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Cloning and spatiotemporal expression of RIC-8 in *Xenopus* embryogenesis

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ABSTRACT

RIC-8 is a highly conserved protein that promotes G protein signaling as it acts as a Guanine nucleotide Exchanging Factor (GEF) over a subset of Gα subunits. In invertebrates, RIC-8 plays crucial roles in synaptic transmission as well as in asymmetric cell division. As a first step to address further studies on RIC-8 function in vertebrates, here we have cloned a *ric-8* gene from *Xenopus tropicalis* (*xtric-8*) and determined its spatiotemporal expression pattern throughout embryogenesis. The *xtric-8* transcript is expressed maternally and zygotically and, as development proceeds, it shows a dynamic expression pattern. At early developmental stages, *xtric-8* is expressed in the animal hemisphere, whereas its expression is later restricted to neural tissues, such as the neural tube and the brain, as well as in the eye and neural crest-derived structures, including those of the craniofacial region. Together, our findings suggest that RIC-8 functions are related to the development of the nervous system.

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Signaling via G protein-coupled receptors (GPCR) is a major regulator of cell communication and, as such, it has been extensively described as a crucial pathway in the differentiation of several cell lineages and in the development of a variety of invertebrate and vertebrate organisms (Parks and Wieschaus, 1991; Moxham et al., 1993; Malbon, 2005). Ligand binding to its corresponding GPCR induces the exchange of GDP by GTP in the Gα subunit and the subsequent dissociation of the Gβγ dimer. Both, Gα-GTP and Gβγ, have the ability to signal through different effectors, such as adenilyl cyclase (AC), some isoforms of phospholipase C, and calcium or potassium channels (Gilman, 1987). The signal is terminated when the GTP is hydrolyzed to GDP by the intrinsic GTPase activity of Gα, which decreases the affinity of Gα for its effectors, leading to its re-association with Gβγ to reconstitute the heterotrimer (Gilman, 1987; Siderovski and Willard, 2005). In addition to GPCRs, G protein signaling is finely modulated by members of the superfamily of regulators of G-protein signaling (RGS) (Hollinger and Hepler, 2002). These proteins include GTPase Accelerating Proteins (GAP), proteins with Guanine Nucleotide Dissociation Inhibitor Activity (GDI), and Guanine nucleotide Exchanging Factors (GEF) (Siderovski and Willard, 2005).

RIC-8, a cytosolic protein highly conserved from invertebrates to vertebrates, was first identified in a genetic screening of *Caenorhabditis elegans* mutants able to resist the neurotoxic effects of inhibitors of cholinesterase (Miller et al., 2000; Tall et al., 2003; Tõnissoo et al., 2003). RIC-8 functions as a receptor-independent GEF, as it interacts directly with the Gβγ-free GαGDP subunit and promotes the GDP for GTP exchange, therefore expanding the lifetime of the signaling, as it prolongs the active state of Gα (Tall et al., 2003; Klattenhoff et al., 2003; Von Dannecker et al., 2005). In mammals, two paralogs of RIC-8 (RIC-8A and RIC-8B) have been identified through yeast two hybrid screening, using different Gα subunits as baits (Tall et al., 2003). RIC-8A has the ability to interact with and promote the nucleotide exchange of Gαq, Gαi1 and Gαo *in vitro* (Tall et al., 2003). In turn, RIC-8B interacts *in vitro* with Gαs and Gαq and potentiates the GPCR-dependent activation of both Gα isoforms (Malnic and Gonzalez-Kristeller, 2009; Nishimura et al., 2006; Nagai et al., 2010). In addition, RIC-8B translocates to the plasma membrane in response to isoproterenol and carbachol, two Gαs and Gαq-coupled GPCR agonists (Klattenhoff et al., 2003; Nishimura et al., 2006).

Along with its function as a GEF, RIC-8 also plays crucial roles in synaptic transmission in worms as well as in mitotic spindle align-

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ment during early embryogenesis in *C. elegans* and *Drosophila*, for what it has also been named "synembryn" (Miller and Rand, 2000; Tõnissoo et al., 2003; David et al., 2005; Hampoelz et al., 2005). In this regard, we have recently proposed that RIC-8 structure is formed almost exclusively by α-helices arranged in an armadillo structure, a configuration allowing the interaction with multiple proteins and that is characteristic of scaffolding proteins of several signaling pathways (Figueroa et al., 2009; Peifer et al., 1994; Coates, 2003). Together, this evidence suggests that RIC-8 could play diverse roles in different time- and tissue-specific contexts.

As a necessary step to address further studies on RIC-8 functions during vertebrate embryogenesis, here we have analyzed the expression of RIC-8 using *Xenopus tropicalis* as model system. To accomplish this aim, we first isolated *X. tropicalis* RIC-8 cDNA (*xtric*-8), and subsequently conducted a detailed analysis of its spatiotemporal expression pattern during embryonic and larval development.

1. Results and discussion

1.1. Cloning and analysis of XtRIC-8 cDNA

In order to identify the *X. tropicalis* ortholog of RIC-8 (*xtric-8*), we first BLAST searched the *X. tropicalis* genome assembly v4.1 gene data base (JGI http://genome.jgi-psf.org/cgi-bin/runAlignment?db=Xentr4&advanced=1) using the *Xenopus laevis* RIC-8 cDNA sequence (XlRIC-8) (*xlric-8* DQ115398), which had been previously cloned in our laboratory (Romo et al., 2008; Figueroa et al., 2009). This comparison identified a gene composed of 10 exons spanning a region of 40,113 bp in the JGI, which was closer related to RIC-8A. A predicted mRNA sequence highly identical (91%) to its *X. laevis* ortholog was employed to design specific primers to amplify the full-length *xtric-8* cDNA. The unique resulting RT-PCR product was subsequently cloned and sequenced. Following this procedure, we obtained a 1620 bp *xtric-8* cDNA coding for a 539 amino acids protein that shares higher percentage of identity (58–60%) with mouse and human RIC-8A, whereas lower identity (27–32%) was observed with the invertebrate orthologs (Fig. 1). Therefore, even though *X. tropicalis* RIC-8 shares sequence similarities with mammalian RIC-8A, the highest sequence identity (95%) was observed for *X. laevis* RIC-8 protein, which has been classified by our group as RIC-8B, as it displays GEF activity over Gαs (Romo et al., 2008). In this regard, it is worth mentioning that a predicted RIC-8B paralog is also annotated in the Ensembl data (Q9NVN3). As for other vertebrate species, this RIC-8B paralog displays about 53% identity with the XtRIC-8 cDNA cloned by us (Q6P4W7). Even though further research has to be made to elucidate the function of these different forms of RIC-8, their highest percentage of identity in the C-terminal region (Fig. 1A) suggests that the structural motifs involved in the differential functions carried out by RIC-8A and B, mediated by their interaction with different G proteins, are likely to be contained within their N-terminal domain.

To analyze the conservation of the *ric-8* gene during evolution, we performed a synteny analysis of genomic regions around *ric-8* from *X. tropicalis* and mice against the human *ric-8* gene. Significant similarities were observed between human and mice at the gene localization. Nevertheless, as shown in Fig. 1C, even though exonic regions of *ric-8* are highly similar, no significant similarities were observed in non-coding and coding genomic regions surrounding the *ric-8* open reading frame from *X. tropicalis*, with respect to human and mice. Interestingly, this low degree of conservation at the gene location and promoter sequence between *X. tropicalis* and other vertebrates has been observed in many other genes, indicating a high divergence in genomic organization during evolution (Chan et al., 2009). Despite these differences, the high percentage of identity observed in the coding region of RIC-8 from different species strongly suggests a conserved function for this protein throughout evolution.

1.2. Temporal expression of XtRIC-8

With the aim of determining the temporal expression of the *xtric-8* gene, we prepared total RNA from different stages of *X. tropicalis* development and performed RT-PCR analyses. We used internal primers to amplify an 856 bp fragment of *xtric-8* along with primers to amplify the *ef1α* elongation factor, used here as an internal control, to allow a semi-quantitative comparison and to ensure the RNA integrity. As shown in Fig. 2A, *xtric-8* is expressed as a maternal and zygotic transcript as it was detected in all the stages analyzed, ranging from unfertilized eggs to stage 42. In support of these findings, *ric-8* mRNA has similarly been detected in *X. laevis* oocytes (Romo et al., 2008). Therefore, we conclude that RIC-8 displays a sustained expression during early *X. tropicalis* embryogenesis.

To globally describe the tissue-specific expression pattern of *xtric-8*, we subjected total RNA samples from different tissues of adult *X. tropicalis* frogs to a similar RT-PCR approach. Our results show that *xtric-8* is widely expressed in various adult tissues, being more abundant in brain, heart and eye. Relatively lower expression levels were detected in other tissues, including skeletal muscle, pancreas, skin, lung and kidney (Fig. 2B). This expression pattern suggests that, in addition to the wider roles that XtRIC-8 could play during early development, this protein may also function in specific tissues later in development. In support of this notion, recent data show that mouse RIC-8 has a GEF activity on several Gα proteins, including Gαolf, Gα-gustducin, and Gαi2, all of which are crucial for the specific function of olfactory sensory neurons, retinal ON bipolar cells and taste bud cells, respectively (Kerr et al., 2008; Dhingra et al., 2008; Fenech et al., 2009). Also in adult invertebrates, *C. elegans* RIC-8 has been shown to exert a positive effect on Gq-mediated neurotransmitter release (Schade et al., 2005; Nishimura et al., 2006). Together, these findings indicate that RIC-8 is likely to maintain GEF functions on several Gα proteins of postnatal organisms to regulate signaling in different specific tissues.

1.3. Spatial expression of XtRIC-8

To precisely describe the distribution of RIC-8 during frog development, we performed whole mount *in situ* hybridization on *X. tropicalis* and *X. laevis* embryos (Figs. 3 and 4). Our results show abundant expression of maternal RIC-8 transcripts at the animal hemisphere during the cleavage and blastula stages of both species (Fig. 3A); in turn, the vegetal hemisphere displayed weak maternal *ric-8* expression (insets in Fig. 3A, panels f and g). At gastrula (stage 10), the developmental stage that marks the onset of mesoderm specification and morphogenetic movements, even though *ric-8* mRNA is weakly expressed at the animal and vegetal regions, a stronger expression was observed at the marginal zone (Fig. 3A, panels h and m). In order to corroborate these findings, we detected the expression of the *Xbra* gene as a control of mesoderm formation (Smith et al., 1997). The expression profile of RIC-8 was remarkably similar to that of Xbra (Fig. 3B), revealing that *Xtric-8* is expressed in the mesoderm. Considering that RIC-8 modulates crucial steps of cell division in invertebrate species (Miller and Rand, 2000; David et al., 2005; Hampoelz et al., 2005), we speculate that the expression of RIC-8 at early stages of *Xenopus* development could similarly be associated to mitosis.

At later developmental stages coinciding with the formation of the nervous system (i.e. neurula, stage 15 and 19), *ric-8* transcripts were detected in almost all neural regions, including the neural

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