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A Small Ras-like protein Ray/Rab1c modulates the p53-regulating activity of PRPK ☆

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Abstract

PRPK phosphorylates serine-15 residue of p53 and enhances transcriptional activity. PRPK possesses a bipartite nuclear localization signal and localizes in nucleus when over-expressed in cells. However, intrinsic PRPK localizes mainly in the cytosol in situ. While studying the mechanisms in the distribution of intrinsic PRPK, we identified a PRPK binding protein, an ubiquitously expressed Small Ras-like GTPase, Rab1c, also named Ray or Rab35. The over-expressed Ray was distributed in the nucleus, cytosol, and cell membrane. Both Ray wild type and GTP-restrictively binding mutant Ray-Q67L, but not guanine nucleotide unstable binding mutant Ray-N120I, partially distributed the over-expressed PRPK to the cytosol and also suppressed the PRPK-induced p53-transcriptional activity profoundly. A Small Ras-like GTPase protein Ray was thus indicated to modulate p53 transcriptional activity of PRPK.
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Keywords: Ray/Rab1c; PRPK; p53; Nucleus; Cytosol

PRPK is a human orthologue of the yeast YGR262c gene product, piD261/Bud32 [1,2]. It was originally identified from the activated T-cell/lymphokine-activated killer (LAK) cell-subtraction library and expected to activate or enhance the ability of adoptive immune cells applied to cancer therapy [1]. YGR262c gene product in yeast plays an important role in cell proliferation [1,3]. In despite of its significant homology with YGR262c, human PRPK does not complement the defective growth of YGR262c-disrupted yeast completely [1,3]. In the PRPK and YGR262c gene product, a dissimilarity was seen in the nuclear localization signal (NLS) in them: human PRPK possesses bipartite NLS but yeast homologue does not. Indeed, human PRPK localizes to the nucleus when it is over-expressed by transfection.

We found that PRPK phosphorylates serine-15 residue of p53 and enhances p53-transcriptional activity in cells [1]. YGR262c gene product also phosphorylates serine-15 residue of p53 [3], however, yeast does not possess p53 equivalent factor [3]. As a result, this phosphorylation activity would be due to its structural resemblance to human PRPK. PRPK-activity thus appears to be "inhibitory" rather than "activating" or "proliferative" in human and mice [1].

☆ *Abbreviations:* PRPK, p53-related protein kinase; NLS, nuclear localization signal; PBS, phosphate-buffered saline; WT, wild type; LAK, lymphokine-activated killer; GST, glutathione-*S*-transferase.

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Transformed culturing cells such as COS-7 monkey fibroblast and HEK293 human kidney fibroblast strongly express PRPK in situ [1]. These cells are highly proliferative in spite of their elevated p53-expression which is functionally suppressed by the viral T-antigen [4–6]. Numerous established culturing cells without viral transformation, such as HeLa human cervical cancer and U-2 OS human osteosarcoma cells, also express PRPK [7]. On the other hand, among normal human tissues, the testis expresses PRPK strongly [1]. The testis is an organ where numerous proliferating cells, i.e., spermatogenic germ cells exist. Though possessing an inhibitory activity, PRPK thus somehow expressed in cells and tissues possessing a high proliferation activity.

In addition, its promoting activity on p53, biological role of PRPK in cell proliferation is still unclear [1]. While addressing its biological significance in cells, we found an intriguing discrepancy in the intracellular distribution of PRPK between intrinsically expressed and extrinsically transfected proteins. When over-expressed, PRPK localizes to the nucleus exclusively, however, the intrinsic PRPK is mainly distributed to the cytosol in situ. To explain this, we sought the binding proteins for PRPK and identified a ubiquitously expressed small Ras-like GTPase protein, Rab1c, also named Ray [8]. We carried out experiments on Ray and PRPK in this study.

Materials and methods

Cloning. To identify PRPK-binding proteins, a pull-down analysis using Glutathione-*S*-transferase-PRPK (GST-PRPK) and HeLa cell lysate was carried out. Recombinant GST-PRPK bound glutathione–Sepharose 4B gel (Amersham Biosciences, Piscataway, NJ), 50 μl, was mixed with 1 ml of HeLa cell lysate at 5×10^6 cells/ml in lysis buffer (0.1% NP-40, 1 mM PMSF, 1× complete protease inhibitor cocktail set (Roche, Mannheim, Germany) in phosphate-buffered saline (PBS)) overnight. After washing with lysis buffer, gel was treated by sample buffer and applied to SDS–PAGE followed by a Coomassie brilliant blue staining. Specific bands were identified and excised from the gel, and binding proteins were analyzed by the peptide mass fingerprinting (PMF) method at the ProteinProspector site (http://prospector.ucsf.edu/) using a MALDI TOF-MS, Voyager DE Pro (Applied Biosystems, Tokyo, Japan).

Ray was cloned using specific primers (RayF-*Bam*HI: 5′-CGC GGATCCATGGCCCGGGACTACGACCACCTCT-3′; RayB-*Eco*RI: 5′-CCGGAATTCGCCATTAGCAGCAGCGTTTC-3′), cDNA prepared from HeLa cells and LA Taq (Takara, Tokyo, Japan) into pcDNA3-HA (Invitrogen, Carlsbad, CA) and pQBI 25-fC3 (Qbiogene, Irvine CA) vectors after digestion by *Bam*HI and *Eco*RI (Fermentas, Vilnuis, Lithuania). Ray mutants were prepared using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). PCR step was carried out using Pfu Turbo DNA polymerase (Stratagene), the cloned Ray vector as a template, and specific primers: N120I-F: 5′-GCCGAATATTAGTGG GTATTAAGAATGACGACCCTGAGC-3′; N120I-B: 5′-GCTCAGGG TCGTCATTCTTAATACCCACTAATATTCGGC-3′, or Q67L-F: 5′-G ACACAGCGGGGCTGGAGCGCTTCCGC-3′; Q67L-B: 5′-GCGGAA GCGCTCCAGCCCCGCTGTGTC-3′. Chemically competent XL1-Blue *Escherichia coli* cells (Stratagene) were transformed by the PCR products after digestion with *Dpn*I (Stratagene). The sequences were confirmed using a 310 Genetic Analyzer (Applied Biosystems), a Big Dye terminator system (Applied Biosystems), and specific sequencing primers.

Recombinant proteins and mutants. GST fusion proteins of PRPK and Ray were prepared by PCR cloning into pGEX-6P-2 vector (Amersham Biosciences) using LA-Taq, specific primers, and pcDNA3-HA-PRPK or -Ray vector as a template. Fusion proteins were induced by the addition of 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 2.5 h after pre-culturing. *E. coli* cells were centrifuged to precipitate and then were dissolved by a freeze and thaw method followed by sharing of genomic DNA using a 18 G needle and syringe, and sonication in lysis buffer (20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM EsDTA, and 0.5% NP-40) with 100 μg/ml lysozyme (Wako, Tokyo, Japan). Glutathione–Sepharose 4B was added to the supernatant of cell lysate (500 μl suspended gel to the 1,000 ml culture) and washed with lysis buffer. GST fusion proteins were eluted from the glutathione–Sepharose 4B gel using elution buffer (20 mM Tris–HCl, pH 9.6, 120 mM NaCl, and 20 mM glutathione). The protein concentrations were estimated using a D/C protein assay kit (Bio-Rad, Tokyo, Japan).

Cells. Cells used in this study were: COS-7 monkey SV40 transformed fibroblast cells, HeLa human cervical cancer, and U2-OS human osteosarcoma cells. These cells were cultivated with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (10% DMEM) and antibiotics at 37 °C in 5% CO_2.

For synchronization of HeLa cells, cell-cycle was blocked in the early S phase by thymidine double block method. HeLa cells were incubated with 2.5 mM thymidine (Sigma–Aldrich, St. Louis, MO) in 10% DMEM for 22 h at 37 °C in 5% CO_2, followed by two washings with PBS, and further incubation for 10 h in 10% DMEM at 37 °C in 5% CO_2. Cells were then incubated with 1 mM hydroxyurea for 15 h at 37 °C, 5% CO_2, followed by two washings with PBS, and released from the block by incubation with fresh medium. The cells were collected hourly.

Antibodies. Anti-PRPK rabbit polyclonal antibody was prepared by immunization to rabbits with recombinant GST fusion PRPK protein. Antiserum was collected and purified by protein G–Sepharose 4FF followed by absorption of anti-GST content using GST–Sepharose 4B. This antibody reacted with both intrinsically expressed and extrinsically transfected PRPK in Western blotting. Anti-SV40 T-antigen (Clone Pab 108) and anti-cyclin B1 (Clone GNS1) mouse monoclonal antibodies for Western blotting and anti-HA rabbit polyclonal antibody for immunoprecipitation were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-α-tubulin (Clone DM1A) mouse monoclonal antibody was purchased from Sigma–Aldrich. Anti-p53 mouse monoclonal (DO-7) and anti-p53-phospho-serine-15 rabbit polyclonal antibodies were purchased from DAKO (Tokyo, Japan) and Cell Signaling Technology (Beverly, MA), respectively.

Transfection experiments. Plasmids were prepared using a Maxi prep kit (Qiagen, Tokyo, Japan). Transfection of cells was carried out using TransFast reagent (Promega, Madison, WI). The cells were grown in a $\phi = 60$ mm tissue culture dish overnight. After the aspiration of medium, a mixed solution of 6.0 μg of plasmid and 18 μl of TransFast reagent in 2.0 ml of serum free DMEM was added, followed by incubation for 1 h at 37 °C in 5% CO_2. Four milliliters of 10% DMEM was then added and cultivated for 2 or 3 days.

Western blotting and immunoprecipitation. Western blotting was carried out using a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) and an ECL detection system (Amersham Biosciences). Samples were subjected to SDS–PAGE and transferred to the membrane using a semi-dry blotter (BioCraft, Tokyo, Japan) for 120 min at 1 mA/cm^2. The membrane was then immersed into blocking solution of 5% skim milk in PBS with 0.1% Tween 20 for 60 min h at room temperature. The membrane was incubated with antibody solution diluted in PBS, 5% bovine serum albumin, and 0.1% Tween 20 (BSA–PBST) for 60 min at room temperature and washed three times with washing buffer, 0.1% Tween 20, 0.15 M NaCl. The membrane was then incubated with horseradish peroxidase conjugated with anti-mouse or anti-rabbit antibody (Santa Cruz Biotechnology) in BSA–PBST for 60 min at room temperature and washed four times with washing buffer. The membrane was developed using ECL solution and exposed to a X-ray film (Hyper Film; Amersham Biosciences).

For preparation of cytosol- and nucleus-rich fractions, cells were treated with 1:10 PBS in water with the addition of 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 1x Complete protease inhibitor

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