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Cell Cycle Control by *Xenopus* p28^{Kix1}, a Developmentally Regulated Inhibitor of Cyclin-dependent Kinases

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We have isolated *Xenopus* p28^{Kix1}, a member of the p21^{CIP1}/p27^{KIP1}/p57^{KIP2} family of cyclin-dependent kinase (Cdk) inhibitors. Members of this family negatively regulate cell cycle progression in mammalian cells by inhibiting the activities of Cdks. p28 shows significant sequence homology with p21, p27, and p57 in its N-terminal region, where the Cdk inhibition domain is known to reside. In contrast, the C-terminal domain of p28 is distinct from that of p21, p27, and p57. In co-immunoprecipitation experiments, p28 was found to be associated with Cdk2, cyclin E, and cyclin A, but not the Cdc2/cyclin B complex in *Xenopus* egg extracts. *Xenopus* p28 associates with the proliferating cell nuclear antigen, but with a substantially lower affinity than human p21. In kinase assays with recombinant Cdks, p28 inhibits pre-activated Cdk2/cyclin E and Cdk2/cyclin A, but not Cdc2/cyclin B. However, at high concentrations, p28 does prevent the activation of Cdc2/cyclin B by the Cdk-activating kinase. Consistent with the role of p28 as a Cdk inhibitor, recombinant p28 elicits an inhibition of both DNA replication and mitosis upon addition to egg extracts, indicating that it can regulate multiple cell cycle transitions. The level of p28 protein shows a dramatic developmental profile: it is low in *Xenopus* oocytes, eggs, and embryos up to stage 11, but increases ~100-fold between stages 12 and 13, and remains high thereafter. The induction of p28 expression temporally coincides with late gastrulation. Thus, although p28 may play only a limited role during the early embryonic cleavages, it may function later in development to establish a somatic type of cell cycle. Taken together, our results indicate that *Xenopus* p28 is a new member of the p21/p27/p57 class of Cdk inhibitors, and that it may play a role in developmental processes.

INTRODUCTION

Progression through the cell cycle is controlled by the cyclin-dependent kinases (Cdks), which comprise a family containing various catalytic subunits and regulatory partners called cyclins. In mammalian cells, Cdk4/cyclin D, Cdk2/cyclin E, Cdk2/cyclin A, and Cdc2/cyclin B act sequentially at different points in the cell cycle (for review, see Draetta, 1993; Sherr, 1993). Although particular details vary, the central mechanisms of cell cycle regulation by Cdks are conserved from yeast to vertebrates.

Cdk activities are strictly controlled to ensure that a cell undergoes cell division cycles only under the appropriate circumstances. The Cdks are regulated by at least three distinct mechanisms: cyclin binding, subunit phosphorylation, and association with Cdk inhibitors. To date, two classes of Cdk inhibitors have been identified in mammalian cells (for review, see Elledge and Harper, 1994; Massagué and Polyak, 1995). The p15/p16 class includes p15^{INK4B} (Hannon and Beach, 1994), p16^{INK4} (Serrano *et al.*, 1993), p18 (Guan *et al.*, 1994), and p19 (Chan *et al.*, 1995). Proteins in this class share considerable sequence homology with each other. They exclusively associate with and inhibit D-type Cdks, and appear to play a role in cellular differ-

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entiation and tumor suppression (reviewed in Elledge and Harper, 1994). A second class of Cdk inhibitors includes p21 (also known as CIP1, WAF1, CAP20, and SDI1) (El-Deiry *et al.*, 1993; Gu *et al.*, 1993; Harper *et al.*, 1993; Xiong *et al.*, 1993; Noda *et al.*, 1994), $p27^{KIP1}$ (Polyak *et al.*, 1994b; Toyoshima and Hunter, 1994), and $p57^{KIP2}$ (Lee *et al.*, 1995; Matsuoka *et al.*, 1995). The N-terminal regions of these three proteins share significant homology; this domain can bind to and inhibit Cdk2/cyclin E, Cdk2/cyclin A, Cdk4/cyclin D, and to a lesser extent, Cdc2/cyclin B. Although p21 and p27 do not directly inhibit Cdk-activating kinase (CAK), they can associate with Cdks and prevent them from being phosphorylated and activated by CAK (Polyak *et al.*, 1994b; Aprelikova *et al.*, 1995). Except for a bipartite nuclear localization signal, the C-terminal domains of these proteins are not strongly conserved: $p21^{CIP1}$ binds proliferating cell nuclear antigen (PCNA; a DNA polymerase δ-subunit), while $p27^{KIP1}$ and $p57^{KIP2}$ do not (Waga *et al.*, 1994; Chen *et al.*, 1995; Luo *et al.*, 1995). Moreover, in the central regions, human $p57^{KIP2}$ has PAPA repeats while mouse $p57^{KIP2}$ has a proline-rich domain followed by acidic repeats (Lee *et al.*, 1995; Matsuoka *et al.*, 1995). The structural diversity among $p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$ suggests that these proteins may play distinct roles in cell cycle regulation.

p21 and p27 participate in diverse regulatory responses. Following radiation-induced DNA damage, the tumor suppressor protein p53 up-regulates p21 mRNA levels (El-Deiry *et al.*, 1993). p21 inhibits Cdk2/cyclin E activity, and thereby prevents DNA replication (Jackson *et al.*, 1995). Although the C-terminal domain of p21 associates with PCNA and blocks PCNA-dependent DNA replication, it does not inhibit PCNA-dependent DNA repair (Li *et al.*, 1994), giving irradiated cells the opportunity to remain in G1 and repair their DNA. Consistent with these observations, embryonic fibroblasts derived from $p21^{-/-}$ mice are significantly deficient in their ability to arrest in G1 in response to DNA damage (Deng *et al.*, 1995). Besides playing a role in the G1 checkpoint, p21 may also be involved in cellular differentiation under normal circumstances. For example, p21 mRNA levels increase in senescent cells (Noda *et al.*, 1994). Also, MyoD, a skeletal-muscle–specific transcriptional regulator, activates the expression of p21 during differentiation in a p53-independent fashion (Halevy *et al.*, 1995). The expression pattern of p21 in the mouse correlates with terminal differentiation and cell cycle withdrawal, suggesting roles in development (Parker *et al.*, 1995). However, $p21^{-/-}$ mice undergo normal development, and do not develop spontaneous tumors (Deng *et al.*, 1995), implying the existence of redundant pathways that ensure proper development and tumor prevention in this organism.

Although the functions of p27 and p57 are less well understood, they appear to play a role in differentiation-mediated cell cycle arrest and possibly in tumor prevention. In the mouse, most of the p57-expressing cells are terminally differentiated (Matsuoka *et al.*, 1995). The human p57 gene is located at a chromosomal region implicated in both sporadic cancers and a familial cancer syndrome, suggesting that p57 is a candidate tumor suppressor (Matsuoka *et al.*, 1995). The regulation of p27 appears to be cell-type dependent. In transforming growth factor β–arrested or contact-inhibited mink epithelial cells, p27 dissociates from Cdk4/cyclin D, and binds to and prevents the CAK-mediated activation of Cdk2/cyclin E (Polyak *et al.*, 1994a,b). In macrophages, cAMP exerts its antimitogenic effects by raising the level of p27, which then associates with Cdk4/cyclin D and prevents its activation by CAK (Kato *et al.*, 1994). During T cell mitogenesis, interleukin 2 signaling activates Cdk2/cyclin E complexes by eliminating the p27 protein, whereas p27 levels fail to drop when the immunosuppressant rapamycin is present (Nourse *et al.*, 1994). In at least some human cell lines, proliferating cells have a lower level of p27 due to an elevated p27-ubiquitinating activity that targets p27 to the ubiquitin-dependent proteasome degradation pathway (Pagano *et al.*, 1995).

Although considerable information about Cdk inhibitors has emerged recently, much remains to be learned about the evolution of these families and the diversity of their functions. Two Cdk inhibitors from *Saccharomyces cerevisiae* (the Cdc28/Cln inhibitor Far1 and the Cdc28/Clb2,5,6 inhibitor $p40^{SIC1}$) and one from *Schizosaccharomyces pombe* (the Cdc2/Cdc13 inhibitor Rum1) have been identified, but these show little homology with p15/p16 or p21/p27 Cdk inhibitors (for review, see Elledge and Harper, 1994). Since cell-free extracts from *Xenopus* eggs faithfully recapitulate many cell cycle events including DNA replication, mitosis, and various checkpoint mechanisms (Dasso and Newport, 1990; Leno and Laskey, 1991; Murray, 1991; Minshull *et al.*, 1994; Kumagai and Dunphy, 1995), it will be valuable to ascertain the extent to which Cdk inhibitors contribute to the regulation of the various Cdks present in this system. Because *Xenopus* embryos are readily available and easy to manipulate, *Xenopus* is also an attractive organism for the study of developmental regulatory mechanisms. Isolation of Cdk inhibitors from *Xenopus* and characterization of their upstream regulators, downstream targets, and expression during embryogenesis will contribute to our understanding of cell cycle regulation and its dynamic changes during development. With these goals in mind, we have searched for Cdk inhibitors in *Xenopus laevis* using a polymerase chain reaction (PCR)-based approach. Here, we report the

isolation and initial characterization of *Xenopus* $p28^{Kix1}$, a p21/p27-class Cdk inhibitor.

MATERIALS AND METHODS

Cloning of Xenopus p28

An internal fragment of *Xenopus* p28 was isolated by PCR using degenerate primers specific to conserved regions among human $p21^{CIP1}$, mouse $p21^{CIP1}$, and human $p27^{KIP1}$ (see Figure 1A). The 5′ primer was (5′) CGCGGATCCTG(C/T)(A/C)G(I/C)(I/C)(G/A)(I/C)(T/C)T(I/C)TT(C/T)GG(I/C)CC(I/C)GT (3′), and the 3′ primer was (5′) CGGGGTACCT(G/C)(I/C)IT(I/C)I(G/C)IAA(G/A)TC-(G/A)AA(A/G)TTCCA (3′). The 5′ end of each primer contains nine extra nucleotides (underlined) to provide restriction sites for *Bam*HI or *Kpn*I, respectively. PCR reactions (50 μl) contained 15 ng of *Xenopus* oocyte cDNA as template (Mueller *et al.*, 1995) and 50 pmol of each primer. PCR reactions were carried out as described (Mueller *et al.*, 1995), except that the first five cycles were 94°C for 1 min, 54°C for 2 min, and 72°C for 1 min, and the remaining 30 cycles were 94°C for 1 min, 57°C for 2 min, and 72°C for 1 min. An ~130-bp DNA fragment was isolated and cloned into the TA cloning vector (Invitrogen, San Diego, CA). After the fragment was sequenced to confirm its identity, it was radiolabeled by PCR and used to screen a *Xenopus* oocyte cDNA library by colony hybridization (Sambrook *et al.*, 1989; Mueller *et al.*, 1995). Approximately 1.2 million colonies were screened. Four positives were identified, two of which encoded the full-length *Xenopus* p28 gene. The entire cDNA was sequenced on both strands by primer walking using Sequenase (United States Biochemical, Cleveland, OH) with the dideoxy chain termination method. The GenBank accession number is U38844.

Subcloning of Xenopus p28 into Protein Expression Vectors

The pAX-NMT plasmid (Mueller *et al.*, 1995) harboring the full-length *Xenopus* p28 cDNA was mutagenized by PCR to create an *Nde*I site at the initiation codon. Briefly, the 5′ primer (5′) GGAAGTCCATATGGCTGCTTTCCACATCGC (3′) containing an *Nde*I site (underlined) and the 3′ primer (5′) CTAGATTCGATTG-GTGCCATGG (3′) containing an *Nco*I site (underlined) were used to amplify 10 ng of the pAX-NMT-p28 plasmid in the presence of 2.5 U of Pfu DNA polymerase and dNTPs in the buffer supplied by the manufacturer (Stratagene, La Jolla, CA). The reactions were heated to 94°C for 2.5 min and 95°C for 0.5 min followed by 20 cycles at 94°C for 1 min, 56°C for 2 min, and 75°C for 5 min. In addition, an extra 5 min at 75°C was added to the last cycle. After verification by sequencing, the PCR product was digested with *Nde*I and *Nco*I, generating fragment A (~650 bp), which included the entire coding region of *Xenopus* p28. Fragment B (~1 kb) containing the 3′-untranslated region of *Xenopus* p28 was obtained by digesting *Xenopus* p28 in the pBlueScript vector (Stratagene) with *Nco*I and *Eco*RI. Finally, the bacterial expression vector pET9-His6 (Kumagai and Dunphy, 1995) and the insect cell expression vector pVL1393-His6 (Tang *et al.*, 1993) were digested with *Nde*I and *Eco*RI, and were ligated with fragments A and B through a three-piece ligation. The resulting plasmids pET9-His6-p28 and pVL1393-His6-p28 were used for production of recombinant proteins. Sequence alignments were performed using the PILEUP program.

Antibody Production

Rabbits were immunized either with a C-terminal peptide from *Xenopus* p28 (CPLEQTPRKKIR) coupled to keyhole limpet hemocyanin or with purified *Xenopus* p28 protein expressed in bacteria (see below). Anti-peptide antibodies were affinity-purified on Affi-Gel 10 columns (Bio-Rad, Hercules, CA) containing covalently attached peptides. Anti-p28 protein antibodies were affinity purified on purified p28 protein coupled to CNBr-activated Sepharose 4B columns (Pharmacia Biotech, Uppsala, Sweden). Affinity-purified anti-*Xenopus* cyclin E1 antibodies and anti-*Xenopus* Cdk2 antibodies were a generous gift from J. Maller (University of Colorado, Denver, CO). Purified monoclonal anti-human PCNA antibodies and polyclonal rabbit anti-human p21 antibodies were purchased from PharMingen (San Diego, CA). Affinity-purified rabbit anti-mouse IgG antibodies were purchased from Cappel (West Chester, PA). Antibodies to *Xenopus* Cdc2, cyclin A1, and cyclin B2 were generously provided by A. Kumagai (Kumagai and Dunphy, 1995; our unpublished results).

Production and Purification of Proteins from Insect Cells and Bacteria

The pET9-His6-p28 and pET3d-His6-human p21 (Xiong *et al.*, 1993) plasmids were transformed into BL21(DE3)pLysS bacteria. The bacteria were grown to mid-log phase and then induced, harvested, and lysed as described (Kumagai and Dunphy, 1991). The lysates were clarified and the p28 protein was purified by nickel-IDA Sepharose chromatography (Kumagai and Dunphy, 1995). In the case of *Xenopus* p28, the protein was further purified using SDS-PAGE followed by electro-eluting in an Elutrap (Schleicher & Schuell, Keene, NH). The pure protein was used to produce rabbit anti-*Xenopus* p28 protein antibodies.

Histidine-tagged *Xenopus* p28, histidine-tagged human cyclin B1 (Kumagai and Dunphy, 1995), and histidine-tagged human cyclin A and cyclin E (Desai *et al.*, 1992; Koff *et al.*, 1992) were purified from insect cell lysates using established protocols (Desai *et al.*, 1992). *Xenopus* Cdc2- or human Cdk2-containing lysates were aliquoted, drop frozen in liquid nitrogen, and stored at −80°C. ^{35}S-labeled His6-p28 was purified from metabolically labeled insect cells using a standard protocol (Kumagai and Dunphy, 1995).

In Vitro Cdk Inhibition Assays

Active Cdk2/cyclin A, Cdk2/cyclin E, and Cdc2/cyclin B complexes were prepared essentially as described previously (Kumagai and Dunphy, 1995). Briefly, 20 μl of histidine-tagged cyclins bound to nickel-IDA beads were agitated with 100 μl of Cdk2- or Cdc2-containing insect cell lysates in the presence of 0.5 mM ATP and 10 mM $MgCl_2$ for 20 min at room temperature. The beads were then washed four times with ice-cold HBS (150 mM NaCl, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.5), and eluted with 20 μl of 300 mM imidazole in HBS. All kinase assays were performed in the linear range. Kinase complexes were mixed with increasing amounts of *Xenopus* p28 or human p21, preincubated on ice for 5 min, and finally histone H1 assays were performed as described in Dunphy and Newport (1989). To assess the effect of *Xenopus* p28 on the activation of the Cdc2/cyclin B complex in insect cell lysates, increasing amounts of p28 were incubated with Cdc2-containing insect cell lysates (2 μl) and purified cyclin B (0.3 μl) at room temperature for 20 min, and the histone H1 kinase activity was measured. Quantitation of kinase assays was performed with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Preparation of Extracts from Xenopus Eggs, Embryos, Oocytes, and Tissue Culture Cells

Xenopus cytostatic factor (CSF)-arrested egg extracts were prepared as described by Murray (1991). Interphase extracts were obtained by activation of CSF extracts with 0.4 mM $CaCl_2$. Freshly squeezed eggs were fertilized in vitro to obtain synchronously developing embryos (Newport and Kirschner, 1982a). Embryos were maintained in 0.2× MMR (Murray, 1991) for the first 6 h, and in 0.1× MMR thereafter. Embryos were staged according to the method of Nieuwkoop and Faber (1967). Typically, 20 embryos were homogenized in 200 μl of ice cold EB (80 mM β-glycerol phosphate, pH 7.3, 20 mM EGTA, and 15 mM $MgCl_2$) containing 1 mM dithiothreitol,

and 10 μg/ml each of pepstatin, chymostatin, and leupeptin (PCL). The homogenate was clarified by centrifugation at 16,000 × *g* for 5 min at 4°C. The crude cytoplasmic fraction was aliquoted and drop frozen in liquid nitrogen. Oocytes were separated from ovary tissue by treatment with collagenase (Cyert and Kirschner, 1988). Oocytes at different stages were manually selected and homogenized as described above for embryos. *Xenopus* tissue culture (XTC) cells were cultured using standard methods (Smith and Tata, 1991). Proliferating cells were harvested, and cell pellets were dissolved in SDS gel sample buffer.

Immunoprecipitation and Western Blotting

Affinity-purified anti-p28 protein antibodies (2 μg) or control rabbit anti-mouse IgG antibodies (2 μg) were incubated with protein A beads (Sigma, St. Louis, MO) in HBS for 1 h at 4°C. In the case of p21 immunoprecipitation, 2 μl of rabbit anti-human p21 antibodies were used. The antibody-coated beads were then incubated with mitotic extracts or interphase extracts for 1 h at 4°C. In some cases, recombinant p28 was added to the extracts, and in these experiments, cycloheximide was also included. Following incubation with the extracts, the beads were collected by centrifugation and washed four times with EB containing 0.1% NP-40, 25 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 10 μg/ml PCL, and then three times with HBS. All washes were done at 4°C. Similar washes were carried out for nickel-IDA beads recovered from the extracts (which had been diluted two-fold in EB containing PCL). To recover p28 using p13 beads, CSF extracts were incubated with a 25% volume of p13 beads (5 mg p13/ml beads) or control beads lacking p13 for 1 h at 4°C. Beads were removed by centrifugation, and the extracts were re-incubated with fresh beads. The p13 beads or the control beads were pooled, and washed as described for protein A beads. Immunoblotting using ^{125}I-labeled protein A (ICN, Irvine, CA) or ^{125}I-labeled sheep anti-mouse IgG antibodies (Amersham, Arlington Heights, IL) was performed as described (Coleman *et al.*, 1993). Alternatively, ECL (Amersham) was performed using horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (Bio-Rad).

Replication Assays

Replication assays were performed essentially as described previously (Dasso and Newport, 1990). Briefly, 40 μl of CSF extracts containing 100 μg/ml cycloheximide, 10 μCi [α-^{32}P]dCTP (ICN), and demembranated sperm nuclei (500 per μl) were incubated with 10 μl of Cdk inhibitors for 5 min at room temperature before activation with $CaCl_2$. At various time points, 3-μl aliquots were taken, mixed with replication sample buffer, and frozen at −20°C. The samples were digested with proteinase K (Boehringer Mannheim, Mannheim, Germany) and separated on a 0.8% agarose gel. Quantitation was performed using a PhosphorImager (Molecular Dynamics).

RESULTS

Isolation of Xenopus p28

To search for Cdk inhibitors from *Xenopus laevis*, we designed degenerate PCR primers based upon conserved residues in the Cdk inhibition domain of human and mouse $p21^{CIP1}$ as well as human $p27^{KIP1}$ (Figure 1A). PCR amplification of *Xenopus* oocyte cDNA yielded a ~130-bp fragment, which was used to isolate the corresponding full-length cDNA from an oocyte library. Several positive clones were identified; the longest (~1.7 kb) encodes a protein of 209 amino acids with a predicted molecular weight of 23,460 Da (Figure 1A). Since the endogenous protein in *Xenopus* extracts migrated at 28 kDa during SDS gel electrophoresis (see below), we have designated this protein as $p28^{Kix1}$ (for cyclin-dependent kinase inhibitor from *Xenopus*).

The N-terminal region of p28 is 35% identical to p21/p27-class Cdk inhibitors (Figure 1A). The most noticeable conservation is within the Cdk inhibition domain (the hatched box in Figure 1B; residues 30–90 in p28), which in the cases of p21, p27, and p57, is sufficient to bind and inhibit Cdks (Polyak *et al.*, 1994b; Luo *et al.*, 1995). The C-terminal regions of p28, p21, and p27/p57 are less well conserved (21–25% identical), but they all have a putative nuclear localization signal. In the case of p28, residues 166–182 (KREITTPITDYFPKRKK; the black box in Figure 1B) fit the consensus for the bipartite nuclear localization signal first found in nucleoplasmin (Dingwall and Laskey, 1991). Moreover, p28 has several Ser/Thr-Pro motifs (stars in Figure 1B) that are potential sites for phosphorylation by various mitotic kinases. Recently, Su *et al.* (1995) have cloned a distinct *Xenopus* Cdk inhibitor (Xic1) that is 90% identical to Kix1 at the amino acid level, indicating that multiple genes for Cdk inhibitors are expressed in this organism.

Recombinant p28 Can Inhibit Cdks via Two Mechanisms

To characterize its biochemical properties, p28 was expressed as a histidine-tagged fusion protein in baculovirus-infected insect cells, and purified using nickel-IDA affinity chromatography (Figure 2). His6-p28 migrated with an apparent molecular weight of 28 kDa, slightly larger than the endogenous *Xenopus* p28 (see Figure 6A).

Because p28 possesses a Cdk inhibition domain similar to that of other p21/p27 Cdk inhibitors, we examined whether p28 could inhibit various recombinant Cdk complexes. In particular, the effect of p28 upon Cdk2/cyclin E, Cdk2/cyclin A, and Cdc2/cyclin B was examined. Active Cdk complexes were prepared by mixing insect cell lysates containing the individual Cdk components under conditions that allowed formation of the complex and its activation by an endogenous insect cell CAK. After Cdk complexes were purified by nickel-IDA chromatography and mixed with either human p21 or *Xenopus* p28, their activities were measured with histone H1 as the substrate (Figure 3A). As expected, p21 inhibited Cdk2/cyclin E in a dose-dependent manner (Figure 3, A and B). In parallel experiments, p28 effectively inhibited the kinase activity of both Cdk2/cyclin E and Cdk2/cyclin A when present in only a fivefold molar excess of the Cdk (Figure 3, A and B). In contrast, p28 displayed little inhibitory activity toward Cdc2/cyclin B even at molar concentrations ~800-fold higher than the Cdk

complex. Finally, like p21 and p27, *Xenopus* p28 is heat stable: heating p28 to 100°C for 5 min had little effect upon its capacity to inhibit Cdk2/cyclin E (Figure 3C). Taken together, these data suggest that p28 is a Cdk inhibitor with a striking preference for the G1/S Cdks over the mitotic Cdk in these in vitro assays.

In addition to directly inhibiting Cdk activity, both the p21/p27 and p15/p16 classes of inhibitors have been observed to exert their effects by preventing CAK-mediated activation of Cdks (Polyak *et al.*, 1994b; Aprelikova *et al.*, 1995). To investigate the possibility that p28 might have a similar function, we added p28 during the step at which the active Cdk complex was prepared. Although p28 did not inhibit pre-activated Cdc2/cyclin B, it nevertheless blocked the formation of the activated Cdc2/cyclin B complex. At a concentration of 160 nM, p28 elicited a 90% reduction in the H1 kinase activity generated by mixing insect cell lysates containing Cdc2 and cyclin B (Figure 3D). This inhibition coincided with a reduction in the level of the threonine-161-phosphorylated (active) form of Cdc2 (our unpublished results), indicating that p28 can interfere with CAK-mediated activation of Cdc2/cyclin B.

p28 Associates with Cyclin-dependent Kinases in Xenopus Extracts

Having characterized the effect of p28 upon recombinant Cdks, we examined whether p28 might associate with any of these Cdks in *Xenopus* egg extracts. As an initial method to monitor the association of p28 with Cdks in egg extracts, we utilized p13-agarose beads, an affinity matrix that binds Cdc2, Cdk2, and associated proteins. p28 was recovered efficiently by p13 beads but not control beads (Figure 4A), suggesting that endogenous p28 is associated with Cdks in egg extracts. To identify which Cdks associate with p28, we immunoprecipitated p28 from egg extracts with anti-p28 whole protein antibodies, and subsequently subjected the immunoprecipitates to immunoblotting with various antibodies. Cyclin E1 and cyclin A1 (Figure 4B), but not cyclin B2, co-immunoprecipitated with p28. We also probed the immunoprecipitates

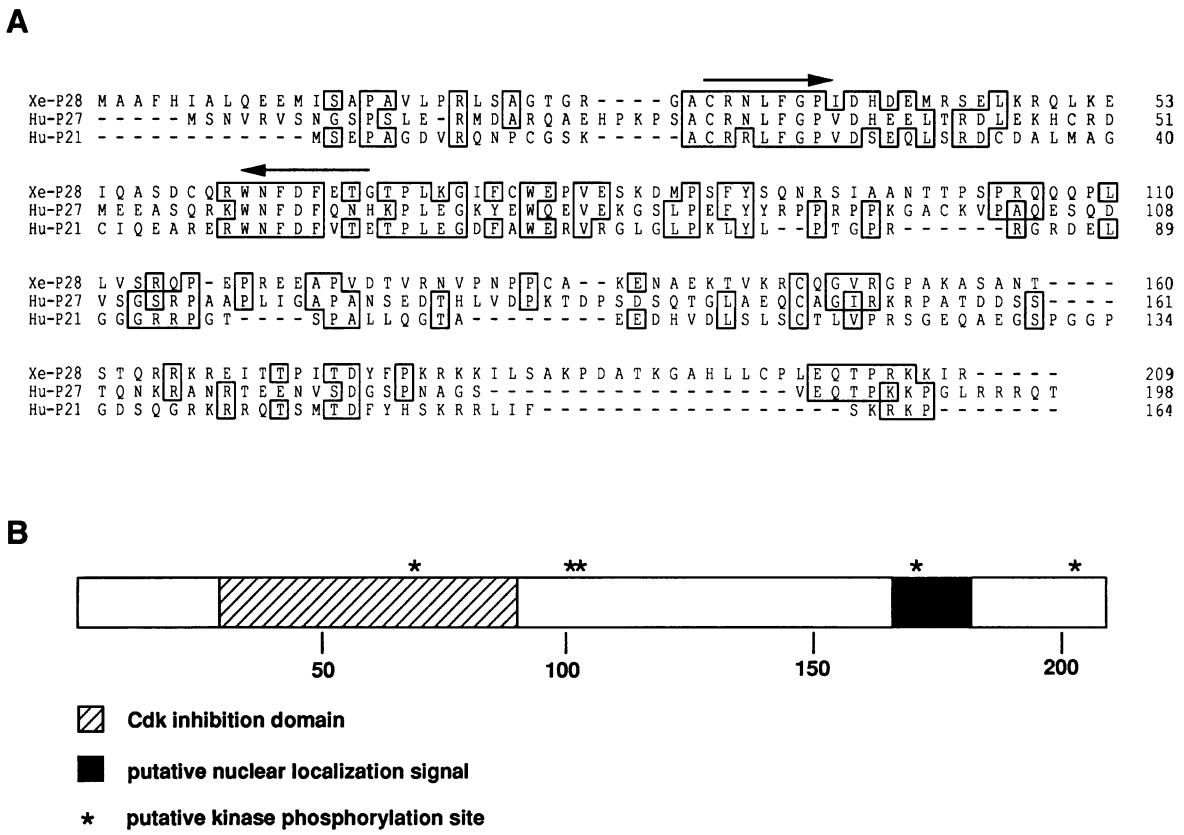

Figure 1. *Xenopus* p28 belongs to the $p21^{CIP1}/p27^{KIP1}$ Cdk inhibitor family. (A) Sequence alignment of *Xenopus* p28, human $p27^{KIP1}$, and human $p21^{CIP1}$. Boxes indicate identical residues shared by two or more sequences. Arrows mark sequences that were used to design degenerate PCR primers. (B) Schematic diagram of the domain structure of *Xenopus* p28. The CDK inhibition domain (hatched box) is conserved among *Xenopus* p28, human and mouse $p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$. A putative bipartite nuclear localization signal (black box) and several potential kinase phosphorylation sites (stars) are indicated. Numbers indicate amino acid residues.

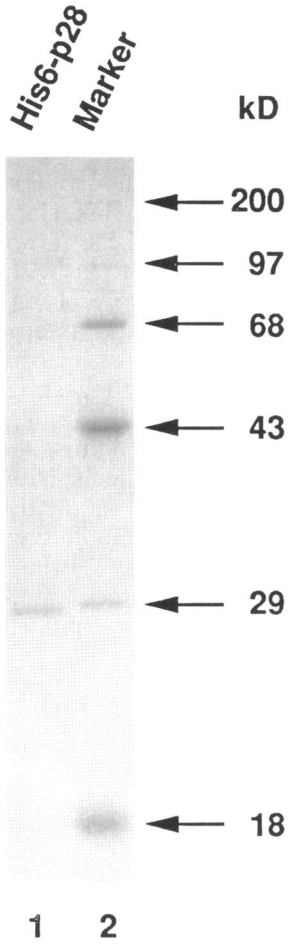

Figure 2. Purification of recombinant *Xenopus* p28. Recombinant His6-p28 was expressed in Sf9 insect cells and purified using nickel-IDA chromatography. Purified His6-p28 (lane 1) and molecular markers (lane 2) were run on a 12.5% SDS gel, and stained with Coomassie brilliant blue.

with anti-p28 antibodies to monitor the endogenous p28 protein (Figure 4B). Using recombinant p28 as a standard, we estimated that p28 was present at a rather low concentration (0.05 ng/μl, or 2 nM) in *Xenopus* egg extracts (our unpublished results). In control experiments with ^{35}S-labeled p28, we verified that the anti-p28 antibodies immunoprecipitated p28 quantitatively under these conditions. In accompanying studies, recombinant p28 was incubated with extracts, and then immunoprecipitated (Figure 4C). Immunoblotting of these immunoprecipitates revealed a significant association of p28 with cyclin E1 and Cdk2, but not cyclin B2. In addition, only small amounts of Cdc2 (perhaps complexed with cyclin A1 or A2) were detected in the anti-p28 immunoprecipitates.

Next, we tested whether p28 was modified during the cell cycle, and if so, whether this could affect its association with Cdks. Radiolabeled recombinant p28 was incubated with either mitotic or interphase extracts. The electrophoretic mobility of p28 incubated in mitotic extracts was reduced compared with p28 from interphase extracts (Figure 4D). Furthermore, the upshifted form of p28 could be shifted down by protein phosphatase 2A (our unpublished results), suggesting that certain kinase(s) in mitotic extracts can phosphorylate p28. Although p28 was differentially phosphorylated during the cell cycle, its association with Cdk complexes did not vary discernibly (Figure 4, B and C).

The C-terminal domain of human p21 associates with the replication and repair factor PCNA (Waga *et al.*, 1994; Chen *et al.*, 1995; Luo *et al.*, 1995). To determine whether p28 could bind PCNA, equivalent amounts (0.12 μg) of recombinant p28 or human p21 (as a positive control) were added to extracts, immunoprecipitated with their respective antibodies, and immunoblotted with anti-human PCNA antibodies, which cross-react well with *Xenopus* PCNA. As anticipated, anti-human p21 antibodies immunoprecipitated PCNA. In contrast, PCNA was not readily detected in anti-p28 immunoprecipitates (Figure 4E), suggesting that either p28 does not bind to PCNA or it binds more weakly than human p21. To explore this issue further, approximately 40-fold more recombinant p28 was added to the *Xenopus* extracts, and was later recovered with nickel-IDA beads. We observed that p28 could associate with PCNA under these conditions, but the amount of PCNA bound to 5 μg of p28 was less than that bound to 0.7 μg of p21 (Figure 4E). Thus, $p28^{Kix1}$, like the recently described Xic1 protein (Su *et al.*, 1995), can associate with PCNA, but not nearly as efficiently as human p21.

p28 Inhibits Chromosomal Replication and Mitosis in a Dose-dependent Manner

To explore further the functional properties of p28, we added recombinant p28 to cell cycle extracts from *Xenopus* eggs (Murray, 1991). Upon activation with Ca^{2+}, CSF-arrested mitotic egg extracts enter interphase, undergo a complete round of semi-conservative DNA replication, and enter mitosis shortly thereafter. Tracer radiolabeled p28 was found to be stable in extracts throughout the duration of such experiments.

We first asked whether p28 would affect chromosomal DNA replication, which is known to require Cdk2/cyclin E activity (Jackson *et al.*, 1995). Using [α-^{32}P]dCTP as a tracer, the extent of DNA replication was assessed at various time points after Ca^{2+} activation. In control extracts treated with buffer only, replication commenced between 30 and 45 min after Ca^{2+} addition, and was essentially complete by 120 min (Figure 5A). However, in extracts containing added p28, there was a strong inhibition of DNA replication (Figure 5A). At the highest concentration of p28 tested (1.6 μM), there was essentially no replication within the first 90 min. At later times, even though a small amount of replication took place, it clearly occurred at a substantially reduced rate relative to the control

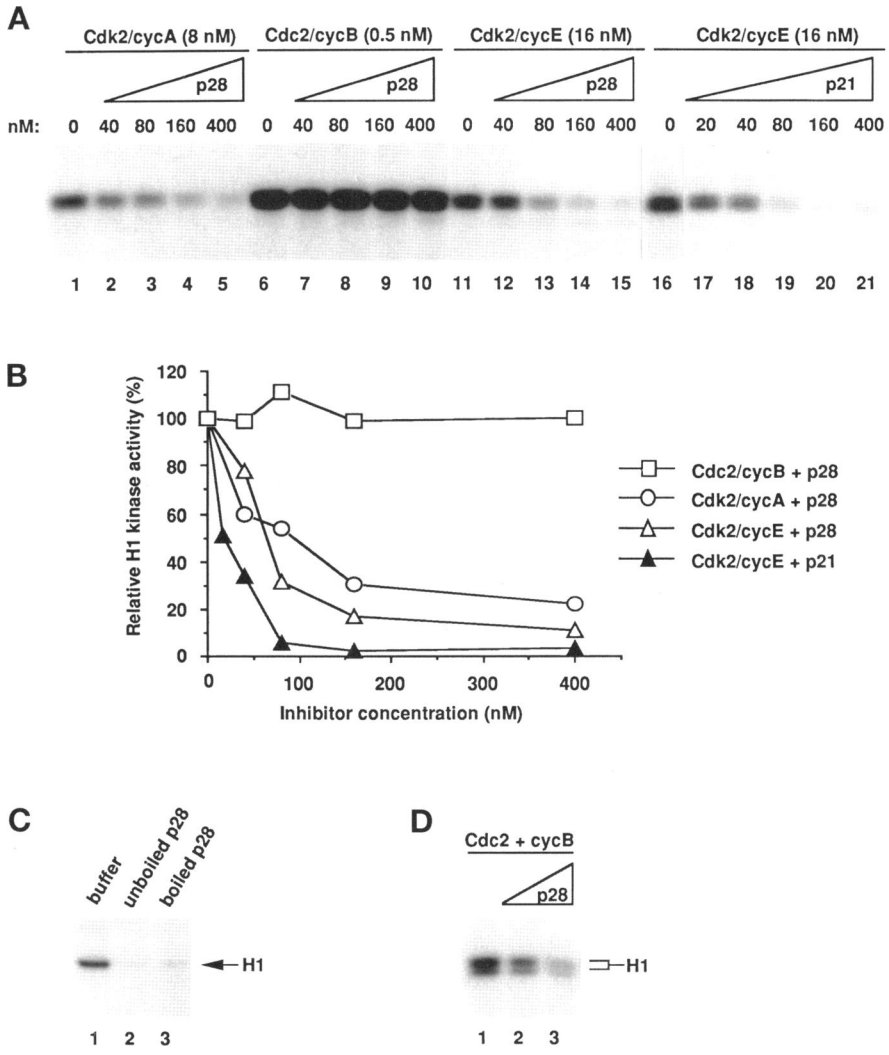

Figure 3. *Xenopus* p28 exhibits differential inhibitory activities toward various Cdk complexes. (A) Pre-activated Cdk2/cyclin A (lanes 1–5), Cdc2/cyclin B (lanes 6–10), and Cdk2/cyclin E (lanes 11–15) were incubated with the indicated amounts of p28. As a control, pre-activated Cdk2/cyclin E was also incubated with the indicated amounts of human $p21^{CIP1}$ under identical conditions (lanes 16–21). The H1 kinase activities of the Cdks were then measured. (B) H1 kinase activities in panel A were quantitated, and are plotted as the percentage of kinase activity in the absence of inhibitors. (C) p28 is heat stable. H1 kinase activities of Cdk2/cyclin E complex (16 nM) in the presence of control buffer (lane 1), native p28 (80 nM, lane 2), or p28 that had been boiled at 100°C for 5 min (80 nM, lane 3) were assayed. (D) p28 inhibits Cdc2/cyclin B activation. Cdc2-containing insect cell lysates were incubated at room temperature with purified cyclin B in the presence of either control buffer (lane 1) or p28 (40 nM, lane 2; 160 nM, lane 3). The mixtures were then subjected to H1 kinase assays.

extracts. A similar phenomenon was observed in extracts supplemented with the same amount of human p21 (Figure 5A). At lower concentrations of p28, the onset of replication was delayed in a dose-dependent fashion (Figure 5, A and B). In particular, replication commenced at 90 min and 60 min at p28 concentrations of 800 nM and 320 nM, respectively. Interestingly, once replication began, it proceeded at a similar rate to that in the control extracts. This observation might suggest that at lower concentrations (≤800 nM), p28 has a preferential effect on initiation versus elongation, whereas at higher concentrations, both processes are compromised. Finally, because chromosomal replication in egg extracts requires that the DNA be properly assembled into a nuclear structure, we verified by phase and fluorescence microscopy that the control and inhibitor-treated extracts were equally competent for nuclear assembly (our unpublished results).

We also examined whether recombinant p28 could affect the entry into mitosis in extracts containing a very low concentration of sperm chromatin (25 demembranated sperm nuclei per microliter of extract). It has been shown previously that this concentration of sperm nuclei is below the threshold necessary to trigger the replication checkpoint (Dasso and Newport, 1990). These extracts allow a direct assessment of the effect of p28 on mitosis independent of its effect on replication. Intriguingly, we observed that p28 elicited a dose-dependent delay of mitosis relative to control extracts (Figure 6A). At a concentration of 800 nM, p28 delayed mitosis by approximately 60 min. In parallel, we examined the effect of p28 (800 nM) on total H1 kinase activity during the cell cycle in egg extracts (Figure 6B). As expected, p28 suppressed the rise in H1 kinase activity that occurred in the control extracts at 90 min. Significantly, exogenously added

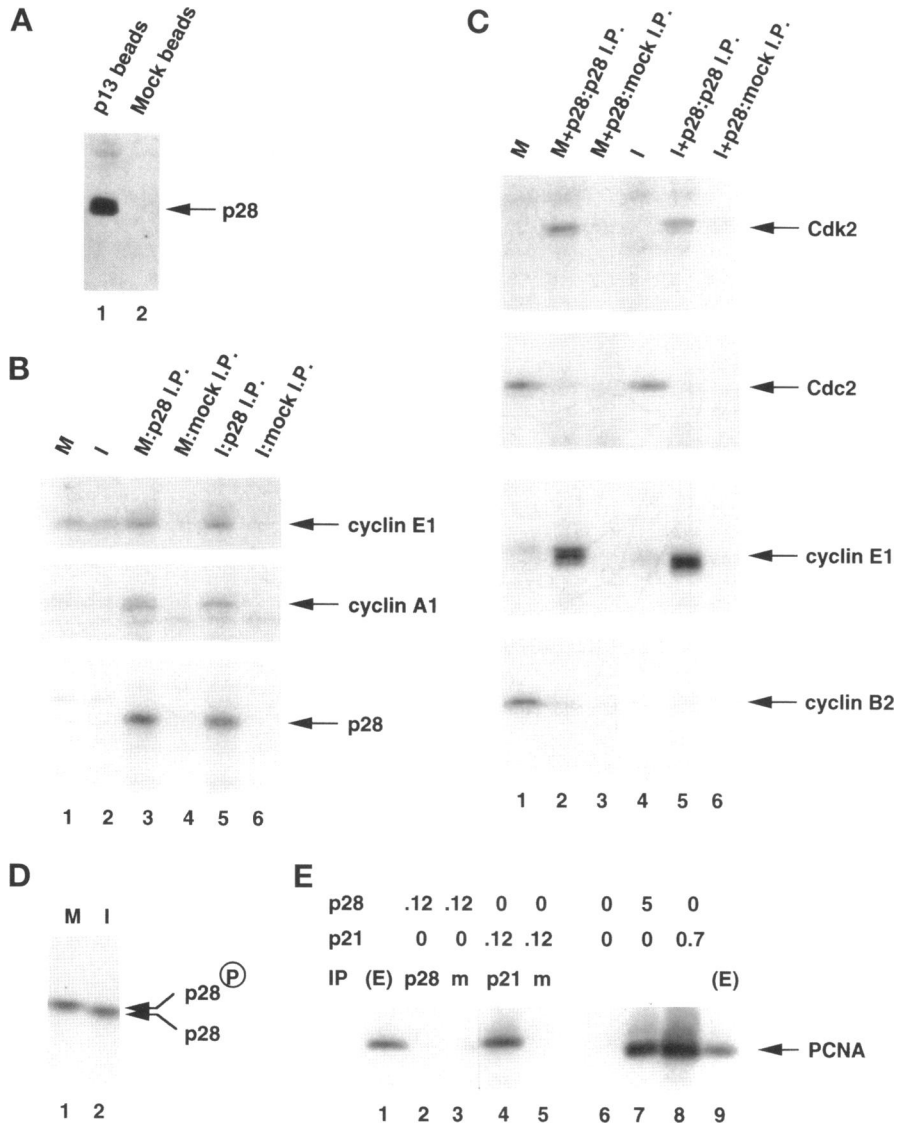

Figure 4. p28 associates with Cdks in *Xenopus* egg extracts. (A) M-phase extracts (160 μl) were rotated with p13 (lane 1) or control (lane 2) agarose beads. The beads were then washed and immunoblotted with anti-p28 peptide antibodies. (B) Mitotic (M-phase) extracts (300 μl, lanes 3 and 4) and interphase (I-phase) extracts (300 μl, lanes 5 and 6) were immunoprecipitated with anti-*Xenopus* p28 whole protein antibodies (lanes 3 and 5) or with control rabbit anti-mouse IgG antibodies (lanes 4 and 6). The total immunoprecipitates or M-phase and I-phase extracts (2 μl, lanes 1 and 2, respectively) were probed with antibodies against *Xenopus* cyclin E1, cyclin A1, and p28 as indicated. (C) Recombinant p28 (10 ng) was added to 100 μl of M-phase (lanes 2 and 3) or I-phase (lanes 5 and 6) extract. The total anti-p28 immunoprecipitates (lanes 2 and 5) and mock immunoprecipitates (lanes 3 and 6) from these extracts or 2 μl of M-phase and I-phase extract (lanes 1 and 4, respectively) were subsequently probed with antibodies against *Xenopus* Cdk2, Cdc2, cyclin E1, and cyclin B2 as indicated. (D) ^{35}S-labeled p28 was added to M-phase (lane 1) and I-phase (lane 2) extracts. The slower-migrating form in lane 1 corresponds to the phosphorylated p28. (E) Lanes 2–5: the indicated amounts (in μg) of His6-p28 or His6-p21 were added to M-phase extracts (50 μl), and immunoprecipitated with anti-*Xenopus* p28 (lane 2), or anti-human p21 (lane 4) antibodies, or mock-precipitated (m) with control rabbit anti-mouse IgG antibodies (lanes 3 and 5). Lanes 6–8: nickel-IDA beads and the indicated amounts (in μg) of His6-p28 or His6-p21 were incubated in 200 μl of two-fold diluted M-phase extract, and the beads were then recovered. The immunoprecipitates, the nickel-IDA beads, or M-phase extracts (E) (0.5 μl, lane 1; 1 μl, lane 9) were probed with anti-human PCNA antibodies.

p28 also depressed the level of H1 kinase activity at early times in the cell cycle (i.e., 30 min) when relatively little active Cdc2/cyclin B would be expected to be present. This effect could be due to inhibition of the Cdk2/cyclin E complex, which shows significant activity throughout the early embryonic cell cycles (Rempel *et al.*, 1995), or, alternatively, another unidentified Cdk. Taken together, our results indicate that at sufficiently high levels, not only does p28 abolish DNA replication, it also strongly inhibits entry into mitosis.

The Abundance of p28 Varies during Development

The abundance of p28 in *Xenopus* eggs is approximately 2 nM, whereas the concentration of recombinant p28 required to either inhibit recombinant Cdks in vitro or affect cell cycle progression in egg extracts is approximately 100-fold higher. To evaluate this paradox, we asked whether p28 might be expressed at higher levels during later stages of development when dividing cells acquire an extended G1 phase or withdraw from the cell cycle. For this purpose, extracts from oocytes, eggs, stage 26 embryos, and somatic XTC cells were immunoblotted with antibodies directed toward a C-terminal peptide of *Xenopus* p28. Although the level of p28 was similarly low in oocytes and mature eggs (~2 nM), it was approximately 100-fold higher in stage 26 embryos and XTC cells where the "somatic" cell cycle has replaced the "embryonic" one (Figure 7A). To pinpoint more precisely at what stage during development p28 begins to be up-

regulated, embryonic lysates were prepared at finer time points. The level of p28 remained low throughout the blastula and early gastrula stages. However, it increased dramatically between stages 12 and 13, and by stage 14, it had peaked to a level that remained relatively constant until at least stage 26 (Figure 7B; our unpublished results). The up-regulation of p28 occurs at ~5 h after the commencement of gastrulation, a time corresponding most closely to the small yolk plug stage and the slit-blastopore stage when the neural plate first becomes discernible.

To verify that the protein detected in these experiments is *Xenopus* p28, a peptide-competition experiment was carried out (Figures 7C). Immunoblots containing recombinant p28 and stage 26 embryonic lysates were treated with anti-p28 antibodies in the presence or absence of the immunizing peptide. The staining of both recombinant and embryonic p28 was abolished by the peptide, whereas that of a background band was unaffected. Taken together, these experiments indicate that the expression of p28 increases as cells acquire a somatic type of cell cycle.

DISCUSSION

To study the potential regulation of the cell cycle by Cdk inhibitors in *Xenopus* egg extracts, we have isolated *Xenopus* p28, a new member of the $p21^{CIP1}$/$p27^{KIP1}$/$p57^{KIP2}$ family of Cdk inhibitors. The Cdk inhibition domain of p28 shows significant sequence homology to those of p21, p27, and p57. Indeed, p28 effectively inhibits pre-activated G1/S Cdks, such as Cdk2/cyclin E and Cdk2/cyclin A, while exhibiting little inhibitory activity toward the mitotic Cdc2/cyclin B complex in vitro, like some other members of the family (Harper *et al.*, 1995; Lee *et al.*, 1995). Consistent with this observation, Cdk2, cyclin A, and cyclin E in egg extracts can be co-immunoprecipitated with p28, whereas the Cdc2/cyclin B complex appears not to be stably associated with p28.

It has been shown previously that Cdk inhibitors in the p21/p27/p57 class are also able to block the phosphorylation and activation of Cdks by CAK without directly binding to CAK or inhibiting CAK activity (Harper *et al.*, 1993, 1995; Polyak *et al.*, 1994b; Aprelikova *et al.*, 1995; Matsuoka *et al.*, 1995). p28 has

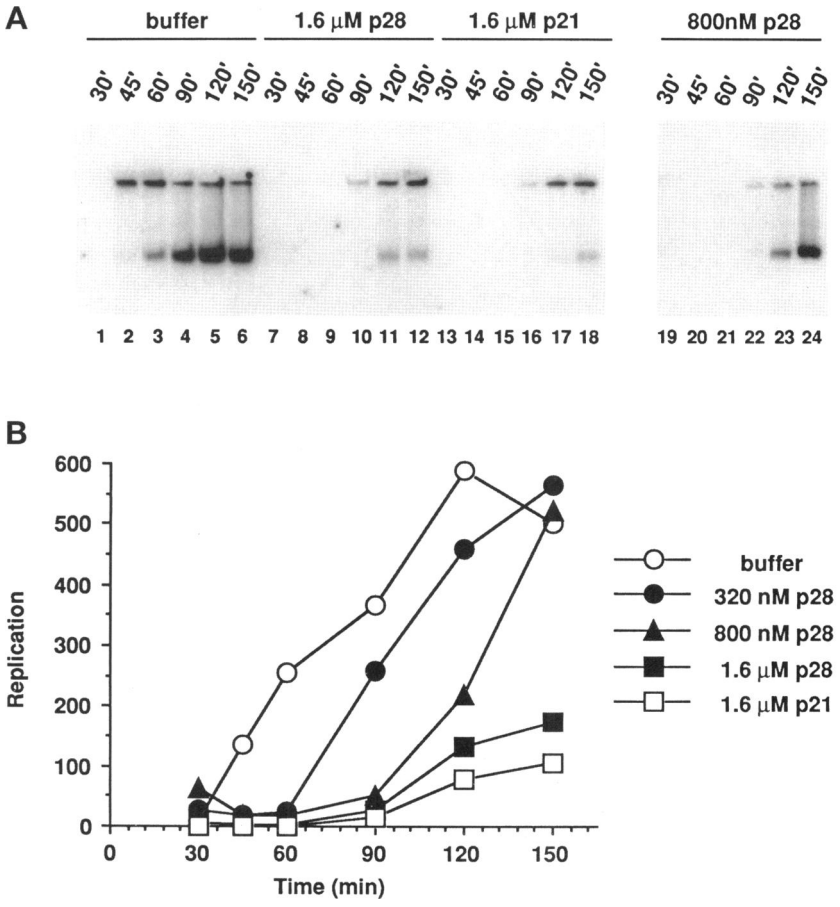

Figure 5. *Xenopus* p28 inhibits chromosomal replication in a dose-dependent manner. (A) CSF-arrested extracts (500 sperm nuclei/μl, 0.2 μCi [α-^{32}P]dCTP/μl) were incubated with buffer (lanes 1–6), *Xenopus* p28 (1.6 μM, lanes 7–12; 0.8 μM, lanes 19–24), or human $p21^{CIP1}$ (1.6 μM, lanes 13–18). The extracts were then activated by $CaCl_2$, and at the indicated time points, samples were taken to assay the extent of replication by monitoring the total incorporation of [^{32}P]dCTP into DNA. (B) Quantitation of various replication assays in arbitrary units (including those presented in part A).

similar properties. In particular, p28 does not bind to recombinant human CAK (our unpublished results), nor does it inhibit the kinase activity of CAK toward the C-terminal peptide of RNA polymerase II (our unpublished results). However, p28 prevents Cdc2/cyclin B activation by CAK, suggesting that despite the apparent preference for G1/S Cdks, p28 could down-regulate mitotic Cdk activities through prevention of CAK-mediated activation. It seems paradoxical that p28 has little affinity for Cdc2/cyclin B or CAK, yet it is able to prevent the latter from activating the former. One possible explanation is based on the observation that multiple molecules of p21 are required to inhibit Cdks and that complexes containing a single p21 molecule are active (Zhang *et al.*, 1994). Thus, it is possible that a single molecule of p28 could bind the Cdc2/cyclin B complex to block CAK-mediated activation, whereas multiple molecules of p28 could not efficiently associate with and inhibit the pre-activated complex. Alternatively, the off-rate of p28 from Cdc2/cyclin B and/or CAK might be fast so that the kinase-inhibitor complexes do not survive successive washing steps in our binding assays. p28 would presumably also inhibit the CAK-mediated activation of Cdk2/cyclin E and Cdk2/cyclin A, since it has a higher affinity for these Cdks.

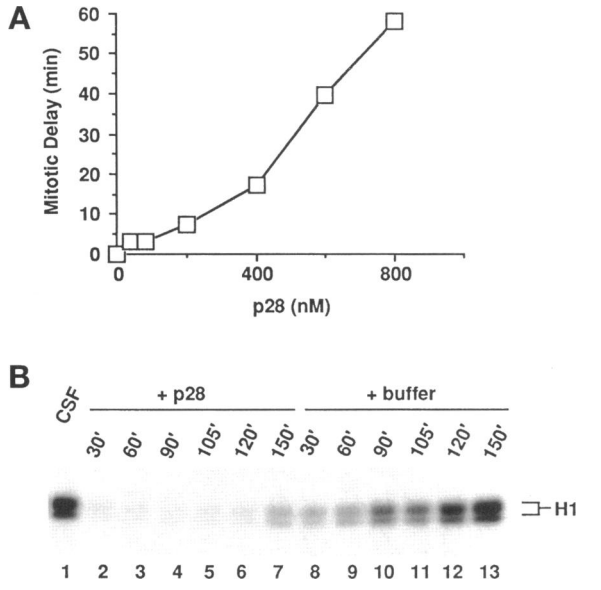

Figure 6. *Xenopus* p28 inhibits mitosis in a dose-dependent manner. (A) CSF extracts (containing 25 sperm nuclei/μl extract) were mixed with various amounts of *Xenopus* p28 protein and then activated. Entry into mitosis was scored visually and was defined as the time point where 50% of the nuclear envelopes had broken down relative to buffer-treated extracts. (B) H1 kinase activities of a CSF extract (lane 1) or activated extracts containing either p28 (800 nM, lanes 2–7) or control buffer (lanes 8–13) were measured at the indicated times after Ca^{2+} addition. The p28-treated and the control extracts entered mitosis at 150 min and 90 min, respectively.

We also analyzed the biochemical functions of p28 by adding recombinant p28 to *Xenopus* egg extracts. Cdk2/cyclin E activity is required for the initiation of chromosomal DNA replication in this system (Jackson *et al.*, 1995). In egg extracts, the concentration of cyclin E1 is ~60 nM (Rempel *et al.*, 1995). At a fivefold molar excess above cyclin E1, p28 elicited a readily observable delay in the onset of replication, consistent with the observed inhibitory effects of p28 on recombinant Cdk2/cyclin E complex. Intriguingly, although there was a delay in the onset of DNA synthesis, once replication began, it proceeded at a rate comparable to that in control extracts. At higher levels of p28, both the rate and the overall extent of DNA replication were severely inhibited. These findings could argue that although p28 preferentially inhibits initiation as opposed to elongation, at higher concentrations, p28 could inhibit elongation as well. An alternative possibility would be that when the level of p28 is high, some replication origins could fire late, giving rise to an extended S phase reminiscent of the somatic cell cycle (for review, see Fangman and Brewer, 1992). Finally, p28 could block initiation of some replication origins, possibly by preventing the transition from pre-replicative foci to initiation complex, a phenomenon previously observed when high levels (1–2 μM) of human p21 were added to extracts (Jackson *et al.*, 1995; Yan and Newport, 1995). Further mechanistic studies will be required to evaluate these possibilities.

We have observed that p28 also elicits a dose-dependent delay of mitosis. In principle, this mitotic delay could result from the replication checkpoint responding to p28-dependent inhibition of DNA synthesis. However, we feel that this explanation is not likely because p28 elicits a mitotic delay even in the presence of a concentration of sperm chromatin (25 sperm/μl of extract) well below the threshold required to trigger the replication checkpoint in this system (Dasso and Newport, 1990). One explanation is that p28 delays mitosis by inhibiting the CAK-mediated activation of Cdc2/cyclin B, which would be consistent with the ability of p28 to block the activation of Cdc2/cyclin B in insect cell lysates. Alternatively, it is conceivable that p28 could be a more potent inhibitor of the endogenous Cdc2/cyclin B in egg extracts than the purified recombinant Cdc2/cyclin B. Finally, it could be that cyclin E1- and/or cyclin A-associated kinase activities play an additional essential role in mitosis, and that by inhibiting the kinase activities associated with these cyclins, p28 impedes the entry into mitosis.

An important characteristic of human p21 is that it can form a stable complex with the replication factor PCNA. The PCNA-interacting domain of p21 has been mapped to the peptide QTSMTDFY (residues 144–151) (Warbrick *et al.*, 1995). The three residues that contribute the most to PCNA binding in p21 (Q144, M147, and F150) are not conserved in *Xenopus* $p28^{Kix1}$

or Xic1 (Su *et al.*, 1995), which could account for the observation that PCNA binds *Xenopus* p28 much less well than it binds human p21. Human and mouse p27 apparently do not interact with PCNA (Luo *et al.*, 1995), raising the possibility that *Xenopus* p28 may belong to the mammalian p21 subfamily. However, the C-terminal domain of p28 does contain a QT motif (LEQTPRK, residues 200–206) that is found in mammalian p27 and p57 (see Matsuoka *et al.*, 1995). Thus, further characterization of p28 will be required to classify this Cdk inhibitor definitively.

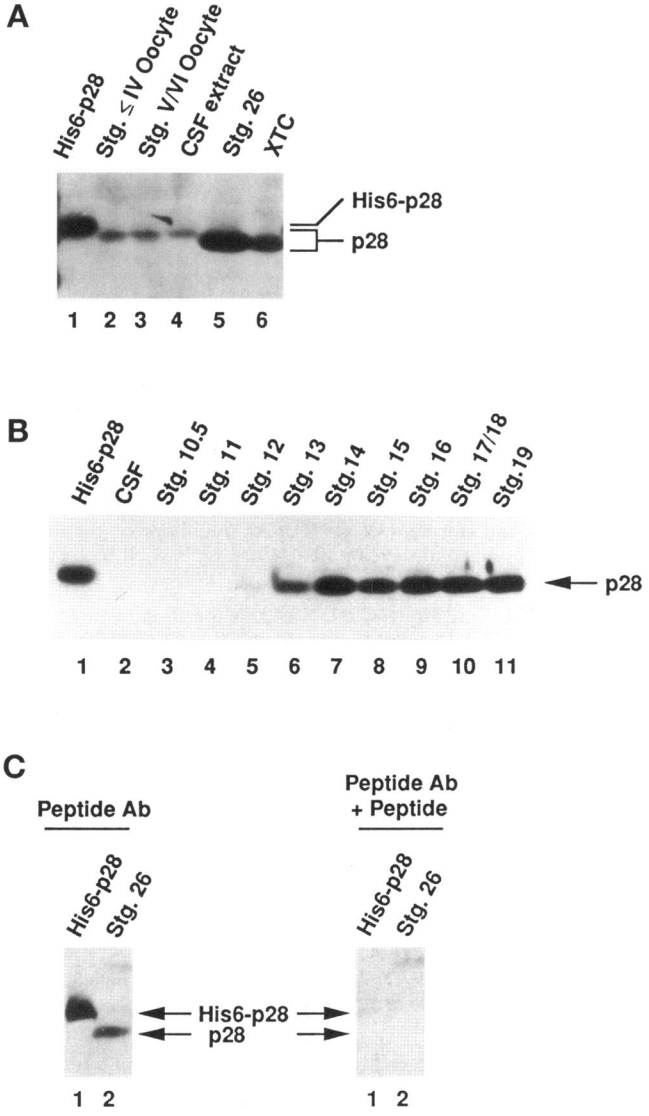

Figure 7. *Xenopus* p28 is developmentally regulated. (A) A comparison of p28 protein levels during *Xenopus* development. His6-p28 (10 ng, lane 1) and extracts made from stage IV or earlier oocytes (100 μg, lane 2), stage V/VI oocytes (100 μg, lane 3), CSF-arrested eggs (100 μg, lane 4), stage 26 embryos (60 μg, lane 5), and XTC cells (60 μg, lane 6) were immunoblotted with anti-p28 peptide antibodies. (B) The p28 protein level is elevated during stages 12 and 13. One nanogram of His6-p28 (lane 1), 100 μg of CSF extract (lane 2), and 60 μg of extract made from embryos at the indicated stages (lanes 3–11) were immunoblotted with anti-p28 peptide antibodies. (C) Peptide-blocking assay. Identical strips containing His6-p28 and stage 26 embryo extracts were immunoblotted with anti-p28 peptide antibodies (400 ng/ml) in the absence (left panel) or presence (right panel) of the p28 C-terminal peptide (200 ng/ml). All blots were visualized using ECL.

The next best candidate, namely cyclin A1, is also more abundant (18 nM-60 nM) than p28 in *Xenopus* eggs (Rempel *et al.*, 1995). Although it is conceivable that p28 might regulate a Cdk distinct from cyclin E1 and cyclin A1, a more plausible scenario is that p28 does not play a rate-limiting role in controlling the onset of replication or mitosis during early embryogenesis. This notion would be consistent with the fact that the early embryonic cleavages occur very rapidly (i.e., approximately every 35 min) without any discernible gaps between S-phase and M-phase. The midblastula transition (stage 8½) defines the first developmental event where the cell cycles in certain embryonic cells begin to slow down and become asynchronous with respect to one another (Newport and Kirschner, 1982a). This transition also coincides with the commencement of zygotic transcription (Newport and Kirschner, 1982b). Later, at stage 10, another major transition occurs during early gastrulation. This early gastrulation transition is marked by the elimination of maternal mRNAs for cyclins A1 and A2 (Howe *et al.*, 1995). In parallel, the amount of cyclin A1 protein drops precipitously to undetectable levels, whereas the level of cyclin A2 protein translated from zygotic cyclin A2 mRNA increases dramatically (Howe *et al.*, 1995; see also Rempel *et al.*, 1995). The level of *Xenopus* p28 protein remains low at both the midblastula transition and early gastrulation transition, but it does increase dramatically at a significantly later time, namely during stages 12 and 13. In principle, the up-regulation of p28 at stages 12 and 13 could mark another developmental transition at late gastrulation in the *Xenopus* embryo. Alternatively, the increase in the levels of p28 could represent a more specialized regulatory mechanism that selectively targets certain cell types. The period encompassing stages 12 and 13 coincides with numerous significant events in development. In the case of neuronal differentiation, primary neuronal precursors undergo their final round of DNA replication (stage 12), the transcript for neuronal signaling molecule X-Delta-1 becomes expressed in prospective neurons (stage 12), and the gene for neuron-specific type-II β-tubulin is turned on in scattered cells in the neural plate (stage 12.5–13) (Hartenstein, 1989; Chitnis *et al.*, 1995). Clearly, it will be highly important to localize

the p28 mRNA and protein by in situ methods to delineate precisely the developmental events that might be related to the up-regulation of p28. In any case, it appears likely that p28, like p21, p27, and p57, may play a role in cellular differentiation. The target of p28 in later embryos has not been established, but cyclin D, the presumed somatic form of cyclin E, or the recently described somatic cyclin A2 are potential candidates (Howe *et al.*, 1995; Rempel *et al.*, 1995).

In summary, we have identified *Xenopus* p28, a new member of the p21/p27 class of Cdk inhibitors. In vitro, p28 inhibits pre-activated Cdk2/cyclin E and Cdk2/cyclin A, and prevents CAK-mediated activation of Cdc2/cyclin B. In *Xenopus* egg extracts, exogenously added p28 inhibits both DNA replication and mitosis. Finally, the level of p28 protein is up-regulated dramatically during stages 12 and 13, which temporally correlates with the earliest events in neural differentiation. In concert with other transitions such as the replacement of embryonic Cdks with somatic Cdks, p28 could play an important role in regulating the somatic cell cycle. Further study of p28 will help us to gain more insight into cell cycle regulation and its connection with developmental regulation.

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