14-3-3 Binding Regulates Catalytic Activity of Human Wee1 Kinase

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Abstract
The mitotic inducer Cdc2 is negatively regulated, in part, by phosphorylation on tyrosine 15. Human Wee1 is a tyrosine-specific protein kinase that phosphorylates Cdc2 on tyrosine 15. Human Wee1 is subject to multiple levels of regulation including reversible phosphorylation, proteolysis, and protein-protein interactions. Here we have investigated the contributions made by 14-3-3 binding to human Wee1 regulation and function. We report that the interactions of 14-3-3 proteins with human Wee1 are reduced during mitosis and are stable in the presence of the protein kinase inhibitor UCN-01. A mutant of Wee1 that is incapable of binding to 14-3-3 proteins has lower enzymatic activity, and this likely accounts for its reduced potency relative to wild-type Wee1 in inducing a G2 cell cycle delay when overproduced in vivo. These findings indicate that 14-3-3 proteins function as positive regulators of the human Wee1 protein kinase.

Introduction
Activation of the Cdc2 protein kinase is an obligate step for entry into mitosis. Cdc2 is regulated by its association with the B-type cyclins and by reversible phosphorylation (1). Throughout the early phases of the cell cycle, Cdc2 exists as an underphosphorylated monomer and is inactive as a protein kinase. During S-phase, the B-type cyclins accumulate, bind to Cdc2, and promote its phosphorylation (2–11). In higher eukaryotic organisms, Cdc2 phosphorylation occurs on three regulatory sites, Thr-14, Tyr-15, and Thr-161 (12–15), whereas in the fission yeast, Cdc2 is not detectably phosphorylated on either Thr-14 or Thr-15. In lysates prepared from Xenopus eggs, Myt1 accounts for the majority of the Thr-14 kinase activity (30). Human Wee1 was originally cloned based on its ability to rescue weel+ mutants in fission yeast (32). Human Wee1 encodes a tyrosine-specific protein kinase that phosphorylates Cdc2 on Tyr-15 (32–38). Although Myt1 and Wee1 share sequence similarities, the two kinases differ in several important ways. Wee1 but not Myt1 is capable of phosphorylating Cdc2 complexed with either cyclin A or E in vitro (38, 39). Furthermore, human Myt1 is localized to the endoplasmic reticulum and Golgi complex (22), whereas Wee1 is reportedly nuclear (35, 40, 41). These differences suggest that Wee1 and Myt1 may serve distinct functions in regulating the cell division cycle. Recently, a second Wee1-like kinase (Wee1B) has been identified and characterized (42). Wee1B is capable of phosphorylating Cdc2 on Tyr-15 in vitro and is highly abundant in testis.

The Wee1 protein kinase is regulated at multiple levels. In fission yeast, Wee1 is phosphorylated and inactivated by the Cdr1/Nim1 protein kinase (43–45). SpWee1 can also be phosphorylated by the Chk1 and Cds1 checkpoint kinases (46, 47). The human and Xenopus Wee1 kinases are negatively regulated by phosphorylation in a cell cycle-specific manner (35, 36, 38, 48, 49). In addition to phosphorylation, human Wee1 is also regulated at the level of protein synthesis and stability (38). Wee1 levels rise during the S and G2 phases of the cell cycle because of increased synthesis, and Wee1 levels fall during M-phase because of decreased synthesis combined with proteolysis. The NH2 terminus of human Wee1 is phosphorylated in a cell cycle-specific manner. NH2-terminal phosphorylation correlates with reduced activity and reduced stability of Wee1 during mitosis. In addition, the NH2 terminus of human Wee1 is important in conferring substrate specificity (36, 37). The COOH terminus of Wee1 contains a 14-3-3 binding site (50, 51). In this study, we have investigated the contribution made by 14-3-3 binding to human Wee1 by characterizing a mutant of Wee1 that cannot bind to 14-3-3.

Results and Discussion
Overproduction of Kinase-active but not Kinase-inactive Wee1 Causes a G2 Cell Cycle Delay. Recombinant adenoviruses encoding human Wee1 were used to infect a population of HeLa cells that were synchronized at the G1-S border by a double thymidine block and release protocol.
Flow cytometry was used to monitor the ability of cells to traverse the cell cycle after release from the block (Fig. 1). Cells infected with a control adenovirus encoding GFP proceeded through the S, G2, and M phases of the cell cycle normally (Fig. 1A). By 10 h after the release, 48% of the cells were already in the G1 phase of the cell cycle. In contrast, a G2-M cell cycle delay was observed in cells infected with recombinant adenovirus expressing Wee1. Depending on the multiplicity of infection used, only 23% (Fig. 1C) or 17% (Fig. 1D) of Wee1-expressing cells were in the G1 phase of the cell cycle by the 10-h time point. Thus, the extent of the G2-M delay correlated with Wee1 expression levels (Fig. 1E).

Similar experiments were performed using a recombinant adenovirus encoding a kinase-inactive form of Wee1. Replacement of lysine at position 328 with arginine generated a kinase-inactive form of Wee1, as evidenced by the inability of this mutant to phosphorylate Cdc2 in vitro (Fig. 1F). Cells expressing kinase-inactive Wee1 did not experience a G2-M cell cycle delay (Fig. 1B), indicating that the delay required the kinase activity of Wee1. Western blotting indicated that the kinase-active and kinase-inactive forms of Wee1 were expressed to similar levels in infected cells (Fig. 1E). Levels of ectopically expressed proteins declined throughout the time course, most dramatically as cells progressed through mitosis, which was 8–10 h for cells expressing kinase-inactive Wee1 and 10–12 h for cells expressing wild-type Wee1.

Mitotic index measurements were made to determine whether cells overproducing Wee1 were arresting in the G2 or M phases of the cell cycle. Cells were synchronized and infected with adenoviruses as described in Fig. 1. Nocodazole was added to trap cells in mitosis. At various times after release from the block, mitotic chromosome spreads were prepared and scored. At least 500 nuclei were counted in each experiment. B, cellular lysates described in Fig. 1 were resolved by SDS-PAGE, and Cdc2 phosphorylation status was monitored by Western blotting. Species a, b, and c represent different electrophoretic forms of Cdc2 (see text for details). m.o.i., multiplicity of infection.

The abbreviations used are: GFP, green fluorescent protein; MCL, mammalian cell lysis; HA, hemagglutinin antigen.
cycle delay required the kinase activity of Wee1 because kinase-inactive Wee1 did not induce a delay. These observations are in contrast to what is observed with the Cdc2 inhibitory kinase Myt1. In this case, overproduction of either kinase-active or kinase-inactive Myt1 induces a G2 cell cycle delay (31, 53). This is attributable to the fact that both kinase-active and kinase-inactive forms of Myt1 bind Cdc2/cyclin B1 and interfere with the nuclear-cytoplasmic shuttling of Cdc2/cyclin B1 complexes (31).

The electrophoretic mobility of Cdc2 can be used as an indicator of cell cycle position and to assess the phosphorylation status of Cdc2 (22, 52). The slowest electrophoretic form of Cdc2 (species a in Fig. 2B) is phosphorylated on both Thr-14 and Tyr-15, whereas the intermediate form (species b) is phosphorylated on Thr-14 or Tyr-15, but not both (52). These two forms of Cdc2 have reduced kinase activity compared with Cdc2 that is not phosphorylated on Thr-14 and Tyr-15 (22). The fastest electrophoretic form of Cdc2 (species c) is not phosphorylated on either Thr-14 or Tyr-15 and represents either the active form of the kinase (phosphorylated on Thr-161 and bound to cyclin B) or Cdc2 that is not bound to cyclin (monomeric, inactive Cdc2). Species c predominates during G1, early S, and in mitosis, whereas species b and c predominate during mid to late S and throughout G2. As seen in Fig. 2B, species c was the predominant form of Cdc2 at the 0-h time point when cells were arrested at the G1-S border. As cells progressed through S and G2 (6- and 8-h time points) species, a and b became more prominent. Cells infected with control virus and kinase-inactive Wee1 proceeded through mitosis and into G1 by 10 h, and in these cells a decrease in levels of species a and b accompanied an increase in species c (the fastest electrophoretic form). The majority of cells expressing kinase-active Wee1 were still in G2 at the 10-h time point, and a decrease in species a and b with a concomitant increase in species c was not evident until the 12-h time point. Also evident in cells expressing kinase-active Wee1 is a higher level of species b, which is likely the Tyr-15-phosphorylated form of Cdc2. These observations are consistent with the conclusion that overproduction of Wee1 induces a G2 cell cycle delay by maintaining Cdc2 in an inactive state through Tyr-15 phosphorylation.

**14-3-3 Binding Mutant of Wee1 Is Not as Effective as Wild-Type Wee1 in Inducing a G2 Cell Cycle Delay.** Human Wee1 binds to 14-3-3 proteins, and phosphorylation of Wee1 on Ser-642 has been shown to be essential for the binding of 14-3-3 proteins to Wee1 (50, 51). We generated a recombinant adenovirus encoding a 14-3-3 binding mutant of Wee1 by substituting alanine for serine at position 642. Wee1(S642A) was assessed for its ability to induce a G2 cell cycle delay as described in Fig. 1. Cells infected with the control adenovirus proceeded through the S, G2, and M phases of the cell cycle normally. By 8 h after the release, 91% of the cells were in the G2-M phase of the cell cycle, and by 10 h, 21% were in G1 (Fig. 3A). Expression of wild-type Wee1 induced a G2 delay, because only 3% of cells had reached G1 by the 10-h time point (Fig. 3B). Cells expressing the 14-3-3 binding mutant experienced a G2 cell cycle delay but not to the same extent as cells expressing wild-type Wee1 (Fig. 3C). By 12 h, 68% of GFP-expressing cells, 18% of Wee1-expressing cells, and 43% of Wee1(S642A)-expressing cells were in the G1 phase of the cell cycle. Thus, 14-3-3 binding enhances the ability of Wee1 to induce a G2 cell cycle delay, suggesting that 14-3-3 proteins are positive effectors of Wee1 function in vivo.

**Interactions between Wee1 and 14-3-3 Are Resistant to UCN-01 Treatment.** We reported previously that treatment of cells with the protein kinase inhibitor UCN-01 results in the rapid dephosphorylation of Cdc25C on Ser-216 and subsequent loss of 14-3-3 binding (54, 55). Chk1 and Chk2, protein kinases that phosphorylate Cdc25C on Ser-216 in vitro, are directly inhibited by UCN-01 (55, 56). Although the kinase activity of Wee1 is diminished in UCN-01-treated cells, UCN-01 does not directly inhibit Wee1 in vitro (57). These findings suggest that UCN-01 effects kinases that function upstream of Wee1 (57). To determine whether UCN-01 would perturb interactions between Wee1 and 14-3-3 in a manner similar to that of Cdc25C and 14-3-3, cells expressing myc-tagged Wee1 were treated with various con-

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**Fig. 3.** 14-3-3 binding mutant of Wee1 is impaired in its ability to induce a G2 cell cycle delay. HeLa cells synchronized by a double thymidine block protocol were infected with recombinant adenoviruses encoding either GFP (A) or with viruses encoding myc-epitope tagged wild-type Wee1 (B) or a mutant of Wee1 containing alanine in place of serine at position 642 (Wee1S642A; C). Cells were harvested at various times (hours) after release from the block, and cellular DNA content was analyzed by flow cytometry. Levels of Wee1 and Wee1(S642A) were determined by immunoblotting (D).
Functional Interactions between 14-3-3 and Human Wee1

centrations of UCN-01 (Fig. 4). Myc-Wee1 was isolated from UCN-01-treated cells, and coprecipitation of 14-3-3 proteins was examined by Western blotting. The electrophoretic mobility of Cdc25C was examined in the same cellular lysate to determine the efficiency of UCN-01 treatment. We demonstrated previously that Ser-216 phosphorylation causes Cdc25C to migrate more slowly on SDS gels (54, 58, 59). Thus, the electrophoretic mobility of Cdc25C can be used as an indirect measure of Ser-216 phosphorylation and 14-3-3 binding. As seen in Fig. 4 (Lane 3) treatment of cells with 300 nM UCN-01 for 2 h caused significant reduction in Ser-216 phosphorylation as indicated by loss of the slower migrating form of Cdc25C. However, Wee1/14-3-3 interactions are stable even under conditions that resulted in the complete loss of Ser-216 phosphorylation and 14-3-3 binding to Cdc25C (Fig. 4, Lanes 4 and 5). These findings demonstrate that UCN-01 does not mediate its negative effects on Wee1 by interfering with 14-3-3 binding. In addition, these findings demonstrate that the interactions between Wee1 and 14-3-3 are regulated by UCN-01-resistant protein kinases. Chk1, a UCN-01-sensitive protein kinase, has been shown to phosphorylate Xenopus Wee1 on its 14-3-3 binding site (60). Our findings indicate that in human cells there are kinases other than, or in addition to, Chk1 that regulate the interactions between Wee1 and 14-3-3. Finally, these results indicate that some of the kinases that regulate interactions between 14-3-3 and Wee1 must be distinct from those regulating interactions between 14-3-3 and Cdc25C.

Reduced Binding of 14-3-3 to Wee1 during Mitosis. Full-length Wee1 and the catalytic domain of Wee1 (p49Wee1) were examined for their ability to bind to 14-3-3 proteins during mitosis. HeLa cells expressing Wee1 (Fig. 5A) and wild-type and mutant forms of p49Wee1 (Fig. 5B) were cultured in the absence or in the presence of nocodazole. The association of 14-3-3 with Wee1 was determined by immunoprecipitating Wee1 and examining these precipitates for the presence of 14-3-3 proteins. As seen in Fig. 5A, interactions between 14-3-3 and Wee1 were markedly decreased in nocodazole-treated cells (Lane 3). This was also seen for both kinase-active and kinase-inactive forms of p49Wee1 (Fig. 5B, Lanes 2 and 4). These results indicate that the interactions between 14-3-3 and Wee1 are cell cycle regulated. Decreased binding of 14-3-3 proteins to Wee1 during mitosis suggests that 14-3-3 proteins might function to positively regulate human Wee1 throughout interphase.

14-3-3 Binding Regulates the Enzymatic Activity of Wee1. Given that the G2 cell cycle delay observed upon Wee1 overproduction requires its kinase activity (Fig. 1), we carried out experiments to assess whether 14-3-3 binding regulated the enzymatic activity of Wee1 (Fig. 6A). Wee1 and the 14-3-3 binding mutant [Wee1(S642A)] were tested for their ability to phosphorylate purified Cdc2/cyclin B1 complexes in vitro. As seen in Fig. 6A, wild-type Wee1 was 3-fold more active than the 14-3-3 binding mutant in phosphorylating Cdc2 in vitro. Bacterially produced Wee1 and Wee1S642A had similar kinase activities, demonstrating that mutation of Ser-642 does not reduce the intrinsic kinase activity of human Wee1 (data not shown). Taken together, these results demonstrate a direct role for 14-3-3 in regulating the enzymatic activity of human Wee1. 14-3-3 binding to Raf1 at Ser-621 (61) and to Xenopus Wee1 at Ser-549 (60) has been shown to serve similar functions.

A previous study by Wang et al. (50) reported that 14-3-3 binding does not affect the intrinsic kinase activity of Wee1 but rather stabilizes the Wee1 protein. In this study, the half-life of ectopically expressed Wee1 was reported to be 25 min when transfected alone and 50 min when cotransfected with 14-3-3β (50). We found that ectopically expressed Wee1 and the 14-3-3 binding mutant of Wee1 were both stable for up to 4 h after transfection in pulse-chase experiments (data not shown). These findings are more in accord with Yu et al. (57), who reported that the half-life of endogenous Wee1 is 4 h (57). Wang et al. (50) also observed that coexpression of 14-3-3β with Wee1 results in higher accumulation of Wee1 protein in vivo. We observed higher accumulation of both Wee1 and the 14-3-3 binding mutant of Wee1 when coexpressed with 14-3-3α in vivo, suggesting an indirect effect of 14-3-3 proteins on Wee1 accumulation under these experimental conditions (Fig. 6B). Our results are in accord with those of Lee et al. (60), who demonstrated that
SDS-PAGE and proteins were transferred to nitrocellulose. 32P-labeled purified cyclin B1/Cdc2(K33R) complexes. Reactions were resolved by (9E10) agarose. Kinase reaction were performed

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\text{My}c-\text{Wee1 and My}c-\text{Wee1(S642A)} \text{ were incubated with anti-}\text{c-Myc}
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modified lysates prepared from HeLa cells infected with adenoviruses encoding a c-Myc polyclonal antibody (Santa Cruz Biotechnology; A-14). Results represented cpm of 32P incorporated into Cdc2.

levels of Wee1 (\text{Xenopus Wee1}).

In summary, results reported in this study support a role for 14-3-3 proteins in positively regulating Wee1 function throughout interphase. The observation that 14-3-3 binding to Wee1 is lost during mitosis is consistent with this conclusion. The positive role of 14-3-3 proteins in Wee1 regulation is in contrast to their negative role in Cdc25C regulation (58). In the case of human Cdc25C, 14-3-3 proteins prevent functional interactions between Cdc25C and Cdc2 by keeping Cdc25C from accumulating in the nucleus throughout interphase (54, 62). Thus, loss of 14-3-3 binding to both Wee1 and Cdc25C during mitosis is expected to lead to reduced inhibition of Cdc2 by Wee1 concomitant with enhanced activation of Cdc2 by Cdc25C. The net effect would be to drive cells into mitosis more efficiently.

Materials and Methods

Antibodies Used for Precipitation and Western Blotting. Anti-c-Myc (9E10) agarose conjugate (Santa Cruz Biotechnology) was used to precipitate myc-tagged proteins. Wee1 was detected with a c-Myc polyclonal antibody (Santa Cruz Biotechnology; A-14) or anti-p49 COOH-terminal peptide antisera (Upstate Biotechnology, Inc.). Cdc25C was detected with a monoclonal antibody (174E10–3). Cdc2 was detected with a monoclonal antibody [Cdc2 p34 (17); Santa Cruz Biotechnology]. K19 antisera (Santa Cruz Biotechnology) was used to detect 14-3-3 proteins. Secondary antibodies included horseradish peroxidase–goat antimouse antibody (ICN/Cappel) and horseradish peroxidase–goat antirabbit antibody (Zymed). Western blots were performed using Amer sham's enhanced chemiluminescence (ECL) protocol.

Bacterial Expression Vectors. Full-length Wee1 was cloned as a BamHI/EcoRI fragment into pGEX-2T (Pharmacia) to generate pGEX2Tp71Wee1Hu. The sequence comprising the BamHI site and the initiating methionine of Wee1 is GGATCCATG. The EcoRI site of pGEX2Tp71Wee1Hu was changed to Hpal to generate pGEX2THp71. pGEX2THp71 was used as template in a three-part PCR reaction using primers 1 and 2 in the first reaction, primers 3 and 4 in the second reaction, and primers 1 and 4 in the third reaction. The sequence of the primers is as follows: 5'-ACCTTCAGCAATGGGACTG (primer 1); 5'-GTATATAGTAAGGGCAGACAGG (primer 2); 5'-CGCTGTTCGCCCTTACTATAC (primer 3); and 5'-TAAA CAAATTGGGTCTCCGCG (primer 4). The final 611-bp PCR product was cloned into pOR 2.1 (Invitrogen) and sequenced. pOR 2.1-1/4#10 containing the appropriate Ser-642 to alanine substitution was digested with XbaI and EcoRI, and a 233-bp fragment was cloned into XbaI/EcoRI-digested pGEX2THp71 to generate pGEX2THp71(S642A). pGEX2Tp49Wee1(37) was used as template to substitute alanine for serine at position 642 and arginine for lysine at position 328. A restriction fragment from pGEX2Tp49Wee1(K328R) containing arginine for lysine at position 328 was used to replace an equivalent restriction fragment in pGEX2Tp71Wee1Hu to generate pGEX2Tp71(K328R).

Mammalian Expression Vectors. pGEX2THp71 was used as template in a PCR reaction with an NH2-terminal primer containing a BamHI site and the myc epitope (5'-CCCGGTATCCACCATGGCGAGAGACAGACTTCACTGCAAGAGA CCTCGATACAAAAACG) and a COOH-terminal primer containing an EcoRI site (5'-CCGGAATCCATGATAGGCGTCGACAGAGC). The PCR product was digested with BamHI and EcoRI and cloned into BamHI/EcoRI-digested pCDNA3 to generate pCDNA3myc-p49Wee1. pGEX2Tp71(K328R) was used as a template in a PCR reaction using the primers and strategy described above to generate pCDNA3myc-p49Wee1(K328R).

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\text{Fig. 6. 14-3-3 binding regulates the enzymatic activity of Wee1. A, clarified lysates prepared from HeLa cells infected with adenoviruses encoding myc-Wee1 and myc-Wee1(S642A) were incubated with anti-c-Myc (9E10) agarose. Kinase reaction were performed in vitro in the presence of purified cyclin B1/Cdc2(K33R) complexes. Reactions were resolved by SDS-PAGE and proteins were transferred to nitrocellulose. 32P-labeled Cdc2 was visualized by autoradiography. Levels of Wee1 (Lanes 1 and 3) and Wee1(S642A) (Lanes 2 and 4) were assessed by immunoblotting with a c-Myc polyclonal antibody (Santa Cruz Biotechnology; A-14). Results from two experiments are shown, and the numbers under each lane represent cpm of }^32\text{P} \text{ incorporated into Cdc2. B, HeLa cells were mock infected (Lane 1) or were coinfected with recombinant adenoviruses encoding wild-type Wee1 and }^\beta\text{-galactosidase (Lane 2); wild-type Wee1 and }^\beta\text{-galactosidase (Lane 4) or Wee1(S642A) and }^\beta\text{-galactosidase (Lane 4) or Wee1(S642A) and }^\beta\text{-galactosidase (Lane 4). Cell lysates prepared 24 h after infection were either resolved directly by SDS-PAGE or were first incubated with anti-c-Myc agarose to isolate myc-tagged Wee1. Lysates and precipitates were monitored for the presence of Wee1 and Wee1(S642A) by immunoblotting with anti-c-Myc polyclonal antibody and K19 antibody, respectively.}
was used as template in a PCR reaction with the NH2-terminal primer described above and COOH-terminal primer containing an EcoRI site and the S642A mutation (5'-CCGGAATTCTCAGTATATGTAAGGCGGACAGCG). The PCR product was digested with BamHI and EcoRI and cloned into BamHI/EcoRI-digested pCDNA3 to generate pCDNA3mycp49Wee1(S642A). To generate NH2-terminal, myc-fusion proteins with full-length Wee1, a modified pCDNA3 vector (Invitrogen), was created. pCDNA3 digested with HindIII and BamHI was ligated with the following linkers: 5'-AGCTTGGTAACAAACCATGGCA-GAGCGAGAAGAGACCTCG (linker 1) and 5'-GATCCAGGCTTCTCTCTGAGATGACCTGCTTGCCATGTTTGGTTACCA (linker 2). The modified vector, pCDNA3myc, contains a Kozak consensus sequence, an ATG start site, and the myc epitope sequence 5' to the BamHI site and lacks the Asp-718 and KpnI restriction sites found in pCDNA3. pGEX2THp71, pGEX2Thp71(K328R), and pGEX2Thp71(S642A) were digested with HindIII and EcoRI, and the 2-kb fragments were subcloned into BamHI/EcoRI-digested pCDNA3myc to generate pCDNA3myc-p71, pCDNA3myc-p71(K328R), and pCDNA3myc-p71(S642A), respectively.

Adenoviral Expression Vectors. Wild-type and mutant forms of Wee1 were cloned as myc-tagged fusions into the adenovirus shuttle vector pAdTrack-CMV (63). pCDNA3myc-p71, pCDNA3myc-p71(K328R), and pCDNA3myc-p71(S642A) were digested with HindIII and EcoRI, and sequences encoding the myc-Wee1 fusions were cloned into EcoRV-digested pAdTrack-CMV to generate pAdTrackCMV myc-p71, pAdTrackCMVmyc-p71(K328R), and pAdTrackCMVmyc-p71(S642A), respectively. To make viruses encoding myc-Wee1 but not GFP, HindIII/EcoRI inserts encoding myc-Wee1 and myc-Wee1(S642A) were cloned into the EcoRV site of pAd5CMV to generate pAd5CMV-mycp71 and pAd5CMV-mycp71(S642A), respectively.

Generation of Recombinant Adenoviruses. The pAdTrack-CMV-based plasmids encoding wild-type and mutant Wee1 myc-fusion proteins were cotransformed with pAdEasy-1 into Escherichia coli B5183 to achieve homologous recombination. Recombinant adenoviruses were generated and propagated using the pAdEasy system as described previously (63). These viruses encode GFP in addition to the Wee1 proteins. Adenoviruses expressing myc-Wee1 and myc-Wee1(S642A) but not GFP were generated by transfecting pAd5CMV-mycP71 and pAd5CMV-mycP71(S642A) with adenoviral DNA into low-passage 293 cells. Plaques were selected to isolate non-GFP recombinant viruses. Control viruses encoding GFP and cells. Plaquing was performed to isolate non-GFP recombinant adenoviruses at various multiplicities of infection in 0.5 ml of serum-free DMEM. Thymidine (2 mM) was included in the infection medium to maintain the G1/S synchronization. At the end of the infection, 4 ml of complete medium containing 2 mM thymidine were added, and cells were incubated for an additional 2 h. Cells were then washed twice with serum-free DMEM and cultured in complete growth medium. Cells were harvested at the desired times by trypsinization. Approximately one-third of the cells were lysed in MCL buffer [50 mM Tris (pH 8.0), 100 mM NaCl, 5 mM EDTA, 0.5% NP40, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 20 μg/ml leupeptin, 5 μg/ml pepstatin, 1 mM Na3VO4, 1 μM microcystin]. Lysate (100 μg) was resolved by SDS-PAGE and analyzed by Western blotting. The remaining cells were fixed in 70% ethanol and stained with 30 μg/ml propidium iodide in PBS containing 1% BSA and 0.25 mg/ml RNase A. Cell cycle profiles were determined by flow cytometry using a Becton Dickinson FACScan, and data were analyzed using CELL QUEST software.

To examine effects of 14-3-3 proteins on Wee1 stability, HeLa cells were mock infected or were coinfectected with recombinant adenoviruses encoding either wild-type Wee1 or the 14-3-3 binding mutant together with viruses encoding β-galactosidase (31) or HA-tagged 14-3-3α (64). Cells were lysed in MCL buffer 24 h after infection. Lysates containing 1 mg of total cellular protein were incubated with 40 μl of anti-c-Myc agarose (1:1 slurry) at 4°C for 2 h. Precipitates were washed three times in MCL buffer.

Mitoic Index Measurements. HeLa cells were subjected to G1/S synchronization and adenoviral infection as described above. Upon releasing cells from the final thymidine block, 0.10 μg/ml nocodazole was added to the medium to trap mitotic cells. Pelleted cells were harvested by trypsinization, washed once with PBS, and then resuspended in 75 mM KCl for 10 min (65). After centrifugation to remove the KCl, cells were treated with fixative (acetic acid:methanol:1:3 v/v). Cells were then resuspended in fixative, spread onto slides, and allowed to air dry. Dried cells were then stained with 1 μg/ml 4′,6-diamidino-2-phenylindole for 5 min at room temperature and then mounted and observed by fluorescent microscopy. A minimum of 500 nuclei were counted for each sample.

Mammalian Cell Transfections. Mammalian cell transfections were carried out using plasmid DNA and Lipofectamine reagent (Life Technologies, Inc.) in a ratio of 1:3 or 1:4 according to the manufacturer’s recommended protocols or using a calcium phosphate transfection protocol (Invitrogen).

Treatment of Cells with UCN-01. HeLa cells were either transfected with empty vector or vector encoding myc-tagged Wee1. Twenty h after transfection, cells were either not treated or were incubated with various concentrations of 2 mM, and cells were cultured for an additional 16 h. Cells were then rinsed twice with PBS and cultured in complete growth medium. Samples were harvested at various times after the release.

To study the effects of overexpressing Wee1 on cell cycle progression, HeLa cells were synchronized at the G1/S border by a second block, -8 × 10⁵ cells were infected for 45–60 min with recombinant adenoviruses at various multiplicities of infection in 0.5 ml of serum-free DMEM. Thymidine (2 mM) was included in the infection medium to maintain the G1/S synchronization. At the end of the infection, 4 ml of complete medium containing 2 mM thymidine were added, and cells were incubated for an additional 2 h. Cells were then washed twice with serum-free DMEM and cultured in complete growth medium. Cells were harvested at the desired times by trypsinization. Approximately one-third of the cells were lysed in MCL buffer [50 mM Tris (pH 8.0), 100 mM NaCl, 5 mM EDTA, 0.5% NP40, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 20 μg/ml leupeptin, 5 μg/ml pepstatin, 1 mM Na3VO4, 1 μM microcystin]. Lysate (100 μg) was resolved by SDS-PAGE and analyzed by Western blotting. The remaining cells were fixed in 70% ethanol and stained with 30 μg/ml propidium iodide in PBS containing 1% BSA and 0.25 mg/ml RNase A. Cell cycle profiles were determined by flow cytometry using a Becton Dickinson FACScan, and data were analyzed using CELL QUEST software.

To examine effects of 14-3-3 proteins on Wee1 stability, HeLa cells were mock infected or were coinfectected with recombinant adenoviruses encoding either wild-type Wee1 or the 14-3-3 binding mutant together with viruses encoding β-galactosidase (31) or HA-tagged 14-3-3α (64). Cells were lysed in MCL buffer 24 h after infection. Lysates containing 1 mg of total cellular protein were incubated with 40 μl of anti-c-Myc agarose (1:1 slurry) at 4°C for 2 h. Precipitates were washed three times in MCL buffer.
UCN-01 for 2 h. Cell lysates were prepared, and 150 μg of cellular lysate were resolved by SDS-PAGE to monitor the electrophoretic mobility of human Cdc25C. Five hundred μg of total cellular protein were incubated with anti-c-Myc agarose at 4°C for 2 h. After precipitation, myc-agarose was pelleted and washed three times with MCL buffer. SDS sample loading buffer was added to each pellet, and the reactions were resolved on 12% polyacrylamide SDS gels. Western blot analysis was performed to detect myc-tagged Wee1Hu and coprecipitating 14-3-3 proteins. Myc-Wee1 fusion proteins were detected using anti-p49 COOH-terminal peptide antisera (Upstate Biotechnology, Inc.).

Interactions between Wee1 and 14-3-3 Proteins during Mitosis. HeLa cells were transfected with plasmids encoding myc-tagged fusions of Wee1 as well as wild-type and mutant forms of p49Wee1. Five h after transfection, 0.15 μg/ml nocodazole was added to some of the cultures. Sixteen h later cells were lysed in MCL buffer at 4°C for 15 min. Lysates were cleared by centrifugation at 4°C and 10,000 rpm for 10 min. Fifty μl of anti-c-Myc agarose (1:1 slurry) were added to the lysate for 2 h at 4°C. Precipitates were washed three times with MCL buffer and then resolved on a 12% polyacrylamide SDS gel. Western blotting was performed to detect myc-tagged Wee1 and coprecipitating 14-3-3 proteins.

Expression and Purification of Proteins Expressed Bacteria for Kinase Assays. Bacterial cells transformed with pGEX2THp71 and pGEX2Tp71(K328R) encoding GST-Wee1 and GST-Wee1(K328R), respectively, were grown at 37°C to an A600 of 0.6. Isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.5 mM. After growing for an additional 3 h at 30°C, cells were pelleted by centrifugation. Cell pellets were washed with PBS buffer and resuspended in STE [100 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA] supplemented with 2 mM phenylmethylsulfonyl fluoride, 0.15 unit/ml aprotinin, 20 μM leupeptin, 20 μM pepstatin, and 0.5 mg/ml lysozyme. After rocking at 4°C for 20 min, Sarkosyl was added to a final concentration of 1.5%, and lysis was accomplished by sonication. Lysates were clarified by centrifugation, and Triton X-100 was added to a final concentration of 2%. Proteins were incubated with glutathione agarose beads for 30 min at 4°C. Precipitates were washed twice with NETN [20 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA, and 0.5% NP40] containing 1 mM NaCl, twice with NETN, and twice with incomplete kinase buffer [50 mM Tris (pH 7.5), 10 mM MgCl2]. Kinase assays were performed as described below.

Phosphorylation of Cdc2 by Wee1 in Vitro. HeLa cells were infected with recombinant adenoviruses encoding myc-Wee1 and myc-Wee1(S642A). Cells were lysed in MCL buffer 24 h after infection. Lysates were incubated with 30 μl of anti-c-Myc agarose (1:1 slurry) at 4°C for 2 h. Precipitates were washed three times in MCL buffer and twice in incomplete kinase buffer. Kinase reactions were carried out in the presence of 1–2 μg of GST-cyclin B1/Cdc2(3K3R) substrate (22) in 40 μl of incomplete kinase buffer supplemented with 1 mM DTT, 0.5 mM Na2VO4, 10 μM ATP, and 10 μCi of [γ-32P]ATP (>4000 Ci/mmol). Reactions were incubated for 5–20 min at 30°C and terminated by the addition of SDS-sample buffer. Proteins were resolved on a 12.5% SDS-polyacrylamide gel, Wee1 levels were determined by immunoblotting, and 32P-labeled Cdc2 was visualized by autoradiography. 32P incorporation was determined by excising radiolabeled Cdc2 from the gel and counting in a scintillation counter.

Acknowledgments

We thank H. Okayama for providing a cDNA encoding human Wee1 and B. Vogelstein for providing recombinant adenovirus encoding HA-tagged 14-3-3 sigma and βI-galactosidase.

References


