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Expression analysis of the peroxiredoxin gene family during early development in *Xenopus laevis*

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ABSTRACT

Development in the frog, *Xenopus laevis*, requires the utilization of yolk glyco-lipo-proteins in a temporally- and spatially-dependent manner. The metabolism of the yolk produces hydrogen peroxide (H_2O_2), a potent reactive oxygen species (ROS). Peroxiredoxins (prdxs) are a family of six anti-oxidant enzymes that, amongst other roles, reduce H_2O_2. Prdxs reduce H_2O_2 through a thiol-redox reaction at conserved cysteine residues which results in the creation of disulfide bonds. Recently the thiol-redox reaction of Prdxs has also been implicated in several cell signaling systems. Here we report the cloning and expression patterns during development of six peroxiredoxin homologs from the frog *X. laevis*. Sequence analysis confirmed their identity as well as their evolutionary relationship with peroxiredoxins from several other species. Using RT-PCR and *in situ* hybridization analysis we have shown that there is early and robust expression of all six homologs during development. All six *X. laevis* peroxiredoxins are expressed in neural regions including the brain, eyes, as well as the somites. Different expression patterns for each peroxiredoxin are also observed in the pronephric region, including the proximal and distal tubules. Expression of several peroxiredoxins was also observed in the blood precursors and the olfactory placode. These results suggest important roles for all six peroxiredoxins during early development. These roles may be restricted to their functions as anti-oxidant enzymes, but may also be related to their emerging roles in redox signaling.

During the oviparous development of the African clawed frog, *Xenopus laevis*, maternal cues are absent and the majority of energy stores are present as glyco-lipo-proteins in the yolk (Jorgensen et al., 2009; Wallace and Selman, 1985). The consumption of yolk stores occurs during specific periods of development and the regulatory signals governing this utilization and the mediation of the ROS by-products are relatively unknown (Armant et al., 1986; Cooper et al., 2007). The fragile environment of the embryo is particularly susceptible to the damaging effects of ROS, affecting physiological and pathological processes during many stages of development and beyond (Inoue et al., 2004; Menon and Rozman, 2007). Most anti-oxidant enzymes, such as catalase and glutathione peroxidase, are expressed at high levels starting at stage 20, when the majority of yolk consumption occurs (Cooper et al., 2007; Fox et al., 2011). However, many developmental processes that utilize metabolism, such as gastrulation, occur before this period. Understanding the sub-cellular management of metabolism before, during, and after yolk consumption is therefore an intriguing target of investigations into the development of many organisms.

Peroxiredoxins (prdxs) are a family of thiol peroxidases that include six vertebrate members (prdx1–6) divided into three subgroups (typical 2-Cys, atypical 2-Cys and typical 1-Cys) (Hall et al., 2009). All prdxs contain either 1 or 2 conserved cysteine residues depending on the subgroup. The primary cysteine, present in all prdxs, is responsible for reducing H_2O_2 and other free radicals. It is oxidized to a sulfenic acid when exposed to H_2O_2 and subsequently forms a disulfide bond with a secondary cysteine residue (Cox et al., 2010). Typical 2-Cys prdxs form dimers caused by disulfide bond formation between the primary and secondary cysteine residues of two different prdxs. Atypical 2-Cys peroxiredoxins form an intramolecular disulfide bond between primary and secondary cysteine residues within the same peptide. 1-Cys prdxs work as heterodimers and form an intermolecular disulfide bond. The final step of this redox reaction is to reduce the disulfide bonds formed by prdxs, which is accomplished by the thioredoxin family of proteins (Cox et al., 2010).

Although H_2O_2 and ROS have historically been considered harmful by-products of normal cellular activity, recent evidence has shown that they can also act within signaling cascades as second messengers. H_2O_2 can be produced transiently by plasma membrane oxidases in response to cytokines (TGFB, TNF-α) and

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growth factors (EDGF, PGF) (Rhee, 2003; Thannickal and Fanburg, 2000). This transient H_2O_2 may oxidize the thiol side group of cysteine residues within proteins and change their conformation and activity. Prdxs are also able to modify cysteine residues in a similar manner. Depending on the redox state of prdxs, they are able to reduce cysteine residues by forming or breaking disulfide bonds between and within various proteins (Iwai et al., 2010; Woo et al., 2010; Yan et al., 2009). Proteins susceptible to this oxidation/reduction include protein tyrosine phosphatases, or transcription factors, which contain cysteine residues in their active sites (Funato et al., 2006; Rhee, 2003; Winterbourn and Hampton, 2008). Though emerging as important regulators of redox signaling, there has not, as of yet, been a comprehensive study of the expression patterns of peroxiredoxins during development.

1. Results and discussion

1.1. Cloning of peroxiredoxin 1–6 from X. laevis

The coding regions of the *X. laevis* prdxs were amplified from adult liver tissues by PCR using primers designed against provisional sequences obtained from the NCBI database (see Section 2.2). The *Xenopus* peroxiredoxins sequences were subsequently compared to the peroxiredoxin family of enzymes from a variety of other organisms (Supplementary Fig. S1 and Supplementary Table S1). This analysis has allowed us to classify the provisional *Xenopus* prdxs as peroxiredoxins 1–6 corresponding to the vertebrate peroxiredoxin gene family members. The *Xenopus* prdxs had open reading frames of 600, 609, 756, 804, 570, and 675 for prdx1–6, respectively and peptide lengths of 199, 202, 251, 267, 189 and 224 amino acids, respectively (GeneBank accession numbers: JF820061, JF820062, JF820063, JF820064, JF820065, JF820066). *Xenopus* prdxs showed high similarity with both mice and human peroxiredoxins at both the DNA and protein level (Supplementary Table S1). Similarity to peroxiredoxins from zebrafish, chicken and fly were also observed (Supplementary Fig. S1). The sequences for *Xenopus* prdx1–4 contain both a primary and a secondary cysteine residue categorizing them as typical 2-Cys peroxiredoxins. *Xenopus* prdx5 contains two cysteine residues, comparable to the atypical 2-Cys peroxiredoxins from other species. *Xenopus* prdx6 has only one cysteine, and is thus a typical 1-Cys peroxiredoxin. The *Xenopus* sequences also contained the appropriate signal and trafficking sequences associated with each prdx (Wood et al., 2003). Prdx3 and five both had cleavable mitochondrial signal sequences, where prdx5 also contained a known peroxisomal signal sequence on the C-terminal (Supplementary Table S1). Prdx4 in other species is known to be a secretory pathway enzyme, and can be secreted. The *Xenopus* prdx4 sequence likewise contains a putative secretory signal (Supplementary Table S1). Based on these data, it is clear that the *Xenopus* prdx sequences fit well into the classical hierarchy and classification of the vertebrate peroxiredoxins.

1.2. Temporal expression pattern

To determine the global expression pattern of the *Xenopus* prdxs during early development, semi quantitative RT-PCR was performed. Developmental stages were selected that preceded and followed the onset of yolk metabolism in most tissues, as well as stages that encompassed significant developmental milestones, including gastrulation, neurulation, cellular differentiation and organogenesis. All *Xenopus* prdxs are present early during embryogenesis (Supplementary Fig. S2). *Xenopus* prdx1, 2, 3 and 6 are present as maternal transcripts at stage 6, prior to gastrulation, and after the activation of the zygotic genome, show relatively consistent levels throughout the stages (6–40) examined. In contrast, *Xenopus* prdx4 starts out with very low levels prior to gastrulation and shows an increase immediately afterwards, while *Xenopus* prdx5 has very low levels of expression throughout the stages examined, except for a peak during stage 28 (Supplementary Fig. S2). Though comprehensive studies have not been done, there is evidence of prdx expression and a developmental role, as all six prdxs are expressed at some level during development in mice (Dammeyer and Arnér, 2011; Donnay and Knoops, 2007; Godoy et al., 2011; Lee, 2003), while Drosophila peroxiredoxin five expression levels vary dramatically from embryos to larvae to pupae (Radyuk et al., 2009). There is also some evidence of expression of peroxiredoxins during bovine oogenesis and embryogenesis (Leyens, 2004; Leyens et al., 2004). Further, NCBI UniGene http://www.ncbi.nlm.nih.gov/unigene/ EST expression analysis demonstrated that *Xenopus tropicalis* peroxiredoxins are, in general, ubiquitously expressed during early developmental stages, while prdx EST expression profiles for *X. laevis* are largely incomplete at this time.

1.3. Comparative tissue analysis of prdx1–6 expression

To determine the spatial expression pattern of *Xenopus* prdxs during development, whole mount *in situ* hybridization analysis was performed on embryos of stages 18–20, 23/24, 27/28, 30, 35/36 and 40/41. Antisense control probes, against Cardiac Troponin I and Islet-I, and negative controls were used to establish specificity in the experiment (data not shown). Whole mount *in situ* hybridization prior to, and during gastrulation (stage 10), revealed general non-specific dorsal signals for all *Xenopus* prdxs (data not shown).

1.3.1. Whole mount in situ hybridization analysis during stage 18–20

Stages 18–20 represent the end of neurulation just prior to the beginning of tail bud stages in *X. laevis*. All six *Xenopus* prdxs were detected in broad dorsal regions of the embryos during these stages (Fig. 1A–F). *Xenopus* prdx1 demonstrated anterior staining in head and presumptive eye structures, but lacked staining in the posterior, including the somitic regions (Fig. 1A). Prdx5 had staining similar to prdx1 with more prominent anterior and head staining (Fig. 1E). Prdx2, 3, 4 and 6 had robust anterior, dorsal, and posterior staining, where 2, 3 and 4 had broader staining in presumptive neural regions and somites (Fig. 1B–D, F). Unique to prdx6 was strong anterior/ventral staining, in regions consistent with blood precursor localization (Fig. 1F). Dorsal views of the same embryos confirmed these observations (Supplementary Fig. S3A–F).

1.3.2. Whole mount in situ hybridization analysis during stage 23/24

Stages 23/24 represent the beginning of tailbud stages. Pre-patterning and cell differentiation in the embryo occurs during these stages as organogenesis also begins. Expression patterns begun during neurulation (Fig. 1A–F) continued during these stages (Fig. 1G–L). *Xenopus* prdx1 showed no dorsal/posterior staining in the somites, but stained the neural tube (Fig. 1G). *Xenopus* prdx1 was strongly present in the anterior neural regions as well as head structures such as the eye, pharyngeal arches and otic placode. Staining for *Xenopus* prdx2, 3, 4, 5 and 6 at these stages was similar to stages 18–20, with very robust somitic staining (Fig. 1H–L). *Xenopus* prdx1, 2, 3 and 4 were also expressed in the pronephric region (Fig. 1G–J). Interestingly the anterior/ventral (blood precursor) staining of prdx6 seen during early stages is not seen at stage 23 (Fig. 1L). Dorsal views of stage 23–24 embryos confirmed these observations (Supplementary Fig. S3G–L).

1.3.3. Whole mount in situ hybridization analysis during stage 27–28

Stages 27–28 represent the end of tailbud stages, organogenesis and the beginning of heart beating. As before, the pattern of