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A- and B-type cyclins differentially modulate substrate specificity of cyclin–cdk complexes

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Both cyclins A and B associate with and thereby activate cyclin-dependent protein kinases (cdks). We have investigated which component in the cyclin–cdk complex determines its substrate specificity. The A- and B-type cyclin–cdk complexes phosphorylated histone H1 and their cyclin subunits in an indistinguishable manner, irrespective of the catalytic subunit, p33*cdk2* or p34*cdc2*. In contrast, only the cyclin A–cdk complexes phosphorylated the Rb-related p107 protein *in vitro*. Likewise, binding studies revealed that cyclin A–cdk complexes bound stably to p107 *in vitro*, whereas cyclin B–cdk complexes did not detectably associate with p107, under identical assay conditions. Binding to p107 required both cyclin A and a cdk as neither subunit alone bound to p107. These results demonstrate that although the kinase subunit provides a necessary component for binding, it is the cyclin subunit that plays the critical role in targeting the complex to p107. Finally, we show that the cyclin A–p33*cdk2* complex phosphorylated p107 *in vitro* at most of its sites that are also phosphorylated in human cells, suggesting that the cyclin A–p33*cdk2* complex is a major kinase for p107 *in vivo*.

Key words: cell cycle/cyclin-dependent kinases/p107/phosphorylation/substrate targeting

Introduction

In higher eukaryotes, cell cycle transitions are regulated by the oscillating activities of at least two homologous cyclin-dependent kinases, p34*cdc2* and p33*cdk2* (Gautier *et al.*, 1989; Fang and Newport, 1991; Meyerson *et al.*, 1992; reviewed in Murray, 1992). The p34*cdc2* protein, a component of maturation promoting factor (MPF), has been implicated mainly in the control of mitosis, whereas p33*cdk2* seems to function earlier in the cell cycle, in S phase (Draetta *et al.*, 1988; Elledge and Spottswood, 1991; Fang and Newport, 1991). Cdks associate with cyclins, assembling active kinase complexes that phosphorylate specific components of the cell division machinery, e.g. lamins (Draetta *et al.*, 1989; Murray and Kirschner, 1989; Westendorf *et al.*, 1989; Peter *et al.*, 1990; Solomon *et al.*, 1990). The mitotic cyclins A and B were first identified as proteins whose abundance oscillates during the cell cycle (Evans *et al.*, 1983). Entry into mitosis requires the induction of p34*cdc2* activity, which involves cyclin binding as well as post-translational modifications (Draetta and Beach, 1988; Gautier *et al.*, 1989; Gould and Nurse, 1989; Krek and Nigg, 1991; Gu *et al.*, 1992; Parker and Piwnica-Worms, 1992).

In vertebrate cells, A- and B-type cyclins appear to have many functional similarities. For example, microinjection of either cyclin A or B into *Xenopus* oocytes induces maturation (Swenson *et al.*, 1986; Pines and Hunt, 1987; Westendorf *et al.*, 1989; Roy *et al.*, 1991). In addition, both A- and B-type cyclin–cdk complexes have very similar substrate specificities *in vitro*: they phosphorylate histones H1 and H2B with comparable efficiencies and moreover, on identical residues (Draetta *et al.*, 1989; Minshull *et al.*, 1990; Parker *et al.*, 1991). Finally, both cyclins can also induce DNA replication in G_1 cell extracts (D'Urso *et al.*, 1990).

In spite of their structural and many functional similarities, cyclins A and B do not seem to be redundant in the regulation of cell division. For example, cyclin A is not present in yeast and in *Drosophila* cyclin B is unable to functionally substitute for the loss of cyclin A (Lehner and O'Farrell, 1990). In addition, cyclin A-dependent kinases exhibit histone H1 kinase activity that increases earlier in the cell cycle than that of cyclin B–cdk complexes, although their activity patterns partially overlap (Minshull *et al.*, 1990; Pines and Hunter, 1990; Dulic *et al.*, 1992). In *Xenopus* oocytes, the activities of cyclin B-, but not of cyclin A-dependent kinases are transiently inhibited by tyrosine phosphorylation, which causes a lag between cyclin B–cdk complex formation and activation (Solomon *et al.*, 1990; Buendia *et al.*, 1991; Clarke *et al.*, 1992). Furthermore, unlike cyclin A, cyclin B stimulates cdc25 tyrosine phosphatase activity *in vitro* (Galaktionov and Beach, 1991) and activates the cyclin destruction pathway (Luca *et al.*, 1991). Perhaps the most striking differences between the A- and B-type cyclins are the observations that cyclin A, but not cyclin B, is sequestered by the transforming adenovirus E1A protein and moreover, is present in an S phase-specific complex together with p33*cdk2*, E2F (a cellular transcription factor) and p107 (an Rb-related protein) (Giordano *et al.*, 1989; Pines and Hunter, 1990; Cao *et al.*, 1992; Devoto *et al.*, 1992; Nevins, 1992; Pagano *et al.*, 1992a; Shirodkar *et al.*, 1992). However, many of the above mentioned results left open the question whether the observed functional differences between the A- and B-type cyclins were directly caused by the individual cyclins, or rather by preferential association of cyclins with different cdk subunits (or a combination).

In order to investigate which subunit in a cyclin–cdk

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complex determines substrate specificity, we have assembled four distinct kinases in which the cyclin A and B subunits, as well as the p33*cdk2* and p34*cdc2* subunits, were exchanged. We have examined the capacity of these cyclin–cdk complexes to bind to and to phosphorylate specific cellular substrates, including p107. Our results suggest that although the cdk subunit provides an essential component for substrate binding, it is the nature of the cyclin subunit, rather than that of the cdk subunit that is critical in targeting the complex to p107. Moreover, we demonstrate that cyclin A and p33*cdk2* bind to p107 *in vitro* in a co-operative manner, as a result of which p107 is efficiently phosphorylated on many sites that are also phosphorylated in human cells.

Results

Binding of cyclin A and p33cdk2 to p107 requires both proteins

In vivo, cyclin A has been shown to associate with p107, presumably in conjunction with a cdk that phosphorylates both p107 and cyclin A (Ewen *et al.*, 1992; Faha *et al.*, 1992). As a first step to elucidate the mechanism of substrate recognition by cyclin–cdk complexes, we investigated the interaction between p107, cyclin A and p33*cdk2*. The p107 protein contains a so-called pocket region that is conserved in Rb and is critical for the association with E2F, adenovirus E1A protein and SV40 LT antigen (Ewen *et al.*, 1991, 1992;

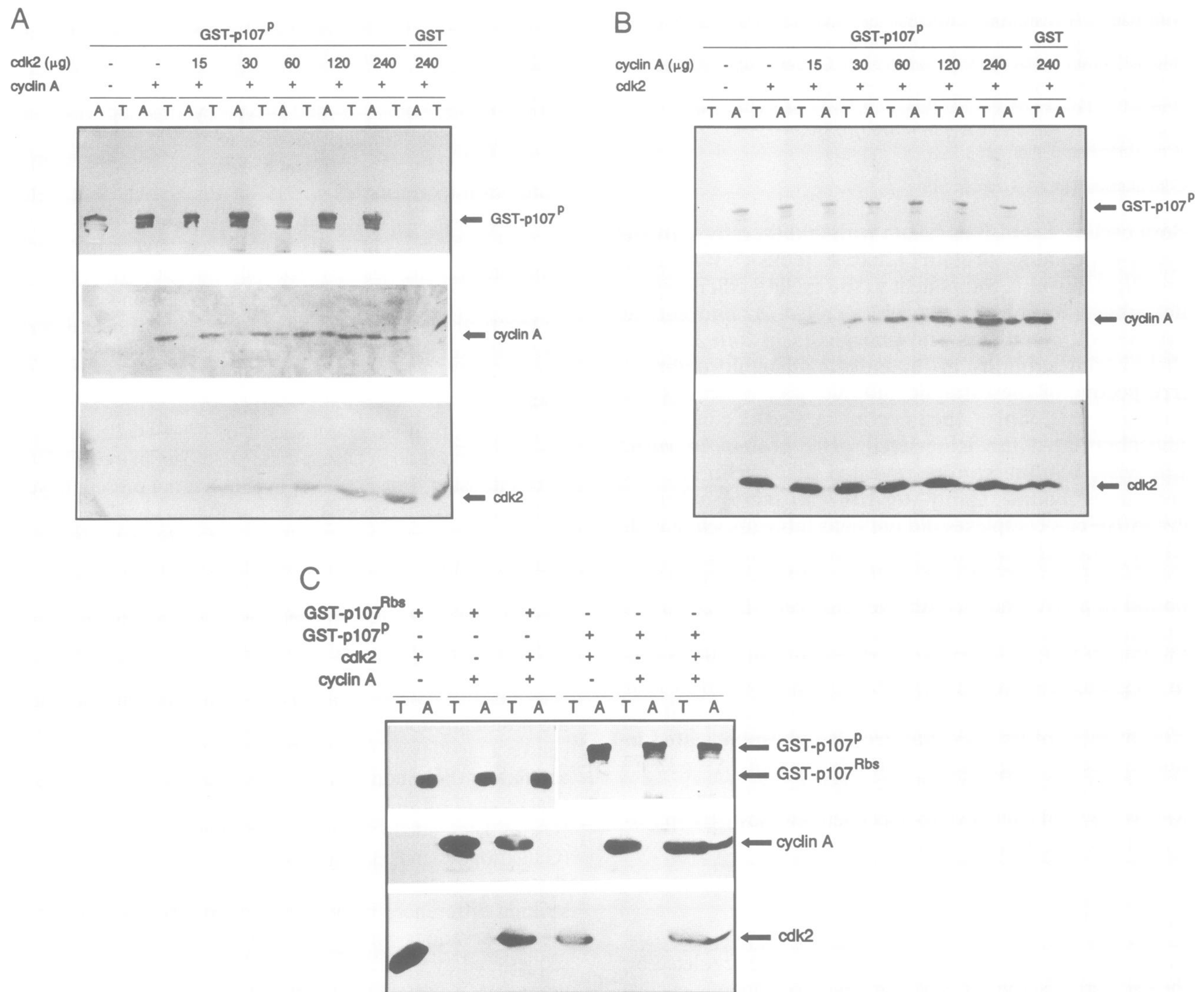

Fig. 1. The p33*cdk2* and cyclin A proteins bind co-operatively to the p107 spacer. GST, GST–p107 pocket (GST–p107P, amino acids 252–816) or GST–p107/Rb spacer (GST–p107Rbs, containing amino acids 252–443 and 654–816 of p107 with Rb residues 572–645 inserted between them) proteins were affinity-purified from *E.coli* and incubated with various amounts of insect cell extracts containing either p33*cdk2* or cyclin A proteins (240 μg extract, unless otherwise indicated). Just prior to the incubation with p107, 10% of the (mixed) insect extracts was removed to analyse the total amounts of p33*cdk2* and cyclin A added to the incubations (indicated as 'T'). 'A'-labelled lanes visualize proteins that were eluted from washed beads after incubation. Proteins were resolved on a 10% SDS–polyacrylamide gel, transferred onto nitrocellulose and probed with antibodies specific for either p33*cdk2* (bottom), cyclin A (middle) or p107 (top). Proper expression of GST was confirmed by staining the nitrocellulose filter with Ponceau S (not shown). **A.** The p107–cyclin A association is dependent on p33*cdk2*. In each lane, purified GST–p107 pocket was incubated with 240 μg of cyclin A-containing extract and the indicated amounts of extract containing p33*cdk2*. **B.** The p107–p33*cdk2* association is dependent on cyclin A. Purified GST–p107 pocket was incubated with p33*cdk2* and increasing amounts of cyclin A, as described for A. **C.** Cyclin A and p33*cdk2* bind to the spacer region in p107. Purified GST–p107/Rb spacer or GST–p107 pocket was incubated with extracts containing p33*cdk2* and/or cyclin A, as indicated.

Kaelin *et al.*, 1991; Faha *et al.*, 1992; Shirodkar *et al.*, 1992).

A GST−p107 fusion protein (amino acids 252−816, comprising the pocket region; Ewen *et al.*, 1992) was affinity-purified from bacteria and incubated with lysates prepared from *Spodoptera frugiperda* (Sf9) cells overproducing p33^{cdk2} or cyclin A. As shown in Figure 1A, the binding of cyclin A to the GST−p107 pocket protein was dependent on the presence of p33^{cdk2}. Moreover, the association of cyclin A with p107 could be enhanced by increasing the levels of p33^{cdk2} in the incubation mixtures. Likewise, the association of p33^{cdk2} with p107 was dependent on the presence of cyclin A (Figure 1B). The binding of cyclin A and p33^{cdk2} to GST−p107 was dependent on the p107 moiety of the fusion protein, since GST did not associate with either protein (Figure 1A and B, the right-hand lanes). As expected, the formation of a ternary p107−cyclin A−p33^{cdk2} complex was specific for the p107 spacer (a region both required and sufficient to bind to cyclin A *in vitro*; Ewen *et al.*, 1992): the p107 spacer alone efficiently associated with cyclin A−p33^{cdk2} (see Figure 2), whereas GST−p107^{Rbs} in which the p107 spacer is replaced by the spacer region of Rb did not (Figure 1C; Ewen *et al.*, 1991, 1992). Identical results were obtained when GST−cyclin A was used to bind to p33^{cdk2} and p107 (data not shown). Thus, neither cyclin A nor p33^{cdk2} alone formed stable complexes with p107 in this assay system. In some experiments we found a weak association of p107 with cyclin A in the absence of overproduced p33^{cdk2} (Figure 2A, left panel). This suggested that some endogenous insect cdks had been recruited by equivalent amounts of overproduced cyclin A to form ternary p107−cyclin A−cdk complexes, which was confirmed by reprobing the Western blot with anti-PSTAIR antibodies (data not shown). From the results shown in Figure 1 we cannot establish whether one of the proteins associates with p107 via the other, or both proteins simultaneously contact p107. Our results argue that cyclin A and p33^{cdk2} must form a complex as a prerequisite to associate with p107, or that both proteins bind to p107 in a strongly co-operative manner.

The cyclin subunit determines binding specificity of cyclin−cdk complexes

The observations that only cyclin A, but not cyclin B, is sequestered by the adenovirus E1A protein and is complexed to E2F together with p107 and p33^{cdk2}, prompted us to investigate what determines the specificity in the binding of cyclin−cdk complexes to cellular substrates. Using baculovirus expression vectors, we generated four stable cyclin−cdk complexes, in which the cyclin A and B, as well as the p33^{cdk2} and p34^{cdc2} subunits were exchanged. In mammalian cells the existence of a cyclin B−p33^{cdk2} complex has not yet been reported. However, affinity-purified GST−cyclin B efficiently bound to p33^{cdk2} in HeLa cell extracts (D.S.Peeper, unpublished results). Furthermore, in agreement with a previous report (Desai *et al.*, 1992) we found that also in insect cells, cyclin B readily associated with p33^{cdk2}, as judged by co-immunoprecipitation and kinase activity (see below). The various cyclin−cdk complexes were then monitored for their ability to stably associate with affinity-purified GST−p107 spacer.

Figure 2A demonstrates that neither cyclin A (consistent with the results shown in Figure 1), nor cyclin B significantly bound to p107 in the absence of p33^{cdk2}. However, in the presence of p33^{cdk2}, cyclin A readily associated with p107 (Figure 2A, left panel). In striking contrast, cyclin B did not detectably associate with p107, under identical assay conditions (Figure 2A, right panel). If the cyclin subunit confers the binding specificity to the cyclin−cdk complex, one might predict that substrate binding would be unaltered upon exchange of the cdk subunit. Indeed, when we

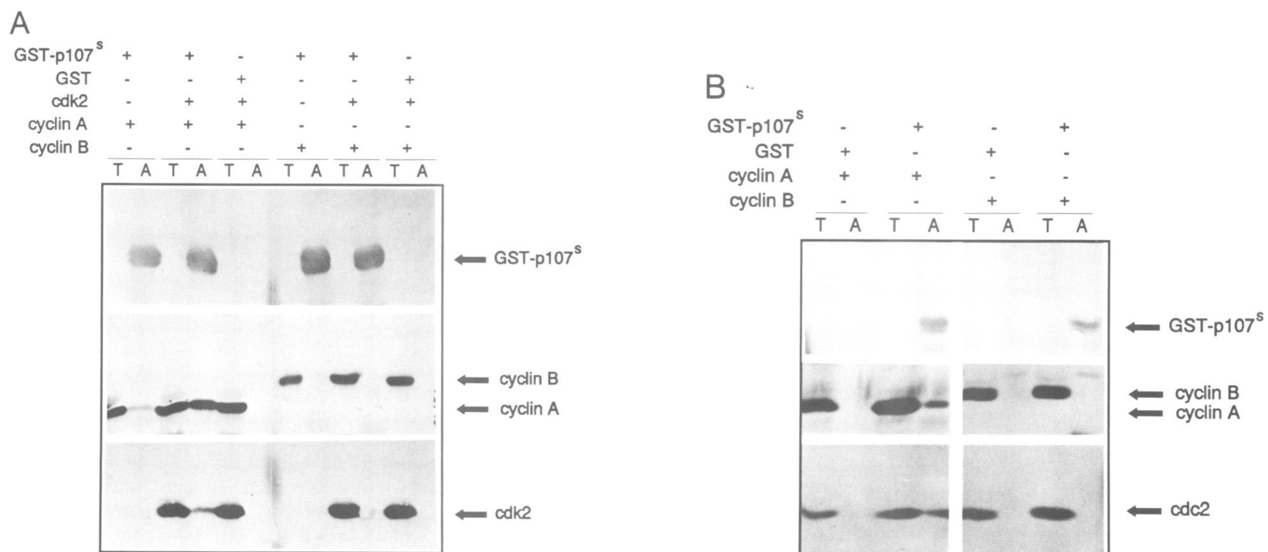

Fig. 2. Targeting to p107 of a cyclin−cdk complex is determined by the cyclin subunit. The experimental approach was the same as described for Figure 1. Samples were electrophoretically separated on duplicate gels that were subsequently used for Western blotting. Cdks and cyclins A and B were visualized on a different filter than the GST−p107 spacer. **A.** Cyclin A−p33^{cdk2} binds specifically to the p107 spacer. GST or GST−p107 spacer (GST−p107^{s}, amino acids 445−654) proteins, affinity-purified from *E.coli*, were incubated with either a cyclin A- or cyclin B-containing insect cell lysate in the absence or presence of a p33^{cdk2} lysate, as indicated. **B.** Cyclin A−p34^{cdc2} binds specifically to the p107 spacer. Affinity-purified GST or GST−p107 spacer proteins were incubated with extracts from insect cells containing p34^{cdc2} and either cyclin A or cyclin B, as indicated.

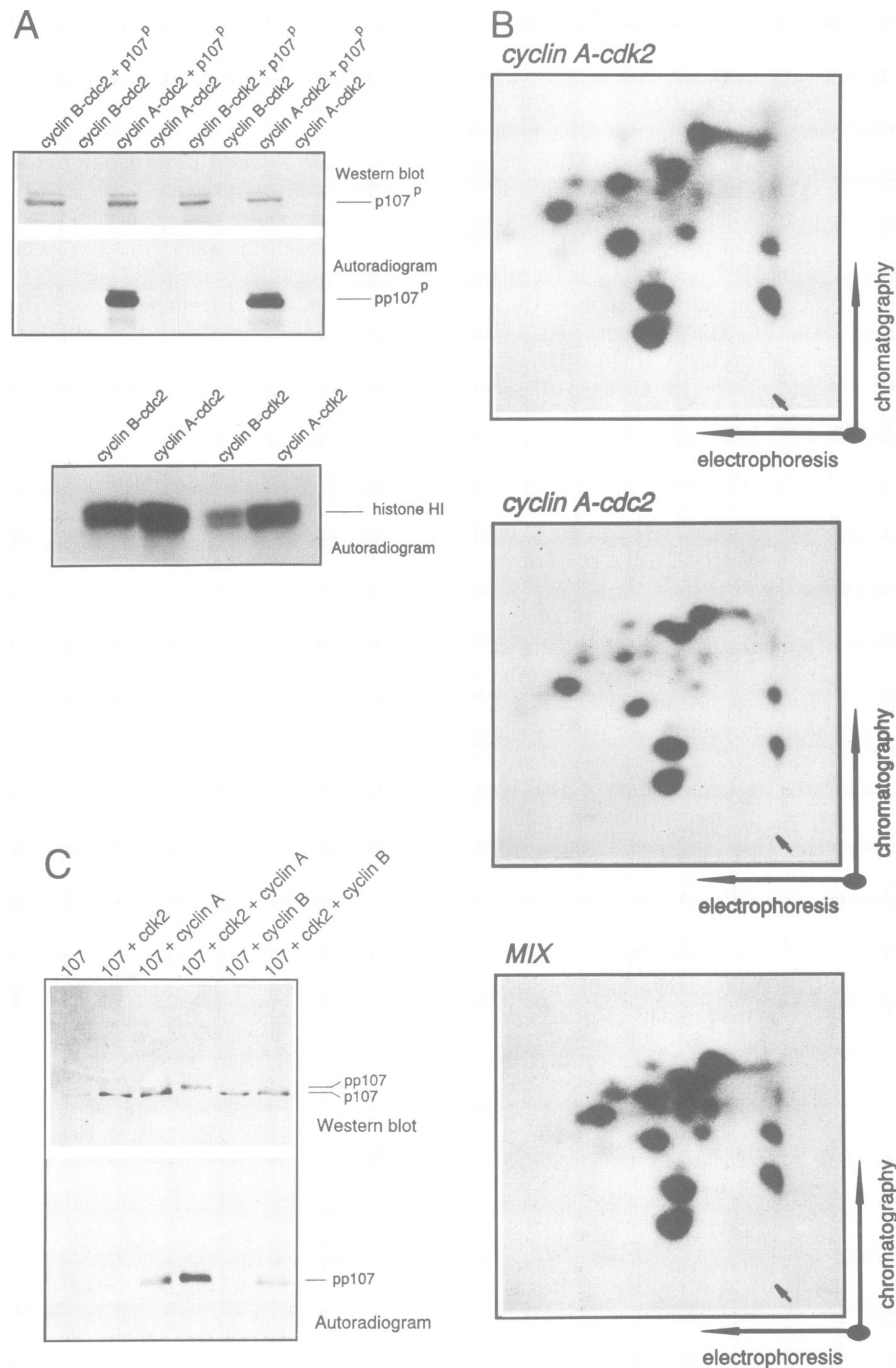

Fig. 3. Cyclin A−cdk complexes specifically phosphorylate p107 *in vitro* and *in vivo*. **A.** The p107 pocket protein is phosphorylated specifically by cyclin A−p33^{cdk2} and cyclin A−p34^{cdc2} complexes *in vitro*. GST−p107 pocket (GST−p107P) and GST−cyclin−cdk complexes as indicated were affinity-purified. Each cyclin−cdk complex was subsequently assayed for kinase activity towards either histone H1 or p107. Top panel shows the p107-specific Western blot; middle panel shows the corresponding autoradiogram. The blot was exposed for 16 h (left-half) or for 48 h (right-half). Lower panel shows histone H1 kinase activity associated with each cyclin−kinase complex. The amount of incorporated $^{32}P_i$ into histone H1 was 1 144 000 c.p.m. (cyclin A−p33^{cdk2}), 439 000 c.p.m. (cyclin B−p33^{cdk2}), 1 993 000 c.p.m. (cyclin A−p34^{cdc2}) or 1 339 000 c.p.m. (cyclin B−p34^{cdc2}); the $^{32}P_i$ incorporation into p107 was 294 000 c.p.m. (cyclin A−p33^{cdk2}), 3000 c.p.m. (cyclin B−p33^{cdk2}), 1 916 000 c.p.m. (cyclin A−p34^{cdc2}) or 19 000 c.p.m. (cyclin B−p34^{cdc2}). **B.** Cyclin A−p33^{cdk2} and cyclin A−p34^{cdc2} phosphorylate p107 on identical sites. The p107 protein was immunoprecipitated from infected insect cells and subjected to phosphorylation *in vitro* by affinity-purified GST−cyclin A−p33^{cdk2} or GST−cyclin A−p34^{cdc2} and tryptic phosphopeptide mapping was performed. The arrow marks the position of sample application. (i) Tryptic phosphopeptide map of p107 phosphorylated by GST−cyclin A−p33^{cdk2} (10 000 c.p.m.) (ii) Tryptic phosphopeptide map of p107 phosphorylated by GST−cyclin A−p33^{cdc2} (10 000 c.p.m.) (iii) Mixture of identical amounts of counts (10 000 c.p.m.) derived from phosphopeptides shown in (i) and (ii). The maps were exposed for 1 day. **C.** The p107 protein is phosphorylated specifically by the cyclin A−p33^{cdk2} kinase upon co-expression in insect cells. The p107 proteins were immunoprecipitated from [^{32}P]orthophosphate-labelled insect cell extracts after (co-)infection as indicated and analysed by SDS−PAGE, followed by Western blotting (top) and autoradiography (bottom). Proper expression of p33^{cdk2} and A- and B-type cyclins was confirmed by Western blotting of total cellular extracts (data not shown).

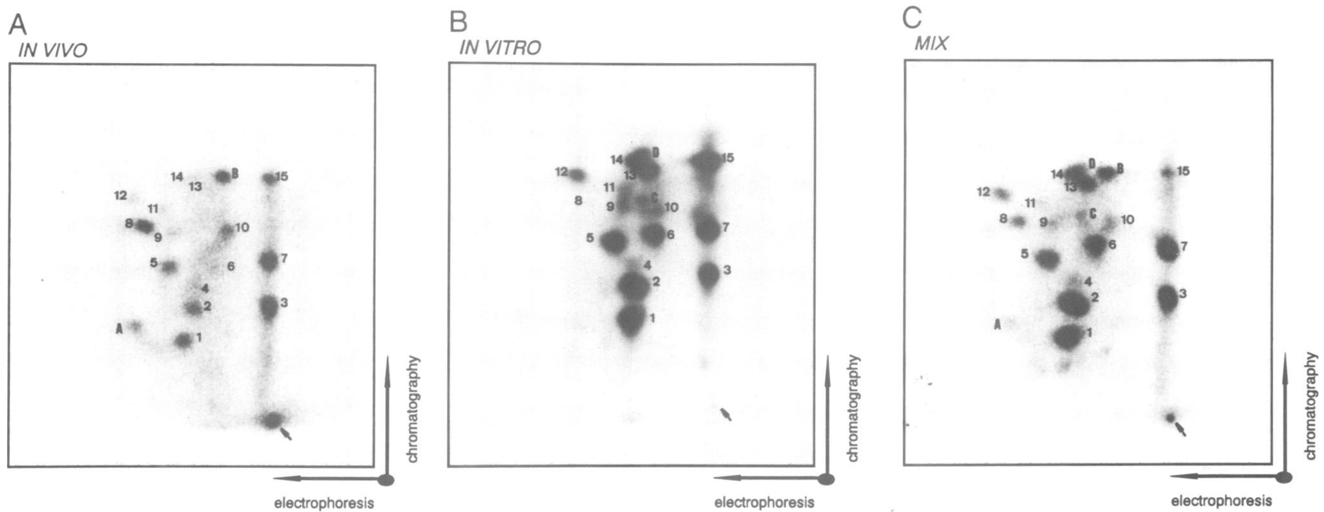

Fig. 4. Cyclin A−$p33^{cdk2}$ is a major kinase that phosphorylates p107 *in vivo*. Tryptic phosphopeptides of p107 that were consistently observed in both *in vivo* and *in vitro* phosphorylations are numbered 1−15. Spots that were unique to *in vivo* phosphorylated p107 are labelled A and B. Spots that were observed exclusively in *in vitro* phosphorylations are indicated as C and D. The arrow marks the position of sample application. **A.** Tryptic phosphopeptide map of p107 isolated from [^{32}P]orthophosphate-labelled 293 cells (200 c.p.m.). **B.** Tryptic phosphopeptide map of p107 that was immunoprecipitated from insect cell extracts and subjected to phosphorylation *in vitro* by purified GST−cyclin A−$p33^{cdk2}$ (1500 c.p.m.). Spot number 8 became more visible on a longer exposure of the same chromatogram. **C.** Mixture of identical amounts of counts (200 c.p.m.) derived from phosphopeptides shown in A and B. The maps were exposed with intensifying screens at −70 °C for 10 (A), 2 (B) and 10 (C) days, respectively.

substituted the $p33^{cdk2}$ subunit for $p34^{cdc2}$, only the cyclin A−$p34^{cdc2}$, but not the cyclin B−$p34^{cdc2}$ complexes associated with p107 (Figure 2B). Thus, these results argue that there is indeed specific substrate recognition by cyclin−cdk complexes and moreover that the specificity of binding resides within the cyclin subunit rather than within the cdk subunit.

The cyclin subunit confers substrate specificity to cyclin−cdk complexes

We next investigated whether the observed differences in the binding specificities between A- and B-type cyclin−cdk complexes could be extrapolated to corresponding differences in catalytic specificities. To determine whether the specific and co-operative binding of cyclin A−cdk complexes to p107 results in the phosphorylation of the latter, labelling experiments were performed both *in vitro* and *in vivo* (Figures 3 and 4, respectively).

First, we studied the ability to phosphorylate p107 *in vitro* for both the $p33^{cdk2}$ and $p34^{cdc2}$ kinase subunits, complexed to either A- or B-type cyclins. To this end, either soluble affinity-purified p107 pocket or histone H1 proteins were mixed together with purified cyclin−cdk complexes and kinase activities were assayed towards both potential substrates. In agreement with previous reports (Minshull *et al.*, 1990; Parker *et al.*, 1991), all four cyclin−cdk complexes similarly phosphorylated histone H1 (Figure 3A). In addition, we found that all cyclin−cdk complexes also similarly autophosphorylated their cyclin subunits (data not shown). However, the A- and B-type cyclin−cdk complexes, in spite of their similar behaviour towards histone H1 and their cyclin subunits, varied dramatically in their ability to phosphorylate p107 (Figure 3A). Both cyclin A−cdk complexes were 1.5- to 2.5-fold more active towards histone H1 than the two cyclin B−cdk complexes (Figure 3A, lower panel), yet they were ~100-fold more active towards p107 (Figure 3A, middle panel). In order to determine the sites on p107 of phosphorylation by the two cyclin A kinases, we performed two-dimensional tryptic phosphopeptide mapping. The maps revealed identical p107 phosphorylation patterns for cyclin A−$p33^{cdk2}$ and cyclin A−$p34^{cdc2}$ (Figure 3B). Thus, p107 formed a very efficient substrate for cyclin A-dependent kinases. However, more importantly and consistent with the results described above, the kinase species associated with the cyclin did not influence the recognition nor the phosphorylation pattern of the substrate.

These results were further supported when we examined the phosphorylation of p107 by cyclin−cdk complexes in co-infected insect cells. The p107 proteins overproduced in insect cells were either non- or hypophosphorylated (Figure 3C). Consequently, they could serve as good substrates for kinase reactions, both in insect cells (upon co-expression of a specific kinase) and *in vitro*, after their purification (Figure 3B and see below). Indeed, overproduced p107 was highly phosphorylated on co-expression with cyclin A and $p33^{cdk2}$, but hardly at all in the presence of cyclin B−$p33^{cdk2}$ complex (Figure 3C, lower panel). As anticipated, the phosphorylation of p107 was dependent on both cyclin A and $p33^{cdk2}$: upon co-expression of p107 and cyclin A, p107 was phosphorylated only to a low extent (consistent with the formation of few p107−cyclin A−cdk complexes in the absence of overproduced $p33^{cdk2}$, Figure 2A), while in the presence of either $p33^{cdk2}$ or cyclin B alone, p107 was not phosphorylated at all. The phosphorylation of p107 correlated with a quantitative and specific shift in its electrophoretic mobility by SDS−PAGE (Figure 3C, upper panel), which was reversed upon exogenous phosphatase treatment (data not shown). Taken together, we demonstrate that indeed, the *in vitro* binding data fully correlate with the *in vitro* and *in vivo* phosphorylation data: the cyclin subunit (either cyclin A or B) activates a given cdk catalytic subunit (either $p33^{cdk2}$ or $p34^{cdc2}$), but

in addition plays an essential role in targeting the kinase activity to a specific substrate.

Cyclin A – p33^{cdk2} *is a major kinase that phosphorylates p107 in vivo*

To determine whether the phosphorylation of p107 by the cyclin A–p33^{cdk2} complex *in vitro* overlapped with the sites of phosphorylation *in vivo*, we again performed two-dimensional tryptic phosphopeptide mapping. For this, p107 was immunoprecipitated with specific antibodies from human adenovirus-transformed 293 cells that had been metabolically labelled with [^{32}P]orthophosphate. Two-dimensional analysis revealed a complex pattern of tryptic phosphopeptides, consisting of 17 spots (Figure 4A). This pattern was specific for p107 and did not contain Rb phosphopeptides, since we used p107-specific antibodies that had been precleared on an Rb affinity column (Shirodkar *et al.*, 1992) and moreover, the same data were obtained with p107 isolated from Rb-deficient 5637 cells (a human bladder carcinoma cell line; data not shown).

Alternatively, overproduced human p107 was immunoprecipitated from insect cell extracts and subjected to phosphorylation *in vitro* by purified cyclin A–p33^{cdk2}. Figure 4B shows that the phosphopeptide map was remarkably similar to the map of p107 labelled *in vivo* in both the number and the location of the spots. The mixing experiment shows that 15 out of 19 spots co-migrated, whereas only two spots were unique for the phosphorylation *in vivo* and two were unique for the phosphorylation *in vitro* (Figure 4C). In agreement with a previous report (Herrmann *et al.*, 1991), phosphoamino acid analysis revealed that p107 was phosphorylated on serine residues and to a lesser extent on threonine residues (data not shown). Taken together, our results strongly suggest that the cyclin A–p33^{cdk2} complex (or a complex with overlapping substrate specificity) is a major kinase that catalyses the phosphorylation of p107 *in vivo*.

Discussion

The increasing number of (characterized) cdk and cyclin family members theoretically allows the assembly of multiple distinct cyclin–cdk complexes. Indeed *in vivo* a number of these complexes, including cyclin A–p33^{cdk2}, cyclin A–p34^{cdc2}, cyclin E–p33^{cdk2}, cyclin E–p34^{cdc2} and cyclin B–p34^{cdc2} (and *in vitro* cyclin B–p33^{cdk2}) have been observed (Fang and Newport, 1991; Koff *et al.*, 1991; Tsai *et al.*, 1991; Desai *et al.*, 1992; Elledge *et al.*, 1992; Pagano *et al.*, 1992b). Ultimately, the role of each of these kinases is predicted to be the phosphorylation of a specific cellular substrate(s). An important question then arises as to how the specificity in these catalytic reactions is achieved. The cyclin subunit has been proposed to be the likely candidate for fulfilling this role (Minshull *et al.*, 1990; Bandara *et al.*, 1991; Pagano *et al.*, 1992a).

To test this hypothesis, we have studied the interactions between cyclin A– and cyclin B–cdk complexes with the p107 protein *in vitro*. Using a baculoviral expression system, we have generated stable complexes between either cyclin A or B and p33^{cdk2} or p34^{cdc2}. We report that the two cyclin A–cdk complexes, but not the cyclin B–cdk complexes, stably associated with p107. These results argue that the cyclin component provides the targeting specificity of the cyclin–cdk complex. Interestingly, only cyclin A, but not cyclin B, is sequestered by the transforming adenovirus E1A protein (Giordano *et al.*, 1989; Pines and Hunter, 1990). *In vitro*, cyclin A does not seem to bind directly to E1A (D.S.Peeper, unpublished results). Our results therefore support a previously proposed model in which the interaction of cyclin A with E1A is thought to be mediated by p107 (Ewen *et al.*, 1992; Faha *et al.*, 1992).

The interaction between cyclin A–cdk complexes and p107 required both cyclin A and a cdk subunit (either p33^{cdk2} or p34^{cdc2}), since neither cyclin A, p33^{cdk2} nor p34^{cdc2} alone formed a stable complex with p107 under our experimental conditions. These results suggest that cyclin A together with p33^{cdk2} or p34^{cdc2} forms a p107 binding site or alternatively that one of the subunits undergoes a conformational change upon association with its partner, which in turn creates a p107 binding site. The finding that the cyclin A–cdk complex rather than the individual components binds to p107 is supported by previously published studies on the transcription factor E2F (Cao *et al.*, 1992; Devoto *et al.*, 1992; Pagano *et al.*, 1992a; Shirodkar *et al.*, 1992). In E2F band-shift assays, antibodies specific for either cyclin A or p33^{cdk2} abolishes the (slowest migrating) E2F–p107–cyclin A–p33^{cdk2} complex, suggesting that both cyclin A and p33^{cdk2}, but not each individual protein, associate with p107–E2F.

A stable physical interaction between cyclin–cdk complexes and their cellular targets is not always a prerequisite for substrate phosphorylation; however, complex formation seems to be essential for the phosphorylation of p107. Whereas cyclin A–cdk complexes were indistinguishable from cyclin B–cdk complexes in terms of histone H1 phosphorylation, only cyclin A–cdk complexes phosphorylated p107 and this correlated with the formation of stable ternary complexes *in vitro*. The pattern of p107 phosphorylation by cyclin A kinases was irrespective of the associated subunit, either p33^{cdk2} or p34^{cdc2}. The phosphorylation of p107 was also evident upon co-production in insect cells of p107 with cyclin A–p33^{cdk2}, but not with cyclin B–p33^{cdk2} complex. The observed specificities in these enzymatic reactions thus fully correlated with the *in vitro* association data. Taken together, these results strongly suggest that it is the nature of the cyclin subunit that is critical in this respect, whereas the cdk subunit can be substituted without influencing the substrate recognition.

The finding that cyclin-dependent kinases exhibit substrate-specific targeting *in vivo* is supported by two recent studies. First, cyclins A and E, but not B1 and B2, were demonstrated to induce Rb phosphorylation and subsequent inactivation *in vivo* (Hinds *et al.*, 1992). Secondly, partially overlapping, but not identical sets of substrates become phosphorylated upon the addition of either cyclin A or truncated cyclin B to *Xenopus* extracts (Thomas *et al.*, 1992). Neither of these studies, however, addressed which component in the cyclin–cdk complex was responsible for the observed targeting.

To date, no physiological substrate(s) has been identified for the cyclin A–p33^{cdk2} kinase. However, its association with E2F and p107 during S phase suggests that p107 and E2F are potential *in vivo* substrates. We have demonstrated that p107 is efficiently phosphorylated by the cyclin A–p33^{cdk2} complex *in vitro* as well as in co-infected insect cells. The question remained as to whether p107 is also a

substrate for this kinase *in vivo*. Our mapping studies argue strongly in favour of this possibility. We detected 17 phosphopeptides upon trypsin digestion of p107 isolated from labelled human cells. Of these phosphopeptides, 15 were also observed upon phosphorylation of p107 by cyclin A–p33^{cdk2} *in vitro*, as was judged from their co-migration on a two-dimensional map. The few dissimilarities between the *in vitro* and *in vivo* maps can be accounted for in several ways. First, for the *in vitro* phosphorylation we used a clone that lacks sequences encoding the precise N-terminus of p107 (Ewen *et al.*, 1991). This domain may contain (an) additional phosphorylation site(s) utilized *in vivo* by the cyclin A–p33^{cdk2} complex. Alternatively, cyclin A–p33^{cdk2} may be the major but not the sole kinase for p107 *in vivo*. Finally, we cannot rule out the possibility that the additional phosphopeptides seen in the maps generated from p107 labelled *in vivo*, are derived from a co-immunoprecipitating cellular phosphoprotein that co-migrates with phosphorylated p107 in SDS–polyacrylamide gels. Our finding that cyclin A–p33^{cdk2} is a genuine p107 kinase *in vivo*, is supported by the observations that during the cell cycle, elevated levels of phosphorylated p107 (present in anti-E1A immunoprecipitates) are seen in S and G_2/M, which coincides with the activity peaks of cyclin A-dependent kinases (Herrmann *et al.*, 1991; Pagano *et al.*, 1992b).

In vivo, the growth-suppressive function of Rb seems to be regulated by cyclin-dependent phosphorylations (Hinds *et al.*, 1992). The release of free E2F from the E2F–Rb complex in late G_1 is believed to play an important role in the activation of genes required for cellular proliferation (Hiebert *et al.*, 1992; Weintraub *et al.*, 1992). It is likely that like Rb, p107 is involved in the accurate modulation of E2F activity. As a part of the upstream regulation, the co-operative binding of cyclin A and p33^{cdk2} to p107 may play an important role in determining the affinity of p107 for E2F. Moreover, based on the E2F bandshift assays described above, p107 does not seem to bind to E2F in the absence of cyclin A and p33^{cdk2} (i.e. outside S phase). This may indeed suggest that p107 must first be associated with a cyclin–cdk complex in order for binding to E2F to occur.

It is tempting to speculate that also the other functions of cyclin A are involved in the regulation of E2F activity: it specifically targets its cdk catalytic subunit to p107 and it induces cdk activity, which results in the efficient phosphorylation of p107. Clearly, it is of interest to know how E2F activity is modulated upon p107–cyclin A–p33^{cdk2} binding and moreover, what the role is of the p107 phosphorylation in this respect. The p107 protein, in turn, may serve as a scaffold to facilitate the interactions between the cyclin A–p33^{cdk2} complex and E2F. The rather unexpected stable character of the p107–cyclin A–p33^{cdk2} complex may suggest that its binding to E2F during S phase serves to phosphorylate the latter protein and/or components of the basal transcription initiation machinery. Indeed, phosphorylation of E2F has been implicated previously in the modulation of E2F DNA-binding activity (Bagchi *et al.*, 1989).

Materials and methods

Generation of recombinant baculoviruses

All procedures relating to cell growth and viral propagation were performed as described previously by Summers and Smith (1987) and Piwnica-Worms (1990). The transfer vectors to generate the various recombinant baculoviruses were constructed as follows. For the construction of a baculovirus encoding p107, a 2.9 kb *Pst*I fragment encoding the nearly full-length p107 cDNA was isolated from Bluescript SK(−) (Ewen *et al.*, 1991) and inserted into the corresponding site in pIC20R. This clone was digested with *Bam*HI and *Sal*I and used to insert a linker encoding an ATG initiation codon. The sequences of the partially complementary linker oligonucleotides were: 5′-GATCCGCAAAATGCG-3′ and 5′-TCGACGCATTTTGCG-3′. Correct insertion of the linker was confirmed by sequencing. A 2.9 kb *Bam*HI–*Bgl*II fragment was subsequently isolated and ligated into the *Bam*HI site of the baculovirus expression vector pAcYM1 (Matsuura *et al.*, 1987). The human cyclin A gene was excised from pCycA (Pines and Hunter, 1990) with *Eco*RI as a 2.2 kb fragment and cloned directly into pVL1392 (Luckow and Summers, 1988) to generate pVLCycA. A p33^{cdk2}-expressing virus was generated by cloning the 1.3 kb *Xho*I–*Bam*HI fragment of pSE1000 (Elledge and Spottswood, 1991) after treatment with Klenow, into the *Sma*I site of the pJR2 baculovirus expression vector (kindly provided by J.Roosien). Generation of a GST–cyclin A virus was as follows: pCycA was digested with *Eco*RI and *Sma*I and the human cyclin A gene was cloned into pGEX1 (Pharmacia) to generate pGEX1CycA. This clone was then digested with *Eco*NI and ligated to two partially overlapping oligonucleotides 5′-ATCTAGATGCCTAT-3′ and 5′-TATAGGCATCTAGA-3′ to generate pGEX1CycA-X. This step inserted a unique *Xba*I site and an initiation codon at the N-terminus of the GST coding sequence. pGEX1CycA-X was digested with *Xba*I and *Eco*RI and the 2.5 kb insert containing the GST–cyclin A fusion gene was ligated into the corresponding sites in pVL1393 (Luckow and Summers, 1988). Generation of baculoviruses encoding the human p34^{cdc2} and cyclin B genes and the GST–human cyclin B fusion gene were as described by Parker *et al.* (1991) and S.Atherton-Fessler, L.L.Parker, R.L.Geahlen and H.Piwnica-Worms (in preparation).

For the p107 and p33^{cdk2} cDNAs, we used linearized pAcRP23-lacZ baculovirus DNA to generate recombinant viruses (kindly provided by R.Possee); for the other viruses, we used wild-type AcNPV DNA. Purified recombinant virus stocks were obtained as described by Peeper *et al.* (1992). Recombinant baculoviruses that had been plaque-purified at least twice were used to infect exponentially growing insect cells in TC-100 medium (Gibco) supplemented with 10% fetal calf serum, 2.5 μg/ml fungizone and 50 μg/ml gentamycin.

In vitro association assays and protein analysis

40 h after infection (m.o.i. of ~10) of Sf9 insect cells with recombinant baculoviruses, extracts were prepared in lysis buffer (50 mM Tris, pH 7.4, 0.5% Nonidet P-40, 5 mM EDTA, 150 mM NaCl, 50 mM NaF, 0.1 mM sodium orthovanadate) containing 1 μg/ml of each aprotinin, pepstatin and leupeptin and 1 mM PMSF. Lysates were clarified by centrifugation. In each incubation we mixed 5–10 μg of *Escherichia coli*-produced GST or GST–p107, purified by binding to glutathione–agarose (Parker *et al.*, 1991; Ewen *et al.*, 1992), with ~240 μg whole-cell extracts from virus-infected insect cells for 60 min at 4 °C. Beads were subsequently washed five times in lysis buffer and once in PBS prior to SDS–PAGE and Western blotting. Nitrocellulose filters were incubated with either anti-p107 serum (Shirodkar *et al.*, 1992), CHLA-1 anti-cyclin A IgGs (Hall *et al.*, 1991), anti-p34^{cdc2} serum (Parker *et al.*, 1991), anti-cyclin B serum (S.Atherton-Fessler, L.L.Parker, R.L.Geahlen and H.Piwnica-Worms, in preparation) or with anti-p33^{cdk2} IgGs (Elledge *et al.*, 1992). Positions of specifically bound antibodies were detected with alkaline phosphatase-conjugated sheep anti-rabbit IgGs (Jackson).

Phosphate labelling and two-dimensional phosphopeptide mapping

Either exponentially growing human 293 cells, human Rb-deficient 5637 cells or infected insect cells (at ~40 h p.i.) were cultured in the presence of [^{32}P]orthophosphate for 3 h, as described by Parker *et al.* (1991). Clarified extracts in lysis buffer were incubated with affinity-purified anti-p107 IgGs and immune complexes were collected on protein A–Sepharose beads. Alternatively, overproduced p107 was immunoprecipitated from insect cell extracts and used for phosphorylation *in vitro* by purified cyclin A–p33^{cdk2} kinase. Proteins were resolved by SDS–PAGE, transferred onto nitrocellulose and identified with specific antibodies. With the help of the autoradiogram the p107 band was localized, excised from either the gel or the filter and treated as described (Boyle *et al.*, 1991). The resulting phosphopeptides were analysed by electrophoresis towards the cathode at pH 1.9 for 30 min at 1000 V followed by ascending chromatography, as described by Boyle *et al.* (1991).

Kinase assays in vitro

GST–cyclin–cdk complexes from insect cell extracts and bacterially produced GST–p107 were purified on glutathione–agarose as described

by Parker et al. (1992). GST–fusion proteins were eluted from the agarose with 20 mM glutathione in 50 mM Tris, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$ and 1 mM $CaCl_2$ for 30 min at 4 °C with agitation. 10 units of thrombin (Sigma) were added to cleave the proteins from their GST–moieties (GST–cyclin A was not cleaved by thrombin since GEX1 does not contain a thrombin cleavage site). Kinase assays were performed by incubating the purified proteins in the presence of 0.2 mCi/ml [γ-^{32}P]ATP for 15 min at 30 °C, as described by Parker et al. (1991). Proteins were analysed by SDS–PAGE, Western blotting and autoradiography.

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