Biochemical characterization of recombinant yeast PPZ1, a protein phosphatase involved in salt tolerance

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Abstract **The *Saccharomyces cerevisiae* gene *PPZ1* codes for a 692-residues protein that shows in its carboxyl-terminal half about 60% identity with the catalytic subunit of mammalian and yeast protein phosphatase-1 and that is involved in salt homeostasis. The complete PPZ1 protein has been succesfully expressed as a soluble glutathione-S-transferase fusion protein. The recombinant protein, after purification by a single affinity chromatography step, displayed phosphatase activity towards a number of substrates, including myelin basic protein, histone 2A and casein, but was ineffective in dephosphorylating glycogen phosphorylase. It was also active towards *p*-nitrophenylphosphate. The activity was severalfold increased by the presence of Mn^{2+} ions and by limited trypsinolysis. The enzyme was inhibited by okadaic acid and microcystin-LR at concentrations comparable to what is found for type 1 protein phosphatase although it was much less sensitive to inhibitor-2. The recombinant protein was phosphorylated in vitro by cAMP-dependent protein kinase, protein kinase C and casein kinase-2. Phosphorylation affected preferentially sites located in the amino-terminal half of the protein and did not alter the activity of the phosphatase.**

Key words: Bacterial expression; Fusion protein; Protein phosphatase; *Saccharomyces cerevisiae*

1. Introduction

Ser/Thr protein phosphatases have been traditionally classified in four groups, namely type 1, 2A, 2B and 2C, on the basis of their enzymological properties. With the exception of type 2C, these enzymes are found as oligomers composed of one catalytic subunit and one or more regulatory subunits. The elucidation of the primary structure of their catalytic subunits revealed that type 1 (PP-1c), 2A (PP-2Ac) and 2B enzymes are related in sequence (see [1] for review) and was the starting point for the identification of a number of DNA clones from mammals, *Drosophila* and both fission and budding yeast encoding proteins structurally related but clearly distinct from the above mentioned enzymes [2,3]. In some cases, these novel phosphatases have been found in a variety of species and share with PP-1c and PP-2Ac the characteristic of being extremely conserved in their primary structure. For instance, this is the situation for PPX, a PP-2Ac-related protein that has been cloned from mammals [4,5], *Drosophila* [5] and higher plants [6]. However, in most cases these novel forms have been only identified so far in a single species. The biological role, as well as the enzymological characteristics and the regulation of these proteins are, almost without exception, completely unknown.

The genome of the budding yeast *Saccharomyces cerevisiae* encodes not less than five of these novel putative phosphatases, namely SIT4/PPH1 [7], PPH3 [8], PPZ1 and PPZ2 [9–11] and PPQ [12], although PCR experiments have suggested the existence of additional related genes [13]. The complete *PPZ1* gene was initially cloned in our laboratory some time ago [9] and found to encode a protein of 692 amino acids whose carboxyl-terminal half could be aligned with PP-1c from a large variety of species (about 60% of identity). On the contrary, its large amino-terminal extension was unrelated to Ser/Thr phosphatases and contained a remarkably high number of Ser residues. A second, closely related gene, named *PPZ2* has been also cloned [10,11]. We have found that simultaneous disruption of both genes results in a phenotype of hypersensitivity to caffeine that leads to cell lysis unless the growth medium was osmotically stabilized [14]. The idea that one of the biological roles of PPZ1/PPZ2 is related to the maintenance of cell integrity is in keeping with the isolation of gene *PPZ2* as a multicopy suppressor of the lytic defect associated with the disruption of the gene *SLT2/MPK1*, which codes for a MAP kinase homologue [11]. Very recently, we have proven that PPZ1/PPZ2 are key determinants in salt homeostasis. Cells lacking PPZ1 and PPZ2 tolerate high concentrations of Na^+ and Li^+ cations as a result of an increase in the efflux of these cations. Our data indicate that the ENA1 gene, encoding the putative P-type ATPase acting as a major determinant of cation efflux in yeast [15] is a molecular target for PPZ1. Our laboratory is presently characterizing the biological role and enzymological properties of the PPZ proteins. As one of the steps in this direction, we report here the bacterial expression of PPZ1 and the characterization of the recombinant enzyme.

2. Materials and methods

2.1. Strains and media

E. coli Strain NM522 was employed for both construction of vectors and expression experiments. Standard methods were used for the construction of plasmids [16]. Bacteria were grown in LB medium (1% tryptone, 0.5% yeast extract and 1% NaCl) supplemented with the appropriate antibiotic when needed for plasmid maintenance.

2.2. Expression of recombinant PPZ1 using the pGEX-KT vector

The complete open reading frame encoding PPZ1 was fused to the GST moiety as follows. PCR reactions were carried out using the PPZ-A plasmid and oligonucleotides 5′ CGGGATCCATGGGTAATTCAAGTTCA 3′ and 5′ CCAAGCTTACTGTTGAGATTCGTT 3′ (underlined are *Bam*HI and *Hin*dIII sites) to create a *Bam*HI site in front of the initiating methionine codon and a *Hin*dIII site right after

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Abbreviations: CK-2, casein kinase-2; GST, glutathione-S-transferase; PK-A, cAMP-dependent protein kinase, PK-C, protein kinase C; PP, protein phosphatase.

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the stop codon of the PPZ1 open reading frame (plasmid PPZ-A is the original 4.5 kbp clone isolated from a size-selected genomic library constructed in pUC19 [9] and contains the complete PPZ1 gene) PCR conditions were: 94°C for 1.5 min, 50°C for 2 min and 72°C for 3 min. A final extension step of 3 min at 72° was added. The PCR product was a single band of about 2.1 kbp that was digested with *Bam*HI and *Hin*dIII and ligated into the *Bam*HI and *Hin*dIII sites of plasmid pSP72 (Promega) to yield pSP72-PPZ1. This plasmid was digested with *Pvu*II and *Bam*HI and ligated into the *Sma*I and *Bam*HI sites of the expression vector pGEX-KT [17,18] creating the pGEX-KT/PPZ1 construct.

Twenty five ml of a culture of NM522 *E. coli* cells carrying the pGEX-KT/PPZ1 plasmid were grown overnight at 37°C and then used to inoculate 1 liter of LB medium containing ampicillin. Growth at 37°C was resumed until the absorbance at 600 nm was 0.8–1. Isopropylthiogalactoside was then added to a final concentration of 1 mM, and the culture was grown for an additional 3 hours at 28°C. The cells were harvested by centrifugation, resuspended in 20 ml of STET buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, Triton X-100 1%) containing 0.1% (v/v) β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM benzamidine, 1 μg/ml leupeptin and 1 μg/ml pepstatin. Bacteria were lysed by sonication and the lysate was clarified by centrifugation at 4°C (10 min, $7000 \times g$). Aliquots of the supernatant were stored at −80°C. For affinity purification of the GST-PPZ1 recombinant protein, 2 ml of bacterial supernatant prepared as described above were mixed with 1 ml of a 50% (v/v) suspension of glutathione-agarose beads (Sigma Chem. Co.) and incubated for 30 min at 4°C with gentle shaking. The agarose beads were washed four times with STET buffer and, finally, once with 50 mM Tris pH 8.0, 1 mM EDTA. The fusion protein was eluted with 10 mM glutathione in 50 mM Tris pH 7.5, 2 mM dithiothreitol, 150 mM NaCl [17,19]. Cleavage of the GST moiety was carried out essentially as described in [18] and was monitored by SDS-polyacrylamide gel electrophoresis.

2.3. Phosphatase assays

Phosphatase substrates were phosphorylated by rabbit skeletal muscle protein kinase A in the presence of 0.2 mM [γ-^{32}P]ATP ($0.8–1 \times 10^6$ cpm/nmol) and 10 mM magnesium acetate. The substrates used were myelin basic protein (6.75 nmol of phosphate/mg of protein), histone 2-A (17 nmol/mg protein), casein (22 nmol/mg protein). Phosphorylase *b* was fully converted to the active *a* form by phosphorylation with purified phosphorylase kinase [20]. The phosphatase assays were carried out according to methods previously described [21] on a final volume of 30 μl. The final concentrations of the substrates were of 135 μg/ml (1 mg/ml for phosphorylase) and the concentration of recombinant PPZ1 in the assay was between 1 and 5 μg/ml. Unless otherwise stated, 25 mM Mn_2Cl was present in the PPZ1 assays. One unit of phosphatase was defined as the amount of enzyme which releases one μmol of phosphate/min from ^{32}P-labeled myelin basic protein. *p*-nitrophenylphosphatase activity was measured as described in [22]. One unit of activity is defined as 1 μmol of *p*-nitrophenylphosphate hydrolyzed/min.

The effect of limited trypsinolysis on phosphatase activity was tested by incubation of the above mentioned mixture (without ^{32}P-labeled substrate) with trypsin (200 μg/ml) for five min at 30°C. Soybean trypsin inhibitor was added to a final concentration of 0.75 mg/ml at the end of the incubation. Phosphatase activity was then determined as described above, after addition of the ^{32}P-labeled substrate.

Tyr-protein phosphatase activity was assayed with modified lysozyme as substrate. The preparation and phosphorylation of the substrate and the assay conditions were as described [23].

2.4. Phosphorylation of recombinant PPZ1

Recombinant GST-PPZ1 was phosphorylated in vitro as follows: 90 ng of the protein were incubated for 30 min at 30°C in a final volume of 20 μl in the presence of 37.5 mM Tris pH 7.5, 0.1 mM [γ-^{32}P]ATP (0.1 μCi/nmol), 1 mM magnesium acetate. The mixture included preparations of either rabbit skeletal muscle PK-A (125 mU), rat liver CK-2 (15 mU) or rat brain PK-C (30 mU). When the latter kinase was employed, 0.1 mM calcium chloride, 0.15 mg/ml of phosphatidylserine and 15 μg/ml of diolein were also included in the reaction mixture. After incubation samples were boiled and analyzed by electrophoresis. In the case of PK-A, the commercial preparations (Sigma Chemical Co.) used for testing the effect of phosphorylation on PPZ1 activity, were subjected to gel filtration in a Superdex 75 FPLC column (Pharmacia) to remove a termostable, low molecular weight inhibitory activity of the phosphatase present in the kinase preparations.

2.5. Other techniques

2.5.1. Electrophoresis of proteins. Protein samples were boiled in 50 mM Tris-HCl pH 6.8, 100 mM dithiothreitol, 2% (w/v) SDS, 0.1% (w/v) Bromophenol blue and 10% (v/v) glycerol. Electrophoresis in SDS-polyacrylamide gels was performed as described [24].

2.5.2. Determination of protein concentration. The concentration of proteins was determined by the Bradford's method [25] using bovine serum albumin as a standard.

2.5.3. Purification of PP-1c and inhibitor-2. The catalytic subunit of PP-1 and inhibitor-2 were isolated from rabbit skeletal muscle as described [26,27]

2.5.4. Cyanogen bromide cleavage and phosphoaminoacid analysis. For cyanogen bromide analysis samples of previously phosphorylated PPZ1 were boiled for five min and digested at room temperature for 24 h in a 70% (v/v) formic acid solution containing 50 mg/ml of CNBr. Samples were evaporated to dryness and electrophoresed in SDS-polyacrylamide gels. In the case of samples phosphorylated with CK-2, the GST-PPZ1 protein was replaced by the GST fragment and run in parallel to identify the radioactive fragments derived from the autophosphorylated kinase. For phosphoamino acid analysis, samples of previously phosphorylated PPZ1 were electrophoresed in SDS-polyacrylamide gels and the fragment of the gel containing the 110 kDa radioactive band was excised, washed with water and incubated at 110°C in the presence of 6 M HCl for 4 h. After evaporation to dryness, samples were supplemented with phosphoaminoacid standards and subjected to two-dimensional thin-layer electrophoresis on cellulose plates at pH 1.9 and 3.5, successively, as described [28].

3. Results and discussion

3.1. Expression of PPZ1 as a GST-fused protein

Initial attempts to express the complete PPZ1 open reading frame, using the pT7-7 system [29], were unsuccesful. An (insoluble) expression product was obtained, however, with a construct containing the putative catalytic domain (residues 297–692). Attempts to renature this fragment yielded a soluble material with a detectable but very low activity towards ^{32}P-labeled myelin basic protein (0.10 mU/mg) that could be enhanced to 0.35 mU/mg by limited trypsinolysis.

Since the results obtained using a T7-based system were not satisfactory, a second strategy was devised, based on the in frame fusion of the complete PPZ1 open reading frame with the 26 kDa fragment of the GST present in the pGEX-KT vector [18]. After induction of the cultures with isopropylthiogalactoside, electrophoresis of bacterial buffered extracts revealed the presence of a soluble protein with an estimated molecular mass of about 110 kDa which was not present in uninduced cultures. This protein was purified in a single step using glutathione-agarose affinity chromatography (Fig. 1). Approximately 2–4 mg of the recombinant protein were recovered from 1 liter of bacterial culture. An important parameter in the expression experiments was the temperature of the culture during induction: expression at temperatures higher than 28°C produced essentially insoluble protein. Some preparations contained variable amounts of a shorter polypeptide with an estimated molecular mass of 32 kDa (i.e. the polypeptide running close to the electrophoretic front in lane 3 of Fig. 1) that consisted of the GST moiety plus the first 45–50 amino acids of the PPZ1 protein. Evidence for the nature of this fragment are that it binds again to the column when submitted to a second chromatography in a glutathione-agarose affinity system. Furthermore, the expression of the N-terminal half of PPZ1 (from amino acid 1 to 319) as a GST-fusion protein using the same

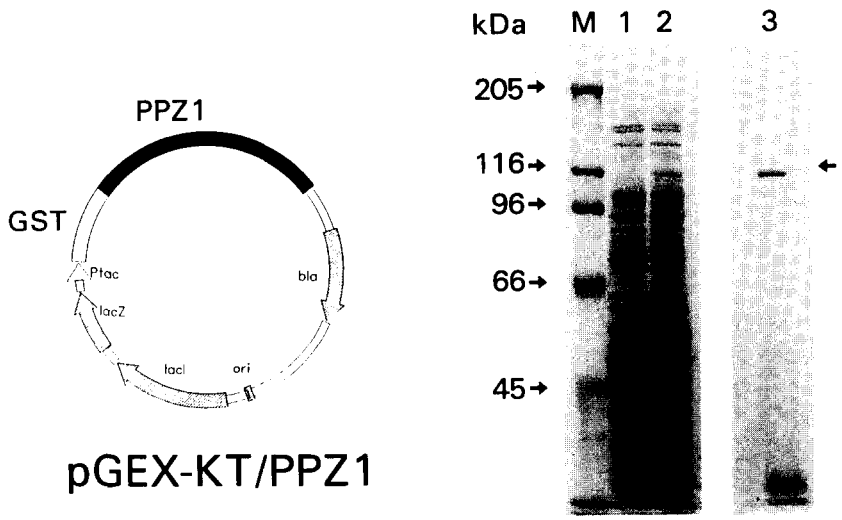

Fig. 1. Expression of recombinant PPZ1 using the pGEX-KT vector. The diagram on the left shows the pGEX-KT/PPZ1 construct used for expression. On the right, a Coomassie blue-stained electrophoretic analysis of *E. coli* extracts from cells transformed with the plasmid pGEX-KT/PPZ1, before induction (1) and after three hours in the presence of 1 mM isopropylthiogalactoside (2) is presented. The recombinant protein was purified by glutathione-agarose chromatography and is shown in lane 3. Note the presence of the 32 kDa protein migrating slightly slower than the front (see the main text). Lane M, standard molecular weight markers.

vector also yields the 32 kDa fragment in addition to the expected expression product (not shown). The 110 kDa GST-PPZ1 protein can be separated from the 32 kDa fragment by ion exchange chromatography in a Mono Q column (Pharmacia).

3.2. Catalytic properties of recombinant PPZ1

The recombinant PPZ1 was active as phosphatase on different substrates, as shown in Table 1. Interestingly, it did not show any activity on glycogen phosphorylase, a well-known substrate for protein phosphatases-1 and 2A. We found the most suitable protein substrate to be myelin basic protein phosphorylated by PK-A. Using this substrate, the effect of partial trypsinolysis on the activity of the enzyme was studied. As shown in Table 1, treatment with trypsin resulted in a 6-fold increase in activity. The recombinant protein also displayed activity towards *p*-nitrophenyl phosphate. A noticeable activity towards *p*-nitrophenyl phosphate has been reported in the case of bacterially expressed PP-1c [30], although there are contradictory reports regarding the ability of native PP-1c to dephosphorylate the mentioned nonprotein substrate. For another Ser/Thr phosphatase (PP-2A) Goris et al. [31] reported a correlation between *p*-nitrophenyl phosphatase activity and phosphotyrosyl activity. However, we observed that PPZ1 was unable to dephosphorylate Tyr-phosphorylated lysozyme, which is a commonly used substrate for Tyr-protein phosphatases [32]. All properties tested of the recombinant GST-PPZ1 polypeptide (for instance: specific activity, incapacity to dephosphorylate phosphorylase, dependence of Mn^{2+}, sensitivity to inhibitors) were essentially identical to those of the product resulting from the cleavage of the thrombin recognition sequence that is present in the boundary between the GST polypeptide and the PPZ1 open reading frame. Therefore, the complete GST-PPZ1 was used routinarily for characterization. The presence of variable amounts of the 32 kDa fragment did not affect the catalytic properties of the recombinant GST-PPZ1, as observed after further purification of the 110 kDa GST-PPZ1 protein by ion exchange chromatography on a Mono Q column.

Table 1
Phosphatase activity of recombinant PPZ1

Substrate	Specific activity (mU/mg protein)
Myelin basic protein	
− trypsin	16.9 ± 0.4
+ trypsin	107.8 ± 14.5
Histone 2A	10.5 ± 0.9
Casein	2.2 ± 1.2
Phosphorylase	n.d.
p-Nitrophenyl phosphate	118.0 ± 9.8
Tyr(P)-lysozyme	n.d.

Substrates were assayed as described in section 2, in the presence of 25 mM of $MnCl_2$. Protein substrates were phosphorylated with cAMP-dependent protein kinase or, in the case of phosphorylase, with phosphorylase kinase. The concentration of *p*-nitrophenol phosphate in the assay was 5 mM. n.d. indicates non-detectable activity. Results are means ± S.E.M. of three different experiments.

The PPZ1 activity could be inhibited by fluoride (IC_{50} = 9.3 ± 0.9 mM) and ATP (IC_{50} = 2.4 ± 0.3 mM). When the dependence on divalent cations was tested using myelin basic protein as substrate (Fig. 2), it was found that Mg^{2+} and Ca^{2+} at a concentration of 1 mM were unable to affect the activity of the phosphatase. However, addition of 1 mM of EDTA (but not EGTA) resulted in a modest (35%) decrease in activity. Interestingly, the activity increased fourfold in the presence of 1 mM Mn^{2+}, and up to sixfold when the concentration of Mn^{2+} was increased in the interval 1–25 mM. No activation was detected at concentrations below 0.1 mM. These concentrations are much higher than those required for bacterially expressed PP-1, which was fully activated by 0.1 mM Mn^{2+} [30]. It is worth noting that in the case of PP-1, the dependence of Mn^{2+} is a characteristic of the bacterially expressed enzyme but not of the native phosphatase. When refolded in the presence of inhibitor-2, a specific cytoplasmic protein inhibitor, bacteri-

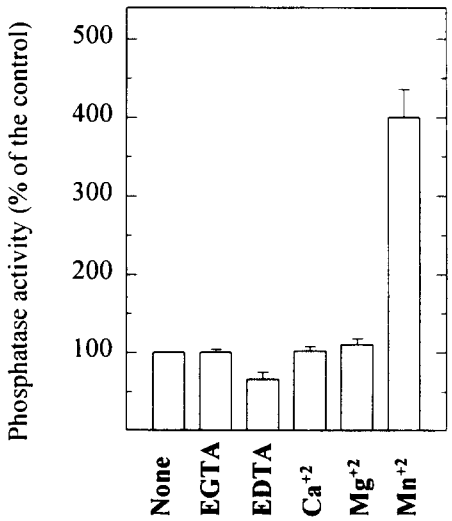

Fig. 2. Effect of different effectors on the recombinant PPZ1 phosphatase activity. 0.05 mU/ml of purified recombinant PPZ1 was assayed (see section 2) on ^{32}P-labeled myelin basic protein in the presence of different effectors at a final concentration of 1 mM. Results are shown as percentage of the activity in absence of the effector and are means ± S.E.M. of 5 experiments.

ally expressed PP-1c became Mn^{2+}-independent [33]. Mn^{2+} was also found to be essential for the renaturation of PP-1c expressed in insect cells, but subsequently the renatured enzyme was independent of Mn^{2+} [34]. The PPZ1 activity displayed towards *p*-nitrophenyl phosphate was completely dependent on the presence of Mn^{2+} ions (not shown).

Protein phosphatases-1 and -2A are sensitive to the well-known inhibitors okadaic acid and microcystin-LR [35,36]. We have found that recombinant PPZ1 is also sensitive to the mentioned toxins. As shown in Fig. 3 (left panel), a significant inhibition was observed at concentrations of okadaic acid of 0.1–1 μM. The phosphatase activity was almost fully inhibited by 10 nM of microcystin. The IC_{50} calculated for okadaic acid and microcystin were of 0.67 μM and 0.65 nM, respectively. When the IC_{50} of these inhibitors was determined under the same conditions for the catalytic subunit of PP1 from rabit skeletal muscle the values obtained were 0.14 μM and 0.69 nM, respectively. These concentrations are within the range of those reported in the literature [37]. Therefore, the sensitivity of recombinant PPZ1 and native PP1 to the inhibitors okadaic acid and microcystin is quite similar. The sensitivity of PP-1c for these inhibitors have been recently attributed to the sequence GEFD present in the carboxyl-terminal domain, which is identical in PPZ1 (residues 629–632), but corresponds to YRCG in PP-2Ac [38]. We have also investigated whether the activity of PPZ1 was affected by inhibitor-2, a specific cytoplasmatic protein inhibitor of PP-1. As illustrated in Fig. 3 (right panel), PPZ1 was only slightly inhibited by micromolar concentrations of inhibitor-2. Contrary to what has been reported for PP-1 holoenzymes (discussed in [37]), the sensitivity of PPZ1 to inhibitor-2 was not increased by prior treatment with trypsin (not shown). However, the low sensitivity to inhibitor-2 may not be an intrinsic property of PPZ1, since it may at least in part be explained by the basic character of the substrate. Indeed, with myelin basic protein as substrate, IC_{50} of inhibitor-2 acting on PP-1c was estimated at 100 nM, in sharp contrast with an IC_{50} of 3 ± 1 nM (n = 3) with phosphorylase as substrate (not shown). A similarly low sensitivity of PP-1c to inhibitor-2 has been noted by others with phosphorylated histone H1 as substrate [33,39].

The primary structure of PPZ1 (C-terminal half) displays more homology with PP-1c than with other Ser/Thr-protein phosphatases. Our current data reveal that this similarity

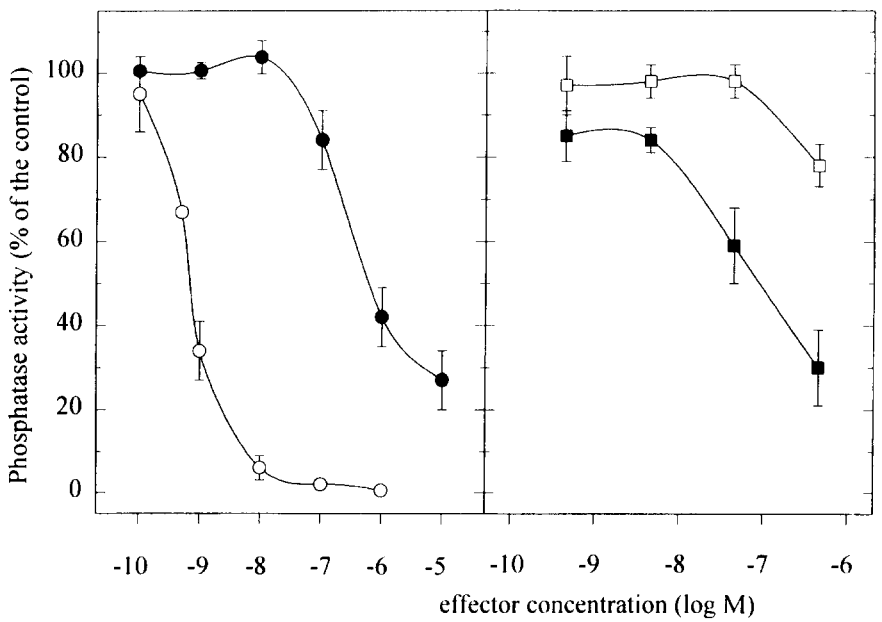

Fig. 3. Inhibition of recombinant PPZ1 by microcystin, okadaic acid and inhibitor-2. (Left panel) Purified recombinant PPZ1 was assayed using ^{32}P-labeled myelin basic protein in the presence of varying amounts of microcystin (open circles) and okadaic acid (closed circles). (Right panel) Rabbit muscle PP-1c (closed squares) and recombinant PPZ1 (open squares) were assayed on ^{32}P-labeled myelin basic protein in the presence of varying amounts of rabbit muscle inhibitor-2. Results are shown as percentage of the activity in the absence of inhibitors and are means ± S.E.M. of 5 experiments.

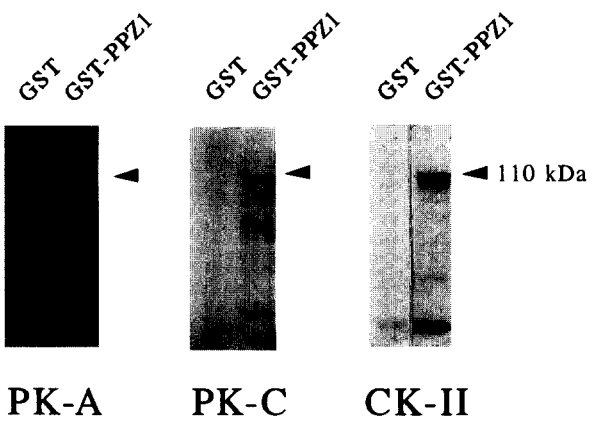

Fig. 4. In vitro phosphorylation of recombinant PPZ1. Recombinant GST-PPZ1 was phosphorylated in vitro by PKA, PKC and CKII as described in section 2. Aliquots were electrophoresed on SDS-polyacrylamide gels and autoradiograms prepared. The arrows indicate phosphorylated GST-PPZ1. Lanes labeled as GST denote control samples prepared from bacterial cells containing the expression vector without the PPZ1 open reading frame. Note the incorporation of radioactive phosphate in the 32 kDa band in the PK-A lane (see main text), and the low-M_r radioactive band in the CK-2 lanes, which corresponds to the autophosphorylation of the kinase preparation.

applies also to the catalytic properties. Thus, the high sensitivity of PPZ1 to microcystin relative to okadaic acid is characteristic of PP-1c but not of PP-2Ac. Also, unlike PP-2Ac, PPZ1 is affected by inhibitor-2, although it was less sensitive then PP-1c in the same assay conditions. The bacterial origin of PPZ1 may account for some unusual properties like its dependence of Mn^{2+} and its high *p*-nitrophenylphosphatase activity. A remarkable difference with both PP-1 and PP-2A is the inability of PPZ1 to dephosphorylate glycogen phosphorylase. We suggest that the N-terminal half of PPZ1 acts as a substrate-specifying domain, in much the same way as the substrate specificity of PP-1c is dramatically altered by interaction with non-catalytic subunits like the G-subunit or the M-subunit [37].

3.3. In vitro phosphorylation of recombinant PPZ1

The analysis of the amino acid sequence of PPZ1 revealed the presence of a large number of consensus sequences for phosphorylation [9]. These included sites for PK-A and PK-C (almost without exception present in the amino-terminal half) and CK-2 (distributed along the whole molecule). As shown in Fig. 4, recombinant PPZ1 was phosphorylated in vitro by all three kinases. Incorporation of phosphate was about 1 mol/mol of PPZ1 in the case of PK-A and PK-C, and slightly lower for CK-2. PK-A and PK-C, but not CK-2, were able to incorporate radioactive phosphate into the 32 kDa band containing a short fragment of the amino-terminal half of PPZ1. Phosphoaminoacid analysis indicated that both PK-A and PK-C phosphorylated exclusively Ser residues, whereas CK-2 was able to phosphorylate both Ser and Thr (not shown). Cyanogen bromide analysis revealed in all cases incorporation of radioactive phosphate into two large fragments of 20 and 21 kDa (Fig. 5). Phosphorylation of the 21 kDa fragment was further confirmed by in vitro phosphorylation of the solubilized polypeptide resulting of expression on a pT7-7 vector of the C-terminal half of PPZ1, which contains the mentioned fragment. It is remarkable that the presence of PK-A or PK-C sites in the 21 kDa CNBr fragment is not evident from computer analysis of the primary sequence of the protein. PK-A, and to a minor extent PK-C, were also able to phosphorylate residues located in the 14 kDa amino-terminal CNBr fragment. The incorporation of radioactive phosphate into the 32 kDa expression product (see Fig. 4) indicates the presence of PK-A and PK-C phosphorylation sites within the 40–50 first residues, which is in accordance with the prediction from the primary sequence of the protein.

The confirmation of the presence of phosphorylation sites in PPZ1 raised the question of whether or not phosphorylation might regulate the activity of the phosphatase. Therefore,we determined phosphatase activity in samples of PPZ1 previously phosphorylated in vitro by the mentioned kinases and we found that phosphorylation of PPZ1 by PK-A, CK-2 or PK-C did not result in changes in phosphatase activity. On the other hand, we have immunoprecipitated PPZ1 from crude extracts pre-

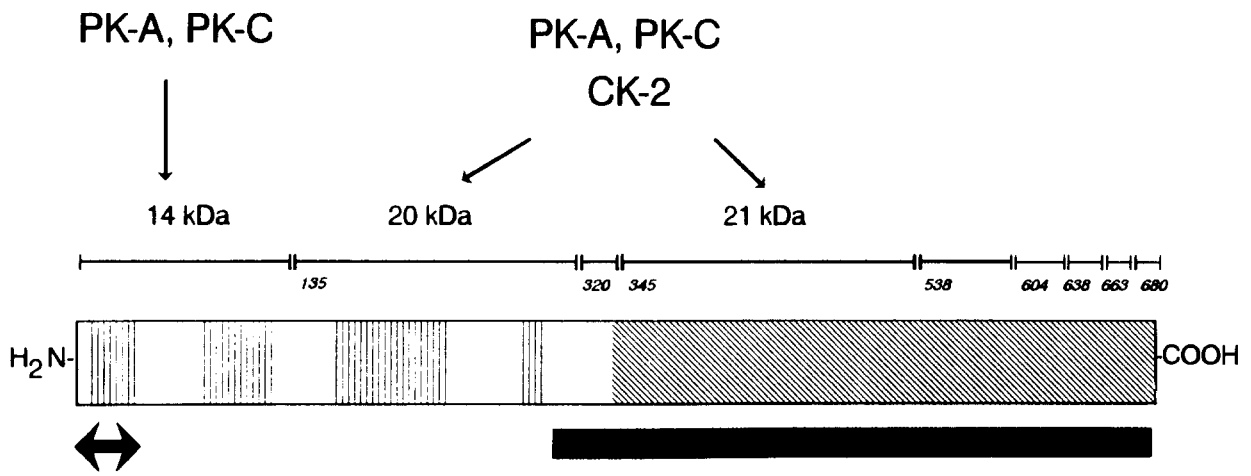

Fig. 5. Schematic representation of the results of the CNBr analysis after phosphorylation of recombinant PPZ1 with different protein kinases. The GST moiety is not represented. The carboxyl-terminal half of PPZ1 is denoted as a diagonally striped box. Vertical stripes in the amino-terminal region indicate a high concentration of predicted phosphorylation sites. The localization of Met residues in the PPZ1 molecule is indicated below the expected fragments resulting from CNBr treatment. The double headed arrow indicates the fragment of PPZ1 attached to the GST moiety in the 32 kDa expression product. The black box denotes the region of PPZ1 included in the product of expression of the pT7-7 based construct.

pared from yeast cells exponentially growing in rich medium containing [^{32}P]phosphate and the results obtained indicate that the PPZ1 protein is not labeled in vivo. These findings might be interpreted as evidence against the idea that phosphorylation is involved in the regulation of the enzyme. However, this possibility cannot be ruled out. For instance, changes in phosphatase activity might occur upon phosphorylation by a kinase(s) other(s) than the enzymes employed here. It might still be possible that phosphorylation might occur in vivo only under specific stimuli and that its biological role would not be the regulation of the enzyme activity but, for instance, the intracellular localization of the protein.

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