

## Structure–function analysis of yeast piD261/Bud32, an atypical protein kinase essential for normal cell life

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The *Saccharomyces cerevisiae* *YGR262c/BUD32* gene, whose disruption causes a severe pleiotropic phenotype, encodes a 261-residue putative protein kinase, piD261, whose structural homologues have been identified in a variety of organisms, including humans, and whose function is unknown. We have demonstrated previously that piD261, expressed in *Escherichia coli* as a recombinant protein, is a Ser/Thr kinase, as judged by its ability to autophosphorylate and to phosphorylate casein. Here we describe a mutational analysis showing that, despite low sequence similarity, the invariant residues representing the signature of protein kinases are conserved in piD261 and in its structural homologues, but are embedded in an altered context, suggestive of unique mechanistic properties. Especially noteworthy are: (i) three unique inserts of unknown function within the N-terminal lobe, (ii) the lack of a lysyl residue which in all other Ser/Thr

kinases participates in the catalytic event by interacting with the transferred ATP  $\gamma$ -phosphate, and which in piD261 is replaced by a threonine, and (iii) an exceedingly short activation loop including two serines, Ser-187 and Ser-189, whose autophosphorylation accounts for the appearance of an upshifted band upon SDS/PAGE. A mutant in which these serines are replaced by alanines was devoid of the upshifted band and displayed reduced catalytic activity. This would include piD261 in the category of protein kinases activated by phosphorylation, although it lacks the RD (Arg-Asp) motif which is typical of these enzymes.

**Key words:** casein, CK2, protein phosphorylation, *Saccharomyces cerevisiae*, Ser/Thr kinase.

### INTRODUCTION

It has been estimated that more than 30% of the proteins in a eukaryotic cell undergo phosphorylation, this event representing the most frequent and general device by which biological functions are reversibly regulated in higher organisms [1]. The enzymes responsible for this reaction, protein kinases, make up a large family of proteins, whose members share a common catalytic domain composed of about 300–350 residues characterized by a number of conserved features, leading to the definition of 12 distinct subdomains. Such structural organization, deduced from the sequence alignment of about 120 protein kinases, either Ser/Thr- or Tyr-specific [2], underlies a common bi-lobal architecture, with the catalytic site between the two lobes, that is found in all the protein kinases whose crystal structures have been solved to date (e.g. [3,4]). This has led to a general understanding of the relationship existing between conserved subdomains, three-dimensional structure and catalytic properties of protein kinases [2].

Sequencing of the genomes of *Saccharomyces cerevisiae*, *Caenorhabditis elegans* and *Homo sapiens* has confirmed the prediction that in eukaryotes protein kinases will constitute one of the largest families of enzymes [5–7]. Interestingly, however, besides a majority of deduced sequences that display the structural features of protein kinases unambiguously, there are a number of putative protein kinase genes whose products do not display all the stigmata of this family of enzymes. Since these highly conserved features are believed to be essential for correct folding and/or catalytic competence, a legitimate question is

whether these proteins are actually operating as *bona fide* protein kinases.

In *S. cerevisiae* one of these putative atypical protein kinases is the product of the *YGR262c* gene [8]; its structural homologues are present in a variety of organisms, from archaeobacteria to humans. Despite several structural abnormalities, the product of this gene, originally termed piD261, when expressed in *Escherichia coli* with a C-terminal His tag in the presence of Mn<sup>2+</sup> displays significant ATP-protein phosphotransferase catalytic activity affecting Ser/Thr residues, as judged from both autophosphorylation and the ability to phosphorylate acidic proteins, notably casein and osteopontin, *in vitro* [9]. The protein appears to be implicated in a general cellular mechanism as its deletion confers to yeast cells a severe slow-growth phenotype [8]. Mutant cells also display several specific defects [9a], which include reduced survival of cells in the stationary phase, the inability of homozygous diploids to enter sporulation, with no visible meiotic division [9a], and alterations in cell-wall structure, as indicated by several specific tests [10]. Diploid *ygr262c* mutants also exhibit random budding [11]. Due to this phenotype the name *BUD32* has been reserved recently at the *Saccharomyces* Genome Database (SGD<sup>TM</sup>; <http://genome-www.stanford.edu/Saccharomyces>) for the *YGR262c* gene. The apparent involvement of piD261/Bud32 in different cellular processes might be explained by its crucial role in a biological pathway endowed with a general function or, alternatively, by its activity as a regulator of different substrates by phosphorylation. In order to define the biochemical characteristics of the protein we started a mutational study aimed at probing the structure–function

Abbreviations used: PKA, cAMP-dependent protein kinase; FSBA, fluorylsulphonylbenzoyladenine.

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relationships of this atypical protein kinase. The results are described in the present paper.

## EXPERIMENTAL

Partially dephosphorylated  $\alpha$ -casein was purchased from Sigma. The CK2 $\beta$  1-77 peptide reproducing the N-terminal segment of the protein kinase CK2  $\beta$  subunit was synthesized as described previously [12]. The pET-261 plasmid encoding the mutant form SS187,189AA of piD261/Bud32 was provided kindly by Dr P. Ghisellini (University of Genoa, Genoa, Italy). The anti-fluorylsulphonylbenzoyl adenosine (FSBA) antibody was a generous gift from Dr P. J. Parker (ICRF, London, U.K.) [13]. Monoclonal antibody anti-His was from Amersham Pharmacia Biotech. Mouse antiserum against piD261/Bud32 was raised using 10  $\mu$ g of purified recombinant protein dissolved in 100  $\mu$ l of Ringer solution: this solution, emulsified previously with Freund's adjuvant (1:1, v/v), was injected intraperitoneally into mice five times at 1 week intervals.

### Strains, media and plasmids

The *E. coli* strains INV $\alpha$ F' (Invitrogen), used for plasmid preparations, and BL21(DE3) (Novagen), used for protein expression, were grown in LB medium (1% bacto-tryptone, 0.5% yeast extract and 0.5% NaCl) or LB containing 50 mg/l ampicillin. The pET-261 plasmid, used for *YGR262c* expression in *E. coli*, has been described in [9].

### Mutagenesis of the *YGR262c/BUD32* gene

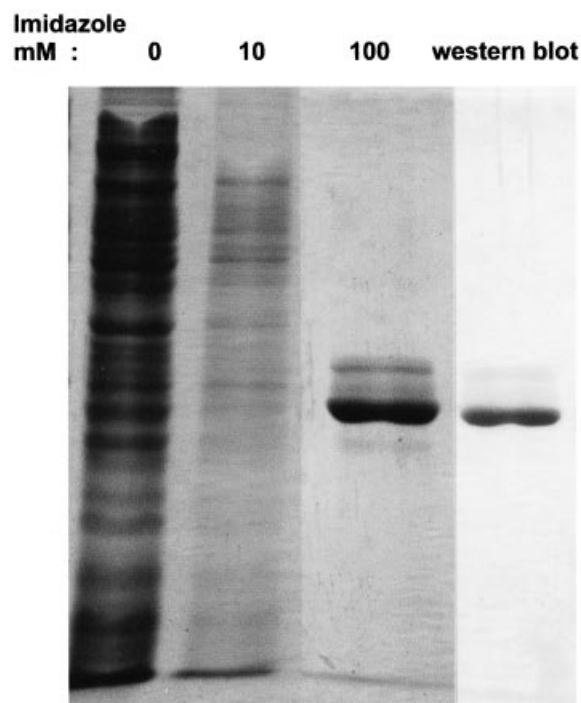
The mutant forms of *YGR262c/BUD32* were obtained using the QuikChange<sup>TM</sup> Site-Directed Mutagenesis Kit (Stratagene, catalogue no. 200518). The mutagenic primers were designed according to the instruction manual. As double-stranded DNA templates we used the pET-261 plasmid construct. The resulting plasmids were controlled by DNA sequencing of the inserts.

### Tryptic digestion and MS

After blotting on PVDF membrane (Millipore), the area corresponding to piD261/Bud32 was cut out and put into a 200  $\mu$ l solution of ammonium bicarbonate [14]. The reaction was started by addition of trypsin (Sigma; trypsin/protein ratio of 1:50). After 2 h an equal amount of fresh trypsin was added and the digestion stopped at the end of 4 h. The membrane was removed and the solution lyophilized several times. The residue was dissolved in water containing 0.1% trifluoroacetic acid and subjected to MS (Maldi-1; Kratos-Schimidzu, Manchester, U.K.) using  $\alpha$ -cyano-4-hydroxycinnamic acid as matrix.

### Expression and purification of piD261-His<sub>6</sub>

The expression of recombinant piD261-His<sub>6</sub> was performed as described previously [9], with some modifications. The *E. coli* strain BL21(DE3), containing plasmid pET-261, was grown in LB medium at 37 °C until the  $D_{600}$  value reached 0.3 and at room temperature until  $D_{600}$  reached 0.7–0.8, when transcription of the *YGR262c* coding sequence was induced with 0.4 mM isopropyl  $\beta$ -D-thiogalactoside. After 16–18 h of incubation at 16 °C bacteria were harvested and resuspended in 10 ml/g of pellet of purification buffer (20 mM Tris/HCl, pH 7.5, 0.3 M NaCl, 10% glycerol, 1 mM 2-mercaptoethanol and 0.2 mM PMSF). piD261-His<sub>6</sub> was purified to homogeneity from the bacterial extract [9] according to the protocol of the manufacturer by an affinity column containing Ni<sup>2+</sup>-nitrilotriacetate-agarose gel



**Figure 1** Purification of recombinant piD261-His<sub>6</sub> by Ni<sup>2+</sup>-nitrilotriacetate affinity chromatography

The soluble bacterial extract was stirred with Ni<sup>2+</sup>-nitrilotriacetate-agarose according to the protocol of the manufacturer (Qiagen). The suspension was added to the column and the resin was subsequently submitted to a two-step elution with purification buffer containing 10 and 100 mM imidazole, respectively. Samples of each eluate (15  $\mu$ l), as indicated, were subjected to SDS/PAGE (11% gel) and proteins were detected with Coomassie Brilliant Blue (lanes 1–3). In lane 4 the same sample as in lane 3 was subjected to Western-blot analysis with anti-piD261 antibodies. For details, see the Experimental section.

(Qiagen). As shown in Figure 1, most contaminating proteins were not retained on the column (Figure 1, lane 1) and/or were eluted by washing the column with the purification buffer containing 10 mM imidazole (Figure 1, lane 2); piD261-His<sub>6</sub> was eluted by 100 mM imidazole as a main 29.5 kDa band accompanied by two faint, slightly less mobile bands (Figure 1, lane 3). These were also recognized by anti-piD261 antibodies (Figure 1, lane 4) consistent with their identification as phosphorylated forms of piD261-His<sub>6</sub> (see the Results section).

### Western-blot analysis

Approx. 200 ng of recombinant piD261-His<sub>6</sub> (wild-type or mutant) were subjected to SDS/PAGE [11% gel; the protein concentration was determined by densitometry (Image Master, Amersham Pharmacia Biotech), using BSA as a standard]. Proteins were blotted on to PVDF membrane and detected by using the mouse antiserum against piD261-His<sub>6</sub> at a 500-fold dilution. This reaction was followed by incubation with a phosphatase-coupled antibody directed against the corresponding IgG type of the first antibody. The same procedure was used in the case of anti-His (diluted 1000-fold) and anti-FSBA (diluted 2000-fold) antibodies. The experimental procedure used to examine the binding of FSBA to wild-type or mutant piD261 was as described in [12], using Mn<sup>2+</sup> instead of Mg<sup>2+</sup>.

## Phosphorylation assay

The protein kinase activity of piD261 and of its mutant forms was assayed routinely by incubating the recombinant His-tagged protein (0.2  $\mu\text{g}/\mu\text{l}$ ) at 37 °C for 15 min in 20  $\mu\text{l}$  of a medium containing 50 mM Tris/HCl, pH 7.5, 10 mM MnCl<sub>2</sub>, 25  $\mu\text{M}$  [ $\gamma$ -<sup>32</sup>P]ATP (Amersham Pharmacia Biotech; specific radioactivity, 2000–3000 c.p.m./pmol) and either the CK2 $\beta$  1-77 peptide (10–100  $\mu\text{M}$ ) or  $\alpha$ -casein (0.5–1  $\mu\text{g}/\mu\text{l}$ ) as phosphorylatable substrates. The reaction was stopped by adding the gel electrophoresis loading buffer and samples were subjected to SDS/PAGE, with either 11 % (casein) or 18 % (CK2 $\beta$  1-77) gels. The dried gels were scanned directly using Cyclone apparatus (Packard).

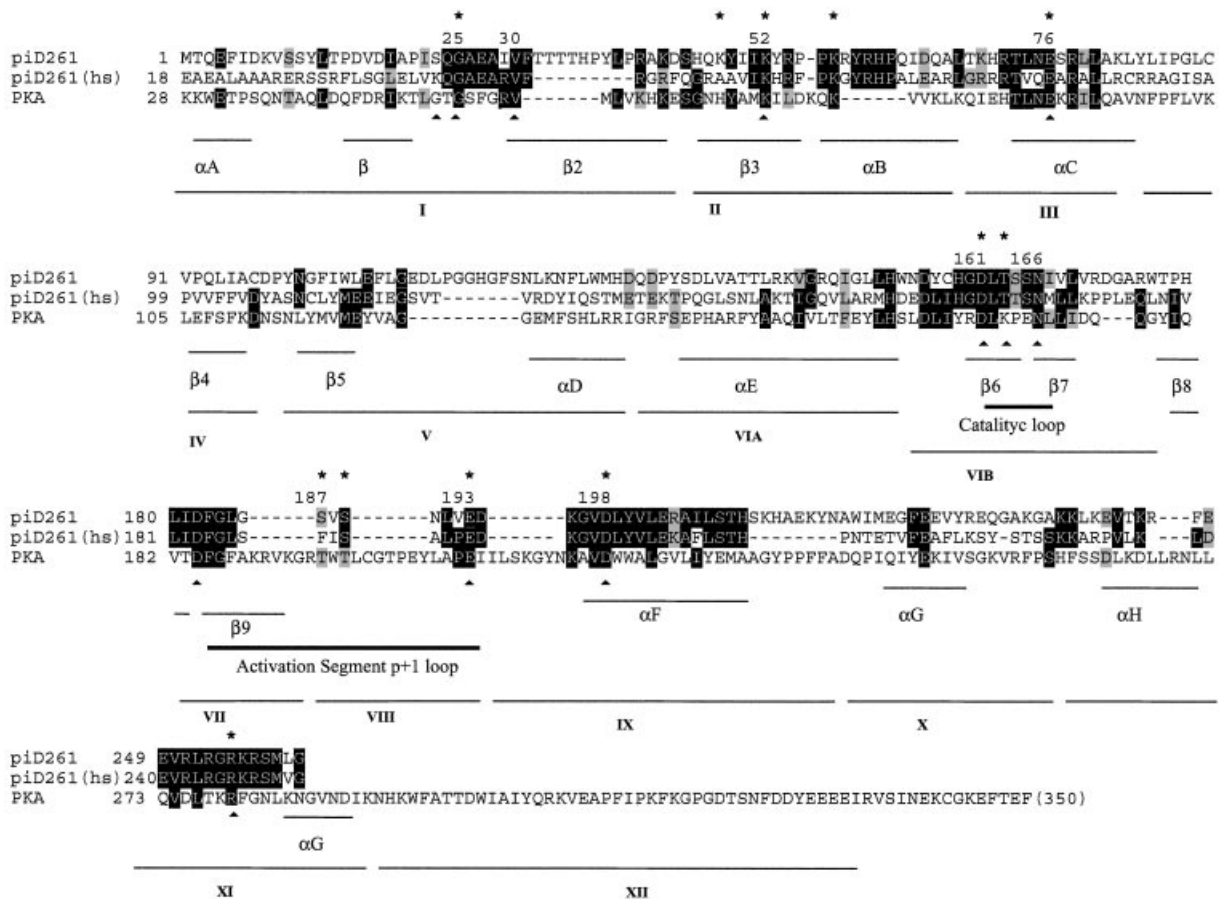
## RESULTS

### Mutational analysis of functionally relevant residues

A novel alignment of piD261 with the prototype kinase, mammalian cAMP-dependent protein kinase (PKA;  $\alpha$ -catalytic subunit), is presented in Figure 2. The previously adopted alignment [9] has been modified partially by manual adjustment corroborated by the mutational analysis presented in this work. To this effect we introduced in piD261 different amino acid substitutions

by mutagenizing the *YGR262c* coding sequence already inserted in the bacterial pET-261 plasmid. The phosphotransferase activities of the different mutant proteins were assayed after expression in *E. coli* and purification as described in [9] and the experimental section. The results are summarized in Table 1.

Starting from the N-terminus, the suspected functional equivalence of Gly-25 with the invariant second glycine of the phosphate anchor motif, GXGXXG (equivalent to PKA Gly-52), was confirmed by mutating it to Val to give a mutant that was fully inactive (Table 1) and also unable to bind the ATP analogue FSBA (Figure 3). Gly-25 is therefore the only glycine of the 'glycine loop' left in piD261. The highly conserved valine responsible for hydrophobic interaction with the adenine moiety of ATP, present five residues downstream from the central glycine of the phosphate anchor in nearly all kinases (PKA Val-56), is also conserved in piD261 (Val-30). In contrast, the previously proposed [9] matching of piD261 Lys-57 with the invariant lysine essential for the correct positioning of the ATP triphosphate group (PKA Lys-72), turned out to be incorrect since Lys-57 could be mutated to alanine without any appreciable loss of activity (Table 1). Mutagenesis of two lysyl residues upstream from Lys-57, Lys-48 and Lys-52, demonstrated that Lys-52 must be the functional equivalent of PKA Lys-72, since its replacement fully suppressed catalytic activity, whereas mu-



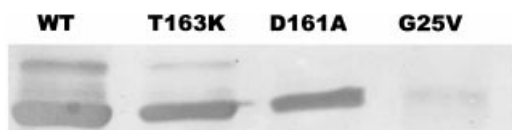
**Figure 2** Alignment of piD261/Bud32 and PKA sequences

The sequences of yeast piD261/Bud32 (piD261; SWISS-PROT accession no. P53323) and human piD261/Bud32 [piD261(hs), NCB accession no. CAC00561] proteins were aligned with that of PKA (SWISS-PROT accession no. P05132) by computer analysis and manual adjustment corroborated by mutational analysis (see the text). Asterisks denote piD261 residues that were mutated to assess their relevance. Arrowheads denote residues that are highly conserved, as they are found in PKA. Black, identity; grey, similarity. The subdomains of protein kinases and functionally relevant structural elements are also indicated.

**Table 1** Mutational analysis of conserved residues in piD261/Bud32

The invariant residues defining protein kinase subdomains are shown (the numbering refers to PKA) together with the putative equivalent in piD261/Bud32. Bold denotes piD261 residues where the mutation combined with sequence alignment denote functional equivalence with conserved kinase residues. The alignment of Arg-255 with PKA Arg-280 is questionable (see the text). The mutations performed and their activities relative to the wild type (100%) are listed in the final two columns. Activity of wild-type and mutant piD261/Bud32 was determined using the CK2 $\beta$  1-77 peptide as a phosphorylatable substrate under the conditions specified in the Experimental section. Activities are means from at least three separate determinations with a S.E. of less than 15%.

Subdomain	Invariant residue (in PKA)	Function	Putative equivalent residue(s) in piD261/Bud32	piD261/Bud32 mutant	Specific activity (% of wild type)
<b>I</b>	<b>Gly-52</b>	Provides space for ATP $\beta$ -phosphate	<b>Gly-25</b>	Gly-25 $\rightarrow$ Val	< 1
<b>II</b>	<b>Lys-72</b>	Co-ordinates $\alpha$ - and $\beta$ -phosphates of ATP	<b>Lys-48</b> <b>Lys-52</b> <b>Lys-57</b>	Lys-48 $\rightarrow$ Ala Lys-52 $\rightarrow$ Ala Lys-57 $\rightarrow$ Ala	68 < 1 90
<b>III</b>	<b>Glu-91</b>	Interacts with Lys-72	<b>Glu-76</b>	Glu-76 $\rightarrow$ Ala	< 1
<b>VIB</b>	<b>Asp-166</b>	Catalytic base	<b>Asp-161</b>	Asp-161 $\rightarrow$ Ala	6
<b>VIB</b>	<b>Lys-168</b>	Transfer of $\gamma$ -phosphate	<b>Thr-163</b>	Thr-163 $\rightarrow$ Lys Thr-163 $\rightarrow$ Ala	70 90
<b>VIB</b>	<b>Asn-171</b>	Co-ordinates $\alpha$ - and $\gamma$ -phosphates of ATP	<b>Asn-166</b>	—	—
<b>VII</b>	<b>Asp-184</b>	Positioning of $\gamma$ -phosphate	<b>Asp-182</b>	—	—
<b>VIII</b>	<b>Glu-208</b>	Correct folding of catalytic core	<b>Glu-193</b>	Glu-193 $\rightarrow$ Ala	20
<b>IX</b>	<b>Asp-220</b>	Correct folding of catalytic core	<b>Asp-198</b>	Asp-198 $\rightarrow$ Ala	10
<b>XI</b>	<b>Arg-280</b>	Correct folding of catalytic core	<b>Arg-255?</b>	Arg-255 $\rightarrow$ Ala	90

**Figure 3** piD261 mutant Gly-25  $\rightarrow$  Val is unable to bind the ATP analogue FSBA

piD261 wild-type (WT) and mutant forms [Thr-163  $\rightarrow$  Lys (T163K), Asp-161  $\rightarrow$  Ala (D161A) and Gly-25  $\rightarrow$  Val (G25V)] were assayed for their ability to bind the ATP analogue FSBA by Western-blot analysis, as described in the Experimental section.

tation of Lys-48 had only modest effects on activity (Table 1). Alignment of piD261 Lys-52 with PKA Lys-72 implies the presence in piD261 of a unique insert of seven amino acids just downstream from the conserved Val-30. Curiously, this insert includes a series of four consecutive threonines which are not found in other protein kinases. Another short insert of six amino acids is present at the end of putative subdomain II, based on the unequivocal alignment of the sequence flanking Glu-76, which matches well the invariant glutamyl residue (PKA Glu-91) that defines subdomain III. As expected, the mutation of this glutamic acid to alanine fully suppressed catalytic activity (Table 1).

A third insert of 10 residues must be introduced into the putative domain V of piD261 to allow the alignment of putative subdomains VIA and VIB. Subdomain VIA displays also in piD261 the typical pattern of hydrophobic residues (o) ending with a histidine (equivalent to PKA His-158), o—o—ooH (where a dash indicates any residue) [2]; domain VIB includes the highly conserved catalytic loop, with its invariant motif DXXXXN, whose actual identification with the 161–166 segment of piD261 (DLTSSN) was validated by showing that the replacement of Asp-161 by Ala nearly abolishes activity (Table 1), consistent with its essential role as a catalytic base. This also assigns piD261 to the small group of 'non-RD' (non-Arg-Asp) protein kinases, where the catalytic aspartate is not preceded by an arginine [4]. Even more unusual is the lack in piD261 of a lysine (Lys-168 in PKA) that is present in all Ser/Thr protein kinases two residues

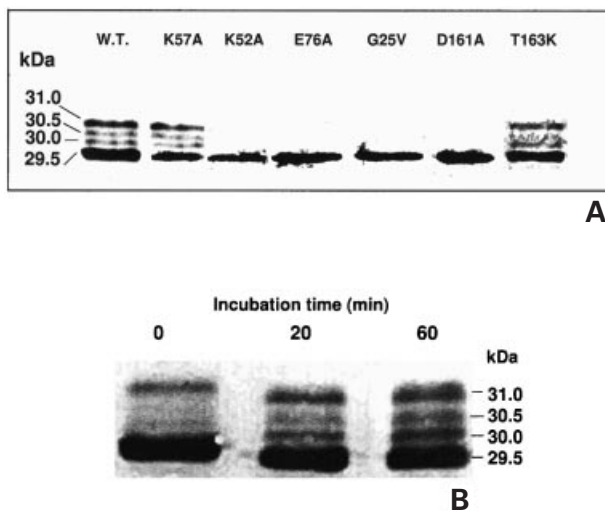
**Table 2** Conserved motifs at the catalytic loop of protein kinases

The piD261/Bud32 sequence is aligned and compared with the consensus determined by Hanks [22]. o denotes hydrophobic consensus. The invariant Asp and Asn residues common to all the motifs are underlined.

Kinase sequence	Subdomain VIB
Ser/Thr kinase general consensus	DoKXXN
piD261/Bud32	<u>DLTSSN</u>
Tyr kinase general consensus	<u>DLAARN</u>
Tyr kinase Src consensus	<u>DLRAAN</u>

downstream from the catalytic base (see Table 2), and which has been shown to play a crucial role in catalysis by interacting with the  $\gamma$ -phosphate of ATP while it is transferred to the phospho-acceptor amino acid [15]. In piD261 this lysine is replaced by threonine (Thr-163). Mutation of this threonine to lysine does not increase catalytic activity, but rather decreases it slightly, whereas its mutation to alanine is not detrimental at all (Table 1). These data, in conjunction with the finding that, conversely, mutation of Lys-168 to Ala in yeast PKA is strongly detrimental [16], suggest that Thr-163 does not play any relevant role in catalysis, unlike its sequence homologue lysine found in all Ser/Thr protein kinases.

Alignment downstream from subdomain VIB becomes problematic due to the reduced size of the C-terminal moiety of piD261. At variance with the previous alignment [9], however, the new match, based on the insertion of three series of gaps in subdomains VII, VIII and IX (Figure 2), would lead to the conservation in piD261 of the four invariant residues which in protein kinases specify subdomains VII, VIII, IX and XI. The reliability of this alignment is supported by mutational analysis showing that both Glu-193 (equivalent to PKA Glu-208) and Asp-198 (equivalent to PKA Asp-220) are important for activity (see Table 1). Both these residues are required for the correct assembly of the catalytic core. The most C-terminal conserved residue, defining subdomain XI in nearly all protein kinases, is an arginine (Arg-280 of PKA) which makes a salt bridge to Glu-208.



**Figure 4** Western-blot analysis of piD261 wild type and mutants

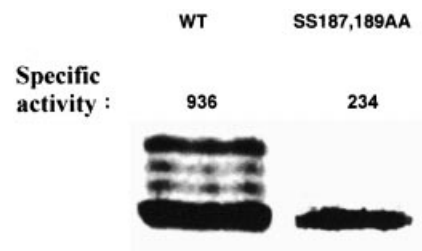
(A) piD261 wild type and mutants (see the Experimental section and Table 1) were subjected to SDS/PAGE (11% gel containing 16% glycerol), transferred to PVDF membrane and immunoreacted with anti-piD261 antibodies, as detailed in the Experimental section. Apparent molecular-mass values were assigned to the immunoreactive bands relative to the migration position of a kit of eight prestained protein markers (molecular masses, 6500–175 000 Da; BioLabs). (B) The slowly migrating bands of wild-type piD261 increase upon incubation with ATP and  $MnCl_2$  for the indicated periods of time. Experimental conditions were as in (A).

The structural equivalent of this arginine in piD261 would be Arg-255. Its mutation to alanine only marginally affects catalytic activity (Table 1), suggesting that the functional equivalent of PKA Arg-280 might be one of the other three arginines clustered together with Arg-255 at the very C-terminal end of piD261.

Taken together, the results of the mutational analysis and computer-assisted alignment, which was refined manually in critical regions, are consistent with a scenario in which the invariant residues of protein kinases are conserved in piD261, although in an altered context.

#### Autophosphorylation of piD261 at its activation loop correlates with increased catalytic activity

Recombinant piD261-His<sub>6</sub> expressed in *E. coli* and purified by Ni<sup>2+</sup>-nitrilotriacetate affinity chromatography gave rise to two faint protein bands that were slightly less mobile than the main band upon SDS/PAGE (see Figure 1). Better resolution was achieved by including 16% glycerol in the gel, giving rise to four bands having apparent molecular masses of 29.5, 30, 30.5 and 31 kDa upon SDS/PAGE (see Figure 4A). All bands were recognized by anti-piD261 antibodies as well as by antibodies raised against the C-terminal histidines (results not shown) and against the ATP analogue FSBA covalently bound to the protein (Figure 3). Given the C-terminal location of the His<sub>6</sub> tag and the vicinity of the ATP/FSBA-binding motif to the N-terminus (see Figure 2), these data argue against the possibility that heterogeneity on SDS/PAGE is due to proteolytic degradation. Conversely, the possibility that heterogeneity is due to variable extents of autophosphorylation occurring in *E. coli* is suggested by the observation that heterogeneity on SDS/PAGE disappears in the case of inactive mutants, notably Gly-25 → Val, Lys-52 → Ala and Glu-76 → Ala; these give rise only to the 29.5 kDa band (Figure 4A). In contrast, heterogeneity is preserved with active mutants, e.g. Thr-163 → Lys and Lys-57 → Ala. These



**Figure 5** Effect of mutating Ser-187 and Ser-189 on piD261 SDS/PAGE pattern and catalytic activity

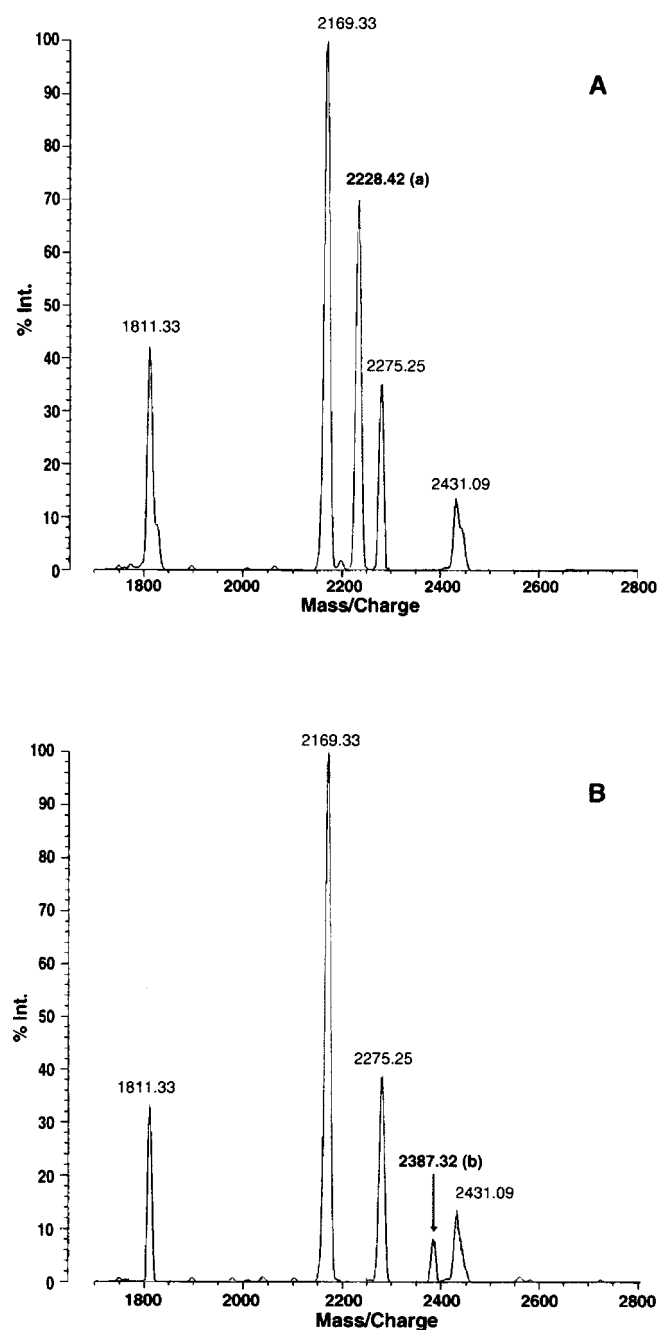
The Western blots of the wild type (WT) and the SS187,189AA mutant are shown, with specific activity expressed as  $\text{pmol of P} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ , determined using the CK2 $\beta$  1-77 peptide as a phosphorylatable substrate (see the Experimental section).

data are consistent with the concept that the upper bands with apparent molecular masses of > 29.5 kDa are indeed generated by multiple autophosphorylation of piD261 expressed in bacteria, by analogy with other kinases, primarily PKA [17,18].

Autophosphorylation of PKA occurs at a threonyl residue (Thr-197) located in the activation loop (also known as the T-loop) [18,19] and is required for full activity; phosphorylation gives rise to an upshifted band on SDS/PAGE (the ‘slow form’) whose catalytic efficiency is much higher than that of the unphosphorylated form (the ‘fast form’) [20]. Likewise, many other protein kinases have been shown to undergo a similar activation mechanism based on the phosphorylation of seryl, threonyl or tyrosyl residues of their activation loops, either by autocatalysis or by heterologous kinases [4]. Similar to PKA, whose isoforms cannot be readily interconverted *in vitro* by incubation with either  $MgATP^{2-}$  or phosphatases [20], suggesting that once the mature protein is assembled the phosphoacceptor site is hardly accessible, the upper band (31 kDa) of piD261 could also not be converted to more mobile band(s) by incubation with either acidic or alkaline phosphatases (results not shown). However, the fast-migrating band of piD261 is significantly, albeit slowly, converted into the upper bands by incubation with  $MnATP^{2-}$  (Figure 4B).

The activation loop of piD261 is abnormally short (see above); however, it includes two phosphorylatable residues, Ser-187 and Ser-189, the latter possibly representing the sequence homologue of PKA Thr-197 (see Figure 2). To check whether these residues might undergo autophosphorylation, which would account for the heterogeneity of piD261, a mutant in which both Ser-187 and Ser-189 had been replaced by Ala (SS187,189AA) was generated and expressed in *E. coli*. As shown in Figure 5 this mutant, similar to the inactive mutants, gives rise almost exclusively to the more mobile 29.5 kDa band, the most upshifted band of 31 kDa being fully undetectable. These data support the concept that Ser-187 and/or Ser-189 do indeed undergo autophosphorylation accounting at least partially for the upshift of piD261 on SDS/PAGE.

The demonstration that in the case of the main upshifted band (31 kDa) this inference is correct was provided by MS analysis of the tryptic fragments obtained from this band and from the 29.5 kDa band of piD261. As shown in Figure 6, the 29.5 kDa band gives rise to a fragment of 2228.4 Da, corresponding exactly to the expected tryptic peptide, including the catalytic loop, encompassing residues between Trp-176 and Lys-195 (theoretical molecular mass, 2228.5 Da). This individual peak is absent in the tryptic digest of the 31 kDa band, where instead a



**Figure 6** Matrix-assisted laser-desorption/ionization-time-of-flight (MALDI-TOF) mass spectrum of piD261 after tryptic digestion

(A) Analysis of the 29.5 kDa band. The mass spectrum region including the signal (a) of the tryptic peptide between residues 176 and 195 (theoretical  $m/z$ , 2228.51 Da) is shown. (B) Analysis of the 31 kDa band. The same mass spectrum region as in (A) is shown. Signal (a) disappears while a novel signal (b) appears corresponding to the bisphosphorylated tryptic fragment (theoretical  $m/z$ , 2388.47 Da).

small yet significant peak of 2389 Da, with the expected molecular mass of the bisphosphorylated tryptic fragment (theoretical molecular mass, 2388.4 Da), is detectable. A similar analysis with the two faint intermediate bands (30.0 and 30.5 kDa) did not provide reliable results due to their very small amounts. It is not clear therefore whether these bands are generated through the individual phosphorylation of Ser-187 and/or Ser-189 or are

**Table 3** Kinetic constants of wild-type piD261 and the SS187,189AA mutant

Catalytic activity was determined at variable concentrations of ATP (10–300  $\mu\text{M}$ ) using the CK2 $\beta$  1-77 peptide as a phosphoacceptor substrate. The peptide concentration (20  $\mu\text{M}$ ) was far above its  $K_m$  value (approx. 1  $\mu\text{M}$  for both the wild-type and mutant proteins). For additional details, see the Experimental section. The data presented are means from three separate determinations with S.E. values of less than 20%.

piD261	$V_{\max}$ (pmol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$ )	$K_m$ (ATP; $\mu\text{M}$ )	Efficiency ( $V_{\max}/K_m$ )
Wild type	1500	25	60
SS187,189AA	460	75	6.1

accounted for by phosphorylation occurring at residue(s) outside the activation loop. This latter hypothesis would be more consistent with the observation that only the two intermediate bands, but neither the 29.5 nor the 31.0 kDa bands, immunoreact with anti-phosphothreonine antibodies (results not shown).

The reduced catalytic activity of mutant SS187,189AA (see Figure 5) supports the idea that the phosphorylation of both or one of these residues is required for optimal activity. As shown in Table 3 the mutant lacking the two serines in the activation loop exhibits both a higher  $K_m$  for ATP and a reduced  $V_{\max}$ , with an overall phosphorylation efficiency 10-fold lower than that of the wild type. Assuming that, as in the case of PKA [18,20], the activity of the SS187,189AA mutant is comparable with that of the unphosphorylated form of the wild type, one would conclude that the experimentally determined catalytic efficiency of wild-type piD261 is an average value, resulting from highly active phosphorylated molecules and less-active non-phosphorylated molecules. Moreover, if it is taken into account that the unphosphorylated 29.5 kDa band accounts for about 80% of piD261 expressed in *E. coli*, whereas the doubly phosphorylated 31 kDa band accounts for just 15% of the whole protein, and considering the negligible contribution of the two intermediate bands (see Figure 5), it can be argued from the data of Table 3 that phosphorylation promotes an up to 60-fold increase in catalytic efficiency. This figure compares quite well with the increase in catalytic activity undergone by PKA upon auto-phosphorylation [20].

## DISCUSSION

The data presented indicate that, despite its small size and low overall similarity with the other members of the protein kinase family, piD261/Bud32 displays all the main signatures of a protein kinase catalytic domain, with special reference to the conservation of invariant residues, whose relevance to piD261 catalytic activity has been confirmed by mutational analysis. These canonical features, however, are dispersed in a deeply altered context, suggesting that in several respects the properties of piD261 are unique. Especially remarkable features are discussed below.

(i) The lack of the N-terminal helix A and C-terminal extension, which are present in PKA and in many other protein kinases. Moreover, the C-terminal stretch of piD261 is unusually basic and includes a seryl residue which displays the consensus sequence for several basophilic protein kinases (e.g. PKA, p70<sup>rsk</sup> and p90<sup>rsk</sup>) [21]. (ii) A striking paucity of residues in the region encompassing subdomains VII and VIII, where only 16 residues connect the end of the putative  $\beta$ 8 strand to helix F, as compared with 35–40 residues in PKA (see Figure 1) and most protein kinases. This is especially remarkable if it is considered that this region includes crucial functional elements, notably the activation

segment and the 'p+1 loop', which in piD261 are shortened drastically (see Figure 2). (iii) The replacement of a lysyl residue (PKA Lys-168), which is strictly conserved in all Ser/Thr protein kinases (see Table 2), where it plays a critical role in the phosphotransferase reaction [15]. In piD261 this lysine is replaced by a threonine (Thr-163) that does not appear to play any critical role in catalysis since its replacement with either Lys (as in all Ser/Thr kinases) or Ala does not significantly affect activity (Table 1). It has to be concluded therefore that Thr-163 is structurally, not functionally, the homologue of conserved PKA Lys-168. This in turn raises the possibility that the mechanism of the phosphotransferase reaction is different from that generally adopted by other Ser/Thr kinases. (iv) Activation through phosphorylation of one or two seryl residues in its abnormally short activation loop, although piD261 does not belong to the category of RD kinases. In these enzymes this kind of regulatory mechanism is considered to be mandatory in order to neutralize the positive charge of the arginine side chain, which otherwise hampers the efficiency of the catalysis accomplished by the adjacent aspartate. It is possible that, as in the case of other kinases [4], the residue(s) of the activation loop phosphorylated in piD261 also interact with basic residues other than the arginine of the RD motif, thus stabilizing the active conformation of the catalytic site. (v) The presence in the piD261 N-terminal moiety of three unique inserts in sharp contrast with the extremely reduced size of the C-terminal lobe. The function of these inserts could be to interact with subunits and/or regulatory proteins that are presently unknown but which are likely to exist, considering the very small size of piD261, shorter than any other protein kinase known.

All the main features of piD261, with the exception of the two inserts in subdomains I and V, are conserved in the structural homologues of higher eukaryotes, notably the human one (see Figure 2), suggesting that they underlie common biological function(s) in distantly related organisms. Apparently these must be related to a very general cellular mechanism, or an early step of a ramified pathway, since in yeast cells disruption of the gene encoding piD261 causes a pleiotropic phenotype that is manifested as severely impaired vegetative growth.

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