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*Short Contribution*

# The cytoskeletal effector xPAK1 is expressed during both ear and lateral line development in *Xenopus*

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**ABSTRACT xPAK1, a probable effector of stress activated MAP-kinase SAPK1/JNK activation and cytoskeletal dynamics, was found to be ubiquitously expressed within the *Xenopus laevis* ear and lateral line system during the development and differentiation of these organs. xPAK1 expression was very strong in the otic placode from its condensation, and expression continued in the otic vesicle up until stage 35/36, after which it abruptly ceased. At stage 29/30 expression occurred specifically in the epithelium of the otic vesicle, which includes the prospective sensorial epithelium. Expression of xPAK1 was also observed in the lateral line system from stage 35/36, at which stage the lateral line primordia have begun to migrate from the region of the otic vesicle. Lateral line expression continued at least until stage 37/38, at which time xPAK1 was noted in association with the differentiating lateral line organs. To our knowledge, xPAK1 is the first ubiquitous lateral line marker that is also expressed in the ear. In the context of previous studies, our data suggest that xPAK1 either plays a role in the differentiation of the mechano-sensors of the auditory system or in the formation of the otic vesicle epithelium and the lateral line primordia.**

KEY WORDS: *Xenopus, ear, lateral-line, xPAK1*

The development of the auditory system in *Xenopus* is a subject of considerable interest, presenting as it does a complex patterning of canals and chambers, the differentiation of mechano-receptors and innervation by the eighth cranial nerve (Nieuwkoop and Faber, 1967; Fritzsch, 1996). The otic placode, the ear primordium, condenses in *Xenopus* by stage 21 and develops into the closed otic vesicle by stage 27. Both auditory and vestibular mechanisms then develop quite rapidly and are probably functional by stage 45. It is not certain at what stage the hair cells, the mechano-receptors or neuromasts of the ear, begin to differentiate but at least some are fully differentiated at stage 45 (Díaz *et al.*, 1995). The lateral line organs are a complex system of epidermal mechano-receptors present in amphibia and fish. They are phylogenically related to the hearing and vestibular apparatus, and consist of small groups of neuromast cells, each very similar to the hair cells of the ear (Nieuwkoop and Faber, 1967; Winklbauer, 1989). In *Xenopus,* each group of neuromasts develops at the base of an epidermal pit. Though molecular markers of ear development have been identified, to our knowledge, only one, Tor70, is known to be expressed both during ear development and during development of the related lateral line organs (Bolce *et al.*, 1992). Tor70 is, however, not expressed in all lateral lines. Thus, none of the known markers to date have a strong potential of encoding common factors in sensorial epithelium or neuromast differentiation.

We have found that the developmentally regulated expression of xPAK1, is associated with ear and lateral line development in *Xenopus*. The p21-activated kinases (PAKs) were one of the first targets of the activated GTPases Rac and Cdc42 to be identified (Manser *et al.*, 1994; Daniels and Bokoch, 1999; Manser and Lim, 1999). PAK is a fairly close relative of the sterile 20 (Ste20p) kinase of yeast implicated in MAP-kinase activation, cytoskeletal reorganization and cell cycle arrest, and is a more distant relative of the *Drosophila* kinase MSN implicated in planar polarity.

### *xPAK1 is expressed throughout early embryogenesis and in the adult*

The sequence of the xPAK1 protein (xPAK1p) deduced from a cDNA identified using the RT-PCR fragment Xltk24 (Islam *et al.*, 1994) differed at only six amino acids (and the cDNA sequence differed at 26 positions) from a clone previously identified (Genbank AF000239) (Faure *et al.*, 1997) (Fig. 1A). Since both xPAK1 cDNAs showed essentially identical 5' and 3' flanking sequences they probably represent the same gene. Alignment with the mammalian PAKs suggested that xPAK1 was most closely related to rPAKα and hPAK1. xPAK1 structure is summarized diagrammatically in Figure 1B. xPAK1 was found to be expressed as a maternal message whose concentration reduced until well past the mid-blastula tran-

*Abbreviations used in this paper:* PAK, p21 activated kinase; MAP-kinase, Mitogen activated protein kinase; SAPK, Stress activated protein kinase; JNK, jun N-terminal kinase.

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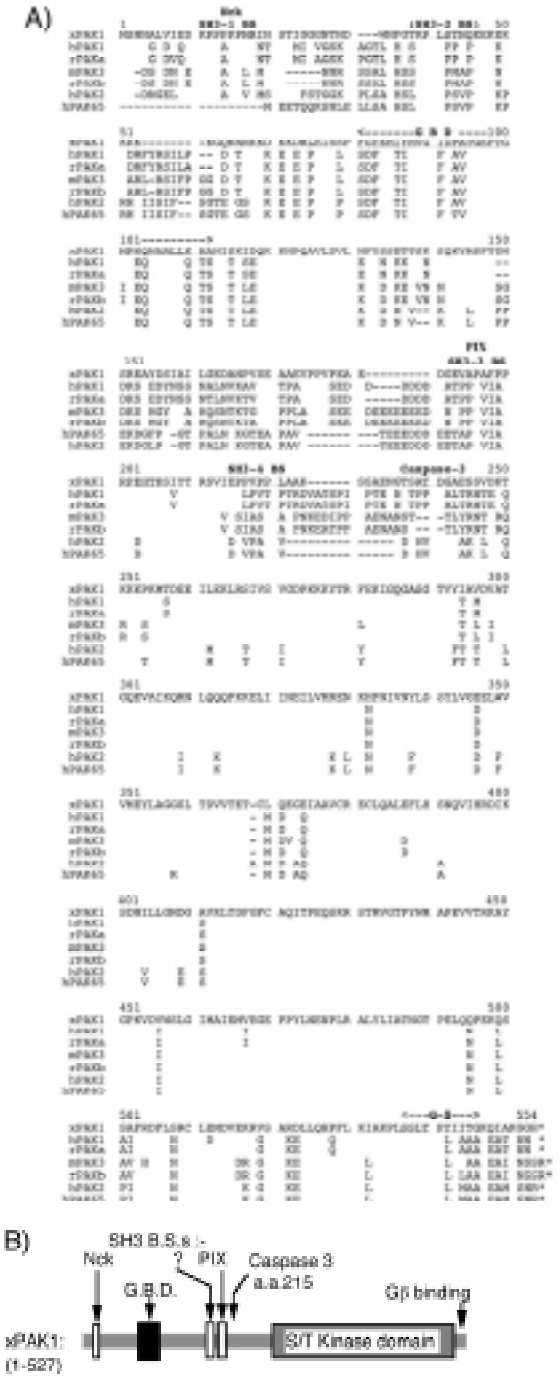


**Fig. 1. Structure of xPAK1 protein (Genbank AF169794). (A)** *The sequence of xPAK1p is shown aligned with those of human PAK (hPAK) -1 (Genbank U24152), -2 (Genbank U24153) and -65, (Genbank U25975), rat PAK (rPAK) -α (Genbank U23443) and -β (Genbank U33314), mouse PAK3 (mPAK3) (Genbank U39738). Only amino acids that differ from those of xPAK1 are shown for the other species.* **(B)** *Schematic structure of xPAK1. In both A and B the positions of known or potential SH3-domain binding sites (SH3 BS) are shown (underlined), as are the p21ras-like GTPase Binding Domain (GBD), caspase 3 cleavage site and the potential G-protein-β (Gβ) recognition peptide. The sequence shown in (A) differs at six a.a. positions from the previously identified xPAK1 sequence Genbank AF000239 (Faure et al., 1997), but both cDNAs show essentially identical 5' and 3' flanking sequences, suggesting that, in fact, they represent the same gene.*

sition (Fig. 2A,B). mRNA concentration was very low at the early gastrula, stage 10 (Nieuwkoop and Faber, 1967), but picked up around neural plate stage, stage 14, and continued to increase at least as far as stages 40 to 45. Present maternally, xPAK1p protein maintained a roughly constant concentration until mid-neurula, stage 17, (Fig. 2C). At stages 21 and 25 xPAK1p was present at a low concentration, but by stage 36 its concentration had increased to well above the maternal level. In the adult, significant levels of both message and protein were present in the spleen, brain and stomach and lower levels were found in the pancreas and stomach (mRNA was not tested) (Fig. 2D,E).

### *Expression in the otic placode and otic vesicle epithelium*

The most striking site of early xPAK1 expression was the developing ear. The otic placode is first defined after neural tube closure at stages 20/21 (Nieuwkoop and Faber, 1967), forms a closed vesicle by stage 27 and by stage 29/30 the otic vesicle is separated from the overlying epidermis. From about stage 22 (Fig. 3A) to at least as far as stage 35/36 (Fig. 4A), xPAK1 mRNA was abundant within the otic placode and later in the otic vesicle. By stage 37/38, however, expression in the ear was significantly attenuated (Fig. 4B). Sectioning of embryos at stage 29/30 showed that xPAK expression occurred only within the epithelium of the otic vesicle. This cell layer includes the sensorial epithelium, which gives rise to the hair cells of the ear. Though the exact timing of hair cell differentiation in *Xenopus* is not known, it probably starts around stage 29 and at least some hair cells are functional by stage 45 (Nieuwkoop and Faber, 1967).

xPAK1 has been associated with apoptosis in *Xenopus* oocytes (Faure *et al.*, 1997). However, in agreement with an earlier study (Hensey and Gautier, 1998), we detected no apoptotic events associated with the development of the otic vesicle (e.g. Fig. 3I,J). Thus, it would not appear that the opening of the otic vesicle occurs by programmed cell death. The function of xPAK1p in the ear would therefore seem to be related either to the formation of the sensorial

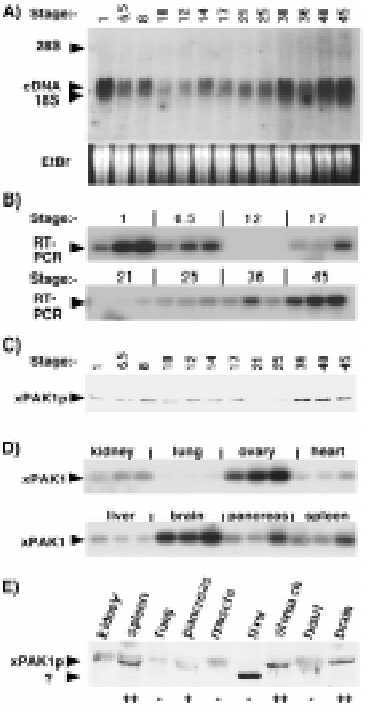


**Fig. 2. Expression of xPAK1 mRNA and protein. (A)** *Northern blot of xPAK1 mRNA during early development. The positions of 18S and 28S rRNAs are indicated and the ethidium bromide stained 18S band shown below the Northern blot.* **(B)** *Quantitative RT-PCR of xPAK1 mRNA from embryos confirms the quantitation by Northern blot and in (D) shows the relative expression of mRNA in isolated adult tissues. For a given RNA source three gel tracks are shown corresponding to PCR reactions with 2 fold increasing aliquots of reverse transcribed RNA. Western analyses of endogenous xPAK1 protein showing its relative abundance* **(C)** *during early development and* **(D)** *in isolated adult tissues. In (D) the relative abundance of xPAK1p is indicated as "-", "+", etc, and "?" indicates an unidentified peptide present in liver.*

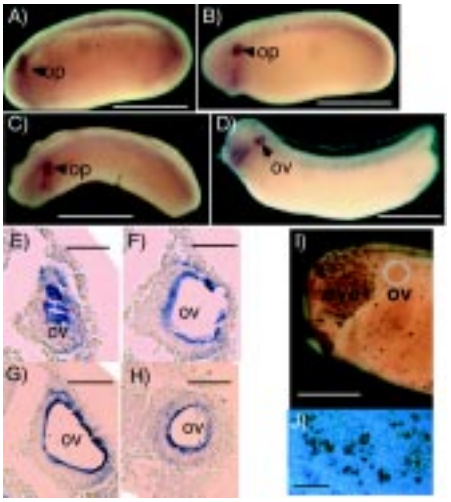


**Fig. 3. Expression of xPAK1 in the otic placode and vesicle.** *Lateral views of whole-mount in situ hybridization of xPAK1 transcripts in X. laevis embryos at* **(A)** *stage 23/24,* **(B)** *stage 25,* **(C)** *stage 26, dorso-lateral view,* **(D)** *stage 29.* **(G-H)** *Sequential sections of a xPAK1 hybridized stage 29/30.* **(I)** *Whole-mount TUNEL staining [(Hensey and Gautier, 1998) as modified by Poitras et al. manuscript submitted] of anterior region of a stage 27 embryo.* **(J)** *Magnified view of TUNEL staining over the eye of a stage 27 embryo showing colocalization of stain with cell nuclei, as revealed by Hoechst 33258 fluorescence. Op, otic placode; ov, otic vesicle. Bar in A to D, 1 mm; in E to H, 50 μm; in I 0.5 mm; and in J 100 μm.*

epithelium, in part a polar epithelium of hair cells (Díaz *et al.*, 1995), or to the differentiation of the hair cells themselves.

### *Expression in the extending and differentiating lateral line system*

Already by stage 35/36, expression of xPAK1 was observed in the elongating lateral line system (Fig. 4A,B), temporally overlapping expression in the ear. The lateral line organs form from primordia which migrate from the region of the ear starting at about stage 33/34 (Nieuwkoop and Faber, 1967). After migration, the primordial cells differentiate into lateral line placodes which along with cells of neural crest origin become the lateral line organs (Collazo *et al.*, 1994). Each lateral line organ consists of small groups of neuromast cells located within pits. xPAK1 was observed to be associated with both the progression of the lateral line primordia and the differentiation of the lateral line organs. Figure 4A shows that xPAK was present in the extending infra- and supra-orbital and dorsal lines at stage 35/36. By stage 37/38, expression the dorsal line had extended caudally, such that the left and right lines almost met dorsally (Fig. 4B,C). Strongest xPAK1 expression was associated with the advancing ends of the dorsal lines. At stage 37/38, expression was also seen in the hyomandibular, ventral and aortic lines (Fig. 4D), and the lateral line organs were visibly forming in the infra- and supra-orbital and the dorsal lines. xPAK1 lateral line staining was restricted to a slightly thickened region of the epidermis, see examples of sections showing infraorbital and dorsal line staining (Fig. 4F-H). Staining in the infra-orbital line (Fig. 4D,E) broke up into distinct cell groups typical of the lateral line organs, each of which are known to contain a small number of mechano-receptor cells (Winklbauer, 1989). Similar observations of xPAK expression during organ formation were made for the dorsal and supraorbital lateral lines, data not shown. (Expression of xPAK1 was also noted in the tail [Fig. 4B], and in the olfactory placode [Fig. 4A-D] during development of the sensorial epithelium of this organ).

### *Possible functions for xPAK1*

xPAK1 provides probably the first developmental marker of the ear which is also expressed ubiquitously during lateral line development. Since this expression also occurs during the process of hair cell and neuromast differentiation, it suggests a role for xPAK1 in the process of differentiation of these mechano-receptors. Both the hair cells and neuromasts develop actin-based stereocilia which rely on specialized myosins for both their development and function (Gillespie and Corey, 1997). PAK1 and xPAK1 have been shown to directly phosphorylate lower eukaryotic myosin I and vertebrate cytoplasmic myosin II *in vitro,* and xPAK1 has also been shown to do so *in vivo* (Tuazon and Traugh, 1984; Wu *et al.*, 1996; Brzeska *et al.*, 1997; Ramos *et al.*, 1997, and Islam *et al.* submitted). Thus, a possible function for xPAK1p lies in the development of the actinomyosin based stereocilia of the neuromast cells. On the other hand, xPAK1 expression appears to be rather evenly distributed over the epithelium of the otic vesicle and it is very probable that not all this surface will generate hair cells. Further, the potential function of the PAK proteins as effectors of the Stress Activated MAP-kinase (SAPK1/JNK) pathway suggests they may fulfill a more general role in the formation of epithelia and possibly polar epithelia, (Noselli and Agnès, 1999), of which the sensorial epithelium of the ear is an example (e.g. see Díaz *et al.*, 1995). The function of xPAK1 in the lateral lines could yet further be related to the migration of the lateral line primordia. Cell migration and the formation of polar epithelia in *Drosophila*, in fact, share common components and both show a dependence on SAPK1/JNK activation, (Noselli and Agnès, 1999). Thus, the role of xPAK1 may rather lay in the formation of a polar

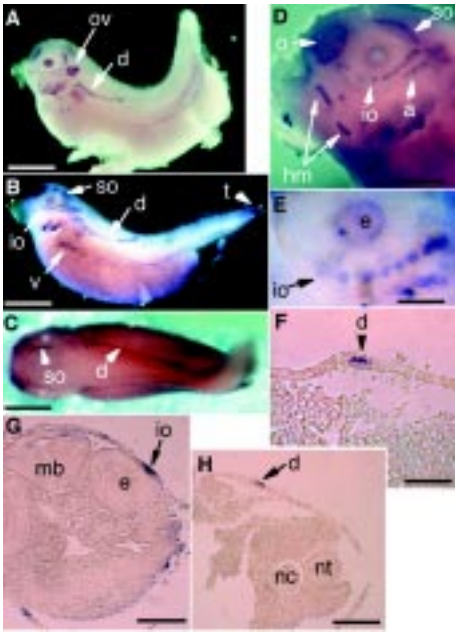


**Fig. 4. Expression of xPAK during lateral line development. (A)** *Stage 35/36,* **(B-H)** *stage 37/38.* **(A-E)** *Whole-mount in situ hybridization.* **(F,G** *and* **H)** *Transverse sections of stage 37/38 whole mount embryos sectioned after hybridization with xPAK1. ov, otic vesicle; o, olefactory placode; nc, notochord; e, eye; a, d, io, so, hm, respectively the aortic, dorsal, infraorbital, supraorbital and hyomandibular lateral lines; t, tail. Bar in A to C, 1 mm; in D, 0.3 mm; in E and F, 0.16 mm; and in G and H, 0.3 mm.*

epithelium and/or cell migration than in the subsequent differentiation of the mechano-receptors of the ear and lateral lines.

## Experimental Procedures

### *Cloning*

A first clone of xPAK1 was isolated by the use of degenerate primers corresponding to kinase domain VIII and IX in a PCR reaction on stage 24 cDNA (Islam *et al.*, 1994). This clone, xltk24, was then transcribed as a riboprobe and used to screen a *Xenopus laevis* embryonic stage 17 λgt10 cDNA bank provided by D. Melton. Screening of this bank was performed in 50% formamide, 5xSSC, 5xDenhardt's and 100 μg/mL$^{-1}$ torula RNA (Sigma) at 42°C. After hybridization, washing consisted of two washes in 6xSSC and two washes in 2xSSC at 42°C for 15 min each. The filters were then treated with RNase A, 20 μg/mL$^{-1}$ and RNase T1, 10 units/mL$^{-1}$ in 2xSSC at 37°C for 30 min before further washing in 0.5xSSC, 0.1% SDS at 55°C for 30 min. A partial cDNA was isolated and used to screen a stage 24 λgt10 cDNA bank established using a degenerate primer to kinase domain IX (Islam *et al.*, 1996). Finally, both clones were completely sequenced, fused by restriction and religation and resequenced.

### *Northern and RT-PCR*

mRNA was isolated from staged embryos and adult tissues using guanidinium isothiocyanide and LiCl precipitation (Cathala *et al.*, 1983). Northern blot was prepared from formaldehyde gel separation (Brown, 1987). The blot contained 20 μg per lane of total RNA from each stage was probed with an [α-$^{32}$P]-RNA antisense probe corresponding to nucleotides 1 to 395 of the xPAK1 cDNA. Hybridization was performed in 50% formamide, 6xSSC, 5xDenhardt's and 100 μg/mL$^{-1}$ of torula RNA (Sigma) at 60°C. After hybridization, washing consisted of two washes in 6xSSC and two washes in 2xSSC at 60°C for 15 min each. The blot was washed in 0.5xSSC, 0.1% SDS and 0.1xSSC, 0.1% SDS at 65°C for 15 min each. Finally, the blot was washed again in 0.1xSSC, 0.1% SDS at 68°C for 15 min. Quantitative RT-PCR reactions were made using degenerate probes to kinase domains VIII and IX on 10 μg of total RNA as previously described (Islam *et al.*, 1994) but probed using the xPAK1 cDNA.

### *Antibody production*

Rabbit polyclonal antibody against xPAK1 was generated by injection of bacterial expressed GST fusion protein containing xPAK1 residues 1 to 63. Two rabbits were injected with 750 μg of GSTxPAK1 twice in an interval of 18 days. Twenty days after the second priming, rabbits were sacrificed and blood was recovered. Blood was kept overnight at 4°C to coagulated. It was centrifuged, the serum was recovered and stored at -80°C.

### Western analysis

Tissues from adult *X. laevis* frogs were homogenized in extraction buffer (250 mM Sucrose, 10 mM Tris pH 7.4, 1 mM EGTA and 1 mM $MgCl_2$) and centrifuged to removed cellular debris. Proteins from tissue lysates were separated on a 8% SDS acrylamide gel and electrophoretically transferred to Hybond-C membrane (Amersham) in 25 mM Tris, 192 mM glycine and 20% methanol. Membranes were blocked in PBS (145 mM NaCl, 10 mM Na-phosphate buffer pH 7.4) containing 2.5% dry milk. The membranes were then incubated with xMLK2 1-154 polyclonal antibody (1/500) in PBS containing 1% dry milk for 2 h at room temperature. After washing with PBS, 1% dry milk, blots were incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG (1/5000) in PBS, 1% dry milk. The blots were developed using Renaissance (NEN-Dupont) western blot chemiluminescence reagents.

### *Whole-mount in situ hybridization*

Whole-mount *in situ* hybridization was carried out on albino *X. laevis* (Nasco) as described (Hemmati-Brivanlou *et al.*, 1990; Jowett and Lettice, 1994; Islam and Moss, 1996). Probes containing either cDNA nucleotides 1 to 395 or 200 to 1800, but gave equivalent results. Sectioning was carried out after whole-mount hybridization by standard paraffin embedding.

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