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Multiple Cdk1 Inhibitory Kinases Regulate the Cell Cycle during Development

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The Wee kinases block entry into mitosis by phosphorylating and inhibiting the activity of the mitotic cyclin-dependent kinase, Cdk1. We have found that the various *Xenopus* Wee kinases have unique temporal and spatial patterns of expression during development. In addition, we have isolated and characterized a new Wee1-like kinase, *Xenopus* Wee2. By both *in vivo* and *in vitro* tests, *Xenopus* Wee2 functions as a Wee1-like kinase. The previously isolated Wee1-like kinase, *Xenopus* Wee1, is expressed only as maternal gene product. In contrast, *Xenopus* Wee2 is predominantly a zygotic gene product, while the third Wee kinase, *Xenopus* Myt1, is both a maternal and zygotic gene product. Concurrent with the changing levels of these Cdk inhibitory kinases, the pattern of embryonic cell division becomes asynchronous and spatially restricted in the *Xenopus* embryo. Interestingly, once zygotic transcription begins, *Xenopus* Wee2 is expressed in regions of the embryo that are devoid of mitotic cells, such as the involuting mesoderm. In contrast, *Xenopus* Myt1 is expressed in regions of the embryo that have high levels of proliferation, such as the developing neural tissues. The existence of multiple Wee kinases may help explain how distinct patterns of cell division arise and are regulated during development.

Key Words: **Wee1; Myt1; Wee2; Cdc2; gastrulation; cell cycle; *Xenopus*; cell division; morphogenesis; embryogenesis.**

INTRODUCTION

The coordination of cell division and morphogenesis is critical for the proper development of an organism. In *Xenopus*, cell division is rapid and synchronous during the cleavage stages, slows during the blastula stages, and becomes spatially restricted and asynchronous during the gastrula and neurula stages. The early cleavage-stage divisions promote a rapid increase in cell number by using a modified cell cycle that consists of little more than alternating S and M phases. These S-M cycles are characterized by their clockwork-like synchrony, their dependence on maternal gene products, and their lack of zygotic transcription and cellular growth (Masui and Wang, 1998; Newport and Kirschner, 1982a). The initial slowing of the cell cycle during *Xenopus* development takes place at the midblastula transition (MBT) concurrent with the onset of zygotic transcription (Frederick and Andrews, 1994; Masui and Wang, 1998; Newport and Kirschner, 1982b). However, the cell cycle remains under the control of maternal transcripts for several hours after MBT (Newport and Dasso, 1989). Just before gastrulation begins at the early gastrula transition (EGT), there is a marked loss or destruction of several maternal cell cycle regulators (Audic *et al.*, 2001; Hartley *et al.*, 1997; Howe *et al.*, 1995; Howe and Newport, 1996; Kim *et al.*, 1999; Stack and Newport, 1997). This allows the zygotically encoded cell cycle regulators to pattern the cell cycle, and the more typical G_1-S-G_2-M cell cycle is observed in many cell types. These zygotically controlled cell divisions must coordinate their rate and position in the cell cycle with morphogenetic events, such as gastrulation and cell-type specification. In the post-EGT embryo, cell divisions are asynchronous and cell-autonomous, and both mitotically active and inactive domains are observed in the embryo (Saka and Smith, 2001).

The establishment of spatially restricted regions of proliferating and nonproliferating cells during development is not unique to *Xenopus*. For example, elegant

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studies in *Drosophila* have shown that there is a predictable spatial pattern of cell division once zygotic transcription begins (Foe, 1989). In most cases, this pattern is preceded by the transcription of the cell cycle activator String, the fly homolog of the phosphatase Cdc25 (Edgar and O'Farrell, 1989). Recent studies have shown that the proper spatial patterning of cell division is required for gastrulation in *Drosophila*. An early event in *Drosophila* gastrulation is ventral furrow formation. This involves the invagination of the presumptive mesodermal cells into the ventral region of the embryo. During this process, the invaginating mesodermal cells must stop proliferating (reviewed in Johnston, 2000). This is accomplished by the regional down-regulation of Cdc25 activity. In mutants or experimental situations that permit the continual proliferation of the presumptive mesodermal cells, ventral furrow formation and gastrulation are blocked (Grosshans and Wieschaus, 2000; Mata *et al.*, 2000; Seher and Leptin, 2000). While a comparable level of understanding the coordination of cell proliferation and morphogenesis in vertebrates is still in its infancy, recent studies have shown that the dorsal mesoderm that involutes during *Xenopus* gastrulation is also devoid of proliferating cells (Saka and Smith, 2001). In this study, we have found that an antagonist of Cdc25, *Xenopus* Wee2 (XWee2), is expressed in the involuting mesoderm of *Xenopus*, suggesting a parallel pathway of cell cycle regulation during gastrulation in frogs and flies.

In all eukaryotic cells, the rate of cell division is ultimately controlled by modulating the activity of the various cyclin-dependent kinases (Cdks). The regulation of Cdk activity is complex, consisting of association with accessory factors and posttranslational phosphorylations of the Cdk (Morgan, 1997). For Cdk1, the Cdk that triggers entry into mitosis, inhibitory phosphorylation of residues in the ATP-binding domain is critical for its regulation (reviewed in Lew and Kornbluth, 1996). Members of the Wee family of kinases add these phosphates and inhibit Cdk1 activity, while members of the Cdc25 family of phosphatases remove these phosphates and restore Cdk1 activity.

The first Wee kinase isolated was Wee1 from fission yeast, *Schizosaccharomyces pombe* (Russell and Nurse, 1987). Subsequently, Wee1 homologs have been found in many other eukaryotes, including humans, *Saccharomyces cerevisiae*, *Xenopus*, and *Drosophila*, and are collectively referred to as Wee1 or Wee1-like kinases (Booher *et al.*, 1993; Campbell *et al.*, 1995; Igarashi *et al.*, 1991; Mueller *et al.*, 1995a; Nakanishi *et al.*, 2000; Watanabe *et al.*, 1995). Wee1 and Wee1-like kinases phosphorylate the tyrosine 15 residue (Tyr15) of Cdk1 (reviewed in Lew and Kornbluth, 1996). Myt1, a related member of the Wee family of kinases, also phosphorylates Cdk1 at Tyr15 as well as the adjacent threonine 14 residue (Thr14) (Booher *et al.*, 1997; Liu *et al.*, 1997; Mueller *et al.*, 1995b). Note that, in this work, we will use the phrases "Wee family of kinases" or "Wee kinases" to include both the Myt1 kinases (Myt1) and the Wee1/Wee1-like kinases (Mik1, Swe1, Wee1, Wee1A, Wee1B, and Wee2) (Booher *et al.*, 1993; Lundgren *et al.* 1991; Mueller *et al.*, 1995a,b; Nakanishi *et al.*, 2000; Russell and Nurse, 1987; and this work).

Xenopus Myt1 (XMyt1) and Wee1 (XWee1) have been shown to play important roles in oogenesis and early development. During oogenesis, XMyt1 activity is required to phosphorylate Cdk1 and maintain the G_2-like arrest of the oocyte in early prophase of meiosis I (Nakajo *et al.*, 2000). When progesterone signals the oocyte to mature into an egg, XMyt1 activity is decreased concomitant with the activation of Cdc25 (Palmer and Nebreda, 2000). During meiosis II, XWee1 protein is synthesized from maternal pools of message (Charlesworth *et al.*, 2000). However, this newly synthesized XWee1 protein is not thought to be required until after fertilization, when it extends the first mitotic cell cycle by phosphorylating Cdk1 (Iwabuchi *et al.*, 2000; Murakami *et al.*, 1999; Nakajo *et al.*, 2000; Walter *et al.*, 2000).

Both XMyt1 and XWee1 proteins are present during the subsequent cleavage-stage cell cycles (Murakami and Vande Woude, 1998; data not shown). Together with the Cdc25 phosphatases, these Wee kinases are thought to be responsible for the periodic changes in the levels of Cdk1 inhibitory phosphorylation that are observed during interphase and mitosis of these synchronous cell cycles (Iwabuchi *et al.*, 2000; Kim *et al.*, 1999). Once the developing embryo has reached MBT, zygotic transcription begins and the cell cycle lengthens and becomes asynchronous (Hartley *et al.*, 1996; Newport and Kirschner, 1982a). At the whole organism level, Cdk1 continues to be phosphorylated on Tyr15 at least through neurulation (Hartley *et al.*, 1996; Murakami and Vande Woude, 1998; data not shown). However, the levels of XWee1 protein begin to decrease at the end of gastrulation and become nearly undetectable after neurulation (Murakami and Vande Woude, 1998; data not shown). This decrease in XWee1 protein levels raises the possibility that another *Xenopus* Wee kinase might be expressed after MBT when zygotic expression begins.

In this study, we have identified a new Wee kinase in *Xenopus*, XWee2. This kinase is expressed concurrently with the acquisition of a somatic-like cell cycle and in regions of the embryo that are devoid of mitotic cells, specifically in the involuting mesoderm. Using both *in vitro* and *in vivo* assays, we have found that XWee2 phosphorylates Cdk1 more efficiently than the maternally encoded XWee1. In addition, XWee2 is more similar to human Wee1 (Watanabe *et al.*, 1995) than to XWee1. Thus, in vertebrates, there are two related families of Wee1-like kinases that are maternally and zygotically expressed. Finally, we have found that each of the Wee kinases in *Xenopus* (Wee1, Myt1, and Wee2) has a unique temporal and spatial pattern of expression during development, suggesting that each plays a unique role in patterning cell proliferation during embryogenesis.

METHODS AND MATERIALS

RNA Isolation and XTC cDNA Production

Total RNA was extracted from various stage *Xenopus* embryos or adult *Xenopus* tissues by using Trizol Reagent (Gibco/BRL/Life Technologies, Gaithersburg, MD) as per the manufacturer's recommendations, except that an additional acidic phenol extraction and a LiCl precipitation were performed to further purify the RNA. Embryos were staged according to Nieuwkoop and Farber (1994). Total RNA and poly $(A)^+$ RNA were prepared from *Xenopus* XTC tissue culture cells (Pudney *et al.*, 1973) as described (Mueller *et al.*, 1988, 1995a). XTC cDNA was prepared from XTC poly $(A)^+$ RNA by using the SuperScript Preamplification System for First-Strand cDNA Synthesis Kit as per manufacturer's suggestions (Gibco/BRL/Life Technologies, Gaithersburg, MD), except that RNasin (Promega, Madison, WI) was added to the reaction.

Isolation of cDNA Encoding Xenopus Wee2 (XWee2)

An internal *Xenopus* Wee1-like kinase fragment was isolated by PCR amplification of XTC cDNA (see above) by using degenerate primers specific to regions of high amino acid similarly shared between the Wee1-like kinases of Human (Igarashi *et al.*, 1991; Watanabe *et al.*, 1995), *Xenopus* (Mueller *et al.*, 1995a), *Drosophila* (Campbell *et al.*, 1995), and sea urchin (Nemer and Stuebing, 1996) (see Fig. 1A). The 5′ primer was CGCGGATCCAA(C/T)(A/G)T(A/C/G/T)AA(C/T)CC(A/C/G/T)TT(C/T)AC(A/C/G/T)CC and the 3′ primer was CGGGGTACCTT(A/G/T)AT(A/C/G/T)GC(A/G)TA(A/G/T)A(A/T)(A/G)CA(A/C/G/T)CC(A/G)TC. The 5′ end of each primer contains nine extra nucleotides (underlined) that provide restriction sites for *Bam*HI or *Kpn*I. A 300-bp DNA fragment was isolated from the PCR, and sequencing of the fragment revealed that it was similar to the Wee1 kinases. However, the sequence was different that the previously isolated isoforms of *Xenopus* Wee1. Two closely related isoforms of Wee1 have been isolated from *Xenopus*. These have been designated XWee1A and XWee1, but collectively share the name XWee1 (Mueller *et al.*, 1995a; Murakami and Vande Woude, 1998). The 300-bp PCR fragment was used to screen a *Xenopus* kidney cDNA library (Stratagene, La Jolla, CA) by plaque hybridization (Sambrook and Russell, 2001). Two clones where obtained, but neither contained a full-length cDNA. The 5′ end of the cDNA was first obtained by using a RACE kit (Clonetech, Palo Alto, CA) as per the supplier's recommendation. This generated a 1.2-kb fragment that was used to screen a stage 42 *Xenopus* cDNA library (a generous gift of M. King) by plaque hybridization (Sambrook and Russell, 2001). From this library, a complete cDNA was obtained and sequenced. This clone was designated *Xenopus* Wee2 (XWee2), and the sequence has been assigned the GenBank Accession No. AF358869.

Baculoviral Expression Plasmids and Vectors

The coding regions of XWee2 and XWee1 (XWee1A) (Mueller *et al.*, 1995a) were amplified by PCR using the PFX DNA polymerase (Gibco/BRL/Life Technologies, Gaithersburg, MD). This amplification changed the sequence immediately upstream of the starting AUG codon so that an *Nde*I site (CATATG) overlapped the start site of translation. The amplified coding regions were subcloned into pFBHT-NdeI; a modified pFastbac plasmid with the *Nco*I site converted into an in-frame *Nde*I site (M. R. Lake and P.R.M., unpublished results). PCR-mediated site-directed mutagenesis was used to create kinase-deficient versions of XWee1 and XWee2 as described (Higuchi *et al.*, 1988). Specifically, Lys253 was changed to Arg in XWee2, and Lys239 was changed to Arg in XWee1. Together, these manipulations created the following plasmids: pFBHT-NdeI-XWee1-WT, pFBHT-NdeI-XWee1-KD, pFBHT-NdeI-XWee2-WT, and pFBHT-NdeI-XWee2-KD. These plasmids were sequenced to confirm that no errors were introduced, and then used subsequently to create all other plasmid constructs (see below), or to create baculoviral stocks of XWee1-WT, XWee1-KD, XWee2-WT, and XWee2-KD as per the FAST-BAC to BAC system (Gibco/BRL/Life Technologies, Gaithersburg, MD). Baculoviral stocks of XCdk1 N133A (Asp133 changed to Ala), XCdk1 N133A, T14A, Y15F (Asp133 changed to Ala, Thr14 changed to Ala, Tyr15 changed to Phe), XCdk1 T161A (Thr161 changed to Ala), and histidine-tagged human Cyclin B1 were produced as described (Kumagai and Dunphy, 1995; Mueller *et al.*, 1995b).

Construction of Expression and in Situ Template Plasmids

The coding regions of the wild type and kinase-deficient XWee2 and XWee1 were isolated from pFBHT-NdeI-based vectors and subcloned into several vectors. These included the *S. pombe* episomal expression vector pRep41 (Maundrell, 1993) to create pRep41-XWee2-WT and pRep41-XWee2-KD, and the *S. pombe* stably integrating expression vector pSULN2 to create pSULN2-XWee2-WT and pSULN2-XWee2-KD. pSULN2-derived vectors integrate into the *ura4* locus of *S. pombe*, complement *his7* deficiency in *S. pombe*, and use the full strength *S. pombe nmtI* promoter to drive expression (W. F. L. and P.R.M., unpublished results). In addition, the coding regions of the wild type and kinase-deficient XWee2 and XWee1 were subcloned into pXenHT to create pXenHT-XWee1-WT, pXenHT-XWee1-KD, pXenHT-XWee2-WT, and pXenHT-XWee2-KD. pXenHT is used for *in vitro* production of mRNA (see below) and is a modified version of pXen1 (MacNicol *et al.*, 1997) that replaces the amino terminal GST tag with an amino-terminal six-histidine tag (M. R. Lake and P.R.M., unpublished results). Furthermore, the coding regions of the wild type and kinase-deficient XWee2 were subcloned into pCS+MT (Turner and Weintraub, 1994) to create pCS2+MT-XWee2-WT and pCS2+MT-XWee2-KD for DNA microinjections (see below). Finally, the coding regions of wild type XWee1, XWee2, and XMyt1 (Mueller *et al.*, 1995b) were subcloned into the pGem4z vector (Promega, Madison, WI) to create pGem4z-XWee1, pGem4z-XWee2, or pGem4z-XMyt1. These pGem-derived plasmids were used for the production of *in situ* probes (see below). Additional plasmids constructs made include pRep1-XCdc25C, which contains the coding region of *Xenopus* Cdc25C (Kumagai and Dunphy, 1992) in the episomal *S. pombe* expression vector pRep1 (Maundrell, 1993), and pGem3z-XChordin, which contains the coding region of *Xenopus* Chordin (Sasai *et al.*, 1994) in the *in situ* probe production vector pGem3z (Promega, Madison, WI).

Expression of XWee2 in Fission Yeast

The *S. pombe* $Wee1^{ts}$ ΔMik1 strain (Lundgren *et al.*, 1991) was transformed by using electroporation (Hood and Stachow, 1995) with the episomal plasmids pRep41-XWee2-WT, pRep41-XWee2-KD, pRep41 (Maundrell, 1993), or pSV40-SpWee1 (Carpenter *et al.*, 1996). Transformants were selected at 25°C on minimal media (PM) (Moreno *et al.*, 1991), supplemented with adenine and thiamine (2 μM), but lacking leucine. Colonies that grew in the absence

of leucine were selected and grown on PM lacking leucine and thiamine at 25 or 35°C. In the absence of thiamine, the *nmt1* promoter is derepressed and the XWee2 constructs are expressed.

The *S. pombe* strain FWP60 (a generous gift of C. Hoffman) (Apolinario *et al.*, 1993) was transformed with linearized pSULN2-XWee2-WT, pSULN2-XWee2-KD, or pSULN2 by using the lithium acetate method (Grallert *et al.*, 1993). Transformants were selected at 25°C on PM supplemented with adenine, leucine, uracil, and thiamine (2 μM), but lacking histidine. From these transformants, homologous integrants where selected on PM in the presence of 5-Fluoroorotic acid (FOA) (1 g/L) at 25°C (Grimm *et al.*, 1988). Stable integration into the *ura4* locus was confirmed by passaging three times on rich media (YES) (Moreno *et al.*, 1991) in the presence of thiamine and by PCR. Liquid cultures of strains that had XWee2-WT, XWee2-KD, and the empty vector stably integrated were grown on PM lacking thiamine for 24 h before being photographed. The strains of *S. pombe* that had XWee2-WT, XWee2-KD, and the empty vector stably integrated into the *ura4* locus were transformed by using electroporation with pRep1-XCdc25C. These transformants were selected on PM supplemented with adenine, uracil, and thiamine (2 μM), but lacking histidine and leucine. Growth of the XCdc25 transformed strains in liquid culture was as described above.

Production and Purification of Recombinant Proteins and in Vitro Kinase Assays

Sf9 insect cells were infected with viral stocks of XWee1-WT, XWee1-KD, XWee2-WT, and XWee2-KD as described (Kumagai and Dunphy, 1995; Mueller *et al.*, 1995a). After 48 h of growth, cells lysates containing the histidine-tagged XWee1-WT, XWee1-KD, XWee2-WT, or XWee2-KD proteins were prepared. Briefly, infected cells were washed with phosphate-buffered saline (PBS), resuspended in 10 ml of lysis buffer [50 mM Na_2HPO_4, 300 mM NaCl, 10 mM imidazole, pH 7.8, 0.5% NP-40, 15 mM 2-mercaptoethanol, and PCL protease inhibitors (10 μg/ml each of pepstatin, chymostatin, and leupeptin)], and sonicated on ice. Lysates were clarified by centrifugation, and the resulting supernatant was added to 100 μl of swelled and washed Ni-IDA beads as described (Kumagai and Dunphy, 1995; Mueller *et al.*, 1995a) and incubated at 4°C for 60 min with rotation. Bound beads were washed four times with 50 bed volumes of wash buffer (50 mM Na_2HPO_4, 750 mM NaCl, 25 mM imidazole, 5 mM EGTA, pH 7.8, 10 mM 2-mercaptoethanol, and PCL protease inhibitors), except that the second, third, and fourth washes each had 5, 2.5, and 0 mM 2-mercaptoethanol, respectively. The purified proteins were eluted from the beads with elution buffer (50 mM Na_2HPO_4, 300 mM NaCl, 250 mM imidazole, 5 mM EGTA, pH 7.2, and PCL protease inhibitors), dialyzed against 10 mM Hepes, 150 mM NaCl, pH 7.4, quantified, and stored at −80°C until use. Recombinant XCdk1 N133A; XCdk1 N133A, T14A, Y15F; XCdk1 T161A; XCdk1 WT; XCdk1 T14A; XCdk1 Y15F; XCdk1 T14A, Y15F; and histidine-tagged human Cyclin B1 proteins were produced and purified as described (Kumagai and Dunphy, 1995; Mueller *et al.*, 1995a,b).

Complexes between histidine-tagged human cyclin B1 and the various forms of *Xenopus* Cdk1 were prepared as described (Kumagai and Dunphy, 1995). Two *in vitro* kinase assays were used to phosphorylate the various forms of Cdk1. In the first, the indicated Cdk1/Cyclin B complexes were phosphorylated in the presence of [^{32}P]ATP (125 μCi/ml) and the indicated amounts of purified recombinant XWee1 or XWee2 protein as described (Mueller *et al.*, 1995a). Following a 20-min incubation at 21°C, the samples were processed for autoradiography, and the results were quantified by using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). In the second assay, the indicated Cdk1/Cyclin B complexes were phosphorylated by XWee2 or XWee1 in the presence of an ATP regenerating system as described (Mueller *et al.*, 1995a,b). Following a 30-min incubation at 21°C, samples were processed for immunoblotting and subsequent H1 kinase assays. Recombinant XWee2, recombinant XCdk1, and tyrosine phosphorylated Cdk1 were detected with antibodies against human Wee1 (H-300; Santa Cruz Biotechnology, Santa Cruz, CA), *Xenopus* Cdk1 (Coleman *et al.*, 1993), and phosphotyrosine (4G10; Upstate Biotechnology, Lake Placid, NY), respectively. H1 kinase assays were performed as described (Mueller *et al.*, 1995a,b).

Microinjection of Protein, mRNA, or DNA into Xenopus Embryos

At the two-cell stage, embryos were transferred to 0.3× MMR, 3% ficoll and microinjected with 23 nl of protein, mRNA, or DNA into one blastomere of the two-cell embryo (Sive *et al.*, 2000). In the case of *in vitro*-translated ^{35}S-radiolabeled protein, both blastomeres of the two-cell embryo were microinjected with 23 nl. Injections were in the animal pole near the middle of the blastomere. Microinjected embryos where maintained in 0.3× MMR, 3% ficoll for 1 h, then transferred to 0.1× MMR, 3% ficoll as described (Kim *et al.*, 1999). Subsequently, embryos were either photographed (for DNA microinjections) or fixed in MEMPFA, dehydrated in 100% ethanol, and photographed (for protein and mRNA microinjections) as described (Sive *et al.*, 2000). The proteins for microinjections were prepared as described above from baculovirus-infected Sf9 cells. Proteins were diluted to 100 pg/nl in 10 mM Hepes, 150 mM NaCl, pH 7.4, before microinjection. The 5′ methyl capped mRNA was prepared by *in vitro* transcription of linearized pXenHT-XWee1-WT, pXenHT-XWee1-KD, pXenHT-XWee2-WT, or pXenHT-XWee2-KD as described (Sive *et al.*, 2000). This mRNA was diluted to 100 pg/nl in H_2O before microinjection. The pCS2+MT-XWee2-WT and pCS2+MT-XWee2-KD plasmids were prepared with Quantum Prep (Bio-Rad, Hercules, CA) and diluted to 25 ng/μl with 0.1× MBS prior to injection (Sive *et al.*, 2000).

The ^{35}S-radiolabeled proteins were prepared in a Rabbit Reticulocyte Lysate System (Promega, Madison, WI) in the presence of Tran[^{35}S]-Label (ICN Biochemicals, Irving, CA) using the 5′ methyl capped mRNA prepared from pXenHT-XWee1-WT and pXenHT-XWee2-WT described above. Before microinjection, unincorporated Tran[^{35}S]-Label was removed by using a Quikspin TE column (Roche, Indianapolis, IN) that was equilibrated with 0.1× MMR and used as per manufacturer's suggested protocol. Embryos were prepared as described above for microinjection, and 23 nl of *in vitro* translated ^{35}S-radiolabeled protein was microinjected into both blastomeres of the two-cell embryo. At the indicated stages of development, embryos were collected, subjected to total protein isolation as described (Hartley *et al.*, 1996), and processed for autoradiography.

Northern Analysis of Total RNA from Embryos and Tissues

Total RNA samples (10 μg each) were resolved by formaldehyde gel electrophoresis (Bryant and Manning, 1998). During the electrophoresis, the gel was photographed to visualize the ethidium bromide-stained 18S and 28S RNA bands. At the end of the

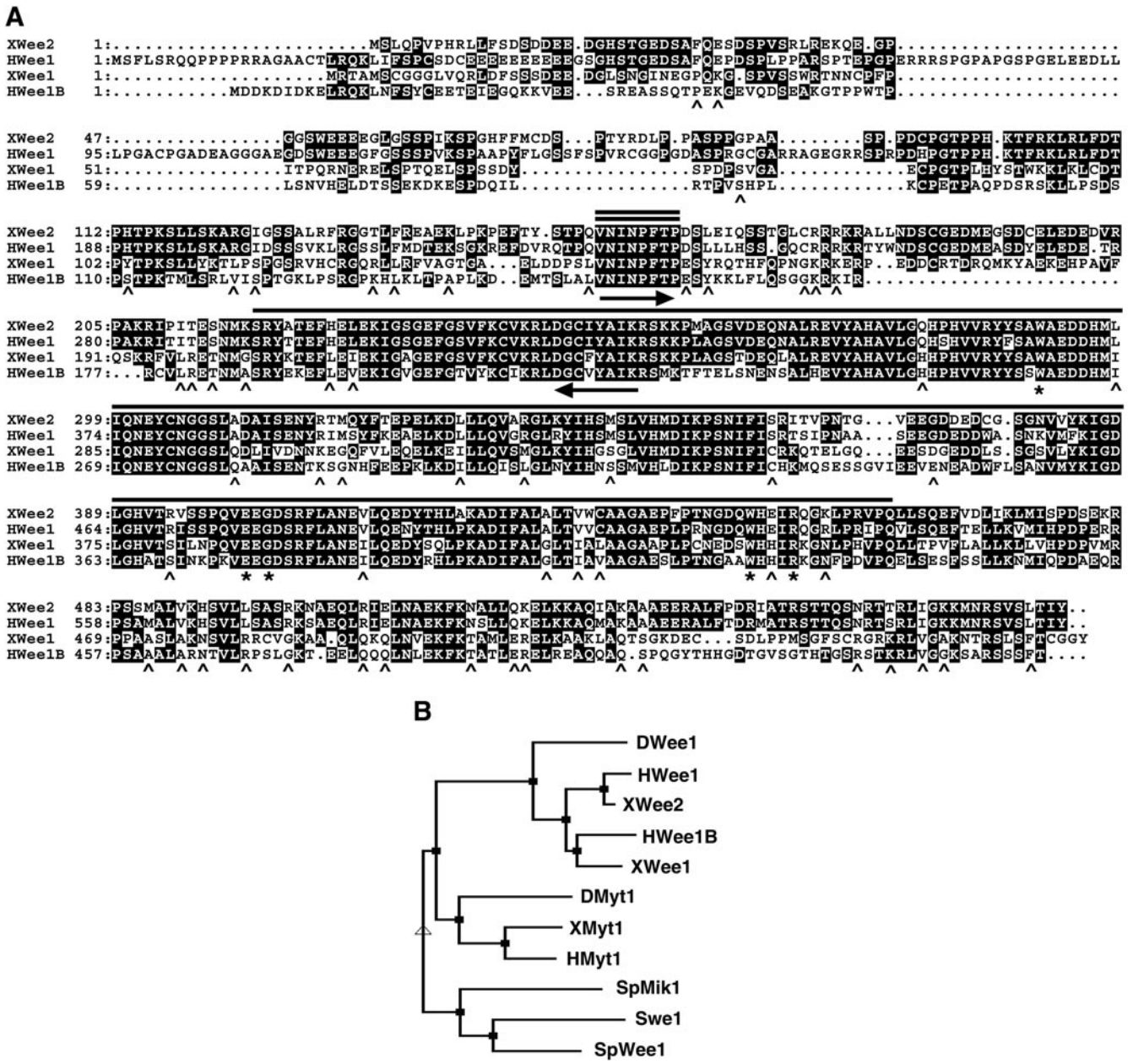

FIG. 1. Sequence analysis of *Xenopus* Wee2 and its relationship to the existing Wee kinase family members. (A) Alignment and comparison of *Xenopus* and human Wee1-like proteins. Identical residues between two or more members are boxed in black. Single overline indicates putative catalytic domains of the kinases. Double overline indicates conserved noncatalytic sequence required for optimal Wee1 kinase activity (Aligue *et al.,* 1997). Asterisks below sequence designate amino acids that are conserved between all known members of the Wee kinase family, but not in other eukaryotic protein kinases (Mueller *et al.,* 1995a). Arrows below sequence indicate sequences that were used to design degenerate PCR primers. Upward pointing carrots below sequence indicate pairs of amino acids that are conserved (identity or similarity) within the maternally or zygotically expressed Wee1-like kinases, but not conserved between these expression groups. Sequence alignment was performed by multi-align Blosum 62-2-2 algorithm (Corpet, 1988) and boxed by MacBoxShade Freeware from M. Baron. (B) Phylogenic tree generated by Multiple sequence alignment with hierarchical clustering (Corpet, 1988) shows that the Wee kinases fall into three major groups: metazoan Wee1-like, metazoan Myt1-like, and yeast Wee1-like. Furthermore, the vertebrate Wee1-like kinases can be further divided into two subgroups consisting of HWee1/XWee2 and HWee1B/XWee1. Sequence comparison and alignments are based on GenBank sequences: SpWee1, *S. pombe* Wee1 (M16508); SWE1, *S. cerevisiae* Wee1 (X73966); SpMik1, *S. pombe* Mik1 (M60834); XWee1, *Xenopus* Wee1A (U13962); XMyt1, *Xenopus* Myt1 (U28931); HWee1, human Wee1 (U10564); DWee1, *Drosophila* Wee1 (AAC46913); HMyt1, human Myt1 (NP004194); DMyt1, *Drosophila* Myt1 (AAF32288); HWee1B, human Wee1B (AAD04726); and XWee2, *Xenopus* Wee2 (AF358869).

electrophoresis, the gel was transferred and UV-crosslinked to a Nytran membrane (Schleicher & Schuel, Keene, NH) as per the manufacturer's recommendations. The blots were hybridized with radioactive probes specific to the 3′ untranslated region of XWee2, or the complete coding regions of XWee1 (XWee1A) (Mueller *et al.,* 1995a) or XMyt1 (Mueller *et al.,* 1995b) in hybridization buffer (0.5 M sodium phosphate, 7% SDS, 1% BSA) for 18 h at 65°C. After hybridization, blots were washed five times at 68°C with washing buffer (50 mM sodium phosphate buffer, 5% SDS, 1 mM EDTA) and exposed on a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA).

Whole-Mount in Situ Hybridization, Immunocytochemistry, and Sections

For whole-mount *in situ* hybridization, albino embryos produced by *in vitro* fertilization were fixed in MEMPFA as described (Sive *et al.,* 2000). Digoxygenin-labeled anti-sense *in situ* probes were prepared by using Megascript kits (Ambion, Austin, TX) as per the manufacturer's recommendation in the presence of digoxigenin-11-UTP (Roche, Indianapolis, IN), and the following linearized templates: pBluescript(ks)-N-CAM (Kintner and Melton, 1987), pGem3z-XChordin, pGem4z-XWee1, pGem4z-XWee2, or pGem4z-XMyt1. *In situ* hybridization was performed as described (Gerber *et al.,* 1999; Patterson and Krieg, 1999) by using BCIP (5-bromo-4-chloro-2-indoyl-phosphate) and NBT (nitro blue tetrazolium) (Roche, Indianapolis, IN) (Sive *et al.,* 2000). For sections of *in situ* hybridized embryos, stained embryos were embedded in OCT (Tissue-Tek, Torrance, CA), sections (16–25 μm) were cut by using a Leica CM3050 cryostat, and then sections were mounted on Colorfrost/Plus slides (Fisher Scientific, Pittsburgh, PA) as described (Fagotto *et al.,* 1997; Fagotto and Gumbiner, 1994).

For whole-mount immunocytochemical detection of phosphorylated histone H3, pigmented embryos were produced by *in vitro* fertilization, then demembranated, fixed 15 min at room temperature in MEMFA, then permeablized overnight in Dent's fix (Sive *et al.,* 2000). If needed, embryos were bleached for 1 h in 1% H_2O_2, 5% formamide, and 0.5× SSC (Sive *et al.,* 2000). Bleached or stained embryos were blocked for 3 h in blocking buffer (5% heat-inactivated goat serum, 10% DMSO, 0.1% Triton X-100, in Tris-buffered saline), then incubated overnight at room temperature with 1 μg/ml of anti-phospho-histone H3 (αPH3) antibody (Upstate Biotechnology, Lake Placid, NY). Embryos were washed 5 times at room temperature in wash buffer (Tris-buffered saline with 0.2% BSA and 0.1% Triton X-100). Detection of αPH3 antibody was accomplished in the blocking buffer overnight by using 1 μg/ml HRP-conjugated anti-rabbit IgG (H + L) (Jackson ImmunoResearch, West Groove, PA). Embryos were then washed 5 times at room temperature in wash buffer. The color reaction was accomplished by first preincubating embryos in 0.3 mg/ml 3′,3′-diaminobenzidine and 0.3 mg/ml NiCl in Tris-buffered saline for 30 min, and then the color reaction was initiated with 0.02% H_2O_2 (final concentration). To generate the composite image in Fig. 7G, 8 serial sections of αPH3 stained stage 18 embryos were photographed on a Zeiss AxioScope and then combined by using Adobe Photoshop 6.01. The edges of each serial section were aligned manually in 8 different layers. Subsequently, this composite image was merged. To preserve the intensity of the internal mitotic nuclei, the contrasted edge of the embryo in the image became black. This dark edge does not represent mitotic nuclei. The XMyt1/phosphohistone H3 double-stained embryos were first prepared for *in situ* analysis and then for immunocytochemistry as described above. Double-stained and immunocytochemistry-stained embryos were embedded in paraffin as described (Sive *et al.,* 2000), sectioned at 10 μM with Leica Jung Histocut microtone, and then mounted on Colorfrost/Plus slides (Fisher Scientific, Pittsburgh, PA).

RESULTS

Isolation of the Xenopus Wee1-like Kinase, Wee2

To clone zygotically expressed Wee1 homologs, we utilized degenerate PCR to amplify segments of *Xenopus* cDNA made from XTC cells, a tissue culture line derived from *Xenopus* tadpoles (Pudney *et al.,* 1973). The primers used in this PCR were designed to anneal to conserved regions of metazoan Wee1 kinases (see Material and Methods; and Fig. 1A). Using these primers, we amplified a 300-bp DNA fragment that is significantly different from either of the previously isolated *Xenopus* Wee1 isoforms, XWee1A and XWee1 (Mueller *et al.,* 1995a; Murakami and Vande Woude, 1998). We used this fragment to clone the complete cDNA from a stage 42 *Xenopus* cDNA library. This clone is 3229 bp in length, contains an open reading frame of 1716 bp, and is predicted to encode a 571-amino-acid protein, with a predicted molecular weight of 64 kDa (Fig. 1A). We have designated this clone *Xenopus* Wee2 (XWee2). Inspection of the protein sequence suggests that XWee2 is member of the Wee family of kinases. It contains the five residues (asterisks) that are conserved in all known Wee kinases and the conserved, noncatalytic VNINPFTP sequence (double overline) needed for optimal Wee1 activity (Alique *et al.,* 1997; Mueller *et al.,* 1995a).

In order to help understand the relationship of the various Wee kinases, we assembled a phylogenic tree (Fig. 1B). There are three main groups on this phylogenic tree: two represented by the metazoan Wee1 and Myt1 kinase families, and a third represented by the yeast Wee1 kinases. In vertebrates, the Wee1 group is further divided into two smaller subgroups. One contains XWee2 and HWee1, while the other contains XWee1 and HWee1B. Alignment of the vertebrate members of the two Wee1 subgroups confirms this grouping (Fig. 1A). At the whole protein level, XWee2 has more sequence similarity to human Wee1 (HWee1) (75% similar) than to the previously isolated *Xenopus* Wee1 (XWee1) (58% similar) (Mueller *et al.,* 1995a; Watanabe *et al.,* 1995). In turn, XWee1 is as similar to a second Wee1 kinase that has been identified in human, HWee1B, as it is to XWee2 (both 58% similar) (Nakanishi *et al.,* 2000). While the kinase domains (single overline) of all four kinases show a high degree of similarity, the subgroups differ in their noncatalytic carboxyl and amino termini. For example, the carboxyl terminus of XWee2 is 92% similar to that of HWee1, but only 32% and 34% similar to that of XWee1 and HWee1B. Additional comparison of these vertebrate Wee1-like kinases indicates that there are 55 conserved differences between the two Wee1-like subgroups (upward pointing carrots in Fig. 1A). In each case, the molecular nature

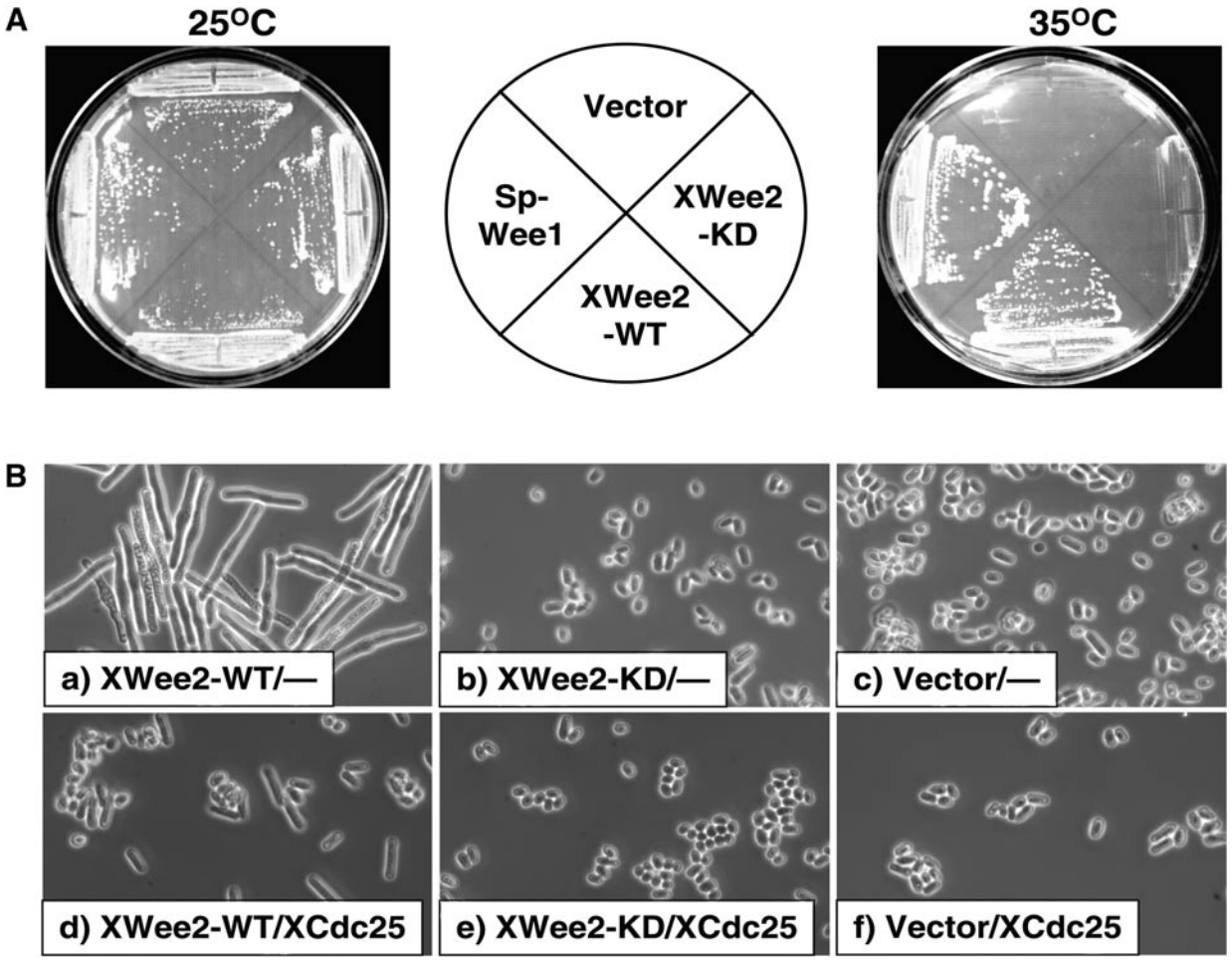

FIG. 2. XWee2 complements Wee1-deficient yeast and causes a G_2 growth arrest when overexpressed. (A) Expression of wild type, but not kinase-deficient XWee2 rescues the Wee1^{ts} ΔMik1 mutant strain of *S. pombe* that lacks Wee1-like kinase activity at 35°C. Wild type XWee2 (XWee2-WT) and kinase-deficient XWee2 (XWee2-KD) were expressed from pRep41, a vector that uses an attenuated *nmt1* promoter to expresses proteins at ~2% the level of the full-strength *nmt1* promoter (Forsburg, 1993; Maundrell, 1993). *S. pombe* Wee1 expressed from pAX-SV40 (Sp-Wee1) and the empty pRep41 construct (Vector) serve as positive and negative controls, respectively. (B) High level expression of XWee2-WT, but not XWee2-KD, caused an elongated phenotype in wild type *S. pombe*. Coexpression of *Xenopus* Cdc25C (XCdc25) rescued the XWee2-WT-induced phenotype. XWee2-WT, XWee2-KD, and Vector (empty expression construct) where stably integrated into the genomic *ura4* locus and expressed from the full-strength *nmt1* promoter. In lower panels, XCdc25 was coexpressed from episomal pRep1 *nmt1* expression vector. See text and Materials and Methods for details.

of each amino acid is identical or similar within the subgroup but differs between the subgroups. Taken together, these comparisons suggest that there are two conserved groups of Wee1-like kinases in vertebrates. In addition, they suggest that XWee2 is the *Xenopus* ortholog of HWee1.

XWee2 Is a Functional Wee1-Like Kinase

We next investigated whether XWee2 is a functional Wee1-like kinase. First, we tested whether XWee2 could complement Wee1-deficient fission yeast. For this purpose, we used a temperature-sensitive, mitotic catastrophe mutant that dies at 35°C due to the absence of both *S. pombe* Wee kinases (Wee1^{ts} ΔMik1) (Lundgren *et al.*, 1991). This and similar mitotic catastrophe strains of *S. pombe* have been used to isolate human and *Drosophila* Wee1 homologs in complementation screens (Campbell *et al.*, 1995; Igarashi *et al.*, 1991). We used an attenuated version of the *S. pombe* *nmt1* promoter to express wild type XWee2 (XWee2-WT) or a kinase-deficient point mutant of XWee2 (XWee2-KD). While XWee2-WT efficiently rescued the mutant at 35°C, neither XWee2-KD nor an empty vector could (Fig. 2A). Thus, XWee2 could functionally replace *S. pombe* Wee1 or Mik1.

In *S. pombe,* the overexpression of the Wee1 kinase or the lack of the Cdc25 phosphatase causes an elongated cell phenotype (Russell and Nurse, 1987). Both situations lead to the sustained phosphorylation of Cdc2 (*S. pombe* Cdk1) on Tyr15, and slowed or arrested progression through the G_2 phase of the cell cycle (Gould and Nurse, 1989). We tested whether XWee2 could cause this phenotype by overexpressing XWee2 in wild type yeast (Fig. 3B; and Material and Methods). Cells overexpressing XWee2-WT, but not XWee2-KD or an empty vector, are clearly elongated, suggesting a G_2 arrest (Fig. 2B). If XWee2 is acting by inhibiting the endogenous Cdk1, then coexpression of the phosphatase Cdc25, a Wee1-like antagonist, should compensate for the XWee2-induced elongation. To test this hypothesis, we expressed *Xenopus* Cdc25C (XCdc25) from an episomal vector (Material and Methods). When both XCdc25 and XWee2-WT were coexpressed, the elongated phenotype is suppressed. As expected, when XCdc25 was coexpressed with either XWee2-KD or the empty vector, a short cell morphology was observed due to the unopposed Cdc25 phosphatase activity (Fig. 2B) (Russell and Nurse, 1987). Together, these experiments show that XWee2 can rescue Wee1-deficient cells and that high level expression of XWee2 in wild type cells slows or blocks entry into mitosis. Furthermore, the suppression of the XWee2-induced growth defect by coexpression of XCdc25C suggests that XWee2 is functioning by phosphorylating *S. pombe* Cdk1 on Tyr15.

Next, we asked whether XWee2 could phosphorylate Cdk1 directly. Recombinant XWee2 and XWee1 proteins were produced and purified by using the Sf9 baculovirus expression system (Materials and Methods). These were tested in an *in vitro* kinase assay for their ability to phosphorylate the Cdk1/Cyclin B complex. Both wild type XWee2 and XWee1 phosphorylate Cdk1, while the kinase-deficient forms of these kinases do not (Fig. 3A, top panel). To test whether this phosphorylation is on specific residues of Cdk1, we repeated the kinase assay with a mutant form of Cdk1 (Cdk1-AF) that cannot be phosphorylated by Wee kinases (Kumagai and Dunphy, 1995). Neither XWee2 nor XWee1 could phosphorylate this mutant substrate, suggesting that, like XWee1, XWee2 phosphorylates Cdk1 on a specific residue in the ATP binding domain of Cdk1 (Fig. 3A, bottom panel).

Interestingly, using equal amounts of XWee1 and XWee2 proteins (Fig. 3C), XWee2 appears to phosphorylate Cdk1 more effectively than XWee1 (compare lanes 2 with 10, Fig. 3A). To further investigate this observation, we repeated the kinase assays over a broad range of XWee1 and XWee2 protein kinase amounts (0.05–100 ng) with a constant amount of Cdk1 substrate. In this experiment, each kinase has an optimal linear range of activity: 3.1–25 ng for XWee1 and 0.2–1.5 ng for XWee2. This difference confirms that XWee2 phosphorylates Cdk1 more efficiently than XWee1 in this assay (Fig. 3B). Quantitation of this and similar experiments with independent preparations of recombinant XWee1 and XWee2 indicate that XWee2 has 10- to 15-fold more Cdk1-directed kinase activity compared with XWee1 per ng of protein (data not shown).

To test whether XWee2 could alter the kinase activity of Cdk1 by phosphorylation, we repeated the Cdk1 kinase assay with active forms of Cdk1 in the presence of an ATP-regenerating system and subsequently measured the Cdk1-associated H1-kinase activity (Fig. 3E). Wild type and three mutant forms of Cdk1 were used in this assay. In this experiment, phosphorylation of Cdk1 was determined by observing the reduced mobility of modified Cdk1 or by immunoblotting with phosphotyrosine antibodies (Mueller *et al.,* 1995b). When treated with active XWee2, both the wild type Cdk1 and the T14A mutant of Cdk1 displayed the characteristic apparent molecular weight shift and reacted with antibodies to phosphotyrosine. In addition, treatment of wild type Cdk1 and T14A Cdk1 with active XWee2 caused an ~90% reduction in their H1 kinase activities. On the other hand, neither the Y15F mutant of Cdk1 nor the T14A, Y15F double mutant of Cdk1 were phosphorylated or appreciably inactivated by XWee2. The kinase-deficient form of XWee2 had no effect on Cdk1 H1 kinase activity (data not shown). Taken together, these experiments indicate that, like other Wee1 kinases, XWee2 phosphorylates Cdk1 on Tyr15 exclusively and that this phosphorylation inhibits Cdk1. Interestingly, XWee2 displayed reduced gel mobility under conditions where Cdk1 could not be inhibited, for example, when the XWee2 was kinase-deficient (KD) or when the Cdk1 lacked a phosphorylatable Tyr15 site (Y15F and AF). This suggests that active XCdk1 can phosphorylate XWee2 in this *in vitro* kinase assay.

Finally, we tested the function of XWee2 *in vivo*. If XWee2 functions by phosphorylating Cdk1 and blocking progression into mitosis, addition or expression of XWee2 in living cells should block or slow division. The blastomeres of cleavage-stage *Xenopus* embryos are rapidly dividing cells that are commonly used to test the function of cell cycle regulators. For example, microinjection of *Xenopus* Cdc25 protein accelerates cleavage, while microinjection of mRNA encoding the *Xenopus* cyclin-dependent kinase inhibitor p27-Xic blocks cleavage (Kim *et al.,* 1999; Ohnuma *et al.,* 1999). In various experiments, we microinjected two-cell embryos with XWee2 or XWee1 recombinant protein, or XWee2 or XWee1 *in vitro* transcribed mRNA (Materials and Methods; Fig. 4). These injections were into one blastomere of the two-cell embryo, so that the noninjected side could serve as a control (Sive *et al.,* 2000). The addition of 2.3 ng of wild type XWee2 protein or mRNA blocks or delays cleavage of the microinjected blastomere (Figs. 4A.1 and 4B.1). A less robust delay was observed with as little as 1.3 ng of wild type XWee2 protein or mRNA (data not shown). On the other hand, microinjection of either wild type XWee1 protein or mRNA had little or no effect even at the highest concentration tested (2.3 ng) (Figs. 4A.3 and 4B.3). Finally, microinjection of either kinase-deficient XWee1 or XWee2 as protein or mRNA had no effect on cleavage (Fig. 4).

While equal amounts of XWee1 and XWee2 protein and

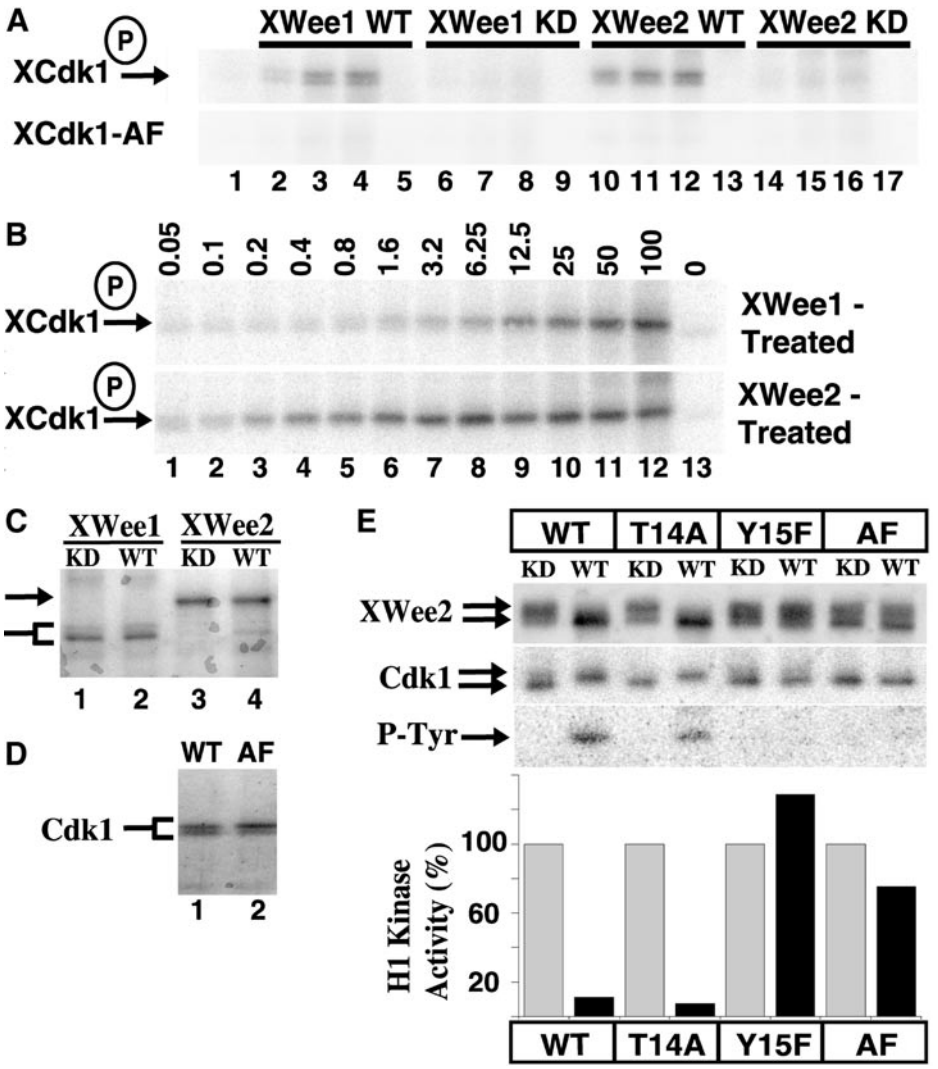

FIG. 3. XWee2 phosphorylates and inhibits the Cdk1/Cyclin B complex. (A) XWee2 phosphorylates Cdk1, but not Cdk1-AF. Purified recombinant XWee1-WT, XWee1-KD, XWee2-WT, and XWee2-KD proteins were tested in an *in vitro* kinase assay with purified recombinant Cdk1/Cyclin B complex (Cdk1) or Cdk1-AF/Cyclin B complex (Cdk1-AF). Both forms of Cdk1 are kinase-deficient due to Asp133 being changed to Ala. In addition, Cdk1-AF has Thr14 changed to Ala and Tyr15 changed to Phe. All lanes contain indicated Cdk1/Cyclin B complex and either buffer (lane 1) or increasing amounts of XWee1 or XWee2: 8 ng (lanes 2, 6, 10, and 14), 32 ng (lanes 3, 4, 11, and 15), or 64 ng (lanes 4, 8, 12, and 16). Lanes 5, 9, 13, and 17 contain 64 ng of the indicated kinase and buffer, but no Cdk1/CyclinB substrate. (B) XWee2 phosphorylates Cdk1 more efficiently than XWee1. *In vitro* kinase assay performed as in (A), except 2-fold increasing amounts of XWee1 or XWee2 were used as indicated and Cdk1-T161A/Cyclin B was used as a substrate (see Material and Methods). Lane 13 contains Cdk1/Cyclin B complex and buffer only. Note, unlike the Cdk1 used in (A), the T161A form of Cdk1 use here runs as a single band since it cannot be phosphorylated by CAK activity present in the Sf9 cell lysates (Kumagai and Dunphy, 1995). (C) Wee1-like kinase loading control for (A) and (B). Purified recombinant XWee1 and XWee2 kinases used in (A) and (B) were processed for gel electrophoresis and Coomassie blue staining. (D) Cdk1 loading control for (A). Purified Cdk1/Cyclin B complexes used in (A) were processed for gel electrophoresis and Coomassie blue staining (only the Cdk1 portion is shown). (E) XWee2 phosphorylates Cdk1 on Tyr15 exclusively and this phosphorylation reduces the H1 kinase activity of Cdk1. A total of 50 ng of recombinant kinase-deficient XWee2 (KD) or wild type XWee2 (WT) was incubated with purified Cdk1/cyclin B complexes containing the indicated wild type (WT) or mutant forms of Cdk1 (T14A, Thr14 changed to ala; Y15F, Tyr15 changed to phe; or AF, double mutant T14A and Y15F change to ala and phe, respectively) in the presence of an ATP-regenerating system. After incubation, samples were processed for immunoblotting or for histone H1 assay. Note: the reduced gel mobilities of Cdk1 and XWee2 is indicative of phosphorylation. The graph shows the percentage of H1 kinase activity (normalized for each mutant; 100% equals the activity of the sample in the presence of kinase-deficient XWee2).

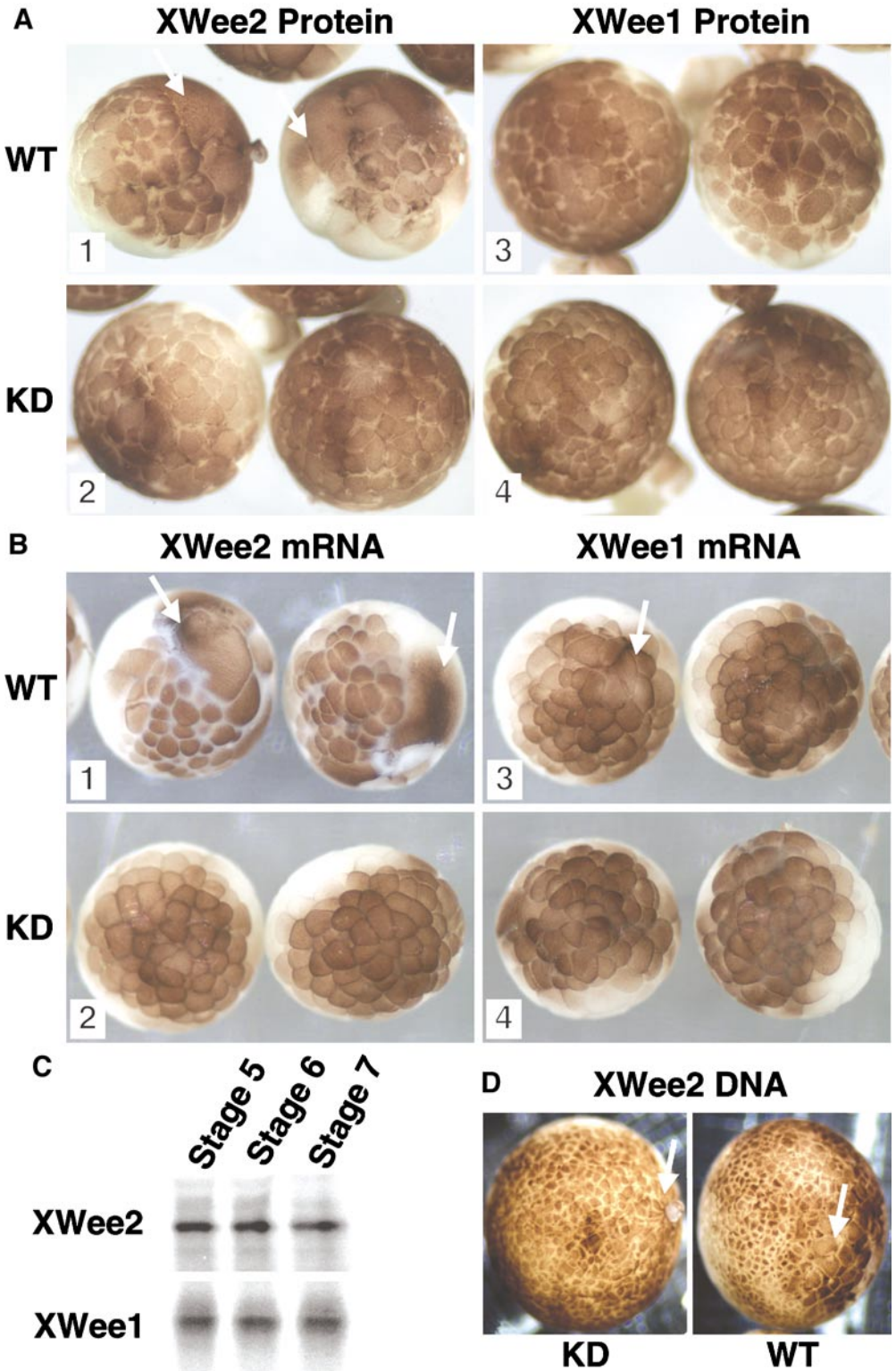

FIG. 4. Microinjection of XWee2 protein, mRNA, or DNA arrests division of cleavage-stage and post-MBT *Xenopus* embryos. (A) 2.3 ng of purified recombinant XWee2-WT (1), XWee2-KD (2), XWee1-WT (3), or XWee1-KD (4) protein was microinjected into one blastomere of a two-cell embryo. XWee2-WT protein caused a delay or arrest in the cleavage of the injected blastomere (arrows), while the other injected proteins did not. (B) 2.3 ng of mRNA encoding XWee2-WT (1), XWee2-KD (2), XWee1-WT (3), or XWee1-KD (4) were microinjected into one

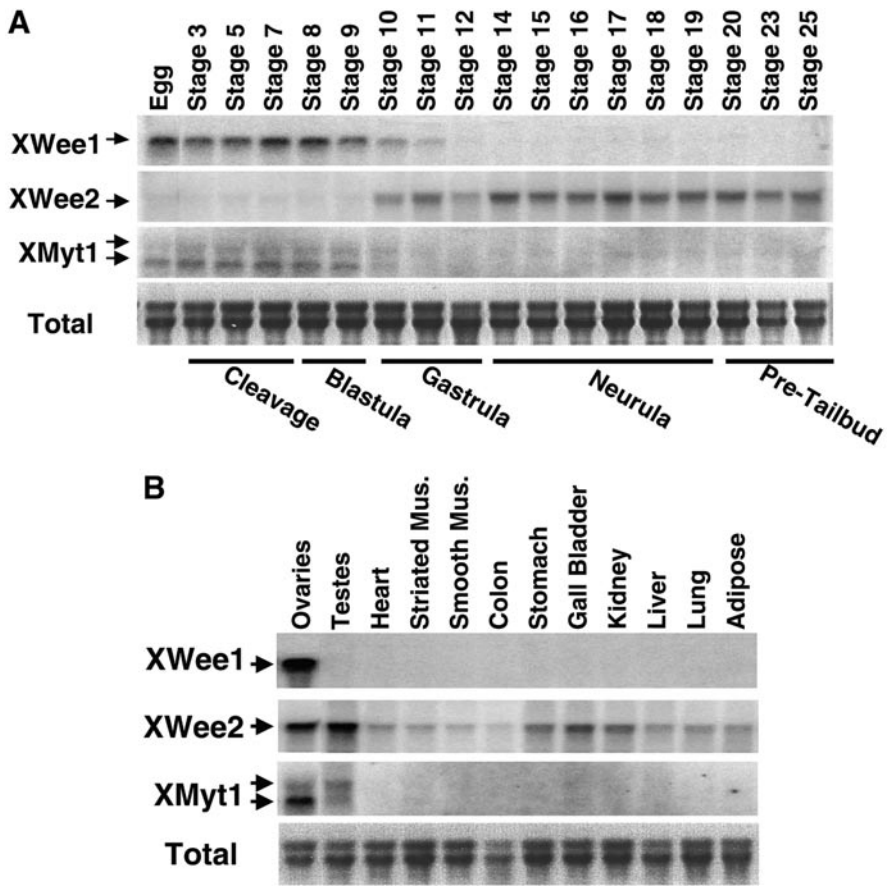

FIG. 5. Expression of XWee2, XWee1, and XMyt1 mRNA is developmentally regulated. (A) Northern blot analysis of 10 μg of total RNA isolated from *Xenopus* egg and embryos stage according to Nieuwkoop and Faber (1994). Labels above panels indicate stages, while labels underneath panels indicate relative period in development. (B) Northern blot analysis of 10 μg of total RNA isolated from indicated tissues. In both (A) and (B), blots were hybridized to radioactive probes corresponding to XWee1, XWee2, or XMyt1 as indicated. Bottom panels in (A) and (B) are loading controls. They show 28S and 18S rRNA in total RNA. See text and Materials and Methods for details.

mRNA were microinjected in the experiments described above, it is formally possible that the differences we observed between XWee2 and XWee1 may reflect different stabilities of the microinjected or translated proteins in the embryos. To test this possibility, we repeated the protein microinjection with ^{35}S-radiolabled XWee1 and XWee2 protein and then monitored the presence of these proteins until stage 7 of development. Both proteins were stable during the time-course of the experiment, indicating that the difference in activity between XWee1 and XWee2 is not caused by differing stability of the proteins in cleaving embryos. Thus, in conjuction with the *in vitro* kinase

blastomere of a two-cell embryo. These injected mRNAs have their endogenous 5′ and 3′ untranslated regions replaced by those of β-globin (MacNicol *et al.*, 1997). XWee2-WT mRNA caused a profound delay in the cleavage of the injected blastomere (arrows), while XWee1-WT mRNA caused a very slight delay in ~50% of the microinjected embryos. The arrow in panel 3 shows one of the strongest cases of this XWee1 induced delay. In both (A) and (B), the embryos were allowed to develop until noninjected control embryos had reach stage 7 before being fixed and photographed. (C) XWee1 and XWee2 protein are equally stable in cleavage-stage embryos. Recombinant ^{35}S-labeled XWee1 or XWee2 protein were microinjected into both blastomeres of a two-cell embryo, and embryos were allowed to develop until the indicated stages before being processed for autoradiography. (D) XWee2 arrest cell division in post-MBT cells. DNA constructs that express wild type (WT) or kinase-deficient (KD) XWee2 were microinjected into one blastomere of a two-cell embryo. Embryos were allowed to develop past MBT before being photographed. See text and Material and Methods for details.

assays (Fig. 3), these results support the conclusion that XWee2 has greater activity than XWee1. In addition, since wild type XWee2, but not kinase-deficient XWee2, could block cleavage in early stage embryos, these results suggest that XWee2 is a functional Wee1-like kinase.

The lack of effect of microinjected XWee1 protein or mRNA was somewhat surprising, since we and others have found that addition of exogenous XWee1 could delay the first mitotic cycle in egg extracts or ionophore-activated eggs (Mueller *et al.,* 1995a; Murakami and Vande Woude, 1998). However, in agreement with those previous studies, we found that microinjection of 2.3 ng of XWee1 mRNA could delay cleavage if we injected it into embryos that were still in their first cell cycle (data not shown). This dependence on the timing of XWee1 injection probably reflects the difference in the first cell cycle vs the subsequent 11 cell cycles of the cleavage-stage embryo (Masui and Wang, 1998). Unlike cycles 2 through 11, there is little Cdc25A phosphatase activity in the first cell cycle (Kim *et al.,* 1999). Therefore, one interpretation of our microinjection results is that XWee1 can delay cleavage only in the absence of appreciable Cdc25A activity, while XWee2 can function in the presence of Cdc25A. It remains to be determined whether this difference reflects unique regulatory pathways controlling XWee1 and XWee2 *in vivo* or differences in the basal kinase activities of XWee1 and XWee2.

To address whether XWee2 could inhibit cell division in post-MBT embryos, we microinjected DNA constructs into one blastomere of a two-cell embryo. Because zygotic transcription begins after MBT in *Xenopus,* these microinjected constructs remain inert until stage 8. Once the embryo has passed MBT, the DNA constructs are transcribed in any cell that has passively inherited the microinjected DNA. As evident by the large cells near the site of injection, expressed wild type XWee2 causes a cell cycle arrest in post-MBT embryos. This arrest is not observed when kinase-deficient XWee2 is microinjected. Thus, XWee2 can inhibit cell division in both cleavage-stage and post-MBT cell cycles of *Xenopus.*

Temporal and Spatial Distribution of Wee Kinases during Development

Having established that XWee2 is a functional Wee kinase, we next investigated the temporal and spatial distribution of XWee2 and the other Wee kinases of *Xenopus,* XWee1 and XMyt1. First, we performed Northern blot analysis on RNA extracted from *Xenopus* embryos at different stages of development to determine the temporal patterns of expression (Fig. 5A). Both XWee1 and XMyt1 mRNA are present during the rapid, synchronous cell cycles of the cleavage- and blastula-stage embryos, but then decrease in abundance at the beginning of gastrulation. We cannot detect XWee1 mRNA after stage 12 by this analysis, but XMyt1 mRNA remains detectable at reduced levels at least through the tadpole stages of development. There are two forms of XMyt1 mRNA: one that runs at the predicted size of ~2 kb and the other that runs at ~3 kb. While XWee2 mRNA is nearly undetectable early in development, its levels increase significantly at the onset of gastrulation. This coincides with the acquisition of slower, asynchronous cell cycles in the post-EGT embryo. From neurulation on, XWee2 mRNA levels remain relatively constant at least through the tailbud stages.

Northern blot analysis of RNA from adult tissues shows that XWee2 is ubiquitously expressed in all tissues examined, albeit at differing levels. Ovaries, testes, stomach, gall bladder, and kidney have the highest levels of XWee2 mRNA (Fig. 5B). On the other hand, XWee1 transcripts are detected only in ovaries, probably due to the high level of transcript in oocytes (Fig. 5B) (Nakajo *et al.,* 2000; data not shown). We observed reproducibly low levels of XMyt1 in adult tissues, but with the exception of ovaries and testes, these levels were near the lower limit of detection. Hence, XWee2 is the predominant Wee transcript present in the *Xenopus* after gastrulation and in adult *Xenopus*. XMyt1 transcripts are also present after gastrulation, but at levels significantly reduced from the pre-MBT levels. Finally, XWee1 is present only in the early cleavage stages of development.

The temporal differences in the levels of the Wee kinases during development lead us to investigate the spatial distribution of these kinases by *in situ* hybridization. Although the cells of the cleavage-stage embryo undergo rapid, synchronous rounds of cell division, this pattern does not continue past MBT. After MBT, the length of the cell cycle increases and, with the onset of gastrulation, distinct spatial patterns of nonmitotic cells are observed (Saka and Smith, 2001). XWee1, XMyt1, and XWee2 have been shown to phosphorylate and inactivate Cdk1 *in vitro* and to block the entry into mitosis *in vivo* (Mueller *et al.,* 1995a,b; Nakajo *et al.,* 2000). Therefore, we hypothesized that the spatial expression pattern of these kinases might correlate with the spatial patterns of nonmitotic cells during development. XWee1 and XMyt1 are both uniformly distributed in the animal cells of the cleavage stage embryo (Fig. 6A; and data not shown). Consistent with the Northern analysis (Fig. 5), XWee1 is no longer detectable by *in situ* hybridization during the rest of development (Figs. 6B–6I). XWee2 is first detected during gastrulation (Figs. 6B and 6C). This expression is in the region of the involuting dorsal and ventral lips. For comparison, the expression pattern of the early dorsal mesoderm marker Chordin is shown (Sasai *et al.,* 1994). By the end of gastrulation, XWee2 is detected predominantly in the dorsal, posterior region of the embryo, but is reduced in the dorsal midline (Fig. 6D). On the other hand, XMyt1 is uniformly detected on the surface of the embryo during gastrulation, but not in the presumptive endodermal cells of the blastopore (Figs. 6B–6D).

During the early neurula stages, both XWee2 and XMyt1 are localized to the dorsal side of the embryo. However, XWee2 is more posterior while XMyt1 is more anterior, with XMyt1 showing predominant staining in the anterior

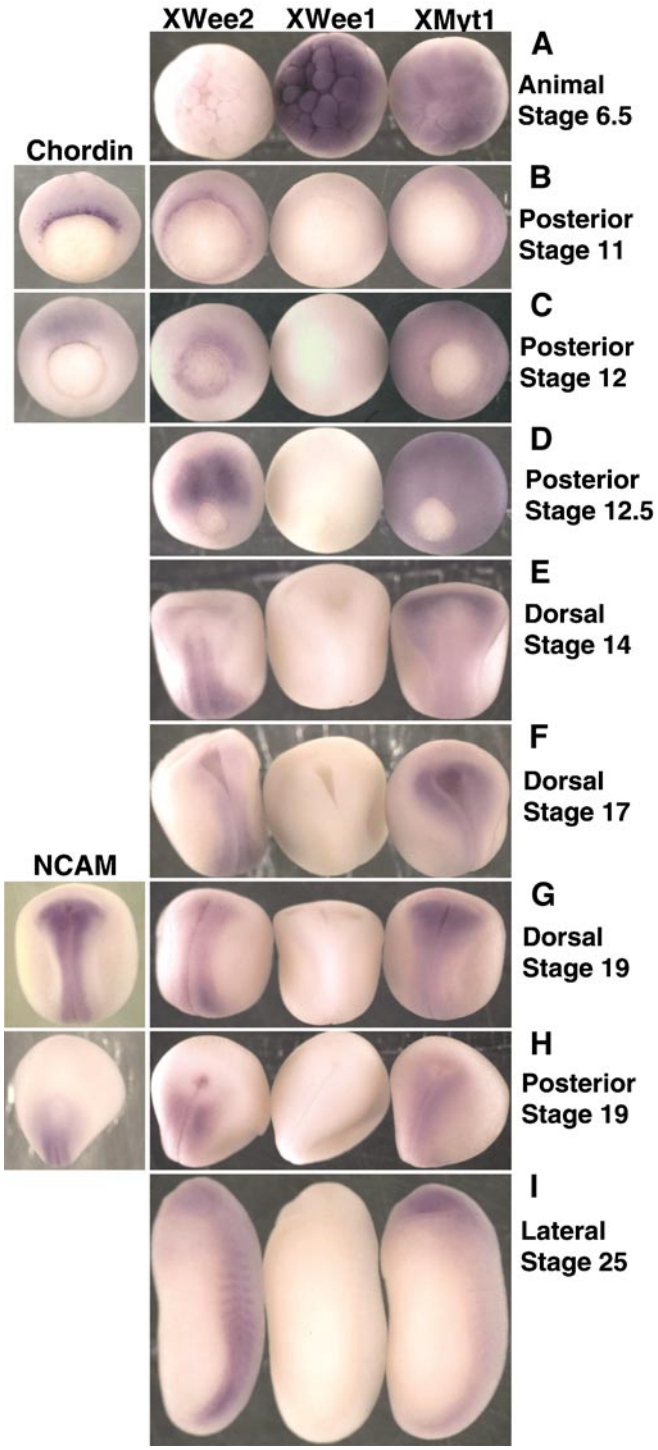

FIG. 6. Whole-mount *in situ* hybridization of *Xenopus* embryos show that each Wee kinase has a specific spatial and temporal developmental profile. *Xenopus* embryos were staged according to Nieuwkoop and Faber (1994), fixed, and subject to *in situ* hybridization for the Wee kinases (XWee2, XWee1, or XMyt1) (A–I), the dorsal mesoderm marker Chordin (B, C), or the neural marker NCAM (G, H). View and stages for each embryo are as indicated. See text and Materials and Methods for details.

neural folds and neural plate (Figs. 6E and 6F). In the late neural-stage embryo, the pattern of XMyt1 localization is similar to that of NCAM, a pan-neural marker, while XWee2 is localized to the posterior dorsal region and the remnant of the blastopore (Figs. 6G and 6H) (Kintner and Melton, 1987). In the pre-tailbud-stage embryo, XWee2 shows a predominant somitic pattern of expression as well as being localized generally to the dorsal side of the embryo (Fig. 6I). At the same stage, XMyt1 is localized strongly in the developing anterior neural structures and eye as well as being present on the dorsal side of the embryo.

To gain a better understanding of the spatial distribution of the Wee kinases during development, we sectioned embryos that had been subjected to *in situ* hybridization. Sagittal sections of midgastrula-stage embryos show that XMyt1 is present in the ectoderm and presumptive neural ectoderm (Fig. 7A). In contrast, sagittal sections show that, near the end of gastrulation, XWee2 is expressed strongly in the involuting dorsal endomesoderm (Fig. 7B). During neurulation, XWee2 expression is further restricted to the dorsal mesoderm, while XMyt1 continues to be expressed in the neural ectoderm and developing neural structures, such as the eye anlage (Figs. 7C and 7D). As expected, we see no XWee1 expression during this period of development (Fig. 7E). In addition, we could not detect XWee1 in sectioned embryos at any point after the initiation of gastrulation (data not shown). Finally, transverse sections of late neurula-stage embryos show that XWee2 is expressed strongly in the somitic mesoderm (Fig. 7F). Taken together, these results show that the *Xenopus* Wee kinases have unique temporal and spatial patterns of expression during development.

Finally, we correlated the spatial expression patterns of XWee2 and XMyt1 with the location of mitotic cells in developing embryos. Histone H3 is phosphorylated on serine 10 from late G_2 through telophase of the cell cycle. This phosphorylation, and thus mitotic cells, can be detected with an anti-phospho-histone H3 (αPH3) antibody that reacts with many eukaryotes including Xenopus (Saka and Smith, 2001). We used whole-mount immunocytochemistry and the αPH3 antibody to visualize mitotic cells in embryos during neurulation. Combining serial sections of these embryos shows that, while cell division is readily observed in developing neural structures, few dividing cells are detectable in the involuting dorsal mesoderm (Fig. 7G). Similar, spatially distributed patterns of mitotic cells have been reported by Saka and Smith (2001). Significantly, XWee2 is expressed in regions of the embryo that lack mitotic cells, such as the involuting dorsal mesoderm (compare Figs. 7D and 7G). On the other hand, XMyt1 does not follow this pattern and is expressed most strongly in regions of the embryo that display high rates of proliferation such as the developing neuronal structures (compare Figs. 7C and 7G). To better observe the colocalization of dividing cells and XMyt1 expression, we combined immunocytochemistry with *in situ* analysis (Fig. 7H). Greater magnification of the eye anlage region shows colocalization of

mitotic cells and XMyt1 expression (Fig. 7I). Thus, while recombinant and misexpressed XWee2 and XMyt1 have both been shown to inactivate Cdk1 and prevent the entry into mitosis, only the expression of endogenous XWee2 correlates with the absence of mitotic cells in embryos during gastrulation and neurulation.

DISCUSSION

We are interested in understanding the role the Wee kinases play in coordinating cell division with morphogenesis during early *Xenopus* development. We have found that the previously isolated Wee1 gene is expressed only as a maternal gene product, and its message becomes undetectable before the completion of gastrulation. This foreshadows the loss of Wee1 protein at neurula stages (Murakami and Vande Woude, 1998). Concomitant with the loss of Wee1 message, the expression of a new Wee1-like kinase, *Xenopus* Wee2 (XWee2), is increased significantly during gastrulation. XWee2 continues to be expressed during embryogenesis and becomes the predominant Wee kinase detected in adult tissues. We have confirmed that XWee2 is a *bona fide* Wee1-like kinase by its ability to rescue Wee1-deficient yeast, phosphorylate and inhibit Cdk1, and block cell division when ectopically expressed in cleavage-stage and post-MBT embryos.

When XWee1 was isolated, it was assumed to be the *Xenopus* ortholog of mammalian Wee1 (Mueller *et al.*, 1995a; Murakami and Vande Woude, 1998). Our results suggest that this is not the case, and that XWee2 is the *Xenopus* ortholog of mammalian Wee1. By sequence comparison, XWee2 and HWee1 share a high degree of similarity. In fact, XWee2 is more similar to HWee1 than it is to XWee1. This is most obvious in comparisons of the kinase domains and carboxyl termini where the proteins are 85 and 92% similar. Furthermore, XWee2 and mouse Wee1 have similar timing in their developmental expression. Both are zygotic transcripts that accumulate at the onset of zygotic transcription (Nakanishi *et al.*, 2000). As for XWee1, our data support the hypothesis that the original *Xenopus* Wee1 is the ortholog of the newly identified human Wee1 gene, HWee1B (Nakanishi *et al.*, 2000). Although XWee1 and HWee1B share less similarity than XWee2 and XWee1, both XWee1 and mammalian Wee1B are expressed as maternal transcripts. Taken together, our results and the published reports suggest that there are at least three families of Wee kinases in vertebrates. The first is made up of the maternally expressed XWee1 and HWee1B kinases, the second is made up of the zygotically expressed XWee2 and HWee1 kinases, and the third is made up of the Myt1 kinases.

Unlike XWee1 and XWee2, XMyt1 is expressed throughout development as both a maternal and zygotic gene product. As a maternal product, XMyt1 is present at high levels during oogenesis, cleavage stages, and blastula stages. Once zygotic expression begins, XMyt1 is observed in gastrula and neural stages, albeit at reduced levels. In adult tissues, XMyt1 is near the lower limit of detection with the exception of testes and ovaries. Interestingly, we observe two sizes of XMyt1 transcripts. Our working hypothesis is that these two forms of XMyt1 are either expressed from the same gene as differentially processed transcripts or that they are expressed from highly similar, but distinct genes. Unlike the situation between XWee1 and XWee2, both forms of XMyt1 are detected with our stringent washing conditions during Northern and *in situ* analysis. Therefore, if the two forms of XMyt1 are from different genes, they are highly similar. Additional studies will be needed to resolve this issue.

The zygotic expression of both XWee2 and XMyt1 is in spatially restricted domains during gastrulation and neurulation. Significantly, XWee2 is expressed in regions of the embryo that lack proliferating cells. For example, XWee2 is strongly expressed in the involuting mesoderm during gastrulation, the dorsal mesoderm during neurulation, and the somites during the pretailbud stages. Each of these tissues is comparatively deficient in mitotic cells as judged by phosphohistone H3 staining (Saka and Smith, 2001). Be-

FIG. 7. XMyt1 and XWee2 are expressed in distinct germ layers of embryos undergoing gastrulation and neurulation. (A) XMyt1 is expressed in the presumptive neural ectoderm. Sagittal section of a gastrulating stage 12 embryo subjected to *in situ* hybridization for XMyt1. Note: the blastocoel has collapsed in this embryo. (B) XWee2 is expressed in the involuting dorsal endomesoderm. Sagittal section of gastrulating stage 12.5 embryo subjected to *in situ* hybridization for XWee2. (C–E) XMyt1 is expressed in developing neural structures, XWee2 is expressed in the dorsal mesoderm, and XWee1 is undetectable. Sagittal sections of neurulating stage 18 embryos subjected to *in situ* hybridization for XMyt1, XWee2, or XWee1, respectively. (F) XWee2 is expressed in the somatic mesoderm. Transverse section of neurulating stage 18 embryo subjected to *in situ* hybridization for XWee2. (G) Composite image of eight serial sagittal sections of a representative stage 18 embryo that was subjected to whole-mount immunocytochemistry with the αPH3 antibody. Black dots indicate mitotic cells. Note the absence of mitotic cells in the involuting dorsal mesoderm. The black ring on the surface of the embryo is caused by the layering of the images and does not represent mitotic cells. (H, I) Stage 18 embryos were subjected to whole-mount XMyt1 *in situ* hybridization and αPH3 immunocytochemistry. Saggital section of a representative embryo and enlargement of the boxed region shows that XMyt1 colocalizes to regions of high proliferation such as the eye anlage. In (I), arrows indicated mitotic nuclei (brown). For all images, positions of sections are indicated as red lines on drawings from Nieuwkoop and Farber (1994). See text for details. Scale bar, 100 μm. Abbreviations: blc, blastocoel; ac, archenteron; yp, yolk plug; pne, presumptive neural ectoderm, dm, dorsal mesoderm; ey, eye anlage; sm, somite; n, notochord.

cause our results show that XWee2 is a potent inhibitor of Cdk1 and cell cycle progression, it is tempting to speculate that XWee2 may play a causative role in blocking cell proliferation in these tissues. In *Drosophila*, a similar cell cycle arrest of mesodermal cells undergoing gastrulation is accomplished by the expression of Tribbles, a protein that down-regulates the activity of Cdc25 (String) (Grosshans and Wieschaus, 2000; Mata *et al.*, 2000; Seher and Leptin, 2000). Since XWee2 is an antagonist of Cdc25, XWee2 may be playing a role that is functionally similar to that of Tribbles in the involuting mesoderm of *Xenopus*. Additional study will be needed to test this hypothesis.

It is important to note that not all nonproliferating tissues express XWee2. For example, XWee2 is not detected in the notochord during late neurula stages, yet this tissue is not dividing. This absence of XWee2 in the notochord suggests that other cell cycle regulators may be blocking cell cycle progression in this tissue. A likely candidate is p27-Xic, the *Xenopus* homolog of the mammalian p27-Kip1 cyclin-dependent kinase inhibitor, since p27-Xic is strongly expressed in the notochord (Hardcastle and Papalopulu, 2000).

In contrast to XWee2, XMyt1 expression is not associated with regions of the embryo that lack mitotic cells. In fact, XMyt1 is expressed in the regions that have high levels of proliferation, such as the presumptive neural ectoderm and developing neural tissues. These tissues have comparatively high amounts of cells undergoing mitosis as judged by phosphohistone H3 staining (Saka and Smith, 2001). This pattern is somewhat surprising since XMyt1 has been shown to inhibit Cdk1 activity *in vitro* (Booher *et al.*, 1997; Liu *et al.*, 1997; Mueller *et al.*, 1995b) and to block entry into meiosis *in vivo* (Nakajo *et al.*, 2000). Regardless, the strong correlation of XMyt1 expression in tissues that are actively proliferating suggests that XMyt1 may be playing a role in cell cycle control that is different than XWee2. While XWee2 expression may cause an overt cell cycle arrest, XMyt1 may function in a more subtle ways to govern progression through the cell cycle or may function as a cell cycle inhibitor in limited cellular contexts. Alternatively, XMyt1 may be expressed in a subset of cells that are not dividing in these developing neuronal tissues. Exactly what role XMyt1 plays in the cell cycle control of the presumptive neuronal tissues of the postgastrulating embryo remains to be determined. However, it is noteworthy that XWee2 and XMyt1 are expressed in different tissue types during embryogenesis.

In conclusion, we have identified and characterized XWee2, a new Wee1-like kinase in *Xenopus*. In both *Xenopus* and mammals, there are at least three conserved families of Wee kinases: a maternally expressed Wee1-like kinase (XWee1/HWee1B), a zygotically expressed Wee1-like kinase (XWee2/HWee1), and a Myt1 kinase (XMyt1/HMyt1). We have found that XWee2 and XMyt1 have unique temporal and spatial patterns of expression during development. Significantly, the distribution of XWee2 in nonmitotic cells suggests that XWee2 may be playing an important role in controlling the pattern of cell proliferation in post-EGT embryos.

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