

Report

The Cyclin-Dependent Kinase (CDK) Family Member PNQALRE/CCRK Supports Cell Proliferation but has no Intrinsic CDK-Activating Kinase (CAK) activity

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cell cycle, cyclin-dependent kinase (CDK), CDK-activating kinase (CAK), Cdk7, PNQALRE/CCRK, apoptosis, RNAi, chemical genetics, analog-sensitive kinase

ABBREVIATIONS

CDK	cyclin-dependent kinase
CAK	CDK-activating kinase
CCRK	cell cycle related kinase
RNAi	RNA interference
siRNA	small interfering RNA
PARP	poly(ADP-ribose) polymerase
T-loop	activation segment

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ABSTRACT

The cyclin-dependent kinases (CDKs) that drive the eukaryotic cell cycle must be phosphorylated within the activation segment (T-loop) by a CDK-activating kinase (CAK) to achieve full activity. Although a requirement for CDK-activating phosphorylation is conserved throughout eukaryotic evolution, CAK itself has diverged between metazoans and budding yeast, and fission yeast has two CAKs, raising the possibility that additional mammalian enzymes remain to be identified. We report here the characterization of PNQALRE (also known as CCRK or p42), a member of the mammalian CDK family most similar to the cell-cycle effectors Cdk1 and Cdk2 and to the CAK, Cdk7. Although PNQALRE/CCRK was recently proposed to activate Cdk2, we show that the monomeric protein has no intrinsic CAK activity. Depletion of PNQALRE by >80% due to RNA interference (RNAi) impairs cell proliferation, but fails to arrest the cell cycle at a discrete point. Instead, both the fraction of cells with a sub-G₁ DNA content and cleavage of poly(ADP-ribose) polymerase (PARP) increase. PNQALRE knockdown did not diminish Cdk2 T-loop phosphorylation in vivo or decrease CAK activity of a cell extract. In contrast, depletion of Cdk7 by RNAi causes a proportional decrease in the ability of an extract to activate recombinant Cdk2. Our data do not support the proposed function of PNQALRE/CCRK in activating CDKs, but instead reinforce the notion of Cdk7 as the major, and to date the only, CAK in mammalian cells.

INTRODUCTION

The cyclin-dependent kinases (CDKs) first emerged as the enzymes controlling the entry to both the DNA synthesis (S) phase and mitosis in the eukaryotic cell division cycle, but they also function in pathways that regulate gene expression. To be fully activated, a CDK must be cyclin-bound, and in most cases phosphorylated by a CDK-activating kinase (CAK) within the activation segment or T-loop (reviewed in Ref. 1). In metazoans, the major CAK is itself a CDK, consisting of the catalytic subunit Cdk7, cyclin H and a RING finger protein, Mat1 (reviewed in ref. 2). Cdk7 has also been implicated in the control of transcription by RNA polymerase (Pol) II as a component of the general transcription factor IIH (TFIIH).³⁻⁵

The requirement for Cdk7 as a CAK has been established by genetic experiments in *Drosophila melanogaster*⁶ and *Caenorhabditis elegans*,⁷ in which *cdk7* mutants are unable to activate Cdk1 and, consequently, cannot enter mitosis. In budding and fission yeast, CAK function is also required for entry into S phase,^{8,9} but in metazoans, dependence of the G₁/S transition on activating phosphorylation of a CDK has not been demonstrated. The existing mutant alleles of *Drosophila cdk7* do not detectably impair activation of Cdk2, the major CDK activated at the onset of S phase.⁶

In contrast to metazoans, the budding yeast *Saccharomyces cerevisiae* contains a single-subunit CAK only distantly related to CDKs, Cak1, which is responsible for activating Cdk1, the principal cell-cycle CDK.¹⁰⁻¹² The fission yeast *Schizosaccharomyces pombe* contains an ortholog of Cak1, Csk1, which activates both Cdk1^{9,13} and the Cdk7 ortholog Mcs6, which is also a CAK.^{13,14} Unlike *CAK1* in budding yeast, *csk1+* is not essential,¹⁵ possibly because of the redundancy of its CAK function with that of *mcs6+*.⁹ Nevertheless, the phenotypes of *csk1Δ* strains indicate that Csk1 has important, if not strictly essential functions in vivo.^{9,16-18}

The absence of a Cak1/Csk1 ortholog in metazoans is surprising, given the relative sizes and complexities of fungal and higher eukaryotic genomes.¹⁹ There is, however, no apparent

need for a CAK other than Cdk7, which is active throughout the cell cycle and capable of phosphorylating all mammalian CDKs known to depend on T-loop modification for full activity (reviewed in ref. 20). Consistent with Cdk7 being the sole CAK in metazoan cells, its quantitative immunodepletion from cell-free extracts removes all detectable CAK activity.^{6,21-23} On the other hand, minor CAK activities, not obviously associated with Cdk7, have been detected upon fractionation of mammalian cell extracts but not identified at the molecular level.^{24,25}

The larger question of whether CAK-mediated T-loop phosphorylation regulates cell cycle progression is unresolved. Several treatments that arrest the mammalian cell cycle in G₁—such as cyclic AMP (cAMP), TGF- β , MAP kinase inhibitors and mevastatin—cause hypophosphorylation of the Cdk2 T-loop.^{24,26-28} This does not mean, however, that these treatments lead directly to down-regulation of CAK. For instance, cAMP-dependent signaling causes increased Cdk2-binding by the CDK inhibitor (CKI) p27^{Kip1}, which passively prevents T-loop phosphorylation,²⁶ presumably through a steric effect common to members of the CKI family.²⁹ Nevertheless, the formal possibility of a regulated (and possibly Cdk2-specific) CAK distinct from Cdk7 remains, and is often invoked when decreased T-loop phosphorylation of a CDK occurs in conjunction with unchanged Cdk7 protein and activity levels.

How a quantitatively minor CAK could regulate CDK activation in vivo when the more abundant Cdk7 remains active is unclear. One possibility is that the two enzymes might work on different populations of CDKs. However, a simple division of labor based on the different CDK catalytic subunits in metazoans cannot be the explanation: Cdk7 phosphorylates purified Cdks 1, 2, 4, 5 and 6;^{21,30-32} and an analog-sensitive (AS) Cdk7 phosphorylates Cdks 1, 2, 4, 5 and 11 in crude mammalian cell extracts.²³ Specialization could be based instead on the substrate preferences of the Cdk7 and Cak1 families for CDK/cyclin complexes and monomeric CDKs, respectively.^{33,34} A putative Cak1-like enzyme was partially purified from human cell extracts with a monomeric Cdk2 phosphorylation assay.³³ *S. pombe* cells possess both a CAK of the Cdk7 class and one that prefers monomers,³⁴ but it is unclear if this feature of the CAK-CDK network is important physiologically.

Recently, a CDK-like protein related distantly to Csk1 (but more closely to Cdks 1, 2 and 7) was reported to be a monomeric CAK required to activate Cdk2 in vivo.³⁵ We can indeed detect weak CAK activity in complexes containing this protein—PNQALRE, also known as p42 or cell cycle-related kinase (CCRK)—isolated from mouse testes extracts, but this could be explained by an associated, rather than an intrinsic activity. PNQALRE binds to Cdk7 both in testes extracts and when the two proteins are overexpressed together in insect cells.

We test the hypothesis that PNQALRE/CCRK is a novel mammalian CAK, by: (1) directly comparing CAK activity of purified, monomeric PNQALRE and Cdk7 complexes; (2) depletion of PNQALRE from human cells in culture by RNA interference (RNAi) and subsequent analysis of CDK phosphorylation, cell proliferation capacity and cell cycle distribution; and (3) measurements of CAK activity in extracts of cells depleted of PNQALRE or Cdk7 by RNAi, with a novel, analog-sensitive Cdk2 activation assay. We find that purified, monomeric PNQALRE expressed with recombinant baculoviruses has no CAK activity towards monomeric or cyclin-bound Cdk2. Cdk7/cyclin H complexes, moreover, show robust activity towards monomeric Cdk2, perhaps obviating any need for a Cak1-like enzyme. Depletion of PNQALRE in human cells by RNA

interference (RNAi) impairs cell proliferation, but does not decrease either the level of activating phosphorylation on endogenous Cdk2 or the capacity of cell-free extracts to activate exogenous Cdk2. Loss of PNQALRE also fails to produce a discrete block to cell cycle progression. Instead, there is an increase in the fraction of cells with a sub-G₁ DNA content and enhanced cleavage of poly(ADP-ribose) polymerase (PARP), possibly suggesting that PNQALRE normally functions to suppress programmed cell death. Consistent with that idea, a recent genome-wide survey of mammalian protein kinases by RNAi-mediated knockdown identified PNQALRE/CCRK as a potential negative regulator of apoptosis.³⁶

MATERIALS AND METHODS

Cloning and protein expression. We identified a human expressed sequence tag (EST) by a BLAST search with the *S. pombe* Csk1 protein sequence. The human EST obtained from ATCC (NCBI Accession number: H17727) was used as a probe to screen a mouse testis cDNA Library (Clontech). A single cDNA clone was identified, which lacked an initiator ATG. We amplified the 5' end of the gene with reverse complementary primers by 5'/3' RACE (Boehringer Mannheim) to generate a full-length mouse cDNA encoding PNQALRE (GenBank Accession number: AAF89089). Nine IMAGE consortium human cDNAs (43754954, 4586360, 4640579, 4639379, 4798672, 5266219, 5270203, 5203849) corresponding to PNQALRE were obtained from Invitrogen and fully sequenced using internal primers designed according to the genomic DNA sequence. Several RT-PCR products obtained with primers located in exons 5 and 10 (forward 5'-CATGCCAACCAACATTGTACATC-3'; reverse 5'-GAATCAGCTCTGG-GTTCAACA-3') were also sequenced.

Antibodies. A 20-amino acid peptide corresponding to sequences near the carboxyl-terminus of human PNQALRE, HDFHVDRPLEESLLNPELIR (SK1103), was used for rabbit immunization (Covance). Crude antisera were affinity-purified with the same peptides. Anti-cyclin H antibodies were described previously.³⁷ Other antibodies were obtained commercially: anti-Cdk7 (C-4), for detection and immunoprecipitation of mouse Cdk7, and anti-Cdk2 (M2) from Santa-Cruz Biotechnology; anti-PARP cleavage product from Cell Signaling Technologies; anti-HA (16B12) from Covance; anti-GAPDH from Biorad; and monoclonal anti-Cdk7 (MO-1.1), specific for human Cdk7, from Sigma.

mRNA expression analysis. A mouse adult tissue blot containing 50 μ g of total RNA from brain, heart, lung, liver spleen, kidney, stomach, small intestine, skeletal muscle, skin, thymus, testis, uterus, and placenta, was obtained commercially (Seegene, Inc.). Full-length mouse cDNA (~1.1 kb) encoding PNQALRE was labeled with [α -³²P]dCTP using the Multiprime DNA labeling system (Amersham Pharmacia Biotech) to generate a probe for hybridization and autoradiographic detection.

Tissue and cultured cell extract preparation. Mouse testes were harvested and immediately frozen in liquid nitrogen and kept at -80°C until lysis with a Dounce homogenizer in prechilled buffer: 25 mM Hepes (pH 7.4), 150 mM NaCl, 0.1% Triton X-100, 1 mM EDTA, 1 mM dithiothreitol (DTT), 50 mM sodium fluoride (NaF), 80 mM β -glycerophosphate, 0.1 mM sodium orthovanadate (Na₃VO₄), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 2 μ g/ml aprotinin, 1 μ g/ml leupeptin. The homogenate was vortexed briefly (30 s), sonicated on ice with 3 x 45 s pulses with a 15 s interval, and centrifuged at 100,000 x g_{av} for 2 h. Supernatants were aliquoted and stored at -80°C. Protein concentration was determined by Bradford assay (Bio-Rad). Cultured cells were harvested by scraping, washed once with phosphate-buffered saline and homogenized in 25 mM Hepes (pH 7.4), 150 mM NaCl, 0.1% Triton X-100, 1 mM EDTA, 1 mM DTT, 50 mM NaF, 80 mM β -glycerophosphate, supplemented with completeTM protease inhibitor cocktail (Roche).

Gel-exclusion chromatography. Approximately 10 mg of total testes extract protein (200–500 μ l) were applied to a Superdex 200 10/30 HR column (Amersham Pharmacia Biotech) equilibrated with 25 mM Hepes (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol. Fractions of

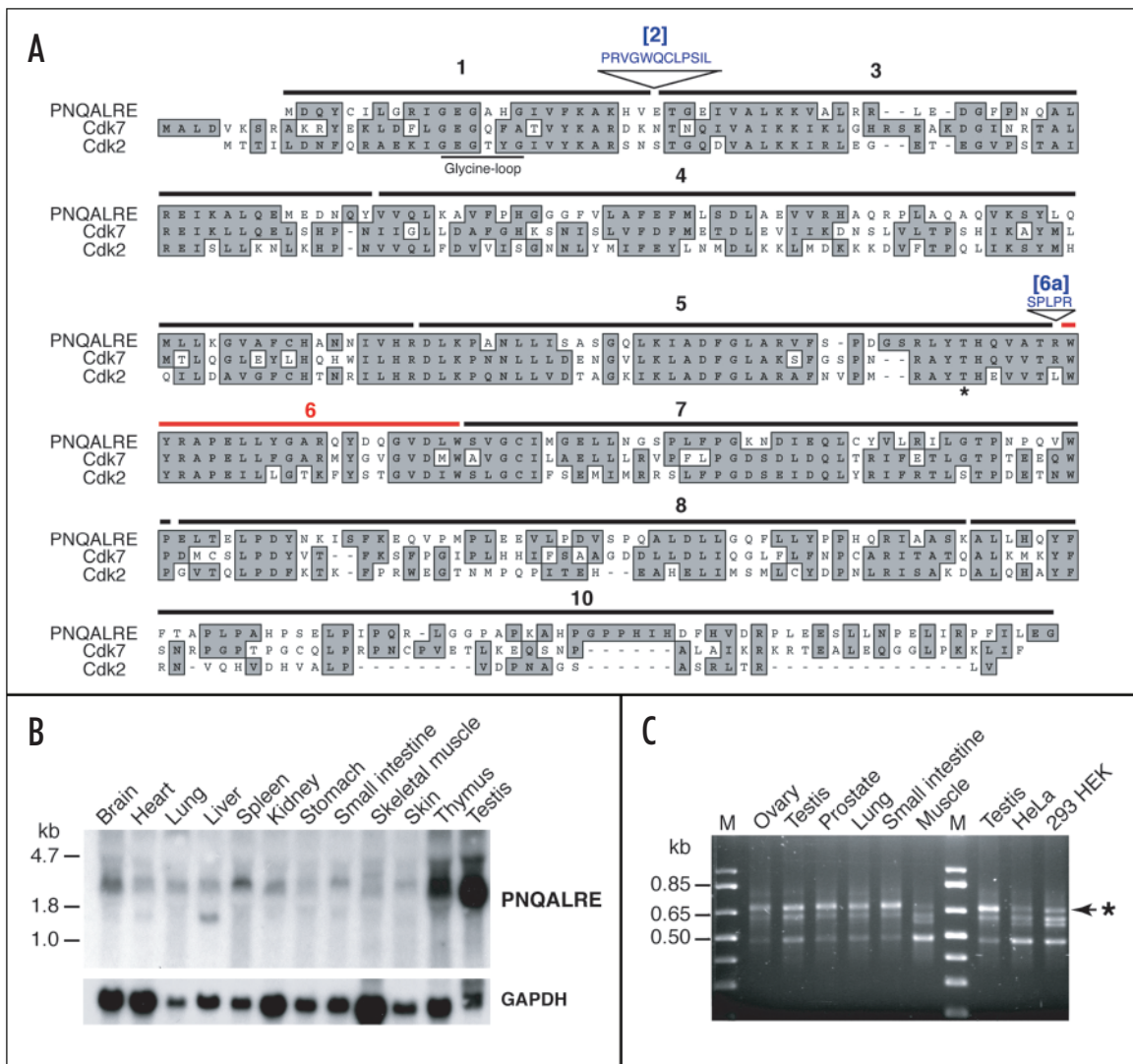


Figure 1. (A) PNQALRE is a CDK family member. Alignment of human PNQALRE with Cdk7 and Cdk2. Exons of PNQALRE splice variant 1 (see Fig. 2) are indicated by solid lines above sequence. Two in-frame insertions of 12 (exon 2) and five (exon 6a) amino acids preceding exons 3 and 6, respectively, are also indicated (see text). Both identities and similarities are boxed. Conserved kinase subdomain I (glycine loop) and the putative site of activating T-loop phosphorylation (Thr161) are indicated by an underline and asterisk, respectively. (B) Northern blot analysis of PNQALRE expression in mouse tissues. (C) Alternative splicing of *PNQALRE* in human tissues and cell lines was detected by RT-PCR amplification with primers located in exons 5 and 10. The PCR product corresponding to the “full-length” splice variant 1 is indicated (*)

500 μ l were collected; 5% and 50% of each fraction were used for immunoblots and immunoprecipitation, respectively.

Immunoprecipitation and kinase assays. For measurements of CAK activity in mouse testes extract, 0.25–1.0 mg of extract was incubated with ~600 ng of anti-PNQALRE (SK1103) or anti-Cdk7 (C4) antibody bound to 20 μ l Protein A- or G-Sepharose (Amersham) for 2 h at 4°C. The immunoprecipitates were washed three times with HBST (10 mM Hepes, pH 7.4, 150 mM NaCl, 0.1% Triton X-100) and twice with HBSD (10 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM DTT). The immunoprecipitates were then incubated with 100 ng Cdk2-HA/His-cyclin A or Cdk1-HA/cyclin B in HBSD in the presence of 400 μ M ATP and 10 mM MgCl₂ in a volume of 20 μ l at 25°C for 15 min. 10 μ l of the supernatant was added to 10 μ l of Histone H1 mix: 25 mM Hepes (pH 7.4), 150 mM NaCl, 10 mM MgCl₂, 5 μ g Histone H1 (Roche), 5 μ Ci [γ -³²P]ATP. Phosphorylation reactions were carried out for 5–10 min at 25°C. Phosphorylated proteins were separated by SDS-PAGE and detected autoradiographically. CAK activity of purified proteins was measured by incubating 10 ng purified enzyme (Cdk7-Flag, PNQ-Flag or Cdk7/Cyclin H) with 2 μ g of substrate

(monomeric Cdk2wt, or Cdk2D145N/His-cyclin A complex) in 20 μ l 25 mM Hepes (pH 7.4), 150 mM NaCl, 10 mM MgCl₂, 200 μ M ATP, 5 μ Ci [γ -³²P]ATP at 25°C for 10 min. Samples were processed as described above.

Cdk2^{AS} activation assay. To test for total CDK-activating capacity in cell lysates, extract containing 3 μ g of protein was preincubated for 5 min at room temperature with ~150 ng Cdk2^{AS}/His-cyclin A in a 17 μ l reaction containing an ATP regenerating system (25 mM Hepes, pH 7.4, 10 mM NaCl, 2 mM MgCl₂, 1 mM ATP, 40 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase). Then, 2 μ Ci [γ -³²P]N⁶ (benzyl)-ATP and 10 μ g histone H1 were added, and the reactions (final volume: 20 μ l) were incubated for another 5 min at room temperature. Labeling was stopped by addition of 2x SDS-PAGE sample buffer. Phosphorylated proteins were separated by SDS-PAGE and detected by autoradiography, and histone H1 phosphorylation was quantified by Phosphorimager.

Baculovirus construction and expression. To construct recombinant baculovirus encoding wild-type, HA-tagged mouse PNQALRE, an NcoI site was introduced at the 5' and 3' ends of the PNQALRE open reading frame in pBluscript SK(-) by oligonucleotide-directed mutagenesis,³⁸ with

the following oligonucleotides: 5'-CCGCGGGCCG-cctggACCAG-3' and 5'-CCAGAGGGGTcctgg-CTGGTCCAGG-3'. The resulting *Nco*I fragment was subcloned in-frame with the HA epitope into the transfer vector pVLCCK2NHA.^{39,40} The construction of recombinant baculoviruses encoding HA-tagged CDK7, untagged cyclin H, and His-tagged Mat1 has been previously described.^{30,37} Viruses encoding carboxyl-terminally tagged human Cdk7 and PNQALRE were generated by cloning the appropriate coding sequence into pFastBac1 (Invitrogen) that had been modified to encode two copies of the Flag peptide sequence in tandem with a hexa-histidine tag. Proteins were purified by metal affinity and conventional chromatography as described previously.^{41,42}

Depletion of PNQALRE by RNA interference (RNAi). Small interfering RNA (siRNA) duplex oligonucleotides containing human *PNQALRE* mRNA sequences: 5'-GGCGGUUGGAGGACGGCUU-3' (si-3) and 5'-GCUUUGUGCUGGCCUUUGA-3' (si-4), or human *CDK7* mRNA sequence 5'-GCCUA-CAUGUUGAUGACUC-3' (si-19), were synthesized by Dharmacon Research. An siRNA duplex of random sequence, 5'-GGUGGACGGCAAGUUUGCU-3', was synthesized as a negative control (si-c). Logarithmically growing cells plated at 0.8×10^5 cells/ml (U2OS) or 2×10^5 cells/ml (HCT116) in 10 cm dishes were transfected twice at intervals of ~30 h with indicated siRNAs and Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. siRNA-transfected cells were harvested 48 h after the second transfection for protein analysis and 72 h after the second transfection for determination of cell number and cell cycle distribution. For measurements of cell proliferation, RNAi- and control-treated cells were trypsinized and counted in a Neubauer chamber using Trypan blue exclusion staining. Cell counts for each treatment were measured and plotted as indicated.

Flow cytometry. DNA content of HCT116 and U2OS cells was measured after fixation in 70% ethanol, digestion with RNase A (100 μ g/ml) and staining with propidium iodide (50 μ g/ml), using a FACScan apparatus (Becton Dickinson).

RESULTS

Identification of a novel CDK family member. Although they diverge in their organization, the CAK-CDK networks have the same basic components in budding and fission yeast, including an evolutionarily conserved, monomeric CAK: Cak1 and Csk1, respectively.^{9,13,43,44} Consistent with interrogations of the complete human genome,¹⁹ we found no obvious ortholog of either kinase in searches of EST databases, but instead detected limited homology to a CDK-like protein (Fig. 1A).

The putative kinase, provisionally named PNQALRE (following the convention for naming suspected but unconfirmed CDKs by the amino acid sequence of α -helix 1), contains all 11 conserved subdomains characteristic of serine/threonine protein kinases,⁴⁵ with homology to Cdk1, 2 and 7 (~35% sequence identity) and, to a lesser extent, Csk1 (~25% identity). All of the critical catalytic residues are conserved, and PNQALRE contains a readily identifiable glycine loop (subdomain I), in contrast to both Cak1 and Csk1, which lack this motif. Also unlike Cak1 and Csk1, PNQALRE contains a threonine residue—Thr-161—in the T-loop region between subdomains VII and VIII, in a position and sequence context identical to those of activating threonines in other CDKs. Finally, PNQALRE contains none of the large loops—stretches of nonconserved amino acids inserted between conserved kinase subdomains—characteristic of Cak1.

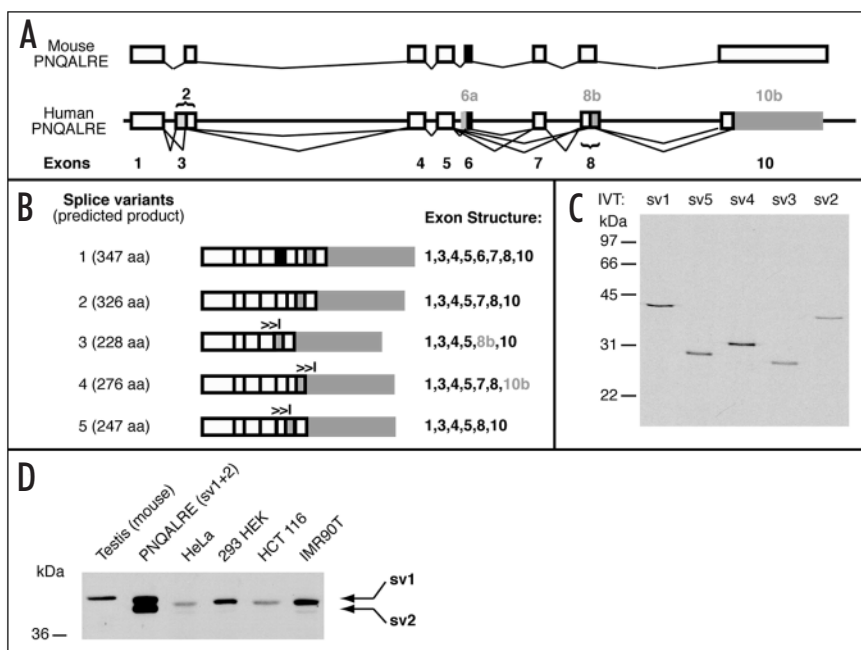


Figure 2. Characterization of *PNQALRE* mRNA and protein expression. (A) Schematic representation of the genomic organization of mouse and human *PNQALRE*. The human gene gives rise to several alternatively spliced isoforms as determined by sequencing of multiple cDNA clones. (B) Exon structure of five *PNQALRE* splice-variants for which cDNAs have been isolated. Splice variant 2 has an in-frame deletion of the conserved APE motif. Splice variants 3–5 are predicted to produce truncated proteins due to early termination caused by frame-shifts (>>I). Only splice variant 1 is predicted to encode an active protein kinase. (C) The splice variants depicted in (B) can be transcribed and translated in reticulocyte lysates in vitro and detected by the incorporation of [³⁵S]-methionine. (D) *PNQALRE* protein expression in mouse testes and human cell lines. Endogenous *PNQALRE* protein can be detected by an antibody raised to a peptide sequence near the carboxyl-terminus of *PNQALRE*. The splice variants 1 and 2 were expressed by transfection of the corresponding cloned DNA. For each indicated cell line, 40 μ g of total protein was loaded compared to 7 μ g of mouse testes protein (lane 1).

By Northern blot hybridization, *PNQALRE* mRNA expression was highest in mouse testes, followed by thymus. All other mouse tissues and cell lines we analyzed contained lower levels of *PNQALRE* mRNA (Fig. 1B and data not shown). By performing RT-PCR amplification of *PNQALRE* mRNA from various human tissues and cell lines, we identified several isoforms presumably generated by alternative splicing of a common precursor mRNA (Fig. 1C). These correspond to five distinct splice variants identified by sequencing nine commercially obtained cDNAs from various human sources (Fig. 2A and B). We detected a full-length cDNA (splice variant 1) encoding a 347 amino acid protein with all conserved kinase subdomains and no insertions, which corresponds to full-length mouse *PNQALRE* (the murine mRNA did not exhibit variable splicing in the sources we analyzed). The other four variants contain internal deletions predicted to render the product catalytically inactive, some with reading-frame shifts (Fig. 2B), but all could be translated in reticulocyte lysates in vitro to produce full-length polypeptides of the predicted sizes (Fig. 2C). In addition, alternative splicing can give rise to *PNQALRE* variants with in-frame insertions of 12 (exon 2) or five (exon 6a) amino acids, which are included in a cDNA sequence deposited in GenBank (accession #AK075325), or in a PCR product we amplified from HeLa cell mRNA, respectively (Figs. 1A, 2A and data not shown).

A polyclonal antibody, raised against a carboxyl-terminal peptide derived from mouse *PNQALRE*, recognized a single polypeptide of ~40 kDa in immunoblots of mouse testes extracts (Fig. 2D). This corresponds closely to the size of the protein expressed upon transient transfection of the full-length *PNQALRE* cDNA (splice variant 1) in human embryonic

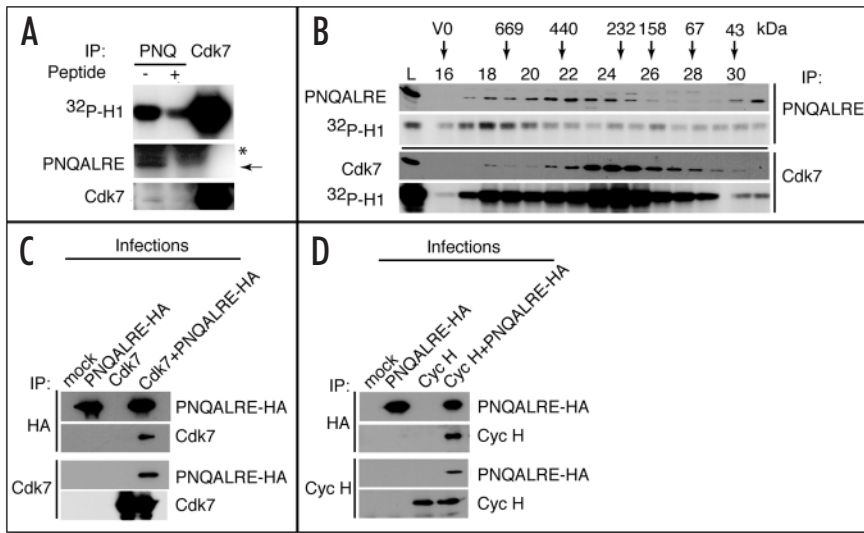
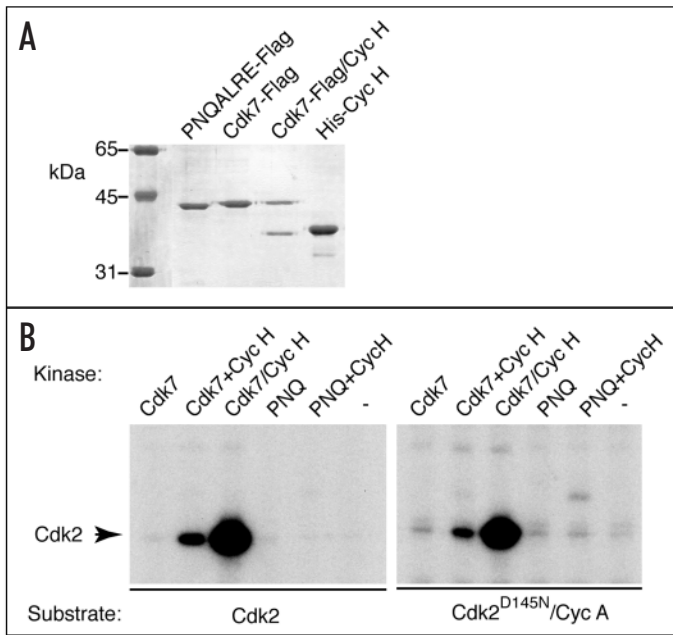


Figure 3. PNQALRE associates with CAK components in vivo. (A) CAK activity is associated with PNQALRE immunoprecipitated from mouse testes extract, measured by the ability to activate Cdk1-cyclin B complexes, which were assayed for the ability to phosphorylate histone H1 (top). Cdk7 is detected in PNQALRE immunoprecipitates by immunoblotting with anti-Cdk7 antibody (bottom). Immunoprecipitation of both PNQALRE (middle) and associated Cdk7 protein and CAK activity can be blocked by an excess of the immunogenic peptide (lane marked "+"). Arrow at right indicates immunoreactive band corresponding to full-length PNQALRE, whereas asterisk at right denotes diffuse bands derived from immunoglobulin heavy chain. (B) Gel-exclusion chromatography of testes extract. Immunoprecipitates from each fraction with antibodies against either PNQALRE (top two panels) or Cdk7 (bottom two panels) were analyzed by immunoblotting with anti-PNQALRE (top) or anti-Cdk7 (third from top) antibody, or tested for CAK activity (second from top, and bottom) as in (A). (C and D) PNQALRE-HA binds Cdk7 (C) and cyclin H (D) when coexpressed in Sf9 insect cells with appropriate baculoviruses, as indicated at top. Immunoprecipitations were performed with antibodies indicated at left, and immunoblots were probed with antibodies indicated at right.

kidney (HEK) 293 cells. PNQALRE in immunoprecipitates from testes extracts, moreover, contained tryptic peptides encoded in exon 6 of splice variant 1 (Fig. 2A and B). This exon encodes kinase subdomain VIII,⁴⁵ including the APE motif highly conserved among Ser/Thr kinases (Liao JC-F, Erdjument-Bromage H, Tempst P, Fisher RP, unpublished observations). The next-longest human isoform, splice variant 2, lacks exon 6. Anti-PNQALRE immunoblots of human cultured cell extracts (Fig. 2D) typically contained major bands intermediate in mobility between those of splice variants 1 and 2. There was variability in electrophoretic mobility among human cell lines, possibly indicating even more extensive splice variation and/or post-translational modifications. That the major immunoreactive bands of ~40 kDa correspond to bona fide products of the *PNQALRE* mRNA was indicated by their diminution after RNAi-mediated knockdown in HEK 293 (data not shown), U2OS osteosarcoma (Fig. 5A) and HCT116 (Fig. 5B) cell extracts.

CAK associates with PNQALRE in vivo. Because of the relatively high level of PNQALRE expression in mouse testes, we focused at first on this tissue as a source of the protein for biochemical assays. Anti-PNQALRE immunoprecipitates from mouse testes extracts were capable of activating recombinant Cdk1/cyclin B complexes, although the yield of CAK was many-fold lower than in anti-Cdk7 immunoprecipitates from the same source (Fig. 3A). A small amount of Cdk7 was precipitated from the testes extract with anti-PNQALRE antibody (Fig. 3A, bottom), which did not cross-react with Cdk1, 2 or 7 (data not shown). Recovery of both CAK activity and Cdk7 protein in the anti-PNQALRE immunoprecipitate was specifically blocked by excess antigenic peptide (Fig. 3A).



The apparent association of PNQALRE with Cdk7 in vivo raised the possibility that the CAK activity in anti-PNQALRE immunoprecipitates might be due to coprecipitating Cdk7. Another indication that this might be the case was the behavior of PNQALRE protein, and of PNQALRE-associated CAK activity, upon gel-exclusion chromatography. Native PNQALRE protein migrated in three discrete peaks, at ~40, ~400 and ~700 kDa. Only the largest complexes had associated CAK activity (Fig. 3B); this was also the only form of PNQALRE that comigrated with a peak, albeit a minor one, of Cdk7.

We analyzed pairwise interactions between PNQALRE and components of the CAK trimer coexpressed in insect Sf9 cells infected with recombinant baculoviruses. PNQALRE associated with a fraction of Cdk7 (Fig. 3C), cyclin H (Fig. 3D) and Mat1 (data not shown). PNQALRE also interacted with cyclin H in a yeast two-hybrid interaction screen (Liao JC-F, Xu W, Fisher RP, unpublished observations). Taken together, the coprecipitation

Figure 4. PNQALRE has no intrinsic CAK activity. (A) PNQALRE-His-Flag, Cdk7-His-Flag, Cdk7-His-Flag/cyclin H complex, and His-cyclin H were purified from appropriately infected insect cells and visualized by staining with Coomassie brilliant blue after 10% SDS-PAGE. (B) Purified, monomeric PNQALRE does not phosphorylate Cdk2. The purified proteins shown in panel (A) were assayed for their ability to phosphorylate either wild-type Cdk2 monomer (left), or the catalytically inactive Cdk2^{D145N}/cyclin A complex (right). Cdk7 was assayed as monomer ("Cdk7"), as Cdk7 mixed with cyclin H just prior to assay ("Cdk7 + Cyc H") or as a dimeric complex formed by insect cell coinfection ("Cdk7/Cyc H"); PNQALRE was assayed as monomer ("PNQ") or mixed with cyclin H just prior to assay ("PNQ + Cyc H"). The substrates were incubated without additional proteins in lanes marked (-).

and chromatographic cofractionation of Cdk7 complex subunits and PNQALRE suggest that the PNQALRE-associated CAK activity might be due to Cdk7.

Monomeric PNQALRE/CCRK lacks intrinsic CAK activity.

To test for CAK activity intrinsic to PNQALRE, we purified the wild-type protein, tagged at its carboxyl-terminus with two Flag epitopes and a hexahistidine motif, by metal-ion affinity and subsequent gel exclusion chromatography to ensure that the protein was monomeric (Fig. 4A). The purified protein had no detectable kinase activity towards either monomeric Cdk2 or a Cdk2/cyclin A complex (Fig. 4B). We tested both forms of Cdk2 as substrates on the premise that a preference for monomeric over cyclin-bound CDK is a criterion by which a metazoan Cak1/Csk1 ortholog might be distinguished from Cdk7.²⁵ Under the conditions of substrate excess tested here, however, purified Cdk7/cyclin H phosphorylated monomeric Cdk2 and the Cdk2/cyclin A complex with apparently equal efficiency (Fig. 4B).

Although PNQALRE is inactive as a monomer, it might acquire CAK activity upon association with a cyclin and/or other modifications. We overexpressed epitope-tagged versions of PNQALRE in mammalian cells but, after recovering the protein by immunoprecipitation, did not detect any associated kinase activity towards CDKs, the Pol II carboxyl-terminal domain (CTD), histone H1 or myelin basic protein (data not shown). Addition of cyclin H, which as expected was able to stimulate the CAK activity of monomeric Cdk7, failed to do so for PNQALRE (Fig. 4B). Attempts to activate PNQALRE by coinfection with cyclin H or cyclin K—which has been reported to associate with a CAK⁴⁶—were also unsuccessful (data not shown). Finally, we increased the amount of PNQALRE in the reaction ten-fold without observing phosphorylation of either substrate above background (data not shown). We therefore cannot conclude that PNQALRE has any intrinsic ability to activate CDKs.

PNQALRE/CCRK depletion does not affect Cdk2 T-loop phosphorylation or cell cycle progression. Depletion of PNQALRE/CCRK in cultured mammalian cells by RNAi was reported to cause a block to cell cycle progression in the G₁ phase, and a reduction in the ability of cells to activate Cdk2 expressed by transient transfection.³⁵ Because our biochemical analysis did not reveal CAK activity intrinsic to PNQALRE, we examined the effects of its depletion on cell proliferation and the T-loop phosphorylation state of endogenous Cdk2. We transfected two human cell lines—HCT116 and U2OS—with two different small interfering RNA (siRNA) oligonucleotides directed against the *PNQALRE* mRNA. One, siRNA 3, targeted the same region as did the reagent described by Galaktionov and coworkers,³⁵ and caused a ~75% reduction in the protein level after two rounds of transfection. In contrast, another oligonucleotide, siRNA 4 (see Materials and Methods), consistently reduced expression levels by close to 90% (Fig. 5A and quantification not shown).

When U2OS or HCT116 cells were subjected to two rounds of transfection with siRNAs 3 or 4, we observed modest but reproducible reductions in cell numbers 48–72 h after the last treatment (Fig. 5B). T-loop phosphorylation of Cdk2, detected either by the increased mobility of this isoform in one-dimensional SDS-PAGE (Fig. 5A) or by immunoblotting with a phosphospecific antibody (data not shown), was not consistently affected by PNQALRE depletion. The cell cycle distribution after PNQALRE knockdown, determined by flow cytometry of fixed cells stained with propidium iodide to measure DNA content, was not appreciably altered compared to mock- or control siRNA-transfected cells (Fig. 5C). There was, however, a modest but reproducible increase in the fraction of cells with a sub-G₁ DNA content (Fig. 5C and data not shown). Corresponding to this increase in the sub-G₁ population, we detected an siRNA-specific increase of PARP cleavage in U2OS cells (Fig. 5A). Taken together, these results suggest that the reduction in U2OS cell numbers

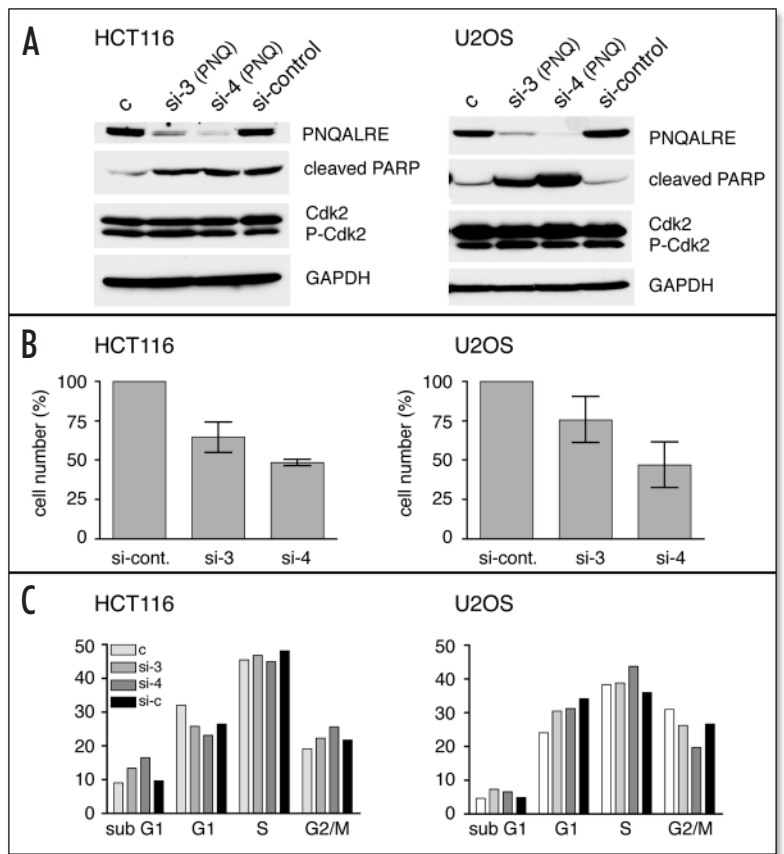


Figure 5. Depletion of PNQALRE by RNAi impairs cell proliferation but does not diminish CDK activation or block cell cycle progression. (A) Immunoblot analysis of PNQALRE, Cdk2, PARP cleavage product and GAPDH (loading control) in HCT116 (left) and U2OS (right) cells following siRNA treatment. The faster-migrating isoform of Cdk2 corresponds to the protein phosphorylated at Thr-160 of the T-loop by CAK (P-Cdk2). (B) Numbers of cells counted 72 h after last siRNA transfection. Values are mean percentages of total cells relative to cells in control siRNA transfection (defined as 100%) \pm standard deviation of four (HCT116) and six (U2OS) independent experiments. (C) The cell cycle distribution (in percent of total cell population) of HCT116 and U2OS cells following siRNA treatment was determined by flow cytometry of PI-stained cell populations. One representative result is shown for each cell line. The different siRNA treatments were as follows: control cells treated with Lipofectamine but no siRNA (c); siRNAs 3 and 4 directed against PNQALRE (si-3 and si-4); and cells treated with a non-targeting control siRNA (si-c).

caused by PNQALRE depletion might be due to increased apoptosis. (Higher levels of PARP cleavage in control siRNA-transfected HCT116 cells make it difficult to say whether this is a general phenomenon.)

PNQALRE/CCRK levels do not correlate with CAK activity. Purified PNQALRE had no measurable CAK activity, and cells lacking PNQALRE due to RNAi-mediated knockdown showed no sign of a CAK malfunction. To test directly whether depletion of PNQALRE or Cdk7 in vivo affected CDK-activating potential of a cell extract, we developed an assay based on the activation of analog-sensitive (AS) Cdk2.⁴⁷ It has been possible to engineer unnatural nucleotide specificity into protein kinases, including Cdk2, enabling them to catalyze transfer of phosphate to target proteins from bulky, derivatized ATP analogs that wild-type kinases cannot accommodate in their active sites.⁴⁸ We can thereby test the ability of mammalian whole-cell extracts to activate preassembled but unphosphorylated Cdk2^{AS}/cyclin A complexes in a single-step reaction without any immunoprecipitations, by including 1 mM unlabeled ATP, an ATP-regenerating system and the radiolabeled analog, *N*6-(benzyl)-ATP. To facilitate detection and quantification, we also included the model CDK substrate histone H1. When a HeLa cell extract immunodepleted of Cdk7 and a mock-depleted

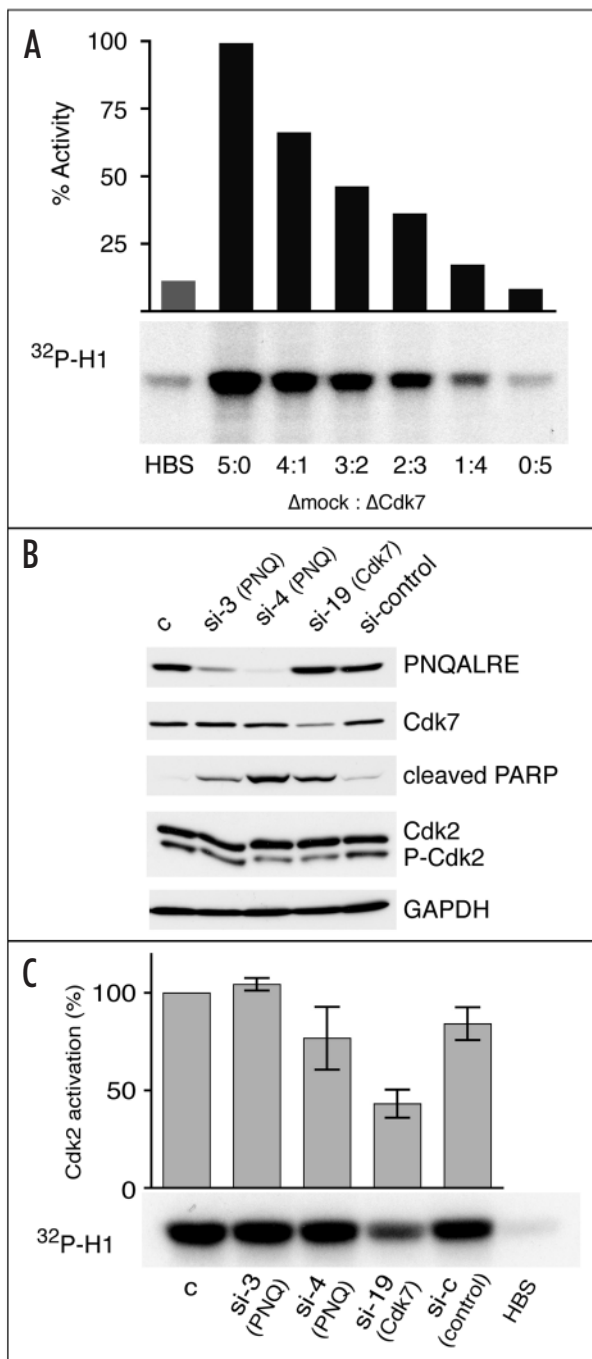


Figure 6. Cellular CAK activity is proportional to Cdk7 levels and unaffected by PNQALRE knockdown. (A) Activation of Cdk2^{AS} by cell extracts is directly proportional to Cdk7 levels. A mock-depleted extract and an extract immunodepleted of Cdk7 were mixed in the indicated ratios and tested for the ability to activate recombinant Cdk2^{AS}/cyclin A complex. Depletion of ~95% of Cdk7 protein reduces Cdk2 activation measured by this assay to or below that obtained with buffer (HEPES-buffered saline, HBS) alone (lane 1). Activity of undiluted, mock-depleted extract is defined as 100%. (B) Immunoblot analysis of PNQALRE, Cdk7, Cdk2/P-Cdk2 (see (Fig. 5) legend), PARP cleavage product and GAPDH loading control in U2OS cells following siRNA treatment. (C) CAK activity was measured in U2OS cells 48 h after second siRNA transfection by the ability of the different cell lysates to activate Cdk2^{AS}/cyclin A complex, which was then tested for its ability to phosphorylate histone H1 with the [γ -³²P]N6(benzyl)-ATP analog substrate. The bar graph shows means \pm standard deviations of three independent experiments. The different siRNA treatments were as follows: control cells treated with Lipofectamine but no siRNA (defined as 100% activity) (c); siRNAs 3 and 4 directed against PNQALRE (si-3 and si-4); siRNA 19 directed against Cdk7 (si-19); and cells treated with a nontargeting control siRNA (si-c).

DISCUSSION

The available biochemical and genetic evidence indicates that the Cdk7 complex is necessary and sufficient to activate cell-cycle CDKs in metazoans. In *S. pombe*, an organism with two CAKs, inactivation of both—Mcs6 and Csk1—is necessary to arrest cell cycle progression.¹³ Thermal inactivation of a temperature-sensitive Cdk7, in contrast, is sufficient to cause arrest at the entry to mitosis in germline and somatic cells of *Drosophila*,⁶ and in *Caenorhabditis* embryos,⁷ arguing against any redundancy of CAK function at the G₂/M transition in metazoans.

In temperature-sensitive *mcs6 csk1* double mutant *S. pombe* strains, prior synchronization of cells in G₁ or G₂ is needed to produce a homogenous arrest at the G₁/S or G₂/M boundary, respectively, upon temperature shift.⁹ A requirement for fly or worm Cdk7 at the G₁/S transition could not be demonstrated with the available conditional mutants, but that should not be interpreted as evidence for another CAK. Instead, different thresholds of CAK activity might be required for mitotic and S phase entry. For example, the T-loop phosphothreonines of Cdk1 and 2 might have different rates of turnover in vivo, and thus differential sensitivity to acute and/or incomplete inactivation of the upstream kinase. Thus, despite the constitutive expression and activity of Cdk7, regulation of its function might still occur through the opposing action of T-loop-specific protein phosphatases, or by changes in substrate accessibility or subcellular localization.²⁰

An alternative theory posits the existence of another CAK that responds directly to anti-proliferative signals to control the state of CDK activation even when Cdk7 is active.²⁴ This idea was originally inspired by the identification of Cak1,¹⁰⁻¹² and by skepticism regarding the assignment of CAK function to metazoan Cdk7, given the inability of the *S. cerevisiae* ortholog Kin28 to perform that function.⁴⁹ No ortholog of Cak1 or Csk1 is present in any sequenced metazoan genome, however, and no convincing functional homolog has emerged from biochemical studies. Recently, human PNQALRE/CCRK was proposed to be a CAK based on: (1) limited homology to Cdk7 and related kinases, including Csk1 and Cak1; (2) a low level of CAK activity towards a GST-Cdk2 fusion protein in vitro; and (3) a cell proliferation defect, apparently accompanied by decreased phosphorylation of Cdk2 Thr-160, upon RNAi-mediated knockdown in human cells.³⁵

extract were added in varying proportions to unphosphorylated Cdk2^{AS}-cyclin A complexes, activation of histone H1 kinase was approximately linear with respect to the Cdk7 concentration (Fig. 6A).

We next tested the effects of RNAi-mediated depletion of PNQALRE or Cdk7 on Cdk2^{AS} activation. Neither siRNA 3 nor siRNA 4—both of which knocked down PNQALRE >75% (Fig. 6B)—had a significant effect on Cdk2^{AS} activation in extracts of either U2OS or HCT116 cells (Fig. 6C and data not shown). We also tested siRNA 19, directed against human Cdk7, which reduced Cdk7 levels in extracts by 50–75% (Fig. 6B and data not shown). Such a treatment consistently reduced Cdk2^{AS} activation by ~60% in U2OS cell extracts (Fig. 6C), in good agreement with its efficacy in depleting cellular Cdk7 (Fig. 6B). This did not correlate with a decrease in endogenous Cdk2 phosphorylation, but was also accompanied by a modest increase in PARP cleavage. We therefore conclude that the Cdk2 activation capacity in crude cell extracts is directly proportional to the level of Cdk7 but not of PNQALRE.

In our studies, we found no evidence that PNQALRE is a CAK. The purified, monomeric protein has no detectable kinase activity towards either monomeric or cyclin-bound CDKs. This clearly distinguishes PNQALRE from Csk1 and Cak1, which in their monomeric, unmodified forms are promiscuous CAKs even towards mammalian CDKs.^{13,14,33} We cannot rule out the possibility that PNQALRE is a CDK that, upon binding an as-yet unidentified cyclin partner, acquires kinase activity. Cdk1, 2 and 7 can all phosphorylate and activate other CDKs⁴² and so, in theory, PNQALRE could also be a cyclin-dependent CAK. We did not recover CAK activity, however, in anti-HA immunoprecipitates from extracts of mammalian cells overexpressing HA-tagged PNQALRE (Liao JC-F, Larochelle S, Fisher RP, unpublished observations). The low levels of CAK activity in PNQALRE-containing complexes isolated from mammalian cells can be explained, moreover, by the association between PNQALRE and a known CAK, Cdk7.

We observed impaired cell proliferation, but no reduction in Cdk2 T-loop phosphorylation, upon PNQALRE depletion (Fig. 5). At present, the mechanism underlying the proliferation defect is mysterious. Increased levels of p27^{Kip1}, which we observed in some of our transfections with siRNA 4 (data not shown), or of p21^{Cip1}, which was induced nonspecifically by another, less potent siRNA directed against PNQALRE (data not shown), could account for decreased proliferation. PNQALRE/CCRK was recently identified in an RNAi-based screen for mammalian kinases or kinase-like proteins that prevent apoptosis,³⁶ and a small molecule inhibitor with specificity for CDK family members, including PNQALRE/CCRK, was shown to promote death of cancer cells in culture.⁵⁰ In U2OS cells, siRNA 4 consistently caused a modest increase in the fraction of cells with a sub-G₁ DNA content, and an increase in PARP cleavage. Although these results appear to support a role (albeit probably a minor one) for PNQALRE in preventing apoptosis, we also detected increased PARP cleavage upon Cdk7 knockdown and, in HCT116 cells, in transfections with the control siRNA. Further experiments will clearly be necessary to determine whether any of these effects is truly specific to PNQALRE depletion.

To understand the role of PNQALRE in vivo, the next steps will be to identify its physiologic binding partners and substrates (if indeed it is an active kinase). The human *PNQALRE* primary transcript is subject to complex alternative splicing. Many (or all) of the splice variants can be translated in vitro, and most encode proteins predicted to be inactive as kinases. These proteins can now be tested for the ability to rescue cell proliferation defects caused by knockdown of PNQALRE.

Consistent with the single-CAK model, the ability of extracts to activate exogenous Cdk2 is directly proportional to the amount of Cdk7 they contain, which can be manipulated in vivo by RNAi, or in vitro by immunodepletion. Similar results have been obtained before with immunodepleted extracts,^{6,21,22} but here we have more closely approximated the situation in vivo with an analog-sensitive Cdk2, which allows direct measurement of CDK activation in a crude extract. Although it could be argued that immunodepletion of Cdk7 might passively deplete another, associated kinase, the similar results of depleting Cdk7 in vivo with RNAi seem to have no alternative interpretation. Moreover, the drop in CAK activity occurs without affecting the steady state level of T-loop phosphorylation on endogenous Cdk2, suggesting that compensatory mechanisms keep levels of Cdk2 activation in the cell buffered against moderate reductions (~60%) in CAK activity.

Evolutionary plasticity is a feature of the CAK-CDK network.^{13,19,44} Connections have been gained and, perhaps, lost, and there has been expansion in metazoans of the number of CDKs that directly regulate the cell cycle.⁵¹ Has there been concomitant contraction in the number of upstream, activating kinases (CAKs), from two to one since the ancestors of fission yeast and metazoans diverged? All the mammalian cell-cycle CDKs are targets of Cdk7 in vitro and, in contrast to Cdk1, Cdk2 can be phosphorylated in both monomeric and cyclin-bound forms by Cdk7.³⁰ Indeed, our results suggest that the lack of a cyclin partner poses no obstacle to Cdk2 T-loop phosphorylation by Cdk7. Thus, an expanded repertoire of effector CDKs might have obviated any need in metazoans for a CAK such as Cak1 or Csk1 with intrinsic preference for the monomer.

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