The existence of two distinct Wee1 isoforms in *Xenopus*: implications for the developmental regulation of the cell cycle

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In eukaryotic cells, the Wee1 protein kinase phosphorylates and inhibits Cdc2, thereby creating an interphase of the cell cycle. In *Xenopus*, the conventional Wee1 homolog (termed Xe-Wee1A, or Wee1A for short) is maternally expressed and functions in pregastrula embryos with rapid cell cycles. Here, we have isolated a second, zygotic isoform of *Xenopus* Wee1, termed Xe-Wee1B (or Wee1B for short), that is expressed in postgastrula embryos and various adult tissues. When ectopically expressed in immature oocytes, Wee1B inhibits Cdc2 activity and oocyte maturation (or entry into M phase) much more strongly than Wee1A, due to its short C-terminal regulatory domain. Moreover, ectopic Wee1B, unlike Wee1A, is very labile during meiosis II and cannot accumulate in mature oocytes due to the presence of PEST-like sequences in its N-terminal regulatory domain. Finally, when expressed in fertilized eggs, ectopic Wee1B but not Wee1A does affect cell division and impair cell viability in early embryos, due primarily to its very strong kinase activity. These results suggest strongly that the differential expression of Wee1A and Wee1B is crucial for the developmental regulation of the cell cycle in *Xenopus*.

Keywords: cell cycle/development/PEST sequence/Wee1 isoforms/*Xenopus*

Introduction

In most eukaryotic cells, the mitotic cell cycle consists of two alternating S and M phases with intervening G₁ and G₂ phases (Murray and Hunt, 1993). The G₂/M transition is a crucial point for progression through the cell cycle and is controlled by the Cdc2 kinase–cyclin B complex (for review see Nurse, 1990; Morgan, 1995). The Wee1 family of protein kinases phosphorylates and inhibits Cdc2 and thus creates an interphase (mainly S and G₂ phases) of the cell cycle (for review see Coleman and Dunphy, 1994; Fattaey and Booher, 1997). The Wee1 family consists of Wee1 (present in all eukaryotes), Mik1 (in fission yeast) and Myt1 (in metazoans). While Myt1, a membrane-associated kinase, phosphorylates both Thr14 and Tyr15 of Cdc2 (Mueller *et al.*, 1995b; Liu *et al.*, 1997), Wee1 and Mik1 (nuclear kinases) phosphorylate Tyr15 almost exclusively (Featherstone and Russell, 1991; Parker *et al.*,

1992; Booher *et al.*, 1993; Lee *et al.*, 1994; Mueller *et al.*, 1995a; Watanabe *et al.*, 1995).

Wee1 homologs have been isolated from a variety of organisms, ranging from yeast through *Drosophila* and *Xenopus* to humans (Russell and Nurse, 1987; Igarashi *et al.*, 1991; Booher *et al.*, 1993; Campbell *et al.*, 1995; Mueller *et al.*, 1995a; Watanabe *et al.*, 1995). Structurally, they commonly consist of a long N-terminal regulatory domain, a central kinase domain and a short C-terminal regulatory domain (Watanabe *et al.*, 1995; Nemer and Stuebing, 1996). During the mitotic cell cycle in many cell types, Wee1 protein is active and relatively stable during G₁, S and early G₂ phases, but is inactive and unstable during late G₂ and M phases (McGowan and Russell, 1995; Mueller *et al.*, 1995a; Watanabe *et al.*, 1995; Aligue *et al.*, 1997; Michael and Newport, 1998; Sia *et al.*, 1998). Phosphorylation of either the N- or C-terminal regulatory domains has been implicated in the negative regulation of Wee1 activity during M phase (Parker *et al.*, 1993; Tang *et al.*, 1993; Mueller *et al.*, 1995a). However, it is poorly understood which regulatory domain determines the stability and the intrinsic kinase activity of Wee1 protein. Wee1 activity is also required for G₂ DNA damage/replication checkpoint control (O'Connell *et al.*, 1997; Raleigh and O'Connell, 2000; Lee *et al.*, 2001), which arrests the cell in G₂ phase until DNA repair/replication is completed (Nurse, 1997; Weinert, 1997). Thus, although its regulation mechanisms remain largely unknown, Wee1 protein is a key molecule in creating an interphase in both the normal cell cycle and the DNA damage/replication checkpoint.

In all metazoans, the cell cycles in oocytes and early embryos differ greatly from those in somatic cells (for review see Edgar, 1995; Sagata, 1996; Bissen, 1997). In *Xenopus*, for example, immature oocytes are arrested at the first meiotic prophase (prophase I or late G₂ phase) and, upon progesterone stimulation, undergo two successive M phases (meiosis I and II) without an S phase and arrest again at metaphase II (Sagata, 1997; Ferrell, 1999; Nebreda and Ferby, 2000). Then, following fertilization, mature oocytes (or eggs) undergo 12 rapid and synchronous divisions during which the cell cycle consists mostly of S and M phases (Graham and Morgan, 1966; Newport and Kirschner, 1984; Hartley *et al.*, 1996). After the 12 divisions or the midblastula transition (MBT), however, the cell cycle lengthens gradually and zygotic transcription is initiated (Newport and Kirschner, 1984; Prioleau *et al.*, 1994). The lengthening of the cell cycle at the MBT, which is due at least in part to the appearance of G₂ phase (Graham and Morgan, 1966; Howe *et al.*, 1995), is most probably caused by the DNA replication checkpoint (Newport and Dasso, 1989; Dasso and Newport, 1990). The cell cycle lengthens further and dramatically after the gastrula stage in a zygotic transcription-

dependent manner (Newport and Dasso, 1989; Howe *et al.*, 1995) and approaches that of somatic cells at the neurula/tailbud stages (Frederick and Andrews, 1994). Similar changes in the cell cycle occur during early development in many other species, such as *Drosophila* (Edgar, 1995), sea urchins (Nemer and Stuebing, 1996) and zebrafish (Kane and Kimmel, 1993). Since the changes in the cell cycle in early development are attributable mainly to the changes in the length of interphase, some developmental regulation(s) of the Wee1 kinase family might play an important role in such changes; however, this potentially important issue has hardly been addressed in any developmental systems.

In *Xenopus*, cDNAs encoding a Wee1 homolog have been isolated from an oocyte cDNA library (Mueller *et al.*, 1995a; Murakami and Vande Woude, 1998). Wee1 protein encoded by the oocyte cDNA is absent or very scarce in immature or maturing (meiosis I) oocytes, but is rapidly synthesized during meiosis II and stably present in mature oocytes arrested at metaphase II (Murakami and Vande Woude, 1998; Iwabuchi *et al.*, 2000; Nakajo *et al.*, 2000). The absence or scarcity of the Wee1 protein during meiosis I is essential for the omission of interphase (or S phase) during the transition to meiosis II (Nakajo *et al.*, 2000). However, the presence of Wee1 protein in mature oocytes is required for the recognizable G₂ phase or Cdc2 Tyr15 phosphorylation in the first embryonic cell cycle (Murakami and Vande Woude, 1998; Walter *et al.*, 2000); at least part of the stored Wee1 protein also seems to be required for the extremely short G₂ phases in cycles 2–12 (Kim *et al.*, 1999; see also Discussion) as well as for the lengthening of the cell cycle at the MBT, which is accompanied by Cdc2 Tyr15 phosphorylation (Ferrell *et al.*, 1991; Hartley *et al.*, 1996). Interestingly, however, the Wee1 protein becomes undetectable after the gastrula stage (Murakami and Vande Woude, 1998), although Tyr15 phosphorylation of Cdc2 can readily be detected even after that time (Ferrell *et al.*, 1991; Hartley *et al.*, 1996). Thus, it is possible that the Wee1 protein present in mature oocytes and pregastrula embryos may be a maternal isoform, and that a second, zygotic isoform of Wee1 might exist and function in postgastrula embryos with somatic-like cell cycles.

In this study, we isolated a second isoform of *Xenopus* Wee1 (Xe-Wee1B), which is expressed in postgastrula embryos and corresponds to human Wee1, and compared its properties with those of the conventional (maternal) isoform of Wee1 (Xe-Wee1A). When expressed in immature oocytes, Wee1A and Wee1B have markedly different kinase activities to inhibit oocyte maturation (and Cdc2 activity) and strikingly different stabilities during oocyte maturation (or during meiosis II), due to their highly divergent C- and N-terminal regulatory domains, respectively. Moreover, and importantly, ectopically expressed Wee1B cannot functionally replace endogenous Wee1A in pregastrula embryos; this is due primarily to its very strong kinase activity. These results strongly suggest that differential expression and different properties of the two Wee1 isoforms play important roles in the regulation of the cell cycle during early *Xenopus* development. We discuss the possibility that developmental regulation of the cell cycle by the two distinct Wee1 isoforms may occur generally in vertebrates.

Results

Cloning of a novel isoform of *Xenopus* Wee1

To clone a possible second isoform of *Xenopus* Wee1, we first performed PCRs against a tailbud embryo cDNA library using degenerate oligonucleotide primers. Using the PCR products obtained, we performed plaque hybridization against the tailbud embryo and liver cDNA libraries. The clones obtained, however, were partial cDNAs (with no initiator ATG codon) so we also performed 5'-RACE against cDNAs derived from several tissues. This approach gave us full information about the coding region of a new Wee1 isolate that was clearly distinct from the one previously described (Mueller *et al.*, 1995a) (Figure 1A). Thus, hereafter, we call the previous and present Wee1 isolates Xe-Wee1A (Wee1A for short) and Xe-Wee1B (Wee1B), respectively.

Wee1B protein has 595 amino acids and, like many other Wee1 homologs, consists of a long N-terminal regulatory domain (NRD), a central kinase domain (KD) and a short C-terminal regulatory domain (CRD) (Figure 1B). Interestingly, at its N-terminus, Wee1B has three tandem repeats of seven amino acids, the third repeat being partial, which are absent in other Wee1 homologs including Wee1A (Figure 1A). These repeats were most probably not due to a cloning artefact, since they were present in other independent Wee1B cDNA clones as well (our unpublished data). Moreover, the NRD of Wee1B has two PEST-like sequences (positions 37–53 and 62–82) typical of many short-lived proteins (Rechsteiner and Rogers, 1996) and also a stretch of basic amino acids (RGRKR; positions 200–204), reminiscent of a nuclear localization signal (NLS) (Mataj and Englmeier, 1998). Sequence comparisons show that in the KD region, Wee1B shares 84% identity with the previously isolated human Wee1 (Wee1Hu; Watanabe *et al.*, 1995) and 69% identity with *Xenopus* Wee1A (Figure 1B). Moreover, even in the NRD and CRD regions, Wee1B shows much greater identity to Wee1Hu (54 and 89%, respectively) than to Wee1A (37 and 37%, respectively). Thus, these results suggest strongly that Wee1B, rather than Wee1A as previously thought (Nemer and Stuebing, 1996), is a genuine *Xenopus* homolog of human Wee1Hu.

Tissue-specific and developmental expression of Wee1A and Wee1B

To determine tissue-specific expression of Wee1B as well as Wee1A, we performed RT-PCR analysis of RNA from various adult *Xenopus* tissues (brain, heart, lung, stomach, intestine, kidney, testis and ovary). Wee1A mRNA was detected strongly in the ovary but not in the other tissues (Figure 2A, upper panel), consistent with it being a maternal mRNA (Nakajo *et al.*, 2000). In contrast, Wee1B mRNA was detected, whether strongly or weakly, in all the tissues examined, including the testis and ovary (Figure 2A, lower panel). Thus, clearly, Wee1B mRNA was expressed ubiquitously while Wee1A mRNA was not.

We next examined expression patterns of Wee1A and Wee1B mRNAs during early development. Wee1A mRNA was present in both immature and mature oocytes, as shown previously (Nakajo *et al.*, 2000), and also in early (or pregastrula) embryos but not in postgastrula embryos

A

ATGAACGTCCAGCCCCGGAATATGAACGTCCAGCCCCGGAATATGAACGTCCAGCCCCGTCGCCACAAGCTCTTC	75
M N V Q P R N M N V Q P R N M N V Q P V R H K L F	25
TTTCAGGCACACAGACGAGGAGGAGGAGGACGACACACGCGGGGAGGACTCGGCTTTTCAAGAGAGCGACTCG	150
F S D T D E E E E D G H S T G E D S A F Q E S D S	50
CCCGTGTCCCGCAGAGAGAGAAGCAGGAAGGGAAGCCCGCGGAGGACCTGGGAGGAGTTGGAGGAGGAGAA	225
P V S R Q R E K Q E G K P P G G T W E E L E E E E	75
GGGTTCGGATCGTCTCCCATCAAAATCGCCCGGGGATTTCTTCATGAGCGACTCCCGTCTTACCGGCAACTGGCT	300
G F G S S P I K S P G D F F M S D S P S Y R Q L A	100
CCCGCTTCCCTCCCGATCCCTCCCAAGGACCCACATCCCTCATCCCGAGTGCCTGGGACCCCGCCCAAG	375
P A S P T R S P Q G P T S P I P E C P G T P P H K	125
ACTTTCCGCAAGCTCCGCCTCTTCGACACCCCGCACACCCCAAGAGTTTATTGTGAAAGCTCGAGGAATCGGC	450
T F R K L R L F D T P H T P K S L L S K A R G I G	150
TCGAGCGCATTGAGGTTTCGAGGCGGGACGCTATTCCGAGAAGCGGAGAAGGCTCCAAAGCCAGAGTTTGTGTAC	525
S S A L R F R G T L F R E A E K A P K P E F V Y	175
AGCACCCCTCAGGTCAACATCAACCCCTTCACCCCGGATTCTTGAAATCCAGTCTTCTGCGGGCTTTGTGCG	600
S T P Q V N I N P F T P D S L E I Q S S A G L C R	200
GGGAGAAAAGGGCACTTTTAAATGACTCTCTGGGGAAGACATGGAAGCAGCGACTGTGAAGTGAAGATGAA	675
G R K R A L L N D S C G E D M E G S D C E L E D E	225
GACATCCGCGCCGGAAGCGGATCCCAATAACAGAAAGCAACATGAAGTCTCGATACGCAACAGAAATCCATGAA	750
D I R P A K R I P I T E S N M K S R Y A T E F H E	250
CTGTGAGAAATCGGTTCCGGAAGAATTCGGCTCTGTGTTTAAATGCGTGAAGAGACTCGATGGCTGCATCTACGCC	825
L E K I G S G E F G S V F K C V K R L D G C I Y A	275
ATAAAGCGATCGAAGAAGCCCTTTCGCGCTCTGTGGATGAGCAGAATGCTTTGAGAGAGGTGTATGCACACGCT	900
I K R S K K P L A G S V D E Q N A L R E V Y A H A	300
GTATTGGGACAGACCCCATGTGGTCCGATACTACTCCGCTTGGGAGAGATGACCACATGCTCATTGAGAAT	975
V L G Q H P H V V R Y Y S A W A E D D H M L I Q N	325
GAGTATTGTAATGGCGGGAGCTTGTCTGATGTCATCAGTGAGAATTACAGGACGATGCAATATTTACGGAGCCA	1050
E Y C N G G S L S D V I S E N Y R T M Q Y F T E P	350
GAAATTAAGGATTTGCTCTTGCAAGTGGCTCGCGCCTGAAATATATTCATCCATGTCACTGGTGACATGGAC	1125
E L K D L L L Q V A R G L K Y I H S M S L V H M D	375
ATAAAGCCGAGTAACATATTCATATCAAGGACGACCCCTCCCAACACGCGCAGTCGAAGAAGCGGACGATGAGAA	1200
I K P S N I F I S R T T L P N T A V E E A D D E E	400
TGTGGCTCCGGAAGTAATCTATAAAATAGGTGATCTTGGTCACGTAACAAGAGTCTCCAGTCCCAAGTGGAA	1275
C G S G K V I Y K I G D L G H V T R V S S P Q V E	425
GAAGGTGACAGTCGGTTTCTTGCCAATGAAGTGCTACAGGAGAACTATACCCACTTGGCAAAAGCTGATATATT	1350
E G D S R F L A N E V L Q E N Y T H L A K A D I F	450
GCCCTGGCGCTGACGTGTGGAGCGCAGCGGAGCAGAGCCCTTCTTACCAATGGAGACAGTGGCAGCAGATC	1425
A L A L T V W S A A G A E P F P T N G D Q W H E I	475
AGACAGGGAAGCTGCCCCGGGTGCCACAGTTACTCTCTCAAGAGTTTGTGGACTTAATCAAGCTTATGATTTCA	1500
R Q G G K L P R V P Q L L S Q E F V D L I K L M I S	500
CCCGATCCAGAAAAGGCTTCGTCCGTGGCCCTGGTCAAGCATTCGGTTCTCTATCCGCGTCTCGCAAGAGC	1575
P D P E K R P S S V A L V K H S V L L S A S R K S	525
GCCGAGCAGCTGCGCATAGAATCGATGCCAGAAGTTTAAATGCACTTCTGCGAGAAGAACTGAAGAAAGCA	1650
A E Q L R I E L D A E K F K N A L L Q K E L K K A	550
CAATAGCCAAAGCAGCGCAGAGGAACGAGCCATTTCCTCCGACCGGATAGCGACTCGATCCAGACTCAGAAC	1725
Q I A K A A A E E R A H F P D R I A T R S T T Q N	575
AACCGGACGACCCGCTCATCGGGAAGAGATGAACCGATCCGTCAGCCTCACTATATACTGA	1788
N R T T R L I G K K M N R S V S L T I Y *	595

B

Xe-Wee1A 1	203	204	480	481	555
	37%		69%		37%
Xe-Wee1B 1	241	242	518	519	595
	54%		84%		89%
Wee1Hu 1	292	293	569	570	646
	NRD	KD	CRD		

Fig. 1. Sequence analysis of Xe-Wee1B. (A) Nucleotide and deduced amino acid sequences of Xe-Wee1B. Only the coding region is shown. The kinase domain is boxed; the N-terminal repeated sequences, a NLS-like sequence (RGRKR) and a putative 14-3-3 binding motif (RSVS; see Discussion) are all denoted with a solid underline; two PEST-like sequences are indicated with a broken underline. The nucleotide sequence data have been deposited in the DDBJ/EMBL/GenBank (accession No. AB071983). (B) Comparison of the predicted amino acid sequence of Xe-Wee1B with those of Xe-Wee1A (Mueller *et al.*, 1995a) and human Wee1 (Wee1Hu; Watanabe *et al.*, 1995). Sequence comparisons (shown by percentage identity) were made by using the DNASIS program for each of the three structural domains, i.e. the NRD, the KD and the CRD, which were tentatively defined according to Hanks *et al.* (1988). Amino acid positions defining the respective domains are also shown.

(Figure 2B). In contrast, Wee1B mRNA was present not only in oocytes and pregastrula embryos but also in postgastrula embryos (at least until the tailbud stage), and, notably, its levels were significantly higher in the latter. In *Xenopus*, zygotic transcription begins at the midblastula

stage and increases greatly after the early gastrula stage (Newport and Kirschner, 1984; Howe *et al.*, 1995). Therefore, at the mRNA level, Wee1A was most probably maternal, but Wee1B was both maternal and zygotic, although principally zygotic.

We also determined expression patterns of Wee1A and Wee1B proteins during development by immunoblot analysis. In agreement with previous reports (Murakami and Vande Woude, 1998; Nakajo *et al.*, 2000), Wee1A protein was absent in immature oocytes but was present in mature oocytes and early embryos; however, it disappeared gradually during gastrulation and was not detected after the neurula stage (Figure 2C, upper panel). By using Wee1B-specific antibody, Wee1B protein (with a predicted size of 67 kDa) was not detected in oocytes or pregastrula embryos (despite the appreciable presence of its mRNA), but became detectable during gastrulation and persisted at least up to the late tailbud stage (Figure 2C, lower panel). Essentially similar results were obtained with anti-Wee1Hu antibody that could recognize Wee1B (see Materials and methods). Thus, except at the immature oocyte stage (for Wee1A) and at the pregastrula stages (for Wee1B), the expression patterns of Wee1A and Wee1B proteins during development were essentially similar to those of their mRNAs (Figure 2B). These results indicate that, at the protein level, Wee1A and Wee1B are maternal and zygotic isoforms, respectively, and that a switchover from Wee1A to Wee1B expression occurs during gastrulation, a period during which the cell cycle lengthens greatly in a zygotic transcription-dependent manner (Frederick and Andrews, 1994; Howe *et al.*, 1995).

M phase-inhibiting kinase activities of Wee1A and Wee1B

We next compared several properties of Wee1A and Wee1B proteins. Ectopically expressed Wee1A can inhibit progesterone-induced oocyte maturation (or entry into the first meiotic M phase) by directly phosphorylating and inhibiting Cdc2 (Murakami and Vande Woude, 1998; Nakajo *et al.*, 2000). Therefore, first we compared M phase-inhibiting activities of Wee1A and Wee1B by ectopically expressing them in immature oocytes (having no endogenous Wee1 protein) and then monitoring the kinetics of maturation or germinal vesicle breakdown (GVBD) after progesterone treatment. We injected immature oocytes with either 25 or 100 pg of Wee1A or Wee1B mRNA, and confirmed that 12 h after the injection (or just prior to progesterone treatment), Wee1A and Wee1B proteins were synthesized at comparable levels and in a dose-dependent manner (see Figure 3A, inset). These proteins, particularly Wee1B, showed upward size shifts, which were found to be due to phosphorylation (data not shown). After progesterone treatment, the oocytes injected with 25 pg of Wee1A mRNA underwent 50% GVBD nearly normally or only 30 min later than uninjected control oocytes, but those oocytes injected with the same amount of Wee1B mRNA showed 50% GVBD very slowly or 3 h later than uninjected oocytes (Figure 3A). On the other hand, the oocytes injected with 100 pg of Wee1A mRNA underwent GVBD essentially with the same kinetics as the oocytes injected with 25 pg of Wee1B mRNA, and those oocytes injected with 100 pg of Wee1B mRNA hardly showed GVBD even long after progesterone treatment (Figure 3A). Thus, interestingly, Wee1B had a significantly (or ~4-fold) greater M phase-inhibiting activity than Wee1A.

We tested whether the different M phase-inhibiting activities of Wee1A and Wee1B could be ascribed to any

Fig. 2. Tissue-specific and developmental expressions of Xe-Wee1A and Xe-Wee1B. (A) Tissue-specific expression of Xe-Wee1A and Xe-Wee1B mRNAs. Xe-Wee1A and Xe-Wee1B transcripts from various adult *Xenopus* tissues were analyzed by RT-PCR. (B) Developmental expression of Xe-Wee1A and Xe-Wee1B mRNAs. Xe-Wee1A and Xe-Wee1B transcripts from immature oocytes, unfertilized eggs and various developing embryos were analyzed by RT-PCR. Nieuwkoop-Faber (N/F) stages are shown at the bottom. (C) Developmental expression of Xe-Wee1A and Xe-Wee1B proteins. Proteins equivalent to one oocyte or embryo were analyzed by immunoblotting using either Xe-Wee1A- or Xe-Wee1B-specific antibodies.

of their regulatory domains. To do this, we injected oocytes with 50 pg of mRNA encoding chimeric Wee1 protein in which either the NRD or CRD (Figure 1B) of one Wee1 isoform was replaced by the counterpart of the other isoform. Wee1A having a substituted Wee1B NRD (Wee1A-NtB) inhibited GVBD more weakly than wild-type Wee1A, while Wee1B having a Wee1A NRD (Wee1B-NtA) inhibited GVBD more strongly than wild-type Wee1B (Figure 3B); thus, somewhat surprisingly, the Wee1B NRD conferred a weaker M phase-inhibiting activity on Wee1 protein than did the Wee1A NRD. Intriguingly, however, Wee1A having a substituted Wee1B CRD (Wee1A-CtB) inhibited GVBD much more strongly than Wee1A (comparable to Wee1B-NtA), while Wee1B having a Wee1A CRD (Wee1B-CtA) inhibited GVBD much more weakly than Wee1B (comparable to Wee1A-NtB) (Figure 3B). Thus, contrary to the NRD, the CRD of Wee1B conferred a much greater M phase-inhibiting activity on Wee1 protein than did the Wee1A CRD, and the Wee1 constructs with the Wee1B CRD consistently showed greater activities than those with the Wee1A CRD. In these experiments, the various Wee1 constructs (all having the same N-terminally located Myc epitope) were expressed at comparable levels in oocytes (see Figure 3B, inset). Thus, these results show that Wee1B has a greater net M phase-inhibiting activity than Wee1A due to its CRD.

We next tested whether the different M phase-inhibiting activities of the various Wee1 constructs were due to their

Fig. 3. M phase-inhibiting and Cdc2-phosphorylating activities of Xe-Wee1A and Xe-Wee1B. (A) Maturation-inhibiting activity of wild-type Xe-Wee1A and Xe-Wee1B proteins in oocytes. Thirty immature oocytes were left uninjected (Cont.) or injected with either 25 or 100 pg of mRNA encoding (Myc-tagged) wild-type Xe-Wee1A or Xe-Wee1B, cultured for 12 h, treated with progesterone (PG), and then cultured and scored for the percentage GVBD. Expression levels of Xe-Wee1A and Xe-Wee1B proteins just before PG treatment were determined by immunoblot analysis using anti-Myc antibody (see inset). (B) Maturation-inhibiting activity of chimeric Wee1 constructs. Thirty immature oocytes were injected with 50 pg of mRNA encoding either the wild-type or the indicated chimeric Wee1 constructs, cultured for 12 h, and then scored for the percentage GVBD. Expression levels of the respective ectopic proteins are shown in the inset. (C and D) Kinase activity of various Wee1 constructs. Oocytes were left uninjected (Cont.) or injected with 1 ng of mRNA encoding either of the Wee1 constructs indicated and were cultured overnight. Their extracts were then subjected either to immunoblot analysis using anti-Myc antibody [to confirm that the expression levels of the respective constructs were comparable; data not shown but see (B)] or to Wee1 kinase assays using [γ -³²P]ATP and Cdc2–cyclin B complexes as substrate (see Materials and methods) (bottom panel). Wee1 kinase activities that were quantitated (see Materials and methods) and averaged from three independent experiments are shown in arbitrary units (see graphs).

different kinase activities to phosphorylate (and inhibit) Cdc2. For this, we incubated an excess of Cdc2–cyclin B complexes (as substrate) and [γ -³²P]ATP with oocyte extracts containing the ectopically expressed Wee1 constructs. In these experiments, the various Wee1 constructs were present in comparable amounts in the respective extracts (data not shown; Figure 3B, inset) and the (Wee1) kinase assays were linear with respect to the incubation time (see Materials and methods). Under these conditions, the extracts containing (wild-type) Wee1B showed ~5-fold greater kinase activity to phosphorylate Cdc2 than those containing Wee1A (above a background activity due presumably to endogenous Myt1), the extracts containing Wee1A-CtB and Wee1B-NtA both had ~2-fold greater kinase activity than those containing Wee1B, and the extracts containing Wee1A-NtB and Wee1B-CtA both showed ~2-fold weaker activity than those containing Wee1A (Figure 3C). Essentially similar results were obtained with *in vitro* kinase assays using immunoprecipitated Wee1 constructs, although the radioactive signals of Cdc2 in these assays were considerably weaker than those in oocyte extracts, presumably due to the low

efficiency of immunoprecipitation (data not shown). Thus, apparently, the kinase activities of the respective Wee1 constructs paralleled well their M phase-inhibiting activities (compare with Figure 3B). Together, these results strongly indicate that Wee1B has a much greater M phase-inhibiting kinase activity than Wee1A, and that the greater activity of Wee1B is attributable to its short (76 amino acids) CRD.

What sequence(s) within the CRD would be responsible for the greater kinase activity of Wee1B? A recent study suggests that Wee1Hu kinase activity may be enhanced by the binding of 14-3-3 proteins to the sequence motif RSVS (the last serine residue being phosphorylated), which is located at the C-terminus of the Wee1Hu CRD (Wang *et al.*, 2000). Notably, the same binding motif (RSVS) exists at the corresponding site of the Wee1B CRD (positions 588–591; Figure 1A). However, a similar motif (RSLS) also exists at the corresponding site of the Wee1A CRD and is implicated in the regulation of Wee1A kinase activity (Lee *et al.*, 2001). To determine whether the 14-3-3 binding motif was responsible for the greater kinase activity of Wee1B, we compared the kinase

activities of the Ser→Ala 14-3-3 binding motif mutants of Wee1B and Wee1A (Wee1B-S591A and Wee1A-S549A, respectively) with those of wild-type Wee1B and Wee1A, in the manner described above (Figure 3C). Wee1B-S591A and Wee1A-S549A had about one-third the kinase activities of wild-type Wee1B and Wee1A, respectively, and Wee1B-S591A still had nearly 2-fold greater activity than wild-type Wee1A (Figure 3D). Thus, although the 14-3-3 binding motif was certainly important for the kinase activity of Wee1B, it was not a critical determinant for the greater kinase activity of Wee1B than Wee1A. We conclude that some other sequence(s) in the CRD is responsible for the greater kinase activity of Wee1B.

Stability of Wee1A and Wee1B during meiosis II

Wee1A protein is absent in immature (meiosis I) oocytes but is present and stable in mature (meiosis II) oocytes (Murakami and Vande Woude, 1998; Nakajo *et al.*, 2000), and the Wee1A protein stored in mature oocytes (arrested at metaphase II) is immediately required for the first embryonic cell cycle (Murakami and Vande Woude, 1998; Walter *et al.*, 2000). Therefore, we next tested whether ectopically expressed Wee1B was also stable during meiosis II (and hence could function immediately after fertilization). For this, we coinjected immature oocytes with 200 pg each of two mRNAs encoding, respectively, (Myc-tagged) kinase-deficient Wee1A and Wee1B (because at this dose of mRNA, wild-type Wee1A and Wee1B inhibited maturation; Figure 3A), treated them with progesterone, and harvested them every 1 h after GVBD for immunoblot analysis. Control Wee1A protein was readily detected even well after 2 h of GVBD (or during meiosis II; Furuno *et al.*, 1994) (Figure 4A, a, lower bands), consistent with it being stable during meiosis II and metaphase II arrest, like endogenous Wee1A (Iwabuchi *et al.*, 2000; Nakajo *et al.*, 2000). In contrast, Wee1B protein, though readily detected 1–2 h after GVBD, decreased 3 h after GVBD and was hardly detected thereafter or during metaphase II arrest; in the absence of progesterone treatment, however, it persisted stably for a prolonged period (Figure 4A, a, upper bands). In these experiments, Wee1B protein underwent progressive upward mobility shifts, probably phosphorylation, after GVBD. We observed that even wild-type Wee1B was degraded during meiosis II, when expressed at much lower levels by injection of 25 pg of mRNA (in which case GVBD occurred, albeit slowly; Figure 3A) (data not shown). Thus, unlike Wee1A, Wee1B was very unstable during meiosis II and could not accumulate in mature oocytes arrested at metaphase II.

We examined whether the different stabilities of Wee1A and Wee1B during meiosis II could be ascribed to either of their regulatory domains, NRD or CRD. For this, we first compared the stabilities of C-terminal chimeric constructs, Wee1A-CtB and Wee1B-CtA (each being kinase deficient), which were coexpressed in the same oocytes. Results showed that Wee1A-CtB and Wee1B-CtA had essentially the same stabilities during meiosis II as parental Wee1A and Wee1B, respectively (compare Figure 4A, b, with Figure 4A, a). We then compared the stabilities of N-terminal chimeric constructs Wee1A-NtB and Wee1B-NtA. Strikingly, while Wee1B-NtA was as stable as Wee1A during meiosis II,

Fig. 4. Stability of various Wee1 constructs during oocyte maturation. (A) Immature oocytes were coinjected with 200 pg each of two mRNAs encoding kinase-deficient full-length Xe-Wee1A and Xe-Wee1B (a), their C-terminal chimeric constructs (b), their N-terminal chimeric constructs (c) or their N-terminally truncated mutants (d), each tagged with three Myc epitopes. One hour later, the oocytes were treated with progesterone and, at every 1 h interval after GVBD, were sampled for immunoblot analysis with anti-Myc antibody. Those injected oocytes that were cultured without progesterone treatment (–PG) until the time corresponding to 8 h after GVBD of progesterone-treated oocytes were also analyzed. MII, meiosis II. (B and C) Immature oocytes were coinjected with 200 pg each of seven mRNAs encoding kinase-deficient full-length (FL) Xe-Wee1B or its N-terminal truncation mutants [as indicated in (B)] or injected, respectively, with 200 pg of mRNA encoding either FL Xe-Wee1B or its N-terminal truncation or internal deletion mutants [as indicated in (C)]. Oocytes were treated with progesterone, sampled at 2 h intervals after GVBD, and processed as in (A).

Wee1A-NtB was as unstable as Wee1B (Figure 4A, c). Thus, the NRD, but not the CRD, of Wee1B appeared to confer instability on Wee1 protein during meiosis II. To confirm this idea, we tested for the stability of NRD-truncated Wee1B (Wee1B-ΔN) as well as NRD-truncated Wee1A (Wee1A-ΔN). Results showed that not only Wee1A-ΔN but also Wee1B-ΔN was very stable during meiosis II (Figure 4A, d). These analyses show clearly that the NRD is responsible for the instability of Wee1B during meiosis II and metaphase II arrest.

We attempted to identify the sequence(s) within the 241 amino acids of the Wee1B NRD that conferred instability during meiosis II. For this, first we tested for the stabilities of a series of N-terminal truncation mutants of Wee1B. Compared with full-length (FL) Wee1B, an N-terminal 40

Fig. 5. Effects of ectopic expression of Xe-Wee1A or Xe-Wee1B on cell division and the development of early embryos. (A) Expression levels of ectopic Xe-Wee1A and Xe-Wee1B proteins. One-cell embryos injected with 250 pg of mRNA encoding either Myc-tagged Xe-Wee1A (+Wee1A) or Xe-Wee1B (+Wee1B) were cultured and, at the indicated stages, were subjected to immunoblot analysis with either anti-Xe-Wee1A antibody (α -Wee1A) or anti-Myc antibody (α -Myc), to detect endogenous (endo-) or exogenous (exo-) Wee1 proteins. (B) External morphology. Embryos ectopically expressing Xe-Wee1A or Xe-Wee1B as in (A) were photographed at the indicated stages of uninjected control embryos (Cont.). (C) DNA content. Total genomic DNA from the embryo ectopically expressing Xe-Wee1A or Xe-Wee1B as in (A) was analyzed at the indicated stages by agarose gel electrophoresis followed by ethidium bromide staining. (D) Tyr15 phosphorylation of Cdc2. Embryos ectopically expressing Xe-Wee1A or Xe-Wee1B as in (A) were analyzed at the indicated stages by immunoblotting with anti-Cdc2 phospho-Tyr15 antibody. (E) Thirty one-cell embryos were injected either with 250 pg of mRNA encoding either one of the Xe-Wee1B constructs indicated or with 250 pg or 1.25 ng (5 $\times$) of mRNA encoding Xe-Wee1A, cultured, and scored for percentage embryonic death at the midgastrula stage.

amino acid-truncated mutant ($\Delta 40$) and other truncation mutants ($\Delta 80$ – $\Delta 240$) were all very stable during meiosis II (Figure 4B), indicating that the sequence(s) required for the instability of Wee1B was located around amino acid 40. Further truncation and internal deletion analyses revealed that while $\Delta 10$ and $\Delta 20$ mutants were as unstable as FL Wee1B and a $\Delta 81$ –100 mutant was significantly unstable (if not comparable to FL Wee1B), $\Delta 30$, $\Delta 41$ –60 and $\Delta 61$ –80 mutants were all very stable (Figure 4C). (In these experiments, $\Delta 20$ and $\Delta 30$ mutants, but not others, showed no upward size shifts after GVBD, suggesting that some sequence(s) around amino acid 20 was required for phosphorylation.) Thus, at least the N-terminal 60-amino-acid sequence (positions 21–80) was essential for the instability of Wee1B during meiosis II. This N-terminal sequence was composed mostly of two PEST-like sequences (positions 37–53 and 62–82; Figure 1A), a sequence typical of short-lived proteins (Rechsteiner and Rogers, 1996), and was largely lacking in the NRD of Wee1A (Mueller *et al.*, 1995a). Thus, it seems that at least the N-terminal 60-amino-acid sequence is a determinant for the instability of Wee1B during meiosis II.

Effects of ectopic Wee1B expression on early embryogenesis

Because Wee1B protein had significantly different properties from Wee1A, we addressed the important question of whether (ectopic) Wee1B could functionally replace Wee1A during early embryogenesis (or at pregastrula stages when only Wee1A is present; Figure 2C). In our initial studies, specific inhibition or ablation of endogenous Wee1A in early embryos and its replacement with ectopic Wee1B turned out to be technically difficult. Therefore, instead, we ectopically expressed Wee1A or Wee1B proteins (at levels comparable to endogenous Wee1A) in early embryos and compared their effects on cell divisions and development. By injection of 250 pg of mRNA encoding either (Myc-tagged) Wee1A or Wee1B into one-cell embryos, ectopic Wee1A or Wee1B proteins were synthesized in amounts comparable to endogenous Wee1A at the morula (stage 6) to initial gastrula (stage 10) stages (Figure 5A). Under these conditions, the embryos expressing ectopic Wee1A developed quite normally throughout early development (at least up to the neurula stage) (Figure 5B). In contrast, the embryos expressing

ectopic Wee1B showed an apparent delay in cell divisions from the early blastula (stage 7½) to initial gastrula stages, failed to progress through gastrulation (retaining a large yolk plug), and eventually died with a dramatic disruption of intercellular contacts (characteristic of apoptosis; Anderson *et al.*, 1997) at the time of the midgastrula stage (stage 11½) of control embryos (Figure 5B). Consistent with the delay in cell divisions, the DNA content in the Wee1B-expressed embryo was 2- to 4-fold lower than that in uninjected control or Wee1A-expressed embryos at the midblastula (stage 8½ or the MBT) to early gastrula (stage 10½) stages (Figure 5C). Moreover, and importantly, Tyr15 phosphorylation of Cdc2 occurred considerably earlier (or 2–3 h before the MBT) in Wee1B-expressed embryos than in uninjected control or Wee1A-expressed embryos (Figure 5D), consistent with Wee1B having a much greater kinase activity (towards Cdc2) than Wee1A (Figure 3). These results suggest that ectopic Wee1B but not Wee1A affects cell division cycles in early embryos due to its strong kinase activity, and thereby leads to apoptosis-like cell death at later gastrula stages.

To ascertain whether the observed effects of ectopic Wee1B on early embryogenesis were indeed due to its very strong kinase activity (conferred by the CRD; Figure 3B and C), we also tested the effects of various Wee1B constructs on early embryogenesis, particularly cell viability. When expressed at levels comparable to endogenous Wee1A, neither kinase-deficient Wee1B (Wee1B-KR) nor CRD-truncated Wee1B (Wee1B-ΔC) had an appreciable effect on embryogenesis (Figure 5E). Moreover, expression of Wee1B-CtA (with a substituted Wee1A CRD and a much lower kinase activity than wild-type Wee1B; Figure 3C) had little effect on early development, whereas that of Wee1B-NtA (with a Wee1A NRD and a greater activity than Wee1B) did induce a delay in cell divisions (not shown) and then embryonic death at the midgastrula stage as efficiently as expression of wild-type Wee1B (Figure 5E). Thus, these results strongly indicate that Wee1B affects early embryogenesis by virtue of its CRD-dependent strong kinase activity. To confirm this idea, we simply expressed wild-type Wee1A ~5-fold more than endogenous Wee1A (or to a kinase activity level comparable to wild-type Wee1B; Figure 3C). Even this overexpression induced a delay in early embryonic cell divisions (not shown) and then efficient embryonic death at the midgastrula stage (Figure 5E). Thus, these results establish that ectopic Wee1B affects cell division in early embryos and then impairs cell viability at the gastrula stage, due primarily to its very strong kinase activity. Taken together, the present results suggest strongly that zygotic Wee1B cannot functionally replace maternal Wee1A in early embryogenesis.

Discussion

Structure and expression of Wee1A and Wee1B

In this study, we have isolated a second isoform of *Xenopus* Wee1. This isoform, termed Xe-Wee1B, has a much greater overall similarity to the human Wee1 homolog (Wee1Hu; Watanabe *et al.*, 1995) than to the conventional *Xenopus* Wee1 homolog (termed here Xe-Wee1A) (Mueller *et al.*, 1995a) (Figure 1B). Thus, Wee1B, rather than Wee1A as previously thought (Nemer

and Stuebing, 1996), is most probably a genuine *Xenopus* homolog of human Wee1Hu. Unlike Wee1Hu, however, Wee1B has three tandem repeats of seven amino acids at its N-terminus. These repeats, which are unlikely to be a cloning artefact (see Results), are identical to each other even at the nucleotide level (see Figure 1A), suggesting that they were evolutionarily generated very recently. The function (if any) of these repeats is currently unknown, although at least it does not seem to be involved in the stability of Wee1B (Figure 4C). Wee1 protein is generally nuclear (Pendergast, 1996), and we indeed found that ectopically expressed Wee1B localizes to the nucleus in oocytes and that the N-terminally located NLS-like sequence (RGRRK; Figure 1A) is required for nuclear localization (our unpublished data).

Wee1B mRNA was present in all the tissues examined, including the ovary and testis, while Wee1A mRNA was detected only in the ovary (Figure 2A). During early development, Wee1B mRNA was detected both in oocytes and throughout embryogenesis, but its protein product was detected only after the gastrula stage (Figure 2B and C), suggesting a translational repression of maternal (but not zygotic) Wee1B mRNA (Curtis *et al.*, 1995). In contrast, Wee1A mRNA was present in oocytes and early embryos until the early gastrula stage (see also Nakajo *et al.*, 2000), and its protein product was detected in mature (meiosis II) oocytes and pregastrula embryos (see also Murakami and Vande Woude, 1998). Thus, clearly, at the protein level Wee1A and Wee1B were maternal and zygotic isoforms, respectively, and the switchover from Wee1A to Wee1B expression occurred during gastrulation, suggesting a role for Wee1B in the greatly expanded, somatic-like cell cycles in postgastrula embryos (see below). The absence of Wee1B protein (as well as Wee1A protein) in meiosis I oocytes is consistent with our recent proposal that absence of Wee1 is essential for the meiotic cell cycle in animal oocytes (Nakajo *et al.*, 2000).

M phase-inhibiting kinase activities of Wee1A and Wee1B

Wee1 phosphorylates Cdc2 on Tyr15 and inhibits entry into M phase. We compared the M phase-inhibiting activities of Wee1A and Wee1B by ectopically expressing them in immature oocytes, and found that Wee1B has a significantly (~4-fold) greater maturation (or M phase)-inhibiting activity than Wee1A (Figure 3A). Consistent with this, Wee1 kinase assays (using Cdc2–cyclin B as substrate) showed that Wee1B has a much (~5-fold) greater kinase activity than Wee1A (Figure 3C). Interestingly, analyses using chimeric mutants revealed that while the NRD of Wee1B confers a somewhat weaker M phase-inhibiting kinase activity on Wee1 protein than does the Wee1A NRD, the short CRD of Wee1B confers a much greater activity than the Wee1A CRD (Figure 3B and C), indicating that the greater net activity of Wee1B is attributable to its CRD. Moreover, and somewhat surprisingly, the CRD-truncated mutants (as well as the NRD-truncated ones) of both Wee1A and Wee1B had little if any kinase activity (our unpublished data; Figure 5E), indicating that the kinase domain alone of either Wee1A or Wee1B is catalytically nearly inactive. Taken together, these results suggest strongly that although both of the CRDs of Wee1A and Wee1B act to positively regulate

Wee1 kinase activity, the CRD of Wee1B does so much more potently than that of Wee1A. In this regard, it is noteworthy that unlike the KD region, the CRD region is highly divergent between Wee1A and Wee1B (~40% identity; Figure 1B).

Interestingly, between Wee1B and human Wee1Hu, the CRD is best conserved among the three domains (NRD, KD and CRD) (~90% identity; Figure 1B), suggesting an important role in the strong kinase activity of somatic-type Wee1. Indeed, the kinase activity of Wee1Hu is as strong as that of Wee1B (our unpublished data). Notably, at the C-terminus of the CRD, both Wee1B and Wee1Hu have the same 14-3-3 binding motif (RSVS) (Figure 1A; Watanabe *et al.*, 1995), which, in the case of Wee1Hu, is suggested to function to enhance kinase activity (Wang *et al.*, 2000). However, at the corresponding site, Wee1A also has a similar binding motif (RSLS) that can enhance kinase activity (Lee *et al.*, 2001). We showed here that mutation of the 14-3-3 binding motif of Wee1B can reduce the kinase activity, but only to the same extent as that of Wee1A (Figure 3D). Thus, some other sequence(s) in the CRD seems to be responsible for the greater kinase activity of Wee1B over Wee1A.

Stability of Wee1A and Wee1B during meiosis II

Wee1A protein is absent or very scarce during meiosis I, but is present and stable during meiosis II in oocytes (Murakami and Vande Woude, 1998; Iwabuchi *et al.*, 2000; Nakajo *et al.*, 2000). Interestingly, however, (ectopic) Wee1B, unlike Wee1A, was very unstable during meiosis II and could not accumulate in mature oocytes arrested at metaphase II (Figure 4A). Analyses of chimeric as well as deletion mutants revealed that the different stabilities of the two Wee1 isoforms are attributable to their highly divergent NRDs (Figure 4A), and that at least the N-terminal 60-amino-acid sequence within the NRD is responsible for the instability of Wee1B during meiosis II (Figure 4B and C). Very interestingly, this N-terminal sequence within the Wee1B NRD contained two PEST-like sequences (typical of short-lived proteins; Rechsteiner and Rogers, 1996) (Figure 1A), which are conserved in Wee1Hu (Watanabe *et al.*, 1995) but are largely lacking in Wee1A (Mueller *et al.*, 1995a). Thus, the PEST-like sequences seem to be involved directly in the instability of (ectopic) Wee1B protein during meiosis II. Generally, Wee1 protein is unstable at M phase of the mitotic (or somatic) cell cycles in diverse species (Watanabe *et al.*, 1995; Aligue *et al.*, 1997; Sia *et al.*, 1998), but an N-terminally truncated form of Wee1Hu (which lacks the PEST-like sequences) is very stable throughout the cell cycle in transfected cells (Wang *et al.*, 2000). Therefore, the PEST-like sequence-dependent degradation of Wee1B during meiosis II may be due to its intrinsic instability during M phase.

Given the instability of somatic-type Wee1 at M phase, it is rather surprising that Wee1A is very stable during meiosis II and metaphase II arrest in mature oocytes. (In cleavage stage embryos, ectopic Wee1A and Wee1B did not show a great difference in their stabilities, due presumably to the very rapid cell cycles; Figure 5A.) Recent studies show, however, that both budding yeast Wee1 and *Xenopus* Wee1A proteins can be degraded in an SCF ubiquitin ligase complex-dependent manner in cells

or cell-free extracts (Michael and Newport, 1998; Sia *et al.*, 1998). It seems possible, therefore, that both Wee1A and Wee1B have the same ability to be degraded by the SCF pathway, but only Wee1A can escape from degradation during meiosis II due to its highly divergent NRD. Hence, Wee1A may be a specialized form of Wee1 to be stored in mature oocytes and used immediately after fertilization (see also below).

Inability of Wee1B to functionally replace Wee1A in early embryos

To determine whether Wee1B could functionally replace Wee1A in pregastrula embryos (in which only Wee1A is present; Figure 2C), we compared the effects of ectopic expression of Wee1A and Wee1B on cell division and development. When expressed in early embryos at levels comparable to endogenous Wee1A, ectopic Wee1B but not Wee1A prematurely induced inhibitory Tyr15 phosphorylation of Cdc2 before the MBT (Figure 5D), slowed cell divisions (by one to two cycles) during the early blastula to initial gastrula stages (Figure 5C), impaired progression through gastrulation, and eventually led to apoptosis-like cell death at the midgastrula stage (Figure 5B). Apparently, these effects were due to the very strong, CRD-dependent kinase activity of Wee1B, as revealed by analyses of various Wee1B constructs (Figure 5E). Thus, these results suggest strongly that ectopic Wee1B cannot functionally replace endogenous Wee1A in early embryos, due primarily to its strong kinase activity.

The dramatic, apoptosis-like death of Wee1B-expressed embryos at the time of the midgastrula stage was somewhat surprising as these embryos could continue to divide, albeit considerably slowly, at least until the onset of gastrulation. A recent study shows that Wee1A can be involved in apoptosis in cell-free extracts, depending on its kinase activity but not on its cell cycle function (Smith *et al.*, 2000). Formally, therefore, it is possible that Wee1B (having a much greater kinase activity than Wee1A) directly induced apoptosis at the gastrula stage. Notably, however, apoptosis can be induced at the gastrula stage by various treatments of cleaving *Xenopus* embryos (Anderson *et al.*, 1997; Hensey and Gautier, 1998; Stancheva *et al.*, 2001). Specifically, inhibiting initiation of zygotic transcription at the MBT can efficiently induce apoptosis at the gastrula stage (Sible *et al.*, 1997), and even slowing the cleavage cell cycles by inhibition of the Wee1-antagonizing Cdc25A phosphatase can induce apoptosis at the gastrula stage (Kim *et al.*, 1999), most likely by perturbing zygotic transcription (Stack and Newport, 1997). The apoptosis-like death of Wee1B-expressed embryos may therefore be a consequence of slowed cell divisions and, hence, of perturbed zygotic transcription. In any case, it seems clear from our study that zygotic Wee1B cannot functionally replace maternal Wee1A in early embryos.

Wee1A and Wee1B: implications for the developmental regulation of the cell cycle

The present results, together with previous results (Murakami and Vande Woude, 1998; Nakajo *et al.*, 2000), enable us to depict how the cell cycles are regulated by members of the Wee1 kinase family in early *Xenopus* development (Figure 6A). In immature prophase I-arrested

Fig. 6. Expression of Wee1/Myt1 kinase family members and Tyr15 phosphorylation of Cdc2 in early *Xenopus* development (A) and the phylogenetic divergence of Wee1 homologs in metazoans (B). In (A), Pro-I, MI, MII and pY15 denote prophase I, meiosis I, meiosis II and Cdc2 Tyr15 phosphorylation, respectively. Myt1 is present throughout oogenesis and early embryogenesis, albeit at lower levels in postgastrula embryos (our unpublished data). The gradual cell-cycle elongation beginning at the MBT is probably due to the activation of the DNA replication checkpoint, while the further and dramatic cell-cycle elongation beginning at the early gastrula transition (EGT; Howe *et al.*, 1995) is dependent on zygotic transcription. See the text for details and relevant references. In (B), sequence comparisons of metazoan Wee1 homologs were made among their CRDs by using the Clustal_W program (Thompson *et al.*, 1994) because the CRD region is highly divergent between Xe-Wee1A and Xe-Wee1B, and is best conserved between Xe-Wee1B and Wee1Hu. The figures in the dendrogram represent bootstrap values. Sequence data were taken from *Drosophila* Wee1 (Dm; Campbell *et al.*, 1995), sea urchin Wee1 (Su; Nemer and Stuebing, 1996), starfish Wee1 (Sf; DDBJ/EMBL/GenBank accession No. AB064523), maternally expressed *Xenopus* Wee1 (Xe-A; Mueller *et al.*, 1995a), zygotically expressed *Xenopus* Wee1 (Xe-B; this work or accession No. AB071983), maternally expressed mouse Wee1 (Mu-m; accession No. AA549285), maternally expressed human Wee1 (Hu-m; Nakanishi *et al.*, 2000) and zygotically expressed human Wee1 (Hu-z; Watanabe *et al.*, 1995).

oocytes, no isoform of Wee1 is present (Murakami and Vande Woude, 1998), but Myt1, a membrane-associated Wee1-related kinase, is present and catalyzes Tyr15 (and Thr14) phosphorylation of Cdc2, thereby arresting the oocyte at G₂ phase (Palmer *et al.*, 1998; Nakajo *et al.*, 2000). Wee1A, a maternal isoform, is then synthesized during meiosis II and is stably present in mature oocytes arrested at metaphase II (Murakami and Vande Woude, 1998; Nakajo *et al.*, 2000); its absence or scarcity during meiosis I is essential for the omission of S phase during the meiosis I/meiosis II transition (Iwabuchi *et al.*, 2000; Nakajo *et al.*, 2000). The Wee1A protein stored in mature oocytes is required for the short G₂ phase in the first embryonic cell cycle (Murakami and Vande Woude, 1998; Walter *et al.*, 2000) and probably also for the extremely short G₂ phases in cycles 2–12 or until the MBT (the Wee1-antagonizing Cdc25A phosphatase is required for these rapid cell cycles) (Kim *et al.*, 1999). Wee1 is also required for the rapid nuclear divisions of early embryogenesis in *Drosophila* (Price *et al.*, 2000). Even if expressed in oocytes, Wee1B, a zygotic isoform, would not be able to functionally replace Wee1A immediately after fertilization, because it is unstable during meiosis II and cannot accumulate in mature oocytes. Between the MBT and the early gastrula stage (cycles 13–15), the interphase is gradually elongated, probably due to activation of the DNA replication checkpoint (Dasso and Newport, 1990; Howe *et al.*, 1995), which, in metazoans, requires Wee1 activity (Price *et al.*, 2000; Lee *et al.*, 2001). This interphase elongation at the MBT can occur in the absence of zygotic transcription (Newport and Dasso, 1989) and should thus require the activity of maternal Wee1A (and probably also Myt1) (Hartley *et al.*, 1996). Apparently, (ectopic) Wee1B cannot functionally replace Wee1A during this period as well as during the cleavage stage, because it does affect cell divisions and, later, cell

viability, due primarily to its very strong kinase activity. After the early gastrula stage (or the early gastrula transition), interphase is further and dramatically elongated in a transcription-dependent manner (Frederick and Andrews, 1994; Howe *et al.*, 1995), coincident with the expression of Wee1B (which replaces Wee1A) (Figure 6A). Thus, Wee1B, which has a very strong kinase activity, would play an important role in expanding the cell cycles after the gastrula stage. In this regard Wee1A, which has a much weaker activity and a different stability to Wee1B, might not be able to functionally replace Wee1B at the postgastrula stages. All together, it seems that the differential expression of the two Wee1 isoforms (as well as Myt1) has crucial implications for the developmental regulation of cell cycles in *Xenopus*.

Evolutionary and functional divergences of Wee1 homologs in metazoans

Recent studies suggest that the expression of a Wee1 homolog might regulate the timing of crucial cell divisions during early development of *Drosophila* (Price *et al.*, 2000), sea urchin (Nemer and Stuebing, 1996) and *Caenorhabditis elegans* (Wilson *et al.*, 1999). The question then arises of whether such Wee1 homologs correspond to Wee1A or Wee1B of *Xenopus*, or are derived from their common progenitor. *Drosophila* Wee1, which seems to be encoded by a single gene (Campbell *et al.*, 1995), is equally distantly related to both Wee1A and Wee1B (see Figure 6B). Interestingly, however, both sea urchin and starfish Wee1 homologs are apparently closer to Wee1B than to Wee1A in the CRD (a region that is highly divergent between Wee1A and Wee1B), although they are related approximately equally to both Wee1A and Wee1B in the entire sequence (data not shown) (Figure 6B). The sea urchin Wee1 mRNA is expressed at least zygotically (Nemer and Stuebing, 1996), and the starfish Wee1 protein

(encoded by a single gene) is expressed both maternally and zygotically (Kishimoto, 1998; and T.Kishimoto, personal communication); thus, echinoderms seem to have a single Wee1 homolog that is probably B-type Wee1. In contrast, it has recently been shown that a Wee1A-like gene (named Wee1B), besides the Wee1B homolog, exists in mice and humans, and that its mRNA is expressed maternally and is replaced zygotically by the Wee1B homolog mRNA (Nakanishi *et al.*, 2000). A Wee1A-like gene also exists in the fish *Carassius* and is expressed in oocytes (DDBJ/EMBL/GenBank accession No. AB051198). Thus, it seems that A-type Wee1 evolved from B-type Wee1 relatively recently or in the vertebrate lineage (Figure 6B). If so, an intriguing question would be why A-type Wee1 was generated only in vertebrates. One answer to this question might be related to the fact that mature oocytes are arrested at metaphase II only in vertebrates (Sagata, 1996), during which time any Wee1 homolog should be stable enough to function immediately after fertilization, as is *Xenopus* Wee1A. In this context, it is interesting to note that the starfish Wee1 homolog, which appears to be B-type Wee1, is also stably present in mature oocytes, which, however, in the case of starfish, are arrested in G₁ phase (Kishimoto, 1998). Further analyses of the regulation and properties of Wee1 homologs in both vertebrates and invertebrates will contribute to our better understanding of cell cycle regulation during metazoan development.

Materials and methods

Preparation, culture, microinjection and treatment of oocytes and embryos

Oocytes and embryos were prepared, cultured and microinjected as described previously (Sagata *et al.*, 1989; Uto and Sagata, 2000). To induce oocyte maturation, stage VI oocytes were treated with progesterone (5 µg/ml). Embryos were staged according to Nieuwkoop and Faber (1956).

cDNA cloning

To clone a cDNA encoding a novel isoform of *Xenopus* Wee1, PCR was first performed against a stage 30 tailbud embryo cDNA library in λZAPII (Stratagene) by using degenerate oligonucleotide primers. The degenerate primers used were derived from kinase domains V and VII of *Xenopus* (Mueller *et al.*, 1995a), human (Igarashi *et al.*, 1991; Watanabe *et al.*, 1995) and *Drosophila* Wee1 (Campbell *et al.*, 1995), and were 5'-GCITGGGCGA(A/G)GA(C/T)GA(C/T)CA(C/T)ATG-3' (where I is inosine) for the 5' primer and 5'-GTIAC(A/G)TGICCA(A/G)(A/G)TC(A/G/C/T)CC(A/G/T)AT(C/T)TT-3' for the 3' primer. A 0.3 kb PCR product was obtained and subcloned into the pGEM-T Easy vector (Promega), sequenced, and found to encode a partial sequence of a novel Wee1 isoform. This partial cDNA was used as a plaque hybridization probe to isolate longer cDNAs from the tailbud embryo cDNA library. The longest cDNA obtained was found to encode a novel *Xenopus* homolog (termed Xe-Wee1B) of human Wee1, but was a 5'-end-truncated one. To obtain the 5' end sequence of Xe-Wee1B cDNA, a 5'-RACE (rapid amplification of 5' cDNA ends) strategy was employed. RNA extracted from embryos, liver or testis was subjected to poly(A)⁺ selection, and a first strand cDNA was synthesized using a Xe-Wee1B-specific primer (5'-CCTGCTTCTCTCTGCGGGACACGGGCG-3') and the Thermoscript RT-PCR System (Gibco-BRL). A second strand cDNA was then synthesized using the Marathon cDNA Amplification Kit (Clontech). The reaction product was amplified by PCR (94°C for 1 min for one cycle, 94°C for 30 s and 72°C for 4 min for five cycles, 94°C for 30 s and 70°C for 4 min for five cycles, 94°C for 30 s and 68°C for 4 min for 25 cycles) using an adaptor primer (5'-CCATCCTAATACG-ACTCACTATAGGGC-3') and a second Xe-Wee1B-specific primer (5'-CCTCCAGGTGCCTCCCGGCGGCTTCCC-3'). The amplified

products were subcloned into the pGEM-T Easy vector, sequenced, and found to have a 5'-end sequence of Xe-Wee1B cDNA.

Construction of recombinant plasmids and in vitro transcription

A cDNA encoding Xe-Wee1A (Mueller *et al.*, 1995a) was isolated by PCR of a *Xenopus* oocyte cDNA library, using 5'-GAAGATCTACCAT-GAGGACGGCCATGTCATGCGGAGGAGG-3' as the 5' primer and 5'-GAAGATCTCAATACCCCTCCGAGGTGAAGCTCAGCG-3' as the 3' primer, and a Xe-Wee1B cDNA with the entire coding region was isolated by PCR of the reconstituted Xe-Wee1B cDNA (see above) using 5'-GAAGATCTCCCGGATCCACCGCCGTCTTCCCC-3' as the 5' primer and 5'-GAAGATCTTCAGTATATAGTGAGGCTGACGGA-TCGGTT-3' as the 3' primer. All of the primers contained an artificial Bgl/II site, and the amplified fragments were subcloned into the pT7G(UKII⁺) vector, a pIBI derivative of the transcription vector pSP64T (Uto and Sagata, 2000). cDNAs encoding 14-3-3 binding motif mutants (Ser549→Ala for Xe-Wee1A and Ser591→Ala for Xe-Wee1B) or kinase-deficient mutants (Lys239→Arg for Xe-Wee1A and Lys277→Arg for Xe-Wee1B) were constructed by site-directed mutagenesis using appropriate primers. For N- or C-terminal chimeric constructs, fragments encoding the Xe-Wee1A NRD (positions 1–203; see Figure 1B), the Xe-Wee1B NRD (1–241), the Xe-Wee1A CRD (481–555) or the Xe-Wee1B CRD (519–595) were substituted for the respective counterparts of Xe-Wee1B or Xe-Wee1A using appropriate primers. N-terminal truncation and deletion mutants, as well as a C-terminal truncation mutant, were made using a PCR-based method with appropriate primers. All constructs were N-terminally tagged with three consecutive Myc epitopes, subcloned into the pT7G(UKII⁺) vector, cut singly with *Not*I, and *in vitro* transcribed into 5'-capped mRNAs using the MEGAscript T7 Kit (Ambion).

RT-PCR

RNA was extracted from oocytes, embryos and various adult tissues using Trizol reagent (Gibco-BRL) and treated with RNase-free DNase I (Takara). cDNAs were synthesized from the extracted RNA using oligo-dT₂₀ primers and the Ready-To-Go Kit (Amersham Pharmacia Biotech). Aliquots of the reaction products were subjected to PCR (94°C for 30 s, 55°C for 30 s, 72°C for 1 min) for 25 cycles; the 5' and 3' PCR primers were, respectively, 5'-GTGCTCTCTATAAGATCGGGGACCTTGGT-CATGTGAC-3' and 5'-CAACTCCTCTCAAGCATGGCCGCTT-GAACTTCTCCA-3' for Xe-Wee1A and 5'-CAAGGACGACCC-TCCCAAACACG-3' and 5'-GCCACGGACGAAGGCCTTTTCTC-3' for Xe-Wee1B. Reaction products were fractionated on 2.5% agarose gels and stained with ethidium bromide.

Antibodies and immunoblotting

Anti-Xe-Wee1B antibodies were raised in rabbits against the C-terminal two-thirds of bacterially produced Xe-Wee1B protein and affinity purified by standard methods. Routinely, proteins equivalent to one oocyte or embryo were subjected to immunoblot analysis with anti-Xe-Wee1B antibody (1 µg/ml), anti-Xe-Wee1A antibody (1 µg/ml; Nakajo *et al.*, 2000), anti-Wee1Hu antibody (1:2000; Santa Cruz), anti-Myc antibody (1:1000; Santa Cruz) or anti-Cdc2 phospho-Tyr15 antibody (1:1000; New England Biolabs). The secondary antibody, a donkey anti-rabbit IgG antibody (1:1000 or 1:500; Amersham), was detected using the enhanced chemiluminescence system (ECL⁺; Amersham). Both anti-Xe-Wee1B antibody and anti-Wee1Hu antibody (raised against the well conserved C-terminal peptide of Wee1Hu; Santa Cruz) were able to recognize endogenous as well as recombinant Xe-Wee1B proteins.

Wee1 kinase assays

Five oocytes were injected with 1 ng of mRNA encoding either Xe-Wee1A or Xe-Wee1B, incubated overnight, and homogenized in 50 µl of an extraction buffer [EB; 1 mM dithiothreitol (DTT), 10 µM pepstatin A, 20 µM leupeptin, 2 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na₂VO₄, 10 mM NaF pH 7.5]. After brief centrifugation, 20 µl of the supernatant were mixed with 10 µl of EB containing 0.5 µg of glutathione bead-bound kinase-dead Cdc2/glutathione S-transferase (GST)-cyclin B2 complexes (a gift from T.Kishimoto; Iwabuchi *et al.*, 2000) and incubated for 15 min at 22°C in the presence of 10 µCi of [γ -³²P]ATP and 10 µM ATP. Upon incubation of the mixtures, the Wee1 kinase assays were linear for at least 30 min. After washing five times with a buffer (0.1% SDS, 0.1 M NaCl, 1 mM EDTA, 1% Triton X-100, 80 mM β-glycerophosphate, 50 mM NaF, 0.5% sodium deoxycholate, 10 µM pepstatin A, 20 µM leupeptin, 2 mM PMSF, 10 mM sodium phosphate pH 7.6), the Cdc2/GST-cyclin B2 complexes bound to

glutathione beads were boiled and subjected to SDS-PAGE. The gel was dried and autoradiographed, and the incorporation of ^{32}P into Cdc2 was quantitated using BAS2000 (Fuji).

Measurement of DNA content

Total DNA was extracted from the embryos as described previously (Furuno *et al.*, 1994), and the (sheared) DNA equivalent to one embryo was electrophoresed on a 1% agarose gel and stained with ethidium bromide. The relative DNA content (in control and experimental embryos) was estimated by serial dilutions of the DNA samples.

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