The transcriptional coactivators Yap and TAZ are expressed during early Xenopus development

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ABSTRACT  The Yap and TAZ genes encode highly conserved domains which bind various transcription factors. Yap and TAZ act as transcriptional coactivators to modulate transcriptional activity. The activities of Yap and TAZ are negatively regulated by Hippo signaling via direct phosphorylation. In this study, we describe the expression patterns of Yap and TAZ during the development of Xenopus tropicalis. The Xenopus tropicalis Yap (xtYap) and Xenopus tropicalis TAZ (xtTAZ) genes are expressed maternally. xtYap is widely expressed throughout embryogenesis, particularly in the facial connective tissues, branchial arch, midbrain-hindbrain boundary, otic vesicle, pronephros, notochord, hindgut and tailbud. xtTAZ expression occurs predominantly in the presomitic mesoderm, facial connective tissues, brain, branchial arch, trunk neural crest cells and migrating hypaxial myoblasts. In the muscle lineage, xtTAZ expression is transient and restricted to proliferating cells, the presomitic mesoderm and the edges of the hypaxial myoblasts, with no expression detected in mature muscle cells. These results provide insights into the functions of Yap and TAZ and their regulation by Hippo signaling during early development in Xenopus.

KEY WORDS: Yes-associated protein, Yap, TAZ, WWtr1, Xenopus

The Hippo pathway was originally identified in the fly as a controller of organ size, and its components are evolutionarily conserved in vertebrates (reviewed in Wang et al., 2009; Badouel et al., 2009). The core components of Hippo, Sav, Wts, and Mats in the fly are conserved in mammals as Mst1/2, WW45, LATS1/2, and Mob1, respectively. Protein interaction screenings in Drosophila revealed that Hippo signaling negatively regulates Yorkie through direct phosphorylation. In the absence of Hippo signaling, Yorkie can promote transcription together with binding partners. The Yes-associated protein (Yap) and transcriptional coactivator with PDZ-binding motif (TAZ) are vertebrate homologs of Yorkie. Yap and TAZ show conservation of multiple functional domains, i.e., the TEF/TEAD binding domain, 14-3-3 binding motif, WW domain, and transactivation domain. In similarity to Yorkie, Yap and TAZ bind to transcription factors and act as transcriptional coactivators.

The overexpression of Yap and TAZ induces cell proliferation, epithelial-mesenchymal transition, and anti-apoptosis (Overholzer et al., 2006; Lei et al., 2008). Gene ablation of Yap results in embryonic lethality, with defects in yolk sac vasculogenesis, chorioallantoic fusion, and embryonic axis elongation in the mouse (Morin-Kensicki et al., 2006). The injection into zebrafish of Yap-specific morpholino oligonucleotides results in the phenotype of small head with smaller eyes, no tail, and cardiac edema (Jiang et al., 2009). TAZ-knockout mice show partial lethality; survivors have renal cysts and slightly shorter skeletons (Hossain et al., 2007; Tian et al., 2007; Makita et al., 2008). TAZ morphants of zebrafish show cardiac edema, ventral curvature, and lack of ossification (Hong et al., 2005; Tian et al., 2007). These studies indicate that the functions of Yap and TAZ and their regulation by Hippo signaling are essential for the development of multiple tissues, and that Yap and TAZ play distinct roles in normal development.

Abbreviations used in this paper: orf, open reading frame; Yap, Yes-associated protein.
vertebrate development.

Previous studies have demonstrated that Tead, Runx, Tbx5, Pax3, and other transcriptional factors are binding partners of Yap and TAZ (reviewed in Wang et al., 2009). These transcriptional factors are differentially expressed and function in several developmental processes. Yap and TAZ may also be required as transcriptional coactivators in each process. Elucidating the spatiotemporal expression patterns of Yap and TAZ in vertebrate embryogenesis is important for understanding how Hippo signaling is involved in differentiation, tissue size control, and cell proliferation. In the present study, we reveal the expression patterns of Xenopus tropicalis Yap and TAZ genes, thereby providing insights into the functions of Yap and TAZ in early development.

Results

Molecular cloning of the xtYap and xtTAZ genes

The sequences of the open reading frames (ORFs) of xtYap and xtTAZ were obtained from the genome databases. We performed PCR-based cloning using the cDNA species derived from stage 10.5 embryos and adult testes, respectively. The full-length ORFs of xtYap and xtTAZ (GenBank accession numbers AB551789 for xtYap and AB551790 for xtTAZ) encode 456 and 390 amino acids, respectively. The deduced amino acid sequences were aligned and compared with their homologs in other vertebrates (Fig. 1-3). The xtYap protein showed 76.4%, 78.0%, and 75.4% identities with the human Yap (hYAP), mouse Yap (mYap), and zebrafish Yap (zYap), respectively. The xtTAZ protein showed 76.2%, 74.0%, and 62.2% identities with hTAZ, mTAZ, and zTAZ, respectively. Although the functional domains of these proteins are conserved across species, a proline-rich motif that is conserved in the N-terminal regions of the mYap and hYAP proteins is not present in the xtYap and zYap proteins.

Expression patterns of xtYap and xtTAZ

We performed semi-quantitative RT-PCR analyses of embryos and adult tissues (Fig. 4). During embryogenesis, xtYap transcripts were detected continuously from the unfertilized egg to the tadpole stages. The levels of xtTAZ transcripts were low from the unfertilized egg to gastrula stages, but gradually increased from the neurula stage to tadpole stage (Fig. 4A). In the adult tissues, xtYap transcripts were found to be abundant in the gut, ovary and testis. In addition, xtYap was expressed at moderate levels in the heart and liver, whereas it was weakly expressed in the eyes and skin. The levels of xtTAZ transcripts were high in the gut, heart, skin, and testes. xtTAZ was also expressed at moderate levels in the eyes, liver and ovaries, whereas it was weakly expressed in the muscles (Fig. 4B).

To examine the spatial expression patterns of these genes during the early development of Xenopus, we performed whole-mount in situ hybridization.

Fig. 1. Comparisons of the Yap protein sequences of Xenopus tropicalis and other vertebrates. The scheme of xtYap is shown on the top. The amino acid sequence of xtYap was aligned with those of human Yap (hYAP; accession no. NP_001123617), mouse Yap (mYap; NP_033560), and zebrafish Yap (zYap; NP_001132952). The dark or light background highlights identical or similar residues, respectively. The domains of Yap are boxed using the following colors and abbreviations; light-green, TB: Tead binding domain; blue, 14-3-3: 14-3-3 binding motif; red, WW: WW domain; light-blue, SH3: SH3 binding motif; yellow, TA: transcriptional activation domain; pink, PDZ: PDZ binding motif.
tion analysis. The xtYap transcripts were localized to the animal hemisphere until the early gastrula stage (Fig. 5 A-C). During gastrulation, the embryo was covered with xtYap-expressing ectodermal cells (Fig. 5D). At the neurula stage, xtYap was mainly expressed in the center of the neural plate and in the cranial neural crest cells (Fig. 5 E,F,J). From the tailbud stage to the tadpole stage, xtYap expression was evident in the facial connective tissues, branchial arch, midbrain-hindbrain boundary, otic vesicle, pronephros, notochord, hindgut, and tailbud (Fig. 5 G-I).

Histologic sectioning of a stage 30 embryo revealed that xtYap was expressed in the ectodermal and mesodermal derivatives (Fig. 5K).

The expression of xtTAZ was initially detected in the paraxial mesoderm at the late neurula stage (Fig. 6 A,B,D). Thereafter, xtTAZ expression was detected in the hindbrain and presomitic mesoderm from the early tailbud stage (Fig. 6 C,E,K,L). It is noted that xtTAZ was not detected in the matured somite (Fig. 6 E,J). In a transverse section of the tailbud stage, xtTAZ expression was observed in the heart, hindbrain, and the trunk neural crest cells (Fig. 6 H-L). At the late tailbud stage, xtTAZ was expressed in the facial connective tissues, midbrain-hindbrain boundary, hindbrain, branchial arch, and trunk neural crest cells. From embryonic stage 35, xtTAZ was expressed in the migrating hypaxial myoblasts, including the facial musculature (Fig. 6 F,G,M,N).

Discussion

The *Xenopus tropicalis* Yap and TAZ proteins contain conserved domains, which include not only transcriptional factor binding sites, such as the Tead binding domain and WW domain, but also other functional domains (Fig. 1,2). However, a proline-rich region that is conserved in the N-terminal regions of the chick, murine, and human Yap proteins is not present in the *Xenopus* or zebrafish Yap proteins. It has been reported that this proline-rich region interacts with the SH3 domain of cYes and other proteins (Sudol, 1994).

The expression patterns of Yap and TAZ during embryogenesis have been studied in mice and zebrafish. Yap transcripts have been detected at the 1-cell stage of mouse development (Nishioka *et al.*, 2009). The murine Yap gene is expressed in almost all tissues, with the exceptions of the visceral and definitive endoderm. In particular, *mYap* is strongly expressed in the extraembryonic ectoderm and epiblast at E6.5. In addition to these regions, *mYap* is expressed in the extraembryonic mesoderm and chorion at E7.5, and at the distal tip of the allantois at E8.5 (Morin-Kensicki *et al.*, 2006). The *mYap* expression patterns at later developmental stages have not been analyzed. In zebrafish, zYap transcripts exist maternally. Zygotic zYap is mainly expressed in the notochord, brain, eyes, branchial arches, and pectoral fins (Jiang *et al.*, 2009). In the present study, we show that xtYap is maternally expressed and is widely expressed throughout the developmental process, with strong expression in the center of neural plate and in the cranial neural crest cells at the neurula stage. We also demonstrate that xtYap is strongly expressed in the facial connective tissues, branchial arch, midbrain-hindbrain boundary, otic vesicle, pronephros, notochord, hindgut, and tailbud at the tailbud stage.

TAZ is expressed from the 1-cell stage of the mouse embryo (Nishioka *et al.*, 2009). TAZ is localized to the forebrain, hindbrain, and somites between E9.0 and E10.5 of mouse embryogenesis (Di Palma *et al.*, 2008). In the present study, we show that xtTAZ is maternally expressed and is expressed in the hindbrain and....
presomitic mesoderm from the neurula stage to the tailbud stage. We also show that \textit{xtTAZ} is expressed in the facial connective tissues, the midbrain-hindbrain boundary, the hindbrain, the branchial arch, and in the trunk neural crest cells at the tadpole stage. Furthermore, we demonstrate that \textit{xtTAZ} is expressed in the presomitic mesoderm and hypaxial myoblasts, but not in the differentiated somite. These observations suggest that \textit{xtYap} and \textit{xtTAZ} are expressed widely, and that in some tissues (e.g., the center of the neural plate and cranial neural crest cells for \textit{xtYap}, and the hindbrain and presomitic mesoderm and hypaxial myoblasts for \textit{xtTAZ}), their expression levels are high. It has been reported that the large amounts of Yap and TAZ produced by overexpression cannot be inhibited by the endogenous Hippo signaling in \textit{Drosophila} and mammalian cells (Dong et al., 2007). There is no information as to when Hippo signaling is active during \textit{Xenopus} development. The region highly expressing \textit{Yap} or \textit{TAZ} may be resistant to be the Hippo signaling.

Yap and TAZ have been shown to function as transcriptional regulators in various cellular processes, including cell growth, proliferation, and differentiation. The expression patterns of \textit{xtYap} and \textit{xtTAZ} during the early development of \textit{Xenopus tropicalis} are shown in Fig. 5.

**Fig. 3 (Left).** Rooted phylogeny of the Yap, TAZ and Yorkie amino acid sequences. The following amino acid sequences were used in the analysis: human Yap (hYAP; accession no. NP_001123617); mouse Yap (mYap; NP_033560); \textit{Xenopus tropicalis} Yap (xtYap; AB551789); zebrafish Yap (zYap; NP_001132962); human TAZ (hTAZ; NP_056287); mouse TAZ (mTAZ; NP_598545); \textit{Xenopus tropicalis} TAZ (xtTAZ; AB551790); and zebrafish TAZ (zTAZ; NP_001032785); Yorkie (NP_001036568).

**Fig. 4 (Right).** Comparison of the \textit{xtYap} and \textit{xtTAZ} expression patterns. (A) Temporal expression profiles of \textit{xtYap} and \textit{xtTAZ}. RNA samples were extracted from embryos at various developmental stages, as indicated above each lane. e, Unfertilized egg. ODC (-) is a negative control for ODC. (B) Expression profiles of \textit{xtYap} and \textit{xtTAZ} in adult \textit{Xenopus} tissues. RNA samples were extracted from \textit{Xenopus} tissues, as indicated above each lane. RT (-), RT-PCR without reverse transcriptase in the testis cDNA synthesis reaction.
coactivators for Tead, Tbx5, and Pax3. Tead family members are major transcriptional factors that interact with Yap and TAZ and induce cell proliferation and epithelial-mesenchymal transition (Vassive et al., 2001, Mahoney et al., 2005, Wang et al., 2009). The mammalian Tead family has four members, i.e., Tead1-4 (NTEF-1, ETEF-1, DTEF-1 and RTEF-1, respectively). However, in Xenopus only two Tead genes have been identified: xTead1 (xNTEF-1) and xTead3 (xDTEF-1); a previous study has characterized the expression patterns of xTead1 and xTead3 (Naye et al., 2007). Some of the expression domains of xTead and xTAZ overlap with those of xTead1 and xTead3. The xTead1 and xTead3 transcripts were predominantly detected in the eyes, embryonic brain, somites, and heart. Therefore, xYap and TAZ can act as binding partners for xTead1 and xTead3 in these tissues. In other regions, in which xYap or xTAZ but not the Tead genes are expressed, these coactivators may function together with other transcriptional factors. For example, in the present study, we show that xTAZ is expressed at the edge of the migrating hypaxial muscle. Pax3 is expressed in Xenopus hypaxial muscle at the same stage, and functions in abdominal muscle formation in the mouse (Martin and Harland, 2001; Tajbakhsh et al., 1997; Tremblay et al., 1998). It has been reported that the transcriptional activity of Pax3 is coactivated by TAZ (Murakami et al., 2006). Therefore, TAZ may act as a coactivator of Pax3 at the edges of proliferating and migratory hypaxial muscles to cancel signaling by the Hippo pathway.

Materials and Methods

Plasmid construction

Using the BLAST search algorithm (Altschul et al., 1990), we identified from JGI Xenopus tropicalis v4.1 the genomic clones that correspond to Xenopus tropicalis Yap and TAZ. The full-length ORFs of xYap and xTAZ were amplified by PCR from the corresponding cDNAs of Xenopus tropicalis embryos and adult testes, respectively. The following primer sets (forward and reverse, respectively) were used:

- xYap: 5'-ATGGGAGCCCGGATCCCAG-3', 5'-CGTTGAGGATGTCGGAACTGAGAG-3'
- xTAZ: 5'-ATGAATCCGAGCCCACATCTCC-3', 5'-CTACAGCCAGGTGAGGTAGGGCTC-3'

Each clone was subcloned into the pGEM-T-easy vector, and pCS2p-xYap and pGEM-xTAZ were used for synthesis of the in situ hybridization probes.

Alignment of sequences and construction of a phylogenetic tree

Multiple protein sequence alignments and the generation of a phylogenetic tree were performed using the CLUSTALW tool of the MacVector software (MacVector Inc.). The following sequences were used: hYAP (accession no. NP_001123617); mYap (NP_033560); zYap (NP_001123952); hTAZ (NP_056287); mTAZ (NP_598545); zTAZ (NP_001032785); and Yorkie (NP_001036568).

Semi-quantitative RT-PCR

Total RNA samples were isolated from various developmental stage embryos and adult tissues using RNAiso (Takara Bio Inc.), and 2 μg of total RNA was used as the template for first-strand cDNA synthesis using SuperScript II Reverse Transcriptase according to the manufacturer's instructions (Invitrogen). The cDNA (1 μl) was used as a template in the PCR. The following primer sets (forward and reverse, respectively) were used:

- xYap: 5'-TCATCCACAGACAGAAAGCACTGAC-3', 5'-CGTTGAGGATGTCGGAACTGAGAG-3'
- xTAZ: 5'-GCCATTACACATACGGGACAGAG-3', 5'-GCAGGGCTGGAATTTGTTTG-3'
- ornithine decarboxylase (ODC) and elongation factor 1 (EF1α) were used as internal controls. The primer sets for ODC and EF1α were described in Sekizaki et al. (2004).

Whole-mount in situ hybridization and histology of embryos

Xenopus tropicalis embryos were obtained by artificial fertilization according to the method of Showell and Conlon (2009) with modifications,
cultured in 10% Steinberg’s solution, and staged according to the scheme of Nieuwkoop and Faber (1994). Whole-mount in situ hybridization analysis was performed as described previously (Harland, 1991). Stained embryos were re-fixed in Bouin’s solution overnight, dehydrated, embedded in paraffin, and sectioned at a thickness of 10 μm.

Acknowledgements

We thank Dr David L. Turner for the generous gift of pCS2 vector. We also thank Eri Torikai for her assistance. This work was supported by a Grant-in-Aid for Young Scientists (to S.T.) from the Japan Society for the Promotion of Science.

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