

Gene expression pattern

Isthmin is a novel secreted protein expressed as part of the *Fgf-8* synexpression group in the *Xenopus* midbrain–hindbrain organizer

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Abstract

Patterning of the central nervous system is regulated by a signaling center located at the midbrain–hindbrain boundary (MHB), or isthmus organizer. Fibroblast growth factors secreted from the MHB are required and sufficient to direct the ordered growth and regionalization of the midbrain and anterior hindbrain. In an unbiased secretion cloning screen of *Xenopus* gastrula embryos we identified a novel gene, which we designated as *Isthmin* (*xIsm*) due to its prominent expression at the MHB. *xIsm* encodes a secreted protein of 449 amino acids containing one copy of the thrombospondin type 1 repeat (TSR). We also found orthologous *Isthmin* genes in human (*hIsm*) and mouse (*mIsm*), as well as a gene encoding an Isthmin-like human unknown protein (*hIsm-l*). The conservation of a unique carboxy-terminal region between *hIsm* and *hIsm-l* suggests that *Isthmin* is the founding member of a new family of secreted proteins. *xIsm* was strongly expressed maternally in the *Xenopus* egg and showed zygotic expression in the ventral blastopore lip, notochord, and MHB. Additional expression domains were detected in neural crest, ear vesicle, and developing blood islands. Interestingly, *xIsm* was co-expressed with *Fibroblast growth factor-8* (*xFgf-8*) at multiple sites including the MHB, indicating that these two genes are part of a synexpression group which also includes *sprouty* and *sef* homologs. © 2002 Published by Elsevier Science Ireland Ltd.

Keywords: Isthmin; Isthmus; Organizer; Blastopore lip; Notochord; Neural crest; Branchial arches; Ear; Diencephalon; Blood islands; Tsp1; Thrombospondin; F-Spondin; Semaphorin; UNC5; Properdin; Sprouty; Sef; Secretion cloning; *Xenopus*

1. Results and discussion

1.1. *Isthmin* is the founder of a new family of secreted proteins

We have recently reported a method, designated secretion cloning, to screen directly for secreted proteins in early *Xenopus* embryos (Pera and De Robertis, 2000). Pools of 16 cDNAs derived from an expression cDNA library of LiCl-dorsalized gastrulae were transfected into human embryonic kidney 293T cells. Proteins were labeled with radioactive ³⁵S-methionine and -cysteine, and supernatants analyzed by SDS gel electrophoresis and autoradiography. During this screening, we identified a novel protein, designated as *Xenopus* Isthmin (*xIsm*), which was secreted as a 60 kDa protein (GenBank accession number pending; Fig. 1A).

The full-length *xIsm* cDNA clone encoded a 449 amino acid protein containing a hydrophobic signal peptide at the N-terminus, three putative O-glycosylation sites, and a centrally located thrombospondin type 1 repeat (TSR; Fig. 1B, D). Lawler and Hynes (1986) originally described the TSR as a domain involved in cell-to-cell and cell-to-matrix interactions. TSR domains are present in a wide set of secreted and membrane-bound proteins including Thrombospondin-1 and -2, the F-Spondin family, the brain-specific angiogenesis inhibitor (BAI), members of the Semaphorin-5 family, the Netrin receptor UNC-5, the vertebrate complement component Properdin, and the thrombospondin-related anonymous protein (TRAP) of *Plasmodium* sporocytes (Adams and Tucker, 2000). The TSR of these proteins contains three conserved motifs that mediate cell and extracellular matrix binding: the WSXW motif, followed by the CSVTCG motif, and basic amino acids including two key arginine residues. These TSR motifs are also present in *xIsm* (Fig. 1C).

Using public sequence databases, we identified the mammalian orthologs of *xIsm*. A human EST (GenBank No. AW873508) led to the identification of a UniGene Clus-

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Abbreviations: BAI, brain-specific angiogenesis inhibitor; FGF, fibroblast growth factor; MHB, midbrain–hindbrain boundary; TRAP, thrombospondin-related anonymous protein; TSR, thrombospondin type 1 repeat

ter (Hs.348287) that partially represented the human *Isthmin* gene (*hIsm*). Two genomic clones deposited in the Sanger Center (GenBank Nos. AL050320 and AL133463) provided missing 5' sequence information, allowing us to reconstruct the full-length open reading frame of *hIsm* (Fig. 1B, D). Human Isthmin protein shares 78% overall amino acid identity with xIsm. The genomic locus of *hIsm* comprises six exons spanning 77.7 kb on chromosome 20p12.1 (Fig. 1E). We also found a murine EST (GenBank No. BF472502) and a UniGene Cluster (Mm.4113) representing the partial sequence of mouse *Isthmin* (*mIsm*, data not shown). *mIsm* maps to chromosome 2.

In addition, we identified a human unknown protein (GenBank No. AAD09622) that is more distantly related to xIsm (43% amino acid identity) and designated human Isthmin-like (*hIsm-l*, Fig. 1B). The *hIsm-l* gene is located on chromosome 14q24.3. *hIsm* and *hIsm-l* share strong sequence conservation with xIsm in the TSR (88 and 60% amino acid identity, respectively) and downstream of the TSR in the carboxy-terminal (CT) region (91 and 58% iden-

tity, respectively; Fig. 1B). Despite the high degree of amino acid similarity, we were unable to detect any known signature in the CT region using available protein domain databases. Thus, Isthmin and Isthmin-like are members of a new family of secreted proteins that are characterized by the presence of a TSR and a unique CT region.

1.2. Expression of *Xenopus Isthmin* during embryonic development

We investigated the temporal and spatial distribution of *xIsm* RNA by whole-mount in situ hybridization in *Xenopus* embryos (Fig. 2). Strong maternal expression was seen at the four-cell stage (Fig. 2A). During gastrulation, *xIsm* transcripts were detected in the ventrolateral blastopore lip (vbl, Fig. 2B–D). In neurula and tailbud embryos, two expression domains were restricted to the paraxial mesoderm (pa) and neural folds (nf) at the posterior end of the body (Fig. 2E, G, I–K). At mid-gastrula, an independent expression domain appeared in the notochord (n) that became strongly positive by the neurula stage (Fig. 2C–F). By neurula stage, three additional expression domains could be seen anteriorly: at the anterior edge of the neural plate (anp), in cranial neural crest cells (nc), and at the level of the future midbrain–hindbrain boundary (MHB) or isthmus (Fig. 2F–I). At tailbud stage, *xIsm* expression was detected in the branchial arches (ba) that are derived from the cranial neural crest (Figs. 2J, K and 3G). Concomitantly, expression in the MHB was maintained (Figs. 2J, K and 3G) and persisted in this structure at least until tadpole stage (stage 40; data

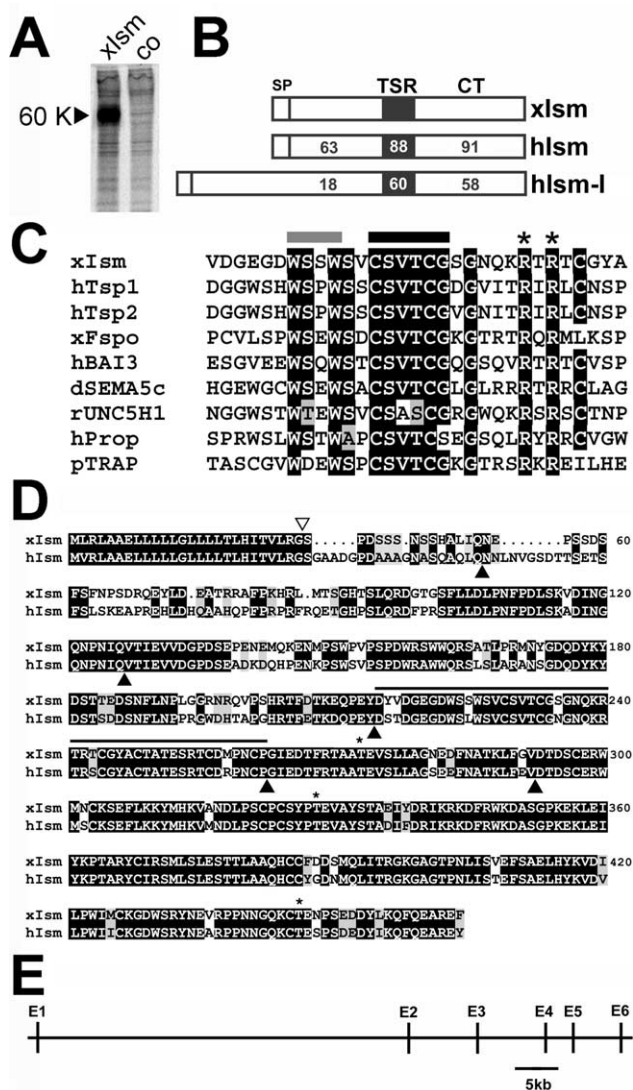


Fig. 1. Isthmin identifies a new family of secreted proteins. (A) Supernatant medium of human 293T cells transfected with *Xenopus Isthmin* cDNA (*xIsm*) or non-secreted green fluorescent protein cDNA as control (*co*). Transfected cells were labeled with ³⁵S-methionine and -cysteine, and their supernatants analyzed by SDS-PAGE and autoradiography. Note that *xIsm* is secreted as a 60 kDa protein. (B) Diagrams of *Xenopus Isthmin* (*xIsm*), human Isthmin (*hIsm*), and human Isthmin-like (*hIsm-l*) proteins. The N-terminal signal peptide (SP), thrombospondin type 1 repeat (TSR; black box), and conserved carboxy-terminal region (CT) are indicated. The numbers represent percentage of amino acid identities between the *Xenopus* and human sequences. (C) Motifs from the TSR of *xIsm* aligned with homologous sequences of other TSR-containing proteins; these sequences are thought to mediate cell and extracellular matrix binding. The WSXW motif is indicated with a gray bar, the CSVTG motif with a black bar, and the two conserved arginine residues with stars. GenBank accession numbers and amino acid residues are as follows: hTsp-1, human Thrombospondin 1 (X14787, 434–464); hTsp-2, human Thrombospondin-2 (L12350, 437–467); xFspo, *Xenopus* F-Spondin (LO9123, 607–637); hBAI3, human brain-specific angiogenesis inhibitor-3 (NP001695, 291–321); dSEMA5c, *Drosophila* Semaphorin-5c (AF198084, 837–867); rUNC5H1, rat UNC5H1 (NP071542, 241–271); hProp, human Properdin (AF005666, 77–107); pTRAP, *Plasmodium* thrombospondin-related anonymous protein (AB0063359, 240–270). (D) Sequence alignment of *Xenopus* and human Isthmin. Identical amino acid residues are shaded in black, and similar residues in gray. The signal peptide cleavage site (open triangle), TSR domain (bar), putative O-glycosylation sites (stars), and exon–intron boundaries of *hIsm* (filled triangles) are indicated. (E) Genomic structure of human *Isthmin*. Note that six exons (E1–6) span a region of 77.7 kb.

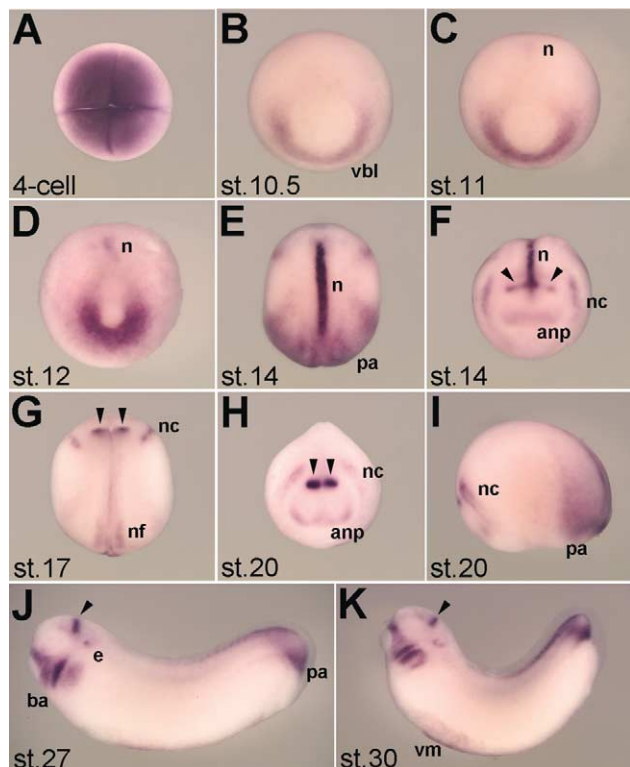


Fig. 2. Expression of *Xenopus Isthmin* by whole-mount in situ hybridization. Embryos are shown in animal (A), vegetal (B–D), dorsal (E,G), anterior (F,H) or lateral (I–K) view. (A) Four-cell stage embryo; note the high level of maternal transcripts. (B–D) Embryos at gastrula stage showing distinct expression in the ventral blastopore lip (vbl), and the anterior end of the notochord (n). (E,F) Early neurulae showing strong expression throughout the notochord, the posterior paraxial mesoderm (pa), cranial neural crest (nc) and anterior edge of the neural plate (anf). The arrowheads mark expression at the prospective midbrain–hindbrain boundary. (G) Late neurula with an additional expression domain in the posterior neural fold (nf). (H,I) Early tailbud stage; note strong expression at the midbrain–hindbrain boundary (arrowheads). (J,K) Tailbud stage; *xIsm* expression is apparent in the branchial arches (ba), ear placode (e) and ventral mesoderm (vm).

not shown). The MHB is an important signaling center in vertebrates and regulates the polarized morphological differentiation of the adjacent tectum and cerebellum (Crossley et al., 1996; Liu and Joyner, 2001; Rhinn and Brand, 2001). By tailbud stage, other *xIsm* expression domains were visible in the posterior diencephalon (di; Fig. 3G), the ear placode (e; Figs. 2J, K and 3G), and ventral mesoderm that later gives rise to blood islands (vm, Fig. 2K). We conclude that *xIsm* is expressed dynamically during early embryonic development, including a specific expression domain in the isthmus organizer.

1.3. *Isthmin* belongs to the *Fgf-8* synexpression group

Studies in chick, zebrafish and mouse embryos have shown that *Fgf-8* is an essential signaling factor that mediates the organizing activity of the MHB (Crossley et al., 1996; Liu and Joyner, 2001; Rhinn and Brand, 2001).

Fig. 3 shows that the expression pattern of *xIsm* closely resembles the pattern previously reported for the *xFgf-8* gene (Christen and Slack, 1997). Expression domains shared by *xIsm* and *xFgf-8* at the gastrula and neurula stages include the ventrolateral blastopore lip (Fig. 3A, D), notochord (Fig. 3B, E), the cranial neural crest, and the MHB (Fig. 3C, F). At the anterior border of the neural plate the expression domains of *xIsm* and *xFgf-8* partially overlap. A comparative analysis of whole-mount in situ hybridizations at the tailbud stage indicated co-expression of *xIsm* and *xFgf-8* at the MHB, branchial arches, the posterior diencephalon, and ear vesicle (Fig. 3G, H). To confirm this, we hybridized embryos with a mixture of probes for both genes. It was found that the hybridization domains at the MHB overlapped exactly (Fig. 3I). In the ear vesicle, *xIsm* and *xFgf-8* were expressed in distinct and complementary expression domains (Fig. 3G–I).

Other genes have previously been demonstrated to be co-expressed with *Fgf-8* in vertebrate embryos. These include further members of the *Fgf* family (*Fgf-17*, *Fgf-18*; Maruoka et al., 1998; Reifers et al., 2000), members of the *sprouty* family (*spry-1*, *spry-2*, *spry-4*; Minowa et al., 1999; Fürthauer et al., 2001) and *sef* (*similar expression to fgf genes*; Tsang et al., 2002; Fürthauer et al., 2002). The term ‘synexpression group’ has been proposed for genes that share a distinct, complex expression pattern and may be involved in the same biological process (Niehrs and Pollet, 1999). Indeed, it has been shown that the intracellular Sprouty proteins and the putative transmembrane protein Sef act as feedback-induced negative regulators of Fgf signaling (Minowa et al., 1999; Fürthauer et al., 2001; Tsang et al., 2002; Fürthauer et al., 2002). To investigate the activity of *xIsm* we microinjected *xIsm* mRNA into early *Xenopus* embryos. We did not detect any apparent phenotypic effect (data not shown). Injected *xIsm* mRNA did not alter the expression of *xFgf-8*, or the expression of the Fgf target gene *Xbra* (data not shown). In conclusion, we have isolated a novel secreted protein, *Xenopus Isthmin*, that is part of the *Fgf-8* synexpression group.

2. Experimental procedures

Embryos were staged according to Nieuwkoop and Faber (1994). An unamplified cDNA library from *Xenopus* LiCl₂-dorsalized stage 11 embryos was prepared by inserting oligo dT-primed cDNA into the *Eco*RI and *Xho*I sites of the expression vector pCS2⁺ (Pera et al., 2001). Secretion cloning was performed as described by Pera and De Robertis (2000). Briefly, 293T cells were transfected with pools of cDNA clones and 1.5 days later labeled with ³⁵S-methionine and -cysteine under serum-free conditions. Secreted proteins were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography, and positive clones individualized in a second transfection step by sib-selection. pβGFP/RN3P (gift of

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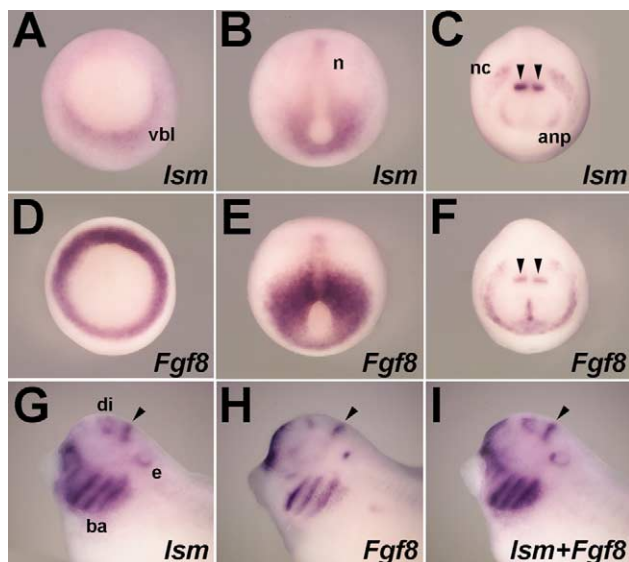


Fig. 3. Comparison of *xIsm* and *xFgf-8* expression. (A,D) Stage 10 embryos in vegetal view; note that *xIsm* and *xFgf-8* expression overlap in the ventrolateral blastopore lip (vbl). (B,E) Stage 11 embryos in dorso-vegetal view showing expression of *xIsm* and *xFgf-8* in the anterior notochord (n). (C,F) Stage 20 embryos in anterior view; note overlapping expression of *xIsm* and *xFgf-8* in cranial neural crest (nc), anterior edge of neural plate (anp), and midbrain–hindbrain border (arrowheads). (G–I) Stage 30 embryos in lateral view. (G) Whole-mount in situ hybridization depicting *xIsm* expression in the branchial arches (ba), diencephalon (di), ear placode (e), and isthmus (arrowhead). (H) *xFgf-8* expression at the same stage. (I) Double in situ hybridization of *xIsm* and *xFgf-8*; note the overlap of expression in branchial arches, diencephalon and isthmus (arrowhead), as well as the complementary expression in the ear placode.

Dr J. Gurdon) was used as a control plasmid. For antisense RNA, pCS2⁺ constructs of *xIsm* (this article) and *xFgf-8* (gift of Dr J. Slack) were linearized with *HindIII* and *XhoI*, respectively, and transcribed with T7 RNA polymerase. Whole-mount in situ hybridizations were performed as described (<http://www.hhmi.ucla.edu/derobertis/>).

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