Xerl, a novel CNS-specific secretory protein, establishes the boundary between neural plate and neural crest

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ABSTRACT A novel gene, Xerl, has been found as a CNS-specific gene encoding a secretory protein. In order to clarify a function of Xerl, we first examined Xerl-expressing areas during early development. Comparison with XISox-2-positive neural plate and ADAM13-positive neural crest showed that Xerl expression was limited within the neural plate area. Microinjection of Xerl mRNA into 2- or 4-cell stage embryos indicated that Xerl overexpression caused the regional expansion of XISox-2- and NCAM-positive neural plate, which was concomitant with the outer shift of ADAM13-positive region. The Xerl injection resulted in incomplete neural closure because of the local overproduction of the neuroepithelium. In contrast, loss of function analysis of Xerl indicated that Xerl inhibition caused the ectopic differentiation of neural crest cells. In the conjugation experiment using chordin-injected animal caps, Xerl promoted chordin-induced XISox-2 expression, whereas Xerl inhibition caused ADAM13 expression even in the injection with a high dose of chordin. Animal cap assays also showed that Xerl expression was induced by chordin. In the functional analysis using truncated forms of Xerl, XerlΔL (lacking LNS domain) worked as a dominant negative form that induced the overproduction of neural crest cells. These results suggest that Xerl is involved in the boundary formation of the neural plate through exclusion of neural crest cell differentiation.

KEY WORDS: Xenopus, Xerl, neural plate, neural crest

Introduction

Vertebrate nervous system consists of central nervous system (CNS) and peripheral nervous system (PNS). The first determination of each fate begins at the end of gastrulation. Vertebrate ectoderm is divided into three sets of cells, neural plate, epidermis and neural crest cells, and finally neural plate cells make CNS and also neural crest cells make PNS (Le Douarin, 1982, Hall and Hörstadius, 1988).

In Xenopus, prospective neural crest cells can be identified at the late gastrula stage (stage 12) by their expression of the zinc finger transcription factors, Xsnail and Xslug (Mayor et al., 1995, Mancilla and Mayor, 1996). These expressions are initially restricted to the crest-forming boundary of the neural plate. Classical experiment showed that medial archenteron roof was grafted into the blastocoel of amphibian embryos, and competent ectoderm could be induced to form both neural plate and neural crest (Raven and Kloos, 1945). In Xenopus embryo, several models explain the neural crest determination. In one model, Raven and Kloos (1945) have postulated the existence of a combined CNS/neural crest “evocator” molecule that is produced at different concentrations across the archenteron roof. Now this model may be explained that BMP receptor (BMPR) activities make a threshold between neural plate and neural crest induction (Mancilla and Mayor, 1996, Marchant et al., 1998). Some neuron-inducible molecules derived from dorsal lip are BMP inhibitors like noggin (Lamb et al., 1993), chordin (Sasai et al., 1994) and follistatin (Fainsod et al., 1997). All of these BMP inhibitors are secretory proteins. It has been shown that low doses (pico gram order) of dominant negative BMPR or low doses of chordin can induce gene expression of neural crest-specific genes, whereas high doses (nano gram order) of these molecules can induce neural plate genes (LaBonne and Bronner-Fraser, 1998, Marchant et al., 1998). However, it is still unknown how secretory inhibitors make such a threshold. If this is the case, how does BMP determine sharp threshold between neural plate and neural crest? There should be a mechanism stocking high level BMP inhibitors in the
CNS or keeping the intermediate activity of BMP inhibitors in the PNS. There is a model about the neural crest development that the encountering between the intermediate level of BMP inhibitor and the neural crest-specific inducing signal causes neural crest cell specification, which have proposed by LaBonne and Bronner-Fraser (1998) and Marchant et al. (1998).

Expected property of the postulated factor may be an extracellular protein and to express at specific CNS. Our previous report has shown that a novel Xenopus gene, Xerl, was expressed in CNS from neurula to early tadpole stage (Kuriyama et al., 2000). The prospective structure of Xerl contains EGF-like repeats (ELRs) and laminin-G-like domain including cell attachment sequence, RGD. ELRs also exist in matrix molecules such as fibronectin (Ruoslahti et al., 1984) and signal controlling molecules such as Notch (Artavanis-Tsakonas et al., 1983). Delta (Fehon et al., 1990). Recently, laminin-G domain was found in a large number of proteins like laminin A, neurexins and sex hormone binding globulins, so this domain is called LNS domain (Misserl and Südhof, 1998). ELR-containing proteins such as Slit (Rothberg et al., 1990) and Crumbs (Tepass et al., 1990) also have the LNS domain. Many proteins with ELRs and LNS domain bind to cell surface receptors, suggesting that the LNS domain is cell-surface recognition elements (Misserl and Südhof, 1998). Therefore, Xerl may function as an extracellular protein during neurogenesis of CNS. Here we examine the possibility that Xerl is involved in the boundary formation between neural plate and neural crest.

Results

Xerl expression in the Central Nervous System

In our previous report, we showed that Xerl was expressed in eye and brain (Kuriyama et al., 2000). In order to know Xerl function, we first examined the expression pattern of Xerl in details during neurogenesis. Before neural folding, Xerl expression was seen in the neural groove along the dorsal midline (Fig. 1A, left) and the inner edge of the anterior neuroectoderm (Fig. 1A, right). As neural folding proceeding, Xerl expression was also seen in the trunk neural tube (Fig. 1B, left). In addition, anterior expression of Xerl was seen in midbrain, diencephalon, and eye corresponding to the future neural retina. The boundary between midbrain and diencephalon was indicated as a narrow expression of Xerl (Fig. 1B, right, opened arrowhead). To confirm Xerl expression only in CNS, we compared Xerl expression with XlSox-2 expression at each corresponding stage (Fig. 1 C,D). XlSox-2 is general neural plate marker, expressed in the neural plate from the end of gastrulation (Mizuseki et al., 1998). Our observation showed that Xerl expression occurred within the XlSox-2-positive area (Fig. 1 A,C). This relationship between Xerland XlSox-2 was more obvious by comparison of gene expression at the narrow region between midbrain and diencephalon (Fig. 1 B,D, right, opened arrowhead).

To recognize the neural crest region, we examined ADAM13 expression at same stages. ADAM13 is metalloproteinase/disintegrin family gene expressing in cephalic neural crest cells (Alfandari et al., 1997, Marchant et al., 1998, Cousin et al., 2000). ADAM13 expression was seen in the outer edge of XlSox-2 expressing anterior neural plate (Fig. 1 C,E and D,F). Consequently, ADAM13-expressing area was located outside of Xerl-positive area (Fig. 1 A,E and B,F, right). Thus, neural crest-specific ADAM13 expression occurred complementary to the cephalic expression of Xerl and XlSox-2 (Fig. 1F, right, arrowhead). These data showed that Xerl expression was specific in CNS before and after neural folding.

Morphological changes in Xerl-overexpressing embryos

For gain-of-function analysis, we performed microinjection of synthesized mRNA including full ORF of Xerl into one blastomere of 4-cell stage embryo. Green Fluorescent Protein (GFP) was used as a lineage tracer of injected blastomeres (Fig. 2A). At the stage of anterior neural plate closure, GFP mRNA-injected embryos indicated normal neurulation at all doses tested (Fig. 2B). However, Xerl mRNA-injected embryos showed incomplete neural closure (Fig. 2 C,D, arrow). About 40% embryos indicated the same phenotype (Table 1). Xerl mRNA was effective in the dorsal side, but the injection of Xerl into the ventral side did not lead any morphological changes (data not shown).

To know how Xerl overexpression causes the abnormal neurulation, we performed histological examination of Xerl-injected embryos. During early stages of neuru-
Phenotype categorized into one group as incomplete neural closure. Of phenotype is the opened anterior neural plate and the small neural fold, which were synthesized mRNAs of 126 (100%) 28 (100%). Total 126 (100%) 28 (100%).

Normal neurogenesis 77 (61%) 27 (96%). Incomplete neural closure 49 (39%) 1 (4%).

First, we examined neural plate marker, Xerl overexpression causes neural plate expansion. Then, in neurulating embryos, XlSox-2 expression was symmetrical at neural plate (Fig. 2A, left). However, in neurulating embryos, XlSox-2 expression was seen in the abnormal cell mass (Fig. 3G, right, arrowhead). In Xerl-injected embryo, posterior shift of neural crest was still recognized at the stage of late neurula, and the exclusion of ADAM13 expression was seen in the abnormal cell mass (Fig. 3F, right, arrow).

**Fig. 2. Effect of Xerl overexpression on anterior neural folding.** (A) Selection of GFP-positive embryos. GFP and Xerl mRNA were co-injected into one dorsal blastomere of 4-cell embryos, and the GFP-positive injected side was recognized under fluorescence microscope at stage 14/15. (B) GFP mRNA-injected control (st.20, anterior view). The closure of the anterior neural plate was completed. (C) Xerl mRNA-injected embryo (st.20, anterior view). The neural fold in the injected side shifted to the distal-posterior from midline as shown by incomplete neural closure (arrow). (D) The same embryo of (C) (st.20, dorsal view). The neural fold in the injected side was smaller than the counter side (arrow). (E) Cross-section of the anterior neural plate of the Xerl-injected embryo (st.15). There was no significant change in the injected side (left). (F) The future mid-hindbrain level section of the anterior neural plate of the Xerl-injected embryo (st.17). Arrowheads indicate the neural plate boundary. Lateral shift of the neural plate boundary occurred in the injected side. (G) Cross-section of the future midbrain of the Xerl-injected embryo (st.20). Abnormal cell mass was observed in the injected side (broken-lined circle and arrowheads). Inj, injected side; npb, neural plate boundary; nc, notochord.

**TABLE 1**

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<tr>
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<td>Incomplete neural closure</td>
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<td>77 (61%)</td>
<td>27 (96%)</td>
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<tr>
<td>Total</td>
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Synthesized mRNAs of Xerl and GFP were injected into one dorsal blastomere of 4-cell embryos. Effect of Xerl on neurogenesis was examined at stage 20. The characteristic of phenotype is the opened anterior neural plate and the small neural fold, which were categorized into one group as incomplete neural closure.

Antisense Xerl injection up-regulates gene expression in the neural crest

Xerl overexpression caused neural plate expansion. Then, in order to examine whether an inhibition of Xerl function causes defective formation of neural plate, we performed antisense Xerl injection. As compared with the normal embryo, the antisense Xerl-injected embryo showed neural fold overgrowth in the injected side (Fig. 4A-C). The histological analysis revealed that overproduction of the neural fold occurred widely in the dorsal ectoderm beyond the neural plate boundary (Fig. 4D, opened arrowhead).

In order to determine the type of cells overproduced in the antisense Xerl-injected side, we examined gene expression of XlSox-2 and ADAM13 in injected embryos. Whole mount in situ hybridization showed that antisense Xerl injection caused a reduction of XlSox-2 expression (Fig. 4E, 56%). In contrast, gene expression of neural crest marker, ADAM13 was clearly en-
hanced in the injected side (Fig. 4F arrowheads, 70%). As compared with the uninjected side, inner shift of ADAM13-positive area was observed in the antisense Xerl-injected side (Fig. 4F, arrows). Also, Xslug expression was increased in the antisense Xerl injected side (Fig. 4F, right, 71%). In order to test whether or not the effect of Xerl on neurogenesis is mediated by chordin, gene expression of chordin was examined in the antisense Xerl-injected embryo. However, as shown in Figure 4G, antisense Xerl did not change the expression pattern of chordin. In order to confirm the inhibitory effect of antisense RNA, double stranded RNA was used as RNAi for suppressing Xerl function. As shown in Table 2, the down regulation of XlSox-2 expression and the inner shift of ADAM13-expressing area were recognized in the Xerl RNAi-injected embryos.

Xerl is essential for neural plate/ neural crest boundary formation

Foregoing results present a possibility that Xerl promotes neural plate formation. However, overexpression of Xerl produced abnormal population having neural plate identity. To examine a function of Xerl in neurogenesis in detail, we considered conjugated animal cap assay (Fig. 5A). In this system, the chordin-injected wild animal cap is the neurogenic center, and this tissue induces gene expression in the conjugated albino animal cap that has been previously injected with test samples. As dispersion of chordin extends from the original cap to the neighboring one (LaBonne and Bronner-Fraser, 1998), the effect of test samples on chordin-induced gene expression can be examined in the albino side. Consequently, in this assay system, stable inducing signal can be obtained from the dose-sensitive chordin, since the inducing tissue is segregated from the reacting tissue. One nano gram of chordin was injected into both blastomeres at 2-cell stage, which can induce neurogenesis in the isolated animal cap. In chordin/control conjugates, Xerl was induced in both caps by chordin (Fig. 5B). Xerl induction by chordin was also confirmed with RT-PCR analysis of the single chordin-injected animal cap (data not shown). Gene expression of XlSox-2 was induced in the chordin-injected animal cap and even in the neighboring area of control cap (Fig. 5C). In chordin/Xerl conjugates, gene expression of XlSox-2 was observed in whole area of the Xerl-injected albino cap (Fig. 5D). In contrast, XlSox-2 expression was not detected in antisense Xerl-injected albino cap of chordin/antisense Xerl conjugates (Fig. 5E). Since XlSox-2 did not induce Xerl, and Xerl could not induce the sufficient expression of XlSox-2 (Fig. 5F,G), these results suggest that the expression of XlSox-2 requires both chordin and Xerl.

In order to know Xerl function in the neural crest formation, we examined ADAM13 expression in this conjugated animal cap assay. Chordin/control and chordin/Xerl conjugates showed no expression of ADAM13 in both caps (Fig. 5H,I). However, in chordin/antisense Xerl conjugates, ADAM13 expression was induced in the antisense Xerl-injected side (Fig. 5J).

These data indicated that Xerl inhibition under the neural induction by chordin could induce the neural crest formation. This result was confirmed by co-injection of chordin and antisense Xerl in animal cap. Chordin could not induce gene expression of ADAM13, whereas co-injection of chordin and antisense Xerl induced ectopic expression of ADAM13 (Fig. 5K,L).

Xerl lacking the LNS domain acts as a dominant negative form

As shown above, antisense RNA injection and RNAi experiment caused the reduction in the neural plate formation or neural fold overgrowth. However, there are many arguments for the
antisense RNA and RNAi methods. To resolve this problem, truncated forms of Xerl were used for the functional analysis. Xerl has two major domains; one is calcium binding EGF-like repeats (cbELRs) and the other is LNS domain. Xerl lacking cbELR3-10 and EGF11 is named as Xerl ΔE. Also Xerl lacking LNS domain is named as Xerl ΔL (see the scheme of Fig. 6A). When truncated-RNAs were injected into one blastomere at 2-cell stage, Xerl ΔE caused the anterior disclosure of the neural plate (Fig. 6B, opened arrowhead). This result is the same as that in the overexpression of Xerl (Fig. 2 C,G). Xerl ΔL did not cause any anterior disclosure (data not shown). In contrast to the injection with Xerl ΔE, the injection with Xerl ΔL caused the inner shift of neural fold (Fig. 6C, opened arrowhead), and the overproduction of the neural fold (Fig. 6C, white arrowhead). These data suggest that Xerl ΔL acts as a dominant negative form of Xerl. To confirm this idea, we examined gene expression in the embryos injected with truncated RNAs. Xerl ΔE caused the distal-posterior shift of ADAM13 expression (Fig. 6D, arrowhead, 65%). In contrast, Xerl ΔL caused the anterior shift and the overgrowth of ADAM13-positive neural fold cells (Fig. 6E, arrowhead, 49%). These data indicate that Xerl ΔL acts as a dominant negative form, which causes the same phenotype as the antisense RNA and RNAi-injected embryos.

Discussion

Xerl excludes neural crest cells

In this study, antisense Xerl injection showed the differentiation of large amount of neural crest cells. Furthermore, antisense Xerl injection induced gene expression of ADAM13 under existence of chordin. Judging from these results, Xerl seems to suppress the neural crest formation. However, overexpression of Xerl did not diminish the neural crest cells, but caused the outer shift of their distribution. In contrast, the inner shift of ADAM13 expression was observed in the antisense Xerl-injected embryo. These results indicate that one of the most important roles of Xerl is to exclude neural crest differentiation from the presumptive neural plate region. If this is the case, Xerl must have a molecular mechanism to suppress

### TABLE 2

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ADAM13

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ADAM13

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Whole mount in situ hybridization analyses were performed on early neurulas (st. 15). One dorsal blastomere of 4 cell embryos was injected. The effect of injected samples was judged by comparison with non-injected control sides. The embryo showing symmetrical expression of each probe was indicated as normal. Effects of the injection were evaluated by quantitative (XlSox-2) or local shift of gene expression (ADAM13, Xslug). NT, not tested.
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the PNS-inducible circumstance. In recent reports, several inducing signals like Wnts or FGFs are involved in the expression of Xslug for neural crest differentiation (Mayor et al., 1997, LaBonne and Bronner-Fraser, 1998). Xerl maybe act as an antagonist of the neural crest-inducing signal, since Xerl has several characteristics as a signaling molecule. One of characteristic domains is calcium binding EGF-like repeat (cbELR). Notch ICD and its downstream gene, Xash3 cause anterior disclosure, and are also involved in neural plate and crest development (Turner and Weintraub, 1994). The signal between Notch and Delta is directly transmitted by cbELRs (Rebay and Fleming, 1991). However, the truncation experiment revealed that Xerl does not need cbELR domains for its function. This is similar to the case of WIF-1. The Wnt-inhibiting molecule has ELR and WIF domain and does not need ELR for Wnt inhibition (Hsieh et al., 1999). They guess that ELR may be required for binding with the extracellular matrix. In contrast, the present study showed that functional domain of Xerl is the LNS domain. There is a report suggesting that LNS domain is also molecular interacting domain on the cell surface (Missler and Südhof, 1998). However, there has been no report connecting LNS molecule with Wnt or FGF signaling. Slit is an axon guidance molecule, which have leucine-rich repeats, ELRs and LNS domain. Also in this case, each domain’s function is still unknown. Recent report showed that LNS domain has a new molecular site interacting with sugars, steroid and proteins (Rudenko et al., 2001). Therefore, Xerl may act as a signaling molecule mediating unknown pathway. Thus, further study is necessary for examining whether Xerl functions as one of antagonistic factors against PNS-inducing signals.

Xerl promotes neural plate formation

In this study, Xerloverexpression caused Xisox-2-positive-neural plate expansion and the opened anterior neural plate, which was due to produce Xisox-2-positive neuroepithelial cell mass. Sox-2 alone is not sufficient for neural induction, but enhances the responsiveness of ectoderm to the neuralizing FGF signals (Mizuseki et al., 1998). Also, in chick embryos, Sox-2 function is related to competence for neural induction (Streit A. et al., 1997). Therefore, one role of Xerl may be to provide the competence for CNS-inducible signals to the neuroepithelium through the promotion of Xisox-2expression. The next question is how Xerl promotes Xisox-2 expression. One
possible mechanism of Xis Sox-2 promotion is that Xerl may interact with chordin directly in the extracellular space. Blitz et al. (2000) showed that chordin-processing by BMP-1 or Xolloid makes the different activity of BMPR. However, BMP-1 expression is ubiquitous, and Xolloid expression is seen in the lateral neural plate in the early neurula embryo (Goodman et al., 1998). If Xerl protects chordin from BMP-1 or Xolloid in the neural plate area, distribution of active chordin could be refined by expression pattern of Xerl in CNS.

Consequently, Xerl must play an important role for neural plate/neural crest boundary formation.

Xerl expression and regulation

Present study showed that chordin induces gene expression of Xerl. In the early neurula stage, Xerl expression was similar to chordin expression (Sasai et al., 1994). However, at the later stage, chordin expression is restricted to only forebrain and chordoneural hinge (Sasai et al., 1994). Whereas, Xerl expression is seen at the ventricular zone of diencephalon and midbrain at the early tadpole stage (Kuriyama et al., 2000). Thus, the expression pattern of Xerl was wider than that of chordin expression. Presumably, Xerl-inducible factor is not only Xerl in the later stage.

Materials and Methods

Embryos

Unfertilized eggs were obtained by intracoelomic injection with gonadotropin hormone (Gestrion; Denka Seiyaku, Tokyo, Japan) into Xenopus adult females and artificial fertilization in vitro were performed as described by Asashima et al. (1990). Fertilized eggs were treated with 1% thiglycoate (pH 10) and cultivated in 0.1x Modified Barth’s Solution (MBS) at 20°C (Moon and Christian, 1989). Developmental stage was according to Nieuwkoop and Faber (1967).

Expression construct and microinjection

A full-frame Xerl expression plasmid, termed pCS2+/Xerl, was constructed in the expression vector pCS2+. The fragment was digested with Avill and Xhol and inserted into the vector. The capped pCS2+/Xerl, pCS2+/chordin and pCS2+/myc-tagged GFP (mtUGP) mRNAs were transcribed using SP6 RNA polymerase as described in mCAP RNA Capping Kit (STRATAGENE). RNAs were injected in a volume of 5 nl at a concentration of 50–400 pg/nl into single blastomere of embryos at stage 2 or 3. Microinjections were done in 0.1 x MBS, 2.5% Ficoll 400 (Amersham Pharmacia Biotech). After gastrulation, injected embryos were transferred to 0.1 x MBS for further incubation (Moon and Christian, 1989). In all experiments, pCS2+/mtUGP mRNA was used as control.

Antisense RNA and RNAi construct

Antisense RNA injection have been used to inhibit the function of target gene in Xenopus (Steinbeisser et al., 1995, Carl et al., 1999). Antisense RNA was synthesized from the first 1.6 kb of 2.9 kb cDNA including 5’ flanking region and the initiating codon. Xenopus RNAi was reported by Nakano et al. (2000). RNAi was synthesized from full-length fragment of Xerl, non-capped antisense RNA and sense RNA were determined by gel-electrophoresis and spectrophotometry. Then, these RNAs were denatured and annealed, and size-up of this double stranded RNA was checked by electrophoresis as described by Li et al. (2000).

Truncated constructs

Truncated constructs of Xerl were made by PCR and replacement of digested cDNA fragment. Xerl ΔE was lacking the position of 323 to 1799 bp fragment. Xerl ΔL was lacking the position of 1630 to 2288 bp fragment.

Histological analysis

All injected embryos were selected under fluorescence microscope. For histological analysis of injected embryos, GFP-positive embryos were selected under fluorescence microscope. Histological analysis of injected embryos, GFP-positive embryos were fixed with 1 x MEMFA for overnight. Embryos were embedded in metacril resin, marked on the injected side and sectioned with ultra microtome. Staining was performed by conventional azofuchsin-anilin blue method as described by Hausen (1988).

In situ hybridization analysis

Whole mount in situ hybridization was performed as described previously (Kuriyama et al., 2000). Minor modification was done in a coloring reaction using BM purple (Roche Diagnostics) at 4°C for overnight. For histological examination of stained embryos, embryos were fixed with 1 x MEMFA for overnight, embedded in metacril resin, and sectioned with 2-8-µm thickness. Bleaching of wild type embryos for in situ hybridization was performed by using 10 % H2O2 in PBS only for samples shown in Fig. 6 D,E.

Conjugated animal cap assay

Animal caps from wild and albino embryos were used to distinguish the donor from the recipient. Wild embryos were injected at the stage 2 with 1 ng chordin mRNA, and albino embryos with 2 ng Xerl or antisense Xerl mRNA. These injected embryos were incubated in 0.1 x MBS to stage 8.5, when they were transferred into 1 x MBS, their vitelline membranes were removed with watchmaker’s forceps, and animal caps were quarrried with tungsten needle.
Wild and albino animal cap were immediately conjugated with each other, washed with 1 x MBS and cultured until stage 17. Except above modification, all procedures of conjugate assay were performed as described by LaBonne and Bronner-Fraser (1998).

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