Inhibitor-resistant type I receptors reveal specific requirements for TGF-β signaling in vivo

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Abstract

Activin/nodal-like TGF−β superfamily ligands signal through the type I receptors Alk4, Alk5, and Alk7, and are responsible for mediating a number of essential processes in development. SB-431542, a chemical inhibitor of activin/nodal signaling, acts by specifically interfering with type I receptors. Here, we use inhibitor-resistant mutant receptors to examine the efficacy and specificity of SB-431542 in Xenopus and zebrafish embryos. Treatment with SB-431542 eliminates Smad2 phosphorylation in vivo and generates a phenotype very similar to those observed in genetic mutants in the nodal signaling pathway. Inhibitor-resistant Alk4 efficiently rescues Smad2 signaling, developmental phenotype, and marker gene expression after inhibitor treatment. This system was used to examine type I receptor specificity for several activin/nodal ligands. We find that Alk4 can efficiently rescue signaling by a wide range of ligands, while Alk7 can only weakly rescue signaling by the same ligands. In whole embryos, nodal signaling during gastrulation can be rescued with Alk4, but not Alk7, while Alk5 can only mediate signaling by ligands expressed later in development. The combination of the ALK inhibitor SB-431542 with inhibitor-resistant ALKs provides a powerful set of tools for examining nodal/activin signaling during embryogenesis.

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Introduction

Signaling by the transforming growth factor β (TGF-β) superfamily of ligands is responsible for regulating a wide range of cellular processes, including proliferation, cell death, differentiation, and development. TGF-β ligands signal by binding to two distinct types of serine-threonine kinase receptors, designated type I and type II. Ligand binding induces phosphorylation of the type I receptor by the type II receptor, which subsequently phosphorylates receptor-regulated Smad proteins, allowing them to translocate to the nucleus and interact with transcription factors to modulate downstream gene expression (reviewed by Shi and Massague, 2003; Whitman, 1998).

The activin/nodal subset of TGF-β superfamily ligands signals through the type I receptors Alk4, Alk5, and Alk7, which leads to phosphorylation of Smad2 and Smad3 (Carcamo et al., 1994; Ryden et al., 1996). Members of this class include early pattern regulators such as nodals as well as later regulators of development and growth such as GDF8/myostatin and canonical TGF-β. During early development, the proper spatiotemporal expression of nodal ligands and the tight regulation of signaling levels are essential for specification of mesendoderm and for left–right patterning (Schier, 2003). In the frog, Alk4 appears to be the type I receptor responsible for the early nodal signaling required for proper patterning of the germ layers during gastrulation as well as for left–right patterning during tailbud stages. In the mouse, loss of Alk4 results in early and lethal defects in egg cylinder organization and gastrulation (Chen et al., 2004; Gu et al., 1998). In vitro evidence suggests that Alk7 may be able to respond to nodal signals in some contexts, but an Alk7 knockout has no phenotype (Jornvall et al., 2004; Reissmann et al., 2001). Alk5 seems to be responsible for later developmental events, such as heart patterning and angiogenesis (Chang et al., 1998; Larsson et al., 2001).
While much has been learned through loss-of-function analysis about the role of activin/nodal ligands and their receptors in development, there are in many cases technical limitations to the interpretation of this analysis. First, late developmental phenotypes are often obscured by disruptions in signaling during early development, and second, signaling components can be partially or completely redundant, making unclear the function of individual components. While tissue-specific gene inactivation in the mouse using the Cre–lox system has provided an approach to the first of these issues, it is limited by the temporal and spatial resolution of gene expression driven by available enhancers, does not easily address the problem of functional redundancy, and is not currently applicable to other embryological model organisms. Chemical inhibitors, which are easily applied to anamniote embryos, can be readily added and removed and can simultaneously inactivate closely related and partially redundant signaling components, providing a potentially useful complement to tissue-specific gene inactivation in the study of temporally specific roles of developmental signals.

A chemical inhibitor of activin/nodal signaling, SB-431542, inhibits Alk5 kinase activity in vitro with an IC50 of 94 nM, and also inhibits Alk4 and Alk7 with similar potency (Callahan et al., 2002; Inman et al., 2002). Studies in cell culture have shown that SB-431542 can inhibit both Smad2/3 phosphorylation and downstream reporter gene expression (Callahan et al., 2002). While inhibitors like SB-431542 are potentially useful probes of activin/nodal signaling function during embryogenesis, a major concern about the use of such inhibitors is their specificity in vivo. A molecule that is designed to bind in the active site of a particular protein may also bind and affect other structurally related but functionally distinct proteins. This is of particular concern for small molecules targeted to ATP binding sites such as SB-431542 (Callahan et al., 2002), since in vitro specificity studies can never fully address the effect of the inhibitor on the full range of nucleotide binding proteins present in vivo.

One means of demonstrating specificity is to show that an inhibitor-resistant target can restore normal signaling and phenotype in the presence of the inhibitor. Although such an approach has not been used before in a complex in vivo system, a mutant of the MAP kinase p38 that is resistant to the inhibitor SB 203580 has been tested in tissue culture cells (Eyers et al., 1998). SB-431542 has great potential as a tool to examine the temporal requirements for nodal signaling throughout embryogenesis. To date, however, it has been used only in tissue culture systems, and its efficacy and specificity in more complex in vivo systems such as the early vertebrate embryo has not been shown. We therefore examined the effect of SB-431542 treatment in Xenopus and zebrafish embryos. We show that treatment with SB-431542 can eliminate both exogenously stimulated and endogenous Smad2 phosphorylation and generates phenotypes strongly resembling those of known perturbations in the nodal signaling pathway. To establish the specificity of SB-431542 action, we constructed a point mutant of Alk4 that is resistant to SB-431542 inhibition. This mutant receptor efficiently rescues Smad2 signaling, developmental phenotype, and marker gene expression in Xenopus and zebrafish upon treatment with SB-431542, demonstrating that the effects of inhibition are indeed specific.

Finally, we used this inhibitor/receptor rescue system in order to determine type I receptor specificity for a number of important ligands and developmental processes. Mutant Alk4 is able to mediate signaling for all ligands tested in Xenopus animal cap explants, but mutant Alk7 can only weakly rescue signaling by the same ligands. The competence of Alk5 to mediate Smad2 signaling appears to be restricted to those ligands acting later in development, such as GDF11 and GDF8/myostatin. In support of these results, we also find that mutant Alk5 is sufficient to rescue p-Smad2 signaling during tailbud but not gastrula stages. Furthermore, while Alk4 can efficiently restore signaling during gastrulation, an equal dose of Alk7 cannot, indicating that Alk7 is not an effective functional substitute for Alk4 during early development.

Materials and methods

Plasmid construction

The Alk4 S275M mutant was generated using a one-step site-directed mutagenesis protocol (Makarova et al., 2000). A single oligonucleotide primer was designed incorporating the point mutation and flanking sequences. The primer sequence (mutations in bold) was 5′-ACC CAG TGG CTT GTT GTT ATG GAC TAT CAT GAG CAT GGA TGG TC-3′. A single-strand PCR reaction was performed using pSP64T-xAlk4 WT as a template. Template was then specifically degraded using DpnI (New England Biolabs). The DpnI-treated PCR product was transformed into DH5α competent cells, and colonies were screened by sequencing for incorporation of the mutation. Alk4-GR constructs were generated by subcloning PCR products encoding the open reading frame of Alk4 S275M or WT upstream of sodins 512–777 of the human glucocorticoid receptor. HA-tagged Alk4 S275M and WT were made by PCR cloning the coding region of Alk4 with the following primers (HA tag sequence in bold): up: 5′-AGA ATT CAT GGC GGA GAT ACC G-3′; down: 5′-TCA ATC TGG TAC ATC ATA TGG ATA GAT TTT CAC ATC TTC-3′. The PCR product was subcloned into pCS4+.

HA-tagged Alk5 S278M and Alk7 S270M were made using the mutagenesis protocol above on wild-type parent plasmids pCS2-rAlk5-6-HA and pCS2-rAlk7-6-HA. The primer for Alk5 S278M was 5′-ACC CAG CTG TGG TTG GTG ATG CAT TAT CAT GAG CAT GGA TGG TC-3′. The primer for Alk7 S270M was 5′-GTC ATC TGG TAC ATC ATA TGG ATA GAT TTT CAC ATC TTC-3′. The PCR product was subcloned into pCS4+.

Xenopus embryo manipulation

Xenopus embryos were fertilized and maintained as previously described (Watanabe and Whitman, 1999). Embryos were staged according to Nieuwkoop and Faber (1967). For animal cap experiments, embryos were injected equally at the 2-cell stage with 10 nl of mRNA in each blastomere. For whole embryo experiments, embryos were injected marginally at 2- to 4-cell stage. For Alk4-GR injections, embryos were injected twice on one side at the 4-cell stage along with GFP mRNA as a tracer, and sorted into left and right side injected embryos based on GFP fluorescence at stage 22 prior to fixation.

Animal cap dissections were performed between stages 8 and 9, and explants were maintained in 0.7× MMR in agarose-coated dishes. For activin protein...
experiments, animal caps were incubated at room temperature with 100 μM SB-431542 (Tocris) or DMSO for 45 min to 1 h followed by treatment with 0.3–2 nM activin protein in 0.1% BSA and 0.02% gelatin for 45 min to 1 h, and harvested immediately afterward for Western blotting. For Alk4-GR experiments, embryos were treated with 10 μM dexamethasone (Sigma) 1 h prior to treatment with SB-431542. For injected ligand experiments, animal caps were incubated overnight at 14°C in 100 μM SB-431542 or DMSO before harvesting at stages 10–10.5.

Zebrafish embryo manipulation

Adult wild-type zebrafish of the AB strain were maintained and embryos collected as previously described (Chan et al., 2001). Embryos were maintained at 24°–33°C and staged according to Kimmel et al. (1995).

For injections, stock mRNA solutions were diluted to working concentrations in 1× Danieau’s solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM Ca(NO3)2, 5 mM HEPES, pH 7.6) with 0.1% phenol red. Embryos were injected in the yolk at the one cell stage with approximately 2 nl of working concentration mRNA.

Embryos were treated with SB-431542 or DMSO at the 16-cell stage unless otherwise noted. SB-431542 was added to a final concentration of 100 μM from stocks of 20 to 100 μM in 80–100% DMSO; DMSO was added to all controls at an equivalent final dilution.

Live embryos were photographed in 2% methylcellulose (Sigma) using a Leica MZFLIII stereomicroscope, Optronics camera, and Magnafire software. In some cases, color balance and contrast were slightly adjusted with Adobe Photoshop 7.

Western blotting

Xenopus animal caps and zebrafish embryos were lysed for Western blotting in modified RIPA buffer (Yeo and Whitman, 2001). 2–2.5 animal caps or 1–1.5 zebrafish embryos were loaded per lane. P-Smad2 antibodies have been described previously (Faure et al., 2000); here, the acid eluate was used at a dilution of 1:100–1:200. For tissue culture cells, commercially available p-Smad2 antibody (Cell Signaling Technologies) was used at a dilution of 1:500. Cytoskeletal actin (AC-40, Sigma) and tubulin (DM1, Sigma) were used as loading controls.

In situ hybridization

Whole-mount in situ hybridization on zebrafish and Xenopus embryos was performed as described previously (Chan et al., 2001; Harland, 1991).

Results

SB-431542 effectively blocks exogenous and endogenous p-Smad2 signaling in embryos

SB-431542 has been shown to block phospho-Smad2 (p-Smad2) signaling downstream of the type I receptors Alk4, Alk5, and Alk7 in tissue culture, but its efficacy in vivo has not been determined (Callahan et al., 2002; Inman et al., 2002). Therefore, we tested whether SB-431542 could attenuate both endogenous and exogenously induced Smad2 phosphorylation in the vertebrate embryo. Treatment with activin protein induces Smad2 phosphorylation in Xenopus animal cap explants; this induction is completely blocked by addition of 100 μM SB-431542 (Fig. 1A). Although doses of SB-431542 as low as 10 μM could block the majority of p-Smad2 signaling in animal caps (data not shown), we have used 100 μM throughout this study since this higher dose was required to elicit p-Smad2 block and phenotypic alterations in whole embryos (see below).

Endogenous p-Smad2 in zebrafish embryos at 50% epiboly is eliminated upon treatment with 100 μM SB-431542 (Fig. 1B). At least 50 μM SB-431542 is required in whole embryos to cause complete block of p-Smad2 and phenotypic alteration; this is approximately 5- to 10-fold more than the concentration required in animal caps and in vitro (Inman et al., 2002). This relatively high dose requirement may reflect the additional complexities of tissue thickness, permeability, and drug efflux pumps in intact embryos.

We also tested whether the effect of SB-431542 could be reversed after removal of the inhibitor. Xenopus embryos treated with SB-431542 were washed out and allowed to recover in inhibitor-free media for 16 h; this treatment allowed p-Smad2 in the tailbud of these embryos to recover to levels comparable to the DMSO-treated control, indicating that SB-431542 treatment is indeed reversible (Fig. 1C).

Phenotypic consequences of SB-431542 treatment in embryos

In intact Xenopus embryos, early SB-431542 treatment blocks endogenous p-Smad2 and causes failure of blastopore lip formation at stage 10 and abnormal, incomplete gastrulation (Figs. 2A and B and data not shown). This phenotype is very similar to that caused by overexpression of known inhibitors of nodal signaling (Agius et al., 1999; Onuma et al., 2002). Treatment of Xenopus embryos at a later, post-gastrulation stage (stage 13) altered left–right patterning as assessed by expression
of the left-side specific marker  \textit{xAntivin} at stage 22 (Figs. 2C and D). Additionally, gut origin and coiling were randomized in embryos treated with SB-431542 from stages 19 to 25 (data not shown).

Zebrafish embryos treated with SB-431542 early in development display reproducible phenotypic alterations consistent with loss of nodal signaling. At 24 h post-fertilization (hpf), treated embryos show significant morphological perturbations, including severe head and midline defects, fewer and more posterior somites, and poor separation and elongation of the yolk extension (Figs. 2E and H). The anterior defects are particularly striking, and range in severity from moderate (some structures deformed or fused, but still recognizable) to severe (no visually identifiable structures) (Figs. 2F, G, and I). This phenotype was highly penetrant, with 90% of embryos showing significant anterior defects (Table 1). The severity of the phenotype depends upon the stage at which SB-431542 is added. Embryos treated at 16-cell stage (Fig. 2J) demonstrate greater loss of anterior structures than those treated at 256-cell stage (Fig. 2K).

In situ hybridization experiments were performed to examine the expression patterns of marker genes at various timepoints after inhibitor treatment. Nodal signaling is important for establishing mesodermal cell fates, particularly dorsally (Doughan et al., 2003). Therefore, we examined the expression of

Table 1

Head phenotypes of 24 hpf embryos treated with 100 \( \mu \text{M} \) SB-431542

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Normal (WT)</th>
<th>Moderate</th>
<th>Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 pg Alk4 S275M +SB</td>
<td>36/65 (55%)</td>
<td>17/65 (26%)</td>
<td>12/65 (18%)</td>
</tr>
<tr>
<td>100 pg Alk4 S275M +DMSO</td>
<td>71/78 (91%)</td>
<td>7/78 (9%)</td>
<td>0/78 (0%)</td>
</tr>
<tr>
<td>100 pg Alk4 WT +SB</td>
<td>4/84 (5%)</td>
<td>32/84 (38%)</td>
<td>48/84 (57%)</td>
</tr>
<tr>
<td>100 pg Alk4 WT +DMSO</td>
<td>88/88 (100%)</td>
<td>0/88 (0%)</td>
<td>0/88 (0%)</td>
</tr>
<tr>
<td>Uninjected +SB</td>
<td>5/51 (10%)</td>
<td>19/51 (37%)</td>
<td>27/51 (53%)</td>
</tr>
<tr>
<td>Uninjected +DMSO</td>
<td>45/45 (100%)</td>
<td>0/45 (0%)</td>
<td>0/45 (0%)</td>
</tr>
</tbody>
</table>

(Results are combined from 3 separate experiments).
several mesodermal markers during gastrulation. Expression of the dorsal mesodermal marker *goosecoid* (*gsc*) at shield stage is completely abrogated or severely reduced in SB-431542-treated embryos (Figs. 3A and B). Expression of the pan-mesodermal marker *no tail/brachyury* (*ntl*) is excluded from the dorsal marginal region, while ventrolateral expression of these genes remains unaffected (Figs. 3C and D). In contrast, SB-431542 treatment had no effect on the ventral mesodermal marker *even-skipped1* (*eve1*) (Figs. 3E and F).

We also examined the expression of several later marker genes known to be affected by nodal signaling. At the end of gastrulation (bud stage), presumptive notochord staining of *ntl* was absent in treated embryos, although the tailbud expression domain remained (Figs. 3G and H). Expression of the floorplate marker *sonic hedgehog* (*shh*) and the notochord marker *axial* were also absent in SB-431542-treated embryos (arrows in Figs. 3I and J, and data not shown). *Shh* expression continued to be severely reduced, with virtually no staining in the anterior part of the embryo and occasional weak, discontinuous staining in the posterior, at the 18-somite stage (Figs. 3K and L).

The phenotype of SB-431542-treated embryos bears a striking resemblance to those of several genetic mutants in the nodal signaling pathway, specifically *cyclops/squint* (*cyc;sqt*) and *one-eyed-pinhead* (*Mzoep*) (Feldman et al., 1998; Gritsman et al., 1999). In addition to the severe disruption of dorsal mesodermal markers and dorso-anterior mesodermal structures (Figs. 2 and 3), we also observe abnormal thickening in the dorsal–medial region of late gastrula embryo (data not shown), which has been attributed to abnormal epiboly and convergence movements in *Mzoep* mutants (Gritsman et al., 1999). Significantly, general anterior–posterior (A–P) patterning of the neural plate did not appear to be affected, as A–P localization of *Pax2.1* and *Krox20* appeared normal (arrowheads in Figs. 3G–J, and data not shown), a result also observed in *Mzoep* mutant embryos (Feldman et al., 1998; Gritsman et al., 1999). Taken together, these data suggest that SB-431542 is indeed functioning by specifically downregulating nodal signaling in the early embryo. In the frog, nodal signaling during early embryogenesis is mediated by the type I receptor Alk4 (Chang et al., 1997). The zebrafish type I receptor
TARAM-A is highly related to Alk4 and is a candidate for the receptor that transduces the early nodal signal in fish (Aoki et al., 2002; Renucci et al., 1996).

**Generation of a mutant Alk4 that is insensitive to SB-431542 inhibition**

To establish the specificity of the inhibitor, we devised a strategy for rescuing SB-431542-treated embryos using an inhibitor-insensitive Alk4. A mutant receptor that is resistant to the p38 inhibitor SB 203580 has been described. Mutation of Thr106 in the ATP-binding pocket of p38 to Met renders it insensitive to inhibition by SB203580; the size of this residue appears to be crucial in determining inhibition efficiency (Eyers et al., 1998). The equivalent residue in the ATP-binding sites of Alk4, Alk5, and Alk7 is a small, conserved serine, suggesting that the inhibitor should bind the wild-type receptor efficiently. Since SB 203580 and SB-431542 are structurally similar, it is likely that their mechanisms of inhibition will also be the same (Callahan et al., 2002). Therefore, using the p38 mutant as a paradigm, we generated a point mutant of Alk4 (Alk4 S275M) in which this serine residue was changed to a large, hydrophobic methionine.

We examined whether Alk4 S275M could restore p-Smad2 signaling in inhibitor-treated animal caps and embryos. *Xenopus* animal cap explants injected with 100 pg of Alk4 S275M showed phosphorylation of Smad2 upon activin treatment, even in the presence of 100 μM SB-431542, whereas those injected with wild-type Alk4 (Alk4 WT) did not (Fig. 4A). Alk4 S275M did not ectopically activate Smad2 signaling in the absence of activin, indicating that, at these doses, the mutant receptor does not non-specifically or constitutively activate the Nodal pathway to an appreciable level in animal caps. Similarly, Alk4 S275M but not Alk4 WT rescued endogenous p-Smad2 in SB-431542-treated zebrafish embryos at 50% epiboly (Fig. 4B).

**Alk4 S275M rescues phenotypic and gene expression defects in SB-431542-treated zebrafish embryos**

Since Alk4 S275M could restore p-Smad2 signaling in embryos, we used it to ask whether the SB-431542-induced phenotype of embryos was likewise specific. Alk4 S275M did indeed rescue the SB-431542 phenotype in zebrafish. 55% (n = 65) of embryos injected with 100 pg Alk4 S275M mRNA and subsequently treated with 100 μM SB-431542 displayed phenotypically normal head development (Table 1). Rescued embryos had two distinct and separate eyes and normal midline structures (Fig. 5A). The unrescued fraction of embryos probably reflects poor or incomplete dispersal of the injected mRNA throughout the entire embryo. In contrast to Alk4 S275M, wild-type Alk4 was unable to rescue inhibitor-induced head defects (Table 1; Fig. 5C). Embryos injected with mRNA encoding either wild type or mutant receptors and treated with DMSO were phenotypically indistinguishable from uninjected embryos, indicating that the receptors alone do not significantly affect phenotype (Figs. 5B and D). A lower dose (50 pg) of mRNA was still able to rescue, but was less efficient (data not shown). Neither mutant Alk5 nor mutant Alk7 were able to rescue SB-431542-induced defects at doses of up to 200 pg mRNA (data not shown).

Mesodermal marker gene expression was also rescued by Alk4 S275M. Whereas SB-431542-treated Alk4 WT embryos showed little or no gsc at shield stage, expression was restored in Alk4 S275M embryos (Figs. 6A–D). Similarly, ntl expression in the dorsal margin was present in SB-431542-treated Alk4 S275M embryos but not in Alk4 WT embryos (data not shown). The notochord expression domain of ntl was restored in SB-431542-treated Alk4 S275M embryos, but not in Alk4 WT embryos (Figs. 6E–H). Midline expression of shh and axial at bud stage in treated embryos could also be rescued by injection of Alk4 S275M (data not shown). At the 18-somite stage, the full range of expression of shh, including the anterior-most domain, was restored by Alk4 S275M but not by Alk4 WT (Figs. 6I–L).

**Alk4 S275M rescues phenotypic and gene expression defects in SB-431542-treated Xenopus embryos**

In *Xenopus* embryos treated with SB-431542 before gastrulation, we found that expression of Alk4 S275M rescues blastopore lip formation (data not shown). We also observed, however, that doses of Alk4 mRNA necessary for rescue of blastopore lip formation in *Xenopus* cause additional, inhibitor-independent defects in post-gastrula patterning. This observation suggests that ectopic Alk4 expression may be sufficient to alter nodal signaling patterns in *Xenopus* embryos. A previous
Many activin/nodal ligands have been implicated in embryonic patterning, including activin, nodals (Xnr1–6 in *Xenopus*), Derriere, Vg1, GDF8/myostatin, GDF11 and canonical TGF-βs (reviewed by Chang et al., 2002; Schier, 2003). Three type I receptors, Alk4, Alk5, and Alk7, are thought to be responsible for mediating the signal produced by these ligands. Previous work on ligand/type I receptor specificity has relied on indirect overexpression techniques, such as co-immunoprecipitation of tagged receptors in tissue culture systems. Our inhibitor/receptor system allows for the selective restoration of a single functional mutant type I receptor after SB-431542 treatment, thus enabling the identification of individual functional ligand/receptor pairs in a systematic manner. *Xenopus* animal caps express no endogenous nodal ligands, so the only nodal signaling source is the introduced ligand of interest. Since SB-431542 blocks signaling through all known activin/nodal type I receptors (thus eliminating background from endogenous receptors), only introduction of the correct mutant receptor should restore signaling by a given ligand, whereas receptors not compatible with this ligand would have no effect.

To facilitate meaningful comparison between the three receptors, we constructed HA-tagged forms of mutant and wild-type Alk4, Alk5, and Alk7. In our experiments, tagged Alk5 protein was 60- to 80-fold more efficiently expressed than either Alk4 or Alk7 (Fig. 8A). Thus, in the experiment shown in Fig. 8B, we injected 3 ng of Alk4, 50 pg of Alk5, and 4 ng of Alk7, such that approximately equal protein levels were obtained. (We have observed that tagged versions of the Alks appear to be much less active than the corresponding untagged constructs (data not shown), hence the apparent discrepancy between the dose of Alk4-HA (3 ng) used here with the lower doses of untagged Alk4 (50–150 pg) used in the experiments described earlier.)

Five activin/nodal ligands with developmental significance were tested in our assay: ActivinβB, Xnr1, Derriere, GDF11, and GDF8/myostatin. We found that Alk4 S275M was capable of efficiently restoring signaling by all five ligands, whereas Alk5 S278M could do so only for GDF11 and GDF8, albeit weakly (Fig. 8B). Alk7 S270M partially rescued pSmad2 activation in response to all the ligands, but rescue by mutant Alk7 was consistently much weaker than that seen with a comparable dose of mutant Alk4 for all ligands tested (Fig. 8B).

In no case was any wild-type receptor able to restore signaling in the presence of SB-431542 (Fig. 8B). Both high doses (1–2 ng, which generate strong, easily photographed, and completely regulated pSmad2 bands) and low doses (100–200 pg, consistent with doses required for mesodermal induction) of Xnr1, Derriere, GDF11, and GDF8 were tested with the same results (Fig. 8B and data not shown).

One possible explanation for the observed differences in rescue potency of different Alks is that the receptors have different levels of intrinsic activity, rather than differences in ability to utilize particular ligands. To address this possibility, we generated constitutively active receptors (Alk*). The S to M
mutation that confers SB-431542 resistance does not affect signaling efficiency for any of the constitutively active receptors (data not shown). When we compared the activity of different constitutively activated Alks at equal protein levels, we find that Alk4* and Alk7* signal equally well. Surprisingly, however, Alk5* is far less active than Alk4* or Alk7* in this system (Fig. 8C). A 100-fold excess (5 ng) of Alk5* is required to approach the activity of the other two receptors (Fig. 8C). (Because of RNA toxicity issues, it was not feasible to increase the dose further). Therefore, in order to correct for signaling efficiency, we repeated the ligand rescue experiment with 5 ng Alk5 S278M or WT. This dose was still unable to elicit rescue of

![Fig. 6](image)

Alk4 S275M rescues marker gene expression in SB-431542-treated embryos. Zebrafish embryos were injected with 50 pg Alk4 S275M or 50 pg Alk4 WT and treated with 100 μM SB-431542 or DMSO at 16-cell stage. Embryos were harvested for in situ hybridization for gsc at shield stage (A–D, dorsal to the right), ntl at bud stage (E–H, anterior to the top), and shh at 18 somite stage (I–L). Alk4 S275M restores gsc expression in SB-431542-treated embryos (A), whereas Alk4 WT did not (C). Notochord expression of ntl at bud stage was restored in Alk4 S275M-injected embryos (arrow in panel E), but not in Alk4 WT embryos (G). Similarly, expression of shh, including anterior domains (arrows in panel I), was rescued in Alk4 S275M embryos (I), but not in Alk4 WT embryos (K). DMSO-treated embryos displayed normal expression patterns for all genes examined (B, D, F, H, J, L).

Table 2

<p>| xAntivin expression at stages 22–23 in the flank of unilaterally injected Alk4GR embryos |
|---------------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Injected side</th>
<th>Left</th>
<th>Right</th>
<th>Both</th>
<th>None</th>
<th>Total (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>150 pg Alk4GR S275M +SB</td>
<td>L</td>
<td>43 (48%)</td>
<td>0 (0%)</td>
<td>1 (1%)</td>
<td>46 (51%)</td>
</tr>
<tr>
<td>150 pg Alk4GR S275M +SB</td>
<td>R</td>
<td>2 (3%)</td>
<td>19 (24%)</td>
<td>3 (4%)</td>
<td>55 (70%)</td>
</tr>
<tr>
<td>150 pg Alk4GR S275M +DMSO</td>
<td>L</td>
<td>24 (69%)</td>
<td>0 (0%)</td>
<td>1 (3%)</td>
<td>10 (29%)</td>
</tr>
<tr>
<td>150 pg Alk4GR S275M +DMSO</td>
<td>R</td>
<td>12 (39%)</td>
<td>9 (29%)</td>
<td>5 (16%)</td>
<td>5 (16%)</td>
</tr>
<tr>
<td>150 pg Alk4GR WT +SB</td>
<td>L</td>
<td>1 (3%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>35 (97%)</td>
</tr>
<tr>
<td>150 pg Alk4GR WT +SB</td>
<td>R</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>23 (100%)</td>
</tr>
<tr>
<td>150 pg Alk4GR WT +DMSO</td>
<td>L</td>
<td>31 (97%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>1 (3%)</td>
</tr>
<tr>
<td>150 pg Alk4GR WT +DMSO</td>
<td>R</td>
<td>22 (81%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>5 (19%)</td>
</tr>
<tr>
<td>Uninjected +SB</td>
<td>–</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>30 (100%)</td>
</tr>
<tr>
<td>Uninjected +DMSO</td>
<td>–</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>1 (3%)</td>
</tr>
</tbody>
</table>

All embryos (except uninjected) were treated with 10 μM dexamethasone starting 1 h before treatment with 100 μM SB-431542 or DMSO at stages 13–14 and sorted into left- and right-side injected prior to fixation by localization of coinjected GFP. Results are combined from 4 separate experiments (except for uninjected—2 experiments).
embryogenesis in SB-431542-treated Xenopus embryos. Embryos were injected on the left side only with 150 pg xAlk4-GR S275M (A, B) or xAlk4-GR WT (C, D) mRNA and treated with 10 μM Dexamethasone at stage 13.5 followed by 100 μM SB-431542 (A, C) or DMSO (B, D) at stage 14. Embryos were processed for in situ hybridization with an xAntivin antisense probe at stage 22. Embryos were photographed with the anterior to the left and the left side showing. Note specific recovery of antivin expression (red arrow) in the left flank in panel A.

signaling by ActB, Derriere, or Xnr1, but completely restored GDF11 and GDF8 signaling, indicating that when intrinsic signaling capacity of the receptors is corrected for, Alk5 is as good a receptor as Alk4 for GDF11 and GDF8, but not for the other ligands tested (Fig. 8D).

Type I receptor specificity in embryos

GDF11 and GDF8 are not expressed before or during gastrulation, but are expressed later in development, suggesting that Alk5 may specifically respond to later ligands. To test this possibility, we asked whether mutant Alk5 could rescue endogenous p-Smad2 signaling during either early or late embryogenesis in SB-431542-treated Xenopus embryos. At gastrulation (st. 10+), 3 ng of mutant Alk5 was unable to rescue any detectable Smad2 phosphorylation in whole embryos (Fig. 8F). We also observed that blastopore lip formation was never restored by Alk5 S278M in the presence of SB-431542, in contrast to the rescue of lip formation observed with Alk4 S275M (data not shown). During early tailbud stages in Xenopus, p-Smad2 signaling is observed in the tail and head regions (D.M.H. and M.W., unpublished observations). In contrast to what we observe during gastrulation, p-Smad2 signaling in the tailbud at stage 26 was blocked by SB-431542 and rescued efficiently by 3 ng of mutant Alk5 (Fig. 8F). The same is true of p-Smad2 signaling in the head at this stage (data not shown).

The experiments shown in Fig. 8B suggest that, although they appear to have the same specificity, Alk4 rescues pSmad2 in response to all ligands tested more efficiently than Alk7 in animal caps. It is possible that the reason for this apparent difference in sensitivity is that mutant Alk7 is not completely resistant to SB-431542. In order to rule out this possibility, we asked whether Alk7* S270M could signal less well in the presence of SB-431542. We find that this is not the case; both Alk7* S270M and Alk4* S275M retain all of their signaling capability even in the presence of SB-431542, indicating that both mutants are equally and completely resistant to inhibition (Fig. 9A). Thus, the decreased ability of Alk7 S270M to rescue signaling relative to Alk4 S275M most likely reflects a true difference in the ability of Alk7 to respond to ligands.

We next asked whether the relative potencies of Alk4 S275M and Alk7 S270M in rescuing SB-431542 in animal caps were recapitulated in whole embryos. High levels of p-Smad2 signaling are required during late blastula and gastrula stages in Xenopus embryos (Lee et al., 2001). p-Smad2 in stage 10+ whole embryos was effectively blocked by SB-431542; this signal could be rescued by mutant Alk4 but not an equivalent dose of mutant Alk7 (Fig. 9B). Indeed, as little as 1.5 ng of Alk4 mRNA was able to rescue signaling, while 6 ng of Alk7 mRNA could not. Phenotypically, blastopore lip formation at stage 10+ was prevented by SB-431542 and rescued by Alk4 S275M but not Alk7 S270M (Figs. 9C–J). Treated Alk4 S275M embryos go on to form complete blastopore lips at stage 10.5, concomitantly with untreated embryos, while SB-431542-treated Alk7 embryos still have no discernible blastopore lip (data not shown). In summary, these experiments demonstrate that there is differential usage of type I receptors by particular ligands and during specific developmental events, and suggest that regulation of ligand/receptor pairing may be important during embryogenesis.

Discussion

A rescuable inhibitor/receptor system for in vivo studies

In zebrafish and Xenopus, loss-of-function analyses are generally carried out using genetic mutants, antisense oligos, or dominant-negative constructs. All of these strategies, with the exception of rare temperature-sensitive mutations, are difficult or even impossible to regulate spatially or temporally. Traditional methods are also insufficient for examining the effects of transient versus sustained signaling during embryogenesis.

Chemical inhibitors of signaling pathways, such as SB-431542, in contrast, can be easily added or removed from media at any time. We show here that SB-431542 is indeed effective as a reversible inhibitor of endogenous and exogenous p-Smad2 signaling in early embryogenesis (Fig. 1). Furthermore, phenotype and marker gene expression are altered in a manner consistent with inhibition of nodal signaling (Figs. 2 and 3).

SB-431542 is a powerful tool for examining the temporal requirements for activin/nodal signaling during development. The relatively high concentration of inhibitor that is required for penetration of embryos, and the additional complexities of multiple cell types and pathways in an intact embryo, however, increase the likelihood of off-target effects in embryos relative to a monolayer of a single cultured cell type. To demonstrate the specificity of inhibition of activin/nodal signaling by SB-431542, we show that introduction of an SB-431542-resistant mutant receptor rescues the inhibitor-induced phenotype and can restore the expression of
pSmad2-dependent marker genes. There is little or no ectopic activation of p-Smad2 upon expression of the mutant receptor at doses sufficient for rescue, since the receptor requires other components such as ligands, type II receptors, and cofactors to signal efficiently. Therefore, signaling that is restored in inhibitor-treated/receptor-rescued embryos most likely reflects endogenous signaling patterns.

Several other type I TGF-β receptor inhibitors that function along the same principles as SB-431542 have recently been identified in vitro (DaCosta Byfield et al., 2004; Gellibert et al., 2004; Kim et al., 2004a,b). Our mutant receptors can be used to test the specificity of these compounds in vivo as they become available.

### Fig. 8. Alk4, Alk5, and Alk7 mediate signaling by different subsets of activin/nodal ligands

(A) Embryos were injected with 3 ng Alk4-HA SM (4M), 50 pg Alk5-HA SM (5M), or 4 ng Alk7-SM (7M) along with 500 pg HA-Smt3C as an internal loading control. α-HA IP-Westerns were performed on stage 10.5 embryos. (B) Embryos were injected with 1 ng Xnr1, 20 pg ActβB, 2 ng Derrière, 2 ng cDsl-hGDF11, or 2 ng cDsl-mGDF8 along with 3 ng xAlk4-HA S275M (4M) or WT (4W), 50 pg rAlk5-HA S278M (5M) or WT (5W), or 4 ng rAlk7-HA S270M (7M) or WT (7W). Animal caps were incubated in 100 μM SB-431542 or DMSO until stages 10–10.5, and subsequently harvested for Western blots against p-Smad2. (C) Constitutively active Alks (Alk4*, Alk5*, and Alk7*) were injected at various doses and pSmad2 signaling was assessed in animal caps. (D) 5 ng of rAlk5-HA S278M or rAlk5-HA WT were coinjected with the same concentrations of ligands used in panel A and p-Smad2 signaling was assayed in SB-431542 or DMSO-treated animal caps. (E) Xenopus embryos were injected with 3 ng Alk5-HA S278M or WT. Embryos were treated with 100 μM SB-431542 at stage 6 and harvested at stage 10+, or treated at stage 14 and harvested (tailbud region only) at stage 26. For all experiments, actin was used as a loading control.

### Determining type I receptor specificity using the inhibitor/receptor rescue system

A number of activin/nodal ligands play essential roles during development and disease. It is unclear, however, which type I receptors these ligands utilize and whether differential usage of type I receptors might provide a mechanism for specificity in the responsiveness of different cells or tissues to ligands. Previous work in mouse and frogs has indicated that Alk4 is essential for mesoderm induction and gastrulation during early development (Chang et al., 1997; Gu et al., 1998). The role of Alk7 is less clear. No phenotype has been reported to date in Alk7 knockout mice, but a previous study has indicated that...
Xnr1 and nodal may be able, in some contexts, to signal through Alk7 and activate transcription of target genes (Jornvall et al., 2004; Reissmann et al., 2001). We find that, in contrast to Alk4, Alk7 is at best an inefficient mediator of p-Smad2 signaling by all the ligands tested in our study, and it could not rescue endogenous signaling at stage 10+ (Figs. 8B and 9). Alk7 may require different cofactors or ligand modifications not present in the pre-gastrula embryo to efficiently mediate signaling. Alternatively, it may be the preferential receptor for a ligand not tested in our study. Our results are consistent with the mouse knockout phenotypes in indicating that Alk4 is the primary type I receptor for activin/nodal signaling during gastrulation.

We also examined the receptor specificity of ligands known to signal through Smad2 after gastrulation. GDF11 is required for anterior–posterior patterning in vertebrates, as well as for other, later functions including neurogenesis (McPherron et al., 1999; Wu et al., 2003). A related ligand, GDF8/myostatin, is a negative regulator of muscle mass (McPherron et al., 1997). Here, we find that both of these ligands, which are active in later development and, at least in the case of myostatin, throughout adulthood, can signal through both Alk4/Alk7 and Alk5 (Figs. 8B and D). Utilization of the inhibitor/receptor rescue system in tissues in which these ligands are active in vivo will shed light on the endogenous functions of the various type I receptors in GDF11/GDF8 signaling. It will be interesting to see whether the usage of different type I receptors by these ligands in vivo is spatially or temporally regulated, and whether Alk4 and Alk5 might mediate different downstream activities.

Intriguingly, the only ligands that can signal through Alk5 in our assay are those that are not expressed during early development, such as GDF11 and GDF8. Consistent with this observation, we find that mutant Alk5 cannot rescue p-Smad2 signaling or phenotype during gastrulation, but can rescue signaling during tailbud stages (Fig. 8F). Alk5 (TβRI) knockout mice do not display gastrulation defects but later develop vascular and circulatory abnormalities (Larsson et al., 2001). Our data are consistent with the mouse knockout phenotype,
indicating that Alk5 is unnecessary for early development and pattern formation and instead is essential for later patterning.

Using the inhibitor/receptor rescue system to examine the basis of receptor specificity

Our results suggest that type I receptor specificity and ligand/receptor pairings may play a role in the regulation of various TGF-β ligand-mediated processes. Previous work on the basis of receptor specificity has relied on cell lines that are deficient in individual Type I receptors. Only a very limited number of such lines are available, and interpretation of data from these lines is complicated by the potential presence of other type I receptors that are functionally redundant with the one that is missing. SB-431542-resistant receptors provide a simple way to systematically examine the role of receptor domains in mediating aspects of TGF–β signaling, as SB-431542 can be used to remove all background signaling from endogenous receptors in virtually any cell line, tissue, or embryo, leaving only the mutant receptor of interest.

There are a number of potential applications of this approach to the study of context-specific function of Type I receptors. General regions or specific residues that are important for interaction of type I receptors with ligands and co-receptors have been identified (Harrison et al., 2003; Yeo and Whitman, 2001). Detailed structure–function analysis, particularly in a developmentally relevant context, is difficult in the absence of cell lines or embryos null for endogenous receptor function. The inhibitor-resistant receptor approach makes convenient the inactivation of endogenous receptors by chemical inhibition, allowing the use of rescue by mutant receptors as a test system for receptor structure–function studies.

This system should also make more accessible the study of signaling pathways used by activin/nodal type I receptors. In addition to signaling through Smads, TGF-β superfamily ligands can signal through a number of Smad-independent pathways, including activation of the EK, JNK, p38 MAPK, and AKT pathways (Derynck and Zhang, 2003). Mutation of the L45 loop of Alks separates Smad-dependent from Smad-independent signaling (Yu et al., 2002); the combination of mutations in this region with our SB-431542-resistant mutation makes straightforward the study of Smad-independent signaling in a background functionally null for endogenous Alk4/5/7 receptors. More generally, the inhibitor-resistant receptors provide a broad approach to studying receptor mutations in the absence of endogenous receptor function.

Using the inhibitor/receptor rescue system to examine temporal and spatial requirements for signaling during development

The inhibitor/receptor rescue system should be useful for the study of activin/nodal signaling in later development. It is difficult to assess the late phenotypes of genetic mutants such as Mzoeper and cysqt because of the confounding effects of the early inhibition of mesodendoderm specification. The injected mRNA that we use here does not persist long enough to allow for the examination of late defects. Therefore, the generation of stable transgenic lines carrying the mutant Alks will facilitate characterization of late defects, since SB-431542 can be specifically applied later in development.

This system can also be used to examine spatial requirements and tissue specificity throughout development. For example, transgenic animals can be generated that express mutant Alks under tissue-specific or inducible promoters, thus providing a means of specifically restoring signaling at given times or places in inhibitor-treated animals. Furthermore, since the mutant receptors do not ectopically activate signaling in the manner of constitutively active receptors, their introduction should not perturb endogenous processes.

The general strategy described here can be used to generate similar inhibitor/rescue systems for the study of other signaling pathways in development and disease. With the advent of high-throughput screening and the availability of large chemical libraries, pharmacological inhibitors for a wide range of pathways have become more and more commonplace. Rigorous proof of inhibitor specificity in vivo, however, must be demonstrated before these compounds can be used for therapeutic applications. The pathway-specific rescue strategy described here can be used to determine the specificity of a wide range of chemical inhibitors in complex in vivo systems.

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