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Mutations of p34^{cdc2} phosphorylation sites induce premature mitotic events in HeLa cells: evidence for a double block to p34^{cdc2} kinase activation in vertebrates

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In vertebrates, entry into mitosis is accompanied by dephosphorylation of p34^{cdc2} kinase on threonine 14 (Thr14) and tyrosine 15 (Tyr15). To examine the role of these residues in controlling p34^{cdc2} kinase activation, and hence the onset of mitosis, we replaced Thr14 and/or Tyr15 by non-phosphorylatable residues and transfected wild-type and mutant chicken p34^{cdc2} cDNAs into HeLa cells. While expression of wild-type p34^{cdc2} did not interfere with normal cell cycle progression, p34^{cdc2} carrying mutations at both Thr14 and Tyr15 displayed increased histone H1 kinase activity and rapidly induced premature mitotic events, including chromosome condensation and lamina disassembly. No phenotype was observed in response to mutation of only Thr14, and although single-site mutation at Tyr15 did induce premature mitotic events, effects were partial and their onset was delayed. These results identify both Thr14 and Tyr15 as sites of negative regulation of vertebrate p34^{cdc2} kinase, and they suggest that dephosphorylation of p34^{cdc2} represents the rate-limiting step controlling entry of vertebrate cells into mitosis.

Key words: cdc2 kinase/cell cycle/mitosis/phosphorylation/regulation

Introduction

Entry of vertebrate cells into mitosis is accompanied by dramatic changes in cell morphology, nuclear structure, and cytoskeletal organization. Concomitantly, major cellular activities such as transcription, translation, and endomembrane traffic, are transiently arrested. Many of these events are believed to be controlled by concerted phosphorylation of key proteins. These phosphorylations are caused, either directly or indirectly, by the activation of p34^{cdc2}, an evolutionarily conserved serine/threonine specific protein kinase (reviewed in Moreno and Nurse, 1990; Pines and Hunter, 1990b; Nigg, 1991). This kinase, named *cdc2* according to its homolog in fission yeast *Schizosaccharomyces pombe* (CDC28 in *Saccharomyces cerevisiae*), is the catalytic subunit of a universal mitotic inducer present in all eukaryotes (for reviews see Dunphy and Newport, 1988; Lohka, 1989; Murray and Kirschner, 1989a; Dorée, 1990; Draetta, 1990; Nurse, 1990).

The regulation of p34^{cdc2} kinase activity is complex. It involves cell cycle dependent physical interaction of the 34 kDa catalytic subunit with other proteins and phosphorylation–dephosphorylation reactions (for reviews, see Draetta, 1990; Nurse, 1990). Prominent among the complex partners of p34^{cdc2} are 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, 1990a; Gautier *et al.*, 1990). These proteins 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). Their association with p34^{cdc2} is required for kinase activation at the G_2/M transition (Minshull *et al.*, 1989; Murray and Kirschner, 1989b; see also Picard *et al.*, 1989), while, conversely, their destruction is necessary for exit from M phase (Murray *et al.*, 1989; Felix *et al.*, 1990).

Although the association of mitotic cyclins with p34^{cdc2} is required for the formation of mitotically active cdc2 kinase, this event is clearly not sufficient (Solomon *et al.*, 1990). Instead, the timing of kinase activation is controlled by post-translational mechanisms, involving the homologs of the *S.pombe* genes *cdc25*, *wee1* and *mik1*. While *wee1* and *mik1* encode protein kinases and negatively regulate p34^{cdc2} activity (Russell and Nurse, 1987; Featherstone and Russell, 1991; Lundgren *et al.*, 1991), the *cdc25* gene almost certainly encodes a phosphatase and functions as an activator of p34^{cdc2} (Russell and Nurse, 1986; Moreno *et al.*, 1990; Ducommun *et al.*, 1990; Kumagai and Dunphy, 1991; Strausfeld *et al.*, 1991). In *S.pombe*, one inhibitory phosphorylation site in p34^{cdc2} was identified as Tyr15, and mutation of this residue to a non-phosphorylatable amino acid caused fission yeast cells to enter mitosis prematurely (Gould and Nurse, 1989). Moreover, *cdc25* mutants could be rescued by expression of a human tyrosine phosphatase (Gould *et al.*, 1990), indicating that dephosphorylation of p34^{cdc2} on Tyr15 is rate-limiting for kinase activation at the G_2/M transition of the fission yeast cell cycle. So far, no evidence has been reported for additional inhibitory phosphorylations in *S.pombe*. In contrast, several studies carried out with higher eukaryotes suggest that activation of p34^{cdc2} kinase at the G_2/M transition might require dephosphorylation of p34^{cdc2} not only on tyrosine but also on threonine (e.g. Morla *et al.*, 1989; Solomon *et al.*, 1990; Krek and Nigg, 1991).

Recently, we have identified four cell cycle regulated phosphorylation sites in chicken p34^{cdc2} (Krek and Nigg, 1991). Of particular interest in the present context, we showed that two residues, Thr14 and Tyr15, are maximally phosphorylated during G_2 phase of the cell cycle but dephosphorylated abruptly at the G_2/M transition, concomitant with activation of p34^{cdc2} kinase. Based on these results we argued that activation of the vertebrate p34^{cdc2} kinase at the G_2/M transition might require removal of a double block imposed by phosphorylation of Thr14 and Tyr15. To test this hypothesis directly using an *in vivo* assay, we have mutated Thr14 and Tyr15 to non-phosphorylatable residues, either individually or in combination, and examined

the phenotypic consequences of expressing wild-type and mutant p34^{cdc2} proteins in HeLa cells. This approach was based on two premises: (i) that the ectopically expressed p34^{cdc2} proteins would be able to compete with the endogenous p34^{cdc2} for cyclin binding; and (ii) that the phosphorylation site mutant proteins might no longer respond to inhibitory controls, and thus produce a dominant phenotype.

Results

Chicken p34^{cdc2} is concentrated within nuclei and associated with centrosomes

The intracellular distribution of p34^{cdc2} has been the subject of some debate (for critical review, see Pines and Hunter, 1990b). Since we intended to use indirect immunofluorescence microscopy to monitor the ectopic expression of chicken p34^{cdc2} in mammalian cells, it was important to address this issue. To this end, we first studied the subcellular distribution of the endogenous p34^{cdc2} protein in chicken DU249 cells. During interphase, p34^{cdc2} was located predominantly within nuclei, nucleoli being stained less intensely (Figure 1a,c,l). Some cytoplasmic labeling could also be seen, and occasionally staining of centrosomes was apparent (Figure 1l). Results were similar when cells were fixed and permeabilized using either formaldehyde/Triton X-100 (Figure 1a,c) or methanol/acetone (Figure 1l), and virtually identical irrespective of whether p34^{cdc2} was localized using an antibody specific for the 18 C-terminal amino acids (Figure 1a) or an antibody raised against the entire protein expressed in *E.coli* (Figure 1c). Finally, staining could be completely abolished by pre-incubation of antibodies with either the synthetic peptide or Sepharose-bound bacterially expressed p34^{cdc2} protein (not shown). The localization of p34^{cdc2} in mitotic cells revealed two interesting features. First, throughout mitosis p34^{cdc2} was associated with spindle poles or caps (Figure 1d–g) and secondly, in anaphase cells, staining was concentrated at the cell periphery, perhaps reflecting an association with the actinomyosin filament system (Figure 1f). These results are consistent with those reported by Riabowol *et al.* (1989) and Bailly *et al.* (1989), but they are at variance with the study by Akhurst *et al.* (1989).

Ectopic expression of wild-type chicken p34^{cdc2} does not interfere with normal cell cycle progression

Transient expression in HeLa cells was achieved by placing the wild-type and mutant chicken p34^{cdc2} cDNAs under the control of the cytomegalovirus (CMV) promoter in the pCMVneo vector (see Figure 4B). Using this system, ~20% of all cells in a culture expressed chicken p34^{cdc2}. The ectopic expression of chicken p34^{cdc2} was monitored by indirect immunofluorescence microscopy (Figure 2A), as well as by immunoprecipitation experiments (Figure 2C). Specific detection of the transfected chicken protein was possible by using an affinity purified antibody raised against a peptide corresponding to the carboxy-terminus of chicken p34^{cdc2} (Krek and Nigg, 1989). This reagent does not recognize the endogenous human p34^{cdc2} (Figure 2).

By immunofluorescence microscopy, the expressed chicken p34^{cdc2} was found to accumulate within nuclei, excluding nucleoli (Figure 2A). Non-expressing cells were unstained, demonstrating the species-specificity of the anti-p34^{cdc2} antibody (Figure 2A and B). While most of the immunoreactive cells showed similar levels of chicken p34^{cdc2} within nuclei, additional cytoplasmic staining could be seen in the most brightly staining transfected cells (not shown). As shown in Figure 2C, the anti-C-terminal peptide antibody immunoprecipitated a major 34 kDa protein from [^{35}S]methionine labeled transfected HeLa cells (Figure 2C, lane 1; arrowhead), but not from untransfected control cells (Figure 2C, lane 3), nor from cells transfected with vector only (not shown). A peptide competition experiment demonstrates that the immunoprecipitated 34 kDa protein indeed corresponds to the *cdc2* gene product (Figure 2C, lane 2). Although it is very difficult to accurately determine the levels of protein expression in a transient transfection assay, the above data suggest that chicken p34^{cdc2} was not grossly overexpressed when compared to the endogenous human homolog.

Figures 2A and B show that ectopic expression of wild-type chicken p34^{cdc2} did not detectably change the morphology of the transfected cells. To determine whether or not HeLa cells expressing this protein were able to proceed normally through mitosis, a careful analysis of mitotic cells in transfected HeLa cultures was undertaken. The distribution of the chicken p34^{cdc2} was determined by immunofluorescence microscopy, the state of chromosome condensation was examined by DNA staining with Hoechst dye, and overall morphology was assessed by differential interference contrast microscopy (DIC). As shown in Figure 3, mitotic cells expressing wild-type chicken p34^{cdc2} could readily be found. During metaphase (Figure 3a) and anaphase (Figure 3b) the transfected chicken p34^{cdc2} protein was distributed throughout the cells, while in telophase it reassociated with nuclei (Figure 3c). In most cells, specific spindle pole staining was difficult to detect. With respect to both chromosome condensation (Figures 3d–f) and morphology (Figures 3g–i), cells expressing wild-type chicken p34^{cdc2} were indistinguishable from untransfected HeLa cells at the corresponding stages of mitosis. Also, we did not observe any significant effect on the mitotic index of HeLa cultures expressing wild-type chicken p34^{cdc2} (see Figure 6). Thus, neither in qualitative nor quantitative terms did overexpression of wild-type chicken p34^{cdc2} protein significantly affect the ability of transfected HeLa cells to proceed through mitosis.

Phosphorylation site mutations enhance p34^{cdc2} histone H1 kinase activity

To study the physiological role of phosphorylation of p34^{cdc2} on Thr14 and Tyr15 *in vivo*, three mutant p34^{cdc2} proteins carrying substitutions of phosphoacceptor sites by non-phosphorylatable residues were constructed by site-directed mutagenesis (Figure 4A). In the T14A mutant Thr14 was changed to alanine, in the Y15F mutant Tyr15 was changed to phenylalanine, and in the T14AY15F double mutant the two changes were combined within the same cDNA. The mutated cDNAs were then subcloned into the pCMVneo vector (Figure 4B), and the functional consequences of these mutations were examined by transfection of HeLa cells.

We first asked whether mutation of Thr14 and/or Tyr15 to non-phosphorylatable residues would affect the timing of p34^{cdc2} histone H1 kinase activation. To this end, transfected HeLa cells were labeled with [^{35}S]methionine,

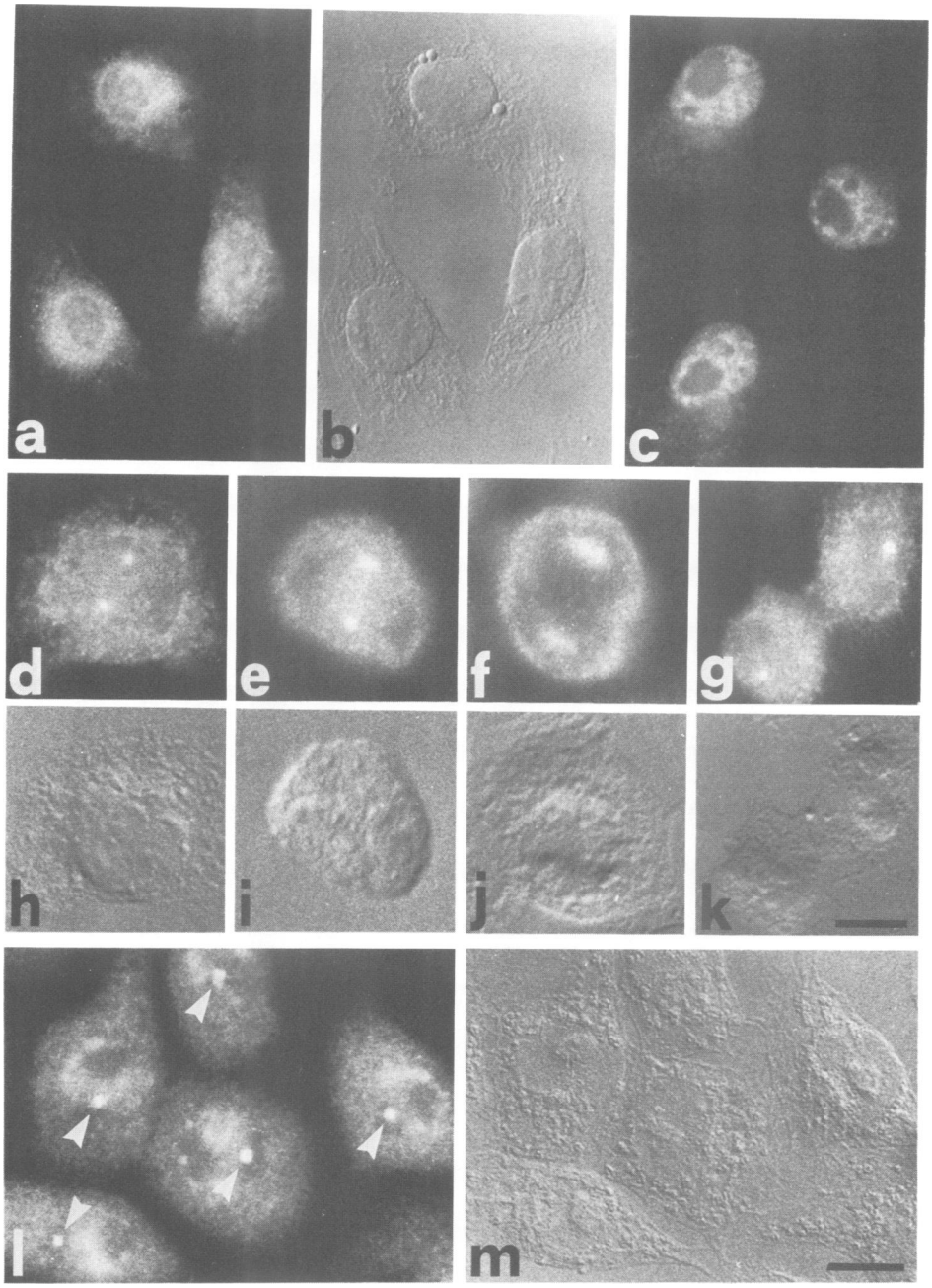

Fig. 1. Immunofluorescent localization of p34^{cdc2} in chicken DU249 cells. Chicken DU249 cells were fixed and permeabilized using either formaldehyde/Triton X-100 (a, c, d, e, f, g) or methanol/acetone (l). They were then incubated with affinity-purified anti-chicken p34^{cdc2} peptide antibodies (a, d, e, f, g) or anti-chicken p34^{cdc2} total protein antibodies (c, l), followed by rhodamine-conjugated goat anti-rabbit IgG. Arrowheads in l point to centrosomes. b, h, i, j, k and m show differential interference contrast (DIC) pictures corresponding to a, d, e, f, g and l respectively. Bar in k: 10 μm. Bar in m: 15 μm.

and at various times after transfection (see Materials and methods) the chicken p34^{cdc2} proteins were immunoprecipitated using the chicken-specific anti-peptide antibody. One half of each immunoprecipitate was then assayed for p34^{cdc2} kinase activity, using histone H1 as an *in vitro* substrate, while the other half was used to quantify the amount of the p34^{cdc2} protein by densitometric scanning. The primary data obtained from one such experiment (a 16 h sample) are illustrated in Figure 5A. The left panel shows that very similar amounts of p34^{cdc2} were recovered from the various transfected cultures, indicating that wild-type and mutant proteins were expressed to comparable levels. The right panel shows the autoradiograph of histone H1 phosphorylated by the immunoprecipitated p34^{cdc2} kinase. For each sample, the extent of histone H1 phosphorylation was then standardized relative to the amount of p34^{cdc2} in the corresponding immunoprecipitate. The histograms shown in Figure 5B summarize the averaged results obtained from several independent experiments, each carried out exactly as described above. These studies show that the histone H1 kinase activity associated with wild-type p34^{cdc2} (lanes 1) and the T14A mutant (lanes 2) was very low at all times after transfection. In contrast, the activity associated with the T14AY15F double mutant rapidly increased with time

(lanes 4). Compared to wild-type levels, the specific activity of this mutant protein increased more than 4-fold within the first 16 h, but prolonged expression produced little further increase in activity (up to 5-fold after 30 h) (lanes 4). The specific histone H1 kinase activity associated with the Y15F mutant p34^{cdc2} protein was also found to increase with time (lanes 3), but remarkably, activation of this mutant followed a different time course. Activity increased only marginally (~1.5-fold) during the first 16 h after transfection before it rose to 3.5 times wild-type level between 16 and 30 h (lanes 3).

Phenotypic consequences of expressing phosphorylation site mutants of p34^{cdc2}

Figure 6 illustrates one of the most readily detectable phenotypic consequences of expressing mutant p34^{cdc2} proteins. While cultures transfected with either wild-type (Figure 6A) or the T14A mutant p34^{cdc2} cDNA (Figure 6B) were indistinguishable from those tranfected with vector only (Figure 6E), cultures transfected for 30 h with T14AY15F double mutant cDNA displayed a large proportion of rounded cells (Figure 6D). A similar effect, albeit less pronounced, was seen in response to the Y15F mutant (Figure 6C). We emphasize that striking increases in the proportion of rounded cells were observed in each of more than fifteen independent transfection experiments.

These observations suggested that simultaneous mutation of Thr14 and Tyr15, and to a lesser extent mutation of only Tyr15, might promote premature entry of transfected cells into mitosis. To substantiate this hypothesis, transfected cells were examined for manifestations of a mitotic phenotype. Specifically, we looked for disassembly of the nuclear lamina and nuclear envelope breakdown, premature chromosome condensation, reorganization of the microtubule network, and dispersal of nucleoli.

When analyzed 30 h after transfection almost all cells expressing wild-type p34^{cdc2} showed a normal interphase phenotype (Figures 7a–d and 8a–c): the chicken p34^{cdc2} protein was present predominantly within nuclei (Figures 7a and 8a), the lamina was typically located at the nuclear periphery (Figure 7b), chromatin was decondensed (Figures 7c and 8c), and the microtubules displayed a characteristic interphase array (Figure 8b). In contrast, 85–90% of the cells expressing the T14AY15F double mutant displayed a phenotype resembling that of mitotic cells: cells were rounded up (Figure 7h), the mutant chicken p34^{cdc2} protein was no longer confined to nuclei but dispersed through the entire cells (Figures 7e and 8d), and the nuclear lamina was disassembled (Figure 7f). These observations indicate that the nuclear envelope had broken down. Moreover, chromatin was strikingly compacted, indicative of premature chromosome condensation (Figures 7g and 8f), and the nucleolar protein No38 was dispersed in a manner typical of untransfected mitotic cells (not shown). Finally, tubulin staining no longer showed the microtubule network characteristic of untransfected interphase cells (Figure 8g), but instead revealed a ring-like concentration of microtubules at the cell periphery (Figure 8e). At no stage of the cell cycle has such a peripheral microtubule distibution been seen in cells transfected with either wild-type p34^{cdc2} or vector alone (not shown). Most importantly, no evidence was obtained for formation of mitotic spindles in the cells transfected by the T14AY15F mutant (Figure 8e). Spindles

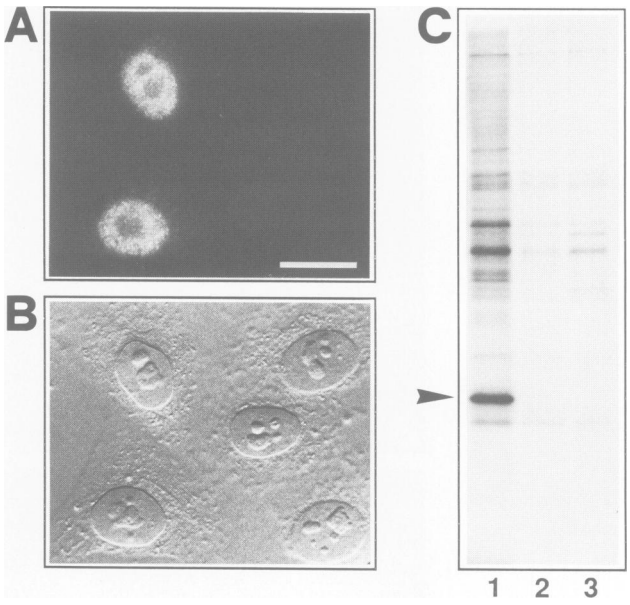

Fig. 2. Localization of ectopically expressed chicken p34^{cdc2} in HeLa cells. (**A**) HeLa cells transfected with wild-type chicken p34^{cdc2} cDNA were stained with the chicken specific affinity-purified peptide antibody R6 (Krek and Nigg, 1989) and examined by indirect immunofluorescence microscopy. Chicken p34^{cdc2} accumulated predominantly within nuclei, consistent with its distribution in chicken cells. (**B**) DIC micrograph corresponding to (A); note the presence of unstained HeLa cells demonstrating that the R6 antibody recognizes only transfected cells. (**C**) Immunoprecipitation of chicken p34^{cdc2} from [^{35}S]methionine labeled HeLa cells transfected with wild-type chicken p34^{cdc2}. Immunoprecipitations with anti-peptide antibody R6 were carried out in the absence (lane 1) or presence of competing peptide (lane 2), or from untransfected cells for control (lane 3). Bar in A: 20 μm.

were readily stained by the anti-tubulin antibodies in either untransfected M phase cells (Figure 8h), or cells transfected with wild-type p34^{cdc2} or vector (not shown). This indicates that activation of the p34^{cdc2} kinase at an inappropriate stage of the cell cycle was not sufficient to trigger mitotic spindle formation, possibly reflecting a requirement for additional events associated with the centriole duplication cycle.

In the next series of experiments, the effects of single-site mutations at Thr14 or Tyr15 were compared to those produced by the double mutant T14AY15F. In view of the results shown in Figure 5, we considered it important to study manifestations of premature mitosis as a function of time after transfection. Cells were therefore examined at 8, 16, 24 and 30 h after transfection with wild-type and mutant p34^{cdc2} proteins (Figure 9). The top panel of Figure 9 illustrates the phenotypes used for classification of the cells, whereas the histograms below each picture summarize the results of a statistical analysis. In cultures transfected by wild-type p34^{cdc2} only occasional cells (<2 per 100) showed a mitotic phenotype. In contrast, expression of the T14AY15F double mutant induced premature entry into mitosis in ~35% of the transfected cells as early as 8 h after transfection, and the proportion of 'mitotic' cells had reached almost 80% by 16 h. Expression of the T14A mutant did not lead to deregulation of the cell cycle, not even after 30 h. Likewise, comparatively minor effects were observed in response to the Y15F mutant, at least at early times after transfection. Manifestations of premature mitosis were

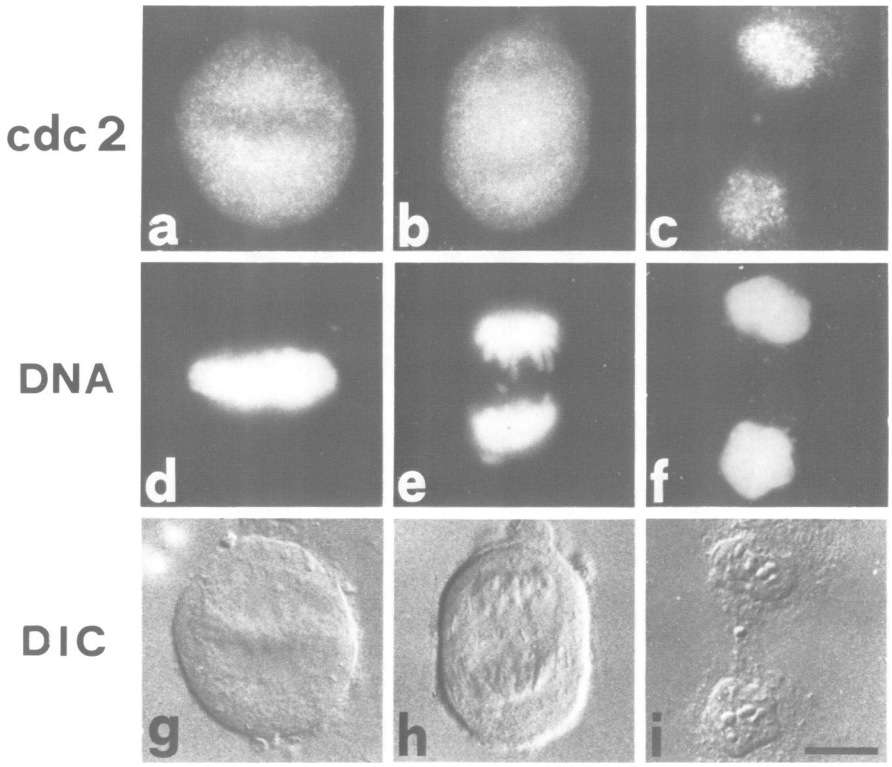

Fig. 3. HeLa cells expressing wild-type chicken p34^{cdc2} undergo normal mitosis. The localization of wild-type chicken p34^{cdc2} in transfected HeLa cells at various stages of mitosis was determined by immunofluorescence microscopy, as described in the legend to Figure 2. (**a**) metaphase; (**b**) anaphase; (**c**) late telophase; **d**–**f** and **g**–**i** represent the corresponding DNA stains with Hoechst dye 33258 and the DIC images, respectively. Note that wild-type chicken p34^{cdc2} is distributed throughout metaphase (a) and anaphase (b) cells but redistributes to the nucleus in telophase (c). Bar in i: 10 μm.

visible in <5% of the transfected cells after 8 h, and even after 16 h a 'mitotic' phenotype was seen in only 25% of the cells. Moreover, although the proportion of 'mitotic' cells in cultures expressing the Y15F mutant increased substantially between 16 and 30 h, we note that lamina disassembly was only partial in the majority of the transfected cells even at the latest time point examined (Figure 9, central part). These results demonstrate that the phenotypic consequences of expressing the T14AY15F double mutant were substantially more rapid and severe than those elicited by the Y15F single mutant.

Discussion

Using a transient transfection assay, we have examined the phenotypic consequences of expressing p34^{cdc2} phosphorylation site mutants in mammalian cells. Ectopic expression of wild-type chicken p34^{cdc2} in HeLa cells did not detectably influence cell cycle progression. In contrast, simultaneous replacement of Thr14 and Tyr15 by non-phosphorylatable residues caused the premature activation of the expressed p34^{cdc2} kinase, and the induction of a mitotic phenotype in the transfected HeLa cells. Premature mitotic events included cell rounding, condensation of chromatin, disassembly of the nuclear lamina, dispersal of nucleoli, and reorganization of the microtubule network. Expression of a p34^{cdc2} protein mutated only at the Thr14 phosphorylation site did not detectably influence any of the parameters studied. Likewise, although p34^{cdc2} mutated exclusively at Tyr15 produced a mitotic phenotype after

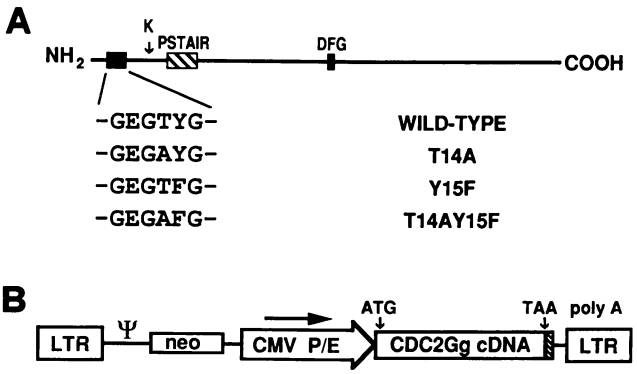

Fig. 4. Schematic representation of wild-type and mutant forms of the chicken p34^{cdc2} protein and the pCMVneo mammalian expression vector. (**A**) Mutations were introduced in the GEGTYG motiv of wild-type chicken p34^{cdc2} by site-directed mutagenesis (see Materials and methods). In the T14A mutant threonine (T) 14 was changed to alanine (A), in the Y15F mutant tyrosine (Y) 15 to phenylalanine (F), and in the T14AY15F mutant both mutations were introduced into the same cDNA. (**B**) Structure of the chicken p34^{cdc2} cDNA expression plasmid used in this study. The chicken wild-type and mutant p34^{cdc2} cDNAs were cloned behind the cytomegalovirus promotor/enhancer element (CMV P/E). A polyadenylation signal (poly A) is provided by the long terminal repeat (LTR) from the mouse retroviral vector (Bender *et al.*, 1987). Ψ denotes the presence of a retroviral packaging signal.

prolonged expression, we note that the effects seen with this mutant were apparent in fewer cells and consistently less severe than those resulting from expression of the T14AY15F double mutant.

A double phosphorylation block to p34^{cdc2} kinase activation in vertebrates?

We have previously identified Thr14 and Tyr15 as major phosphorylation sites in chicken p34^{cdc2} (Krek and Nigg, 1991). Moreover, we have shown that both residues were maximally phosphorylated during G_2 phase, but dephosphorylated abruptly upon activation of p34^{cdc2} kinase at the G_2/M transition. Taken together with biochemical studies suggesting that maximal *in vitro* activation of p34^{cdc2} might require dephosphorylation on both threonine and tyrosine (Morla *et al.*, 1989, Solomon *et al.*, 1990), these results indicated that phosphorylation of Thr14 and Tyr15 might constitute a double block against premature activation of p34^{cdc2} kinase during the vertebrate cell cycle. Our present findings strongly support this hypothesis. Although we cannot strictly eliminate the possibility that mutations of Thr14 and Tyr15 might have affected p34^{cdc2} kinase activity via mechanisms unrelated to phosphorylation, the most straightforward interpretation of our results is that both Thr14 and Tyr15 function as phosphoacceptor sites for inhibitory pathways, imposing cell cycle regulation upon activity of the wild-type p34^{cdc2} kinase. That the T14AY15F mutant protein escaped from negative regulation would then indicate that dephosphorylation of Thr14 and Tyr15 is rate-limiting for activation of the vertebrate p34^{cdc2} kinase at the G_2/M transition.

This conclusion is also supported by the results of an independent study by Norbury *et al.* (1991). These authors examined the activation of mutant versions of human p34^{cdc2} in a *Xenopus* egg extract. They found that double mutation of Thr14 and Tyr15 caused premature activation of human p34^{cdc2}, while both single-site mutant p34^{cdc2} proteins were activated concomitantly with the endogenous *Xenopus* p34^{cdc2} kinase. These *in vitro* results agree very well with our present findings in that they emphasize the existence of a double block to mitotic activation of vertebrate p34^{cdc2} kinase. However, in contrast to the situation in transfected HeLa cells (this study), the levels of exogenous mutant p34^{cdc2} expressed in the *Xenopus* egg extract were extremely low and insufficient to promote any detectable phenotypic consequences (Norbury *et al.*, 1991).

Several aspects of the phenotype of the transfected HeLa cells indicates that expression of the mutant p34^{cdc2} proteins caused premature entry into a pseudo-mitotic state, rather than inhibiting exit from naturally occurring mitoses. First, these cells displayed chromatin structures at various stages of condensation (compare for instance, Figure 8f with 7g and 9), rather than *bona fide* mitotic chromosomes (Figures 3d,e). Second, although lamina disassembly was complete in most of the T14AY15F transfected cells, it was only partial in the majority of the cells transfected by the Y15F mutant. Third, in the cells expressing the T14AY15F double mutant, the onset of a pseudo-mitotic phenotype was rapid. A lag of 8 h before a phenotype was manifest in ~35% of the transfected cells is not unexpected, considering the need for expression of sufficient amounts of exogenous p34^{cdc2} protein, and the need for accumulation of complexes between chicken p34^{cdc2} and human cyclins. Finally, we emphasize that cells expressing activated chicken p34^{cdc2} did not display mitotic spindles, consistent with the view that they had prematurely entered a pseudo-mitotic state. Mitotic spindles could readily be visualized in cells that were blocked in mitosis due to overexpression of a non-destructible mutant cyclin B2 protein and concomitant stabilization of mitotically activated wild-type p34^{cdc2} kinase (P.Gallant and E.A.Nigg, manuscript in preparation).

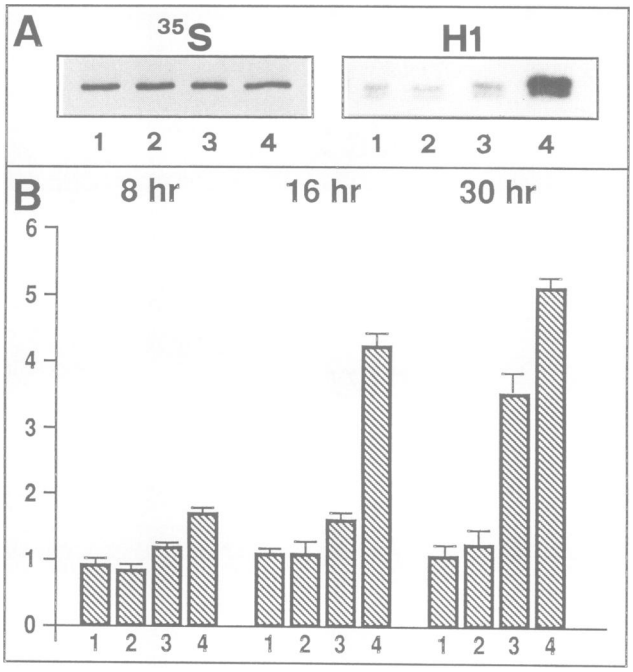

Fig. 5. Histone H1 kinase activities associated with ectopically expressed chicken p34^{cdc2} wild-type and mutant proteins. Following transfection of chicken p34^{cdc2} wild-type, T14A, Y15F and T14AY15F cDNAs, HeLa cells were labeled with [^{35}S]methionine as indicated in Materials and methods. Chicken p34^{cdc2} proteins were then immunoprecipitated 8, 16 and 30 h after removal of the calcium phosphate–DNA precipitate, and the washed immunobeads were divided into two samples. One aliquot was used to assess the specific histone H1 kinase activity associated with individual p34^{cdc2} proteins, the other to quantify the amount of p34^{cdc2} protein recovered. (**A**) Representative primary data from one such experiment, corresponding to a 16 h post-transfection assay. Lanes 1–4 show the [^{35}S]methionine labeled p34^{cdc2} (^{35}S panel) and the corresponding histone H1 kinase activity (H1 panel) recovered in immunoprecipitates of wild-type (lane 1), T14A (lane 2), Y15F (lane 3) and T14AY15F (lane 4) chicken p34^{cdc2} proteins. (**B**) Histone H1 kinase activities were standardized relative to the level of ^{35}S-labeled p34^{cdc2} protein present in each immunoprecipitate. For each time point, results from 2–5 independent experiments were averaged and represented in the form of a histogram. Hours refer to the time between removal of the calcium phosphate–DNA precipitate and the analysis of histone H1 kinase activities in the immunoprecipitates. Activities are expressed in arbitrary units. Lane 1: wild-type p34^{cdc2}; lane 2: T14A; lane 3: Y15F; lane 4: T14AY15F.

Our results are in agreement with the observation that microinjection of active starfish cdc2 kinase into rat fibroblasts did not cause the formation of mitotic spindles, although it elicited the transient manifestation of other mitotic events (Lamb *et al.*, 1990). Thus, unscheduled activation of p34^{cdc2} may not be sufficient to trigger all events characteristic of mitosis. Instead, entry into mitosis may require a co-operation of cdc2 kinase with other factors. Prominent candidates for such factors include the kinase encoded by the *nimA* gene of *Aspergillus* (Osmani *et al.*, 1988), and members of the MAP kinase family (e.g. Gotoh *et al.*, 1991).

Thr14 and Tyr15 may not be functionally equivalent

For technical reasons, it was not possible to determine directly the phosphorylation state of the mutant p34^{cdc2} proteins in the transfected cultures. Phosphopeptide mapping

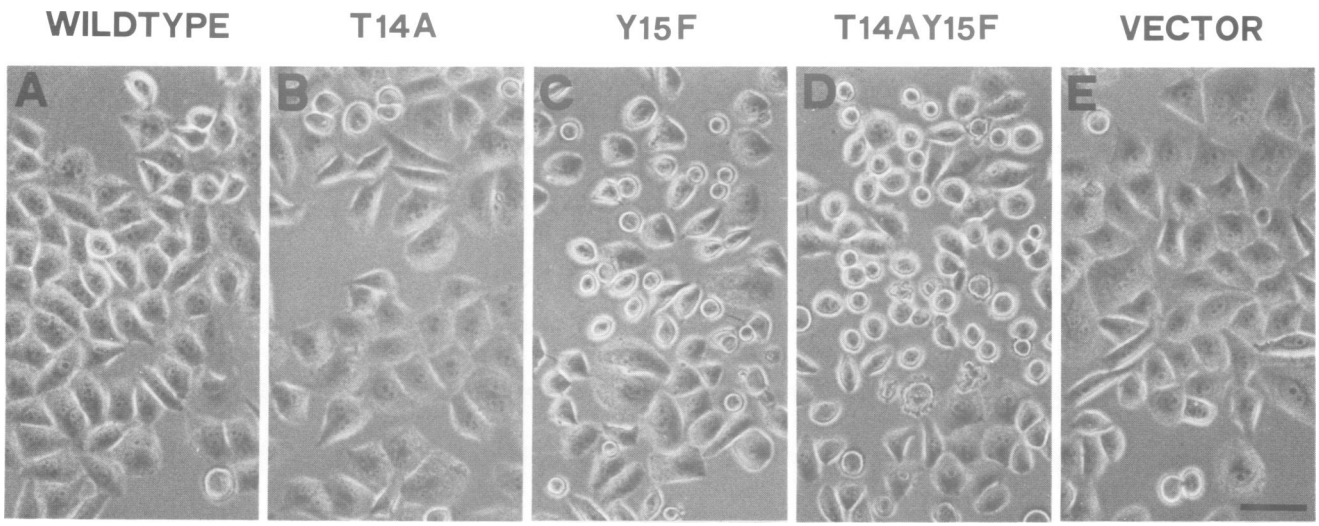

Fig. 6. Induction of cell rounding by expression of mutant chicken p34^{cdc2}. HeLa cells were transfected with the various chicken p34^{cdc2} constructs or with the expression vector alone. 30 h after removal of the calcium phosphate-DNA precipitate the cultures were examined by phase contrast microscopy. The following cDNAs were transfected: (**A**) wild-type chicken p34^{cdc2}; (**B**) T14A mutant; (**C**) Y15F mutant; (**D**) T14AY15F mutant; (**E**) expression plasmid pCMVneo. In parallel samples, indirect immunofluorescence microscopy was used to control for comparable transfection efficiencies (not shown). Bar in E: 20 μm.

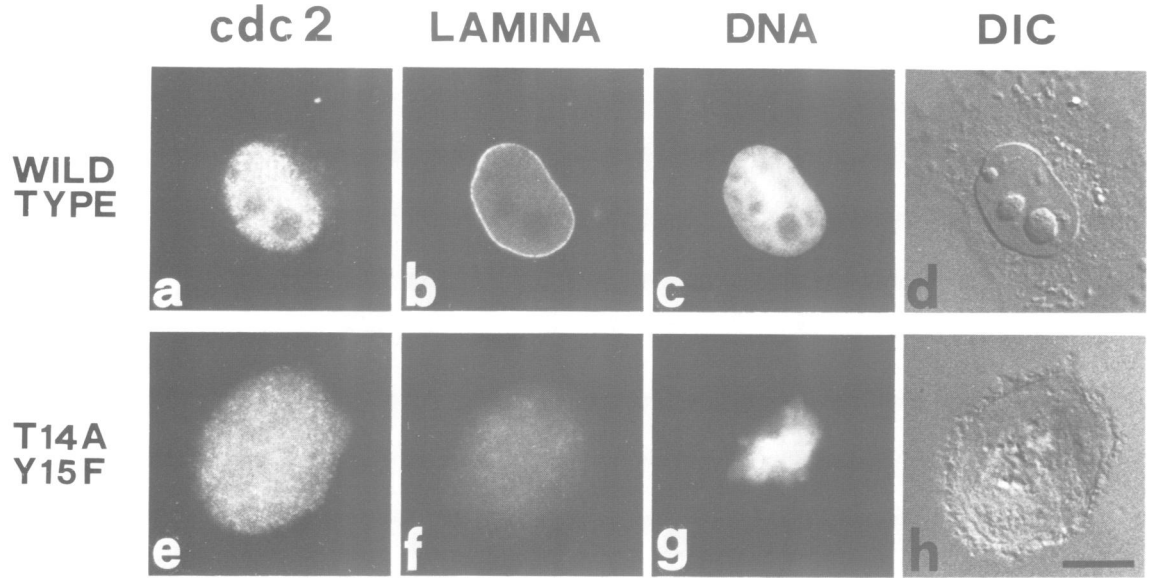

Fig. 7. Nuclear envelope breakdown, lamina disassembly and chromatin condensation induced by expression of the T14AY15F phosphorylation site mutant. HeLa cells were processed for indirect immunofluorescence microscopy 30 h after transfection with chicken p34^{cdc2} constructs. The phenotypes shown are representative of HeLa cells transfected with wild-type chicken p34^{cdc2} (a–d), or with the T14AY15F mutant (e–h). respectively. To reveal the localization of p34^{cdc2}, the state of lamina assembly and chromatin condensation simultaneously, triple labelings were carried out using rabbit anti-p34^{cdc2} antibodies (a, e), mouse anti-lamin A antibodies (b, f) and Hoechst dye 33258 (c, g). The corresponding DIC images are shown in d, h. Bar in h: 15 μm.

experiments could not be carried out because the amounts of material available for analysis were very limited, due to the asynchronous expression of the mutant p34^{cdc2} proteins and the transient nature of the phosphorylations occurring on Thr14 and Tyr15 (see Krek and Nigg, 1991). Moreover, while transient phosphorylation might in principle be detected by short pulse-labeling with [^{32}P]orthophosphate, such experiments are not practical because of the need to equilibrate cellular ATP pools. On the other hand, gel electrophoretic mobility shift assays could not be used for monitoring the expected rapid changes in the phosphorylation states of p34^{cdc2} proteins, because the mobility of chicken p34^{cdc2} does not detectably change in response to phosphorylation (Krek and Nigg, 1991). Nevertheless, we are confident that transient phosphorylation of Thr14 and Tyr15 did occur in the Y15F and T14A mutant proteins, respectively. First, our earlier studies on chicken cells have shown that Thr14 and Tyr15 were phosphorylated to similar levels in wild-type p34^{cdc2}; moreover, Thr14 was clearly phosphorylated in the absence of phosphorylation of Tyr15 and *vice versa*, suggesting that the two sites need not be phosphorylated according to an obligatory order (Krek and Nigg, 1991). Second, the failure of the T14A mutant to produce a detectable phenotype in HeLa cells is interpreted

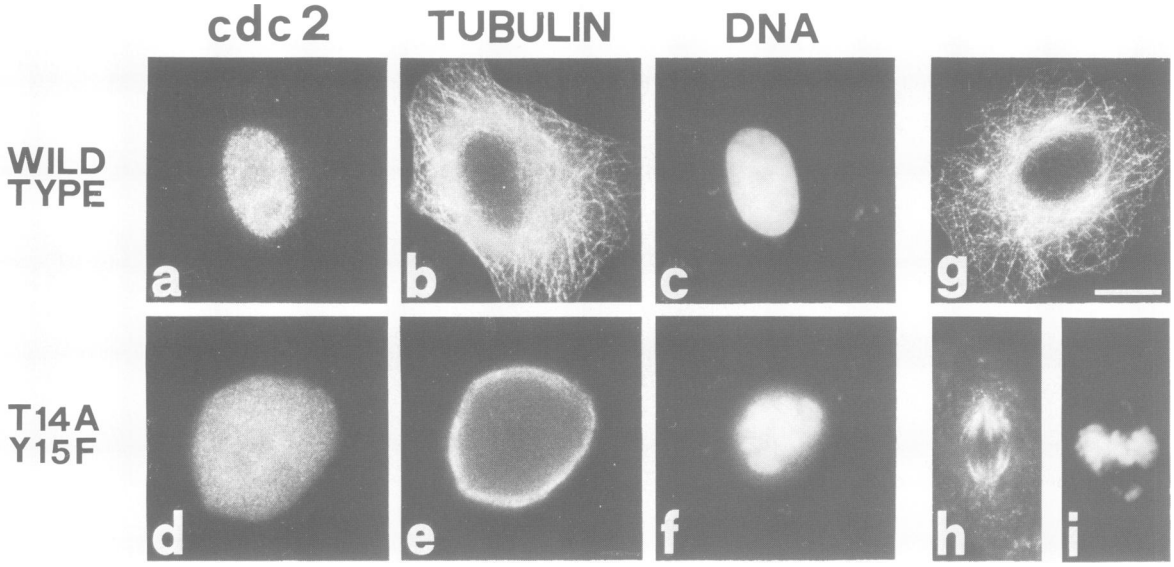

Fig. 8. Reorganization of the microtubule network induced by expression of the T14AY1F p34^{cdc2} mutant. HeLa cells were transfected with either wild-type chicken p34^{cdc2} (**a**–**c**) or the double mutant T14AY15F (**d**–**f**). 30 h after transfection cells were processed for indirect immunofluorescence microscopy using triple labeling with rabbit anti-chicken p34^{cdc2} antibodies (a, d), rat anti-tubulin antibodies (b, e) and the DNA stain Hoechst dye 33258 (c, f). For comparison, (**g**) shows tubulin staining of an untransfected interphase HeLa cell. (**h**) and (**i**) illustrate tubulin (h) and DNA staining (i), respectively, of an untransfected metaphase HeLa cell. Bar in g: 15 μm.

best by assuming that this mutant p34^{cdc2} protein was inhibited by phosphorylation on Tyr15. Likewise, the delayed onset and reduced magnitude of the phenotype associated with the Y15F mutant strongly suggest that inhibitory phosphorylation of Thr14 had occurred, at least transiently. Third, in the cell-free *Xenopus* system discussed above, electrophoretic mobility shifts could be used to show that human p34^{cdc2} mutated at either Thr14 or Tyr15 was readily phosphorylated on the respective neighboring residue (Norbury *et al.*, 1991).

According to a strict interpretation of the double block model, one might have predicted that the presence of either Thr14 or Tyr15 should have prevented premature activation of the single-site mutant p34^{cdc2} proteins. This prediction was met in the *in vitro* studies of Norbury *et al.* (1991) where regulatory systems were challenged with only radiochemical amounts of p34^{cdc2} proteins. In contrast, when p34^{cdc2} single-site mutant proteins were overexpressed *in vivo* (this study), Tyr15 alone was sufficient to confer correct cell cycle regulation to p34^{cdc2}, but Thr14 alone was not. The presence of this residue in the Y15F mutant substantially delayed the onset of p34^{cdc2} kinase activation when compared to the T14AY15F double mutant, but it did not completely prevent premature activation. It is possible that phosphorylation of Thr14 may produce an intrinsically lower inhibitory effect than phosphorylation of Tyr15. Although the two residues are both located within the putative ATP binding site of the p34^{cdc2} kinase, they are expected to display different spatial orientations. Thus, the turnover and/or the structural effects of phosphates attached to these residues may be different. Alternatively, the kinase(s) modifying Thr14 may be less efficient than those acting on Tyr15, either due to lower abundance or activity, or a temporally narrower window of action during the cell cycle. Conversely, phosphatase(s) may act more efficiently on Thr14 than on Tyr15. In either case, expression of mutant p34^{cdc2} protein to sufficient levels would eventually lead to overpowering of the kinase(s) phosphorylating Thr14, and unscheduled activation of the Y15F mutant p34^{cdc2} kinase would occur. Whatever the correct explanation, our observations suggest that Thr14 and Tyr15 may be functionally distinct phosphorylation sites.

Regulation of p34^{cdc2} in S.pombe and vertebrates

It is interesting to compare the regulation of p34^{cdc2} kinase activation in fission yeast and vertebrates. Studies in *S.pombe* clearly demonstrate that the phosphorylation state of p34^{cdc2} Tyr15 depends on the balance between the inhibitory influence of the protein kinases encoded by *wee1* and *mik1* versus the stimulatory effect of the *cdc25* gene product (Russell and Nurse, 1986, 1987; Ducommun *et al.*, 1990; Moreno *et al.*, 1990; Featherstone and Russell, 1991; Lundgren *et al.*, 1991). The *wee1* and *mik1* kinases may either phosphorylate Tyr15 directly, or they may maintain the activity of another tyrosine kinase (Lundgren *et al.*, 1991). The protein encoded by *cdc25*, on the other hand, is likely to be a phosphatase, and functions as an activator of p34^{cdc2} (Kumagai and Dunphy, 1991; Strausfeld *et al.*, 1991).

In *S.pombe*, activation of the *cdc25* gene, and hence dephosphorylation of p34^{cdc2}, has been linked to a regulatory pathway monitoring the completion of DNA replication (Enoch and Nurse, 1990). Moreover, the available evidence indicates that dephosphorylation of Tyr15 is sufficient to activate p34^{cdc2} at the G_2/M transition (Gould and Nurse, 1989; Gould *et al.*, 1990). In contrast, full activation of the vertebrate p34^{cdc2} kinase clearly requires dephosphorylation of Thr14 in addition to dephosphorylation of Tyr15 (Norbury *et al.*, 1991; this study). Thus, an additional level of regulation of the p34^{cdc2} kinase may have arisen during evolution. Alternatively, it may be premature to exclude the possibility that phosphorylation of Thr14 may actually occur in fission yeast, but may have escaped detection.

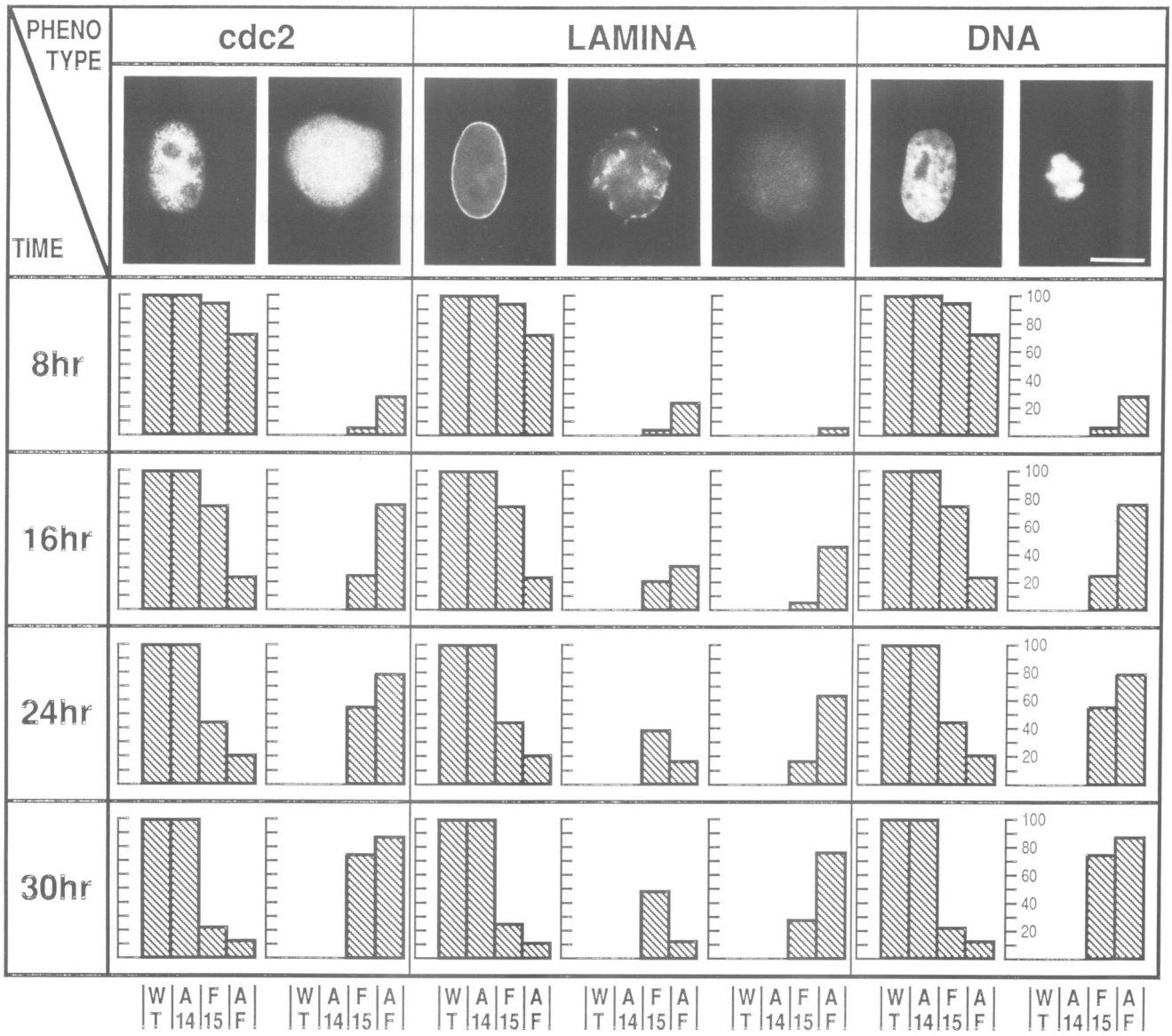

Fig. 9. Kinetics of induction of mitotic phenotypes by p34^{cdc2} phosphorylation site mutants. To provide a quantitative analysis of the kinetics of induction of mitotic events by the various chicken p34^{cdc2} proteins, transfected cells were examined for manifestations of the phenotypes illustrated in the top panel (Bar in the far-right field: 15 μm). Specifically, cells were classified depending on whether the ectopically expressed chicken p34^{cdc2} was confined to nuclei (first column) or distributed throughout the cell (second column), whether the nuclear lamina displayed a typical ring staining (third column), a partial disassembly (fourth column), or a complete disassembly (fifth column), and whether chromosomes were decondensed (sixth column) or condensed (seventh column). The histograms below each picture indicate the percentages of cells displaying the indicated phenotypes in wild-type (WT), T14A (A14), Y15F (F15) and T14AY15F (AF) transfectants, examined at the indicated times after transfection. Data were collected from two independent experiments. In each case, at least 100 cells were counted in random fields.

In vertebrates, the phosphorylation state of Tyr15 is expected to be under the control of the homologs of the yeast genes discussed above. However, the identity of the protein kinase(s) and phosphatase(s) acting on Thr14 remains to be determined. Recent *in vitro* studies suggest that *wee1* may phosphorylate both Tyr and Ser/Thr residues (Featherstone and Russell, 1991), and similarly, the cdc25 phosphatase may act on both phospho-Tyr and phospho-Thr (Kumagai and Dunphy, 1991). Thus, phosphorylation-dephosphorylation of Thr14 and Tyr15 may be controlled in a concerted fashion. Alternatively, however, it would be premature to exclude that *in vivo* the phosphorylation of Thr14 and Tyr15 might be controlled by distinct pathways. Our present observation that T14A and Y15F single-site mutants produced different phenotypic consequences would be consistent with a dual control model. If correct, two kinases and/or two phosphatases might need to be regulated at the G_2/M transition. This in turn might reflect a requirement to pass multiple checkpoints before vertebrate p34^{cdc2} is activated and mitosis ensues.

The subcellular distribution of vertebrate p34^{cdc2}

Finally, in addition to providing information on the regulation of p34^{cdc2} kinase activity, our present results also bear on the subcellular localization of this protein. This issue has been the subject of some debate. While p34^{cdc2} was localized to the nucleus in fission yeast (Booher *et al.*, 1989), in mammalian cells it was described as nuclear (Riabowol

et al., 1989), exclusively cytoplasmic (Akhurst *et al.*, 1989), or nuclear and cytoplasmic (Bailly *et al.*, 1989). Our present results demonstrate that p34^{cdc2} is located predominantly in the nucleus, although significant amounts are present also in the cytoplasm and occasional staining of interphase centrosomes could be seen. The extent of cytoplasmic labeling was somewhat dependent on the protocol used for cell fixation. While cytoplasmic staining was low in aldehyde-fixed cells, it was more prominent in methanol/acetone fixed cells. No evidence was obtained for major redistributions of p34^{cdc2} during interphase. In transfected HeLa cells, chicken p34^{cdc2} was also located predominantly within the nucleus. Moreover, in those cells in which they did not cause a mitotic phenotype, all phosphorylation site mutants analyzed here were distributed in a pattern indistinguishable from that of wild-type p34^{cdc2}. Thus, phosphorylation of Thr14 and Tyr15 is unlikely to play a role in controlling the subcellular distribution of p34^{cdc2}.

Materials and methods

Site-directed mutagenesis and plasmid constructions

For oligonucleotide-directed mutagenesis the following constructions were carried out: a 1041 bp *NcoI*–*SspI* fragment encoding chicken p34^{cdc2} was isolated from the original recombinant pGEM-3Zf(−) plasmid (Krek and Nigg, 1989), filled up with Klenow polymerase and blunt-end ligated into pGEM-3Zf(−) (Promega), which had been cut with *SmaI*. From this plasmid, a 1062 bp *SacI*–*BamHI* fragment was excised and cloned into the double-stranded form of M13mp18, which had been cut with *SacI*–*BamHI*. The single-stranded M13mp18 containing the wild-type chicken p34^{cdc2} cDNA was then used as a template for second-strand synthesis using a site-directed mutagenesis kit (BioRad) and appropriate mutant oligonucleotides as primers. Point mutations were detected by sequencing of the single-stranded M13mp18 recombinants. The appropriate mutant inserts were cloned back into pGEM-3Zf(−) as 1062 bp *SacI*–*BamHI* fragments, and the introduced mutations were confirmed again by double-stranded sequencing. Then, the 1062 bp *SacI*–*BamHI* fragments were re-isolated, made blunt-end with T4-DNA polymerase and cloned into the *HpaI* digested, alkaline phosphatase-treated mammalian expression vector pCMVneo. This vector was derived from pLNL-XHC as described by Bender *et al.* (1987). The resulting plasmids were propagated in DH5α and purified by alkaline SDS lysis followed by two rounds of centrifugation in CsCl gradients. All cloning and sequencing procedures were carried out as described in Krek and Nigg (1989).

DNA transfections

After several unsuccessful attempts to express chicken p34^{cdc2} cDNAs under the control of the SV40 early promoter, the transferrin receptor promoter or the inducible MMTV promoter, transient expression in HeLa cells was achieved by placing the wild-type and mutant chicken p34^{cdc2} cDNAs under the control of the cytomegalovirus (CMV) promoter in the pCMVneo vector. None of these vectors has so far allowed us to obtain stably transformed cell lines expressing p34^{cdc2}. Transfections into HeLa cells were carried out according to the method of Chen and Okayama (1987). Briefly, HeLa cells were grown in DMEM supplemented with 5% heat-inactivated fetal calf serum and 100 U/ml penicillin/streptomycin (Gibco) in a 7% CO_2 incubator at 37°C. 20 h prior to transfection, 9×10^4 cells were plated in 2 ml medium in 18 mm tissue culture dishes containing glass coverslips. 3 h before addition of the DNA the growth medium was removed, fresh pre-warmed medium was added and the cells were transferred to a 37°C incubator with CO_2 set to 3%. For preparation of the calcium phosphate–DNA precipitate 5 μg of plasmid DNA in a volume of 90 μl H_2O was mixed well with 10 μl of a 2.5 M $CaCl_2$ solution and 100 μl of 2×BBS [50 mM BES (*N,N*-bis(2-hydroxyethyl)-2-aminoethane-sulfonic acid; pH 6.95), 280 mM NaCl, 1.5 mM Na_2HPO_4]. After 15 min incubation at room temperature, the mixture was added slowly to the cultures and incubations were continued for 16 h at 37°C under 3% CO_2. Then, the medium was removed, cells washed 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)], refed with growth medium and incubated for the times indicated at 37°C under 7% CO_2. Thus, time zero for expression of p34^{cdc2} is defined as the moment when the calcium phosphate–DNA precipitate was removed.

Indirect immunofluorescence microscopy

Cells were fixed for 5 min in 3% formaldehyde/2% sucrose in PBS, rinsed quickly three times with PBS, incubated another 5 min with Triton X-100 buffer [0.5% Triton X-100, 20 mM HEPES (pH 7.4), 50 mM NaCl, 3 mM $MgCl_2$, 300 mM sucrose] and washed again quickly three times with PBS. Methanol/acetone fixation was carried out exactly as described previously (Nigg *et al.*, 1985). For tubulin staining, cells were post-fixed for 5 min with −20°C methanol immediately after the Triton X-100 buffer treatment, in order to stabilize microtubules.

Affinity-purified rabbit IgG raised against a C-terminal peptide of chicken p34^{cdc2} (serum R6; Krek and Nigg, 1989) were used at 10 μg/ml (for chicken cells) and at 1 μg/ml (for transfected HeLa cells). Affinity-purified rabbit IgG raised against bacterially expressed chicken p34^{cdc2} (serum R8; Krek and Nigg, 1991), were used at 5 μg/ml (for chicken cells). Mouse monoclonal antibodies L3–4B4 (against lamin A; Stick *et al.*, 1988) and rat monoclonal antibody YOL/34 (against tubulin; Kilmartin *et al.*, 1982) were used at 1:1000 dilutions. Incubations of primary antibodies were carried out for 30 min at room temperature. After two quick and three 5 min washes in PBS, secondary antibodies (diluted 1:500) were applied together with Hoechst dye 33258 diluted 1:1000 from a 5 mg/ml stock solution. All dilutions were prepared using PBS. Secondary reagents were affinity-purified rhodamine-conjugated goat anti-rabbit IgG (Pierce), fluorescein-conjugated goat anti-mouse IgG (Sigma) and fluorescein-conjugated goat anti-rat IgG (Sigma). After 15 min incubation with the secondary reagents, cells were washed as described above, mounted in 90% glycerol/10% 1 M Tris–HCl (pH 9.0) and viewed with a Polivar fluorescence microscope, using a ×100 oil immersion objective.

Metabolic labeling and immunoprecipitations

For *in vivo* labeling of transfected HeLa cells, 2×10^5 cells were plated out in 60 mm dishes in 5 ml medium and transfected as described, except that 15 μg DNA in 225 μl H_2O was mixed with 25 μl 2.5 M $CaCl_2$ and 250 μl 2× BBS before being added to the cells. After 16 h the calcium phosphate–DNA precipitate was removed and cells were refed with normal growth medium for appropriate time periods. Then, they were washed twice with methionine-free DMEM and incubated for 4 h in 3 ml of a solution containing 90% methionine-free DMEM supplemented with 10% dialyzed fetal calf serum (Gibco) and 0.1 mCi/ml [^{35}S]methionine (Amersham), and 10% normal growth medium. Immunoprecipitation of expressed [^{35}S]labeled chicken p34^{cdc2} was done exactly as described earlier (Krek and Nigg, 1991), except that 1.0 μg affinity-purified serum R6 was used per 500 μl of lysate. For competition experiments, 200 μM C-terminus peptide was added to the immunoprecipitation reaction. Samples were analysed by electrophoresis on 10% SDS–polyacrylamide gels, followed by fluorography.

Histone H1 kinase assays

Wild-type chicken p34^{cdc2} or the phosphorylation site mutants T14A, Y15F and T14AY15F were individually expressed in HeLa cells and labeled with [^{35}S]methionine as described above. Subsequently, chicken p34^{cdc2} was selectively immunoprecipitated using the species-specific anti-peptide antibody. The washed immmunobeads were divided into two aliquots. One was used to determine the amount of [^{35}S]labeled chicken p34^{cdc2} in each immunoprecipitate by densitometric scanning of fluorographs, the other was used to measure histone H1 kinase activity. Kinase assays were carried out as described previously (Krek and Nigg, 1991), except that histone H1 was from Boehringer-Mannheim (FRG). Reactions were stopped by the addition of 50 μl of 3× gel sample buffer. Samples were then applied to 10% SDS–polyacrylamide gels, dried and autoradiographed. Phosphorylated histone H1 was cut out of the dried gels and phosphorylation was quantified by scintillation counting (Cerenkov).

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