Identification of sds22 as an inhibitory subunit of protein phosphatase-1 in rat liver nuclei

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Abstract sds22 was originally identified in yeast as a regulator of protein phosphatase-1 that is essential for the completion of mitosis. We show here that a structurally related mammalian polypeptide (41.6 kDa) is part of a 260-kDa species of protein phosphatase-1. This holoenzyme, designated PP-1N_{sds22}, could be immunoprecipitated with sds22 antibodies and was retained by microcystin-Sepharose. PP-1N_{sds22} is a latent phosphatase, but its activity could be revealed by the proteolytic destruction of the noncatalytic subunit(s). PP-1N_{sds22} accounted for only 5–10% of the total activity of PP-1 in rat liver nuclear extracts. A synthetic 22-mer peptide, corresponding to a leucine-rich repeat of sds22, specifically inhibited the catalytic subunit of PP-1, showing that at least part of the latency stems from the interaction of the sds22 repeat(s) with PP-1_C.

Key words: Cell cycle; Dephosphorylation; Mitosis; Protein phosphatase

1. Introduction

The Ser/Thr protein phosphatases of type 1 (PP-1) comprise a conserved and widely distributed family of enzymes with functions in various cellular processes (reviewed in [1,2]). They all contain the same catalytic subunit (PP-1_C), which is structurally related to that of other protein phosphatases like PP-2A and PP-2B, but can easily be distinguished from the latter enzymes by its substrate specificity and sensitivity to inhibitory polypeptides and toxins. All known PP-1 holoenzymes contain one or two regulatory subunits that control the activity and substrate specificity of the phosphatase, and target the holoenzyme to its physiological substrate(s). The best characterized cytoplasmic targeting subunits of PP-1 are the G- and M-subunits that anchor the phosphatase to the glycogen particles and to myofibrils, respectively.

The nucleus of mammalian cells contains two major holoenzymes of PP-1, designated PP-1N_{R41} and PP-1N_{R111} [3]. They are both heterodimers of the catalytic subunit and inhibitory polypeptides of 41 and 111 kDa, respectively. R41 was identified as NIPP-1, a phosphopolypeptide with structural features like that of some proteins implicated in RNA processing [4]. Other nuclear polypeptides that have been shown to interact with the catalytic subunit include the retinoblastoma protein [5], the p53-binding protein p53BP2 [6] and the splicing factor PSF [7], but it is not clear yet whether these polypeptides represent real subunits of PP-1. In addition, we have cloned a mammalian protein of 42 kDa that is enriched in the nucleus and is structurally related (46% identity) to the yeast protein sds22 [8]. The similarity includes a tandem array of 11 leucine-rich repeat structures of 22 residues. sds22 has been genetically identified as a positive regulator of PP-1 in yeast [9]. Both immunoprecipitation and two-hybrid analysis have shown that sds22 is physically associated with PP-1_C in yeast [10–12].

There is no information on the physiological role of sds22 in higher eukaryotes or on its potential interaction with PP-1. We show here that sds22 is an inhibitory subunit of a rare nuclear species of PP-1 in rat liver and provide initial evidence that this inhibition is, at least partially, mediated by interaction of the catalytic subunit with the repeat structure of sds22.

2. Materials and methods

2.1. Materials

PP-1_C [13], PP-$2A_C$ [14], inhibitor-2 [15], and phosphorylase *b* [16] were purified from rabbit skeletal muscle. Protein kinase $p34^{cdc2}$ was purified from *Xenopus* oocytes by affinity chromatography on p13-Sepharose [17]. Microcystin-LR, histone IIA, myelin basic protein and the catalytic subunit of the cAMP-dependent protein kinase from beef heart were purchased from Sigma. Histone H1 and the digoxigenin protein labeling and detection kit were obtained from Boehringer. Protein A coupled to TSK beads was obtained from Affiland (Liège, Belgium). Affi-T-agarose, used for the purification of immunoglobulins, was purchased from KemEnTtec (Denmark). Poros 20 HQ was delivered by PerSeptive Biosystems (Framingham, MA, USA). A kit for the development of Western blots by enhanced chemiluminescence was purchased from Amersham Corp. Aminoethanethiol-microcystin (0.5 mg) was prepared and coupled to activated CH-Sepharose 4B (3 ml) according to the procedure of Moorhead et al. [18]. Synthetic peptides were synthesized with a Milligen 9050, using the *N*-(9-fluorenyl)methoxycarbonyl mode.

Phosphorylase *b* was phosphorylated in the presence of [^{32}P]ATP by purified phosphorylase kinase [19]. Myelin basic protein and histone IIA were phosphorylated by the catalytic subunit of cAMP-dependent protein kinase [20], and histone H1 was phosphorylated by $p34^{cdc2}$ [17].

2.2. Preparation of nuclear extracts and partial purification of PP-1N_{sds22}

Intact rat liver nuclei were prepared as described by Jagiello et al. [3]. The nuclei were first washed by resuspension with a Dounce homogenizer (A pestle) in a buffer containing 20 mM Tris-HCl at pH 7.4, 1 mM $MgCl_2$ and 0.25 M sucrose, and subsequent resedimentation by centrifugation for 3 min at 3500×*g*. The nuclear pellet was resuspended with the B pestle in buffer A (consisting of 50 mM glycylglycine/NaOH at pH 7.4, 5 mM 2-mercaptoethanol and 0.5 mM dithiothreitol) plus 0. 3 M NaCl and 0.5% (v/v) Triton X-100. After an incubation for 30 min at 4°C on a rotating wheel, the nuclear particulate fraction was sedimented by centrifugation (10 min at

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Abbreviations: PP-1, protein phosphatase-1; PP-1_C, catalytic subunit of protein phosphatase-1; PP-1N_{sds22}, PP-1 containing sds22; PP-$2A_C$, catalytic subunit of protein phosphatase-2A

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13000×*g*). The supernatant is further referred to as the 'nuclear extract'.

The nuclear extract prepared from the liver of 15 rats was dialyzed against buffer A plus 50 mM NaCl, and applied to a Poros 20 HQ column (1.7 ml), equilibrated in the same buffer. Bound proteins were eluted with 34 ml of a linear salt gradient (50–1000 mM NaCl in buffer A). The fractions were assayed for trypsin-revealed phosphorylase phosphatase activities. In agreement with previous data [3], this assay revealed two phosphatase activity peaks. The fractions of the first eluted phosphatase, which contain mainly PP-1N_{R41} (see Section 3), were pooled, concentrated on a Biomax-10 ultrafiltration membrane (Millipore) until 200 μl, and applied to a Superdex-200 gel filtration column (24 ml), equilibrated with buffer A plus 150 mM NaCl. Fractions of 0.25 ml were collected and assayed for spontaneous and trypsin-revealed phosphorylase phosphatase activities and immunodetectable sds22 (Fig. 1).

2.3. Immunological techniques and assays

Antibodies against a synthetic peptide encompassing the 11 carboxyl-terminal residues of NIPP-1 from bovine thymus were raised in a rabbit and purified by chromatography on Affi-T-agarose [3]. A synthetic peptide with a sequence corresponding to the 14 C-terminal residues of human sds22 [8], plus an additional N-terminal cysteine, was coupled to keyhole limpet hemocyanin using the Pierce immunogen conjugation kit. Rabbit polyclonal antibodies to the hemocyanin-coupled peptide were purified by chromatography on Affi-T-agarose. The NIPP-1 and sds22 antigenic peptides do not show any similarity and their antibodies did not cross-react. For Western analysis the antibodies were used at a final dilution of 10 μg/ml, and the peroxidase-labeled secondary antibodies were detected by enhanced chemiluminescence. For immunoprecipitation, 100 μl PP-1N_{sds22}, purified until after Superdex-200 (Fig. 1), was incubated for 2h at 4°C with 10 μl anti-sds22 IgG fraction (10 mg/ml) in the presence of 0.3 mM phenylmethanesulfonyl fluoride, 50 μM 1-chloro-3-tosylamido-7-amino-2-heptanone (TLCK), 50 μM L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) and 5 μM leupeptin. Subsequently, 30 μl of protein-A TSK was added, and the mixture was incubated during 30 min at 4°C on a rotating wheel. Following centrifugation (5 min at 10000×*g*), the pellet was washed twice with 0.2 ml of buffer A plus 50 mM NaCl, and resuspended in the same medium to a final volume of 50 μl.

Far-Western blotting with digoxigenin-bound PP-1_C was performed according to Jagiello et al. [3]. The spontaneous and trypsin-revealed ('total') protein phosphatase activities were measured as described previously [20].

3. Results and discussion

3.1. Identification of PP-1N_{sds22}

Fractionation of a hepatic nuclear extract on Mono Q [3] or Poros HQ (not shown) revealed the presence of two major peaks of phosphorylase phosphatase activity eluting at about 0.2 M and 0.3 M NaCl, which correspond to the previously characterized PP-1N_{R41} and PP-1N_{R111}, respectively [3]. Screening of the Poros HQ fractions by Western analysis with antibodies against the C-terminus of sds22 showed that this protein co-eluted with PP-1N_{R41} (not illustrated). However, subsequent gel filtration on Superdex-200 resulted in a nearly complete separation between PP-1N_{R41} and a small peak of phosphorylase phosphatase activity, termed PP-1N_{sds22} (Fig. 1A). The latter phosphatase migrated like a protein of 260 kDa and co-eluted with immunodetectable sds22 (Fig. 1B). In contrast, NIPP-1 co-migrated with PP-1N_{R41}, as indicated by Western analysis with NIPP-1 antibodies (not shown) and by far-Western blotting with digoxigenin-labeled $PP1_C$ (Fig. 1C). The phosphorylase phosphatase activity of PP-1N_{sds22} could only be detected after prior proteolysis with trypsin. This phosphatase activity stemmed from PP-1, as indicated by its complete inhibition with 0.1 μM inhibitor-2 (not shown), a specific inhibitor of PP-1 [1]. Since the phosphatase activity of the free catalytic subunit of PP-1 is hardly affected by trypsin under the adopted conditions (Table 1 and [3]), the latency of PP-1N_{sds22} must be explained by association of the catalytic subunit with one or more trypsin-sensitive inhibitory polypeptides. Using phosphorylase as a substrate,

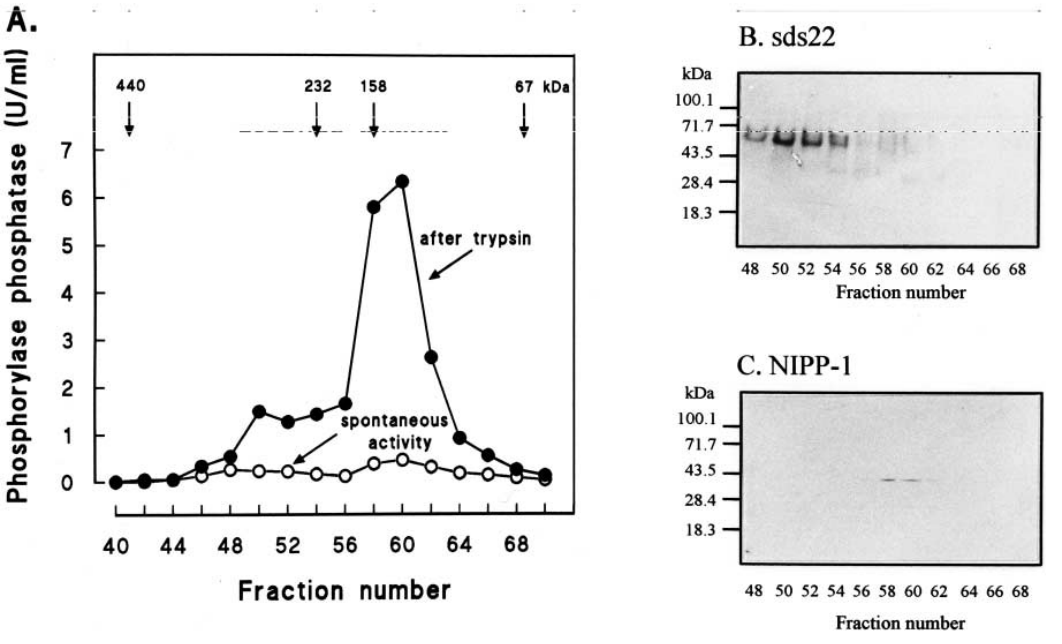

Fig. 1. Separation of PP-1N_{sds22} and PP-1N_{R41} by gel filtration. As described in Section 2, the 0.2 M NaCl pool from the Poros HQ column, containing mainly PP-1N_{R41}, was applied to a Superdex-200 column. The fractions were assayed for spontaneous (○) and trypsin-revealed (●) phosphorylase phosphatase activities (A), for immunodetectable sds22 (panel B) and for NIPP-1 by far-Western blotting with digoxygenin-labeled $PP1_C$ (C). For the immunodetection of sds22, the samples were first concentrated 10-fold by lyophilization. The arrows indicate the elution position of marker proteins, i.e. ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa) and bovine serum albumin (67 kDa).

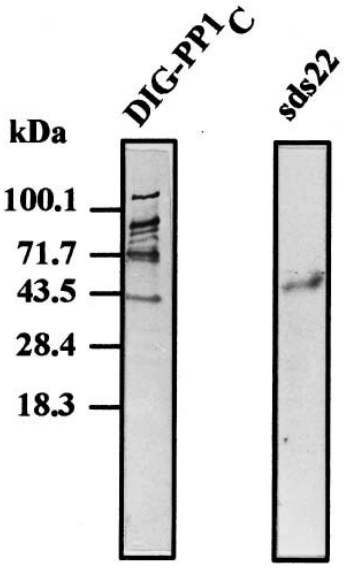

Fig. 2. PP-1N_{sds22} is retained by microcystin-Sepharose. A nuclear extract from the livers of 15 rats was applied to a microcystin-Sepharose column (3 ml), equilibrated in buffer A containing in addition 0.25 M NaCl. After washing of the column with 10 volumes of the same buffer, the retained proteins were eluted with 10 volumes of buffer A containing 3 M KSCN. The eluate was concentrated to 200 μl on a Biomax-10 ultrafiltration membrane (Millipore) and analyzed by far-Western blotting with digoxigenin-labeled PP-1_C (left lane) and by Western blotting with sds22 antibodies (right lane).

the total (i.e. trypsin-revealed) activity of PP-1N_{sds22} was estimated to account for 5–10% of the total activity of PP-1 in a rat liver nuclear extract (not shown).

Direct evidence for an association between sds22 and PP-1_C came from chromatography of a nuclear extract on microcystin-Sepharose, which specifically and covalently binds PP-1_C [18]. We found that sds22 was retained by this affinity matrix, as detected by Western analysis of the 3 M KSCN eluate (Fig. 2). Interestingly, microcystin-Sepharose also bound other nuclear species of PP-1, as indicated by the large fraction of phosphorylase phosphatase activity that was retained (ca. 80% of the total applied activity) and by far-Western blotting with digoxigenin-labeled catalytic subunit. Among the proteins that were visualized by an overlay with catalytic subunit were polypeptides of 41 and 111 kDa, corresponding to NIPP-1 and R111, respectively. Remarkably, far-Western blotting did not visualize sds22 (see also Fig. 1C), which migrated as a slightly larger polypeptide than NIPP-1 during glycine SDS-PAGE (Fig. 2). This suggests that sds22, like inhibitor-2 [3], does not retain its PP-1 binding properties after denaturing electrophoresis. In view of the low abundance of PP-1N_{sds22} it cannot be ruled out, however, that the concentration of sds22 was limiting for detection by far-Western blotting.

Table 1
Effect of trypsin on the protein phosphatase activities of PP-1_C and PP-1N_{sds22}

Substrate	Protein phosphatase activity after trypsin (% of spontaneous activity)	
	PP-1_C	PP-1N_{sds22}
Phosphorylase *a*	116 ± 17	1869 ± 343
Histone H1	127 ± 13	257 ± 32
Histone IIA	110 ± 12	1281 ± 263
Myelin basic protein	125 ± 3	1320 ± 227

PP-1N_{sds22}, purified until after Superdex-200, was immunoprecipitated with sds22 antibodies, as described in Section 2. The washed immunoprecipitate and purified PP-1_C were assayed from spontaneous and trypsin-revealed protein phosphatase activities. The results are expressed as a percentage of the spontaneous activity and represent the means ± S.E.M. for 3–6 assays.

Additional evidence that sds22 is a part of PP-1N_{sds22} came from observations that a fraction of the holoenzyme (38 ± 4%; $n = 3$) could be immunoprecipitated with sds22 antibodies. The immunoprecipitated phosphatase was latent, but the activity was increased 2–18-fold by prior proteolysis, depending on the substrate used (Table 1). In contrast, the free catalytic subunit (Table 1) and the fraction of PP-1N_{sds22} that was not immunoprecipitated (not illustrated) showed only a small activity increase after trypsin treatment. Since the sample before immunoprecipitation was completely latent (see Fig. 1), this suggests that the non-immunoprecipitable PP-1N_{sds22} represents a fraction that was proteolyzed or dissociated during immunoprecipitation. A similar sensitivity to proteolysis during immunoprecipitation has also been noted before for R111 [3].

3.2. *Inhibition of PP-1_C by a synthetic leucine-rich repeat unit of sds22*

Leucine-rich repeats are short sequence motifs of variable length present in various polypeptides with diverse functions and cellular locations [21]. They consist of a short β-strand and an α-helix arranged approximately parallel to one another. All proteins containing these repeats are thought to be involved in protein-protein interactions. Stone et al. [10] have previously shown that the interaction of sds22 with PP-1_C in fission yeast is dependent upon the presence of some leucine-rich repeats. We have found that a synthetic peptide (ENLSNLHQLQMLELGSNRIRAI) with a primary structure corresponding to that of the sixth repeat (residues

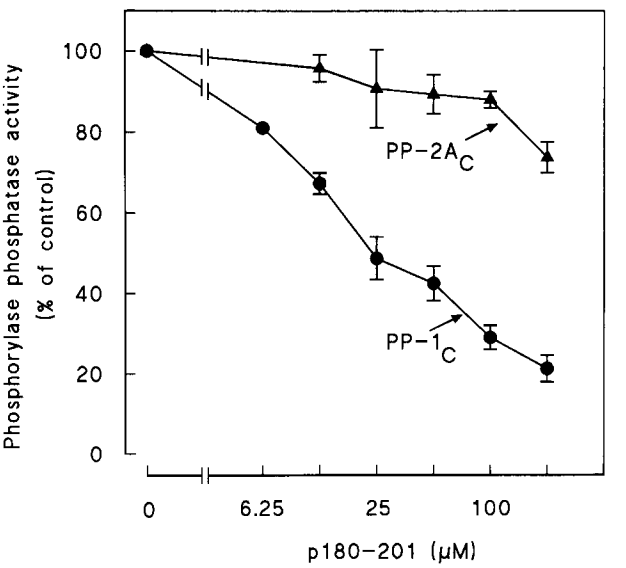

Fig. 3. Effects of a synthetic leucine-rich repeat of sds22 on the activities of PP-1_C and PP-$2A_C$. The phosphorylase phosphatase activity of the catalytic subunits of PP-1 and PP-2A was assayed in the presence of the indicated concentrations of a synthetic peptide (p180–201) encompassing residues 180–201 of human sds22. Results are expressed as a percentage ± S.E.M. of the control activity ($n = 4$).

180–201) of human sds22 was inhibitory to the phosphorylase phosphatase activity of $PP1_C$ (Fig. 3). Half-complete inhibition was obtained at 25 μM. Interestingly, the same concentrations of p180–201 did not affect $PP\text{-}2A_C$, providing additional evidence that the interaction of sds22 with the type-1 catalytic subunit is specific and mediated by the repeat structure. Although these results suggest that sds22 inhibits $PP\text{-}1_C$, we have up to now not been able to obtain sufficient native or recombinant sds22 for functional studies.

3.3. Conclusions

We show here that sds22 is a subunit of a latent high-molecular-weight species of PP-1. The main evidence is that sds22 copurifies with PP-1 during anion exchange chromatography (not illustrated), during gel filtration (Fig. 1) and during affinity chromatography on microcystin-Sepharose (Fig. 2). In addition, we have shown that PP-1 co-immunoprecipitates with sds22 (Table 1). In retrospect, it is easy to understand why $PP\text{-}1N_{sds22}$ escaped our previous attention, since its activity accounts only for 5–10% of the total nuclear activity of PP-1 and can only be revealed after trypsinolysis. Since hepatocytes are nearly all in the G_0 phase of the cell cycle, our data show that sds22 is phosphatase-associated in resting cells. It cannot be excluded, however, that this association is cell cycle regulated.

The estimated mass of $PP\text{-}1N_{sds22}$ (260 kDa) is much higher than expected from the combined masses of $PP\text{-}1_C$ and sds22 (ca. 80 kDa). Although we cannot rule out that the holoenzyme is a polymer of these subunits, it seems more likely that $PP\text{-}1N_{sds22}$ contains additional regulatory subunit(s). This is in agreement with a preliminary biochemical analysis in fission yeast, where sds22 was found to be associated with both $PP1_C$ and a phosphoprotein of 25 kDa [10]. Obvious candidates as additional regulatory subunits of $PP\text{-}1N_{sds22}$ are p53BP2, the retinoblastoma protein and PSF, which have previously already been identified as nuclear regulators of PP-1 (see Section 1).

sds22 has been genetically identified as a positive regulator of PP-1 in yeast [9]. Our finding that $PP\text{-}1N_{sds22}$ is a latent enzyme and that this latency is, at least partially, mediated by the leucine-rich repeat motif of sds22 is somewhat surprising, but not necessarily at variance with its proposed role as a 'positive' effector of PP-1. Indeed, it is quite possible that the effect of sds22 is substrate-dependent, as indicated by the lower degree of latency of the holoenzyme with histone H1 as substrate (Table 1). This is in keeping with the known effects of other regulatory subunits of PP-1 [1,2]. For example, the glycogen-binding G-subunit is inhibitory to the phosphorylase phosphatase activity of the catalytic subunit, but is required to recognize glycogen synthase as a substrate.

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