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PITALRE, a nuclear CDC2-related protein kinase that phosphorylates the retinoblastoma protein *in vitro*

(cell division cycle 2/cyclin-dependent kinase/myelin basic protein/serine-threonine protein kinase)

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ABSTRACT **Members of the cell division cycle 2 (CDC2) family of kinases play a pivotal role in the regulation of the eukaryotic cell cycle. In this communication, we report the isolation of a cDNA that encodes a CDC2-related human protein kinase temporarily designated PITALRE for the characteristic Pro-Ile-Thr-Ala-Leu-Arg-Glu motif. Its deduced amino acid sequence is 47% identical to that of the human cholinesterase-related cell division controller (CHED) kinase, which is required during hematopoiesis, and 42% identical to the *Saccharomyces cerevisiae SGV1* gene product, a putative kinase involved in the response to pheromone via its guanine nucleotide-binding protein α subunit. PITALRE expression is ubiquitous, but its expression levels are different in various human tissues. PITALRE is an ≈43-kDa protein that associates with three cellular polypeptides of 80, 95, and 155 kDa. PITALRE is localized primarily to the nucleus. In addition, we have identified a retinoblastoma protein kinase activity associated with PITALRE immunocomplexes that cannot phosphorylate histone H1, suggesting that the target phosphorylation site of PITALRE differs from that of CDC2 kinase. Interestingly, the retinoblastoma kinase activity associated with PITALRE does not oscillate during the cell cycle.**

The cell cycle in eukaryotes is regulated by a sequence of restriction points. In yeast, the first restriction point occurs during the G_1 phase prior to the DNA synthesis and the second occurs before the initiation of mitosis. In *Saccharomyces cerevisiae*, the cell division cycle 28 (CDC28) kinase controls both restriction points through association with the CLN cyclins in G_1 and with CLB cyclins in G_2/M (1). In vertebrate cells, the regulatory mechanisms involved in cell cycle progression are more complex. CDC2 kinase, in association with cyclin B, appears to be a universal regulator of the eukaryotic entry into mitosis. However, in G_1, just before the onset of DNA synthesis, cyclin-dependent kinase 2 (CDK2), but not CDC2, is required (2, 3). Additional mammalian CDC2-related kinases have been isolated that share >40% identity at the amino acid level (4–11). At least two of them, CDK4 (previously named PSK-J3; ref. 5) and CDK5 (also called PSSALRE for its Pro-Ser-Ser-Ala-Leu-Arg-Glu motif; ref. 9), have been shown to associate with D-type cyclins. *In vitro* assembled CDK4–cyclin D complexes are capable of phosphorylating the retinoblastoma protein. However, the same complexes cannot phosphorylate histone H1. This indicates that CDK4–cyclin D complexes possess a different phosphorylation specificity than the CDC2 kinase. Nevertheless, no kinase activity has been detected in CDK4 immunocomplexes (12). The association of CDK5 with cyclins D1/D3 and with proliferating cell nuclear antigen (PCNA) suggests a role for this kinase in the cell cycle (13). However, the high levels of expression of *cdk5* found in neurons, cells no longer dividing, indicate a role for CDK5 in terminally differentiated cells (11). The study of CDC2 and CDC2-related kinases over the past few years has revealed a key role for these kinases in the regulation of the cell cycle. Most recently, an involvement in differentiation processes has also been proposed (8, 11).

With the aim of isolating additional putative controllers of the mammalian cell cycle, we performed a combination of PCR amplification and low-stringency screening of a human cDNA library. By using this strategy, we have isolated and characterized a CDC2-related protein kinase,§ temporarily named PITALRE for the characteristic motif Pro-Ile-Thr-Ala-Leu-Arg-Glu. We have determined its subcellular localization, identified several associated proteins, and demonstrated kinase activity in its immunocomplexes. We have also studied the regulation of this kinase activity during the cell division cycle. These studies define an additional protein kinase that may be involved in cell cycle control or in differentiation of specific cell types.

MATERIALS AND METHODS

cDNA Cloning. Two degenerate oligonucleotides were used in the polymerase chain reaction (PCR) to amplify ≈500-bp fragments related to the *cdc2* family of genes. A mouse embryonic cDNA library was used as a source of cDNA. The 5′ oligonucleotide (5′-GCAGGATCCGA-RAARATYGGNGARGGNACNTA-3′) corresponds to the CDC2 region of amino acid sequence Glu-Lys-Ile-Gly-Glu-Gly-Thr-Tyr and the 3′ oligonucleotide (5′-CGGCTGCAGAR-NAYYTCNGGNGMNCKRTACCA-3′) corresponds to the CDC2 region of amino acid sequence Trp-Tyr-Arg-Ser-Pro-Glu-Val-Leu (R = G or A, Y = T or C, N = G, A, T, or C, M = A or C, and K = G or T). PCR was carried out for 25 cycles (1 min at 94°C, 2 min at 55°C, and 3 min at 72°C, followed by a final 8-min incubation at 72°C) following manufacturer directions (Perkin–Elmer/Cetus). The nucleotide sequence of several fragments was determined. With one of these *cdc2*-related PCR-amplified fragments as a probe, a human CEM cDNA library (in Lambda ZAP II; Stratagene) was screened at low stringency (38% formamide containing 0.1% SDS, 150 μg of herring sperm DNA per ml, 5× Denhardt's solution (1× = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin), and 5× SSPE (1× = 0.18 M NaCl/10 mM

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Abbreviations: PITALRE, kinase with Pro-Ile-Thr-Ala-Leu-Arg-Glu motif; CDC, cell division cycle; CDK, cyclin-dependent kinase; CHED, cholinesterase-related cell division controller; GST, gluthathione *S*-transferase; MBP, myelin basic protein; PSTAIRE, kinase with Pro-Ser-Thr-Ala-Ile-Arg-Glu motif; RB, retinoblastoma.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. L25676).

phosphate, pH 7.4/1 mM EDTA). Hybridization was performed at 37°C for 16 h, and low-stringency washes were carried out at 37°C for 20 min in 0.30 M NaCl/0.030 M sodium citrate, pH 7/0.1% SDS. Two positives contained ≈1.4-kb (PK10) and ≈1.5-kb (PK14) inserts. Double-stranded DNA sequence determination was performed by using Sequenase 2.0 (United States Biochemical) and oligonucleotide primers. Comparison of the sequences with the major data bases showed that the clone of 1.4 kb corresponded to the PSK-J3/CDK4 (5) and the clone of 1.5 kb encoded a previously unknown CDC2-related putative kinase.

Biological Reagents. The coding region of clone PK14 starting at nucleotide 65 was PCR-amplified and subcloned in pGEX-2T (Pharmacia) linearized with *Bam*HI/*Sma* I. Expression of the fusion protein was performed as described (14, 15). Bacterially expressed glutathione *S*-transferase (GST)-PITALRE fusion protein was used to immunize rabbits. Positive rabbit serum was affinity-purified essentially as described by Koff *et al.* (16) with GST and GST-PITALRE columns. Preparation of anti-C-terminal peptide antibodies to CDC2 (G6) and CDK2 has been described (17, 18).

Cell Culture and Biological Assays. Cells were obtained from the American Type Culture Collection. Cell culture, cell labeling, and centrifugal elutriation were performed as described by Giordano *et al.* (17, 19). HeLa cells were synchronized by serum deprivation and hydroxyurea treatment essentially as described by Ashihara and Baserga (20). Flow cytometric analysis was performed with an Epics Elite system (Coulter). Nuclei from HeLa cells were obtained essentially as described by Li *et al.* (21). Immunoprecipitations were performed as described by Harlow and Lane (22). Immunoprecipitation–reprecipitation experiments were done as described (3). V8 partial digestion mapping was performed as described by Cleveland *et al.* (23). Enhanced chemiluminescence (ECL; Amersham) was used in immunoblot experiments. Kinase assays from immunoprecipitated complexes were performed at 30°C for 20–30 min in 20 mM Hepes/10 mM magnesium acetate/1 mM dithiothreitol/10–100 μM ATP/5 μCi (1 μCi = 37 kBq) of [γ-^{32}P]ATP (DuPont) containing 1–5 μg of the following substrates: myelin basic protein (MBP) and casein (Sigma), histone H1 (Boehringer Mannheim), p56 retinoblastoma (RB) bacterially expressed protein, and several GST fusion proteins (total volume, 25 μl).

RESULTS AND DISCUSSION

Isolation of a Human cDNA Encoding an Additional Member of the CDC2 Family of Protein Kinases. With the aim of isolating new members of the CDC2 family of serine/threonine protein kinases, cDNA from a mouse embryonic library was PCR-amplified by using degenerate oligonucleotides. Next we used a unique PCR clone as a probe to isolate two human cDNAs (see *Materials and Methods*). One of them was PSK-J3, previously isolated by Hanks (5), which recently has been renamed CDK4 because of its association with the D-type cyclins (12). The second cDNA was found to be 1461 bp long and contained an open reading frame of 1181 bp (Fig. 1). A putative start site for translation was found at nucleotides 65–67 (24). Starting at this methionine, the predicted translation product is a 372-amino acid protein with an expected relative molecular mass of ≈43 kDa (Fig. 1). The 3′ noncoding region does not contain a poly(A) tail. The deduced amino acid sequence contains the 11 conserved regions characteristic of the protein kinase catalytic domain (25), and the putative ATP-binding site is identical to that of SGV1 (Fig. 2), a putative kinase required for a guanine nucleotide-binding protein α subunit-mediated adaptive response to pheromone in *S. cerevisiae* (26). A PSTAIRE (Pro-Ser-Thr-Ala-Ile-Arg-Glu)-like motif, PITALRE, is found at residues 60–66 that is also closely related to the motifs of SGV1 and

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cgggacccgagcaggagcggcggcacgagcagctgggggcggcggcgcgttggaggc   60
ggccatggcaaagcagtacgactcggtggagtgccctttttgtgatgaagtttccaaata  120
    M  A  K  Q  Y  D  S  V  E  C  P  F  C  D  E  V  S  K  Y   19
cgagaagctcgccaagatcggccaaggcaccttcggggaggtgttcaaggccaggcaccg  180
 E  K  L  A  K  I  G  Q  G  T  F  G  E  V  F  K  A  R  H  R   39
caagaccggccagaaggtggctctgaagaaggtgctgatggaaaacgagaaggaggggtt  240
 K  T  G  Q  K  V  A  L  K  K· V  L  M  E  N  E  K  E  G  F   59
ccccattacagccttgcgggagatcaagatccttcagcttctaaaacacgagaatgtggt  300
 P  I  T  A  L  R  E  I  K  I  L  Q  L  L  K  H  E  N  V  V   79
caacttgattgagatttgtcgaaccaaagcttcccctataaccgctgcaagggtagtat  360
 N  L  I  E  I  C  R  T  K  A  S  P  Y  N  R  C  K  G  S  I   99
atacctggtgttcgacttctgcgagcatgaccttgctgggctgttgagcaatgttttggt  420
 Y  L  V  F  D  F  C  E  H  D  L  A  G  L  L  S  N  V  L  V  119
caagttcacgctgtctgagatcaagagggtgatgcagatgctgcttaacggcctctacta  480
 K  F  T  L  S  E  I  K  R  V  M  Q  M  L  L  N  G  L  Y  Y  139
catccacagaaacaagatcctgcatagggacatgaaggctgctaatgtgcttatcactcg  540
 I  H  R  N  K  I  L  H  R  D  M  K  A  A  N  V  L  I  T  R  159
tgatggggtcctgaagctggcagactttgggctggcccgggccttcagcctggccaagaa  600
 D  G  V  L  K  L  A  D  F  G  L  A  R  A  F  S  L  A  K  N  179
cagccagcccaaccgctacaccaaccgtgtggtgacactctggtaccggcccccggagct  660
 S  Q  P  N  R  Y  T  N  R  V  V  T  L  W  Y  R  P  P  E  L  199
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 L  L  G  E  R  D  Y  G  P  P  I  D  L  W  G  A  G  C  I  M  219
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 L  I  S  Q  L  C  G  S  I  T  P  E  V  W  P  N  V  D  N  Y  259
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 E  L  Y  E  K  L  E  L  V  K  G  Q  K  R  K  V  K  D  R  L  279
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tcccgccaccaccaaccagacggagtttgagcgcgtcttctgagggccggcgcttgccac 1200
 P  A  T  T  N  Q  T  E  F  E  R  V  F                       372
tagggctcttgtgtttttttcttctgctatgtgacttgcatcgtggagacagggcattt 1260
gagtttatatctctcatgcatattttatttaatccccaccctgggctctgggagcagccc 1320
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tccttgctggttttctggatggttcccagagggtttccatggggtaggaggatgggctcg 1440
cccaccagtgacttttcccg...                                      1461
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FIG. 1. Nucleotide sequence of PITALRE cDNA and deduced amino acid sequence (in single-letter code). The ATP-binding site, the PSTAIRE-like motif, and the putative nuclear localization signal are underlined and appear in this order. Nucleotides and amino acids are numbered on the right.

CHED. We tentatively named this protein "PITALRE," until more functional information allows for more precise classification. PITALRE has the two regulatory threonine residues corresponding to positions 14 and 161 in CDC2, but as in SGV1, the residue corresponding to Tyr-15 of CDC2 is not conserved, thus suggesting an alternative mode of regulation. Table 1 shows the percentages of identities among different members of the family of CDC2-related protein kinases. PITALRE is 47% identical to CHED, a human homolog of CDC2 required in hematopoiesis (8). PITALRE shares ≈41–43% identity (61–65% similarity) with the *S. cerevisiae* SGV1 kinase and the human CDC2, CDK2, CDK3, and CDK5 kinases, but, as mentioned above, certain amino acid clusters are better conserved in relation to SGV1 (Table 1 and Fig. 2). The protein also contains short extensions at the amino- and carboxyl-terminal ends that may have specific regulatory functions, such as substrate recognition or subcellular localization.

Expression of PITALRE in Human Tissues. Recently, CDC2-related protein kinases whose expression is limited to certain tissues or cell types have been isolated (9–11). To determine whether PITALRE is also a tissue-specific kinase, we performed RNA (Northern) blot experiments (Fig. 3). At least two transcripts of ≈2.8 kb and ≈3.2 kb are observed in all tissues tested, which indicates that PITALRE expression is ubiquitous (see below). However, PITALRE expression is highest in liver and placenta, which suggests that PITALRE may be involved in specialized functions in certain cell types. Similarly, high levels of CDK5 have been detected in neurons, cells no longer in the cell cycle (11); on the other hand, CDK5 associates with the D-type cyclins and with proliferating cell

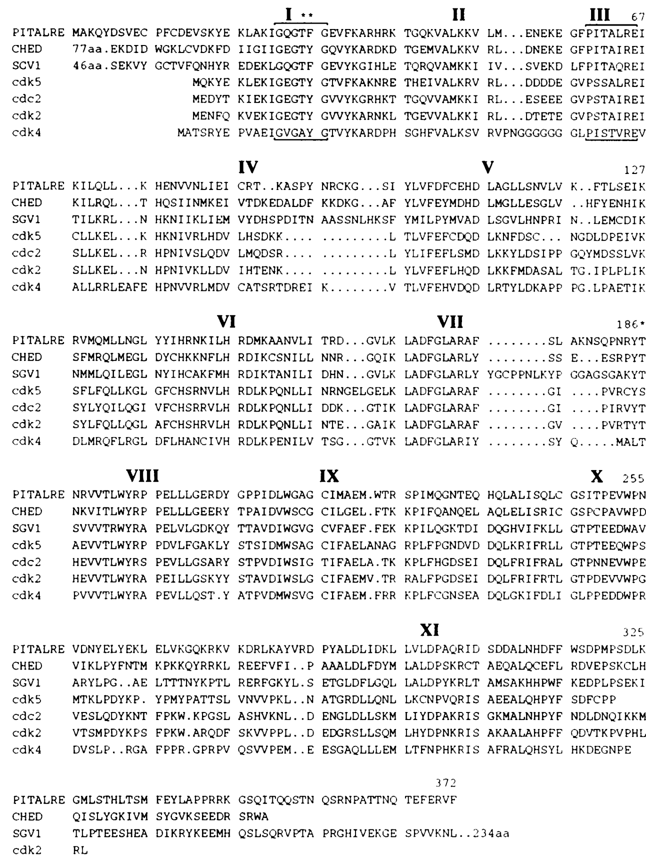

FIG. 2. Comparison of the predicted amino acid sequences of PITALRE and other CDC2-related kinases: CHED, SGV1, CDK5, CDC2, CDK2, and CDK4 kinases. Brackets indicate the ATP binding site and the PSTAIRE-like motif. The conserved phosphorylation sites of CDC2 are indicated (*). Amino acids are numbered on the top-right of the PITALRE sequence. The 11 subdomains conserved in the catalytic domain of the protein kinases are indicated.

nuclear antigen (13), which paradoxically suggests a G_1 cell cycle function. Moreover, other transcripts of higher molecular mass can be detected in some tissues. This may be due to the presence of partially processed RNA or alternative splicing or to the existence of related genes. The difference in size between the RNA transcripts and the ≈1.5-kb PITALRE cDNA is probably due to the presence of long 5′ and/or 3′ extensions and/or the poly(A) tail.

Affinity-Purified Antibody Recognizes a Cellular Protein of ≈43 kDa. To identify the cellular protein encoded by the PITALRE cDNA, we performed immunoprecipitation/reimmunoprecipitation experiments. Fig. 4*A* shows that affinity-purified anti-PITALRE antibodies recognized directly

Table 1. Percent amino acid sequence identity of PSTAIRE-like kinases

	PITALRE	CHED	SGV1	CDK5	CDC2	CDK3	CDK2	CDK4
PITALRE	—	47	42	43	42	41	41	38
CHED		—	40	41	42	44	43	39
SGV1			—	37	42	41	39	36
CDK5				—	57	62	61	45
CDC2					—	66	66	44
CDK3						—	76	47
CDK2							—	47
CDK4								—

The percentages have been obtained by comparing the deduced amino acid sequences corresponding to the catalytic domain of cdc2 among the PSTAIRE-like kinases. All of the sequences used are from human kinases except SGV1, which was from *S. cerevisiae*.

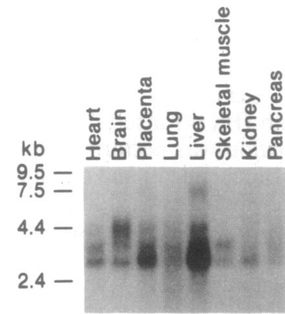

FIG. 3. Expression pattern of PITALRE in various human tissues (Clontech human tissue blot). Each lane contains 2 μg of poly(A)$^+$ RNA. The size of molecular markers is indicated on the left. Nick-translated *Eco*RI full-length fragment or a random primer *Sca* I/*Eco*RI fragment (380 3′ nucleotides) were used as probes for the blot-hybridization that followed manufacturer directions.

a single polypeptide of ≈43 kDa, and no other cross-reacting bands are observed. The ≈43-kDa band was detected in immunoprecipitates of lysates from many cell lines at similar levels, including ML-1, CEM, HeLa, WI38, Col38, 293, SAOS-2, and WERI cells, which is consistent with ubiquitous expression (see above). Immunoblots of affinity-purified anti-PITALRE immunoprecipitates also showed a band of 43-kDa (Fig. 4 *B* and *C*). To determine whether or not the PITALRE cDNA encodes a full-length protein, we transcribed *in vitro* the cDNA and translated the cRNA in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine. The *in vitro* synthesized polypeptide had the expected molecular mass and was immunoprecipitated specifically by the affinity-purified antibody (Fig. 4*D*). To demonstrate that the *in vitro* translated product and the protein immunoprecipitated by the antibody from ^{35}S-labeled cell lysates were, in fact, the same polypeptide, we performed partial digestion with V8 protease from the excised bands. The pattern of the V8 partial digestion was identical (Fig. 4*E*).

We tested the ability of the PITALRE antisera and the affinity-purified antibody to recognize related polypeptides by using *in vitro* translated proteins. PITALRE antibodies were not able to immunoprecipitate *in vitro* translated CDC2, CDK2, CDK4, and CDK5 (data not shown). This observation, together with the immunoprecipitation/reimmunoprecipitation experiment, indicates that the anti-PITALRE antibodies are specific and suitable for the biological characterization of PITALRE function. On the other hand, to immunoprecipitate *in vitro* translated PITALRE we used several antibodies raised against members of the CDC2 family of protein kinases: G6, G8, anti-PSTAIRE, and anti-CDC2-CT antibodies against CDC2; C-terminal CDK2, CDK3, CDK4, and CDK5 anti-peptide antibodies; and anti-ERK1 and anti-ERK2. Only anti-PSTAIRE antibodies were able to immunoprecipitate this polypeptide (data not shown).

Subcellular Localization of PITALRE. To gain additional circumstantial evidence as to the physiological role of PITALRE in cells, we determined its subcellular location by subcellular fractionation followed by Western blotting. Fig. 4*F* shows that PITALRE is primarily, if not exclusively, a nuclear protein. The PITALRE primary sequence contains a putative nuclear localization signal (Fig. 1), which agrees with the consensus sequence Lys-(Arg or Lys)-Xaa-(Arg or Lys) present in many nuclear proteins (27). Similar signals were found in the CHED- and PCTAIRE-type kinases (8, 10), but their subcellular localization is still unknown.

PITALRE-Associated Proteins. The activity of the CDC2 and CDC2-related protein kinases is regulated by phosphorylation and by association with cyclins. The kinases that interact physically with cyclins are called CDKs. Some of these kinases are also known to associate with the tumor

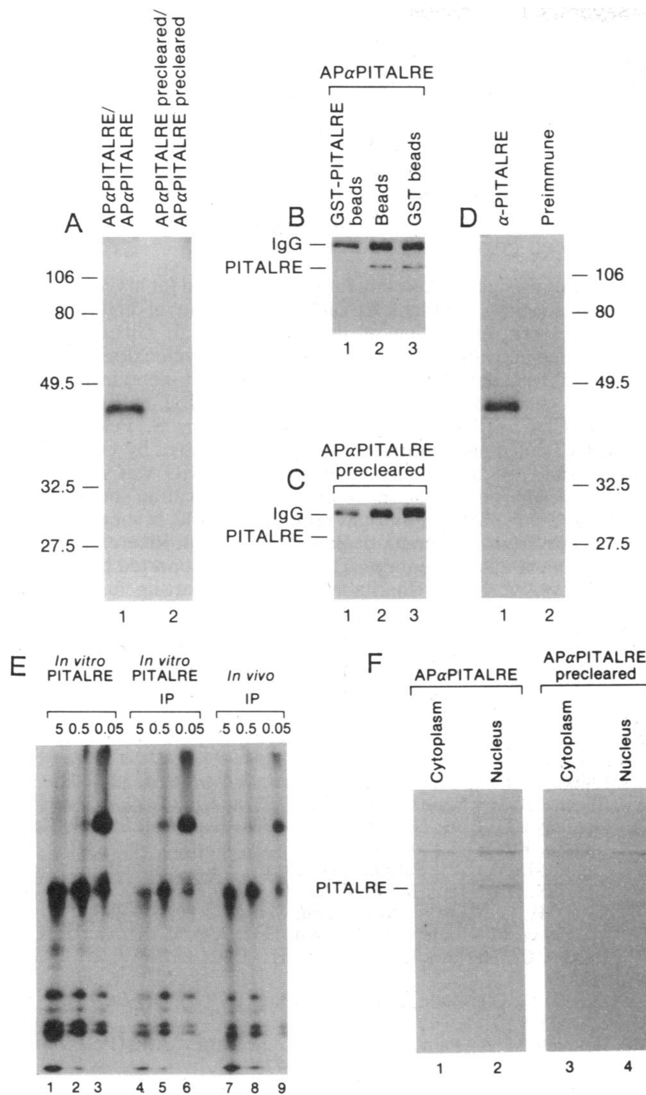

FIG. 4. Characterization of the PITALRE protein kinase by means of anti-PITALRE antibodies. (*A*) [^{35}S]Methionine-labeled ML-1 cells were immunoprecipitated/reimmunoprecipitated by affinity-purified (AP) anti-PITALRE (α-PITALRE) (lane 1) or affinity-purified anti-PITALRE precleared by using GST-PITALRE-treated beads (lane 2). (*B* and *C*) ML-1 cells were immunoprecipitated with affinity-purified anti-PITALRE precleared with GST-PITALRE-treated beads (lanes 1), nontreated beads (lanes 2), and GST-treated beads (lanes 3). The immunoprecipitates were analyzed by SDS/PAGE and immunoblotted with affinity-purified anti-PITALRE (*B*) or with affinity-purified anti-PITALRE precleared with GST-PITALRE (*C*). (*D*) *In vitro* translated PITALRE was immunoprecipitated by anti-PITALRE (lane 1) but not by preimmune serum (lane 2). (*E*) Partial *Staphylococcus aureus* V8 proteolytic mapping of *in vitro* translated PITALRE (lanes 1, 2, and 3), *in vitro* translated PITALRE immunoprecipitated with anti-PITALRE (lanes 4, 5, and 6), and PITALRE from [^{35}S]methionine-labeled ML-1 cells (lanes 7, 8, and 9). The amount of V8 protease used is indicated in each lane (expressed in μg). (*F*) Subcellular localization. Protein (60 μg) from the cytoplasmic and membrane fractions (lanes 1 and 3) and the nuclear fraction (lanes 2 and 4) were separated by 10% SDS/PAGE and immunoblotted by affinity-purified anti-PITALRE (lanes 1 and 2) or affinity-purified anti-PITALRE precleared by using GST-PITALRE (lanes 3 and 4). Relevant proteins are indicated.

supressor gene product pRB or the related protein p107. To search for known or unknown proteins that associate with PITALRE, we performed immunoprecipitations of ^{35}S-labeled cell lysates. Three proteins with molecular masses of ≈155, ≈95, and ≈80 kDa were coimmunoprecipitated with PITALRE (Fig. 5). The absence of these proteins in the immunoprecipitation/reimmunoprecipitation experiment (Fig. 4*A*) and in the immunoblot (Fig. 4*B*) indicates that they are associated and are not cross-reacting proteins. These associated polypeptides are not any of the known cyclins including cyclin X (28). It is conceivable that one of these associated proteins is a regulatory subunit related to the cyclin family.

PITALRE-Associated RB Protein Kinase Activity. To investigate further the function of this putative kinase, we determined the kinase activity associated with its immunocomplexes. PITALRE immunocomplexes showed a strong $p56^{RB}$ kinase activity (Fig. 6*A*). The immunocomplexes also phosphorylated MBP (Fig. 6*B*) and casein (data not shown), but at a lower level than when the $p56^{RB}$ was used as a substrate. Interestingly, histone H1 was not phosphorylated, which suggests that the site of phosphorylation is different from that recognized by CDC2 and CDK2. This observation suggests that all three of these kinases may regulate target molecules through phosphorylation in non-overlapping signal transduction networks. Other exogenous substrates, including CDKs and cyclins, were not phosphorylated (data not shown). The associated kinase activity was also able to phosphorylate PITALRE and its associated proteins (Fig. 6*C*), which suggests that these proteins may be substrates of the PITALRE kinase. The identity of PITALRE, which runs slightly slower in the SDS polyacrylamide gel, was confirmed by reimmunoprecipitation (data not shown). This fact suggests the possibility that PITALRE may autophosphorylate, but we cannot be sure that PITALRE is the only kinase present in the complex.

CDC2 and CDK2 kinases have a cell cycle-regulated kinase activity that can be monitored by using different exogenous substrates. To examine whether or not PITALRE shares this cell cycle-modulated behavior, we performed *in vitro* kinase assays of PITALRE immunocomplexes during the cell cycle. After serum deprivation and blocking with hydroxyurea, cells were allowed to progress through the cell cycle in a synchronous fashion (Fig. 6*D*). Cell fractions were lysed, and the

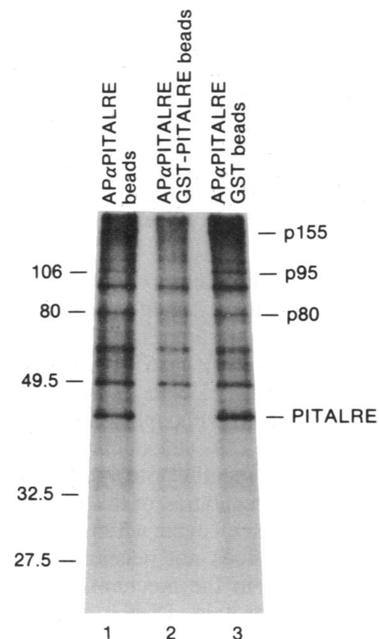

FIG. 5. PITALRE-associated proteins. [^{35}S]Methionine-labeled HeLa cell lysates were immunoprecipitated with affinity-purified anti-PITALRE precleared with untreated beads (lane 1), GST-PITALRE-treated beads (lane 2), and GST-treated beads (lane 3). Relevant proteins are indicated.

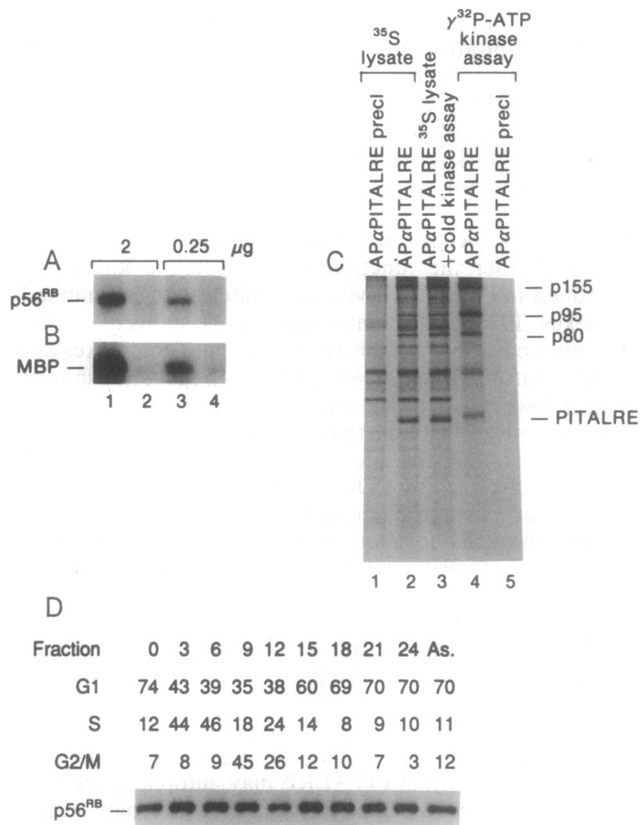

FIG. 6. Kinase activity associated to PITALRE immunocomplexes. (*A* and *B*) HeLa cell lysates were immunoprecipitated with affinity-purified anti-PITALRE (lanes 1 and 3) and affinity-purified anti-PITALRE precleared by using GST-PITALRE (lanes 2 and 4). PITALRE immunocomplexes were assayed for their ability to phosphorylate bacterially expressed $p56^{RB}$ (1-hr exposure) (*A*) or MBP (8-hr exposure) (*B*) as exogenous substrates (0.25 μg of $p56^{RB}$ or 2.5 μg of MBP per lane; 2 μg of antibody in lanes 1 and 2 and 0.25 μg in lanes 3 and 4). (*C*) Either [^{35}S]methionine-labeled HeLa cell lysates (lanes 1, 2, and 3) or nonradioactive HeLa cell lysates (lanes 4 and 5) were immunoprecipitated with affinity-purified anti-PITALRE (lanes 2, 3, and 4) or affinity-purified anti-PITALRE precleared by using GST-PITALRE (lanes 1 and 5). Kinase assays were performed with nonradioactive ATP (lane 3) or [γ-^{32}P]ATP (lanes 4 and 5). (*D*) Percentage of HeLa cells in each phase of the cell cycle is indicated. Lysates of HeLa cells corresponding to each fraction were immunoprecipitated by affinity-purified anti-PITALRE, and kinase activity was assayed *in vitro* by using $p56^{RB}$ as exogenous substrate. Relevant proteins are indicated.

protein extracts were immunoprecipitated with affinity-purified anti-PITALRE to determine the kinase activity towards $p56^{RB}$ exogenous substrate. Fig. 6*D* shows an invariable pattern of phosphorylation of $p56^{RB}$. Similar results were obtained with lysates of ML-1 cells separated by centrifugal elutriation (data not shown). Phosphorylation of RB protein during the G_1 phase of the cell cycle occurs at several different sites. The presence of specific sites for different kinases suggests a multifactorial regulation of this protein. The lack of regulation throughout the cell cycle when RB protein is added as exogenous substrate does not necessarily mean that PITALRE is not involved in the mechanisms controlling cell cycle regulation of RB protein. CLN3, a G_1 cyclin from *S. cerevisiae* does not change in abundance during the cell division cycle, and its associated kinase activity also remains invariant (29). Recently, a MBP kinase activity has been described associated to mcs2, a fission yeast cyclin. This activity is also constant throughout the cell cycle (30).

Several CDC2-related kinases have been identified during the last few years. The physiologic role of some of these kinases is now becoming clear. Many of the members of the CDC2 family of kinases control different points of the cell cycle. The tissue-specific expression of some of the members of this family also indicates the involvement of these kinases in specialized cell functions. Further investigation of the function of PITALRE and other CDC2-related kinases will help to understand better the mechanisms of cell cycle control, cell growth, and differentiation.

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