Cloning and Characterization of a p53-related Protein Kinase Expressed in Interleukin-2-activated Cytotoxic T-cells, Epithelial Tumor Cell Lines, and the Testes*

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A human protein kinase, p53-related protein kinase (PRPK), was cloned from an interleukin-2-activated cytotoxic T-cell subtraction library. PRPK appears to be a homologue of a growth-related yeast serine/threonine protein kinase, YGR262c. However, a complementation assay using YGR262c-disrupted yeast indicated that PRPK is not functionally identical to the yeast enzyme. PRPK expression was observed in interleukin-2-activated cytotoxic T-cells, some human epithelial tumor cell lines, and the testes. The intrinsic transcriptional activity of p53 was up-regulated by a transient transfection of PRPK to COS-7 cells. PRPK was shown to bind to p53 and to phosphorylate p53 at Ser-15. These results indicate that PRPK may play an important role in the cell cycle and cell apoptosis through phosphorylation of p53.

Under stimulation by phytomagglutinin (PHA), interleukin-2 (IL-2) drives the proliferation of T-cells in peripheral blood mononuclear cells so that they become potent cancer killers (1). The IL-2-activated cytotoxic T-cells established by this method express the membrane lymphoxygen (mLT), complexes of the soluble protein lymphoxygen-a and the membrane protein lymphoxygen-β (2–4). We previously established novel cytotoxic T-cells that are devoid of mLT expression, i.e. mLT- cytotoxic T-cells (5, 6). mLT- cytotoxic T-cells have one main characteristic that distinguish them from mLT+ cytotoxic T-cells; while both mLT+ and mLT- cytotoxic T-cells show cell-membrane expression of phenotypes such as CD3, CD4, and CD8, as well as short term cytotoxic activity against cancer cells in vitro, mLT- cytotoxic T-cells lack cytolytic activity, i.e.

long term cancer cell killing activity based on cytokine production (5, 6). In the present study, we conducted cDNA subtraction between these two types of cells and obtained many interesting cDNA fragments using a polymerase chain reaction (PCR)-based method developed by Lisitsyn et al. (7). Among them, a consensus cDNA fragment containing a serine/threonine kinase motif was identified, and a full-length cDNA was cloned.

We deposited this kinase data in GenBank™ (accession number AB017505). In 2001, Ramsay reported a postulated human protein, dJ28H20.2 (CAC00561) that was identical to this kinase and had a chromosomal location of 20q13.1, based on the genomic data of a BAC clone of 2HS20 (GenBank™ accession number: CAC00561, published only in the data base). Because we had not published our data at that time, their report was released first. In the present study, we report on our continued experiments into the important characteristics of this kinase.

EXPERIMENTAL PROCEDURES

Cytotoxic T-cell Subtraction Library—A cytotoxic T-cell cDNA subtraction fragment library was prepared using a PCR-based method (7, 8). Peripheral blood mononuclear cells (PBMC) were separated from a healthy donor with heparinization by discontinuous density gradient centrifugation using Ficoll-Conray (1.077). PBMC were cultivated with RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 400 IU/ml IL-2 and 0.4 μg/ml PHA at 37 °C, 5% CO2. On the fourth day of culture, cells were passaged with a medium containing IL-2 at a cell concentration of 0.5 × 10^6/ml and were then passaged again every 2 days (3). The phenotypic expression of CD3 antigen on the IL-2-activated cytotoxic T-cells was >95%. After 7 days of culture, the cells were used for experiments as mLT- cytotoxic T-cells, i.e. mLT+ cytotoxic T-cells, cytotoxic T-cells were washed twice with PBS and cultured in medium without IL-2 for 18 to 24 h at 37 °C, 5% CO2. The hyperactivated mLT+ cytotoxic T-cells were made by incubating the activated cytotoxic T-cells with 100 ng/ml phorbol myristate acetate (PMA; Sigma) for 3 h at 37 °C, 5% CO2. The differences in the activities and phenotypic expressions between the activated and deactivated cytotoxic T-cells were previously reported (6). The mRNA was separated from both the activated and deactivated cytotoxic T-cells, and double-stranded cDNA was prepared using kits (Amersham Pharmacia Biotech). After the digestion of double-stranded cDNA by Sau3AI (Toyobo, Osaka, Japan) at 37 °C for 3 h, cDNA fragments from both activated (sense) and deactivated (substractor) cytotoxic T-cells were ligated with the R-primer set (B-Bgl 24: 5′-AGACTTCTCCACGCTTCGCGCAGCTG-3′; B-EcoR I: 5′-GATCTGGGTTAATACGACTCACTATAGGG-3′) at both ends using T4 DNA ligase (Toyobo, Tokyo, Japan). The PCR products were digested by Sau3AI, and the sense fragments were then

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AB017505 and AB028045.

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3 The abbreviations used are: PHA, phytohemmagglutinin; PRPK, p53-related protein kinase; mLT, membrane lymphoxygen; IL-2, interleukin-2; PBMC, peripheral blood mononuclear cells; PMA, phorbol myristate acetate; RT, reverse transcription; PCR, polymerase chain reaction; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; PK, protein kinase; NLS-BF, bipartite nuclear localization signal; FISH, fluorescent in situ hybridization; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate.
ligated with the J-primer set (J-Bgl 24: 5'-ACCGACGTCGACTATCC- AGAAACA-3' ; J-Bgl12: 5' -GATCTGCTTGACAGCCATC-3') using T4 DNA ligase after primer annealing. The sense fragments ligated with the J-primer sets were mixed with an excess amount of subfragment, and the mixture was heated up to 94 °C for 90 s after annealing at 67 °C for 20 h. The mixture was then diluted by 1/1000 (they were washed once with PCR subtraction), and the product was then treated again with SssI/3AL. After the removal of digested small fragments, sense fragments were ligated with the N-primer set (N-Bgl 24: 5'-AGCAGCTGTGATCTGCTTGT-GAGGAA-3' ; N-Bgl12: 5'-GATCTCCCTCCG-3') using T4 ligase at both ends, and a PCR-based subfragment was carried out using N-Bgl 24 primer and an excess amount of subfragment fragments. The final PCR products were cloned using a pGEM-T cloning system (Promega, Madison, WI), and transformation was carried out using chemically competent JM109 Escherichia coli cells. Random sequencing of the library was carried out, and the data were analyzed by a BLAST search on the genome net search launch page (www.genome.ad.jp) on the Internet. Consensus cDNA fragments encoding a serine/threonine kinase were identified.

Cloning of PRPK—Cloning of the human p53-related protein kinase (PRPK) was carried out using the Rapid Screen cDNA library panel of the human spleen (Origene Technologies Inc., Rockville, MD). A master plate of the human spleen origin was screened by PCR using a specific primer set (N2F2: 5'-GGCTGCTGTGCTCCGCTTCT-3' ; N2B2: 5'-GGACCTTCTCCACTCTCCAAAA-3') that was designed from the above sequence and used in the first PCR. Clones were selected using the second PCR product and vector using a NucleoSpin Extract kit (Qiagen, Tokyo, Japan). Several colonies were picked up, and sequencing analyses were carried out using a 310 Genetic Analyzer. One clone was selected (pCDNA3RGS-PRPK). The plasmid was purified by a Maxi prep kit (Qiagen, Tokyo, Japan). A kinase-negative PRPK expression vector (pcDNA3RGS-PRPK-D183N) was made using a QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. The PRPK expression vector (pcDNA3RGS-PRPK-KN-B: 5'-AAGACGTGAAATCCTGCTCAAACTTGGGGCT- GAGTTTCTTTCTGCTC-3' and pcDNA3RGS-PRPK vector as a template. Chemically competent XL-Blue E. coli cells (Stratagene) were transformed by the PCR product after digestion with BamHI. Several colonies were picked up, and sequence analyses were carried out with a 310 Genetic Analyzer.

Transfection of 293T and COS-7 cells was carried out using TransFast reagent (Promega, Madison, WI). Cells were grown in a α = 60-mm culture dish overnight. After the culture medium was aspirated, a mixed solution of 5.0 μg of plasmid and 20 μl of transfection reagent in 1.4 ml of Dulbecco's modified Eagle medium was added to the plate, followed by incubation for 1 h at 37 °C, 5% CO 2. Three milliliters of Dulbecco's modified Eagle medium's supplement with 10% fetal bovine serum was then added. After culturing for 48 h, the cells were harvested for analyses.

PRPK-stable transfectants were established using pcDNA3RGS-PRPK and COS-7 cells. After digestion of pcDNA3RGS-PRPK vector with DpnI, the LcDNA was transfected to COS-7 cells was carried out using TransFast reagent. After culturing for 48 h, 500 μg/ml G418 (Sigma) was added and cultured for a week. The G418-selected cells were cloned by a limiting dilution method using COS-7 cells as feeder.

Northern Blot Analysis—Northern blotting was carried out using 2 μg of poly(A) + RNAs isolated from different human normal tissues that had been fractionated by denaturing agarose gel electrophoresis and then transferred onto the charged nylon membranes (OriGene Technologies). The blots were hybridized to a PRPK probe, which was labeled using a Megaprime kit (Amersham Pharmacia Biotech) and [α-32P]ATP (111 Ci/mmol) for 2 h at 37 °C. The blots were washed twice with 0.1× SSC and 0.1% SDS at 65 °C. The blots were then exposed on an X-ray film for 2 h. The signal intensity was analyzed using NIH Image software.

In vitro Kinase Assay—In vitro protein kinase activity was estimated using a recombinant GST-PRPK fusion protein and [γ-32P]ATP (0.11 teraBq/mmol, 3.0 Ci/mmol; Amersham Pharmacia Biotech). Recombinant GST-PRPK or GST gel was pelleted out by centrifugation (10,000 g for 5 min) and then incubated at 30 °C with COS-7 cell lysate (2 × 10^6 cells/ml, 2 mg/ml) in 1× kinase buffer (25 mM HEPE, 25 mM β-glycerophosphate, 25 mM MgCl2, 2 mM dithiothreitol, 1 mM EDTA, 0.1 mM Na3VO4) with the addition of 1% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride. After washing four times with 1× kinase buffer, GST-PRPK or GST gel with or without the addition of dephosphorylated casein (100 μg/ml, Sigma) in 1× kinase buffer was incubated at 30 °C for 30 min.
The enzymatic reaction was stopped by the addition of SDS-PAGE sample buffer and subjected to SDS-PAGE analysis. After electrophoresis, proteins in the gel were transferred to a nitrocellulose membrane (Bio-Rad Laboratories) using a semidy blotter (BioCraft, Tokyo, Japan) for 60 min at 1 mA/cm² and then exposed to x-ray film (BioMax film; Amersham Pharmacia Biotech).

Cells—Cell lines used in this study were HT-29 (American Type Culture Collection [ATCC] HTB 38) and WiDr (ATCC CCL 28) human colon epithelial malignant tumor cells; Daudi (ATCC CCL 213) and RPMI 1788 (ATCC CCL 156) human B-cell lymphomas; HUT 78 (ATCC CRL 1753) human T-cell lymphoma; AsPC-1 (ATCC CRL 1682), MIA PaCa-2 (ATCC CRL 1420) and PANC-1 (ATCC CRL 1469) human pancreatic epithelial malignant tumor cells; 293T (human fetal ovary) and tsA-201 (human embryonic kidney) monkey cell lines; and several colonies were picked up and subjected to sequence analysis. BL21 E. coli cells were transformed, and recombinant material was prepared as described above. Recombinant GST-p53 peptide was eluted by 100 mM Tris-HCl, pH 8.8 with 20 mM glutathione (Sigma) and was analyzed by SDS-PAGE. After incubation with COS-7 lysate in 1× kinase buffer as described for the in vitro kinase assay, recombinant GST-P6PRK or GST gel was incubated with recombinant p53 peptide at 30 °C for 30 min in a 1× kinase buffer supplemented with 300 μM ATP. Sample buffer was added and heated up to 100 °C for 5 min. Samples were subjected to SDS-PAGE and electroblotted to a nitrocellulose or polyvinylidene difluoride membrane. Immunodetection was carried out using anti-p53 phospho-Ser-15-specific rabbit antibody and an ECL detection system.

Immunohistochemical Staining—COS-7 cells transiently transfected with pDNA3/SGS-P6PRK were cultured on a Chamber Slide System (Nalgene Nunc International, Tokyo, Japan) for 20 h. Cells on the slide were washed with PBS and fixed with 3.7% paraformaldehyde solution for 20 min at room temperature. The solution was then changed to 0.1% Triton X-100 in PBS and incubated for 10 min. Cells were washed with PBS three times and incubated with 1% bovine serum albumin, and 0.1% Tween 20 (BSA-PBST) for 1 h at room temperature.

An in vitro phosphorylation assay based on immunoblotting was carried out using gel-bound GST-P6PRK and recombinant p53 peptide. For the establishment of recombinant GST-p53 peptide, a PCR reaction was carried out using specific primers (Recp53F-BamHI: GCGCGATCCTGAGGAGGAGCCGACGTCG and Recp53BB-EcoRI: CCGGAAATCTAAGGACTGCTGTTGCG) and pcDNA3-p53 vector that had been made earlier in our laboratory as a template. The PCR product was digested by BamHI and EcoRI and ligated with pGEX-2TK vector. DH10B E. coli cells were transformed by electroporation, and several colonies were picked up and subjected to sequence analysis. BL21 E. coli cells were transformed, and recombinant material was prepared as described above. Recombinant GST-p53 peptide was eluted by 100 mM Tris-HCl, pH 8.8 with 20 mM glutathione (Sigma) and was analyzed by SDS-PAGE. After incubation with COS-7 lysate in 1× kinase buffer as described for the in vitro kinase assay, recombinant GST-P6PRK or GST gel was incubated with recombinant p53 peptide at 30 °C for 30 min in a 1× kinase buffer supplemented with 300 μM ATP. Sample buffer was added and heated up to 100 °C for 5 min. Samples were subjected to SDS-PAGE and electroblotted to a nitrocellulose or polyvinylidene difluoride membrane. Immunodetection was carried out using anti-p53 phospho-Ser-15-specific rabbit antibody and an ECL detection system.
Cambridge, UK). The primers used in the PCR were N2F2 and N2B2. Data were analyzed using the Sanger Center home page at www.sanger.ac.uk/RHserver/RHserver.shtml.

Transcriptional Activity Assay—An intracellular transcriptional activity assay of p53, Myc, Rb, and E2F was carried out using a Mercury Pathway Profiling System (CLONTECH, Palo Alto, CA) according to the manufacturer’s instructions to examine the activity of cell signaling pathways. COS-7 cells were transfected with pcDNA3RGS-PRPK or mock vector on the first day. Vectors containing cis-acting enhancer elements were then transfected on the second day. On the third day of culture, the cells were harvested using a lysis buffer in a luciferase reporter assay kit (CLONTECH), and their luminescent activities were analyzed by exposing the reaction mixture to BioMax film. The intensities of the spots were calculated using PhotoShop software (Adobe Systems Inc., San Jose, CA).

Yeast Disruption Study—Disruption of the YGR262c gene in yeast was carried out as reported previously (10). The DNA cassette for YGR262c disruption was prepared by three-step PCR using specific primers of P1: 5'-CATATTGACAGAAGAATTC-3' and P4: 5'-TCGCTGATGTATGCTACAC-3'. To confirm the disruption, the URA3 gene was used as a marker. The cassette was used to replace the target YGR262c gene in the genomic DNA. For the galactose-dependent on-off switching of YGR262c expression, the promoter region of YGR262c was replaced by a GAL10 promoter in the same manner as described above. Finally, a pADNSΔE vector with the open reading frame of YGR262c or human PRPK was prepared, and the GAL10 promoter-replaced yeast cells were transformed by these vectors.

Data Analysis—Data were analyzed in this study using MacVector and DNAsis software on a SuperMac S900 computer (UMAX, Tokyo, Japan), and also at the Internet home page of Genome Net (www.genome.ad.jp/). Alignment analysis was carried out using the ExPaSy home page (www.expasy.ch/tools/scnpsit1.html) and the BCM search launcher page (dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html).

RESULTS

Cloning Human and Mouse PRPK—Human and mouse PRPK were cloned from a human spleen and a mouse fetal tissue library, respectively (Fig. 1). Human PRPK mRNA encodes 253 amino acids, while mouse PRPK mRNA encodes 244. These two proteins show 83% identity and 89% homology. The main difference between them occurs in 9 amino acids at the N-terminal domain. Amino acid sequence alignment of human PRPK and the yeast serine/threonine kinase, YGR262c was carried out (Fig. 2A). Human PRPK and yeast YGR262c show 32% identity and 64% homology. As shown in Fig. 2A, both proteins either lack or have mutations for some typical features of protein kinases. The GXGXXG pattern in the first motif was changed to KXXGXX in human PRPK and SXGXXG in

FIG. 2. Comparisons of PRPK and YGR262c. A, the amino acid sequence alignment of PRPK and yeast serine/threonine kinase, YGR262c. The kinase subdomains are indicated on the bottom, and the typical common amino acid residues are indicated by boxes and shaded areas, respectively. B, schematic representations of YGR262c and human PRPK. The amino acid residue numbers are indicated on the top. PK, protein kinase domain; NLS-BP, bipartite nuclear localization signal.
The second motif, AMK, was changed to FPK and PPK in PRPK and YGR262c, respectively. The RDLXXXN pattern was changed to GDLXXXN in both proteins, and the APE pattern in the VIII motif was changed to VLE in both. Both proteins lack the XI motif. Thus, some characteristics of a protein kinase are partially lost in a very similar manner.

**Motifs and Phylogenetic Analysis of PRPK and YGR262c**

The motifs of YGR262c and human and mouse PRPK were analyzed (Fig. 2B). YGR262c possesses only one motif of a protein kinase (PK), while both human and mouse PRPK also possess the bipartite nuclear localization signal (NLS-BP). This suggests that PRPK is a nuclear protein. Phylogenetic analysis of PRPK, YGR262c, and their hypothetical relative proteins in various species showed that human and mouse PRPKs are the most closely related among them (Fig. 3A). The alignment of these proteins in their central regions are depicted (Fig. 3B).

**Northern Blot and RT-PCR Analysis**

The Northern blot analysis was carried out using normal human tissues (Fig. 4). Testicular tissue markedly expressed PRPK mRNA. The size of mRNA was compatible with the cloned PRPK data of 1 kilobase pair. While PRPK was expressed faintly in heart, kidney, and spleen tissues, it did not appear to be expressed in any other normal human tissues.

RT-PCR analysis was carried out using the cDNA of human cancer cell lines and mLT-positive and mLT-negative cytotoxic T-cells (Fig. 5). In cancer cell lines, pancreatic cancer cells such as AsPC-1, PANC-1, and MIA PaCa-2 cells highly expressed...
PRPK mRNA. MDA-MB-231 human breast cancer cells and LNCaP human prostatic cancer cells also expressed PRPK weakly. HT-29 and WiDr human colon cancer cell lines or lymphotoxin-α-producing B-cell lines of Daudi and RPMI 1788 did not appear to express PRPK mRNA. Thus, some human adherent cancer cell lines of epithelial origin but not B-cell lymphoid tumor cell lines expressed PRPK. The activated form of mLT-positive cytotoxic T-cells highly expressed PRPK mRNA, while the deactivated form of mLT-negative cytotoxic T-cells did not. The hyperactivated form of mLT double positive cytotoxic T-cells, i.e. PMA-stimulated cytotoxic T-cells, also expressed it. Because IL-2-activated cytotoxic T-cell preparation does not consist of 100% CD3-positive cells, but contains less than 5% of NK-originated cells, panning was carried out using anti-CD3 mouse monoclonal antibody, followed by RT-PCR analysis. RT-PCR revealed that the panned cells also highly expressed PRPK mRNA (data not shown).

Western Blotting—The anti-PRPK antibody was employed in the Western blot analysis (Fig. 6). A band was seen at 31 kDa in the untransfected 293T cells. By transfection of the pcDNA3RGS-PRPK vector to 293T cells, a band of RGS-His6-tagged PRPK was revealed having almost the same size as the natural PRPK. Anti-RGS-His antibody showed a responsible band of the same size in the transiently transfected 293T cells (data not shown). PRPK protein expression was also seen in MIA PaCa-2, PANc-1, and MDA-MB-231 cells of epithelial origin, IL-2-activated cytotoxic T-cells, with or without panning by anti-CD3 monoclonal antibody, also expressed PRPK protein (data not shown). Finally, PRPK protein was also expressed in HUT 78 human T-cell lymphoma (data not shown).

In Vitro Kinase Assay—Recombinant PRPK was used for the in vitro kinase assay in the gel-bound form after activation by COS-7 lysate. Phosphorylation of the casein was evident with Mg2+ (Fig. 7) but not Mn2+ (data not shown). No autophosphorylation band of GST-PRPK was seen. Thus, PRPK was shown to be kinetically active in vitro. When using the nonactivated form of recombinant PRPK, only a faint band was seen (data not shown).

Intracellular Localization of PRPK—RGS-His-tagged PRPK was transfected to COS-7 cells, and the cells were stained by anti-RGS-His monoclonal antibody in situ (Fig. 8A). PRPK protein expression was localized in the nucleus, forming a clump. Anti-p53 antibody revealed homogenous nuclear staining of the COS-7 cells with (Fig. 8B) or without (data not shown) PRPK co-transfection. As a control staining for the identification of the nucleus and cytosol of COS-7 cells, hematoxylin-eosin staining was carried out (Fig. 8C). The control samples using nonspecific mouse IgG did not reveal any reaction in this procedure (data not shown). This result was consistent with the existence of a bipartite nuclear localization signal motif in the PRPK molecule (Fig. 2B).

Yeast Complementation Assay—Using the YGR262c-disrupted yeast, a complementation assay was carried out (Fig. 9). The YGR262c disruptant showed severely defective growth as reported previously (11). The GAL10 promoter was replaced with the promoter region of YGR262c, and then transformed by the pADNS vector inserted with or without YGR262c or human PRPK. The mock transfectant showed normal growth on the galactose plate when YGR262c was not expressed (Fig. 9A). When cultured on the glucose plate, the yeast showed severely defective growth (Fig. 9B). YGR262c clearly restored the growth, but PRPK did not (Fig. 9B).

FISH Analysis—The location of the human PRPK gene was determined using a cloned human BAC. The initial experiment using a specific BAC clone showed labeling on the long arm of a group F chromosome that was thought to be chromosome 20 on the basis of the size, morphology, and banding pattern. The second experiment was then carried out using a biotin-labeled specific probe for the centromere of chromosome 20, and it was cohybridized with a BAC clone 233L17 (Fig. 10). The specific labeling of the centromere in red and the long arm in green of chromosome 20 were observed. Measurements of 10 specifically labeled chromosome 20 demonstrated that the clone 233L17 is located 69% of the way from the centromere to the telomere of chromosome arm 20q, an area, which corresponds to band...
20q13.2. A total of 80 metaphase cells were analyzed with 72 specific labeling. This result was almost compatible with Ramsey's report of dJ28H20.2 at 20q13.1 (GenBank™ accession number: CAC00561, only published in the data base).

A radiation hybrid analysis showed that the PRPK gene is located at AFM165xh2, which is compatible with the location at 20q13.2 of the PRPK gene (data not shown).

Transcriptional Activity Analysis—An intrinsic transcriptional activity analysis using vectors possessing specific cis-acting enhancer elements was carried out (Fig. 11). A significant up-regulation of p53 activity was repeatedly evident in the PRPK-transfected COS-7 cells. No significant difference was seen, however, in the transcriptional activities of Myc, E2F, or Rb. In addition, no significant difference was seen in the negative control using pTA vector.

Phosphorylation of p53 in Vivo—Expression levels of PRPK in COS-7 cells with or without transient PRPK transfection were compared using anti-PRPK specific antibody, and phosphorylation of p53 in COS-7 cells was analyzed using phospho-specific antibody (Fig. 12). When COS-7 cells were transfected with pcDNA3RGS-PRPK or kinase-negative pcDNA3RGS-PRPK-D183N vector, PRPK protein expression levels detected by the anti-PRPK antibody were highly elevated (Fig. 12A).

Anti-phospho-Ser-15-specific rabbit polyclonal antibody revealed a band in the PRPK-transfected COS-7 cells. The low expression of [Ser(P)-15]p53 in the kinase-negative PRPK-transfected COS-7 cells confirmed this up-regulation (Fig. 12B).

The expressions of p53 and [Ser(P)-15]p53 were analyzed using PRPK-stable transfectants established by COS-7 cells (Fig. 13). A correlation was seen between the expressions of transfected RGS-His-tagged PRPK and the phosphorylation levels of p53 at Ser-15. The total p53 expression levels were comparable within these cells.

Binding of PRPK to p53—The binding activity of PRPK with p53 in vitro was analyzed using gel-bound recombinant GST-tagged PRPK and COS-7 lysate. GST-PRPK evidently bound to p53, while GST protein did not (Fig. 14). A binding analysis using anti-PRPK antibody to COS-7 cell lysate also showed this association (data not shown).

Phosphorylation of p53 in Vitro—In vitro phosphorylation of p53 was examined using gel-bound recombinant PRPK, recombinant p53 peptide-(1−80) and anti-phospho-Ser-15 specific antibody. PRPK phosphorylated p53 at Ser-15 (Fig. 15). Recombinant PRPK was also shown to be bound to p53 peptide-(1−80) in vitro (data not shown).

DISCUSSION

In this study, a protein kinase, PRPK, was identified from the cytotoxic T-cell subtraction library, and the cDNA of both human and mice were cloned. The translated protein showed that PRPK is closely related with the yeast serine/threonine kinase YGR262c (11, 12). The BLAST analysis of the cDNA data also suggested a close relationship between these two kinases (data not shown). YGR262c protein is known to be a growth-related protein kinase of yeast because the disruptant of this gene causes severely defective growth (11). Therefore, PRPK function was expected to be related to proliferation of cells.

Some of the typical amino acid sequence motif patterns of protein kinases are partly absent in both PRPK and YGR262c (11). These were seen in the I, II, VIB, and VIII subdomains. For example, the GXGXXG pattern in the first subdomain of PRPK is replaced by KXXXXA. In YGR262c, this same pattern was replaced by SXGXXA (11). These deficient patterns closely resemble one another. In addition, both kinases are completely
devoid of the XI motif. This evidence also strongly supports the idea that PRPK is a human and mouse homologue of the yeast YGR262c.

YGR262c is classified as a unique kinase of *Saccharomyces cerevisiae* (12). It has been shown to have homologues in other species, i.e. the apple tree calcium/calmodulin-binding protein (PIR:JQ2251) and *O*-sialoglycoprotein endopeptidase of *Methanococcus jannaschii* (12). Short homologous kinases of YGR262c/PRPK, which could form a new kinase family, have recently been identified in other species. Although PRPK and YGR262c are structurally similar, the present yeast complementation assay showed that they are not necessarily functional homologues. The severely defective growth of the YGR262c disruptant was not complemented by a transformation of PRPK. This evidence may indicate that the substrate of YGR262c is not phosphorylated by PRPK or that the subcellular localization of these kinases is different. Both human and mouse PRPK possess a bipartite nuclear localization signal (NLS-BP), but YGR262c does not (13). Our results showed that PRPK is localized in the nucleus, a finding that is compatible with this signal motif. Many protein kinases work within the nucleus (14). Although YGR262c is predicted to be a membrane protein (12), there is no actual data on the subcellular localization of YGR262c in the yeast at present.

YGR262c kinase requires Mn$^{2+}$ or Ca$^{2+}$ ions for its catalytic activity (11), while PRPK works with Mg$^{2+}$. The functions of YGR262c may be associated with the Golgi apparatus, where
the Ca$^{2+}$/Mn$^{2+}$/Mg$^{2+}$-dependent Golgi casein kinase functions (11). We could not show any catalytic activity in PRPK with Mn$^{2+}$ or Ca$^{2+}$ ions (data not shown). PRPK is also different from YGR262c in this respect.

The chromosomal localization of the human PRPK gene was found to be 20q13.2 by FISH analysis using a BAC clone. The radiation hybrid support resulted this finding. This result was nearly compatible with Ramsay’s localization at the dJ28H20.2 gene at 20q13.1 (published only in the data base). Several protein kinases have been found on human chromosome 20: the PTK6 protein tyrosine kinase at 20q13.3 (15), as well as a Src family HCK hematopoietic cell kinase (16), hemopoietic cell kinase, protein tyrosine kinase at 20q13.3 (15), as well as a Src family found to be 20q13.2 by FISH analysis using a BAC clone. The YGR262c was close to those of STK 4 (20q11.2–q13.2), STK 6 (20q13.2–q13.3) and STK 15 (20q13.2–13.3).

The preactivation of recombinant PRPK was necessary for its phosphorylation activity. When the non-activated form was used in these experiments, the level of phosphorylation was marginal (data not shown). This result may indicate that phosphorylation of some critical amino acid of PRPK could be necessary for the enzymatic activity, or that some binding partner could be necessary for the PRPK activity.

The intrinsic transcriptional activity assay in the cells showed that PRPK up-regulates p53 activity. This finding was confirmed by the evidence of phosphorylation of p53 at Ser-15 both in vivo and in vitro, which is important for p53 activation. Thus, the functional role of PRPK appears to be quite different from that of its structural homolog, yeast YGR262c. When the PRPK-stable COS-7 transfectant highly expressed PRPK, the rate of cell growth was slow (data not shown). Established PRPK-expressing T-cell lymphoma lines also showed a cell growth rate that was quite slow, and were found to be as prone to death as when the terminal proliferation phase of IL-2 activated cytotoxic T-cells, which highly express PRPK (data not shown). This evidence may support the conclusion that PRPK phosphorylates and activates p53. In contrast, the specific substrate for YGR262c was not identified in the yeast, which does not possess a p53 homolog.

The arrest of the cell cycle through the phosphorylation of p53 at Ser-15 was induced by ATM, ATR, and DNA-PK (18–20). Both p38 and ERK have been reported to phosphorylate p53 at Ser-15 (21). In addition to these kinases, PRPK may also modulate apoptosis and cell-cycle arrest by phosphorylating Ser-15 of p53. The expression of PRPK was evident in some cancer cell lines of epithelial cell origin. PRPK may play an important role in the epithelial malignant tumor cells. On the other hand, PRPK was evidently not expressed in the B-cell lymphoid tumor cell lines in this study. Though some human T-cell lymphoma and erythroleukemic cell lines do express this kinase (data not shown), the role of PRPK in lymphoid cells and granulocytes should be studied further.

mRNA expression revealed by Northern blot analysis showed that PRPK may be functioning considerably in the testes in vivo. Testis-specific protein kinases have been reported (22–24). Both tssk1 and tssk2 appear in the adult testis and may play a role in the last stage of spermatid maturation (24). TESK1, which is found in mouse and rat testicular germ cells, is mainly expressed in round spermatids (25). PRPK may function as an apoptosis inducer or cell-cycle modulator via p53 activation in the spermatogenesis.

PRPK is a short kinase similar to Cdk and some others (26, 27). Cdk must bind with individual cyclin partners in order to exert its activity (27). It is not known whether PRPK requires a binding partner for the phosphorylation of p53. Our recent work indicates that PRPK has a binding protein (data not shown). Further study of this binding partner could elucidate the regulatory mechanism of PRPK activity.

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