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Differential Regulation of Cyclin-Dependent Kinase 4 (CDK4) and CDK6, Evidence that CDK4 Might Not Be Activated by CDK7, and Design of a CDK6 Activating Mutation[▽]

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The homologous cyclin-dependent kinases (CDK) CDK4 and CDK6 integrate mitogenic and oncogenic signaling cascades with the cell cycle. Their activation requires binding to a D-type cyclin and then T-loop phosphorylation at T172 and T177 (respectively) by the only CDK-activating kinase identified in animal cells, cyclin H-CDK7. At odds with the existing data showing the constitutive activity of CDK7, we have recently identified the T172 phosphorylation of cyclin D-bound CDK4 as a crucial cell cycle regulatory target. Here we show that T172 phosphorylation of CDK4 is conditioned by its unique proline 173 residue. In contrast to CDK4, CDK6 does not contain such a proline and, unexpectedly, remained poorly phosphorylated and active in a variety of cells. Mutations of proline 173 did not adversely affect CDK4 activation by CDK7, but in cells they abolished CDK4 T172 phosphorylation and activity. Conversely, substituting a proline for the corresponding residue of CDK6 enforced its complete, apparently cyclin-independent T177 phosphorylation and dramatically increased its activity. These results lead us to propose that CDK4 might not be phosphorylated by CDK7 in intact cells but is more likely phosphorylated by another, presumably proline-directed kinase(s). Moreover, they provide a new model of a potentially oncogenic activating mutation of a CDK.

Cyclin-dependent kinase 4 (CDK4) and its functional homologue CDK6 act as master integrators in the G_1 phase, coupling with the cell cycle mitogenic and antimitogenic signals as well as with their oncogenic perversions in cancer cells (7, 60, 61). They phosphorylate and inactivate the cell cycle/tumor suppressor proteins of the pRb family ($p105^{Rb}$, p107, and $p130^{Rb2}$) (3, 22, 42, 69) and Smad3 (47). This leads to both E2F-dependent transcription of essential cell cycle enzymes and regulators (50) and assembly of the prereplication complex (9, 26). CDK4/CDK6 activity is deregulated through various mechanisms in many human tumors (27, 52, 61), and it was recently found to be crucial for various oncogenic transformation processes (39, 49, 58, 66). With regard to the interface between mitogenic/oncogenic signaling cascades and autonomous cell cycle orchestration, understanding the molecular mechanisms of CDK4/CDK6 regulation thus remains of fundamental importance.

The activation of CDK4/CDK6 is a multistep process that absolutely requires first the binding of a D-type cyclin (D1, D2, or D3) and then an activating phosphorylation in the T-loop at T172 for CDK4 (8, 38) and T177 for CDK6 (34). It is generally considered that mitogens activate CDK4/CDK6 by inducing D-type cyclins to concentrations that allow an inhibitory threshold imposed by INK4 CDK4/CDK6-inhibitory proteins to be overcome (59). Nevertheless, various molecular features of CDK4/CDK6 activation, including the regulation of their association with cyclins (11, 19) and of their T-loop phosphorylation (8, 37, 53), as well as the complex roles of Cip/Kip CDK "inhibitors" (p21, p27, and p57) in these processes (2, 8, 17, 18, 37, 56, 62), remain poorly known or debated. At variance with the coexistence of several CDK-activating kinases (CAK) in fungi and plants (32, 67, 68), in animal cells one single CAK constituted of the cyclin H-CDK7-Mat1 complex is considered to be responsible for activating phosphorylation of the various cell cycle CDKs (23, 24, 30), including CDK4 (46). Besides its role in CDK activation, CDK7 as a component of the general transcription factor TFIIH phosphorylates the C-terminal domain of RNA polymerase II and is thus required for its activity (23). Since the presence, activity, and nuclear localization of CAK (CDK7) are generally constitutive and nonregulated during cell cycle or mitogenic stimulations (8, 15, 46, 55, 57, 65), activating phosphorylation of CDK4/CDK6 is assumed to passively result from CDK4/CDK6 binding to a cyclin D and subsequent nuclear import (20). Binding of p27 to cyclin D-CDK4/CDK6 also impairs their phosphorylation by CDK7 (34, 37, 56).

The activating T172/T177 phosphorylations of CDK4/CDK6 have been infrequently investigated in intact cells because of a lack of methodological tools. Using two-dimensional (2D) gel electrophoresis (isoelectric focusing and sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]) to separate phosphorylated and nonphosphorylated forms of CDK4 combined with mutagenesis and characterization of a first pilot production of a T172-phospho-specific CDK4 antibody, we have recently identified the T172 phosphorylation of cyclin D-bound CDK4 as a crucial direct target of various extracellular mitogenic or antimitogenic factors and their signaling cascades, determining CDK4 activity, pRb phosphorylation, and cell cycle progression (8, 14, 53, 55, 57). In various cell types, the regulation of T172 phosphorylation is clearly dissociated from modulations of CDK4 binding to cyclins and p27

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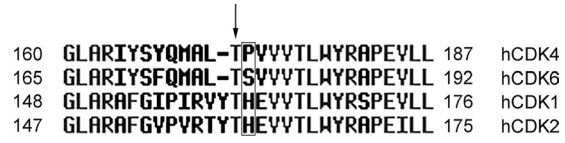

FIG. 1. Alignment of proteic sequences surrounding the phosphoacceptor residues of human CDK4 (T172), CDK6 (T177), CDK1 (T161), and CDK2 (T160). The box outlines the residues that directly follow the phosphorylated threonines.

or p21 and contrasts with the absence of regulation of CDK7 activity (7).

Also at odds with an implication of CAK (CDK7) in regulated CDK4 phosphorylation is that, in T98G glioma cells, the presence of serum induced the phosphorylation of cyclin D3-bound CDK4 but not that of cyclin D3-bound CDK6, which remained poorly phosphorylated (8). We now describe a similar difference in other cell systems and situations and hypothesize that it could be determined by the only difference in the sequence surrounding phosphorylation sites of CDK4 and CDK6: the phosphorylated threonine is followed by a proline in CDK4 but by a serine in CDK6 (Fig. 1). At variance with the observation that CDK recognition by CAK (CDK7) does not depend on a consensus sequence around the phosphoacceptor residues (23, 40), we show here that the mutation of the proline 173 residue of CDK4 abolishes its T172 phosphorylation and activity in intact cells, suggesting that CDK4 might be activated by other proline-directed kinase(s). Moreover, the mutation of the corresponding residue of CDK6 into proline enforces apparently cyclin-independent T177 phosphorylation of CDK6 and dramatically increases its activity, providing the first example of such an activating mutation of a CDK.

MATERIALS AND METHODS

Cloning and mutagenesis. For transfections, cDNAs encoding hemagglutinin (HA)-tagged human CDK4, X-press-tagged human cyclin D3, Flag-tagged human cyclin D1, and untagged human CDK6 were subcloned by PCR into mammalian expression vectors (pcDNA3.1His for cyclin D3-X-press, pcDNA3.1Myc-His for CDK4-HA, PCS2 Flag for cyclin D1-Flag, and pcDNA6 for untagged CDK6 [Invitrogen]). The initial constructs encoding the human untagged cyclin D3 (pXD3), cyclin D1 (pXD1), CDK4, and CDK6 were kindly provided by Jiri Lukas and Jiri Bartek. Site-directed mutagenesis (CDK4T172A, CDK4P173S, CDK4P173H, CDK6S178P, and CDK6S178H) was performed by two-step PCR using oligonucleotide primers containing the desired mutation. All the constructions were verified by sequencing.

Cell culture and transfections. T98G human glioblastoma cells (American Type Culture Collection, Manassas, VA) and HEK (human embryonic kidney) cells were cultured in Dulbecco's modified Eagle's medium, CHO (Chinese hamster ovary) cells were cultured in Ham's F12 medium, HCT116 (human colorectal carcinoma) cells were cultured in McCoy's 5A medium, and, finally, CEM cells (human T lymphoblastoid cell line) were cultured in suspension in RPMI 1640 medium. All the media were supplemented with antibiotics and 10% fetal calf serum (FCS). Cells were transfected with 6 μg of each construct (or with empty vector added to achieve a total of 12 μg DNA) by the use of Lipofectamine (Invitrogen) (T98G cells) or Fugene (Roche Diagnostics, Mannheim, Germany) (CHO and HCT116 cells) or by calcium phosphate precipitation (HEK cells) and harvested 24 h (HCT116, HEK, and T98G cells) or 48 h (CHO cells) after transfection.

Immunoprecipitations and Western blotting. For the analysis of protein complexes and pRb-kinase activity, cells were lysed and homogenized in 1 ml of NP-40 lysis buffer as described previously (53). Lysates (typically from 10 to 50 μg of proteins for transfected cells and about 1 mg for untransfected cells) were incubated at 4°C for 3 h with protein A-Sepharose (Amersham Biosciences, Uppsala, Sweden) that had been preincubated overnight with 2 μg of the following antibodies: polyclonal antibodies against CDK6 (C-21), p21 (C-19), or p27 (C-15) (all from Santa Cruz) or monoclonal antibodies against cyclin D1 (DCS-11), cyclin D3 (DCS-28), CDK6 (DCS-83) (all from Neomarkers [Fremont, CA]), HA epitope (Santa Cruz), or Flag epitope (M2 from Sigma).

Whole-cell extract proteins were separated on SDS-PAGE (10%) according to molecular mass and immunoblotted. The polyclonal and monoclonal antibodies used were as described above, except that cyclin D3 DCS-22 antibody (NeoMarkers) was used.

pRb-kinase assay. Exactly as described previously (8, 53), immunoprecipitated protein complexes were incubated with ATP and a recombinant pRb fragment (QED Bioscience) before SDS-PAGE separation of the incubation mixture and Western blotting detection of the phosphorylated pRb fragment by the use of phospho-specific pRb antibodies from Cell Signaling Technology (S780, S807/811, and S795) or Biosource (T826, T821, and S807). Membranes were then reprobed using the antibodies described above for detection of CDK4, CDK6, and cyclin D3.

Separation of phosphorylated CDK4 and CDK6 by 2D gel electrophoresis. Exactly as described previously (8), immunoprecipitated protein complexes were denatured in a buffer containing 7 M urea and 2 M thiourea. Proteins were separated on immobilized linear pH gradient (pH 3 to 10) strips (Amersham Biosciences) by isoelectric focusing. After SDS-PAGE separation and blotting, CDK4 was immunodetected using a sample of a noncommercialized polyclonal phospho-specific CDK4 (T172) antibody from Cell Signaling Technology (Beverly, MA) that we fully characterized previously (8) or C-22 polyclonal CDK4 antibody (Santa Cruz). CDK6 was detected using phospho-specific CDK4 (T172) antibody (which also recognizes the CDK6-activating phosphorylation at T177 [8]), DCS-83 monoclonal antibody (NeoMarkers), or C-21 polyclonal CDK6 antibody (Santa Cruz). Membranes detected with the phospho-specific antibodies were reprobed for detection of total CDK4 or CDK6.

Dephosphorylation of cyclin D3-CDK4/CDK6 complexes. Immunoprecipitates containing CDK4 or CDK6 were washed four times in NP-40 buffer and once with water. They were then incubated for 1 h at 30°C in λ-phosphatase buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 0.1 mM EGTA, 2 mM dithiothreitol, 0.01% Brij 35, 2 mM $MnCl_2$) in the presence of 400 units of λ-phosphatase (New England Biolabs).

In vitro activation of cyclin D3-CDK4/CDK6 complexes by CAK. Proteins obtained from transfected cells were immunoprecipitated in NP-40 lysis buffer as described above. The immunoprecipitations were washed three times with NP-40 lysis buffer and then three times with CAK buffer (80 mM β-glycerophosphate [pH 7.3], 15 mM $MgCl_2$, 20 mM EGTA, 5 mM dithiothreitol [46]). The beads were resuspended in 40 μl of CAK buffer containing protease and phosphatase inhibitors and 2 mM ATP with or without 1 μg of recombinant CDK7-cyclin H-MAT1 complex (Upstate, Charlottesville, Virginia) and incubated at 30°C for 30 min. After six washes in the appropriate buffer, the immunoprecipitated proteins were either prepared for 2D gel electrophoresis analysis of CDK4 and CDK6 or were assayed for pRb kinase activity.

Impact of CDK4 mutations on mitogenesis. During starvation in 0.2% FCS, T98G cells were transfected for 12 h using Lipofectamine with 2 μg of HA-tagged CDK4 constructs/ml. Cells were then stimulated for 16 h using 15% FCS, with BrdU added during the last 30 min. For double immunofluorescence detection of incorporated BrdU and the HA epitope of CDK4 by the use of two mouse monoclonal antibodies, cells were fixed with 2% paraformaldehyde for 90 s at 4°C and then with methanol for 10 min at −20°C, permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (pH 7.5) at room temperature, and blocked for 30 min with 5% normal sheep serum. BrdU was unmasked by a 30-min incubation with 2 M HCl. After washings, cells were then incubated overnight at 4°C with anti-HA antibody (Santa Cruz) and then for 2 h with Cy3-conjugated anti-mouse immunoglobulin (Jackson Immunoresearch). Washed cells were then successively incubated for 30 min with 1% normal mouse serum, for 2 h with an unconjugated anti-immunoglobulin G Fab fragment (Jackson Immunoresearch) (50 μg/ml), and then overnight at 4°C with mouse anti-BrdU monoclonal antibody (Becton-Dickinson), followed by biotinylated anti-mouse immunoglobulin (Amersham) and fluorescein-conjugated streptavidin (Amersham).

Indirect immunofluorescence. Double-labeling immunofluorescence detections were performed exactly as described previously (8, 14). Cyclin D3 was detected using DCS-22 (hybridoma supernatant kindly provided by J. Bartek), and CDK4 and CDK6 were simultaneously revealed using a selected batch of the C-22 CDK4 polyclonal antibody or the C-21 CDK6 polyclonal antibody (Santa Cruz). CDK7 was detected using the C-4 monoclonal antibody, and cyclin H was simultaneously revealed using the C-18 polyclonal antibody (both from Santa Cruz).

All the experiments were reproduced at least two times with very similar results.

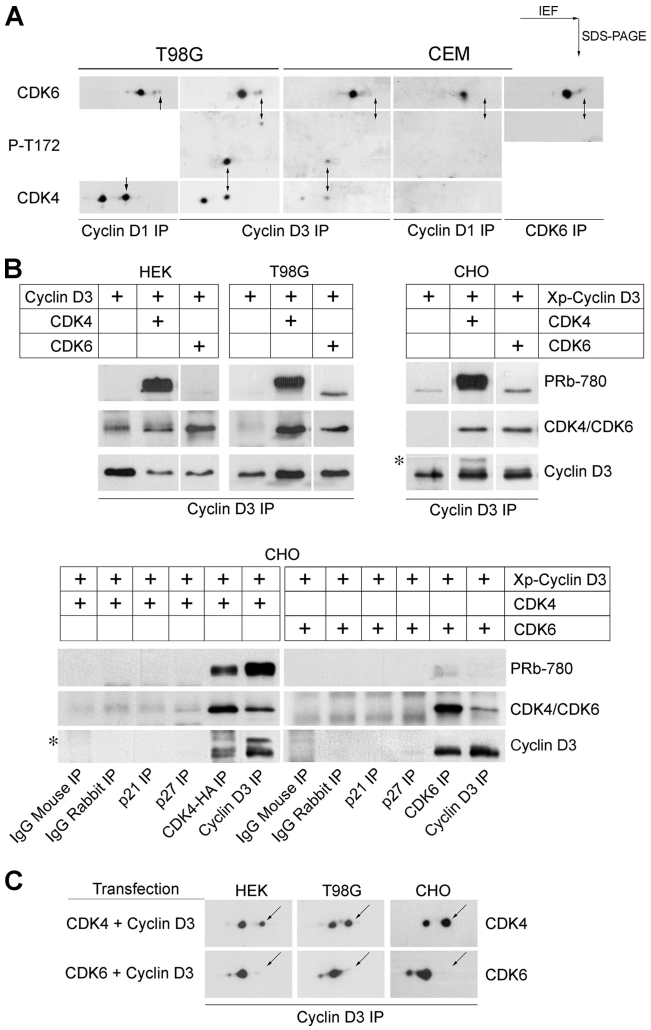

FIG. 2. Activating phosphorylations of CDK4 and CDK6 are differentially regulated. (A) Cyclin D3, cyclin D1, or CDK6 coimmunoprecipitates (IP) from serum-stimulated T98G or CEM cells were separated by 2D gel electrophoresis and electroblotted for immunodetection using the phospho-CDK4 (T172) antibody (P-T172), which also recognizes T177-phosphorylated CDK6. The same membranes were then reprobed for detection of total CDK4 and CDK6 by the use of anti-CDK4 and anti-CDK6 antibodies. Double arrows indicate the positions of the forms of CDK4 phosphorylated at T172 and CDK6 phosphorylated at T177. IEF, isoelectric focusing. (B) Extracts from HEK, T98G, or CHO cells transfected using plasmids encoding untagged cyclin D3 or cyclin D3-X-press (Xp-Cyclin D3) with or without CDK4-HA or untagged CDK6 were coimmunoprecipitated using cyclin D3 antibody (upper panel) or using antibodies against HA-tag (CDK4-HA IP), CDK6, cyclin D3, p21 (C-19 from Santa Cruz), or p27 (C-15 from Santa Cruz) or mouse and rabbit control immunoglobulins (IgG) (lower panel). The immunoprecipitates were assayed for pRb kinase activity, separated by SDS-PAGE, and immunoblotted. The pRb-kinase activity shown by in vitro phosphorylation of the pRb fragment at S780 was detected using a phosphospecific antibody (PRb-780). Cyclin D3 and CDK4 or CDK6 (in the appropriate transfections) were detected using specific antibodies. The upper band in the panel depicting the detection of Xp-Cyclin D3 (*) resulted from its phosphorylations by CDK4 during incubation with ATP for the pRb kinase activity assay. (C) The same extracts from transfected HEK, T98G, and CHO cells were coimmunoprecipitated using cyclin D3 antibody, separated by 2D gel electrophoresis, and electroblotted for immunodetection using anti-CDK4 or anti-CDK6 antibody. The positions of T172-phosphorylated CDK4 and T177-phosphorylated CDK6 are indicated by arrows.

RESULTS

In contrast to CDK4, CDK6 is poorly phosphorylated, restricting its activity in a variety of systems. Using 2D gel electrophoresis and a phospho-specific antibody that recognizes both T172-phosphorylated CDK4 and T177-phosphorylated CDK6, we previously observed that serum induces the activating T172 phosphorylation of endogenous cyclin D3-bound CDK4 in T98G glioma cells, whereas the analogous T177 phosphorylation of cyclin D3-bound CDK6 remains weak and not stimulatable (8) (Fig. 2A). We extended this observation to cyclin D1-bound CDK4 and CDK6 (Fig. 2A), and we confirmed it even in cells that strongly express CDK6 and very weakly express CDK4, such as CEM T-lymphoblastoid cells. Indeed, in these cells the greatly preponderant CDK6, whether associated with cyclin D3 or cyclin D1, was not detectably phosphorylated, whereas more than 50% of the weakly expressed CDK4 associated with cyclin D3 was phosphorylated at T172 (as judged from the proportion of the most negatively charged form recognized by the T172-phospho-specific CDK4 antibody) (Fig. 2A).

This quite unexpected situation was reproduced in various transfected cell systems. In CHO, HEK, and T98G cells (Fig. 2B) as well as in NIH3T3 and HCT116 cells (not shown), ectopically expressed cyclin D3-CDK4 presented much stronger pRb-kinase activity in vitro than similarly expressed cyclin D3-CDK6. Ectopic cyclin D1-CDK4 was also much more active than cyclin D1-CDK6 in T98G and HCT116 cells (see Fig. 4D and 8B below). In these various cells, coexpression of cyclin D3 or cyclin D1 allowed abundant T172 phosphorylation of CDK4, whereas the T177 phosphorylation of CDK6 remained either almost undetectable (Fig. 2C) or weak (see Fig. 9D below) in the case of cyclin D1-bound CDK6 in T98G cells. In these experiments, overexpressed CDK4 and CDK6 complexes were equivalently free of endogenous CIP/KIP proteins, as shown in CHO cells by undetectable or very weak coimmunoprecipitation of CDK4 or CDK6 by the use of antibodies against p21 or p27, respectively (Fig. 2B, lower panel). Ectopic cyclin D3 and CDK4 or CDK6 also presented similar overall cellular localization results (Fig. 3A). Moreover, no other (inhibitory) phosphorylation of CDK6 was detected. Since the T177 phosphorylation of CDK6 is absolutely required for its activity (34), the observed lack of this phosphorylation is sufficient to explain the very weak in vitro activity of ectopic cyclin D3/D1-CDK6 complexes.

This difference in the regulation of the activating phosphorylations of CDK4 and CDK6 was unexpected, since they present a high degree of homology, especially in the sequence surrounding their phosphoacceptor sites. However, CDK4 is clearly distinct, as its T172 site is followed by a proline whereas a serine follows the phosphorylated T177 residue of CDK6. In the other cell cycle CDKs, the phosphoacceptor threonine is followed by a histidine (Fig. 1).

The adjacent proline of CDK4 determines its T172 phosphorylation in cells and is essential for mitogenesis. To directly evaluate the importance of the adjacent proline 173 for T172 phosphorylation and activation of CDK4, we mutated this residue into serine (P173S [mimicking in CDK4 the phosphorylation site of CDK6]) or histidine (P173H [as in CDK2 and CDK1]). These mutants were well expressed in CHO cells

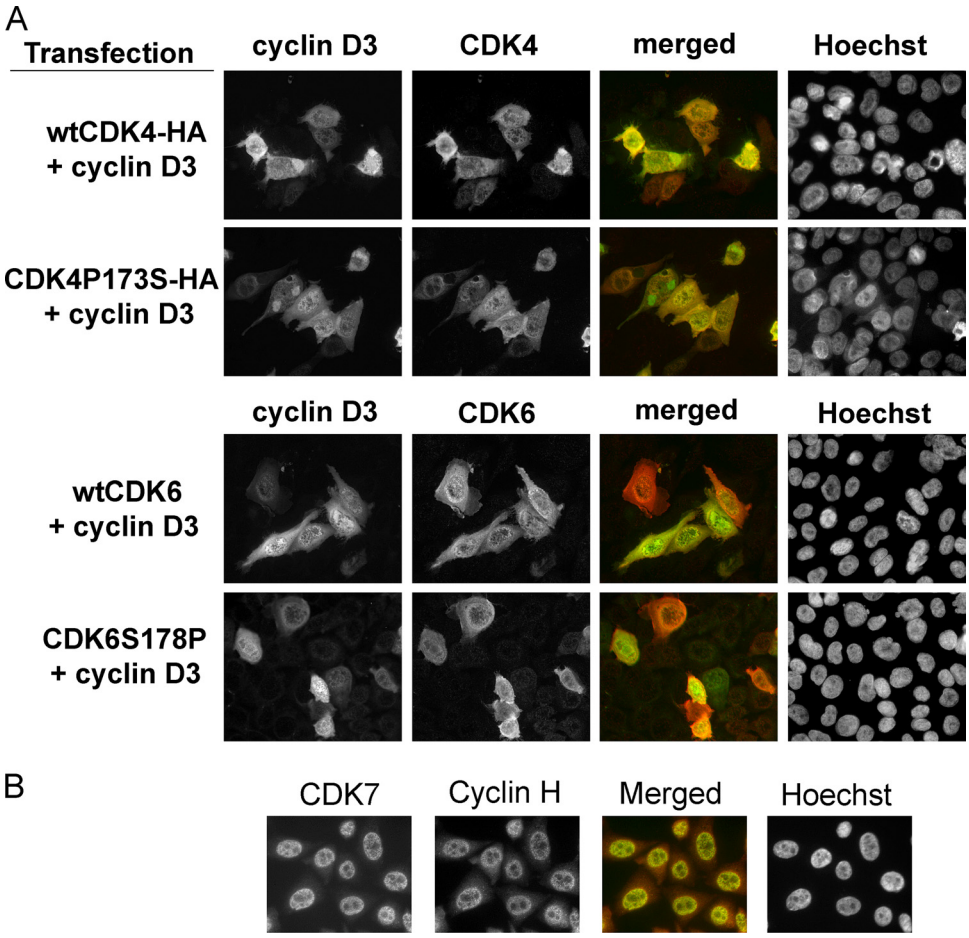

FIG. 3. Ectopic cyclin D3, CDK4, and CDK6 and each respective mutant display an overall cellular location that partly differs from the mostly nuclear compartmentalization of endogenous CAK (cyclin H and CDK7). (A) Indirect immunofluorescent codetection of cyclin D3 (green) and either CDK4 or CDK6 (red) from CHO cells transfected using plasmids encoding cyclin D3-X-press and wtCDK4-HA, CDK4P173S-HA, wtCDK6, or CDK6S178P. (B) Indirect immunofluorescent codetection of CDK7 (green) and cyclin H (red) from nontransfected CHO cells. For panels A and B, nuclei were counterstained using Hoechst 33258 dye.

(Fig. 4A, lanes 2 and 4; see the detection results obtained using whole-cell extracts shown in the lower right panel). They correctly associated with ectopic cyclin D3 (Fig. 4A, lanes 10 and 14), and the resulting complexes were mostly devoid of endogenous p21 or p27 (Fig. 4A, lower left panel). The P173S mutation also did not affect the overall cellular localization of CDK4 (Fig. 3A). However, P173S and P173H CDK4 mutants were totally devoid of in vitro kinase activity toward all the assessed phosphorylation sites of pRb by CDK4, including those at S780, S795, S807, S811, and T826 (lanes 9 and 10 and lanes 13 and 14 in Fig. 4A; Fig. 4B). As shown in Fig. 4C, the lack of pRb-kinase activity of mutated CDK4 (P173S and P173H) was entirely explained by the absence of the T172-phosphorylated form in 2D gel separations. Identical observations were obtained for CDK4P173S complexed to cyclin D3 or cyclin D1 in HEK, T98G, and HCT116 cells (Fig. 4D and E) and NIH3T3 cells (not shown). Overexpressed CDK4P173S prevented serum-induced DNA synthesis in T98G cells, thus likely behaving like nonphosphorylatable CDK4T172A as a dominant-negative competitor that titrates D-type cyclins (Fig. 5).

The adjacent proline of CDK4 does not determine its T172 phosphorylation by CAK (CDK7). CDK6 bound to D-type cyclins can be readily phosphorylated and activated by CAK (cyclin H-CDK7) in vitro (1, 8, 34). The CDK6-mimicking P173S mutation of CDK4 would therefore not be expected to affect its phosphorylation and activation by CAK. Indeed, as shown in Fig. 6A, in vitro incubation of inactive CDK4P173S-cyclin D3 (lanes 7 and 10) with 2 mM ATP and recombinant CAK (cyclin H-CDK7-Mat1 complex) did raise its pRb-kinase activity (lane 8) up to the levels observed with similarly treated wild-type (wt) CDK4 (wtCDK4)-cyclin D3 (lane 6). CAK also activated CDK4-P173H-cyclin D3 (lanes 11 and 12). This in vitro activation was due to T172 phosphorylation of CDK4, as CAK abundantly phosphorylated cyclin D3-bound CDK4P173S but not cyclin D3-bound CDK4T172A (Fig. 6B).

Others have failed to phosphorylate D-type cyclin-bound CDK4 with CAK under in vitro conditions that efficiently allow CAK phosphorylation of CDK6 and CDK2 complexes (34, 51). We have previously observed that coimmunoprecipitated cyclin D3-CDK6 from quiescent T98G cells can be phosphorylated by CAK under suboptimal conditions (low Mg-ATP con-

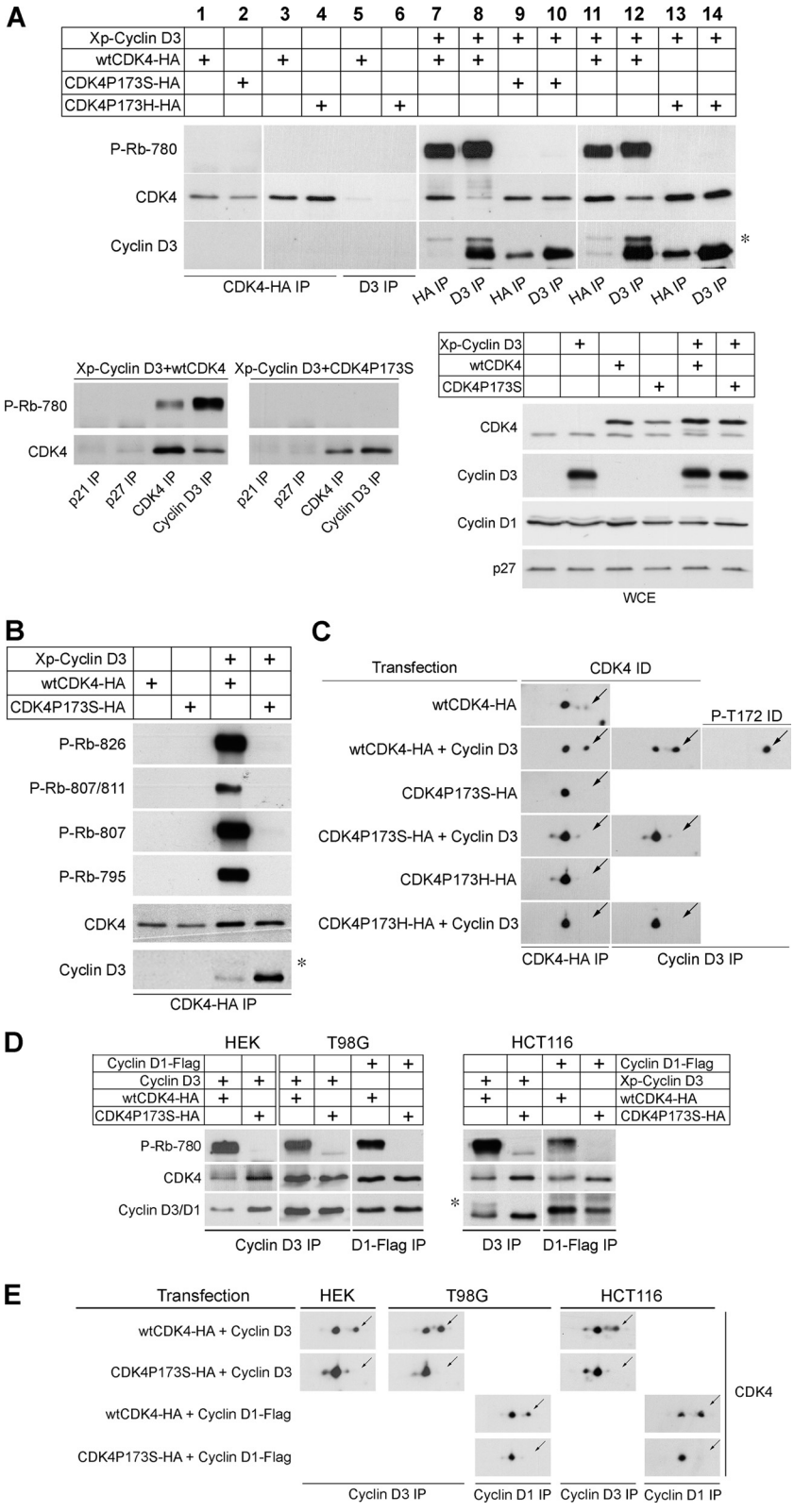

FIG. 4. Proline 173 is essential for T172 phosphorylation and activity of CDK4 in intact cells. (A) Extracts from CHO cells transfected using plasmids encoding cyclin D3-X-press (Xp-Cyclin D3) and wtCDK4-HA, CDK4P173S-HA, or CDK4P173H-HA, alone or in combination, were coimmunoprecipitated (IP) using anti-HA (HA IP) or anti-cyclin D3 (D3 IP) antibody or (as controls; lower left panel) using antibodies against p21 or p27 antibodies, assayed for pRb kinase activity, separated by SDS-PAGE, and immunoblotted. The in vitro S780 phosphorylation of the pRb fragment (P-Rb-780) was detected using a phosphospecific antibody. CDK4 and cyclin D3 were also detected. In the lower right panel, whole-cell-extract (WCE) detections of ectopic and endogenous proteins from the same samples are shown. CHO cells endogenously express

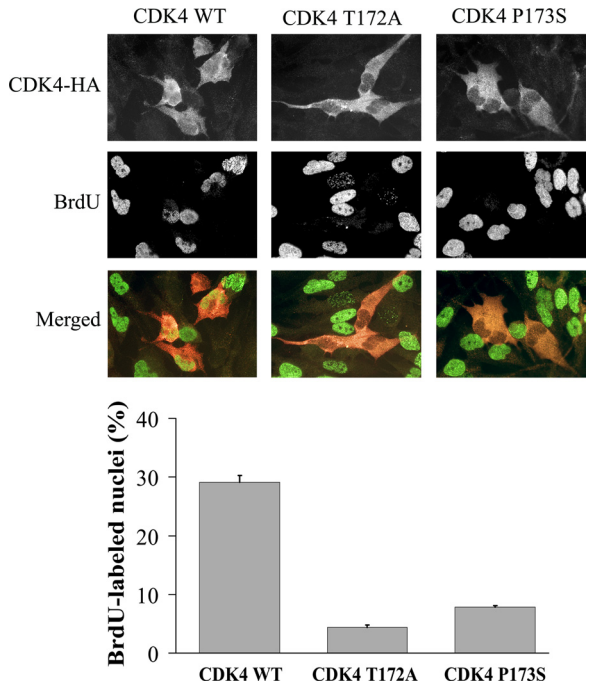

FIG. 5. CDK4 proline 173 is essential for mitogenesis. Quiescent T98G cells were transfected with plasmids encoding wt CDK4-HA, T172ACDK4-HA, or P173SCDK4-HA and stimulated with 15% FBS for 16 h with BrdU during the last 30 min. In duplicate dishes, DNA synthesis was evaluated in 100 cells/dish displaying the HA epitope by counting the proportion of nuclei showing incorporation of BrdU. The upper panel illustrates the double immunofluorescence detection of the HA epitope (red) and BrdU (green).

centrations) that do not allow T172 phosphorylation of cyclin D3-CDK4 from the same source and that CDK4 phosphorylation by CAK requires a high ATP concentration (8). On this basis, CDK6 thus appears to be a "better" substrate for CAK in vitro than is CDK4, reconciling divergent observations by Matsuoka et al. (46) and Kaldis et al. (34), who used different ATP concentrations. We thus wanted to more carefully assess the impact of the CDK6-mimicking P173S mutation of CDK4 with respect to its vitro activation by CAK by the use of the cyclin D3-CDK4/CDK6 complexes ectopically produced in CHO cells. This experiment required prior dephosphorylation of wtCDK4. As recently reported (64), this could be achieved by incubation of the cyclin D3-CDK4 complexes with λ-phosphatase (Fig. 7A). After λ-phosphatase incubation, both wtCDK4 and CDK4P173S complexed to cyclin D3 could be phosphorylated by CAK in the presence of 2 mM ATP, though the yield of CDK4P173S phosphorylation was slightly lower (Fig. 7A). Nevertheless, we observed that lower (0.05 to 0.5 mM) ATP concentrations could support a partial activation by CAK of CDK4P173S-cyclin D3, as seen for wtCDK6-cyclin D3, whereas activation of wtCDK4-cyclin D3 required elevated (2 to 4 mM) ATP concentrations (Fig. 7B).

These experiments thus establish that substituting a Ser for the Pro173 residue of CDK4 does not adversely affect its suitability as a substrate for activation by CAK (CDK7), in agreement with the conception that recognition of CDKs by CAK is relatively independent of a consensus sequence around the phosphoacceptor residues (23, 25). This sharply contrasts with the complete ablation of CDK4 phosphorylation and activity by P173S and P173H mutations in intact cells.

Substituting a proline at the +1 position enforces T177 phosphorylation and activity of CDK6. To further establish the importance of the Pro173 residue of CDK4 as the determinant of the differential regulation of CDK4 and CDK6, we next mutated the Ser178 residue of CDK6 into proline, thus mimicking the phosphorylation site of CDK4 in CDK6 (Fig. 1). This mutation did not affect the overall cellular localization of CDK6 and cyclin D3 (Fig. 3A). As shown in Fig. 8A (left panel), in CHO cells ectopic wtCDK6 presented a weak activity that depended on cyclin D3 coexpression. By contrast, even without cyclin D3 coexpression CDK6S178P displayed low but readily detected pRb-kinase activity in vitro (as also seen in Fig. 9A, lane 9). When coexpressed with cyclin D3, CDK6S178P was highly active (Fig. 8A, left panel; Fig. 9A, lanes 17 and 19 versus lanes 13 and 15). This dramatically elevated pRb-kinase activity was observed with a variety of pRb phosphorylation sites (S780, S795, S807/811, T821, and T826) and thus was not due to a modification of the site specificity of CDK6 (Fig. 8A, left panel). The S178P mutation also dramatically increased the pRb-kinase activity of cyclin D3-CDK6 in HEK, T98G, and HCT116 cells (Fig. 8B) and NIH3T3 cells (not shown), as well as the activity of CDK6 bound to ectopic cyclin D1 in T98G cells (Fig. 8B). Of note, in these different cell lines, this pRb-kinase activity was even more impressive when considered in relation to the concentrations of CDK6S178P and its complex with cyclin D3, which were much reduced compared to those of their wtCDK6 counterparts (Fig. 8A and B; Fig. 9A). These lower levels of CDK6S178P were especially observed in CHO cells cotransfected with cyclin D3 constructs (Fig. 8A, left panel). Whether they might reflect an increased degradation of hyperactive CDK6 within cyclin D3 complexes is unknown. Cyclin D3 levels were also much reduced in that situation, mostly in CHO cells (Fig. 8A, lower right panel).

CDK4 (lower band in CDK4 detection), cyclin D1, and p27 but not cyclin D3. (B) In the same experiment, the in vitro phosphorylation of the pRb fragment was analyzed using phosphospecific antibodies directed against phosphorylated T826 (P-Rb-826), S807 and S811 (P-Rb-807/811), and S807 (P-Rb-807) and S795 (P-Rb-795). (C) The same extracts as those described for panel A were coimmunoprecipitated using HA or cyclin D3 antibody, separated by 2D gel electrophoresis, and electroblotted for immunodetection using anti-CDK4 or the phospho-CDK4 (T172) antibody (P-T172) followed by detection of total CDK4 on the same membrane. (D and E) Extracts from HEK, T98G, or HCT116 cells transfected as described for panel A, or with a plasmid encoding cyclin D1-Flag instead of cyclin D3, were coimmunoprecipitated using anti-cyclin D3 (Cyclin D3 IP or D3 IP) or anti-Flag (D1-Flag IP) antibodies and assayed as described above for pRb kinase activity (P-Rb-780) (D) or separated by 2D gel electrophoresis and electroblotted for immunodetection using anti-CDK4 antibody (E). The upper band in the panels depicting detection of Xp-Cyclin D3 (* in panels A, B, and D) resulted from its in vitro phosphorylations by CDK4 during the pRb kinase activity assay. Arrows in panels C and E indicate the position of the T172-phosphorylated form of CDK4.

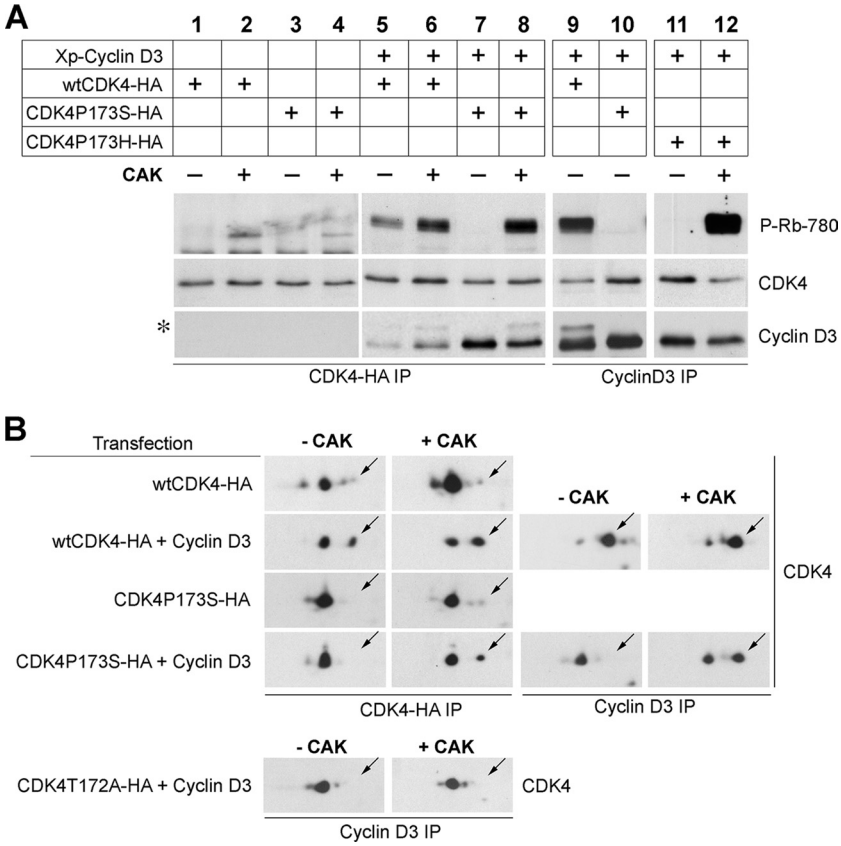

FIG. 6. Proline 173 is not essential for in vitro activation of CDK4 by CAK (CDK7). Extracts from CHO cells transfected using plasmids encoding cyclin D3-X-press (Xp-Cyclin D3) and wt CDK4-HA, CDK4P173S-HA, CDK4P173H-HA, or CDK4T172A-HA, alone or in combination, were coimmunoprecipitated (IP) using anti-HA (HA IP) or anti-cyclin D3 (D3 IP) antibody. The immunoprecipitated complexes were incubated without (−) or with (+) recombinant cyclin H-CDK7-Mat1 complex (CAK) and ATP, assayed for their pRb kinase activity (P-Rb-780) as described for Fig. 4A (A), or separated by 2D gel electrophoresis and electroblotted for immunodetection using anti-CDK4 (B). Arrows indicate the position of the T172-phosphorylated form of CDK4.

Incubation of CDK6S178P-cyclin D3 complexes with recombinant CAK (cyclin H-CDK7-Mat1 complex) did not further increase their pRb-kinase activity (Fig. 9A, lanes 17 to 20), whereas it activated wtCDK6-cyclin D3 complexes (lanes 13 to 16). As shown using 2D gel electrophoresis, incubation with recombinant CAK of wtCDK6 induced the appearance of its phosphorylated form, which was recognized by the T172-phospho-specific CDK4 antibody (Fig. 9B), as previously shown (8). Unexpectedly, the abundant phosphorylation of wtCDK6 by CAK was in large part independent of cyclin D3 coexpression and thus of its binding to a D-type cyclin (Fig. 9B), since CDK6 was also not detectably associated with endogenous D-type cyclins (Fig. 9B, lower panel). This result was at variance with the phosphorylation by CAK of wtCDK4 and CDK4P173S, which strictly depended on their binding to cyclin D3 (Fig. 6B). In sharp contrast with wtCDK6, CDK6S178P was almost entirely phosphorylated at T177 in CHO cells (Fig. 9B), as demonstrated by (i) its isoelectric point shift in 2D gels and comigration with CAK-phosphorylated wtCDK6, (ii) detection by the T172-phospho-specific CDK4 antibody, and (iii) the fact that it was not further phosphorylated by recombinant CAK (Fig. 9B), whereas (iv) its incubation with λ-phosphatase induced a reverse isoelectric point shift and comigration with nonphosphorylated wtCDK6 (Fig. 9C, upper panel).

Very interestingly, the complete phosphorylation of CDK6S178P was observed even in the absence of cyclin D3 coexpression (Fig. 9B) or of detectable binding to endogenous cyclin D1 or cyclin D3 (Fig. 9B, lower panel). This suggests that its occurrence could be independent of cyclin binding, at variance with findings showing the more incomplete phosphorylation of wtCDK4, which absolutely depends on its association with D-type cyclins (see Fig. 6B) (8, 38). Nevertheless, the activity of the mutated CDK6 remained largely dependent on the presence of cyclin D3 (Fig. 8A and B; Fig. 9A), likely because binding to the pRb substrate is ensured by the presence of the cyclin (21, 36). The complete T177 phosphorylation of CDK6S178P was also intriguing, as it only very partially colocalized with mostly nuclear CDK7 and cyclin H (Fig. 3A and B).

The complete phosphorylation of CDK6S178P did explain its dramatically elevated pRb-kinase activity. Indeed, incubation of CDK6S178P-cyclin D3 with λ-phosphatase ablated its pRb-kinase activity (Fig. 9C, lower panel). Dephosphorylated CDK6S178P-cyclin D3 was readily phosphorylated and reactivated by CAK (Fig. 9C). At variance with the situation observed with wtCDK4-cyclin D3 (Fig. 7B), the reactivation of

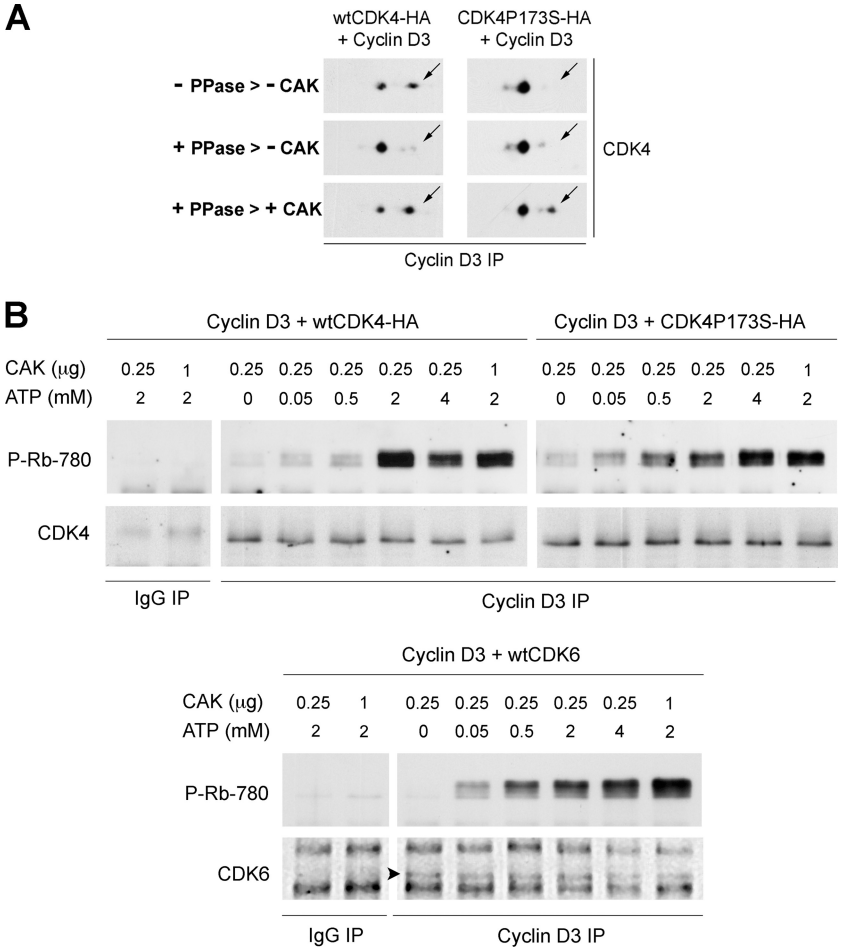

FIG. 7. In vitro activation of cyclin D3-bound CDK4, CDK4P173S, and CDK6 by CAK in the presence of different ATP concentrations. Extracts from CHO cells transfected using plasmids encoding cyclin D3-X-press and wt CDK4-HA, CDK4P173S-HA, or wt CDK6 were coimmunoprecipitated (IP) using anti-cyclin D3 antibody. (A) The immunoprecipitated complexes were preincubated without (−) or with (+) λ-phosphatase (PPase), extensively washed, and then incubated without or with 1 μg of recombinant cyclin H-CDK7-Mat1 complex (CAK) and 2 mM ATP, separated by 2D gel electrophoresis, and electroblotted for immunodetection using anti-CDK4. Arrows indicate the position of the T172-phosphorylated form of CDK4. (B) Cyclin D3 complexes (Cyclin D3 IP) or mock immunoprecipitates (IgG IP) processed using the same CHO cell extracts were dephosphorylated by preincubation with λ-phosphatase, extensively washed, and then incubated with 0.25 or 1 μg of CAK in the presence of different ATP concentrations. After thorough washings, the activation of cyclin D3-CDK4/CDK6 complexes was assessed by their pRb-kinase activity (P-Rb-780) codetected with CDK4 or CDK6 (arrowhead).

CDK6S178P-cyclin D3 was maximal with low ATP concentrations (0.05 to 0.5 mM) (Fig. 9C, lower panel). Since the T-loops of wtCDK4 and CDK6S178P are almost identical, this indicates that sequences outside the T-loop could also influence the differential recognition of CDK4 and CDK6 by the CAK ATP-enzyme complex.

Most of CDK6S178P was also phosphorylated in transfected HEK and T98G cells (Fig. 9D) and HCT116 and NIH3T3 cells (not shown). On the other hand, replacing the Ser178 residue with a histidine (as in CDK1 and CDK2) did not increase CDK6 phosphorylation (Fig. 9E). The S178P mutation thus enforces the activating phosphorylation of CDK6, providing the first such example of a CDK-activating mutation.

DISCUSSION

The two major results of our study are (i) the observation that CDK6 can be readily phosphorylated and activated in vitro by CAK (CDK7), but is poorly or not phosphorylated and activated in intact stimulated cells, and that one mutation suffices to make it constitutively active and (ii) the discovery that mutations of a critical proline preclude phosphorylation and activation of CDK4 in intact cells, but not its activation by CAK (CDK7), which might argue for the existence of (an)other CDK4-activating kinase(s).

As for CDK1 and CDK2, the activity of CDK4 and CDK6 absolutely requires their T-loop phosphorylation. At variance with the situation seen in fission yeast (32) and plants (68), the constitutively active cyclin H-CDK7-Mat1 complex is the only CAK identified so far in animal cells (23, 70). Though a second CAK activity has been partially purified from human cells (35), CDK7 is generally accepted to be responsible for the activating phosphorylations of the various cell cycle CDKs, including CDK4 (38, 46). Using a chemical genetic approach, CDK7 was indeed recently confirmed to be required for activation of

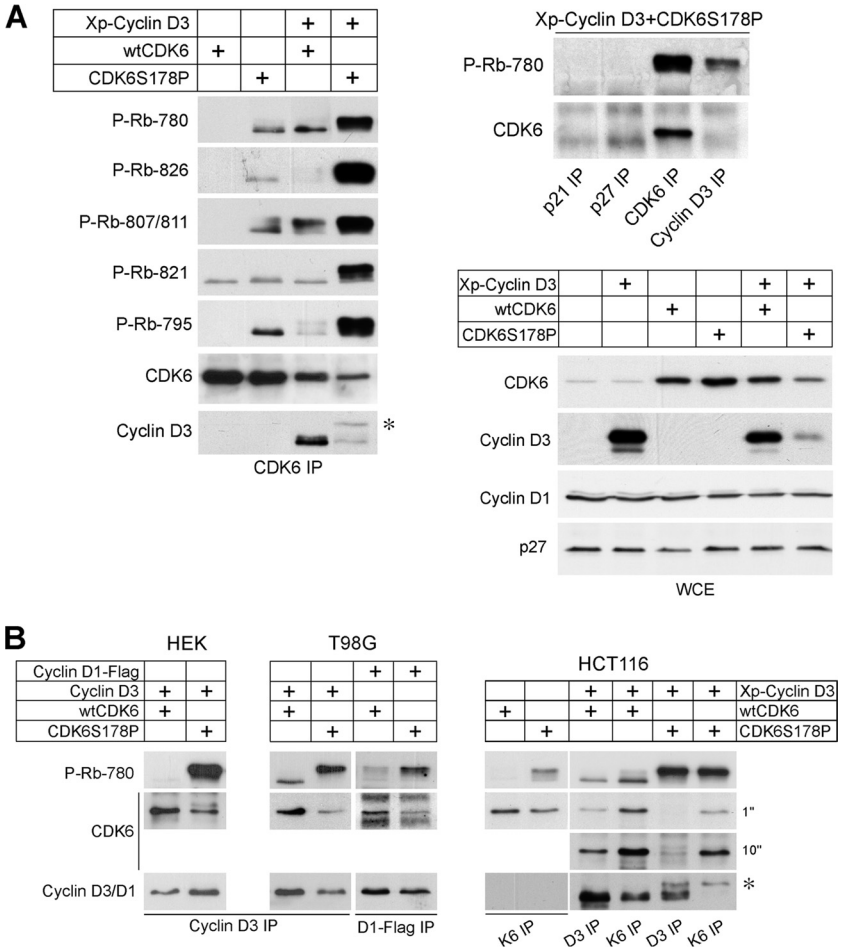

FIG. 8. S178P mutation activates CDK6. (A) Extracts from CHO cells transfected using plasmids encoding cyclin D3-X-press (Xp-Cyclin D3) and wt CDK6 or CDK6S178P, alone or in combination, were coimmunoprecipitated (IP) using anti-CDK6 antibody or anti-cyclin D3 antibody or (as controls; upper right panel) using antibodies against p21 or p27 antibodies, assayed for pRb kinase activity, separated by SDS-PAGE, and immunoblotted. The in vitro phosphorylation of the pRb fragment was analyzed using phosphospecific antibodies directed against phosphorylated S780 (P-Rb-780), T826 (P-Rb-826), S807 and S811 (P-Rb-807/811), T821 (P-Rb-821), and S795 (P-Rb-795). CDK6 and cyclin D3 were also detected from the same membranes. In the lower right panel, whole-cell-extract (WCE) detections of ectopic and endogenous proteins from experiments using the same samples are shown. (B) Extracts from HEK, T98G, and HCT116 cells, transfected using plasmids encoding cyclin D3-X-press (Xp-Cyclin D3) or cyclin D1-Flag and wtCDK6 or CDK6S178P, alone or in combination, were coimmunoprecipitated using anti-cyclin D3 (Cyclin D3 IP or D3 IP), anti-Flag (D1-Flag IP), or anti-CDK6 (K6 IP) antibody, assayed for pRb kinase activity, separated by SDS-PAGE, and immunoblotted. The in vitro phosphorylation of the pRb fragment (P-Rb-780), CDK6, and cyclin D3 or cyclin D1 (in the appropriate transfections) was then detected using membranes from the same experiment. The upper band in the panels depicting detection of Xp-Cyclin D3 (*) resulted from its in vitro phosphorylation by CDK6S178P during the pRb kinase activity assay.

CDK2 and CDK1 in HCT116 colorectal cancer cells (41). To our knowledge, no such direct evidence has been reported for CDK4 and CDK6. In this and our previous study (8), we have confirmed that CDK7 phosphorylates and activates both CDK4 and CDK6 in vitro, as shown by others (1, 6, 34, 46). However, CDK6 was clearly a better CDK7 substrate than CDK4, being phosphorylated by CDK7 at lower Mg-ATP concentrations that do not allow CDK4 phosphorylation (8, 34) (Fig. 7B). Moreover, CDK6, like CDK2 (24, 48), could be efficiently phosphorylated by CDK7 in the absence of a cyclin, whereas CDK4 phosphorylation by CDK7 absolutely requires its binding to a cyclin D (8, 38). This is at variance with a previous report (34), but others have also seen significant phosphorylation of cyclin-free CDK6 by CDK7 (1).

In intact cells, a very different situation is demonstrated by our comparison of T172/T177-activating phosphorylations of CDK4 and CDK6. We recently found that T172 phosphorylation is highly regulated within D-type cyclin-CDK4 complexes, determining their pRb-kinase activity, pRb phosphorylation, and passage through the G_1 restriction point (7). This was observed with a variety of cell types and in response to diverse mitogenic and antimitogenic stimuli, including cyclic AMP-dependent mitogenesis of normal thyroid epithelial cells (53, 54), cell cycle inhibition by cyclic AMP in thyroid carcinoma cells (57), G_1-phase arrest by transforming growth factor β (8, 14), and cell cycle triggering by serum in T98G cells (8) through mechanisms that depend on the activity of both MEK and mTOR pathways (55). In all these systems the presence and activity of cyclin H-CDK7 were unchanged (see the references cited above). On the other hand, a recent in vitro study

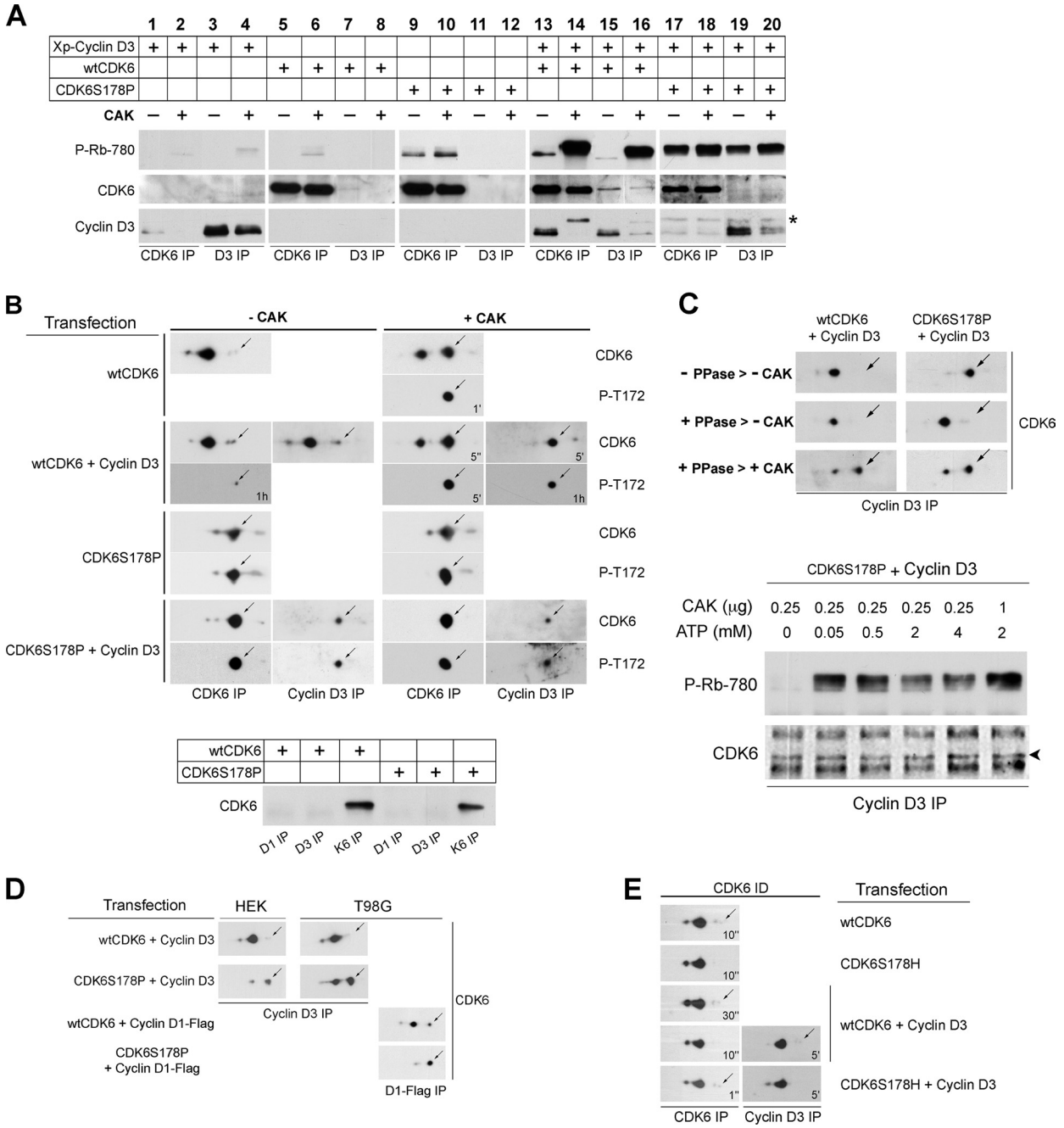

FIG. 9. S178P mutation enforces T177 phosphorylation and activity of CDK6. (A to C) Extracts from CHO cells transfected using plasmids encoding cyclin D3-X-press (Xp-Cyclin D3) and wt CDK6 or CDK6S178P, alone or in combination, were coimmunoprecipitated (IP) using anti-CDK6 (CDK6 IP) or anti-cyclin D3 (D3 IP) antibody. The immunoprecipitated complexes were incubated without (−) or with (+) recombinant cyclin H-CDK7-Mat1 complex (CAK) and ATP. (A) The complexes were then assayed for pRb kinase activity, which was detected using the S780 pRb phospho-specific antibody (P-Rb-780). CDK6 and cyclin D3 were then detected on the same membrane. The upper band in the panel depicting detection of Xp-Cyclin D3 (*) resulted from its in vitro phosphorylations by CDK6 during the pRb kinase activity assay. (B, upper panel) The immunoprecipitated complexes incubated without or with CAK as described for panel A were separated by 2D gel electrophoresis and electroblotted for immunodetection using the phospho-CDK4 (T172) antibody (P-T172) (which detects T177-phosphorylated CDK6) or the CDK6 antibody for the detection of total CDK6. (B, lower panel) The same extracts from cells transfected using plasmids encoding wt CDK6 or CDK6S178P were coimmunoprecipitated using anti-cyclin D1 (D1 IP), anti-cyclin D3 (D3 IP), or anti-CDK6 (K6 IP) antibodies, separated by SDS-PAGE, and electroblotted for immunodetection using the CDK6 antibody. (C) The immunoprecipitated complexes were preincubated without (−) or with (+) λ-phosphatase (PPase), extensively washed, and either incubated without or with 1 μg CAK and 2 mM ATP, separated by 2D gel electrophoresis, and electroblotted for immunodetection using anti-CDK6 (upper panel) or incubated with 0.25 or 1 μg CAK in the presence of different ATP concentrations, and the activation of cyclin D3-CDK6S178P was assessed by its pRb-kinase activity (P-Rb-780) codetected with CDK6 (lower panel). (D) Extracts from HEK and T98G cells transfected using plasmids encoding cyclin D3-X-press or cyclin D1-Flag and wt CDK6 or CDK6S178P were coimmunoprecipitated using cyclin D3 or Flag (D1-Flag IP) antibodies, separated by 2D gel electrophoresis, and electroblotted for immunodetection using anti-CDK6 antibody. (E) Extracts from CHO cells transfected using plasmids encoding wt CDK6 or CDK6S178H and cyclin D3-X-press (Cyclin D3) were coimmunoprecipitated using anti-CDK6 or anti-cyclin D3 antibody, separated by 2D gel electrophoresis, and electroblotted for immunodetection using the CDK6 antibody. Arrows indicate the position of the T177-phosphorylated form of CDK6.

has suggested that D-type cyclin-CDK4 complexes would be prevented from being activated by CDK7 by their association with p27 unless p27 is Y phosphorylated (56). However, nonphosphorylated CDK4 from inactive cyclin D3 complexes that are largely associated with p27 in serum-deprived T98G cells is perfectly competent for efficient phosphorylation and activation by cyclin H-CDK7 from different sources (8, 55, 57). Moreover, in nontransformed cells, Y phosphorylation concerns only a small minority of p27 molecules, even during cell cycle progression (28, 31), which contrasts with the abundance of T172-phosphorylated CDK4 enriched in p27-containing complexes (30 to 80% of p27-bound CDK4) (8, 14, 55). Thus, binding of CDK4 complexes to p27 and p27 Y phosphorylations might not be able to explain the modulation of CDK4 phosphorylation in our models.

No such regulation is observed for T177 phosphorylation of CDK6. Even in the CEM T-lymphoblastoid cells that predominantly express CDK6 and express little CDK4, CDK6 phosphorylation remained weakly detectable, in agreement with the previous conclusion that active CDK6 represents only a small minority of the total CDK6 in these cells (44). Using five different cell lines cultured with serum (CHO, HEK, T98G, HCT116, and NIH3T3), we now show that expression of cyclin D3 or cyclin D1 induced the abundant T172 phosphorylation of ectopic CDK4 but not (or only very weakly) the phosphorylation of CDK6. In all these cell lines, overexpressed cyclin D3/D1-CDK6 complexes thus remained almost inactive compared to similar CDK4 complexes. This unexpected observation suggests that CDK4 might be the main functional and regulated D-type cyclin-dependent kinase in most cells and that CDK6 might play a minor role, at least in cells that express even a small amount of CDK4. This might explain the findings that in CDK2 knockout mice, genetic inactivation of CDK6 brings almost no additional phenotype (45) whereas CDK4 knockout dramatically reduces pRb phosphorylation and arrests embryonic development at midgestation (5).

The generally accepted model that both CDK4 and CDK6 are activated by CDK7 does not predict such very different phosphorylation levels of CDK4 and CDK6. Sequences around the T172/T177 phosphoacceptor sites of CDK4 and CDK6 are almost identical, with the notable presence at the +1 position of a proline in CDK4 instead of a serine in CDK6. This difference has been previously discussed by others (38) and is considered one argument suggesting that CDK recognition by CDK7 does not depend on a consensus sequence around the phosphoacceptor site (23, 25, 40). In agreement with this conception, we indeed show here that the P173S mutation of CDK4 does not impair its T172 phosphorylation and activation by CDK7. However, the +1 proline of CDK4 is a hallmark conserved throughout evolution and already found in echinoderms. We now demonstrate that P173S and P173H mutations of CDK4 abolished its T172 phosphorylation and activity in all the presently assayed cells, whereas, conversely, S178P but not S178H mutation of CDK6 did dramatically induce its T177 phosphorylation and activity. Though not all amino acid substitutions were tested, this suggests that the unique presence of a proline at the +1 position is critical for CDK4-activating phosphorylation in vivo.

This diametrically different impact of P173S/H-CDK4 mutations in intact cells and on in vitro phosphorylation by CDK7 raise additional questions as to whether CDK7 could still be maintained as the sole, or the main, CDK4-activating kinase. It could be argued that the present mutations of CDK4 and CDK6 could affect their affinity for CAK (CDK7) in such a way that the changes are not perceived in vitro but could lead to profound alterations of their phosphorylation by CAK in cells, e.g., owing to their overexpression relative to endogenous CAK in face of competing reactions and/or different compartmentalizations. However, ectopic CDK4 and CDK6 and their mutants displayed similar overall cellular locations and only partially colocalized with nuclear cyclin H-CDK7. Moreover, wtCDK6 and even CDK4P173S appeared to be better CAK substrates than wtCDK4 under suboptimal conditions such as limiting ATP concentrations. If CDK7 should notwithstanding prove to be the kinase responsible for the abundant or complete phosphorylation of wtCDK4 or CDK6S178P in intact cells, then a mechanism that could totally prevent it from also phosphorylating wtCDK6 and CDK4P173S/H would have to be conceived. The present data thus lead us to propose that CDK4 might not be phosphorylated by CDK7 in intact cells but more likely by other, presumably proline-directed kinase(s). In addition to the exquisite regulation of T172 phosphorylation, other observations recently called for a reappraisal of the role of CDK7 in CDK4 activation (7), including (i) the enrichment of T172-phosphorylated CDK4 in p27-containing complexes (8, 14), while p27 prevents CDK4/CDK6 phosphorylations by CDK7 (34, 37, 56) (but not by yeast monomeric Cak1/Csk1 [34, 56]), and (ii) the differential regulation of phosphorylation and activity of CDK4 bound to cyclin D1 or cyclin D3 (54, 57). Considering in addition to this second point the variety of signaling pathways already shown to impact CDK4 phosphorylation (PKA, transforming growth factor β, mTOR, and MEK/Erk) (8, 14, 53, 55, 57), we surmise that several regulated proline-directed kinases might well be able to perform the crucial phosphorylation of CDK4.

By enforcing almost complete T177 phosphorylation of CDK6 apparently independently of cyclin binding by an undefined proline-directed kinase(s), the CDK6S178P mutation would constitute the first identified activating mutation of a CDK in animal cells (in addition to the weak phosphomimetic T172E mutation of CDK4 [8]). A single base transition can generate the S178P mutation. Further studies should examine whether it could be found in natural situations. Every constituent of the INK4/D-type cyclin/CDK4/CDK6/pRb pathway has turned out to be an important oncogene(s) or tumor suppressor(s) (3, 8, 27, 61). CDK6 is overexpressed by gene amplification, chromosomal translocations, or epigenetic mechanisms in lymphomas and gliomas (10, 12, 13, 43). Interestingly, CDK6 is also hyperactivated independently of CAK by the viral cyclin encoded by the Kaposi's sarcoma-associated herpesvirus (33). The R24C mutation of CDK4 that precludes its inhibition by $p16^{INK4A}$ causes human melanomas and various tumors in mice (63). At variance with CDK4, CDK6 also appears to exert dedifferentiating activities in various cell types (29). Further studies should thus evaluate the oncogenic potential of the CDK6S178P-activating mutation, including that in quiescent differentiated cells that express high amounts of cyclin D3 (4, 19).

In all the cell cycle regulation models that we have recently investigated (8, 14, 53–55, 57), pRb phosphorylation and DNA

replication onset perfectly correlated with CDK4 T172 phosphorylation but not with the concentration of any of the CDK4/CDK6 regulatory proteins (cyclin D1, cyclin D3, p27, and p21) that are most generally considered to be endpoints of mitogenic and antimitogenic signal transduction cascades. Recent determinations of the crystallographic structure of D-type cyclin-CDK4 complexes have indicated that their structural activation mechanisms diverge markedly from those of cyclin A-CDK2 complexes. Specifically, at variance with the cyclin A-CDK2 complex, cyclin binding may not be sufficient to drive the CDK4 active site toward an active conformation, and it also does not preclude the accessibility of the phosphorylated T-loop to solvent and λ-phosphatase (16, 64), as also observed here for both CDK4-cyclin D3 and CDK6S178P-cyclin D3. As CDK4 T172 phosphorylation is emerging as a determining cell cycle regulator, major efforts should be devoted to the understanding of mechanisms responsible for its regulation, including the identification of the putative CDK4-activating proline-directed kinase(s) that we are proposing and the delineation of signaling cascades that might control them.

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We have no conflict of interest to disclose.

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