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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.

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

cocktail set. After incubation for 15 min on ice, cells were homogenized using a Dounce tissue homogenizer (Wheaton, Millville, NJ) with occasional microscopic observation. After homogenization, the nuclei were precipitated by centrifugation at 500*g* for 5 min at 4 °C. SDS–PAGE sample buffer was added to the total cell, cytosol, and nucleus fractions up to the same final volume per used cell number (1 ml for 5×10^6 cells).

For immunoprecipitation, the cell lysate was prepared as follows: cells were washed once with ice-cold PBS and treated with lysis buffer consisting of 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 1× Complete protease inhibitor cocktail set at 5×10^6 cells/ml. The cells were sonicated and centrifuged at 16,000*g* for 12 min at 4 °C. The supernatants of cell lysates were incubated with the indicated antibodies at 4 °C overnight. Protein G–Sepharose 4FF gel was added and incubated for 1 h at 4 °C. The precipitates were washed three times with lysis buffer and subjected to SDS–PAGE followed by immunoblotting as described above.

Pull-down analysis using GST fusion proteins was carried out as follows: COS-7 cell lysate at 10^6 cells/ml with transfection of expression vectors was mixed with GST fusion proteins bound to glutathione–Sepharose 4B gel suspension, 20 μl, overnight at 4 °C. After washing four times with lysis buffer, sample buffer was added to each sample and subjected to SDS–PAGE followed by an immunodetection using antibodies as indicated.

Fluorescent immunohistochemical analysis. Cells were grown on 4-well chamber culture slides (Nalgene Nunc International, Tokyo, Japan). The cells were washed once with PBS and fixed with methanol at −20 °C for 10 min. After fixation, cells were incubated with 3% bovine serum albumin in PBS with 0.01% NaN_3 (BSA–PBS) for 30 min at room temperature. The primary antibodies used in this study were: anti-PRPK, anti-p53-phospho-serine-15 rabbit polyclonal antibodies and anti-p53 mouse monoclonal antibody. Antibodies were diluted in BSA–PBS and incubated for 30 min at room temperature. After washing four times with PBS, secondary antibody solution in BSA–PBS was added and incubated for 30 min at room temperature. The secondary antibodies used were: Alexa Fluor 488- and 568-conjugated anti-mouse or rabbit IgG (Molecular Probes, Eugene, OR). DNA was stained with 0.5 μg/ml of 4′,6-diamidino-2-phenylindole (DAPI). Fluorescent fusion proteins were expressed using pQBI 25-fC3 or pHcRed-Tandem-C3 (Evrogen, Moscow, Russia) vector. Immunofluorescent microscopy was performed using a BX51 immunofluorescent microscope with DP70 digital camera (Olympus, Tokyo, Japan). Images were managed using an Apple PowerMac G4 computer with Adobe Photoshop 7.0 software (Adobe Systems, San Jose, CA).

GTP-binding assay. GTP-binding activity of Ray and its mutants were analyzed according a previous report [9] using [α-^{32}P]GTP (Amersham Biosciences). Shortly thereafter, recombinant GST fusion proteins were subjected to SDS–PAGE and blotted to a PVDF membrane as described above. A set of membrane was stained by CBB and destained by 70% ethanol, 7% acetic acid. Another set of membrane was blocked in 10 μM $MgCl_2$, 2 mM dithiothreitol, 0.2% Tween 20, and 4 μM ATP in PBS (GTP-binding buffer) and incubated with [α-^{32}P]GTP, 1 μC/ml in GTP-binding buffer at room temperature for 2 h. The membrane was washed with GTP-binding buffer for 1 h with four buffer changes. After drying, the membrane was exposed to a X-ray film (Hyper Film) overnight.

Flow cytometric analysis. Synchronized HeLa cells were harvested by trypsinization and fixed with 70% ethanol at −20 C. After washing with PBS, the cells were mixed with RNase, 100 U/ml (Sigma–Aldrich), and 10 μg/ml of propidium iodide (Sigma–Aldrich) and then subjected to the flow cytometric analysis using a FACSCalibur (BD Biosciences, San Jose, CA).

p53 transcriptional activity reporter assay. An intracellular transcriptional activity of p53 was estimated using pGluc vector system (Tarteting systems, CA). The p53 binding promoter sequence (5′-GGA CATGCCCGGGCATGTCC-3′) [10] was cloned into pGluc-Basic-1 vector. COS-7 cells were transfected with pcDNA3-HA-Ray and its mutants, HA-PRPK or mock vectors on the first day. The pGluc containing p53-promoter binding sequence was transfected on the second day to these cells. On the fourth day of culture, supernatants were collected and their luminescent activities were analyzed using a Luminescencer JNR AB2100-R (ATTO, Tokyo, Japan).

Results and discussion

Extrinsic and intrinsic PRPK expressions in cells

PRPK possesses a bipartite NLS in the middle of molecule [1]. In fact, PRPK localizes in the nucleus when over-expressed using mammalian expression vectors (Fig. 1A-a) [1]. When expression levels of the transfected PRPK are very high, it forms some aggregates in the nucleus [1]. Only a small amount of transfected PRPK allocates in the cytosol (Fig. 1A). To analyze the intrinsic expression of PRPK in situ, we made an antibody that could be used for the Western blotting and fluorescent immunohistochemical analysis of PRPK. This antibody revealed intriguing evidence that PRPK does not evidently localize to the nucleus but it rather distributes throughout the cytosol in HeLa human cervical cancer cells (Fig. 1A). The same results were observed when using other cells such as U-2 OS and COS-7 cells. To confirm the cytosolic distribution of PRPK in situ, a Western blotting analysis of cytosol- and nucleus-rich fractions of COS-7 cells was carried out using SV40 T antigen as a nucleus marker (Fig. 1B). This confirmed that intrinsic PRPK localizes mainly in the cytosol rather than the nucleus. Intrinsic PRPK thus distributes mainly to the cytosol and only weakly to the nucleus in situ. In contrast, over-expressed PRPK localized to the nucleus, somehow.

Protein kinases possess functional bipartite NLS have been identified [11–14]. Some will go through the nuclear membrane depending on Ran-importin system [13,14]. Human PRPK also possesses bipartite NLS at residues 78–95 [1]. However, as presented herein, the intrinsic PRPK localized mainly to the cytosol in situ. Some binding proteins will support moving PRPK from the nucleus to the cytosol or they will inhibit transportation of PRPK into the nucleus. We then sought binding proteins for PRPK.

Cloning Ray and pull-down analysis

Using recombinant GST-PRPK protein and HeLa cell lysate, binding proteins were investigated. A specific band at 28 kDa was evident in SDS–PAGE (Fig. 1C). Protein identification using the peptide mass fingerprinting method with a MALDI TOF-MS machine revealed a small Ras-like GTPase protein, Rab1c (Rab35) also named Ray (Fig. 1D). Cloning of Ray was then carried out using HeLa cell cDNA as a template. HA-tagged Ray vector was transfected to COS-7 cells and lysate was mixed with GST-PRPK- or GST-glutathione gel. A pull-down analysis showed that Ray binds to PRPK (Fig. 1E). A reverse binding analysis was carried out using intrinsic PRPK and recombinant GST-Ray fusion protein. Intrinsic PRPK in COS-7 cells were pulled down by GST-Ray fusion protein (Fig. 1E). Ray was thus shown to bind to PRPK by a pull-down analysis.

Several Ras-like small GTPase proteins bind to protein kinases. Rab8 binds to the mitogen activated protein

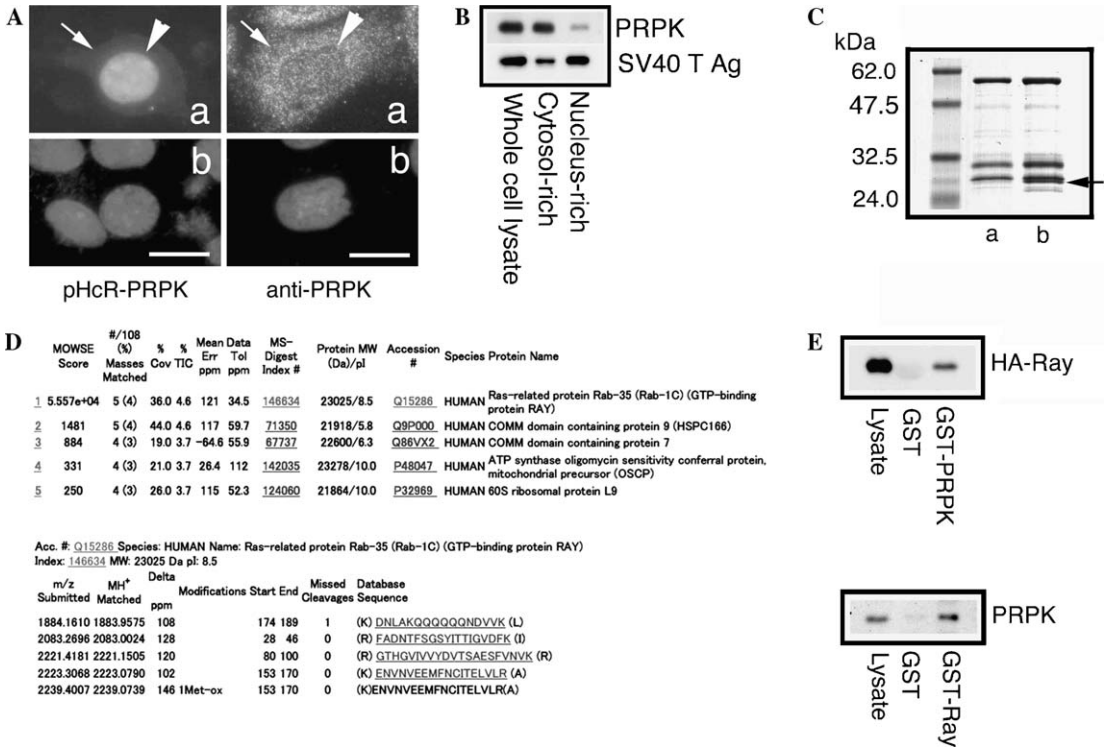

Fig. 1. Nuclear and cytosolic distribution of PRPK, and the identification of PRPK binding protein, Ray/Rab1c. (A) PRPK was transfected to HeLa cells using a fluorescent fusion protein expression vector, pHcRed (pHcR-PRPK, a). Intrinsic expression of PRPK was immunofluorescently detected by anti-PRPK rabbit IgG (anti-PRPK, a). Nucleus was stained by DAPI (b). White bar indicates 20 μm. (B) A Western blotting analysis of intrinsic PRPK expression in the cytosol- and nucleus-rich fractions of COS-7 cells. Each fraction was prepared by hypotonic cell lysis followed by a Dounce homogenization. SV40 T antigen was used for a nucleus maker. The intrinsic PRPK localizes mainly to the cytosol rather than nucleus. (C) Identification of PRPK-binding proteins using GST-PRPK and HeLa cell lysate. After performing the pull-down procedure, gel was subjected to SDS–PAGE and stained by Coomassie brilliant blue. The arrow indicates the specific band of PRPK binding protein. (D) The analyzed data obtained from the excised gel using MALDI TOF-MS and the mass fingerprinting method. The protein was identified to be Ray/Rab1c. (E) A pull-down assay using HA-Ray transfected COS-7 cell lysate and GST-PRPK (upper panel). Ray was detected by anti-HA antibody. A reverse pull-down assay was carried out using intrinsic PRPK and GST-Ray (lower panel). PRPK was detected by anti-PRPK antibody. As a result, Ray was shown to bind to PRPK.

kinase kinase kinase kinase 2 (MAPKKK2) and it acts as a vesicular transporter [15]. Rab13 binds to PKA and inhibits its kinase activity [16]. Rab11 binds to phosphatidylinositol 4-kinase β and regulates the membrane transport from Golgi complex to the plasma membrane [17]. A small Ras-like GTPase protein, Ray, has been shown to bind to PRPK. Ray was initially identified from human fetal skeletal muscle [8]. According to that report, Ray was found to be ubiquitously in human tissue [8]. Because of general implementations of Rab proteins in the intracellular transport, we investigated the role of Ray in the intracellular localization and biological activity of PRPK.

Expression of Ray and its mutants in cells

The alignment of Ray/Rab1c and its close relatives, Rab1a and Rab1b, was analyzed by the CLUSTALW method at http://align.genome.jp/ (Fig. 2A). When compared to Rab1a and Rab1b, Ray was unique in its base-rich short amino acid sequence, KRKKR, at C-terminus. This type of C-terminal polybasic region (PBR) in small GTPase proteins has been identified in several Ras-like proteins. This sequence can be attributed to the nuclear localization, membrane association, and interaction with binding proteins [18]. Theoretically, the short basic amino acid sequence in Ray can also be associated with these three possibilities. To address the role of this basic region at C-terminal, we made a Ray mutant that possesses QQQQQ (Ray-PBRQ) instead of KRKKR and analyzed the intracellular distribution by transfection experiment. While wild type Ray (Ray-WT) distributed throughout the cytosol, nucleus, and membrane, Ray-PBRQ localized to the cytosol exclusively (Fig. 2B). This short basic sequence contributes at least to the nuclear localization of Ray. Another unique point in the Ray molecule is the existence of a poly-glutamine sequence, QQQQQQ, at the near C-terminus, however, its significance is unclear at this moment.

We then carried out transfection experiments to see the intracellular localization of Ray concerning guanine nucleotide binding status. For this analysis, we prepared Ray mutants, one is an unstable guanine nucleotide binding mutant, Ray-N120I (dominant negative form), and the other is a GTP-restrictively binding mutant, Ray-Q67L (dominant active form). These mutants were created based on a sequence similarity with Rab1a and Rab1b [19–21].

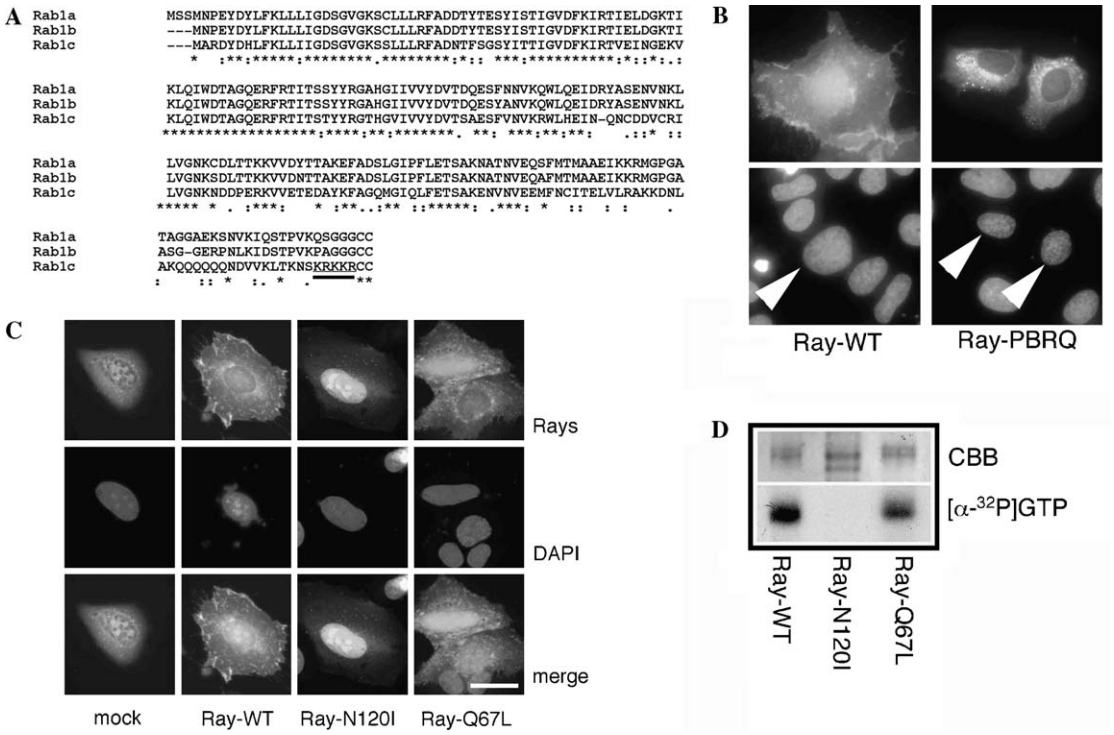

Fig. 2. Alignment of Rab1 proteins with Ray/Rab1c and intracellular localization of Ray. (A) Rab1a and Rab1b was aligned with Ray/Rab1c by the CLASTALW method. Ray was unique for its short polybasic region (PBR, underlined) at the C-terminal. (B) The intracellular localization of Ray and its mutant, where PBR was replaced by neutral amino acids of QQQQQ (Ray-PBRQ), in U-2 OS cells were analyzed using GFP vector pQBI 25. Nucleus was stained by DAPI. While Ray-WT express in the nucleus, cytosol and plasma membrane, Ray-PBRQ mainly in the cytosol. White bar indicates 20 μm. (C) Unlike the localization of Ray-WT, Ray-N120I, a guanine nucleotide unstable binding type, accumulated into the nucleus. Ray-Q67L, GTP-restrictively binding type, distributed in the nucleus, cytosol and plasma membrane as Ray-WT. Nucleus was stained by DAPI. White bar indicates 20 μm. (D) GTP binding activity of Ray and its mutants. After SDS–PAGE, PVDF membrane-blotted recombinant GST fusion proteins were stained by Coomassie brilliant blue (CBB). Another set of blot was incubated with [α-^{32}P]GTP, 1 μCi/ml in GTP-binding buffer. After washing, the membrane was exposed to a X-ray film overnight and then was developed.

Ray and its mutants were transfected to the cells (Fig. 2C). When over-expressed in cells, Ray-WT distributed throughout the cytosol, nucleus, and the membrane as was also shown in Fig. 2B. The membrane association of Ray-WT can be clearly recognized when compared to mock-transfectant. When guanine nucleotide was not stably associated with this molecule as Ray-N120I, it accumulated into the nucleus strongly. GTP-restricted mutant Ray-Q67L distributed throughout the cytosol, nucleus, and to the plasma membrane similar to Ray-WT. As a result, Ray is basically distributed to the nucleus, cytosol, and cell membrane.

To confirm the GTP binding activities of Ray and its mutants, GTP binding assay was carried out using PVDF membrane-blotted proteins (Fig. 2D). As shown in the figure, Ray-WT and Ray-Q67L but not Ray-N120I bound to GTP.

Rab1 localizes to ER and Golgi, and it regulates antegrade protein transport from ER to Golgi [19–22]. Rab1a and Rab1b have not been found in the nucleus and they will not act in shuttling through the nuclear membrane. In contrast, Ray/Rab1c is distributed to the cytosol, nucleus, and plasma membrane. These data indicate that Ray may shuttle between the cytosol and nucleus, where the polybasic sequence and GTP binding status both play an important role [18].

Ray partially distributes PRPK to the cytosol

We then carried out immunoprecipitation analyses using transfected Ray and PRPK in cells to see their binding in vivo (Fig. 3A). Ray and its mutants were thus shown to bind to PRPK in cells. As seen in the figure, Ray-N120I, guanine nucleotide unstable binding type, showed some faster mobility than the other two forms in the SDS–PAGE, which was also seen in the GST-fusion proteins (Fig. 2D). This was repeatedly observed and thought to be due to some conformational change in this form.

Immunofluorescent histochemistry was carried out to see the localization of both PRPK and Ray (Fig. 3B). When over-expressed, PRPK strongly accumulated to the nucleus as seen in the figure "a" of mock transfectants. When Ray-WT was co-expressed, PRPK protein re-localized to the cytosol. However, this typical figure was seen in about 5% to 10% of both the PRPK- and Ray-transfected cells. About 40% of the transfected cells showed both the nuclear and cytosolic distribution of PRPK, while the other 50% of the cells showed the nuclear localization of PRPK. Ray-Q67L

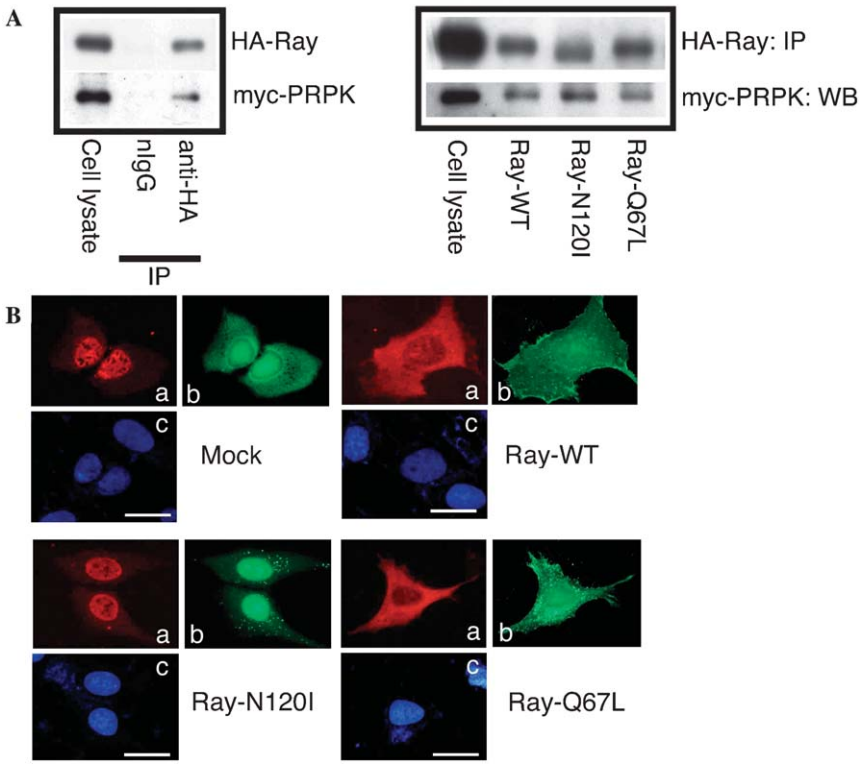

Fig. 3. Binding analysis of Ray with PRPK by immunoprecipitation and effect of Ray on the localization of PRPK. (A) Myc-PRPK and HA-Rays were co-transfected to COS-7 cells and immunoprecipitated using anti-HA rabbit polyclonal antibody. Samples were subjected to SDS–PAGE, and PRPK was detected by anti-myc antibody. As shown, Ray bound to PRPK irrespective to these three forms. (B) Ray and PRPK were transfected to U-2 OS cells using mammalian expression vectors. pHcRed-PRPK (a) and pQBI 25-Rays (b) were co-transfected to U-2 OS cells. Nucleus was stained by DAPI (c). PRPK protein expression was re-localized to the cytosol in 5–10% of Ray-WT transfected cells (Ray-WT). Similar effect on PRPK localization was observed in Ray-Q67L-transfected cells (Ray-Q67L). On the other hand, PRPK remained in the nucleus in Ray-N120I transfected cells (Ray-N120I). The white bar indicates 20 μm.

was also shown to re-distribute some of the transfected PRPK from the nucleus to the cytosol as Ray-WT. Please note that no cytosolic localization of PRPK was observed in the mock or Ray-N120I co-transfectants.

Cell-cycle and PRPK

Because PRPK enhances the p53 transcriptional activity, this kinase can be related with the cell-cycle. The intrinsic expression of PRPK in cell-cycle was analyzed by Western blotting using synchronized HeLa cells and anti-PRPK antibody (Fig. 4A). Expression of PRPK was upregulated in G1-phase, as seen at 0–3 h after the release from cell-cycle block. PRPK was thus suggested to be a G1-kinase. Via activating p53, PRPK may exert cell-cycle regulating activity at G1-phase.

The DNA damage by radiation and chemotherapeutics activates p53 and the cell-cycle is arrested at G1 phase via p21 activation [23]. Both ATM and ATR kinases activate p53 by phosphorylation of serine-15 residue responding to DNA damages [24,25], and Chk1 and Chk2 phosphorylate serine-20 residue to further stabilize p53 [26]. DNA-PK also activates p53 by DNA damage [27,28]. These kinases play important roles in the regulation of the cell-cycle. PRPK also phosphorylates serine-15 residue and activates p53 transcriptional activity [1]. As shown here, the expression of PRPK protein is strongly induced at the G1-phase. In addition to ATM/ATR and Chk1/Chk2 [29], PRPK could suppress the G1/S transition via the p53–p21 axis.

Ray inhibits PRPK-induced p53 transcriptional activity

To observe the biological effect of Ray on the PRPK-activity, an immunofluorescent histochemical analysis was carried out using anti-S15-p53 phospho-specific antibody (Fig. 4B). When over-expressed, PRPK localized to the nucleus and induced the phosphorylation of S15-p53. The co-transfected Ray-WT suppressed PRPK-induced S15-p53 phosphorylation and some PRPK protein is re-localized to the cytosol. On the other hand, Ray-N120I did not affect the phosphorylation status of S15-p53 and did not change nuclear localization of PRPK. GTP-restricted mutant, Ray-Q67L, dispersed PRPK somewhat to the cytosol and suppressed p53 phosphorylation as was wild type. Please note that the some PRPK protein still remains in the nucleus of Ray-O67L transfected cells but S15-p53 is not evidently phosphorylated.

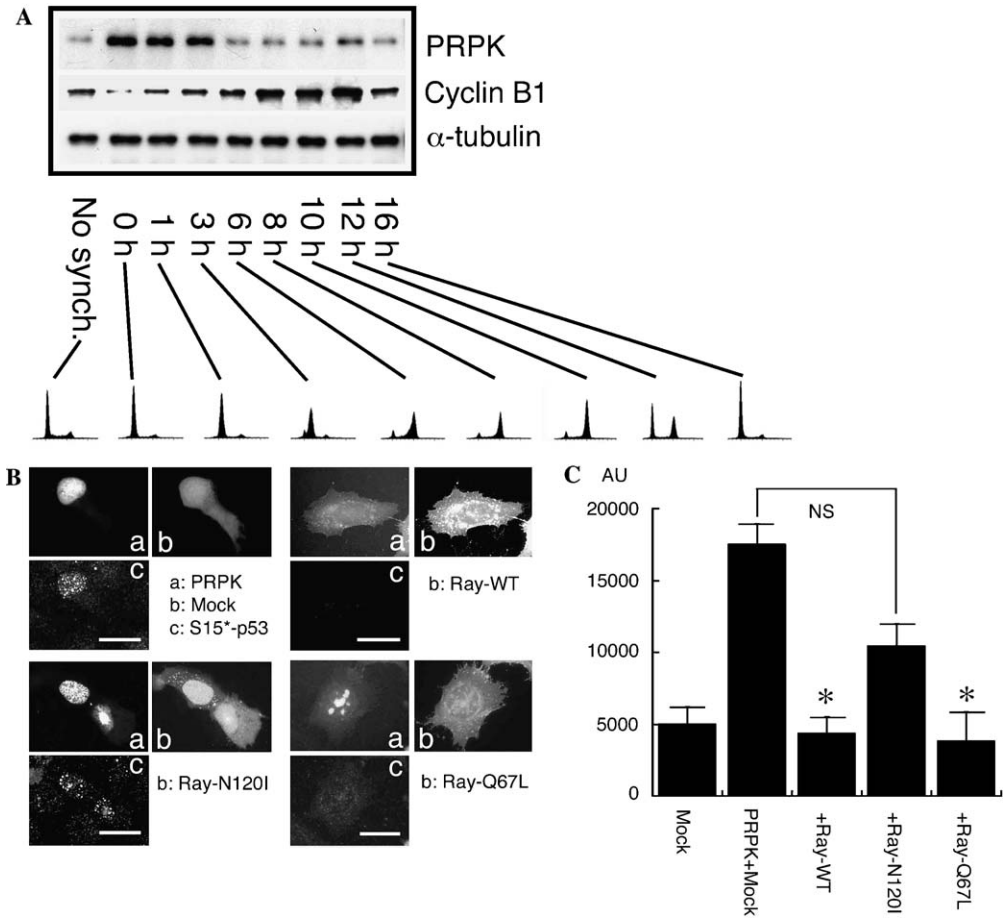

Fig. 4. G1-expression of PRPK protein in cell-cycle and inhibition of PRPK-induced p53 transcriptional activity by Ray. (A) PRPK, cyclin B1 and α-tubulin expressions in the synchronized HeLa cells using Western blotting. PRPK expression was enhanced at G1-phase as seen at 0 to 3 h after cell-cycle release. The expression of mitotic kinase, cyclin B1, was elevated at G2/M phase. (B) pHcRed-PRPK and pcDNA3HA-Rays were co-transfected to U2-OS cells and immunofluorescent histochemistry was carried out using anti-phospho-specific S15-p53 antibody. Phosphorylation of S15-p53 was observed in the mock and Ray-N120I transfectants, while no evident phosphorylation was seen in the Ray-WT and Ray-Q67L transfectants. White bar indicates 20 μm. (C) PRPK and Rays were co-transfected to COS-7 cells and the p53-promoter activity was estimated using pGluc-p53 luciferase reporter vector. After the transfection of these vectors, culture supernatant was collected and luciferase activity was estimated ($n = 4$ for each sample). Error bars indicate the standard deviations. Ray-WT and Ray-Q67L suppressed the PRPK-induced p53 promoter activities significantly. On the other hand, the suppression by Ray-N120I was not statistically significant. Both Ray-WT, Ray-N120I, and Ray-Q67L by themselves did not evidently affect to the p53 transcriptional activity in this assay. $^*p < 0.05$. NS, not significant.

This evidence was supported by the result of transcriptional activity assay using pGluc luciferase reporter vector possessing p53-binding sequence (Fig. 4C). When PRPK was over-expressed in COS-7 cells, transcriptional activity of p53 was enhanced as reported previously [1]. Ray-WT suppressed this activity down to the basal level, which was statistically significant. On the other hand, Ray-N120I did not significantly suppress it. Ray-Q67L suppressed the PRPK-induced p53 transcriptional activity as Ray-WT. Ray-WT, Ray-N120I, and Ray-Q67L by themselves did not evidently affect the p53-transcriptional activity (data not shown). In comparison to the PRPK-re-localizing effect from the nucleus to the cytosol, the suppressive effect of Ray on the PRPK-activity appears quite potent. This indicates that the re-localization of PRPK will not be necessary for the suppression of PRPK-activity. As shown in the Ray-Q67L-transfected cells in Fig. 4B, some remaining PRPK in the nucleus did not evidently phosphorylate S15-p53. Therefore, the binding of Ray to PRPK itself appears to be sufficient for the functional suppression of PRPK. The precise mechanism of this suppression is unclear at this moment.

Oxidative stress induces nuclear re-localization of the intrinsic PRPK

We found an intriguing result in the localization of intrinsic PRPK after oxidative stress. HeLa cells were treated with hydrogen peroxide and immunofluorescent histochemistry was carried out using anti-PRPK antibody (Fig. 5). The oxidative stress induced re-localization of the intrinsic PRPK from the cytosol to the nucleus. Nuclear re-localization of PRPK can be important for the enhancement of PRPK activity on p53. Ray could take some part in the shuttling of PRPK to the nucleus during this phenomenon.

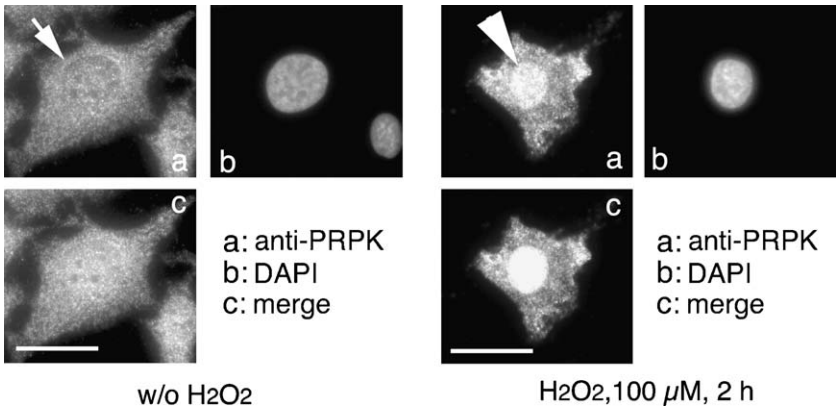

Fig. 5. Re-localization of PRPK to the nucleus by oxidative stress. HeLa cells were treated by 100 μM H_2O_2 for 2 h and localization of the intrinsic PRPK was detected using anti-PRPK antibody. The nucleus was stained by DAPI. After H_2O_2 treatment, PRPK accumulated into the nucleus from the cytosol as indicated by the arrowhead. The white bar indicates 20 μm.

In this study, a ubiquitously expressing Rab protein, Ray/Rab1c, was shown to bind to PRPK. Unlike two other relatives (Rab1a and Rab1b), Ray is unique for its polybasic sequence at C-terminal and it appears to go through the nuclear membrane. In spite of its NLS, PRPK somehow localizes to the cytosol in situ, and this localization may at least be partially carried out by Ray. The intracellular localization of PRPK can be important for its activity on p53. Helping to re-distribute PRPK from the nucleus to the cytosol, Ray may suppress PRPK-activity. Our data also indicated that the binding of Ray to PRPK by itself can be enough for functional suppression of PRPK. The elevated p53 transcriptional activity by the PRPK-over expression in cells was clearly suppressed to the basal level by a co-transfection of Ray.

Some p53 is distributed in the cytosol as well as nucleus in situ [30,31]. How p53 is transported into the cytosol and go back to the nucleus is not yet clearly understood. Nuclear shuttling of p53 by Hsp90-related system was reported [32]. We speculate that Ray may also play some role in the p53 shuttling via PRPK. PRPK binds to p53 [1] and Ray binds to PRPK. As shown in this study, PRPK accumulates in the nucleus by an oxidative stress.

The tissue distribution of Ray mRNA expression was ubiquitous [8]. It can also be referred to in databases such as at http://hal.hgc.jp/cgi-bin/gene_ncbi.cgi?id=11021 or http://www.dsi.univ-paris5.fr/genatlas/unigene.php?UNIGENE=94308&SYMBOL=RAB35&NAME=. Considering such wide tissue distribution, this small GTPase could have other roles than modulating PRPK-activity, because PRPK is exclusively expressed in the testis [1].

Recently, Ray was identified to be one of the proteins associated with the Nucleophosmin-anaplastic lymphoma kinase (NPM-ALK) which may function as an oncogenic process of anaplastic large cell lymphoma [33]. Therefore, Ray may also play a role in moving kinases such as NPM-ALK between the nucleus and cytosol with some partners such as NIPA [34]. Our preliminary result indicates that Ray works as an absorber of PRPK-induced p53-activity. When cells are treated by various types of stresses such as hypertonic pressure, oxygen, and UV irradiation, p53-transcriptional activity elevates. To such cells, over-expressed Ray protein suppressed p53-transcriptional activities in our preliminary data (data not shown). Ray could work as a p53-modulating molecules through binding to the p53 activating factors such as PRPK or other p53-related kinases. This is only an initial study on Ray which is indicated to play an important role in the regulation of p53 activity. Further analyses will clarify the biological significance of this small GTPase.

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