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# Identification of a 95-kDa WEE1-like tyrosine kinase in HeLa cells

**(cell cycle/cdc2 protein/phosphorylation)**

LAURA L. PARKER*, PAUL J. SYLVESTRE†, MICHAEL J. BYRNES III‡, FENG LIU‡, AND HELEN PIWNICA-WORMS‡§

*Department of Molecular Genetics, Pfizer Central Research, Eastern Point Road, Groton, CT 06340; †Division of Signal Transduction, Beth Israel Hospital, Alpert Building, 200 Longwood Avenue, Boston, MA 02115; and ‡Department of Cell Biology and Physiology and Howard Hughes Medical Institute, Washington University Medical School, Campus Box 8228, 600 South Euclid Avenue, St. Louis, MO 63110-1093.

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**ABSTRACT** **Human *WEE1* (*WEE1Hu*) was cloned on the basis of its ability to rescue *wee1*$^+$ mutants in fission yeast [Igarashi, M., Nagata, A., Jinno, S., Suto, K. & Okayama, H. (1991) *Nature (London)* 353, 80–83]. Biochemical studies carried out *in vitro* with recombinant protein demonstrated that *WEE1Hu* encodes a tyrosine kinase of ≈49 kDa that phosphorylates p34$^{cdc2}$ on Tyr-15 [Parker, L. L. & Piwnica-Worms, H. (1992) *Science* 257, 1955–1957]. To study the regulation of *WEE1Hu* in human cells, two polyclonal antibodies to bacterially produced p49WEE1Hu were generated. In addition, a peptide antibody generated against amino acids 361–388 of p49WEE1Hu was also used. Unexpectantly, these antibodies recognized a protein with an apparent molecular mass of 95 kDa in HeLa cells, rather than one of 49 kDa. Immunoprecipitates of p95 phosphorylated p34$^{cdc2}$ on Tyr-15, indicating that p95 is functionally related to p49WEE1Hu, and mapping studies demonstrated that p95 is structurally related to p49WEE1Hu. In addition, the substrate specificity of p95 was more similar to that of fission yeast p107$^{wee1}$ than to that of human p49WEE1. Finally, the kinase activity of p95 toward p34$^{cdc2}$/cyclin B was severely impaired during mitosis. Taken together, these results indicate that the original *WEE1Hu* clone isolated in genetic screens encodes only the catalytic domain of human WEE1 and that the authentic human WEE1 protein has an apparent molecular mass of ≈95 kDa.**

In *Schizosaccharomyces pombe*, *wee1*$^+$ functions as a dose-dependent inhibitor of mitosis (1). Biochemical studies demonstrated that *wee1*$^+$ encodes a protein kinase of 107 kDa, which autophosphorylates on serine, threonine, and tyrosine residues (2–4). The physiologic target of p107$^{wee1}$ is p34$^{cdc2}$, a serine/threonine-specific protein kinase whose activity is required for the entry of cells into mitosis. p107$^{wee1}$ phosphorylates p34$^{cdc2}$ on Tyr-15, a modification that is sufficient for the inactivation of the p34$^{cdc2}$/cyclin B complex (4). Tyr-15 phosphorylation is also catalyzed by Mik1, a tyrosine kinase that functionally compensates for loss of *wee1*$^+$ in fission yeast (5, 6). Acting in opposition to *wee1*$^+$ and *mik1*$^+$ is the mitotic inducer *cdc25*$^+$. *cdc25*$^+$ encodes a protein phosphatase that dephosphorylates Tyr-15 of p34$^{cdc2}$, thereby activating the p34$^{cdc2}$/cyclin B complex (7–12). Functional homologs of *wee1*$^+$ and *cdc25*$^+$ have been isolated from higher eukaryotic cells.

A human homolog of *wee1*$^+$ (*WEE1Hu*) was isolated on the basis of its ability to functionally compensate for loss of *wee1*$^+$ function in fission yeast (13). *WEE1Hu* encodes a tyrosine kinase of 49 kDa, denoted p49WEE1Hu (14–16). Structurally, p49WEE1Hu consists primarily of a catalytic domain, lacking the large amino-terminal regulatory domain present in its fission yeast counterpart. The kinase domain of p49WEE1Hu is 29% identical to that of the *S. pombe* protein p107$^{wee1}$. Like p107$^{wee1}$, recombinant p49WEE1Hu phosphorylates p34$^{cdc2}$ on Tyr-15 and inactivates the p34$^{cdc2}$/cyclin B complex (15, 16). However, there are notable differences between the human and yeast enzymes. Whereas p107$^{wee1}$ shows strict substrate specificity for the cyclin-bound form of p34$^{cdc2}$ (4), p49WEE1Hu will also phosphorylate monomeric p34$^{cdc2}$ and a peptide containing Tyr-15.

All of the above-mentioned studies were carried out with recombinant p49WEE1Hu. To both characterize and study the regulation of the human WEE1 kinase *in vivo*, we generated two polyclonal antibodies to recombinant p49WEE1Hu. In addition, a peptide antibody generated against amino acids 361–388 of p49WEE1 was used. These antibodies specifically recognized a protein of 95 kDa in HeLa cells rather than one of 49 kDa. p95 was found to be structurally and functionally related to p49WEE1.

## MATERIALS AND METHODS

Procedures relating to propagation of virus; culturing of insect cells; isolation of the glutathione *S*-transferase (GST) fusion proteins GST-p49WEE1Hu and GST-p107$^{wee1}$ and the Lys-33 → Arg mutant p34$^{cdc2}$ (K33R)/cyclin B complex; and analysis of phosphoamino acids have been described (3, 4, 15, 17).

**Mapping.** $^{32}$P-labeled WEE1Hu immunoprecipitates derived from both HeLa cells and insect cells infected with recombinant baculoviruses were transferred to nitrocellulose in transfer buffer supplemented with 0.1% SDS. Nitrocellulose membranes containing the proteins of interest were incubated for 30 min at 37°C in 0.5 ml of 0.5% polyvinylpyrrolidone-40 in 100 mM acetic acid and then washed six times in water. Chymotrypsin digestions were performed as described previously for trypsin (18). Phosphopeptides were separated in the first dimension by thin-layer electrophoresis at pH 1.9, buffered with water/acetic acid/formic acid in a volume ratio of 800/150/50. Phosphopeptides were separated in the second dimension by ascending chromatography, with 1-butanol/pyridine/acetic acid/water in a volume ratio of 75/50/15/60 used as a solvent. V8 protease mapping of [$^{35}$S]methionine-labeled proteins was performed as described previously (18).

**Culturing and Labeling of HeLa Cells.** HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) (attached cultures) or RPMI 1640 medium (suspension cultures) supplemented with 10% calf serum and 1 mM glutamine. Cells were grown at 37°C in the presence of 5% $CO_2$/95% air. For the [$^{35}$S]methionine labeling experiments, four 100-mm tissue culture dishes of HeLa cells at 70–80% confluency were labeled for 3 hr in the presence of [$^{35}$S]me-

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Abbreviation: GST, glutathione *S*-transferase.

§To whom reprint requests should be addressed.

thionine at 0.2 mCi/ml (1 mCi = 37 MBq). Cells were lysed in HeLa cell lysis buffer as described below and lysates were pooled. Pooled lysates (≈7 mg of protein) were divided in half. One sample was incubated with preimmune TF5 serum and the other with immune TF5 serum as described below.

**Antibodies.** *Anti-p49WEE1Hu serum.* GST-p49WEE1Hu was produced in bacteria as described (15). Bacteria were lysed by sonication in 20 mM Tris·HCl, pH 8.0/100 mM NaCl/1 mM EDTA/0.5% Nonidet P-40 (NETN buffer). The lysate was centrifuged for 15 min at 10,000 × *g*, the insoluble pellet was boiled in SDS/sample buffer, and proteins were resolved by SDS/PAGE on a 10% polyacrylamide gel. The region of the gel containing GST-p49WEE1Hu was excised and was used to immunize two New Zealand White rabbits (TF5 and TF6).

*Peptide antibody.* The peptide antibody generated against amino acids 361–388 of p49WEE1Hu was a kind gift of Steven Pelech (Kinetek Biotechnology, St. Louis). This antibody binds in immunoblots but does not immunoprecipitate WEE1Hu.

**Binding of Antibody to Sepharose CL-4B-Protein A.** Sepharose CL-4B coupled with staphylococcal protein A and TF5 serum (either immune or preimmune) were incubated for 2 hr at 4°C in 1 ml of HeLa cell lysis buffer [50 mM Tris·HCl, pH 7.4/5 mM EDTA/250 mM NaCl/0.1% Brij 35/5 mM NaF/1 mM sodium pyrophosphate ($NaPP_i$)] at a ratio of 8 μl of packed Sepharose per μl of serum. The antibody/bead mixture was washed twice in HeLa lysis buffer, twice in LiCl buffer (0.5 M LiCl/50 mM Tris·HCl, pH 8.0), and twice more in HeLa lysis buffer. The antibody/bead mixture was resuspended in HeLa lysis buffer (at a 1:1 ratio of buffer to packed beads).

**Immunoblotting of p95 and GST-p49WEE1Hu.** Uninfected insect cells, insect cells infected with recombinant baculovirus encoding GST-p49WEE1Hu, and HeLa cells were lysed in HeLa lysis buffer supplemented with 1 μM microcystin, 2 mM phenylmethanesulfonyl fluoride, aprotinin at 0.15 unit/ml, 20 μM leupeptin, 20 μM pepstatin, and 1 mM sodium orthovanadate. Lysates were centrifuged at 10,000 × *g* for 10 min at 4°C. Samples containing 6 mg of total cell protein from HeLa cell lysates and uninfected insect cell lysates and 2.5 mg from infected insect cell lysates were cleared by incubation with 25 μl of packed Sepharose CL-4B-protein A beads for 30 min at 4°C. Cleared lysates were then incubated with 100 μl of antibody/bead mixture (prepared as described above) for 2.5 hr at 4°C. Immunoprecipitates were centrifuged and washed twice in HeLa lysis buffer, twice in LiCl buffer, twice again in HeLa lysis buffer, and once in phosphate-buffered saline (PBS; 137 mM NaCl/2.7 mM KCl/4.3 mM $Na_2HPO_4$/1.4 mM $KH_2PO_4$, pH 7.3). Reaction products were resolved by SDS/PAGE on a 7% polyacrylamide gel. Proteins were transferred to nitrocellulose and immunoblotted with affinity-purified antibodies to p49WEE1Hu (raised against either recombinant p49WEE1Hu or a peptide containing amino acids 361-388 of p49WEE1Hu). Proteins were visualized by enhanced chemiluminescence (ECL; Amersham).

**Autophosphorylation Reactions of p95 and p49WEE1Hu.** HeLa cells and insect cells expressing p49WEE1Hu were washed twice in PBS and were then lysed in HeLa lysis buffer supplemented as described above at protein concentrations of 4 mg/ml (HeLa cells) and 1 mg/ml (insect cells). Lysates were clarified by centrifugation at 4°C for 10 min at 10,000 × *g*. One milliliter of each lysate was cleared by incubation with Sepharose CL-4B-protein A beads (10 μl of packed Sepharose CL-4B-protein A beads per mg of protein in lysate) for 30 min at 4°C. Cleared lysates were incubated at 4°C for 2 hr with TF5 serum bound to Sepharose CL-4B-protein A beads (100 μl of antibody/bead mixture per 5–6 mg of HeLa cell protein and per 2–5 mg of insect cell protein). Immunoprecipitates were washed twice in HeLa lysis buffer, twice in LiCl buffer, and twice in incomplete kinase buffer (50 mM Tris·HCl, pH 8.0/10 mM $MgCl_2$/2 mM dithiothreitol). Complete kinase buffer [50 mM Tris·HCl, pH 7.4/10 mM $MgCl_2$/2 mM dithiothreitol/10 μM ATP and ≈20 μCi of [$\gamma$-$^{32}$P]ATP (>4000 Ci/mmol) per reaction] was added and reaction mixtures were incubated at 30°C for 15–20 min. Reaction products were washed three times in LiCl buffer, once in HeLa lysis buffer, and once in PBS and then boiled in sample buffer. Reaction products were resolved by SDS/PAGE on an 8% polyacrylamide gel and phosphoproteins were visualized by autoradiography. Phosphorylated proteins were isolated and subjected to two-dimensional phosphopeptide mapping and to phosphoamino acid analysis.

**Phosphorylation of GST-Cyclin B/p34$^{cdc2}$(K33R).** HeLa cells and insect cells expressing GST-p49WEE1Hu were lysed in HeLa lysis buffer supplemented as described above. Lysates were clarified by centrifugation at 10,000 × *g* for 10 min at 4°C. One milliliter of HeLa cell lysate (4 mg of total cell protein) and 1 ml of insect cell lysate (2 mg of total cell protein) were cleared by incubation with 100 μl of a preimmune serum bead mixture (prepared as described above) for 1 hr at 4°C. Cleared lysates were incubated at 4°C for 4 hr with 100 μl of TF5 antibody/bead mixture. Immunoprecipitates were washed twice in LiCl buffer, twice in HeLa lysis buffer, and twice in incomplete kinase buffer. GST-cyclin B/p34$^{cdc2}$(K33R) isolated on glutathione agarose beads (4) was added. Kinase reactions were initiated by the addition of complete kinase buffer and reaction mixtures were incubated at 30°C for 15 min. Reaction products were washed three times in LiCl buffer and once in PBS. Proteins were resolved by SDS/PAGE on a 10% polyacrylamide gel and phosphoproteins were visualized by autoradiography.

**Purification of Monomeric p34$^{cdc2}$(K33R) and Cyclin B/p34$^{cdc2}$(K33R) Complex.** Approximately 5 × 10$^7$ insect cells infected with recombinant virus encoding p34$^{cdc2}$(K33R) were washed in PBS and then lysed in 4 ml of Tris lysis buffer (25 mM Tris·HCl, pH 7.5/1 mM EDTA, pH 8.0/0.01% Brij/1 mM dithiothreitol). Lysates were clarified by centrifugation at 10,000 × *g* for 12 min at 4°C. Clarified lysates were incubated with 1 ml of packed ATP-Sepharose (Sigma; 11-atom spacer) that had been washed five times in Tris lysis buffer at 4°C for 1 hr. A column was poured and washed with 4 bead volumes of Tris lysis buffer. The column was then washed in Tris lysis buffer containing 125 mM NaCl. p34$^{cdc2}$ was eluted in ≈500 μl of Tris lysis buffer containing 225 mM NaCl, was adjusted to 10% sucrose, and was stored at −80°C. The purification of cyclin B/p34$^{cdc2}$(K33R) complex is described in detail by Lee *et al.* (6).

**Substrate Specificity of p95 and p49WEE1Hu.** Immunoprecipitates of p95 from HeLa cells and of p49WEE1Hu from insect cells were prepared by using TF5 sera. Glutathione-agarose precipitates were prepared from bacteria overexpressing GST-p49WEE1Hu (15). Precipitates were washed twice in LiCl buffer, twice in HeLa lysis buffer, and twice in incomplete kinase buffer. Kinase reactions (50 μl total reaction volume) were performed in the presence of ≈2 μg of purified cyclin B/p34$^{cdc2}$(K33R) and ≈2 μg of purified monomeric p34$^{cdc2}$(K33R) or 40 μg of a peptide containing Tyr-15 (3). Kinase reaction products were resolved by SDS/PAGE on 12% polyacrylamide gels or in the case of peptides on 20% polyacrylamide gels. Phosphorylated peptides were visualized by exposing the wet gel to film. The 12% gels were stained and dried, and phosphorylated proteins were visualized by autoradiography.

**Synchronization of HeLa Cells.** Nocodazole-arrested cells were obtained by incubating cells in the presence of nocodazole (Sigma) at 0.1 μg/ml for 15 hr. Mitotic cells were then collected by mitotic shake-off. S-phase-arrested cells were obtained by incubating cells in the presence of hydroxyurea at 0.5 mg/ml for 15 hr. Cells were lysed in HeLa lysis buffer supplemented with 1 μM microcystin, 2 mM phenylmethanesulfonyl fluoride, aprotinin at 0.15 unit/ml, 20 μM leupeptin,

20 μM pepstatin, and 1 mM sodium orthovanadate. Lysates were centrifuged at 10,000 × *g* for 10 min at 4°C. Samples containing 1.5 mg of total cell protein were cleared by incubation with 3 μl of preimmune TF5 serum and 25 μl of packed Sepharose CL-4B-protein A beads for 1 hr at 4°C. Cleared lysates were then incubated with 3 μl of TF5 serum for 4 hr at 4°C, 25 μl of packed Sepharose CL-4B-protein A beads was added, and reaction mixtures were incubated for a further 2 hr. Immunoprecipitates were centrifuged and washed twice in RIPA/Tris buffer (3), twice in LiCl buffer, and twice more in HeLa lysis buffer. One set of reaction products was then resolved by SDS/PAGE on a 7% polyacrylamide gel. The second set of reaction products was washed twice in incomplete kinase buffer and then kinase reactions were performed in the presence of purified $p34^{cdc2}$/cyclin B for 15 min at 30°C. Kinase reaction products were resolved by SDS/PAGE on a 10% polyacrylamide gel.

## RESULTS

Three polyclonal antibodies were used to characterize WEE1Hu in HeLa cells. Two of the antibodies (TF5 and TF6) were generated against bacterially produced GST-p49WEE1Hu (15) and the third was generated against a peptide consisting of amino acids 361–388 within subdomain 10 of the catalytic region of p49WEE1Hu. To characterize the antibodies, lysates prepared from insect cells overproducing GST-p49WEE1Hu were immunoprecipitated with preimmune serum (Fig. 1*A*, lane 6) or with TF5 immune serum (Fig. 1*A*, lane 5). Proteins were resolved by SDS/PAGE and then immunoblotted with the peptide-specific antiserum. As expected, a GST fusion protein of ≈79 kDa was specifically precipitated with the immune serum. Unexpectantly, when the same experiment was performed with HeLa cell lysates, a protein of 95 kDa rather than one of 49 kDa was detected (Fig. 1*A*, lane 3). Identical results were obtained with TF6 serum (data not shown). As a second approach to monitor for the presence of WEE1 in HeLa cells, labeling experiments were performed. Lysates prepared from $^{35}S$-labeled HeLa cells were immunoprecipitated with preimmune (Fig. 1*B*, lane 1) or immune (Fig. 1*B*, lane 2) TF5 serum. A predominant protein of 95 kDa rather than one of 49 kDa was observed when immune serum was used.

To investigate the relationship between p49WEE1Hu and the 95-kDa protein detected in HeLa cell lysates, immune complex kinase assays were performed *in vitro* (Fig. 2*A*). Lysates prepared from insect cells overproducing p49WEE1Hu were immunoprecipitated with TF5 serum (lane 2) and immune complex kinase assays were performed. As expected, a 49-kDa phosphoprotein was detected. In contrast, when the same experiment was performed with HeLa cell lysates, a protein of 95 kDa was detected (lane 1). Two additional minor phosphoproteins of 110 kDa and 120 kDa were also observed. The 120-kDa protein binds nonspecifically to Sepharose CL-4B-protein A and its appearance is sporadic. The identity of the 110-kDa protein (also seen in Figs. 1*B* and 3*A*) is unknown.

Mapping studies were performed to determine whether p49WEE1Hu and p95 were related. Phosphorylated p49WEE1Hu and p95 were digested with chymotrypsin and the phosphopeptides were resolved in two dimensions. As seen in Fig. 2*B*, p95 and p49WEE1Hu share three major phosphopeptides, indicating that p95 is structurally related to p49WEE1Hu. Results from one-dimensional V8 protease mapping on $^{35}S$-labeled proteins also indicated that p95 and p49WEE1Hu are structurally related. As seen in Fig. 2*C*, at the highest concentrations of V8 protease two characteristic sets of doublets are observed for both p49WEE1Hu and p95.

Kinase reactions were performed *in vitro* to determine whether p95 was capable of phosphorylating $p34^{cdc2}$ in complex with cyclin B. As seen in Fig. 3*A*, immunoprecipitates of p95 readily phosphorylated $p34^{cdc2}$ (lane 1), as did immunoprecipitates of GST-p49WEE1Hu (lane 3). Phosphoamino acid analysis of $p34^{cdc2}$ revealed phosphotyrosine (data not shown), and two-dimensional tryptic phosphopeptide mapping studies demonstrated that Tyr-15 was the site of phosphorylation (Fig. 3*B*). The substrate specificities of p49WEE1 and p95 toward monomeric $p34^{cdc2}$, a peptide containing Tyr-15 as well as the cyclin-bound form of $p34^{cdc2}$, were also compared. As seen in Fig. 4, p95 showed a strong preference for the cyclin-bound form of $p34^{cdc2}$. This is in contrast to p49WEE1, which phosphorylated monomeric $p34^{cdc2}$, a peptide containing Tyr-15 as well as the cyclin-bound form of $p34^{cdc2}$.

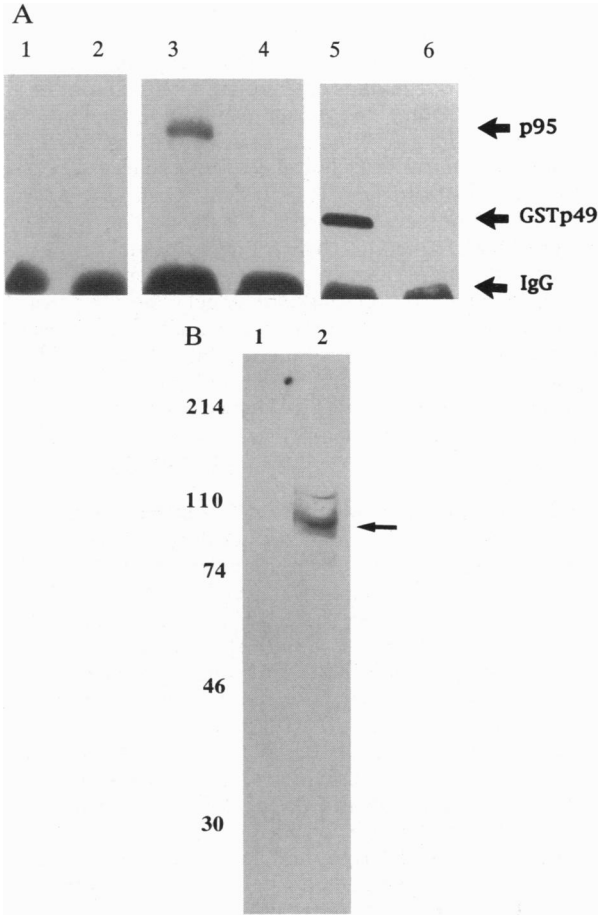


FIG. 1. Immunoblotting and labeling experiments indicate that the human WEE1 protein is ≈95 kDa in HeLa cells. (*A*) Lysates were prepared from uninfected insect cells (lanes 1 and 2), from HeLa cells (lanes 3 and 4), or from insect cells infected with recombinant baculovirus encoding p49WEE1Hu fused to GST (lanes 5 and 6). Lysates were incubated with preimmune serum (lanes 2, 4, and 6) or with antibody specific for WEE1Hu (lanes 1, 3, and 5). Immunoprecipitates were resolved by SDS/PAGE. Proteins were transferred to nitrocellulose and immunoblotted with antibody specific for WEE1Hu. Proteins were visualized by chemiluminescence (ECL). (*B*) HeLa cells were incubated with [$^{35}S$]methionine. Lysates were prepared and incubated with either preimmune (lane 1) or immune (lane 2) TF5 serum. Immunoprecipitates were resolved by SDS/PAGE on a 10% polyacrylamide gel. Labeled proteins were visualized by autoradiography. The arrow denotes p95. The numbers to the left indicate the migration of molecular mass standards (kDa).

We have previously reported that in HeLa cells and in extracts prepared from *Xenopus* eggs, the activity of the Tyr-15 kinase is inhibited coincident with entry into mitosis (19). These assays were performed using crude extracts that had been prepared from synchronized *Xenopus* egg extracts or HeLa cells. To examine the activity of WEE1Hu in a more

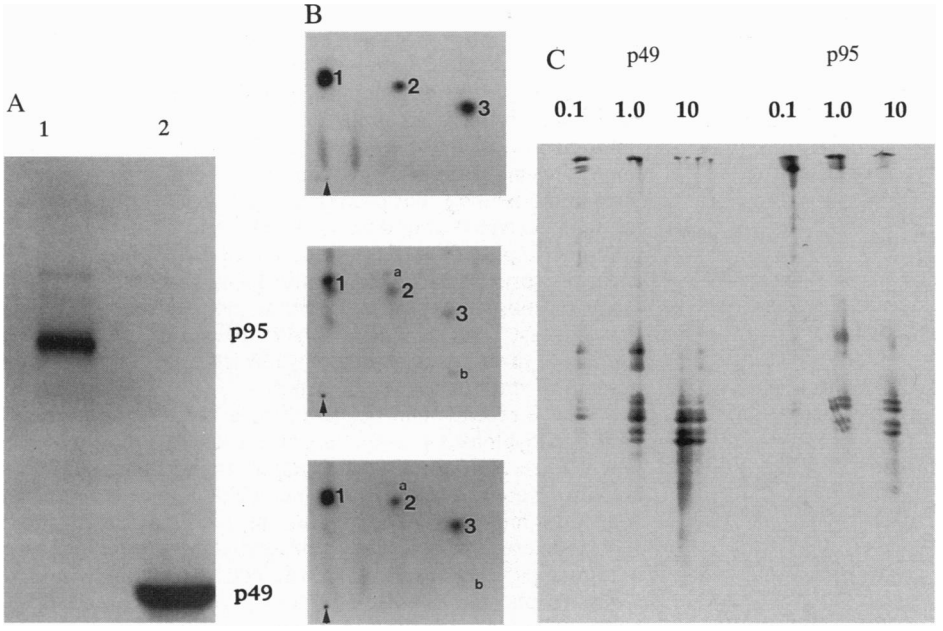


FIG. 2. Immune complex kinase assays indicate that the human WEE1 protein is ≈95 kDa in HeLa cells. (*A*) Lysates prepared from HeLa cells (lane 1) or from insect cells expressing p49WEE1Hu (lane 2) were immunoprecipitated by using an antibody raised against bacterially produced p49WEE1Hu. Immune complex kinase assays were performed *in vitro*. Immunoprecipitates were resolved by SDS/PAGE and phosphoproteins were visualized by autoradiography. (*B*) $^{32}$P-labeled p49WEE1Hu and p95 were subjected to two-dimensional chymotryptic phosphopeptide mapping. (*Top*) p49WEE1Hu. (*Middle*) p95. (*Bottom*) Mix of p49WEE1Hu and p95 phosphopeptides. Arrows denote the origins. (*C*) Gel slices containing $^{35}$S-labeled p49WEE1Hu and p95 were incubated with 0.1, 1.0, or 10 μg of V8 protease. Peptides were resolved in one dimension by SDS/PAGE on a 20% polyacrylamide gel.

pure system, lysates were prepared from asynchronously growing HeLa cells (Fig. 5, lanes 1) or from HeLa cells arrested either in S phase with hydroxyurea (Fig. 5, lanes 2) or in M phase with nocodazole (Fig. 5, lanes 3). Lysates were immunoprecipitated with WEE1-specific antibody and were examined both for WEE1 levels by immunoblotting (*A*) and for their ability to phosphorylate the cyclin-bound form of p34$^{cdc2}$ (*B*). As seen in Fig. 5, immunoprecipitates of WEE1Hu from mitotic cell lysates were severely impaired in their ability to phosphorylate p34$^{cdc2}$. This finding is consistent with our previous results with whole cell lysates (19).

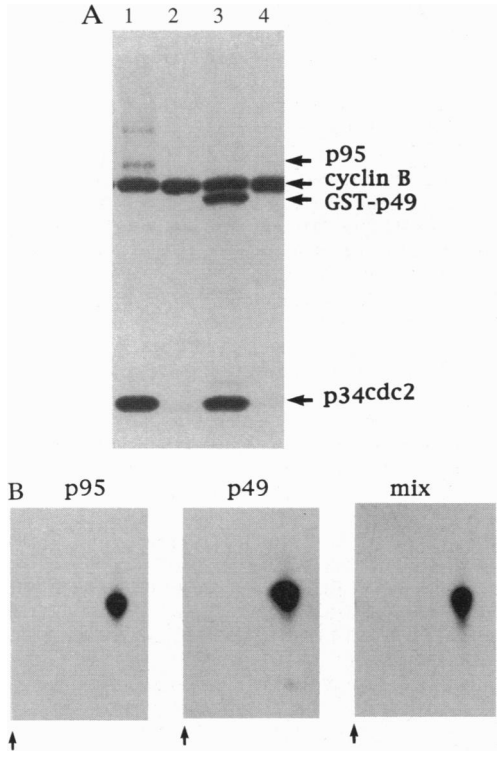


FIG. 3. Immunoprecipitates of HeLa cell p95 phosphorylate p34$^{cdc2}$ on Tyr-15. (*A*) Lysates prepared from HeLa cells (lanes 1 and 2) or from insect cells infected with p49WEE1Hu fused to GST (lanes 3 and 4) were incubated with Sepharose CL-4B-protein A beads for clearing. The cleared supernatants were incubated with antibody specific for WEE1Hu (lanes 1 and 3) or with preimmune serum (lanes 2 and 4). p34$^{cdc2}$(K33R)/GST-cyclin B complexes purified on glutathione beads were added and kinase assays were performed *in vitro*. Reaction products were resolved by SDS/PAGE and $^{32}$P-labeled proteins were visualized by autoradiography. (*B*) Two-dimensional phosphopeptide mapping of p34$^{cdc2}$ phosphorylated *in vitro* by GST-p49WEE1Hu (*Center*), by p95 (*Left*), or a mixture of phosphopeptides shown in *Left* and *Center* (*Right*). Arrows denote the origins.

## DISCUSSION

To date, six eukaryotic kinases that phosphorylate p34$^{cdc2}$ on Tyr-15 have been reported. These include Wee1 (107 kDa) and Mik1 (68 kDa) from fission yeast, WEE1Hu (49 kDa) from humans, Swe1 (95 kDa) from budding yeast, and Wee1Xe from *Xenopus* (3, 4, 6, 20, 21). Using antibodies raised against either recombinant human p49WEE1 or a peptide derived from p49WEE1Hu, we have detected a protein of ≈95 kDa in

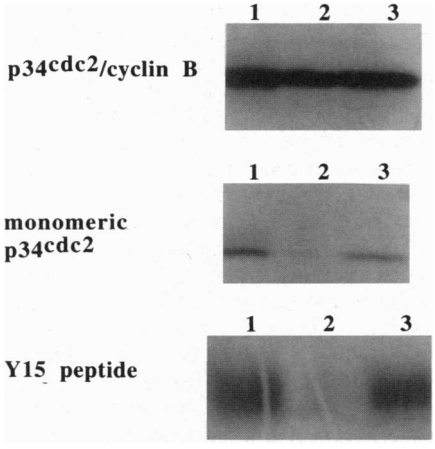


FIG. 4. Substrate specificity of p95 and p49WEE1Hu. Lysates were prepared from bacteria overproducing GST-p49WEE1Hu (lanes 1), from HeLa cells (lanes 2), and from insect cells overproducing p49WEE1Hu (lanes 3). p95 and p49WEE1Hu were immunoprecipitated by using an antibody raised against bacterially produced p49WEE1Hu. Bacterially produced GST-p49WEE1Hu was isolated on glutathione-agarose beads. Kinase assays were performed *in vitro* in the presence of p34$^{cdc2}$(K33R)/cyclin B (*Top*), monomeric p34$^{cdc2}$ (*Middle*), or a peptide containing Tyr-15 (*Bottom*).

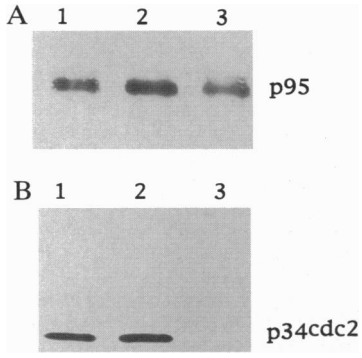


FIG. 5. Kinase activity of p95 is inhibited during mitosis. p95 was immunoprecipitated from lysates prepared from asynchronously growing HeLa cells (lanes 1); HeLa cells arrested in S phase with hydroxyurea (lanes 2); or HeLa cells arrested in M phase with nocodazole (lanes 3). One half of each immunoprecipitate was resolved by SDS/PAGE, transferred to nitrocellulose, and immunoblotted with antibody specific for WEE1Hu (*A*). The second half of each immunoprecipitate was tested for its ability to phosphorylate purified $p34^{cdc2}$(K33R)/cyclin B complexes *in vitro* (*B*).

HeLa cells. Mapping studies indicated that p95 is structurally related to p49WEE1Hu, and immunoprecipitates of p95 phosphorylated $p34^{cdc2}$ on Tyr-15. These results raise the question of whether HeLa cells contain a distinct but immunologically related member of the Wee1/Mik1 family of tyrosine kinases or whether p49 represents only the catalytic domain of a larger human WEE1 protein.

Interestingly, the original cDNA reported for WEE1Hu does not contain a termination codon in the 5′ noncoding region, leaving open the possibility that the original human WEE1 clone isolated in genetic screens is an incomplete cDNA (13). This appears to be the case, as a longer cDNA-encoded WEE1Hu recently appeared in the GenBank/EMBL data base (accession no. X62048). This cDNA contains a termination codon upstream of the initiating methionine and is predicted to encode a protein of ≈71 kDa (216 amino acids longer at its amino terminus than p49WEE1Hu). The sequence of the full-length clone contains a stretch of 10 glutamic acid residues that is also found in budding yeast Swe1 but which is absent from fission yeast Wee1 and Mik1. This leaves open the question of whether p95 is the protein product of this longer clone. p95 may represent a posttranslationally modified form of the 71-kDa protein. Alternatively, the stretch of glutamic acid residues may cause p71 to migrate with an apparent molecular mass of 95 kDa in SDS gels. Finally, p95 may represent a distinct Wee1/Mik1 family member present in HeLa cells. Interestingly, p71 expressed either transiently in HeLa cells or in insect cells after infection with recombinant baculoviruses migrates in SDS/polyacrylamide gels with an apparent molecular mass of 95 kDa, lending support to one of the first two models proposed above (M.J.B. and H.P.-W., unpublished observation). The question arises as to how many Wee1/Mik1-like kinases exist in higher eukaryotic cells. It is clear from studies conducted in *Xenopus* that at least two distinct Tyr-15 kinases exist. One kinase (represented by Wee1Xe) phosphorylates $p34^{cdc2}$ exclusively on Tyr-15, and a second kinase appears to be a membrane-associated dual-specificity protein kinase that phosphorylates $p34^{cdc2}$ on both Thr-14 and Tyr-15 (19, 22).

We have previously reported that a recombinant protein consisting only of the carboxyl-terminal catalytic domain of $p107^{wee1}$ (lacking the entire amino terminus) does not recognize its physiologic target ($p34^{cdc2}$/cyclin B) as a substrate *in vitro* but will phosphorylate enolase (4). In contrast, full-length $p107^{wee1}$ does not recognize enolase as a substrate *in vitro* but quite readily phosphorylates the cyclin-bound form of $p34^{cdc2}$. Furthermore, full-length $p107^{wee1}$ does not phosphorylate monomeric $p34^{cdc2}$ or a peptide containing Tyr-15 (4). These data suggest that the amino terminus of $p107^{wee1}$ may be involved in conferring substrate specificity to the kinase domain. This notion is reinforced in the present study, where we have demonstrated that p49WEE1, in addition to phosphorylating the cyclin B-bound form of $p34^{cdc2}$, also phosphorylates monomeric $p34^{cdc2}$ and a peptide containing Tyr-15. In contrast, p95 prefers the cyclin-bound form of $p34^{cdc2}$. Thus, in terms of substrate specificity, p95 is more closely related to fission yeast $p107^{wee1}$ than is p49WEE1 and lends further support to the conclusion that p49 consists only of the catalytic domain of the human WEE1 kinase.

We have recently characterized the activities of the Thr-14 and Tyr-15 kinases present in crude extracts prepared from synchronized HeLa cells and *Xenopus* egg extracts (19). Both activities were shown to diminish coincident with the entry of cells into mitosis. These results are consistent with those reported here, where we demonstrate that immunoprecipitates of human p95 prepared from mitotic cells are impaired in their ability to phosphorylate $p34^{cdc2}$ compared with immunoprecipitates prepared from either unsynchronized cells or cells arrested in S phase with hydroxyurea (Fig. 5).

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1. Russell, P. & Nurse, P. (1987) *Cell* **49,** 559–567.
2. Featherstone, C. & Russell, P. (1991) *Nature (London)* **349,** 808–811.
3. Parker, L. L., Atherton-Fessler, S., Lee, M. S., Ogg, S., Falk, F. L., Swenson, K. I. & Piwnica-Worms, H. (1991) *EMBO J.* **10,** 1255–1263.
4. Parker, L. L., Atherton-Fessler, S. & Piwnica-Worms, H. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 2917–2921.
5. Lundgren, K., Walworth, N., Booher, R., Dembski, M., Kirschner, M. & Beach, D. (1991) *Cell* **64,** 1111–1122.
6. Lee, M. S., Enoch, T. & Piwnica-Worms, H. (1994) *J. Biol. Chem.* **269,** 30530–30537.
7. Galaktionov, K. & Beach, D. (1991) *Cell* **67,** 1181–1194.
8. Gautier, J., Solomon, M. J., Booher, R. N., Bazan, J. F. & Kirschner, M. W. (1991) *Cell* **67,** 197–211.
9. Kumagai, A. & Dunphy, W. G. (1991) *Cell* **64,** 903–914.
10. Millar, J. B. A., McGowan, C. H., Lenaers, G., Jones, R. & Russell, P. (1991) *EMBO J.* **10,** 4301–4309.
11. Strausfeld, U., Labbe, J. C., Fesquet, D., Cavadore, J. C., Picard, A., Sadhu, K., Russell, P. & Doree, M. (1991) *Nature (London)* **351,** 242–245.
12. Lee, M. S., Ogg, S., Xu, M., Parker, L. L., Donoghue, D. J., Maller, J. L. & Piwnica-Worms, H. (1992) *Mol. Biol. Cell* **3,** 73–84.
13. Igarashi, M., Nagata, A., Jinno, S., Suto, K. & Okayama, H. (1991) *Nature (London)* **353,** 80–83.
14. Honda, R., Ohba, Y. & Yasuda, H. (1992) *Biochem. Biophys. Res. Commun.* **186,** 1333–1338.
15. Parker, L. L. & Piwnica-Worms, H. (1992) *Science* **257,** 1955–1957.
16. McGowan, C. H. & Russell, P. (1993) *EMBO J.* **12,** 75–85.
17. Atherton-Fessler, S., Parker, L. L., Geahlen, R. L. & Piwnica-Worms, H. (1993) *Mol. Cell. Biol.* **13,** 1675–1685.
18. Xu, M., Sheppard, K.-A., Peng, C.-Y., Yee, A. S. & Piwnica-Worms, H. (1994) *Mol. Cell. Biol.* **14,** 8420–8431.
19. Atherton-Fessler, S., Liu, F., Gabrielli, B., Lee, M. S., Peng, C.-Y. & Piwnica-Worms, H. (1994) *Mol. Biol. Cell* **5,** 989–1001.
20. Booher, R. N., Deshaies, R. J. & Kirschner, M. W. (1993) *EMBO J.* **12,** 3417–3426.
21. Mueller, P. R., Coleman, T. R. & Dunphy, W. G. (1995) *Mol. Biol. Cell* **6,** 119–134.
22. Kornbluth, S., Sebastian, B., Hunter, T. & Newport, J. (1994) *Mol. Biol. Cell* **5,** 273–282.