

Direct neural induction and selective inhibition of mesoderm and epidermis inducers by Xnr3

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SUMMARY

During gastrulation in amphibians, secreted factors from Spemann's organizer act on dorsal ectoderm to induce the central nervous system. A number of secreted factors produced by Spemann's organizer have recently been identified. The TGF β family member Xnr3 is similar in amino acid sequence to the mouse factor nodal and is expressed in a restricted group of cells in the superficial layer of Spemann's organizer. Xnr3, unlike the related factors nodal, Xnr1 and Xnr2, lacks mesoderm-inducing activity. We report here that Xnr3 can directly induce neural tissue in *Xenopus* ectoderm explants (animal caps). Injection of animal caps with either Xnr3 RNA or plasmids induces the expression of the pan-neural genes NCAM and *nrp1*, as well as the anterior neural marker *Cpl1*. A growing body of evidence suggests that neural induction in *Xenopus* proceeds as the default in the absence of epidermis inducers. The best candidates for the endogenous epidermis inducers are BMP-4 and BMP-7. The neural inducing activity of Xnr3 can be inhibited by overexpression

of BMP-4, as has been observed with the neural inducers noggin, chordin and follistatin. Furthermore, Xnr3 can block mesoderm induction by BMP-4 and activin, but not by Xnr2. The structural basis underlying the divergent activities of Xnr2 and Xnr3 was analyzed using site-directed mutagenesis. Mutations introduced to the conserved cysteine residues characteristic of the TGF β family were found to inactivate Xnr2, but not Xnr3. The most unique feature of Xnr3 is the absence of a conserved cysteine at the C terminus of the protein. This feature distinguishes Xnr3 from other TGF β family members, including Xnr2. However, we observed that changing the C terminus of Xnr3 to more closely resemble other TGF β family members did not significantly alter its activity, suggesting that other structural features of Xnr3 distinguish its biological activity from Xnr2.

Key words: *Xenopus*, neural induction, Xnr3, BMP-4, mesoderm, epidermis

INTRODUCTION

A principle mechanism regulating embryonic development in animals is induction (Slack, 1993). One of the most studied sources of inductive signals is Spemann's organizer (Spemann, 1938). During amphibian gastrulation, secreted signals from Spemann's organizer act on dorsal ectoderm to induce the central nervous system, and on dorsal mesoderm to induce somites (Kessler and Melton, 1994; Lettice and Slack, 1993; Smith and Slack, 1983). Numerous secreted inducing factors have been identified in the gastrulating *Xenopus* embryo, and pattern formation at gastrulation appears to involve the interaction of a complex set of stimulatory and inhibitory signals. Several lines of evidence now support a model in which neural-inducing signals function by inhibiting epidermis induction, suggesting that neural development proceeds as the default in the absence of epidermis-inducing factors (Sasai et al., 1995; Wilson and Hemmati-Brivanlou, 1995). It was first observed that dissected *Xenopus* animal pole ectoderm (animal caps), which normally form epidermis when cultured in isolation, will spontaneously develop as neural tissue when dissociated (Grunz and Tacke, 1989; Wilson and Hemmati-Brivanlou,

1995). According to the model, dispersal of the cells causes an endogenous epidermis-inducing factor to diffuse from the cells to sub-inducing concentrations.

The epidermis-inducing factor in the gastrula embryo appears to be either, or both, of the bone morphogenetic proteins-4 and -7 (BMP-4 and BMP-7) (Hawley et al., 1995; Sasai et al., 1995; Wilson and Hemmati-Brivanlou, 1995). While both BMP-4 and BMP-7 are expressed throughout the blastula ectoderm, BMP-4 becomes excluded from the future neural plate during gastrulation (Fainsod et al., 1994; Hawley et al., 1995; Hemmati-Brivanlou and Thomsen, 1995). Overexpression of BMP-4 in animal caps overrides neural-inducing signals and promotes the formation of epidermis (Sasai et al., 1995; Wilson and Hemmati-Brivanlou, 1995). Treatments that disrupt BMP-4-like function lead to neural development, which is consistent with the identification of a BMP-4-like activity as the epidermis-inducing factor. Injection of RNA encoding a dominant negative BMP receptor causes neural induction in *Xenopus* animal caps (Sasai et al., 1995; R. H. Xu et al., 1995). Similarly, injection of RNAs encoding dominant negative versions of BMP-4 or BMP-7 also induce neural tissue in *Xenopus* animal caps (Hawley et al., 1995).

Finally, the neural-inducing activity of a dominant negative activin receptor (Hemmati-Brivanlou and Melton, 1994) appears to be explained by its ability to inactivate the BMPR (Schulte-Merker et al., 1994; Wilson and Hemmati-Brivanlou, 1995).

BMP-4 (or factors with similar activities) also appears to play a role in patterning mesoderm at gastrulation (Jones et al., 1996). Overexpression of BMP-4 will convert dorsal mesoderm to ventral (Dale et al., 1992; Jones et al., 1992), while disruption of BMP signaling with a dominant negative BMPR is sufficient to promote the expression of dorsal mesoderm markers from explanted ventral mesoderm (Graff et al., 1994). This result suggests that dorsal development in the mesoderm, like neural development in the ectoderm, may be the default state.

Spemann's organizer produces at least three secreted factors with direct neural-inducing activity: noggin, follistatin and chordin (Hemmati-Brivanlou et al., 1994; Lamb et al., 1993; Sasai et al., 1995). In neural induction assays, all three factors induce anterior-type neural tissue, as assessed by the expression of brain-specific molecular markers. Basic FGF (bFGF) has been reported to have direct posterior neural-inducing activity (Kengaku and Okamoto, 1993; Lamb and Harland, 1995), although other studies have failed to detect this activity (Cox and Hemmati-Brivanlou, 1995). However, there is general agreement that bFGF can promote the expression of posterior markers from explants previously fated to develop as anterior neural tissue (Cox and Hemmati-Brivanlou, 1995; Lamb and Harland, 1995). Recently, it has been reported that inhibiting FGF signaling in animal caps with a dominant negative FGF receptor blocks neural induction by both noggin and explanted Spemann organizers (Launay et al., 1996). Therefore, the inducing activity of bFGF stands apart from those of noggin, follistatin and chordin.

A common mechanism has been proposed by which follistatin, chordin and noggin induce neural tissue by inhibiting BMP-4. Both noggin and chordin have been shown to directly bind BMP-4 in solution, thus preventing it from interacting with its receptor (Piccolo et al., 1996; Zimmerman et al., 1996). In *Xenopus* neural induction assays, it is thus proposed that exogenously added noggin or chordin binds the endogenous BMP-4 (or BMP-4-like activity) present in the animal cap, and that this alone, by suppression of epidermis induction, is sufficient to initiate the pathway of neural development. It is also assumed that follistatin, which is known to bind activin with high affinity, works in a similar way, although it is unclear if it binds BMP-4 or closely related molecules (Wilson and Hemmati-Brivanlou, 1995). Both noggin and chordin are reported to dorsalize ventral mesoderm explants (Sasai et al., 1994; Smith et al., 1993), indicating that an identical mechanism is important in patterning mesoderm at gastrulation (i.e., inhibition of BMP-4-like activity by Spemann's organizer factors).

We report here on the neural-inducing activity of another organizer-specific gene, the nodal-related gene *Xnr3*. *Xnr3* was isolated independently in two screens, one using an activity based assay to isolate cDNAs with dorsal-inducing activity (Smith et al., 1995), and the other using a differential screening strategy (Ecochard et al., 1995). Here we show that *Xnr3*, which lacks mesoderm-inducing activity, is able to directly induce the expression of neural markers in *Xenopus* animal

caps. The present results indicate that the mechanism of *Xnr3* neural induction is similar to that of noggin, chordin and follistatin. *Xnr3* and BMP-4 have mutually antagonistic activities. The neural-inducing activity of *Xnr3* is inhibited by overexpression of BMP-4, while *Xnr3* overexpression is able to inhibit mesoderm induction by BMP-4. However, *Xnr3* is also able to partially block mesoderm induction by activin, an activity that distinguishes *Xnr3* from noggin and chordin.

MATERIALS AND METHODS

Xenopus embryos and explants

Induced ovulation of female *Xenopus* and fertilization of eggs was as described previously (Condie and Harland, 1987). Embryos were staged according to Nieuwkoop and Faber (1994). 15-20 animal caps were cultured together in approximately 100 μ l of 75% normal amphibian media (3/4 NAM: 82.5 mM NaCl, 1.5 mM KCl, 0.75 mM $\text{Ca}(\text{NO}_3)_2$, 0.75 mM MgSO_4 , 75 μ M EDTA, 1.5 mM sodium phosphate (pH 7.5), 0.75 mM NaHCO_3) with 25 mg/ml gentamycin and BSA added to 1 mg/ml. Where indicated, activin containing COS cell media (from the same batch described previously; Lamb and Harland, 1995) was diluted in 3/4 NAM with BSA before adding to animal caps.

Plasmid constructs

A constitutively active version of the type I BMP receptor (the ALK-3 receptor; Koenig et al., 1994; ten Dijke et al., 1994) was made by changing glutamine at residue 233 to aspartic acid. Details of the construction will be published elsewhere (R. Wieser and J. Massagué, personal communication). For the present study, the activated receptor was subcloned into the *EcoRI* and *XbaI* sites of pCS2+ (Turner and Weintraub, 1994). The construction of the human BMP-4/pCSKA plasmid has been described previously (Jones et al., 1996).

RNA synthesis and injection

Capped RNAs were synthesized from linearized plasmid templates using the mMessage mMachine (Ambion). The integrity of the transcripts was checked by formaldehyde/agarose gel electrophoresis. The preparation of the noggin, *Xnr3* and β -galactosidase plasmid templates was as described previously (Green et al., 1992; Smith and Harland, 1991, 1992; Smith et al., 1995). The *Xnr2* template consisted of a 1.4 kb cDNA insert (clone UDL3) in pBluescript SK- (Stratagene) (Jones et al., 1995). The *Xnr2* plasmid was linearized by *XhoI* digestion and RNA transcribed with T3 RNA polymerase. RNA transcripts were made from the activated BMPR/CS2+ construct with SP6 RNA polymerase after digestion of the plasmid with *NotI*.

RNA extraction and analysis

Total RNA was isolated from animal cap explants and embryos using Trizol Reagent (Gibco). For northern analysis, 15-20 caps were used per sample and electrophoresed on formaldehyde-containing agarose gels (Sambrook et al., 1989). Gels were transferred to Hybond-N nylon membrane (Amersham) by capillary action overnight and hybridized with QuikHyb (Stratagene). Random primed ^{32}P -labeled probes were prepared using the Prime-a-gene system (Promega) with isolated fragments from NCAM (Kintner and Melton, 1987), EF1 α (Krieg et al., 1989), muscle actin (also referred to as cardiac actin) (Dworkin-Rastl et al., 1986), *Xbra* (Smith et al., 1991) and epidermal keratin (Jamrich et al., 1987) plasmids.

Ribonuclease (RNase) protection assays (Melton et al., 1984) were performed on the total RNA isolated from 30 animal caps per sample using the HybSpeed RPA kit (Ambion). Yeast RNA was used as a negative control. NCAM protection RNA probes were made from a plasmid containing a 752 bp *EcoRI* to *PvuII* fragment of the 5' end

of the *Xenopus* cDNA (Kintner and Melton, 1987) cloned into pSP70 (Promega). Probes were synthesized from *Hind*III linearized templates with SP6 RNA polymerase. The protected NCAM fragment was 199 bp. Protection RNA probes for Cpl1 (lipocalin) were prepared from plasmid p13-8 (Richter et al., 1988). The plasmid was digested with *Ssp*I, and protection probes transcribed with T3 RNA polymerase. The protected Cpl1 fragment was 140 bp. For EF1 α protection probes, the plasmid pEF1aEH (Krieg et al., 1989) was cut with *Hinf*I, and transcribed using SP6. The protected fragment was 114 bp. For HoxB9, nucleotides 148-624 of a previously described HoxB9 cDNA (Sharpe et al., 1987, referred to in manuscript as XLHbox6) were ligated into pGEM2 (Promega). For probe synthesis, this plasmid was digested with *Sma*I and transcribed with T7 RNA polymerase. The protected fragment was 238 bp.

Immunostaining

Formaldehyde-fixed *Xenopus* embryos and animal caps were stained using the neural-specific monoclonal antibody 6F11 (Jones and Woodland, 1989) and peroxidase-conjugated secondary antibody as described previously (Hemmati-Brivanlou and Harland, 1989).

Site-directed mutagenesis

Oligonucleotide-mediated site directed mutagenesis was performed using a *dut- ung-* strain of *E. coli* (Kunkel, 1985). Single-stranded uracil-containing templates were prepared from Xnr3 and Xnr2 and annealed with the following oligonucleotides to make the indicated mutants: Xnr3/cys+: 5'-GAAGAATGTGGATGCGCATAGATGTAA-CACAGA-3';

C290S: 5'-ACCCGAGTCCAAGAAGGT-3';

C328S: 5'-GCATATAGATCTGAATCCAC-3';

C365S: 5'-TGTCCCTCCTCTGTCCCCATG-3';

Xnr2/C333S: 5'-TGCCTATAGGTCGGAGGGAG-3'.

The presence of the desired mutations was confirmed by dideoxy sequencing using Sequenase (US Biochem). All mutants were also examined by in vitro translation with rabbit reticulocyte lysate (Promega). One of the Xnr3/cys+ mutants was observed to give a truncated protein due to a mutation at a site distal to the targeted mutation. This construct, named Xnr3/trunc, was used as a negative control.

RESULTS

Xnr3 directly induces neural tissue in *Xenopus* animal caps

Xnr3 RNA was injected into the animal pole of 1-cell-stage *Xenopus* embryos at amounts ranging from 5-0.04 ng/embryo. Control embryos received either no injection or 5 ng β -galactosidase RNA. At stage 8, the animal pole ectoderm (animal cap) was dissected from the embryos. The animal caps were grown until the equivalent of early tail-bud stage (stage 25; Nieuwkoop and Faber, 1994), and then RNA was extracted for analysis. The RNA from 20 animal caps was analyzed by hybridizing northern blots with three probes: NCAM (to detect the presence of neural tissue; Kintner and Melton, 1987), EF1 α (as a control for total amount of RNA loaded; Krieg et al., 1989) and muscle actin (to detect the presence of dorsal mesoderm; Dworkin-Rastl et al., 1986). To compare the results with other known inducers, an identical injection series was performed with noggin RNA.

The resulting blots are shown in Fig. 1A. Consistent with our previous observations that Xnr3 lacks of mesoderm-inducing activity (Smith et al., 1995), muscle actin transcript

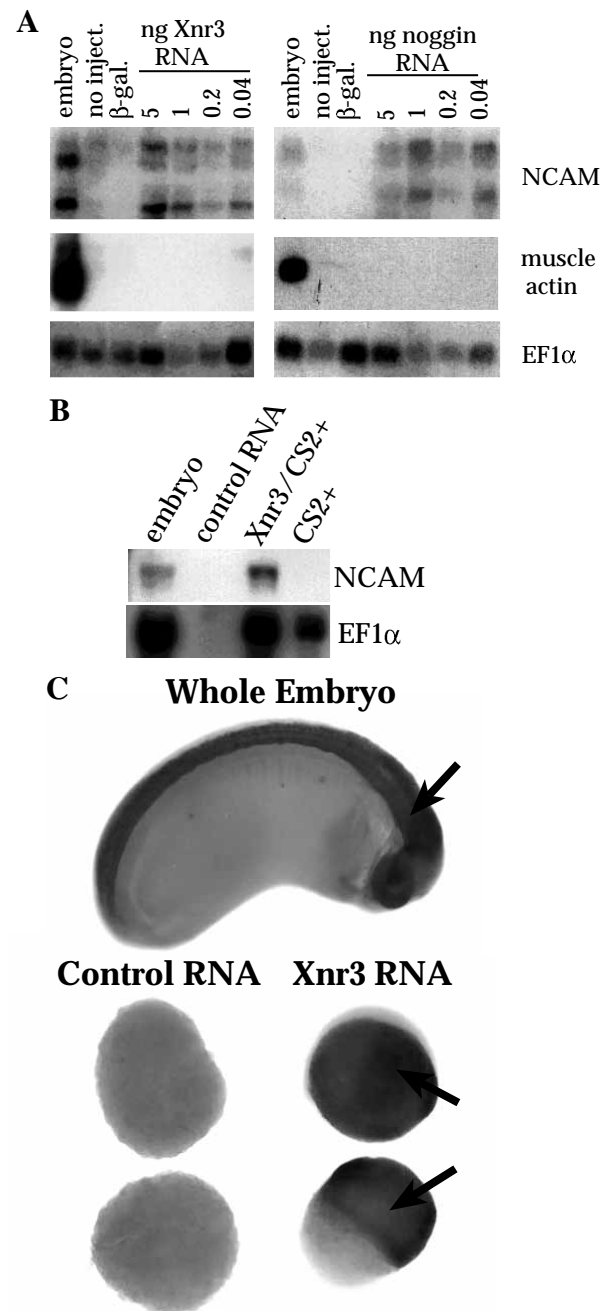


Fig. 1. NCAM (neural cell adhesion molecule) RNA is induced after injection of Xnr3 mRNA or plasmid. 1-cell embryos were injected at the animal pole with indicated RNAs or plasmids. Animal caps dissected at late blastula (stage 8) were grown until sibling embryos reached early tailbud (about stage 25). (A) Northern blot analysis of animal caps receiving injections of 0.04 to 5 ng Xnr3 and noggin mRNA. The response of animal caps to Xnr3 is similar to noggin in that it induces neural tissue, without inducing mesodermal markers such as muscle actin. EF1 α serves as a loading control and embryos injected with β -galactosidase (β -gal.) or uninjected (no inject.) are negative controls. (B) Animal caps dissected from embryos injected with 200 pg Xnr3 expression plasmid (Xnr3/CS2+), or control plasmid (CS2+). Shown is an RNase protection assay for NCAM and EF1 α at about stage 25. Yeast RNA was used as a control for nuclease digestion. (C) Whole-mount immunostaining of *Xenopus* animal caps and embryos with the neural-specific antibody 6F11. Animal caps were either injected with Xnr3 or β -galactosidase RNAs (2.5 ng). Specific staining is indicated by arrows

was not induced at any level of Xnr3 RNA injection. Previous reports have shown that noggin also lacks mesoderm-inducing activity (Lamb et al., 1993). In contrast, NCAM transcripts were detected at all of the injected doses of Xnr3 RNA. The induction of NCAM transcript at the higher three doses was observed consistently in numerous experiments, while induction at the lowest dose (0.04 ng), was more variable [note also that, in the data shown, the loading control (EF1 α) for the 0.04 ng dose is significantly higher than the other doses]. The induction of nrp1, which is expressed throughout the nervous system like NCAM, was also observed in response to Xnr3 (data not shown). The extent of NCAM induction seen with Xnr3 RNA is very similar to that seen with injected noggin RNA (Fig 1A), and significantly higher than that seen with equivalent amounts of injected follistatin RNA (data not shown). No induction of NCAM transcript was seen in either uninjected or β -galactosidase-injected animal caps (in addition, a mutated Xnr3 RNA encoding a truncated protein also lacked NCAM-inducing activity, see Fig. 5B).

To test the neural-inducing activity of Xnr3 in a context more similar to its normal pattern of expression, the Xnr3 cDNA was ligated into the plasmid CS2+, which contains the CMV promoter (Turner and Weintraub, 1994). The CMV promoter starts transcription at the mid-blastula transition, as does the *Xnr3* gene (Smith et al., 1995) (although the CMV promoter directs transcription beyond the gastrula stage, unlike the *Xnr3* gene). 1-cell-stage embryos were injected at their animal poles with 200 pg of Xnr3/CS2+, or CS2+ lacking an inserted cDNA (negative control) and 20 animal caps were assessed for neural-specific gene expression, as in the previous experiments. The results of an RNase protection assay for NCAM and EF1 α are shown in Fig. 1B. NCAM transcript was detected in the animal caps injected with Xnr3/CS2+, but not with the empty CS2+ vector. Although the reduced EF1 α level indicates that the CS2+ control had a significantly lower amount of total RNA, an extended exposure of the protection assay gel failed to detect NCAM transcript in the control.

It was previously reported that when normal *Xenopus* embryos were injected with Xnr3 RNA, the most pronounced responses were reduction in the head and the appearance of multiple tube-like protrusions (Smith et al., 1995). Antibody staining indicated that the protrusions contained variable amounts of muscle or neural tissue. To determine if only limited patches of NCAM-positive cells were induced in the Xnr3-injected animal caps, immunocytochemistry was performed using the neural-specific antibody 6F11. The results in Fig. 1C show that extensive staining was seen throughout the animal caps.

Xnr3 induces anterior neural tissue

To further characterize the neural-inducing activity of Xnr3, animal caps injected with Xnr3 RNA were assayed by RNase protection assay at the equivalent of stage 25 for expression of the anterior neural marker Cpl1 (a member of the lipocalin family; Knecht et al., 1995; Richter et al., 1988), and the posterior marker HoxB9 (also known as XIHbox6; Wright et al., 1990). Only the anterior marker Cpl1 was seen in Xnr3 RNA-injected animal caps, which is identical to the result seen with noggin (Fig. 2).

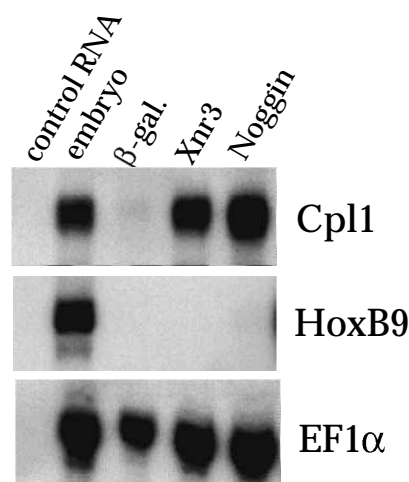


Fig. 2. Xnr3 induces expression of the anterior neural marker Cpl1, but not the posterior neural marker HoxB9. 5 ng of the indicated RNAs were injected into the animal poles of 1-cell-stage embryos. Dissected animal caps were grown to the equivalent of about stage 25. The presence of induced transcripts for the anterior neural marker Cpl1 and the posterior marker HoxB9 were detected by RNase protection assay. EF1 α serves as a control for total RNA recovered per sample. Yeast RNA was used as a control for nuclease digestion.

Neural induction by Xnr3 is inhibited by BMP-4

A number of recent reports indicate that BMP-4 (or other molecules with similar activity) plays a central role in the differentiation of epidermis and neural fates from the ectodermal lineage (Sasai et al., 1995; Wilson and Hemmati-Brivanlou, 1995). According to this model, BMP-4-like activity promotes the differentiation of epidermis, while its inhibition induces neural development. To test if Xnr3 induces neural tissue by a similar mechanism, embryos were co-injected with Xnr3 RNA and a BMP-4 expression plasmid under the control of the cytoskeletal actin promoter plasmid, pCSKA (Harland and Misher, 1988). Control embryos were injected with the empty pCSKA vector alone. Animal caps were dissected from the embryos and processed as before. As shown in Fig. 3A, the BMP-4 expression plasmid was effective at completely suppressing the induction of NCAM transcript by Xnr3 RNA. Conceivably, BMP-4 expression plasmids could inhibit Xnr3 neural induction by converting the animal caps to mesoderm. Epidermal keratin is shown to indicate that the animal caps remain ectodermal in the presence of the injected BMP4 plasmid (additionally, we failed to detect any induction of globin by the BMP-4 expression plasmid, data not shown). Mesoderm inducers such as activin completely suppress epidermal keratin expression (Green et al., 1992). Therefore the inhibition appears to be the result of a competition between two ectodermal fates (neural versus epidermis) and not between mesoderm and ectoderm.

To further characterize the interaction between Xnr3 and BMP-4, animal caps were dissected from embryos injected with Xnr3 RNA along with RNA encoding an activated type I BMP receptor (actBMPR). In an initial characterization of the actBMPR, we observed that injection of RNA for the actBMPR was able to induce brachyury (Xbra) in *Xenopus* animal caps (data not shown). In neural induction assays, we observed that

the actBMPR was equally effective as the BMP-4 expression plasmid at suppressing neural induction by Xnr3 (Fig. 3B).

Xnr3 can inhibit mesoderm induction by BMP-4 and activin, but not by Xnr2.

These results clearly show an inhibitory relationship between Xnr3 and BMP-4. Noggin, chordin and, presumably, follistatin act by directly binding the endogenous BMP-4-like activity present in animal caps to promote neural induction. Unlike these other neural inducers, Xnr3 is a member of the TGF β gene family, suggesting that the mechanism by which Xnr3 inhibits BMP-4 may be different. Xbra and muscle actin induction assays were used to determine if Xnr3 was capable of inhibiting the mesoderm-inducing activity of BMP4 and other TGF β family members. BMP-4 can induce ventral mesoderm in *Xenopus* animal caps (Dale et al., 1992; Köster et al., 1991). The ability of Xnr3 to inhibit the mesoderm-inducing activity of BMP-4 was assessed by dissecting animal caps from embryos injected with three different amounts of BMP-4 RNA (0.5, 1 and 2.5 ng) either alone, or with co-injected Xnr3 RNA (2.5 ng). Animal caps dissected at stage 8 were grown to the equivalent of stage 10.5 (mid-gastrula) and assayed for the presence of Xbra (Smith et al., 1991) transcripts by northern blotting. Induction of Xbra transcripts was seen at both 1 and 2.5 ng of injected BMP-4 RNA (Fig. 4A). Co-injection of Xnr3 RNA was able to cause a complete inhibition of Xbra induction, even when equal amounts (2.5 ng each) of the two RNAs were injected. We also observed that Xnr3 RNA injection was able to partially inhibit muscle actin (Fig. 4B) and Xbra (not shown) induction in animal caps by activin-containing COS cell media at a range of concentrations.

Xnr3 has greatest homology to two TGF β family members also expressed in the gastrulating embryo, Xnr1 and Xnr2.

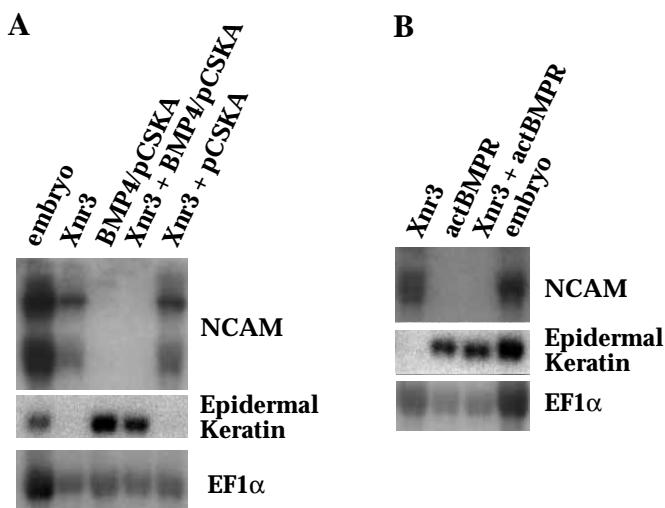


Fig. 3. Neural induction by Xnr3 is inhibited by BMP-4 and the activated type I BMP receptor (actBMPR). (A) *Xenopus* animal caps received either 1 ng Xnr3 RNA, 100 μ g BMP-4 expression plasmid (BMP-4/pCSKA), or both. Control animal caps received the empty plasmid vector (pCSKA). (B) *Xenopus* animal caps received either 1 ng Xnr3 RNA, 2.5 ng actBMPR RNA or both. For both experiments, the induction of NCAM and epidermal keratin transcripts were detected at about stage 25 by northern blotting. EF1 α serves as a control for total RNA loaded per sample.

Unlike Xnr3, Xnr1 and Xnr2 have strong mesoderm-inducing activity (Jones et al., 1995). However, Xnr3 was not able to

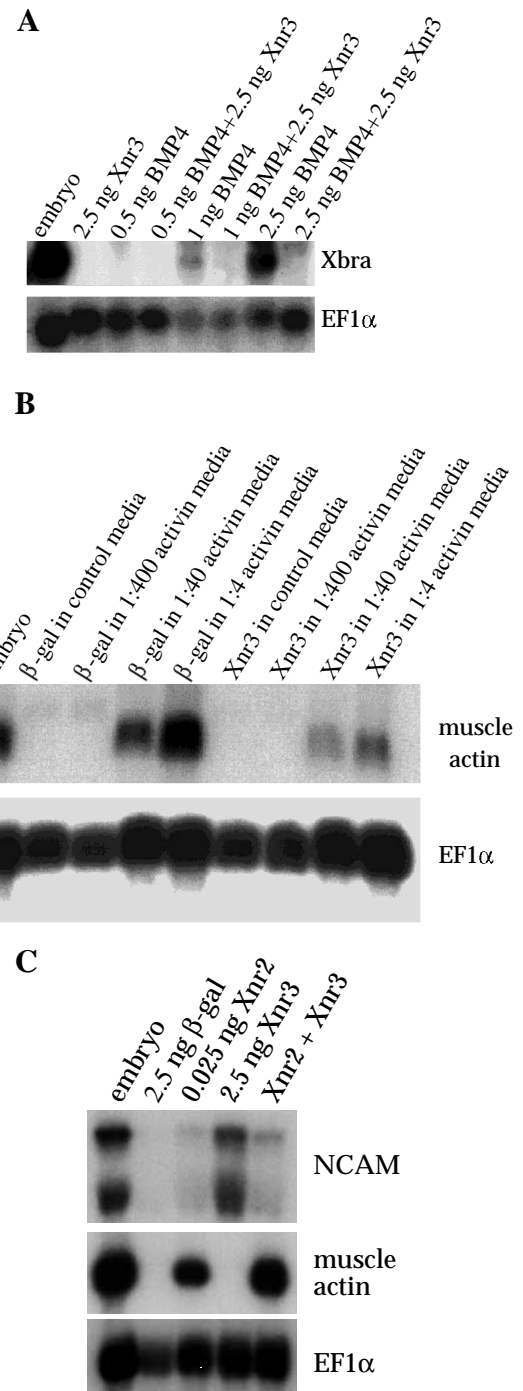


Fig. 4. Xnr3 inhibits mesoderm induction by BMP4 and activin, but not Xnr2. Mesoderm induction in *Xenopus* animal caps was determined by northern blots for either brachyury (Xbra) at stage 10.5 or muscle actin at about stage 25. (A) Induction of Xbra in animal caps by injected BMP-4 RNA is inhibited by co-injection of Xnr3 RNA. (B) Xnr3 is able to partially inhibit mesoderm induction by activin. Animal caps were first dissected from embryos injected with either 2.5 ng Xnr3 or control (β -gal.) RNAs and then treated with various dilutions of activin-containing COS cell media. Shown is a northern blot for muscle actin expression at about stage 25. (C) Xnr3 RNA does not inhibit muscle actin induction by Xnr2 RNA.

inhibit mesoderm induction by Xnr2, even when Xnr3 RNA was injected at a 100-fold excess, as determined by the induction of muscle actin (Fig. 4C) and Xbra (not shown) transcripts. These results show that while Xnr3 is capable of inhibiting mesoderm induction by BMP-4 and activin, it is not capable of inhibiting mesoderm induction by all TGFβ family members. NCAM is shown in this figure as a positive control for the activity of Xnr3. The small amount of NCAM seen in the presence of Xnr2 is likely to be the result of secondary neural induction.

Directed mutations reveal different structural requirements for the activity of Xnr3 and Xnr2

While Xnr3, Xnr2, Xnr1 and nodal constitute a subgroup of closely related growth factors within the TGFβ family, the activity of Xnr3 stands out from the other three. Xnr2, Xnr1 and nodal all have mesoderm-inducing activity, while Xnr3 has direct neural-inducing activity. The deduced amino acid sequence of Xnr3 has both structural features that are common to all TGFβ family members and features that are unique. Using site-directed mutagenesis, an analysis was undertaken to further characterize the divergent activities of Xnr3 and Xnr2. One highly conserved feature in TGFβ family members is a group of seven cysteine residues in the C terminus of the protein. The C terminus comprises the cleaved, active domain of the protein (Kingsley, 1994). It has been previously reported for activin that mutation of any of these seven cysteines results in an inactive protein (Mason, 1994). Site-directed mutagenesis was used to systematically change the first, second and fifth of the conserved cysteines of Xnr3 to serine, generating mutants C290S, C328S and C365S, respectively (Fig. 5A). In vitro RNA transcripts made from the Xnr3 mutants and wild-type Xnr3 were microinjected into one-cell stage embryos. Animal caps were dissected from the injected embryos at stage 8. At approximately stage 25, the animal caps were assayed for the induction of NCAM by northern blotting. Each of the Xnr3

cysteine mutants retained neural-inducing activity, although at reduced potency relative to wild-type Xnr3 (Fig. 5A,B). Because of previous reports on the importance of the conserved cysteines for biological activity of activin (Mason, 1994), the retention of biological activity by the Xnr3 cysteine mutants was surprising. To determine if corresponding mutations gave similar results in the related gene Xnr2, the second conserved cysteine of Xnr2 was mutated to serine, making mutant Xnr2/C333S, which is analogous to Xnr3 mutant C328S (Fig. 5A). Unlike the cysteine mutants in Xnr3, this mutation completely eliminated the mesoderm-inducing activity of Xnr2, as assayed by its ability to induce Xbra (Fig. 5C).

The most divergent feature of Xnr3 compared to other TGFβ family members is found at the C terminus. Xnr1, Xnr2, nodal and almost all other known TGFβs end with the sequence CXCX. In Xnr3, the sequence CGFKDM is found at the C terminus (note that Xnr3 is lacking the C-terminal cysteine found in other TGFβ family members). The C-terminal

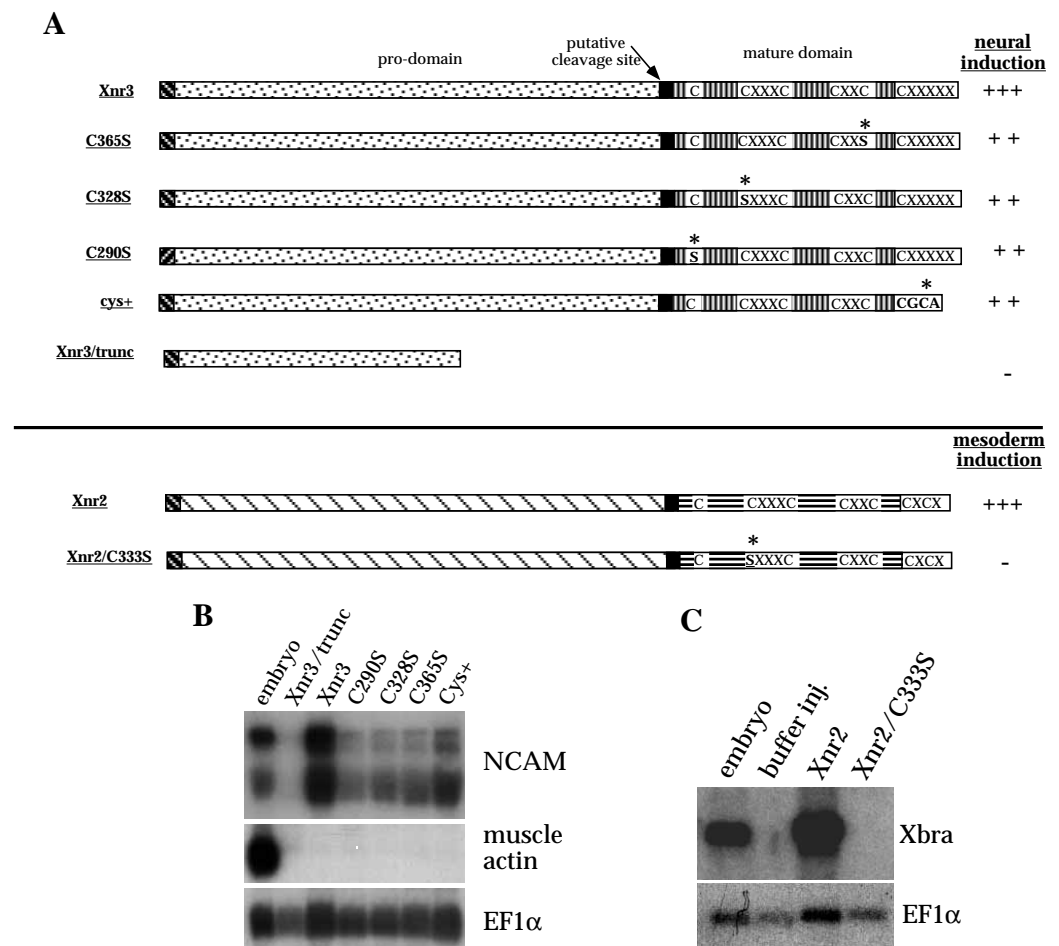


Fig. 5. Functional analysis of Xnr2 and Xnr3 mutants. (A) Summary of site-directed mutations introduced in Xnr3 and Xnr2. The altered amino acids are indicated by asterisks. The neural-inducing activity of the Xnr3 mutants, and the mesoderm-inducing activity of the Xnr2 mutant, are indicated. (B) The neural-inducing activity of Xnr3 is retained, although at a reduced level, by mutations introduced to the conserved cysteine residues (C290S, C328S and C365S) and by addition of a cysteine to the C terminus (Cys+). A mutated Xnr3 RNA encoding a truncated protein (Xnr3/trunc) is a negative control. (C) The mesoderm-inducing activity of Xnr2 is completely eliminated by the Xnr2/C333S mutation. Embryos injected with a buffer (buffer inj.) serve as a negative control.

sequences found in Xnr1, Xnr2 and nodal are CGCS, CGCN and CGCL, respectively. Site-directed mutagenesis was used to change the C terminus of Xnr3 to CGCA, making the mutant Xnr3/cys+ (Fig. 5A). Because there was no conservation of the C-terminal amino acids in Xnr1, Xnr2 and nodal, alanine was chosen for the mutant because it facilitated screening for mutated plasmids by the addition of a *FspI* restriction site (additionally, *Xenopus* activin has an alanine in this position; Thomsen et al., 1990). The Xnr3/cys+ mutant was assayed for neural-inducing activity along with the other Xnr3 cysteine mutants. Surprisingly, this mutant also retained neural-inducing activity, and did not confer muscle actin-inducing activity, a characteristic of Xnr1, Xnr2 and nodal.

DISCUSSION

Xnr3 has direct neural-inducing activity

Xnr3 is a member of the TGF β family that is expressed exclusively within the superficial layer of Spemann's organizer. The predicted amino acid sequence of Xnr3 is most similar to mouse nodal (Zhou et al., 1993), and two other *Xenopus* gene transcripts, Xnr1 and Xnr2 (Jones et al., 1995). However, Xnr3 has structural features, particularly in the C terminus, that distinguish it from other TGF β s. Initial reports on Xnr3 primarily characterized the response of whole embryos to microinjection of Xnr3 RNA, and it was demonstrated that Xnr3 expression plasmids could induce muscle from ventral mesoderm explants (Smith et al., 1995). It was also reported that Xnr3 did not induce mesoderm in *Xenopus* animal caps, unlike nodal, Xnr1 and Xnr2 (Jones et al., 1995). Here we report the further characterization of the response of animal caps to Xnr3. Xnr3 RNA or expression plasmids can induce the expression of the neural-specific genes *NCAM* and *nrp1* in *Xenopus* animal caps. Both *NCAM* and *nrp1* are expressed throughout the nervous system. In the embryo, the source of neural-inducing factors is the dorsal mesoderm. Because Xnr3 lacks mesoderm-inducing activity (Smith et al., 1995 and Figs 1A, 4A-C), Xnr3 is most likely acting directly on ectoderm to induce neural tissue. The neural-inducing activity of Xnr3 appears to be similar to that of noggin, chordin and follistatin because it induces anterior-type neural tissue only, as indicated by the induction of the anterior marker *Cpl1*, but not the posterior marker *HoxB9*.

Spatially and temporally, the inductive signal of Xnr3 is very different than those of noggin, chordin or follistatin. Two models of signaling between the organizer and the overlying presumptive neural plate have been proposed (see review, Doniach, 1993). Dorsal mesoderm from the organizer, as it involutes during gastrulation, may induce the overlying neural plate (Hemmati-Brivanlou et al., 1990; Saha and Grainger, 1992). This has been termed vertical signaling. However, it has been shown that properly formed neural plates still develop in *Xenopus* explants, which never undergo involution (Doniach et al., 1992; Ruiz i Altaba, 1990). In this case, the signal between the organizer and the presumptive neural plate must be passing through the plane of the tissue connecting the organizer and the ectoderm. These two models are not mutually exclusive. If Xnr3 plays a role in neural induction, it probably functions only as a planar inducing signal. The Spemann organizer has distinct domains of gene expression on the axes extending from the

surface (superficial) to deep layers and from the animal-vegetal poles (Vodicka and Gerhart, 1995). Unlike noggin, chordin and follistatin, Xnr3 is expressed exclusively within the superficial layer and only during gastrulation (Smith et al., 1995). The expression of noggin, follistatin and chordin extends into the neurula stage, and later. The unique pattern and timing of expression of Xnr3 is likely to be significant for its role in the gastrula embryo. Xnr3 would appear to be unable to function as a vertical signal for two reasons. The first is that cells stop expressing Xnr3 as soon as they involute at gastrulation (Smith et al., 1995; Vodicka and Gerhart, 1995). In addition, even if the cells continued to secrete Xnr3 for a limited time after the transcript is no longer detectable, the Xnr3-expressing cells are fated to the archenteron roof and thus are separated from the neural plate by the axial mesoderm (Keller, 1975).

Xnr3 may be a naturally occurring dominant negative or receptor antagonist

The mechanism by which Xnr3 induces neural tissue remains to be determined. Xnr3 could bind a unique receptor that initiates a signaling pathway that leads to neural induction. In this way, Xnr3 would not be directly interacting with BMP-4 or its receptor. Suppression of the neural-inducing activity of Xnr3 by BMP-4 expression plasmids would be the result of competition between the two inductive pathways for neural versus epidermal fate. This pathway of neural induction would be very different than that proposed for noggin, follistatin and chordin, in which induction is achieved simply by inhibiting a soluble epidermis-inducing factor.

A similar argument could be made for the ability of Xnr3 to suppress mesoderm induction by BMP4 and activin (i.e., that Xnr3 directs all of the animal caps to neural tissue, thus not allowing mesoderm induction). However, the observation that Xnr3 was not able to inhibit the mesoderm-inducing activity of Xnr2 argues against this possibility. Furthermore, addition of noggin to activin-treated animal caps does not suppress mesoderm induction; on the contrary, it potentiates the induction of dorsal mesoderm by low doses of activin (Smith and Harland, 1992; José De Jesus-Escobar and Jeremy Green, personal communication). Likewise, chordin acts in conjunction with FGF to promote the development of dorsal mesoderm (Sasai et al., 1994). Because the induction of mesoderm precedes neural induction (Kessler and Melton, 1994), it is not surprising that noggin and chordin do not block mesoderm induction. These results suggest that Xnr3 is able to selectively block mesoderm induction by certain growth factors, rather than simply redirecting animal caps to other fates. Xnr3 is expressed during gastrulation, and this may be too late to have role in mesoderm induction. However, the ability of Xnr3 to block BMP-4 mesoderm induction may be indicative of its mechanism of action in neural induction, where BMP-4 inhibition is known to be involved.

There are several possible mechanisms by which Xnr3 could induce neural tissue by a mechanism similar to noggin or chordin (i.e., by directly blocking the activity of the endogenous BMP-4-like activity). Such mechanisms are consistent with the ability of the activated BMPR to inhibit neural induction by Xnr3. One possibility is that in neural induction assays, exogenous Xnr3 forms a nonfunctional heterodimer with the endogenous BMP-4-like activity. In this case, Xnr3 would be functioning as a naturally occurring dominant

negative. A similar mechanism has been reported to explain the antagonistic behavior of activin and inhibin (Xu et al., 1995). The activin beta/beta homodimer can bind the activin type II receptor and activate the association of the type I receptor, while inhibin, made up of an alpha/beta heterodimer, can bind the type II receptor but cannot recruit the type I receptor. BMP4 can heterodimerize with BMP7 in an expression system (Hazama et al., 1995). However, the formation of heterodimers between members of the TGF β family as distantly related as Xnr3 and BMP-4 has not been reported. In addition, Xnr3 can partially inhibit mesoderm induction by soluble activin protein. Because the dimerization of TGF β s occurs intracellularly, it would be very unlikely that Xnr3 is forming a heterodimer with activin in our experiments. A remaining possibility is that Xnr3 could be a BMPR antagonist. Mutations in three of the conserved cysteines residues in Xnr3 resulted in only partial reduction its activity. These results contrast sharply with those from similar mutations made in activin (Mason, 1994), and Xnr2 (shown here). Together, the results indicate significant differences in the structure and function of Xnr3 compared to other TGF β family members. One possibility is that Xnr3 may retain structural features for receptor binding, but not activation. Accordingly, if Xnr3 is a partially inactivated ligand, mutations such as those to the conserved cysteine residues may have less pronounced effects.

Spemann's organizer expresses multiple genes with similar or identical activities

Spemann's organizer expresses the genes encoding at least four soluble factors (noggin, follistatin, chordin and Xnr3) with neural-inducing activity. The reason that the organizer expresses multiple structurally unrelated molecules, all with apparently similar or identical activities and mechanisms of action, is not clear. One obvious reason could be to provide functional redundancy, thus ensuring fidelity in early pattern formation. In the mouse, early development proceeds normally in the absence of follistatin, indicating that, at least in the mouse, not all of these genes are required (Matzuk et al., 1995). Similarly, while the zebrafish has a gene homologous to noggin, it is not expressed within the organizer (Scott Stachel and Richard Harland, personal communication). Finally, while the mouse node expresses nodal, the activity of nodal is very different than Xnr3 and attempts to identify a cDNA from the mouse encoding a protein with greater similarity to Xnr3 have been unsuccessful (Smith et al., 1995). Together, these results indicate that there is considerable variation among vertebrates in the expression of inducing factors at gastrulation. These differences may reflect both the redundancy of these factors, as well as subtleties that give rise to divergent morphologies of these various species.

The restricted domain of Xnr3 expression also raises the possibility that expression of multiple factors in Spemann's organizer that have BMP-4-inhibiting activity could result in a gradient of inhibition. Xnr3 is expressed only in the superficial layer of Spemann's organizer, which until involution at gastrulation, is at the extreme dorsal side of the embryo. Significantly, the superficial layer of Spemann's organizer is fated to contribute to definitive endoderm, as compared to the deeper layer, which contributes to axial mesoderm (Keller, 1975, 1976). The superficial layer of Spemann's organizer also has a different inductive activity to the deeper component. Specifi-

cally, the superficial layer is a stronger inducer of organized convergent extension behavior (Shih and Keller, 1992). We have speculated that Xnr3 may play a role in this activity of the superficial layer (Smith et al., 1995). Whether this activity is also a manifestation of the inhibition of the ventralizing BMP-4-like activity in gastrula-stage embryos is not known.

Both noggin and chordin RNAs give very similar phenotypes when injected into *Xenopus* embryos. Both can completely restore axis development to UV-ventralized embryos and at high concentrations can result in hyperdorsalized embryos (Sasai et al., 1994; Smith and Harland, 1992). The phenotype seen with Xnr3 RNA injection is very different. Only partial rescue of UV-ventralized embryos was reported. In addition, injection of Xnr3 RNA resulted in the appearance of multiple tube-like projections from the surface of the embryos (Smith et al., 1995). These differences clearly indicate that responses to Xnr3 are distinct from those of noggin or chordin. This may reflect responses to a signaling cascade initiated by a putative Xnr3 receptor. Alternatively, if Xnr3 acts as a dominant negative or receptor antagonist, this may reflect differences in the specificity of the inhibition. For example, noggin and chordin may be specific for BMP-4 and related molecules such as BMP-7, whereas Xnr3 may be inhibitory for a broader set of TGF β family members, such as BMP-4 and activin-like molecules.

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