

Interaction of protein phosphatase type 1 with a splicing factor

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Abstract A gizzard cDNA library was screened by the two-hybrid system using as bait the δ isoform of the catalytic subunit of protein phosphatase 1 (PP1 δ). Among the proteins identified was a fragment of the polypyrimidine tract-binding protein-associated splicing factor (PSF) and for 242 residues was 97.1% identical to the human isoforms. Binding of PSF and PP1 δ was confirmed by inhibition of phosphatase activity and by an overlay technique. The PP1 δ binding site was contained in the N-terminal 82 residues of the PSF fragment. PSF may therefore act as a PP1 target molecule in the spliceosome.

Key words: Protein phosphatase type 1; Two-hybrid system; Human splicing factor PSF

1. Introduction

The formation of mRNA from its precursor, pre-mRNA, involves the removal of introns. This reaction occurs in the nucleus within a dedicated, high-molecular mass assembly, termed the spliceosome [1,2]. This assembly involves small nuclear ribonucleoprotein particles (snRNPs) and other splicing factors [3]. The spliceosomes assemble on pre-mRNA as discrete complexes and four functional intermediates have been identified, i.e. E $\rightarrow$ A $\rightarrow$ B $\rightarrow$ C [2,3]. The products of the initial catalytic step (exon 1 and lariat-exon 2) are associated with the C complex. The exons are then rapidly ligated in the second catalytic step and released from the C complex. After the binding of U1 snRNP to pre-mRNA the subsequent events required ATP [1] and it is likely that phosphorylation reactions are involved. Several lines of evidence support this assumption, including the use of ATP analogs [4], application of phosphatase inhibitors [4–6] and purified phosphatases [5,6], and the phosphorylation of several spliceosome components, notably those containing the RS-rich domain [7–9]. In general, details of the phosphorylation-dependent mechanisms are not known. With respect to the phosphatases involved the specific substrates are not identified and there is no information on phosphatase-binding, or target, molecules.

The concept that such target molecules may regulate phosphatase activity is based on their ability to bind to the catalytic subunit and to the substrate, the classical example being that of the G-subunit involved in glycogen metabolism [10]. To search for potential target molecules in smooth muscle, the two-hybrid system [11] was applied using the δ isoform of the catalytic subunit of type 1 phosphatase (PP1 δ) as bait to screen a chicken gizzard cDNA library [12]. One of the interacting proteins identified was a splicing factor, the polypyri-

midine tract-binding protein-associated splicing factor, termed PSF [13]. This binds to the polypyrimidine tract of mammalian introns [14] and was suggested to be essential for the second catalytic step occurring on the short-lived C complex [15]. This study documents the interaction of PSF and PP1 and suggests that PSF could act as a target molecule in the spliceosome.

2. Materials and methods

2.1. Two hybrid screening

This system was established [12] using PP1 δ to screen a chicken gizzard cDNA library. The library plasmid for clone 24 was recovered and examined for interaction with various bait constructs, i.e. pAS1-PP1 δ (nomal bait) and two control constructs to screen for false positives [12]. Y190 cells were transformed with these plasmids alone or with these bait plus pGAD424-clone 24 and assayed for β -galactosidase activity [16]. Only the pAS1-PP1 δ plus pGAD424-clone 24 pair gave measurable activity.

2.2. Expression of the PSF fragment

The fragment of chicken PSF (crPSF fragment) was expressed as a hexahistidine-tagged protein in the pQE *E. coli* system (Qiagen). The clone 24 insert was amplified by PCR using primers designed to introduce *Bam*HI and *Pst*I sites. The *Bam*HI and *Pst*I-digested insert was ligated with vector, pQE 32. The nucleotide sequence of this construct was determined and was identical to the pGAD424-clone 24. It encoded a 237 residue fragment (residues K349 through M585 of human PSF) plus the hexahistidine tag (MRGSHHHHHGIP) at the N-terminus and an 8 residue peptide at the C-terminus (DLQPSLIS). Both additional peptides were derived from the vector. Expression and purification of crPSF fragment were as described previously for recombinant PP1 δ [12].

2.3. Cleavage of the PSF fragment

Cleavage at the single C residue, corresponding to residue 431 of human PSF [13] was achieved with the 5,5'-dithiobis(2-nitrobenzoic acid)-CN complex (DTNB-CN) as described [17]. After cleavage the mixture was dialyzed against 20 mM Tris-HCl (pH 8.0) and 0.1 M KCl, clarified by centrifugation at 25000 $\times g$ for 15 min, and the supernatant applied to a Ni-agarose column and washed with dialysis buffer. The C-terminal peptide was recovered in the flow through and the bound protein (N-terminal peptide plus uncleaved molecule) eluted with 0.25 M imidazole-HCl (pH 8.0) in dialysis buffer. This fraction contained a molar ratio of N-terminal peptide to parent molecule of 11:1. Further purification of the N-terminal peptide was hindered by the tendency of the peptide to aggregate at lower ionic strengths.

2.4. Expression of full-length recombinant PSF

The full-length human PSF was expressed as a hexahistidine-tagged protein as described earlier [13]. The initial stages of purification followed the previous protocol [12] but after elution from the metal affinity column (Talon, Clontech) the procedure was modified. The bound protein was eluted by 0.1 M imidazole, 20 mM Tris-HCl (pH 8.0) 0.1 M NaCl and 6 M guanidine-HCl. When the column eluate was dialyzed directly to remove guanidine, most of the hrPSF precipitated. Therefore, the column eluate was first rapidly diluted with 50 vols. of 20 mM Tris-HCl (pH 8.0), 1 M NaCl, 0.05% Tween 20, 5 mM dithiothreitol, 25 μ g/ml leupeptin, 0.5 mM diisopropyl fluorophosphate and then dialyzed against 20 mM Tris-HCl (pH 8.0), 0.1 M

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KCl, 0.2 mM EDTA, 0.03% Brij 35, 0.5 mM dithiothreitol. Using this procedure, most of the hrPSF remained soluble. The dialysate was concentrated by centrprep-30 and microcon-30 concentrators (Amicon). The purity was about 60%. The concentration of hrPSF was estimated on SDS-PAGE after staining with Coomassie brilliant blue R-250 (Sigma) with bovine serum albumin as standard.

2.5. Other procedures

The recombinant PP1 δ (rPP1 δ) was expressed as described [12]. The catalytic subunit of PP2A (PP2Ac) was isolated from bovine heart [18]. An N-terminal fragment of the large phosphatase subunit (rN130) was prepared as described [19]. Other protein preparations and routine procedures were as listed previously [12]. Phosphatase activity was assayed using 32 P-labelled 20 kDa light chain [12] and conditions are given in the figure legends. Thrombin digestion of rPSF in 20 mM Tris-HCl (pH 7.5), 2 mM dithiothreitol was carried out at 25°C with thrombin (Sigma, 120 U thrombin/mg rPSF) for 2 h. The reaction was stopped by addition of 4-amidinophenylmethanesulfonyl fluoride to 0.75 mM. The hydrolysis products were separated on the Ni-agarose column as described above for DTNB-CN cleavage. For the PP1c overlay assay proteins were transferred [12] onto polyvinylidene difluoride membrane (Immobilon-P, Millipore), blocked with 5% nonfat dry milk in 0.05% Tween 20, 20 mM Tris-HCl (pH 7.4) and 0.15 M NaCl for 3 h and incubated for 12 h at 4°C with 3.7 μ M rPP1 δ in 2% nonfat dry milk, 0.03% Brij 35, 20 mM Tris-HCl (pH 7.4), 0.1 M KCl, 0.2 mM EDTA, 1 mM MnCl₂, and 0.5 mM dithiothreitol. The bound PP1 δ was detected using a rabbit polyclonal PP1 δ antibody [20] and horseradish peroxidase-conjugated anti-rabbit IgG antibody (Sigma).

3. Results and discussion

3.1. Two-hybrid screening

PP1 δ was used as bait to screen a gizzard cDNA library.

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1  GAATTCGGCAGCAGCGCAAGGCGAAGCTGGACGACACCCCATGCGGGGCCGACAGCTC
348      A K A E L D D T P M R G R Q L

61  CGTGTTCGGTTTSCAACGCACGCTGCCGCGTGTCTAGTCGTAACCTTCGCTTACGTG
363 R V R F A T H A A A L S V R N L S P Y V

121  TCCAACGAGTTACTGGAGGAGGCTTCTCCGAGTTCGGTCCGTTGGAGAGAGCTGTGTG
383 S N E L L E E A F S Q F G P V E R A V V
      I

181  ATTGTAGATGATCGAGGTAGATCAACGAAAGGCGATTGTTGAATTTGCATCAAGGCCA
403 I V D D R G R S T G K G I V E F A S K P

241  GCTGCCAGAAAAGCGTTTGAACGGTGACTGAGGGAGTGTTCTTGTGACAACTACTCT
423 A A R K A F E R C T E G V F L L T T T P
      S

301  AGGCCAGTTATTGTGAACCTTTGAGCAACTGGATGATGAAGATGGTCTTCCAGAAAAG
443 R P V I V E P L E Q L D D E D G L P E K

361  CTTGCTCAGAAGAACCCGATGTATCAAAAGGAAAGAGAACTCTCCCGTTTGTCTCAG
463 L A Q K N P M Y Q K E R E T P P R F A Q

421  CCTGGCAGTTTGTAGTTTGTAGTATTCAGAGAGATGGAATCTTTAGATGAAATGGAAAA
483 P G S F E F E Y S Q R W K S L D E M E K
      H T Y

481  CAACAGAGAGAACAGTGGCAAAAACATGAAAGATGCCAAGGACAACTTGAGAGTGA
503 Q Q R E Q V A K N M K D A K D K L E S E
      E

541  ATGGAAGATGCTTATCATGAGCATCAGGCAAACTTTTGCCTCAAGACCTTATGAGCGT
523 M E D A Y H E H Q A N L L R Q D L M R R

601  CAGGAGGAATGAGACGTATGGAAGAACTCCATCAAGAAATGCAGAAACGCAAGGAA
543 Q E E L R R M E E L H N T Q E M Q K R K E

661  ATTCAAGCTCAGGCAGGAGGAGCGTCGACAGCGGAGGAGGAAATGATGATCCGTCAG
563 I Q L R Q E E E R R R R E E E M M I R Q
      M

721  CGAGAAATGGAAGACAGATGAGAGAGAGAGAGAGAGAACTAGTCTCGACCTGCAGAG
583 R E M E E Q M

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Fig. 1. Nucleotide sequence and derived amino acid sequence of clone 24. The nucleotide sequence of clone 24 is shown from the 5'-ligation site (EcoRI). The amino acid sequence was deduced in-frame continuing from the GAL4 activation domain. Underlined sequences are adaptor sequences. The numbering of residues in clone 24 corresponds to the sequence of human PSF [13]. In the human PSF sequence 7 residues are different. These are shown below the clone 24 sequence.

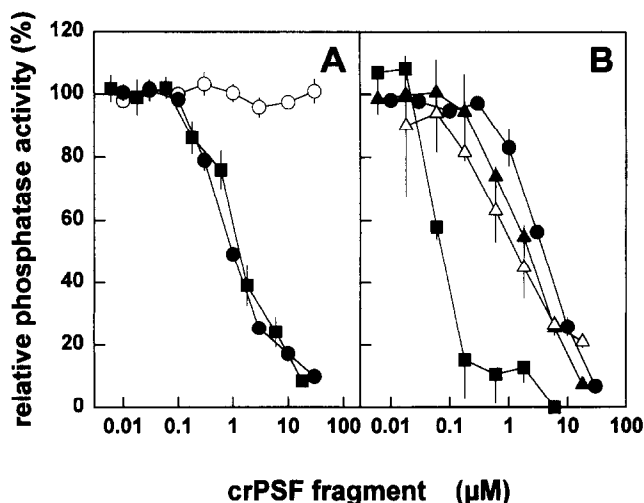


Fig. 2. Effect of the recombinant cPSF fragment on phosphatase activity. (A) Assays carried out minus Co²⁺; (B) assays carried out plus Co²⁺ (0.3 mM CoCl₂). Other assay conditions: 50 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 0.1 mg/ml bovine serum albumin, 15 mM KCl, 5 μ M 32 P-labelled gizzard LC 20, varying crPSF fragment. Gizzard PP1c (●); rPP1 δ (■); bovine PP2Ac (○); rabbit skeletal muscle PP1c (△); myosin-bound phosphatase (▲). Data are means $\pm$ S.E.M. (n not less than 3).

Several proteins were detected [12], including clone 24. The nucleotide and derived amino acid sequence of clone 24 is shown in Fig. 1 and is compared to a partial sequence (residues 348–589) of human PSF [13]. For the 242 residues, 235 were identical (97.1% identity). The nucleotide sequence identity over this region was 81%. It is concluded, therefore, that clone 24 encodes part of the chicken PSF molecule and will be referred to as 'cPSF fragment'. The cPSF fragment is predominantly hydrophilic and more acidic (theoretical $pI=5.5$) than the parent human molecule (theoretical $pI=10.3$).

3.2. Inhibition of phosphatase activity by crPSF fragment

To obtain an independent criterion for the interaction of PP1 δ and the PSF fragment, the effect of crPSF fragment on phosphatase activity was determined. In the absence of Co²⁺ the crPSF fragment inhibited PP1c from turkey gizzard and rPP1 δ (Fig. 2A). Half-maximal inhibition was at about 1 μ M crPSF fragment. In contrast, PP2Ac was not inhibited at concentrations up to 30 μ M. In the presence of 0.3 mM CoCl₂ (Fig. 2B) PP1c from gizzard (predominantly 35 kDa species) and rabbit skeletal muscle (predominantly 38 kDa species) were inhibited similarly (half-maximal inhibition at about 3 μ M). Inhibition of rPP1 δ by crPSF showed half-maximal inhibition at about 0.1 μ M. The trimeric myosin-bound phosphatase was also inhibited by crPSF (Fig. 2B). This PP1 preparation is Co²⁺-dependent [21]. It is interesting that the presence of the two non-catalytic subunits did not block inhibition. The recombinant full-length PSF also inhibited the activity of rPP1 δ . However, its effectiveness as an inhibitor was reduced compared to the crPSF fragment and in the presence of Co²⁺ the IC₅₀ was about 3 μ M.

Basic kinetic parameters were estimated from a double-reciprocal plot of v versus substrate (light chain) for rPP1 δ in the absence and presence of 1.8 μ M crPSF fragment in the absence of Co²⁺. The major effect was on V_m and this was

reduced from 0.84 ± 0.06 to 0.4 ± 0.01 nmol/min per mg protein by addition of crPSF. K_m values changed slightly from 1.2 ± 0.3 μ M (control) to 2.2 ± 0.5 μ M.

Inhibition of PP1 by inhibitors 1 and 2 and DARPP-32 [22,23] occurs in the nanomolar range and it is therefore unlikely that the unmodified form of PSF functions as an *in vivo* inhibitor. In this context, it should be noted that the non-phosphorylated DARPP-32 inhibited PP1c with an IC_{50} about 1 μ M [24]. It is not known whether the properties of PSF are altered by post-translational modification, but a phosphorylated form of PSF has been detected in HeLa nuclear extracts (Patton, J.G., unpublished observations).

The intent of the phosphatase assays was to confirm the results of the two-hybrid screen and to demonstrate by an independent method binding of PSF fragment and PP1. This has been shown both for recombinant PP1c and for native PP1.

3.3. Overlay with PP1 catalytic subunit

A second method to confirm binding of crPSF fragment and PP1c was an overlay procedure using rPP1 δ . Binding of rPP1 δ was detected with the crPSF fragment and with the N-terminal fragment of the 130 kDa phosphatase subunit [19]. The latter served as a positive control.

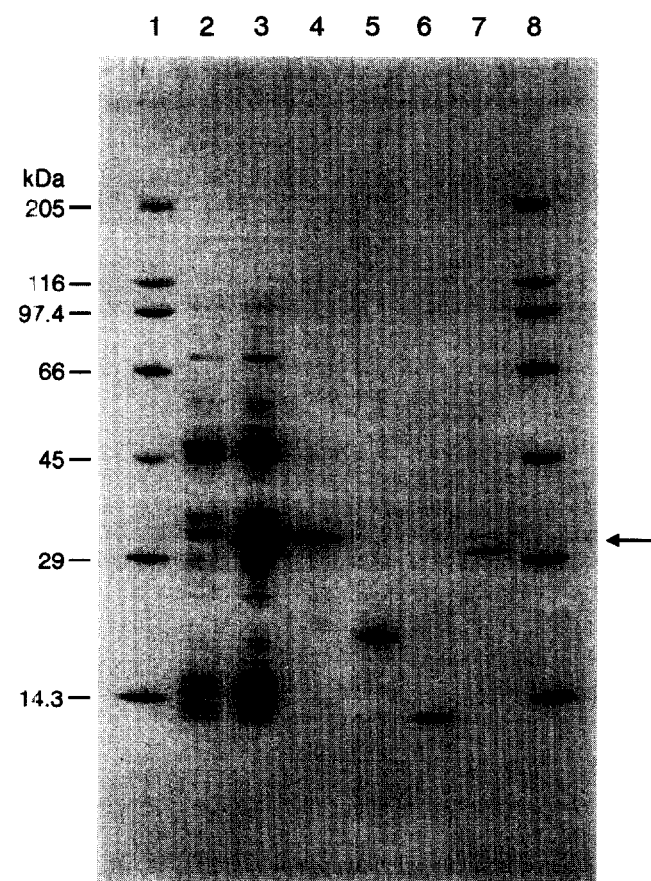


Fig. 3. Overlay binding assay and SDS-PAGE patterns. (A) Lanes: 1, crPSF fragment (4 μ g); 2, bovine serum albumin (4 μ g); 3, rN130 (4 μ g). (B) Lanes: 1,8, molecular weight markers; 2,3, bacterial homogenates before and after induction of crPSF fragment, respectively; 4, crPSF fragment; 5,6, C- and N-terminal peptides of crPSF fragment, respectively; 7, thrombin digest of crPSF fragment. Position of parent crPSF indicated by arrow.

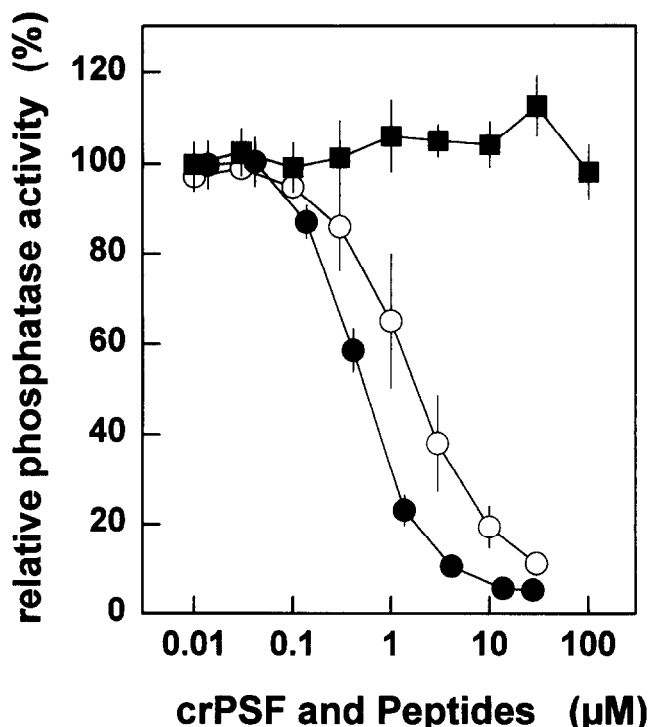


Fig. 4. Effect of crPSF fragment and DTNB-CN cleavage products on gizzard PP1c. Parent crPSF fragment ($\circ$); C-terminal peptide ($\blacksquare$); N-terminal peptide plus residual crPSF fragment, 11:1 molar ratio ($\bullet$). Assay conditions given in Fig. 2, except that additional imidazole added with the N-terminal peptide was accounted for in controls. Data are means $\pm$ S.E.M. ($n=4$).

3.4. Cleavage of the rPSF fragment

In order to localize the binding site with a shorter sequence, various methods of cleavage were investigated. Limited proteolysis of rPSF fragment with α -chymotrypsin, trypsin and thermolysin resulted in loss of binding (as judged by inhibition assays). Cleavage with thrombin at a site near the C-terminus generated a peptide of 29.7 kDa (Fig. 3) that retained inhibitory activity, but was only slightly smaller than the parent molecule.

Non-proteolytic cleavage at the single C residue by DTNB-CN was investigated. Two peptides were generated of 13.4 and 19.5 kDa (Fig. 3) corresponding to N-terminal and C-terminal fragments, respectively. The two peptides were separated and assayed for inhibition of phosphatase activity (Fig. 4). The smaller N-terminal peptide retained the inhibitory effect. This peptide is composed of residues 349–430 of the human PSF [13] plus the hexahistidine tag. It is known from other constructs [12] that the hexahistidine tag itself is not inhibitory.

Patton et al. [13] showed that two isoforms of PSF exist, differing at the C-terminal end (after residue 662). Thus, both isoforms would contain the inhibitory sequence. Comparison of this sequence with other PP1 inhibitors (inhibitors 1 and 2 and DARPP-32) and the PP1-binding proteins, L5 [12] did not reveal a common motif indicative of a PP1c-binding sequence.

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