Regulation of Cyclin D-Dependent Kinase 4 (cdk4) by cdk4-Activating Kinase

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The accumulation of assembled holoenzymes composed of regulatory D-type cyclins and their catalytic partner, cyclin-dependent kinase 4 (cdk4), is rate limiting for progression through the G₁ phase of the cell cycle in mammalian fibroblasts. Both the synthesis and assembly of D-type cyclins and cdk4 depend upon serum stimulation, but even when both subunits are ectopically overproduced, they do not assemble into complexes in serum-deprived cells. When coexpressed from baculoviral vectors in intact Sf9 insect cells, cdk4 assembles with D-type cyclins to form active protein kinases. In contrast, recombinant D-type cyclin and cdk4 subunits produced in insect cells or in bacteria do not assemble as efficiently into functional holoenzymes when combined in vitro but can be activated in the presence of lysates obtained from proliferating mammalian cells. Assembly of cyclin D-cdk4 complexes in coinfecting Sf9 cells facilitates phosphorylation of cdk4 on threonine 172 by a cdk-activating kinase (CAK). Assembly can proceed in the absence of this modification, but cdk4 mutants which cannot be phosphorylated by CAK remain catalytically inactive. Therefore, formation of the cyclin D-cdk4 complex and phosphorylation of the bound catalytic subunit are independently regulated, and in addition to the requirement for CAK activity, serum stimulation is required to promote assembly of the complexes in mammalian cells.

Mammalian D-type cyclins form complexes with various cyclin-dependent kinases (cdks) during the G₁ interval to form active holoenzymes that facilitate progression through the G₁ phase of the cell cycle into S phase (reviewed in reference 42). Three D-type cyclins (D1, D2, and D3) are differentially expressed in proliferating cells in response to various growth factor-mediated signals (1, 3, 5, 24, 25, 31, 47), and they interact combinatorially with cdks 2, 4, 5, and 6 (6, 29, 33, 49). The enforced overexpression of cyclin D1 in fibroblasts is itself sufficient to shorten their G₁ interval by several hours and partially relieves cells of their growth factor dependency (39). Conversely, microinjection of antibodies or antisense plasmids to cyclin D1 into fibroblasts stimulated with serum to reenter the cell cycle prevents their entry into S phase; injections performed several hours prior to the G₁/S boundary can prevent G₁ exit, whereas those performed at or after the G₁/S transition are without effect (5, 39). Together, these results indicate that cyclin D1 in fibroblasts carries out a critical function late in G₁ which is both necessary and rate limiting for entry into S phase.

In macrophages and fibroblasts where complexes between cyclin D1 and cdk4 predominate (5, 30, 39, 49), growth factor stimulation of quiescent cells induces cyclin D1 synthesis early in G₁, whereas cdk4 is induced several hours later (29, 30). Induction of cdk4 facilitates the assembly of cyclin D1-cdk4 complexes, which are active in phosphorylating the retinoblastoma gene product (pRb), but not histone H1 or casein (15, 23, 29, 30). In peripheral blood T cells activated by mitogens and cyclins D2 and D3, but not D1, are induced during G₁ (1) and these form active pRb kinases in association with cdk6 (33). In each of these cell systems, cyclin D-dependent pRb kinase activity first appears in mid-G₁ and reaches a maximum near

the G₁/S transition (30, 33). In asynchronously growing fibroblasts engineered to ectopically overproduce D-type cyclins together with cdk4, the levels of cyclin D-cdk4 pRb kinase activity are significantly elevated, but even in the face of constitutive overproduction of both subunits, the timing of cyclin D-cdk4 assembly and the rate of activation of the resulting holoenzymes remains unchanged when cells reenter the cycle from quiescence (30). Therefore, the assembly of enzymatically active cyclin D-cdk4 complexes must be governed by additional regulatory molecules whose activities also depend upon growth factor-induced signals.

One potential regulator of cyclin-cdk activity is cdk-activating kinase (CAK) (10, 44). The activities of complexes formed by p34^{cdc2} or p33^{cdk2} with cyclin A or B are regulated both positively and negatively by phosphorylation of the bound catalytic subunits (reviewed in reference 35). Activation of the holoenzymes requires CAK-mediated phosphorylation of a single threonine residue corresponding to Thr-161 in cdc2 (Thr-167 in fission yeast species) and Thr-160 in cdk2 (7, 8, 11, 13, 17-19, 26-28, 34, 44). The recently solved crystal structure of cdk2 reveals that Thr-160 sits in a loop at the mouth of the substrate binding cleft and below the cyclin binding interface, suggesting that its phosphorylation releases an inhibitory constraint which otherwise prevents activation of the enzyme (9). Phosphorylation of Thr-161/167 in p34^{cdc2} may help to stabilize its binding to cyclin A (13), but this modification is not required for formation of stable complexes between cdc2 and cyclin B, cdk2 and cyclin A, or cdk2 and cyclin B (4, 10, 44).

The CAK that phosphorylates cdc2 and cdk2 is in part composed of a catalytic subunit (now designated MO15), which is itself structurally related to the known cdks, together with an as-yet-uncharacterized regulatory subunit(s) (16, 38, 43). Despite the fact that CAK is itself structurally reminiscent of cyclin-cdk complexes, its substrate specificity appears restricted to cdks, and unlike the cyclin-cdks which themselves recognize Thr-Pro-X-basic motifs, neither Thr-161 in cdc2 nor Thr-160 in cdk2 contains adjacent Pro-X-basic residues. The

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sites in cdc2 and cdk2 phosphorylated by CAK are analogous in position and context to Thr-172 in cdk4. We now provide evidence that the kinase activity of cdk4 similarly depends upon a CAK activity that specifically phosphorylates this threonyl residue. cdk4 mutants containing nonphosphorylatable amino acids at codon 172 can stably assemble with D-type cyclins to form inactive holoenzymes in intact insect cells but do not efficiently associate with cyclin D *in vitro*, suggesting that assembly and cdk4 phosphorylation are independently regulated processes.

MATERIALS AND METHODS

Preparation of cdk4 mutants and manipulation of baculoviruses. cdk4 mutants were generated by a two-step PCR with the following oligonucleotide primers: 5'-ATTAACCTCACTAAAGGGA-3' (upstream primer from pBluescript vector), 5'-CATCTCTGCAAAGATACAGCC-3' (downstream primer inversely complementary to codons 200 to 207 of cdk4 cDNA), 5'-ATGGCCCTCGCGCTGTGGTG-3' (T172A; 5' to 3'), 5'-CACCACAGGCGCGAGGGCCAT-3' (T172A; 3' to 5'), 5'-ATGGCCCTCTCGCTGTGGTG-3' (T172S; 5' to 3'), 5'-CACCACAGGCGAGAGGGCCAT-3' (T172S; 3' to 5'), 5'-ATGGCCCTCGAGCCTGTGGTG-3' (T172E; 5' to 3'), and 5'-CACCACAGGCTCGAGGGCCAT-3' (T172E; 3' to 5'). Triplets corresponding to codon 172 are underlined. The template was mouse cdk4 cDNA cloned into pBluescript (29). PCR was first performed with either the upstream primer and the 3'-to-5' oligonucleotides encoding codon 172 or with the downstream primer and the T-172 5'-to-3' oligonucleotides. The two PCR products of 0.6 and 0.1 kb were purified from gels using GeneClean (Bio 101, La Jolla Calif.). Each set of amplified fragments was mixed and used as a template for another PCR performed with the upstream and downstream primers, thereby generating fused products of 0.7 kb. The final PCR products were digested with *SacI* to yield a 0.55-kb cDNA fragment which was substituted for the corresponding wild-type cdk4 *SacI* fragment in the cdk4 pBluescript vector. The resulting plasmids were sequenced over both strands to confirm the absence of adventitious mutations. Construction of a kinase-deficient (K35M) mutant of cdk4, in which the ATP binding site (Lys-35) was converted to a methionine residue, was previously described (23, 29).

Spodoptera frugiperda (Sf9) cell culture, manipulation of baculoviruses, and the preparation of recombinant vectors encoding D-type cyclins and cdk4 were described previously (23, 29) and are based on the detailed published procedures of others (45). cDNA fragments encoding mutant cdk4 proteins were cloned into the baculovirus transfer vector pAcYM1 (32) and cotransfected into Sf9 cells with linearized baculovirus DNA (PharMingen, San Diego, Calif.). Recombinant viruses were expanded and assayed for expression of their encoded proteins by metabolic labeling and immunoprecipitation or by immunoblotting as described previously (23, 29). Sf9 cells infected with recombinant viruses were harvested 40 h postinfection and lysed at 4°C in kinase buffer (50 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; pH 7.5], 10 mM MgCl₂, 1 mM dithiothreitol [DTT]) containing 2.5 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-N-N'-N'-tetraacetic acid], 0.1 mM phenylmethylsulfonyl fluoride, aprotinin (5 μg/ml; Sigma Chemicals, St. Louis, Mo.), 10 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, and 0.1 mM NaF. Cell lysates were clarified by centrifugation at 4°C for 10 min at 10,000 × g in a Microfuge.

Metabolic labeling, immunoprecipitation, and protein detection. Sf9 cells (1 × 10⁶) infected with the indicated recom-

binant viruses were metabolically labeled 32 h after infection for 8 h with 50 μCi of [³⁵S]methionine (1,000 Ci/mmol; Trans ³⁵S-label; ICN, Irvine, Calif.) per ml in 1 ml of methionine-free Grace's medium supplemented with 5% dialyzed fetal bovine serum or with 1 mCi of carrier-free ³²P_i (9,000 Ci/mmol; Amersham) per ml in 1 ml of phosphate-free medium. Labeled cells were lysed for 1 h at 4°C in 1 ml of Tween 20 immunoprecipitation buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1 mM EDTA, 0.1% Tween 20) containing protease and phosphatase inhibitors (see the description of the kinase buffer above). Centrifuged lysates were incubated with protein A-Sepharose beads precoated with monoclonal antibodies to D-type cyclins (46) or with rabbit antisera to cyclins or cdk4 (29, 31) for 3 h at 4°C. Pelleted beads were then washed four times with Tween 20 immunoprecipitation buffer, and the proteins were denatured and separated on polyacrylamide gels containing sodium dodecyl sulfate (SDS) and detected by autoradiography (2). For immunoblotting, immunoprecipitates recovered from unlabeled lysates were separated on denaturing gels, transferred to nitrocellulose, and blotted with antibodies followed by ¹²⁵I-protein A to detect sites of antibody binding (12).

Tryptic phosphopeptide and phosphoamino acid analyses. ³²P-labeled proteins were eluted from gels and subjected to digestion with trypsin; phosphopeptides were separated in two dimensions by electrophoresis at pH 1.9 followed by ascending chromatography (40). Unfractionated trypsin digests of radio-labeled cdk4 or individual phosphopeptides eluted from thin-layer plates were hydrolyzed in 6 N HCl at 110°C for 1 h, mixed with phosphoamino acid standards, and separated electrophoretically at pH 3.5 (40). The positions of phosphoamino acids were visualized by ninhydrin staining of the plates.

Expression and purification of recombinant proteins from bacteria. Glutathione S-transferase (GST) fusion proteins containing human cyclin A and mouse cdk4 or hexahistidine (His)-tagged mouse cyclin D2 and cdk2 were constructed by subcloning fragments containing the entire coding sequences of each molecule into pGEX (Pharmacia) or pQE (Qiagen, Chatsworth, Calif.) vectors, respectively. Recombinants were transformed into *Escherichia coli* BL21(DE3) (for GEX plasmids; Pharmacia) or M15(pREP4) (for pQE plasmids; Qiagen), and fresh bacterial cultures were grown at 37°C to A₆₀₀ of 2.0. Expression of recombinant proteins was induced with 100 μM isopropyl-β-D-thiogalactoside (IPTG) at 23°C for 18 h. Cells were harvested and lysed by sonication in buffer A (20 mM Tris HCl [pH 7.5], 150 mM NaCl, 0.5% Tween 20, 1 mM phenylmethylsulfonyl fluoride), and the lysates were clarified by centrifugation. GST fusion proteins were adsorbed to glutathione-Sepharose beads (Pharmacia), washed, and eluted with 5 mM reduced glutathione (Sigma). His-tagged cdk2 was purified by a modification of the procedures described by Hoffman and Roeder (22). In brief, cleared lysates were incubated with Ni²⁺-nitrilotriacetic acid (NTA)-agarose (Qiagen). The agarose beads were washed three times with buffer A and twice with buffer B (20 mM Tris HCl [pH 7.5], 150 mM NaCl), and His-tagged cdk2 was eluted with 40 mM Tris HCl, pH 9.2, containing 250 mM imidazole. Eluted proteins were dialyzed in buffer C (20 mM Tris HCl [pH 7.5], 20 mM NaCl, 0.5 mM EDTA, 0.25 mM DTT). Because of its relative insolubility, His-tagged cyclin D2 was purified under denaturing conditions. Harvested cells were lysed and solubilized in 20 mM Tris HCl, pH 7.5, containing 150 mM NaCl and 6 M guanidine hydrochloride, and the cleared lysate was incubated with Ni²⁺-NTA-agarose. The agarose beads were washed twice with the same buffer and twice with 20 mM Tris HCl, pH 7.5, containing 150 mM NaCl and 6 M urea. His-tagged cyclin D2

was eluted in the same buffer containing 250 mM imidazole, dialyzed to remove imidazole, renatured by sequential dialysis against 20 mM Tris HCl [pH 7.5]–150 mM NaCl, with stepwise twofold reductions in urea concentrations from 6 to 0.75 M (at least 6 h per step), and finally dialyzed against buffer lacking urea. The renatured proteins were dialyzed against buffer C and clarified by centrifugation. The purified proteins were separated on denaturing gels and stained with Coomassie brilliant blue to estimate their purity and their concentration by comparison to defined quantities of marker proteins.

CAK assay. For *cdk2* CAK assays, NIH 3T3 cells or infected Sf9 cells used as a source of CAK activity were lysed by sonication in 80 mM sodium- β -glycerophosphate, pH 7.3, and 15 mM MgCl₂ containing 5 μ g of leupeptin (Sigma) per ml, 1 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 mM sodium orthovanadate, and 10 μ g of soybean trypsin inhibitor per ml, and the lysates were clarified by centrifugation. For *cdk4* CAK assays, the lysis solution was supplemented with 10 mM EGTA and 2 mM DTT. Cell lysates (corresponding to 5×10^5 NIH 3T3 or Sf9 cells) were mixed with purified recombinant cyclin and cdk fusion proteins produced in bacteria (either 0.1 μ g each of cyclin A and *cdk2* or 1 μ g each of cyclin D2 and *cdk4*) in a final volume of 50 μ l of lysis solution and incubated at 23°C for 1 h in the presence of 1 mM ATP. Assembled and modified cyclin-cdk complexes were recovered with glutathione-Sepharose beads (for GST-*cdk4*) or Ni²⁺-NTA-agarose beads (for His-*cdk2*) and washed six times with lysis buffer containing EDTA and DTT (see the description above). Cyclin D-*cdk4* kinase was measured by suspending the washed beads in kinase buffer supplemented with 50 μ M ATP–1 mM DTT–10 μ Ci of [γ -³²P]ATP (6,000 Ci/mmol; New England Nuclear) containing 0.5 μ g of purified GST-pRb substrate (23, 29, 30). For cyclin A-*cdk2* kinase, 2 μ g of histone H1 (Boehringer Mannheim, Indianapolis, Ind.) was substituted for GST-pRb as the substrate. Samples were incubated for 30°C for 1 h, denatured in SDS sample buffer, and separated on denaturing polyacrylamide gels before autoradiography of the dried slab gels.

RESULTS

Cyclin D-*cdk4* complexes assemble inefficiently in vitro.

Lysates of Sf9 cells coinfecting with baculoviruses encoding *cdk4* together with any one of three D-type cyclins, but not those expressing individual regulatory or catalytic subunits alone, exhibited readily detectable protein kinase activities able to phosphorylate a recombinant pRb fusion protein produced in bacteria (Fig. 1, lanes 1 to 3 versus 7 to 10) (23). However, when lysates from an equivalent number of cells containing either of the cyclin Ds and *cdk4* subunits were mixed and preincubated for 60 min in vitro, only 1 to 5% of the expected kinase activity was recovered (Fig. 1, lanes 4 to 6). Immunoblotting and metabolic labeling analyses were each used to confirm that coinfecting lysates contained approximately equimolar quantities of *cdk4* and D-type cyclins and to prospectively ensure that the quantities of individual subunits mixed in vitro corresponded to those expressed in the coinfecting cells (23). Similar data were obtained when subunits were mixed at different temperatures ranging from 4 to 37°C, either with or without the addition of 1 mM ATP (data not shown).

To compare the levels of cyclin D-*cdk4* complexes formed after coinfection or after mixing the individual subunits in vitro, lysates from cells coinfecting with cyclin D1 and *cdk4* (Fig. 2A, lanes 1, 2, and 10) or admixed lysates containing both components (lanes 3, 4, and 11) were immunoprecipitated with

FIG. 1. Formation of functional cyclin D-*cdk4* kinases in insect cells. Insect Sf9 cells were coinfecting with baculoviruses encoding mouse D-type cyclins and *cdk4* (lanes 1 to 3) or infected with vectors encoding the individual subunits alone (lanes 7 to 10). Immunoblotting and metabolic labeling were used to quantitate the levels of the various subunits produced in different cultures, which varied no more than threefold overall (23). D-type cyclins and *cdk4* derived from cultures producing the individual subunits were mixed in vitro (lanes 4 to 6) so that the ratio of D-type cyclin to *cdk4* was 1:1 and the final concentrations approximated those achieved in the coinfecting cells. Mixed lysates were incubated for 60 min at 4°C; similar results were obtained with incubations performed at different temperatures ranging from 24 to 37°C in the presence or absence of exogenous (1 mM) ATP. All lysates were assayed for pRb kinase activity, and the labeled product was resolved electrophoretically on denaturing gels and detected by autoradiography (exposure time, 30 min). /, coinfection.

various antisera (Fig. 2A, IP), and the precipitates were immunoblotted with antiserum to *cdk4*. Of the total *cdk4* detected in cells coexpressing cyclin D1 (lane 10), 30 to 40% was bound to cyclin (lane 2). In contrast, when lysates containing *cdk4* and D1 were mixed in vitro (lane 11), complexes between them were not readily detected (lane 4). The antiserum to cyclin D1 did not precipitate *cdk4* itself (lane 9), and proteins related to *cdk4* could not be precipitated from cells expressing cyclin D1 alone (lane 7). Similar results were obtained in parallel experiments using cyclin D2 (Fig. 2B) or D3 (Fig. 2C) in place of D1. Comparable data were also obtained by immunoblotting *cdk4* precipitates with antibodies to each of the D-type cyclins (data not shown). Therefore, the low levels of kinase activity detected in mixed cell lysates appeared to reflect the relatively inefficient formation of complexes between D-type cyclins and *cdk4*.

Because coexpression of D-type cyclins with *cdk4* in intact cells facilitated their assembly into active complexes, we reasoned that endogenous cellular factors that regulate cyclin D-*cdk4* complex formation must be rate limiting under conditions in which both subunits are produced at high levels. In agreement, further addition of extracts from uninfected insect cells together with ATP to constant amounts of admixed *cdk4* and cyclin D enables subunit assembly and the activation of the resulting complexes to proceed somewhat more efficiently in vitro, although generally not to the levels achieved in coinfecting cells (data not shown, but see Fig. 6 for analogous experiments with mammalian cell lysates). It is therefore likely that prolonged coinfection of Sf9 cells with baculoviruses producing both subunits serves to overcome the block to assembly and activation.

Cyclin D-bound *cdk4* is phosphorylated on threonine 172.

On the basis of studies with p34^{cdc2} and p33^{cdc2}, one rate-limiting regulator of *cdk4* activation might be a CAK similar to the enzyme which phosphorylates cyclin-bound p34^{cdc2} and p33^{cdc2} on Thr-161/160 (10, 16, 38, 43, 44). It has been suggested that Thr-161/160 phosphorylation might serve to

FIG. 2. Assembly of cyclin D-cdk4 complexes in intact Sf9 cells or in vitro. Sf9 cells were coinfectd (/) with baculoviruses encoding D-type cyclins and cdk4 (lanes 1, 2, and 10) or infected with vectors encoding the individual subunits (lanes 5 to 9 and 12). The levels of subunits were quantitated, and mixtures (+) of D-type cyclins and cdk4 (lanes 3, 4, and 11) were prepared as described in the legend of Fig. 1. Lysates containing the various components (indicated at the top of each panel) were precipitated (IP) with rabbit antisera to the indicated components or with nonimmune rabbit serum (NRS), and the washed immunoprecipitates were electrophoretically separated on denaturing polyacrylamide gels, transferred to nitrocellulose, and blotted with an antiserum to cdk4. Results with cyclins D1, D2, and D3 are illustrated in panels A, B, and C, respectively. Sites of antibody binding were visualized with ^{125}I -protein A. The autoradiographic exposure times were 8 h.

stabilize certain cyclin-cdk complexes (13) (but not others [4, 10, 44]), thereby contributing to both their assembly and subsequent enzymatic activation in the former case. Data shown below indicate that cyclin D-cdk4 assembly and activation of the complex via threonine phosphorylation of cyclin D-bound cdk4 are independently regulated processes.

To determine whether cdk4 was regulated by phosphorylation, Sf9 cells infected with the cdk4 vector alone or together with a cyclin D1 baculovirus were metabolically labeled with $^{32}\text{P}_i$, and monomeric cdk4 or cdk4 coprecipitated with antiserum to cyclin D1 was subjected to phosphoamino acid analysis. Significantly more ^{32}P was incorporated into the cyclin D-bound species (data not shown, but see the explanation below). Monomeric cdk4 primarily contained phosphoserine, whereas phosphothreonine was the predominant phosphoamino acid recovered from cyclin D-bound cdk4; phosphotyrosine was not detected in either case (Fig. 3A). Trypsin digestion of ^{32}P -labeled monomeric cdk4 yielded three phosphopeptides (Fig. 3B, spots A to C), but when cdk4 was recovered from complexes with cyclin D1, the relative intensity of spot A was greatly increased (Fig. 3C). Phosphoamino acid

analysis revealed that spot A contained phosphothreonine, whereas spots B and C contained phosphoserine (data not shown). Minor spots (labeled a to e in Fig. 3C) are likely to represent products of partial tryptic cleavage of cyclin D-bound cdk4, because they contained only phosphothreonine; digestion with higher concentrations of trypsin or for longer times reduced their relative intensities and increased that of spot A. Identical results were obtained with cdk4 isolated from complexes containing cyclin D2 and D3 (data not shown). Therefore, assembly of cyclin D-cdk4 complexes correlated with cdk4 threonine phosphorylation on a single major phosphopeptide.

Comparison of the deduced amino acids of p34^{cdc2}, p33^{cdc2}, and p34^{cdk4} reveals that Thr-172 in cdk4 occurs in a position and context corresponding to the unique site of CAK phosphorylation in each of the other cdk4s (Fig. 4A). Although the amino acids immediately adjacent to Thr-172 in cdk4 differ from those in the other two proteins, highly conserved residues are found C-terminal to the putative CAK phosphorylation site. Site-directed mutagenesis was used to generate cdk4 variants in which Thr-172 was replaced with a nonphosphorylatable residue (Ala), a different phosphorylatable residue (Ser), or a negatively charged amino acid (Glu) that might in principle mimic the function of phosphothreonine.

When expressed in $^{32}\text{P}_i$ -labeled Sf9 cells and recovered in complexes with cyclin D1, the cdk4 (T172A) protein was poorly phosphorylated (Fig. 4B, lane 3). Control experiments demonstrated that virtually identical amounts of cdk4 protein were coprecipitated under these conditions (see, for example, Fig. 5C, lanes 3, 5, and 7). The cyclin D-bound cdk4 (T172A) protein contained only phosphoserine (data not shown) and failed to yield phosphotryptic peptide A (Fig. 4C, panel 2). In contrast, cyclin D1-bound cdk4 (T172S) (Fig. 4B) yielded a phosphopeptide map very similar to that of the wild-type protein (Fig. 4C, panel 3). Phosphoamino acid analysis revealed that the peptide designated A' in panel 3 contained only phosphoserine, and a mixing experiment confirmed that peptide A (from wild-type cdk4) and A' (from the T172S mutant) had virtually identical mobilities in the two-dimensional separation system (Fig. 4C, panel 5), as would be expected from their very similar amino acid compositions and phosphorylations. The concomitant loss of both phosphothreonine and peptide A from the T172A mutant and the substitution of P-Thr by P-Ser in peptide A' in the T172S mutant demonstrate that T-172 is a major and possibly unique site of threonine phosphorylation in cdk4.

Importantly, threonine phosphorylation of cdk4 in complexes with cyclin D did not depend on intrinsic cyclin D-cdk4 kinase activity itself. Complexes composed of a catalytically inactive cdk4 mutant (K35M) and cyclin D can readily assemble in Sf9 cells (23) (also see Fig. 5C) and undergo phosphorylation on peptide A (Fig. 4C, panel 4). A CAK present in Sf9 cells must therefore be able to phosphorylate cyclin D-bound cdk4 on Thr-172. As previously reported (31), cyclin D1 can also be phosphorylated on serine, whether free or bound to cdk4 (Fig. 4B), and the function of this modification, if any, remains unclear.

P-Thr-172 is required for cdk4 kinase activity but not for complex formation with D cyclin. Sf9 cells were infected with vectors encoding the wild-type or mutant cdk4 proteins, either alone or with a vector encoding cyclin D1, and both the coinfectd and mixed cell lysates were assayed for pRb kinase activity. Like wild-type cdk4, the cdk4 (T172S) mutant yielded high levels of pRb kinase activity when coexpressed with cyclin D1 (Fig. 5A, compare lanes 1 and 5) but generated significantly lower levels when mixed with equivalent amounts of cyclin D1

FIG. 3. Phosphoamino acid composition and tryptic phosphopeptide maps of cdk4. Sf9 cells infected with a baculovirus encoding cdk4 alone (CDK4) or coinfecting (+) with vectors encoding cdk4 and cyclin D1 (CDK4 + D1) were labeled for 8 h with $^{32}\text{P}_i$, and cell lysates were precipitated with antiserum directed to cdk4 (CDK4) or to cyclin D1 (CDK4 + D1). The immunoprecipitated products were electrophoretically separated on denaturing polyacrylamide gels and visualized by autoradiography. Radiolabeled cdk4 was eluted and hydrolyzed in acid (panel A) or digested with trypsin (panels B and C). Phosphoamino acids were separated electrophoretically in a single dimension (panel A) together with internal standards, which were detected with ninhydrin (positions noted at the left). Tryptic peptides were resolved in two dimensions (panels B and C) by electrophoresis (left to right) and ascending chromatography (bottom to top) and visualized by autoradiography. The positions of major phosphopeptides are denoted by capital letters. Elution of the individual peptides, hydrolysis, and phosphoamino acid analysis demonstrated that peptide A (as well as the minor peptides denoted by lowercase letters) contained phosphothreonine, whereas peptides B and C contained phosphoserine. Autoradiographic exposure times for all panels were 18 h.

in vitro (lanes 2 and 6). The T172A mutant was completely inactive (lanes 3 and 4), indicating that phosphorylation of Thr-172 (or of substituted serine in this position) is required for enzyme function. Importantly, both the T172A and T172S mutants were equally able to form complexes with cyclin D1 in the coinfecting cells (Fig. 5B). Cyclin D-cdk4 complexes were readily detected in coinfecting cells when these cell lysates were immunoprecipitated with an antiserum to cyclin D1 and the precipitated proteins were blotted with antiserum to cdk4 (Fig. 5B, lanes 1, 3, and 5) but not after admixing of Sf9 lysates containing individual subunits (lanes 2, 4, and 6). Similar levels of cdk4 were expressed in each of the coinfecting or mixed lysates (lanes 11 to 16), and the efficiency of complex formation was high in cells coinfecting with the cdk4 mutants (lanes 3 and 5). The two forms of cdk4 of 34 and 35 kDa observed in Fig. 5B and C arise from differential translational termination in Sf9 cells; only the 34-kDa species is produced in mammalian cells (23, 29).

Similarly, when coinfecting lysates metabolically labeled with [^{35}S]methionine were immunoprecipitated with antiserum to cyclin D1, nearly equivalent quantities of wild-type or mutant cdk4 proteins coprecipitated with cyclin (Fig. 5C, bottom panel, odd-numbered lanes). An immune complex kinase assay again confirmed that the cdk4 T172A mutant was enzymatically inactive (Fig. 5C, top panel, lane 5), whereas the T172S mutant functioned as well as the wild-type protein (compare lanes 3 and 7). This experiment also shows results with the T172E mutant, which, while very poorly active, retained some ability to phosphorylate pRb when bound to cyclin D1 (lane 9). Thus, assembly of cdk4 with cyclin D1 can proceed in coinfecting cells in the absence of cdk4 Thr-172 phosphorylation, implying that cdk4 CAK activity is not required for cdk4-cyclin D complex formation. The fact that assembly of cyclin D-cdk4 complexes proceeds inefficiently in vitro does not, then, de-

FIG. 4. Tryptic phosphopeptide analysis of cdk4 mutants. (Panel A) Amino acid sequences in single-letter code of residues flanking CAK phosphorylation sites in mammalian cdc2 (Thr-161) and cdk2 (Thr-160). Amino acids in the same position and context in both murine and human cdk4 are aligned below. (Panel B) Insect cells infected with baculoviruses encoding cyclin D1 alone (lane 1) or coinfecting with vectors encoding cyclin D1 plus wild-type cdk4 (lane 2) or the indicated mutants (lanes 3 and 4) were metabolically labeled with $^{32}\text{P}_i$. Cell lysates were precipitated with antiserum to cyclin D1, and the precipitates were separated on denaturing gels and visualized by autoradiography (exposure time, 2 h). (Panel C) Radiolabeled cdk4 was eluted from gels (panel B), digested with trypsin, and separated as described in the legend to Fig. 3. Results with wild-type cdk4 (C1) and those obtained with the indicated Thr-172 substitution mutants (C2 and C3) are shown. Results with a catalytically inactive cdk4 mutant (C4; not shown in panel B) and results of mixing experiments performed with tryptic peptides from wild-type cdk4 and the T172S mutant (C5) are illustrated. Exposure times were 18 h. A, phosphothreonine-containing peptide; A', phosphoserine-containing peptide; C, peptide C.

FIG. 5. Assembly and pRb kinase activity of enzymes formed between cyclin D1 and cdk4 mutants. Insect cells were infected with baculoviruses encoding the indicated enzyme subunits or were coinfecting (/) with cdk4 variants and cyclin D1. Mixtures of lysates (+) were prepared as described in the legend to Fig. 2. (A) Lysates were assayed for pRb kinase activity, and the radiolabeled pRb product was resolved on denaturing gels (exposure time, 30 min). (B) Lysates were immunoprecipitated with antiserum to cyclin D1 (lanes 1 to 10) or cdk4 (lanes 11 to 16). The immune complexes were separated on denaturing gels, blotted with antiserum to cdk4, and detected with ¹²⁵I-protein A (exposure time, 8 h). (C) Insect cells infected with the indicated vectors were metabolically labeled with [³⁵S]methionine, lysed, and immunoprecipitated with antiserum to cyclin D1. Portions of the immunoprecipitates were assayed for pRb kinase activity (top panel), and the remaining portions were separated on denaturing gels and visualized by autoradiography (bottom panel). The positions of cyclin D1 and coprecipitating cdk4 are noted at the right. Exposure times were 30 min for the top panel and 6 h for the bottom panel. The two forms of cdk4 (34 and 35 kDa) observed in panels B and C result from alternative translational termination from the vector-encoded mRNA in Sf9 cells, and only the major 34-kDa species is detected in mammalian cells (23, 29).

pend upon CAK but may instead depend upon the presence of another regulatory protein or "assembly factor" whose concentration is rate limiting in Sf9 cells.

Assembly and activation of cyclin D-cdk4 complexes by lysates from mammalian cells. Reasoning that mammalian cells, but not bacterial cells, contain the regulators responsible for efficient cyclin D-cdk4 assembly and activation, we produced either GST- or His-tagged cyclins A and D2 and cdk2 and cdk4 in bacteria. The individual recombinant proteins were isolated by affinity chromatography on nickel-agarose or glutathione-Sepharose and eluted from beads, and different combinations of soluble cyclins and cdks were incubated with ATP for 60 min at 23°C in vitro, either in buffer alone or with

FIG. 6. Reconstitution of cdk kinase activities in vitro. Indicated tagged recombinant cyclins and cdks were purified from bacteria and mixed in vitro in the absence (-) or presence (+) of lysates from proliferating NIH 3T3 cells. After addition of ATP, the mixtures were incubated at room temperature for 60 min and cdks together with any bound proteins were recovered by affinity chromatography on beads, washed, and assayed for the presence of histone H1 (A) or pRb (B and C) kinase activities. The radiolabeled products were separated on denaturing gels and detected by autoradiography (exposure times, 15 min [A] and 4 h [B and C]).

lysates from asynchronously proliferating NIH 3T3 cells. The "marinated" tagged cdks, together with any bound proteins, were then reisolated on nickel-agarose or glutathione-Sepharose and assayed for either histone H1 or pRb kinase activity. Note that this assay for enzyme activation should depend upon complex formation as well as CAK activity.

As expected, mixtures of bacterial cyclin A plus cdk2 or cyclin D2 plus cdk4 lacked detectable histone H1 or pRb kinase activity, respectively (Fig. 6A, lane 3, and 6B, lane 4). In contrast, their preincubation with cell lysates from proliferating NIH 3T3 cells generated highly active kinases (Fig. 6A, lane 4, and 6B, lane 5), implying that assembly and CAK-mediated modification of the complexes can occur under these conditions. Activation of the cyclin D2-cdk4 holoenzyme occurred less efficiently than that observed for cyclin A-cdk2 (note the longer autoradiographic exposure times for Fig. 6B and C versus 6A), but we suspect that these differences might simply reflect the purification of His-tagged cyclin D2 under denaturing conditions (see Materials and Methods). Preincubation of the same quantities of cyclin A, cdk2, or cyclin D2 alone with NIH 3T3 cell lysates yielded negligible levels of enzyme activity (Fig. 6A, lanes 1 and 2, and 6B, lane 2) that were no greater than that obtained with marinated GST-beads alone (Fig. 6B, lane 1). However, preincubation of cdk4 alone with NIH 3T3 cell lysates yielded low but detectable levels of pRb kinase activity (Fig. 6B, lane 3). This activity can most

likely be attributed to interactions of cdk4 with the pool of unbound D-type cyclins expressed in proliferating NIH 3T3 cells (39). cdk4 lacks pRb kinase activity when expressed in Sf9 cells lacking D-type cyclins (Fig. 1), and it does not productively interact with cyclins A, B, and E (23), which are expressed in both insect and mammalian cells in association with other cdk. Lysates from serum-starved, contact-inhibited NIH 3T3 cells, which do not express D-type cyclins (39), did not activate cdk4 pRb kinase in this assay (negative data not shown), although in this case, the failure to recover an active enzyme might be due to the absence of the putative assembly factor and/or cdk4 CAK in the extracts or to the presence of inhibitory activities for cdk4. The addition of bacterial cyclin D2 to GST-cdk4 in the presence of lysates from proliferating cells reproducibly generated at least 10-fold-greater levels of pRb kinase activity than did cdk4 alone (Fig. 6B, lane 5), indicating that additional active holoenzyme complexes were assembled *in vitro* under these conditions. Lysates from other proliferating mammalian cell lines (e.g., Rat2 fibroblasts and BAC1.2F5 macrophages), but not those obtained from their quiescent, growth factor-deprived counterparts, were equally active in supporting the formation of pRb kinases from bacterial cyclin D2 and cdk4 subunits (data not shown).

In a separate series of experiments, we tested various cdk4 mutants for their abilities to be activated in this assay (Fig. 6C). Neither the T172A nor K35M mutants of cdk4 underwent activation in the presence of mammalian cell lysates (Fig. 6C, lanes 1 and 2), which were again required for activation of complexes containing wild-type cdk4 (lanes 3 and 4). Given the ability of the T172A mutant to physically interact with D-type cyclins (Fig. 5B and C), activation of the wild-type cdk4 kinase must depend at least in part upon a cdk4 CAK activity present in the mammalian cell lysates.

DISCUSSION

When both cyclin D and cdk4 are constitutively overexpressed in mammalian cells, complexes between regulatory and catalytic subunits do not assemble in serum-starved cells but, like endogenous complexes, begin to accumulate in mid-G₁ and are maximally expressed at the G₁/S transition (30). Whether the individual subunits are encoded by the endogenous genes or are ectopically expressed, the kinetics of appearance of cyclin D-CDK4 kinase activity temporally correlate with complex formation. Because assembly of cyclin D-CDK4 complexes and the concomitant appearance of their kinase activities in mammalian cells remain dependent on growth factor stimulation, upstream regulators must govern the formation of the active holoenzymes. Here, we provide evidence that cyclin D-CDK4 complex formation followed by cdk4 phosphorylation and accompanying holoenzyme activation are subject to dual controls, at least one of which must be growth factor dependent.

CAK activity for cdk4. When coexpressed in Sf9 cells, cyclin D and cdk4 can assemble into complexes, and the catalytic subunit undergoes phosphorylation on threonine 172, resulting in the formation of an enzymatically active pRb kinase. By contrast, monomeric cdk4 subunits produced in insect cells contained significantly lower levels of phosphothreonine, suggesting that this modification occurs much more efficiently when cdk4 is bound to cyclin D. A cdk4 mutant containing an alanine-for-threonine substitution at codon 172 was equally able to form complexes with D-type cyclins in intact insect cells but was not phosphorylated on threonine or enzymatically activated. By contrast, substitution of a phosphorylatable serine residue for Thr-172 yielded complexes that acquired

additional serine phosphate and were active. In the latter case, tryptic phosphopeptide mapping demonstrated that the single phosphothreonine-containing peptide (peptide A) characteristic of wild-type cyclin D-bound cdk4 was replaced by another phosphoserine-containing tryptic peptide (peptide A'), whose similar mobility in the two dimensional peptide separation system was consistent with replacement of P-Thr by P-Ser at residue 172. Substitution of glutamic acid for Thr-172 generated a holoenzyme that was very weakly active as a pRb kinase, indicating that a negative charge at this position could inefficiently substitute for the presence of covalently linked phosphate. The ability of a cyclin-bound, catalytically inactive cdk4 mutant to undergo threonine phosphorylation on peptide A argues that the modification is not a consequence of cdk4 activation but is instead catalyzed by a CAK, which is expressed in Sf9 cells. Together, these results indicate that (i) cyclin D-CDK4 assembly and phosphorylation are independently regulated processes; (ii) cdk4 CAK specifically phosphorylates Thr-172 in assembled cyclin D-CDK complexes but is poorly active on monomeric cdk4; (iii) CAK-mediated phosphorylation is not required for cyclin D-CDK4 assembly, at least in coinfecting insect cells where both components are produced at relatively high levels; and (iv) Thr-172 phosphorylation is required for the enzymatic activity of cyclin D-CDK4 holoenzymes.

These conclusions are in general agreement with those emanating from studies of cdk2 and cdc2 CAK. At least for cyclin A-CDK2, cyclin B-CDK2, and cyclin B-cdc2, CAK activity is not required for assembly of the complexes (4, 10, 44). The crystal structure of cdk2 localizes the CAK target site (Thr-160) to the apex of a loop which blocks the substrate binding cleft (9). Phosphorylation of this residue is thought to induce a conformational change that removes a structural constraint to substrate binding, enabling enzyme activation. Because cyclin binds to the region of the cdk2 N-terminal lobe above the substrate binding cleft, it might also affect the conformation of these structures to form part of the recognition site for CAK-mediated phosphorylation and could potentially affect substrate recognition by cdk2 itself. In the case of D-type cyclin complexes with either cdk4 or cdk2, their respective preferences for substrates (pRb or histone H1) are determined by both the regulatory and catalytic subunits (23, 30).

The substrate specificity of cdk2 and/or cdc2 CAK appears to be highly restricted (16, 38, 43). Although the catalytic subunit (MO15) of cdk2 and/or cdc2 CAK is itself related in its predicted amino acid sequence to known cdk, the sites phosphorylated by CAK within p34^{cdc2} and p33^{cdk2} lack adjacent prolyl residues characteristic of cdk phosphorylation sites. By contrast, T-172 in cdk4 lies directly adjacent to a prolyl residue and might in principle be recognized by a different enzyme. When purified *Xenopus* CAK (generously provided by Mark Solomon, Yale University, New Haven, Conn.) was added to mixtures of cdk4 and D-type cyclins produced in insect cells, pRb kinase activity was not generated, most probably because the mixed subunits assemble very poorly *in vitro*. However, *Xenopus* CAK was also unable to superactivate preassembled cyclin D-CDK4 complexes derived from intact insect cells engineered to coexpress both components, implying that all preformed complexes were already maximally phosphorylated on Thr-172 or, alternatively, that the *Xenopus* enzyme cannot efficiently phosphorylate cyclin D-bound cdk4 (data not shown). Because we have not identified a phosphatase that removes the Thr-172 phosphate from catalytically active cyclin D-CDK4 complexes, we have so far been unable to distinguish between these alternatives. These results leave open the possibility that CAK for cdk4 differs from that which

acts on cdc2 and/or cdk2, in terms of either its subunit composition or its posttranslational modification.

What governs cyclin D-cdk4 assembly? Because cyclin D-cdk4 complexes accumulate to high levels in Sf9 cells coinfectected with baculoviruses encoding both subunits but cannot be as readily assembled *in vitro*, assembly of the holoenzyme is also a rate-limiting process potentially subject to other forms of regulation. The simplest idea is that an assembly factor is required to join or stabilize the cyclin D-cdk4 complex, making it a suitable substrate for CAK and thereby enabling the subsequent activation of the functional holoenzyme. Consistent with this hypothesis, a mutant cdk4 subunit containing alanine at codon 172 can assemble with D-type cyclins in coinfectected insect cells, despite the fact that it remains catalytically inactive. Lysates from proliferating mammalian cells appear to contain all that is necessary to generate active complexes *in vitro*, because recombinant cyclin D and cdk4 subunits produced in bacteria can be activated as pRb kinases when marinated in such extracts. Even if CAK were to serve a dual function in facilitating both phosphorylation and cyclin D-cdk4 assembly, formation of such complexes would not depend upon CAK-mediated phosphorylation. In intact mammalian NIH 3T3 or Rat2 fibroblasts engineered to ectopically express D-type cyclins and cdk4, only free subunits accumulate in serum-depleted, contact-inhibited cells (30), suggesting that the activity of the factor(s) governing assembly is serum dependent. Facilitators of cyclin D-cdk4 assembly might include molecules homologous to those of suc1/CKS, whose recently determined crystal structure is consistent with such a function (36). Alternatively, assembly of the holoenzymes might be prevented by any of a number of recently identified cdk inhibitors, whose interactions with unbound catalytic subunits might prevent their association with cyclins (14, 20, 21, 37, 41, 48–50). In particular, one such protein (p16) binds at the expense of D-cyclins to cdk4 (41). Whatever the mechanisms, it will be important to establish the molecular identities of the factors controlling cyclin D-cdk assembly as well as the nature of cdk4 CAK in order to better understand how activation of cyclin D-dependent kinases is regulated by growth factors.

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