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Differential phosphorylation of vertebrate p34^{cdc2} kinase at the G1/S and G2/M transitions of the cell cycle: identification of major phosphorylation sites

W.Krek and E.A.Nigg[1]

Swiss Institute for Experimental Cancer Research (ISREC), 155 Chemin des Boveresses, CH-1066 Epalinges, Switzerland

[1]Corresponding author

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The cdc2 kinase is a key regulator of the eukaryotic cell cycle. The activity of its catalytic subunit, p34^{cdc2}, is controlled by cell cycle dependent interactions with other proteins as well as by phosphorylation-dephosphorylation reactions. In this paper, we examine the phosphorylation state of chicken p34^{cdc2} at various stages of the cell cycle. By peptide mapping, we detect four major phosphopeptides in chicken p34^{cdc2}; three phosphorylation sites are identified as threonine (Thr) 14, tyrosine (Tyr) 15 and serine (Ser) 277. Analysis of synchronized cells demonstrates that phosphorylation of all four sites is cell cycle regulated. Thr 14 and Tyr 15 are phosphorylated maximally during G2 phase but dephosphorylated abruptly at the G2/M transition, concomitant with activation of p34^{cdc2} kinase. This result suggests that phosphorylation of Thr 14 and/or Tyr 15 inhibits p34^{cdc2} kinase activity, in line with the location of these residues within the putative ATP binding site of the kinase. During M phase, p34^{cdc2} is also phosphorylated, but phosphorylation occurs on a threonine residue distinct from Thr 14. Finally, phosphorylation of Ser 277 peaks during G1 phase and drops markedly as cells progress through S phase, raising the possibility that this modification may contribute to control the proposed G1/S function of the vertebrate p34^{cdc2} kinase.

Key words: cdc2 kinase/cell cycle/G1 phase/phosphorylation/regulation

Introduction

The serine/threonine specific protein kinase p34^{cdc2} plays a pivotal role in eukaryotic cell cycle regulation (reviewed by Cross *et al.*, 1989; Lohka, 1989; Murray and Kirschner, 1989a; Draetta 1990; Nurse, 1990; Pines and Hunter, 1990a). Genetic evidence obtained from the fission yeast *Schizosaccharomyces pombe* demonstrates that the *cdc2* gene product is required for entry into mitosis (M phase) as well as for induction of DNA synthesis (S phase) (Nurse and Bissett, 1981, reviewed in Hayles and Nurse, 1986). A similar function can be attributed to CDC28, the homolog of cdc2 in budding yeast *Saccharomyces cerevisiae* (Hartwell *et al.*, 1974; Pringle and Hartwell, 1981; Beach *et al.*, 1982; Pigott *et al.*, 1982; Lörincz and Reed, 1984; Reed and Wittenberg, 1990; Wittenberg *et al.*, 1990). In higher eukaryotes, the importance of the *cdc2* gene at the G2/M transition is well established (Draetta, 1990; Nurse, 1990; Pines and Hunter, 1990a) but evidence for a function at earlier stages of the cell cycle is only beginning to emerge (Blow and Nurse, 1990; D'Urso *et al.*, 1990).

The activity of p34^{cdc2} is regulated both by physical interaction with other proteins and by posttranslational modification (for reviews see Draetta, 1990; Nurse, 1990). Prominent among the complex patterns of p34^{cdc2} are proteins called cyclins (Booher *et al.*, 1989; Draetta *et al.*, 1989. Giordano *et al.*, 1989. Labbé *et al.*, 1989; Meijer *et al.*, 1989; Moreno *et al.*, 1989; Pines and Hunter, 1989; Gautier *et al.*, 1990). Cyclins were first identified on the basis of their characteristic accumulation during interphase, followed by abrupt destruction at mitosis (Evans *et al.*, 1983; Swenson *et al.*, 1986; for review see Hunt, 1989). In *Xenopus*, they were shown to be required for activation of p34^{cdc2} at the G2/M transition (Minshull *et al.*, 1989a; Murray and Kirschner, 1989b; see also Picard *et al.*, 1989), while their destruction was reported to be necessary for exit from M phase (Murray *et al.*, 1989; Félix *et al.*, 1990a). Based on cDNA sequence analysis of cyclins from numerous species, these can be classified as either A- or B-type (reviewed in Minshull *et al.*, 1989b). Differences in the subcellular location and the precise timing of accumulation and destruction of A- and B-type cyclins suggest that they may carry out different functions (Pines and Hunter, 1989, 1990b; Westendorf *et al.*, 1989; Whitfield *et al.*, 1989, 1990. Lehner and O'Farrell, 1990a; Minshull *et al.*, 1990), a notion supported by mutational analysis in *Drosophila* (Lehner and O'Farrell, 1989, 1990a). Furthermore, several novel members of the cyclin protein family have recently been characterized in *Saccharomyces cerevisiae* (Hadwiger *et al.*, 1989a), and the properties of these cyclin-like proteins suggest that they may play a role in regulating p34^{cdc2} activity at the G1/S transition (Richardson *et al.*, 1989; Wittenberg *et al.*, 1990). Despite the rapid accumulation of structural information on cyclin proteins, it remains largely unknown what mechanisms control the formation of various cyclin-p34^{cdc2} complexes and how these complexes function during the cell cycle.

Another protein implicated in the regulation of p34^{cdc2} kinase activity is the product of a gene called *suc1* in *S.pombe* (Hayles *et al.*, 1986; Brizuela *et al.*, 1987; Moreno *et al.*, 1989). Homologous genes have been identified also in *S.cerevisiae* (where the gene is called *CKS1*; Hadwiger *et al.*, 1989b) and in vertebrates (Richardson *et al.*, 1990). Again, the precise function of these proteins remains obscure, but genetic as well as biochemical studies demonstrate that the *suc1/CKS1* gene product interacts physically with p34^{cdc2} (Brizuela *et al.*, 1987), and that these interactions contribute to regulate p34^{cdc2} kinase activity (for references see Dunphy and Newport, 1989; Draetta, 1990; Jessus *et al.*, 1990; Nurse, 1990).

Finally, numerous studies demonstrate that cdc2 kinase activity is subject to control by phosphorylation and dephosphorylation. Substrates for these modifications are both p34^{cdc2} (Draetta and Beach, 1988; Draetta *et al.*,

1988; Lee *et al.*, 1988; Dunphy and Newport, 1989; Gautier *et al.*, 1989; Gould and Nurse, 1989; Morla *et al.*, 1989; Pondaven *et al.*, 1990), as well as cyclin subunits (Booher *et al.*, 1989; Félix *et al.*, 1990b; Labbé *et al.*, 1989; Meijer *et al.*, 1989; Pines and Hunt, 1987; Standart *et al.*, 1987). The most direct information on the role of phosphorylation of $p34^{cdc2}$ in the regulation of its kinase activity stems from recent studies on fission yeast (Gould and Nurse, 1989). Activation of cdc2 kinase at the G2/M transition was shown to require dephosphorylation of $p34^{cdc2}$ on Tyr 15, and mutation of this residue to phenylalanine caused premature entry of cells into mitosis (Gould and Nurse, 1989). However, $p34^{cdc2}$ is phosphorylated not only on tyrosine, but also on threonine and serine residues (Simanis and Nurse, 1986; Lee *et al.*, 1988; Draetta *et al.*, 1988; Gould and Nurse, 1989; Morla *et al.*, 1989; Norbury and Nurse, 1990; Picard *et al.*, 1989). To understand fully the regulation of the cdc2 kinase it is important to identify the various phosphorylation sites on $p34^{cdc2}$ and to establish at what stages during the cell cycle phosphate groups are added to or removed from individual sites. This information will be indispensable for a rational design of experiments aimed at establishing functional consequences of changes in $p34^{cdc2}$ phosphorylation during the cell cycle.

In this study, we have examined the phosphorylation state of chicken $p34^{cdc2}$ at various stages of the cell cycle. We have used a combination of peptide mapping procedures to identify the major *in vivo* phosphorylation sites on this protein, and we have monitored changes in the phosphorylation state of each individual site as cells progress through the cell cycle.

Results

Characterization of anti-$p34^{cdc2}$ antibodies

Throughout this study, two different anti-$p34^{cdc2}$ sera were used. A first reagent (serum R6) was raised against an 18 amino acid synthetic peptide corresponding to the carboxy-terminus of chicken $p34^{cdc2}$, its monospecificity for the functional chicken homolog of the fission yeast *cdc2* gene product has been documented previously (Krek and Nigg, 1989). The second antibody used here (serum R8) was raised against the full-length chicken $p34^{cdc2}$ protein expressed in *Escherichia coli* (Krek and Nigg, 1989); its specificity is illustrated in Figure 1. This antiserum specifically immunoprecipitated a 34 kd protein from whole chicken cell lysates, irrespective of whether cells had been metabolically labeled with [^{35}S]methionine (Figure 1A, lane 1) or [^{32}P]orthophosphate (Figure 1B, lane 1). No reactivity was detectable in the corresponding pre-immune serum (Figure 1A and B, lanes 2). Anti-$p34^{cdc2}$ antibodies were further purified by affinity-chromatography on bacterially expressed chicken $p34^{cdc2}$ immobilized on Sepharose. As shown by immunoblotting, affinity-purified immunoglobulins reacted exclusively with $p34^{cdc2}$ when probed on total chicken cell lysates (Figure 1C, lane 1). Likewise, they only reacted with a single 34 kd protein when assayed on *E.coli* lysates prepared from cells expressing chicken $p34^{cdc2}$ protein (Figure 1C, lane 3). No reactivity was observed with lysates of *E.coli* transformed by the expression vector lacking the chicken $p34^{cdc2}$ cDNA insert (Figure 1C, lane 2).

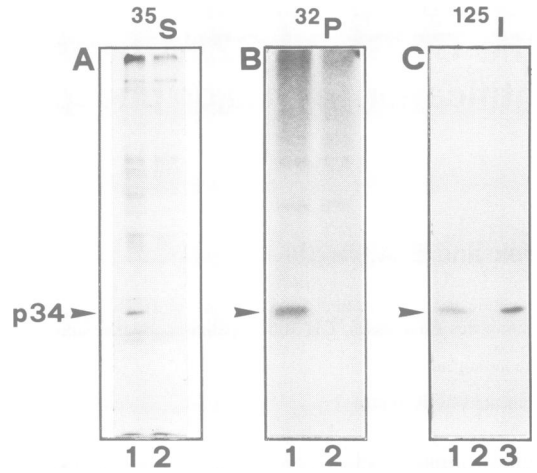

Fig. 1. Specificity of antibodies against chicken $p34^{cdc2}$. Immunoprecipitation of chicken $p34^{cdc2}$ from [^{35}S]methionine (**A**) or [^{32}P]orthophosphate (**B**) labeled DU249 cell lysates. Lanes 1: precipitation with rabbit serum R8. Lanes 2: precipitation with corresponding pre-immune serum. (**C**) Immunoblotting of chicken $p34^{cdc2}$, using affinity purified R8 antibodies. Lane 1: whole DU249 cell lysate. Lane 2: total *E.coli* lysate. Lane 3: total lysate of *E.coli* induced to express chicken cdc2 protein.

Quantitative changes in $p34^{cdc2}$ phosphorylation and histone H1 kinase activity during the cell cycle

In order to determine the state of phosphorylation of $p34^{cdc2}$ at various stages of the cell cycle, DU249 cells were synchronized. For this purpose, they were released for various time periods from either an aphidicolin block at the G1/S boundary or a nocodazole block in M phase. Synchrony of the cultures was monitored by FACS analysis (Figure 2A) and by measuring [^{3}H]thymidine incorporation (Figure 2B). At the times indicated cells were labeled for 4 h with [^{3}P]orthophosphate, $p34^{cdc2}$ was isolated by immunoprecipitation, and its phosphorylation state was assessed by autoradiography and immunoblotting (Figure 2C and D). This analysis shows that, within the limits of temporal resolution of these assays, $p34^{cdc2}$ was phosphorylated at all stages of the cell cycle, although phosphorylation was substantially reduced during M phase (Figures 2C and D).

Next, we correlated the observed quantitative changes in $p34^{cdc2}$ phosphorylation with kinase activity in $p34^{cdc2}$ immunoprecipitates (Figure 3A and B). To demonstrate the presence of similar amounts of $p34^{cdc2}$ protein in the various kinase reactions, aliquots of the individual immunoprecipitates were processed for immunoblotting (Figure 3C and D). Using histone H1 as a substrate, maximal $p34^{cdc2}$ kinase activity was observed during M phase (i.e. 12 h after aphidicolin release or during nocodazole block); no activity could be seen during G1 and G2 phases (Figure 3A and B), and activity during S phase (i.e. at 4 h after aphidicolin release) was marginal (Figure 3A). These results are consistent with previous data on the cell cycle dependent variation of $p34^{cdc2}$ kinase activity in other species. We also note that phosphorylation of a 45 kd protein was readily detectable in M phase arrested cells (Figure 3B, arrowhead); in analogy to results obtained in other systems, we presume that this phosphoprotein represents cyclin B co-precipitating with $p34^{cdc2}$.

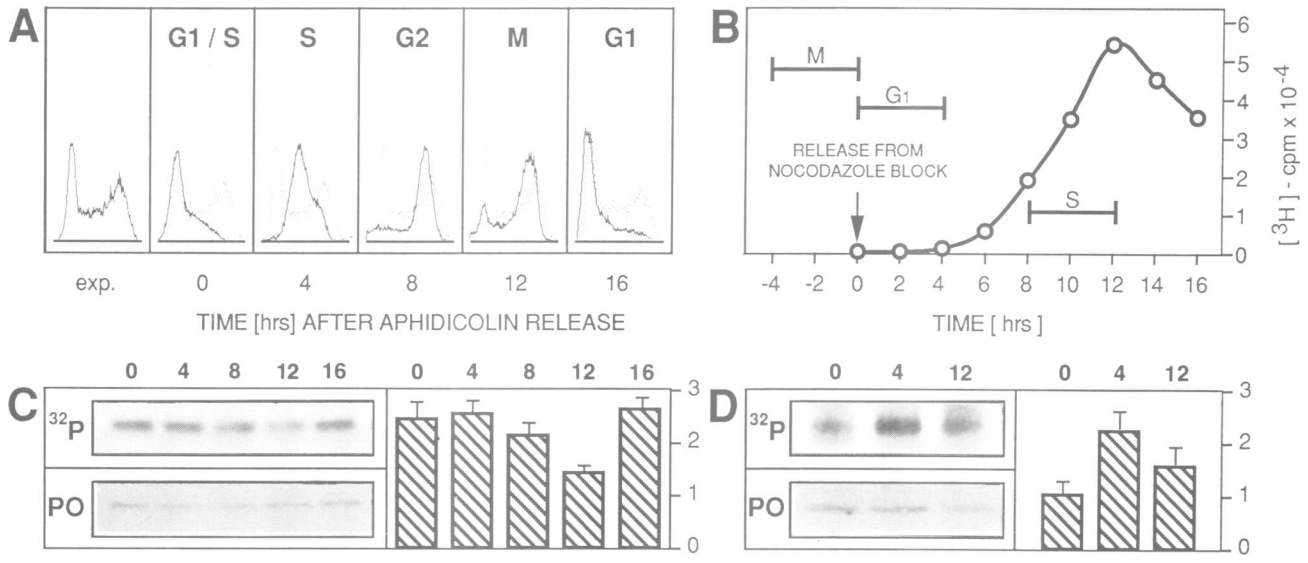

Fig. 2. Quantification of cell cycle dependent phosphorylation of $p34^{cdc2}$ in chicken DU249 cells. (**A**) Flow cytometric (FACS) analysis of cell synchrony. DU249 cells were synchronized by aphidicolin treatment. Following release from aphidicolin arrest, samples were taken at the times indicated, stained with propidium iodide, and analyzed as described in Materials and methods. The observed fluorescence intensity profiles are plotted as continuous lines; for comparison, the fluorescence intensity profiles corresponding to an asynchronously growing population of cells are shown superimposed (dotted lines). Shown are (from left to right) results obtained for an asynchronously growing cell population (exp.), aphidicolin arrested cells (G1/S phase), cells released for 4 h (S phase), 8 h (G2 phase, no mitotic cells visible by phase contrast microscopy), 12 h (M phase; phase contrast microscopy showed detachment of cells from the culture dish), and 16 h (subsequent G1 phase; phase contrast microscopy showed re-attachment of cells to the culture dish). (**B**) DNA synthesis, measured by [^{3}H]thymidine incorporation, after release from a nocodazole arrest. Cells were blocked in M phase by nocodazole treatment for 10 h. They were then collected, washed and replated into fresh medium. At 2 h intervals after nocodazole removal, aliquots of cells were pulse-labeled (for 1 h) with [^{3}H]thymidine. As judged by adherence to the culture dish, all cells had entered G1 phase by 4 h after release. (**C**) and (**D**) Phosphorylation of $p34^{cdc2}$ during the cell cycle. Cells were released from an aphidicolin block (C) or a nocodazole block (D); at the times indicated, aliquots of cells were labeled (for 4 h each) with [^{32}P]phosphate. Lysates were prepared from the various cell populations and equalized for protein content by measurement of OD_{280}. Then $p34^{cdc2}$ was immunoprecipitated with R8 serum. After SDS–PAGE, samples were transferred to nitrocellulose and $p34^{cdc2}$ was visualized by immunoblotting with affinity-purified R8 antibodies, using peroxidase staining (PO); the phosphorylation state of $p34^{cdc2}$ in the very same samples was determined by autoradiography (^{32}P). For quantification of phosphate incorporation into $p34^{cdc2}$, autoradiographs were subjected to laser densitometric scanning. Histograms represent the results normalized to the relative amounts of $p34^{cdc2}$ as determined by immunoblotting (average of two independent experiments).

Identification of four major phosphopeptides in $p34^{cdc2}$ of exponentially growing chicken cells

As a first step toward the identification of individual phosphoacceptor sites in $p34^{cdc2}$, exponentially growing DU249 cells were labelled with [^{32}P]orthophosphate, then $p34^{cdc2}$ was isolated by immunoprecipitation and subjected to both tryptic phosphopeptide mapping and phosphoamino acid analysis. These experiments revealed four major tryptic phosphopeptides in $p34^{cdc2}$ (Figure 4A). Phosphorylation occurred on serine, threonine and tyrosine residues in approximate ratios of 2:1:2 (Figure 4B). Very similar results were obtained also when analyzing $p34^{cdc2}$ isolated from chick embryo fibroblasts or from murine NIH 3T3 cells (not shown). To determine which amino acids were phosphorylated in each of the four phosphopeptides detected in DU249 cells, these were eluted individually, hydrolyzed and subjected to phosphoamino acid analysis. The results of this analysis are summarized in Figure 4C: peptide 1 contained similar amounts of phospho-threonine and phospho-tyrosine, peptide 2 contained predominantly phospho-tyrosine with little phospho-threonine, peptide 3 contained only phospho-serine and peptide 4 contained phospho-threonine with a trace of phospho-serine. As indicated by experiments aimed at phosphosite identification, the trace of phospho-serine in phosphopeptide 4 almost certainly represents a contaminant (results not shown).

Phosphorylation of individual sites in $p34^{cdc2}$ displays differential regulation during the cell cycle

To examine the contribution of each individual phosphopeptide to the observed quantitative changes in $p34^{cdc2}$ phosphorylation (Figure 2), $p34^{cdc2}$ was immunoprecipitated at various stages of the cell cycle and its phosphorylation state determined by both phosphoamino acid analysis (Figure 5A) and phosphopeptide mapping (Figure 5B). These experiments revealed that phosphorylation of all four sites on $p34^{cdc2}$ followed a precise temporal pattern, with phosphorylation of individual sites displaying differential cell cycle regulation: during G1 phase, phosphorylation of $p34^{cdc2}$ occurred almost exclusively on serine (phosphopeptide 3); low levels of phospho-threonine were present (attributable to phosphopeptide 2), but phospho-tyrosine was undetectable. During S phase, serine phosphorylation (on phosphopeptide 3) diminished, while phospho-threonine increased and phospho-tyrosine was first detectable. In G2 phase, we observed no phospho-serine, but extensive phosphorylation on tyrosine and threonine (phosphopeptides 1 and 2). Strikingly, phosphopeptides 1 and 2 completely disappeared upon entry of cells into M phase. Thus, all M phase phosphorylation of $p34^{cdc2}$ could be attributed to phosphopeptides 4 (phospho-threonine) and 3 (phospho-serine).

From this analysis we draw the following conclusions:

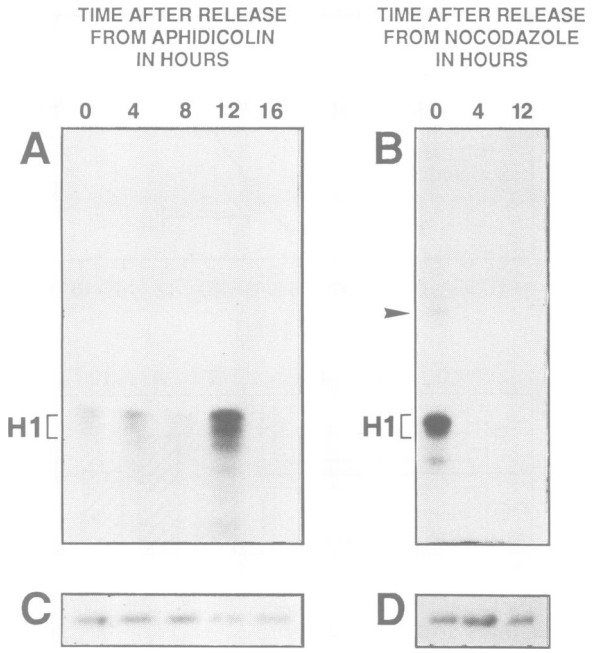

Fig. 3. Cell cycle dependent histone H1 kinase activity in chicken $p34^{cdc2}$ immunoprecipitates. (**A**) and (**B**) Histone H1 kinase activity in $p34^{cdc2}$ immunoprecipitates. Cells were synchronized by aphidicolin at the G1/S boundary or by nocodazole in M phase, and samples were taken at the indicated times after release from these blocks. Following immunoprecipitation of $p34^{cdc2}$ from P-RIPA lysates, *in vitro* kinase reactions were carried out as described in Materials and methods, using histone H1 as a substrate. (**C**) and (**D**) Immunoblots of aliquots of the $p34^{cdc2}$ immunoprecipitates used for the histone H1 kinase assays shown in (A) and (B), respectively. These controls confirm the presence of similar amounts of $p34^{cdc2}$ in the various immunoprecipitates. Immunoblots were probed with affinity-purified R8 antibodies, followed by ^{125}I-labeled secondary antibodies.

first, entry of cells into mitosis is accompanied by dephosphorylation of $p34^{cdc2}$ on both threonine and tyrosine residues (phosphopeptides 1 and 2). Second, G1 phase $p34^{cdc2}$ is phosphorylated largely on serine (phosphopeptide 3). Third, some level of phosphorylation of $p34^{cdc2}$ on threonine persists throughout the cell cycle. However, whereas threonine phosphorylation during S, G2 and M phase is attributable to phosphopeptide 4, during G1 phase it is due to phosphopeptide 2.

Recent studies indicate the existence, in several organisms, of a family of $p34^{cdc2}$ like proteins; these proteins, although structurally related, are not functionally equivalent (Jimenez *et al.*, 1990; Lehner and O'Farrell, 1990b; Paris *et al.*, 1990; see also Pines and Hunter, 1990b). Thus, although only a single $p34^{cdc2}$ protein has so far been detected in somatic chicken cells (Krek and Nigg, 1989), we have considered the possibility that the 34 kd proteins isolated here from cells synchronized at various stages of the cell cycle might represent several immunologically related proteins. Specifically, we were concerned that the 34 kd protein found to be phosphorylated on serine during G1 phase might be distinct from the *bona fide* $p34^{cdc2}$ described earlier (Krek and Nigg, 1989). Two lines of evidence argue against this possibility. First, identical tryptic phosphopeptide maps and phosphoamino acid compositions were determined for the G1 $p34^{cdc2}$ protein, irrespective of whether immunoprecipitations were carried out using the antibody raised against the entire protein expressed in *E.coli* (serum R8),

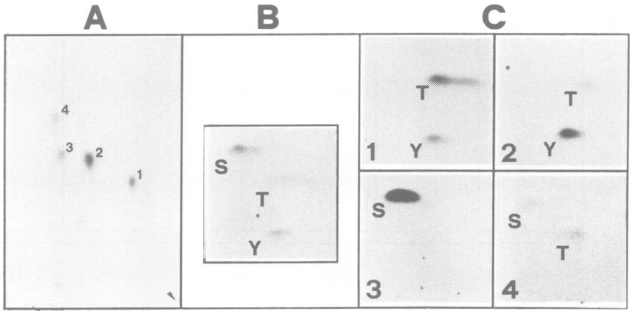

Fig. 4. Tryptic phosphopeptide and phosphoamino acid analysis of chicken $p34^{cdc2}$ from exponentially growing cells. (**A**) Two-dimensional tryptic phosphopeptide map of $p34^{cdc2}$ isolated from [^{32}P]phosphate labeled exponentially growing chicken DU249 cells. A tryptic digest of immunoprecipitated $p34^{cdc2}$ was analyzed by electrophoresis at pH 1.9 in the horizontal dimension (anode to the right), followed by ascending chromatography as described in Materials and methods. Numbers from 1–4 indicate individual tryptic phosphopeptides. Small arrowheads mark the positions of sample application. (**B**) Phosphoamino acid composition of $p34^{cdc2}$ isolated from ^{32}P-labeled exponentially growing DU249 cells. S: phospho-serine; T: phospho-threonine; Y: phospho-tyrosine. (**C**) Phospho-amino acid analysis of the four tryptic phosphopeptides shown in panel A. Individual phosphopeptides were eluted from thin layer plates, hydrolyzed and subjected to phosphoamino acid analysis. The numbering of the panels corresponds to the numbering of the individual phosphopeptides resolved in panel A.

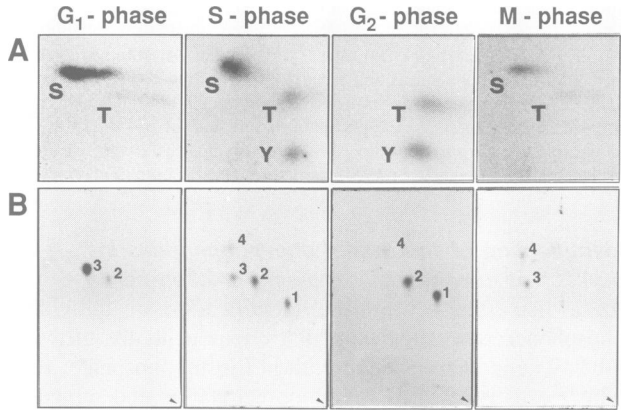

Fig. 5. Analysis of the phosphorylation state of $p34^{cdc2}$ at different cell cycle stages. DU249 cells were synchronized in G1, S, G2 and M phase and labeled with [^{32}P]phosphate as described in the legend to Figure 2. (G1 and S phase samples were prepared using cells released from a nocodazole block, G2 phase samples using cells released from an aphidicolin block, and M phase samples using cells arrested by nocodazole; results obtained when analyzing cells blocked with either aphidicolin or hydroxyurea were indistinguishable from those shown for the S phase sample). Then, $p34^{cdc2}$ was immunoprecipitated and subjected to either phosphoamino acid analysis (**A**) or tryptic phosphopeptide mapping (**B**). S: phospho-serine; T: phospho-threonine; Y: phospho-tyrosine. Numbering of tryptic peptides is as in Figure 4A. Small arrowheads mark the positions of sample application. Note that phospho-threonine and -tyrosine may be somewhat underrepresented in the phosphoamino acid analyses, due to preferential stability of phospho-serine during acid hydrolysis (Cooper *et al.*, 1983).

or the antibody raised against the carboxy-terminus of $p34^{cdc2}$ (serum R6; not shown). Second, as shown in Figure 6, the 34 kd protein recognized by R8 antibody could be quantitatively re-precipitated by R6 antibody, when released from the primary immune complex. For this experiment,

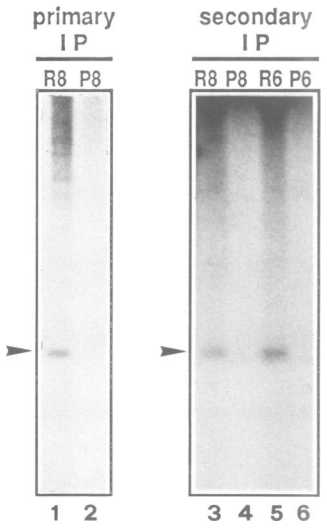

Fig. 6. Sequential immunoprecipitation of chicken p34^{cdc2} from G1 phase cells, using two different antibody reagents. DU249 cells were labeled with [^{32}P]phosphate and synchronized in G1 phase. Then, p34^{cdc2} was isolated by immunoprecipitation using serum R8. An aliquot of this immunoprecipitation was subjected to SDS−PAGE (lane 1); the result obtained with the corresponding pre-immune serum P8 is shown in lane 2. The remainder of the isolated and washed R8 immune complex was then boiled in SDS in order to release p34^{cdc2} (see Materials and methods). Following reconstitution of P-RIPA conditions, the sample was divided into four aliquots and subjected to a second immunoprecipitation, using either R8 (lane 3), P8 (lane 4), antibodies directed against the carboxy-terminus of chicken p34^{cdc2} R6 (lane 5), or the corresponding pre-immune serum P6 (lane 6). The immunoprecipitated p34^{cdc2} is indicated by an arrow.

[^{32}P]phosphate labeled DU249 cells were synchronized in G1 phase and p34^{cdc2} was immunoprecipitated using the antibody (serum R8) raised against the total p34^{cdc2} protein expressed in *E.coli* (Figure 6, lane 1); lack of reactivity of the corresponding pre-immune serum is shown in Figure 6, lane 2. Subsequently, p34^{cdc2} was released from the immunobeads and re-precipitated using either the same R8 antibody (Figure 6, lane 3) or the antibody directed against the 18 residue carboxy-terminus of p34^{cdc2} (Figure 6, lane 5; serum R6). As can be seen, both antibody preparations efficiently re-precipitated the G1 form of p34^{cdc2} (arrowheads), whereas the corresponding pre-immune sera contained no activity (Figure 6, lanes 4 and 6, respectively). These results demonstrate that the G1 form of p34^{cdc2} studied here shares multiple epitopes, including the carboxy terminus, with *bona fide* p34^{cdc2}.

Identification of phosphopeptides 2 and 1 as singly and doubly phosphorylated peptides containing tyrosine 15 and threonine 14

The presence of phosphotyrosine in two tryptic phosphopeptides (1 and 2, see Figure 4C) suggested that either p34^{cdc2} was phosphorylated on two distinct tyrosine residues, or, alternatively, that phosphopeptides 1 and 2 were structurally related. In support of this latter possibility, we note that the relative migrations of phosphopeptides 1 and 2 on thin layer chromatography (TLC) plates were consistent with the idea that phosphopeptide 1 might represent a doubly phosphorylated form of phosphopeptide 2 (see Figure 4A). In addition, the relative electrophoretic migrations of phosphopeptides 1 and 2 in various buffer systems (differing notably in pH), as well as their responses to secondary digestions with chymotrypsin, strongly suggested that they might be structurally related (data not shown). Examination of the theoretical charges predicted for various tryptic peptides led us to suspect that phosphopeptides 1 and 2 might both result from phosphorylation of the amino-terminal peptide IGEGTYGVVYK. (This peptide spans residues 10−20 in chicken p34^{cdc2} and its sequence is conserved from fission yeast to man; Krek and Nigg, 1989). As a preliminary test of this idea, ^{32}P-labeled p34^{cdc2} was immunoprecipitated and cleaved with CNBr, a procedure yielding a 20 kd fragment representing the central part of the protein (residues 86−267). Phosphoamino acid analysis of this fragment revealed only phospho-threonine, indicating that phospho-tyrosine (as well as phospho-serine, see below) must lie within the flanking amino- or carboxy-terminal domains (data not shown). To provide more direct evidence for the identity of phosphopeptides 1 and 2, we synthesized an 18-mer peptide of the structure shown in Figure 7C (hereafter called GEGTYG peptide). In an *in vitro* reaction using immunoprecipitated tyrosine kinase p60^{src}, this peptide could readily be phosphorylated (Figure 7A); as expected, phosphorylation occurred exclusively on tyrosine (Figure 7D). The resulting phosphopeptide was then cleaved with trypsin and subjected to two-dimensional analysis on TLC plates (Figure 7B, panel 1). For comparison, a tryptic phosphopeptide map of p34^{cdc2} immunoprecipitated from exponentially growing chicken cells is shown in Figure 7B, panel 2. As demonstrated by mixing of the two samples, the tryptic fragment derived from the GEGTYG peptide comigrated precisely with phosphospot 2 (Figure 7B, panel 3, arrow).

The presence of roughly equal amounts of phospho-threonine and phospho-tyrosine in phosphopeptide 1 (see Figure 4C) was consistent with the idea that phosphopeptide 1 might represent the doubly phosphorylated form of phosphopeptide 2. To confirm this notion, the two phosphopeptides were isolated separately, and phospho-peptide 1 was then incubated *in vitro* with purified phosphatase 2A (Stone *et al.*, 1987). This enzyme was expected to dephosphorylate phosphopeptide 1 on threonine but not on tyrosine. Dephosphorylation of phosphopeptide 1 on threonine could thus be expected to produce a shift in the migration of the phosphatase 2A treated peptide on TLC plates. Moreover, if the above hypothesis was correct, the shifted peptide was expected to co-migrate precisely with phosphopeptide 2. Figure 8 illustrates that this was indeed observed. For comparison, Figure 8A and B show the migrations on TLC plates of the isolated phosphopeptides 1 (Figure 8A, filled triangle) and 2 (Figure 8B, open triangle). As shown in Figure 8C (open triangle), treatment of phosphopeptide 1 with phosphatase 2A induced the bulk of the material to migrate closer to the cathode, as well as to a more hydrophobic position. Concomitantly, free phosphate (P_i) was released (Figure 8C). Most importantly, as demonstrated by mixing phosphatase 2A treated phospho-peptide 1 with untreated phosphopeptide 2, the two samples comigrated precisely (Figure 8D).

We note that the tryptic GEGTYG peptide contains a single threonine (residue 14) but two different tyrosines (residues 15 and 19; see Figure 7C). To determine which of the two potential tyrosine phosphoacceptor sites was actually utilized, the doubly phosphorylated tryptic

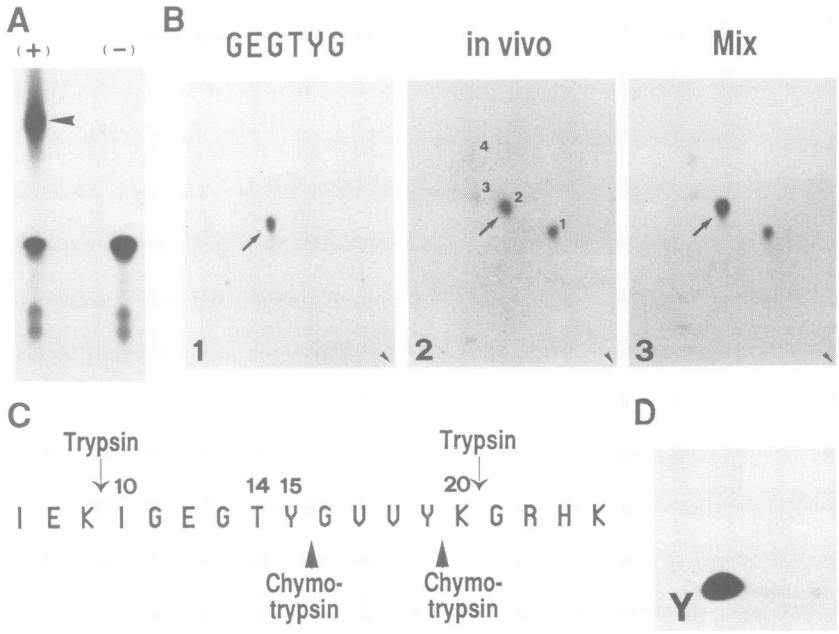

Fig. 7. Identification of the tyrosine phosphorylation site in $p34^{cdc2}$. (**A**) *In vitro* phosphorylation of the synthetic GEGTYG-peptide by immunoprecipitated $p60^{v\text{-}src}$, and subsequent analysis of the phosphorylation product by chromatography in buffer C was carried out as described in Materials and methods. The (+) and (−) signs refer to the presence (+) or absence (−) of peptide substrate in the reaction. The position of the phosphorylated peptide is indicated by an arrowhead; the multiple spots near the positions of sample application are derived from [γ-^{32}P]ATP. (**B**) The phosphorylated GEGTYG-peptide was eluted from chromatography plates and digested with trypsin. The migration of the resulting tryptic peptide on a TLC plate is shown in panel 1 (GEGTYG). For comparison, a tryptic digest of *in vivo* ^{32}P-labeled $p34^{cdc2}$ is shown in panel 2 (*in vivo*). Panel 3 (Mix) illustrates the result of mixing equal amounts (Cerenkov counts) of the tryptic digests of the *in vitro* phorphorylated synthetic peptide and *in vivo* labeled $p34^{cdc2}$. The numbers in panel 2 correspond to those introduced in Figure 4A. The arrows indicate comigration of the tryptic GEGTYG peptide with *in vivo* phosphopeptide 2. (**C**) Sequence of the synthetic peptide corresponding to amino acids 7−26 of chicken $p34^{cdc2}$. Arrows and arrowheads indicate the positions of trypsin and chymotrypsin cleavage sites, respectively. The numbers indicate the positions, from the N-terminus, of the corresponding amino acids in the sequence of chicken $p34^{cdc2}$. (**D**) Phosphoamino acid analysis of the GEGTYG peptide after phosphorylation by $pp60^{v\text{-}src}$. The phosphorylated peptide was eluted from a TLC plate, hydrolyzed and subjected to phosphoamino acid analysis. Y marks the position of phosphotyrosine, as visualized by ninhydrin staining of standards.

phosphopeptide 1 was subjected to secondary digestion with chymotrypsin. Given that this treatment produced a single phospholabeled cleavage product (results not shown), both phospho-threonine and phospho-tyrosine must lie on the same side relative to the chymotrypsin cleavage sites within the GEGTYG peptide (see Figure 7C). These results thus identify Thr 14 and Tyr 15 as the phosphoacceptor sites within the GEGTYG peptide.

Based on these results we assign the major phosphorylated residue in phosphopeptide 2 to Tyr 15; phosphopeptide 1 then corresponds to the same tryptic peptide but contains phosphate groups on both Tyr 15 and Thr 14. Interestingly, phosphospot 2 also contains significant amounts of phospho-threonine (see Figure 4), indicating that it actually consists of a mixture of two phosphopeptides, one (the quantitatively predominant species) being phosphorylated exclusively on Tyr 15, the other (the minor species) being phosphorylated exclusively on Thr 14.

Identification of serine 277 as the phosphorylated residue in phosphopeptide 3

Based on CNBr cleavage experiments and electrophoresis of phosphopeptide 3 at different pH values (results not shown), we concluded that the major site of serine phosphorylation must reside in the carboxy-terminus of $p34^{cdc2}$, i.e. downstream of residue 267. Consideration of sequence conservation among different species focused our attention on a predicted tryptic peptide flanking serine residue 277. To examine whether or not phosphopeptide 3 might arise from phosphorylation of this residue, we synthesized a peptide of the structure AKRISGKMA (Figure 9D). This peptide could readily be phosphorylated *in vitro* with purified protein kinase C (Marais and Parker, 1989) (Figure 9A, lane 1), a result consistent with the known substrate requirements (i.e a preference for basic residues flanking the target site) of this kinase (Kemp and Pearson, 1990). No phosphorylation occurred when peptide was omitted from the kinase reaction (Figure 9A, lane 2). However, when the *in vitro* phosphorylated peptide was eluted, digested with trypsin and analyzed by electrophoresis, we did not observe the shift in migration that would have been expected to result from removal of two positive charges by trypsin (Figure 9B, compare lanes 1 and 2). We interpreted this result to indicate that *in vitro* phosphorylation interfered with trypsin cleavage of the peptide at one of the expected sites (i.e. between R and I, see Figure 9D). To circumvent this problem, we digested the AKRISGKMA peptide with the endoproteinase Arg-C prior to *in vitro* phosphorylation. Although the cleaved peptide was a comparatively poor substrate, it could still be phosphorylated by protein kinase C (Figure 9A, lane 3), and, as expected, phosphorylation occurred on serine (Figure 9E). Following elution of the cleaved phosphorylated peptide, it was digested with trypsin and analyzed by electrophoresis; this time the expected shift in migration (due to loss of two positive charges) could readily be observed (Figure 9B, compare lanes 2 and 3). The migration of the phosphorylated ISGK peptide on a TLC plate is illustrated in Figure 9C, panel 1. For comparison, the migration of

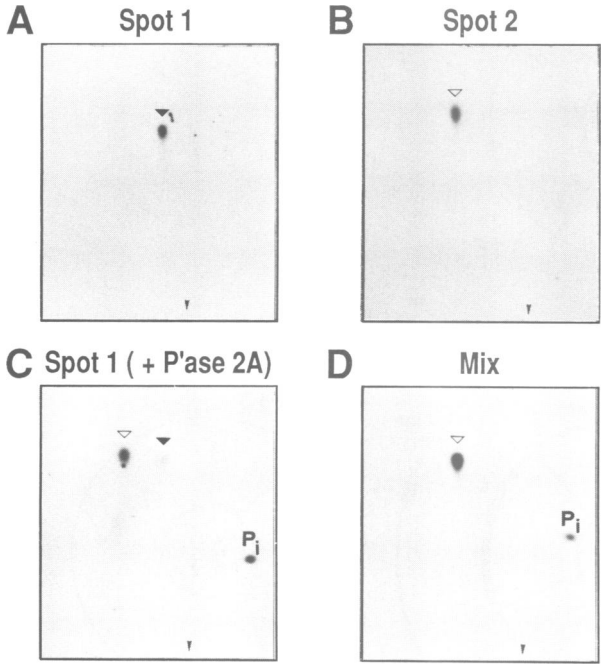

Fig. 8. Phosphatase 2A treatment converts phosphopeptide 1 to phosphopeptide 2. (**A**) and (**B**). To examine the relationship between the two phospho-threonine and phospho-tyrosine containing tryptic peptides 1 and 2 (see Figure 4), these were isolated separately from TLC plates and re-run. Their migrations are marked by a filled triangle (Spot 1) and open triangle (Spot 2), respectively. Phosphopeptide 1 was then treated for 20 min with purified phosphatase 2A (Stone *et al.*, 1987); as demonstrated in panel **C**, this dephosphorylation resulted in a shift of the phosphopeptide toward the cathode, and it increased the peptide's hydrophobicity [compare the migration of the phosphatase 2A treated peptide (open triangle) with that of residual fully phosphorylated phosphopeptide 1 (filled triangle)]. As illustrated by mixing equal Cerenkov counts of Spot 2 (panel B) and phosphatase 2A treated Spot 1 (panel C), the two samples comigrated exactly (**D**). The spot labeled P_i in panels C and D indicates free phosphate released from phosphopeptide 1 by phosphatase 2A treatment. Little arrowheads mark the positions of sample application.

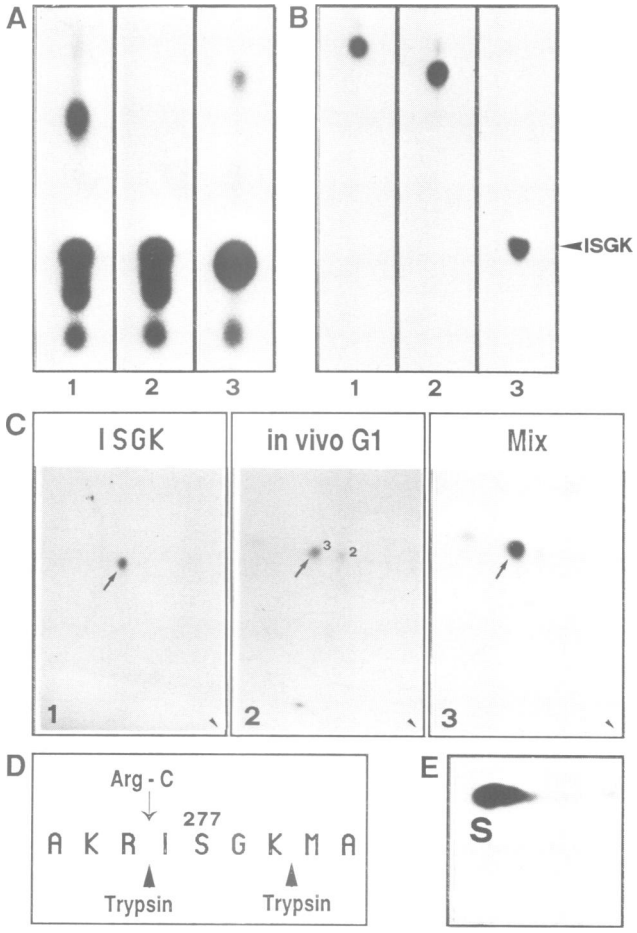

Fig. 9. Identification of serine 277 as the major phosphoacceptor site of G1 phase p34cdc2. (**A**) The synthetic peptide AKRISGKMA (lane 1) as well as the endoproteinase Arg-C cleavage product ISGKMA (lane 3) were phosphorylated *in vitro* with purified protein kinase C (Marais and Parker, 1989) and analyzed by chromatography as described in the legend to Figure 7A. For control, lane 2 shows the result of carrying out the reaction in the absence of peptide substrate. (**B**) The phosphorylated peptide AKRISGKMA (shown in panel A, lane 1) was eluted from the TLC plate and subjected to electrophoresis at pH 1.9 either before (lane 1) or after (lane 2) digestion with trypsin. Note the absence of a dramatic shift in electrophoretic mobility in response to trypsin treatment (see text for further explanation). Lane 3 shows the result of electrophoretic analysis of the phosphorylated peptide ISGKMA after trypsin digestion. The migration of the resulting phosphorylated peptide (ISGK) is marked by an arrowhead. (**C**) The migration of the phosphorylated ISGK peptide on a TLC plate is shown in panel 1 (ISGK). For comparison, panel 1 (*in vivo*) illustrates the phosphopeptide map of p34cdc2 ^{32}P-labeled *in vivo* during G1 phase. The result of mixing equal amounts (Cerenkov counts) of the tryptic digests shown in panels 1 and 2 is illustrated in panel 3 (Mix). Note the exact comigration of the ISGK peptide with phosphopeptide 3 (indicated by arrows). Little arrowheads mark the positions of sample application. (**D**) Sequence of the synthetic peptide corresponding to amino acids 273–281 of chicken p34cdc2. Arrowheads indicate the positions of trypsin cleavage sites and the arrow points to the single endoproteinase Arg-C cleavage site. The number indicates the position of the serine residue in the amino acid sequence. (**E**) Phosphoamino acid analysis after phosphorylation of the endoproteinase Arg-C cleavage product ISGKMA by protein kinase C. The phosphorylated peptide was eluted from a TLC plate, hydrolyzed and analyzed as described in the legend to Figure 4B. S marks the position of phospho-serine, as visualized by ninhydrin staining of standards.

phosphopeptide 3 (obtained from a G1 cell population) is shown in Figure 9C, panel 2. As can be seen from the result of mixing the two samples (Figure 9C, panel 3), the phosphorylated ISGK peptide comigrated precisely with phosphopeptide 3 obtained from *in vivo* labeled p34cdc2. From these results we conclude that G1 phase serine phosphorylation of p34cdc2 occurs on residue 277. Implicit in this assignment is the assumption that trypsin did cleave the intact p34cdc2 protein at the expected site amino-terminal of Ser 277 *in vivo*, although it did not cleave the phosphorylated synthetic AKRISGKMA peptide *in vitro*. Such a differential specificity may be explained by conformational differences between protein and peptide.

Discussion

We have shown that in exponentially growing chicken cells, p34cdc2 is phosphorylated on four major sites. Three of these sites were identified as Thr 14, Tyr 15 and Ser 277, respectively. As yet, we have not been able to identify unequivocally the site corresponding to phosphopeptide 4. Results of sequential cleavage of isolated tryptic phosphopeptide 4 with *N*-chloro-succinimide and V8 protease led us to conclude that this phosphopeptide almost certainly contains Thr 161 as the phosphorylated residue (W.Krek and E.A.Nigg, unpublished results). So far, it has not been possible to obtain direct proof because a synthetic peptide

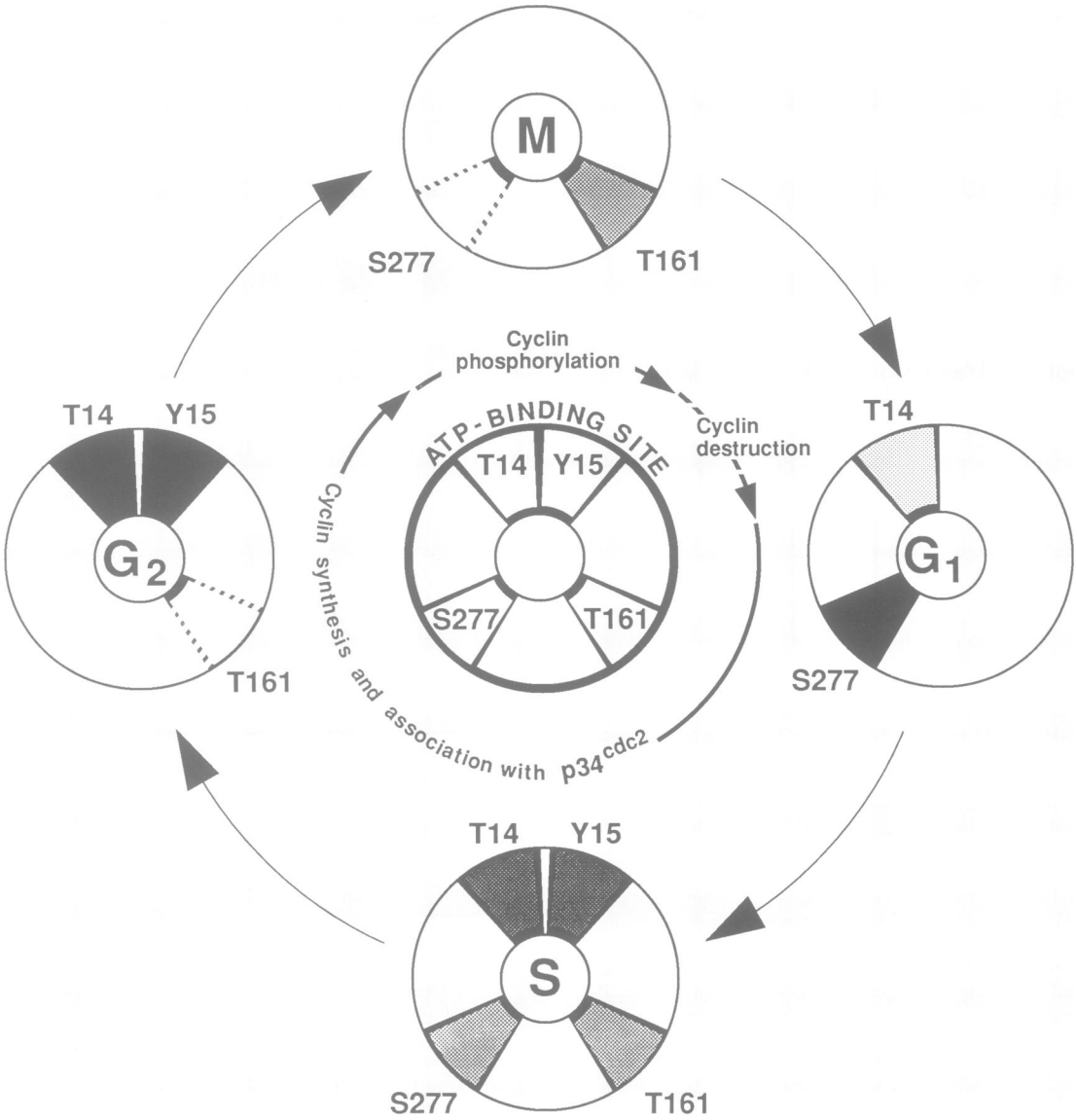

Fig. 10. Diagram summarizing the cell cycle dependent phosphorylation of $p34^{cdc2}$. This representation draws attention to the likely involvement of Thr 14 and Tyr 15 in ATP binding, and it includes a simplified summary of the interactions between $p34^{cdc2}$ and cyclin proteins during the cell cycle. Extensive phosphorylation of any particular site is indicated in black, whereas various degrees of shading are meant to provide a semi-quantitative representation of substoichiometric phosphorylations. Broken lines are used to indicate that the apparent phosphorylation of Thr 161 during G2 phase and of Ser 277 during M phase may be due to contamination of the corresponding samples by cells from adjacent stages in the cell cycle. We note that identification of Thr 161 as a phosphorylation site is tentative; also, if phosphorylation of this site were due to an autocatalytic reaction, the observed low levels of phosphorylation might lead to an underestimate of the actual phosphorylation stoichiometry. Analogy to results obtained with protein kinase A (Shoji *et al.*, 1981) would suggest that phosphate groups added by autophosphorylation might display a low turnover; this in turn would lead to inefficient labeling *in vivo*. For further discussion, see text.

mimicking the putative Thr 161 site was completely insoluble. Thus, definitive identification of the fourth phosphorylation site in vertebrate $p34^{cdc2}$ may have to await results of site-directed mutagenesis.

Based on the analysis of $p34^{cdc2}$ isolated from chicken cells synchronized at various stages of the cell cycle, we have further shown that phosphorylation of all four sites on $p34^{cdc2}$ is cell cycle regulated. To facilitate the subsequent discussion, the major results of this cell cycle analysis are summarized schematically in Figure 10. Although this scheme includes information of a semi-quantitative nature, we note that we do not presently know the exact stoichiometry of $p34^{cdc2}$ phosphorylation, nor the rate of turnover of phosphate at the various sites. Also, we emphasize that no quantitative studies have so far been reported on the partitioning of $p34^{cdc2}$ among the various protein complexes known to co-exist in the cell (see Introduction). To address the question to what extent the differentially phosphorylated $p34^{cdc2}$ proteins described here interact preferentially with one or the other complex partner, we are currently raising antibodies against chicken cyclins and suc1 proteins.

Recently, proteins closely related to $p34^{cdc2}$ have been identified in both invertebrate (Lehner and O'Farrell, 1990b; Jimenez *et al.*, 1990) and vertebrate species (Paris *et al.*, 1990; see also Pines and Hunter, 1990b). These observations raise the possibility that structurally distinct $p34^{cdc2}$-like proteins may carry out different functions during the cell cycle. In this context, we emphasize that the serine phosphorylated 34 kd protein isolated from G1 cells

was recognized equally well by antibodies raised against either the entire chicken p34^{cdc2} gene product expressed in *E.coli*, or the 18 carboxy-terminal amino acids of p34^{cdc2} (Krek and Nigg, 1989). Given that all *cdc2*-related gene products identified so far differ in their carboxy-termini from the corresponding functional cdc2 homologs (Lehner and O'Farrell, 1990b; Paris *et al.*, 1990; Pines and Hunter, 1990b), we are confident that the 34 kd protein studied here is most probably the product of a single gene.

Activation of p34^{cdc2} at the G2/M transition

Several studies have emphasized the importance of tyrosine dephosphorylation for activation of the p34^{cdc2}–cyclin complex at the G2/M transition (Dunphy and Newport, 1989; Gould and Nurse, 1989; Morla *et al.*, 1989). The recent identification of residue 15 as the tyrosine phosphoacceptor site in p34^{cdc2} of *S.pombe* provides a rationalization for the inhibitory effect of tyrosine phosphorylation (Gould and Nurse, 1989). Tyr 15 is in fact located within a characteristic motif (GXGXXG) implicated in ATP binding by protein kinases (Hanks *et al.*, 1988), and it is plausible that phosphorylation of this residue might interfere with kinase function. Consistent with this view, mutation of Tyr 15 to a non-phosphorylatable residue caused *S.pombe* cells to enter mitosis prematurely (Gould and Nurse, 1989).

Although no evidence was obtained for additional inhibitory phosphorylation events in *S.pombe*, studies in vertebrates have suggested that dephosphorylation of tyrosine alone is insufficient for activation of p34^{cdc2} (Morla *et al.*, 1989). The identification of Thr 14 as an additional phosphorylation site in vertebrate p34^{cdc2} (this study, and Norbury,C.J., Blow,J.J. and Nurse,P., personal communication) provides an explanation for these observations. Given that both Thr 14 and Tyr 15 lie within the proposed ATP binding site of p34^{cdc2} kinase, it may be expected that dephosphorylation of both residues is required for full activation of the vertebrate p34^{cdc2} kinase. Interestingly, phosphospot 2 was found to contain a mixture of peptides phosphorylated on either Thr 14 or Tyr 15 in a mutually exclusive way. This indicates that phosphorylation of the two sites does not occur according to an obligatory sequence of events. On the other hand, considering that in G1 samples we detected phosphorylation of Thr 14 but not of Tyr 15 (see Figure 5), Thr 14 phosphorylation may begin at an earlier stage of the cell cycle than Tyr 15 phosphorylation.

What is the role of G1 phosphorylation of serine 277?

Although in earlier studies no evidence was reported for phosphorylation of p34^{cdc2} during G1 phase (Draetta and Beach, 1988), chicken p34^{cdc2} was found to be highly phosphorylated during this stage of the cell cycle. G1 phosphorylation occurred almost exclusively on a single, evolutionarily conserved residue (Ser 277). The function of this modification remains to be elucidated, but several interesting possibilities come to mind. Despite its prominence in G1 phase cells, Ser 277 phosphorylation might relate to the well established G2/M function of p34^{cdc2}. For instance, phosphorylation of Ser 277 might inactivate the cdc2 kinase upon exit from mitosis and keep the kinase inactive until appropriate stimuli reactivate it for the subsequent cell cycle. Alternatively, it is most intriguing to consider phosphorylation of Ser 277 in the context of the proposed function of p34^{cdc2} at the G1/S transition. In support of a role prior to S phase, phosphorylation of p34^{cdc2} on serine was observed also in NIH 3T3 cells which were stimulated to exit from quiescence; in these experiments, serine phosphorylation was shown to precede DNA replication (Lee *et al.*, 1988; Norbury and Nurse, 1990).

Irrespective of whether phosphorylation of Ser 277 plays a role in p34^{cdc2} function during G1/S, S or G2/M phases, it is attractive to propose that this modification may regulate interactions between p34^{cdc2} and other proteins, e.g. A- or B-type cyclins, the *suc1* gene product, or, if they exist in higher vertebrates, homologs of the *S.cerevisiae* G1 cyclins. That the phosphorylation state of p34^{cdc2} does contribute to control protein–protein interactions is strongly suggested by circumstantial evidence from several other systems (e.g. Pines and Hunter, 1989, 1990b; Giordano *et al.*, 1989). With the availability of appropriate mutants and antibody reagents it should in future be possible to test this notion directly.

Is M phase phosphorylation of p34^{cdc2} autocatalytic?

Previous studies were widely interpreted to indicate that p34^{cdc2} is completely dephosphorylated during mitosis (Gautier *et al.*, 1989; Labbé *et al.*, 1989). Contrary to this view, we detect a low but significant level of phosphorylation of p34^{cdc2} during M phase (see Figures 2 and 5). This M phase phosphorylation could be attributed to two sites, Ser 277 and a threonine residue (phosphopeptide 4) which most likely corresponds to Thr 161 (see above). While it is difficult to exclude rigorously the possibility that M phase phosphorylation of Ser 277 may be due to contamination of M phase cells by G1 phase cells, we are confident that M phase phosphorylation of phosphopeptide 4 (presumably Thr 161) is real and functionally significant. This view is strongly supported by the observation that mutation of the corresponding residue (Thr 167) in *S.pombe* inactivates p34^{cdc2} (Booher and Beach, 1986), and that Thr 167 is phosphorylated in *S.pombe* (K.L.Gould and P.Nurse, personal communication).

Thr 161 is located within a domain which in other kinases is subject to autophosphorylation (Shoji *et al.*, 1979, 1981; Snyder *et al.*, 1983; Weinmaster *et al.*, 1984). In support of the possibility that phosphorylation of Thr 161 may be autocatalytic in p34^{cdc2}, we observed a correlation between strong phosphorylation of phosphopeptide 4 and histone H1 kinase activity in p34^{cdc2} immunoprecipitates (W.Krek and E.A.Nigg, unpublished results). Phosphorylation of Thr 161 might thus exert a positive influence on p34^{cdc2} kinase activity and contribute to the autocatalytic activation of p34^{cdc2} at the G2/M transition.

In conclusion, we have identified four major cell cycle regulated phosphopeptides in vertebrate p34^{cdc2}. Three phosphorylation sites were mapped to Thr 14, Tyr 15, and Ser 277, respectively. The fourth site most probably corresponds to Thr 161 (W.Krek and E.A.Nigg, unpublished results). All of these phosphorylation sites are highly conserved in evolution (Krek and Nigg, 1989). Thr 14 is phosphorylated during S and G2 phase, and, in parallel with Tyr 15, is dephosphorylated upon activation of the cdc2 kinase at the G2/M transition. Accordingly, it appears that full activation of cdc2 kinase in vertebrates may require release from a double-block imposed by phosphorylation of

both Thr 14 and Tyr 15. However, we emphasize that $p34^{cdc2}$ is phosphorylated also during M phase. This M phase phosphorylation occurs on a threonine residue distinct from Thr 14 (probably Thr 161) and may be due to an autocatalytic reaction. Finally, we demonstrate that phosphorylation of $p34^{cdc2}$ on Ser 277 is maximal during G1 phase. This latter finding raises the possibility that serine phosphorylation may contribute to control a function of $p34^{cdc2}$ at an early stage of the cell cycle, possibly the G1/S transition. Our results set the stage for a future mutational analysis aimed at elucidating the role of phosphorylation in regulating $p34^{cdc2}$ function during the cell cycle. In addition, they may allow us to design specific substrates for a biochemical characterization of the various kinases and phosphatases acting on $p34^{cdc2}$.

Materials and methods

Cell culture and virus infection

Rous sarcoma virus (RSV) strain PR-A (Sefton *et al.*, 1980) was kindly provided by Dr B.Sefton (Salk Institute, San Diego, CA). Virus stocks for infection of cultured cells were prepared by collecting medium from fully infected cultures of chicken embryonic fibroblasts (CEF), followed by passage through a 0.4 μm milipore filter and freezing at −70°C in 1 ml aliquots. Virus-free (VALO-SPF) chicken eggs were obtained from Lohmann Tierzucht Ges.M.B.H (Cuxhaven FRG) and incubated at 37°C. CEF were prepared by trypsinization of skins obtained from 11 day old chicken embryos and cultured in DMEM supplemented with 2% fetal calf serum, 1% chicken serum, 2% tryptose phosphate broth and 1% glutamine and antibiotics (penicillin/streptomycin, 100 U/ml). Chicken hepatoma cells (DU249; Langlois *et al.*, 1974) were cultured as reported previously (Nakagawa *et al.*, 1989).

For transformation by RSV, CEF were seeded at low density into 60 mm dishes 6 h prior to infection. Then, the medium was removed, 400 μl of virus stock was added, and virus absorption was carried out for 30 min at 37°C in the CO_2 incubator. Subsequently, fresh medium was added and the cultures were grown for 2−3 days. When morphological transformation was apparent, cells were trypsinized, reseeded at low density, and cultured for an additional 3−4 days. At that time transformation was normally complete.

Cell cycle synchronization and metabolic labeling

Cells were synchronized at the G1/S boundary by the addition of 4 μg/ml aphidicolin (Sigma; 100× stock solutions in DMSO) for 12 h. To release cells from the aphidicolin block, cultures were rinsed three times with phosphate buffered saline [PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2HPO_4, (pH 7.2)], followed by addition of fresh medium. The cell cycle synchrony of the various cultures was monitored by flow cytometric (FACS) analysis. For this purpose, 10^6 cells were washed twice in ice-cold PBS and resuspended in 0.3 ml of PBS containing 1% fetal calf serum, 0.1% glucose and 0.3 mM EDTA. The cells were then fixed by slow addition of 0.9 ml of 95% ethanol (−20°C) while vortexing. After fixation, cells were washed twice in cold PBS and finally resuspended in 0.5 ml PBS. Ten microliters of a 10 μg/ml solution of boiled RNase A (Sigma) and 50 μl of a 50 μg/ml propidium iodide solution in 50 mM sodium citrate (pH 7.6) were then added. After incubation for 30 min on ice the fluorescence intensities of the samples were measured, using a FACS II (Becton & Dickinson) instrument.

For synchronization at metaphase, cells were incubated for 10 h with 500 ng/ml nocodazole (Janssen, added from a 100× stock in DMSO), and mitotic cells were collected by gentle pipetting. To release them from the nocodazole block, they were washed twice in medium and replated in fresh medium. As judged by re-attachment to the culture dishes, cells entered G1 phase by 4 h after release from the nocodazole block. DNA synthesis at various times after release was determined by 1 h pulse labeling with 5 μCi/ml [^{3}H]thymidine (Amersham), as described elsewhere (Freytag, 1988).

For *in vivo* labeling of proteins with [^{35}S]methionine or [^{32}P]inorganic phosphate, subconfluent 10 cm dishes of DU249 cells were incubated for 4 h in 5 ml of either methionine-free DMEM containing 10% dialyzed fetal calf serum, 1% dialyzed chicken serum and 0.1 mCi/ml [^{35}S]methionine (Amersham) or phosphate-free DMEM containing 10% dialyzed fetal calf serum, 1% dialyzed chicken serum and 0.8 mCi/ml [^{32}P]orthophosphate (Amersham).

Preparation and affinity purification of anti-chicken $p34^{cdc2}$ antibodies

Full-length chicken $p34^{cdc2}$ was overexpressed in *E.coli* (Krek and Nigg, 1989), using the T7 polymerase expression system (Studier and Moffatt, 1986), and the protein was purified essentially as described by Potashkin and Beach (1988). For intramuscular injection into rabbits, purified $p34^{cdc2}$ was emulsified with either Freund's complete adjuvant (for the first injection) or with incomplete adjuvant (for all subsequent injections). Approximately 0.5−1.0 mg of $p34^{cdc2}$ was administered at 2 week intervals, for a total of 5 months. Bleedings were done at biweekly intervals.

For affinity purification of antibodies, 1 mg of bacterially expressed, purified $p34^{cdc2}$ was covalently coupled to CNBr-activated Sepharose 4B (Pharmacia), as described by the manufacturers. Following pre-equilibration of the affinity matrix in 0.5% Tween 20 in PBS, 3 ml of immune serum was added and the sample was gently agitated overnight at 4°C. Then the material was poured into a column (BioRad Econocolumn, 0.5 × 5 cm) and washed twice with 10 ml of 0.5% Tween 20 in PBS, followed by 40 ml of PBS. Specifically bound antibodies were eluted with 0.1 M glycine−HCl (pH 2.5), and 0.75 ml fractions were immediately neutralized by the addition of 0.25 ml of 1 M K_2HPO_4.

Immunochemical techniques

Immunoblotting of proteins was carried out as described previously (Krek and Nigg, 1989). For immunoprecipitation, cells from a 10 cm dish were washed twice in ice-cold PBS and immediately lysed in 1 ml of phosphate-buffered RIPA [P-RIPA; 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 20 mM sodium phosphate (pH 7.2), 100 mM NaCl, 20 mM NaF, 0.3 mM sodium orthovanadate, 0.02% NaN_3] containing 1% Trasylol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 70 μg/ml peptstatin A, 30 μg/ml DNase I and 30 μg/ml RNase A. After 30 min on ice, extracts were centrifuged for 10 min at 15 000 *g*, and supernatants were pre-incubated for 60 min at 4°C with 50 μl of a 50% (v/v in P-RIPA) suspension of protein A−Sepharose (Pharmacia), followed by centrifugation. Where appropriate, aliquots of the supernatants were removed for protein quantification. Then 4 μl of serum R8 or 8 μl of serum R6 were added to the lysates. After incubation on ice for 1 h (R8 serum) or 8 h (R6 serum), 50 μl of protein A−Sepharose was added and incubation was continued for another hour on ice. Immune complexes were collected by centrifugation and washed four times in P-RIPA, and once in 50 mM Tris−HCl, pH 7.5. Pellets were resuspended in 40 μl of 3× gel sample buffer [60 mM Tris−HCl (pH 6.8), 10% glycerol, 2% SDS, 5% (v/v) β-mercaptoethanol, 0.01% bromophenol blue], boiled for 10 min, centrifuged and analyzed on 10% SDS−polyacrylamide gels. Alternatively, for secondary immunoprecipitations, pellets were boiled for 7 min in 100 μl of 50 mM Tris−HCl (pH 8.0), 1% SDS, 1 mM DTT, followed by addition of 900 μl SDS-free P-RIPA. Such samples were then treated with antisera and immune complexes were isolated as described above. Polyacrylamide gels were either dried and exposed for autoradiography or subjected to immunoblotting as described (Krek and Nigg, 1989).

Immunoprecipitation of $p60^{v\text{-}src}$ from PR-RSV-A transformed CEF was carried out essentially as described above, except that monoclonal antibody 327 (Lipsich *et al.*, 1983) was used and that immune complexes were collected with goat anti-mouse IgG coupled to agarose (Sigma).

In vitro kinase assay

Immunoprecipitates of $p34^{cdc2}$ were prepared as described above, and immune complexes were washed three times in kinase assay buffer (50 mM Tris−HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT). Kinase reactions were carried out in a total volume of 50 μl in assay buffer supplemented with 4 μM ATP, 400 μg/ml of histone H1 (Sigma) and 20 μCi of [γ-^{32}P]ATP (Amersham). Reactions were initiated by the addition of ATP, incubated at 30°C for 20 min, and stopped by the addition of 50 μl of 3× gel sample buffer.

In vitro phosphorylation of synthetic peptides

Peptides to be used for *in vitro* phosphorylation assays were prepared by solid phase synthesis. They were used at a final concentration of 0.5 mM in total reaction volumes of 30 μl. Phosphorylation of the AKRISGKMA and ISGKMA peptides were carried out with purified protein kinase C (Marais and Parker, 1989) as described previously (Peter *et al.*, 1990). *In vitro* phosphorylation of the GEGTYG-peptide with immunoprecipitated $p60^{v\text{-}src}$ was done as described by Gould and Nurse (1989). Reactions were stopped by the addition of 15 μl of acetic acid, and 1 μl of each sample was analyzed on Merck TLC plates (Nr5716) by either electrophoresis in

buffer B (acetic acid, formic acid and H_2O at a ratio of 195:65:1040) or chromatography in buffer C (isobutyric acid, pyridine, acetic acid, butanol and H_2O at a ratio of 65:5:3:2:29).

Purification of phosphopeptides for phosphatase and chymotrypsin treatments

Purification of phosphopeptides 1 and 2 for phosphosite identification was carried out as follows: ^{32}P-labeled peptides 1 and 2 were prepared by tryptic digestion of immunoprecipitated p34^{cdc2} and resolved on cellulose TLC plates. Following localization by autoradiography, they were scraped from the TLC plates and eluted with 500 μl of buffer B (see above). The eluates were spun for 15 min at 12 000 *g*, and the supernatants were dried by centrifugal evaporation using a Speedvac. Peptides were then re-dissolved (in 500 μl of H_2O) and re-dried for twice more, and finally dissolved in buffers suitable for either phosphatase treatments or chymotrypsin digestions.

For phosphatase treatment, phosphopeptide 1 was dissolved in 30 μl substrate buffer [50 mM Tris−HCl (pH 7.5), 1 mM EDTA, 0.1% β-mercaptoethanol], and the phosphatase reaction was carried out as follows: 2 μl of phosphatase 2A purified from rabbit skeletal muscle (Stone *et al.*, 1987) was diluted 1:10 in substrate buffer containing 1 mg/ml BSA, and, following the addition of 20 μl of substrate buffer containing 3 mM $MnCl_2$, the phosphatase was incubated for 2 min at 30°C. The reaction was then started by adding 20 μl of purified phosphopeptide 1 substrate. After 10 and 20 min, respectively, 30 μl aliquots were removed and mixed with 10 μl acetic acid. Individual samples were lyophilized, taken up in 500 μl buffer B and dried. They were then resuspended in 5 μl of buffer B and spotted on TLC plates for two-dimensional analysis (Peter *et al.*, 1990).

For chymotrypsin digestion, purified phosphopeptides 1 or 2 were incubated with 3 μg chymotrypsin (Boehringer Mannheim) in 50 μl of ammonium bicarbonate (pH 8.0) at 37°C for 5 h. Endoproteinase Arg-C digestion of the synthetic peptide AKRISGKMA was done under the same conditions, except that digestion was carried out for 8 h. After addition of 500 μl H_2O, peptides were dried by centrifugal evaporation (Speedvac).

Miscellaneous techniques

Protein was quantified using the BioRad protein determination assay, with bovine serum albumin (BSA) as a standard. Tryptic phosphopeptide mapping and phosphoamino acid analyses were carried out exactly as described in Peter *et al.* (1990). For charge estimation of phosphorylated peptides electrophoresis was carried out at pH 6.5 in 10% (v/v) pyridine, 0.5% (v/v) acetic acid. For CNBr cleavage, immunoprecipitated *in vivo* ^{32}P-labelled p34^{cdc2} was resolved by SDS−PAGE, eluted and precipitated as described (Peter *et al.*, 1990). Lyophilized proteins were resuspended in 90 μl 70% formic acid. Cleavage was initiated by addition of 10 μl CNBr from a 100 mg/ml stock in 70% formic acid and allowed to proceed for 16 h at room temperature in the dark. The sample was then lyophilized, resuspended in 500 μl of H_2O, redried, taken up in 50 μl 3× sample buffer and analyzed by SDS−PAGE on a 15% gel. Subsequent phosphoamino acid analysis of the 20 kd CNBr cleavage product of p34^{cdc2} was as described above.

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References

Beach,D., Durkacz,B. and Nurse,P. (1982) *Nature*, **300**, 706−709.
Blow,J.J. and Nurse, P. (1990) *Cell*, **62**, 855−862.
Booher,R. and Beach,D. (1986) *Mol. Cell Biol.*, **6**, 3523−3530.
Booher,R., Alfa,C., Hyams,J. and Beach,D. (1989) *Cell*, **58**, 485−497.
Brizuela,L., Draetta,G. and Beach,D. (1987) *EMBO J.*, **6**, 3507−3514.
Cooper,J.A., Sefton,B.M. and Hunter,T. (1983) *Methods Enzymol.*, **99**, 387−402.
Cross,F., Roberts,J. and Weintraub,H. (1989) *Annu. Rev. Cell Biol.*, **5**, 341−395.
Draetta,G. (1990) *Trends Biochem. Sci.*, **15**, 378−383.
Draetta,G.and Beach,D. (1988) *Cell*, **54**, 17−26.
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.
Dunphy,W.G. and Newport,J.W. (1989) *Cell*, **58**, 181−191.
D'Urso,G., Marraccino,R.L., Marshak,D.R. and Roberts,J.R. (1990) *Science*, **250**, 786−791.
Evans,T., Rosenthal,E.T., Youngbloom,J., Distel,D. and Hunt,T. (1983) *Cell*, **33**, 389−396.
Freytag,S.O. (1988) *Mol. Cell Biol.*, **8**, 1614−1524.
Félix,M.-A., Labbé,J.-C., Dorée,M., Hunt,T.and Karsenti,E. (1990a) *Nature*, **346**, 379−382.
Félix,M.-A., Cohen,P. and Karsenti,E. (1990b) *EMBO J.*, **9**, 675−683.
Gautier,J., Matsukawa,T., Nurse,P. and Maller,J. (1989) *Nature*, **339**, 626−629.
Gautier,J., Minshull,J., Lohka,M., Glotzer,M., Hunt,T. and Maller,J.L. (1990) *Cell*, **60**, 487−494.
Giordano,A., Whyte,P., Harlow,E., Franza,Jr.,B.R., Beach,D. and Draetta,G. (1989) *Cell*, **58**, 961−990.
Gould,K.L. and Nurse, P. (1989) *Nature*, **342**, 39−45.
Hadwiger,J.A., Wittenberg,C., Richardson,H.E., De Barros Lopes,M. and Reed,S.I. (1989a) *Proc. Natl. Acad. Sci. USA*, **86**, 6255−6259.
Hadwiger,J.A., Wittenberg,C., Mendenhall,M. and Reed,S. (1989b) *Mol. Cell Biol.*,**9**, 2034−2041.
Hanks,S.K., Quinn,A.M. and Hunter,T. (1988) *Science*, **241**, 42−52.
Hartwell,L.H., Culotti,J., Pringle,J.R. and Reid,B.J. (1974) *Science*, **183**, 46−51.
Hayles,J. and Nurse,P. (1986) *J. Cell Sci. Suppl.*, **4**, 155−170.
Hayles,J., Beach,D., Durkacz,B. and Nurse,P. (1986) *Mol. Gen. Genet.*, **202**, 291−293.
Hunt,T. (1989) *Curr. Opinion Cell Biol.*, **1**, 268−274.
Jimenez,J., Alphey,L., Nurse ,P. and Glover,D.M. (1990) *EMBO J.*, **9**, 3565−3571.
Jessus,C., Ducommun,B. and Beach,D. (1990) *FEBS Lett.*, **266**, 4−8.
Kemp,B.E. and Pearson,R.B. (1990) *Trends Biochem. Sci.*, **15**, 342−346.
Krek,W. and Nigg,E.A. (1989) *EMBO J.*, **8**, 3071−3078.
Labbé,J.-C., Capony,J.-P., Caput,D., Cavadore,J,.C., Derancourt,J., Kaghad,M., Lelias,J.-M., Picard,A. and Dorée,M. (1989) *EMBO J.*, **8**, 3053−3058.
Langlois,A.J., Lapis,K., Ishizaki,R., Beard,J.W. and Bolognesi,D.P. (1974) *Cancer Res.*, **34**, 1457−1464.
Lee,M.G., Norbury,C.J., Spurr,N.K. and Nurse,P. (1988) *Nature*, **333**, 676−679.
Lehner,C.F. and O'Farrell,P.H. (1989) *Cell*, **56**, 957−968.
Lehner,C.F.and O'Farrell,P.H. (1990a) *Cell*, **61**, 535−547.
Lehner,C.F. and O'Farrell,P.H. (1990b) *EMBO J.*, **9**, 3573−3581.
Lipsich,L.A., Lewis,A.J. and Brugge,J.S. (1983) *J. Virol.*, **48**, 352−360.
Lohka,M.J. (1989) *J. Cell Sci.*, **92**, 131−135.
Lörincz,A.T. and Reed,S.I. (1984) *Nature*, **307**, 183−185.
Marais,R.M. and Parker,P.J. (1989) *Eur. J. Biochem.*, **182**, 129−137.
Meijer,L., Arion,D., Golsteyn,R., Pines,J., Brizuela,L., Hunt,T. and Beach,D. (1989) *EMBO J.*, **8**, 2275−2282.
Minshull,K., Blow,J. and Hunt,T. (1989a) *Cell*, **56**, 947−956.
Minshull,J., Pines,J., Golsteyn,R., Standart,N., Mackie,S., Colman,A., Blow,J., Ruderman,J.V., Wu,M. and Hunt.T. (1989b) *J. Cell Sci. Suppl.*,**12**, 77−97.
Minshull,J., Golsteyn,R., Hill,C.S. and Hunt,T.(1990) *EMBO J.*, **9**, 2865−2875.
Moreno,S., Hayles,J. and Nurse,P. (1989) *Cell*, **58**, 361−372.
Morla,A.O., Draetta,G., Beach,D. and Wang,J.Y.J. (1989) *Cell*, **58**, 193−203.
Murray,A.W. and Kirschner,M.W. (1989a) *Science*, **246**, 614−621.
Murray,A.W. and Kirschner,M.W. (1989b) *Nature*, **339**, 275−279.
Murray,A.W., Solomon,M.J. and Kirshner,M.W. (1989) *Nature*, **339**, 280−286.
Nakagawa,J., Kitten,G.T. and Nigg,E.A. (1989) *J. Cell Sci.*, **94**, 449−462.
Norbury,C.J. and Nurse,P. (1990) *Proto-oncogenes in Cell Development*. Ciba Foundation Symposium, **150**, 168−177.
Nurse,P. and Bissett,Y. (1981) *Nature*, **292**, 558−560.
Nurse,P. (1990) *Nature*, **344**, 503−508.
Paris,J., Le Guellec,R., Couturier,A., Le Guellec,K., Omilli,F., Camonis,J., McNeill,S. and Philippe,M. (1990) *Proc. Natl. Acad. Sci. USA*, in press.
Peter,M., Nakagawa,J., Dorée,M., Labbé,J.C. and Nigg,E.A. (1990) *Cell*, **61**, 591−602.
Picard,A,. Capony,J.P., Brautigan,D.L. and Dorée,M. (1989) *J. Cell Biol.*, **109**, 3347−3354.

Pigott,J., Rai,R. and Carter,B. (1982) *Nature*, **298**, 391–393.
Pines,J. and Hunt,T. (1987) *EMBO J.*, **6**, 2987–2995.
Pines,J. and Hunter,T. (1989) *Cell*, **58**, 833–846.
Pines,J. and Hunter,T. (1990a) *New Biol.*, **2**, 389–401.
Pines,J. and Hunter,T. (1990b) *Nature*, **346**, 760–763.
Pondaven,P., Meijer,L. and Beach,D. (1990) *Genes Dev.*, **4**, 9–17.
Potashkin,J.A. and Beach,D.H. (1988) *Curr. Genet.*, **14**, 235–240.
Pringle,J.R. and Hartwell,L.H. (1981) In Strathern,J., Jones,E. and Broach,J. (eds) *The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance*, Cold Spring Harbor Laboratory Press, NY. pp. 97–142.
Reed,S.I. and Wittenberg,C. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 5697–5701.
Richardson,H.E., Wittenberg,C., Cross,F. and Reed,S.I. (1989) *Cell*, **59**, 1127–1133.
Richardson,H.E., Stueland,C.S., Thomas,J., Russel,P. and Reed,S.T. (1990) *Genes Dev.* **4**, 1332–1344.
Sefton,B.M., Hunter,T., Beemon,K. and Eckhart,W. (1980) *Cell*, **20**, 807–816.
Shoji,S., Titani,K., Demaille,J.G. and Fischer,E.H. (1979) *J. Biol. Chem.*, **254**, 6211–6214.
Shoji,S., Parmelee,D.C., Wade,R., Kumar,S., Ericsson,L.H., Walsh,K.A., Neurath,H., Long,G.L., Demaille,J.G., Fischer,E.H. and Titani,K. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 848–851.
Simanis,V. and Nurse,P. (1986) *Cell*, **45**, 261–268.
Snyder,M.A., Bishop,J.M., Colby,W.W. and Levinson,A.D. (1983) *Cell*, **32**, 891–901.
Standart,N., Minshull,J., Pines,J. and Hunt,T. (1987) *Dev. Biol.*, **124**, 248–258.
Stone,S.R., Hofsteenge,J. and Hemmings,B. (1987) *Biochemistry*, **26**, 7215–7220.
Studier,F.W. and Moffat,B.A. (1986) *J. Mol. Biol.*, **189**, 113–130.
Swenson,K., Farrell,K. and Ruderman,J. (1986) *Cell*, **47**, 861–870.
Weinmaster,G., Zoller,M.J., Smith,M., Hinze,E. and Pawson,T. (1984) *Cell*, **37**, 559–568.
Westendorf,J.M., Swenson,K.I. and Ruderman,J.V. (1989) *J. Cell Biol.*, **108**, 1431–1444.
Whitfield,W.G.F., Gonzalez,C., Sanchez-Herrero,E. and Glover,D.M. (1989) *Nature*, **338**, 337–340.
Whitfield,W.G.F., Gonzales,C., Maldonado-Codina,G. and Glover,D.M. (1990) *EMBO J.*, **9**, 2563–2572.
Wittenberg,C., Sugimoto,K. and Reed,S.I. (1990) *Cell*, **62**, 225–237.

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