Functional homology between yeast piD261/Bud32 and human PRPK: both phosphorylate p53 and PRPK partially complements piD261/Bud32 deficiency

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Abstract **Yeast piD261/Bud32 belongs to the piD261 family of atypical protein kinases structurally conserved, from Archaea to human. The disruption of its gene is causative of severely defective growth. Its human homologue, PRPK, interacts with and phosphorylates the oncosuppressor p53 protein, which is lacking in yeast. Here we show that on one hand piD261/Bud32 interacts with and phosphorylates human p53 in vitro, on the other hand PRPK can partially complement the phenotype of yeast lacking the gene encoding piD261/Bud32. These data indicate that, despite considerable structural divergence, members of the piD261 family from distantly related organisms display a remarkable functional conservation.**

Key words: piD261/Bud32; Protein kinase; Protein phosphorylation; Surface plasmon resonance; p53; *Saccharomyces cerevisiae*

1. Introduction

Members of the piD261 atypical protein kinase family are present along the whole evolutionary scale, from Archaea to human, but not in eubacteria, suggesting that they make up a primordial branch of the eukaryotic protein kinase superfamily [1]. Consistent with this the human member of the piD261 family, PRPK, occupies the position closest to the origin in the dendrogram of the human 'kinome' [2]. At protein level the best characterized member of the piD261 family is *Saccharomyces cerevisiae* piD261/Bud32, which has been expressed as recombinant protein in *Escherichia coli* and phosphorylates a number of acidic proteins in vitro [3,4]. A mutational analysis of piD261/Bud32 has led to the conclusion that, despite low sequence similarity with the other members of the ePK family, the invariant residues representing the signature of this latter are conserved in piD261/Bud32 as well, but are embedded in an altered context [5]. In yeast the disruption of the gene encoding piD261 is causative of severe growth defects [6], including low survival of cells in stationary phase, inability of homozygous diploids to enter sporulation [7] and tendency to undergo random budding (whence the acronym Bud32) [8].

The only other member of the piD261 family having been characterized at protein level to some extent is human PRPK which was 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 [9]. PRPK binds to and phosphorylates p53 at Ser15 [9], whence the acronym PRPK (p53 related protein kinase). In yeast bona fide p53 homologue(s) are not present and whether or not are there in yeast proteins which may surrogate p53 functions is still a matter of debate.

These premises raised the question whether or not piD261/Bud32 and PRPK play at least partially interchangeable functions, despite their structural similarity that is rather modest (30% identity). Here we show that indeed piD261/Bud32 interacts with the carboxy-terminal domain of human p53 and phosphorylates its amino-terminal domain, at Ser15 (the same as affected by PRPK) and Ser37, while conversely, PRPK partially restores the normal phenotype of yeast strains where the gene encoding piD261/Bud32 had been disrupted.

2. Materials and methods

2.1. Yeast strains

W303-1B +/*ΔYGR262c*/*BUD32* (a/α, *ade2-1*/*ade2-1*, *his3-11,15*/*his3-11,15*, *leu2-3,112*/*leu2-3,112*, *trp1-1*/*trp1-1*, *ura3-1*/*ura3-1*, *can1-100*/*can1-100*; *BUD32*/*bud32::kanMX4*); W303-1B *ΔYGR262c*/*BUD32* (*Mat a*; *ade2-1*; *his3-11,15*; *leu2-3,112*; *trp1-1*; *ura3-1*; *can*^{R}; *bud32::kanMX4*). Yeast Media (YPD, SD) were prepared according to [10]; adenine-limiting medium was prepared by adding adenine 10 mg l^{-1} to SD medium. Media components were from Difco, amino acids from Sigma.

2.2. Cloning of the *PRPK* and YGR262c/BUD32 coding sequence

The PRPK and *BUD32* coding sequences were inserted in the *Bam*HI/*Kpn*I sites of the centromeric galactose-inducible pYeDP1/8.2 vector [11], giving the PRPK-pYeDP and BUD32-pYeDP plasmids, respectively. The PRPK coding sequence was amplified from the I.M.A.G.E. clone ID 3899629 using the 5′-PRPK (5′-AGCTGTTGCGCCGAGGATCCATGGCG-3′) and the 3′-PRPK (5′-TGTGGTACCCATACACACATTCTACCC-3′) primers; the *BUD32* coding sequence was amplified from yeast genomic DNA using the 5′-BUD32 (5′-CGCTTGGATCCTTATGACGCAAGAA-3′) and 3′-BUD32 (5′-TACCCTAGGCATGACGGACATATATACG-3′) primers, which introduce the restriction sites *Bam*HI and *Kpn*I (underlined). The BUD32-pASZ11 plasmid was described elsewhere [Lopreiato, R., Facchin, S., Sartori, G., Casonato, S., Ruzzene, M., Pinna, L.A., Carignani, G. (2003) A study on functional partners of

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piD261/Bud32, an atypical protein kinase of *S. cerevisiae* conserved during evolution, submitted to Biochem J.]

2.3. Phenotype complementation assay

Yeast cells of strain W303-1B *ΔBUD32* contained the BUD32-pASZ11 (adenine) plasmid: the presence in it of the *BUD32* coding sequence under the control of the *BUD32* promoter allows the protection of yeast cells from the damages due to the deletion of the *BUD32* gene.

The 'protected' yeast cells were co-transformed with the pYeDP1/8.2 [11] plasmid alone and pYeDP1/8.2 plasmids containing the yeast *BUD32* or the human PRPK coding sequences, under the control of the GAL10 promoter (inducible on galactose medium). The co-transformed cells were first grown in glucose medium (without adenine to maintain the plasmidic protection) and then shifted to adenine-limiting galactose medium, which induces the specific loss of the protection plasmid (evaluable by a red color of colonies) and the overexpression of the Bud32/piD261 and PRPK proteins. The red colonies, still carrying the pYeDP vectors, were grown at 28°C on galactose selective medium for 4 days.

2.4. Surface plasmon resonance (SRP) analysis

For the SRP analysis, a BIAcoreX system was used, as described in [12], with the following modifications. piD261 was covalently immobilized on the surface of a flow cell in a CM5 sensor chip, to a final density of 2600 resonance units (RU), while a flow cell with no immobilized protein was used as a control. The reported values of kinetic data are the mean of three separate experiments, with S.E.M. values lower than 10%.

2.5. Peptide synthesis

The peptides p53(325–356), p53(361–393), p53(344–376) were synthesized by automatic solid phase procedures. The synthesis were performed on a 431 model (Applied Biosystems) peptide synthesizer under conditions described elsewhere [4]. After cleavage with TFA the peptides were purified to homogeneity by RP-HPLC on a Waters prepNova-Pak HR C18 column with a linear gradient of 10% to 45% acetonitrile at 12 ml min^{-1}. The molecular weights of the peptides were confirmed by mass spectroscopy.

2.6. Expression and purification of p53 and piD261/Bud32

p53-GST: the bacterial clones of BL21 overexpressing p53-GST and p53(1–363) were kindly provided by Dr. M. Cordenonsi (Padova, Italy). The p53-His-tagged truncated forms (p53 1–286, p53 264–393, p53 287–393, p53 340–393) were kindly provided by Dr. M. Montenarh (Homburg, Germany) and were obtained as described in [13,14].

The expression and purification of His-tagged recombinant piD261/Bud32 were performed as previously described [5].

2.7. Phosphorylation assay

Phosphorylation of 500–1000 ng of CK2β, p53-GST, p53(Δ363–393)-GST, p53(1–286)His-tag, and p53(Δ340–393)-His-tag by piD261/Bud32 (200 ng) was performed by incubation at 37°C with [γ^{33}P]ATP under conditions described previously [5]. Phosphorylation of p53(340–393) His-tag by protein kinase CK2 was performed by incubation under conditions described elsewhere [15]. The reaction was stopped by addition of gel electrophoresis loading buffer and samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), with 11% gels. The dried gels were directly scanned on the Cyclone apparatus (Packard).

2.8. Western blot analysis

Approximately 200 ng of recombinant p53-GST were subjected to 11% SDS–PAGE; proteins were blotted onto PVDF membrane (Millipore) and detected by using the phospho-p53 antibody anti-phospho Ser(Ser6, Ser9, Ser15, Ser20, Ser37, Ser46 and Ser392) purchased from Cell Signaling Tech. at a 1000-fold dilution. This reaction was followed by incubation with a HRP-coupled antibody directed against the corresponding IgG type of the first antibody.

2.9. Far-Western blot (FWB) analysis

The overlay method was used to provide evidence of interaction between piD261/Bud32 and p53 and the protein fragments. Approximately 200 ng of purified recombinant protein p53-GST, of truncated forms and 50–100 ng of peptides (as indicated in Fig. 3) were subjected to SDS–PAGE or directly spotted onto nitrocellulose membrane (BioRad). The overlay method was performed as described elsewhere [12].

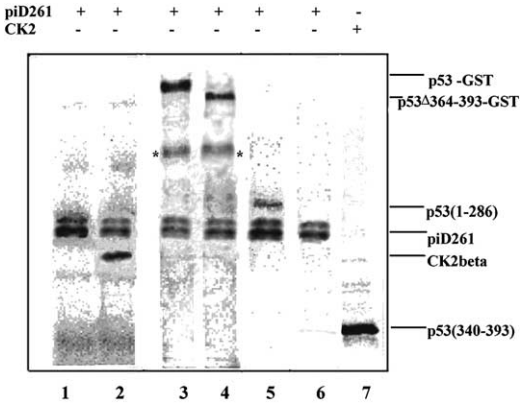

Fig. 1. Phosphorylation of full-size and truncated forms of p53 by piD261 (lanes 3–6) and by CK2 (lane 7). For comparison phosphorylation of β-subunit of CK2 by piD261 (lane 2) and autophosphorylation of piD261 alone (lane 1) are also shown. Phosphorylation was performed and evaluated as described in Section 2.7. Substrate addition in the incubation medium were: none (lane 1), β-subunit of CK2 (lane 2), p53-GST (lane 3), p53Δ364–393 (lane 4), p53(1–286) (lane 5), p53(340–393) (lanes 6 and 7). The band denoted by an asterisk in lanes 3 and 4 corresponds to a degradation product of p53, as judged from its reaction with anti-p53 antibodies (not shown). The faint bands between CK2β and p53(340–393)in lane 7 were bacterial contaminations of the recombinant p53(340–393) fragment as they are not detectable in the control without p53(340–393) (not shown).

3. Results

As shown in Fig. 1 full-size p53 is readily phosphorylated by piD261/Bud32 with an efficiency comparable to that of other protein substrates previously used for its characterization. A similar phosphorylation is observed if C-terminally truncated forms of p53, lacking the site affected by CK2 (Ser392), are used as substrates for piD261/Bud32. Consistent with this a C-terminal fragment of p53 encompassing residues 340 to 393, which is phosphorylated by CK2, is not affected to any appreciable extent by piD261 (Fig. 1, compare lanes 6 and 7).

To gain information about the p53 residues phosphorylated by piD261 advantage has been taken of specific anti-phosphosite antibodies. As shown in Fig. 2, p53 previously phosphorylated by piD261 immunoreacted with anti-phospho Ser15 and phospho Ser37 Ab., but not with a number of antibodies that recognize other phosphosites. It can be concluded therefore that Ser15 (the same also affected by PRPK) and Ser37 are the main targets of piD261. Note that these two phosphoacceptors sites appear to be functionally related as they are targeted by the same set of kinases, notably Chk1, DNA-PK and ATR.

Full-size p53 also physically interacts with piD261, as judged from FWB analysis (Fig. 3). No interaction however is observed between piD261 and the truncated form of p53 lacking its C-terminal region (p53(1–286)). This demonstrates that distinct structural elements along the sequence of p53 are responsible for physical interaction with and phosphorylation by piD261.

In order to map more precisely the C-terminal segment of

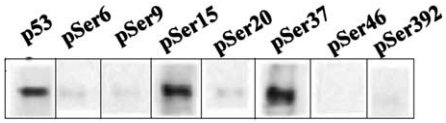

Fig. 2. Mapping the p53 residues phosphorylated by piD261. Identical aliquots of p53 phosphorylated by incubation with ATP-Mn^{2+} and piD261 and resolved by SDS–PAGE (see Section 2.7) were immunodetected either with anti-p53 antibodies or with antibodies that specifically recognize individual phosphoresidues, as indicated (for details see Section 2.8).

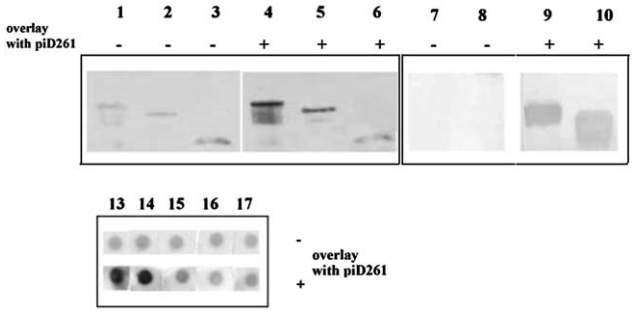

Fig. 3. Mapping the p53 region interacting with piD261 by FWB analysis. The following (poly)peptides were either run on gel electrophoresis and trans-blotted to nitrocellulose membrane (upper panels) or directly spotted on nitrocellulose (lower panel): full-size p53 (lanes 1 and 4), p53(1–363) (lanes 2 and 5), p53(1–286) (lanes 3 and 6), p53(264–393) (lanes 7 and 9), p53(287–393) (lanes 8 and 10), p53(325–356) (spot 13), p53(340–393) (spot 14), p53(361–393) (spot 15), p53(387–393) (spot 16), p53(344–376) (spot 17). The lanes/spots were overlayed with a solution containing or not containing piD261, as indicated. After extensive washing bound piD261 was revealed by immunoreaction with specific anti-piD261 Abs as detailed in Section 2.9. Electrophoretic runs were either in 11% SDS–PAGE (lanes 1–6), or 18% SDS–PAGE (lanes 7–10).

p53 responsible for physical interaction with piD261 a number of peptides variably encompassing the C-terminal domain of p53 have been synthesized and probed for their ability to interact by FWB. As shown in Fig. 3 a clear signal was observed with peptides 264–393, 287–393, 340–393 and 325–356, but not with peptides 361–393, 387–393 and 344–376. These data, in conjunction with the observation that at variance with the truncated form p53(1–286) (which does not interact) a truncated form with a shorter deletion (p53(1–363)) still interacts with piD261, map between residues 340 and 355 (within the tetramerization domain) the structural element indispensable for the main contacts.

These conclusions are in substantial agreement with the outcome of a SRP analysis performed using a sensor chip with bound piD261 and either full-length p53 or its fragments as analytes. The sensorgrams presented in Fig. 4 disclose regular interactions of piD261 with both full-size p53 and its C-terminal 340–393 fragment but not with p53 having a C-terminal deletion p53(Δ287–393). The K_d values calculated for full-size p53 and its C-terminal fragment (2.3×10^{-6} and 8.5×10^{-7} M, respectively) are consistent with the view that the main contacts are concentrated into the C-terminal segment of p53.

The ability of piD261 to interact with and phosphorylate p53, a typical property of human PRPK, suggests that, despite the lack of bona fide p53 homologues in yeast and the overall modest sequence identity between the two protein kinases, these latter have conserved similar functions. This concept was corroborated by complementation experiments indicating that human PRPK behaves as a functional homologue of yeast Bud32, i.e. it is able to partially alleviate the slow-growth phenotype of yeast cells caused by deletion of the *YGR262c/BUD32* gene, which encodes piD261/Bud32. This result was obtained by overexpressing the PRPK gene in a yeast strain deleted of the chromosomal copy of the *YGR262c/BUD32* gene (*ΔYGR262c/BUD32*). Mutant yeast cells, 'protected' by the presence of a plasmid expressing wild-type *YGR262c/BUD32*, were transformed with a pYeDP1/8.2 expression plasmid containing the human PRPK gene under the control of the *GAL10* promoter and, in parallel, with the same plasmid containing the yeast *YGR262c/BUD32* gene or with the plasmid without insert. After loss of the protection plasmid, transformed cells were grown on galactose medium. Fig. 5 shows that overexpression of PRPK in *ΔYGR262c/BUD32* yeast cells partially suppresses their slow-growth phenotype, consistent with a partial conservation of cellular functions between the yeast and human proteins. This outcome is in apparent contradiction with the report of Abe et al. [9] claiming that PRPK could not restore the slow-growth phenotype of *YGR262c/BUD32* disrupted yeast. This discrepancy may be due to the different experimental procedures used in the two experiments.

4. Discussion

Our results show that two members of the atypical protein kinase family piD261, present in distantly related organisms such as yeast and human, display, despite considerable structural divergence, a remarkable functional conservation.

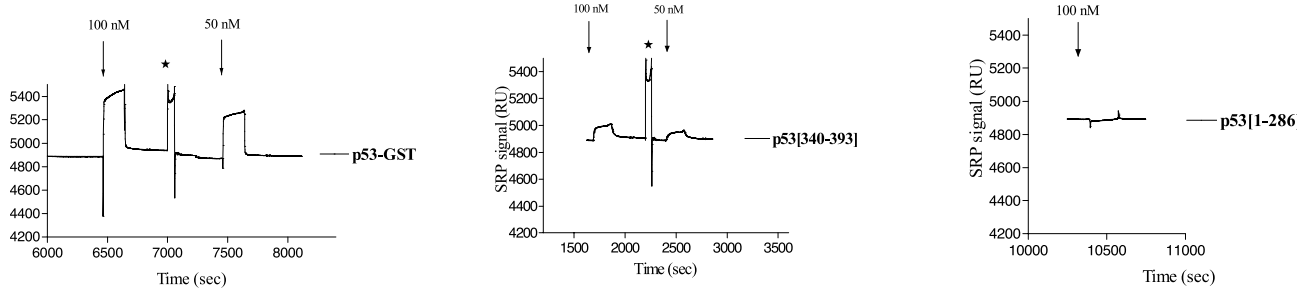

Fig. 4. Analysis of piD261/p53 interaction by SRP signal. Shown are representative sensorgrams (time course of the SRP signal) obtained with the SRP sensor system BIAcoreX, by injection of p53 and its deletion mutants, at the indicated concentrations, over a sensor surface where piD261 was covalently immobilized. The responses shown are the differences (RU) obtained by subtraction of the signal detected over a control surface (without piD261). The arrows mark the start of the injections; the regeneration step is indicated by the asterisk (*).

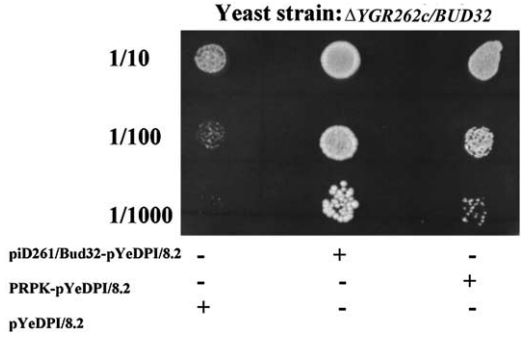

Fig. 5. Complementation of the slow-growth phenotype of *ΔYGR262c/BUD32* yeast cells by human PRPK. *ΔYGR262c/BUD32* yeast cells transformed with plasmids pYeDP1/8.2, piD261-pYeDP1/8.2 or PRPK-pYeDP1/8.2 were grown until stationary phase in galactose liquid medium to induce overexpression of the gene inserted in the plasmid. Cultures were then spotted in serial dilutions (1/10, 1/100, 1/1000) on selective galactose solid medium and growth was observed after 4 days of incubation at 28°C. It is possible to observe that overexpression of PRPK partially restores the phenotype of *ΔYGR262c/BUD32* yeast cells.

Yeast piD261/Bud32 and human PRPK, which share an overall identity of 32%, have both been shown to phosphorylate acidic substrates in vitro [4,9]; also, PRPK interacts with and phosphorylates, both in vitro and in vivo, the tumor suppressor gene product p53 [9]. Despite p53 having no structural homologue in yeast, overexpression of human p53 (as well as of other apoptotic inducers) leads to growth arrest both in *S. cerevisiae* [16] and in *Schizosaccharomyces pombe* [17], where the protein is phosphorylated at the same sites as in mammalian cells. This and other observations indicate the presence in yeast of machinery able to promote the basic steps of apoptosis [18]. Mammalian p53 can also function in yeast as a transcription factor in the presence of p53-specific promoters, and this has allowed the identification of yeast proteins that functionally interact with p53, such as NuA4, a multisubunit HAT complex, probably present also in animals, involved in transcription regulation and essential for cell cycle progression [19–21].

We demonstrate here that yeast piD261/Bud32 has conserved the property of human PRPK to interact with and phosphorylate p53 at Ser15, an event that leads, in answer to DNA damage, to p53 stabilization and increased activity. The yeast protein phosphorylates p53 at Ser15 and at Ser37, while its interaction with p53 has been mapped to the C-terminal region of the protein, in particular to a stretch encompassing residues 340–355, which are included in the tetramerization domain and partially overlap the nuclear export sequence.

On the other hand human PRPK must functionally interact with some yeast proteins, as it significantly alleviates the slow-growth phenotype caused by the absence of piD261/Bud32. It should be noted in this respect that piD261/Bud32 shares with PRPK nuclear localization, despite the fact that it lacks a bipartite nuclear localization motif (Lopreiato et al., as noted in Section 2.2), which is present in the PRPK molecule [9].

Since the only known physiological substrate of PRPK is p53, which is absent in yeast, the ability of the human protein to partially complement the mutant phenotype due to the deletion of the yeast *YGR262c/BUD32* gene indicates that PRPK is able to phosphorylate and/or to interact with one or more of the physiological substrate/partners(s) of piD261/Bud32. It should be noted that piD261/Bud32 associates with a variety of proteins, as disclosed by a recent yeast proteome analysis [22] and by a 2-hybrid system scrutiny (Lopreiato et al. as noted in Section 2.2). It will be interesting to investigate these interactions at molecular level, and to see if PRPK also interacts with (some of) the protein partners of piD261/Bud32.

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