Embryonic Expression and Function of the Xenopus Ink4d Cyclin D-Dependent Kinase Inhibitor

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Abstract

Here we report the cloning and functional characterization of the cyclin D-dependent kinase 4 and 6 (Cdk4/6) inhibitory protein Cdkn2d/p19Ink4d of Xenopus laevis (Xl-Ink4d). Xl-Ink4d is the only Ink4 family gene highly expressed during Xenopus development and its transcripts were detected maternally and during neurulation. The Xl-Ink4d protein has 63% identity to mouse and human Cdkn2d/p19Ink4d and its function as a negative regulator of cell cycle traverse is evolutionarily conserved. Indeed, XI-Ink4d can functionally substitute for mouse Cdkn2d in binding to mouse Cdk4 and inhibiting cyclin-D1-dependent Cdk4 kinase activity. Further, enforced expression of XI-Ink4d arrests mouse fibroblasts in the G1 phase of the cell cycle. These findings indicate that Cdkn2d/p19Ink4d is conserved through vertebrate evolution and suggest XI-Ink4d may contribute to the development of Xenopus laevis.

Keywords: Xenopuslaevis; Cyclin-dependent kinase inhibitor; Ink4d; Cdkn2d; Cell cycle

Introduction

Regulation of the cell cycle is intimately involved in development, where cell cycle arrest is coordinately controlled with terminal differentiation. Progression through the cell cycle is driven by cyclin-dependent kinases (Cdks) and their obligatory binding partners, the cyclins. Cyclin/Cdk complexes are regulated by two families of Cdk inhibitory proteins (CKIs), the Cip/Kip family (Cdkn1) and the Inhibitors of Cdk4 (Ink4) family (Cdkn2) [1]. Enforced expression of Cki1s induces cell cycle arrest by binding to and inhibiting cyclin-dependent Cdk activity. Specifically, Cip/Kip family members negatively regulate cyclin E-Cdk2, cyclin A-Cdk2, and cyclin B-Cdk1 complexes, whereas Ink4 proteins bind to and inhibit Cdk4 and Cdk6 kinases, by preventing their binding to D-type cyclins [1]. In turn, the retinoblastoma tumor suppressor protein Rb cannot be phosphorylated, and this prevents the release of E2F transcription factors that regulate cell cycle re-entry of sensory hair cells within the organ of Corti, followed by apoptosis [13]. Moreover, p19Ink4d is required for proper mouse tooth development [14, 15]. Further, Ink4d and Cdkn1b/p27Kip1 together are necessary to maintain cerebral cortex neurons and retinal progenitor cells in a post-mitotic state, and for postnatal survival [16, 17]. In the mouse, p18Ink4c is induced during myogenic differentiation [18], is transiently expressed in granule neuron progenitors to time their exit from the cell cycle [19], and is required to maintain the hematopoietic stem cell progenitor pool [20]. Thus, in mice both p16Ink6 and p15Ink4 contribute to the induction and/or maintenance of a post-mitotic state in differentiated tissues.

Here we evaluated the expression and function of Ink4 genes in early Xenopus laevis development. Only one Ink4 gene is highly expressed during Xenopus development and this encodes a protein, XI-Ink4d, that was highly similar to the mouse orthologue p19Ink4d, where XI-Ink4d can bind to and inhibit mouse Cdk4 kinase activity on Rb, and is sufficient to provoke G1 arrest in mouse fibroblasts.

Materials and Methods

Cloning of Xenopus Ink4 genes

A 139 base pair fragment was amplified from Xenopus laevis...
genomic DNA by PCR using published degenerate oligonucleotide primers predicted to amplify the first 139 bp region of exon 2 of Ink4-like genes [3]. This fragment was used as a probe to screen a Xenopus laevis adult spleen library (Lamda Zap Express) using standard methods [21]. Several clones were obtained and sequenced; however, only a single Ink4cDNA sequence was found (noted X-Ink4d1) that was similar to mouse and human Cdkn2d/p19Ink4d. Sequence analysis was performed using the DNA star software package (Lazergene).

**Production of GST-Xl-Ink4d and GST-Mm-Ink4d fusion proteins and Cdk4 binding assays**

The Xl-Ink4d1and mouse Ink4d(Mm-Ink4d) coding sequences were cloned in frame with an N-terminal GST-tag into pGEX-5X-1 and pGEX-2T, respectively (Amersham). The pGEX plasmids were transformed into BL21-D bacteria and their expression was induced with Isopropyl β-d-Thiogalactoside (IPTG) (0.1 mM) for 2 hour according to the manufacturer’s instructions. GST-tagged proteins were purified using glutathione-sepharose (Amersham) according to the manufacturer’s instructions. The coding sequence of Xenopuslaevis Cdk4 (Xl-Cdk4) was cloned into pCMVTNT™(Promega) and transcribed and translated in the presence of [35S]-methionine using TNT® Coupled Reticulocyte Lysate Systems according to the manufacturer’s instructions (Promega).

GST pull down assays were performed as described [22]. Briefly, in vitro transcribed and translated XI-Cdk4 (20 µl) was incubated with 1 µg of purified GST, GST-Xl-Ink4d1 (Xenopus), or GST-Mm-Ink4d (mouse) proteins immobilized on glutathione sepharose. The mixture was incubated at 4°C for 2 hour and washed several times in IP kinase buffer (50 mM HEPES pH 7.5, 10 mM MgCl2, 1 mM DTT, 2.5 mM EGTA, 10 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM MnNaF). Bound proteins were denatured and separated on a 12% (w/v) polyacrylamide-SDS gel and visualized by autoradiography [23].

**In vitro kinase assays**

In vitro Cdk4 kinase assays were performed as described [22,24], with minor modifications. Briefly, Spodoptera frugiperda S9 cells were infected with baculovirus encoding mouse Cdk4 and cyclin D1. Lysates from these cells were immuno-precipitated with Protein A-Sepharose pre-adsorbed to a Cdk4 antibody (C-22, Santa Cruz Biotechnology). After overnight incubation at 4°C, increasing amounts of GST-Xl-Ink4d1, GST-Mm-Ink4d4, or GST proteins were added to the reactions and incubated for 2 hour at 4°C. Immuno-precipitates were washed in IP kinase buffer (50 mM HEPES pH 7.5, 10 mM MgCl2, 1 mM DTT, 2.5 mM EGTA, 10 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM MnNaF) and in vitro kinase assays were performed using GST-Rb as substrate and [γ-32P]-ATP. The reactions were resolved by electrophoresis on 12.5% (w/v) poly-acrylamide-SDS gels, and analyzed by autoradiography [23].

**Virus infection and cell cycle analysis**

Retroviruses were generated as described [25], including control MSCV-IRES-GFP virus as well as a MSCV-Xl-Ink4d1-IRE-SFPend MSCV-Mm-Ink4d1-IRE-SFP virus. NIH-3T3 mouse fibroblast cells were infected with retroviruses and cultured for 24 hour. Cells were trypsinized, permeabilized and stained with propidium iodide to stain DNA. The DNA content of GFP-positive cells was measured by fluorescence-activated cell sorting (FAC scaler) and the data were analyzed using Cell quest software (Becton Dickinson).

**Xenopus embryo manipulations**

Xenopus laevis embryos were obtained, fertilized and microinjected as described [26]. Briefly, female frogs were induced to lay eggs by gonadotropin injection, fertilized in vitro with macerated testis and de-jellied with 3% (w/v) cyanide hydrochloride. Embryos were staged according to the Xenopus laevis normal tables of development [27].

**Reverse transcription PCR and quantitative RT-PCR**

Total RNA was isolated from Xenopus embryos at stages 2-41 using QiaShredder and Qiasey RNA isolation kit (Qiagen). RNA was reverse transcribed with Superscript II polymerase primed with oligoDT and PCR amplified with Hot Star Taq DNA polymerase (Qiagen). For RT-PCR trace [α32P]-dCTP was included in the reaction to allow detection of the PCR product by autoradiography. Reactions were separated on pre-cast 10% (w/v) polyacrylamide Tris–Borate–EDTA gels (BioRad), fixed, dried and exposed to X-ray film. For relative quantitative RT-PCR, reactions were performed on an Cycler thermocycler using iQ SYBR Green Supermix (Bio-Rad) and primers for Ink4d1 and ODC (as an internal control). ODC C values were subtracted from Ink4d1 C values (ΔCt) to normalize for input cDNA. Relative RNA levels were calculated by subtracting the ΔCt/endogenous from ΔCt/ODC and using the calculation 2-ΔΔCT. Primers used to amplify Xenopus Ink4d1 (forward; 5'-TTGTAGGATGTCGAGAATC-3', reverse; 5'-TGTAACCGGATACCTTGAC-3'), Ink4d-Q (quantitative) (forward; 5'-TCTCTGTCATACATCTTCTTGCCCT-3', reverse; 5'-TGGACAGTGTGTGTAATCT-3'), Cyclin-D1 (forward; 5'-ATCTGGAGACCTACAGATCTC-3', reverse; 5'-GGACAACTGCATTCCGACCG-3'), Cdk4 (forward; 5'-CAGCTGTGAGACCTACAGATCTC-3', reverse; 5'-ATGCGTGGACTGCATTCCGACCG-3'), and ODC (forward; 5'-GCTGCAATGTGTGTGTAATCT-3', reverse; 5'-CCATTCCGCTCTCTCCTGAGACCG-3')

**Immunoblotting**

Embryos were lysed in ice-cold lysis buffer (120 mM NaCl, 50 mM Tris-HCl [pH 8.0], 0.5% NP-40, 1 mM EDTA, and Complete protease inhibitors [Roche]). Lysates were cleared of lipid and yolk by Freon extraction (http://stop.colorado.edu/~klym/) and protein concentration was determined using a BCA Protein Assay Kit (Pierce). Equal amount of protein was resolved on 15% (w/v) polyacrylamide-SDS gels and transferred onto nitrocellulose membranes. To detect Xl-Ink4d4 protein, we raised a rabbit polyclonal antibody to the C-terminal peptide of Xl-Ink4d1 (amino acid sequence: SQQAAILDRLPASFELST) and affinity-purified the antibody using the same peptide. This peptide is unique to the predicted Xenopus/Xl-Ink4d4 protein and its sequence is shared between XI-Ink4d1, XI-Ink4d2 and the Ink4d of X-Tropical. It is not but those of Fugu, Mouse or Human. Thus the antibody does not cross react with mouse p19Ink4d protein (negative data not shown). Membranes were probed with an antibody against α-tubulin (Sigma) as a control for protein loading. Goat anti-rabbit HRP-conjugated secondary antibodies (Amersham) and Super Signal Dura (Pierce) were used to develop the blots.

**In situ hybridization of whole embryos**

In situ hybridization was performed as described [26]. Anti-sense probes were generated as described by Kelley et al.[29]. Briefly, anti-sense probes were synthesized with digoxigenin-coupled UTP (Roche) and detected with alkaline phosphatase coupled to anti-digoxigenin Fab fragments (Roche) followed by the chromogenic reaction with NBT/BCIP (Vectstain).

**Results and Discussion**

We identified a cDNA, Xl-Ink4d1 that was most similar to mouse.
p19\textsuperscript{inkd}(Mm-Ink4d). Blast search of the NCBI EST database using the Xl-Ink4d\textsubscript{1} sequence identified a second allele (Xl-Ink4d\textsubscript{2}) and the predicted amino acid sequences of these proteins was compared to the Ink4d proteins from other species (Figure 1). Alignment of the Ink4d proteins from Homo sapiens (human), Mus musculus (mouse), Xenopus tropicalis, and Fugu rubripes showed a high degree of amino acid conservation (63% amino acid identity between mouse and Xenopus Ink4d), suggesting a conserved function for this gene throughout evolution.

Two Ink\textsubscript{4} genes are expressed during mouse embryogenesis, Ink\textsubscript{kc} and Ink\textsubscript{kd}, whereas Ink\textsubscript{ka} and Ink\textsubscript{kb} are only expressed in adult and aging animals [8,9]. As three Ink\textsubscript{4} genes were identified in Xenopus tropicalis, we evaluated their expression by RT-PCR and real-time PCR in developmentally staged Xenopus\textsubscript{laevis} embryos (Figure 2a and 2b). Xl-Ink4d\textsubscript{1} transcripts were readily detected by RT-PCR and real-time PCR indicating high-levels of this transcript during early development (Figure 2a and 2b). Xl-Ink4b transcripts were detected only by real-time PCR and their detection required five additional amplification cycles compared to that of Xl-Ink4d\textsubscript{1} mRNA; thus, significantly lower levels of Xl-Ink4b vs. Xl-Ink4d\textsubscript{1} are expressed during early Xenopus\textsubscript{laevis} development. Moreover, Xl-Ink4c was undetectable by RT-PCR at any stage of embryonic development (not shown). This finding is in accord with the lack of Xl-Ink4c transcripts in publically available Xenopus tropicalis embryonic tissue EST libraries (Unigene (www.ncbi.nih.gov/Unigene) and in the Gurdon Institute Xenopus tropicalis EST database (http://informatics.gurdon.com.ad.uk)). Collectively, these data indicate that Xl-Ink4d\textsubscript{1} is the predominant Ink4 gene expressed during Xenopus development.

![Figure 1](image)

The Xl-Ink4d\textsubscript{1} transcript was maternally expressed in the egg and was evident before the mid-blastula transition (MBT, stage 8) when zygotic transcription begins. Xl-Ink4d\textsubscript{1} transcript levels decreased at the beginning of gastrulation (stage 10.5), remained low through neurulation (stage 22) and then increased at the end of neurulation (stage 25) (Figure 2a and 2b). In situ hybridization performed with in vitro transcribed antisense riboprobes revealed low levels of Xl-Ink4d\textsubscript{1} expression in the dorsal anterior region of the developing tadpole (Figure 2c). At neurula stages, Xl-Ink4d\textsubscript{1} was expressed in the neural plate (Figure 2c i-vi). By the late tail bud stage (stage 32a) Xl-Ink4d\textsubscript{1} expression was most prevalent in the brain, somite field, and eye and throughout the head (Figure 2c vii). In situ analysis using a probe that selectively detects the Xl-Ink4d\textsubscript{1} allele established that its expression was identical to that of Xl-Ink4d\textsubscript{1} indicating that expression of the two alleles is regulated in a similar manner (data not shown). Interestingly, mouse p19\textsuperscript{inkd} is expressed in the brain, spinal cord and dorsal root ganglia at embryonic day 13.5[9], suggesting a conserved function for Ink4d in neural development.
A rabbit polyclonal antibody generated against the C-terminus of Xl-Ink4d1, which is conserved between Xl-Ink4d1 and Xl-Ink4d2, detected the Xenopus protein by immuno-blotting with high specificity (Figure 2d). Immunoblotting of whole embryo extracts from staged embryos demonstrated equal levels of Xl-Ink4d protein in the unfertilized egg and through early development (up to stage 18) indicating that although mRNA levels fluctuated during development the protein levels did not change.

To determine if Xl-Ink4d functioned in a manner akin to that of mouse p19 Ink4d, we tested its activity in a series of in vitro and ex vivo experiments. Mammalian Ink4 proteins inhibit the kinase activity of Cdk4 by binding to Cdk4 and preventing its interactions with D-type cyclins[30]. We assessed the binding of Xl-Ink4d1, and of mouse Ink4d (Mm-Ink4d), to Xenopus laevis Cdk4 (Xl-Cdk4) by GST-pull down experiments (Figure 3a). Both GST-Xl-Ink4d1 and GST-Mm-Ink4d fusion proteins bound to Xl-Cdk4, whereas, as expected, GST alone failed to interact with Xl-Cdk4 (Figure 3a).

Cdk4 phosphorylates and inactivates the function of the Rb tumor suppressor [1]. To test the ability of the Xl-Ink4d1 protein to inhibit Cdk4 kinase activity, mouse Cdk4-cyclin-D1 complexes were immuno-precipitated from Sf9 insect cell lysate that were co-infected with baculoviruses expressing mouse cyclin-D1 and Cdk4 [31], and these complexes were then incubated with increasing concentrations of the purified GST-Ink4d1 fusion proteins. Both Xl-Ink4d1 and Mm-Ink4d inhibited Rb phosphorylation in a dose-dependent fashion (Figure 3b). Therefore, Xl-Ink4d1 can inhibit the kinase activity of mouse cyclin-D1/Cdk4 complexes.

Cdk4 kinase activity drives cell cycle progression through the G1 phase of the cell cycle and inhibition of Cdk4 activity by overexpression of mouse Ink4d arrests cells in this phase [21]. To test if Xl-Ink4d1 could also arrest mouse fibroblasts in G1 phase we infected NIH-3T3 mouse fibroblasts with retroviruses encoding Xl-Ink4d1 or Mm-Ink4d under the control of the MSCV promoter together with GFP, which is expressed from the same transcript through an Internal Ribosomal Entry Site (IRES) present in the MSCV-IRES-GFP retroviral vector [32]. Infected cells were analyzed 32 hours after infection by Fluorescence Activated Cell Sorting (FACS) and the DNA content of GFP-positive cells was determined by propidium iodide staining. Overexpression of either Xl-Ink4d1 or Mm-Ink4d significantly reduced the percentage of cells in S phase compared to cells infected with control retrovirus.
(from 34% to 16% and 24%, respectively) and led to corresponding increases in the percentage of cells in G1 phase (from 49% to 72% and 57%, respectively), indicative of an accumulation of cells arrested in G1 (Figure 3c). Therefore, the *Xenopus*laevis*Xl*-Ink4d1 protein can functionally substitute for the mouse *Mm*-Ink4d protein by binding to mouse Mm-Cdk4, inhibiting its cyclin D-dependent kinase activity and arresting mouse fibroblasts in G1 phase.

Altogether, our data demonstrate that *Xl*-Ink4d1 is a bona fide *Xenopus* orthologue of mammalian p19 Ink4d that is remarkably conserved throughout evolution. This is underscored by the facts that, similar to mouse Mm-Ink4d, *Xl*-Ink4d1 protein by binding to mouse Mm-Cdk4, inhibiting its cyclin D-dependent kinase activity and arresting mouse fibroblasts in G1 phase.

Acknowledgments

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**Figure 3:** *Xl*-Ink4d is a cyclin D-dependent Cdk4 inhibitor. (a) The indicated GST fusion proteins were expressed in bacteria and purified on glutathione sepharose. The adsorbed sepharose was incubated with *in vitro* transcribed and translated [*35S-*labeled *Xenopus* Cdk4 (*Xl*-Cdk4), washed and separated on an SDS-polyacrylamide gel. 50% of the input *Xl*-Cdk4 that was used for pull-downs was run to estimate the percent of starting material that bound to the GST fusion protein (50% input). (b) Cdk4 kinase reactions were run with increasing amounts of purified GST-*Xl*-Ink4d1 and GST-*Mm*-Ink4d (mouse Ink4d) fusion proteins. Active mouse Cdk4/cyclinD1 kinase complexes were generated from SF9 cells infected with baculoviruses encoding mouse Cdk4 and cyclin-D1. Kinase assays were performed with [*γ*-32P]-ATP, using purified GST-Rb as substrate. (c) The cell cycle profile of NIH-3T3 mouse fibroblasts overexpressing vector-alone (black bars), *Xenopus* *Xl*-Ink4d1 (dark grey bars) or mouse *Mm*-Ink4d (light grey bars). NIH-3T3 cells were infected with retroviruses that co-express the indicated Ink4d genes and GFP, and the DNA content of GFP-positive cells was measured by propidium iodide staining and FACS analysis. The percentage of cells in G1/G0, S and G2/M phase is presented. Expression of either *Xl*-Ink4d1 (*p=0.006) or *Mm*-Ink4d (***p=0.05) shows a significant decrease in S phase compared to vector alone.
References


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