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Molecular cloning and characterization of a novel inhibitor of apoptosis protein from *Xenopus laevis*

Kwang-Hoon Song,[a] Tae-Moon Kim,[b] Han-Jong Kim,[a] Jung Woo Kim,[c] Hong-Hee Kim,[d] Hyuk-Bang Kwon,[a] Won Sun Kim,[b] and Hueng-Sik Choi[a,*]

[a] *Hormone Research Center, Chonnam National University, Kwangju 500-757, Republic of Korea*
[b] *Department of Life Science, Sogang University, Seoul, Republic of Korea*
[c] *Department of Biology, Seonam University, Namwon, Republic of Korea*
[d] *School of Dentistry, Chosun University, Kwangju, Republic of Korea*

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Abstract

A novel inhibitor of apoptosis protein family member termed SIX was identified in *Xenopus* containing a single baculoviral IAP repeat (BIR) domain and no COOH-terminal RING finger domain. It exhibited striking amino acid sequence similarity with human *survivin*, mouse *TIAP*, and recently found *Xenopus* survivin, especially a part of BIR domain was highly conserved. Interestingly, *SIX* interacted with RXRα through the AF2 domain in the absence of ligand, which was weakened when the ligand was present. Northern blot analysis demonstrated that *SIX* mRNA was not detectable in adult with exception of the ovary and testis, and *whole-mount in situ* hybridization and Northern blot analyses revealed strong and homogeneous expression of SIX in the developing oocytes. In the embryos, the expression of *SIX* was observed in the animal hemisphere from one-cell to yolk plug stages and high level of expression was detected in the future brain and dorsal region of the neural tube at the neurula stage and early tail-bud stage. These results strongly support the fact that survivin is evolutionarily conserved in structure and *SIX* is likely to be the *Xenopus* counterpart of human and mouse survivin.

Apoptosis or programmed cell death is a genetically regulated process that plays a crucial role in development, tissue homeostasis: elimination of redundant cells during embryogenesis, cell atrophy upon endocrine withdrawal or loss of essential growth factor or cytokines, tissue remodeling and repair, and removal of cells that have sustained genotoxic damage [1–4]. Dysregulation of cell death pathways occurs in cancer, autoimmune and immunodeficiency diseases, reperfusion injury after ischemic episodes, and in neurodegenerative disorders [5]. It has been reported that products of different genes, which act as inhibitors or stimulators of apoptosis, which is associated with activation of intracellular cysteine proteases with aspartate-specificity, are the so-called caspases [6,7]. Recently, several apoptosis inhibitors related to baculovirus *iap* gene have been identified in silkworm [8], fruit fly [9,10], mouse [11], and human [12]. All members of the IAP gene family isolated from different species are characterized by a common structural domain of ~70 amino acids, termed the baculovirus IAP repeat (BIR) that is present up to three tandem copies, and IAPs also contain another common feature, RING finger domain, near their COOH termini, whose function still remains controversial [13].

Moreover, another intriguing member of IAP gene, survivin, has been reported from mouse [11], human [12], and frog [14] that contains only a single BIR and no RING finger domain. In contrast to other IAP members, which are broadly distributed in normal adult tissues [13,15], survivin mRNA is abundantly expressed only in fetal tissues, but not in normal adult tissues except placenta, thymus, and testis [11,12]. In addition survivin is over-expressed in most of common human cancers [12], thus making it an exciting new tumor marker.

* Corresponding author. Fax: +82-62-530-0500.
E-mail address: hsc@chonnam.chonnam.ac.kr (H.-S. Choi).

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These IAPs can directly bind and inhibit several members of the caspase family including caspase-3, -7, and -9 [15–19]. Furthermore, changes in survivin expression and function by antisense or phosphorylation-defective mutations cause apoptosis at least in some tumor cell lines [20,21], implying that survivin expression is important for cell survival and thus applicable for cancer therapy. Furthermore, in mouse embryos, prominent and nearly ubiquitous expression of survivin has been detected at embryonic day (E) 11.5, whereas survivin expression has been limited to only a few locations at E15–E21 [11], suggesting that the survivin expressed during embryonic and fetal development may contribute to tissue homeostasis and differentiation, while it becomes inactive in most normal adult tissues.

While first identified in baculovirus, the IAP family has been conserved evolutionarily from virus to human. Here we report a novel IAP gene from *Xenopus laevis* via yeast-two hybrid screening, which we termed *SIX* (survivin in *Xenopus*). *SIX* encodes a protein with a single BIR domain and no RING finger domain, exhibiting 49% identity and 66% similarity to human *survivin* and mouse *TIAP*. High expression of SIX was detected during embryogenesis and only in ovary and testis in adult.

Materials and methods

Animals. Adult frogs of *X. laevis* were purchased from Nasco (Fort Alkinson, WI) and were maintained in glass or plastic boxes. The photoperiod and temperature were kept at 12L:12D and 20 °C, respectively. The frogs were fed Nasco Frog Brittle at approximately 1% of their body weight twice a week. Under these conditions, females remain in reproductive condition throughout the year, with ovaries containing a heterogeneous population of oocytes. The animals were killed and the tissues were removed for Northern blot analysis. Oocytes were obtained from sexually mature female frogs by surgically removing parts of the ovary. The oocytes in the excised ovarian tissue were staged according to Dumont [22]. Eggs for artificial insemination were obtained from both wild-type and albino females of *X. laevis* primed with 600 U of human chorionic gonadotropin (hCG, Sigma). Ovulated eggs were inseminated with the sperm suspension prepared from minced testes. Fertilized eggs were dejellied in 2.5% cysteine (pH 8.0), maintained in 0.4× Marc's modified ringer (MMR; [23]) until stage 4, and then transferred to 0.1× MMR supplemented with 50 μg/ml gentamicin sulfate (Sigma). Embryos were staged according to Nieuwkoop and Faber [24] and fixed with MEMFA (0.1 M MPOS, pH 7.2, 2 mM EGTA, 1 mM $MgSO_4$, and 3.7% formaldehyde).

Plasmids. The plasmids for pCDNA3-RXRα, Gal4-tk-luciferase reporter, and RARE-tk-Luc reporter plasmid containing three copies of the direct repeat 5 (DR-5) motif from the promoter of the mouse RAR gene were donated by Dr. David Moore (Bayler College of Medicine, Huston, TX). The full-length cDNA of SIX was obtained by 5′ RACE-PCR, using the stage cDNA library of *Xenopus* embryos (Clontech) as a template. The sequence of SIX was submitted to GenBank under Accession No. AY174765. B42AD fused SIX was constructed by insertion of the *Eco*RI–*Xho*I digested polymerase chain reaction (PCR) fragment into *Eco*RI–*Xho*I digested pJG4-5 vector. A PCR amplified fragment of the RXRα-LBD (ligand binding domain), RXRα-LBDΔ AF2 (AF2 domain deletion mutant), and RARα was cloned into *Eco*RI and *Xho*I sites of pL202PL for the LexA fusion expressions in yeast. Gal4-RXRα was subcloned by in-frame insertion full-length RXRα into pCMX-GAL4N vector and VP16-SIX was constructed by in-frame insertion of SIX full-length into pCMX-VP16N vector. Expression vectors were confirmed by sequencing prior to use.

Yeast two-hybrid screening. Yeast two-hybrid screening was carried out according to manufacturer's instructions (Clontech). Yeast strain EGY48 (Clontech) was cotransformed with the bait plasmid, pLexA-RXRα LBD (LexA DBD fused mouse RXRα ligand binding domain) and with the plasmid of B42 AD fused *Xenopus* embryo stage cDNA library (Clontech). Briefly, transformants were grown in selective medium without uracil, histidine, and tryptophan (Ura^-, His^-, Trp^-) for 3 days at 30 °C and replated on selection plates (Ura^-, His^-, Trp^-, and Leu^-). The positive colonies were picked, streaked on X-gal plates containing galactose, and confirmed on the X-gal plates containing glucose. The nucleotide sequences of inserts in true positive hybrid plasmids were generated and the identity of the cDNAs was determined through a homology search against known sequences in GenBank.

Protein–protein interaction assays. Yeast and mammalian two-hybrid assays (Clontech) were used to examine protein–protein interactions in vivo. Yeast mating approach was used in yeast two-hybrid assays to study protein–protein interactions as described in the protocol of Clontech Laboratories, Inc. Briefly, the yeast strain EGY48 was transformed with LexA (DNA-BD vector only as a negative control) and LexA target plasmids (for RXRα, LBD, RXRαΔ AF2, and RARα), and B42AD vector-SIX transformants were grown in the galactose containing medium without uracil, histidine, and tryptophan at 30 °C for 2 days in the absence or in the presence of respective cognate ligands at the following concentrations: 10 μM all-*trans*-retinoic acid for RARα; and 10 μM of 9-*cis*-retinoic acid for RXRα, and assayed for liquid β-galactosidase assay. The uracil, histidine, and tryptophan-positive colonies were further analyzed for β-galactosidase activity by liquid β-galactosidase activity measurement as essentially described in the protocol. Mammalian two-hybrid assays in CV-1 cells were performed according to the procedures described in manufacturer's guidelines.

Northern blot analysis. Total RNA from tissues and oocytes of *Xenopus* was isolated using TRI Reagent (Sigma). Twenty micrograms of total RNA was fractionated by electrophoresis on 1.2% agarose gel containing formaldehyde and was transferred to a nylon membrane (Zeta-probe, Bio-Rad, Richmond, CA) by capillary blotting with 10× sodium citrate–sodium chloride (SSC). After a UV cross-linking and prehybridization, membranes were hybridized 24 h at 42 °C in solution containing 50% formamide, 10% dextran sulfate, 5× SSC, 1 mM EDTA, 10 mg/ml denatured salmon sperm DNA, and a total of $2–4 \times 10^6$ cpm of α-^{32}P-labeled SIX full-length cDNA. After hybridization, membranes were washed twice for 5 min at room temperature in 2× SSC and 0.1% sodium dodecyl sulfate (SDS), followed by 1 h at 65 °C in 0.5× SSC and 0.1% SDS. Membranes were then exposed using Kodak RX films (Eastman Kodak, Rochester, NY) for 12–24 h at −70 °C. The 28S ribosomal RNA was used as a loading control.

Whole-mount in situ hybridizaton. Whole-mount in situ hybridization (WISH) was performed as described by Harland [25] and Wilkinson [26]. The pretreatment and hybridization were carried out according to Harland's protocols and the detection was carried out according to Wilkinson's protocol. The specimens in 100% ethanol were rehydrated through a descending series of methanol/PBT (75%, 50%, and 25%) and PBT for 3–5 min, bleached in 3% hydrogen peroxide in PBT under fluorescent light for 1 h (except albino specimens), and washed three times in PBT. Specimens were then permeabilized with proteinase K in PBT (10 μg/ml) for 15 min and washed with 0.1 M triethanolamine twice for 5 min. After washing, acetylation and neutralization of free amines were done by incubating specimens for 5 min in acetic anhydride–triethanolamine (2.5 μl/ml). After this step, specimens were postfixed in MEMFA for 20 min and washed twice for

5 min in PBT. After washing specimens were equilibrated with prehybridization buffer (50% formamide, 5× SSC, pH 4.5, 1% SDS, 50 mg/ml tRNA, 50 mg/ml heparin, and 1× Denhart's) for 4 h at 62 °C. Later specimens were hybridized with DIG-labeled riboprobe in the prehybridization buffer at 62 °C for overnight. Hybridized specimens were washed with solution I (50% formamide, 4× SSC, pH 4.5, and 1% SDS) at 62 °C and with solution III (50% formamide, 2× SSC, pH 4.5) at 60 °C for 30 min, respectively, three times. Thereafter the specimens were washed three times in TBST (140 mM NaCl, 2.7 mM Tris–Cl, pH 7.5, and 0.1% Tween 20) with 2 mM levamisole. Following washes, specimens were preblocked for 2.5 h in 10% heat-inactivated sheep serum/TBST/2 mM levamisole. To prevent non-specific binding of antibody, the anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (Anti-DIG-AP, Boehringer–Mannheim) were preabsorbed with heat-inactivated *Xenopus* embryo powder suspended in heat-inactivated sheep serum in TBST/2 mM levamisole. Preabsorption lasted for 2.5 h at 4 °C and the antibody mixture was centrifuged at 10,000*g* for 10 min at 4 °C to remove any particular material. Specimens were then incubated in supernatant containing antibody at 4 °C for overnight. The specimens were washed with TBST/2 mM levamisole 3× for 10 min, 5× for 1 h, and overnight at 4 °C. These washes were followed by three washes with NTMT (100 mM NaCl,

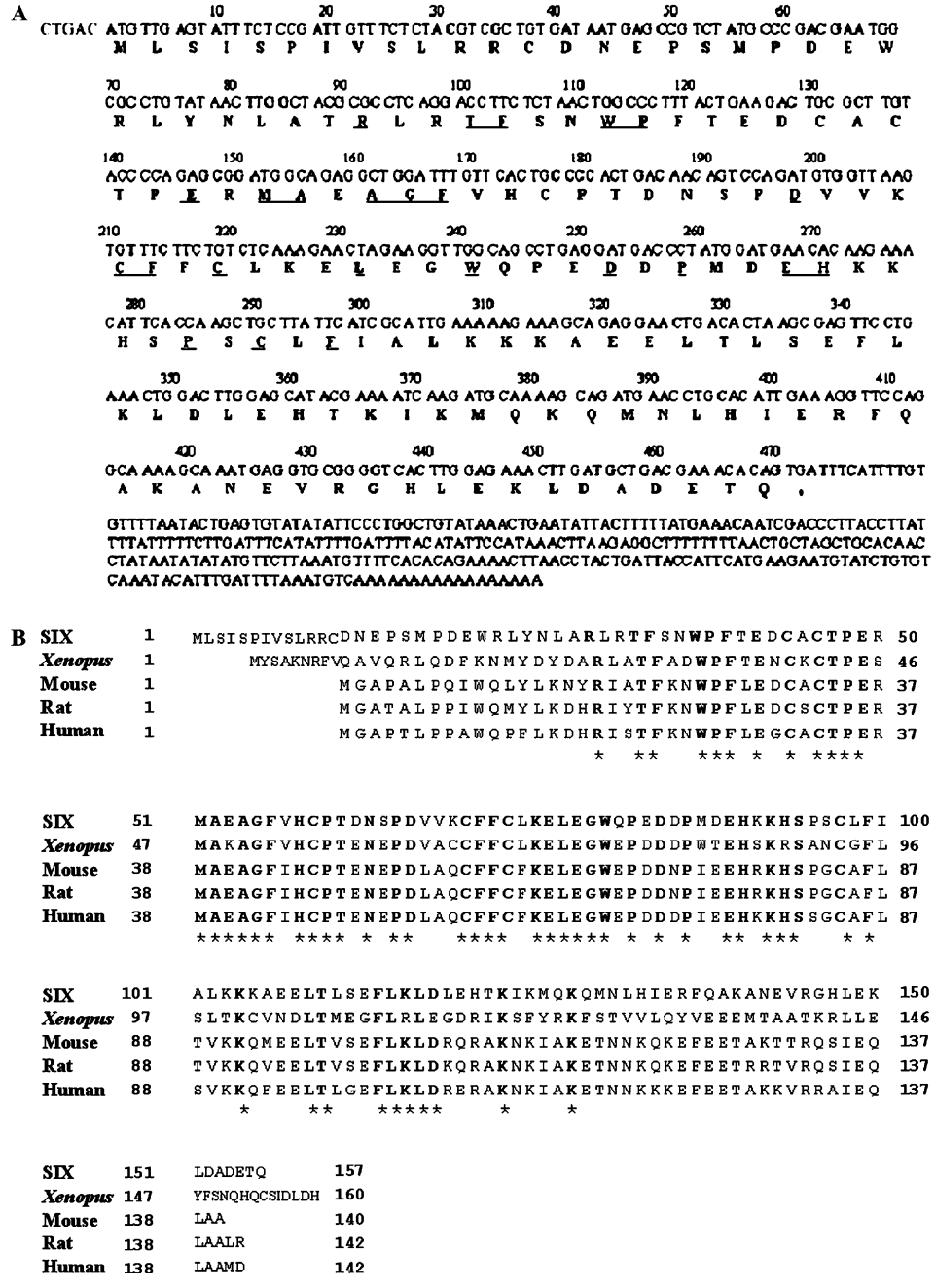

Fig. 1. Nucleotide and deduced amino acid sequences of the SIX cDNA, and sequence alignment. (A) The conserved amino acids of the BIR domain are underlined. (B) Homology between SIX and *Xenopus* and human survivin, mouse TIAP, and rat survivin. Bold letters and asterisks indicate identical amino acids among members.

100 mM Tris, pH 9.5, 0.1% Tween 20, and 50 mM $MgCl_2$)/2 mM levamisole for 10 min each. Color reaction was carried out using BM purple alkaline phosphatase substrate (Boehringer–Mannheim) in dark with gentle rocking for 9–14 h. The reaction was stopped by three washes of PBT/1 mM EDTA and stored in the same solution in the dark place at 4 °C before viewing and photography.

Transient transfection and β-galactosidase assay. Twenty-four hours before transfection, CV-1 cells were plated in 24-well culture dishes at a density of 2.5×10^4 cells/well. Transfection was performed using SuperFect Transfection Reagent (Qiagen, Germany), according to manufacturer's instructions with Gal4-tk-Luc, Gal4-DBD, Gal4-RXR, VP16-AD, VP16-SIX, and internal control pCMV-β-gal. After 48 h, cells were lysed with 100 μl of 1% Triton X-100, 25 mM GLY–GLY, pH 7.5, 15 mM $MgSO_4$, and 2 mM EGTA for 15 min. Twenty microliters of the cell lysates was assayed for luciferase activity with a Dual-luciferase Reporter assay system (Promega, Madison, WI) and determined with a MLX microtiter luminometer (Dynex). The lysates were transferred into 96-well microtiter plates for β-galactosidase assay by using the *o*-nitrophenyl-β-D-galactopyranoside (ONPG) (Sigma) as a substrate as described previously [27]. The luciferase activities were normalized to the β-galactosidase activity expressed from the co-transfected pCMV-β-gal plasmid and reported as means ± SE in relative luciferase units (RLU). All transfection experiments were performed for at least five times in duplicate.

Results and discussion

Molecular cloning of SIX

To identify novel genes that interact with the nuclear receptor RXRα, a yeast two-hybrid screening was performed, using RXRα LBD as bait with a *Xenopus* embryo cDNA library. Among 32 independently identified interacting clones, 2 clones encoded sequences similar to the apoptosis inhibitor protein. The cDNA encodes an ORF of 157 amino acids, named SIX, survivin in *Xenopus*, with a predicted molecular mass of 18–19 kDa (Fig. 1A). SIX encodes a protein with a single BIR domain and no RING finger motif, exhibiting 49% identity to human survivin and mouse TIAP; especially, a part of BIR domain was highly conserved (Fig. 1B), although there is considerable divergence in structure in the N-terminal including 13-residue insertion and little sequence homology over the C-terminus. Moreover, overall protein identity of SIX to recently found *Xenopus* survivin [14] was 40%. These results strongly support the fact that survivin is evolutionarily conserved in structure and SIX is likely to be the second *Xenopus* counterpart of human and mouse survivin.

Interaction of RXR and SIX

To confirm the interaction domain between RXRα and SIX, we performed a liquid β-galactosidase assay (Fig. 2A). Interestingly, RXRα interacted more strongly with SIX without the ligand and the interaction was weakened with the addition of ligand. In contrast to wild-type RXRα, AF2 domain deletion mutant of RXRα did not show any significant interaction with SIX, suggesting that SIX interacts with the AF2 domain of RXRα. Retinoic acid receptor α (RARα), which is also activated by retinoic acid and has similar structural features to RXRα, did not interact with SIX with or without ligand. Retinoids, ligand of RXRα, have been shown to regulate complex gene networks involved in morphogenesis, organogenesis, growth, cellular differentiation, and homeostasis [28–31]. Although we cloned SIX as a RXRα interacting protein, the interaction between RXRα and SIX was stronger in the absence of 9-*cis*-retinoic acids, and SIX did not interact with RARα, suggesting that SIX is differentially involved in the control of retinoic acid-induced cellular responses.

To investigate whether RXR interacts with SIX in vivo, we applied mammalian two-hybrid assay in CV-1 cells (Fig. 2B). CV-1 cells were transiently co-transfected with expression vector Gal4-DBD or Gal4-RXRα,

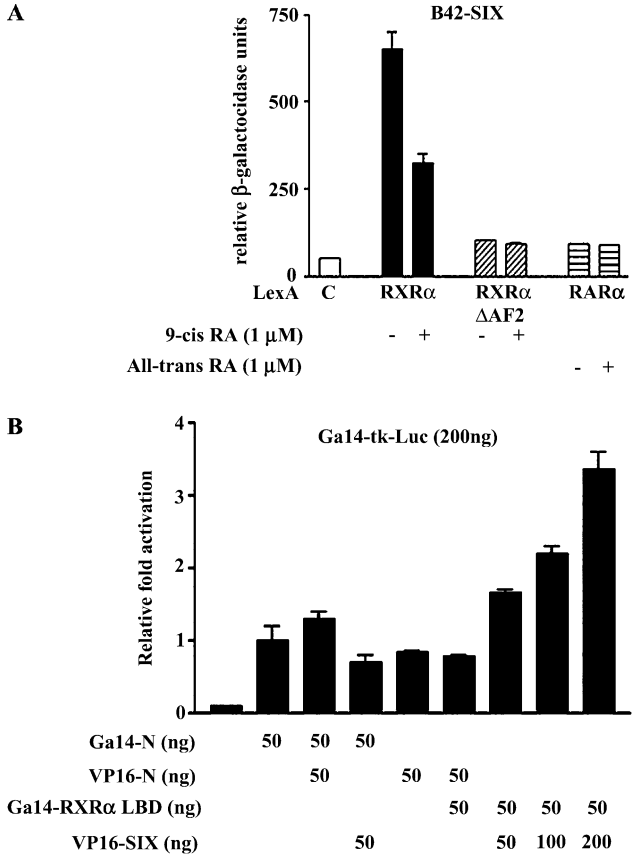

Fig. 2. Interaction between nuclear receptor RXR and SIX. (A) Yeast two-hybrid assay for interaction. B42-SIX was transformed into EGY48 cells along with LexA alone, LexA-RXRα LBD, LexA-RXRα LBD ΔAF2, or LexA-RARα LBD. Interaction was measured by liquid β-galactosidase assay as described in Materials and methods. (B) Mammalian two-hybrid assay. Gal4 alone (Gal4-N) or Gal4-RXRα LBD was transfected into CV-1 cells along with VP16-SIX, or VP16 alone (VP16-N). Luciferase activity was normalized for transfection efficiency to be the corresponding β-galactosidase activity. Data shown represent means of three independent experiments (±SEM).

together with either VP16 AD or VP16-SIX. Co-transfection of Gal4-RXRα and VP16-SIX increased luciferase activity, suggesting that RXRα interact with SIX in vivo.

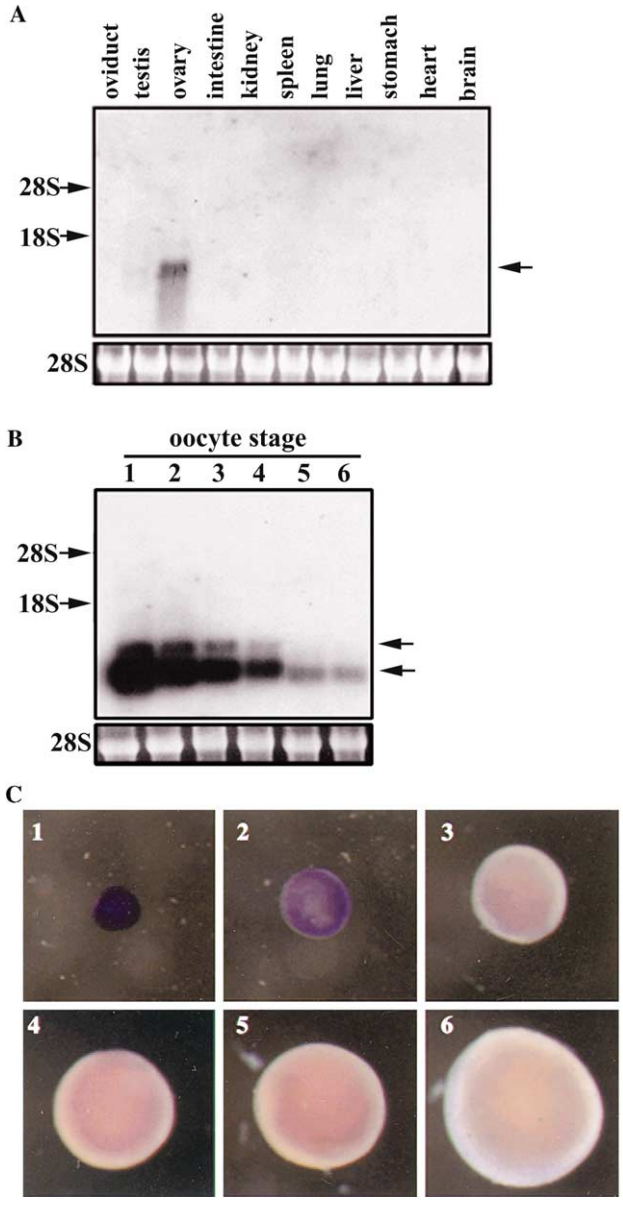

Fig. 3. Expression of SIX. The tissue distribution of SIX was determined by Northern blot analysis (A, B) and whole-mount in situ hybridization (C). Total RNA (20 μg) was analyzed by Northern blotting using a cDNA probe for SIX. The migration distances of 28S and 18S ribosomal RNA (left) and SIX transcripts (right) are indicated by arrow. The 28S ribosomal RNA was used as a loading control. The expression profile of SIX in the growing oocytes. The SIX expression was examined by whole-mount in situ hybridization using the DIG-labeled antisense riboprobe. Somewhat different colors of hybridization signals are due to varying illumination conditions in the photographing process. Oocytes at stage I (1) and stage II (2). A strong and homogeneous expression of SIX is observed in the oocytes but its intensity declines at stage II. (3–6) Oocytes at stage IV (3,4) and stage VI (5,6).

Expression of SIX

We examined the expression of SIX in various *Xenopus* tissues by Northern blot analysis using the full-length SIX as a probe. Northern blot analysis revealed approximately 1.4 kb transcripts in the ovary and weak transcripts in the testis of adult *Xenopus* (Fig. 3A). The expression pattern of SIX is compatible with that of TIAP [11], which is expressed in thymus, testis, and spleen of adult mice. SIX mRNA was also detected in all stages of developing oocyte of *Xenopus*. In the oocyte, the expression of SIX was decreased gradually in the successive stages of oogenesis (Fig. 3B). Interestingly, there were two transcripts of 1.4 kb and much fainter 1.6 kb. Recent reports indicate that there are several types of splice variants of the survivin genes [32,33], which might determine the response to proapoptotic stimuli in fetal tissues and other types of cancer, and survivin plays

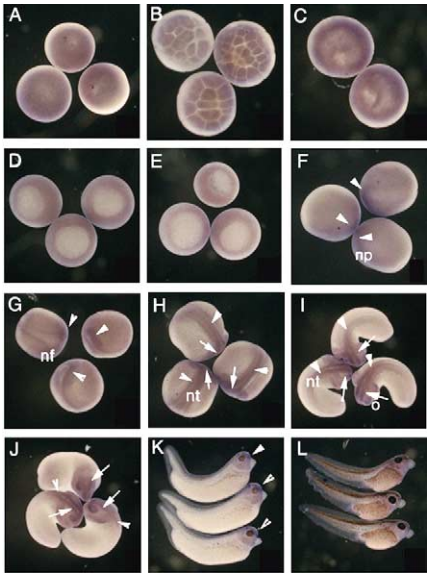

Fig. 4. The expression profile of SIX in the developing *Xenopus* embryos. The SIX expression was examined by whole-mount in situ hybridization using the DIG-labeled antisense riboprobe. (A–E, I) Dorsal view; (F–H) dorso-lateral view; (J–L) lateral view. Scale bar: 1 mm; np, neural plate; nf, neural fold; nt, neural tube; o, optic vesicle. (E) Embryos at stage 1 (A), stage 6 (B), stage 8 (C, mid-blastula stage), stage 10 (D, early gastrula stage), and stage 12 (E, yolk plug stage), respectively. The SIX expression is detected in the animal hemisphere at stages 1, 6, and 8. The SIX expression is also detected in the animal hemisphere at stages 10 and 12 but the expression level is very weak in the animal cap. (F–H) Embryos at stage 14 (F, neural plate stage), stage 17 (G, neural fold stage), and stage 21 (H, neural tube stage). The SIX expression is detected in the neural plate (arrowheads) at stage 14, in the neural fold (arrowheads) at stage 17, and in the future head region (arrows) and the neural tube (arrowheads) at stage 21. (I, J) Embryos at stage 24, The expression of SIX is strong in the head region (arrows) and at the dorsal mid-line of the neural tube (arrowheads). (K) Embryos at stage 35. Low level of signal is detected in the head region (arrowheads). (L) Embryos at stage 41. The SIX expression disappears completely at this stage (only background level of signal is present).

a complex role in regulating apoptosis. Moreover, WISH revealed that the temporal expression profile of SIX in developing *Xenopus* oocyte is similar to Northern blot analysis results (Fig. 3C). Strong and homogeneous expression of SIX was observed in the oocytes at stages I and II. However, its expression level started to decline after stage I. The SIX expression became localized to the animal hemisphere of oocyte from stage III and the same expression pattern was maintained up to stage VI. Moreover, the level of hybridization signal became even lower than those of the previous early stages. The expression profile of SIX was consistent along three batches of *Xenopus* oocytes examined by WISH procedure. Throughout the entire stages, only background level of hybridization signal was detected with sense probe (data not shown). These results indicate that SIX may be involved in the regulation of oocyte development.

In a series of developing *Xenopus* embryos, the spatiotemporal expression profile of SIX was also examined via WISH procedure. The examined stages are as follows; one cell (stage 1), 32–64 cells (stage 6), mid-blastula (stage 8), early gastrula (stage 10), yolk plug (stage 12), neural plate (stage 14), neural fold (stage 17), neural tube (stage 21), early tail bud (stage 24), hatching larva (stage 35), and swimming larva (stage 44). The SIX expression was observed in the animal hemisphere from stage 1 to stage 12 and intensity of hybridization signal was higher than that of oocytes at stages III–VI (Figs. 4A–E). At the neural plate stage, a reduced level of SIX expression was detected in the neural plate and the future neural fold (Fig. 4F). At the neural tube stage, the SIX expression became strong in the future head region and neural tube. Especially, at this stage, not only the intensity of hybridization signal was higher but also the expression domains were broader than those in the previous stages (Fig. 4H). At the early tail-bud stage, the expression was high in the head and dorsal midline of the neural tube (Figs. 4I and J). At the tadpole stage, the SIX expression was detected only in the head region but the level of signal was low compared to the previous stage (Fig. 4K). At stage 41, only a background level of signal was detected (Fig. 4L). The expression profile of SIX was consistent along the four batches of developing *Xenopus* embryos and very little variation was detected among sibling embryos in their SIX expression levels. Throughout the entire developmental stages, only a background level of hybridization signal was observed with sense probe (data not shown). The serial section of WISH stained *Xenopus* embryos showed that the SIX was expressed in the cortex layer of animal hemisphere from the blastula to the yolk plug stages. The expression of SIX was observed in the epithelial layer of future head region and neural tube at the neurula stage and the epidermal layer of head at the early tail-bud stage (data not shown). Therefore, the SIX is expressed throughout the entire oocyte stage and maintained up to the larva stage (stage 35), and the expression of SIX is dynamic during the embryonic development, especially during neurulation. These results indicate that SIX plays an important role during oogenesis and neurogenesis. Along this line, it is interesting to note that the expression domains of SIX overlap the apoptosis domains of embryos at neural stages to some degree [34].

The apoptotic program is set up at fertilization and is present in all cells during early embryogenesis [35]. Apoptosis is actively suppressed in the blastomeres of *Xenopus* embryos at early cleavage stages by maternally encoded inhibitors [36]. The above results are consistent with the expression pattern of SIX during early development in the present study. The expression pattern of SIX is similar to that of Dentrin, an inhibitor of apoptosis in *Drosophila* [10]. In situ hybridization detected the Dentrin transcript distributed throughout the embryo prior to stage 4, consistent with a putative maternal source. As embryonic development proceeds after the onset of zygotic transcription, the Dentrin expression is strong with a more restricted pattern especially in the brain and central nervous system. Recently, a protein involved in regulation of cell cycle has also been shown to have a similar embryonic expression pattern. For example, small minded (smid) is initially expressed throughout the whole embryo, but after the extended germ band stage, it is restricted to neurogenic ectoderm and gonad [37]. Smid-null mutants exhibit an abnormally small central nervous system, due to defective mitosis of post-embryonic neuroblast and their subsequent apoptosis.

In summary, we have described a novel survivin homologue from *Xenopus*, SIX. The amino acid sequence of SIX shows considerable conservation between human, mouse, and rat with BIR coding region. The expression of SIX is detectable only in gonad in adult, but during embryonic development its expression domain is very wide. Furthermore, SIX interacts with nuclear receptor RXRα, suggesting that it may be involved in retinoic acid mediated cellular response.

Acknowledgments

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