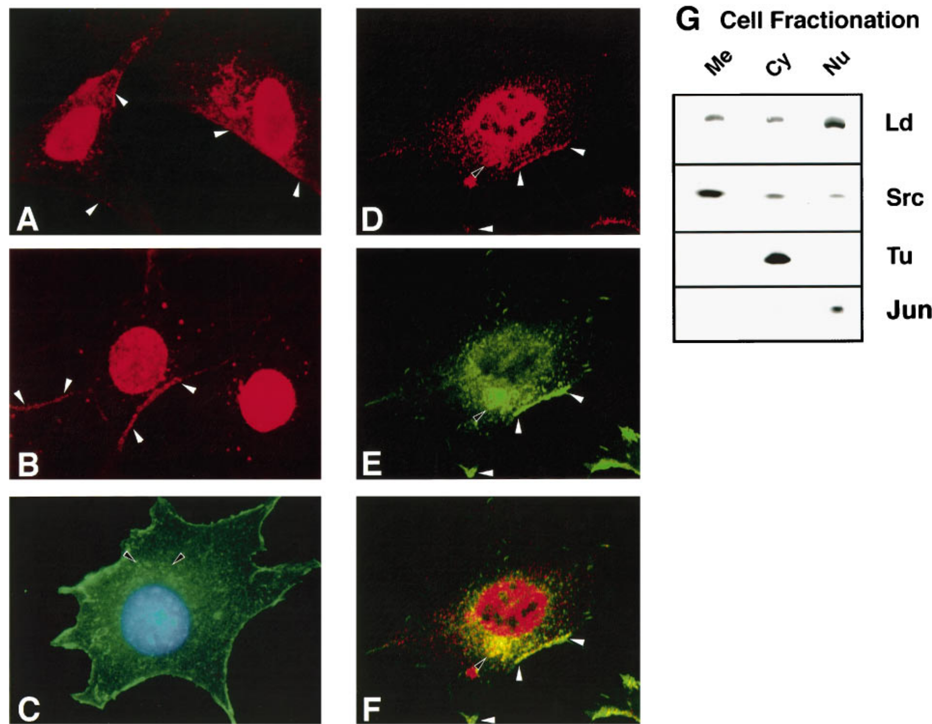

FIG. 4. **A fraction of Ld proteins is membrane-associated and co-localizes with the c-Src tyrosine kinase in cultured cells.** *A*, localization of the Ld antigen in cultured primary chicken embryonic fibroblasts. Note that chicken embryonic fibroblasts consist of mixed fibroblast populations. Only cells containing significant levels of non-nuclear Ld proteins are shown. *B,* localization of Ld proteins in NIH3T3 fibroblasts. Note that the cell located to the left contains non-nuclear Ld proteins and that fewer such proteins are detected in the cell located on the right. *A* and *B, white arrowheads* point to membrane-associated Ld antigens. *C,* c-Src distribution in wild-type chicken c-Src expressing NIH3T3 fibroblasts (31). Note the predominant localization to plasma and perinuclear membranes (*white lined arrowheads*). Small amounts of antigen are detected in cell nuclei (counterstained with Hoechst 33258). *D-F,* co-localization of Ld and c-Src proteins in optical sections of c-Src expressing NIH3T3 cells using confocal laser microscopy. The cell shown is representative for the average co-localization of the two antigens (see also *panel B*). *White arrowheads* point to membrane-associated co-localization, *white lined arrowheads* point to perinuclear co-localization of both antigens (see F). *D,* distribution of Ld antigen. *E,* distribution of the c-Src antigen. *F,* co-localization (yellow) of Ld (red, *panel D*) and c-Src antigens (green, *panel E*) in parts of the plasma (*white arrowheads*) and perinuclear membranes (*white lined arrowheads*). *G,* biochemical fractionation of NIH3T3 fibroblasts reveals the presence of membrane-associated Ld proteins (180 kDa). Cells were fractionated as described under "Experimental Procedures" and equal amounts of total proteins (about 50 μg) analyzed by immunoblotting using different types of antibodies. Note that Ld proteins are detected in all three fractions with levels being highest in the nuclear and lowest in the cytosolic fraction. c-Src proteins are most abundant in the membrane fraction. The c-Src proteins detected in the nuclear fraction most likely correspond to residual contamination by perinuclear membranes. *Me*, membrane fraction; *Cy*, cytosolic fraction; *Nu*, nuclear fraction; *Ld*, Ld protein; *Src*, c-Src tyrosine kinase; *Tu*, tubulin, marker to assess cytosolic fraction and possible cross-contamination; *Jun*, c-Jun protein, marker to assess nuclear fraction and possible cross-contamination.

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.[4] 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

[4] R. Zeller, unpublished observations.

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 proteins 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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