Annexin IV (Xanx-4) has a functional role in the formation of pronephric tubules

Rachel A. Seville, Sarbjit Nijjar, Mark W. Barnett, Karine Massé and Elizabeth A. Jones*

Cell and Molecular Development Group, Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK

*Author for correspondence (e-mail: eoliver-jones@dna.bio.warwick.ac.uk)

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SUMMARY

Vertebrate kidney organogenesis is characterised by the successive formation of the pronephros, the mesonephros and the metanephros. The pronephros is the first to form and is the functional embryonic kidney of lower vertebrates; although it is vestigial in higher vertebrates, it is a necessary precursor for the other kidney types. The Xenopus pronephros is a simple paired organ; each nephron consists of a single large glomus, one set of tubules and a single duct. The simple organisation of the pronephros and the amenability of Xenopus laevis embryos to manipulation make the Xenopus pronephros an attractive system in which to study organogenesis. It has been shown that pronephric tubules can be induced to form in presumptive ectodermal tissue by treatment with RA and activin. We have used this system in a subtractive hybridisation screen that resulted in the cloning of Xenopus laevis annexin IV (Xanx-4). Xanx-4 transcripts are specifically located to the developing pronephric tubules, and the protein to the luminal surface of these tubules. Temporal expression shows zygotic transcription is upregulated at the time of pronephric tubule specification and persists throughout pronephric development. The temporal and spatial expression pattern of Xanx-4 suggests it may have a role in pronephric tubule development. Overexpression of Xanx-4 yields no apparent phenotype, but Xanx-4 depletion, using morpholinos, produces a shortened, enlarged tubule phenotype. The phenotype observed can be rescued by coinjection of Xanx-4 mRNA. Although the function of annexins is not yet clear, studies have suggested a role for annexins in a number of cellular processes. Annexin IV has been shown to have an inhibitory role in the regulation of epithelial calcium-activated chloride ion conductance. The enlarged pronephric tubule phenotype observed may be attributed to incorrect modulation of exocytosis, membrane plasticity or ion channels and/or water homeostasis. In this study, we demonstrate an in vivo role for annexin IV in the development of the pronephric tubules in Xenopus laevis.

Key words: Annexin, Pronephros, Kidney, Xenopus, Morpholino

INTRODUCTION

Vertebrate kidney morphogenesis involves the progressive development of three kidney forms the pronephros, mesonephros and the metanephros (Saxén, 1987). Each kidney type arises from the intermediate mesoderm by an inductive process that is dependent for its formation on the previous temporal form. In amphibia, the pronephros is the functional larval kidney and is the first to form. It is a paired organ that consists of a single non-integrated nephron found in a lateral position of the embryo (Saxén, 1987; Vize et al., 1995; Brändli, 1999). There are three identifiable components that together form the functional pronephros: the glomus, the pronephric tubules and the collecting duct. The glomus filters waste into the coelom, where it is collected into the coiled tubules via the ciliated nephrostomes. Water balance is controlled as the waste passes down the tubules, along the duct and to the exterior via the cloaca. The simplicity of this organ coupled with the fact that it displays the same basic organisation and function as the more complex mesonephros and metanephros make this an attractive model to study the earliest events in vertebrate kidney morphogenesis (Vize et al., 1997).

We, and others, have started to establish some of the biological parameters that control pronephros formation. The temporal specification of all three components of the kidney have been established and all occur between stages 12.5 and 14 (Brennan et al., 1998; Brennan et al., 1999). A molecular map of many of the early markers of the pronephric field and pronephric anlagen is also being built up in order to unravel the molecular control of early kidney induction and patterning. In previous studies, more than 30 genes have been shown to be expressed in the pronephros and their temporal and spatial patterns of expression established (http://golgi.anal.eds.ac.uk/kidhome.html) (Wallingford et al., 1999; Carroll and Vize, 1999; Carroll et al., 1999a; Carroll et al., 1999b; Brändli, 1999; Sato et al., 2000; Onuma et al., 2000; McLaughlin et al., 2000). Many of these genes have also been shown to be expressed in mesonephric and metanephric kidneys in higher vertebrates. Evidence from knockout mutant mice has shown that Lim1, Pax2 and Wt1 are essential for pronephros development. The role of known growth factors in the process has also been
investigated (Moriya et al., 1993; Uochi and Asashima, 1996; Brennan et al., 1999).

However, the initial events in kidney organogenesis are not yet fully understood and Xenopus provides an ideal vertebrate system to identify and establish the functional role of genes involved in early kidney development. It is relatively easy to establish the domains of expression of novel genes expressed in particular regions of interest, and then it is possible, by overexpression, antisense depletion or morpholino treatment to perturb normal expression pattern and get a developmental handle on function (Zhang et al., 1998; Summerton and Weller, 1997; Heasman et al., 2000). Furthermore, direct expression cloning is proving an invaluable tool in identifying novel genes with defined roles in developmental patterning and organogenesis (Smith and Harland, 1991; Smith and Harland, 1992; Smith et al., 1995; Hsu et al., 1998; Grammer et al., 2000).

In order to identify novel genes that may be involved in pronephros development, we have adopted a subtractive hybridisation strategy that increases the levels of those genes expressed early in kidney development. This approach is based on the observations that animal caps treated with a combination of retinoic acid and activin develop in vitro into differentiated kidney tubules. Recent studies in our laboratory have also shown that glomus can be induced in animal caps by treatment with retinoic acid and activin or retinoic acid and FGF (Brennan et al., 1999). No combinations of RA, activin or bFGF have been found to induce pronephric duct at high frequency (E. A. J., unpublished). Evidence from histological studies indicates that these tubules have normal morphology (Moriya et al., 1993) (H. C. Brennan and E. A. J., unpublished). Furthermore, these tubules express differentiation markers characteristic of the correct developmental stage and in the correct developmental sequence (Uochi and Asashima, 1996). The kidney tubules formed have been reported to rescue pronephric function in tadpoles in which the pronephros has been extirpated by dissection. During the course of our experiments a similar strategy has been used by others to successfully clone the pronephros specific genes XcIRP (Uochi and Asashima, 1998) and XSMP-30 (Sato et al., 2000). In vitro induction followed by differential display has recently resulted in the isolation of Xsal-3 that is also expressed in the pronephros (Onuma et al., 2000).

We describe the isolation of Xenopus annexin IV (Xanx-4) via a subtractive hybridisation strategy designed to increase the levels of tubule-specific genes, which are expressed specifically at high levels in the pronephric tubules. We have established the temporal and spatial expression patterns of both mRNA and protein in embryos. We have established the mRNA expression pattern by northern analysis in the adult frog. Finally, we have used morpholino oligonucleotides to specifically inhibit the translation of Xanx-4 and show that a tubule phenotype results, which can be rescued by the addition of wild-type message. The tubules appear less coiled and have a diameter that is significantly greater than that seen in control embryos. These results indicate that Xanx-4 plays an important role in morphogenesis of the pronephric tubules.

MATERIALS AND METHODS

Preparation of subtracted probe
Animal caps were isolated from stage 9 Xenopus laevis embryos by manual dissection and divided into three groups each containing approximately 400 animal caps. The first group was incubated in Barth X media containing 1/40 dilution of WEHI factor, a kind gift from Professor J. Slack (Bath) (source of activin A at ~10 ng/ml) and 10^{-5} M retinoic acid, RA. The second group was incubated in media containing WEHI factor alone and the third group in media containing 10^{-5} M RA. Each group of caps was harvested at stage 20 and total RNA prepared as described by Barnett et al. (Barnett et al., 1998). A small part of each sample was used for spectrophotometric quantification. PolyA+ RNA was prepared from total RNA using an Oligotex mRNA Kit (Hybaid) according to the manufacturer’s protocol.

Tester cDNA was prepared from 2 μg of group one polyA+ RNA. The polyA+ RNA from groups two and three were pooled and Driver cDNA was prepared from 2 μg of this pool. The subtracted probe was prepared by subtracting Driver cDNA from Tester cDNA, according to manufacturer’s protocol using Clontech PCR-Select cDNA Subtraction Kit (K1804-1).

Library screening and clone sequencing
Whole embryo stage 13 Xenopus laevis λgt 11 cDNA library (gift from I. Dawid) was plated at a density of 2.5×10⁶ plaques on 25 cm² plates and duplicate plaque lifts taken on Hybond N+ (Amersham) nylon filters. The subtracted probe was hybridised at 62°C (0.1% SDS, 5×SSC, 0.1% BSA, 0.1% poly vinyl-pyrollidone, 0.1% Ficol, type 400). The first wash was performed at 62°C (2×SSC, 0.1% SDS), followed by two further washes (1×SSC/ 0.1% SDS, 0.5×SSC/0.1% SDS and 0.2×SSC/0.1% SDS). The 1131 bp K2 (annexin IV) cDNA insert was subcloned into pGEM-T Easy (Promega) and both strands were sequenced using an Applied Biosystems 373A instrument.

Embryo culture and dissection
Embryos were obtained by in vitro fertilisation of hormonally stimulated Xenopus laevis and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994). Standard embryological procedures were used as described by Jones and Woodland (Jones and Woodland, 1986). Embryos were dejellied in 2% cysteine hydrochloride pH 8.5 and cultured in 1/10 BarthX. Dissected animal caps were cultured in BarthX and staged using whole embryo controls.

Expression clones, mRNA Synthesis and micro-injection
The full-length Xanx-4 cDNA was removed by digestion with EcoRI from pGEM-T Easy plasmid and cloned into the RN3 pBluescript vector (a gift from J. B. Gurdon, Cambridge) at the EcoRI sites. Xanx-4 mRNA was synthesised from Xanx-4 pRN3 plasmid template, linearised with SfiI, using the mMessage mMachine (T3 RNA polymerase, Ambion). For the Myc-tagged Xanx-4 expression construct, a PCR cloning approach was used. Primers were designed to contain BamHI restriction sites (U-gccgggatcccatggcagcactc, D- cgccgctagctctacctccctcgg) and the resulting product cloned into pCS3+MT vector (a gift from H. Benisek, Michigan). Myc-tagged Xanx-4 mRNA was synthesised by linearising the Xanx-4/M3T construct with EcoRI and transcribed using SP6 RNA polymerase mMessage mMachine Kit. The Sox17β mRNA and Myc-tagged constructs were a gift from D. Clements (Warwick). Approximately 0.5 ng of mRNA was injected into dejellied embryos at the one-cell stage, alone or in combination with MOs (as specified in the text), under 3% Ficol in BarthX.

Morpholinos
Xanx-4 and Xsox17β (gift from D. Clements) morpholinos (MO) were designed and supplied by GeneTools, LLC (Corvallis, OR):

\[ \text{Xanx-4: } 5'\text{-acctctattcagttccgtgct-3'} \]
\[ \text{Xsox17β: } 5'\text{-cctctacaactcaggtcacttat-3'} \]

The MOs were dissolved in double distilled H₂O to a stock
RT-PCR
Total RNA from whole embryos was isolated and used for RT-PCR as described by Barnett et al. (Barnett et al., 1998). Primers used in this study are as follows.

Xanx-4: U-AGCGGCACGATGAAGATG and D-TCATTCAGGGTGCGTCTCTG (this work)

Xlim-1: U-GAAGGATGACCGTGGTG and D-CAGCCTACGGTGCTCTG (Accession Number, AF179301)

XPlx-2: U-CTCGGAAAGGTGGTGCTAC (this work) and D-GTTATCCATGCAGTCTCATTCTCC (Watta and Sato, 1997)

XPlx-8: U-CCACCGACGATCAGATC (this work) and D-CAATGACACCTGCGAGGTA (Accession Number, AF179301)

Whole-mount in situ hybridisation
Whole-mount in situ hybridisation was carried out as described elsewhere (Harland, 1991). The embryos were fixed in MEMFA (0.5 M MOPS, pH 7.4, 100 mM EGTa, 1 mM MgSO4, 4% formaldehyde) and hybridised with RNA probes produced from cDNA clones. The Xanx-4 antisense probe was transcribed with SP6 RNA polymerase from the full-length Xanx-4 in pGEMT-Easy. The XPlx-8, Xlim-1 and XPlx-7 were kind gifts from T. Carroll (Texas). Probes were synthesised and labelled using a DIG labelling kit (Boehringer) and visualised using anti-DIG-alkaline phosphatase secondary and NBT/BCIP for the colour reaction according to manufacturer’s recommendations (Boehringer).

Immunohistochemistry
Whole-mount immunohistochemistry was performed using standard methods on MEMFA fixed embryos. The primary antibodies used were pronephric tubule-specific monoclonal antibody 3G8 and pronephric duct specific monoclonal antibody 4A6 (Vize et al., 1995), and an anti-annexin IV monoclonal antibody BL7B1, a kind gift from D. Massey-Harroche, Marseille (Massey et al., 1991). The secondary antibodies were alkaline phosphatase-conjugated goat anti-mouse (Sigma) and FITC-conjugated goat anti-mouse (Sigma). BCIP/NBT (Boehringer) or Fast Red TR/Naphol AS/MX (Sigma) was used for the colour reaction, according to manufacturer’s recommendations.

Nuclear staining was carried out using Hoechst stain (33258) at a concentration of 1 μg/ml on acrylamide embedded sections, mounted in glycerol and viewed on Nikon microscope and using a u.v. filter.

Acrylamide embedding and cryostat sectioning
Embryos were fixed in MEMFA, rinsed in phosphate-buffered saline (PBS) and incubated at 4°C for 5 hours in embedding acrylamide (8.4 g acrylamide, 13.4 mg bis-acrylamide, 700 μl TEMED to 100 ml in PBS). The embryos were embedded in acrylamide using 5 μl/10% ammonium persulphate to polymerise overnight at 4°C. The acrylamide blocks were frozen in iso-pentane over liquid nitrogen for 5 minutes. The blocks were then allowed to warm to –20°C and sectioned on a cryostat at 12 μm. The sections were lifted onto 0.1% gelatin subbed slides (300 bloom), fixed in acetone and mounted in 50% PBS/glycerol.

XWnt-4: U-GAGTGAATTGACAGGTC (this work) and D-TACACTTGCCGACAGGTC (Accession Number, U13183)


wT1: U-CAAGGACGGGTCTCTG and D-TGGATGTGTGTGATGACG (Carroll and Vize, 1996)

ODC: U-GAGCTCAAGTGTGGAGA and D-TCAAGTGGCAGTGTGTC (Bassee et al., 1990)

EF1α: U-CAGATGGTGCTCTGAGTATGC and D-CACTGGCTTACCTCTCTGCT (Mohun et al., 1989)

Annexin IV and pronephros development

**Fig. 1.** Amino acid sequence and motif diagram of Xanx-4A. (A) Predicted amino acid sequence of Xanx-4 compared with human (72%), rat (72%), mouse (71%), cow (74%) and medaka (67%). ‘*’ indicate identical amino acids. (B) Xanx-4 motif diagram showing N-terminal PKC phosphorylation site (P), myristoylation site (m) and C-terminal core domain of four annexin repeats (shaded boxes). which contain calcium-binding sites.

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In vitro and in vivo translation of Myc-tagged construct mRNA

mRNA (10 ng) was translated in vitro in the Rabbit Reticulocyte Lysate System (Promega) using the according to manufacturer’s protocol. Reactions (5 μl) were denatured at 95°C in 2x SDS loading buffer (Harlow and Lane, 1988) and subjected to western immunoblot analysis.

For in vivo analysis, 0.5 ng of mRNA was microinjected into one-cell stage embryos, which were then cultured to the appropriate stage. Groups of five embryos were homogenised in 50 μl of homogenisation buffer (0.1 M NaCl, 1% Triton X-100, 1 mM PMSF, 20 mM Tris-Cl pH 7.6) at 4°C and centrifuged in a bench top microcentrifuge at 4°C for 10 minutes at 10,000 g. The cytosolic layer was removed, 5 μl of each sample was denatured at 95°C in 2x SDS loading buffer and subjected to western analysis.

Western analysis

SDS-PAGE was performed on 12% (w/v) resolving gel using a vertical minigel apparatus for 1 hour at 20 mA. The proteins were transferred to nitrocellulose membrane (Amersham) according to Harlow and Lane (Harlow and Lane, 1988) for 2 hours at 350 mA or overnight at 50 mA. After transfer the nitrocellulose was incubated for 1 hour at room temperature in TBS-Tween (0.15 M NaCl, 10 mM Tris-Cl, pH 7.4, 0.1% Tween-20) containing 3% (w/v) powdered milk. The blots were then incubated in 1:1000 anti-Myc monoclonal antibody (gift from D. Stott, Warwick) in TBS-Tween overnight at 4°C. The blots and were washed at room temperature in 0.1% SDS, 2x SSC, twice for 15 minutes at 42°C, and then for 15 minutes at 65°C in 1x SSC, 0.1% SDS. Autoradiographs were obtained by exposure of Fuji super RX films with intensifying screens at ~80°C.

RESULTS

Cloning and sequence analysis of Xenopus laevis annexin IV (Xanx-4)

In order to identify novel molecules involved in the early events of pronephric tube development, we have used a subtractive hybridisation approach. We have generated a subtracted probe based upon the results reported in other experiments (Moriya et al., 1993) and our own data (H. C. Brennan and E. A. J., unpublished). It has been shown that treatment of animal cap ectoderm, dissected from blastula stage Xenopus embryos, with 5 ng/ml activin A and 10⁻⁵ M retinoic acid (RA) results in the formation of pronephric tubules at high frequency, whereas, treatment of animal caps with activin A or RA alone does not. cDNA synthesised from poly A+ mRNA extracted from animal caps treated with RA and activin was therefore subtracted with cDNA synthesised from polyA+ mRNA synthesised from animal caps treated with RA alone pooled with that prepared from activin alone (see Materials and Methods). This allowed the production of a probe enriched for molecules expressed at the time of induction and specification of pronephric tubules. Our laboratory has shown that pronephric tubules are specified at stage 12.5 (Brennan et al., 1998), therefore we used the subtracted probe to screen a Xenopus laevis stage 13 cDNA library in order to identify genes expressed early in pronephric development. K2 was one of the clones isolated in this screen. Sequencing revealed K2 to be a clone of 1131 nucleotides, containing an in-frame coding sequence corresponding to Xenopus laevis annexin IV (Xanx-4) of 963 bases, including a 50 base 5’UTR and a 118 base 3’UTR (Accession Number, AY039235). Conceptual translation of Xanx-4 yielded a
predicted amino acid sequence of 321 amino acids that displays identity with annexin IV in other vertebrate species of between 67-74% (Fig. 1A).

Analysis of the predicted amino acid sequence (Prosite) indicated that Xanx-4 contains the archetypal conserved annexin motifs, the four-fold repeating containing calcium and phospholipid binding sites, myristoylation sites, N-glycosylation sites, and a PKC phosphorylation site (Thr6) (Fig. 1B).

**Temporal expression of Xanx-4**

The temporal expression profile of Xanx-4 transcripts was revealed by RT-PCR (Fig. 2). Maternal expression of Xanx-4 was detected in the egg but subsequently declined rapidly and was substantially reduced by the 32-cell stage. Zygotic expression was detected at a very low level between stages 9-12.5 and at a significantly increased level at stage 13. This coincides with the time of pronephric tubule specification (Brennan et al., 1998). Expression then continues at a similar level up to and beyond the stage when the pronephros becomes functional (stage 37). ODC was used as a loading control.

**Expression of Xanx-4 transcripts and protein is restricted to the pronephric tubules in embryos and to specific organs in the adult**

Whole-mount in situ hybridisation using a DIG labelled Xanx-4 antisense probe in albino embryos detected transcripts in the pronephric anlagen from stage 26 onwards and more specifically in pronephric tubules as they continue to develop through to stage 42 (Fig. 3A,B, arrows). Low levels of Xanx-4 transcript were also detected in the otic vesicle at stage 26 (data not shown). A sense Xanx-4 control probe showed no staining pattern in embryos at any stage (data not shown).

The monoclonal anti-annexin IV antibody BL7B1, raised against rabbit annexin IV, was used in whole-mount immunohistochemistry to establish the protein distribution of Xanx-4. This antibody has been shown to react specifically with rabbit annexin IV (Massey et al., 1991), and crossreacts with the Xanx-4 protein. The staining observed indicates that Xanx-4 protein is specifically localised to the pronephric tubules of *Xenopus laevis* embryos from stage 38 onwards (Fig. 4A). Anti-annexin IV staining of transverse sections of stage 40 embryos reveals Xanx-4 is located on the luminal side of the tubule and is confined to the apical membrane of the pronephric tubule epithelium (Fig. 4B).

Northern blot analysis of mRNAs isolated from dissected adult organs was carried out (Fig. 5). Transcripts of approximately 1.6 kb were detected at high levels in the gall bladder and intestine, at lower levels in tadpole, lung, kidney, ovary, testis, stomach, bladder, spleen and pancreas. No transcripts were detected in neural tissues, heart, liver, muscle or skin. All the organs in which transcripts were detected contain significant quantities of polarised epithelial tissue, thus correlating with the observed polarised expression of the Xanx-4 protein.

**Perturbation of the expression of Xanx-4 using morpholino technology**

Recently, morpholino technology has been used to knock out endogenous gene expression in *Xenopus* and other organisms by blocking translation of specific genes (Heasmann et al., 2000; Nasevicius and Ekker, 2000; Yang et al., 2001). An MO complementary to Xanx-4 was designed and tested in vitro and in vivo for its ability to specifically perturb Xanx-4 translation. In order to control for the effects of the MO, a number of antiannexin IV antibodies were tested by western blotting and immunoprecipitation for their ability to detect Xanx-4. Unfortunately, none of these reagents was able to detect Xanx-4 by western analysis, although the antibodies did perform in immunohistochemistry. Therefore, a Xanx-4-Myc-tagged expression construct was prepared (see Materials and Methods). Myc-tagged Xanx-4 mRNA was synthesised and used in the following assays to demonstrate the effectiveness and specificity of the MO.

**Xanx-4 MO depletion of the translation of Xanx-4 mRNA in vitro**

Initially the ability of the Xanx-4 MO to deplete Xanx-4 translation was established in vitro. Myc-tagged *Sox17β* mRNA (a kind gift from D. Clements, Warwick) was used as a control to test for the Xanx-4 MO specificity. A combination of 10 ng of Myc-tagged *Sox17β* mRNA and 10 ng of Myc-tagged Xanx-4 mRNA was incubated either alone
or in combination with 1 µg, 5 µg or 10 µg of Xanx-4 MO in the reticulocyte lysate system. The lysates were subjected to SDS-PAGE and western blotting using anti-Myc antibody (Fig. 6A). Both Myc-tagged Sox17β mRNA and Myc-tagged Xanx-4 mRNA were successfully translated at similar levels (lanes 1, 3 and 5) but when incubated with 1 µg, 5 µg or 10 µg Xanx-4 MO, Xanx-4 translation was blocked, whereas Sox17β translation was not (lanes 2, 4 and 6). In a similar experiment, the Xanx-4 MO was also shown to have no effect on the translation of XEZ (Barnett et al., 2001) mRNA (data not shown). This shows that the Xanx-4 MO preferentially depletes Xanx-4 mRNA in vitro.

Xanx-4 MO depletion of the translation of Xanx-4 mRNA in vivo

Embryos were injected at the one-cell stage with 0.5 ng Myc-tagged Xanx-4 mRNA alone or in combination with Xanx-4 MO and cultured to stage 9, 13 or 21. The embryos were homogenised, fractionated and the cytosolic fraction subjected to SDS-PAGE and western blotting using anti-Myc antibody. As specificity controls, 0.5 ng Myc-tagged Sox17β mRNA was also injected alone or in combination with Sox17β MO or Xanx-4 MO. The results (Fig. 6B) show that Myc-tagged Xanx-4 is translated in vivo (lane 1) and that its translation is blocked by 5 ng (lane 2), 10 ng (lane 3) or 20 ng (lane 4) Xanx-4 MO at all stages examined (stage 9, 13 and 21). For the specificity control, injected Myc-tagged Sox17β alone is translated (lane 6) and translation is depleted by Sox17β MO (lane 7). However, the expression of Myc-tagged Sox17β is not affected by the Xanx-4 MO (lane 8). Uninjected, control embryos do not react with the anti-Myc antibody at any of the stages tested (lane 5). Therefore, we show that the Xanx-4 MO preferentially depletes Myc-tagged Xanx-4 mRNA in vivo. Myc-tagged Sox17β mRNA injected embryos could not be analysed at later stages, owing to lethality at late gastrula/early neurula stages of this level of message.

Xanx-4 depletion using Xanx-4 MO produces pronephric tubules with an enlarged diameter

In order to examine the activity of Xanx-4 in vivo, we have used overexpression and depletion to perturb endogenous expression of Xanx-4. Embryos were injected at the one-cell stage with 10 ng control MO, 10 ng Xanx-4 MO or 0.5 ng Xanx-4 mRNA. The injected embryos were cultured to stage 40 and whole-mount antibody stained with 3G8 and 4A6, which are tubule- and duct-specific monoclonal antibodies, respectively. Although no obvious effect on pronephric tubule or duct morphology was observed after overexpression of Xanx-4 mRNA or on injection of control MO (two experiments, counting each pronephros individually, total number of animals scored n=116, n=102), a clear effect on tubule morphology was observed after Xanx-4 MO injection. On whole-mount inspection, the normal coiled tubule and duct morphology was observed after overexpression of Xanx-4 mRNA or on injection of control MO (two experiments, counting each pronephros individually, total number of animals scored n=116, n=102), a clear effect on tubule morphology was observed after Xanx-4 MO injection. On whole-mount inspection, the normal coiled tubule and duct morphology was observed after overexpression of Xanx-4 mRNA or on injection of control MO (two experiments, counting each pronephros individually, total number of animals scored n=116, n=102), a clear effect on tubule morphology was observed after Xanx-4 MO injection.
two independent experiments; Fig. 7). In some embryos, the tubules were reduced in overall size and in some cases missing completely (8/56, 12/60 in the same independent experiments). In order to investigate the phenotype, a random sample of six immunostained embryos from each treatment group were acrylamide embedded, frozen and cryostat-sectioned (12 μm). The slides were counterstained with Hoechst, inspected under light and u.v. illumination. (A) A schematic representation of B showing pronephric tubule sections illustrating true transverse sections, which were scored (filled) and partial longitudinal sections which were not counted (unfilled). (C,D) Normal uninjected control embryo. (E,F) Embryo injected with 10 ng Xanx-4 MO showing enlarged pronephric tubule phenotype. (G,H) embryo injected with 0.5 ng Xanx-4 mRNA and 10 ng Xanx-4 MO showing partial rescue of pronephric tubule phenotype. C,E,G are viewed under UV illumination to identify Hoechst nuclei staining. B,D,F,H are viewed under partial white light and u.v. illumination to identify 3G8 pronephric tubule staining and Hoechst nuclei staining.

In order to quantify the extent of the MO phenotype, complete serial sections of the pronephroi from each of the six samples for each treatment were inspected. Approximately 24 sections were counted from each pronephros scored. Tubules were positively identified by the light microscopic identification of 3G8-positive immunostain, and nuclei counted under u.v. illumination to identify Hoechst staining (Fig. 8A,B). Owing to the coiled nature of the pronephric tubules, many of the tubule sections could be seen as elongated or oval shaped cross-sections. An example of a section with true transverse (counted) and a partial longitudinal section (not counted) is shown in Fig. 8A,B. Any section of a pronephric tubule considered not to be a true transverse section on the basis of such cross-sectional shape was discounted. All cells of each sectioned pronephric tubule, on both sides of the embryo and in each section were counted. The mean cell count, together with the standard deviation for each treatment was calculated. The numbers of cells contributing to normal control tubules were remarkably consistent, averaging nine cells and with a range of 6-15 cells. Inspection of the sections of the Xanx-4 MO-injected embryos for the number of cells making up a tubule, however, revealed that the pronephric tubules had an enlarged lumen, but appeared normal in all other respects. The cells of the tubules retained normal cell integrity and shape, the organisation of the tubule epithelium was one cell in diameter, as in normal controls. The average number of cells contributing to the tubule, however, was 25 and the range was 6-48. The wide range observed reflects the observation that the tubule width at the start and the finish of each tubule domain was of normal size. This represents a significant difference from either uninjected control values or from control MO-injected values. Injection of either Xanx-4 mRNA or a control MO had no effect on the phenotype of the tubules. Representative results are presented in Fig. 8C-F and numerical data shown in Fig. 9A. Duct staining was carried out using specific monoclonal antibody 4A6 (Vize et al., 1995): no effect on duct morphology was observed.

**Xanx-4 depleted phenotype can be rescued by co-injection with Xanx-4 mRNA**

We have used Xanx-4 mRNA co-injected with Xanx-4 MO, in order to rescue the phenotype caused by injection of the Xanx-4 MO. Embryos were injected at the one-cell stage with 0.5 ng Xanx-4 mRNA alone or in combination with 5 ng, 10 ng or 20 ng of Xanx-4 MO. Equal volumes were injected into all embryos. The embryos were cultured to stage 40 and whole-mount antibody immunostained with tubule-specific 3G8. Embryos injected with Xanx-4 MO were compared with the co-injected and to the uninjected controls. The embryos were examined initially as whole-mount specimens (data not shown).

As previously described, pronephric tubules of the embryos injected with the Xanx-4 MO appeared in general to be wider, shorter and less coiled than those of the normal control embryos. It was also observed that the most severe phenotype occurred in those embryos injected with the higher concentration of MO. Apart from the aberrant tubule phenotype, the embryos appeared to be of normal morphology. Those embryos co-injected with 5 ng Xanx-4 MO and Xanx-4 mRNA appeared almost completely rescued and had almost normal tubules (Fig. 8, compare C-F with G,H). The 10 ng and 20 ng Xanx-4 MO embryos co-injected with Xanx-4 mRNA showed significant, although incomplete, rescue. The tubules appeared somewhat less normal and displaying some larger and less coiled tubules. Seemingly the rescue was not complete at the higher concentrations of MO. Other than the pronephric tubule phenotype described above, the gross phenotype of the embryos in all groups appeared normal.

In order to quantify this phenotype, a random sample of six
with 10 ng of MO were rescued to a 5 ng MO phenotype (mean=12, range=6-24) and 20 ng was rescued to an intermediate phenotype between 10 ng and 20 ng. The data collected from the serial sections of each of six randomly chosen embryos from each group is shown as a graphical representation in Fig. 9B. Similar results were obtained from a repeat experiment (data not shown).

Analysis of the expression of pronephric molecular markers in Xanx-4 over-expression and Xanx-4 MO treated embryos

In order to assess whether either the observed phenotype or other unidentified earlier events was related to other genes known to be expressed in the pronephros, the following experiments were carried out. Both semi-quantitative RT-PCR and in situ hybridisation were performed for a selection of genes whose mRNAs have been shown to play an early role in kidney development. Embryos were injected at the one-cell stage with 0.5 ng Xanx-4 mRNA or 10 ng Xanx-4 MO, cultured to stage 25. Groups of five embryos (in duplicate) were analysed by RT-PCR (Barnett et al., 1998) using primers designed against a selection of genes known to be expressed in the pronephros (see Materials and Methods). EF1α is used as a loading control. No effect on expression of any of the marker genes was observed (Fig. 10). Embryos injected with 0.5 ng Xanx-4 mRNA or 10 ng Xanx-4 MO were cultured to stages 26-28 and subjected to in situ hybridisation using RNA probes prepared from cDNA clones of pronephric marker genes XPax-8, Xlim-1 and xWT1. The expression pattern of each marker gene RNA in the embryos injected with Xanx-4 mRNA and Xanx-4 MO1 were compared with uninjected control embryos (Fig. 11). No apparent change in XPax-8 or Xlim-1 expression pattern was observed in either group with perturbed Xanx-4 expression, compared with uninjected control. However, a reduced field of xWT1 expression was observed in 27% (n=12/44) of the embryos that overexpressed Xanx-4, compared with controls. This apparent reduction may have been due to a more dispersed xWT1 expression domain, as no detectable reduction in xWT1 expression was observed by RT-PCR. No altered xWT1 field of expression was seen in the embryos injected with Xanx-4 MO1. It seems perturbation of expression of Xanx-4 is not linked to any of the tubule markers analysed. We also assume from these results that the Xanx-4 depletion phenotype is not associated with more or less tissue being specified to become pronephric tubule in character.

DISCUSSION

The aim of this work was to use a subtractive hybridisation strategy to identify novel molecules involved in the early events of pronephric tubule development in Xenopus laevis larval kidney. We report the cloning and characterisation of the Xenopus orthologue of annexin IV (Xanx-4), a pronephric tubule specific gene with an important role in the morphogenesis of the pronephric tubules in Xenopus laevis.

Cloning of Xanx-4

A gradient of the TGFβ superfamily member activin A will induce different kinds of mesodermal tissues in dissociated cells (Green and Smith, 1990; Green and Smith, 1991; Green
pronephric tubules with high frequency, our approach of preparing a subtracted probe allowed the elimination of molecules that were upregulated by RA or activin alone. Thus, we have created a probe specific for molecules upregulated only by the combination of activin and retinoic acid, which would include those expressed at the time of induction and specification of pronephric tubules. In our study, we chose to screen an early *Xenopus* cDNA library, stage 13, based on the work carried out in our laboratory that demonstrated that the tubules were specified at stage 12.5 (Brennan et al., 1998).

In choosing to screen an early library, we were able to select for genes not only specific for pronephric tubule specification, but also genes that are involved in early events of pronephric differentiation, patterning and development.

The zygotic upregulation of *Xanx-4* occurs at stage 12.5-13. RT-PCR carried out on dissected stage 13 embryos (dorsal, lateral and ventral domains) revealed that *Xanx-4* is expressed in the lateral domain at this stage (data not shown). *Xanx-4* expression is maintained through all the stages of pronephric tubule development to and beyond the time when the pronephros becomes functional. This implies an important and specific role for *Xanx-4* not only in the development, but also perhaps in the function of the pronephros. In this work, we have described the highly restricted distribution of both *Xanx-4* mRNA and protein during embryogenesis. The expression patterns of *Xenopus annexin II* and *annexin VII* are less restricted, being expressed in various neural and mesodermal tissues (Izant and Bryson, 1991; Srivastava et al., 1996).

We have shown by northern blot analysis that the expression pattern of *Xanx-4* in the adult frog is restricted to epithelial tissues. In agreement with this result, *annexin IV* displays polarised expression in adult epithelial tissues of other animal species: lungs (Sohma et al., 1995), intestines and pancreas (Massey et al., 1991), liver (Boustead et al., 1993) and kidney (Massey-Harroche et al., 1995; Kojima et al., 1994). It appears that the common theme for localisation of *annexin IV* expression in adult tissues is that of polarised epithelial tissue.

### The effects of altered expression of *Xanx-4*

We have shown that *Xanx-4* is expressed at the right time in the right place to have a functional role in pronephric tubule development. To investigate this, we have perturbed the expression of *Xanx-4* in *Xenopus* tadpoles and identified a clear phenotype on pronephric tubule morphology in whole-mount and in section.

Depletion of *Xanx-4* protein by

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**Fig. 10.** Embryos were injected at the one-cell stage with 0.5 ng *Xanx-4* mRNA or 10 ng *Xanx-4* MO and cultured to stage 25. Groups of five embryos were then subjected to RT-PCR using primers designed against a range of pronephric molecular markers. No effect on expression of any of the markers was observed. *EF1α* was used as a loading control.

**Fig. 11.** Analysis of the effects of *Xanx-4* depletion or overexpression on the expression of pronephric marker genes by in situ hybridisation. Embryos were injected at the one-cell stage with 0.5 ng *Xanx-4* mRNA or 10 ng *Xanx-4* MO and cultured to stage 27. The embryos were then subjected to in situ hybridisation using specific probes prepared from pronephric molecular markers *Xlim-1* (A,D,G), *XPax-8* (B,E,H) and *xWT1* (C,F,I). No effect on expression of *XPax-8* or *Xlim-1* was observed; however, a reduced field of *xWT1* expression was observed in the embryos injected with *Xanx-4* mRNA (compare C with F).
MO causes an enlarged, shortened and uncoiled pronephric tubule phenotype. The effect of Xanx-4 MO can be rescued by co-injection of Xanx-4 mRNA. Longevity of the MO is suggested to be considerable (Summerton and Weller, 1997; Nutt et al., 2001), and may be still acting at much later times than that of the ectopic mRNA. The rescue of the MO phenotype observed may be due to action of the Xanx-4 mRNA antagonising the action of the Xanx-4 MO on co-injection. We have shown that 0.5 ng of injected message will completely rescue the tubule phenotype seen in 5 ng Xanx-4 MO embryos, whereas at higher concentrations of Xanx-4 MO, the phenotype is only partially rescued. Interestingly Xanx-4 mRNA injected alone does not produce a phenotype. This enables a rescue experiment to be performed that will return the MO phenotype to the normal range, rather than to producing an overexpression phenotype. The lack of overexpression phenotype may be due to the relatively late requirement of Xanx-4 in kidney morphogenesis and the inability of injected mRNA to survive to this point. We are currently investigating the potential of transgenics to overcome this problem.

The pronephric tubules of the Xanx-4 MO-injected embryos, although enlarged in diameter, were constructed and organised in the normal way. The tubules consisted of a lumen, albeit enlarged, and the walls were constructed of one epithelial cell layer, though they contained more cells in their circumference. The pronephroi of Xanx-4-depleted embryos consisted of the normal complement of pronephric components, including capsule, nephrostomes, tubules and duct, as shown by the expression of marker genes (Fig. 11). It appears that the pronephroi were functional, maintaining body water and electrolyte homeostasis, as none of the components appeared cystic and no general oedema was observed. Recent work (Drummond et al., 1998) has isolated 18 independent recessive mutations that affect pronephric development from a large scale ENU mutagenesis screen (Driever et al., 1996). A common theme for the phenotypes of these mutants was the appearance of fluid-filled cysts in the pronephric region followed by general oedema. The authors suggest that the phenotypes observed are the consequence of pronephric failure and altered osmoregulation. Histological analysis of the double bubble mutant showed that the glomerulus was loose and distorted, swelling was apparent, and the cells appeared flattened. The glomerulus overall architecture was disorganised and the basement membrane was severely distorted (Drummond et al., 1998). No such tissue disorganisation, cell shape distortion or fluid filled cysts were observed in any of the Xanx-4-depleted embryos.

In counting all cells in serial sections, we have shown that the number of cells making up the circumference of the pronephric tubules in normal controls and Xanx-4 MO-injected embryos differs. A transverse section of the tubules does not, in general, provide perfect circles. By using cell counting as a measure of the phenotype observed, we have taken a measure of the size of tubule sections, thereby removing errors that could complicate the analysis and introduce errors.

The pronephric tubule markers tested were not affected by perturbation of Xanx-4 expression. We suggest that the phenotype observed is not due to a change in the amount of pronephric tubule tissue specified but is due to an alteration in the morphological process during tubulogenesis. Preliminary studies of the pronephric tubule morphology of Xanx-4 MO-depleted embryos at earlier stages (35-36) revealed no aberrant tubule phenotype (data not shown). It appears that the enlarged tubule phenotype, caused by depletion of Xanx-4, may only manifest itself at later stages of tubulogenesis during tubule maturation and elongation.

Previous studies have shown that co-expression of either XPax-2 or XPax-8 with Xlim-1 results in a synergistic effect producing increased pronephric tubule complexity, enlarged tubules and ectopic tubules, while expression of either XPax-2 or XPax-8 alone has a moderate effect (Carroll and Vize, 1999). We did not see complex or ectopic tubules under any of the conditions tested. We have also shown that perturbation of Xanx-4 expression does not affect the expression of either XPax-8 or Xlim-1. Ectopic expression of xWT1, which is required for metanephric development in vivo, inhibits pronephric tubule development, probably by repressing tubule specific gene expression in the region of the pronephros fated to become tubules (Wallingford et al., 1999). Depletion of Xanx-4 does not effect the expression of xWT1. The effect of Xanx-4 overexpression on xWT1 was unexpected, as no Xanx-4 overexpression phenotype was previously observed. The fact that RT-PCR analysis gave similar levels of xWT1 expression in controls and Xanx-4 overexpressing embryos suggests that the effect of Xanx-4 overexpression results in more diffuse expression of xWT1. This is consistent with the results observed in the xWT1 in situ hybridisation of embryos overexpressing Xanx-4, where in some embryos the xWT1 expression domain was more dispersed. These results place Xanx-4 downstream of Xlim-1/XPax-2/8 and xWT1 in a molecular pathway of tubulogenesis. Further experiments will be required to confirm the relative positions of these pronephric genes.

It is thought that annexins participate in calcium homeostasis, regulation of ion channel activities and membrane traffic events. Annexins have been well characterised with regard to their binding activities. They have been shown to bind various proteins, including proteoglycans, F-actin and collagen, in addition to calcium and phospholipids (Lecat and Lafont, 1999; Seaton and Dedman, 1998). Annexins have been shown to bind phospholipid membranes in ordered arrays, and it has been suggested that annexin complexes may function to modify membrane structure (Oling et al., 1999). A role for annexin IV in epithelial membrane integrity, adhesion or plasticity could account for the aberrant tubule phenotype observed. Depletion of Xanx-4 in the pronephric tubules may cause a remodelling of tubule morphology during development. Annexin IV has been shown to be involved in calcium-activated cellular signal transduction events (Raynal et al., 1996), exocytosis (Sohma et al., 2001) and in the regulation of calcium-activated epithelial chloride ion channel activity (Kaetzel et al., 1994; Chan et al., 1994; Jorgensen et al., 1997). The primary function of the pronephric tubules is the transport of ions, water and other molecules, and as the site of Xanx-4 localisation (apical) in the pronephric tubules is in agreement with a possible role in these events, further studies will be directed at ascertaining its function directly. Although, as previously discussed, a role in osmoregulation seems unlikely, the enlarged tubule phenotype observed could well be attributed to incorrect modulation of exocytosis, ion channel activity or other calcium signalling events. Direct investigations of these processes in MO-treated embryos will
form the basis of future work. This study represents a study of the role of Xanx-4 in the formation of pronephric tubules in Xenopus laevis.

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