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$p107^{wee1}$ is a dual-specificity kinase that phosphorylates $p34^{cdc2}$ on tyrosine 15

(cell cycle/baculovirus expression)

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ABSTRACT **$p107^{wee1}$ is a protein kinase that functions as a dose-dependent inhibitor of mitosis through its interactions with $p34^{cdc2}$ in *Schizosaccharomyces pombe*. To characterize the kinase activity of $p107^{wee1}$, its carboxyl-terminal catalytic domain was purified to homogeneity from overproducing insect cells. The apparent molecular mass of the purified protein ($p37^{wee1}$KD) was determined to be ≈37 kDa by gel filtration, consistent with it being a monomer. Serine and tyrosine kinase activities cofiltered with $p37^{wee1}$KD, demonstrating that $p107^{wee1}$ is a dual-specificity kinase. *In vitro*, $p107^{wee1}$ phosphorylated $p34^{cdc2}$ on Tyr-15 only when $p34^{cdc2}$ was complexed with cyclin. Neither monomeric $p34^{cdc2}$ nor a peptide containing Tyr-15 was able to substitute for the $p34^{cdc2}$/cyclin complex in this assay. Furthermore, the phosphorylation of $p34^{cdc2}$ by $p107^{wee1}$ *in vitro* inhibited the histone H1 kinase activity of $p34^{cdc2}$. These results indicate that $p107^{wee1}$ functions as a mitotic inhibitor by directly phosphorylating $p34^{cdc2}$ on Tyr-15 and that the preferred substrate for phosphorylation is the $p34^{cdc2}$/cyclin complex.**

The cell cycle regulator $p107^{wee1}$ was first identified in the fission yeast *Schizosaccharomyces pombe* as a dose-dependent inhibitor of mitosis (1, 2). Genetic analysis suggests that $p107^{wee1}$ functions by inhibiting the activity of $p34^{cdc2}$, a serine/threonine protein kinase required for entry of cells into mitosis (1, 3). Sequence analysis revealed a significant degree of homology between $p107^{wee1}$ and the serine/threonine class of protein kinases (4). However, recent evidence has implicated $p107^{wee1}$ in the phosphorylation of $p34^{cdc2}$ on Tyr-15 (an inhibitory phosphorylation), despite its lack of homology to known tyrosine kinases. First, coexpression of $p34^{cdc2}$ with cyclin and $p107^{wee1}$ in insect cells leads to the stoichiometric phosphorylation of $p34^{cdc2}$ on Tyr-15. In this system, the tyrosine phosphorylation of $p34^{cdc2}$ is absolutely dependent upon the kinase activity of $p107^{wee1}$, as a kinase-deficient mutant of $p107^{wee1}$ does not induce the tyrosine phosphorylation of $p34^{cdc2}$ (5). Second, although deletion of the $wee1^+$ gene of *S. pombe* leads to no detectable change in the phosphorylation state of $p34^{cdc2}$ (6), deletion of $wee1^+$ and a related gene, $mik1^+$, leads to a rapid decrease in tyrosine phosphorylated $p34^{cdc2}$ and mitotic lethality (3). These genetic findings suggest that $wee1^+$ and $mik1^+$ encode partially redundant proteins that cooperate to control the tyrosine phosphorylation of $p34^{cdc2}$. However, to date, reconstitution of these interactions *in vitro* has not been reported.

Several proteins have recently been identified that possess intrinsic serine/threonine as well as tyrosine kinase activities. These proteins belong to a class of dual-specificity kinases and include such proteins as ERK 1 and ERK 2, STY1/clk, MCK1 (previously named YPK1), and SPK1 (7–15). Recent evidence has indicated that $p107^{wee1}$ may also belong to this class of kinases. First, immunoprecipitates of $p107^{wee1}$ from insect cells overproducing $p107^{wee1}$ or from budding and fission yeast overproducing $p107^{wee1}$ contain serine and tyrosine kinase activities, whereas a kinase-deficient mutant of $p107^{wee1}$ lacks both activities (5, 16). Second, both activities copurify through gel filtration and density gradient centrifugation of $p107^{wee1}$ (16). Although these results suggest that $p107^{wee1}$ possesses both enzymatic activities, they do not rule out the possibility that $p107^{wee1}$ tightly associates with and activates an endogenous insect cell tyrosine kinase.

To fully characterize the kinase activity of $p107^{wee1}$, we purified the catalytic domain of $p107^{wee1}$ to homogeneity. Serine and tyrosine kinase activities copurified with the catalytic domain of $p107^{wee1}$, demonstrating that $p107^{wee1}$ is a dual-specificity kinase. In addition, incubation of $p34^{cdc2}$/cyclin complexes with $p107^{wee1}$ *in vitro* resulted in the phosphorylation of $p34^{cdc2}$ on Tyr-15 concomitant with an inhibition in histone H1 kinase activity.

MATERIALS AND METHODS

All procedures relating to virus propagation, protein analysis, immunoprecipitations, $p13^{suc1}$ precipitations, phosphoamino acid analysis, and tryptic phosphopeptide mapping have been described (5).

Generation of Recombinant Baculoviruses. Recombinant baculoviruses encoding $p34^{cdc2}$, cyclin A, $p107^{wee1}$, and $p34^{cdc2}$ (Phe-15) were constructed as described (5). A recombinant virus encoding the kinase domain of $p107^{wee1}$ was constructed as follows: pJ3-W1 containing a *Bam*HI cloning site in place of *Xho* I (5) was digested with *Nco* I and *Bam*HI, and the 2.5-kilobase (kb) fragment encoding the predicted kinase domain of $p107^{wee1}$ was isolated and cloned into a derivative of pVL941 (17) containing an *Nco* I and *Bam*HI cloning site. A kinase-inactive version of the $p107^{wee1}$ kinase domain was similarly engineered.

A recombinant baculovirus encoding a fusion protein between glutathione *S*-transferase (GST) and $p107^{wee1}$ was created as follows: pJ3-W1 containing a *Bam*HI cloning site in place of *Xho* I (5) was digested with *Bam*HI, and the 4.8-kb fragment encoding $p107^{wee1}$ was cloned into the *Bam*HI site of pGEX-2T (Pharmacia LKB) to generate pGEX-2Twee. The unique *Eco*NI site of pGEX-2Twee was changed to *Xba* I. The resulting plasmid was digested with *Xba* I and *Nhe* I, and the 3.6-kb fragment encoding a fusion protein between GST and $p107^{wee1}$ was cloned into the *Nhe* I site of pBluebac (Invitrogen).

Generation of recombinant baculoviruses encoding kinase-deficient $p34^{cdc2}$ (Lys-33 changed to Arg), GST, and human

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Abbreviation: GST, gluthathione *S*-transferase.

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cyclin B as a fusion protein with GST will be described elsewhere.

Purification of p37^{wee1}KD. Insect cells (2.7×10^8), expressing the kinase domain of p107^{wee1}, were collected by centrifugation at $1000 \times g$ for 10 min at 4°C and were washed once in phosphate-buffered saline (PBS). Cells were lysed by freeze-thawing once; this was followed by Vortex spinning in 20 mM triethanolamine (pH 7.2) containing 2 mM phenylmethylsulfonyl fluoride, 0.15 unit of aprotinin per ml, 20 μM leupeptin, and 5 μg of pepstatin per ml. Lysates were clarified at $10,000 \times g$ for 10 min at 4°C followed by centrifugation at $100,000 \times g$ for 45 min. The supernatant was collected and 2 ml (≈15 mg) was passed over a FPLC 5/5 Mono Q column equilibrated in 20 mM triethanolamine (pH 7.2). The bound proteins were eluted with a linear gradient of 0–600 mM NaCl in 20 mM triethanolamine (pH 7.2) in a total volume of 45 ml. Fractions (0.5 ml) were collected and analyzed by Coomassie blue staining following SDS/PAGE. p37^{wee1}KD eluted at 260 mM NaCl.

Fractions containing p37^{wee1}KD were pooled, adjusted to 1.5 M ammonium sulfate, and centrifuged at $10,000 \times g$ for 10 min to remove particulates. The supernatant (≈2 mg) was passed over a FPLC 5/5 phenyl-Superose column equilibrated in 50 mM sodium phosphate (pH 7.0) containing 1.5 M ammonium sulfate. Bound proteins were eluted with a linear gradient of 1.5–0 M ammonium sulfate in a total volume of 45 ml. Fractions (0.5 ml) were collected and analyzed by SDS/PAGE followed by Coomassie blue staining. p37^{wee1}KD eluted at 225 mM ammonium sulfate. Approximately 100 μg of purified protein was recovered from the initial 15 mg of crude lysate. All chromatographic steps were performed at 4°C. The kinase domain of p107^{wee1} (Leu-596) was similarly purified. This protein contains leucine in place of lysine at position 596 and is kinase inactive (5). Kinase activity did not copurify with this mutant (data not shown).

Superose 12 Chromatography. Fractions from the phenyl-Superose column containing p37^{wee1}KD were pooled, adjusted to 70% (wt/vol) ammonium sulfate, and centrifuged at $10,000 \times g$ for 10 min at 4°C. Precipitated proteins were resuspended in 0.2 ml of buffer A (50 mM Tris, pH 7.4/50 mM NaCl) and recentrifuged at $10,000 \times g$ for 10 min. The 0.2-ml supernatant (≈100 μg) was passed over a FPLC 30/10 Superose 12 column equilibrated in buffer A. Fractions (0.5 ml) were collected and analyzed by SDS/PAGE. p37^{wee1}KD eluted between 12.5 and 13.5 ml. Molecular mass standards (Sigma) included carbonic anhydrase (29 kDa), albumin (66 kDa), and alcohol dehydrogenase (150 kDa).

Kinase Assays Using Purified p37^{wee1}KD. Fractions (10 μl) from the Superose 12 column (in buffer A) were adjusted to 10 mM $MnCl_2$ or 10 mM $MgCl_2$, 1 mM dithiothreitol, and 10 μM ATP and were then incubated with 1.0 mCi of [γ-^{32}P]ATP per ml (1 Ci = 37 GBq) at 30°C for 15 min (final reaction volume, 100 μl). When kinase assays were performed on fractions from the phenyl-Superose column, the proteins were first desalted on a Bio-Rad disposable desalting column according to instructions from the manufacturer using buffer A as the elution buffer. Kinase assays were then performed as described above. When included, acid-denatured enolase was added to a final concentration of 250 μg/ml.

Phosphorylation of p34^{cdc2} *in Vitro* by p107^{wee1}. *Kinase assays using p13^{suc1} beads.* Insect cells were infected with recombinant viruses as described in the legend to Fig. 3. Cells were lysed in RIPA/Tris buffer (5) supplemented with 2 mM phenylmethylsulfonyl fluoride, 0.15 unit of aprotinin per ml, 20 μM leupeptin, and 1 mM sodium orthovanadate. Lysates prepared from cells infected with p107^{wee1} were divided into thirds. One-third was mixed with a lysate prepared from cells expressing p34^{cdc2} alone, one third was mixed with a lysate prepared from cells coexpressing p34^{cdc2} (Phe-15) and cyclin A, and the final third was mixed with a lysate prepared from cells expressing wild-type p34^{cdc2} and cyclin A. Mixed lysates were precipitated with p13^{suc1} beads, and the precipitates were washed once in Ripa/Tris buffer, twice in LiCl wash buffer (5), and once in incomplete kinase buffer (50 mM Tris, pH 7.4/10 mM $MnCl_2$). Kinase assays were carried out in 50 mM Tris, pH 7.4/10 mM $MnCl_2$/1 mM dithiothreitol/10 μM ATP/1.0 mCi of [γ-^{32}P]ATP per ml at 30°C for 15 min.

Kinase assays using glutathione beads. Insect cells were infected with viruses encoding either GST or GST-p107^{wee1} or were coinfected with viruses encoding GST-human cyclin B and p34^{cdc2} (Arg-33). Cells were lysed in Nonidet P-40/Tris buffer (5) and lysates were mixed as described in the legend to Fig. 4. Proteins were precipitated with glutathione agarose beads (Sigma). Precipitates were washed twice in Nonidet P-40 lysis buffer and twice in incomplete kinase buffer. Kinase assays were performed as described above.

Regulation of p34^{cdc2} Kinase Activity by p107^{wee1}. Insect cells were infected with viruses encoding either GST or GST-p107^{wee1} or were coinfected with viruses encoding GST-human cyclin B and p34^{cdc2}. Cells were lysed as described above and lysates were mixed as described in the legend to Fig. 5. Proteins were precipitated with glutathione agarose beads. The precipitates were washed twice in Nonidet P-40 lysis buffer and twice in incomplete kinase buffer. Kinase assays were performed as described above except that 1 mM unlabeled ATP was substituted for the [γ-^{32}P]ATP. Kinase reaction mixtures were washed three times in incomplete kinase buffer. Part of each reaction was resolved by SDS/PAGE and analyzed for levels of p34^{cdc2} by immunoblotting. Histone H1 kinase assays were performed on the remainder of each sample.

Preparation of Anti-p107^{wee1} Serum (R1902-R1905). Plasmid pJ3-W1 was digested with *Hin*dIII and *Xho* I and the 1.7-kb fragment containing the putative kinase domain of p107^{wee1} was cloned into the *Hin*dIII/*Xho* I sites of plasmid pBSK (Stratagene) in frame with the β-galactosidase gene to generate the plasmid pBSK(weeKD). The lon$^-$ bacterial strain SG931Q was transformed with pBSKweeKD (18). Recombinant protein was induced and antibodies were generated as described (5).

RESULTS

Purification of p37^{wee1}KD. We have previously reported that immunoprecipitates of p107^{wee1} (overproduced in insect cells using a baculoviral expression system) contain serine/threonine as well as tyrosine kinase activities (5). To determine which of these activities were intrinsic to p107^{wee1}, we expressed a 37-kDa carboxyl-terminal truncated form of p107^{wee1} in insect cells. The 37-kDa derivative of p107^{wee1} contained all of the canonical sequences present in protein kinases (4) and, as shown in Fig. 1*B*, retained kinase activity.

When expressed in insect cells, the kinase domain of p107^{wee1} was completely soluble, represented ≈5% of total cellular protein, and was easily purified using classical biochemical fractionation techniques (Fig. 1*A*). The purification scheme consisted of a high-speed spin ($100,000 \times g$, Fig. 1*A*, lane 1) followed by chromatography on Mono Q (lane 2) and phenyl-Superose (lane 3) columns. A similar strategy was employed to purify full-length p107^{wee1}. Unfortunately, the majority of p107^{wee1} pelleted during the $100,000 \times g$ spin and attempts to resolubilize it were unsuccessful. As shown in Fig. 1*A*, the 37-kDa kinase domain was purified to apparent homogeneity after the phenyl-Superose column (lane 3). To test for kinase activity, fractions containing p37^{wee1}KD were pooled after the Mono Q column (Fig. 1*A*, lane 2) or the phenyl-Superose column (Fig. 1*A*, lane 3), p37^{wee1}KD was further purified by immunoprecipitation with anti-p107^{wee1} serum and kinase assays were performed *in vitro*. As shown in Fig. 1*B*, a single phosphopeptide of 37 kDa was detected

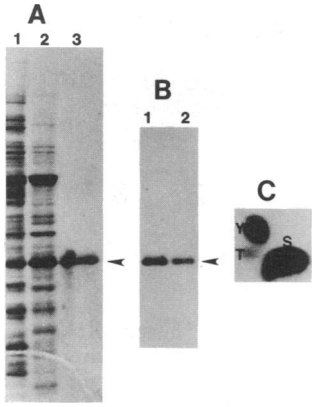

FIG. 1. Purification of the p107^{wee1} kinase domain. (*A*) Coomassie blue stain of p37^{wee1}KD at various stages of purification. Lane 1, lysates prepared from cells overproducing p37^{wee1}KD were centrifuged at 100,000 × *g* for 45 min (20 μg); lane 2, pooled fractions from Mono Q column (10 μg); lane 3, pooled fractions from phenyl-Superose column (2 μg). (*B*) Pooled fractions from the Mono Q column (lane 1) and the phenyl-Superose column (lane 2) were immunoprecipitated with anti-p107^{wee1} serum and kinase assays were performed *in vitro*. Reaction products were resolved by SDS/PAGE. Arrowheads indicate p37^{wee1}KD. (*C*) Phosphoamino acid analysis of p37^{wee1}KD from *B*, lane 2.

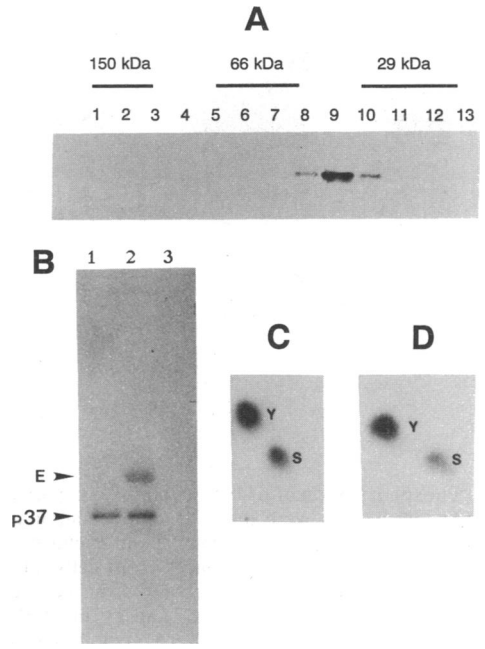

FIG. 2. Kinase activities associated with monomeric p37^{wee1}KD. (*A*) Purified p37^{wee1}KD was chromatographed on a Superose 12 gel filtration column and 0.5-ml fractions were collected beginning at 9.0 ml (lane 1) and ending with 15 ml (lane 13). p37^{wee1}KD was detected by immunoblotting. Positions of molecular mass markers are listed above the lanes: alcohol dehydrogenase (150 kDa), albumin (66 kDa), carbonic dehydrogenase (29 kDa). (*B*) The 13-ml fraction containing p37^{wee1}KD (*A*, lane 9) was assayed for kinase activity *in vitro* in the absence (lane 1) and in the presence (lane 2) of acid-denatured enolase (E). Lane 3, enolase assayed in the absence of p37^{wee1}KD. (*C*) Phosphoamino acid analysis of p37^{wee1}KD from *B*, lane 1 (2000 cpm loaded, exposed for 16 hr). (*D*) Phosphoamino acid analysis of enolase from *B*, lane 2 (2000 cpm loaded, exposed for 16 hr).

in both cases. Phosphoamino acid analysis of p37^{wee1}KD from Fig. 1*B* (lane 2) demonstrated that p37^{wee1}KD was phosphorylated primarily on serine and tyrosine residues, although low levels of phosphothreonine were also detected (Fig. 1*C*). No detectable differences were observed when $MgCl_2$ was substituted for $MnCl_2$ in the kinase assays.

To verify that p37^{wee1}KD was purified as a monomer, pooled fractions of p37^{wee1}KD from the phenyl-Superose column were chromatographed on a Superose 12 gel filtration column. The apparent molecular mass of p37^{wee1}KD by gel filtration was determined to be ≈37 kDa (Fig. 2*A*). Kinase activity cofiltered with p37^{wee1}KD (Fig. 2*B*, lane 1). Phosphoamino acid analysis revealed phosphotyrosine and phosphoserine (Fig. 2*C*). Several substrates were tested for their ability to be phosphorylated by purified p37^{wee1}KD (enolase, histone H1, casein, monomeric p34^{cdc2}, a peptide containing Tyr-15, and p34^{cdc2}/cyclin complex from overproducing insect cells). Acid-denatured enolase was the only substrate that was detectably phosphorylated by p37^{wee1}KD (Fig. 2*B*, lane 2) and the phosphorylation occurred on serine and tyrosine residues (Fig. 2*D*).

Interactions Between p34^{cdc2} and p107^{wee1} *in Vitro*. To study the interactions between p107^{wee1} and p34^{cdc2} *in vitro*, p107^{wee1} was tested for its ability to phosphorylate monomeric p34^{cdc2}, p34^{cdc2} in complex with cyclin A, or a mutant of p34^{cdc2} [p34^{cdc2} (Phe-15) that encodes phenylalanine rather than tyrosine at position 15] in complex with cyclin A (Fig. 3*A*). Neither monomeric p34^{cdc2} (Fig. 3*A*, lane 1) nor p34^{cdc2} (Phe-15) (Fig. 3*A*, lane 2) was phosphorylated under these conditions. However, wild-type p34^{cdc2} in complex with cyclin was recognized as a substrate for phosphorylation (Fig. 3*A*, lane 3). The phosphorylation occurred exclusively on tyrosine residues (Fig. 3*B*). Also seen in Fig. 3*A* is the phosphorylation of p107^{wee1} (lanes 1–3) and the phosphorylation of clam cyclin A by p34^{cdc2} (lane 3) or by p34^{cdc2} (Phe-15) (lane 2). As reported previously, phosphorylated clam cyclin A is resolved into three or four phosphoproteins by SDS/PAGE (5).

To confirm that the 34-kDa phosphoprotein (seen in Fig. 3*A*, lane 3) was indeed p34^{cdc2}, two-dimensional phosphopeptide mapping was performed. As seen in Fig. 3*C*, one phosphopeptide was detected upon digestion with trypsin [designated phosphopeptide 3 according to Parker *et al.* (5)]. A Tyr-15-containing peptide of p34^{cdc2} was labeled *in vitro* with pp60$^{v\text{-}src}$ and likewise separated in two dimensions (Fig. 3*D*). When tryptic peptides of the 34-kDa protein were mixed with the Tyr-15-containing phosphopeptide, a single phosphopeptide was observed, indicating that the 34-kDa protein was p34^{cdc2} and that the phosphorylation was on Tyr-15 (Fig. 3*E*).

The assay shown in Fig. 3 was performed using p13^{suc1} beads. Interestingly, p107^{wee1} was precipitated by p13^{suc1} beads. We have found the interaction between p107^{wee1} and p13^{suc1} beads to be independent of p34^{cdc2}. In addition, p107^{wee1} can be dissociated from p13^{suc1} beads by the addition of soluble p13^{suc1} protein. As an independent means of assaying for the ability of p107^{wee1} to phosphorylate p34^{cdc2} *in vitro*, p107^{wee1} was overproduced in insect cells as a fusion protein with GST. In this way, large quantities of biologically active GST-p107^{wee1} could be purified in a single step using glutathione agarose beads (Fig. 4*A*, lanes 2 and 5). Human cyclin B was also overproduced in insect cells as a fusion protein with GST. In this way, large quantities of p34^{cdc2}/GST-cyclin B complex could be purified with glutathione agarose beads rather than with p13^{suc1} beads (Fig. 4*A*, lanes 3–5). In this experiment, a kinase-deficient mutant of p34^{cdc2} (generated by replacing Lys-33 with Arg) was used to eliminate the p34^{cdc2}/cyclin-specific phosphorylations that are evident in Fig. 3*A*. As a control, a recombinant virus encoding GST was also generated (Fig. 4*A*, lanes 1 and 4). As shown in Fig. 4*B*, p34^{cdc2} (Arg-33) in complex with GST cyclin B was phosphorylated upon incubation with GST-p107^{wee1} (lane 5) but not in the absence of GST-p107^{wee1} (lane 3) or when GST was substituted for GST-p107^{wee1} (lane 4). The phosphorylation of p34^{cdc2} occurred exclusively on tyrosine (Fig. 4*C*), whereas the phosphorylation of GST-

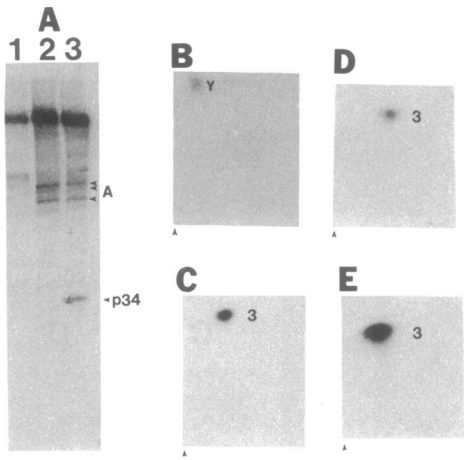

FIG. 3. Phosphorylation of $p34^{cdc2}$/cyclin A complex *in vitro* by $p107^{wee1}$. (*A*) Insect cells were infected with recombinant baculoviruses encoding $p34^{cdc2}$, $p107^{wee1}$, $p34^{cdc2}$ and cyclin A, or $p34^{cdc2}$ (Phe-15) and cyclin A. Lysates prepared from cells infected with $p107^{wee1}$ were mixed with lysates prepared from cells expressing either $p34^{cdc2}$ (lane 1), $p34^{cdc2}$ (Phe-15) and cyclin A (lane 2), or $p34^{cdc2}$ and cyclin A (lane 3). Proteins were precipitated with $p13^{suc1}$ beads and kinase assays were performed *in vitro*. Reaction products were resolved by 12% SDS/PAGE. (*B*) Phosphoamino acid analysis of $p34^{cdc2}$ from *A*, lane 3. (*C*) $p34^{cdc2}$ from *A*, lane 3, was excised from the gel and digested with trypsin, and the phosphopeptides were resolved in two dimensions. (*D*) A synthetic peptide containing Tyr-15 was phosphorylated *in vitro* by $pp60^{v\text{-}src}$, digested with trypsin, and then resolved as described above. (*E*) Mixture of material in *C* and *D*.

$p107^{wee1}$ occurred primarily on serine and tyrosine residues (Fig. 4*D*). A low level of GST cyclin B phosphorylation was detected, which may be due to the interaction of GST cyclin B with endogenous insect cell $p34^{cdc2}$ (Fig. 4*B*, lanes 3 and 4).

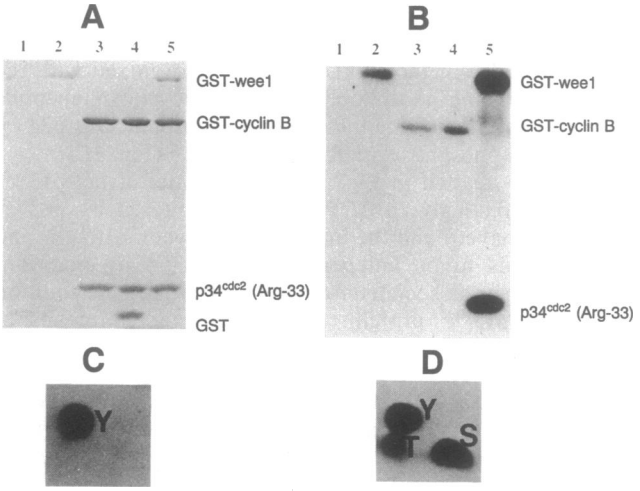

FIG. 4. Phosphorylation of $p34^{cdc2}$ (Arg-33)/GST-cyclin B complex *in vitro* by GST-$p107^{wee1}$. Insect cells were infected with recombinant baculovirus encoding GST or GST-$p107^{wee1}$ or were coinfected with viruses encoding $p34^{cdc2}$ (Arg-33) and cyclin B fused to GST (GST-cyclin B). Lysates were prepared, proteins were precipitated with glutathione agarose beads, and kinase assays were performed *in vitro*. Precipitates were from lysates containing GST only (lane 1), GST-$p107^{wee1}$ only (lane 2), or $p34^{cdc2}$ (Arg-33) and GST-cyclin B (lane 3). Alternatively, lysates prepared from cell coexpressing $p34^{cdc2}$ (Arg-33) and GST-cyclin B were mixed with those expressing either GST (lane 4) or GST-$p107^{wee1}$ (lane 5) prior to precipitation. (*A*) Coomassie blue-stained gel. (*B*) Autoradiograph of gel shown in *A*. (*C*) Phosphoamino acid analysis of $p34^{cdc2}$ from *B*, lane 5. (*D*) Phosphoamino acid analysis of GST-$p107^{wee1}$ from *B*, lane 2.

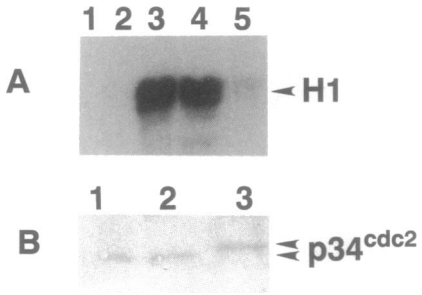

FIG. 5. Inhibition of $p34^{cdc2}$ histone H1 kinase activity *in vitro* by $p107^{wee1}$. Insect cells were infected with viruses encoding either GST or GST-$p107^{wee1}$ or were coinfected with viruses encoding GST-cyclin B and $p34^{cdc2}$. Lysates were prepared and in some cases were mixed. Proteins were precipitated with glutathione agarose beads and kinase assays were performed in the presence of 1 mM unlabeled ATP. Reaction mixtures were washed and histone H1 kinase assays were performed on one-fourth of each sample (*A*). The remainder of each sample was resolved by SDS/PAGE, blotted onto nitrocellulose, and probed for levels of $p34^{cdc2}$ (*B*). (*A*) GST only (lane 1), GST-$p107^{wee1}$ only (lane 2), $p34^{cdc2}$ and GST-cyclin B (lane 3), mixture of lanes 1 and 3 (lane 4), mixture of lanes 2 and 3 (lane 5). (*B*) Lanes 1–3 coincide with samples 3–5 respectively, shown in *A*.

Interestingly, GST cyclin B phosphorylation was less evident when GST-$p107^{wee1}$ was present (lane 5) and may indicate inhibition of the cyclin B kinase by GST-$p107^{wee1}$.

Inhibition of $p34^{cdc2}$ Kinase Activity *in Vitro* by $p107^{wee1}$. To determine what effect the tyrosine phosphorylation of $p34^{cdc2}$ by $p107^{wee1}$ *in vitro* had on the kinase activity of $p34^{cdc2}$, histone H1 kinase assays were performed (Fig. 5). $p34^{cdc2}$/cyclin B complexes were isolated from overproducing insect cells on glutathione beads as described in the legend to Fig. 4. The complexes were incubated in kinase buffer alone (Fig. 5*A*, lane 3) or in kinase buffer supplemented either with GST (Fig. 5*A*, lane 4) or with GST-$p107^{wee1}$ (Fig. 5*A*, lane 5). Reaction mixtures were then washed, and part of each was resolved by SDS/PAGE, blotted onto nitrocellulose, and probed for $p34^{cdc2}$ (Fig. 5*B*); the remainder of each reaction mixture was assayed for histone H1 kinase activity (Fig. 5*A*). As seen in Fig. 5*B* (lane 3), the phosphorylation of $p34^{cdc2}$ by GST-$p107^{wee1}$ *in vitro* resulted in an alteration in the electrophoretic mobility of $p34^{cdc2}$ to the retarded electrophoretic form, indicative of tyrosine phosphorylation. Concomitant with the shift in electrophoretic mobility was an inhibition in histone H1 kinase activity (Fig. 5*A*, lane 5). As expected, there was no histone H1 kinase activity associated either with GST (Fig. 5*A*, lane 1) or with GST-$p107^{wee1}$ (Fig. 5*A*, lane 2).

DISCUSSION

$p37^{wee1}$KD was purified to homogeneity from overproducing insect cells in three steps: ultracentrifugation (100,000 × *g*), Mono Q chromatography, and phenyl-Superose chromatography. The apparent molecular mass of the purified protein was judged to be ≈37 kDa by gel filtration, consistent with it being a monomer. In kinase assays performed *in vitro*, $p37^{wee1}$KD was phosphorylated predominantly on serine and tyrosine residues. Similarly, acid-denatured enolase was phosphorylated on serine and tyrosine residues by purified $p37^{wee1}$KD. These results demonstrate that $p107^{wee1}$ is a dual-specificity kinase.

Recently, several kinases have been identified that fall into this category. These kinases challenge a central dogma that kinases recognize either serine/threonine residues or tyrosine residues, but not both. Two of the kinases, MCK1 (previously named YPK1) and SPK1 (12–15), were isolated from budding yeast, whereas three others, ERK1, ERK2 (7–9), and STY1/clk1 (10, 11), were isolated from mammalian

systems. Thus, dual-specificity kinases appear to be ubiquitous in eukaryotes. To date no tyrosine-specific protein kinase has been isolated from yeast, suggesting that dual-specificity kinases may have been the evolutionary precursor of tyrosine-specific protein kinases. It has been shown that many of these dual-function kinases have a significant degree of homology in subdomain XI of the catalytic domain (9). Comparison of domain XI of $p107^{wee1}$ to domain XI of other dual-specificity kinases indicated the greatest similarity between $p107^{wee1}$ and the mammalian kinases ERK1 and ERK2 (57%) and STY1 (50%).

We have previously reported the use of a baculoviral expression system to study the interactions between $p34^{cdc2}$ and $p107^{wee1}$. In insect cells, a small fraction (≈10%) of $p34^{cdc2}$ becomes phosphorylated on Tyr-15 when coproduced with $p107^{wee1}$. However, coproduction of cyclin with $p34^{cdc2}$ greatly enhances the $p107^{wee1}$-dependent tyrosine phosphorylation of $p34^{cdc2}$ (≈90%). These data suggest that the $p34^{cdc2}$/cyclin complex rather than monomeric $p34^{cdc2}$ is the preferred substrate for tyrosine phosphorylation and implicate $p107^{wee1}$ in regulating the phosphorylation of $p34^{cdc2}$ on Tyr-15. In an attempt to recreate these interactions *in vitro*, we tested for the ability of $p37^{wee1}$KD and $p107^{wee1}$ to phosphorylate several substrates (histone H1, enolase, casein, a peptide containing Tyr-15, monomeric $p34^{cdc2}$, and $p34^{cdc2}$/cyclin complex). Enolase was recognized as a substrate by the catalytic domain of $p107^{wee1}$ ($p37^{wee1}$KD) but not by full-length $p107^{wee1}$, whereas the $p34^{cdc2}$/cyclin complex (from overproducing insect cells) was recognized by full-length $p107^{wee1}$ but not by $p37^{wee1}$KD. These results suggest that the amino terminus of $p107^{wee1}$ may play a role in substrate recognition. It is interesting that $p107^{wee1}$ did not phosphorylate the Tyr-15-containing peptide or monomeric $p34^{cdc2}$ *in vitro*. This result points to the high degree of substrate specificity of the $p107^{wee1}$ kinase.

It remains formally possible that a kinase other than $p107^{wee1}$ is responsible for the phosphorylation of the $p34^{cdc2}$/cyclin complex *in vitro*. However, this kinase would have to copurify with $p107^{wee1}$ or the $p34^{cdc2}$/cyclin complex on $p13^{suc1}$ beads and on glutathione beads. In addition, as the tyrosine phosphorylation of $p34^{cdc2}$ is absolutely dependent upon the kinase activity of $p107^{wee1}$, this copurifying kinase would have to be activated by $p107^{wee1}$. As $p107^{wee1}$ is a tyrosine kinase, there is no compelling reason to invoke this more complicated mechanism of regulation.

In higher eukaryotes, $p34^{cdc2}$ is phosphorylated on Thr-14 as well as Tyr-15 (in fission yeast Thr-14 phosphorylation has not been detected) (19). Both residues are located within the putative ATP binding site of $p34^{cdc2}$, and the phosphorylation of Tyr-15 has been shown to be inhibitory to $p34^{cdc2}$ kinase activity (20). Because $p107^{wee1}$ is a serine/threonine and tyrosine kinase, it is reasonable to predict that $p107^{wee1}$ would phosphorylate $p34^{cdc2}$ on Thr-14 as well as on Tyr-15, yet we have not observed any threonine phosphorylation of $p34^{cdc2}$ by $p107^{wee1}$ either *in vivo* (in insect cells) or *in vitro*. However, our studies have been carried out with human $p34^{cdc2}$ and *S. pombe* $p107^{wee1}$. It is possible that the human homolog of $p107^{wee1}$ in similar assays would catalyze the phosphorylation of human $p34^{cdc2}$ on Thr-14 and Tyr-15. It is also possible that the phosphorylation of $p34^{cdc2}$ on Thr-14 is catalyzed by a kinase that has yet to be identified.

The biochemistry reported in this paper confirms certain predictions made from the genetic studies conducted in fission yeast. Genetics indicated that $wee1^+$ encodes a negative regulator of the cell cycle through its interactions with $p34^{cdc2}$. The biochemistry reported here suggests that $wee1^+$ negatively regulates the entry of cells into mitosis by directly phosphorylating $p34^{cdc2}$ on Tyr-15, an inhibitory modification. Recent biochemical studies suggest that the mitotic control gene $cdc25^+$ (a positive regulator of the cell cycle that acts in opposition to $wee1^+$) encodes a phosphatase that directly dephosphorylates the $p34^{cdc2}$/cyclin complex on Tyr-15, thereby activating it (21–24).

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