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Biochemical Characterization of the Human Cyclin-dependent Protein Kinase Activating Kinase

IDENTIFICATION OF p35 AS A NOVEL REGULATORY SUBUNIT*

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The activation of cyclin-dependent protein kinases (Cdks) is dependent upon site-specific phosphorylation and dephosphorylation reactions, as well as positive and negative regulatory subunits. The human Cdk-activating protein kinase (Cak1) is itself a Cdc2-related cyclin-dependent protein kinase that associates with cyclin H. The present study utilized specific anti-Cak1 antibodies and immunoaffinity chromatography to identify additional Cak1-associated proteins and potential target substrates. Immunoprecipitation of metabolically labeled human osteosarcoma cells revealed a number of Cak1-associated proteins, including p95, p37 (cyclin H), and a 35-kDa protein that was further characterized herein. Microsequence analysis obtained after limited proteolysis revealed peptide fragments that are similar, but not identical to, human and yeast cyclins, thus identifying p35 as a cyclin-like regulatory subunit. The greatest sequence similarity of human p35 is with *Mcs2*, a yeast cyclin that is essential for cell cycle progression. Immunoaffinity chromatography performed under nondenaturing conditions afforded the isolation of enzymatically active Cak1 from cell lysates, enabling studies of kinase autophosphorylation and comparative substrate utilization. Immunoaffinity-purified Cak1 phosphorylated monomeric Cdc2 and Cdk2, but not Cdk4; the phosphorylation of both Cdc2 and Cdk2 were increased in the presence of recombinant cyclin A. These studies indicate that the Cak1 catalytic subunit, like Cdc2 and Cdk2, associates with multiple regulatory partners and suggests that subunit composition may be an important determinant of this multifunctional enzyme.

Critical transitions in the eukaryotic cell cycle are regulated by patterns of protein phosphorylation events governed predominantly by the catalytic activities of cyclin-dependent protein kinases (Cdks)[1] (Norbury and Nurse, 1992; Meyerson *et al.*, 1992; Reed, 1992; Pines, 1993; Dorée and Galas, 1994). The activities of these Cdks are themselves regulated by site-specific phosphorylation-dephosphorylation reactions (Krek and Nigg, 1991; Norbury *et al.*, 1991; Solomon, 1993) as well as the association with positive (*i.e.* cyclins) (Hunt, 1991; Wu *et al.*, 1993; Sherr, 1993; Draetta, 1994) and negative (*i.e.* Cdk inhibitors) (Elledge and Harper, 1994; Peter and Herskowitz, 1994; Xiong *et al.*, 1993) regulatory subunits. An essential phosphoregulatory site, deduced from genetic studies in yeast, was identified within the T-fold of kinase subdomain VIII (Marcote *et al.*, 1993; DeBondt *et al.*, 1993) of Cdc2 (at Thr-161) and Cdk2 (at Thr-160) and whose phosphorylation is required for the generation of Cdc2/Cdk2 kinase activity (Krek *et al.*, 1992; Gu *et al.*, 1992; Connell-Crowley *et al.*, 1993). Subsequent studies have identified a distinctive Cdc2/Cdk2-activating protein kinase (CAK) in *Xenopus* oocytes, MO15 (Shuttleworth *et al.*, 1990; Poon *et al.*, 1993; Solomon *et al.*, 1993), and human tumor cells (Solomon *et al.*, 1993; Williams *et al.*, 1993a, 1993b). The catalytic subunit of the human CAK (Cak1) has been cloned and is found to be structurally related to Cdc2(Hs) (Wu *et al.*, 1994). Human Cak1 has been shown to complex with at least one regulatory subunit, cyclin H (Fisher and Morgan, 1994; Mäkelä *et al.*, 1994), revealing a vectorial cascade of cyclin-dependent protein kinases.

Other recent studies suggest that Cak1 functions in transcriptional regulation, DNA repair, and tumor suppression, in addition to its role in regulating cell cycle progression.

However, numerous questions remain regarding the subunit configuration(s), biochemical activation, substrate specificity, and physiological targets of this key regulatory enzyme. Pronounced disparities in the published literature concerning the molecular mass of the Cak1 complexes (Solomon *et al.*, 1992; Poon *et al.*, 1993; Williams *et al.*, 1994; Wu *et al.*, 1994), the nature and identity of its regulatory subunit(s) (Mäkelä *et al.*, 1994; Tassan *et al.*, 1994), the phosphorylation of putative target substrates, including monomeric Cdc2 (Dorée and Galas, 1994; Fisher and Morgan, 1994) and Cdk4 (Kato *et al.*, 1994; Matsuoka *et al.*, 1994), and the regulation of its enzymatic activity (Fisher and Morgan, 1994; Matsuoka *et al.*, 1994; Williams *et al.*, 1994), suggest that our understanding of this key regulatory enzyme system is still incomplete.

Using the deduced amino acid structure of (Hs)Cak1 (Wu *et al.*, 1994), we developed anti-peptide antibodies that are selective for the p42^{cak1} subunit and have characterized the performance of these antibodies for both immunoprecipitation and Western blotting. In this study, we used anti-Cak1 antibodies and immunoaffinity chromatography to investigate the enzyme activities of purified Cak1 and to further characterize Cak1-associated proteins. In addition to verifying high molecular

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[1] The abbreviations used are: Cdk, cyclin-dependent protein kinase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatography.

weight complexes reported previously (Williams *et al.*, 1993a, 1993b) and the association of a 37-kDa protein identified as cyclin H (Mäkelä *et al.*, 1994; Fisher and Morgan, 1994), we detected a distinctive 35-kDa protein in association with $p42^{cak1}$. Isolation, fragmentation, and microsequence analysis of p35 revealed that this Cak1-associated protein exhibits amino acid sequence homologies to human cyclin H and yeast *Mcs2*; indicating that p35 is indeed a cyclin-like regulatory subunit that is related to, but distinct from, cyclin H.

EXPERIMENTAL PROCEDURES

Materials

Cell Cultures and Cell Synchronization—Human MG-63 and TE-85 osteosarcoma cells were obtained from the American Type Cell Culture Collection (ATCC; Rockville, MD). The MG-63 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.), penicillin, and streptomycin, and the TE-85 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics, at 37 °C, in 5% CO_2 humidified air. MG-63 cells were synchronized by serum deprivation, as described previously (Carbonaro-Hall *et al.*, 1993). Hela S-3 cells, grown as large scale suspension cultures, were obtained from the University of Southern California Norris Comprehensive Cancer Center, frozen in liquid nitrogen, and stored at −70 °C prior to use.

Recombinant Plasmids and Synthetic Peptides—The expression vectors encoding the glutathione *S*-transferase fusion proteins glutathione *S*-transferase-Cdc2 and glutathione *S*-transferase-Cdk2, and the epitope-tagged HA-Cdk4, have been described elsewhere (Connell-Crowley *et al.*, 1993). Polymerase chain reaction-derived full coding sequences of human cyclin H (Fisher and Morgan, 1994; Mäkelä *et al.*, 1994) were ligated into the pET-28 expression vector (Novagen) and maintained in XL Blue strain of *E. coli*. For expression of the His-tagged human cyclin H fusion protein, the plasmid was transformed into BL21(DE3) strain *E. coli*, and recombinant protein expression was induced by incubation in the presence of isopropyl-1-thio-β-D-galactopyranoside. Anti-peptide antisera, directed against the first 15 amino acids of human cyclin H (Fisher and Morgan, 1994) was raised in rabbits. The synthetic peptide corresponding in sequence to residues 151–170 of the human $p34^{cdc2}$ (Lee and Nurse, 1987), as well as the Ala-162 congener, was chemically synthesized and coupled to Affi-Gel 10 as described previously (Williams *et al.*, 1994).

Methods

Metabolic Labeling, Immunoprecipitation, and Immunoblotting—For ^{35}S labeling of cellular proteins, TE-85 and MG-63 osteosarcoma cells in log phase growth were washed twice with prewarmed methionine-free and cysteine-free Dulbecco's modified Eagle's medium (ICN) and were preincubated in this media supplemented to 0.5% dialyzed fetal bovine serum (Life Technologies, Inc.) for 30 min at 37 °C. [^{35}S]Met/Cys (1 mCi/ml; ICN) was added directly to the culture media to effect a final concentration of 100 μCi/ml, and the cell cultures were incubated for 4 h at 37 °C. Following metabolic labeling, the cells were washed twice to remove exogenous label prior to cell lysis, immunoprecipitation, and visualization of the immune complexes by SDS-PAGE and autoradiography. Cell lysates were prepared from fresh monolayer cultures as follows. The cells were washed twice in Hanks' balanced salt solution and lysed in a small volume (1 ml/75-cm_2 culture flask) of IP buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EGTA, 25 mM sodium fluoride, 25 mM β-glycerolphosphate, 0.1 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 0.2% (v/v) Triton X-100, 0.3% (v/v) Nonidet P-40) and clarified by centrifugation (20,000 × *g*) for 10 min at 4 °C. The supernatant was collected for Western blotting, *in vitro* kinase assays, immunoprecipitation studies, and column chromatography.

For immunoprecipitations, MG-63 and TE-85 cells were cultured and lysed as described above, and the lysate was clarified by incubation with 50 μl (1:1 slurry in (1×) PBS) of protein A-Sepharose CL-4B on ice for 15 min. Immunoprecipitations and affinity precipitations were performed by incubating aliquots of the precleared lysate (supernatant) with either no antibodies (control), specific antibodies, or specific peptide-blocked antibodies for 20 min in ice with periodic mixing. The resulting immune complexes were collected by the addition of 50 μl (1:1 slurry in (1×) PBS) of protein A-Sepharose CL-4B, incubation in ice for 20 min, followed by centrifugation. After washing each pellet 4 times with 750 μl of ice-cold Fast Q Buffer (20 mM Tris-HCl, pH 7.6, 1 mM dithiothreitol, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 0.1 mM sodium orthovanadate) containing 50 mM NaCl, and once in 750 μl of ice-cold kinase assay buffer, each pellet was resuspended in 50 μl of (1×) SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 40 mM dithiothreitol, 2% SDS, 0.025% bromphenol blue, 10% glycerol) containing 10 mM *N*-ethylmaleimide (to minimize dissociation of IgG chains), heat-denatured for 2 min at 95 °C, and the solubilized proteins were analyzed by SDS-PAGE and Western blotting, as described by Williams *et al.* (1992). The specific immunoreactivity of the newly developed anti-Cak1 antibodies were validated by competition of the antibody with the immunizing peptide as follows: approximately 400 μg of the immunizing peptide was mixed with anti-Cak1 IgG and preincubated for 30 min at 30 °C prior to separate parallel incubations of both blocked and nonblocked antibodies for 30 min on Western blots. The resulting Western blots were developed under identical conditions.

Development and Application of Anti-Cak1 Antibodies—The C-terminal amino acids (residues 328–346) of Cak1 were chemically synthesized (Microchemical Core Facility, University of Southern California Norris Cancer Center) and conjugated to KLH (Pierce, Rockford, IL) for the immunization of rabbits to raise antisera directed against Cak1. Portions of the collected antisera were used to prepare two subsets of polyclonal antibodies: an affinity-purified fraction and an IgG fraction. To generate the affinity-purified polyclonal antibodies, the antigenic Cak1 peptide was coupled to Affi-Gel 10 (Bio-Rad, Richmond, CA) beads preequilibrated in 20 mM KPO_4, pH 7.4, at room temperature, with gentle rocking for 2 h. The Affi-Gel beads were collected by centrifugation (3000 rpm × 5 min), and the reaction was quenched by the addition of 0.2 M ethanolamine and then transferred to a small column and washed with 20 volumes of filtered (1×) phosphate-buffered saline. Anti-Cak1 antisera was diluted 1:4 in 1 × PBS, filtered through 0.45 μm, and passed over the Affi-Gel beads 3 times. The beads were washed extensively with 10 volumes filtered (1×) PBS, and the anti-Cak1 antibodies were eluted using 0.1 M glycine-HCl, pH 2.7, collected in 0.5-ml fractions. The fractions were monitored for protein content (Bio-Rad), and those containing affinity-purified antibodies were pooled and aliquoted for storage at −20 C until use. To purify polyclonal anti-Cak1 IgG fractions, protein A-Sepharose CL-4B (1 ml bed volume) was equilibrated with 20 volumes of 100 mM Tris-HCl, pH 8.0. Anti-Cak1 antisera was adjusted to pH 8, diluted 1:4 with equilibration buffer, and filtered through 0.45 μm for extraction by protein A-Sepharose CL-4B as above. The antisera was passed over the resin twice, and the resin was washed with 10 volumes of 100 mM Tris-HCl, pH 8.0, followed by 10 volumes of 10 mM Tris-HCl, pH 8.0, before eluting with 100 mM glycine, pH 3.0. Fractions containing IgGs were pooled, aliquoted for later use, and stored at −20 °C.

Immunoaffinity Chromatography—To prepare an anti-Cak1 immunoaffinity column, 1 ml (bed vol) of protein A-Sepharose CL-4B resin was equilibrated in 100 mM Tris-HCl, pH 8.0, and several ml of anti-Cak1 antisera was passed over it twice. The resin was washed extensively with 0.1 M sodium borate, pH 9.0, and dimethyl pimelimidate (Pierce Chemicals) was added to 20 mM to covalently bind the IgG to the resin (30 min, at room temperature, with gentle rocking). The resin was collected by centrifugation, washed with 0.2 M ethanolamine, pH 8.0, and allowed to block in ethanolamine for 2 h. The resin was again collected by centrifugation (2500 rpm × 5 min) and washed extensively with filtered (1×) PBS, pH 7.4. The column was preeluted with 100 mM glycine, pH 3.0, to remove noncovalently bound IgG prior to immunoaffinity chromatography.

Hela S-3 cells (5–7 g pellets) were stored at −70 °C until use. The cells were lysed in 20 volumes of IP buffer at 4 °C and cleared by centrifugation (10 min × 20,000 × *g*) and filtered through 0.45 μm prior to immunoaffinity column chromatography. After the entire clarified cell lysate has passed through the column, the column was developed as follows: a 10-volume wash with IP buffer followed by 5 volumes of 1 × PBS, followed by 10 volumes of 0.3 M NaCl in IP buffer, followed by 10 volumes of 0.3 M NaCl, 30% ethylene glycol in IP buffer, followed by 5 volumes of 1 × PBS, followed by 10 volumes of 1 M $MgCl_2$ in IP buffer, followed by 5 volumes of 1 × PBS, followed by elution with 0.1 M glycine, pH 2.7 (collected in 0.5-ml fractions). The glycine-eluted fractions containing proteins were pooled and concentrated in Amicon microconcentrators, and samples of each wash were analyzed by Western blotting. Enzymatically active Cak1 was recovered in both the NaCl/ethylene glycol and the $MgCl_2$ eluates, as well as the NaCl wash, indicating that a substantial proportion of the anti-Cak1 peptide antibodies were low to moderate in their affinity (Kellogg and Alberts, 1992) and enabling the purification of Cak1 under nondenaturing conditions.

In Vitro Protein Kinase Assays—MG-63 and TE-85 cells were cultured, lysed, and clarified as described for immunoprecipitation. After aliquots of the clarified lysates were incubated either without primary

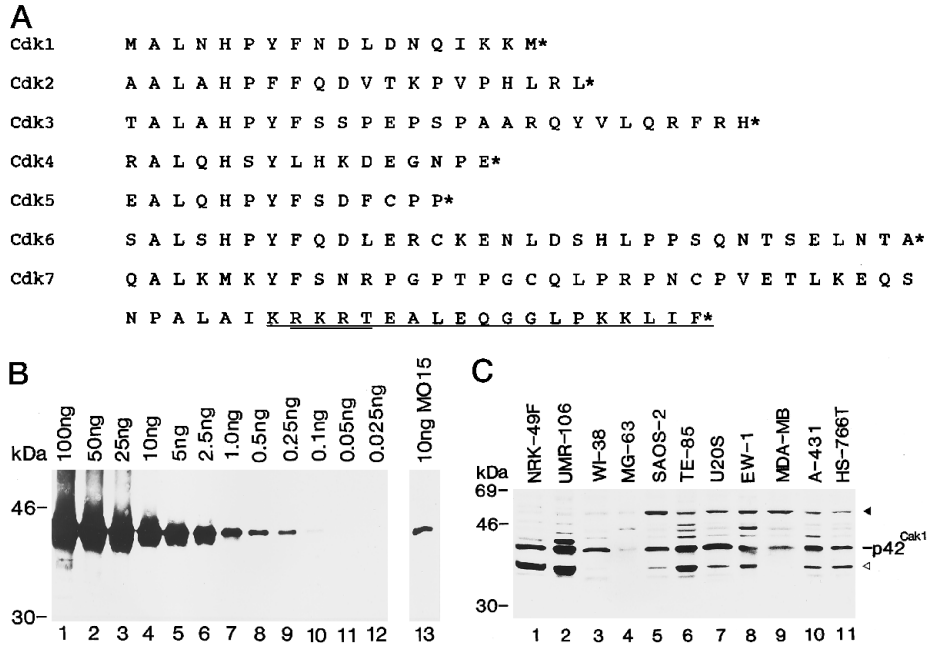

FIG. 1. **Design and application of specific anti-Cak1 antibodies.** *A*, alignment of the C-terminal domains adjacent to the conserved subdomain XI of identified human Cdks. The primary structure of the synthetic peptide used to raise specific anti-Cak1 antibodies is *underlined*. *Double underlined* is the putative PKA phosphorylation site located within the bipartite nuclear localization motif. *B*, titration of Cak1 detection upon Western blotting by polyclonal anti-Cak1 antibodies raised against the immunizing peptide. Note comparative cross-reactivity with *Xenopus* M015, which exhibits a similar but nonidentical C terminus (see Shuttleworth *et al.*, 1990). *C*, Western blotting of cell lysates obtained from a variety of normal and neoplastic cell lines with affinity-purified anti-Cak1 antibodies. Each *lane* was loaded with 10 μg of protein of the respective detergent lysate: normal rat kidney fibroblast (*NRK-49F*), rat osteosarcoma (*UMR-106*), normal human diploid fibroblast (*WI-38*), human osteosarcoma (*MG-63, SAOS-2, TE-85, U2OS*), Ewings sarcoma (*EW-1*), mammary carcinoma (*MDA-MB*), epithelioid carcinoma (*A-431*), pancreatic carcinoma (*HS-766T*). While the anti-Cak1 antibodies detected a protein of the expected size ($p42^{cak1}$) in all samples, additional bands of immunoreactivity migrating at ~36 and ~55 kDa were pronounced in the majority of the human cancer-derived cell lines (see *lanes 5–11*). Also note the additional band at ~43 kDa that appears to be unique to the rat osteosarcoma cell line (*lane 2*).

antibodies (control) or with anti-Cak1 antibodies (IgG fraction), the resulting immune complexes were collected and washed as described above. Each pellet was resuspended in 50 μl of kinase assay buffer and incubated with 100 μM [^{32}P]ATP (specific activity at 500-1000 cpm/pmol), 10 mM magnesium acetate, with and without recombinant renatured Cdk substrates, for 30 min at 30 °C with continuous agitation. The kinase reactions were terminated by the addition of 5 × SDS sample buffer; the proteins were denatured by heating at 95 °C for 5 min and then analyzed by SDS-PAGE, Western blotting, and autoradiography at −70 °C using Lightning Plus or Quanta III intensifying screens (Dupont).

Protein Fragmentation and Microsequence Analysis—Cak1-associated proteins were isolated using Anti-Cak1 antibodies and immunoaffinity chromatography. Hela S-3 cells (7 g wet weght) were lysed in 20 volumes of ice-cold IP buffer, cleared by centrifugation, and filtered through 0.45 μm prior to application to the immunoaffinity column. After extensive washing, the bound proteins were eluted with low pH buffer (100 mM glycine, pH 2.7). The eluate was pooled and concentrated in Amicon Centricon 10 microconcentrators, and samples were analyzed by SDS-PAGE and Western blotting prior to protein microsequencing. The concentrated eluates from the immunoaffinity column were loaded directly onto a 12% SDS-polyacrylamide gel for electrophoresis as described previously. The gel was stained with 0.05% Coomassie Brilliant Blue (Sigma), destained, and soaked in distilled water for 1 h. The protein band, migrating at approximately 35 kDa, was excised from the gel and subjected to in-gel digestion with Achromobacter endoprotease K for 24 h at 30 °C, as described previously (Kawasaki *et al.*, 1990). The digests were cleared by centrifugation followed by filtration through a 0.22-μm membrane (Ultrafree-MC, Millipore). The peptides were separated by reverse-phase HPLC (Hewlett Packard 1090) on a Vydac C18 column (2.1 × 250 mm, 5 μm, 300 A) attached to an anion-exchange precolumn (Brownlee GAX-013, 3.2 × 15 mm) and eluted with an acetonitrile gradient as their absorbances at 214, 280, 295, and 550 nm were monitored. Amino acid sequencing was performed on an automated microsequencer (ABI model 470) with an on-line HPLC (ABI model 120A) for the analysis of phenylthiohydantoin-tagged amino acids.

RESULTS

Characterization and Application of Specific Anti-Cak1 Antibodies—The high degree of homology conserved within the family of Cdc2-related enzymes (*i.e.* Cdks) necessitated the development of specific immunochemical reagents. Therefore, we developed antipeptide antibodies directed against a unique sequence in the C terminus of (Hs)Cak1 (see Fig. 1*A*). The resulting antipeptide antibodies readily recognized the recombinant protein on Western blots (Fig. 1*B*), exhibiting detection limits of ~0.1 ng under standard conditions (see "Experimental Procedures"). The capability of these anti-Cak1 antibodies to detect the $p42^{cak1}$ protein in Western blots of crude lysates of various rat and human cell lines was demonstrated in Fig. 1*C*. The levels of $p42^{cak1}$ protein in the various cell lines were estimated to be ~1 ng of protein kinase subunit/10 μg of cell lysate, which is remarkably in close agreement to the levels (1:10,000) predicted by protein purification studies (Williams *et al.*, 1994; Fisher and Morgan, 1994). Comparatively high levels of $p42^{cak1}$ were detected in rat and human osteosarcoma cells (Fig. 1*C*), both of which exhibited additional immunoreactive protein band(s) at ~44 and ~55 kDa, possibly representing alternatively spliced or closely related isoforms of the Cak1 kinase (see Shuttleworth *et al.* (1990)).

The anti-Cak1 antibodies efficiently precipitated the native protein, enabling further studies of Cak1-associated proteins. Immunoprecipitation experiments of [^{35}S]Met/Cys-labeled osteosarcoma cells revealed two prominent proteins, at 37 and 35 kDa, respectively, that associate with Cak1 in potentially stoichiometric amounts (Fig. 2*A*), and the former has been identified as cyclin H (Mäkelä *et al.*, 1994; Fisher and Morgan, 1994). A heavily labeled protein of 80–90 kDa, as well as several proteins in the 50–55-kDa range, were also identified as po-

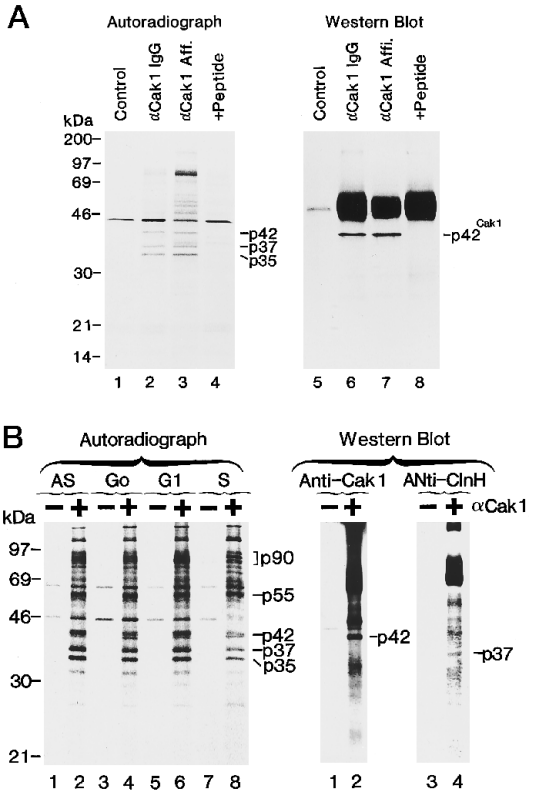

FIG. 2. **Identification of Cak1-associated proteins in human osteosarcoma cells.** *A*, asynchronous cultures of TE-85 osteosarcoma cells were metabolically labeled with [^{35}S]Met/Cys, and the washed cells were lysed and immunoprecipitated with anti-Cak1 antibodies (IgGs, *lanes 2* and *6*) or affinity-purified anti-Cak1 antibodies (*Affi*, *lanes 3* and *7*). The specificity of the immunoprecipitation was confirmed by minus antibody (*Control*, *lanes 1* and *5*) and by precipitation with peptide-blocked primary antibodies (+*Peptide*, *lanes 4* and *8*). The resulting immune complexes were analyzed by autoradiography (*left panel*). Of particular interest are the three prominent bands migrating at ~35, 37, and 42 kDa, respectively, as well as several proteins of higher molecular mass. Western analysis of the resulting immune complexes (*right panel*) identifies the 42-kDa protein as Cak1 and further indicates that p35 and p37 are Cak1-associated proteins. *B*, cell cycle dependence was examined in highly synchronized MG-63 cells (Carbonaro-Hall *et al.*, 1993), which were lysed and analyzed by immunoprecipitation with (+) and without (−) anti-Cak1 antibodies. Based on SDS-PAGE and autoradiography (*left panel*), Cak1 (p42) appears to be constitutive in the cell cycle, as are its associated proteins, p37 (*i.e.* cyclin H), p35, p55, and p90, although p90 undergoes appreciable shifts in electrophoretic mobility in the transition from G_1 to S phase. Western analysis (*right panel*) identifies the Cak1-associated p37 as cyclin H.

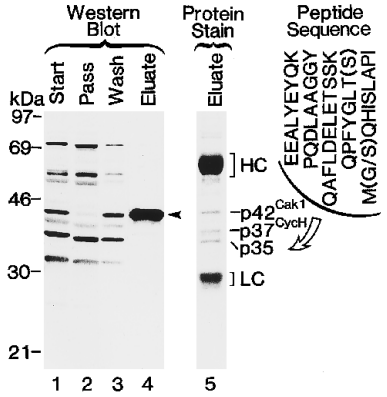

FIG. 3. **Immunoaffinity chromatography isolates Cak1 complexes from Hela cell lysates.** Approximately 7 g (wet weight) of frozen Hela cells were lysed, filtered, and applied to an anti-Cak1 affinity matrix, as described under "Experimental Procedures." The affinity-purified protein complexes were eluted with 100 mM glycine, pH 2.7 (*lane 4*), followed by Western analysis of the various fractions and eluates for p42/Cak1 immunoreacted with the anti-Cak1 antibodies. The efficiency of Cak1 in depleting p42/Cak1 from the crude lysates is shown (compare *lanes 1* and *2*). Protein staining of part of the eluate indicate the relative abundance of the three proteins bands of particular interest: p42, identified as Cak1; p37, identified as Cyclin H; and p35, which remains to be characterized. The band of protein at ~35 kDa was excised and subjected to limited proteolysis by endoprotease K, followed by HPLC separation of the digests and microsequencing of the resulting peptides, as described under "Experimental Procedures." Five peptide sequences were obtained, as shown. (*Parentheses* indicate uncertainty.)

tential Cak-associated proteins. The specificity of the immunoprecipitation assay was confirmed by the use of affinity-purified antibodies (Fig. 2*A*, *lane 3*) and peptide-blocked antibodies (Fig. 2A lane 4), as well as minus antibody controls. Western blotting of the immunoprecipitates confirmed that p42^{cak1} was the only immunoreactive protein recognized by these antibodies (Fig. 2*A*, *lanes 5–8*) further indicating that the profile of proteins represented are physically associated with Cak1 (see "Discussion"). Further studies of Cak1-associated proteins in highly synchronized MG-63 osteosarcoma cells (Fig. 2*B*) confirmed the constitutive expression of p42^{cak1} (Wu *et al.*, 1994) and demonstrated that both p37 (*i.e.* Cyclin H) and p35 remain constant in their association with Cak1 throughout the cell cycle. However, the relative abundance of the 90–95-kDa protein, as well as its electrophoretic migration pattern, appears to shift as cells proceed from G_1 to S phase.

Isolation and Characterization of a Cak1-associated Protein—The immunoprecipitation experiments were scaled up in the form of immunoaffinity column chromatography in which 7 g (~7 × 10^9 cells) of Hela cells were lysed and applied to an anti-Cak1 affinity matrix. The column effectively depleted a majority of the Cak1 and Cak1-associated proteins (see Fig. 3). After extensive washing and elution of bound proteins (see "Experimental Procedures"), the adsorbed immune complexes were analyzed by SDS-PAGE. As shown in Fig. 3, *lane 5*, the amounts of p37 and p35 appear similar. The p35 protein band was excised from the gel and subjected to endoproteinase K digestion prior to analysis by HPLC fractionation and microsequencing of the resultant peptide fragments. Five polypeptide sequences were obtained, as follows: peptide K17, -EEALYEYQK-; peptide K6, -PQDLAAGGYK-; peptide K23, -QAFLDELETSSK-; peptide K10, -M(G/S)QHISLAPI(H)-; and peptide K12, -QPFYGLT(S)-. The amino acids within parentheses represent an uncertain reading near the detection limits of the instrument. Three of the five polypeptides aligned well with sequences of identified cyclins, as shown in Fig. 4. These sequences lie within the designated cyclin box region (Hunt, 1991), with closest similarity to the yeast cyclin *Mcs2*, followed by human cyclin H and human cyclin C, respectively.

Immunoaffinity Purification and Elution of Enzymatically Active Cak1 under Nondenaturing Conditions—The anti-Cak1 antibodies, which selectively immunoprecipitate Cak1 and its associated proteins from cell lysates, were used to construct immunoaffinity chromatography matrices for the purification of active Cak1 complexes from Hela S-3 cells. After extensive washing of the affinity column, immunopurified Cak1 complexes were eluted from the column under a series of increasingly harsh elution conditions to ascertain the overall affinity of the anti-peptide antibodies and to obtain a purified fraction of enzymatically active Cak1 complexes under nondenaturing conditions. As shown in Fig. 5*A*, the immunoaffinity matrix depleted the majority of the Cak1 present in Hela cell lysates (*lanes 1* and *2*). A significant amount of bound Cak1 could be eluted from the affinity matrix with buffer containing 0.3 M NaCl and 30% ethylene glycol (*lane 4*), while the bulk of the remaining bound Cak1 could be eluted by 1 M $MgCl_2$ (*lane 5*), leaving only traces of the kinase (*lane 6*) on the column. The

Peptide K-17	E	E	A	L	_	Y	E	Y	Q	K			
MCS 2 (Sp)	E	E	V	L	E	Y	E	F	N	V			
Cyclin H (Hs)	E	Q	I	L	E	Y	E	L	L	L			
Cyclin C (Hs)	N	H	I	L	E	C	E	F	Y	L			
Peptide K-23	Q	A	F	L	_	D	E	L	E	T	S	S	K
MCS 2 (Sp)	Q	G	F	L	L	D	C	_	Q	T	V	L	P
Cyclin H (Hs)	E	G	F	L	I	D	L	K	T	R	Y	P	I
Cyclin C (Hs)	L	Q	Y	V	Q	D	M	G	Q	E	D	M	L
Peptide K-10	M	G(S)	Q	H	I	S	L	A	_	P	I	H	
MCS 2 (Sp)	L	S	S	A	L	S	L	_	_	P	T	H	
Cyclin H (Hs)	F	C	S	V	F	K	P	A	M	P	R	S	
Cyclin C (Hs)	L	G	E	H	L	K	L	_	_	R	Q	Q	

FIG. 4. **Amino acid sequence alignment of peptides derived from the Cak1-associated 35-kDa protein with identified cyclins.** The amino acids sequences of three of the five peptides obtained are aligned with three of their nearest cyclin relatives: the yeast *Mcs2* (Molz and Beach, 1993), the human cyclin H (Fisher and Morgan, 1994), and human cyclin C (Fisher and Morgan, 1994). The homology to *Mcs2* is greater than the homology to human cyclin H and human cyclin C, suggesting that p35 may be a human homologue of the yeast cyclin, *Mcs2*.

enzymatic activity of the immunoaffinity-purified Cak1 was confirmed by use of the Cdc2 Thr-161-immobilized peptide substrate assay (Williams *et al.*, 1994), in which the NaCl/ethylene glycol eluates exhibited a 2-fold higher kinase activity (per μl) than the $MgCl_2$ eluates, revealing an apparent specific activity approximately 10-fold higher (data not shown). In agreement with studies of purified preparations (Williams *et al.*, 1994), substitution of the histidine residue with an alanine in the test peptide reduced the peptide kinase activity by ~90% (data not shown), confirming that the adjacent histidine residue may be an important determinant of Cak1 substrate specificity.

The immunoaffinity-purified Cak1 preparations exhibited phosphorylation of the $p42^{cak1}$ monomer in *in vitro* kinase assays (Fig. 5*B*), resulting presumably from autophosphorylation, although heterologous phosphorylation by a tightly associated Cak1 kinase cannot be ruled out. Furthermore, the immunopurified Cak1 preparations were active in phosphorylating Cdc2 and Cdk2, but not Cdk2/160-Ala, even in the presence of recombinant cyclin A (Connell-Crowley *et al.*, 1993). Phosphorylation of both Cdc2 and Cdk2 was markedly increased by the addition of recombinant Cyclin A to the reaction mixtures. In agreement with recent reports using purified materials (Dorée and Galas, 1994), but in contrast to those using reconstituted Cak1·cyclin H complexes (Fisher and Morgan, 1994), these immunopurified Cak1 preparations phosphorylated both monomeric Cdc2 and Cdk2 but not Cdk4 (see Fig. 5*C*), suggesting that they may include regulatory and/or targeting subunits that are missing from the reconstituted Cak1·cyclin H complexes.

DISCUSSION

Subunit configuration and site-specific phosphorylation reactions regulate the enzymatic activities of the cyclin-dependent protein kinases $p34^{cdc2}$ and $p33^{cdk2}$. Our previous studies of Cdc2 phosphorylation led to the isolation (Williams *et al.*, 1993a, 1993b) and molecular cloning (Wu *et al.*, 1994) of the human Cdk-activating kinase, $p42^{cak1}$, a homologue of *Xenopus* MO15 (Shuttleworth *et al.*, 1990) that targets the phosphoregulatory sites within subdomain VIII of Cdc2 and Cdk2 (Solomon, 1993, 1994; Solomon *et al.*, 1993; Poon *et al.*, 1993). The striking

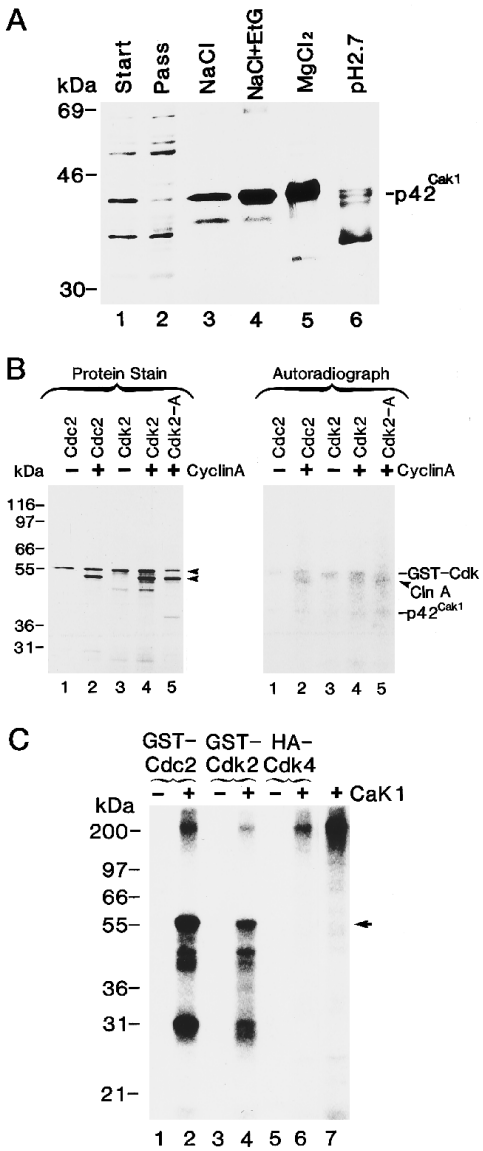

FIG. 5. **Immunoaffinity chromatography yields active Cak1 complexes from Hela cell lysates.** *A*, approximately 7 g (wet weight) of frozen Hela cells were lysed, filtered, and applied to an anti-Cak1 affinity matrix, as described under "Experimental Procedures"; the column was washed with 300 mM NaCl (*lane 3*), and the affinity-purified protein kinase complexes were eluted sequentially with 300 mM NaCl plus 30% ethylene glycol (*lane 4*), 1 M $MgCl_2$ (*lane 5*), and 100 mM glycine, pH 2.7 (*lane 6*), followed by Western analysis of the eluates for $p42^{cak1}$. The efficiency of Cak1 depletion from the crude lysates (compare *lanes 1* and *2*), taken together with the elution of the bulk of the bound enzyme under nondenaturing conditions, indicates that the anti-Cak1 antibodies were uniformly low to moderate in their affinity. *B*, immunoaffinity-purified Cak1 ($MgCl_2$ fractions) were utilized as a source of enzyme for *in vitro* kinase assays performed in the presence (+) or absence (−) of recombinant cyclin A with either glutathione *S*-transferase-Cdc2, glutathione *S*-transferase-Cdk2, or an Ala-160 mutant of glutathione *S*-transferase-Cdk2 as substrates for Cak1. The migration of the respective recombinant proteins upon SDS-PAGE was visualized by Coomassie Blue stain (*arrows*, *left panel*), while the kinase reaction was evaluated by autoradiography (*right panel*). Under these conditions, both Cdc2 and Cdk2 are more readily phosphorylated by Cak1 in the presence of cyclin A (compare *lanes 1* and *2* and *lanes 3* and *4*), while the Cdk2-A mutant was enzymatically active toward cyclin A (see *lane 5*) without Thr-160 phosphorylation (Connell-Crowley *et al.*, 1993). Note discernable autophosphorylation of immunopurified $p42^{cak1}$. *C*, comparative utilization of recombinant Cdc2, Cdk2, and Cdk4 as Cak1 substrates was examined by incubation of the respective recombinant Cdk substrates in the presence (+) or absence (−) of immunoaffinity-purified Cak1 followed by SDS-PAGE and autoradiography. Under these conditions, monomeric Cdc2 and Cdk2, but not Cdk4 was readily phosphorylated by Cak1 complexes.

homology of (Hs)Cak1 to (Hs)Cdc2 (Wu *et al.*, 1994), together with the identification of Cyclin H as a prospective regulatory partner (Fisher and Morgan, 1994; Mäkelä *et al.*, 1994), support the concept that Cdks participate in a vectorial kinase cascade.

Recent studies have indicated that Cak1·MO15, by association with the TFIIH transcription factor, may also function as the RNA polymerase II C-terminal domain kinase (Roy *et al.*, 1994; Feaver *et al.*, 1994), linking Cak1 activity to the control of transcription, as well as cell cycle progression. Taken together with the seminal observation that M015 mRNA is, by virtue of alternative start sites (Shuttleworth *et al.*, 1990), capable of yielding two different forms of the kinase, these findings raise the possibility that structurally and functionally distinct isoforms of Cak1 may participate in transcriptional regulation *vis à vis* cell cycle control pathways. Alternatively, the association of Cak1 with multiple regulatory subunits (*i.e.* cyclin-like proteins) may provide additional regulatory and or targeting features, as has been demonstrated for Cdk2 (see Peeper *et al.* (1993)).

Immunoaffinity Purification of Enzymatically Active Cak1—Anti-Cak1 antibodies afforded the precipitation of Cak1-associated proteins (see Fig. 2*A*), revealing proteins at 37 (cyclin H) and 35 kDa that appear to be stoichiometric, and a series of higher molecular mass proteins at ~80–90 and 50–55 kDa that may correspond to the high molecular mass complexes observed upon extensive purification of Cak1 (Wu *et al.*, 1994; Williams *et al.*, 1994). Indeed, the report that Cak1 associates with the TFIIH transcription-DNA repair complex and, by hyperphosphorylation, regulates the function of RNA polymerase II (Roy *et al.*, 1994; Feaver *et al.*, 1994) supports the concept that these coprecipitated proteins, in addition to Cdks, may be physiological substrates for Cak1 (Williams *et al.*, 1994).

The utility of the antipeptide antibodies in immunoprecipitation studies was further exploited in immunoaffinity chromatography. The ability of these antipeptide antibodies to release enzymatically active Cak1 complexes under nondenaturing conditions indicate that the bulk of the precipitable antibodies within the IgG fractions were low to moderate in affinity (Kellogg and Alberts, 1992). Immunoaffinity-purified Cak1 complexes were capable of phosphorylating monomeric Cdc2 and Cdk2, but not Cdk4 (see Fig. 5*C*), which is consistent with observations based on purified materials (Williams *et al.*, 1994; Dorée and Galas, 1994) but is strikingly different from the substrate specificity of reconstituted Cak1 (Fisher and Morgan, 1994), indicating additional regulatory features that remain to be identified.

Identification of a New Cak1 Subunit—Cak1 is associated with at least two auxiliary subunits, $p37^{Cyclin\ H}$ and a slightly lower molecular mass subunit previously designated p35 (Mäkelä *et al.*, 1994). Metabolic labeling of cellular proteins with [^{35}S]methionine followed by immunoprecipitation using specific anti-Cak1 antibodies revealed a number of Cak1-associated proteins: (i) a high molecular mass complex (~90 kDa), (ii) a series of multiple protein bands at ~55 kDa, and (iii) two Cak1-associated proteins at ~35 and 37 kDa, respectively. The 37-kDa protein has previously been identified as cyclin H (Mäkelä *et al.*, 1994; Fisher and Morgan, 1994). In this study, we further characterized the primary structure of the 35-kDa protein. Following SDS-PAGE, protein isolation, endoprotease K digestion, and HPLC fractionation of the resultant peptides, microsequence data was obtained. The results indicate that the 35-kDa protein represents a cyclin-like regulatory subunit that associates either directly or indirectly with Cak1. The novel regulatory subunit, represents a new member in a growing family of cyclin-like proteins that are related to cyclin C (see Fig. 4). Sequence alignment of three of the five peptides indicates appreciable homology to the yeast *Mcs2* protein (a yeast cyclin) as well as to human cyclin H and cyclin C, which suggests that p35 may be a human homologue of the *Mcs2* gene of *S. pombe* (Molz and Beach, 1993). The finding that Cak1 may form complexes with multiple regulatory partners (*i.e.* cyclin-related subunits) is reminiscent of other cyclin-dependent kinases, such as Cdc2 and Cdk2, that associate with different partners in the performance of specific functions.

These studies, taken together with other recent findings, support the concept that the Cdk-activating kinase system is highly regulated and multifunctional. The suggestion that Cak1 may be a primary RNA polymerase II C-terminal domain Kinase (Feaver *et al.*, 1994; Roy *et al.*, 1994) points to a major role in transcriptional regulation. CAK activity is associated with the transcription initiation factor TFIIH, which also functions in nucleotide excision repair (Hanawalt, 1994). Thus, in addition to its classic role as the upstream activator for Cdks, and hence crucial to cell cycle progression, Cak1 appears to have a function in DNA transcription, fidelity, and repair mechanisms. Thus, the demonstration that the Cak1 catalytic subunit forms stable complexes with multiple cyclin-like regulatory partners may provide a mechanistic basis for substrate targeting and/or specificity (see Peeper *et al.*, 1993) that would be predicted for a multifunctional enzyme system.

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