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EXPRESSION OF RECOMBINANT XVAX2 PEPTIDE-104 OF *XENOPUS LAEVIS* AND PREPARATION OF THE ANTIBODY

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Abstract: An N-terminal polypeptide (XVAX2-P104) of the XVAX2 protein, which is involved in controlling the dorsoventral patterning of the retina in *Xenopus laevis*, was expressed and purified as a His-tagged fusion protein (pHis-XVAX2-P104), and it was employed to generate an anti-XVAX2 antibody in New Zealand white rabbits. ELISA analysis shows that the titer of the antibody is as high as ~1:100,000. The antibody could specifically recognize the full-length XVAX2 protein in extracts of *Xenopus* embryos, as determined by Western Blotting.

INTRODUCTION

A homeobox gene, *Vax2*, previously isolated, is involved in regulating eye polarity at different levels, such as regional specification of ocular structures, execution of correct morphogenetic movements and proper establishment of the dorsal-ventral axis of the retina [1-3]. In *Xenopus*, an orthologous gene *Xvax2* also displays an expression pattern in the ventral retina and in the optic disc and stalk, as well as in the ventral telencephalon and diencephalon [4,5]. The overexpression of *Xvax2* in *Xenopus* embryos leads to an aberrant eye phenotype and, in particular, determines a ventralizing effect on the developing eye. So far, however, only expression of *Xvax2* mRNA has been analyzed, and the cellular and molecular mechanisms remain unclear. To further investigate the function of *Xvax2*, it is necessary to study temporal-spatial expression patterns of the *XVAX2* protein.

Previously, we prepared an N-terminal polypeptide of a member of POU subfamily, qBrn-2, and raised a specific antiserum to study the protein expression pattern in developing quail embryos [6,7]. Similarly, in view of high conservation of the homeodomain and toxicity of the full-length transcription factor to host cells [8], a relatively divergent region of XVAX2 (residues 1-104, XVAX2-P104) was expressed and used for preparation of an anti-XVAX2 antibody. In this work, XVAX2-P104 was expressed as a hexahistidine (6xHis) fusion protein in *E. coli* and purified by metal affinity chromatography. Subsequently, an anti-XVAX2 antibody was generated using the purified XVAX2-P104 polypeptide, and shown to specifically recognize the XVAX2 protein in western blot of proteic extracts of *Xenopus* embryos.

MATERIALS AND METHODS

1.1. Materials

Full length *Xvax2* was previously cloned from *Xenopus laevis* (GenBank access No. AJ238649) [3]. The expression vector pET15b and bacterial strain BL21(DE3) were from Novagen. NucleoSpin Extraction Kit and NucleoBond Plasmid Midi Kit (BD Biosciences Clontech) were used to extract PCR products and the recombinant plasmids, respectively. Thrombin, AccuTaq polymerase and isopropyl-β-D-thiogalactoside (IPTG) were from Sigma. Shrimp alkaline phosphatase (SAP) and T4 DNA ligase came from Roche Company. The Chelating Sepharose Fast Flow for affinity chromatography and the nitrocellulose membrane came from Pharmacia Biotech. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was from Jackson Inc. Other reagents were all analytic grade without further purification before used.

1.2. Construction of pET15b-Xvax2-P104 plasmid

For amplification of the insert, PCR was conducted by using the forward primer (5'-GGG AAT TCC ATA TGT TTG ATC AAG CTA CTA G-3') and reverse primer (5'-AAC CGC CGC TCG AGG GGT CGA TCC AGA TCC AGG C-3'). The PCR conditions consisted of one cycle of 94°C for 5 min, 35 cycles of 94°C for 1 min, 53°C for 1 min, 72°C for 1 min, and one cycle of 72°C for 7 min. After digestion with *Nde*I and *Xho*I, the PCR products were ligated in frame into the *Nde*I-*Xho*I fragment of pET15b plasmid. Transformation and amplification was performed as described by Ausubel, et al [9]. The recombinant plasmid was confirmed by sequencing (Sangon Biotech Company).

1.3. Expression and purification

For expression of pHis-XVAX2-P104 in BL21(DE3), an overnight culture was used to inoculate (1:50, v:v) Luria Bertani (LB) medium containing 100 μg/mL ampicillin, and incubated at 37°C until the absorbance at 600 nm reached 0.6. The expression was induced by adding IPTG to a final concentration of 0.4 mM. After a 4-hour growth at 37°C with shaking, the bacteria were collected by centrifugation (5000g, 10 min, 4°C). The pellet was lysed in 100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea (pH 8.0) and the protein expression was detected by SDS-PAGE.

For large scale purification of pHis-XVAX2-P104 under native conditions, the bacteria pellet from one litre IPTG-induced culture was washed once with phosphate-buffered saline (PBS), pH 7.4, resuspended in 25 mL suspension-buffer (25 mM NaH_2PO_4, 75 mM Na_2HPO_4, 0.5 M NaCl, pH 7.4) containing 1% Triton X-100, 0.2 mM DTT, 10 mM β-mercaptoethanol and 2 ug/umL aprotinin, and then lysed by sonication on ice for a few of bursts (15 sec. each). The lysate was centrifuged (10,000g, 4°C, 15 min), and the clarified supernatant was loaded onto pre-equilibrated Ni^{2+}-chelating Sepharose column (1×8cm). Rinsing was performed with 45 mM imidazole in suspension-buffer until the absorbance of the flow-through at 280 nm was lower than 0.01, and the protein of interest was eluted with 200 mM imidazole in suspension-buffer. The absorbance at 280 nm of each fraction was measured by spectrometry (Perkin Elmer-λ7), and the yield was estimated by Advanced American Biotechnology (AAB) Software. Fractions containing pHis-XVAX2-P104 were pooled, dialyzed against one liter of suspension-buffer (4°C, 4 times, 1 hour each), and then stored at -70°C with 2 ug/umL aprotinin and 1 mM EDTA.

1.4. Immunization and Western blot analysis

The pHis-XVAX2-P104 was SDS-PAGE purified, adjusted to 1 mg/mL in double distilled water and employed to raise antiserum in rabbits. Briefly, 8-week-old male New Zealand white rabbits were immunized subcutaneously with 1 mL protein emulsified with complete Freund's adjuvant (1:1 v/v). One month later, animals were boosted with the same amount of pHis-XVAX2-P104 in incomplete Freund's adjuvant (IFA), and further boosted twice with 0.5 mL protein in IFA at a two-week interval. Individual immune serum was collected one week after each boosting. The titers of the antisera were determined by ELISA [9]. For western blotting, extracts of *Xenopus* embryos at stage 10 and 24 were prepared [10,11], resolved along with the bacterial extracts and pHis-XVAX2-P104 antigen by 15% SDS-PAGE and then transferred onto nitrocellulose membrane. After blocked with 5% (w/v) skim milk in PBS containing 0.2% Tween-20 (PBST), the membrane was incubated with the antiserum (1:1000), washed with PBST, and then reacted with HRP-conjugated goat anti-rabbit IgG (1:1000). The bands were visualized with 400 μg/mL DAB and 0.03% hydrogen peroxide in PBS.

RESULTS AND DISCUSSION

2.1. Cloning and analyzing

The homology analysis of *Xvax* genes shows that only one amino acid residue within the homeodomain (60 residues) of XVAX2 is different from that of XVAX1b, while homeodomains of XVAX2 and XVAX1 differ for four residues [3,4]. However, the N-terminal region of XVAX2 is significantly divergent from those of XVAX1 and XVAX1b (GenBank, tBlastn). Thus, DNA fragment encoding XVAX2-P104 was subcloned from pCS2-*Xvax2* plasmid. The PCR product exhibited a single band with an expected length (312 bp) in agarose gel electrophoresis. It was recovered and then digested with *Nde*I and *Xho*I, before inserted into *Nde*I-*Xho*I digested pET15b vector, to generate an open reading frame of 441bp containing the *Xvax*2-P104 fragment fused in frame to 6xHis tag, thrombin recognition site and terminator. The sequence of the resultant plasmid had been confirmed by DNA sequencing.

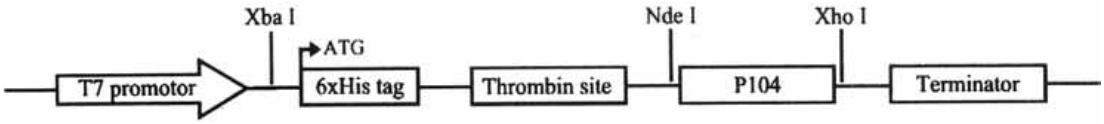

Fig. 1. Structure of the expression plasmid pET15b-*Xvax*2-P104. ATG: first translation codon; P104: N-terminal fragment of XVAX2 (amino acid residues 1-104).

2.2. Expression and purification

Based on the open reading frame of pET15b-*Xvax*2-P104 plasmid, the pHis-XVAX2-P104 fusion protein has a predicted size of 147 amino acid residues with a theoretical molecular mass of 15.8 kD, containing extra 20 amino acids at the N-terminus and extra 23 amino acids at the C-terminus of the XVAX2-P104, respectively (Fig.1). Compared with BL21(DE3) control bacteria, the induced pET15b-*Xvax*2-P104-harboring cells produced a protein showing a distinct band of 19.2 kD in SDS-PAGE. Nevertheless, the corresponding band was not detected in the extracts, either non-induced pET15b-*Xvax*2-P104-harboring cells or induced cells transformed with empty pET15b (Fig 2). For nickel-affinity chromatography, 45 mM imidazole was used for unspecific washing, followed by specific elution with 200 mM imidazole. As shown in Figure 3, the eluted pHis-XVAX2-P104 was predominant and the yield was as high as 15 mg per liter culture.

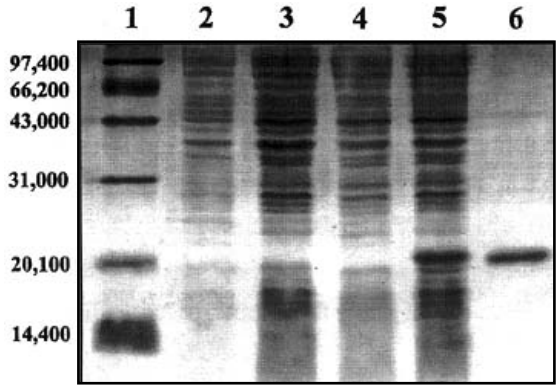

Fig. 2. Expression of the recombinant XVAX2-P104 by pET15b vector in *E. coli*. Lanes represent molecular weight marker (lane 1), total bacterial extracts of BL21(DE3) (lane 2), those of BL21(DE3) harboring pET15b after induction (lane 3), those of BL21(DE3) harboring pET15b-*Xvax*2-P104 plasmid before (lane 4) and after (lane 5) IPTG induction and purified pHis-XVAX2-P104 (lane 6), respectively.

The thrombin recognition site between 6xHis tag and XVAX2-P104 was used to identify the product. The resultant shorter polypeptide with an apparent molecular mass of 18.0 kD (Fig. 4) was confirmed to have a sequence corresponding to the N-terminal portion (10 residues) of the predicted XVAX2-P104 by sequencing.

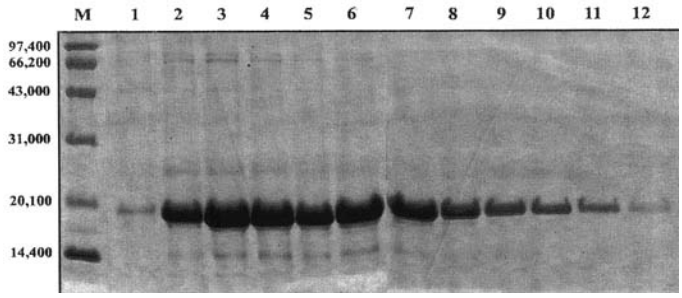

Fig. 3. Elution of pHis- XVAX2-P104 with 200 mM imidazole. Lane M represents molecular weight marker. Lanes 1-12 represents the eluted fractions

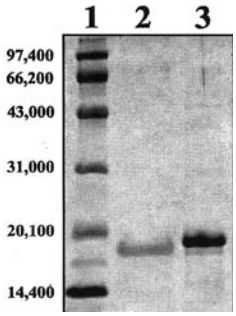

Fig. 4. Thrombin digestion of pHis-XVAX2-P104. Lane 1: Molecular weight marker; lane 2: Digested pHis-XVAX2-P104; lane 3: pHis-XVAX2-P104 as control.

2.3. Immunizing and Western blotting

The SDS-PAGE purified pHis-XVAX2-P104 was used as antigen to immunize male New Zealand white rabbits. ELISA showed that the anti-XVAX2 serum, obtained after four consecutive injections, got a titer up to 1:100,000. The specificity of the polyclonal antibody was examined by western blotting. As shown in Fig.5, the antiserum could specifically recognize the pHis-XVAX2-P104 in extracts of recombinant bacterial cells harboring the pET15b-*Xvax2*-P104 plasmid. Further, it was investigated whether the anti-XVAX2 serum could recognize the full-length XVAX2 protein in embryos. Expression of *Xvax*2 *in vivo* begins at early neurula stage (st. 13, as staged by Nieuwkoop and Faber [10]), and remains in adult [3,4]. Consistently, western blots of proteic extracts from *Xenopus* embryos (st. 24) exhibited a single band, whose molecular mass (36.0 kD) corresponded to that of a full-length XVAX2 protein. In contrast, no band could be detected in extracts from embryos at early gastrula stage (st. 10, Fig. 6), as expected. It was suggested that the anti-XVAX2 could specifically recognize the wild-type XVAX2 protein from *Xenopus* embryos, indicating that the antibody is useful in study of the expression pattern and the function of XVAX2 protein during *Xenopus* development.

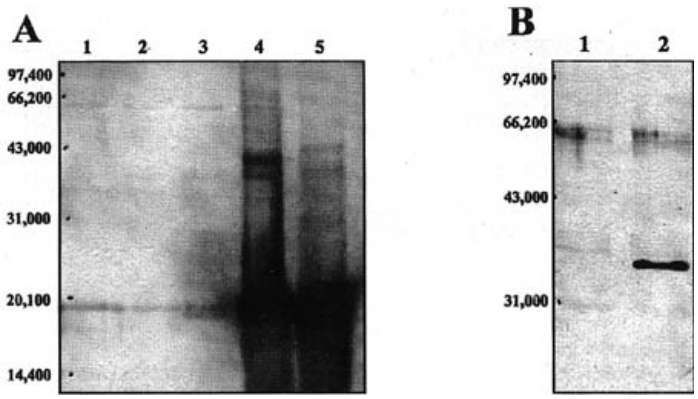

Fig. 5. Western blots with anti-XVAX2 serum of proteic extracts from bacterial cells harboring the pET15b-*Xvax*2-P104 plasmid (A) and *Xenopus* embryos at stage 10 and stage 24 (B). A. Lanes represent total bacterial extracts of BL21(DE3) (lane 1), those of BL21(DE3) harboring pET15b after induction (lane 2), those of BL21(DE3) harboring pET15b-*Xvax*2-P104 plasmid before (lane 3) and after (lane 4) IPTG induction and purified pHis-XVAX2-P104 (lane 5). B. Extracts of whole embryos at st. 10 (lane 1) and st. 24 (lane 2) were western blotted, respectively.

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