Biochemical evidence that *Saccharomyces cerevisiae YGR262c* gene, required for normal growth, encodes a novel Ser/Thr-specific protein kinase

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Abstract ***Saccharomyces cerevisiae YGR262c*** **gene, whose disruption causes severely defective growth, encodes a putative protein kinase shorter than any other protein kinase biochemically characterized to date and lacking some of the conserved features of these enzymes. Here we show that the product of the *YGR262c* gene, piD261, expressed in *E. coli* with a C-terminal $(His)_6$ tag, is a bona fide Ser/Thr protein kinase as judged from its capability to autophosphorylate and to phosphorylate casein and osteopontin in the presence of [γ-^{32}P]ATP. In contrast, no phosphorylation of histones, myelin basic protein, phosvitin, bovine serum albumin and poly(Glu/Tyr)4:1 could be detected. Mn^{2+} or, less effectively, Co^{2+} are required for piD261 catalytic activity, which is conversely undetectable in the presence of Mg^{2+}, a behaviour unique among Ser/Thr protein kinases.**

Key words: Protein kinase; piD261; Casein; Osteopontin; (*Saccharomyces cerevisiae*)

1. Introduction

The reversible phosphorylation of seryl, threonyl and tyrosyl residues of proteins has turned out to be the most frequent and general mechanism by which nearly all cellular functions are regulated in eukaryotes. The enzymes responsible for protein phosphorylation, protein kinases, make up a large superfamily of proteins, sharing catalytic domains with remarkable similarities [1] and a common bilobal architecture as judged from the protein kinase crystal structures solved to date (reviewed in [2–4]). According to their residue specificity they fall into two main subfamilies of Ser/Thr and Tyr-specific kinases, respectively, exhibiting distinctive features in their sequences.

The analysis of yeast (*Saccharomyces cerevisiae*) genome has revealed that out of 5800 genes, 113 (about 2%) encode for putative protein kinases, as judged from the presence in their deduced amino-acid sequences of the typical signatures of this family of enzymes [5]. Part of these genes correspond to those of protein kinases already known at the protein level. The majority however are still awaiting for characterization and the catalytic activity of their products is just inferred from their deduced sequences. Among these, piD261, whose gene is localized to the right arm of chromosome VII [6], is especially attractive for a number of reasons. Firstly disruption of the *YGR262c* gene (encoding piD261) in haploid yeast cells is causative of severely defective growth (unpublished data). Secondly piD261, if really endowed with ATP:protein phosphotransferase activity, would be, with its 261 residues, the shortest protein kinase known to date, entirely lacking the C-terminal subdomain XI (see Fig. 1). If it is considered moreover that the C-terminal 44 residues sequence, including a unique stretch of basic residues, does not display any significant homology with the members of the protein kinase family, it should be concluded that also subdomain X and part of subdomain IX are lacking or deeply altered in piD261. Thirdly some of the conserved features of other protein kinases are absent and/or altered in piD261, notably only the second glycine of the GXGXXG loop and the glutamic acid of the 'APE' motif that defines subdomain VIII are conserved, while the invariant Asp defining subdomain IX (D-220 in PKA) is conservatively replaced by Glu (see Fig. 1). Intriguingly moreover the lysyl residue in the DLKPEN motif, diagnostic of Ser/Thr protein kinases, is substituted by Thr, a feature which could be suggestive of Tyr-specific protein kinases, where this lysine is not conserved.

These observations prompted us to start a study aimed at the biochemical characterization of piD261. Here we show that recombinant piD261, expressed in bacteria, is indeed a bona fide Ser/Thr protein kinase capable to autophosphorylate and to phosphorylate casein in vitro using ATP as phosphate donor and Mn^{2+} as activator.

2. Material and methods

2.1. Materials

Casein, partially dephosphorylated α-casein, phosvitin, myelin basic protein, mixed histones, random polymer poly(Glu/Tyr)4:1 and heparin were from Sigma. Recombinant osteopontin was a generous gift from Dr. Prince (Birmingham, Alabama). Nitrocellulose membrane (0.2 μm) was from Bio-Rad. [γ-^{32}P]ATP (2 mCi/ml) was from Amersham Corp.

2.2. Cloning, Expression and Purification of piD261-His_6

The *YGR262c* coding sequence was amplified by PCR from plasmid pLA635, which contains a 6764 base pairs long *Xho*I-*Bam*HI fragment of *S. cerevisiae* chromosome VII in pGEM-7Zf(+) vector (Promega) [6]. *Pfu* DNA polymerase (Stratagene) and oligonucleotide primers *Nde*I-piD261 (CCATATGACGCAAGAATTCATTG) and piD261-*Xho*I (ATACTCGAGTAGCATACTTCTCTTACGAC) were employed. The amplified fragment was digested with *Nde*I and *Xho*I and inserted into the expression vector pET-20b (Novagen). The resulting plasmid, pET-261, allowed synthesis of piD261-His_6 in a bacterial T7 expression system [7]. The *E. coli* strain BL21(DE3), containing plasmid pET-261, was grown in LB medium (0.5% NaCl, 0.5% Difco yeast extract, 1% Difco bacto tryptone) at 20°C until A_{600} reached 0.7, when transcription of the *YGR262c* coding sequence

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Abbreviations: PKA, cyclic AMP-dependent protein kinase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; FMOC, 9-fluorenylmethoxycarbonyl; Cdk, cell cycle-dependent protein kinase

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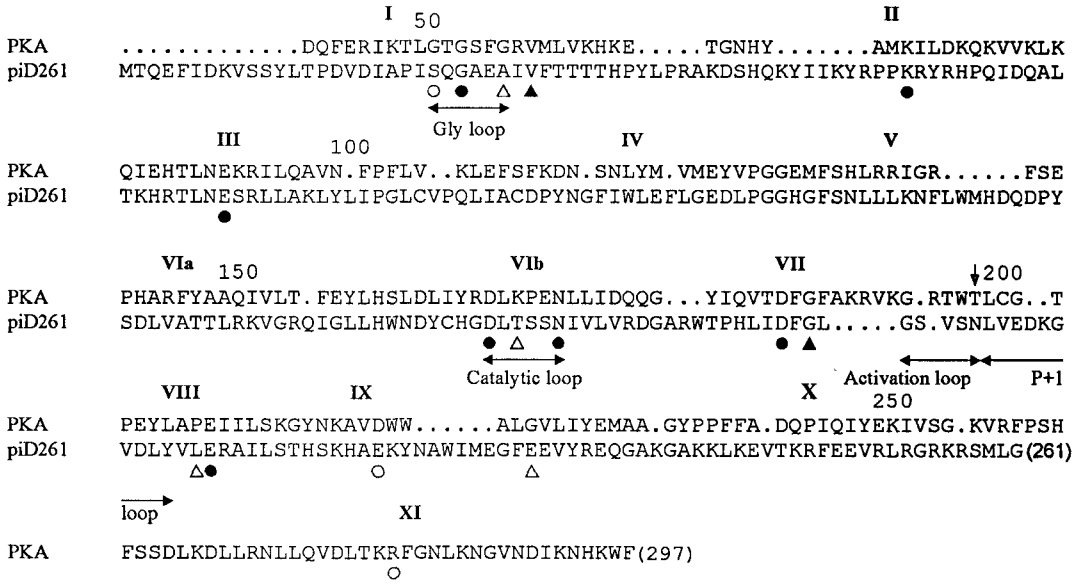

Fig. 1. Amino-acid sequence of piD261. The sequence is aligned to that of PKA. The circles and the triangles denote the 10 invariant (or nearly invariant) residues of protein kinases and the other highly conserved residues, respectively. Closed symbols indicate residues that are conserved in piD261, open symbols those which are not. The conserved regions (I–XI) as defined by Hanks and Hunter [1] are also shown. The 'Gly loop", the 'catalytic loop' and the 'activation loop" plus 'P+1 loop' are highlighted. PKA Thr-197, whose autophosphorylation leads to activation, is denoted by an arrow.

was induced with 0.4 mM IPTG. After 12–16 h of further incubation, bacteria were harvested and resuspended in 50 ml of purification buffer (20 mM Tris-HCl, pH 8, 0.3 M NaCl, 10% glycerol, 0.2 mM PMSF) per liter of culture. Purification was performed according to the protocol of the manufacturer by an affinity column containing Ni-NTA-agarose (Quiagen). piD261-His_6 was eluted with 100 mM imidazole. Protein concentration was determined by the method of Bradford [8] using bovine serum albumin as a standard.

2.3. Immunodetection of piD261

Rabbit antiserum against piD261 was raised using a 16aa peptide reproducing the piD261 sequence 231–245 with the addition of a N-terminal cysteine, according to manufacturer (Pierce). The peptide was obtained by solid-phase peptide synthesis (FMOC protocol) using an automatic peptide synthesizer (Model 431-A Applied Biosystems). Approximately 500 ng of recombinant piD261-His_6 was subjected to 12% SDS-PAGE [9], protein was blotted onto nitrocellulose membrane and for detection the rabbit antiserum against piD261 was diluted 1000-fold. The reaction with first antibody was followed by incubation with a phosphatase-coupled antibody direct against the corresponding IgG type of the first antibody.

2.4. Phosphorylation assays

piD261 protein kinase activity was routinely assayed by incubation at 37°C for 10 min in a final volume of 20 μl in the presence of 50 mM Tris HCl pH 7.5, 10 mM $MnCl_2$, 50 μM [γ-^{32}P]ATP (specific activity 700–2000 cpm/pmol), casein (0.25–1 mg/ml) and recombinant His-tagged piD261 (10 μg/ml). In similar experiments casein was replaced by other protein substrates, as indicated in Table 1. The reaction was stopped by addition of gel electrophoresis loading buffer and samples were subjected to 12% SDS-PAGE [9] followed by autoradiography. The radioactive bands were quantified by a Instant Imager apparatus (Packard).

Autophosphorylation of piD261 was performed and measured by incubation in the same medium described above, except for the absence of any protein substrate. Alkali lability of ^{32}P incorporated in either casein or piD261 was assessed by treating gels, after the electrophoretic run, with a solution of 2 M NaOH at 56°C for 2 h and

Table 1
Protein substrates specificity and responsiveness to various effectors of piD261

Substrates	Protein kinase activity
Casein	100
Casein plus GTP (0.5 mM)	101
Casein plus staurosporine (200 μM)	95
Casein plus heparin (0.1 μg/ml)	102
Casein plus heparin (1 μg/ml)	61
Casein plus heparin (10 μg/ml)	54
Casein plus heparin (50 μg/ml)	32
Myelin basic protein	<1
Poly(Glu/Tyr)4:1	<1
Histones	<1
Osteopontin (0.05 mg/ml)	161
α-Casein dephosphorylated (0.12 mg/ml)	275
Phosvitin	<1
Bovine serum albumin	<1
Ovalbumin	<1
CK2 peptide (RRRAADSDDDDD)	<1
CK1 peptide (RRKDLHDDEEDEAMSITA)	<1

The concentration of all substrates was 1 mg/ml, except otherwise indicated. Activities are expressed relative to casein (= 100).

comparing the radioactivity present in the protein bands before and after this treatment. Phosphoamino acids were isolated after partial hydrolysis of the eluted radiolabeled proteins by high voltage paper electrophoresis as previously described [10]. Kinetic constants were determined by double reciprocal plots, constructed from initial-rate measurements fitted to the Michaelis-Menten equation. The values reported are the means of three separate experiments with a standard error less than 15%.

3. Results

The deduced amino-acid sequence of piD261 is shown in Fig. 1. The protein was cloned and expressed in *E. coli* with a tag of six tandem histidines (His_{6}) to the carboxyl terminus to facilitate purification from bacterial extracts. The cloning strategy used for adding the tag region (see the experimental procedures in Section 2) has produced the substitution of the last glycine present at the C terminus with a leucine, the latter being followed by a glutamic acid preceding the six histidines. As shown in Fig. 2A, after induction of piD261-His_6 expression, a prominent protein band of the expected 31 kDa is present in bacterial extracts (lane 1). After affinity chromatography on nickel-nitrilotriacetic acid (Ni-NTA)-agarose column, the 31-kDa band was purified to near homogeneity as judged from SDS-PAGE (Fig. 2A, lane 2). This band is recognized by the regio-specific antibodies raised against the EQGAKGAKKLKEVTK peptide sequence localized near the C terminus of piD261 (Fig. 2A, lane 3), and the signal is suppressed by the presence of an excess of the peptide reproducing the same sequence (not shown). The identity of this band as recombinant piD261-His_6 was further confirmed by N-terminal sequencing, which revealed the expected (M)TQEFIDKV sequence, and by its M_r (30 798) determined by MALDI, which matches almost perfectly the theoretical mass of piD261-His_6 (30 813).

Upon incubation at pH 7.5 in a medium containing 50 mM [γ-^{32}P]ATP and 10 mM MnCl2 for 10 min at 37°C piD261 underwent autophosphorylation giving rise to a doublet of radiolabeled bands (Fig. 2B). Such a radiolabeling increased linearly with the increase of piD261 concentration, consistent with an intra- rather than an inter-molecular process. As shown in Fig. 2C, piD261 catalyzes also the phosphorylation of added casein. By contrast no significant phosphorylation of myelin basic protein, mixed histones, phosvitin, bovine serum albumin, ovalbumin and poly(Glu/Tyr)4:1 and of two specific peptide substrates of protein kinases CK1 (casein kinase 1) and CK2 (casein kinase 2) could be detected (Table 1). Interestingly partially dephosphorylated α-casein is phosphorylated more readily than the native protein suggesting that some of the serines that are phosphorylated in native casein

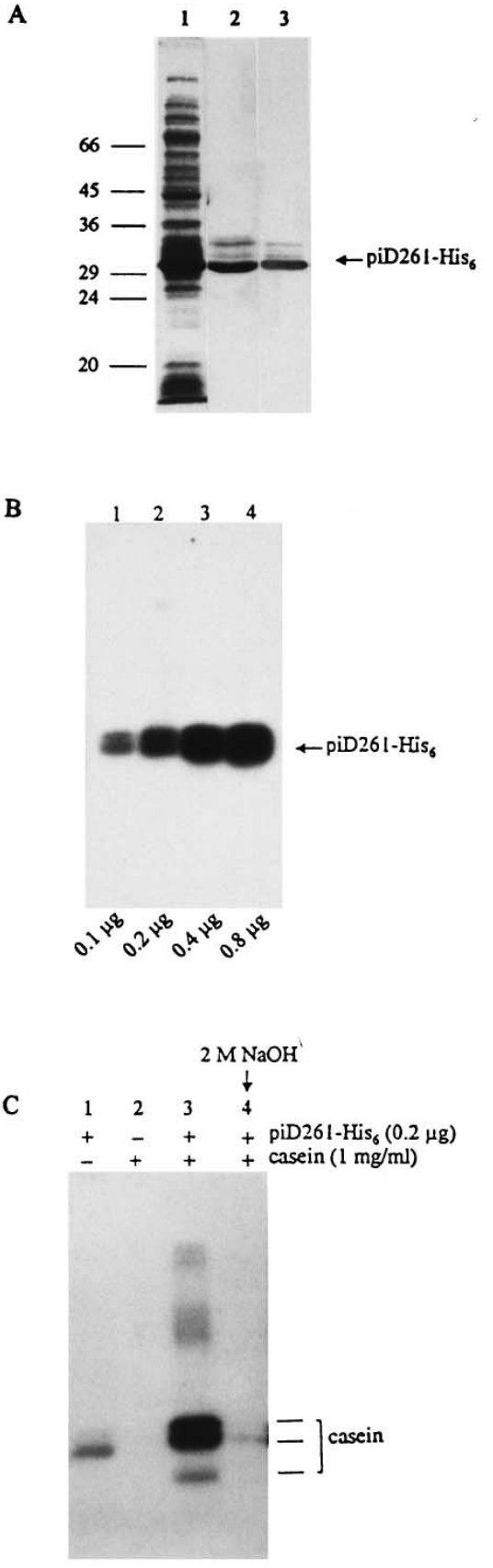

Fig. 2. Purification of recombinant piD261-His_6 and demonstration that it exhibits protein kinase activity. In (A) the Coomassie staining (lanes 1 and 2) and Western blotting (lane 3) of soluble bacterial crude extract (lane 1) and purified piD261 (lane 2 and 3) after SDS-PAGE are shown. Positions migrated by marker proteins are indicated on the left. In (B) and (C) the autoradiographs are shown of gels where either increasing amounts (as indicated) of piD261 alone (B) or piD261 plus casein (C), previously incubated with [γ^{32}-P]ATP, were run. Lane 4 in panel C is equivalent to lane 3 except for treatment of the gel with 2 M NaOH before exposure. Details are given in the experimental procedures in Section 2. Position of main casein fractions (α, β and κ-casein) are indicated on the right in panel C. The radioactive bands of piD261 (lane B3) and casein (lane C3) were eluted and analysed for their content in phosphoamino acids (see the experimental procedures in Section 2). In both Ser-P was predominant (76% and 80%, respectively) over Thr-P. No Tyr-P could be detected.

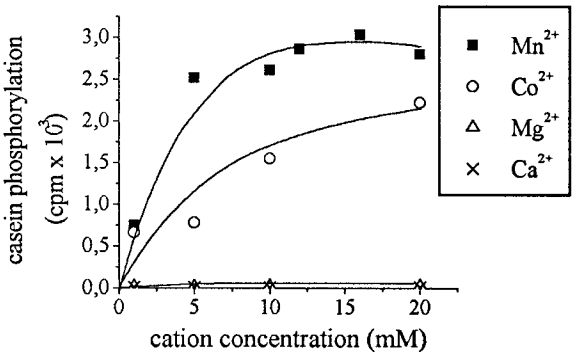

Fig. 3. Divalent cation requirement for piD261 protein kinase activity.

are also affected by piD261. Consistent with this, osteopontin, which includes many potential phosphoacceptor sites displaying the motif phosphorylated in casein fractions (S/T-X-E) [11] is also readily phosphorylated by piD261 (Table 1).

Radiolabeling of both piD261 and casein disappeared upon incubation of the gel in 2 M NaOH (Fig. 2C, lane 4), suggesting that phosphorylation is not accounted for by phosphotyrosine. Radioactive phosphoserine and, to a lesser extent, phosphothreonine could be isolated after partial acid hydrolysis of either autophosphorylated piD261 or phosphorylated casein, by high voltage paper electrophoresis (not shown, for further details see legend of Fig. 2). Time course phosphorylation experiments showed that phosphorylation of casein proceeds linearly for 30 min and is proportional to the amount of piD261 added, as expected for an enzymatic reaction (not shown). From the initial rates of phosphorylation measured by varying the concentration of the substrates and double reciprocal plot analyses the K_m values for ATP and casein were calculated to be 9.5 μM and 1.1 μM, respectively.

GTP cannot replace ATP as phosphate donor, as judged from isotopic dilution experiments in which a 10-fold excess of unlabeled GTP added to 50 μM [γ-^{32}P]ATP did not significantly reduce the radiolabeling of casein (Table 1). As also shown in Table 1 staurosporine, a powerful inhibitor of many, albeit not all, protein kinases is almost ineffective on piD261, exhibiting an $IC_{50} > 100$ μM, a huge value as compared to IC_{50} values in the low nmolar range typical of the majority of protein kinases [12]. In contrast phosphoradiolabeling of casein by piD261 is inhibited by heparin, with an IC_{50} value of 10 μg/ml (see Table 1).

Quite unexpectedly for a Ser/Thr protein kinase, piD261 is strictly Mn^{2+}-dependent, while Mg^{2+} is totally unable to sustain the catalytic activity, even if added at a concentration up to 20 mM (Fig. 3). Ca^{2+} is also ineffective while Co^{2+} triggers kinase activity, though less efficiently than Mn^{2+}.

4. Discussion

piD261 is the product of a yeast gene whose disruption is causative of severely defective growth both in terms of reduced division rate and of lower cell concentration at stationary phase (unpublished data). Since the deduced primary structure of piD261 displays evident similarity with the catalytic domain of protein kinases, it was tempting to correlate its functional role to its putative ATP: protein phosphotransferase activity. On the other hand piD261 is shorter than any other protein kinase known to date, since it completely lacks subdomain XI, and displays some unique features as well, notably a very basic C-terminal segment sharing no apparent homology with protein kinase subdomain X, a single glycine left in the glycine loop, proline replaced by leucine in the APE motif and the lysyl residue which is diagnostic of Ser/Thr kinases within the DLKPEN sequence substituted by a threonyl residue (see Fig. 1). It was therefore important to check if piD261 is really endowed with protein kinase activity, either Ser/Thr or Tyr-specific. To this aim we have cloned and expressed piD261 in *E. coli* and provided the evidence that it is indeed a bona fide protein kinase, capable to autophosphorylate and to catalyze the phosphorylation of casein and osteopontin at seryl and threonyl residues. Despite its somewhat anomalous Gly loop piD261 exhibits a quite low K_m for ATP (9.5 μM), comparable to the K_m values of highly efficient protein kinases, like PKA and CK2, and significantly lower than the K_m values of other kinases, e.g. cdc2 kinase (around 100 μM). This did not entirely come as a surprise considering that the main residues implicated in ATP binding by PKA [13] are also conserved in piD261. piD261 also displays a quite low K_m, 1.1 μM, for the phosphate acceptor protein, casein. This is remarkable in the light of the fact that bona fide casein kinase from lactating mammary gland as well as ubiquitous 'casein kinases" of the CK1 and CK2 types display quite higher K_m values toward casein, around 150μM [14] and 50 μM [15,16], respectively. The finding that partially dephosphorylated casein and osteopontin are phosphorylated by piD261 more readily than native casein itself, while a number of other proteins are not, may suggest that piD261 recognizes a motif identical or similar to the S-X-E triplets, which are present both in casein (where they are mostly occupied by endogenous phosphate, however) and in osteopontin, and which are specifically affected by the Golgi apparatus casein kinase (G-CK) as well [17,18].

Pertinent to this could be the observation that some properties of piD261, notably its substrate specificity, insensitivity to staurosporine and use of Mn^{2+} instead of Mg^{2+} as activator, are intriguingly reminiscent of the Golgi casein kinase (G-CK) which is specifically localized to and tightly associated with the Golgi apparatus of several tissues [18] and whose amino-acid sequence is still unknown. In accordance with the observed low level of transcription of the YGR262c gene [6], the amount of piD261 in wild type yeast cells is below the threshold of detectability by the antibodies used in this study; its subcellular localization therefore could not be determined. By contrast to G-CK, piD261 is inhibited by heparin, though less efficiently than CK2 ($IC_{50} = 10$ vs 0.1 μg/ml). This may reflect the presence in piD261 of a basic triplet (^{70}KHR^{72}) which is homologous to three residues of a basic quartet (^{74}KKKK^{77}) of CK2 responsible for sensitivity to heparin inhibition [19–21].

Quite strikingly piD261 is absolutely dependent on Mn^{2+} for activity, whereas the Golgi casein kinase by far prefers Mn^{2+} but it works with Mg^{2+} as well [14]. This unique feature, not shared to our knowledge by any other Ser/Thr protein kinase, raises the question as to how does piD261 operate in vivo, considering that in living organisms Mn^{2+} is 100–200-fold more diluted than Mg^{2+}. It is conceivable therefore that in some subcellular compartments Mn^{2+} is rate limiting for piD261 activity. Interestingly while $[Mn^{2+}] \geq 10$ mM in the culture medium is detrimental to normal growth of wild

type yeast cells, this concentration is better tolerated by yeast overexpressing piD261 (unpublished data). This observation discloses the possibility that piD261 in yeast could operate as a 'Mn^{2+} gauge', triggering reactions that lead to a decrease of Mn^{2+} intracellular concentration or to the neutralization of its toxic effects, whenever the concentration of this cation tends to become too high. The implication of protein phosphorylation events in Mn^{2+} homeostasis in yeast would not come as a surprise since protein phosphatase 2B has been shown to be required for tolerance to manganese [22].

Given the unique molecular and catalytic properties of piD261 and its requirement for yeast normal growth it will be interesting to check if kinase(s) identical or similar to it are present in higher organisms as well. Experiments are in progress aimed at assessing this possibility.

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