Vol. 184, No. 1, 1992
April 15, 1992

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 93-99

Expression of Thymosin β4 Gene during *Xenopus laevis* Embryogenesis

Masaya Yamamoto, Akihito Shoda, Naoto Minamino*, Hisayuki Matsuo*
Shin-ichiro Nishimatsu, Naoto Ueno[1] and Kazuo Murakami

Institute of Applied Biochemistry, University of Tsukuba, Ibaraki 305, Japan

* National Cardiovascular Center Research Institute, Suita, Osaka 565, Japan

Received Feburary 24, 1992

Summary: In order to investigate the molecular events which take place during gastrulation, extracts from developing *Xenopus* embryos were analyzed for temporal peptide distribution by high performance liquid chromatography. One peptide peak which became increasingly dominant after gastrulation was purified and partially characterized. The amino acid sequence of enzymic digests showed the peptide is extremely similar to mammalian thymosin β4. The peptide was capable of binding actin monomers like mammalian counterparts. Cloning of the *Xenopus* thymosin β4 cDNA showed that only three amino acid substitutions occurred between amphibian and bovine. Northern blot analysis revealed the mRNA is maternally present at a low level and the transcript becomes abundant after gastrulation, supporting the distribution of the peptide.

Thymosin β4 is a peptide isolated originally from calf thymus and composed of 43 amino acids (1). Although it has been reported to function as an immunomodulator, the abundant expression in a wide variety of tissues unrelated to immunological function (2) and its cytoplasmic distribution (3) indicated that the peptide may act as an essential intracellular component rather than a hormonal factor. Safer et al. (4) has recently reported that an actin-sequestering peptide designated as Fx is identical to thymosin β4, indicating that thymosin β4 may have an important role in the regulation of actin polymerization. It is of special interest that the gene for thymosin β4 is induced significantly in association with differentiation of many cell types (5,6), for example, neurite extension of PC12 cells treated with nerve growth factor and basic fibroblast growth factor (7). As recent reports

[1] To whom correspondence should be addressed.

0006-291X/92 $1.50

(8-11) suggest the involvement of growth factors in developmental regulations in *Xenopus*, it is possible that part of such actions by growth factors are mediated by the regulation of thymosin β4 gene.

To investigate the role of the thymosin β4 in early development, especially in gastrulation and neurulation, we have cloned a cDNA for *Xenopus* thymosin β4 and examined relative abundance of its transcript in embryogenesis.

MATERIALS AND METHODS

Analysis of peptides by HPLC: Developing *Xenopus* embryos were collected at stage 8 (blastula), 10 (early gastrula) and 12 (late gastrula), and extract was prepared as described (12) from each pool of the staged embryos. Two hundred μl of each extract equivalent to 20 embryos was separated on an analytical C4 column (0.45 X 25cm, Vydac) by a gradient of acetonitrile from 16 to 48% in 0.1% trifluoroacetic acid. The abundance of peptides separable in the gradient was monitored by absorption at 210nm. Large scale purification was carried out similarly starting with 25 ml of extract except that semi-preparative C4 column (0.9 X 15cm, Vydac) was used. The peak which increased upon gastrulation was collected and further purified by the analytical column described above.

Partial characterization: The purified peptide was digested with lysyl endopeptidase (EC3.4.21.50, Wako Pure Chemicals) as previously described (13) and the resulting fragments were separated by HPLC. Amino acid sequence of each peptide was determined by an automated gas phase sequencer (ABI, Model 477A).

Cross-linking of the peptide to G-actin: Binding ability of the purified peptide to G-actin (SIGMA) was tested according to the method of Safer et al (4).

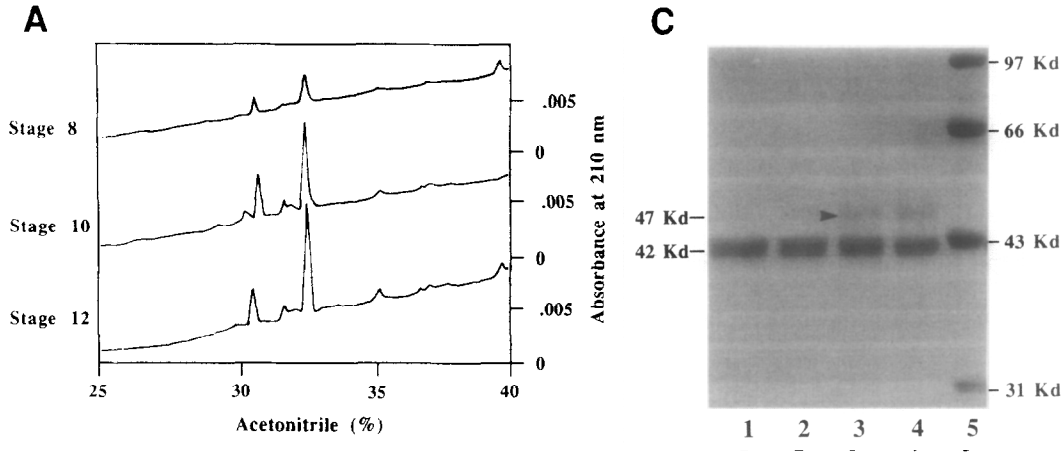
Cloning of Xenopus thymosin β4 cDNA: Specific DNA primers 5'-CTGCGC AACCATGTCTGACA-3' and 5'-CTGCGCACGCCTCATTAC-3' for human thymosin β4 were synthesized by automated DNA synthesizer (MilliGen/Biosearch, Cyclon Plus) and used for PCR in which human placental cDNA was used as template DNA. The amplified product was subcloned into pUC19 and the insert (154 b.p.) was used as a probe to clone *Xenopus* thymosin β4 cDNA. A lambda ZAP-based cDNA library for *Xenopus* embryos of stage 5 and 6, generously supplied by Dr. Ken Cho, University of California, Irvine, was screened with the [^{32}P] (Amersham) labeled probe. Nucleotide sequence of cloned cDNA, pXTβ4A was determined by the double-stranded dideoxynucleotide chain termination method.

Northern blot analysis: Cytoplasmic RNA was purified from each pool of staged embryos by the method of Chomczynski (14). The total RNA (12 μg/lane) was electrophoresed through a 1.2% agarose gel and transferred to nylon membrane by passive capillary blotting. The membrane was hybridized to [^{32}P] labeled insert of pXTβ4A. After washing in 0.3 M sodium chloride, 0.03M sodium citrate and 0.2% SDS at 60°C, the membrane was autoradiographed

RESULTS

Purification and characterization of a gastrulation-associated peptide

Extracts form *Xenopus laevis* embryo staged 8, 10 and 12 were each analyzed on HPLC (Fig.1A). A linear gradient of acetoritrile from 16 to 48% was used for the separation of biologically active peptides whose abundance changes during gastrulation. It was found that two peaks eluted at 30.8 and 32.5% of acetonitrile became greater as development proceeds from stage 8 (blastula) to 12 (gastrula). One of the peaks which is more abundant and eluted at higher acetonitrile concentration was further purified by applying a larger volume of the extract of stage 12 embryos on a semi-preparative HPLC



B

```
                    1                   10                  20
Bovine thymosin β4  S D K P D M A E I E K F D K S K L K K T E
Xenopus peptide     • • • • • • • • • • • • • X X X X • • •

                                30                  40      43
Bovine thymosin β4  T Q E K N P L P S K E T I E Q E K Q A G E S
Xenopus peptide     • • • • • • • • • • • • • • • • • • S T • •
```

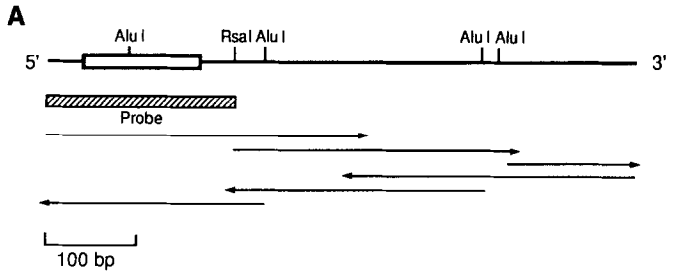
Fig.1. Biochemical properties of the gastrulation-associated peptide from *Xenopus laevis*. (A) Reversed-phase HPLC profile of extracts from developing embryos on an analytical C4 column. (B) Amino acid sequence of the *Xenopus* peptide and comparison with bovine thymosin β4. Amino acids are indicated by one-letter symbols. Identical amino acid residues are indicated by dots. "X" denotes amino acid not determined. (C) Cross-linking of *Xenopus* peptide to G-actin. G-actin was incubated with increasing concentrations (lane 1, control without peptide, lane 2, 0.025 μg, lane 3, 0.25 μg, lane 4, 2.5 μg) of the *Xenopus* thymosin β4, chemically cross-linked, and analyzed by 10% SDS-PAGE under reducing condition. Molecular weight markers were run on lane 5.

column with the same gradient condition. The isolated peptide was found to be blocked at the N-terminus because no significant peak of PTH-amino acid was yielded in the first cleavage cycle. Alternatively, 10 peptidic fragments were yielded by an enzymic digestion with lysyl endopeptidase (13). The N-termial structure was determined by sequencing a trace amount of unblocked peptide which was copurified with blocked peptide. Homology search of the obtained amino acid sequence of each fragment revealed that the isolated peptide was likely to be a homologue of mammalian thymosin β4 (1). Only two amino acid substitutions were evident in the aligned sequence of entire 43 amino acid (Fig. 1B). It is known for mammalian thymosin β4 that N-terminal methionine is acetylated and then removed (15). Similar modification seems to occur for of *Xenopus* thymosin β4 because the *Xenopus* sequence also lacks the N-terminal methionine.

Recently, an actin sequestering peptide, Fx (4) has been shown to be identical to thymosin β4. In order to examine whether the *Xenopus* thymosin has similar activity to bind G-actin, the purified preparation was mixed with G-actin, chemically cross-linked, and analyzed by SDS-PAGE. As shown in Fig 1C, when increasing amount of *Xenopus* thymosin β4 is mixed with G-actin, the ratio of higher molecular weight of actin which represents thymosin-actin complex, indicated by an arrow, increased concomitantly. The result indicates the *Xenopus* thymosin β4 is also an actin-binding peptide.

Cloning of cDNA for Xenopus thymosin β4

Twenty positive clones were obtained after screening 25,000 plaques of a library for *Xenopus* embryos staged 5 and 6 using 154 b.p. fragment of PCR-cloned human thymosin β4 as a probe. The structure and nucleotide sequence of one of the clones designated as pXTβ4A were shown in Fig 2A and 2B, respectively. The pXTβ4, 600 b.p. in length, encodes the entire peptide sequence in 5' region, having approximately 400 b.p. of untranslated region followed by poly(A) sequence in 3' region. The deduced amino acid sequence (Fig. 2B) has demonstrated itself to be essentially identical to the predicted sequence obtained from purified peptide (Fig.1B). One additional amino acid substitution was found at position 15 where Ala (Ser for bovine) is predicted from the cDNA structure.



B

```
CGACGCTTGTGAGGGCTGCCTCTCTGAACATACCACAAAATGTCTGACAAACCAGATATG 60
                                        M  S  D  K  P  K  M

GCTGAGATTGAGAAATTCGATAAGGCCAAGCTGAAGAAGACAGAAACACAAGAAAAGAAC 120
 A  E  I  E  K  F  D  K  A  K  L  K  K  T  E  T  Q  E  K  N

CCACTCCCATCAAAAGAAACAATTGAGCAAGAGAAGCAATCAACTGAGTCATAATGAATC 180
 P  L  P  S  K  E  T  I  E  Q  E  K  Q  S  T  E  S STP

CCCATCACCCCAATATGCACTGTACATTCCACAAGCATTGCCTTCTTATTTTACTTTTAG 240
CTGTTTAACTTTGTAAGAAATAACAAATACAAAGAATTTTGGAGAACATGTTCTGTGCTG 300
CCCTGTTACACCAAAAGAAAGGAATAAGAACTACTGAACACGGAATGAAGGCGCTGCCTT 360
TCCATCTACCTGTCTGGCTGGCTTTGATTCTGGTGGCATTGAAATATCTCGCATGATTAT 420
CTAACGAGAAATGGTGTGGAAATAAAAGAACTAAAGTAACATGCTCTGATGTGGGTGGAA 480
TGAGAGCTGCACCAAGGTCCAGCAAGCTGTAAAATACGGTCAAATCGAGTGCCATTTTAT 540
TTGTTCAAAATGAATTGAATTATTGGAATGCACAATTTTTTTTAATATGCAAATAAAAAT 600
AATAAAACGTTAAAAAAAAAAAAAAA 626
```

Fig.2. Structure and sequence of *Xenopus* thymosin β4. (A) Restriction enzyme map and sequencing strategy for *Xenopus* thymosin β4 cDNA. The arrows indicate the direction of sequence analysis. The open box represents peptide coding region and the hatched box denotes the sequence used as a probe to detect embryonic mRNA in Fig.3. (B) Nucleotide sequence of the cDNA, pXTβ4A encoding *Xenopus* thymosin β4. The deduced amino acid sequence was indicated by one-letter symbols. Polyadenylation signal sequences are underlined.

Expression of Xenopus thymosin β4 gene

Peptide analysis performed by HPLC showed that *Xenopus* thymosin β4 becomes abundant during and after gastrulation (Fig 1A). In order to correlate the presence of the peptide with the transcription of the gene, Northern blot analysis was performed using specific cDNA probe for *Xenopus* thymosin β4. As shown in Fig.3, an mRNA of approximately 600 b.p. was found to be present in unfertilized eggs at a low level and it increased gradually after gastrulation. It should be noted that the transcript increases further after neurulation to tail bud embryos.

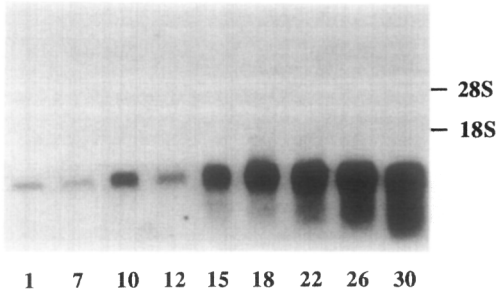


Fig. 3. Detection of *Xenopus* thymosin mRNA in developing embryos. Total RNA was purified and separated on a 1.2% agarose gel. The RNA was transferred to a nylon membrane and hybridized with the *Xenopus* thymosin β4 cDNA fragment (see Fig. 2A) labeled with [^{32}P].

DISCUSSION

In this study, we have identified a gastrulation-associated peptide in *Xenopus* embryos as an ubiquitous peptide, thymosin β4 and demonstrated that the *Xenopus* thymosin is also capable of binding actin. Thymosin family peptides were originally purified from calf thymus and defined as peptide which have immunomodulatory effects (1). Later, it became apparent that thymosins are expressed in a diverse array of mammalian and other vertebrate tissues (2) and thus the biological significance is obscure. Cloning of *Xenopus* thymosin β4 cDNA has shown that like mammalian thymosin β4, the translation product has neither signal sequence nor precursor polypeptide, suggesting the role of the peptide is limited to intracellular function(s). Based on binding ability of thymosin β4 to G-actin, it is suggested that the peptide plays a role in the regulation of actin polymerization

As shown in this study, mRNA for *Xenopus* thymosin β4 is present in unfertilized eggs and embryos before gastrula at trace levels as a maternal factor and the level becomes higher as development proceeds, suggesting that thymosin may have important biological action(s) related to morphogenesis of early embryos. In rat embryos, thymosin β10 is extensively studied. The thymosin β10 gene is induced to high levels in the embryonic nervous system and declines rapidly in the brain during the postpartum period. Thus the thymosin β10 is thought to be an important probe to study the molecular mechanism underlying neural development (16,17).

One of the advantages of using *Xenopus laevis* as experimental animal is that embryos are relatively large and manipulatable. Therefore, it would provide a suitable system to correlate ontogeny of thymosin family peptides with particular developmental events such as gastrulation, and neurulation.

ACKNOWLEDGMENTS

We thank Dr. Ken Cho for the gift of cDNA library and Mr. A. Suzuki for helpful discussions and technical advice. This work is supported by a grant-in-aid from Ministry of Education, Science and Culture, Japan (N.U., 0383301).

REFERENCES

1. Low, T.L.K., Hu, S.-K. and Goldstein, A.L. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 1162-1166.
2. Horecker, B.L. and Morgan, J. (1984) Lymphokines. 9, 15-35.
3. Watts, J.D., Cray, P.D., Sautiere, P., and Crane-Robinson, C. (1990) Eur. J. Biochem. 192, 643-651.
4. Safer, D., Elzinga, M., and Nachmias, V. T. (1991) J. Biol. Chem. 266. 4029-4032.
5. Gondo, H., Kudo, J., White, J.W., Barr, C., Selvanayagam, P.and Saunders, G.F. (1987) J. Immunol. 139. 3840-3848.
6. Baxevanis, C.N., Reclos, G.J., Perez, S., Kokkinopoulos, D., and Papamichail, M. (1987) Immunopharmacology. 13, 133-141.
7. Leonard, D.G.B., Ziff, E.B., and Greene, L.A., (1987) Mol. Cell. Biol. 7, 3156-3167.
8. Kimelman, D. and Kirschner, M. (1987) Cell. 51, 869-877.
9. Rosa, F., Roberts, A.B., Danielpour, D., Dart, L.L., Sporn, M.B.and Dawid, I.B. (1988) Science. 239, 783-785.
10. Asashima, M., Nakano, H., Shimada, K., Kinoshita, K., Ishii, K., Shibata, H. and Ueno, N. (1990) Roux's. Arch. Dev. Biol. 198, 330-335.
11. Thomsen, G., Woolf, T., Whitman, M., Sokol, S., Vaughan, J., Vale, W. and Melton, D.A., (1990) Cell. 63, 485-493.
12. Sagata, N., Watanabe, N., Woude, G.F.V., and Ikawa, Y. (1989) Nature. 342, 512-518.
13. Aburaya, M., Hino, Jun., Minamino, N., Kangawa, K., and Matsuo, H.(1989) Biochem. Biophys. Res. Commun. 163, 226-232.
14. Chomczynski, P. and Sacchi, N. (1987) Ana. Biochem. 162, 156-159.
15. Wodnar-Filipowicz, A., Gubler, U., Furuichi, Y., Richardson, M., Nowoswiat, E.F., Poonian, M.S., and Horecker, B.L., (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 2295-2297.
16. Lin, S.-C. and Morrison-Bogorad, M. (1990) J. Mol. Neurosci. 2, 35-44.
17. Lugo, D.I., Chen, S.-C., Hall, A.K., Ziai, R., Hempstead, L.J., and Morgan, J.I., (1991) J. Neurochem. 56. 457-461.