Biochemical properties of amphibian bone morphogenetic protein-4 expressed in CHO cells

Atsushi SUZUKI, Shin-ichiro NISHIMATSU, Akihito SHODA, Kimiko TAKEBAYASHI, Kazuo MURAKAMI and Naoto UENO*

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

The biochemical properties of recombinant amphibian bone morphogenetic protein-4 (BMP-4), the cDNA of which has been cloned recently by screening of a *Xenopus* cDNA library, was characterized. The protein was expressed by the transfection of Chinese hamster ovary (CHO) cells with the cDNA cloned into expression vectors bearing a cytomegalovirus promoter or a simian virus 40 promoter. Northern-blot analysis showed that the latter vector was more efficient for *Xenopus* BMP-4

expression. Specific antiserum against *Xenopus* BMP-4 peptide demonstrated that the protein is synthesized as a large precursor, processed to the mature form and then secreted from the cells as a homodimer. Analysis of the biological activity in the conditioned medium revealed that *Xenopus* BMP-4 has a potent alkaline phosphatase-inducing activity on mouse osteoblastic cells.

INTRODUCTION

Bone morphogenetic proteins (BMPs) are polypeptides which have been purified from guanidium chloride extract of bovine bone on the basis of their ability to induce cartilage and bone formation *in vivo* (Urist, 1965; Urist et al., 1973). So far, seven members of this family, namely BMP-1–BMP-7, have been identified and the structures of all members except BMP-1 are found to be related to each other and also to the superfamily of transforming growth factor β (TGF- β) proteins (Wozney et al., 1988; Celeste et al., 1990). Although TGF- β was originally identified as a polypeptide growth factor with either growth-stimulating or -inhibiting functions depending on the cell type (Derynck et al., 1985), it was later found to be a multifunctional factor controlling the differentiation of a variety of cell types (Rizzino, 1988) including developmental regulation in early embryogenesis.

Activin is also a member of the TGF- β superfamily, which stimulates the secretion of follicle-stimulating hormone (Vale et al., 1986; Ling et al., 1986) as well as erythroid differentiation (Eto et al., 1987; Murata et al., 1988) in adult animals. Recently, activin has been shown to trigger morphogenetic events, such as mesoderm induction in early *Xenopus laevis* embryos (Asashima et al., 1990; Smith et al., 1990; Thomsen et al., 1990). This discovery prompted us to identify activin or activin-related genes in *Xenopus* embryos. In the course of screening for activin-related genes from a cDNA library prepared from *Xenopus* embryos, we identified three genes that encode homologues of mammalian BMP-2, -4 and -7 (Nishimatsu et al., 1992). Northern-blot analysis and reverse transcription PCR revealed that mRNAs for BMP-2 and -4 are widely distributed and detected in a variety of tissues (Wozney et al., 1990; Suzuki et al., 1993) as well as early embryos (Nishimatsu et al., 1992). These results indicate that BMPs may have as yet unknown function(s) unrelated to bone formation. Moreover, the function and biochemical nature of embryonic BMPs are even more obscure. One of the difficulties of studying the biological activities of BMPs is

that the supply of a pure preparation of BMPs is limited because of a relatively low efficiency of production. Since most of the TGF- β family peptides need to be dimerized to exhibit their biological activities, it is necessary to produce the dimeric proteins at high efficiencies. Moreover, correct enzymic processing to obtain the mature proteins is required because many of the TGF- β family proteins with precursor sequences attached appear to be much less active than their respective mature forms.

In this report, we describe the expression of amphibian *Xenopus* BMP-4 in Chinese hamster ovary (CHO) cells, as an initial approach to investigating the structural and functional aspects of the protein.

MATERIALS AND METHODS

Materials

Restriction enzymes and modification enzymes were purchased from Toyobo Biochemicals Co. (Tokyo, Japan). Methotrexate (MTX), all-*trans*-retinoic acid, cell culture media and supplements were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Serum-free medium S-Clone (SF-O2) was supplied by Sanko Pure Chemical Co. (Tokyo, Japan). MC3T3-E1, an osteoblastic cell line derived from newborn mouse calvaria, was a kind gift from Dr. Kumegawa (Meikai University, Saitama, Japan).

Construction of expression vectors

In order to express the *Xenopus* BMP-4 protein in CHO cells, two types of vectors were used. Plasmid pCVD(X), which contains both the human cytomegalovirus (CMV) promoter and the mouse dihydrofolate reductase (*dhfr*) gene under the control of the simian virus 40 (SV40) promoter, was constructed as schematically outlined in Figure 1(a). Briefly, the pCVD(X) was prepared from pSD(X) (Murata et al., 1988) as follows: the plasmid was completely digested with *Pvu*II and partially digested with *Bam*HI. The resulting *Pvu*II–*Bam*HI fragment (5.3 kb)

Abbreviations used: BMP, bone morphogenetic protein; TGF- β , transforming growth factor β ; CHO, Chinese hamster ovary; CMV, cytomegalovirus; SV40, simian virus 40; MTX, methotrexate; FBS, fetal bovine serum; MEM, minimum essential medium; TBS, Tris-buffered saline; ALPase, alkaline phosphatase.

* To whom correspondence should be addressed.

Figure 1 Construction of two expression vectors

(a) Schematic diagram for the construction of pCVD(X). This expression vector carries a CMV promoter to drive the expression of exogenous cDNA and an SV40 promoter-driven *dhfr* gene for gene amplification. (b) Construction of pSVD(X) was by transformation of the *Bam*HI site of plasmid pSVD into the *Xho*I site.

which contains the mouse *dhfr* gene was separated on an agarose gel and ligated to the *Nru*I–*Bam*HI fragment (1.6 kb) derived from plasmid pCDM8 (Seed, 1987) harbouring the CMV promoter and *Xho*I site to yield the pCVD(X) plasmid. Figure 1(b) shows the structure of the plasmid pSVD(X) which resulted from transformation of the *Bam*HI site in pSVD (Hosoi et al., 1991) to the *Xho*I site. A 1.3 kb *Eco*RI–*Xho*II cDNA fragment containing the entire coding sequence of *Xenopus* BMP-4 (Nishimatsu et al., 1992) was blunt-ended with T4 DNA polymerase and ligated with *Xho*I linkers. The resulting DNA construct was inserted to the *Xho*I site of these two vectors for *Xenopus* BMP-4 cDNA expression.

Cell culture and transfection of DNAs

CHO (*dhfr*[−]) cells were cultured in Ham F12 medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin. Transfections of the plasmids pCVD(X)/BMP-4 and pSVD(X)/BMP-4 were carried out by the calcium phosphate coprecipitation method using the CellPfect transfection kit (Pharmacia, Uppsala, Sweden). Selection of CHO transformants expressing the *dhfr* gene was accomplished by plating in Dulbecco's modified Eagle's medium supplemented with 10% dialysed FBS, 0.1 mM minimum essential medium (MEM) non-essential amino acids, 100 units/ml penicillin and 100 µg/ml streptomycin. The colonies that appeared after culturing for 10–14 days in the selection medium were isolated using cloning cylinders. The cloned cells were then amplified for both Northern-blot analysis and Western-blot analysis. For gene amplification, one of these clones, S-6, was

adapted to increasing concentrations of MTX (up to 1600 nM) in the selection medium and the resulting clone, designated S-6-1600, was established.

RNA isolation and Northern-blot analysis

Total RNA from CHO transformants were prepared by the SDS/urea method (Gough, 1988). Total RNA (15 µg) was electrophoresed on 1.2% agarose gel and transferred to nitrocellulose membrane. The membrane was hybridized with a ³²P-labelled *Eco*RI–*Not*I fragment (308 bp) from the BMP-4 cDNA as described previously (Asashima et al., 1991). To confirm the quality and quantity of RNA, the same filter was rehybridized with a ³²P-labelled mouse β-actin probe (Tokunaga et al., 1986).

Western-blot analysis

Western-blot analysis of recombinant BMP-4 was done essentially as described (Ueno et al., 1992). Samples were prepared as follows: serum-free conditioned media obtained from CHO cells transfected with the expression vector with or without the BMP-4 cDNA were dialysed against 10 mM Tris/HCl, pH 7.4. After lyophilization, the materials were solubilized in 1×SDS sample buffer and then separated on a 15% (w/v) polyacrylamide gel. After electrophoresis, proteins were transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA, U.S.A.) by a semi-dry blotting apparatus (Millipore; Milliblot SDE). The membrane was blocked with 5% (w/v) non-fat dried milk in Tris-buffered saline (TBS) and allowed to react with purified anti-*Xenopus* BMP-4 antibodies Ab97 (Nishimatsu

et al., 1993) diluted 25-fold with a dilution buffer [150 mM NaCl, 1 % (v/v) Nonidet P40, 0.5 % (w/v) sodium deoxycholate, 0.1 % (w/v) SDS, 50 mM Tris/HCl, pH 7.4, 1 % (w/v) non-fat dried milk] at 4 °C overnight. The filter was then washed twice with TBS containing 0.1 % (v/v) Tween 20 and once with TBS, and allowed to react with peroxidase-conjugated goat anti-rabbit IgG antibody (Jackson Immunoresearch Laboratories, West Grove, PA, U.S.A.) for 6 h at 4 °C. After being washed with TBS as above, proteins were visualized by the addition of 0.05 % (w/v) 4-chloro-1-naphthol and H₂O₂.

Alkaline phosphatase (ALPase) assay

The measurement of ALPase activity was carried out using mouse MC3T3-E1 cells as previously described (Hashimoto et al., 1992). Briefly, MC3T3-E1 cells were cultured in 48-well culture plates at 2×10^4 cells/well in 500 μ l of α -MEM containing 10 % FBS for 4 days to a confluent state. The cells were washed once with the medium and cultured further in the presence of conditioned media from CHO transformants. After 48 h the cells were washed twice with PBS, and then 200 μ l of a reaction mixture containing 0.56 M 2-amino-2-methyl propan-1-ol, 1 mM MgCl₂ and 10 mM disodium *p*-nitrophenyl phosphate was added. After incubation for 1 h at 37 °C, the reaction was terminated by the addition of 200 μ l of 1 M NaOH, and samples of the mixture were tested for absorption at 405 nm. ALPase activity was calculated from the standard curve and expressed as nmol of *p*-nitrophenol produced per 10^4 cells during the 1 h incubation.

RESULTS AND DISCUSSION

Expression efficiency of *Xenopus* BMP-4

Two expression vectors shown in Figure 1 were used to express *Xenopus* BMP-4 cDNA. pCVD(X) was constructed from pSD(X) by switching the SV40 promoter to the CMV promoter derived

from pCDM8. Both vectors carry the *dhfr* gene for amplification of the introduced DNA by MT treatment (Kaufman et al., 1985). The full-length cDNA of *Xenopus* BMP-4 (Nishimatsu et al., 1992) was inserted into the two vectors and the respective constructs were transfected into CHO cells by the calcium phosphate transfection method. Some 10–14 days after the transfection, single cells were isolated from each CHO cell population and cultured further to obtain the clones. Figure 2 shows the Northern-blot analysis of BMP-4 transcript in each CHO cell clone. Interestingly, a significant amount of BMP-mRNA was detected in the cells transfected with the pSVD(X) vector whereas hybridization was very weak in those transfected with the pCVD(X) vector. In an independent experiment, *Xenopus* BMP-7 was also expressed using these two vectors, and the efficiency of expression was much higher with the pCVD(X) vector than with the pSVD(X) vector (A. Suzuki, S. Nishimatsu, A. Shoda, K. Takebayashi, K. Murakami and N. Ueno, unpublished work). Therefore, the low efficiency of pCVD(X) is not due to the weak promoter activity of CMV and suggests that *Xenopus* BMP-4 gene has a preference for the SV40 promoter over the CMV promoter. This result was also confirmed by Western-blot analysis with specific antibodies to BMP-4, which showed that the immunoreactive protein is more abundant in the conditioned medium from the pSVD(X)/BMP-4 construct (results not shown).

On the basis of the efficiency of BMP-4 transcription, the strain designated as S-6 in Figure 2 was subjected to amplification by MTX. The concentration of MTX in the culture medium of S-6 was gradually increased to a final concentration of 1600 nM, and a high-yield clone, S-6-1600, was obtained. The concentration of *Xenopus* BMP-4 in the medium of S-6-1600 was calculated to be approximately 20 ng/ml on the basis of intensity of immunoreactivity of known concentrations of recombinant human BMP-4. This expression rate was rather low compared with other proteins unrelated to BMP, such as renin (Poorman et al., 1986) which was produced in CHO cells with the same expression vector. However, it has been shown by others that the production of TGF- β -family-related proteins in mammalian cells is less efficient than other proteins (ten Dijke et al., 1990; Hammonds et al., 1991). The inefficiency is probably due to the fact that biosynthesis of these proteins involves post-translational modification, such as dimerization of polypeptides and processing of large precursor proteins into the mature forms.

Predicted structure of *Xenopus* BMP-4 protein

In order to investigate the biochemical properties of the recombinant *Xenopus* BMP-4, the culture medium was subjected to SDS/PAGE followed by Western-blot analysis. As shown in Figure 3(a) (lane 1), under non-reducing conditions, four distinct molecular species of 120 kDa, 80 kDa, 40 kDa and 20 kDa were detected with BMP-4-specific antibodies. In contrast, two intense staining bands (lane 2) at 60 kDa and 20 kDa were detected under reducing conditions. As deduced from molecular cloning of BMP cDNAs by us and others (Wozney et al., 1988; Köster et al., 1991; Nishimatsu et al., 1992), BMPs are synthesized as larger precursor proteins. The precursor of *Xenopus* BMP-4 consists of 401 amino acids and is assumed to form a homodimer which is then enzymically processed to yield a mature dimeric protein of 114 amino acids in each polypeptide chain. Since there are several potential N-linked glycosylation sites in both the precursor and the mature region of BMP, the molecular masses of the precursor and mature proteins were estimated to be 60 kDa and 20 kDa respectively in their monomeric forms (Wang et al., 1990). On the basis of the location of the putative

Figure 2 Northern-blot analysis of CHO transformants

Total RNA from CHO cells transfected with the expression vectors that contained the *Xenopus* BMP-4 cDNA was electrophoresed and hybridized with *Xenopus* BMP-4 probe as described in the text. The clones from pCVD(X)/BMP-4 transformants and pSVD(X)/BMP-4 transformants were designated C and S respectively. The RNA from CHO cells transfected with pSVD(X) without the DNA insert was electrophoresed in the control lane. To ensure that the same amount of RNA was loaded into each lane, the filter was rehybridized with a ³²P-labelled β -actin probe.

Figure 3 Identification of recombinant *Xenopus* BMP-4 in the conditioned medium from CHO cells transfected with *Xenopus* BMP-4

(a) A 1 ml equivalent of conditioned medium from the transfected cells was separated by SDS/PAGE (15% gels), and immunoreactive *Xenopus* BMP-4 proteins were detected by Ab97 antibody. Molecular masses (kDa) of the immunoreactive proteins are indicated by arrow heads on the left margin, and molecular-mass markers (kDa) are indicated on the right. Lanes 1 and 2 represent conditioned medium from clone S-6-1600 under non-reducing and reducing conditions respectively. Lane 3 represents conditioned medium from CHO cells transfected with pSVD(X) as control. (b) Predicted structures of BMP-4 proteins secreted from CHO cells transfected with BMP-4 cDNA. The precursor region is represented by an open bar and the mature region represented by a closed bar. The BMP-4 peptides form a dimer through any one of the cysteine residues in the mature region.

processing site R-X-K-R (Hosaka et al., 1991) in the deduced primary sequence of the BMP-4 precursor, the immunoreactive species in Figure 3(a) can be represented schematically as in Figure 3(b). The largest 120 kDa species is probably a homodimer of two 60 kDa precursors, the 80 kDa species is a heterodimer of the 60 kDa precursor and the 20 kDa mature peptide and the 40 kDa species is a homodimer of two mature peptides. These results show that dimerization and processing of *Xenopus* BMP-4 polypeptide can occur correctly in the CHO cells, similarly to the biosynthesis of BMP *in vivo*.

Biological activity of *Xenopus* BMP-4

BMPs are known to induce ectopic cartilage and bone formation when transplanted into rat muscle *in vivo*. However, this *in vivo* bioassay requires several weeks to obtain the biological results. Therefore we employed an *in vitro* bioassay using an osteoblastic cell line, MC3T3-E1, to measure the induction of ALPase activity by BMP-4 because another member of the BMP family, named BMP-2, was previously demonstrated to induce this enzyme activity (Katagiri et al., 1990; Takuwa et al., 1991), like other

Figure 4 Dose-dependent effect of recombinant *Xenopus* BMP-4 on ALPase activity

MC3T3-E1 cells were cultured in α -MEM plus conditioned media from CHO transformants for 48 h. Media from CHO transformants were as follows: $\circ$, pSVD(X); $\bullet$, pSVD(X) + 300 pM retinoic acid; $\triangle$, pSVD(X)/BMP-4; $\blacktriangle$, pSVD(X)/BMP-4 + 300 pM retinoic acid. After being washed, the cells were treated with a reaction mixture which lyses the cells to release the ALPase activity. The concentration of 300 pM retinoic acid represents the final concentration of retinoic acid added to the medium. Values are means $\pm$ S.E.M. for three determinations. Where no S.E.M. is shown, it is because it was so small as to be within the area of the symbol.

various stimuli (Kumegawa et al., 1984). As shown in Figure 4, recombinant *Xenopus* BMP-4 alone was found to induce ALPase activity in a dose-dependent fashion, whereas medium from CHO cells transfected with pSVD(X) without the *Xenopus* BMP-4 DNA insert showed no such activity. The induction of ALPase activity is not due to the proliferative effect of BMP-4 on the cells because no significant increase in DNA synthesis, measured by thymidine uptake, was detected in BMP-treated cells (results not shown). This finding also applies to BMP-2 (Takuwa et al., 1991). The ALPase-inducing activity was highly potentiated by the presence of 300 pM-retinoic acid. Retinoic acid itself was previously shown to stimulate the differentiation of MC3T3-E1 cells to induce ALPase activity (Nakayama et al., 1990). However, the effect of retinoic acid with BMP-4 appears to be synergistic rather than additive because *Xenopus* BMP-4 containing medium alone or 30 pM retinoic acid added to control medium from pSVD(X) vector-transfected cells induced rather low levels of ALPase activity. Recently, retinoic acid was shown to induce BMP-4 transcription in human prostate carcinoma cells (Harris et al., 1991). Therefore our observation of synergistic action may be explained by concomitant induction of BMP gene(s) by retinoic acid in the cells. Also, the effect of *Xenopus* BMP-4 on MC3T3-E1 cells is equivalent to that of human BMP-4 (results not shown) because they share more than 90% amino acid sequence homology in the predicted mature region. Although little is known about the difference in biological activity among BMPs, we have previously reported that *Xenopus* BMP-4 is more potent than *Xenopus* BMP-2 in the ALPase-inducing assay when both proteins are expressed transiently in COS cells (Nishimatsu et al., 1992).

Our screening of the *Xenopus* genomic library with a probe for activin, a member of the TGF- β superfamily of proteins, resulted in the identification of homologous genes to those of mammalian BMPs. In the present study, we have successfully demonstrated that at least one of the amphibian BMPs, *Xenopus* BMP-4, is

biosynthesized similarly to mammalian BMP. Northern- and Western-blot analysis of BMP genes in early embryos showed that not only their mRNAs but also their proteins are present as maternal factors before bone is first produced in the animals (Nishimatsu et al., 1992; Ueno et al., 1992). Although the amphibian BMP-4 has been shown to exert the same activity on osteoblastic cells as mammalian BMP-4, the question relating to the function of BMPs in early embryos remains to be investigated. Pure BMP preparation obtained from a large-scale production followed by the development of other bioassays that can correlate activity with early developmental phenomena is required to address this question.

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