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KAP: A dual specificity phosphatase that interacts with cyclin-dependent kinases

(cell cycle/CDK2/cdc2)

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ABSTRACT **The cyclin-dependent kinases are key cell cycle regulators whose activation is required for passage from one cell cycle phase to the next. In mammalian cells, CDK2 has been implicated in control of the G_1 and S phases. We have used a two-hybrid protein interaction screen to identify cDNAs encoding proteins that can interact with CDK2. Among those identified was a protein (KAP), which contained the HCXX-XXGR motif characteristic of protein tyrosine phosphatases. KAP showed phosphatase activity toward substrates containing either phosphotyrosine or phosphoserine residues. Since KAP is not significantly similar to known phosphatases beyond the catalytic core motif, it represents an additional class of dual specificity phosphatase. KAP interacted with cdc2 and CDK2 in yeast. In mammalian cells, KAP also associated with cdc2 and CDK2 but showed a preference for cdc2. The ability of KAP to bind multiple cyclin-dependent kinases suggests that it may play a role in cell cycle regulation.**

In fission and budding yeasts, control of both the G_1/S and G_2/M transitions is accomplished through the activity of a single cyclin-dependent kinase known as cdc2 in *Schizosaccharomyces pombe* or CDC28 in *Saccharomyces cerevisiae* (reviewed in ref. 1). In mammalian cells, the cyclin-dependent kinase family consists of at least five members: cdc2 (CDK1) and CDK2–CDK5 (refs. 2–4; reviewed in ref. 1). These associate with an equally diverse family of positive regulatory subunits known as cyclins, and each cyclin–CDK complex may play a distinct role in cell cycle regulation (see ref. 5 for a recent review).

Several lines of evidence suggest that CDK2 participates in control of the G_1 and S phases. First, microinjection of CDK2 antibodies into mammalian cells caused cell cycle arrest prior to S phase (6, 7). Second, CDK2 complexes with cyclins that act early in the cell cycle—namely, cyclins A, D, and E (2, 4, 8). Of these, cyclins D and E have demonstrated roles in the control of G_1 (9, 10). Cyclin A executes at least one of its functions during S phase as indicated both by its periodic expression and by the results of antibody microinjection experiments (11, 12). Numerous studies have suggested that passage through G_2 and M is regulated by a different cyclin-dependent kinase, cdc2, and its associated cyclins A and B (reviewed in refs. 13–15).

Coimmunoprecipitation experiments have revealed that cyclin-dependent kinases bind a number of proteins in addition to cyclins. For example, cdc2/cyclin B forms a stable complex with cdc25, a dual-specificity phosphatase that regulates cdc2 activity (16). Also, proliferating cell nuclear antigen and a protein known as p21 are universal components of cell cycle kinase complexes (4, 17). On the premise that proteins that associate with cyclin-dependent kinases are likely to function in cell cycle regulation, we have used the two-hybrid screen (18) to isolate cDNAs encoding proteins that can interact with CDK2. Among the proteins identified by this procedure was a previously uncharacterized dual-specificity protein phosphatase, which we have termed KAP. KAP binds to cdc2 and CDK2 in mammalian cells, suggesting that it may play a role in cell cycle control.§

MATERIALS AND METHODS

Construction of cDNA Libraries and Two-Hybrid Screens. Construction of the HeLa cell cDNA library and two-hybrid screening were exactly as described (19).

Primary positives were tested for target specificity by retransformation of library plasmids into the tester strain in conjunction with a number of different GAL4 DNA binding domain–target fusions. These included fusions with cdc2, CDK2, CDK4, CDK5, SNF1, and pim1. The SNF1 fusion was a gift from Stan Fields, State University of New York, Stony Brook; the CDK4 plasmid was provided by Yue Xiong, Cold Spring Harbor Laboratory; the CDK5 fusion was provided by Hui Zhang, Cold Spring Harbor Laboratory; and the pim1 fusion was a gift from Harriet Feilotter, Cold Spring Harbor Laboratory.

Phosphatase Assays. The entire coding sequence of the KAP cDNA was inserted into the bacterial expression vector pGEX-KG to form a plasmid that directed the expression of a fusion between glutathione *S*-transferase (GST) and KAP (pGST-KAPw). We also constructed a similar plasmid encoding a fusion between GST and a mutant KAP protein in which Cys-139 had been changed to Ser (pGST-KAPm). These plasmids were transformed into *Escherichia coli* BL21 for fusion protein expression. Cells containing each plasmid were grown at 37°C to a density of OD_{600} = 0.5; the cultures were then shifted to 23°C–25°C, and isopropyl β-D-thiogalactopyranoside was added to 0.4 mM. After 12–14 hr, cells were harvested, washed once in phosphate-buffered saline, and resuspended in GCB [50 mM Tris·HCl, pH 8.0/200 mM NaCl/1 mM EDTA, 1% Triton X-100/1 mM dithiothreitol (DTT)/1× protease inhibitors (2 μg of leupeptin per ml/2 μg of aprotinin per ml/0.3 μg of benzamide per ml/10 μg of soybean trypsin inhibitor per ml/100 μg of L-1-tosylamido-2-phenylethyl chloromethyl ketone per ml/50 μg of 7-amino-1-chloro-3-tosylamido-2-heptanone per ml)]. Cells were lysed by sonication and fusion proteins were prepared as described (19).

Preparation of phosphatase substrates and phosphatase assays were essentially as described (20). For phosphotyrosine dephosphorylation, a standard assay included 3–6 μg

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Abbreviations: GST, glutathione *S*-transferase; RCML, reduced carboxyamidomethylated and maleylated lysozyme; HA, hemagglutinin.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. L27711).

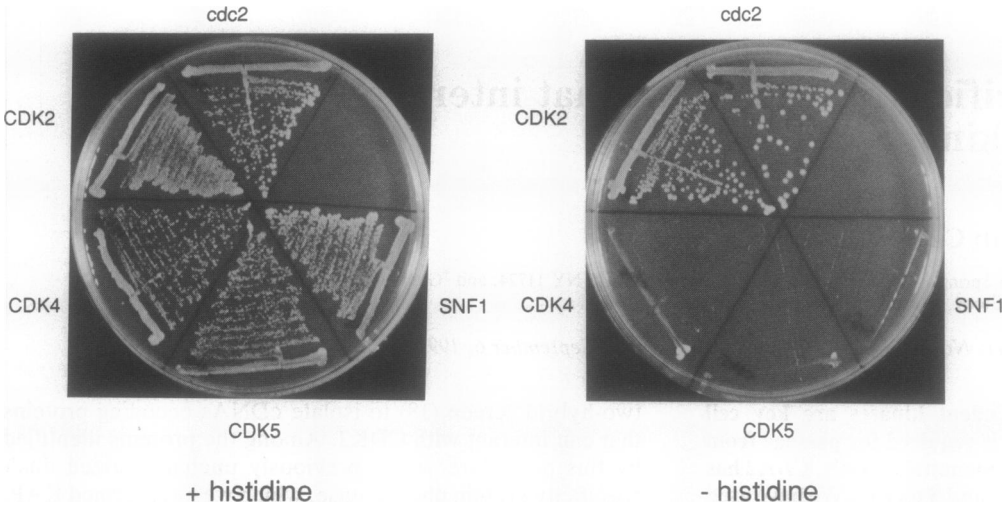

FIG. 1. Interaction of KAP with cdc2 and CDK2 in yeast. A yeast strain containing a GAL4-dependent *HIS3* gene was transformed with a plasmid encoding a fusion between the GAL4 activation domain and KAP. This strain was simultaneously transformed with plasmids encoding fusions between the GAL4 DNA binding domain and cdc2, CDK2, CDK4, CDK5, or SNF1 (as indicated). Transformants were restreaked on plates lacking leucine and tryptophan (+ histidine) or lacking leucine, tryptophan, and histidine (− histidine).

of GST–KAPw or GST–KAPm in 60 μl containing 2 μM [^{32}P]tyrosine reduced carboxyamidomethylated and maleylated lysozyme (RCML), 0.1 mg of bovine serum albumin per ml, 2 mM DTT, and 50 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 6.5). Phosphoserine dephosphorylation reactions using 2 μM [^{32}P]serine RCML required a 10-fold higher concentration of GST–KAPw or GST–KAPm and were carried out at pH 6.0. Reactions were started by the addition of enzyme and terminated after 10 min (unless otherwise indicated) by the addition of 290 μl of stop solution [0.9 M HCl/90 mM $Na_4P_2O_7$/2 mM NaH_2PO_4/1–2% (vol/vol) Celite/6% (vol/vol) Norit-A charcoal]. The phosphorylated substrate bound to the charcoal and was removed by centrifugation. Released phosphate was measured by scintillation counting of the supernatant. In all cases, a similarly treated reaction mixture lacking enzyme served as a blank. For testing the effects of inhibitors, reactions proceeded for 10 min in the presence of 1 mM Na_3VO_4/1 μM okadaic acid/1 mM tetramizole/5 mM sodium tartrate/1 mM EDTA/1 mM EGTA, or 1 mM NaF. *N*-Ethylmaleimide (10 mM) and iodoacetic acid (10 mM) were preincubated with the enzyme for 5 min at 30°C.

Transfection, Immunoprecipitation, and Western Blotting. For use in transfection experiments, we constructed a KAP cDNA in which three copies of the hemagglutinin epitope tag (HA; ref. 21) were inserted at amino acid 3 of the KAP sequence. This modified KAP cDNA was inserted downstream of the cytomegalovirus promoter in the expression vector pCMV5 (22) to form the plasmid pCMV-HA-KAP. COS-1 cells were grown to confluence and then transfected with this construct (3 μg of DNA per 6-cm plate) using lipofectamine (BRL) according to the manufacturer's instructions. At 48 hr posttransfection, cells were metabolically labeled with [^{35}S]methionine. Proteins were immunoprecipitated from cell lysates with 1 μl of anti-HA (12CA5, Babco, Emeryville, CA), 1 μl of anti-cdc2 (G6; ref. 23), or 1 μl of anti-CDK2 (K. Galaktionov, personal communication) antibody as described (4). Proteins were released from the Sepharose by boiling in SDS sample buffer and were electrophoresed on a 12.5% polyacrylamide gel. Western blotting and partial proteolytic mapping were performed exactly as described (4).

RESULTS AND DISCUSSION

Isolation of CDK2 Binding Proteins. To search for previously uncharacterized proteins with a role in cell cycle regulation, we have used a two-hybrid screen (18, 24) to isolate cDNAs encoding proteins that can physically interact with CDK2 (19). Using this procedure, we isolated three classes of cDNAs. The first encoded a previously characterized protein known as CKS1 (25). CKS1 is a human homolog of fission yeast p13^{Suc1}, a protein that binds to fission yeast cdc2. The second corresponded to the adenovirus E1A-associated p130, a newly discovered member of the *RB* gene

A

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1   GCACGAGCTGCAGAGGGAGGCGGCACTGGTCTCGACGTGGGGCGGCCAGCGATGAAGCCG
1                                                        MetLysPro

61  CCCAGTTCAATACAAACAAGTGAGTTTGACTCATCAGATGAAGAGCCTATTGAAGATGAA
4   ProSerSerIleGlnThrSerGluPheAspSerSerAspGluGluProIleGluAspGlu

121 CAGACTCCAATTCATATATCATGGCTATCTTTGTCACGAGTGAATTGTTCTCAGTTTCTC
24  GlnThrProIleHisIleSerTrpLeuSerLeuSerArgValAsnCysSerGlnPheLeu

181 GGTTTATGTGCTCTTCCAGGTTGTAAATTTAAAGATGTTAGAAGAAATGTCCAAAAAGAT
44  GlyLeuCysAlaLeuProGlyCysLysPheLysAspValArgArgAsnValGlnLysAsp

241 ACAGAAGAACTAAAGAGCTGTGGTATACAAGACATATTTGTTTTCTGCACCAGAGGGGAA
64  ThrGluGluLeuLysSerCysGlyIleGlnAspIlePheValPheCysThrArgGlyGlu

301 CTGTCAAAATATAGAGTCCCAAACCTTCTGGATCTCTACCAGCAATGTGGAATTATCACC
84  LeuSerLysTyrArgValProAsnLeuLeuAspLeuTyrGlnGlnCysGlyIleIleThr

361 CATCATCATCCAATCGCAGATGGAGGGACTCCTGACATAGCCAGCTGCTGTGAAATAATG
104 HisHisHisProIleAlaAspGlyGlyThrProAspIleAlaSerCysCysGluIleMet

421 GAAGAGCTTACAACCTGCCTTAAAAATTACCGAAAAACCTTAATACACTGCTATGGAGGA
124 GluGluLeuThrThrCysLeuLysAsnTyrArgLysThrLeuIleHisCysTyrGlyGly

481 CTTGGGAGATCTTGTCTTGTAGCTGCTTGTCTCCTACTATACCTGTCTGACACAATATCA
144 LeuGlyArgSerCysLeuValAlaAlaCysLeuLeuLeuTyrLeuSerAspThrIleSer

541 CCAGAGCAAGCCATAGACAGCCTGCGAGACCTAAGAGGATCCGGGGCAATACAGACCATC
164 ProGluGlnAlaIleAspSerLeuArgAspLeuArgGlySerGlyAlaIleGlnThrIle

601 AAGCAATACAATTATCTTCATGAGTTTCGGGACAAATTAGCTGCACATCTATCATCAAGA
184 LysGlnTyrAsnTyrLeuHisGluPheArgAspLysLeuAlaAlaHisLeuSerSerArg

661 GATTCACAATCAAGATCTGTATCAAGATAAAGGAATTCAAATAGCATATATATGACCATG
204 AspSerGlnSerArgSerValSerArgEnd

721 TCTGAAATGTCAGTTCTCTAGCATAATTTGTATTGAAATGAAACCACCAGTGTTATCAAC

781 TTGAATGTAAATGTACATGTGCAGATATTCCTAAAGTTTTATTGACAAAAAAAAAAAAAA
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B

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KAP      Y R K T L I HC Y G G L G RS C L V A A C L L L Y L S D
VH1      N E P V L V HC A A G V N RS G A M I L A Y L M S K N K
3CH134   G G R V F V HC Q A G I S RS A T I C L A Y L M R T N R
VHR      N G R V L V HC R E G Y S RS P T L V I A Y L M M R Q K
BVP      G M L V G V HC T H G I N RT G Y M V C A Y L M H T L G
YVH1     R G A V F A HC Q A G L S RS V T F I V A Y L M Y R Y G
IphP     D G A V L F HC T A G K D RT G I I A G L L L D L A G V
HCDC25A  R V I V V F HC E F S S E RG P R M C R Y V R E R D R L
PTP-1B   H G P V V V HC S A G I G RS G T F C L A D T C L L L M
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FIG. 2. Sequence of KAP. (*A*) Complete sequence of KAP cDNA and deduced amino acid sequence of KAP protein are shown. The HCXXXXGR motif characteristic of protein tyrosine phosphatases is underlined. (*B*) Region surrounding the HCXXXXGR motif in KAP is aligned with the analogous region from a number of other phosphatases. These are VH1 (vaccinia virus), 3CH134(MKP) (human), VHR (human), BVP (baculovirus *Autographa californica*), YVH1 (*S. cerevisiae*), IphP (cyanobacterium *Nostoc commune* UTEX 584), HCDC25A (human), and PTP-1B (human) (16, 20, 26–32). Amino acids conserved among all of these proteins are boxed.

family (19). The third class, described here, encoded a dual specificity protein phosphatase, which we have designated KAP (CDK associated phosphatase; see below). This protein was capable of interacting in yeast either with CDK2 or with human cdc2 but not with CDK4, CDK5, *S. cerevisiae* SNF1 kinase, or *Sc. pombe* pim1 (Fig. 1; data not shown).

The complete sequence of the KAP cDNA and the deduced amino acid sequence of the KAP protein are shown in Fig. 2*A*. Two lines of evidence suggest that we have identified the complete KAP open reading frame. First, Northern blot analysis indicated that the KAP mRNA was approximately equal in size to the KAP cDNA that we had isolated (unpublished results). Second, we have raised a polyclonal antiserum specific for KAP, and this serum recognized in cell lysates a protein that roughly comigrated with KAP protein produced by *in vitro* translation of our cDNA (unpublished results).

Searches of available data bases failed to identify significant similarity between KAP and any previously characterized protein. However, the KAP sequence was found to contain a motif, HCXXXXGR, characteristic of the catalytic core of protein tyrosine phosphatases (see below; reviewed in refs. 33 and 34; see Fig. 2).

KAP Is a Dual Specificity Phosphatase. To determine whether KAP was, in fact, a protein tyrosine phosphatase, the entire KAP protein was produced in bacteria as a fusion with GST and tested for activity against a model tyrosine phosphatase substrate, tyrosine phosphorylated RCML. Upon treatment of [^{32}P]tyrosine RCML with KAP, a substantial release of phosphate was detected (Fig. 3*A*). KAP tyrosine phosphatase activity was dependent on both enzyme concentration and time for the first 10 min of the reaction. Tyrosine dephosphorylation showed a sharp pH optimum around pH 6.5; virtually no activity was seen above pH 7.0 or below pH 6.0 (unpublished results). Phosphatase activity was sensitive to orthovanadate, a known inhibitor of protein tyrosine phosphatases, but it was not affected by inhibitors of acid phosphatase (tartrate), phosphatase 1A (okadaic acid), alkaline phosphatase (tetramizole), or the general serine/threonine phosphatase inhibitor fluoride (Fig. 3*B*). The HCXXXXGR motif forms the catalytic core of protein tyrosine phosphatases, and the cysteine present in this motif is directly involved in the dephosphorylation reaction, forming a thiolphosphate intermediate (see refs. 33 and 34). Thus, modification or mutation of this cysteine abolishes the activity of known tyrosine phosphatases. Consistent with the

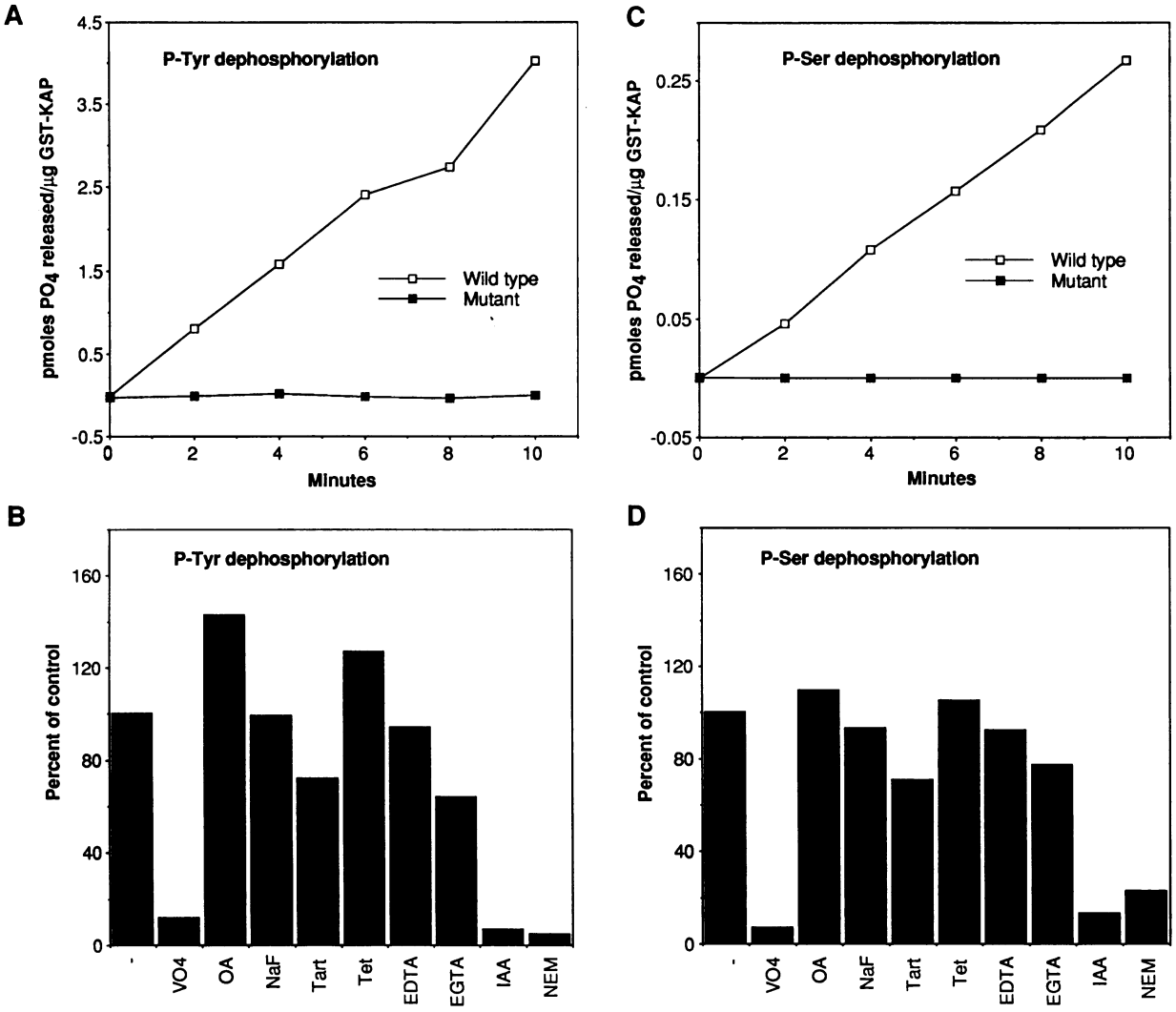

FIG. 3. KAP is a dual-specificity protein phosphatase. (*A*) Bacterially produced GST–KAPw or a mutant GST–KAPm (C139S) was used to dephosphorylate a model tyrosine phosphatase substrate RCML phosphorylated on tyrosine (20). Time course of the reaction is shown. (*B*) Several known phosphatase inhibitors (VO4, Na_3VO_4; OA, okadaic acid; Tart, tartrate; Tet, tetramizole; NEM, *N*-ethylmaleimide; IAA, iodoacetic acid) were tested for their effect on the ability of GST–KAP to dephosphorylate tyrosine phosphorylated RCML. Activity in the presence of inhibitors is expressed as percentage of control. (*C* and *D*) Same as *A* and *B* except that a model serine phosphatase substrate, serine phosphorylated RCML, was used. Preparation of phosphatase substrates was essentially as described (20).

notion that KAP acts through a similar catalytic mechanism, treatment of KAP with sulfhydryl modifying reagents, *N*-ethylmaleimide or iodoacetic acid, abolished KAP activity (Fig. 3*B*). Furthermore, mutation of the putative catalytic cysteine to serine (C139S) resulted in an inactive enzyme (Fig. 3*A*).

Several members of the tyrosine phosphatase family can dephosphorylate not only phosphotyrosyl but also phosphoseryl and phosphothreonyl residues (26, 27, 35–37). To determine whether KAP was a member of this group of dual-specificity phosphatases, we tested its ability to dephosphorylate phosphoseryl RCML. As shown in Fig. 3*C*, GST–KAP showed significant activity toward this substrate. Approximately 10-fold more KAP enzyme was required to achieve phosphate release from phosphoseryl RCML, which equaled that seen with the phosphotyrosyl substrate. Such differences in activity toward model substrates have previously been observed for other dual specificity phosphatases (see ref. 36, for example). These probably do not reflect differences in the intrinsic ability of the enzymes to dephosphorylate phosphoserine versus phosphotyrosine residues but instead likely reflect the ability of the enzyme to recognize phosphorylated residues in different contexts within artificial substrates. The serine phosphatase activity of KAP showed a spectrum of sensitivity to inhibitors identical to that observed for its tyrosine phosphatase activity (Fig. 3*D*). Also, mutation of Cys-139 abolished activity, suggesting that dephosphorylation of phosphotyrosine and phosphoserine proceeds through a similar mechanism (Fig. 3*C*).

Based on these results, we conclude that KAP is a dual-specificity phosphatase. A detailed comparison of the sequence of KAP to known phosphatases revealed no significant homology beyond the HCXXXXGR motif (Fig. 2*B*). Thus, KAP represents an additional class of dual-specificity phosphatase within the tyrosine phosphatase family.

KAP Associates with cdc2 and CDK2 in Mammalian Cells. KAP interacted both with CDK2 and with cdc2 in yeast (Fig. 1). To ask whether KAP also associated with these proteins in mammalian cells, we expressed in COS cells a KAP protein bearing three copies of the HA epitope tag at its N terminus (HA–KAP). In anti-HA immunoprecipitates from metabolically labeled, transfected cells, we observed a doublet corresponding to the epitope-tagged KAP protein and a prominent band of ≈34 kDa, which roughly comigrated with cdc2 and CDK2 immunoprecipitated from lysates of the same transfected cells (Fig. 4*A*). Similar bands were not observed in anti-HA immunoprecipitates from untransfected cells or from cells transfected with a control plasmid (unpublished results). The interaction of KAP with cyclin-dependent kinases in the two-hybrid screen suggested the possibility that

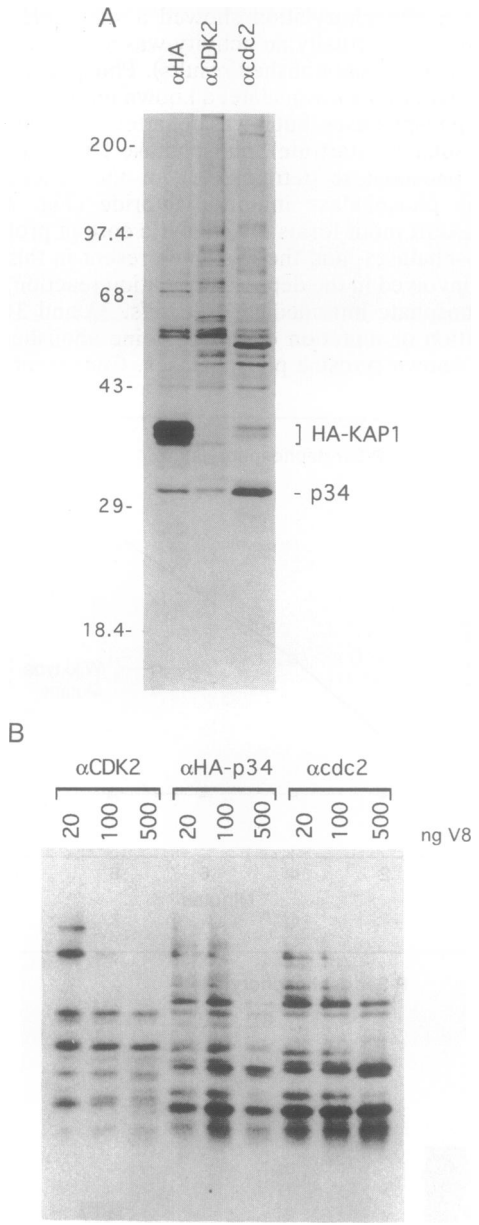

FIG. 4. Interaction of KAP with cdc2 in mammalian cells. (*A*) COS cells were transfected with a plasmid directing the expression of a KAP protein bearing three copies of the HA epitope tag at its N terminus (HA–KAP). At 48 hr posttransfection, cells were metabolically labeled with [^{35}S]methionine. Proteins were immunoprecipitated from cell extracts by using antibodies directed against the HA epitope (αHA), human cdc2 (αcdc2), or human CDK2 (αCDK2), and precipitated proteins were analyzed by electrophoresis. For reference, positions of protein markers (kDa) electrophoresed in parallel are shown. Positions of the epitope-tagged KAP protein (HA–KAP), cdc2, and CDK2 are indicated. (*B*) CDK2, cdc2, and KAP-associated p34 were electrophoretically purified from immunoprecipitates similar to those shown in *A*. These polypeptides were then subjected to partial V8 protease digestion with the indicated amounts of enzyme. Resulting peptide fragments were fractionated on a 17.5% polyacrylamide gel.

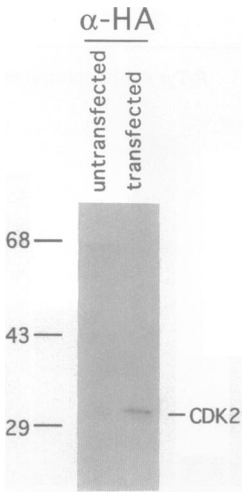

FIG. 5. KAP associates with CDK2 in mammalian cells. Lysates were prepared from COS cells transfected with a plasmid directing the expression of HA–KAP and from an untransfected control. Proteins present in anti-HA (α-HA) immunoprecipitates from each of these cell extracts (as indicated) were electrophoresed on a 12.5% polyacrylamide gel, transferred to nitrocellulose, and analyzed by Western blotting with a monospecific CDK2 antiserum. Positions of CDK2 and of protein size markers (kDa) (electrophoresed in parallel) are indicated.

one or more of these proteins might be the KAP-associated p34. Consistent with this possibility, in anti-cdc2 immunoprecipitates from transfected cells, we observed a doublet of bands that comigrated with the two HA–KAP species (Fig. 4*A*). In addition, we detected HA–KAP by Western blotting in similar cdc2 immunoprecipitates (unpublished results). To definitively determine the nature of the KAP-associated p34, the protein was excised from a polyacrylamide gel and subjected to partial digestion with V8 protease. For comparison, immunoprecipitated cdc2 and CDK2 were analyzed in parallel. As shown in Fig. 4*B*, the KAP-associated p34 showed a pattern of V8 peptides that corresponded to those generated upon V8 digestion of cdc2. The slight differences between the V8 patterns of the KAP-associated p34 and cdc2 could result from differences in the cdc2 phosphorylation state or could indicate that the KAP-associated p34 contains a mixture of cdc2 and CDK2. The latter possibility is suggested by the fact that when similar immunoprecipitates were examined by Western blotting, both cdc2 and CDK2 antisera recognized the ≈34-kDa protein (Fig. 5; data not shown). Considered together, these results indicate that KAP can associate with cdc2 and CDK2 in mammalian cells. Interestingly, cyclins known to associate with cdc2 and CDK2 were not observed in KAP–CDK complexes, suggesting that KAP does not require the presence of a cyclin subunit for CDK binding.

Protein phosphorylation is a central mechanism by which the cell cycle is regulated. Phosphorylation of key substrates by activated cyclin-dependent kinases is thought to promote passage through cell cycle transitions (reviewed in refs. 1 and 5). These kinases are themselves regulated by phosphorylation at several sites. For example, cdc2 is subject to inhibitory phosphorylation of Thr-14 and Tyr-15 and requires an activating phosphorylation at Thr-161 (ref. 38; reviewed in ref. 1). The conservation of these residues in other members of the CDK family suggests that they may be similarly regulated. Several cyclins are also phosphorylated *in vivo*, but the significance of these modifications is unknown (39). It is likely that the net level of phosphorylation of each of these proteins is dictated by the balanced activities of protein kinases and protein phosphatases. This has been shown to be the case for Thr-14 and Tyr-15 of cdc2 where the overall level of phosphorylation is controlled by the opposing activities of the wee1 and mik1 kinases and the dual-specificity phosphatase cdc25 (40). The association of KAP with cdc2 and CDK2 suggests that KAP may play a role in cell cycle control, probably by regulating the phosphorylation state of a CDK or CDK-associated protein.

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