Casein Kinase 1δ-dependent Wee1 Protein Degradation*

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Background: Wee1 is an essential gene in mammals that encodes the cell cycle and cancer associated protein Wee1 kinase.

Results: Inhibition of CK1δ kinase increases the levels of Wee1 protein kinase.

Conclusion: Casein kinase 1δ is required for Wee1 degradation in HeLa cells and mouse embryonic fibroblasts.

Significance: This is a previously unappreciated role for CK1δ in controlling Wee1 degradation and cell cycle progression.

Eukaryotic mitotic entry is controlled by Cdk1, which is activated by the Cdc25 phosphatase and inhibited by Wee1 tyrosine kinase, a target of the ubiquitin proteasome pathway. Here we use a reporter of Wee1 degradation, K328M-Wee1-luciferase, to screen a kinase-directed chemical library. Hit profiling identified CK1δ-dependent Wee1 degradation. Small-molecule CK1δ inhibitors specifically disrupted Wee1 destruction and arrested HeLa cell proliferation. Pharmacological inhibition, siRNA knockdown, or conditional deletion of CK1δ also reduced Wee1 turnover. Thus, these studies define a previously unappreciated role for CK1δ in controlling the cell cycle.

Ubiquitin-mediated proteolytic pathways regulate critical cell cycle transitions by targeting specific inhibitors for degradation by the proteasome. Substrate targeting to the proteasome requires recognition by a specific E3 ubiquitin ligase that works in conjunction with an E2 ubiquitin-conjugating enzyme to transfer ubiquitin to the substrate (1). When a sufficient number of ubiquitins have been added to the substrate, it is recognized by the proteasome, and degradation ensues. The SCF ubiquitin ligases represent one of the largest classes of E3 ubiquitin ligases. These ligases contain the proteins Skp-1, Cul-1, and a substrate recognition molecule termed the F-box protein. F-box proteins contain a 40 amino acid F-box domain that is required for interaction with Skp-1 and ~70 F-box proteins are present in the vertebrate genome (2). SCF substrate degradation is generally initiated by posttranslational phosphorylations that are necessary for initiating ubiquitylation. This allows binding by the F-box protein component of the SCF complex. When recognition occurs, the substrate is brought into close proximity to the E2 enzyme, which initiates ubiquitin transfer.

One of the most important SCF substrates degraded in a phosphorylation-dependent manner is Wee1, an inhibitor of mitotic entry (3). Wee1 is a highly conserved kinase that inactivates the mitosis-specific kinase Cdk1-cyclin B1 complex during the S and G2 phases of the cell cycle by phosphorylating Cdk1 at tyrosine 15. Wee1 activity is antagonized by the phosphatase Cdc25, which removes Cdk1 phosphorylation of tyrosine 15 involved in activation of Cdk1-cyclin B1 (4). Not surprisingly, the control of Wee1 degradation is an important regulator of mitotic entry (5–9). Degradation of nuclear Wee1 (5, 10) during G2 phase of the cell cycle increases the activity of Cdk1, leading to mitotic entry (7, 8, 11, 12). To identify kinases that control Wee1 phosphorylation and subsequent degradation, we screened a 16,000-member, kinase-directed, small molecule library available at Scripps Florida and a reporter of Wee1 turnover, K328M-Wee1-luciferase (13). Highly specific and potent CK1δ small molecule inhibitors were identified, which uncovered an essential role for casein kinase 1δ in controlling Wee1 degradation and cell cycle progression.

EXPERIMENTAL PROCEDURES

Luciferase Assays—HeLa cells expressing K328M-Wee1-luciferase or luciferase alone were treated with SR-653234 or SR-1277 for 24 h, after which britelite was added to detect luciferase or K328M-Wee1-luciferase levels. We have previously described similar assays (12, 13). N-cyclin B-luciferase, p21cip1-luciferase, or p27kip1-luciferase assays were performed as described previously (12, 13).

SR-653234 and SR-1277 Synthesis—SR-653224 and SR-1277 synthesis has been described previously (14).

In Vitro Kinase Assays—An in vitro kinase assay to detect CK1 or FLT3 activity, CK1 and FLT3 assays, as well as a complete kinase profile of 296 kinases was performed by Reaction Biology Corp. For FLT3, 20 μM final Abltide was used (sequence EAIYAAPFAKKK). For CK1 (all isoforms), 20 μM CK1 final Abltide was used (sequence KRRRAL(pS)VASLPGL).
in a standard kinase assay with \(^{32}\)P-ATP (PerkinElmer Life Sciences, 3000 Ci/mmol, 5mCi/ml) and purified kinase. Incorporation of \(^{32}\)P-ATP into the peptide was measured after a filter-binding assay.

**In Vitro Phosphorylation of Wee1 with CK1δ—293T cells were transfected with pCS2-FLAG-Wee1 K328M in a 10-cm tissue culture dish and incubated for 48 h in a tissue culture incubator (37 °C, 10% CO\(_2\)). Cells were collected, washed in PBS, and resuspended in lysis buffer (PBS containing 0.1% IGEPAL CA-630, 10% glycerol, 5 mM NaF, microcystin LR, and protease inhibitor mixture). Lysates were clarified by centrifugation and incubated with one-tenth the volume of packed EZview Red anti-FLAG M2 affinity gel beads (Sigma-Aldrich, catalog no. F2426) overnight at 4 °C. Beads were isolated by centrifugation and washed three times in wash buffer (PBS, 150 mM NaCl, 10% glycerol, 0.1% IGEPAL CA-630). Beads were then incubated with 1× kinase buffer (25 mM Tris (pH 8.5), 0.01% Brj-35, 10 mM MgCl\(_2\), 1 mM EGTA, and 10 mM ATP) and 20 units of CSNK1D (Invitrogen, catalog no. PV3665) for 20 min at 30 °C. Laemmli sample buffer was added to terminate the reactions, and the samples were boiled and resolved by SDS-PAGE. Bands corresponding to FLAG-Wee1 K328M were excised and processed for mass spectrometry. FLAG-Wee1 K328M incubated with CK1δ or buffer was analyzed by mass spectrometry, and phosphorylated peptides obtained in each case were observed. An identical protocol was utilized for**

**Cell Synchronization—Mitotic entry assays were performed essentially as described previously (11). Briefly, HeLa cells were treated with 2 mM thymidine for 18 h, and then they were released from the thymidine block for 8 h. 2 mM thymidine was then incubated again on the cells for an additional 8 h. In the case of mitotic entry in the presence of compounds, thymidine was washed away, and then compound or DMSO\(^3\) along with 330 mM nocodazole was added to the cells. Cells were processed for phospho-histone H3 (catalog no. sc-8656-R, Santa Cruz Biotechnology) and Skp1 (catalog no. sc-7163, Santa Cruz Biotechnology) immunoreactivity. Cells were also isolated at 2-h intervals and processed for FACS analysis (catalog no. P3566, Invitrogen).**

**Cycloheximide Degradation Assay—100 μg/ml cycloheximide or DMSO was added to HeLa cells 2 days after they were transfected with plasmids or siRNAs or treated with compounds. Cells were harvested at specific time points, and extracts were prepared as described above, followed by SDS-PAGE and Western blotting.**

| Abbreviation | Description |
|--------------|-------------|
| CK1δ/e | and LKB1 Depletion—HeLa cells were transfected with siRNAs to target CK1δ, CK1ε, and LKB1 and processed for Wee1 degradation as described previously (11). The following siRNAs were used in this study: GFP (GFP-22 siRNA, 5 nmol, Qigen, catalog no. 1022064), CK1δ (Hs-CSNK1D_5 FlexiTube siRNA, 5 nmol, Qigen, catalog no. S100287406), CK1ε (ONTARGETplus human CSNK1D siRNA, 5 nmol, Thermo Scientific, catalog no. L-003478-01-0005), CK1ε (Thermo Scientific, catalog no. L-0003479), and LKB1 (Thermo Scientific catalog no. L-005035). Wee1 Western blot analyses were processed as described previously (11). |
| CK1δ/e Deletion in Mouse Fibroblasts—Double-floxed mutant fibroblasts were grown in DMEM supplemented with 10% FBS. The fibroblasts were infected with adenoviral gfp or adenoviral cre in 100-mm dishes when the cells reached ~70% confluence, and the cultures were then split twice over ~5 days. At the third split, the cells were transferred to 60-mm dishes, (15) infected with wild-type CK1δ or no virus as indicated, and harvested 24 h later. |
Cell Extract Preparation, Antibodies, and Coimmunoprecipitation Assay—Cells were homogenized and extracts were prepared using lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1× protease inhibitor mixture, and 1 μM Microcystin LR). Cells were lysed by the freeze/thaw method (liquid nitrogen/37 °C water bath) followed by two passages through a 20.5-gauge needle. The soluble fraction was recovered by centrifugation at 14,000 rpm for 20 min at 4 °C. Protein concentration was measured with a BCA protein assay kit (Pierce), and 30 μg of protein from each sample was resolved by SDS-PAGE. The resolved bands were transferred onto a nitrocellulose membrane by Western blotting and then probed with relevant antibodies.

Primary antibodies used were as follows: anti-CK1δ antibody (C-8) from Santa Cruz Biotechnology (catalog no. sc-55553), anti-CK1ε γ2 antibody (26-P) from Santa Cruz Biotechnology (catalog no. sc-130365), anti-CK1α antibody (C-19) from Santa Cruz Biotechnology (catalog no. sc-6477), anti-Skp1 p19 antibody (H-163) from Santa Cruz Biotechnology (catalog no. sc-7163), anti-p-histone H3 (Ser-10)-R antibody from Santa Cruz Biotechnology (catalog no. sc-8566-R), anti-Wee1 antibody from Cell Signaling Technology (catalog no. 4936S), anti-CK1ε antibody from BD Transduction Laboratories (catalog no. 610445), anti-V5 HRP antibody from Invitrogen (catalog no. R961–25), anti-FLAG M2-HRP antibody from Sigma-Aldrich (catalog no. A8592–1MG), anti-cyclin B1 from Abcam (catalog no. ab72), and anti-phospho-tyrosine-15-cdc2 from Cell Signaling Technology (catalog no. 911). Secondary antibodies used were as follows: anti-mouse IgG-HRP antibody from GE Healthcare (catalog no. NXA931), anti-rabbit IgG-HRP antibody from GE Healthcare (catalog no. NA9340V), and donkey anti-goat IgG-HRP antibody from Santa Cruz Biotechnology (catalog no. sc-2020).

For the coimmunoprecipitation assay, cell lysates were incubated with one-tenth the volume of packed EZview Red anti-FLAG M2 affinity gel beads (Sigma-Aldrich, catalog no. F2426) on a rotary shaker overnight at 4 °C. The next day, beads were washed with TBS, and the bound FLAG fusion proteins were eluted by boiling at 92 °C for 5 min in Laemmli sample buffer. The supernatants were collected and analyzed by SDS-PAGE and Western blotting.

RESULTS

SR-653234 Selectively Stabilizes Wee1—Wee1 degradation during mitosis is dependent upon Plk1, Cdk1, and CK2 activity. They phosphorylate multiple N-terminal residues to initiate recognition by an SCF ubiquitin ligase containing the F-box protein β-TrCP (7, 8). Wee1 is turned over in cell extracts isolated from HeLa cells in S/G2 phase cells (8, 12). Furthermore, overexpression of nonphosphorylatable versions of Wee1 limits its mitotic entry and arrests cells in G2 phase (8, 12). Thus, there must be regulatory mechanisms and specifically kinases controlling Wee1 turnover during S and G2 phase. We utilized a chemical biology approach to identify kinases responsible for Wee1 degradation during interphase. We expressed K328M Wee1-luciferase (a kinase-inactive version of Wee1-luciferase) in HeLa cells and subsequently incubated these cells with compounds from a 16,000-member, kinase-directed chemical library. Using this strategy, we isolated a class of compounds that specifically induced Wee1 stabilization relative to another protein turned over via the ubiquitin proteasome pathway, N-cyclin B1-luciferase (16) (Fig. 1, A and B). Of these screening hits, SR-653234 was the most potent in stabilizing K328M-Wee1-luciferase as well as endogenous Wee1 (Fig. 1, B–D). Importantly, SR-653234 specifically inhibited turnover of K328M-Wee1-luciferase, suggesting that it limits Wee1 turnover and not expression (Fig. 1, E and F).

SR-1277 Is a Potent and Selective Inhibitor of Wee1 Degradation and CK1δ—Because similar purine compounds (17) (Fig. 1, A and B) inhibit kinase activity, we profiled SR-653234 kinase activity against a panel of kinases. The results of these assays demonstrated that SR-653234 is a potent inhibitor of casein kinase 1δ, casein kinase 1ε, LKB1, and FLT3 relative to the DMSO control or to the highly promiscuous kinase inhibitor staurosporine (Fig. 2, A and B). Importantly, kinases implicated previously in Wee1 turnover, such as Cdk1-cyclin B1, CK2, PLK1, or Cdk2-cyclin A2, were not inhibited by SR-653234 (7, 8, 12, 18). Dose response measurements of kinases that were inhibited by SR-653234 by 65% or more in single-dose measurements provided evidence that the IC50 is for inhibiting LKB1, FLT3, CK1δ, and CK1ε were 92, 100, 161, and 540 nM, respectively (Fig. 2, A and B) (14). Notably, SR-653234 is a more active inhibitor of CK1δ and CK1ε than the broad-spectrum kinase inhibitor staurosporine (14). By contrast, staurosporine was a more potent inhibitor of FLT3 activity than SR-653234 (14). These studies suggest that SR-653234-mediated stabilization of Wee1 is due to inhibition of LKB1, FLT3, and CK1δ and/or CK1ε activities.

SR-653234 optimization suggests that CK1δ and/or CK1ε controls Wee1 degradation. To distinguish between LKB1, FLT3, and CK1δ and CK1ε activities, we generated a panel of SR-653234 analogs (14) and tested their activities against LKB1, FLT3, CK1δ, and CK1ε in vitro. As shown in Fig. 2, A–D, SR-1277 was a more potent inhibitor of CK1δ than SR-653234 (CK1δ IC50 of 161 nM for SR-653234 and 49 nM for SR-1277) and a weaker inhibitor of FLT3 and LKB1 (FLT3 IC50 of 100 nM for SR-653234 and 305 nM for SR-1277; SR-1277 was inactive toward LKB1). Further, SR-1277 was inactive toward kinases implicated previously in Wee1 turnover as well as all kinases tested, with the exception of CK1δ, CK1ε, and FLT3 (Fig. 2B). SR-1277 stabilization of K328M-luciferase was more pronounced than that observed with SR-653234, suggesting that the improved CK1δ or CK1ε inhibitory activity of SR-1277 correlated with more potent K328M-luciferase degradation inhibition. SR-1277 did not affect the degradation of other proteins turned over via the ubiquitin proteasome pathway, including N-cyclin B1-luciferase, p27kip1-luciferase, or p21cip1-luciferase, suggesting that its activity was directed toward K328M-Wee1-luciferase (Fig. 2C). SR-653234 or SR-1277 treatment also inhibited the degradation of endogenous Wee1, suggesting that inhibiting CK1δ and/or CK1ε activity is required for endogenous Wee1 turnover (Fig. 2D).

CK1δ Activity Is Required for Wee1 Degradation—Comparison of CK1δ, CK1ε, FLT3, and LKB1 inhibition mediated by SR-653234 or SR-1277 suggested that CK1δ or CK1ε were responsible for Wee1 degradation. To test this model, we incu-
bated HeLa cells expressing K328M-Wee1-luciferase with a known CK1δ/ε or FLT3 inhibitor. As shown in Fig. 3A, incubation with the CK1δ/ε inhibitor D4476 stabilized K328M-Wee1-luciferase but did not affect levels of luciferase alone (the CK1 inhibitors PF-670462 and IC261 were not used because of multiple off-target effects (14)). By contrast, a known inhibitor of FLT3, Sutent (19), had no measurable effect on Wee1 turnover (Fig. 3E). SR-653234 inhibits K328M-Wee1-luciferase degradation. HeLa cells transfected with K328M-Wee1-luciferase were incubated with cycloheximide along with either DMSO or 5 μM SR-653234, and the extent of Wee1 degradation was measured after luminescence detection. The 0 time point was set to 100%. F. SR-653234 does not affect luciferase degradation. HeLa cells were transfected with PGL3 cDNA encoding luciferase and subsequently incubated with cycloheximide along with DMSO or 5 μM SR-653234. The extent of degradation was measured as in E. For all luciferase assays, one representative luciferase assay performed in quadruplicate is shown.

The stabilization of Wee1 activity by SR-653234, SR-1277, and D4476 suggests that CK1δ and/or CK1ε is required for Wee1 turnover. To test this possibility, we first used two siRNAs that target CK1δ and assayed Wee1 turnover. CK1δ siRNA overexpression depleted CK1δ levels and specifically reduced Wee1 turnover relative to GFP siRNA (Fig. 4A). By contrast, depletion of CK1ε did not affect Wee1 proteolysis (Fig. 4B). Consistent with these findings, conditional deletion of CK1δ and ε in mouse embryonic fibroblasts increased Wee1 levels, which could be reversed by CK1δ overexpression (Fig. 4C). Collectively, these studies suggest that CK1δ is required for Wee1 degradation in both HeLa cells and mouse embryonic fibroblasts.

**CK1δ Phosphorylates Wee1 in an N-terminal Region Required for Turnover**—Our pharmacological and genetic studies suggested that CK1δ may directly control Wee1

![FIGURE 1. SR-653234 selectively stabilizes Wee1. A, SR-653234 stabilizes Wee1-luciferase but not N-cyclin B1-luciferase. HeLa cells transfected with either K328M-Wee1-luciferase or N-cyclin B1-luciferase were incubated with increasing concentrations of SR-653234. The percentage of signal relative to the proteasome inhibitor MG132 is shown. B, structures of SR-653234 analogs that stabilized K328M-Wee1-luciferase with corresponding EC50s for stabilization of K328M-Wee1-luciferase or N-cyclin B1-luciferase. C, MG132 stabilizes K328M-Wee1-luciferase. D, a class of SR-653234 analogs selectively stabilizes endogenous Wee1. Shown is a Western blot analysis of endogenous Wee1 or Skp1 (loading control) of HeLa cells incubated with 5 μM of indicated compounds for 24 h. E, SR-653234 inhibits K328M-Wee1-luciferase degradation. HeLa cells transfected with K328M-Wee1-luciferase were incubated with cycloheximide along with either DMSO or 5 μM SR-653234, and the extent of Wee1 degradation was measured after luminescence detection. The 0 time point was set to 100%. F, SR-653234 does not affect luciferase degradation. HeLa cells were transfected with PGL3 cDNA encoding luciferase and subsequently incubated with cycloheximide along with DMSO or 5 μM SR-653234. The extent of degradation was measured as in E. For all luciferase assays, one representative luciferase assay performed in quadruplicate is shown.**
We hypothesized that CK1δ controls Wee1 turnover via phosphorylation because several CK1δ consensus motifs can be found in human Wee1 (NetPhos 2.0). To determine whether CK1δ directly phosphorylates Wee1, we incubated recombinant CK1δ with immunopurified FLAG-Wee1 K328M or a version of Wee1 lacking its N terminus, FLAG-Wee1 K328M ΔN214, in an in vitro phosphorylation assay containing [γ-32P]ATP. As shown in Fig. 5A, CK1δ phosphorylated FLAG-Wee1 K328M, but not FLAG-Wee1 K328M ΔN214, suggesting that the N terminus of Wee1 is required for CK1δ-dependent phosphorylation. This was confirmed by mass spectrometry, wherein we identified the Wee1 site serine 212 as specifically phosphorylated in the presence of recombinant CK1δ (Fig. 5, B and C). Collectively, these results suggest that CK1δ phosphorylates Wee1 on its N terminus.

Prior studies demonstrated that multiple N-terminal phosphorylations initiate Wee1 turnover (7, 20). Thus, we predicted that serine 212 phosphorylation is part of a motif that induces Wee1 recognition by ubiquitin ligases. We mutated serine 211 and serine 212 to alanine (FLAG-Wee1 K328M GAAL) and measured the degradation of the mutated version relative to FLAG-Wee1 K328M. Serine 211 phosphorylation occurred under control conditions in the absence of CK1δ and in the presence of GSK3-β (Fig. 5, D and E). As shown in Fig. 5F, wild-type Wee1 was turned over within 2 h. However, FLAG-Wee1 K328M GAAL was not appreciably degraded within that time frame (the single site K328M GSAL and K328M GASL mutants were degraded with similar kinetics as FLAG-Wee1 K328M). This stabilization did not affect the levels of other cell cycle markers, such as cdc27, cyclin B1, and phospho-histone 3 (serine 10 phosphorylation). By contrast, reduced degradation of Wee1 is likely related to binding to β-TrCP because FLAG-Wee1 K328M GAAL did not associate as well as FLAG-Wee1K328M with β-TrCP-V5 (Fig. 5G). These studies suggest that CK1δ-dependent phosphorylation is required for Wee1 turnover via SCF-β-TrCP. It is possible
that, in addition to inhibiting degradation, CK1δ may affect Wee1 kinase activity. However, CK1δ phosphorylation of Wee1 does not affect Wee1 activity, as determined by an *in vitro* Wee1 activity assay containing recombinant Wee1 and CK1δ (Fig. 5H).

**Casein Kinase 1 δ Activity Is Required for Cell Cycle Progression**—Our pharmacological, genetic, and biochemical studies suggest that CK1δ targets Wee1 for destruction to initiate progression through S or G2/M phase because Wee1 has been implicated in these cell cycle phases (6, 23–26). To test a possible role for CK1δ in S to G2/M phase progression, we examined the levels of CK1δ in cells progressing from S phase into mitosis (Fig. 6, A and B). We synchronized HeLa cells using a double thymidine block, which arrests them at the G1/S phase transition, released them into nocodazole-containing medium, and measured CK1δ levels via Western
blot analysis as cells progressed into mitosis. CK1δ levels increased as cells progressed into mitosis (Fig. 6, A and B). By contrast, CK1α, CK1ε, or CK γ2 levels either remained constant or decreased as cells proceeded from S to G2/M phase (Fig. 6, A and B).

Because CK1δ levels increased during S and G2 phase, we hypothesized that CK1δ may play a role in progression through these phases. Inhibition of CK1δ activity by SR-653234 or SR-1277 treatment in asynchronous HeLa cells induced an accumulation of cells in both S and G2/M phase, suggesting that CK1δ mediates S and G2/M phase progression (Fig. 6, A and B). To further analyze a possible role in S and G2/M phase progression, we synchronized HeLa cells using a double thymidine protocol and subsequently added DMSO, SR-653234, or SR-1277 before shifting the cells into nocodazole-containing media (Fig. 6E). To further analyze a possible role in S and G2/M phase progression, we synchronized HeLa cells at the G1/S phase transition using a double thymidine protocol and subsequently added DMSO, SR-653234, or SR-1277 before shifting the cells into nocodazole-containing media (Fig. 6E). Subsequently, we collected cells at 2-h intervals and measured the effect of SR-653234 or SR-1277 treatment upon mitotic entry using phospho-histone H3 Western blot analysis. As shown in Fig. 6D, mitotic entry was decreased sharply in cells treated with either SR-653234 or SR-1277, which was accompanied by Wee1 stabilization. DMSO-treated cells entered mitosis within 6 h after release from the double thymidine release. By contrast, SR-653234- or SR-1277-treated cells entered mitosis within 10 or 12 h after release, respectively. However, inhibition of mitotic entry could be due to an inability of cells to progress through S phase in the presence of SR-653234 or SR-1277. Thus, these studies suggest that CK1δ inhibition via SR-653234 or SR-1277 treatment significantly inhibits progression through S and G2/M phase.

**DISCUSSION**

This study demonstrates a novel role for CK1δ kinase in Wee1 regulation. Several lines of evidence suggest that CK1δ controls Wee1 destruction. First, novel small molecule inhibitors of CK1δ regulate Wee1 kinase levels. Second, the well-characterized CK1δ/ε inhibitor D4476 stabilized Wee1. Third, SR-653234 or SR-1277 treatment upon mitotic entry using phospho-histone H3 Western blot analysis. As shown in Fig. 6D, mitotic entry was decreased sharply in cells treated with either SR-653234 or SR-1277, which was accompanied by Wee1 stabilization. DMSO-treated cells entered mitosis within 6 h after release from the double thymidine release. By contrast, SR-653234- or SR-1277-treated cells entered mitosis within 10 or 12 h after release, respectively. However, inhibition of mitotic entry could be due to an inability of cells to progress through S phase in the presence of SR-653234 or SR-1277. Thus, these studies suggest that CK1δ inhibition via SR-653234 or SR-1277 treatment significantly inhibits progression through S and G2/M phase.

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CK1δ Controls Wee1 Destruction

CK1δ depletion by siRNA-mediated transfection in HeLa cells or conditional deletion of CK1δ/e in mouse embryonic fibroblasts stabilizes Wee1. Fourth, CK1δ phosphorylated Wee1 in vitro in the N-terminal region required for turnover. Finally, CK1δ levels increase as cells progress through S and G2/M phase, suggesting a role for CK1δ in controlling Wee1 levels when its activity must be regulated.

Because CK1δ phosphorylates and promotes degradation of Wee1, CK1δ control of the cell cycle could be linked to different processes controlled by Wee1, including the G2/M transi-
CK1δ Controls Wee1 Destruction

![Diagram](image)

**FIGURE 5.** Casein kinase 1δ is required for S and G2/M phase progression. SR-653234 or SR-1277 treatment inhibits S and G2/M phase progression. A, CK1δ protein levels rise as cells enter mitosis. HeLa cells synchronized in S phase by a double thymidine arrest were released into medium containing nocodazole, and cells were collected at the time points shown. Cell extracts were prepared and immunoblotted for the indicated proteins. B, percentage of cells in each phase of the cell cycle analyzed in A as measured by flow cytometry. C, SR-653234 or SR-1277 treatment inhibits S and G2/M phase progression. Asynchronous HeLa cells were treated with the indicated amounts of either SR-653234 or SR-1277 and processed for PI-FACS. The plot represents the mean ± S.E. of three independent experiments. D, asynchronous HeLa cells were treated with the indicated amounts of either SR-653234 or SR-1277, and levels of cyclin B1, phospho-tyrosine-15-cdc2, or the loading control Skp1 were determined. One representative Western blot analysis performed in triplicate is shown. E, SR-653234 and SR-1277 inhibit S/G2/M phase progression in synchronized cells. HeLa cells were synchronized at the G1/S phase transition using a double thymidine synchronization procedure. Subsequently, cells were released from the block by washing away thymidine. Cells were then incubated in the presence of DMSO, 100 nM SR-653234, or 50 nM SR-1277 along with 330 nM nocodazole for 14 h. Cells were collected every 2 h and processed for Western blot or FACS analysis. Western blot analyses were performed for Wee1, phospho-histone H3 (Ser-10), or Skp1 (loading control).

**FIGURE 6.** Casein kinase 1δ-mediated N-terminal Wee1 phosphorylation is required for interaction with the F-box protein β-TrCP. A, autoradiogram (Autorad) showing in vitro phosphorylation of FLAG-Wee1 K328M (WT-Wee1) and FLAG-Wee1 K328M Δ214 (Δ214-Wee1) performed in the presence of recombinant CK1δ. Anti-FLAG Western blot analysis confirmed the expression of both constructs. The anti-FLAG Western blot analysis was the loading control for the autoradiograph. B, MS/MS spectra of human Wee1 identifying serine 212 as phosphorylated after incubation with recombinant CK1δ. C, identification of the Ser-211 and Ser-212 sites on Wee1 by mass spectrometry. Ser-212 was only present when CK1δ was added. Equivalent amounts of commercially purchased CK1δ (New England Biolabs) were used for in vitro kinase reactions. The control was a buffer only control. Protein Pilot was used to search for acetylation, phosphorylation, and known biological modifications. Mascot was used to search for phosphorylation and acetylation. With the exception of Ser-444 (given its high confidence), only peptides that were phosphorylated in both the Mascot/Protein Pilot searches are shown. Chromatographic retention in time shifts from unmodified to modified peptides was also used for validation. pT (phospho-threonine) was only identified in GSK3 treatment, pS (phospho-serine) was found only in CK1 treatment, pS was not identified in the control. The residues shown in boldface have already been identified in other publications (as reported in Uniprot). Peptide was only identified in Protein Pilot but with very high confidence (99%). D, MS/MS spectra of human Wee1 identifying serine 212 as phosphorylated after incubation with recombinant GSK3β. E, schematic of the position of serines 211 and 212 in the Wee1 N-terminal (N-term) domain. Ser-211 and Ser-212 are shown in red. F, mutation of serines 211 and 212 to alanine reduces Wee1 turnover. HeLa cells were transfected with FLAG-Wee1 K328M or FLAG-Wee1 K328M GAAL, and the extent of degradation was determined after a cycloheximide (CHX) degradation assay. Protein levels of Wee1 and cell cycle markers such as cdk2, cyclin B1, and phospho-histone 3 (serine 10) were assessed. Transfection efficiency was routinely judged to be greater than 80% on the basis of cotransfection with an enhanced GFP-expressing plasmid. G, mutation of serines 211 and 212 to alanine reduces the association with β-TrCP-V5. HeLa cells were transfected with FLAG-Wee1 K328M or FLAG-Wee1 K328M GAAL along with β-TrCP-V5. The extent of β-TrCP-V5 association was determined after anti-β-TrCP-V5 Western blot analysis. H, CK1δ does not affect Wee1 activity. CycLex assay was used to measure Wee1 activity in the presence of either buffer (−) or CK1δ. The Wee1 inhibitor MK-1775 and staurosporine were used as positive controls to inhibit Wee1 activity. All reactions contained 40 μM recombinant Wee1.
phase as an epigenetic modifier that phosphorylates histone 2b at Tyr-37 and inhibits transcription of the histone cluster Hist1 (27). Thus, these complex roles for Wee1 may explain why we observed an increase in S and G2/M phase cells after CK1δ inhibition. For instance, CK1δ could promote the degradation of Wee1 and, therefore, induce cyclin A-Cdk2 and cyclin B-Cdk1 activation as well as histone 2b-dependent transcription. In turn, higher cyclin A-Cdk2 activity could potentially promote S phase progression, whereas Cdk1-cyclin B1 activation might induce the G2/M transition. By contrast, increased Wee1 after CK1δ inhibition or depletion may stall cells in S phase because Wee1 functions in the S phase checkpoint (28) or in G2 because Wee1 degradation is required for the G2-to-M transition (10, 11). However, further studies are required to delineate these complex roles for CK1δ and Wee1.

Our novel small molecule CK1δ inhibitors are useful tools to uncover these roles. Although other CK1 inhibitors have been shown to have off-target effects via p38 MAPK or tubulin inhibition (29, 30), SR-653234 and SR-1277 are not likely to modulate these targets. Our in vitro kinase profiling using multiple methods demonstrate specificity for CK1δ, and we did not observe p38 MAPK inhibition at any SR-653234 and SR-1277 concentrations tested (14). Further, SR-653234 and SR-1277 treatment did not arrest cells at prometaphase or metaphase, as determined by live cell imaging,4 and, thus, SR-653234- and SR-1277-mediated inhibition of tubulin is unlikely. It is possible that the effects of SR-653234 and SR-1277 on the cell cycle and Wee1 destruction are due to inhibition of both CK1δ and CK1ε. However, we observed effects on cell cycle progression at a concentration of SR-1277 of 50 nM, which is similar to the in vitro IC50 of CK1δ (49 nM) but not CK1ε (258 nM). Thus, we favor a model where SR-653234 and SR-1277 inhibit cell cycle progression and Wee1 destruction via CK1δ inhibition.

We find that CK1δ is a major kinase controlling Wee1. Although several mammalian isoforms of CK1 have been reported (α, γ1, γ2, γ3, δ, ϵ1, ϵ2, and ϵ3), our inhibitors specifically target the CK1δ and CK1ε isoforms and are more selective for CK1δ (31) (Fig. 2, A and B). Further, CK1δ is unique among CK1 isoforms because it increases from S to G2/M phase. Thus, CK1δ may be one of the only CK1 isoforms that controls Wee1 degradation, although depletion of all CK1 isoforms is required to compare their relative contribution to Wee1 destruction. At least under our experimental conditions, depletion of CK1ε did not affect Wee1 turnover,4 suggesting that CK1δ, and not CK1ε, is responsible for Wee1 proteolysis.

We propose that CK1δ controls Wee1 turnover by phosphorylating Wee1 (8) and targeting the SCF-β-TrCP ubiquitin ligase. This is supported by CK1-dependent phosphorylation of Wee1 in a region required for interaction with β-TrCP as well as turnover. CK1δ-dependent targeting of Wee1 to β-TrCP is similar to CK1δ phosphorylation of Per2 and Mdm2, which initiates β-TrCP recognition and degradation (20, 21). Unlike Per2 and Mdm2, however, Wee1 is an essential cell cycle protein because weel−/− cells and mice are not viable prenatally (22). Similarly, Csnk1d (CK1δ) knockout mice are not viable, suggesting that CK1δ may also be an essential cell cycle gene (31).

Our studies are the first to implicate CK1δ in control of the core mammalian cell cycle machinery. Our results suggest that CK1δ controls Wee1 during S and G2 phase, although we cannot rule out regulation during other cell cycle phases. Treatment of cells synchronized at G1/S transition with highly specific small molecules reduced mitotic entry of cells progressing through S and G2 phase, as judged by phospho-histone H3 analysis (Fig. 6). By contrast, PI-FACS analysis of SR-653234-, SR-1277-, or DMSO-treated cells progressing into mitosis from G1/S phase showed a similar profile for all cells.4 This may suggest that SR-653234 or SR-1277 treatment reduces G2/M transition, which can be observed by phospho-histone H3 analysis but not PI-FACS because the latter cannot distinguish G2 from mitosis. We were unable to deplete CK1δ by siRNA and subsequently synchronize cells at G1/S transition because of cytotoxicity upon synchronization.4 However, CK1δ depletion in asynchronous cells increased the number of cells in the S and G2/M phases of the cell cycle, as judged by PI-FACS analysis.

CK1δ’s control of the cell cycle is due, in part, to the ability of CK1 to target Wee1 for destruction, although other CK1δ substrates involved in cell cycle progression likely exist. This is supported by our observation that inhibition of Wee1 activity does not overcome mitotic entry defects observed after SR-653234 and SR-1277 treatment, although this may be due to toxicities observed with Wee1 inhibition alone. Thus, further studies including conditional inhibition of Wee1 in vitro and in vivo will be required to examine the relative contribution of the CK1δ-Wee1 pathway to cell cycle progression. Nonetheless, our studies strongly implicate CK1δ in controlling eukaryotic cell cycle transitions in part through regulating Wee1 turnover.

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