Cyclin promotes the tyrosine phosphorylation of p34^{cdc2} in a *wee1*⁺ dependent manner

Laura L.Parker, Sue Atherton-Fessler,
Margaret S.Lee, Scott Ogg, Jennifer L.Falk,
Katherine I.Swenson¹ and
Helen Piwnicka-Worms

Department of Physiology, Tufts University School of Medicine,
Boston, MA 02111 USA, and ¹Department of Anatomy and Cellular
Biology, Harvard Medical School, Boston, MA 02115, USA

Communicated by A.Smith

The regulation of p34^{cdc2} was investigated by over-producing p34^{cdc2}, cyclin (A and B) and the *wee1*⁺ gene product (p107^{wee1}) using a baculoviral expression system. p34^{cdc2} formed a functional complex with both cyclins as judged by co-precipitation, phosphorylation of cyclin *in vitro*, and activation of p34^{cdc2} histone H1 kinase activity. Co-production of p34^{cdc2} and p107^{wee1} in insect cells resulted in a minor population of p34^{cdc2} that was phosphorylated on tyrosine and displayed an altered electrophoretic mobility. When p34^{cdc2} and p107^{wee1} were co-produced with cyclin (A or B) in insect cells, there was a dramatic increase in the population of p34^{cdc2} that was phosphorylated on tyrosine and that displayed a shift in electrophoretic mobility. The phosphorylation of p34^{cdc2} on tyrosine was absolutely dependent upon the presence of kinase-active p107^{wee1}. Tyrosine-specific as well as serine/threonine-specific protein kinase activities co-immunoprecipitated with p107^{wee1}. These results suggest that cyclin functions to facilitate tyrosine phosphorylation of p34^{cdc2} and that p107^{wee1} functions to regulate p34^{cdc2}, either directly or indirectly, by tyrosine phosphorylation.

Key words: baculovirus/cdc2/cell cycle/cyclin/*wee1*⁺

Introduction

The control of the eukaryotic cell cycle at the G₂/M transition centers on the kinase activity of a 34 kd serine/threonine protein kinase (p34^{cdc2}), the product of the *cdc2*⁺ gene of *Schizosaccharomyces pombe* (Nurse and Bissett, 1981; Simanis and Nurse, 1986). In yeast and possibly higher eukaryotes, p34^{cdc2} also functions at the G₁/S transition (Reed, 1980; Nurse and Bissett, 1981; Blow and Nurse, 1990; Furukawa *et al.*, 1990). The regulation of p34^{cdc2} has been shown to be quite complex, involving subunit rearrangement as well as molecular modification. p34^{cdc2} physically associates with a class of proteins called the cyclins (Draetta *et al.*, 1989). The abundance of the cyclins oscillates as a function of the cell cycle (Evans *et al.*, 1983). The cyclins fall into two classes designated A and B which can be distinguished by sequence as well as by kinetics of accumulation and degradation throughout the cell cycle (Swenson *et al.*, 1986; Pines and Hunter, 1989, 1990;

Westendorf *et al.*, 1989). Association with the cyclins is a necessary step in the activation of p34^{cdc2} and for entry of cells into mitosis (Minshull *et al.*, 1989; Murray and Kirschner, 1989).

Several mitotic control genes have been identified in *S.pombe* that are thought to regulate p34^{cdc2} function by altering its state of phosphorylation. One of these genes, *wee1*⁺, encodes a negative regulator of mitosis (Russell and Nurse, 1987). Based on sequence comparisons with known protein kinases, *wee1*⁺ is predicted to encode a serine/threonine specific protein kinase and may exert its inhibitory effects by regulating the phosphorylation of p34^{cdc2} (Russell and Nurse, 1987; Hanks *et al.*, 1988).

Activation of p34^{cdc2} in mitotic metaphase is correlated with its dephosphorylation on tyrosine (Dunphy and Newport, 1989; Gautier *et al.*, 1989; Gould and Nurse, 1989; Morla *et al.*, 1989). p34^{cdc2} has been shown to be phosphorylated on tyrosine 15 in fission yeast (Gould and Nurse, 1989). When phenylalanine is substituted for tyrosine at position 15, cells advance into mitosis prematurely indicating that the dephosphorylation of tyrosine 15 may be a key step in the activation of p34^{cdc2} function in *S.pombe* (Gould and Nurse, 1989).

Despite the tremendous advances recently made in our understanding of p34^{cdc2} regulation, the temporal sequence of events (phosphorylation/dephosphorylation; cyclin association/cyclin dissociation) that leads to p34^{cdc2} activation remains unresolved. To investigate the regulation of p34^{cdc2}, we have overproduced human p34^{cdc2} as well as several of its regulators (cyclins A and B and the *wee1*⁺ gene product from *S.pombe*) using a baculoviral expression system and have studied the interactions of these proteins in insect cells. One amazing feature of the cell cycle regulatory proteins is their functional conservation across a billion years of evolution. This allows functional studies of these regulatory proteins to be conducted in heterologous systems (Beach *et al.*, 1982; Lee and Nurse, 1987; Murray and Kirschner, 1989; Murray *et al.*, 1989; Russell *et al.*, 1989). Our results suggest that the p34^{cdc2}–cyclin complex rather than monomeric p34^{cdc2} is the preferred substrate for tyrosine phosphorylation and that the tyrosine phosphorylation of p34^{cdc2} in insect cells is absolutely dependent upon the presence of kinase-active p107^{wee1}.

Results

p34^{cdc2} is phosphorylated on tyrosine when co-expressed with p107^{wee1} in insect cells

Human p34^{cdc2} produced in insect cells migrated as a single species with an apparent molecular weight of 34 kd (Figure 1A, lanes 1–4). Interestingly, when p34^{cdc2} and *S.pombe* p107^{wee1} were co-produced in insect cells an additional slower electrophoretic form of p34^{cdc2} was detected (Figure 1A, lanes 5–8). Densitometric scanning of several immunoblots similar to that seen in Figure 1A,

indicated that this species constituted ~10% of the total p34^{cdc2}.

This result suggested that p107^{wee1} might alter the phosphorylation state of p34^{cdc2}. Phosphamino acid analysis of the faster migrating species of p34^{cdc2} showed mainly phosphothreonine (Figure 1B). Surprisingly, the slower migrating species was phosphorylated primarily on tyrosine (Figure 1C). The low levels of phosphoserine detected in each case may not be derived from p34^{cdc2} because we detected phosphoserine when control areas of the gel were excised and subjected to phosphoamino acid analysis. In addition, we never detected phosphoserine when individual phosphotryptic peptides were examined (see Figure 3).

Cyclin promotes the tyrosine phosphorylation of p34^{cdc2} in the presence of p107^{wee1}

To investigate the contribution made by cyclin to p34^{cdc2} phosphorylation, triple infections were carried out with recombinant viruses encoding p34^{cdc2}, p107^{wee1} and clam cyclin (either A or B). As shown in Figure 2A, immunoblots indicated that the slower electrophoretic form of p34^{cdc2} was dramatically increased in the presence of cyclin A (Figure 2A, lanes 4, 11 and 12) as well as cyclin B

Fig. 1. Phosphorylation of p34^{cdc2} on tyrosine when co-produced with p107^{wee1} in insect cells. Panel A. Sf9 cells were infected with recombinant virus encoding p34^{cdc2} alone (lanes 1–4) or p34^{cdc2} together with p107^{wee1} (lanes 5–8) or wild-type virus (lane 9). Lysates were prepared and various amounts of each lysate were resolved by electrophoresis through a 12% SDS–polyacrylamide gel. Proteins were transferred to nitrocellulose and the blot was probed with anti-p34^{cdc2} serum (Draetta *et al.*, 1987). Lane 1, 5 µg; lane 2, 7 µg; lane 3, 10 µg; lane 4, 20 µg; lane 5, 5 µg; lane 6, 10 µg; lane 7, 20 µg; lane 8, 30 µg; lane 9, 30 µg of total cell protein. Panels B and C. Sf9 cells co-infected with recombinant viruses encoding p34^{cdc2} and p107^{wee1} were labeled with ³²P_i. Lysates were prepared and precipitated with p13 conjugated Sepharose 6B. The two forms of p34^{cdc2} were excised from the gel and subjected to two dimensional phosphoamino acid analysis. Panel B, faster electrophoretic form; panel C, slower electrophoretic form. Panels B and C, 350 c.p.m., 7 day exposure.

(Figure 2A, lane 8). The appearance of this slower electrophoretic form of p34^{cdc2} was absolutely dependent upon the presence of kinase-active p107^{wee1} as no shift in mobility was observed when p34^{cdc2} was co-produced with cyclin A alone (Figure 2A, lane 3) or cyclin B alone (Figure 2A,

Fig. 2. Cyclin enhances the tyrosine phosphorylation of p34^{cdc2}. Panel A. Sf9 cells were infected with recombinant virus encoding either p34^{cdc2} (lane 1); p34^{cdc2}(Phe15) (lane 2); or were co-infected with recombinant virus encoding p34^{cdc2} and cyclin A (lane 3); p34^{cdc2}, cyclin A and p107^{wee1} (lane 4); p34^{cdc2}(Phe15) and cyclin A (lane 5); p34^{cdc2}(Phe15), cyclin A and p107^{wee1} (lane 6); p34^{cdc2} and cyclin B (lane 7); p34^{cdc2}, cyclin B and p107^{wee1} (lane 8); p34^{cdc2}(Phe15) and cyclin B (lane 9); p34^{cdc2}(Phe15), cyclin B and p107^{wee1} (lane 10); p34^{cdc2}, cyclin A and p107^{wee1} (lanes 11 and 12); p34^{cdc2}, cyclin A and kinase-negative p107^{wee1} (lanes 13 and 14). Lysates were prepared, resolved by SDS–PAGE and immunoblotted with p34^{cdc2} specific antibody (R1267). 10 µg (lanes 1 and 2) or 20 µg (lanes 3–14) of total cell protein were loaded per lane and were resolved on a 12% gel. Panel B. Triple infections with viruses encoding p34^{cdc2}, cyclin B and p107^{wee1} were carried out. Cell lysates were prepared, proteins were resolved by SDS–PAGE and then blotted onto nitrocellulose. Blots were probed with antibodies specific for p34^{cdc2} (R1267) (lane 1), or phosphotyrosine (lane 2). Panel C. Sf9 cells infected with recombinant viruses encoding cyclin A (lane 1); p107^{wee1} (lane 2); p34^{cdc2} (lane 3); p34^{cdc2}(Phe15) (lane 4); or co-infected with recombinant viruses encoding p34^{cdc2} and cyclin A (lane 5); p34^{cdc2}, cyclin A and p107^{wee1} (lane 6); p34^{cdc2}(Phe15) and cyclin A (lane 7); p34^{cdc2}(Phe15), cyclin A and p107^{wee1} (lane 8); p34^{cdc2}(Phe15), cyclin A and pp60^{v-src} (lane 9); p34^{cdc2}, cyclin A and pp60^{v-src} (lane 10); or p34^{cdc2} and p107^{wee1} (lane 11) were labeled *in vivo* with ³²P-labeled inorganic phosphate. Lysates were prepared and precipitated with p13 conjugated Sepharose 6B. The precipitates were resolved by electrophoresis through a 12% SDS–polyacrylamide gel. Some cyclin A non-specifically precipitates with the p13 beads in the absence of p34^{cdc2} (Figure 2C, lane 1) and p13 beads precipitate other phosphorylated species, which are especially evident in precipitates from cellular lysates containing both p34^{cdc2} and cyclin (Figure 2C, lanes 5–10). These may represent degradation products of cyclin or cellular proteins whose phosphorylation is enhanced by the expression of kinase-active complex (p34^{cdc2}–cyclin) in insect cells. The apparent faster electrophoretic mobility of p34^{cdc2} and the mutant protein in lanes 3 and 4 are likely due to an overloading of protein in these lanes. Panel D. Triple infections were carried out with viruses encoding p34^{cdc2}, cyclin A and p107^{wee1} and infected cells were labeled *in vivo* with ³²P-labeled inorganic phosphate. Cell lysates were prepared, immunoprecipitated with p13 beads and proteins were resolved by SDS–PAGE. The slower electrophoretic form of ³²P-labeled p34^{cdc2} (similar to that shown in panel C, lane 6) was excised from the gel and subjected to two-dimensional phosphoamino acid analysis.

lane 7) or when p34^{cdc2} was co-produced with cyclin A in the presence of a kinase-negative mutant of p107^{wee1} (Figure 2A, lanes 13 and 14). Densitometric scanning indicated that when p34^{cdc2} was co-produced with p107^{wee1} and cyclin A or cyclin B, the slower electrophoretic form of p34^{cdc2} constituted 50%–90% of the total p34^{cdc2}. This is in contrast to 10% in the absence of cyclin.

In vivo labeling with ³²P_i confirmed a prediction of panel A, that cyclin stimulated the phosphorylation of p34^{cdc2}. A low level of phosphorylation of p34^{cdc2} was observed when p34^{cdc2} was made alone (Figure 2C, lane 3) or when it was co-expressed with cyclin A (Figure 2C, lane 5). When p34^{cdc2} was co-produced with p107^{wee1}, two phosphorylated forms of p34^{cdc2}, differing in electrophoretic mobility were observed (Figure 2C, lane 11). The phosphorylation of p34^{cdc2} was greatly enhanced when p34^{cdc2} was co-produced with both cyclin A and p107^{wee1} (Figure 2C, lane 6). The same results were obtained with cyclin B (data not shown). The increase of phosphorylation was entirely in the slower electrophoretic form of p34^{cdc2}. Also evident in Figure 2C is the co-precipitation of ³²P-labeled cyclin A with p34^{cdc2} (Figure 2C, lanes 5–10). The ability of other tyrosine kinases to substitute for p107^{wee1} was tested by co-producing p34^{cdc2} and cyclin with pp60^{v-src} in insect cells. As shown in Figure 2C (lane 10) pp60^{v-src} was unable to substitute functionally for p107^{wee1} in co-infections with p34^{cdc2} and cyclin A.

Two lines of evidence indicate that phosphorylation was on tyrosine. Firstly, phosphoamino acid analysis demonstrated that the slower electrophoretic form of p34^{cdc2} contained primarily phosphotyrosine although low levels of phosphothreonine were also detected (Figure 2D). Secondly, although antibodies against p34^{cdc2} reacted with both

electrophoretic forms of p34^{cdc2} (Figure 2B, lane 1), antibodies specific for phosphotyrosine reacted only with the slower species (Figure 2B, lane 2).

Phosphorylation of p34^{cdc2} on tyrosine 15

Phosphorylation of p34^{cdc2} on Tyr15 in *S.pombe* is a key regulatory step controlling entry into mitosis (Gould and Nurse, 1989). If p34^{cdc2} was phosphorylated on Tyr15 when co-produced with p107^{wee1} in insect cells, substitution of phenylalanine for tyrosine at residue 15 of p34^{cdc2} should abolish its phosphorylation. To test this, we generated a recombinant virus encoding Phe for Tyr at residue 15. The electrophoretic mobility of the Tyr15 mutant protein (Figure 2A, lane 2) was indistinguishable from that of wild-type p34^{cdc2} (Figure 2A, lane 1) and like wild-type p34^{cdc2}, the Tyr15 mutant was capable of forming a complex with cyclin A (Figure 2C, lanes 7, 8 and 9). Unlike wild-type p34^{cdc2}, however, the electrophoretic mobility of the mutant was not altered as a result of co-expression with p107^{wee1} and cyclin A (Figure 2A, lane 6) or cyclin B (Figure 2A, lane 10). In addition, no increase in phosphorylation of the mutant was seen when it was co-produced with cyclin and p107^{wee1} (Figure 2C, lane 8) or with cyclin and pp60^{v-src} (Figure 2C, lane 9).

Phosphotryptic mapping of p34^{cdc2} and the mutant protein was also performed (Figure 3). When p34^{cdc2} was produced alone (Figure 3A), one major species (2) and one minor species (1) were found. Species 1 was often resolved into two spots. Phosphoamino acid analysis of both species showed only phosphothreonine (data not shown). Tryptic digests of the slower electrophoretic form of p34^{cdc2} (from cells co-producing p34^{cdc2} and p107^{wee1}) generated one major phosphopeptide (Figure 3B, species 3) which mixing

Fig. 3. Phosphotryptic maps of p34^{cdc2} and p34^{cdc2}(Phe15). Cells were infected with recombinant virus and labeled as described for Fig. 2C. ³²P-labeled p34^{cdc2} was excised from the gel, digested with trypsin and the phosphopeptides were resolved in two-dimensions. Panel A, p34^{cdc2} produced alone; panel B, p34^{cdc2} co-produced with p107^{wee1} (slower migrating species); panel C, p34^{cdc2} co-produced with p107^{wee1} (faster migrating species); panel D, p34^{cdc2}(Phe15) produced alone; panel E, p34^{cdc2}(Phe15) co-produced with p107^{wee1}; panel F, p34^{cdc2} co-produced with cyclin A; panel G, p34^{cdc2} co-produced with cyclin A and p107^{wee1} (slower migrating species). Panel H. A synthetic peptide containing tyrosine 15 was phosphorylated *in vitro* by pp60^{v-src}, digested with trypsin, and was resolved as described above. Panel I. mixture of G and H. Panels A–F, 300 c.p.m., 3 day exposure. Panels G and H, 100 c.p.m., 16 h exposure. Panel I, 700 c.p.m. each F and G, 16 h exposure.

Fig. 4. Cyclins as substrates for the activated $p34^{cdc2}$ kinase. Panel A. Cells infected with wild-type virus (lane 1), or virus encoding $p34^{cdc2}$ (lane 2); cyclin A (lane 3); $p34^{cdc2}$ and cyclin A (lane 4); cyclin B (lane 5); or $p34^{cdc2}$ and cyclin B (lane 6) were lysed, immunoprecipitated with p13 conjugated beads, and kinase assays were performed *in vitro*. Three-fourths of each reaction was resolved by SDS-PAGE on a 12% gel and transferred to nitrocellulose. The blot was exposed to film (upper panel) and then probed with antibodies specific for either cyclin A (lower panel, lanes 1–4) or cyclin B (lower panel, lanes 5 and 6). Panel B. The remainder of the reaction products (from A above) were resolved by SDS-PAGE on a 12% gel and the gel was exposed to film. The phosphorylated species (denoted as a, b and c in panel A, lane 4) were excised and subjected to phosphopeptide mapping. Arrows mark origins. 1000 c.p.m. were loaded and films were exposed for 16 h. Panel C. Phosphoamino acid analysis of phosphorylated cyclin A (species a, b and c) from panel A (lane 4) and of cyclin B from panel A (lane 6).

experiments showed to be distinct from species 1 and 2. Tryptic digests of the faster electrophoretic form of $p34^{cdc2}$ (from cells co-producing $p34^{cdc2}$ and $p107^{wee1}$) consisted primarily of the threonine containing peptides (species 1 and 2) but species 3 was sometimes detectable (Figure 3C). Since immunoblotting with anti-phosphotyrosine sera (Figure 2B, lane 2) did not detect the faster electrophoretic form of $p34^{cdc2}$, the presence of phosphopeptide 3 was likely due to cross-contamination during isolation. Co-expression of cyclin A with $p34^{cdc2}$ did not change the phosphorylation state of $p34^{cdc2}$ (Figure 3F). Although cyclin increased the amount of the slower electrophoretic form of $p34^{cdc2}$ in the presence of $p107^{wee1}$, peptide analysis showed that cyclin did not change the sites of phosphorylation of the slower electrophoretic form of $p34^{cdc2}$ in the presence of $p107^{wee1}$ (Figure 3G).

The phosphorylation of the Tyr15 mutant was unaffected by $p107^{wee1}$ and it was never detectably phosphorylated on tyrosine. The phosphotryptic map of the Tyr15 mutant (Figure 3D) was indistinguishable from that of $p34^{cdc2}$ (Figure 3A) and was unaltered as a result of co-expression with $p107^{wee1}$ (Figure 3E). The mutant, whether produced alone or co-produced with $p107^{wee1}$, was phosphorylated on threonine residues (data not shown).

These results suggested that $p34^{cdc2}$ is phosphorylated on tyrosine 15. To address this further, we obtained a peptide corresponding to amino acids 7–26 of *S.pombe* $p34^{cdc2}$ which yields a tryptic fragment identical to human $p34^{cdc2}$ (Lee and Nurse, 1987; Gould and Nurse, 1989). This peptide contains two tyrosines (tyrosines 15 and 19), however, $pp60^{v-src}$ phosphorylates tyrosine 15 *in vitro* (Gould and

Nurse, 1989). The mixing experiment shown in Figure 3I, demonstrated that the migration of this $pp60^{v-src}$ labeled synthetic peptide (Figure 3H) was identical to the migration of the ^{32}P -labeled tryptic fragment obtained when $p34^{cdc2}$ was co-produced with $p107^{wee1}$ and cyclin A (Figure 3G).

Kinase activity of $p34^{cdc2}$ in response to cyclin and $p107^{wee1}$

Functional complexes between $p34^{cdc2}$ and cyclin would be expected to show cyclin phosphorylation (Figure 4). Phosphorylation of p13 precipitates *in vitro* showed that both cyclin A (Figure 4A, upper panel, lane 4) and cyclin B (Figure 4A, upper panel, lane 6) were phosphorylated. A single phosphoprotein that co-migrated with baculovirus produced cyclin B (shown by blotting, Figure 4A, lower panel, lane 6) was detected. As seen in panel A (lane 4), three distinct phosphoproteins were detected in $p34^{cdc2}$ –cyclin A precipitates. These same species were detected in immunoblots (Figure 4A, lower panel, lane 4). The degree of phosphorylation of each species relative to one another varied between experiments and in some experiments a fourth species was also detected. Each of these phosphoproteins shared a similar phosphopeptide map (Figure 4B). Although each electrophoretic form of cyclin A shared several phosphopeptides (species 1–4), other phosphopeptides (species 5 and 6) were unique to the two slower electrophoretic forms of cyclin A. In addition, the level of phosphorylation of individual phosphopeptides varied between the different electrophoretic forms of cyclin A. For example, there was a reduction of the phosphorylation of species 2 and 4 and an enhancement in the phosphorylation

Fig. 5. Histone H1 kinase activity. Panel A. Cells were infected with wild-type virus (lane 1); or with recombinant virus encoding either p34^{cdc2} alone (lane 2); or cyclin B alone (lane 3). In addition cells were co-infected with virus encoding p34^{cdc2} and cyclin B (lane 4) or p34^{cdc2} and cyclin A (lane 5). Cells were lysed, immunoprecipitated with anti-p34^{cdc2} antibody (R1267), and histone H1 kinase assays were performed *in vitro*. Reaction products were resolved by SDS-PAGE on a 12% gel. Panel B. Cells were infected with recombinant virus encoding either p34^{cdc2} alone (lane 1); or were co-infected with viruses encoding p34^{cdc2} and cyclin A (lane 2); or p34^{cdc2}, cyclin A and p107^{wee1} (lane 3). In addition, cells were infected with recombinant virus encoding p34^{cdc2}(Phe15) alone (lane 4); or were co-infected with viruses encoding p34^{cdc2}(Phe15) and cyclin A (lane 5); or p34^{cdc2}(Phe15), cyclin A and p107^{wee1} (lane 6). Cells were lysed, immunoprecipitated with p34^{cdc2} antibody (R1267), and histone H1 kinase assays were performed *in vitro*. Reaction products were resolved by SDS-PAGE on a 12% gel.

of species 1 and 3 as the electrophoretic mobility of cyclin A decreased. Both cyclins were phosphorylated on serine and threonine residues (Figure 4C). Interestingly, the fastest electrophoretic form of cyclin A (species c) was phosphorylated predominantly on serine residues whereas the slower electrophoretic forms (species a and b) demonstrated enhanced levels of threonine phosphorylation.

These results suggested that p34^{cdc2} was capable of forming a complex with cyclins A and B and that both cyclins were substrates for the activated p34^{cdc2} kinase. Histone H1 kinase assays showed that p34^{cdc2}-cyclin complexes had enhanced kinase activity *in vitro* (Figure 5). Immunoprecipitates of p34^{cdc2} (panel A, lane 2; panel B, lane 1) or the p34^{cdc2}(Phe15) mutant (panel B, lane 4), had little histone H1 kinase activity. Immunoprecipitates prepared from cells expressing cyclin B alone (panel A, lane 3) or cyclin A alone (data not shown) were indistinguishable from controls (panel A, lane 1). In contrast, when p34^{cdc2} and cyclin A were co-produced, the immunoprecipitates had an ~200-fold increase in histone H1 kinase activity (panel A, lane 5), while co-production of p34^{cdc2} and cyclin B resulted in an ~50-fold increase (panel A, lane 4). The histone H1 kinase activity measured for p34^{cdc2}-cyclin A complexes and p34^{cdc2}-cyclin B complexes was always dramatically increased but varied between experiments. This variability may represent differences in the levels of cyclin

Fig. 6. Phosphorylation of p107^{wee1}. Panels A-C. Sf9 cells were infected with wild-type virus (panels A and B, lane 1) or with recombinant virus encoding either p107^{wee1}(Leu596) (panel A, lane 2 and panel B, lane 3) or p107^{wee1} (panel A, lane 3 and panel B, lane 2). Lysates were prepared and immunoprecipitated with anti-p107^{wee1} serum. The immune complexes were assayed for kinase activity *in vitro*. The reaction products were then divided in half. One half was analyzed directly on an 8% SDS-polyacrylamide gel (panel A). The other half was similarly resolved by SDS-PAGE, then blotted onto nitrocellulose and probed with an anti-p107^{wee1} serum (panel B). Phosphorylated p107^{wee1} was excised from a gel similar to that shown in panel A and was subjected to two-dimensional phosphoamino acid analysis (panel C). Panel C, 200 c.p.m., 7 day exposure. Panel D. Sf9 cells were infected with wild-type virus (lane 1) or with recombinant virus encoding either p107^{wee1} (lane 2) or p107^{wee1}(Leu596) (lane 3). Cells were labeled with [³⁵S]methionine, lysates were prepared and immunoprecipitated with anti-p107^{wee1} serum. Immunoprecipitates were resolved by SDS-PAGE on a 10% gel.

Fig. 7. Effects of vanadate on the tyrosine phosphorylation of p34^{cdc2}. Duplicate plates of cells were infected with virus encoding p34^{cdc2} alone (lanes 1 and 2); p34^{cdc2} and cyclin A (lanes 3 and 4); or p34^{cdc2}, cyclin A and p107^{wee1} (lanes 5 and 6). One set of infections was allowed to proceed in the absence (-) of vanadate (lower panel) whereas vanadate was included (+) in the second set (upper panel). At 40 h after infection, cell lysates were prepared and proteins were resolved on wide lanes by SDS-PAGE. Proteins were transferred to nitrocellulose and each lane was cut in half. One half of each lane was probed with antibody specific for p34^{cdc2} (R1267) (lanes 2, 4 and 6), the second half of each lane was probed with antibody specific for phosphotyrosine (lanes 1, 3 and 5).

A or cyclin B protein produced between infections. Co-production of cyclin A with the p34^{cdc2}(Phe15) mutant also resulted in an increase in kinase activity (panel B, lane 5). These results suggested that p34^{cdc2} forms a functional

complex with both cyclin A and cyclin B in insect cells. Interestingly, co-expression of p107^{wee1} reduced (by ~5-fold) the kinase activity associated with the wild-type p34^{cdc2}–cyclin complex (panel B, lane 3) but not with the mutant p34^{cdc2}(Phe15)–cyclin complex (panel B, lane 6).

Phosphorylation of p107^{wee1}

p107^{wee1} phosphorylation was examined both *in vitro* and *in vivo* and was compared with the phosphorylation of a mutant of p107^{wee1} containing leucine in place of lysine at position 596 (Figure 6). This mutant should be unable to bind ATP and function as a kinase (Russell *et al.*, 1989). As shown in Figure 6A, immunoprecipitates of wild-type protein using anti-p107^{wee1} serum, showed p107^{wee1} phosphorylation (panel A, lane 3). Immunoblotting of wild-type p107^{wee1} (panel B, lane 2) also revealed forms of decreasing electrophoretic mobility that may reflect differences in phosphorylation. p107^{wee1} was phosphorylated *in vitro* primarily on serine and tyrosine residues although a minor amount of phosphothreonine was also detected (Figure 6C). The mutant p107^{wee1} protein was not phosphorylated *in vitro* (panel A, lane 2) nor did it display as many electrophoretic forms as wild-type protein (panel B, lane 3) although blotting revealed amounts of protein similar to wild-type p107^{wee1}. Immunoprecipitates of p107^{wee1}, and the kinase-negative mutant of p107^{wee1} from lysates of cells labeled *in vivo* with [³⁵S]methionine were indistinguishable (Figure 6D). [³⁵S]methionine-labeled p107^{wee1} was resolved into a doublet. This may be due to differences in the phosphorylation state of p107^{wee1} as p107^{wee1} is phosphorylated during its production in insect cells.

Both wild-type p107^{wee1} and the mutant protein were phosphorylated *in vivo*, primarily on serine and threonine residues (data not shown). Wild-type p107^{wee1}, but not the kinase-deficient mutant, was also phosphorylated to a low level on tyrosine and could be immunoprecipitated with an anti-phosphotyrosine antibody (data not shown).

Vanadate does not substitute for either p107^{wee1} or cyclin in insect cells

We considered the possibility that p34^{cdc2} was phosphorylated on tyrosine in the absence of p107^{wee1} in insect cells and that the phosphorylation was unstable due to the presence of an endogenous phosphotyrosine specific protein phosphatase. In this case, p107^{wee1} could function by inactivating the phosphatase (thereby stabilizing the phosphotyrosine on p34^{cdc2}). Alternatively, cyclin might stabilize the tyrosine phosphorylated form of p34^{cdc2} perhaps by inhibiting/preventing dephosphorylation of p34^{cdc2}. We tested these possibilities by determining whether the phosphotyrosine specific protein phosphatase inhibitor vanadate, could substitute for either cyclin or p107^{wee1} in insect cells (Figure 7). If either p107^{wee1} or cyclin were acting through the phosphatase pathway then p34^{cdc2} should be stably phosphorylated on tyrosine in the presence of vanadate and in the absence of either cyclin or p107^{wee1}. As shown in Figure 7, phosphotyrosine-containing p34^{cdc2} was not detected when p34^{cdc2} was produced in insect cells in the presence of vanadate but in the absence of p107^{wee1} (upper panel, lanes 1 and 2) nor was it detected when p34^{cdc2} was produced in insect cells in the presence of cyclin and vanadate but in the absence

of p107^{wee1} (upper panel, lanes 3 and 4). Further, vanadate did not alter the levels of phosphotyrosine-containing p34^{cdc2} detected when p34^{cdc2} was co-produced with both cyclin and p107^{wee1} (both panels, lanes 5 and 6).

Discussion

We have used a baculoviral expression system to over-produce several of the key cell cycle regulatory proteins. We have made the surprising and intriguing observation that human p34^{cdc2} becomes phosphorylated on tyrosine when co-produced with *S.pombe* p107^{wee1} in insect cells but not when co-produced with a kinase-deficient mutant of p107^{wee1}. Results from mutagenesis studies as well as peptide phosphorylation and mapping studies indicated that phosphorylation occurred on tyrosine 15. In addition, clam cyclin (either A or B) greatly facilitated the phosphorylation of p34^{cdc2} on tyrosine in the presence of p107^{wee1}. These results suggest that the cyclin–p34^{cdc2} complex rather than monomeric p34^{cdc2} is the preferred substrate for tyrosine phosphorylation. Further, these results suggest a role for p107^{wee1} in the regulation of p34^{cdc2} by tyrosine phosphorylation and are supported by our findings that immunoprecipitates of p107^{wee1} possess tyrosine specific protein kinase activity.

In insect cells, both cyclin A and B formed a functional complex with p34^{cdc2} as judged by co-precipitation and activation of p34^{cdc2} kinase activity towards histone H1. In addition, both cyclin proteins were substrates *in vitro* for the activated p34^{cdc2} kinase. Our data do not address the question of what is regulating the association between p34^{cdc2} and cyclin in insect cells however, our data do suggest that a functional complex is formed. The production of functional cyclin A and B in insect cells was further indicated by their ability to induce meiotic maturation when injected into *Xenopus* oocytes (Roy *et al.*, in press). These results suggest that cyclins A and B complex with p34^{cdc2} in insect cells and somehow make p34^{cdc2} a better substrate for tyrosine phosphorylation. Perhaps cyclin association induces a conformational change in p34^{cdc2} such that tyrosine 15 becomes accessible to phosphorylation.

The large amount of p34^{cdc2} produced in insect cells yet the low level of label incorporated into p34^{cdc2}, suggest that the majority of p34^{cdc2} produced in insect cells is not phosphorylated. The low level that does occur is on threonine residues. This phosphothreonine-containing population of p34^{cdc2} (faster electrophoretic form) appears unaffected by the presence of p107^{wee1} (Figure 2C, compare p34^{cdc2} in lane 3 to faster electrophoretic form of p34^{cdc2} shown in lane 11). It is possible that the unphosphorylated form of p34^{cdc2} is the preferred substrate for tyrosine phosphorylation and that the threonine-containing population of p34^{cdc2} remains stable and is unaffected by the presence of p107^{wee1}. Although the major effect of cyclin production was the enhancement of phosphorylation of p34^{cdc2} on tyrosine, low levels of phosphothreonine were also detected in the slower electrophoretic form of p34^{cdc2} (Figure 2D). Perhaps cyclin associates with the unphosphorylated form of p34^{cdc2}, induces conformational changes that make p34^{cdc2} accessible to tyrosine phosphorylation, and the conformational change also exposes threonine residues which are in turn phosphorylated to a low level by insect cell kinases that are still active at the time of labeling.

There is evidence that association of p34^{cdc2} with cyclin is not enough for the full activation of p34^{cdc2} (Draetta and Beach, 1988; Pines and Hunter, 1989). As p34^{cdc2} has also been shown to be regulated by its state of phosphorylation, it is likely that additional post-translational modifications are also required for full activation of the complex. Our data suggest that at least one of these modifications involves tyrosine phosphorylation. After association with cyclin, p34^{cdc2} must undergo tyrosine phosphorylation on residue 15 and eventual dephosphorylation on tyrosine 15. We have no data regarding the contribution of threonine phosphorylation to this process, although threonine phosphorylation has been implicated in the activation process as well (Lewin, 1990).

Although the presence of both cyclin A and cyclin B has been described in clams, *Drosophila*, *Xenopus* and humans, essentially nothing is known about their functional differences (Swenson *et al.*, 1986; Lehner and O'Farrell, 1989; Minshull *et al.*, 1989; Pines and Hunter, 1989, 1990; Westendorf *et al.*, 1989; Whitfield *et al.*, 1989). Both cyclins A and B from clam have been reported to associate with p34^{cdc2} (Draetta *et al.*, 1989). In contrast, human cyclin B protein but not cyclin A protein has been reported to associate with p34^{cdc2} (Pines and Hunter, 1989, 1990). We find that both clam cyclins associate with human p34^{cdc2} and have observed no functional differences so far between the two types of complex. However, the baculoviral expression system has provided a rich source of both p34^{cdc2}-cyclin A and p34^{cdc2}-cyclin B complexes (purified from one another) and should facilitate studies aimed at determining function differences between these two types of complexes.

Dephosphorylation of p34^{cdc2} on tyrosine correlates with its activation and of entry of cells into mitosis (Dunphy and Newport, 1989; Gautier *et al.*, 1989; Morla *et al.*, 1989). Localization of the phosphorylation site to tyrosine 15, which is part of the putative ATP binding domain of p34^{cdc2}, suggests inhibition by abolishing enzyme activity (Gould and Nurse, 1989). Our findings that the kinase activity of the p34^{cdc2}-cyclin complexes isolated from cells also expressing p107^{wee1} (and therefore phosphorylated on tyrosine) is reduced compared with complex isolated from cells lacking p107^{wee1} (and therefore not phosphorylated on tyrosine) is consistent with this proposed mechanism of p34^{cdc2} inactivation (Figure 5). Although the activity of the p34^{cdc2}-cyclin complex was clearly reduced in response to co-production with p107^{wee1}, it was not ablated as there are three forms of p34^{cdc2} present in the immunoprecipitates: monomeric p34^{cdc2}, p34^{cdc2}-cyclin complex (containing phosphotyrosine) and p34^{cdc2}-cyclin complex (lacking phosphotyrosine). The latter form of the kinase is active.

Genetic data from *S.pombe* indicate that p107^{wee1} functions as a mitotic inhibitor perhaps through its interactions with p34^{cdc2} (Russell and Nurse, 1987). The gene for p107^{wee1} has been cloned and its sequence predicts that it is a kinase (Russell and Nurse, 1987). A possible way to link the genetics of p107^{wee1} with the biology of p34^{cdc2} would be if p107^{wee1} regulated the tyrosine phosphorylation of p34^{cdc2}. Our data clearly demonstrate that the tyrosine phosphorylation of p34^{cdc2} in insect cells is absolutely dependent upon the presence of kinase-active p107^{wee1}. Neither a kinase-deficient mutant of p107^{wee1} nor pp60^{v-src} could substitute for kinase-active p107^{wee1} in inducing the

tyrosine phosphorylation of p34^{cdc2} in the presence of cyclin (Figure 2). This argues for specificity in the reaction as pp60^{v-src} is fairly promiscuous and will phosphorylate many non-physiological substrates, given the opportunity.

Three models could be invoked to explain these results. One model would predict that *wee1*⁺ encodes a tyrosine kinase. However, there has been a bias against this model based on computer-derived sequence comparisons (Hanks *et al.*, 1988; Murray, 1989). The *wee1*⁺ gene product is predicted to encode a serine/threonine protein kinase based on sequence comparisons with known protein kinases (Russell and Nurse, 1987; Hanks *et al.*, 1988). However, low quantities of p107^{wee1} in yeast has hindered a direct biochemical confirmation of this prediction. A second model would predict that *wee1*⁺ encodes a serine/threonine kinase which in turn activates a tyrosine kinase (perhaps through phosphorylation) and it is this intermediary kinase which phosphorylates p34^{cdc2} on tyrosine 15. A third model would predict that p107^{wee1} inactivates a phosphotyrosine specific protein phosphatase (perhaps through phosphorylation of the phosphatase), thereby stabilizing the phosphate group on tyrosine 15. If p107^{wee1} functions by inactivating a phosphotyrosine specific protein phosphatase (model 3), vanadate would be predicted to substitute for p107^{wee1} in insect cells. In this case, tyrosine phosphorylation of p34^{cdc2} would be expected in the absence of p107^{wee1} but in the presence of vanadate (whether or not cyclin was also present). This was not the case suggesting that p107^{wee1} does not function by inhibiting an endogenous phosphatase nor does cyclin function by stabilizing the tyrosine phosphorylated form of p34^{cdc2} (Figure 7).

Although it is clear that cyclin facilitates the tyrosine phosphorylation of p34^{cdc2}, the question remains as to the identity of the kinase responsible for the phosphorylation (models 1 and 2). To characterize the kinase activity of p107^{wee1}, we performed immune complex kinase assays *in vitro*. p107^{wee1} was phosphorylated both on serine and tyrosine residues although a low level of phosphothreonine was also detected. To date, we have been unable to dissociate the serine/threonine specific protein kinase activity from the tyrosine specific protein kinase activity present in p107^{wee1} immunoprecipitates. This is even after using lysis and washing conditions that totally removed all activity associated with a kinase-deficient mutant of p107^{wee1} *in vitro* (Figure 6A). This argues that the tyrosine kinase activity present in p107^{wee1} immunoprecipitates (if not due to p107^{wee1} itself), is due to a tightly complexed host-derived tyrosine kinase whose activity is absolutely dependent upon p107^{wee1} kinase activity. Recently a kinase has been identified from budding yeast by screening a *Saccharomyces cerevisiae* λ gt11 library with an anti-phosphotyrosine antibody (Stern *et al.*, 1991). This kinase possesses both serine/threonine specific as well as tyrosine specific protein kinase activity. Further studies will be required to determine whether p107^{wee1} has similar capabilities.

We are unable to say whether p107^{wee1} is directly responsible for phosphorylation of p34^{cdc2} on tyrosine or whether it acts through an intermediary kinase (models 1 and 2). However, one argument in favor of p107^{wee1} itself catalyzing the reaction comes from the biology of the baculoviral expression system. We estimate that p34^{cdc2} represents ~5% of total cellular protein and anywhere from 50 to 90% of this p34^{cdc2} becomes phosphorylated on

tyrosine in response to co-production with both cyclin and p107^{wee1}. Thus, if p107^{wee1} is acting through an intermediary kinase to phosphorylate this much p34^{cdc2}, this intermediary kinase must be extremely active. In addition, this intermediary kinase must be extremely stable as baculoviral infections result in the degradation of cellular chromatin and termination of cellular transcription within one hour of infection. Thus, this intermediary kinase must be present at the time of infection and stable up until the time of recombinant protein production which occurs late in the viral life cycle (recombinant protein production is under the control of the viral polyhedrin promoter, a promoter which is activated at late times after viral infection). These properties of the baculoviral expression system, in conjunction with our results, are supportive of model 1, that p107^{wee1} is directly responsible for the tyrosine phosphorylation of p34^{cdc2}.

Materials and methods

Generation of viruses

All procedures relating to cell growth and viral propagation were performed as described (Summers and Smith, 1987; Piwnicka-Worms, 1990). Recombinant virus encoding pp60^{v-src} was a gift from Frank McCormick. Recombinant viruses encoding cyclins A and B were generated as described (Roy *et al.*, in press). The cDNA encoding the wild-type *wee1*⁺ gene product of *S.pombe* was cloned into the plasmid pVL941 (Luckow and Summers, 1988) by the insertion of a *Bam*HI linker into the unique *Xho*I site of pJ3-W1 (Russell and Nurse, 1987), digesting the resulting plasmid with *Bam*HI, and ligating the 4.2 kb *Bam*HI fragment (encoding the *wee1*⁺ gene product) into the *Bam*HI site of pVL941 to generate pVL(*wee1*⁺). A presumptive kinase-negative mutant of *wee1*⁺ was cloned into pVL941 as follows: plasmid pWG-L596 (Russell *et al.*, 1989) was digested with *Sph*I and a *Bam*HI linker was inserted. The resulting plasmid was digested with *Bam*HI and the 4.2 kb fragment encoding the kinase-deficient mutant of *wee1*⁺ was cloned into the *Bam*HI site of pVL941 to generate pVL*wee1*(Leu596). The gene encoding the human homolog of p34^{cdc2} was cloned into plasmid pVL941 by digestion of plasmid pOB231 (Lee and Nurse, 1987) with *Bam*HI, and ligating the 2.0 kb *Bam*HI fragment (containing the *cdc2* gene) into the *Bam*HI site of pVL941 to generate pVL941 (*cdc2*).

Site-directed mutagenesis of the codon for tyrosine 15 of p34^{cdc2} was performed using a mutagenesis kit by Amersham, which was based on the method of Sayers *et al.* (1988). A 2.0 kb *Bam*HI fragment encoding the human *cdc2* gene was excised from pOB231 (Lee and Nurse, 1987) and inserted into the polylinker of pGC52 to generate pSAF1. pGC52 was provided by B.Drucker and is a derivative of pGC1 (Myers *et al.*, 1985). The *Pvu*II site of pSAF1 was then replaced by an *Eco*RI site to generate pSAF2. A 1.2 kb *Bam*HI–*Eco*RI fragment containing the *cdc2* gene was then cloned into the pGC52 polylinker to form pSAF10. A 679 bp *Bam*HI–*Hind*III N-terminal fragment of *cdc2*, was then cloned into pGC62, also provided by B.Drucker, to generate pSAF20. Single-stranded pSAF20 was mutagenized using the oligo 5' AGAAGGTACCTTTGGAGTTGT-GTA 3', where the underlined codon is residue 15. This codon now encodes Phe rather than Tyr. The wild-type *cdc2* N-terminus of pSAF10 was then replaced by a 634 bp *Bam*HI–*Bgl*III fragment containing the mutant N-terminus to generate pSAF40. Mutants were verified by sequencing. A 1.2 kb *Bam*HI–*Eco*RI fragment encoding p34^{cdc2}(Phe15) was excised from pSAF40 and ligated into the polylinker of pVL1393 to produce pVL1393 *cdc2*(Phe15).

Analysis of protein production

Sf9 cells (3×10^6) were infected with either wild-type virus, or recombinant virus at an m.o.i. of ~10. Cell lysates were prepared in NP-40/Tris buffer [50 mM Tris pH 7.4, 0.25 M NaCl, 50 mM NaF, 10 mM sodium pyrophosphate (NaPPi), 0.1% NP-40, 10% glycerol] supplemented with 2 mM phenylmethylsulfonyl fluoride (PMSF), 0.15 U/ml aprotinin, 1 mM sodium orthovanadate and 20 μ M leupeptin. Proteins were resolved by SDS–PAGE, transferred to nitrocellulose and the blots were developed using the enzymatic color reaction kit from Promega Biotec.

Preparation of lysates for immunoprecipitations

Cells (3×10^6) were infected with virus at an m.o.i. of 10. At ~40 h after infection, cell lysates were prepared in RIPA/Tris buffer (20 mM Tris pH 7.4, 130 mM NaCl, 10% glycerol, 0.1% SDS, 0.5% sodium desoxycholate, 1% Triton X-100) supplemented with 2 mM PMSF, 0.15 U/ml aprotinin, 1 mM sodium orthovanadate and 20 μ M leupeptin. Lysates were clarified at 10 000 g for 10 min at 4°C.

Kinase assays in vitro

p107^{wee1}. 500 μ g of protein were immunoprecipitated by incubation for 2 h at 4°C with anti-p107^{wee1} serum coupled to Sepharose [coupling of anti-p107^{wee1} serum with Sepharose C14B–protein A (Sigma Chemical Co.) was as follows: beads and antibody were incubated at 4°C for 1 h, followed by washing three times in RIPA/Tris buffer].

Cyclin. Lysates were precipitated with p13 beads for 2 h at 4°C.

Histone H1. Lysates were immunoprecipitated with anti-p34^{cdc2} serum (R1267) for 2 h at 4°C. Sepharose C14B–protein A beads were added and the incubation was continued for another hour.

Immunoprecipitates were washed twice in RIPA/Tris buffer and twice in LiCl₂ wash buffer (0.5 M LiCl₂/20 mM Tris pH 8.0). When kinase assays were to be performed the immunoprecipitates were washed once in kinase buffer, otherwise they were washed in PBS. Kinase assays were performed in 50 mM Tris pH 7.4, 10 mM MgCl₂, or 10 mM MnCl₂, 1 mM dithiothreitol, 10 μ M ATP and 0.5–1.0 mCi/ml γ -³²P-labeled ATP at 30°C for 10–30 min. When performing histone H1 kinase assays, histone was added at a concentration of 400 μ g/ml.

Phosphorylation of synthetic peptide

Sf9 cells (3×10^6) were infected with virus encoding pp60^{v-src}, lysed in RIPA/Tris buffer and immunoprecipitated with EC10 antibody (Parsons *et al.*, 1984). Immunoprecipitates were washed as described above and combined with 1 μ g of a peptide of the sequence IEKIGEGTYGVVYK-GRHKTT (Gould and Nurse, 1989). Kinase assays were performed as described above and the supernatant was resolved by SDS–PAGE (25%). The phosphorylated peptide was excised from the gel, and was subjected to trypsin digestion and phosphoamino acid analysis as described below.

Labelings in vivo

Sf9 cells (3×10^6) were infected with virus at an m.o.i. of ~10. At 36 h post-infection, cells were rinsed once in phosphate-free media and then incubated for 3 h in 1.5 ml of phosphate-free medium supplemented with 2 mM glutamine, 1.5% dialyzed calf serum and 4.0 mCi/ml ³²P-labeled inorganic phosphate. [³⁵S]methionine labeling was carried out as described above using methionine-free media, and 0.1 mCi/ml [³⁵S]methionine.

Phosphopeptide mapping and phosphoamino acid analysis

Cells were labeled and lysates were prepared and immunoprecipitated as described above. Tryptic digests were performed as described previously (Piwnicka-Worms *et al.*, 1987). Tryptic peptides were separated in the first dimension by thin layer electrophoresis at pH 1.9, buffered with water/acetic acid/formic acid in a ratio of 800/150/50. Peptides were separated in the second dimension by ascending chromatography, with *n*-butanol/pyridine/acetic acid/water in a ratio of 75/50/15/60 used as a solvent. Two-dimensional phosphoamino acid analysis was performed as described (Draetta *et al.*, 1988).

Vanadate experiments

Cells were infected with virus encoding p34^{cdc2}, p34^{cdc2} and cyclin A; p34^{cdc2} and p107^{wee1}; or p34^{cdc2}, cyclin A and p107^{wee1} (in duplicate). One set of infections was allowed to proceed in the absence of vanadate whereas vanadate was included in the second set [After viral attachment, vanadate was added at a concentration of 50 μ M. Old medium was removed and fresh medium (containing vanadate at a concentration of 50 μ M) was added every 12 h until 40 h post-infection when cells were harvested]. At 40 h after infection, cell lysates were prepared and 60 μ g of total cell protein was resolved on wide lanes by SDS–PAGE. Proteins were transferred to nitrocellulose and each lane was cut in half. One half of each lane was probed with antibody specific for p34^{cdc2} (R1267), the second half of each lane was probed with antibody specific for phosphotyrosine.

Preparation of p13 Sepharose 6B

Purified p13 was isolated from an overexpressing strain of *Escherichia coli* as described previously (Brizuela *et al.*, 1987). p13 (5 mg/ml of gel) was coupled to Cyanogen bromide activated Sepharose 6B according to directions from the manufacturer (Pharmacia).

Antibodies

EC10 sera is specific for avian pp60^{src} (Parsons *et al.*, 1984). The anti-p107^{wee1} antibody, provided by Paul Russell, is a rabbit polyclonal antibody raised against a bacterially derived C-terminal fragment of *S.pombe* derived p107^{wee1}. Affinity-purified anti-cyclin A and anti-cyclin B antibodies were produced and characterized as described by Swenson *et al.* (1986) and Westendorf *et al.* (1989). The anti-phosphotyrosine antibody was a murine monoclonal antibody generated using phosphotyramine (Druker *et al.*, 1989). The anti-p34^{cdc2} antibody provided by G.Draetta and D.Beach is a rabbit polyclonal antibody and was generated as described (Draetta *et al.*, 1987).

The second source of anti-p34^{cdc2} serum (R1267 and R1268) was produced as follows: pTR1340 was cut with *XbaI* and *SalI* and the 4 kb backbone was isolated (Pallas *et al.*, 1986). pSP68-4 (RBS) was cut with *XbaI* and *SalI* and the pp60^{src}-containing fragment (1.6 kb) was isolated and cloned into the pTR1340 backbone to generate pLONCm (Piwnica-Worms *et al.*, 1986). pLONCm was cut with *NcoI* and *SalI* and the 4 kb backbone (devoid of pp60^{src}) was isolated. This backbone contains a strong *tac* promoter and a chemically synthesized Shine-Dalgarno sequence to direct the synthesis of p34^{cdc2} in bacteria (Pallas *et al.*, 1986). Placement of the *cdc2* gene into pLONCm was done as follows: pSAF-1 [contains a 2.0 kb *BamHI* fragment encoding the human *cdc2* gene from pOB231, (Lee and Nurse, 1987)] was cut with *NcoI* and *SalI* and two fragments were isolated (a 1262 bp *NcoI-SalI* fragment and a 358 bp *NcoI-NcoI* fragment). The 1262 bp fragment (encoding the terminal 94 amino acids of human p34^{cdc2}) was cloned into the pLONCm backbone to generate pML1. pML1 was linearized with *NcoI* and the 358 bp fragment was inserted to generate pML2. pML2 encodes a 23 kd truncated form of p34^{cdc2}.

A *lon*⁻ bacterial strain SG931Q (Chin *et al.*, 1988) was transformed with pML2. Fresh colonies were inoculated into 10 ml of Luria broth/ampicillin (100 µg/ml)/kanamycin (30 µg/ml) and were grown overnight at 30°C. The 10 ml was used to inoculate 200 ml of L broth and bacteria were grown to an OD₅₅₀ of 0.25–0.4 at 30°C. Protein was induced by the addition of 0.5 mM IPTG for 3–5 h at 37°C. Bacteria were pelleted at 3000 g for 15 min, the bacterial pellets were rinsed with PBS, and then suspended in 50 ml of NP-40/Tris buffer supplemented with aprotinin (0.15 U/ml); leupeptin (20 µM); PMSF (2 mM). Cells were lysed with a probe tip sonicator and the insoluble protein was pelleted at 3000 g for 15 min, and then resuspended in boiling buffer. Total protein (2–3 mg) was loaded on 15% polyacrylamide gels and p34^{cdc2} (23 kd) was excised. Two white New Zealand rabbits were immunized with a primary injection of 0.5 mg of protein with complete Freund's adjuvant, followed by boosts every 3 weeks with 0.2 mg protein in incomplete Freund's adjuvant. Antisera (R1267 and R1268) both immunoblotted and immunoprecipitated human p34^{cdc2} produced in insect cells.

Acknowledgements

The authors acknowledge Paul Russell for his contribution of pJ3-W1, PWG-L596 and anti-wee1 serum. We also wish to thank Paul Nurse for pOB231; Giulio Draetta and David Beach for the bacterial p13 overproducer and anti-p34 serum; Brian Druker for pGC52, pGC62 and the anti-phosphotyrosine antibody; Max Summers for pVL1393 and Frank McCormick for recombinant baculovirus encoding pp60^{v-src}. In addition, we thank Kathy Gould and Paul Nurse for the tyrosine 15 containing peptide and Barrett Rollins for his assistance in the densitometry scanning. We thank Jim Maller, Brian Schaffhausen, Tom Roberts and Lew Cantley for their support and their comments on the manuscript. This work was supported by a Whitaker Health Sciences Fund Award, PHS grant CA50767 and ACS JFRA-290 to H.P.-W. and an ACS postdoctoral fellowship (MA division) to L.L.P.

References

- Beach, D., Durkacz, B. and Nurse, P. (1982) *Nature*, **300**, 706–709.
- Blow, J. and Nurse, P. (1990) *Cell*, **62**, 855–862.
- Brizuela, L., Draetta, G. and Beach, D. (1987) *EMBO J.*, **6**, 3507–3514.
- Chin, D.T., Goff, S.A., Webster, T., Smith, T. and Goldberg, A.L. (1988) *J. Biol. Chem.*, **263**, 11718–11728.
- Draetta, G. and Beach, D. (1988) *Cell*, **54**, 17–26.
- Draetta, G., Brizuela, J., Potashkin, J. and Beach, D. (1987) *Cell*, **50**, 319–325.
- Draetta, G., Piwnica-Worms, H., Morrison, D., Druker, B., Roberts, T. and Beach, D. (1988) *Nature*, **336**, 738–744.
- Draetta, G., Luca, F., Westendorf, J., Brizuela, L., Ruderman, J. and Beach, D. (1989) *Cell*, **56**, 829–838.

- Druker, B.J., Mamon, H.J. and Roberts, T.M. (1989) *New Engl. J. Med.*, **321**, 1383–1391.
- Dunphy, W. and Newport, J. (1989) *Cell*, **58**, 181–191.
- Evans, T., Rosenthal, E.T., Youngblom, J., Distel, D. and Hunt, T. (1983) *Cell*, **33**, 389–396.
- Furukawa, Y., Piwnica-Worms, H., Ernst, T.J., Kanakura, Y. and Griffin, J.D. (1990) *Science*, **250**, 805–808.
- Gautier, J., Matsukawa, T., Nurse, P. and Maller, J. (1989) *Nature*, **339**, 626–629.
- Gould, K. and Nurse, P. (1989) *Nature*, **342**, 39–44.
- Hanks, S., Quinn, A. and Hunter, T. (1988) *Science*, **241**, 42–52.
- Lee, M. and Nurse, P. (1987) *Nature*, **327**, 31–35.
- Lehner, C.F. and O'Farrell, P.H. (1989) *Cell*, **56**, 957–968.
- Mewin, B. (1990) *Cell*, **61**, 743–752.
- Luckow, V.A. and Summers, M.D. (1988) *Bio/Technology*, **6**, 47–55.
- Minshull, J., Blow, J.J. and Hunt, T. (1989) *Cell*, **56**, 947–956.
- Morla, A., Draetta, G., Beach, D. and Wang, J. (1989) *Cell*, **58**, 193–203.
- Murray, A.W. (1989) *Nature*, **342**, 14–15.
- Murray, A.W. and Kirschner, M.W. (1989) *Nature*, **339**, 273–280.
- Murray, A.W., Solomon, M.J. and Kirschner, M.W. (1989) *Nature*, **339**, 280–286.
- Myers, R.M., Lerman, L.S. and Maniatis, T. (1985) *Science*, **229**, 242–247.
- Nurse, P. and Bissett, Y. (1981) *Nature*, **292**, 558–560.
- Pallas, D.C., Schley, C., Mahoney, M., Harlow, E., Schaffhausen, B.S. and Roberts, T.M. (1986) *J. Virol.*, **60**, 1075–1084.
- Parsons, S.J., McCarley, D.J., Ely, C.M., Benjamin, D.C. and Parsons, J.T. (1984) *J. Virol.*, **51**, 272–282.
- Pines, J. and Hunter, T. (1989) *Cell*, **58**, 833–846.
- Pines, J. and Hunter, T. (1990) *Nature*, **346**, 760–763.
- Piwnica-Worms, H. (1990) In Ausubel, F., Brent, R., Kingston, R., Moore, D., Seidman, J., Smith, J. and Struhl, K. (eds) *Current Protocols in Molecular Biology*. Greene Publishing Associates, Brooklyn, New York, Vol. 2. pp.16.8.1–16.11.7.
- Piwnica-Worms, H., Kaplan, D.R., Whitman, M. and Roberts, T.M. (1986) *Mol. Cell. Biol.*, **6**, 2033–2040.
- Piwnica-Worms, H., Saunders, K., Roberts, T.M., Smith, A.E. and Cheng, S.H. (1987) *Cell*, **45**, 75–82.
- Reed, S. (1980) *Genetics*, **95**, 561–577.
- Roy, L.M., Swenson, K.I., Walker, D.H., Gabrielli, B.G., Li, R.S., Piwnica-Worms, H. and Maller, J.L. (1991) *J. Cell Biol.*, in press.
- Russell, P. and Nurse, P. (1987) *Cell*, **45**, 559–567.
- Russell, P., Moreno, S. and Reed, S. (1989) *Cell*, **57**, 295–303.
- Sayers, J.R., Schmidt, W. and Eckstein, F. (1988) *Nucleic Acids Res.*, **16**, 791–802.
- Simanis, V. and Nurse, P. (1986) *Cell*, **45**, 261–268.
- Stern, D.F., Zheng, P., Beidler, D.R. and Zerillo, C. (1991) *Mol. Cell. Biol.*, **11**, 987–1001.
- Summers, M. and Smith, G. (1987) *A manual for methods for baculovirus vectors and cloned insect cell culture procedures*. Texas Agricultural Experimental Station Bulletin No. 1555, College Station, TX.
- Swenson, K., Farrell, K. and Ruderman, J. (1986) *Cell*, **47**, 861–870.
- Westendorf, J., Swenson, K. and Ruderman, J. (1989) *J. Cell Biol.*, **108**, 1431–1443.
- Whitfield, W.G.F., Gonzalez, C., Sanchez-Herrero, E. and Glover, D.M. (1989) *Nature*, **338**, 337–340.

Received on December 10, 1990; revised on January 18, 1991