The type I serine/threonine kinase receptor Alk8/Lost-a-fin is required for Bmp2b/7 signal transduction during dorsoventral patterning of the zebrafish embryo

Hermann Bauer1, Zsolt Lele1, Gerd-Jörg Rauch2, Robert Geisler2 and Matthias Hammerschmidt1,*

1Hans-Speemann Laboratory, Max-Planck Institut für Immunbiologie, Stuebeweg 51, D-79108 Freiburg, Germany
2Max-Planck Institut für Entwicklungsbiologie, Spemannstrasse 35, D-72076 Tübingen, Germany
*Author for correspondence (e-mail: hammerschmid@immunbio.mpg.de)

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SUMMARY

Ventral specification of mesoderm and ectoderm depends on signaling by members of the bone morphogenetic protein (Bmp) family. Bmp signals are transmitted by a complex of type I and type II serine/threonine kinase transmembrane receptors. Here, we show that Alk8, a novel member of the Alk1 subgroup of type I receptors, is disrupted in zebrafish lost-a-fin (laf) mutants. Two alk8/laf null alleles are described. In lafm110, a conserved extracellular cysteine residue is replaced by an arginine, while in lafm100, Alk8 is prematurely terminated directly after the transmembrane domain. The zygotic effect of both mutations leads to dorsalization of intermediate strength. A much stronger dorsalization, similar to that of bmp2b/swirl and bmp7/snailhouse mutants, however, is obtained by inhibiting both maternally and zygotically supplied alk8 gene products with morpholino antisense oligonucleotides. The phenotype of laf mutants and alk8 morphants can be rescued by injected mRNA encoding Alk8 or the Bmp-regulated transcription factor Smad5, but not by mRNA encoding Bmp2b or Bmp7. Conversely, injected mRNA encoding a constitutively active version of Alk8 can rescue the strong dorsalization of bmp2b/swirl and bmp7/snailhouse mutants, whereas smad5/somatabun mutant embryos do not respond. Altogether, the data suggest that Alk8 acts as a Bmp2b/7 receptor upstream of Smad5.

Key words: Alk8, Bmp2b, Bmp7, Smad5, Dorsoventral patterning, Lost-a-fin, Zebrafish, Morpholino antisense oligonucleotides

INTRODUCTION

During establishment of the body plan, signaling events regulate cellular behavior and specification. Members of the transforming growth factor β (Tgfβ) superfamily of signaling molecules have been shown to be crucial mediators of a variety of such processes (reviewed by Massagué, 1998). In target cells, Tgfβ signaling is transduced by transmembrane receptors of the serine/threonine kinase family, which themselves regulate the activity of members of the family of Smad transcription factors. Two types of receptors can be distinguished, type I and type II, which form heteromeric complexes upon ligand binding. Ligand binding occurs either in a sequential or a cooperative manner. The sequential mode, as characteristic for Tgfβ and activin receptors, involves direct binding of the ligand to high-affinity type II receptors and subsequent recruitment of type I receptors to the complex, whereas ligands bound in a cooperative mode, such as the bone morphogenetic proteins Bmp2, Bmp4 and Bmp7 or the growth differentiation factor Gdf5, display low affinity to both type I and type II receptors alone, but high affinity to the two receptors together (reviewed in Massagué, 1998). The type II receptors have constitutive kinase activity, leading to phosphorylation and activation of the type I receptor after ligand-induced complex formation. Activated type I receptors themselves phosphorylate and activate Smad proteins, which thereafter enter the nucleus to regulate transcription of target genes.

In line with their distinct functions, type I and type II receptors differ in several structural features. Both have a relatively short (approx. 150 amino acids) extracellular domain that contains 10 or more cysteines to determine the general folding of this region. Three of these cys-residues near the transmembrane domain constitute a specific cluster, the cysteine box, which is conserved in all serine/threonine transmembrane kinase receptors (Wrana et al., 1994). A unique feature of type I receptors is the GS domain, which precedes the kinase domain in the cytoplasmic part of the receptors. The GS domain contains several serine residues that can be phosphorylated by the type II receptors during signal transduction and type I receptor activation. Another special region of type I receptors is the L45 loop, a stretch of 10 amino acids of the kinase domain that determines the specificity of the receptors to the various receptor-regulated Smad proteins (Chen et al., 1998a; Chen et al., 1998b). The sequence of this L45 loop is highly divergent between different subgroups of
type I receptors, such as the Tgfβ receptors (Alk5/TβRI, Alk4/ActRIB, Alk7, etc.), the Bmp receptors (Alk3/BmpRIA, Alk6/BmpRIB and Drosophila Thick veins) and the Alk1 group (Alk1, Alk2/ActRI A and Drosophila Saxophone), whose endogenous ligands have been a controversial subject (see Discussion). Among the various members of each subgroup, however, the L45 sequences are highly conserved (Chen et al., 1998b).

Members of the Bmp subgroup of Tgfβ proteins are implicated in many different developmental processes, such as early dorsoventral pattern formation of the vertebrate embryo (reviewed in Hogan, 1996). In Xenopus laevis, overexpression of bmp2, bmp4 and bmp7 leads to ventral specification within ectoderm and mesoderm of the embryo (Dale et al., 1992; Jones et al., 1992; Schmidt et al., 1995; Wilson and Hemmati-Brivanlou, 1995). In contrast, disruption of Bmp signaling by cleavage resistant, dominant negative ligands (Hawley et al., 1995) or C-terminally truncated, dominant negative receptors (Graff et al., 1994; Maeno et al., 1994; Suzuki et al., 1994; Schmidt et al., 1995) causes dorsalization of the embryo.

Genetic evidence that graded Bmp activity is required for a balance between ventral and dorsal development in vertebrates has been provided by studies in zebrafish. The molecular analysis of the dorsoventral mutations swirl, snailhouse and somitabun (Mullins et al., 1996) has shown that zebrafish Bmp2b (Kishimoto et al., 1997; Nguyen, 1998b), Bmp7 (Schmidt et al., 2000; Dick et al., 2000) and the Bmp signal transducer Smad5 (Hild et al., 1999) are necessary for ventral development, while dorsal development requires the secreted protein Chordin, an inhibitor of Bmps (Piccolo et al., 1996; Hammerschmidt et al., 1996b) that is disrupted in the ventralized dino mutant (Hammerschmidt et al., 1996a; Schulte-Merker et al., 1997). On the ventral side, the activity of Chordin is attenuated by the metalloprotease Bmp1/Tolloid/Minifin (Connors et al., 1999), which cleaves Chordin, thereby promoting Bmp activity (Piccolo et al., 1997; Blader et al., 1997). In contrast to bmp2b, bmp7 and smad5, null mutations in tolloid cause only a very mild dorsalized phenotype (Connors et al., 1999).

Here, we show that the dorsalized phenotype of lost-a-fin zebrafish mutants is caused by null mutations in Alk8 (Yelick et al., 1998), a novel member of the Alk1 subgroup of type I serine/threonine kinase receptors. We provide genetic evidence that Alk8, although clearly distinct from the thus far described ‘classical’ Bmp type I receptors, is required for the transmission of Bmp2b/7 signaling during dorsoventral pattern formation.

MATERIALS AND METHODS

Cloning of zebrafish alk8

RT-PCR with total RNA from early gastrula stage embryos and oligonucleotides corresponding to conserved regions of serine/threonine kinase receptors was used to isolate cDNA fragments encoding various zebrafish type I and type II receptors. With degenerated oligonucleotides encoding YGEVWRG (sense) and KLMKECW (antisense), a 780 bp fragment was amplified, which strongly hybridized with a degenerated oligonucleotide deduced from the internal highly conserved sequence HENILGF. The PCR fragment was cloned using a TA cloning kit (Invitrogen) and used to screen gastrula stage cDNA libraries as described (Bauer et al., 1998), resulting in the isolation of three overlapping clones encoding the type I receptor serine/threonine kinase Alk8 (Accession Number AF292028; Yelick et al., 1998, Accession Number AF038425).

Mapping, linkage analysis, genotyping and RT-PCR analysis

alk8 was mapped on a radiation hybrid panel that was anchored to the genetic zebrafish map (Geisler et al., 1999). Primers used to amplify a genomic alk8 fragment were AAAACCGACTGCTGAGGAGA (sense) and ATTTGTAGGGCCTCTGTCTCC (antisense). The laf mutation was mapped via its recessive zygotic effect, causing C1-C2 dorsalization in homozygous embryos. A laf<sup>ml110</sup> carrier fish of the Tü background was crossed with a WIK wild-type fish (Rauch et al., 1997). PCR analysis of genomic DNA from laf mutant F<sub>2</sub> embryos for SSLP markers (Knapi et al., 1998) put laf<sup>ml110</sup> between the markers z17291 (four recombinations in 192 meioses=2.1 cM) and z9234 (2 recombinations in 192 meioses=1.0 cM) on linkage group 2.

For direct linkage analysis, alk8 cDNA was amplified from single laf mutant embryos and wild-type siblings via RT-PCR with total RNA, using the following conditions and primers: 5 minutes 95°C; 30× (30 seconds at 95°C, 30 seconds at 58°C, 3 minutes at 72°C); 7 minutes at 72°C; sense primer, GTCGAGATGTCATGTGAGGAC; antisense primer GTGACCCGTACGATTCGTCG. For laf<sup>ml110</sup>, the 1597 bp PCR product was digested with FspI, resulting in three fragments of 1188, 309 and 100 bp for the mutant allele and two fragments of 1497 and 100 bp for the wild-type allele. In the case of laf<sup>ml100</sup>, 40 PCR cycles and a SsoI digest were performed, yielding fragments of 1126 and 471 bp (mutant) or the uncleaved 1597 bp fragment (wild type). Alternatively, linkage analysis of laf<sup>ml100</sup> was carried out via PCR with genomic DNA of single embryos. Primers were AGCAAGGCGCATGACCTGCTC (sense) and CAGCAGTGAAGTGGGGTCT (antisense). PCR conditions were 5 minutes at 95°C; 40× (30 seconds at 95°C, 30 seconds at 58°C, 20 seconds at 72°C); 7 minutes at 72°C. FspI digestion of the 111 bp amplification product resulted in a 44 bp and a 67 bp fragment for the mutant allele, whereas the wild-type allele was not cleaved. The same protocol was used to genotype experimental laf<sup>ml100</sup> mutants. The bmp2b allele swrt<sup>72</sup> and the bmp7 allele snbl<sup>68</sup> were genotyped as previously described (Hild et al., 1999; Dick et al., 2000).

Developmental RT-PCR analysis was carried out as previously described (Bauer et al., 1998). To avoid cross reaction with other type I receptors, primers were derived from the highly divergent extracellular domains. alk8 primers used were ATGGG-GCAATGCCACCC (sense) and GCAAGTGTCGTTCCACAC (antisense). The resulting 533 bp fragment was blotted and hybridized with the internal oligonucleotide AGCAAGGCGCATGACCTGCT (antisense) and CATCAAGAAAAACGGCACCTGC (internal). BmpR1/alk3 primers used were GCATACGGAGATCTAC-GGCAG (sense), ATACGTTGGCTTAATGGGAGG (antisense) and CATACGAAAGAAAAACGGCACCTGC (internal); BmpR1/alk6 primers were TAAGGCAAGCCGGCTCGTG (sense), ATCTCA-GTGTCAGATGGCG (antisense) and ACTACTGTAAGCCAC-CAGC (internal).

Generation of constructs

All constructs used for in vitro transcription were cloned into pCS2+ (Rupp et al., 1994). The coding region of wild-type and mutant forms of alk8 was amplified by PCR with a proofreading polymerase (Pfu, Stratagene), introducing 5’ and 3’ EcoRI sites and a Kozak sequence upstream of the start codon. Truncated Alk8 and receptors terminating one or 13 amino acid residues after the transmembrane domain were generated via PCR. For the dominant negative version Alk8(K232R), for the constitutively active Alk8CA and for introducing the laf<sup>ml110</sup> mutation into the truncated Xenopus Bmp receptor IA, PCR-based site specific mutagenesis was applied as described (Hild et al., 1999).

RNA synthesis, microinjection and in situ hybridization

pCS2-based constructs were linearized with NotI, pSP64TS-based constructs with BamHI (alk3) or XbaI (bmp2b). Capped mRNA was
synthesized using the message machine kit (Ambion), and injected into one- to four-cell stage embryos as described (5 nl per embryo; Hammerschmidt et al., 1999). The sequence of the morpholino antisense oligonucleotide alk8morph1 was CAACTCTCAATGGACTCTCAACCG, complementary to the region from −38 to −17 in the 5'UTR of alk8 cDNA, alk8morph2 was GATTCATGTTGTTGTCATTTTGC, complementary to −130 to −105 of the alk8 cDNA, and the two corresponding four-mismatch control morpholino oligonucleotides 4mm-alk8morph1,2 were CAAAGTGACTCTCAACCG and GATACATGTTGTTGTCATTTTGC. Morpholino oligonucleotides were injected at a concentration of 1–3 mg/ml in 1× Danieau buffer (5 nl per embryo; 58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM Ca(NO3)2, 5 mM Hepes pH 7.6). Whole-mount in situ hybridization was carried out as previously described (Hammerschmidt et al., 1996a).

RESULTS

Cloning of alk8

Using a degenerate approach to isolate serine/threonine kinase receptors (see Materials and Methods), several cDNA clones were isolated, three of which encode Alk8, a novel type I serine/threonine kinase receptor whose sequence was published during the course of our analysis (Yelick et al., 1998). The extracellular domain of Alk8 is rather divergent from other receptors (30% amino acid identity to the most closely related receptor, human Alk2), whereas the kinase domain is more highly conserved (84% identity with hALK2; Yelick et al., 1998). Within the 45 loop of the kinase domain, the motif that determines the specificity of type I receptors to Smad proteins (Chen et al., 1998a; Chen et al., 1998b), the Alk8 sequence, matches the consensus sequence of the Alk1/2 subgroup of receptors, which is clearly distinct from the subgroup of the classical Bmp receptors (see Introduction; Fig. 1A).

Developmental RT-PCR analysis (Fig. 1B) and whole-mount in situ hybridization (see Fig. 4C and Yelick et al., 1998) revealed that alk8 is ubiquitously and uniformly expressed from the two-cell stage until day 5, the latest stage examined. Thus, alk8 displays both maternal and zygotic expression, similar to the putative Bmp type I receptors Alk3/BR1A and Alk6/BR1B (Nikaido et al., 1999a; Nikaido et al., 1999b; Fig. 1B).

Loss of Alk8 function leads to dorsalized, gain of Alk8 function to ventralized phenotypes

As a first step to investigate which signals are mediated by the Alk8 receptor, loss- and constitutively active versions of Alk8 were injected into one- to four-cell stage embryos as described (5 nl per embryo; Hammerschmidt et al., 1999). The sequence of the morpholino antisense oligonucleotide alk8morph1 was CAACTCTCAATGGACTCTCAACCG, complementary to the region from −38 to −17 in the 5'UTR of alk8 cDNA, alk8morph2 was GATTCATGTTGTTGTCATTTTGC, complementary to −130 to −105 of the alk8 cDNA, and the two corresponding four-mismatch control morpholino oligonucleotides 4mm-alk8morph1,2 were CAAAGTGACTCTCAACCG and GATACATGTTGTTGTCATTTTGC. Morpholino oligonucleotides were injected at a concentration of 1–3 mg/ml in 1× Danieau buffer (5 nl per embryo; 58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM Ca(NO3)2, 5 mM Hepes pH 7.6). Whole-mount in situ hybridization was carried out as previously described (Hammerschmidt et al., 1996a).

activates signal transduction in the absence of ligand. Such versions can be engineered by exchanging an amino acid residue at a specific position near the GS domain to aspartate (e.g. Wieser et al., 1995). Injection of alk8CA mRNA encoding the corresponding (Q204D) mutant receptor into wild-type embryos led to a strong ventralization, morphologically characterized by the absence of head and notochord, and by enlarged blood islands (Fig. 2B). This phenotype was indistinguishable from that obtained upon overexpression ofbmp2, bmp4 or bmp7 (Nikaido et al., 1997; Dick et al., 2000; Schmid et al., 2000).

Conversely, dorsalized phenotypes were obtained upon inhibition of Alk8 using two different approaches. The first was injection of mRNA encoding dominant negative versions of the receptor. C-terminally truncated Alk8 receptors (Alk8ΔC) that lack the cytoplasmic kinase domain only caused weakly dorsalized phenotypes (up to C1) at rather low frequencies (Table 1). In contrast, stronger dorsalized phenotypes up to C5 were obtained with a potential kinase-dead Alk8 version carrying a Lys→Arg exchange in the ATP-binding domain, as originally described for a mutant Tgfβ type I receptor (Bassing et al., 1994; alk8(K232R); Table 1; Fig. 2C).

For both dominant negative Alk8 versions, it cannot be ruled out that their effect is caused by interfering with the signaling of other type I receptors, e.g by titrating out shared co-receptors or ligands. For a specific targeting of Alk8, we used a novel antisense approach, injecting antisense
morpholino oligonucleotides against the 5’UTR of the alk8 cDNA. According to recent results obtained in Xenopus and zebrafish (Heasman et al., 2000; Nasevicius and Ekker, 2000), such morpholino oligonucleotides lead to a specific inhibition of both maternally and zygotically supplied gene products by interfering with the translation of the corresponding mRNAs. Injection of two distinct alk8 antisense morpholino 25mer oligonucleotides (alk8morph1,2) into wild-type zebrafish embryos resulted in dorsalization up to C5 strength, as judged by morphology and the krox20 expression pattern (Table 1, Fig. 2D,F-H). Co-injection of wild-type alk8 mRNA, which by itself had no effect, led to a significant reduction of dorsalization, whereas co-injected alk3 mRNA had no rescuing effect (Table 1). Furthermore, embryos injected with morpholino oligonucleotides carrying four nucleotide exchanges, compared with alk8 morphology1,2, developed normally (4mm-alk8morph1,2; Table 1, Fig. 2E). Altogether, these data indicate that the effect of the alk8 antisense morpholino oligonucleotides is specific; and that Alk8 is an essential component involved in the specification of ventral

### Table 1. Injection studies

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<th>Concentration (ng/μl)</th>
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<th>C4 (%)</th>
<th>C3 (%)</th>
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*Classification of the strength of dorsalization (from weak (C1) to strong (C5)) and ventralization (from weak (V1) to strong (V4)) was according to Mullins et al., 1996 and Kishimoto et al., 1997. Used alleles were lost-a-fin laf+/10, snailhouse snh+/68, swirl swrplemented and somitabun stb+/75.

†Frequency of mutant or morphant embryos showing a response to the injected RNA. Response was defined as a shift of the mutant phenotype to an at least two classes weaker phenotypic strength, e.g. C5 to at least C3 for swr, C4 to at least C2 for snr or snb, C2 to WT for laf. Response frequencies were calculated relative to the expected frequencies of mutant embryos in the various crosses, or relatively to the obtained frequencies in the morpholino oligonucleotide single injection (alk8morph1 or bmp2bmorph).

‡Injected alk8 mRNA leads to a general dorsalization of wild-type embryos.

For injections of bmp2b, bmp7 and smad5 mRNA into offspring of two laf carriers, embryos with the laf-characteristic tail phenotype were scored as C2 or C1, independent of ventralized traits in other regions of the body (see text and Fig. 3). In morpholino oligonucleotide/RNA co-injections, injected mRNAs did not contain the 5’UTR sequences targeted by the morpholinos.

alk8CA, constitutively active Alk8; alk8AC, C-terminally truncated Alk8; alk8(K232R), Alk8 with Lys-Arg exchange in ATP binding domain; alk8 antisense morpholino oligonucleotides; 4mm-alk8morph1,2, control morpholino oligonucleotides with four mismatches compared with alk8morph1,2; n, number of scored embryos; iXBRA1, C-terminally truncated Xenopus BmpR1A.
Fig. 2. alk8 is implicated in ventral specification. All embryos, unless stated otherwise, are genetically wild type. Embryos in A-D are at 36 hours post fertilization, lateral view, head to the left. (A) Uninjected control. (B) Strongly ventralized embryo (V3) after injection of alk8CA mRNA. Arrowhead indicates absent head region, arrow indicates enlarged blood islands. (C-D) Strongly dorsIALIZED embryo (C4) after injection of alk8(K232R) mRNA (C) or alk8 antisense morpholino oligonucleotide alk8morph2 (D). Arrows indicate wound-up trunk. Note that the phenotype is much stronger than that of the laf mutant (see Fig. 3B). (E) Uninjected control; (F) injected with alk8morph2; (G) swirl/bmp2b mutant swirlK168/alk8morph2-injected and bmp2b mutant embryo display a ventral fusion of both krox20 stripes (arrows), indicative of C5 dorsralization (compare with Dick et al., 2000).

*alk8* is mutated in the dorsIALIZED mutant *lost-a-fin* (*laf*)

We next sought to identify a zebrafish *alk8* mutant. During the large-scale mutant screen, dorsIALIZED mutations defining six genes required for ventral specification were isolated, *swirl*, *snailhouse*, *somitabun*, *piggy-tail*, *lost-a-fin* and *minifin* (Mullins et al., 1996). For *swirl*, *snailhouse*, *somitabun* and *minifin*, the mutated genes have been identified (see Introduction). As a first step to investigate whether *alk8* might be mutated in one of the two thus far unresolved dorsIALIZED complementation groups *lost-a-fin* (*laf*) or *piggytail*, wild-type *alk8* mRNA was injected into *laf*mut110 and pgy140 mutants. *laf* mutant embryos display dorsализation of intermediate strength (C1-C2), characterized by a complete loss of the ventral tail fin and an enlarged heart cavity at 36 hours postfertilization (Fig. 3B), while the phenotype of *pgy* mutants can be slightly stronger (up to C3; Mullins et al., 1996). The phenotypes of both mutants are significantly weaker than those caused by *alk8* antisense morpholino oligonucleotides (compare with Fig. 2D) and by mutations in *bmp2b*, *bmp7* or *smad5* (C4-C5; for *smad5*, see Fig. 3M). Injection of *alk8* mRNA led to a striking rescue of the *laf* phenotype (Table 1, Fig. 3C), but not of the *pgy* phenotype (data not shown), while injection of mRNAs encoding the putative Bmp receptors Alk3/BR1A and Alk6/BR1B had no rescuing effect (Table 1).

As a next step, the *alk8* gene and the *laf* mutation were genomically mapped. Using a radiation hybrid panel of the zebrafish genome (Geisler et al., 1999), *alk8* was mapped to LG2 between the markers z3743 and z17291. The *laf* allele *laf*mut110 (see below) was mapped to the same region (Fig. 4A). In addition, direct linkage analysis between the *laf*mut110 mutation and the *alk8* gene was carried out, taking advantage of a restriction fragment length polymorphism (RFLP) generated by the *laf*mut110 mutation. No recombination between *laf*mut110 and *alk8* was found in 280 meioses, indicating that they are located within 0.4 cM. Similarly, no recombination was found between *alk8* and the *laf*mut110 allele (see below) in 50 meioses.

Finally, *alk8* cDNA was cloned and sequenced from mutant embryos of the two existing *laf* alleles, *laf*mut110 (isolated during the Tübingen screen; Mullins et al., 1996), and *laf*mut110 (isolated during the Boston screen and initially named *grinch*; Solnica-Krezel et al., 1996). The two alleles behave very similarly. They cause phenotypes of identical strengths. In addition, both are zygotic recessive, and no dominant maternal or dominant zygotic effect has been observed.

Sequence analysis of *alk8* cDNA from *laf*mut110 mutant embryos revealed a TGC→CGC mutation causing a Cys→Arg exchange at amino acid position 91 in the extracellular domain of the Alk8 receptor (Fig. 4B). The mutated cysteine is part of a conserved motif, the cysteine-box, which is present in all serine/threonine kinase receptors. In contrast to wild-type *alk8* mRNA (see above), injected *laf*mut110 mutant *alk8* mRNA could not rescue the *laf* phenotype, even when double the amount of mRNA was injected (Table 1). The consequences of the Cys→Arg exchange of the *laf*mut110 allele were also examined in a second assay. A C-terminally truncated Xenopus Bmp receptor (XtBRIA; Graff et al., 1994) has been reported to dorsализre zebrafish embryos (Hammerschmidt et al., 1996b), presumably by titrating out Bmp ligand or type II co-receptor. Introduction of the Cys→Arg mutation into this truncated receptor abolished its dorsализing potential: while injecting wild-type truncated receptor led to severe dorsализation (up to C4), no sign of dorsализation was observed after injection of Cys→Arg mutant truncated receptor (Table 1). This demonstrates that the *laf*mut110 mutation abolishes the ability of the truncated type I receptor to act in a dominant negative fashion, presumably by reducing its ability to bind ligand or type II co-receptor. Together, these results indicate that *laf*mut110 is a strong hypomorph or a null mutation.

Sequence analysis of the *laf*mut110 allele revealed a
CGA→TGA nonsense mutation, introducing a premature stop codon at position 145, two amino acids after the transmembrane domain (Fig. 4B). This leads to a C-terminally truncated protein, similar to the dominant negative *Xenopus* receptor XtBR1A and the engineered truncated Alk8AC receptors described above. The resulting protein lacks the entire kinase domain, which is necessary to transmit the signal into the cell. Thus, the lafm allele should have no residual activity and, as expected, injected lafm8 mRNA was not able to rescue the laf phenotype (Table 1). Similar to Alk8AC (see above), but unlike XtBR1A (see above), injection of lafm8 mRNA into wild-type embryos had no dorsalizing effect, indicating that in contrast to the truncated *Xenopus* type I receptor, truncated Alk8 does not act in a dominant negative manner. This is consistent with the genetic behavior of the mutation, which does not display any dominant effect (Solnica-Krezel et al., 1996). Thus, lafm10, like lafm110, appears to be an amorph rather than an antimorph.

One explanation for the missing antimorphic character of the lafm100 mutation could be instability of mutant alk8 mRNA (see Discussion for alternative explanation). Both RT-PCR analyses and whole-mount in situ hybridization revealed strongly reduced levels of alk8 transcripts in lafm100 mutant embryos (Fig. 4C,D). In contrast, no such reduction was observed in lafm110 mutant embryos, ruling out an autoregulatory mechanism (data not shown). Consistently, RT-PCR and restriction analyses of the alk8 mRNA population in lafm100/sbamorph2 mutant, injected with smad5 mRNA (G), co-injection of bmp2b mRNA and the alk8 antisense morpholino oligonucleotide alk8morph2 (H), injection of smad5 mRNA (I), or co-injection of smad5 mRNA and alk8morph2 (J). The embryos in G, I, J display strong ventralization (V3; arrowheads indicate absent head and enlarged blood island); the embryo in H shows strong dorsallization (C4; arrow indicates wound-up trunk), similar to the alk8morph2- injected embryo shown in Fig. 2D. (K) Rescued bmp2b mutant sbn, injected with alk8CA mRNA. (L) Rescued bmp7 mutant sbn, injected with alk8CA mRNA. Arrows in K, L indicate reduced or absent ventral tail fin, indicating mild dorsalization (C1); arrowheads indicate smaller head, indicating weak ventralization. (M, N) Sibling embryos from sbn mother; displaying C4 dorsalization, characterized by wound-up tail and trunk (arrows); (M) un.injected embryo; (N) alk8CA mRNA-injected embryo.

**Fig. 3.** *alk8* acts downstream of *bmp2b* and *bmp7* and upstream of *smad5*. All embryos are shown at 36 hours post-fertilization, lateral view, head towards the left. The laf, swr and snh embryos shown in panels C-F, K, L were genotyped after photography. (A) Wild-type sibling. (B) lafm100 mutant. Arrows indicate absent ventral tail fin and enlarged pericardial cavity. (C) Rescued lafm100 mutant, injected with alk8 mRNA. (D) Rescued lafm110 mutant, injected with smad5 mRNA; arrow indicates starting swelling of the pericardial cavity. (E) lafm110 mutant, injected with bmp2b mRNA. (F) lafm110 mutant, injected with bmp7 mRNA. Arrowheads indicate small head and enlarged blood islands, both signs for ventralization; arrow indicates absent ventral tail fin and enlarged heart cavity, both laf-characteristic features (compare with B). (G-J) Genetically wild-type embryos after injection with bmp2b mRNA (G), co-injection of bmp2b mRNA and the alk8 antisense morpholino oligonucleotide alk8morph2 (H), injection of smad5 mRNA (I), or co-injection of smad5 mRNA and alk8morph2 (J). The embryos in G, I, J display strong ventralization (V3; arrowheads indicate absent head and enlarged blood island); the embryo in H shows strong dorsallization (C4; arrow indicates wound-up trunk), similar to the alk8morph2 -injected embryo shown in Fig. 2D. (K) Rescued bmp2b mutant sbn, injected with alk8CA mRNA. (L) Rescued bmp7 mutant sbn, injected with alk8CA mRNA. Arrows in K, L indicate reduced or absent ventral tail fin, indicating mild dorsalization (C1); arrowheads indicate smaller head, indicating weak ventralization. (M, N) Sibling embryos from sbn mother; displaying C4 dorsalization, characterized by wound-up tail and trunk (arrows); (M) un-injected embryo; (N) alk8CA mRNA-injected embryo.

**Rescued laf mutants develop later defects**

While injection of wild-type alk8 mRNA into lafalk8 mutants led to embryos with wild-type appearance at the first day of development (see above; Fig. 3C), these embryos started to develop severe pericardiac edema around 36 hours after fertilization, and died between day 5 and day 10 of development (see Fig. 3D for an example of the starting phase of this phenotype; 10/10 larvae with normal ventral tail fins, but edemas at day 5 of development were genotyped as lafm110 homozygotes). This edema phenotype of injected embryos was observed for both laf alleles and trans-heterozygotes (24% of offspring of lafm110/+ x lafm110/+ cross, n=350; 28% of offspring of lafm100/+ x lafm100/+ cross, n=108; 27% of offspring of lafm110/+ x lafm100 cross, n=218), suggesting that it is not due to closely linked second site mutations. When raising alk8-injected offspring of two lafm110 carriers and genotyping them as adults, not a single homozygote could be
identified among over 150 tested fish. Therefore, no laf homozgyous adult females were available to study whether the laf mutation has a recessive maternal effect.

**alk8 acts downstream of bmp2b/bmp7 and upstream of smad5**

The dorsalized phenotype of the laf mutant suggests that Alk8 acts in a Bmp signal transduction pathway. To further test this notion, injection experiments were carried out in various dorslized zebrafish mutants. First, we tested the ability of different components of the Bmp signal transduction pathways to rescue the laf mutant phenotype. Overexpression of smad5, encoding a transcription factor supposed to act downstream of the Bmp receptors, led to a normalization of the laf dorsalization to wild-type condition (Table 1; Fig. 3D).

In contrast, bmp2b or bmp7 mRNA, both encoding potential Alk8 ligands, failed to rescue the phenotypic traits of laf mutants (Table 1; Fig. 3E,F). Although both mRNAs led to strong ventralization of laf mutant embryos characterized by a loss of dorsoanterior fates, the same embryos still displayed severe edema and the absence of the ventral tail fin, the characteristics of laf mutants. This demonstrates that, despite their strong ventralizing activity, both Bmp proteins are unable to rescue the defects of laf mutant embryos. Consistent results were obtained when mRNA was co-injected with morphorlino antisense oligonucleotides in order to interfere with the aforementioned strong dorsalization caused by the maternal-zygotic alkah knockdown (see Fig. 2D). bmp2b or bmp7 mRNA, although strongly ventralizing in parallel single injections (Fig. 3G; Table 1), had hardly any effect in co-injections with the dorsalizing alkah morphorlino antisense oligonucleotides (Fig. 3H; Table 1), indicating that a gain of Bmp2b or Bmp7 function cannot compensate for the effect caused by the loss of Alk8 function. In contrast, co-injection of smad5 mRNA and alkah morphorlino antisense oligonucleotides led to ventralized phenotypes (Fig. 3J; Table 1), as after injection of smad5 mRNA alone (Fig. 3I; Table 1). Altogether, the data suggest that Alk8 is essential to mediate Bmp2/Bmp7 signaling. In contrast, Smad5 action is not blocked by the loss of Alk8 function, suggesting that Smad5 acts downstream of Alk8 or in a parallel pathway.

To distinguish between the two possibilities, the constitutively active form of Alk8 described above (Fig. 2B) was applied to bmp2b, bmp7 and smad5 mutant embryos. Injection of alkahCA mRNA into swirl/bmp2b (Kishimoto et al., 1997; Nguyen et al., 1998b; Fig. 3K) or snailhouse/bmp7 (Schmid et al., 2000; Dick et al., 2000) mutant embryos (Fig. 3L) led to an almost complete rescue of the strongly dorsalized phenotype (Table 1). Consistently, alkahCA mRNA had a strong ventralizing effect even when co-injected with (normally strongly dorsalizing) bmp2b antisense morphorlino oligonucleotides (Table 1). In contrast, somitabun/smad5 mutants (Hild et al., 1999) showed no response to alkahCA (Fig. 3M,N; Table 1), indicating that Alk8 requires Smad5 to mediate Bmp signaling.

**DISCUSSION**

The dorsalization of laf mutants is caused by null mutations in Alk8

In the past two years, two Bmp ligands (Bmp2b, Bmp7) and one downstream transcription factor (Smad5) have been shown to be essential for ventral development during dorsoventral pattern formation of the zebrafish embryo. Here, we have identified a receptor, the serine/threonine kinase Alk8, as an additional essential component. Two mutant alleles are described: lost-a-fin lafm110 from the Tübingen screen and lafm100 from the Boston screen, both of which appear to be null mutations. lafm100 encodes a C-terminally truncated receptor that lacks its entire cytoplasmic kinase domain. In contrast to similar versions of other type I receptors such as Xenopus and zebrafish Alk3/BRIA (Graff et al., 1994; Nikaido et al., 1999a), however, truncated lafm100 Alk8 does not act in a dominant fashion. This could be due to a specific degradation of mutant lafm100 alkah RNA caused by the premature stop codon, in line with the strongly reduced lafm100 alkah mRNA levels found in mutant embryos. Recent findings indicate that such nonsense-mediated decay is a widely used mechanism to eliminate mRNAs with nonsense mutations (reviewed in Czapinski et al., 1999). Alternatively, the absent dominant effect of the lafm100 mutation could be explained by differences in the mechanisms of signal transduction used by Alk8 and other type I receptors, such that only Alk8 requires its intracellular domain to bind ligand and/or co-receptor. Such a role of the cytoplasmic domain would explain why even the engineered C-terminally truncated versions of Alk8, which lack all possible 3’ elements associated with nonsense-mediated decay (Czapinski et al., 1999), had hardly any dorsalizing effects, whereas the kinase-dead version Alk8(K232R), which carries just a single amino acid exchange in the ATP-binding domain, was strongly dorsalizing.

**Alk8 acts as a Bmp2b/7 receptor upstream of Smad5**

Previously, the function of Alk8 and potential ligands was unknown. No alkah orthologs have been described from other vertebrate species (Yelick et al., 1998). Sequence comparison with other type I receptors revealed that Alk8 is rather divergent from the hitherto described Bmp receptors Alk3/BmpRIA and Alk6/BmpRIB. Alk8 shares highest similarity with Alk2, previously known as ActRIA, and the other members of the Alk1 subgroup of Tgfβ receptors (Massagué, 1998; Chen and Massagué, 1999). For Alk2, a role during signaling by Bmps as well as by other Tgfβ proteins has been suggested. It has been shown to bind activin and Tgfβ1, and to mediate activin-like responses in cell culture studies (Attisano et al., 1993; Ebner et al., 1993; ten Dijke et al., 1994; Yamashita et al., 1995). In addition, Alk2 is capable of binding Bmp7 (ten Dijke et al., 1994; Liu et al., 1995) and possibly Bmp2/4, although with lower affinity (Liu et al., 1995). Functional studies in Xenopus indicate that Alk2 is involved in the induction of ventral cell types, similar to Bmp2 and Bmp4 (Armes and Smith, 1997). In line with these findings, it was shown that Alk2, despite its different L45 loop (see Introduction), can phosphorylate and activate Smad1, a transducer of Bmp2/4 signals, but not Smad2, a transducer of Tgfβ and activin signals (Macias-Silva et al., 1998; Chen and Massagué, 1999), similar to the Smad specificities of the classical Bmp receptors Alk3/BmpRIA and Alk6/BmpRIB (Kretschmar et al., 1997).

Another prominent member of the Alk1 subgroup of Tgfβ receptors is Drosophila Saxophone (Massagué, 1998).
Previous reports have described Saxophone as a receptor of the Drosophila Bmp2/4 homolog Decapentaplegic (Dpp, Brummel et al., 1994; Nellen et al., 1994). However, more recent findings indicate that Saxophone mediates signaling by the more distantly related Tgfβ protein Screw (Neul and Ferguson, 1998; Nguyen et al., 1998a) and the Bmp7-related protein Gbb-60A (Chen et al., 1998a; Khalsa et al., 1998; Haerry et al., 1998), while Dpp signaling is transduced by the serine/threonine kinase receptor Thick veins (Neul and Ferguson, 1998; Nguyen et al., 1998). This suggests that Saxophone might be a receptor of Bmp7-like ligands. Interestingly, Dpp and Screw have synergistic roles during embryonic dorsoventral patterning (Neul and Ferguson, 1998; Nguyen et al., 1998a), and the same is true for Dpp and Gbb-60A during wing formation of Drosophila (Khalsa et al., 1998; Haerry et al., 1998), similar to the equivalent roles of the related vertebrate proteins Bmp2b and Bmp7 during dorsoventral patterning of the zebrafish embryo (Dick et al., 2000; Schmid et al., 2000). In summary, there are hints pointing to Alk8-related proteins as a distinct class of Bmp receptors, while other data suggest other functions.

Here, we provide genetic data pointing to a role of zebrafish Alk8 as a receptor for Bmp2b and Bmp7. The alk8 loss-of-function phenotype indicates that Alk8 is required for the specification of ventral fates during dorsoventral pattern formation, similar to Bmp2b, Bmp7 and the Bmp signal transducer Smad5 (Kishimoto et al., 1997; Nguyen et al., 1998b; Schmid et al., 2000; Dick et al., 2000; Hild et al., 1999). In addition we found that Smad5, but not Bmp2b and Bmp7 can rescue the alk8 loss-of-function phenotype, while Alk8 can rescue the bmp2b and bmp7, but not the smad5, mutant phenotypes, demonstrating that Alk8 acts downstream of Bmp2b and Bmp7 and upstream of Smad5. These data, however, do not exclude the possibility that Alk8 serves as a receptor for other ligands such as Bmp4. Biochemical studies are in progress to clarify this point.

**The requirement of maternally supplied alk8 mRNA**

The phenotypes caused by the zygotic effect of null mutations in alk8 are relatively weak compared with those caused by the loss of the two putative Alk8 ligands Bmp2b and Bmp7 (Kishimoto et al., 1997; Nguyen et al., 1998b; Dick et al., 2000; Schmid et al., 2000) and the putative downstream transcription factor Smad5 (Hild et al., 1999). This indicates that some, but not all Bmp2b/7 signaling depends on zygotic Alk8, a notion supported by our results obtained in injection studies and double mutant analyses. Injected bmp2b and bmp7 mRNA, although unable to rescue the Alk8-dependent development of the ventral tail fin, can ventralize all other body regions of laf mutant embryos. Similarly, loss of the Bmp2/7 inhibitor Chordin (Hammerschmidt et al., 1996b; Schulte-Merker et al., 1997; Dick et al., 2000) in din/laf double mutants leads to reduced head sizes and enlarged blood islands as in din single mutants and after bmp2/7 overexpression, while the laf-specific ventral tail fin deficiency is maintained (H. and M. H., unpublished observation). Altogether, these data indicate that – except during ventral tail fin specification – Bmp2b/7 signals can use receptors that act in parallel to those encoded by zygotically generated alk8 mRNA.

It appears that these partially redundant receptors are encoded by maternally supplied alk8 mRNA (see Fig. 1B), similar to the situation found for the putative downstream component Smad5: loss of zygotic smad5 gene products leads to a very weak dorsalization (C1); loss of maternal gene products to a very strong dorsalization (C4-5; Hild et al., 1999; T. Mayr and M. H., unpublished observations). To study a possible recessive maternal effect of the laf mutations, homozygous laf mutant females need to be generated, which unfortunately has failed so far, due to later lethality of alk8 mRNA-injected laf embryos (see below). As an alternative approach to target both maternally and zygotically supplied alk8 mRNA, we injected antisense morpholino oligonucleotides. alk8morph injections led to dorsalization up to C5, which is as strong as that caused by the loss of bmp2b, bmp7 or smad5 function, and significantly stronger than the C1/C2 phenotype.
dorsalization caused by the zygotic effect of the \textit{laf} mutations. In contrast to the \textit{C1/C2 laf} mutants (see above), the \textit{C5 alk8} morphants do not respond to co-injected \textit{bmp2b} or \textit{bmp7} mRNA. This indicates that dorsoventral patterning and Bmp2b signal transduction does indeed depend on maternally supplied \textit{alk8} gene products that can partly compensate for the loss of zygotic \textit{alk8} in mutant offspring from heterozygous mothers. According to this notion, the missing zygotic supply of wild-type \textit{alk8} mRNA leads to a progressively increasing reduction in the overall concentration of Alk8 receptors that affects only those cell fates specified the latest and/or requiring strongest Bmp signaling (as is indeed the case in \textit{laf} mutants).

A second reason for the observed weak phenotype of \textit{laf} mutants could be partial redundancy of Alk8 with other receptors such as the ‘classical’ Bmp type I receptors Alk3/BmpR1A and Alk6/BmpR1B, whose mRNAs are present during all relevant stages. However, this possibility appears quite unlikely in the light of preliminary results, according to which targeting of either receptor with morpholino antisense oligonucleotides has no dorsalizing or \textit{alk8}-enhancing effect (H. B. and M. H., unpublished observations).

Possible later functions of \textit{laf}/\textit{alk8}

In addition to dorsoventral pattern formation, \textit{alk8} might be required for later developmental processes. This is indicated by results obtained in our \textit{laf} rescuing experiments. Upon injection of \textit{alk8} mRNA, the \textit{laf}-specific dorsoventral defects were normalized in most of the injected \textit{laf} mutants. However, in contrast to all other studied dorsoventral mutants, none of these embryos survived to adulthood. Rather, they developed pericardiac edema and died at larval stages. More detailed analyses of such \textit{alk8} mRNA-injected \textit{laf} mutants are under way to address possible later functions of the Alk8 receptor.

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NOTE ADDED IN PROOF

In the accompanying paper (Mintzer et al., 2001), similar results on the essential role of \textit{Alk8}/\textit{Laf} during dorsoventral pattern formation of the zebrafish are described. Mintzer et al. succeeded in raising and breeding homozygous \textit{laf} mutant females. The offspring of such homozygous mothers are strongly dorsalized, which correlates well with our results obtained using morpholino antisense oligonucleotide injections.

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