Regulation of cyclin-dependent kinase (Cdk) 2 Thr-160 phosphorylation and activity by mitogen-activated protein kinase in late G₁ phase

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Mitogen-activated protein (MAP) kinases, p42^{MAPK} and p44^{MAPK}, are central components of growth-promoting signalling pathways. However, how stimulation of MAP kinases culminates in cell-cycle progression is still poorly understood. Here we show that mitogenic stimulation of NIH 3T3 cells causes a sustained activation of MAP kinases, which lasts until cells begin progressing through the G₁/S boundary. Furthermore, we observed that disruption of the MAP-kinase pathway with a selective MEK (MAP kinase/extracellular-signal-regulated protein kinase) inhibitor, PD98059, prevents the activation of cyclin-dependent kinase (Cdk) 2 and DNA synthesis, even when added during late G₁ phase, once the known mechanisms by which MAP kinase controls G₁ progression, accumulation of G₁ cyclins

and degradation of Cdk inhibitors have already taken place. Moreover, we provide evidence indicating that MAP kinases control Cdk2 Thr-160 activating phosphorylation and function, possibly by regulating the activity of a Cdk-activating kinase, thus promoting the re-initiation of DNA synthesis. These findings suggest the existence of a novel mechanism whereby signal-transducing pathways converging on MAP kinases can affect the cell-cycle machinery and, ultimately, participate in cell-growth control.

Key words: Cdk-activating kinase, cell-cycle control, MEK inhibitors, PDGF.

INTRODUCTION

The exposure of quiescent cells to mitogens causes the rapid activation of a number of signalling pathways, which, in turn, control the nuclear expression of genes involved in the re-initiation of DNA synthesis and cell proliferation. One such pathway involves the activation of the small GTP-binding protein Ras and the stimulation of a protein-kinase cascade that includes, sequentially, Raf, MEK [mitogen-activated protein (MAP) kinase/extracellular-signal-regulated protein kinase (ERK) kinase], and p42 and p44 MAP kinases, also known as Erk-2 and Erk-1, respectively, referred to herein as MAP kinases [1–3]. Indeed, activation of the Ras-MAP-kinase pathway appears to be central for the proliferative response to many mitogenic growth factors [4], including platelet-derived growth factor (PDGF) [5], and persistent activation of this signalling route can be sufficient for oncogenesis [6,7].

The vast majority of growth factors act during the G₁ phase of the cell cycle, eventually promoting cells to progress through the G₁/S phase transition [8]. Cyclin-dependent kinase (Cdk) 4 and Cdk6, in association with D-type cyclins, and Cdk2, in association with cyclin E, positively regulate this transition by phosphorylating the retinoblastoma protein (pRb), which results in the release of E2-promoter-binding factor (E2F)-related proteins and the enhanced transcription of genes participating in cell-cycle progression [8]. This complex series of events is controlled by several co-ordinate regulatory mechanisms, which ensure the correct order and timing of cell-cycle transitions and the fidelity of DNA replication. While Cdk levels tend to remain constant, their catalytic activity is tightly regulated: this regulation depends both on the levels of cyclin subunits and Cdk inhibitors bound to the Cdks, and on the post-translational modification of the Cdk subunits through phosphorylation [9,10]. Specifically, the activity

of Cdks can be regulated negatively by phosphorylation of C-terminally located inhibitory residues, represented by Thr-14 and Tyr-15 in Cdk2, by Wee1-related kinases, or positively, by dephosphorylation of these amino acid residues by Cdc25-related phosphatases [9,10]. Furthermore, activating phosphorylation by Cdk-activating kinases (CAKs) of a threonine residue laying in the T loop of the Cdks is strictly necessary for the activity of these enzymes as, when unphosphorylated, this residue blocks the access of the substrates to the catalytic site of Cdks [9,10].

The Ras-MEK-MAP-kinase pathway is a key regulatory component of biochemical routes controlling cell-cycle progression, as it regulates cyclin D expression and p27 degradation in early and mid-G₁ phases [5,11–15]. Specifically, constitutively active forms of both Ras and MEK1 proteins are able to up-regulate the expression of cyclin D1, as well as to down-regulate p27 levels by repression of its synthesis and acceleration of its degradation [14,16,17]. Confirming these data, dominant negative forms of Ras and a potent MEK inhibitor, PD98059, have been demonstrated to inhibit cyclin D synthesis and p27 degradation [5,12,13,18]. In addition, a recent available report indicates that the MAP-kinase pathway is also required in G₂/M transition [19].

Here, we demonstrate that a sustained MAP-kinase activity is required for the final commitment to re-initiate DNA synthesis in NIH 3T3 cells, as inhibition of MAP-kinase activity at the G₁/S boundary prevents Cdk2 activation without affecting p27 degradation or the level of Cdk2-bound cyclin E. This effect is reflected by the inability of Cdk2 to phosphorylate one of its physiological substrates, pRb. Interestingly, we show that inhibition of the MAP-kinase-dependent activation of Cdk2 correlates with a decrease in the level of its Thr-160 activating phosphorylation, thus suggesting the existence of a novel role for the MAP-kinase signalling pathway in the control of the activity of CAKs.

Abbreviations used: Cdk, cyclin-dependent kinase; MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase/extracellular-signal-regulated protein kinase; PDGF, platelet-derived growth factor; pRb, retinoblastoma protein; CAK, Cdk-activating kinase; HA, haemagglutinin; PP, protein phosphatase.

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MATERIALS AND METHODS

Cell culture and transfections

NIH 3T3 fibroblasts were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY, U.S.A.) supplemented with 10% calf serum (BioWhittaker). Subconfluent cells, plated on 0.5 $\mu\text{g}/\text{cm}^2$ fibronectin (Boehringer Mannheim), were synchronized by 36 h of serum starvation with Dulbecco's modified Eagle's medium containing 10 mM Hepes, pH 7.5, 6 ng/ml sodium selenite and 6 $\mu\text{g}/\text{ml}$ transferrin (all from Life Technologies). Cells were either left untreated or stimulated with 10 ng/ml PDGF-BB (Upstate Biotechnology, Lake Placid, NY, U.S.A.). Where indicated, cells were also treated with 60 μM PD98059 (Calbiochem) or 10 μM U0126 (Promega). 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. 293T cells were transfected by Lipofectamine Plus Reagent, according to the manufacturer's instructions (Life Technologies), with the different expression plasmids. Cells were washed in cold PBS, and lysed at 4 °C in a buffer containing 20 mM Hepes (pH 7.5), 10 mM EGTA (pH 8.0), 40 mM β -glycerophosphate, 1% Nonidet P-40, 2.5 mM MgCl_2 , 1 mM dithiothreitol, 2 mM sodium vanadate, 1 mM PMSF, 20 $\mu\text{g}/\text{ml}$ aprotinin and 20 $\mu\text{g}/\text{ml}$ leupeptin. The lysates were centrifuged at 14000 g for 20 min at 4 °C and then assayed for protein concentration by BCA (bicinchoninic acid) protein assay reagents (Pierce).

Kinase assays and immunoprecipitations

For kinase assays, proteins (400 μg) from clarified supernatants were immunoprecipitated with the specific antibodies for 2 h at 4 °C, and immunocomplexes were recovered with Gamma-bind G (Pharmacia). Bound proteins were washed three times at 4 °C with PBS supplemented with 1% Nonidet P-40 and 2 mM sodium vanadate, once with 0.5 M LiCl in 100 mM Tris (pH 7.5), and once with the specific kinase reaction buffer [25 mM Hepes (pH 7.5), 20 mM MgCl_2 , 20 mM β -glycerophosphate, 0.1 mM sodium vanadate and 2 mM dithiothreitol for MAPK assays; 50 mM Hepes (pH 7.5), 10 mM MgCl_2 , 1 mM dithiothreitol, 2.5 mM EGTA, 5 mM MnCl_2 , 10 mM β -glycerophosphate, 0.5 mM NaF and 0.4 mM sodium vanadate for Cdk2 assays]. Reactions were performed in a 30- μl volume of kinase reaction buffer containing 1 μl of [γ - ^{32}P]ATP per reaction, unlabelled ATP (20 μM for MAPK assays; 2.5 mM for Cdk2) and the specific substrates [40 $\mu\text{g}/\text{reaction}$ myelin basic protein (Sigma) for MAPK; 20 $\mu\text{g}/\text{reaction}$ histone H1 (Sigma) for Cdk2], at 30 °C for 30 min. Reactions were terminated by addition of 5 $\times$ Laemmli buffer. Samples were boiled and proteins were separated by SDS/PAGE. Phosphorylated substrates were visualized by autoradiography and quantified by Phosphor-Imager analysis (Molecular Dynamics).

Western-blot analysis and antibodies

Lysates of total cellular proteins or anti-Cdk2 immunoprecipitates were analysed by protein immunoblotting after SDS/PAGE with the specific rabbit antisera or mouse monoclonal antibodies. Immunocomplexes were visualized by enhanced chemiluminescence (ECL[®]) detection (Amersham) with the use of goat antiserum to rabbit or mouse IgG coupled to horseradish peroxidase (Cappel, West Chester, PA, U.S.A.). Rabbit polyclonal antiserum to Erk1/Erk2, Cdk2, cyclins E, A, D1, D2 and D3, p21, p27, p57 and goat polyclonal antiserum to Cdk2 were purchased from Santa Cruz Laboratories (Santa Cruz, CA, U.S.A.); monoclonal antibodies to phospho-Rb

(Ser795) were purchased from New England Biolabs; monoclonal anti-phospho-Tyr antibodies were purchased from Upstate Biotechnology; anti-haemagglutinin (HA) monoclonal antibody (HA.11) was purchased from the Berkeley Antibody Company; anti-pRb-phosphoserine-780 polyclonal antiserum was provided kindly by A. M. Senderowicz, National Cancer Institute, Bethesda, MD, U.S.A.; specific anti-phospho-Cdk2 (Thr-160) polyclonal antiserum was provided kindly by New England Biolabs.

Cell-cycle analysis

Control and treated cells were harvested, washed in cold PBS and fixed in 70% ethanol. DNA was stained by incubating the cells in PBS containing propidium iodide (50 $\mu\text{g}/\text{ml}$) and RNase A (0.1 mg/ml) for 30 min at 4 °C. The DNA content of the cells was analysed for DNA fluorescence on a Becton Dickinson FACScan, using the Cell Quest software (Becton Dickinson Immunocytometry Systems, San Jose, CA, U.S.A.).

Thymidine-incorporation assays

NIH 3T3 cells were grown in 24-well plates and synchronized as described above. Cells, treated as indicated, were pulsed 14 h after PDGF stimulation with [^3H]thymidine (1 $\mu\text{Ci}/\text{well}$; ICN) for 4 h, fixed (5% trichloroacetic acid) and cellular DNA was solubilized (0.5 M NaOH) prior to scintillation counting. Experiments were performed in triplicate.

DNA constructs

Expression vectors encoding HA-epitope-tagged wild-type Cdk2 and the A14F15 and A160 mutants were provided kindly by Dr D. O. Morgan (University of California, San Francisco, CA, U.S.A.) [20]. A plasmid encoding cyclin E cDNA was provided kindly by Dr R. A. Weinberg (Whitehead Institute, Cambridge, MA, U.S.A.).

RESULTS AND DISCUSSION

PDGF-induced MAP-kinase activity in NIH 3T3 cells is sustained until late G₁ phase

Most of the known signalling events elicited by mitogenic stimulation, including the activation of the Ras-MEK-MAP-kinase pathway, occur within few min of exposure of quiescent cells to growth factors. However, it has been shown that microinjection of Ras-neutralizing antibodies in late G₁ phase is able to block G₁-to-S-phase transition [21] and, more recently, that the level of active GTP-bound Ras in cell-cycle-synchronized HeLa cells remains high even until late G₁ phase [22]. Thus to establish the time frame where the MAP-kinase pathway acts, we first examined the enzymic activity of the endogenous protein in response to PDGF stimulation of NIH 3T3 cells, a potent mitogen in these murine fibroblasts [23]. As shown in Figure 1(A), addition of PDGF to serum-starved NIH 3T3 cells caused a rapid increase of the endogenous MAP-kinase activity, peaking at 5–15 min and decreasing thereafter. However, MAP-kinase activity was sustained above control levels for up to 12 h (Figure 1A), at which time most of the PDGF-stimulated NIH 3T3 cells were just beginning to enter the S phase (Figure 1B). These results suggest that, in addition to their well-documented function in early gene expression, MAP kinases may also play a role in the late G₁ phase, possibly at the G₁/S boundary.

Figure 1 PDGF causes the sustained activation of MAP kinase during early and late G₁ phases

(A) Time course of MAP-kinase activity in response to PDGF stimulation. Serum-deprived NIH 3T3 cells were treated with PDGF (10 ng/ml) and then lysed at the indicated time points (m, min). MAP-kinase activity was assessed in immunocomplex kinase assays, and the protein levels of MAP kinase were determined by Western-blot (WB) analysis. Data are representative of five independent experiments. (B) Cell-cycle progression of NIH 3T3 cells after PDGF stimulation. Serum-deprived G₀-synchronized NIH 3T3 cells were treated with PDGF (10 ng/ml) for the indicated times and analysed subsequently by flow cytometry. Similar results were obtained in two additional experiments. MBP, myelin basic protein, PI, propidium iodide.

Effect of PDGF stimulation on cell-cycle regulatory molecules

To begin exploring a possible role for MAP kinases at the G₁/S transition, we investigated the temporal expression and activity of cell-cycle-regulating molecules upon addition of PDGF to quiescent serum-starved NIH 3T3 cells. As shown in Figure 2(A), an increase in endogenous Cdk2 activity was observed 6–8 h after PDGF stimulation, reaching a peak between 12 and 15 h, a time frame in which cells had already passed the G₁ restriction checkpoint and initiated a round of *de novo* DNA synthesis (Figure 1B). Cdk2 protein levels did not show significant variations at the different time points (Figure 2A). These data confirmed a direct correlation between PDGF-stimulated Cdk2 activity and G₁-S progression in this cellular setting.

As expected [24], parallel experiments revealed that growth-factor stimulation led to an increase in the protein levels of the G₁ cyclins, most remarkably D1 and D2, which peaked after 3 h, and cyclin E, whose expression was clearly increased after 6 h and reached a maximum 8–10 h after PDGF stimulation (Figure 2B). Thus, the elevation of G₁ cyclins preceded, but did not correlate with, Cdk2 activation. In contrast, the protein levels of cyclin A, which is involved in both S phase and the G₂/M cell-cycle transition [25], began to increase 14 h after PDGF treatment, reaching a plateau at 15 h (Figure 2B). Thus the expression of this cyclin may correlate with the peak of activation of Cdk2

Figure 2 Effect of PDGF stimulation on cell-cycle regulatory molecules

Quiescent NIH 3T3 cells were treated with PDGF (10 ng/ml) and lysed at the indicated time points. (A) Cellular lysates were analysed for Cdk2 activity in immunocomplex kinase assays. Cdk2 protein levels, analysed by Western blotting (WB) using specific antisera, were substantially unchanged at the different time points. (B) Protein levels of cyclins E, A, D1 and D2, and (C) the Cdk-inhibiting proteins p27, p21 and p57 were examined in lysates from PDGF-treated cells by Western-blotting analysis with specific antisera, as indicated. m, min. Results are representative of 3–5 independent experiments. H1, histone 1.

14 h after mitogenic stimulation. However, based on the time course of cyclin A expression and previous reports [25], this cyclin is likely to regulate the activity of Cdk2 during the S phase, once the G₁ restriction checkpoint has already been passed.

Cdk2 activity has also been shown to be regulated by the inhibitory activity of members of the CIP/KIP family of cyclin-dependent kinase-inhibitor proteins (p21, p27 and p57) [10,26]. In particular, p27 degradation has been shown to be a prerequisite for Cdk2 activation and G₁/S transition [27]. When we examined the effect of PDGF stimulation on this class of proteins, only p27 showed a dramatic decrease, reaching a minimal level between 6 and 8 h after PDGF addition (Figure 2C). Collectively, these data indicate that the temporal pattern of Cdk2 activation and DNA synthesis does not correlate with the elevation of G₁ cyclins or with the degradation of Cdk-inhibitory proteins, thus suggesting that Cdk2 is regulated by additional controlling mechanisms.

Effect of MEK inhibition on DNA synthesis and cell-cycle-dependent events in mitogen-stimulated NIH 3T3 cells

As an approach to examine the role of MAP kinases in cell-cycle regulation, we took advantage of the availability of synthetic inhibitors of MEK, PD98059 and U0126, which block MAP-kinase stimulation by preventing its phosphorylation by the upstream activating kinase [28,29] without affecting any other tested kinase, including proline-directed Ser/Thr kinases, such as

Figure 3 Effect of MEK inhibition on DNA synthesis and cell-cycle-dependent events in mitogen-stimulated NIH 3T3 cells

Effect of (A) PD98059 and (B) U0126 on PDGF-induced MAP-kinase activity. NIH 3T3 cells were serum-deprived for 15 h and then stimulated with PDGF (10 ng/ml). Where indicated, cells were treated with different concentrations of the PD98059 (A) and U0126 (B) or vehicle (—) 20 min prior to PDGF addition. Cells were lysed and MAP-kinase activity was assessed in immunocomplex kinase assays. MAP-kinase protein levels were determined by Western-blotting (WB) analysis. Data are representative of two independent experiments. (C) Schematic representation of the experimental design. NIH 3T3 cells were synchronized in G_0 by serum deprivation and stimulated with PDGF (10 ng/ml; time, 0 h). At the time points indicated, cells were treated with 60 μ M PD98059 and then lysed 15 h post PDGF stimulation, or examined for [3 H]thymidine incorporation from 15 to 18 h after PDGF addition. In the case of PD98059 treatment at time 0, the drug was added 20 min prior to PDGF stimulation to allow entry into the cells. (D) Anti-proliferative effect of PD98059 on PDGF-stimulated NIH 3T3 cells. Quiescent NIH 3T3 cells were left untreated (control) or stimulated with PDGF (10 ng/ml), and treated, as described in (C), with 60 μ M PD98059 or not (c), and then analysed for [3 H]thymidine incorporation. (E) PD98059 treatment in late G_1 phase of NIH 3T3 cells prevents Cdk2 activation without affecting p27 degradation. Serum-starved NIH 3T3 cells were treated with PDGF (10 ng/ml) and subsequently with PD98059 (60 μ M) at different times, as indicated, and Cdk2 kinase activity and protein levels (WB) of Cdk2 and p27 were examined at 15 h post-stimulation, as depicted. Similar results were obtained in three other

JNKs, p38 and Cdk2 [28,30]. We first sought to confirm the ability of these compounds to inhibit PDGF-induced MAP-kinase activation. As shown in Figures 3(A) and 3(B), both drugs were able to abolish PDGF-induced MAP-kinase activation in a concentration-dependent manner. For further experiments we chose to use PD98059 initially, based on its wider use for the study of the MAP-kinase signalling pathway, and available results were supported further by the use of U0126, a structurally unrelated MEK inhibitor [28], in subsequent experiments.

As an experimental design, PD98059 was added either 20 min prior to PDGF or at different time points (1–14 h) after PDGF treatment (Figure 3C). Expression level and activity of key cell-cycle regulatory molecules and incorporation of [³H]thymidine into cellular DNA were then assessed at 15 h and from 15 to 18 h, respectively, after stimulation with PDGF, a time frame of maximal activity for Cdk2, and when the majority of the cells would be expected to have already passed the G₁/S restriction point and initiated DNA synthesis (see above). Supporting a role for the Ras-MAP-kinase pathway in the regulation of early and mid-G₁ events, treatment of cells with PD98059 prior to PDGF or from 1 to 8 h after exposing cells to this growth factor prevented cell proliferation (Figure 3D, and results not shown). Of interest, however, was that the MEK inhibitor still prevented the onset of DNA synthesis when added between 8 and 12 h after PDGF treatment (Figure 3D). These data suggest an unexpected role for MAP kinase in the regulation of a critical step in late G₁ phase, which, as shown above, occurs after accumulation of G₁ cyclins and degradation of Cdk inhibitors, but prior to the G₁/S transition and DNA synthesis. This is also consistent with the remarkable sustained pattern of MAP-kinase activation (see above). In contrast, PD98059 was of limited effect when added 14 h after mitogenic stimulation (Figure 3D), thus demonstrating that this inhibitor does not affect directly the synthesis of DNA. This observation also suggested that the activity of MAP kinase is dispensable once DNA synthesis is initiated.

To help to define the target for MAP-kinase action in late G₁ phase, we examined whether treatment with PD98059 affects Cdk2 activity, an essential requirement for G₁/S transition in all mammalian cells [31]. As shown in Figures 3(E) and 3(F), treatment of NIH 3T3 cells with PD98059 up to 8 h after the addition of PDGF greatly reduced Cdk2 activity when measured at 15 h post-stimulation, but the MEK inhibitor exerted a much smaller effect on Cdk2 when added 12 or 14 h after PDGF. Noteworthy is that PD98059 treatment up to 1 h after PDGF stimulation prevented p27 degradation, as reported previously [16], but at 8 h post-PDGF the treatment with PD98059 did not affect the already decreased levels of p27 (Figure 3E). Thus inhibition of MAP-kinase activity prevents the activation of Cdk2 even during late G₁ phase (8–12 h), once the already described mechanisms by which the Ras-MAP-kinase pathway controls G₁ progression, p27 degradation and stimulation of G₁ cyclins [5,11,12] have already taken place.

To confirm that the MEK inhibitor prevents Cdk2 activation *in vivo*, we made use of site-specific anti-phospho-pRb antibodies. As shown in Figure 3(G), the use of an anti-phospho-pRb serum raised against the phosphorylated serine-780 of pRb, a Cdk4/Cdk6 phosphorylation site [32], revealed that PDGF stimulation (15 h) readily induced the appearance of phosphoserine-780 pRb, and that treatment with PD98059 8 h post-stimulation did

not affect pRb phosphorylation at this site. In contrast, under identical conditions, an anti-phospho-pRb antibody that recognizes pRb phosphorylated in Ser-795, a Cdk2 phosphorylation site [33], demonstrated the complete absence of pRb phosphorylation when PD98059 was added 8 h after PDGF (Figure 3G, lower panel). Together, these data suggest that after 8 h of mitogenic stimulation, corresponding to the late G₁ phase, MAP kinase does not regulate the activity *in vivo* of Cdk4 or Cdk6, but still exerts a controlling effect on the activation of Cdk2, as judged by both its activity *in vitro* and its ability to phosphorylate *in vivo* one of its natural substrates, pRb.

PD98059 treatment of NIH 3T3 cells in late G₁ phase does not affect the level of cyclin E and cyclin A bound to Cdk2 and Cdk2 Tyr-15 phosphorylation

As shown in Figures 4(A) and 4(B), addition of PD98059 8–14 h after mitogenic stimulation did not have any demonstrable effect on the amount of cyclin E and cyclin A bound to Cdk2, although it did abolish Cdk2 activation specifically when added at 8 h after PDGF treatment. Moreover, we have already shown in Figure 3(E) that the addition of PD98059 8 h after mitogenic stimulation did not affect the already reduced levels of p27 protein. Collectively, these data indicate that a MAP-kinase target other than p27, cyclin E and cyclin A controls Cdk2 activation in late G₁ phase. In this regard, besides binding to G₁ cyclins, full Cdk2 activation requires the dephosphorylation of its Thr-14 and Tyr-15 sites by Cdc25 phosphatase(s) [9,10], Tyr-15 being more extensively phosphorylated *in vivo* than Thr-14 [20]. As Tyr-15 is the only tyrosine-phosphorylated residue in the Cdk2 molecule [9], we therefore sought to investigate whether the MEK inhibitor affects the status of tyrosine phosphorylation of Cdk2 by using general anti-phosphotyrosine antibodies to perform Western blots on anti-Cdk2 immunoprecipitates from PDGF-stimulated NIH 3T3 cells. As shown in Figure 4(C), although Tyr-15 phosphorylation is inhibitory for Cdk2 activity, cellular Cdk2 was observed to be increasingly phosphorylated after mitogenic stimulation, as reported previously [20]. However, PD98059 treatment was found to have little or no effect on the phosphorylation of Cdk2 at Tyr-15, thus suggesting that changes in Cdc25 activity might not account for the inhibitory effect of the MEK blocker on Cdk2 activity.

Inhibition of MAP-kinase activity by PD98059 and U0126 treatment, in late G₁ phase, prevents Cdk2 Thr-160 activating phosphorylation

Phosphorylation of a conserved threonine residue (Thr-160 in Cdk2) is strictly required for the activation of all Cdk2s [9,10,34]. Enzymic activities phosphorylating this critical residue in Cdk2 have been purified from different organisms and termed CAKs [34]. In mammalian cells, CAK activity has been attributed to a complex between Cdk7 and cyclin H, which is also a component of the basal transcription complex TFIIF, required for both transcription initiation and nucleotide excision repair [34]. However, homologous proteins in budding yeast (Kin28/Ccl1) [35] have also been shown to have a role in transcription, but cannot act as CAKs either *in vitro* or *in vivo* [34]. Instead, this function in yeast has been attributed to a newly discovered kinase, Cak1

experiments. (F) Effect of PD98059 treatment on Cdk2 activity. Cells were processed as in (E), and Cdk2 activity was quantified by PhosphorImager analysis. Bars represent means $\pm$ S.E.M. of three independent experiments performed in duplicate and expressed as Cdk2 phosphorylating activity relative to untreated cells (control). (G) Effect of PD98059 treatment on phosphorylation *in vivo* of pRb by Cdk4/6 and Cdk2. Total cellular lysates from serum-starved NIH 3T3 cells, treated or not with PDGF (10 ng/ml) for 15 h, and with PD98059 (60 μ M) 8 h post-stimulation, as indicated, were analysed by Western blotting with antisera that specifically recognized pRb when phosphorylated at Ser-780 [32] or Ser-795 (New England Biolabs), as indicated.

Figure 4 Effect of PD98059 treatment on the Cdk2-bound cyclin E and cyclin A levels, and Cdk2 Tyr-15 phosphorylation

Lysates from NIH 3T3 cells, treated as described in Figure 3, were immunoprecipitated (IP) with specific anti-Cdk2 antibodies and immunoblotted with (A) anti-cyclin E (cycE), (B) anti-cyclin A (cycA) and (C) anti-phosphotyrosine (α -PY) antibodies, as indicated. The same blots were stripped and re-probed with anti-Cdk2 antibodies to confirm the Cdk2 protein level in the corresponding immunoprecipitates. Similar results were obtained in four independent experiments. WB, Western blotting.

(or CIV1 for CAK *in vivo*) [36–38], which lacks transcriptional regulation activity [34]. Furthermore, recently available information also suggests the existence of a mammalian Cak1 homologue, distinct from the Cdk7/cyclin H complex [39,40]. Together, these findings raised the possibility that the regulation of Cdk2 by phosphorylation of Thr-160 occurs through a kinase different from Cdk7/cyclin H, possibly a Cak1-related kinase [34]. Although the identity of the biologically relevant mammalian CAK might still be unclear, it is, nevertheless, possible to assess its function *in vivo* by following the phosphorylation of Cdk2 in Thr-160. To this end, we chose to use as an experimental approach a recently developed anti-Thr-160-phospho-Cdk2-specific antiserum, and also followed the electrophoretic mobility shift of Cdk2, which is known to occur upon phosphorylation of Cdk2 on Thr-160 [20].

Initially, we sought to confirm the ability of the anti-phospho-Cdk2 antibodies to recognize Cdk2 only in its Thr-160-

Figure 5 Inhibition of Cdk2 Thr-160 activating phosphorylation by MEK inhibitors in late G₁ phase

(A) Expression vectors for HA-epitope-tagged Cdk2 wild-type (WT), Cdk2 T14A-Y15F and Cdk2 T160A mutants (1 μ g each) were co-transfected in 293T cells by the Lipofectamine Plus method, along with a plasmid encoding cyclin E protein (1 μ g). Following transfection (24 h), cells were lysed and subjected to immunoprecipitation with anti-HA antibodies. Immunoprecipitates were analysed by Western blot (WB) using anti-phospho-Thr-160-specific Cdk2 antibodies. Expression of the different proteins was examined in the same membrane by stripping the antibodies and re-probing with anti-HA antibodies. Equal expression of cyclin E in each sample was confirmed (results not shown). GFP, green fluorescent protein. (B) Quiescent NIH 3T3 cells were treated as described in Figure 3. Lysates were immunoprecipitated with anti-Cdk2 antibodies and then immunoblotted with anti-phospho-Thr-160-specific Cdk2 antibodies, as indicated. Alternatively (C), total cellular lysates were immunoblotted using anti-Cdk2 antibodies. P-Cdk2 indicates the faster-migrating form of Cdk2 that corresponds to Cdk2 phosphorylated at Thr-160 [20]. Results are representative of two independent experiments, performed in triplicate. (D) Quiescent NIH 3T3 cells were treated as described in Figure 3, but using 10 μ M U0126 as a pharmacological inhibitor of MEK. Lysates were immunoprecipitated with anti-Cdk2 antibodies and then immunoblotted with anti-phospho-Thr-160-specific Cdk2 antibodies, as indicated.

phosphorylated form. For these experiments, we transfected 293T cells with HA-epitope-tagged wild-type Cdk2, a double mutant Cdk2 in which threonine-14 and tyrosine-15 were

Scheme 1 Schematic representation of cell-cycle regulatory events affected by the MAP-kinase signalling pathway

The Ras-Raf-MEK-MAP-kinase pathway regulates the expression of G₁ cyclins and the formation of Cdk–cyclin complexes [5,47], the degradation of Cdk-inhibitory molecules, including p27 [13], and the activation of Cdk-activating kinases (CAK; this study), thereby leading to the formation of active cyclin E–Cdk2 complexes which, in turn, promote the re-initiation of DNA synthesis. The nature of additional targets for MAP kinase once Cdk2 is fully active, and prior to the initiation of DNA synthesis, is under current investigation. CycE, cyclin E.

replaced with alanine and phenylalanine, respectively (Cdk2 T14A-Y15F), and a Cdk2 mutant in which the threonine-160 was replaced with alanine (Cdk2 T160A). As shown in Figure 5(A), in actively growing 293T cells the anti-phospho-Thr-160 Cdk2 antiserum readily recognized Cdk2 in anti-HA immunoprecipitates. Furthermore, the immunodetection of Cdk2 was not affected by mutations affecting the phosphorylation of the N-terminal regulatory sites on Cdk2. However, this antiserum failed to recognize the T160A mutant form of Cdk2 (Figure 5A), thus supporting its specificity for phospho-Thr-160 Cdk2. Although whether this antiserum can also recognize other phosphorylated Cdk2s has not been thoroughly tested yet, the ability to immunoprecipitate Cdk2 using anti-epitope antibodies or anti-Cdk2 antibodies should ensure, nonetheless, the specificity of this approach.

Stimulation of quiescent NIH 3T3 cells with PDGF caused the accumulation of Thr-160-phosphorylated Cdk2, as judged by the use of anti-phospho-Thr-160 Cdk2 antibodies (Figures 5B and 5D) and by the appearance of faster-migrating Cdk2 species (Figure 5C). This increase started to take place around 6–8 h after stimulation and reached its maximal intensity between 12 and 15 h post-stimulation ([41], and results not shown), the time of maximal Cdk2 activation (see above). These data are consistent with the activation of a cellular CAK activity upon mitogenic stimulation. Interestingly, PD98059 treatment of NIH 3T3 cells 8–10 h after PDGF stimulation nearly abolished the later Thr-160 activating phosphorylation of Cdk2 (Figures 5B and 5C), but no significant effects were observed when adding the drug at later time points (12 and 14 h after PDGF stimulation; Figures 5B and 5C). Similar results were obtained when using U0126 as a MEK inhibitor (Figure 5D). These data further support the notion that phosphorylation of Thr-160 is an essential step in the activation of Cdk2, and suggest that this phosphorylation and the Cdk2-phosphorylating activity, CAK, is under the control of MAP kinases.

The state of Thr-160 Cdk2 phosphorylation depends on a balance between the activities of specific kinases and phosphatases [34,42]. Thus activation of Cdk2 phosphatases could mediate the inhibition of Thr-160 phosphorylation on Cdk2 upon MEK blockade. However several lines of evidence suggest that this is unlikely. Thus far, two phosphatases, Cdk-associated phosphatase (KAP) and protein phosphatase (PP) A1, have been shown to dephosphorylate Cdk2 on Thr-160 [42]. However, KAP can only recognize phospho-Thr-160 on monomeric Cdk2 [42], and would not be expected to regulate Cdk2 function after 8 h of PDGF stimulation, when cyclin E is at its highest level and stably associated with Cdk2. On the other hand, PPA1 can dephosphorylate Cdk2 Thr-160 on both monomeric and cyclin E-associated Cdk2 [42], but the observation that PD98059 does not affect the state of Thr-160 phosphorylation when added 10–12 h after PDGF stimulation strongly suggests that the inhibition of the MAP-kinase pathway does not result in PPA1 activation. In addition, recent work suggests that type-2C PP2Cs are the predominant phosphatases acting on human Cdk2 in HeLa cell extracts [43]. Nevertheless, the constitutive expression of PP2C throughout the cell cycle [44], and the evidence that PP2C dephosphorylates Cdk2 only upon cyclin degradation [43], make unlikely the involvement of PP2C-related enzymes in mediating the PDGF effects on Thr-160 Cdk2 phosphorylation in late G₁ phase.

In contrast, our present findings suggest that the Thr-160 phosphorylation on Cdk2 by endogenous CAK is the target for MAP-kinase regulation. This phosphorylation requires the previous cyclin binding [45], the dissociation of p27 from Cdk2–cyclin complexes and the consequent degradation of p27 [16]. Thus the temporal order of these events could explain why the initial peak in MAP-kinase activity, at a time in which cyclin E is absent in the cells (see above), does not result in the immediate Cdk2 Thr-160-phosphorylation. It can also explain why a sustained MAP-kinase activity is strictly necessary until the very

late G₁ phase, when p27 degradation and cyclin E association with Cdk2 renders the Cdk2 molecule apt to be phosphorylated at the Thr-160 residue, and thereby to be activated by CAK, in a MAP-kinase dependent fashion.

In all, the picture emerging from this study and from recently published reports is that environmental signals transmitted to the nucleus through the Ras-Raf-MEK-MAP-kinase pathway regulate key molecules controlling cell-cycle progression in a distinct temporal pattern, which includes the expression of G₁ cyclins in early G₁ [11], the timely degradation of Cdk inhibitors in mid-G₁ [13] and the phosphorylation-dependent activation of Cdk2 in late G₁ phase (see Scheme 1). In addition to MAP kinase, these cell-cycle events might also be affected, positively or negatively, by the activity of additional signalling pathways [46], including those involving highly related protein kinase cascades, a possibility that is under intense investigation. We can postulate that this increasingly complex regulatory network regulating the activating phosphorylation of Cdk2 might allow the cells to 'sense' and integrate growth-stimulatory as well as inhibitory signals even just prior to the final commitment to DNA synthesis, thus providing a novel mechanism whereby signal-transducing pathways can affect the cell-cycle machinery and, ultimately, participate in cell-growth control.

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