Cell. Mol. Life Sci. 64 (2007) 2680 – 2689
1420-682X/07/192680-10
DOI 10.1007/s00018-007-7179-7
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Cellular and Molecular Life Sciences

Research Article

Phosphorylation and activation of the atypical kinase p53-related protein kinase (PRPK) by Akt/PKB

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Received 12 April 2007; received after revision 30 July 2007; accepted 7 August 2007
Online First 23 August 2007

Abstract. p53-related protein kinase (PRPK), the human homologue of yeast Bud32, belonging to a small subfamily of atypical protein kinases, is inactive unless it is previously incubated with cell lysates. Here we show that such an activation of PRPK is mediated by another kinase, Akt/PKB, which phosphorylates PRPK at Ser250. We show that recombinant PRPK is phosphorylated *in vitro* by Akt and its phospho-form is recognized by a Ser250-phospho-specific antibody; that cell co-transfection with Akt along with wild-type PRPK, but not with its Ser250Ala mutant, results in increased PRPK phosphorylation; and that the phosphorylation of p53 at Ser15, the only known substrate of PRPK, is markedly increased by co-transfection of Akt with wild-type PRPK, but not PRPK dead mutant, and is abrogated by cell treatment with the Akt pathway inhibitor LY294002. Our data disclose an unanticipated mechanism by which PRPK can be activated and provide a functional link between this enigmatic kinase and the Akt signaling pathway.

Keywords. PRPK, Bud32, piD261, PKB/Akt, protein kinases.

Introduction

The term piD261, initially applied to a putative protein kinase composed of only 261 residues encoded by the yeast *YGR262c* gene [1] denotes a family of protein kinases with atypical features whose members are present throughout the evolutionary scale from Archaea to human, but not in eubacteria, suggesting that they make up a primordial branch of the eukaryotic protein kinases superfamily [2].
The best-characterized member of the whole family is the yeast kinase, renamed Bud32, in the *Saccharomyces* genome database, which can be expressed in bacteria in a catalytically active form, as judged from its ability to autophosphorylate and to phosphorylate, albeit with low efficiency, casein and other proteins and peptides *in vitro* [3, 4]. This has made possible a thorough structure-function analysis of Bud32, outlining a number of unusual biochemical features, notably the misplacement of the essential lysine in subdomain II and the replacement of the homolog of highly conserved PKA Lys168 by a threonine [5]. It was also possible to show that Bud32 catalytic activity is required *in vivo* to fully rescue the severe slow-growth phenotype due to disruption of its gene [5, 6], although catalytically inactive mutants can also partially restore the hormonal phenotype [6]. Functional information has been provided by the identification of yeast protein complexes including Bud32 [7] and by a

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two-hybrid approach [6]. The panel of interacting proteins disclosed by both approaches include, among others, a putative glycoprotease (Kae1) and glutaredoxin 4. This latter is also readily phosphorylated *in vitro* by Bud32, suggesting that it represents one of its physiological targets. On the other hand Kae1 and Bud32 participate with other small proteins in an evolutionary conserved complex named EKC (endopeptidase kinase complex) [8] or KEOPS (kinase, putative endopeptidase and other proteins of small size) [9]. Two independent studies have demonstrated a role for this complex both in transcription regulation [8] and telomere maintenance/homeostasis [9].

The functional homology between Bud32 and its human counterpart, p53-related protein kinase (PRPK), is supported on one hand by the ability of PRPK to partially complement the Bud32-deficiency phenotype in yeast, on the other by the ability of Bud32 *in vitro* to phosphorylate human p53 at Ser15 [10], reported to be a target of PRPK [11]. At variance with Bud32, however, recombinant PRPK is entirely devoid of catalytic activity, and consequently classified among "pseudokinases" [12], a circumstance that has until now hampered any kind of biochemical characterization. Detectable catalytic activity is conferred to immobilized GST-PRPK (bound to glutathione-Sepharose) by treatment with COS-7 cells lysates [11], suggesting that the cellular environment contains component(s) capable to activate PRPK.

This prompted us to start an investigation aimed at disclosing the mechanism(s) by which PRPK is upregulated in living cells. Here we report on experiments showing that PRPK is phosphorylated both *in vitro* and *in vivo* by Akt/PKB at a highly conserved C-terminal residue (Ser250) and we present evidence that such a phosphorylation correlates with increased PRPK catalytic activity

Materials and methods

Reagents and plasmids

GST-PRPK [wild-type (wt) and mutagenized forms], GFP-PRPK, p53-GST and PRPK-His_6 were purified as described below, or as described by Facchin et al. [10]. Akt1/PKBα was either purchased from Upstate (NY), or kindly provided by Dr., H. McLauchlan and Dr., J. Hastie (Dundee). In this latter case, Akt was expressed in bacteria and activated by exhaustive phosphorylation at Thr308 and Ser473; inactive (non-phosphorylated) Akt was used as a negative control. LY294002 inhibitor, phospho-(Ser/Thr) Akt substrate antibody, total p53, phospho-p53 Ser15, phospho-GSK3β Ser9, phospho-Akt Thr308 antibodies were purchased from Cell Signaling Technology. Anti-c-myc was purchased from Sigma. Phospho-Akt Ser473, total Akt1 and phospho p70 S6 kinase antibodies were from Santa Cruz Biotechnology.

Anti-PRPK and anti-phospho Ser250 PRPK antibodies were generated using the peptide CEVRLRGRKRSMLG or the phosphopeptide CRGRKRpSMLG, respectively. Peptides were synthesized using an automated peptide synthesizer (Model 432 Applied Biosystems, CA) and 9-fluorenylmethoxycarbonyl (Fmoc) chemistry [13]. Phosphoserine was coupled as Fmoc-Ser[PO(OBzl)OH]-OH (Novabiochem, Switzerland).

The antibodies, conjugated to keyhole limpet hemocyanin, were raised in New Zealand rabbits, against the two synthetic peptides. The antisera were purified using an immobilized peptide affinity resin (Sulfo Link Coupling Gel, Pierce, UK) according to manufacture's instructions. The plasmid pCMV6-HA-Myr-AKT1 was kindly supplied by Dr Sandra Marmiroli (Modena).

Cloning of PRPK coding sequence

The PRPK coding sequence was amplified by PCR from the, I.M.A.G.E. clone ID 3899629 using the PRPK-S (5'-CGGATCCTCATGGCGGCGGCCAGAGC) and the PRPK-AS (5'-GCTCGAGCTTCTACCCAACCATGGACC) primers, which introduce the restriction sites *Bam*HI and *Xho*I (underlined), respectively, at the 5' and 3' end of the PRPK cDNA. The PCR product was directly cloned in the pCR-Blunt II TOPO vector using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen). This recombinant plasmid, TOPO-PRPK, was used in all subsequent cloning steps.

Overexpression of PRPK and PRPK Ser250Ala

The PRPK cDNA sequence, isolated from TOPO-PRPK plasmid by digestion with the *Bam*HI and *Xho*I enzymes, was ligated into the pcDNA 3.0 plasmid (Invitrogen) digested with the same enzymes. The resulting recombinant plasmid (pcDNA-PRPK) was used as DNA template to obtain the S250A PRPK and D163A PRPK mutants by the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene cat. no. 200518). The mutagenic primers were designed according to the instruction manual. The constructs obtained were verified by DNA sequencing and used to overexpress the wt and mutagenized forms of PRPK in mammalian cells.

Expression and purification of PRPK from bacterial cells

Purification of 6His-PRPK. The same *Bgl*II-*Sal*I fragment containing the PRPK coding sequence and

previously used to obtain the pGFP-PRPK plasmid (see above) was ligated into the pQE 81L plasmid (Qiagen) digested with the same enzymes. The resulting pQE-PRPK recombinant plasmid was used to induce the overexpression of a 6His N-terminal tagged form of the PRPK protein in bacterial cells. The, *E. coli* strain BL21(DE3), transformed with pQE-PRPK plasmid, was grown in LB medium at 16°C until OD_{600} reached 0.7, when transcription of PRPK cDNA coding sequence was induced with 0.15 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After 12–16 h of further incubation, bacteria were harvested and resuspended in 25 ml purification buffer (20 mM Tris-HCl pH 7.6, 0.3 M NaCl, 10 % glycerol, 10 mM imidazole, 0.02 mM PMSF) per liter of culture. Purification was performed according to the protocol of manufacturer using an affinity column containing Ni-NTA-agarose (Qiagen). PRPK-His_6 was eluted with 200 mM imidazole.

Purification of GST-PRPK. The PRPK cDNA sequence, isolated from TOPO-PRPK plasmid by digestion with *Bam*HI and *Xho*I enzymes, was ligated into the pGEX plasmid (GE Healthcare) digested with the same enzymes. The resulting recombinant plasmid, pGEX-PRPK allows the inducible overexpression in bacterial cells of the PRPK protein fused, at the N-terminal end, to the glutathione S-transferase (GST) protein. The, *E. coli* strain BL21(DE3), transformed with pGEX-PRPK plasmid, was grown in LB medium at 16°C until OD_{600} reached 0.7, when transcription of the PRPK cDNA coding sequence was induced with 0.15 mM IPTG. After 12–16 h of further incubation, bacteria were harvested and resuspended in 30 ml PBS and 1 % Triton X-100 per 4 g pellet. Purification was performed according to the protocol of the manufacturer using an affinity column containing glutathione-Sepharose 4B (GE Healthcare). GST-PRPK was eluted with 20–30 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0 and finally dialyzed and concentrated in 20 mM Tris-HCl pH 7.5 and 50 % glycerol.
Protein concentration was determined by the method of Bradford, using bovine serum albumin as a standard.

Cell culture and transfection
The human leukemia Jurkat T cell and human embryonic kidney epithelial HEK 293T cells were maintained in the appropriate medium, RPMI 1640 (Sigma) for Jurkat, Dulbecco's modified Eagle's medium (Sigma) for HEK 293T, supplemented with 10 % fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μM streptomycin, in an atmosphere containing 5 % CO_2. HEK 293T cells, plated onto 60-mm-diameter dishes at about 80 % confluency, were transiently transfected with 1–3 μg cDNA by standard calcium phosphate procedure. The transfection mixture was removed after 8–16 h, and cells were lysed 24–48 h after transfection.

Cell lysis and Western blot analysis
For lysate preparation, cells were lysed as described by Di Maira et al. [14]. For nuclear extract preparation, cells were lysed by the addition of hypotonic buffer, followed by extraction of nuclear proteins with a hypertonic buffer, as described by Ruzzene et al. [15]. Equal amount of protein were loaded on 11 % SDS-PAGE, blotted on Immobilon-P membranes (Millipore), and processed by Western blot with the indicated antibody, detected by ECL (enhanced chemiluminescence, Amersham Biosciences). Quantitation of the signal was obtained by chemiluminescence detection on Kodak Image Station 440cf and analysis with the Kodak 1D image software.

PRPK-myc construct and immunoprecipitation experiments
The PRPK cDNA sequence was amplified by PCR from the TOPO-PRPK plasmid (see above) using the PRPKMyc-S (5'-CTCGAGATGGCGGCGGCCAGAGCTAC) and PRPKMyc-AS (5'-AAGCTTCCCAACCATGGACCTC) primers that introduce the *Xho*I and *Hind*III restriction sites (underlined), respectively, at the 5' and 3' end of the PRPK cDNA. The PCR product was cloned again in a PCR-Blunt II TOPO vector, isolated from the latter recombinant plasmid by digestion with the *Xho*I and *Hind*III enzymes and then ligated into the pcDNA 3.1 plasmid (Invitrogen) digested with the same enzymes. The resulting construct, pcDNA-PRPK-cMyc/His was verified by DNA sequencing and used to overexpress a cMyc/6His C-terminal tagged form of the PRPK protein in mammalian cells.
Immunoprecipitations were performed by incubation of 80–100 μg cell lysate with the indicated antibody at 4°C overnight followed by addition of protein A-Sepharose beads (Sigma) as described by Sarno et al. [16].

***In vitro* phosphorylation of GST-PRPK and His-PRPK by Akt**
Purified recombinant GST-PRPK (0.5 μg) or purified His-PRPK (0.5 μg) were incubated with recombinant Akt (0.06 μg) in the presence of 50 mM Tris-HCl, pH 7.5, 12 mM $MgCl_2$, 62 μM [γ-^{33}P]ATP (~5000 cpm/pmol), in a total volume of 20 μl, for 15 min at 30°C. Proteins were resolved by SDS-PAGE and analyzed by autoradiography.

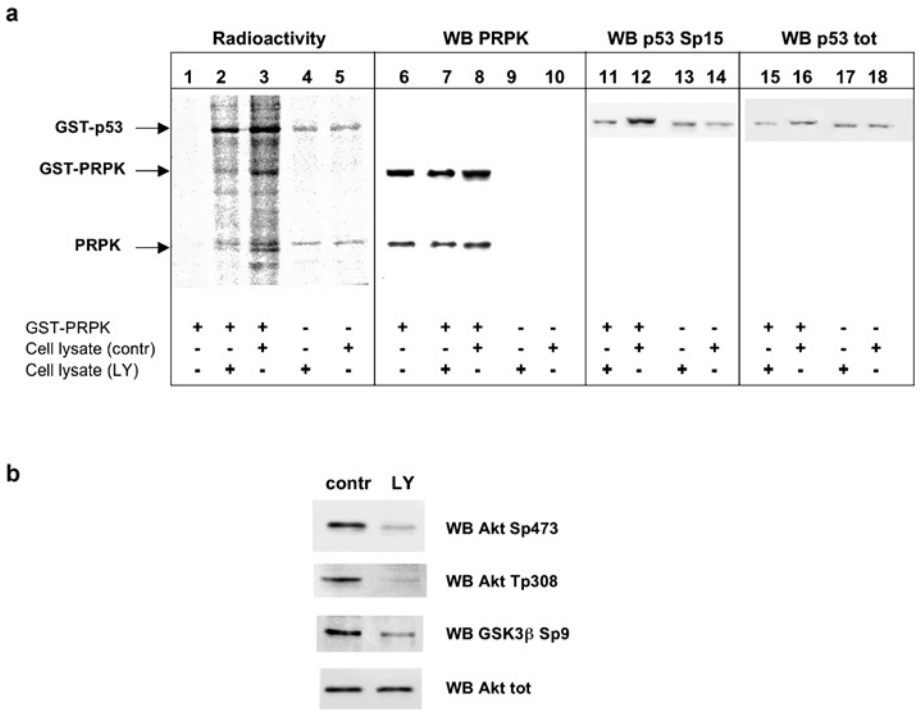

Figure 1. Adverse effect of cell treatment with the Akt pathway inhibitor LY294002 on the ability of cell lysates to evoke catalytic activity of PRPK. (*a*) Recombinant GST-PRPK, bound to glutathione-Sepharose beads, was incubated for 30 min with cell lysis buffer (lanes 1 and 6) or lysates from Jurkat cells (80 μg proteins, lanes 2–5 and 7–18) either treated or not with LY294002, as indicated. Beads were washed and incubated with GST-p53 and radioactive phosphorylation mixture. Proteins were separated by SDS-PAGE followed by blotting, and analyzed by autoradiography (lanes 1–5), Western Blot (WB) for PRPK (lanes 6–10), Western Blot for p53, either pSer15 (lanes 11–14) or total protein (lanes 15–18). Protein migrations, as detected by Western blots, are indicated on the left side of the figure. Apparently partial cleavage of GST-PRPK occurs during incubation, giving rise to free PRPK whose position is also indicated. (*b*) Lysates (10 μg proteins) from Jurkat cells, treated (LY) or not (contr) with LY294002, as in (*a*), were analyzed by Western blot with the indicated antibodies.

Treatment of GST-PRPK with Jurkat cell lysates

Recombinant GST-PRPK (1.5 μg), bound to glutathione-Sepharose beads, was incubated with cell lysis buffer or 80 μg of total proteins from lysate of Jurkat cells, previously treated or not for 6 h with 25 μM LY294002 inhibitor. Beads were then washed two times with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.25 M saccharose, 5 mM EDTA, 1 μM PMSF and one time with 50 mM Tris-HCl, pH 7.5, and incubated for 20 min at 30°C in presence of 50 mM Tris-HCl, pH 7.5, 10 mM $MnCl_2$, 50 μM [γ-^{33}P]ATP and 0.6 μg GST-p53. Proteins were separated by SDS-PAGE followed by blotting, and analyzed by autoradiography.

Statistical analysis

Each experiment was performed at least four times. Data were evaluated for their statistical significance with the paired Student's *t*-test. p values <0.05 were considered statistically significant.

Results

Activation of recombinant PRPK by cell lysates is abrogated by down-regulation of endogenous Akt

At variance with its yeast homologue, Bud32, whose recombinant form expressed in bacteria displays a significant, albeit modest, catalytic activity, detectable both in terms of autophosphorylation and using exogenous substrates like casein and p53 [3, 4], its human homologue, PRPK, under the same conditions is catalytically inactive, unless its recombinant GST fusion protein, bound to glutathione-Sepharose, is previously incubated with COS-7 cell lysates [11]. As also shown in Figure 1a, such a treatment is absolutely required to evoke the phosphoradiolabeling of p53, as well as of additional bands, two of which are accounted for by GST-PRPK and free PRPK, as judged from the corresponding Western blots. These radioactive bands seemingly reflect autophosphorylation of GST-PRPK and PRPK, which is partially cleaved under our experimental conditions. Phosphorylation of p53 was also monitored with a phospho-specific antibody that recognizes pSer15 (Fig. 1a, lanes 11–14), confirming that Ser15 is the phosphorylated p53 residue.

Considering that in Jurkat cells Akt is constitutively active, owing to lack of PTEN expression, and that the C-terminal segment of PRPK, highly conserved throughout the members of the piD261/Bud32 family of atypical protein kinases (see Fig. 2), includes a serine residue (Ser250) in the context of an Akt consensus (R-x-R-x-xS/T-Hyd), we wanted to check if inactivation of endogenous Akt could influence the activation of GST-PRPK by Jurkat cell lysates. Jurkat cells were, therefore, treated with LY294002, an inhibitor of the Akt pathway [17]. As shown in Figure 1b, this treatment actually resulted in a drastic drop in Akt activity, as judged from decreased phosphorylation of both Akt residues responsible for its activation (Thr308 and Ser473) and of the S9 of GSK 3β, a typical endogenous Akt substrate. Interestingly, the same treatment resulted in a significant drop in the ability of the cell lysate to promote PRPK activation, as judged from reduced phosphorylation of p53 and PRPK autophosphorylation (Fig. 1a, lanes 2, 3 and 11, 12). The residual phosphorylation of p53 is likely contributed by protein kinases different from PRPK, present in the cell lysate.

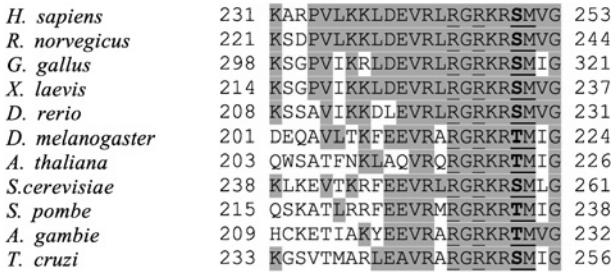

Figure 2. Alignment of the C-terminal segment of human PRPK with those of its homologues from other organism reveals the conservation of an Akt consensus site (underlined). Amino acid sequence of human PRPK, *H. sapiens* (GenBank accession No. AB017505) is aligned with: *R. norvegicus* (GenBank accession no. XM 342580); *G. gallus* (GenBank accession no. XM 425703); *X. laevis* (EMBL accession no. AAH97750.1); *D. rerio* (EMBL accession no. AAH95119.1); *D. melanogaster* (EMBL accession no. CG10673-PA); *A. thaliana* (GenBank accession no. NM 122512.2); *S. cerevisiae* (EMBL accession no. P53323); *S. pombe* (EMBL accession no. SPAP27G11.07); *A. gambie* (GenBank accession no. XM 308396); *T. cruzi* (GenBank accession no. XM 816481.1). The putative phosphoacceptors site is denoted in bold, gray boxes denote identity.

In vitro phosphorylation of PRPK by Akt at Ser250

The above results supported the working hypothesis that activation of PRPK is promoted by Akt, possibly through the phosphorylation of Ser250, sitting in a typical Akt consensus sequence (see Fig. 2). To corroborate this hypothesis, *in vitro* phosphorylation experiments were performed, taking advantage of recombinant protein kinases and either a radioactive assay or a phospho-specific antibody that recognizes phospho-Ser250. Firstly, we were able to show that indeed both GST-PRPK and His-tagged PRPK were phospho-radiolabeled upon incubation with [γ-^{33}P]ATP, provided active Akt was also present (Fig. 3a). Next, the same experiment was run with cold ATP, and the phosphorylation of Ser250 was revealed using a phospho-specific antibody, which reacts with PRPK phosphorylated at Ser250 but not with its unphosphorylated form (Fig. 3b). A signal was evident that was entirely dependent on the presence of Akt, demonstrating that Akt phosphorylates PRPK at Ser250. As previously reported [11] and mentioned above, recombinant PRPK is catalytically inactive. In an attempt to correlate *in vitro* phosphorylation of recombinant PRPK with induction of its phosphotransferase activity, time course experiments were performed to increase the phosphorylation stoichiometry of PRPK by recombinant Akt. However, even on prolonging incubation for up to 2 h with repeated additions of Akt, only very low phosphorylation stoichiometries (ranging between 3% and 5%) were achieved (not shown), not sufficient to justify hardly reproducible appearance of PRPK activity, monitored on p53. It has to be assumed, therefore, that Akt-dependent PRPK activation observed using cell lysate, as described in the previous paragraph, is optimized by co-factors, which are lacking in the *in vitro* experiments run with just the two recombinant proteins.

Phosphorylation of PRPK at Ser250 in HEK 293T cells

Having determined that PRPK can be phosphorylated *in vitro* by Akt at its C-terminal highly conserved Ser250, we wanted to check if this phosphorylation also takes place in cells and might be related to the observed activation of PRPK upon incubation with cell lysates (see Fig. 1).

We, therefore, initially transfected HEK 293T with myc-tagged PRPK, either alone or in combination with Akt, in its form that is susceptible to myristoylation and thus constitutively activated. Immunoprecipitated myc-PRPK was then probed for Ser250 phosphorylation by Western blot with anti-pSer250 antibodies. As shown in Figure 4a, a weak immunoreaction was revealed also in the absence of transfected Akt, the intensity of which, however, was significantly increased in cells co-transfected with Myr-Akt. In this respect, in our experiments, endogenous Akt was already activated as cells were not deprived of serum.

In a parallel approach, advantage was taken of a PRPK mutant in which Ser250 was replaced by a non-phosphorylatable residue (S250A). In this case, phosphorylation of PRPK was detected using a commer-

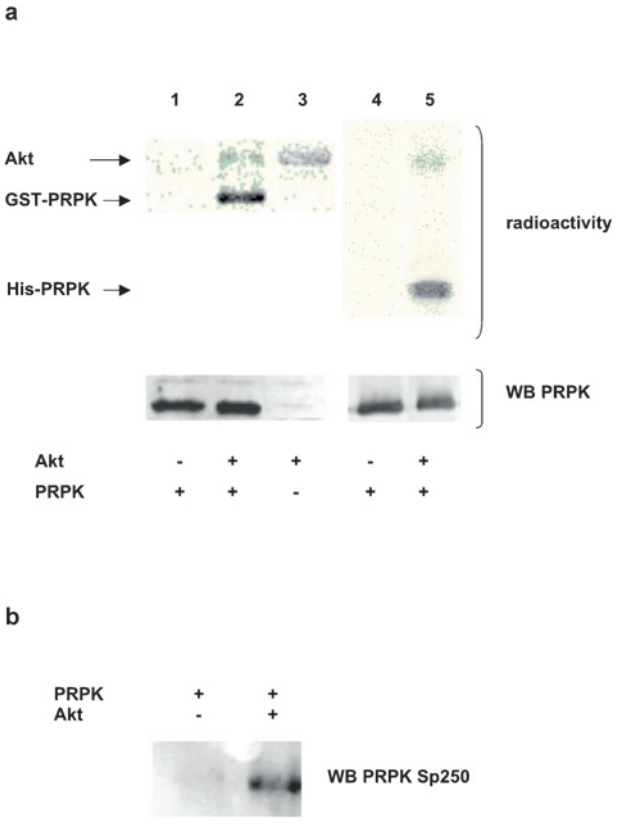

Figure 3. *In vitro* phosphorylation of PRPK by Akt. (*a*) GST-PRPK (lanes 1, 2) or PRPK-His-tagged (lanes 4, 5) were incubated with recombinant active Akt (from Upstate, NY) and a radioactive phosphorylation mixture, as described under Materials and methods. Lane 3: Control with Akt alone. Proteins were resolved by SDS-PAGE and analyzed by autoradiography or Western blot for PRPK, as indicated. Similar results were obtained using recombinant Akt, expressed in bacteria and activated by phosphorylation (see Materials and methods). No PRPK phosphorylation was observed using inactive Akt. (*b*) Blot corresponding to lanes 1 and 2 of (*a*) was analyzed by Western blot with PRPK phospho-specific pSer250 antibodies.

cially available antibody, which recognizes phosphorylated Akt substrates in general. As shown in Figure 4b, upon co-transfection with Akt, wt PRPK was recognized by the Akt phospho-substrate antibody, while the S250A mutant was not.

The role of Akt in the phosphorylation of PRPK at Ser250 in cells was demonstrated on one hand by the negative effect of the upstream inhibitor LY294002 (Fig. 4c); on the other hand, rapamycin, a specific blocker of mTOR [18], failed to reduce the phosphorylation of PRPK Ser250, ruling out the possibility that other kinases downstream of PI3K were involved.

Akt is directly responsible for PRPK activation in cells through phosphorylation of Ser250

Collectively taken, the above results show that, in HEK 293T cells, PRPK is phosphorylated by Akt, and that this phosphorylation mainly, if not exclusively, occurs at Ser250. They do not answer the question, however, as to whether such a phosphorylation is related to the activation of PRPK.

To gain information about this point, we designed experiments aimed at linking the phosphorylation state of endogenous p53 Ser15 (the only known physiological target of PRPK) [11, 19] to conditions known to alter the level of PRPK Ser250 phosphorylation by Akt. In a first set of experiments, cells were transfected with PRPK, either wt or S250A mutant, in the absence or presence of the Akt pathway inhibitor LY294002. The phosphorylation of p53 Ser15 was then analyzed, using the phospho-specific antibody, in the nuclear extract of transfected cells, given the nuclear localization of ectopically expressed PRPK [19]. The results, displayed in Figure 5, show that transfection with wt PRPK significantly increases the phosphorylation of p53 at its Ser15 residue. This effect is much less pronounced when cells are transfected with the S250A PRPK mutant, instead of wt PRPK. Importantly, moreover, LY294002 drastically reduces the stimulatory effect of wt PRPK on p53 Ser15 phosphorylation, while displaying no effect on the residual p53 Ser15 phosphorylation occurring in cells transfected with the PRPK S250A mutant, consistent with the concept that Ser250 is essential to mediate the stimulatory effect of Akt. In this respect, LY294002 also inhibits the phosphorylation of p53 Ser15 in non-transfected cells, suggesting that the stimulatory effect of Akt is also exerted on the endogenous PRPK. On the other hand, the finding that transfection with PRPK S250A increases the phosphorylation of p53 Ser15 in the presence of LY294002, (compare lanes 1, 2 and 5, 6) suggests that, in a cellular environment, PRPK is endowed with a low, yet significant, basal activity regardless of its phosphorylation at Ser250. In a parallel set of experiments, the mTOR inhibitor rapamycin and the upstream Akt pathway inhibitor LY290042 were compared for their efficacy on p53 Ser15 phosphorylation (the phosphorylation state of Thr421/Ser424 p70 S6kinase and Ser473 Akt were analyzed as controls for the efficacy of cell treatment with rapamaycin and LY290042, respectively). As shown in Figure 5, lanes 7–9, rapamycin, already shown to be ineffective on PRPK Ser250 phosphorylation (see Fig. 4c), also had no effect on p53 Ser15 phosphorylation, while suppressing, as expected, the phosphorylation of p70 S6 kinase. These data, in conjunction with drastic inhibition of both PRPK Ser250 (Fig. 4) and p53 Ser15 (Fig. 5) phosphorylation by LY290042 and *in vitro* experiments, strongly support the view that phosphorylation and up-regulation of PRPK by Akt is a direct event, not mediated by other kinase(s).

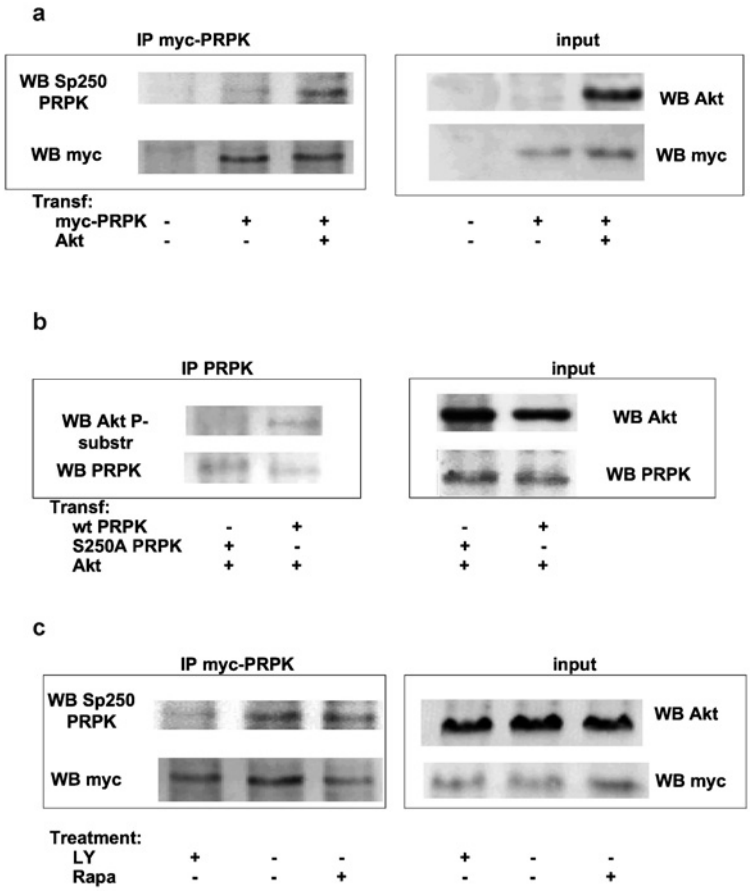

Figure 4. Akt promotes the phosphorylation of PRPK at Ser250 in HEK 293T cells. (*a*) HEK 293Twere transfected with empty vector (-), myc-PRPK or myc-PRPK plus Akt, as indicated. PRPK was immunoprecipitated from cell lysate by an anti-myc antibody, and pellets were probed by Western blot with anti-Sp250 PRPK followed by anti-myc. (*b*) HEK 293T cells were transfected with Akt plus wt PRPK, or Ser250Ala PRPK mutant, as indicated. PRPK was immunoprecipitated from cell lysate (by an anti-PRPK antibody), and pellets were analyzed by Western blot with a phospho-(Ser/Thr) Akt substrate antibody followed by anti-PRPK antibodies. (*c*) HEK 293T cells were transfected with Akt plus wt PRPK, and treated, where indicated (+) with 25 μM LY294002 or 0.25 μM rapamycin during transfection, 2 h before lysis. PRPK was immunoprecipitated from cell lysate by an anti-myc antibody, and pellets were probed by Western blot with anti-Sp250 PRPK followed by anti-myc. To assess transfection, 20 μg total cell lysates were analyzed by Western blot with the indicated antibodies, as shown in the right part of each panel (input).

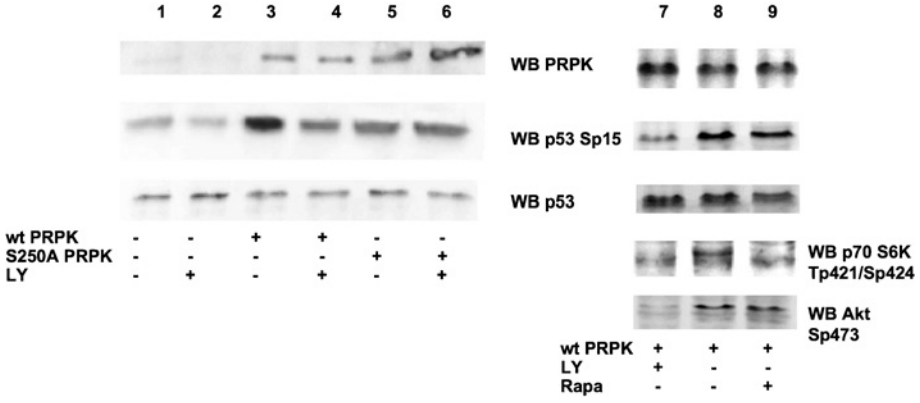

Figure 5. PRPK-dependent phosphorylation of p53 Ser15 in HEK 293T cells. HEK 293T cells were transfected with empty vector (-), wt PRPK, or Ser250Ala PRPK mutant, as indicated. Where present (+) 25 μM LY294002 or 0.25 μM rapamycin were added to the cells during transfection, 2 h before lysis. Aliquots corresponding to 20 μg of nuclear extracts were loaded on SDS-PAGE and analyzed by Western blot with the indicated antibodies. Anti-phospho-p70 S6 kinase and anti-phospho-Akt were used as a control for the treatment with rapamycin and LY294002, respectively. Results shown in lanes 1–6 and lanes 7–9 derives from two different set of experiments.

Further support for the concept that endogenous p53 Ser15 phosphorylation by PRPK is enhanced by Akt, *via* phosphorylation of PRPK Ser250, was provided by experiments in which HEK 293T cells were co-transfected with Myr-Akt. We chose this cell line because it expresses large amounts of stabilized p53 protein [20]. Akt was transfected in its form susceptible to myristoylation, and is thus constitutively active; the endogenous Akt present in HEK 293T cells was also active since we did not deprive cells from serum. This accounts for the stimulation of p53 Ser15 phosphorylation upon transfection of PRPK alone (Fig. 6, compare lanes 1 and 2). Interestingly, transfection with Akt potentiates the beneficial effect exerted by wt PRPK alone on p53 Ser15 phosphorylation, while having no effect on the modest stimulation promoted by the PRPK S250A mutant. The beneficial effect observed upon transfection of Akt alone (lane 6 as compared to lane 1), on the other hand, is likely due to the phosphorylation of endogenous PRPK Ser250, as it is abrogated by co-transfection with the PRPK S250A mutant (compare lanes 5 and 4, see also the bar graph for values normalized to the p53 total protein amount), possibly acting as a dominant negative in this respect.

To corroborate the concept that PRPK catalytic activity was responsible for the phosphorylation of p53 Ser15, a PRPK dead mutant was generated by replacing Asp163 (homologue to essential Bud32 Asp166 acting as the catalytic base [5]) with Ala. As shown in Figure 7, at variance with wt PRPK, transfection of which correlates with increased p53 Ser15 phosphorylation, transfection with the PRPK D163A dead mutant fails to promote any similar effect. Indeed, the weakness of the p53 pSer15 signal observed upon PRPK D163A mutant transfection suggests that even endogenous phosphorylation is reduced, consistent with a behavior of the dead mutant as a dominant negative.

In Figure 7 it is also evident that the kinase dead mutant of PRPK is not only unable to induce the p53 Ser15 phosphorylation, but also totally unresponsive to the cotransfection of Akt

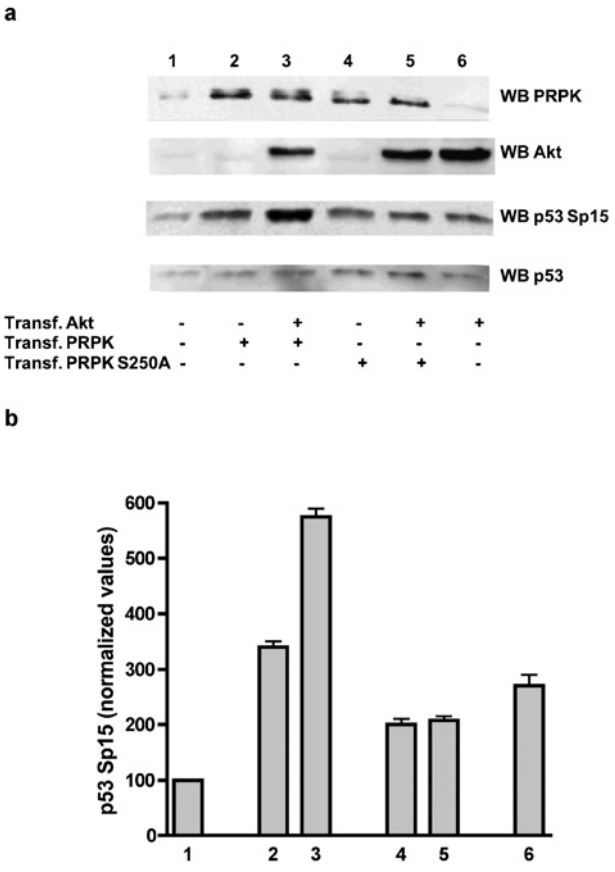

Figure 6. Akt potentiation of p53 Ser15 phosphorylation by wt PRPK but not by PRPK Ser250Ala mutant. (*a*) HEK 293T cells were transfected with empty vector (-), wt PRPK, or PRPK Ser250Ala mutant, alone or in combination with Akt, as indicated. Aliquots corresponding to 20 μg nuclear extracts were loaded on SDS-PAGE and analyzed by Western blot with the indicated antibodies. (*b*) Bars show the quantification of pSer15 p53 signal from lanes as in (*a*), obtained by Kodak Image Station 440CF. Values have been normalized to the p53 total protein amount, and are shown in arbitrary units, assigning 100 to the p53 pSer15 of non-treated, non-transfected cells (lane 1) (means values ± SEM from four experiments).

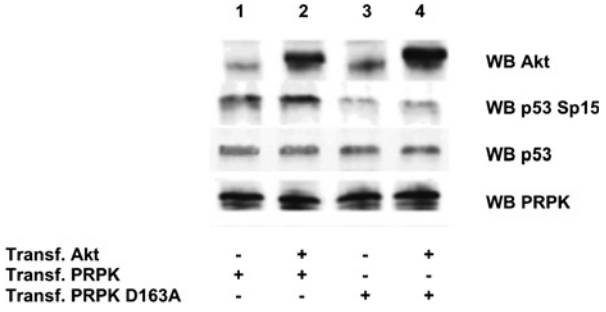

Figure 7. PRPK catalytic activity is required for p53 Ser15 phosphorylation. HEK 293T cells were transfected with empty vector (-), wt PRPK, or PRPK Asp163Ala mutant, alone or in combination with Akt, as indicated. Aliquots corresponding to 20 μg nuclear extracts were loaded on SDS-PAGE and analyzed by Western blot with the indicated antibodies.

Discussion

While a considerable amount of information is already available concerning the biochemical properties of yeast Bud32 and the nature of several proteins interacting with it [4–9], our knowledge about the mechanistic features and biological role(s) of its human homologue, PRPK, are very limited, owing to the difficulty to obtain this putative kinase in a catalytically active form. PRPK was firstly cloned

from an interleukin-2-activated cytotoxic T cell subtraction library and shown to up-regulate transcriptional activity of p53 once transfected in COS-7 cells [11], from which its acronym derives, "p53-related protein kinase". Recombinant GST-PRPK was also shown to interact with p53 and, although devoid *per se* of catalytic activity, it proved able to phosphorylate p53 at Ser15 once immobilized to glutathione-Sepharose and incubated with COS-7 cell lysates [11].

The aim of this work was to gain information about the mechanism(s) responsible for such an activation of PRPK by cell lysates, which is presumably also responsible for up-regulation of PRPK in living cells. Our attention was attracted by the unique structural features of the PRPK C-terminal segment. Although this is outside the *bona fide* kinase domain, it is almost 100 % conserved between PRPK and its yeast homologue Bud32 (as well as the other members of the piD261 family) (Fig. 2), while the overall identity between the kinase domains is about 30 %. Interestingly, this highly conserved stretch includes a serine (Ser250 in PRPK) displaying the consensus of typical Akt phosphoacceptor sites (R-x-R-x-x-S-Hyd). This prompted us to elaborate on the working hypothesis that Akt might be implicated in the activation of PRPK. A preliminary observation supporting the consistency of this hypothesis was provided by a set of experiments run by analogy to those of Abe et al. [11], in which the lysates used to activate the immobilized GST-PRPK were obtained from Jurkat cells treated with LY294002, a compound expected to prevent the activation of endogenous Akt [17]. At variance with lysates from control cells, these proved unable to promote PRPK-dependent phosphorylation of p53 (see Fig. 1). In Jurkat cells the Akt pathway is constitutively on, due to the lack of PTEN, the lipid phosphatase committed to the dephosphorylation of PIP3, which triggers the first step of Akt activation by recruiting the kinase to the membrane; an effective way to turn off this pathway is to block PIP3 synthesis by the PI3K-specific inhibitor LY294002. Therefore, the adverse effect of LY294002 treatment on the ability of cell lysates to activate PRPK was a strong indication for the implication of the Akt pathway in such an activation.

The direct implication of Akt itself, on the other hand, is consistent with *in vitro* experiments showing that Akt is indeed able to phosphorylate PRPK at the expected phosphoacceptor site, as judged from detection with phospho-specific anti pSer250 antibodies.

The ability of Akt to promote the phosphorylation of PRPK at Ser250 has been confirmed in HEK 293T cells transfected with either PRPK or its Ser250Ala mutant, alone or in conjunction with Akt. All data obtained are consistent with a scenario whereby the conserved C-terminal segment of PRPK is also phosphorylated by Akt in living cells, since phosphorylation is enhanced by co-transfection with Akt and is abrogated by mutating PRPK Ser250 to alanine. This latter outcome (see Fig. 4b) supports the view that Ser250 is the main, if not the only, target for Akt in PRPK.

In addition, we were able to show that phosphorylation by Akt leads to an up-regulation of PRPK catalytic activity, as judged from p53 phosphorylation at its Ser15 residue. This latter reached its maximal level under conditions where wt PRPK, but not its S250A mutant, is expressed in the absence of LY294002, which otherwise would prevent Akt activation. The level of Ser15 p53 phosphorylation, moreover, could be further increased by co-transfecting cells with Akt and wt PRPK, but not with its Ser250Ala mutant (Fig. 6, compare lanes 2/3 and 4/5). The finding that LY294002 also reduces p53 Ser15 phosphorylation in the absence of ectopically expressed PRPK (Fig. 5, lanes 1 and 2) may be held as an indication that endogenous PRPK is responsible for such a residual phosphorylation of p53 Ser15. On the other hand, cell treatment with rapamycin, a specific inhibitor of mTOR [18], fails to prevent the phosphorylation of either PRPK Ser250 or p53 Ser15, consistent with the view that PRPK is directly targeted by Akt in the cells.

This would be also consistent with the beneficial effect of transfecting cells with Akt alone (Fig. 6, lane 6), which would saturate with phosphate Ser250 of endogenous PRPK. Although it is possible that another kinase activated through the Akt pathway is also implicated, the crucial importance of PRPK catalytic activity is highlighted by the negligible p53 Ser15 phosphorylation observed upon transfection of the PRPK dead mutant D163A (see Fig. 7). Our data provide the first incontrovertible evidence that PRPK operates as a *bona fide* protein kinase, since the replacement of the aspartate (D163) homologue to the catalytic base of the other protein kinases [5] suppresses its phosphotransferase activity.

Our data are consistent with a model in which PRPK is also endowed with a basal activity in the cellular environment independently of Akt. In fact, the overexpression of the S250A PRPK mutant induces a significant increase of p53 Ser15 phosphorylation, which in some experiments is not much lower than that induced by overexpression of wt PRPK (see for example results shown in Fig. 6), in particular whenever Akt is inactivated (see lanes with LY294002 treatment in Fig. 5). However, the significant difference between the activity of wt PRPK and S250A PRPK mutant, observed in case of Akt co-expression, strongly indicates that a further marked activation of

PRPK is promoted by an Akt-dependent mechanism, strictly requiring the presence of the Ser250 target site. In summary, our data definitely correlate PRPK functionality to the Akt pathway and show that direct phosphorylation of p53 on Ser15 also occurs *in vivo*, thus providing evidence for a novel link between Akt and p53, independent of MDM2 [21], as well as of FOXO3a *via* MnSOD, as recently proposed [22]. Assuming that PRPK plays a role similar to its yeast homologue Bud32 in forming complexes such as the telomere regulator KEOPS [9], it would be tempting to include PRPK, under the supervision of the Akt pathway, in the list of kinases, such as ATM/ATR [23] and Akt itself [24, 25], related to telomere disfunctions. It will be also interesting to check whether Bud32, whose C-terminal Akt consensus site is highly conserved, is also susceptible to phosphorylation and up-regulation by the yeast Akt homologue, Sch9, as suggested by recent results in our laboratory (Peggion et al., manuscript in preparation).

Acknowledgments. This work was supported by grants from: Associazione Italiana per la Ricerca sul Cancro (AIRC), European Commission (EC) PRO-KINASE-RESEARCH 503467 and University of Padova, Progetto di Ateneo 2005.

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