Expression, purification and kinetic behaviour of fission yeast low M_r protein-tyrosine phosphatase

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Abstract A gene named *stp1*+, coding for a 17.5-kDa protein, that rescues *cdc25-22* when overexpressed, has been previously isolated from fission yeast. Here we describe the expression and purification of Stp1 protein as a fusion with the glutathione *S*-transferase in *E. coli* and its kinetic characterisation. Stp1 deduced protein sequence shows an high homology to members of a class of cytosolic low M_r protein phosphatase previously known to exist only in mammalian species. Stp1 has a kinetic behaviour that appears to be intermediate with respect to the two isoenzymatic forms of low M_r protein tyrosine phosphatases present in mammalian tissues. These differing kinetic characteristics are mainly due to the sequence 45–56 that is spatially close to the active site pocket.

Key words: Phosphotyrosine protein phosphatase; Fission yeast; Active site

1. Introduction

Protein tyrosine phosphorylation plays a crucial role in regulating many aspects of cell division, proliferation and differentiation [1–3]. The cellular phosphotyrosine level is regulated by the relative activities of phosphotyrosine protein kinases (PTKs) and phosphotyrosine protein phosphatases (PTPases). PTKs activity is enhanced by external stimuli (i.e. growth factors and hormones) and this leads to increased phosphorylation levels in proteins involved in signal transduction. The importance of tyrosine-phosphorylation regulation system is well demonstrated in yeast cell cycle control. The phosphorylation of cdc2 (a component of the maturation promoting factor) on tyrosine, carried out by two protein tyrosine kinases, wee1 and nik1, is an inhibitory signal to mitosis [4,5]. On the other hand, dephosphorylation of cdc2 predominantly performed by the cdc25 protein phosphatase leads to restore mitosis [6–10].

A gene named *stp1*+ coding for a 17.5-kDa protein was isolated by screening a cDNA expression library for clones that were able to rescue *cdc25-22* [11]. Stp1 deduced protein sequence shows a high similarity to a family of mammalian low

M_r protein tyrosine phosphatase (LMW-PTP). The LMW-PTP is a cytosolic enzyme expressed in all eukaryotic organisms showing specific PTPase activity on tyrosine phosphorylated proteins and peptide substrates [12–15] and have the active site motif $CysX_5Arg$, present in all known types of protein tyrosine phosphatases. Site directed mutagenesis and kinetic studies have proven that the Cys^{12} residue in CX_5R is directly involved in the catalytic mechanism of the enzyme: the $Cys^{12}Ser$ mutant shows, in fact, a total loss of activity [16].

Two isoforms of the enzyme have been isolated [17,18] in various mammalian tissues. They originated from a single gene through an alternative splicing mechanism and they differ in 40–73 zone. The two isoforms of the LMW-PTP called PTPfast and PTPslow in human and ACP1 and ACP2 in rat liver, were isolated in our and other laboratories [17–19]. On the basis of sequence similarity, kinetic properties and cyclic guanosine monophosphate- (cGMP) dependent activation, these enzymes were classified in two distinct groups: IF1 (isoform-1) which includes PTPfast and ACP1, and IF2 (isoform-2) including PTPslow, ACP2 and bovine liver PTPase. The biological functions of mammalian LMW-PTPs are still unknown but the discovery of a yeast homologue suggests that they must play an important role because of their high sequence conservation through evolution.

The purpose of this study was to investigate the relationship between structure and function of LMW-PTPs previously isolated in our laboratories and the recombinant Stp1. For this reason we cloned the *stp1*+ gene in a suitable *E. coli* expression vector to produce Stp1 protein. The recombinant product was analysed in order to compare this enzyme to IF1 and IF2. We also describe the mutagenesis of Stp1 protein on Cys^{11} residue to Ser on the basis of LMW-PTP mutagenesis previously reported [16]. The results confirm the involvement of Cys^{11} in the active site of Stp1 molecule.

2. Materials and methods

2.1. Construction of pGEX-STP1 and pGEX-STP1C11S plasmids

Two synthetic primers were synthesised: a direct one (5′-GGCCG-

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CGGATCCACCAAGAATATTCAAGTA) whose 3' ends was identical to the sequence coding for the first six amino acid residues in Stp1 coding sequence cloned in Bluescript(KS) plasmid, and a reverse one (5'-GGCCGCGAATTCAAACTTAAACTAAGCA) whose 3' ends was complementary to the 3' non -coding region of the $stp1^+$ gene (kind gift of Dr. Paul Russell, La Jolla, CA). These primers, harbouring the restriction site for BamHI and EcoRI, respectively, were used in a PCR experiment to obtain the codifying sequence for the expression of Stp1 as fusion protein. The template for this amplification was the Bluescript(KS) plasmid harbouring the $stp1^+$ gene. In order to create the Cys^{11} mutant to Ser, a direct primer where the TGC codon for Cys^{11} was substituted with the TCT codon for Ser, was synthesised and used in the PCR experiment. The resulting fragments from PCR, coding for the entire sequence of Stp1 protein and for the Stp1C11S mutated protein were purified on agarose gel and digested with BamHI and EcoRI restriction enzymes. These fragments were separately ligated into the pGEX-2T vector previously digested with the same enzymes to create the corresponding sticky ends. The fragments were consequently inserted downstream and in frame with the glutathione S-transferase coding sequence. The recombinant plasmids named pGEX-STP1 pGEX-STP1C11S were separately used to transform the DH5 E. coli strain.

2.2. Screening of recombinant clones

Transformants were analysed in order to isolate clones harbouring the recombinant plasmids (pGEX-STP1 and pGEX-STP1C11S, respectively) and expressing either the recombinant Stp1 fusion protein or the Stp1C11S mutant. Twenty clones, from the recombinant Stp1 and from the Stp1C11S mutated protein, were assayed for the expression of fusion protein. Colonies were inoculated in 1 ml of LB and grown for 1 h at 37°C. After the induction of expression adding 0.2 mM IPTG, cells were harvested by centrifugation, resuspended in 200 μl of 20 mM 3,3-dimethylglutarate buffer pH 7.0, and sonicated for a few seconds. The clear lysates were assayed on 12% SDS-PAGE according to Laemmli [20]. The clones showing an evident sharp band of the molecular weight corresponding to the fusion proteins, were isolated. Double-stranded DNA sequencing was performed in order to control that PCR amplification did not introduce any undesired mutation in Stp1 sequence and in the mutated Stp1C11S one. These clones were used for massive expression of the two recombinant proteins.

2.3. Expression and purification of recombinant and mutated proteins

TB1 E. coli strains was transformed with pGEX-STP1 and pGEX-STP1C11S separately. 5 ml overnight culture were added to 500 ml of LB containing 100 mg/l of ampicillin. Culture were grown at 37°C under shaking and after two hours were supplemented with 0.2 mM IPTG to induce the expression of the recombinant proteins. The culture were grown for 6 h at 37°C under shaking. After centrifugation at 8000 × g for 30 min at 4°C, the pellets were resuspended in 1/100 (v/v) of 20 mM 3,3-dimethylglutarate buffer pH 7.0, 200 mM NaCl, 10 mM 2-mercaptoethanol and 1 mM EDTA. Cells were disrupted by sonication at 20 kHz and 100 W and after centrifugation, as previously described by Modesti et al. [21], the two clear lysates containing the recombinant and mutated proteins respectively were separately applied to a glutathione-agarose affinity column equilibrated with a 20 mM 3,3-dimethylglutarate buffer pH 7.0, containing 200 mM NaCl, 10 mM 2-mercaptoethanol and 1 mM EDTA. The proteins were purified with an affinity chromatography procedure as described by Taddei et al. [22] and the recombinant proteins were separately eluted with 5 mM reduced glutathione. Protein concentration was measured by bicinchoninic acid assay.

2.4. Thrombin cleavage

The fusion proteins were incubated for 1 h at room temperature with 1:500 (w/w) bovine thrombin in 50 mM Tris-HCl buffer pH 8.0, containing 150 mM NaCl and 2.5 mM $CaCl_2$. The Stp1 protein was separated from GST and uncleaved fusion protein by a second affinity chromatography on the same column equilibrated with the 50 mM Tris-HCl buffer pH 8.0.

2.5. Enzyme assay and kinetic parameters

The main kinetic parameters of the recombinant Stp1 protein and of the Stp1C11S mutant enzyme were determined by measuring the initial hydrolysis rate on p-nitrophenylphosphate (pNPP) and L-phospho-

tyrosine (L-P-Tyr) as follows: the substrates were dissolved in 1 ml of 0.1 M acetate buffer pH 5.5, 1 mM EDTA and incubated in water bath at 37°C. The reaction started by adding a suitable samples volume and stopped by 1 ml of 1 M NaOH. The phenols produced by enzymatic activity was measured spectrophotometrically using ε_{400} = 18,000 $M^{-1} \cdot cm^{-1}$ for p-nitrophenol and $\varepsilon_{293.5}$ = 2,330 $M^{-1} \cdot cm^{-1}$ for tyrosine in alkali solution. K_m and V_{max} were determined in duplicate by plotting the initial substrate hydrolysis rate versus its initial concentration, using a non-linear fitting. The activation of the enzyme at saturating cGMP concentration was determined according to Cirri et al. [23]. Free phosphate generated by hydrolysis of Tyr-phosphorylated peptides was evaluated with malachite green assay [24].

3. Results and discussion

3.1. Cloning, mutagenesis and expression of $stp1^+$ and $stp1^+C11S$ genes

In order to verify that the activity of $stp1^+$ gene product was similar to the related mammalian proteins, we have expressed the Stp1 protein in E. coli as fusion protein with GST: this system allows rapid purification and was demonstrated to be very efficient with mammalian LMW-PTP. To do that, a PCR amplification was performed on Bluescript(KS) harbouring the $stp1^+$ gene previously isolated by Mondesert et al. [11], introducing a BamHI and a EcoRI restriction sites at the two ends of the amplified sequences: the corresponding sites on the pGEX-2T vector were used for cloning this fragment.

In parallel to that we have also created a Cys^{11}-to-Ser mutant, corresponding to the Cys^{12}-to-Ser mutant of LMW-PTP, that we have demonstrated to have lost any catalytic activity [12]. For this purpose an oligonucleotide direct mutagenesis was performed, followed by the direct cloning of the mutated amplified sequence in the pGEX-2T vector. In this way the Stp1 and Stp1C11S sequences were placed downstream and in frame
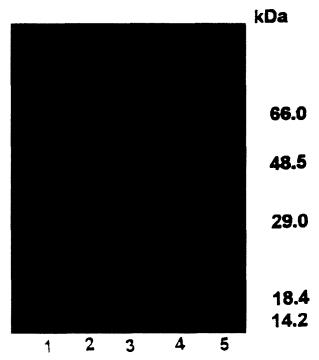

Fig. 1. SDS-PAGE analysis of recombinant proteins. Lanes 1 and 2 = Stp1 and Stp1C11S cleaved proteins; lanes 3 and 4 = Stp1 and Stp1C11S recombinant fusion proteins; lane 5 = molecular weight standard.

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         40        50        60        70
IF1  ACP1  RIDSAATSTYEVGNPPDYRGQNCMKKHGIHMQHI
     Bfast  -V------G--I---------S---R---P-S-V


STP1         ----CG-GAWH---R--P-TLEVL--N---TK-L


IF2  ACP2  A---S-V-DWN--R---P-AV--LRN---STA-K
     Bslow  V---G-V-DWN--RS--P-AVS-LRN----TA-K
     Bov-PTP  V---G-V-DWN--RS--P-AVS-LRN---NTA-K
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Fig. 2. Sequence alignment of the 40–73 zone of LMW-PTP and Stp1. ACP1 and ACP2 are from rat liver, Bslow and Bfast are from human erythrocytes, bov-PTP is from bovine liver, Stp1 is from fission yeast. Dashes indicate identity with ACP1. Numbers are refer to amino acids position in the proteins.

with the sequence coding for GST. DH5 *E. coli* cells were transformed with the two recombinant plasmids and assayed for protein expression: the supernatants of *E. coli* cells expressing the two recombinant fused proteins were separated on a 2% SDS-PAGE together with a control from cells transformed with the pGEX-2T vector alone. Sharp bands corresponding to the expected fusion protein molecular weight are evident for the clones harbouring the two recombinant plasmids (data not shown). Clones with the correct insertion were completely checked by DNA sequencing with the Sanger method to control any undesired mutation in the sequences due to PCR amplification. The two recombinant plasmids pGEX-STP1 and pGEX-STP1C11S, were used to transform the TB1 strain in order to enhance, after induction with IPTG, the expression of the two proteins.

The two recombinant fusion proteins were cleaved with thrombin as reported in section 2. The cleavage time was reduced as much as possible in order to achieve a high cleavage yield avoiding significant enzyme denaturation. The two recombinant proteins were separated from GST by a second affinity chromatography. Fractions containing the pure proteins were pooled, concentrated by ultrafiltration and checked for purity by SDS-PAGE as shown in lanes 1 and 2 of Fig. 1 in comparison with the recombinant fusion proteins (lanes 3 and 4). With this method it is possible to obtain up to 20 mg/l of purified fusion protein. This value is very similar to that obtained for LMW-PTP, confirming this method to be extremely efficient for the production of this class of proteins.

3.2. Kinetic parameters of the recombinant Stp1 and mutated Stp1C11S proteins

LMW-PTP activity was assayed on pNPP and L-P-Tyr. Dif-

ferences between IF1 and IF2 isoforms can be shown using these substrates. Moreover, IF2 activity can be enhanced by cGMP addition. In this work our purpose was to: (i) verify that Stp1 has a PTPase activity similar to LMW-PTP; and (ii) which of the two LMW-PTP isoforms shows a more similar behaviour respect to Stp1. Concerning the first point, the main kinetic constants (k_{cat} and K_m) of the recombinant Stp1 protein, were determined with two different substrates: pNPP and L-P-Tyr. The K_m and k_{cat} on pNPP show lower values for Stp1 with respect to those from IF1 and IF2 isoenzymes; on L-P-Tyr the K_m for Stp1 is equal to the same value from IF1 meanwhile the *kcat* value is lower with respect to both IF1 and IF2 (Table 1). This values are in any case comparable to those of both isoforms indicating that Stp1 has an enzymatic activity very similar to that of LMW-PTP. Stp1 is also totally inactive of other phosphorylated amino acids, such as P-Ser and P-Thr: this fact should exclude a dual specificity of this enzyme, in analogy with LMW-PTP. In addition, Stp1 activity was tested on a Tyr-phosphorylated peptide. For this purpose a synthetic peptide covering the sequence 767–776 of the PDGF-R molecule was treated with the enzyme and free phosphate was evaluated. In analogy to LMW-PTP characteristics, Stp1 is active also on this substrate (data not shown).

Analysis of the activity of the Stp1C11S mutant that we have created, confirms that the activity of this PTPase is very similar to that of LMW-PTP: no enzymatic activity was detectable, indicating that this mutation, in analogy with the C12S mutated LMW-PTP, completely abolishes the functionality of the enzyme. It is very likely that also in the case of Stp1, the Cys contained in the CXXXXXR motif is essential in the catalytic mechanism of the enzyme. Further experiments will clarify whether also in this case the inability to form thiophosphate intermediate is causing this effect, in order to demonstrate the identity of the mechanism of action with respect to LMW-PTP.

For what the similarity of Stp1 with the two different LMW-PTP is concerned, we have to notice that the amino acid residues within the 40–73 divergent sequence of mammalian sequences are involved in the differing kinetic behaviour of the two isoenzymes. These structural differences, in fact, might distinguish important in vivo roles for these isoenzymes (Cirri et al. [25]; manuscript submitted). Comparison the portion of Stp1 sequence 40–73 corresponding to the 40–72 divergent sequence of IF1 and IF2 (Fig. 2), shows a comparable percentage of identity with both isoforms (51% and 45%, respectively), highlighting Stp1 as a third type of PTPase intermediate between mammalian IF1 and IF2 isoforms.

In order to clarify the relationship between Stp1 with IF1 and IF2 isoforms, we have consider the k_{cat}/K_m ratio. The k_{cat}/K_m is an apparent second-order rate constant that refers to the properties and the reactions of the free enzyme and free sub-

Table 1
Catalytic parameters values with p-NPP and L-P-Tyr of IF1, IF2 and Stp1 (refer to the text for details)

	pNPP			P-Tyr			Maximum activation cGMP
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1}mM^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1}mM^{-1})	
F1	15.0 ± 0.5	0.070 ± 0.01	214	14.6 ± 0.6	0.6 ± 0.04	24.3	1.3
F2	29.2 ± 1.1	0.19 ± 0.03	154	11.9 ± 0.5	5.8 ± 0.10	2.0	9.0
STP1	5.3 ± 0.2	0.028 ± 0.01	189	5.0 ± 0.1	0.6 ± 0.05	8.4	5.0

cGMP maximum activation values are also indicated. Values are means of at least three independent determinations. S.E.M. are indicated.

strate. Results in Table 1 show that Stp1 k_{cat}/K_m ratios on both substrates have a value that is intermediate in comparison with those of the two isoforms. With the same purpose we also measured the cGMP-dependent activation of Stp1. This nucleotide increases IF2 catalytic activity but has a poor effect on IF1 [23]. Stp1 display again an intermediate position between the two isoforms, showing a cGMP activation ratio higher with respect to IF1 but lower with respect to IF2 (Table 1).

Recently, by means of site directed mutagenesis, we have demonstrated that the differing kinetic behaviour of IF1 and IF2 was mainly due to amino acids in position 49 and 50 (Cirri et al. [25], manuscript submitted). In particular the Asn[50] residue of IF2 is both responsible of cGMP activation ratio and differing kinetic constant values between IF1 and IF2. Data obtained for Stp1 confirm this findings: residue 50 of Stp1 is an His while in IF1 and IF2 there are Glu and Asn, respectively. Accordingly Stp1 kinetic characteristic are different from both IF1 and IF2 ones. Furthermore the Trp[49] of IF2 (Fig. 2) is partially involved only in cGMP activation. In position 49 Stp1 has a Trp residue and consequently is able to partially maintain the capability of being activated by cGMP (Table 1). Hence, Stp1 appears to be an intermediate isoform between eukaryotic IF1 and IF2. Probably this in vitro kinetic differences may reflect in vivo different regulation pathway and substrate specificity of this class of enzyme.

In conclusion, Stp1 can be considered as a new member of the LMW-PTP family, on the basis of its enzymatic activity and of the key role that the Cys of the characteristic CXXXXXR motif shows. The fact that Stp1 demonstrate to have intermediate characteristics with respect to mammalian IF1 and IF2 do not exclude that other members of this family could be expressed in fission yeast, as suggested also by the fact that killing of the *stp1*[+] gene do not cause any apparent phenotype in yeast [11]: further investigation are necessary to try to clarify this point.

References

[1] Krebs, E.G. (1996) in: The Enzymes (Boyer, P.D. and Krebs, E.G. eds.) vol. XVII, Academic Press, New York.

[2] Yarden, Y. and Ullrich, A. (1988) Annu. Rev. Biochem. 57, 443–478.

[3] Bishop, J.M., (1991) Cell 64, 235–248.

[4] Nurse, P. (1990) Nature 344, 503–508.

[5] Russell, P. and Nurse, P. (1987) Cell 49, 559–567.

[6] Dunphy, W.G. and Kumagai, A. (1991) Cell 67, 189–196.

[7] Gautier, J., Solomon, M.J., Booher, R.N., Bazan, J.F. and Kirschner, M.W. (1991) Cell 67, 197–211.

[8] Lee, M.S., Ogg, S., Xu, M., Parker, L.L., Donoghue, D.J., Maller, J.L. and Piwinica-Worms, H. (1992) Mol. Biol. Cell 3, 73–84.

[9] Millar, J.B.A., McGowan, C.H., Lenaers, G., Jones, R. and Russel, P. (1991) EMBO J. 10, 4301–4309.

[10] Russell, P. and Nurse, P. (1986) Cell 45, 145–153.

[11] Mondesert, O., Moreno, S. and Russell, P. (1994) J. Biol. Chem. 269, 27996–27999.

[12] Waheed, A., Laidler, P.M., Wo, Y.Y.P. and Van Etten, R.L. (1988) Biochemistry 27, 4265–4273.

[13] Chernoff, J. and Lee, H.C. (1985) Arch. Biochem. Biophys. 240, 135–145.

[14] Boivin, P. and Galand, C. (1986) Biochim. Biophys. Res. Commun. 134, 557–564.

[15] Ramponi, G., Manao, G., Camici, G., Cappugi, G., Ruggiero, M. and Bottaro, D.P. (1989) FEBS Lett. 250, 469–473.

[16] Chiarugi, P., Marzocchini, R., Raugei, G., Pazzagli, C., Berti, A., Camici, G., Manao, G., Cappugi, G. and Ramponi, G. (1992) FEBS Lett. 310, 9–12.

[17] Dissing, J., Johnsen, A.H. and Sensabaugh, G.F. (1991) J. Biol. Chem. 266, 20619–20625.

[18] Manao, P., Pazzagli, L., Cirri, P., Caselli, A., Camici, G., Cappugi, G., Saeed, A. and Ramponi, G. (1992) J. Protein Chem. 11, 333–345.

[19] Manao, G., Camici, G., Cappugi, G., Tremori, E., Pazzagli, L. and Ramponi, G. (1991) Proceedings of 6th National Meeting 'Protein '91', Trieste, May 22–24, p. 96.

[20] Laemmli, U.K. (1970) Nature 277, 680–685.

[21] Modesti, A., Taddei, N., Bucciantini, M., Stefani, M., Colombini, B., Raugei, G. and Ramponi, G. (1995) Protein Expression and Purification, accepted for pubblication.

[22] Taddei, N., Chiarugi, P., Cirri, P., Fiaschi, T., Stefani, M., Camici, G., Raugei, G. and Ramponi, G. (1994) FEBS Lett. 350, 328–332.

[23] Cirri, P., Caselli, A., Manao, G., Camici, G., Polidori, R., Cappugi, G. and Ramponi, G. (1995) Biochim. Biophys. Acta 1243, 129–135.

[24] Baykov, A.A., Evtushenko, O.A. and Avaeva S.M. (1988) Anal. Biochem. 171, 266–270.

[25] Cirri, P., Fiaschi, T., Chiarugi, P., Camici, G., Manao, G., Raugei, G. and Ramponi, G. (1995) J. Biol. Chem., in press.