XSIP1, a Member of Two-Handed Zinc Finger Proteins, Induced Anterior Neural Markers in Xenopus laevis Animal Cap

Akira Eisaki,* Hiroki Kuroda,* Akimasa Fukui,* and Makoto Asashima*†
*Department of Life Science (Biology) and †CREST, Japan Science and Technology Corporation (JST), University of Tokyo, 3-8-1 Komaba, Meguro, Tokyo 153-8902, Japan

Received March 6, 2000

We characterized Xenopus SIP1 (XSIP1), Smad interacting protein, from activin-treated animal caps by differential screening. The XSIP1 is very similar to mouse SIP1 in the protein coding region including the zinc finger domain and homeodomain. The expression pattern was analyzed by RT-PCR and whole mount in situ hybridization. XSIP1 expression was initially restricted to the dorsal marginal zone in the late gastrula and was subsequently expressed at the lateral edge of neural plate and, in the tailbud stage, in the forebrain, neural tube, and eye. Overexpression of XSIP1 at the animal caps resulted in activation of anterior neural markers without mesodermal markers. Ectopic expression of XSIP1 induced enlargement of neural cells and disordered eye formation. In addition to abnormal head phenotypes, many embryos were short-tailed. Our findings suggest that XSIP1 is a transcriptional repressor, which may be involved in the activin-dependent signal pathway.

Key Words: SIP1; neural induction; Xenopus laevis; zinc finger.
FIG. 1. (A) Nucleotide sequence of XSIP1. Predicted ORF is boxed. (B) Amino acid sequence comparison between mouse SIP1 and Xenopus XSIP1. Gray boxes represent identical or biochemically similar amino acids. The position of the C2H2 type zinc finger in STP1 is indicated by bold underlining, other zinc fingers in SIP1 are indicated by thin double underlining. The dotted line indicates the Smad binding domain (SBD).
embryos failed to form eye vesicles. Ectopic expression of XSIP1 induced anterior neural markers suggesting that XSIP1 plays a role in early neurogenesis.

MATERIALS AND METHODS

Eggs and embryos. Xenopus eggs were obtained by injecting adult males and females with human chorionic gonadotropin (Gestrin) at a dose of 600 U. Fertilized eggs were dejellied by treatment with 4.5% cysteine hydrochloride in Steinberg's solution (pH 7.8) with kanamycin sulfate (100 mg/l; Banyu Pharmaceutical Co.), then washed thoroughly with Steinberg's solution (pH 7.4). Embryos were transferred to culture dishes containing Steinberg's solution according to Nieukoop and Faber (12).

Differential screening. Animal caps were cut from the late blastula and treated with 100 ng/ml activin A (supplied by Dr. Yuzuru Eto, Central Research Laboratories of Ajinomoto Co. Inc., Japan) for 1 h, then cultured for 7–10 h in Steinberg's solution. Poly(A)-tailed RNA was extracted from 2000 animal caps cultured in Steinberg's solution for 7–10 h after activin A treatment. In brief, total RNA was extracted according to the guanidine thiocyanate method (10). Then Poly(A)-tailed RNA was extracted from total RNA by binding to oligo d(T) cellulose. A cDNA library was constructed using this RNA as a template. The library, consisting of approximately 100,000 clones, was screened using 32P-labeled probes generated from stage-10 whole embryo cDNA. Negative clones were sequenced and compared with known sequences, and novel clones were examined for their mRNA localization in embryos by hole-mount in situ hybridization.

In vitro transcription and microinjection. Capped sense RNAs for microinjection were synthesized (11) by using Ambion Megascript (Austin, TX) SP6 and T7 kits. The clone used was as follows: XSIP1 linearized by NotI and transcribed by Sp6; with embryos fertilized in vitro, dejellied, cultured, and injected with solutions as described (12). Embryos were injected in the animal cap with 100 pg to 1 ng at the 2-cell stage into both or one blastomere or at the 4-cell stage into the left side blastomeres.

Whole mount in situ hybridization. Whole mount in situ hybridization was performed as described in Harland (13): Embryos obtained from albino females were used. Digoxigenin (DIG)-labeled antisense RNA corresponding to 4953 bp of XSIP was synthesized in vitro. Antidigoxigenin antibodies were purchased from Boehringer Mannheim. To prepare sections, embryos stained by whole mount in situ hybridization were dehydrated in ethanol, transferred to xylene and embedded in paraffin and sectioned at 10 μm.

RT-PCR. Total RNA was isolated from various developmental stages and mRNA was injected animal caps using ISOGEN (NIPPON GENE). Two micrograms of total RNA were used as a template to generate first strand cDNA. One tenth of this cDNA was used as a template in subsequent RT-PCR (Reverse Transcription-polymerase chain reaction) analysis. The primers used were: XSIP (forward, 5'GGGAGCCCCTACCTCTTCT-3'; reverse, 5'ATCCGCCAGATCTCTTGCT-3'), ODC (14), ms-actin (15), F-spondin (16), XAG1 (17, 18), NCAM (19) and X1HBOX6 (20).

FIG. 2. RT-PCR analysis was performed at various stages. Stages according to Niewcoop and Faber (1956) are shown over the lanes. ODC (lower panel) is indicated as a loading control.

FIG. 3. Whole mount in situ hybridization with an XSIP antisense probe. (A) Stage 9 embryo, vegetal view. XSIP was not detected. (B and C) Stage 10.5 embryo, dorsovegetal view and vegetal view. XSIP1 is detected in the dorsal ectoderm. (D) Stage 12 embryo, dorsal view. (E) Stage 17 embryo, dorsal view. (F) Stage 26 embryo. Large white arrowhead and small white arrowhead indicate the telencephalon and mesencephalon. d, dorsal; v, ventral; b, blastopore.
RESULTS AND DISCUSSION

Animal caps treated with activin A differentiated into mesodermal or endodermal tissues depending on concentration, and treated animal caps mimic the organizer and induced a variety of tissues in other untreated animal caps with contact. For example, animal caps, treated with 100 ng/ml activin A for 1 h and then cultured for 7–10 h, showed strong induction of neural tissues into sandwiching animal caps (data not shown). In order to isolate the genes responsible for activating neural determination, we carried out differential screening between activin A treated animal caps and late blastula whole embryos. We isolated a number of clones, up-regulated in animal caps treated with activin and cultured but not in whole embryo blastulas. One clone had a two-handed zinc finger/homeodomain class gene, but lacked the 5'-sequence. We screened the gastrula library again and isolated this one (Fig. 1A). In comparison with other vertebrate zinc finger genes, this clone showed marked similarities to mouse Smad Interaction Protein 1 (SIP1), with 77% amino acid identity (Fig. 1B), and to the vertebrate δ-crystalline enhancer binding protein (δEF1), with 39% identity. The homologies between mouse SIP1 and this gene were 68%, at the nucleotide level, and 83%, at the biochemically similar amino acid level, (Fig. 1B). Notably, these proteins share the same amino acids in the zinc finger domain and certain similarities in their Smad binding domain (SBD). These findings suggest that this clone is a homologue of SIP1 in Xenopus, and for that reason the clone was named XSIP1.

The length of XSIP1 was 4529 bp and conceptual translation of the 3642 bp ORF yielded a 1214 amino acid sequence containing a two-handed zinc finger and a homeodomain. This sequence also contains 230 bp of the 5'-untranslated region and 653 bp of the 3'-untranslated region. The homeodomain has conserved arginine and asparagine at the same position, which is
critical for DNA binding. However, mSIP1, XSIIP1 and ßEF1 do not contain these critical amino acids. This suggests they are not able to bind DNA directly and that they are the same type of two-handed zinc finger/homeodomain class gene.

To examine the XSIIP1 gene expression pattern in embryos at various developmental stages, RT-PCR analysis and whole mount in situ hybridization were carried out (Fig. 2). We designed XSIIP1 specific primers to discriminate another clone, which was for several of the first amino acids. This clone had almost the same sequence (97% similarity to XSIIP1) but there was no effect on embryos with the injection of its mRNA (data not shown), suggesting that it might be a splicing isofom. The single product was not detected from the unfertilized egg to the stage 8 embryo, and then first appeared at stage 9 and its expression was maintained through the progression of development, gradually increasing through the early neurula stage, and was then sustained in the tail-bud stage. ODC confirmed equal loading of PCR products in each lane.

To determine localization of XSIIP1 expression, we performed whole mount in situ hybridization using a digoxygenin-labeled XSIIP1 sense and antisense RNA probe on whole embryos. There was no detectable signal in stage 9 embryos (Fig. 3A), based on RT-PCR detection. The first detectable expression of XSIIP1 RNA was observed in the dorsal ectoderm at stage 10.5 (Figs. 3B and 3C). From the early neurula stage onwards, XSIIP expression had entered a second phase with restriction to the lateral edge of the neural plate (Fig. 3D), i.e., the presumptive dorsal neural tube and neural crest. Then, XSIIP1 was expressed in the neural plate and tube along the anteroposterior axis of the developing central nervous system (CNS) (Fig. 3E). After the tailbud stage, expression was seen mainly in the telencephalon and diencephalon of the forebrain, and more posteriorly along the neural tube and eye (Figs. 3F and 3G).

Since many zinc finger genes expressed in the early gastrula through neurula stages induce neural genes like those of the zinc family (21), we induced overexpression of XSIIP1 by microinjection of synthesized XSIIP1 mRNA to test the neuralizing activity. XSIIP1 mRNA and control mRNA (pCS2) were transcribed in vitro, then dissolved in Gurdon’s buffer (88 mM NaCl, 1 mM KCl, 15 mM Tris-HCl, pH 7.5). First, we injected XSIIP1 mRNA into one blastomere of 2-cell stage embryos to over express XSIIP1 hemilaterally. In almost all cases, the XSIIP1 injected side was enlarged and showed poor eye formation (Fig. 4B) or ocular defects (Figs. 4C and 4G), whereas the uninjected control side was normal (Fig. 4A). The sections through the head regions of injected embryos showed neural tissues to be markedly thickened of the injection side (Figs. 4D and 4F). Hyperplastic neural walls, as well as distorted eyes and neural tissues characterized the injected side. Particularly, no lens induction occurred on the injected side and retinal pigment cells were variably diminished. In addition to the abnormal head phenotypes, embryos injected with 1 ng of XSIIP1 in both blastomeres developed short tails (Fig. 4G), probably due to inhibition of Xbra (7).

As has been noted, XSIIP1 plays important roles in early neural development. To clarify the effect of injected XSIIP1 mRNA in molecular marker gene expression, after the injection of mRNA into the animal pole of 2-cell stage embryos, the animal caps from these embryos at stage 9 were dissected and total RNA from the caps, which had been cultured for 2 days, was extracted. We carried out RT-PCR analysis, demonstrating that XSIIP1 mRNA injection caused up-regulation of N-CAM (a general neural marker), and XAG1 (cement gland marker), but no up-regulation of F-spondin, X1Hbox6 (trunk/tail neural marker), or ms-actin (mesodermal marker) (Fig. 5). The structures induced by XSIIP1 injection suggest that XSIIP1 is an early activin response gene. When animal caps are cultured in a medium containing activin A, neural tissue is secondarily induced, owing to primarily induced mesodermal tissue, resulting in expression of N-CAM. Mouse SIP1 interacts with Smads via the SBD and by binding the Xbra-2 enhancer to regulate its expression and down-regulating Xbra expression (8). XSIIP1 is induced by activin A (data not shown), thereby inducing the neural marker without mesodermal formation and suppresses Xbra expression indicate the possibilities...
that XSIPI makes neuroectoderm from neural rather than mesodermal tissue. XSIPI injected animal caps expressed neural marker but did not form neural tissue (data not shown). This, it is probable that other signals, which can induce neural tissue in animal caps with activin/Smad 2 signaling, cooperate with XSIPI to induce the formation of neuroectoderm from neural tissue.

ACKNOWLEDGMENTS

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, and by CREST (Core Research for Evolutional Science and Technology) of the Japan Science and Technology Corporation. We thank Dr. Yuzuru Eto of Central Research Laboratories of Ajinomoto Co. Inc. for providing human recombinant activin A.

REFERENCES