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Short Report

Ol-KIP, a cyclin dependent kinase inhibitor, is expressed in developing and adult brain of the Medaka *(Oryzias latipes)*

EVA MARÍA CANDAL SUÁREZ*[,1], VÂN NGUYEN[2], JEAN-STÉPHANE JOLY[2] and FRANCK BOURRAT[2]

[1]*Facultad de Biología, Departmento de Biología Fundamental, University of Santiago de Compostela, Spain and*
[2]*Jeune Equipe INRA "Morphogenèse du Système Nerveux des Chordés", CNRS, Institut Fessard, France*

ABSTRACT We have isolated Ol-KIP, a new member of CIP/KIP family of cyclin-dependent kinase inhibitors (CDIs), from an expression screen in the medaka (Teleost), and then analyzed its expression pattern in developing and adult brain by *in situ* hybridization (ISH). To better define the expression profile of this CDI during cell differentiation, we performed a double labeling with Ol-KIP mRNA and antibodies against proliferating cell nuclear antigen (PCNA). In the optic tectum, expression of RNA encoding Ol-KIP during early embryogenesis is largely associated with the start of cell differentiation in proliferative zones; later in development, its expression is closely related to cell cycle exit. In the adult brain, transcripts appeared also in postmitotic cells long after they have exited the cell cycle. So, they may play a role in maintenance of the quiescent state throughout life.

Spatial and temporal control of cell proliferation (including its arrest), is required to proper development of a multicellular organism, via an intricate network of extracellular and intracellular signals. The final target of many of these signals are cyclin-dependent kinases (Cdks), that catalyze events required for the cell cycle transition. Although much attention has been focused on how cells enter the cell cycle, less is known concerning the strategies that they employ to exit the cell cycle, which are of great importance given that the vast majority of cells are in a non-dividing state throughout adult life. In this context, several proteins known as Cdk inhibitors (CDIs) act as potential mediators of the cell cycle exit and the maintenance of the postmitotic state. In mammals, two structurally defined classes of Cdk inhibitors have been described (revised in Zindy *et al.,* 1999): the INK4 family ($p16^{INKa}$, $p15^{INKb}$, $p18^{INKc}$, $p19^{INKd}$), and the CIP/KIP family ($p21^{CIP}$, $p27^{KIP1}$, $p57^{KIP2}$). Each of these inhibitors also present specific patterns of expression during embryonic development and in adult, where they act at different moments of the cell determination and differentiation process. The great diversity of these specific patterns suggests that Cdk inhibitors have additional roles to cell cycle regulation *per se*. The study of the expression of INK4 and CIP/KIP genes during development, and postnatally as animals grow and age, is therefore a prerequisite step for dissecting their functions.

Materials and Methods

For isolation and sequencing of Ol-KIP, we used total mRNA from stage 25 embryos and from adult medaka optic tectum to synthesize cDNA by RT-PCR as described in the SMART RACE cDNA amplification kit (Clontech). The resulting PCR product was cloned into the PCRII-TOPO vector and sequenced. Sense and antisense probes were prepared following the procedure described in Joly *et al.,* 1997. For ISH, embryos and adult brains were fixed in 4 % paraformaldehyde (PFA), and processed according to Joly *et al.,* 1997. Proteinase K treatment and developing times were extended in adult brains. For PCNA immunohistochemistry, embryos were fixed in Clark's solution, wax-embedded, sectioned (8 μm) and processed using the Vectastain© Universal ABC Kit. For double labeling experiments, embryos and adults were fixed in 4% PFA, and processed to ISH. After development, samples were refixed in PFA, dehydrated, wax-embedded and processed for PCNA immunohistochemistry. To optimize antigen retrieval, microwave pre-treatment was performed (3 heat pulses for 5 minutes, with cooling intervals at RT for 5 minutes, in PBS), before incubation in primary antibody .

Results

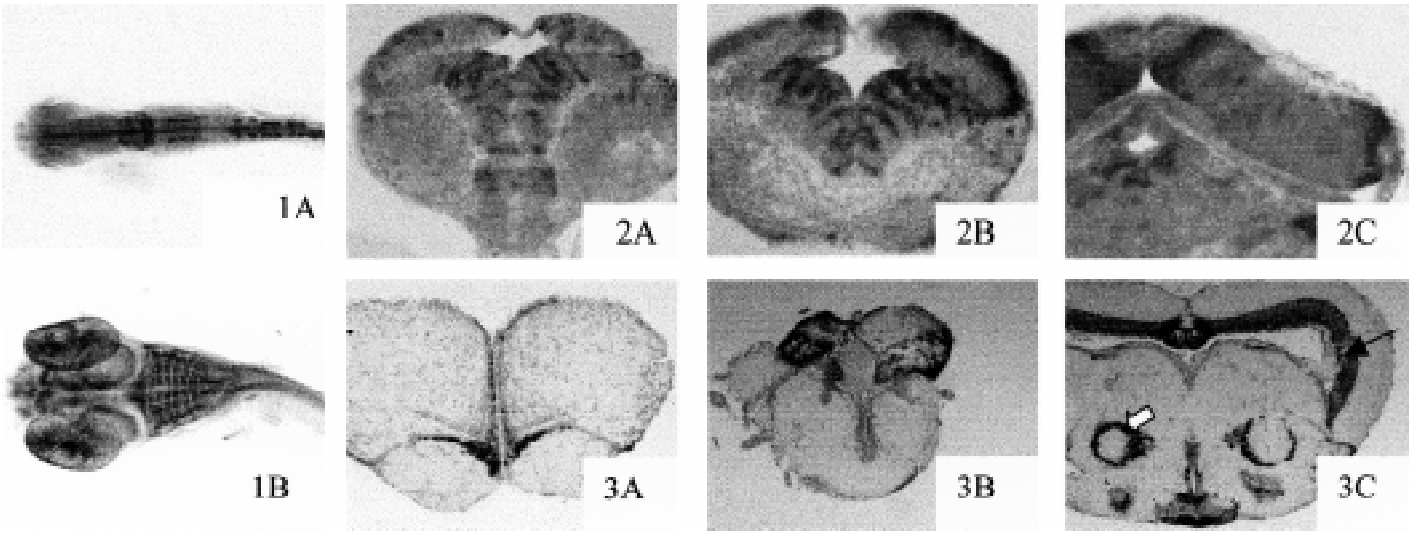
Isolation and sequence analysis of Oryzias latipes Ol-KIP

We have obtained a full-length cDNA which encoded a protein (210 amino acids, predicted MW of 23,775 Da) that we have named Ol-KIP (for Kinase Inhibitor Protein from *Oryzias latipes*). Ol-KIP presents a significant identity to the KIP subfamily of cyclin-dependent kinase inhibitors. Its N-terminal region is 41% identical to $p27^{KIP1}$ and 52% identical to $p57^{KIP2}$, and includes the inhibition domain that is the most conserved region in all CIP/KIP proteins; this domain binds to specific CDK/cyclin complexes inhibiting their function, or preventing them from being phosphorylated and so activated by Cyclin activating kinases (Polyak *et al.,* 1994). The C-terminal regions of these proteins are less well conserved but they all have a putative nuclear localization signal. Ol-KIP also contains several Ser/Thr-Pro motifs (potential phosphorylation sites for various mitotic kinases that could affect its association with CDKs), several amino acidic residues that are critical for the binding to PCNA (Watanabe *et al.,* 1998), and a C-terminal region known as QT domain the function of which remains unknown.

Medaka brain development

Ol-KIP mRNA is first detected in the hindbrain at stage 22 concurring with the beginning of brain organogenesis (Fig. 1A), and shortly after in the midbrain and forebrain. Later in development, Ol-KIP is clearly associated to differentiating cell populations (Fig. 1B).

***Address correspondence to:** Eva María Candal Suárez. Facultad de Biología, Departmento de Biología Fundamental, University of Santiago de Compostela, Spain. e-mail: bfcasual@usc.es



Results in the optic tectum

In early development Ol-KIP mRNA is evident in the proliferative periventricular zone (Fig. 2A). As neurogenesis proceeds, the size of this zone diminishes as cells exit the cell cycle and undergo terminal differentiation, occupying the most superficial layer. Ol-KIP is expressed in these superficial cells undergoing terminal differentiation (Fig. 2 B,C). Double labeling for PCNA and Ol-KIP mRNA shows that Ol-KIP is expressed between the proliferative and postmitotic zones.

Ol-KIP expression in the adult brain

Ol-KIP expression is high in the adult brain, where mRNA is detected not only associated to proliferative zones, but also to fully differentiated neurons, for example in the granular layer of the olfactory bulb (Fig. 3A), the habenula (Fig. 3B), posterior glomerular nucleus (white arrow in Fig. 3C), periventricular gray stratum of optic tectum (black arrow in Fig. 3C) and cerebellum.

Conclusion

In embryos and young fries, *Ol*-KIP mRNA was largely associated to the limit between proliferative (PCNA-immunopositive) and differentiated zones, so its expression pattern was consistent with a role of this protein in the regulation of the cell cycle exit. Nevertheless, in the adult medaka brain, numerous nuclei expressed *Ol*-KIP long after they have exit the cell cycle, suggesting that it could play roles different from the regulation of cell cycle withdrawal, such as the control of pathways that lead to a terminal cell differentiation in the adult CNS.

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