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The Molecular Basis of Src Kinase Specificity during Vertebrate Mesoderm Formation*

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Members of the Src family of non-receptor tyrosine kinases play a critical role in mesoderm formation in the frog, *Xenopus laevis*, acting as required mediators downstream of the fibroblast growth factor receptor. At least four members of this gene family, Src, Fyn, Yes, and Laloo, are expressed during early embryonic development. Ectopic expression of Laloo and Fyn, but not Src, induce mesoderm in ectodermal explants, indicating that these factors are non-redundant during early vertebrate development. Here we investigate the basis for the differential activity of the Src and Laloo kinases during mesoderm formation. We demonstrate that although both Src and Laloo physically interact with the substrate protein SNT-1/FRS2α only Laloo phosphorylates SNT-1, an event previously shown to be required for the activity of the latter and for mesoderm induction in *vivo*. We show that Src is enzymatically capable of stimulating mesoderm formation, as an activated Src construct both phosphorylates SNT-1 and induces mesoderm in explant cultures. However, a chimeric Laloo construct containing a Src C-terminal tail is inactive, suggesting that the early embryo contains a specific Laloo-activating, or Src-inactivating, factor. Finally, through further chimeric analysis, we provide evidence to suggest that differences in Laloo and Src activity are also mediated by the SH2, SH3, and kinase domains of these molecules.

The mesodermal germ layer plays a fundamental role in organizing the vertebrate body axes and gives rise to the skeletal, muscular, and circulatory systems. In the frog, *Xenopus laevis*, members of both the transforming growth factor-β and fibroblast growth factor (FGF) ligand families have been shown to play critical roles during mesoderm formation (1). In recent years, a model has emerged in which members of the Src family of non-receptor tyrosine kinases serve as central mediators of the signal transduction cascade initiated by activation of the FGF receptor. Both the *Xenopus* Src kinase Laloo and the related *Xenopus* Fyn induce mesoderm in ectodermal (animal cap) explants (2–4). Induction by these factors is blocked by reagents that inhibit signaling downstream of the FGF receptor; conversely, Src family kinase activity is required for mesoderm induction by FGF (3, 4).

The epistasis studies described above indicate that the Src kinases function as required components of the cascade triggered by the FGF receptor during mesoderm formation. The molecular interactions underlying this requirement have begun to emerge. A physical association between Laloo and the FGF receptor-associated scaffolding protein SNT-1/FRS2α has recently been demonstrated (5, 6). SNT-1 contains six tyrosine residues, which upon phosphorylation serve as binding sites for both the Grb2 adaptor protein and the Shp2 phosphatase (7–9). An unphosphorylatable form of SNT-1 prevents mesoderm induction by FGF downstream of Laloo, suggesting that Laloo functions to recruit Shp2 and Grb2 to SNT-1 by phosphorylation of SNT-1 (5, 6). The demonstration that an activated Laloo mutant can phosphorylate exogenous SNT-1 supports such a role for endogenous Laloo (5).

An initial challenge in elucidating the mechanism of Src kinase function during mesoderm formation has been to determine which members of this sizeable protein family are directly involved in mediating the mesoderm-inducing signal. In addition to Laloo, the Src, Fyn, and Yes kinases are also expressed during early *Xenopus* development (2, 10, 11). Although *Xenopus* Fyn activity is indistinguishable from that of Laloo, *Xenopus* Src, with a primary sequence very similar to that of Fyn, is completely inactive in mesoderm induction assays (4).

We have attempted to dissect the molecular basis for differences in the behavior of these related proteins, anticipating that such studies would provide us with valuable insights on the mechanisms by which these factors function during early development. Here, we demonstrate that Src, like Laloo, physically associates with SNT-1; however, although Laloo effectively phosphorylates tyrosine residues on SNT-1, Src does not. We then demonstrate that SNT-1 may serve as a Src substrate under some conditions, as an activated form of Src both induces mesoderm and phosphorylates SNT-1. We provide evidence suggesting that Src inactivity during early development is due to the action of a factor that specifically recognizes the C-terminal tail of Src but not a similar motif in Laloo. Finally, we...
demonstrate that the SH3, SH2, and kinase domains of Laloo may additionally contribute to differences in activity to the two molecules.

**EXPERIMENTAL PROCEDURES**

**RNA Preparation, Explant Dissection, and Cell Culture—**RNA was synthesized in vitro in the presence of the cap analog using the mMessage mMACHINE kit (Ambion). Microinjection, explant dissection, and culture were performed as described (12, 13).

**Preparation of Laloo, Src, and SNT-1 Constructs—**SNT-1-Myc and Laloo-FLAG construction were described previously (6, 14). Laloo-FLAG includes the sequence DYKDDDDK at the Src C terminus. SrcY526F was generated by PCR-based mutagenesis (TAC→TTG) at nucleotide 1579 of _Xenopus_ Src. Domain-swap hybrids were constructed by PCR; domains were defined as corresponding to the following amino acids (Laloo accession number AAC31209; _Xenopus_ Src accession number AAA99692): Laloo SH2, 14–55; Laloo SH3, 56–117; Laloo SH3, 118–232; Laloo kinase, 233–483; Laloo tail, 484–496; Src SH2, 1–85; Src SH3, 86–147; Src SH2, 148–255; Src kinase, 256–517; Src tail, 518–532.

**RT-PCR—**RT-PCR was performed as described (3, 13). All primer sequences are as described (3, 12, 15).

**Xenopus Co-immunoprecipitation Assay—**50 pg of SrcY526F and 1 ng each of Laloo-FLAG, Src-FLAG, and/or SNT-1-Myc RNA were injected into the animal poles of early cleavage stage embryos. Embryos were lysed after 6 h in 500 μl of lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, and 1 Complete mini tablet/10 ml of buffer (Roche)). For studies examining tyrosine phosphorylation of SNT-1, lysis buffer included 1 mM sodium orthovanadate. After 30 min of incubation on ice, lysates were centrifuged twice, 2 min each at 4 °C, at 14,000 g. After each centrifugation, only the clear lysate was retained. Lysates were incubated overnight at 4 °C with 1:1000 antibody (anti-FLAG M2 monoclonal or anti-Myc tag monoclonal 9E10 (Sigma)), followed by incubation with protein A/G PLUS-agarose (Santa Cruz Biotechnology) for 1 h at 4 °C. After four washes in lysis buffer, protein was eluted in 0.1 M glycine, pH 3.5, neutralized with wash buffer (0.05% Tris, pH 7.4, 0.15% NaCl), and subjected to standard SDS-PAGE and Western blotting protocols.

**RESULTS**

**Src Is Expressed but Inactive in the Mesoderm during Gastrulation—**The expression patterns and activities of _Xenopus_ FGFRs are consistent with a model in which FGFR pathway activation within the gastrula stage marginal zone is required for the proper response to and maintenance of mesoderm induction. Although both maternal and zygotic _Xenopus_ src transcripts have been identified (10, 15), the localization of _Xenopus_ src during gastrulation has not been characterized. As shown in Fig. 1A, src is present throughout the gastrula stage embryo, including in the cells of the dorsal and ventral marginal zone (Fig. 1A). Thus, _Xenopus_ src, like _laloo_, is expressed in the cells of the presumptive early mesoderm, consistent with a role for these factors in mesoderm formation.

Although previous studies of Src function in mesoderm formation have been performed by introducing src transcripts into competent ectoderm, it is conceivable that Src, unlike Laloo, requires a mesodermal context for activation. To address this possibility, we examined Src activity in embryonic mesoderm. It was not possible to assay for Src function directly in the marginal zone, the site of endogenous mesoderm formation, because of the high levels of mesoderm-specific marker genes already expressed in these cells; this strong baseline expression precluded detection of additional mesoderm induction by ectopic Src. As an alternative approach, we examined whether ectopic Src could boost mesoderm formation by low levels of the mesoderm-inducing factor, basic FGF (bFGF). As shown in Fig. 1B, 25 ng/ml bFGF is a potent inducer of both the panmesodermal marker _Xbra Chunyu_ (Xbra) and the ventrolateral mesodermal marker _Xwnt8_. (Fig. 1B, lane 1; Refs. 16–18). 1 ng/ml bFGF induces low levels of these markers (Fig. 1B, lane 2). Expression of high doses of src mRNA does not induce expression of these markers (Fig. 1B, lane 3; Ref. 4); furthermore, Src expression does not enhance mesoderm induction by 1 ng/ml bFGF (Fig. 1B, compare lanes 2 and 4). Thus, Src misexpression cannot stimulate mesoderm formation in either an ectodermal or a mesodermal context.

**Src Interacts with the Laloo Kinase Substrate SNT-1/FRS2α—**Laloo physically interacts with SNT-1 and phosphorylates tyrosine residues on SNT-1 (5, 6). Because phosphorylatable SNT-1 is required for mesoderm induction by Laloo (5, 6), we examined whether Src inactivity in the mesoderm induction assay could be due to an inability to bind SNT-1. Towards this end, we expressed epitope-tagged SNT-1 with either tagged Laloo or Src in early cleavage stage embryos and performed co-immunoprecipitation assays on lysates from these embryos that were harvested at blastula stages. As shown in Fig. 2, both Laloo and Src physically associate with exogenous SNT-1 (Fig. 2, lanes 4 and 5). These results suggest both that endogenous Src interacts with SNT-1 and that differences in SNT-1 affinity alone are unlikely to underlie the difference in activity of these kinases during mesoderm induction.

**Src Does Not Phosphorylate SNT-1—**Studies using an activated Laloo construct suggest that Laloo phosphorylates SNT-1 on one or more of the tyrosine residues that serve as SH2 and Grb2 binding sites (5). As shown in Fig. 3, expression of wild-type Laloo similarly triggers the tyrosine phosphorylation of SNT-1 (Fig. 3, lane 2). Notably, however, ectopic expression of Src does not induce any appreciable tyrosine phosphorylation of exogenous SNT-1 (Fig. 3, lane 2). Because SNT-1 tyrosine phosphorylation is required for mesoderm induction by Laloo...
and in vivo, this result strongly suggests that Src fails to induce mesoderm because it does not phosphorylate tyrosine residues on SNT-1.

Role of the Src C-terminal Tail—The inability of Src to phosphorylate SNT-1 suggests that SNT-1 is an incompatible substrate for Src and/or that Src activity is inhibited during mesoderm formation. To distinguish between these possibilities, we constructed an activated Src mutant. All Src family kinases contain a C-terminal tyrosine residue that when phosphorylated significantly inhibits the function of these proteins (19). We generated a tyrosine-to-phenylalanine mutation in the corresponding residue (Tyr-526) of *Xenopus* Src. mRNA synthesized from this construct, SrcY526F, was first examined for its ability to induce phosphorylation of SNT-1. In co-expression assays, SrcY526F consistently phosphorylated SNT-1 on tyrosine (Fig. 4A, lane 2). Levels of SNT-1 phosphorylation were not as robust as that seen following co-expression of SNT-1 and Laloo; this may be due to differences in the levels of Laloo and SrcY526F protein expression.

We next examined whether SrcY526F was active in the mesoderm induction assay. As shown in Fig. 4B, injection of 50 pg of SrcY526F RNA is sufficient to induce both Xbra and Xwnt8 expression (Fig. 4B, lane 5); 4 ng of wild-type src RNA, in contrast, produced no induction of either marker (Fig. 4B, compare lanes 1 and 5; Ref. 4). SrcY526F RNA is thus able to induce mesoderm at doses similar to those observed for activated Laloo (3).

In order to explore further the role of the Src C terminus in the regulation of mesoderm formation, we generated Src-Laloo chimeric molecules in which the C-terminal tails of the two proteins, including the regulatory tyrosine residue, were exchanged. These reagents were then tested for mesoderm-inducing activity in the animal cap assay. As shown in Fig. 4C, both Laloo with the Src tail (lane 1, LST) and Src with the Laloo tail
(lane 2, SLT) are inactive. This inactivity is not due solely to the elimination of native sequence, because deletion of the tail regions of either Src or Laloo produce activated molecules of comparable potency to LalooY492F and SrcYS26F (data not shown). Taken together, these results demonstrate that the Src kinase is capable of SNT-1 phosphorylation and mesoderm induction and suggest that the Src C terminus inhibits Src activity in the early embryo.

Activity of Additional Src-Laloo Chimeras—Although the experiments described above indicate that the activated Src kinase can induce mesoderm, the inactivity of SLT suggests that there may be other regions of Laloo that preferentially activate the molecular cascade that ultimately results in mesoderm formation. To examine this possibility, we generated additional chimeric molecules in which the SH4, SH3, SH2, and kinase domains of Laloo and Src were individually exchanged. mRNA derived from these constructs were then tested in the mesoderm induction assay. As shown in Fig. 5, exchange of the SH4 domains of Laloo and Src had no effect; i.e. Laloo with the Src SH4 (Fig. 5A, lane 2, S4L) domain remained active, and Src with the Laloo SH4 (Fig. 5A, lane 1, L4S) domain remained inactive. However, Src chimeras that contain either the Laloo SH3 (Fig. 5B, lane 2, S(L3)S); SH2 (Fig. 5C, lane 2, S(L2)S); or kinase (Fig. 5D, lane 2, S(LK)S) domains induced the expression of both Xbra and Xwnt8 at doses similar to those required for wild-type Laloo. Laloo chimeras with the Src SH3 (Fig. 5B, lane 1, L(S3)L); SH2 (Fig. 5C, lane 1, L(S2)L); or kinase (Fig. 5D, lane 1, L(SKL)S) domains remained active. These data, summarized in Fig. 5E, indicate that the Laloo SH3, SH2, and kinase domains are independently sufficient to activate Src in the mesoderm induction assay but are not specifically necessary for Laloo activity.

Regulation of Src-Laloo Chimeras—The chimeric analyses described above suggest important roles for multiple Src homology domains during Src kinase-mediated mesoderm induction. It remains possible, however, that the Src-Laloo chimeras are regulated differently and/or induce mesoderm via molecular mechanisms distinct from those that govern, or are governed by, the endogenous Src kinases. Such a possibility complicates the interpretation of these data. To address these issues, we next examined the upstream regulation of the Src-Laloo domain-swap mutants. We decided to focus on those Src constructs containing an activating Laloo region, because these display a striking alteration of function mediated solely by the exchange of a single functional domain. Thus, they are the constructs for which it is most critical to determine faithful intracellular regulation.

We have previously demonstrated that mesoderm induction by the Src-related kinases is inhibited by co-expression of a dominant inhibitory FGF receptor construct (XFD) (3, 4, 20). However, a constitutively active Laloo mutant in which the C-terminal regulatory tyrosine is mutated to phenlyalanine (Y492F) is insensitive to FGF receptor blockade, suggesting that signals downstream of the receptor dephosphorylate the C-terminal tyrosine residue and activate wild-type Laloo (3). As shown in Fig. 6, inhibition of FGF receptor signaling significantly inhibits mesoderm induction by the domain-swap mutants S(L3)S (compare lanes 1 and 2), S(LK)S (compare lanes 3 and 4), and S(L2)S (compare lanes 5 and 6). These data suggest that the chimeric molecules are not constitutively active and are regulated in a manner similar to that of Laloo.

We next examined the molecular targets of the activated domain-swap mutants. We have shown that Laloo, but not Src, phosphorylates tyrosine residues on SNT-1 and that SNT-1 mutants lacking tyrosine phosphorylation sites block mesoderm induction by the Src kinases (Fig. 3 and Ref. 6). These data strongly suggest that the Src-related proteins induce mesoderm via the phosphorylation of SNT. In Fig. 7, we demonstrate that the activated Src domain-swap constructs S(L3)S (lane 3), S(LK)S (lane 5), and S(L2)S (lane 6), like Laloo (lane 1) but unlike Src (lane 2), phosphorylate SNT-1. Furthermore, mesoderm induction by these chimeric proteins is inhibited by co-expression of 6YF, an unphosphorylatable form of SNT-1 (data not shown). These results suggest that the activated domain-swap constructs used in this study induce mesoderm through the phosphorylation of SNT-1, in a manner similar to that of wild-type Laloo.

DISCUSSION

Members of the Src kinase family play an essential role in vertebrate mesoderm formation, functioning as mediators of a
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Chordin is a dorsal mesodermal marker.

lysates (bottom lanes). The tagged SNT-1 protein was determined by Western blot analysis of cell lysis and P. Wilson for critical reading of the manuscript.

EF1-α was used as a loading control. Xbra is a ventrolateral mesodermal marker.

SNT-1 phosphorylation by activated Src-Laloo domain-swap hybrids. Western blot analysis of SNT-1 immunoprecipitation. Synthetic RNA from epitope-tagged constructs was injected into early cleavage stage embryos; 1 ng each of S(L3)S, S(L2)S, and S(LK)S RNA, and 2 ng of a truncated FGF receptor construct (XFD, Ref. 20) were injected as listed. EF1-α was used as a loading control.

Src family proteins, including Laloo.

negative regulation of the Src kinases is mediated by a number of intramolecular interactions involving the SH3, SH2, and C-terminal domains of these molecules (22). The activity of our Laloo-Src chimeras thus likely reflects alterations in both intra- and intermolecular stability. LST may be inactive in part because the phosphorylated Src tail binds with more affinity than the Laloo tail to the Laloo SH2 domain. However, because activated Src but not SLT induces mesoderm, the Src SH2 domain appears to bind both the Laloo and Src tails with sufficient affinity to inhibit kinase function in these constructs. Further complicating such a scenario, L(S2)L is active, suggesting that SLT inactivity is not only due to interactions between the Src SH2 domain and the Laloo tail.

When considered in aggregate, our results point to the contribution of inter- as well as intramolecular interactions underlying the differences in the ability of Src and Laloo to participate in the activation of a mesoderm-forming signaling cascade. SNT-1 is a substrate of Src family proteins, including Laloo.

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FIG. 6. Inhibition of Src-Laloo domain-swap hybrids by a dominant negative FGF receptor construct. RT-PCR analysis of animal caps dissected at late blastula stages and cultured until midgastrula stages. 1 ng each of S(L3)S, S(L2)S, and S(LK)S RNA, and 2 ng of a truncated FGF receptor construct (XFD, Ref. 20) were injected as listed. EF1-α was used as a loading control. Xbra is a panmesodermal marker. Chordin is a dorsal mesodermal marker. Xwnt8 is a ventrolateral mesodermal marker.

FIG. 7. SNT-1 phosphorylation by activated Src-Laloo domain-swap hybrids. Western blot analysis of SNT-1 immunoprecipitation. Synthetic RNA from epitope-tagged constructs was injected into early cleavage stage embryos; 1 ng each of SNT-1-Myc, Laloo, Src, S(L3)S, S(LK)S, and S(L2)S RNA were injected as listed. Cell lysates were collected at late blastula stages. Myc-tagged SNT-1 protein was immunoprecipitated using an anti-Myc antibody. SNT-1 tyrosine phosphorylation was detected by Western blot (top lanes). Expression of Myc-tagged SNT-1 protein was determined by Western blot analysis of cell lysates (bottom lanes).
