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SARP, a new alternatively spliced protein phosphatase 1 and DNA interacting protein

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PP1 (protein phosphatase 1) is a ubiquitously expressed serine/threonine-specific protein phosphatase whose activity towards different substrates appears to be mediated via binding to specific proteins that play critical regulatory and targeting roles. In the present paper we report the cloning and characterization of a new protein, termed SARP (several ankyrin repeat protein), which is shown to interact with all isoforms of PP1 by a variety of techniques. A region encompassing a consensus PP1-binding motif in SARP ($K^{354}VHF^{357}$) modulates endogenous SARP–PP1 activity in mammalian cells. This SARP–PP1 interaction motif lies partially within the first ankyrin repeat in contrast with other proteins [53BP2 (p53 binding protein 2), MYPT1/M_{110}/MBS (myosin binding protein of PP1) and TIMAP (transforming growth factor β inhibited, membrane-associated protein)], where a PP1-binding motif precedes the ankyrin repeats. Alternative mRNA splicing produces several isoforms of SARP from a single human gene at locus 11q14. SARP1 and/or SARP2 (92–95 kDa) are ubiquitously expressed in all tissues with high levels in testis and sperm, where they are shown to interact with both PP1γ_1 and PP1γ_2. SARP3 (65 kDa) is most abundant in brain where SARP isoforms interact with both PP1α and PP1γ_1. SARP is highly abundant in the nucleus of mammalian cells, consistent with the putative nuclear localization signal at the N-terminus. The presence of a leucine zipper near the C-terminus of SARP1 and SARP2, and the binding of mammalian DNA to SARP2, suggests that SARP1 and SARP2 may be transcription factors or DNA-associated proteins that modulate gene expression.

Key words: ankyrin repeat, DNA binding protein, leucine zipper, protein phosphatase 1, protein serine/threonine phosphatase.

INTRODUCTION

PP1 (protein phosphatase 1) is a serine/threonine-specific phosphatase that controls many aspects of mammalian cell physiology. It is expressed ubiquitously and found in multiple subcellular locations [1]. In mammalian genomes, three genes encode the PP1 catalytic subunits: PP1α, PP1β (also termed PP1δ) and PP1γ, and alternatively spliced isoforms of PP1α and PP1γ have been described [2]. Although the PP1 isoforms exhibit differential tissue and subcellular distribution [3–6], they have broad substrate specificities *in vitro* and their cellular actions are largely determined by the binding of the catalytic subunit to many different proteins that act as regulatory and targeting subunits [1,7,8]. Several earlier studies confirm that the PP1-targeting subunits direct the catalytic subunit to specific subcellular locations, thus restricting the actions of the catalytic subunit to the substrates present there [9,10]. Most PP1-binding proteins interact with the PP1 catalytic subunit through a common PP1-binding motif termed the RVxF motif with the consensus $(R/K)X_{A(0-1)}(V/I)X_B$ (F/W), where X_A is any amino acid and X_B is any amino acid except proline [1,11–13]. Although this canonical motif occurs in numerous proteins, limited accessibility to the motif may prevent PP1 binding. A refinement of the motif has been suggested which decreases the sequence variation of the motif in order to identify good PP1 interactors [14]. The interaction of PP1 with the RVxF motif is believed to initiate binding and additional weaker interactions may then stabilize the PP1 complexes but in some cases a second binding site may also retain interaction with PP1 [1,15]. The existence of a common interaction site explains why the binding of most regulatory subunits is mutually exclusive; however, trimeric and multicomponent complexes of PP1 can also occur [16–18]. Since the regulatory and/or targeting subunits are responsible for controlling PP1 activity and determining the disparate physiological roles of the different PP1 complexes, the key to understanding the function of PP1 lies in identifying and studying the cellular roles of the PP1-binding subunits. In the present study we have focused on the identification of novel PP1 regulatory proteins through yeast two-hybrid methodology using PP1γ isoforms as bait [19,20]. The gene encoding PP1γ undergoes alternative splicing to generate a ubiquitous PP1γ_1 protein and a testis-enriched PP1γ_2 variant, which differ solely at their extreme C-termini [4,21,22]. In the present paper we report a novel protein possessing several ankyrin repeats (termed SARP, several ankyrin repeat protein), which forms a complex not only with both PP1γ_1 and PP1γ_2, but also with PP1α and PP1β and interacts with DNA.

MATERIALS AND METHODS

Yeast two-hybrid analysis

Microbial strains and methods for yeast two-hybrid screening of human peripheral lymphocyte or human testis cDNA libraries using human PP1 have been described previously [19,20]. Positive colonies were obtained on selective medium from which pACT plasmids were recovered into *Escherichia coli* and

Abbreviations used: 53BP2, p53 binding protein 2; ECL, enhanced chemiluminescence; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione S-transferase; HA, haemagglutinin; HEK-293, human embryonic kidney 293; HRP, horseradish peroxidase; IVT, *in vitro* translation; MBP, maltose-binding protein; MYPT1/M_{110}/MBS, myosin-binding protein of PP1; PP1, protein phosphatase 1; SARP, several ankyrin repeat protein; ssDNA, single stranded DNA; TBS, tris-buffered saline; TIMAP, transforming growth factor β inhibited, membrane-associated protein.

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their cDNA inserts sequenced. Screening of human Universal (Stratagene) and Marathon-Ready (Clontech) cDNA libraries was performed according to the manufacturers' protocols. Automated DNA sequencing was performed by the sequencing services at the University of Dundee and University of Aveiro, using oligonucleotide primers. Analyses of protein sequence domains were performed using SMART (the Simple Modular Architecture Research Tool algorithm; http://smart.embl-heidelberg.de/) and the ExPASy Proteomic PSORT II prediction algorithm (http://www.expasy.org/).

Cloning and bacterial expression of epitope-tagged SARP

To construct the fusion vector for expression of GST (glutathione S-transferase)–SARP1(240–832), the H2 cDNA was cleaved out of pACT using BglII and ligated into the BamH1 restriction site of the vector pGEX-3X (Pharmacia) and pMAL-HA [plasmid pMAL-c2 modified to contain a HA (haemagglutinin)-tag and additional restriction sites; New England Biolabs]. Fusion vectors for the expression of SARP1(708–832), SARP1(240–708) and SARP1(240–333) were constructed by PCR using appropriate oligonucleotide primers and a H2 template, followed by ligation into pCR2.1 TOPO (Invitrogen) and subcloning into pGEX and pMAL vectors. The cDNA encoding the 40Q3 cDNA was directionally subcloned into EcoRI/XhoI-digested pET28a to express His_6-SARP2(66–847). Another construct [His_6-SARP2-(331–847)] was produced by PCR amplification using a primer starting at Met^{331}. SARP1(240–832) was subcloned into the pEBG vector for expression in mammalian cells, and mutagenesis to generate pEBG-SARP1(F357A) was performed using Stratagene's QuikChange™ II site-directed mutagenesis kit. All constructs were verified by DNA sequencing and expressed in *E. coli* BL21 (DE3) pLysS. Soluble GST-fusion and MBP (maltose binding protein)-fusion proteins were affinity purified on glutathione–Sepharose (Amersham Biosciences) and amylose resin (New England Biolabs) respectively. Insoluble fusion proteins located in *E. coli* inclusion bodies were washed in 1 % Triton X-100/ 1 M NaCl, solubilized using either 1 % SDS or 8 M urea with subsequent removal of the urea by dialysis.

Northern blot analysis

The 40Q3 cDNA was purified by low melting-point agarose gel electrophoresis, labelled by the random primed method using a commercial kit (Roche) and [α-^{32}P]dCTP (Amersham Biosciences) and purified by passage through a NucTrap column (Stratagene) to remove unincorporated nucleotides. A multiple tissue Northern blot (Clontech) was incubated in the presence of the denatured radiolabelled DNA and hybridizing mRNAs were detected using a PhosphorImager (Bio-Rad Laboratories). After probe stripping in 0.5 % SDS at 90–100 °C for 10 min, the same blot was re-used with a β-actin probe as a control. Complete probe removal was verified before use.

Production of antibodies and immunoblotting

Antisera were raised in sheep against GST–SARP1(240–832) at the Scottish Antibody Production Unit (Carluke, Lanarkshire, Scotland, U.K.) and affinity purified against MBP–SARP1(240–832), MBP–SARP1(240–708), MBP–SARP1(708–832) or SARP1(240–333) from *E.coli* inclusion bodies. Affinity purified anti-PP1α, anti-PP1β, anti-PP1γ, anti-PP1γ_1 and anti-PP1γ_2 antibodies have been described previously [4,23–25]. For immunoadsorption studies, anti-PP1γ_1 antibodies (200 μg) raised in sheep were non-covalently coupled to Protein G–Sepharose (200 μl) by incubation at 4 °C for 1 h in 1 ml of 50 mM Tris/HCl (pH 7.5) and 0.15 M NaCl, followed by washing twice with the same buffer. Alternatively anti-PP1 antibodies and anti-SARP(240–333) antibodies were covalently coupled to Protein G–Sepharose using dimethylpimelimidate [26]. Immunoblotting was performed using anti-SARP or anti-PP1 primary antibodies and HRP-(horseradish peroxidase) conjugated anti-sheep or anti-rabbit secondary antibodies coupled to ECL (enhanced chemiluminescence) detection.

Blot overlay analysis

A single colony expressing His-tagged SARP2(331–847) was selected and grown overnight in 3 ml of Luria–Bertani/ampicillin at 37 °C. Aliquots (0.5 ml) were transferred to 50 ml Luria–Bertani/ ampicillin until A_{600} reached 0.6. Expression was induced with 50 μl of 1 M isopropyl β-D-thiogalactoside at 21 °C, for different periods of time with shaking. Samples (1 ml) were collected every hour, the cells were recovered by centrifugation (12 000 ***g*** for 1 min at 4 °C), resuspended in 0.5 ml 1 % SDS and 30 μl of the bacterial extracts were loaded onto SDS/PAGE (12 % gels). The proteins were subsequently transferred to a nitrocellulose membrane that was then overlaid with purified PP1γ_2 protein, prepared as described previously [27]. After washing to remove excess protein, bound PP1γ_2 was detected by incubating the membrane with a rabbit anti-PP1γ_2 antibody, raised against a specific C-terminal peptide [4,24]. Immunoreactive bands were revealed by incubating with a HRP-conjugated secondary antibody followed by ECL detection [20].

Immunocytochemical analysis of COS-7 cells

COS-7 cells were grown as previously described [28] in Dulbecco's modified Eagle's medium containing 10 % heat-inactivated fetal calf serum (Invitrogen). After fixation with 4 % paraformaldehyde, cells were permeabilized with methanol for 2 min and washed with PBS. Cells were incubated with the anti-SARP primary antibodies for 1 h at room temperature (20 °C), followed by FITC-conjugated anti-sheep secondary antibody. After thorough washing with PBS, preparations were mounted using Fluoroguard (Bio-Rad) and visualized in an Olympus IX-81 inverted epifluorescence microscope.

Preparation of tissue and cell extracts

Tissue and cultured cell lysates for assays were prepared as previously described [29]. For immunoblotting, tissues were ground under liquid nitrogen and cell cultures were harvested and heated at 100 °C for 5 min in gel loading buffer containing 1 % SDS or 4 % lithium dodecyl sulphate before being analysed by denaturing PAGE. HeLa cell nuclear extract was obtained commercially from the Computer Cell Culture Centre (Mons, Belgium).

Protein phosphatase assays

Rabbit skeletal muscle glycogen phosphorylase was phosphorylated to a stoichiometry of 1 mole phosphate per mole subunit using phosphorylase kinase and [γ-^{32}P]ATP. PP1 assays were performed in the presence of 3 nM okadaic acid and either 0.5 mM Mn^{2+} (PP1γ_1) or in the absence of divalent cations (endogenous PP1) as described in [30]. Immunoadsorption and sedimentation of endogenous SARP–PP1 complexes was performed using anti-SARP(240–333) antibodies covalently coupled to Sepharose. Subsequent phosphatase assays were performed in a shaking incubator in the presence of 2 μM PP1-complex dissociating peptide (GKRTNLRKTGSERIAMGMRVKFNPLALLLDSC) or 2 μM control peptide (NLRKTGSERIAMGMRVKANPLALLL-DSC) as previously described [29]. Trypsin treatment of pellets

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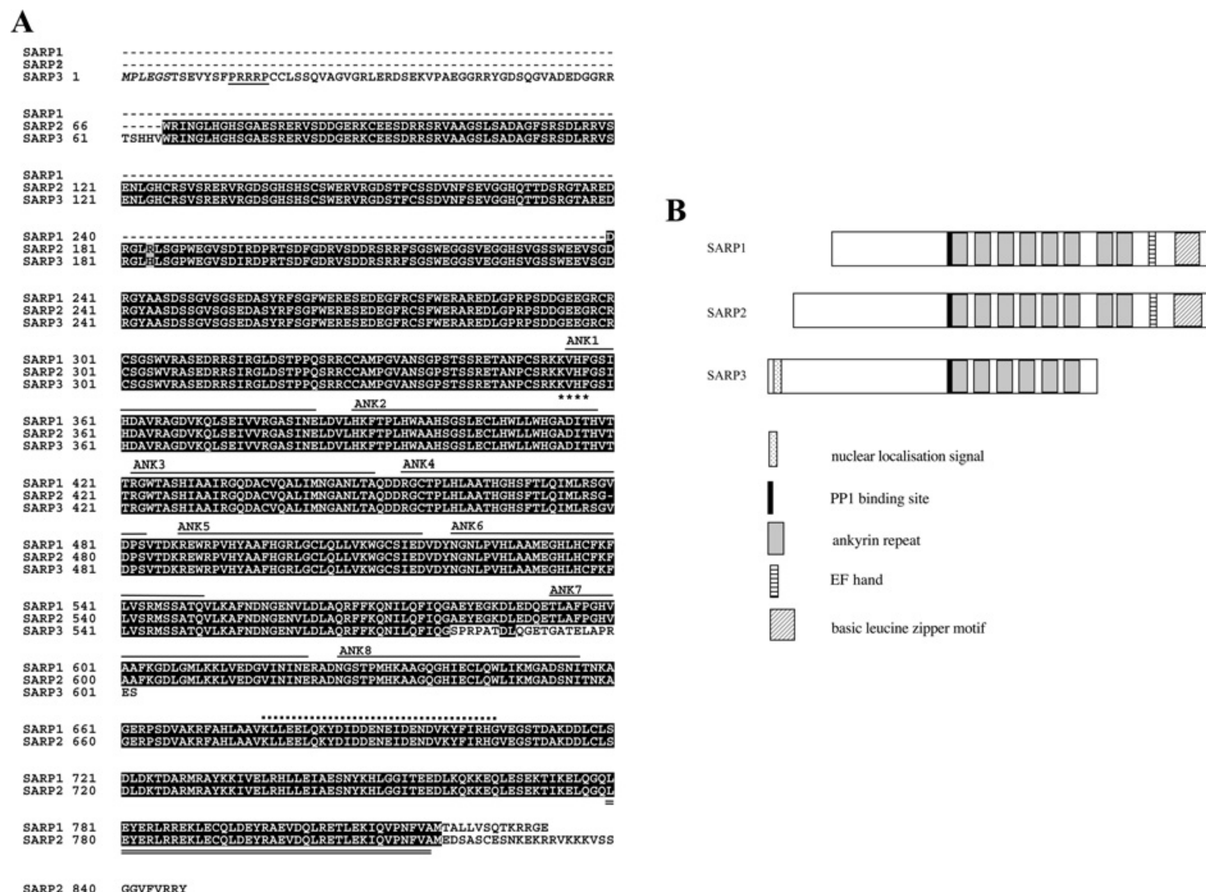

Figure 1 SARP protein isoforms

(**A**) Comparison of the SARP1, SARP2 and SARP3 amino acid sequences derived from the three cDNAs (DQ508815, EF041819, DQ508934). The six N-terminal amino acids of SARP3, not encoded in cDNA but predicted from the gene sequence, are shown in italics. The putative nuclear localization signal (amino acids 14–18) is underlined. The molecular masses of SARP1 and SARP2 on SDS/PAGE are consistent with these isoforms possessing the same N-terminal sequence as SARP3. The PP1-binding motif is underlined with asterisks. The eight ankyrin repeats (ANK1–ANK8) are indicated by a single line above the sequences. The dotted line above the sequences indicates a putative EF hand in SARP1 and SARP2. A double underline indicates a putative leucine-zipper domain present in SARP1 and SARP2. Note that only one clone of SARP3 was sequenced and the encoded PP1-binding motif was KMHF. Since PCR of this region in several libraries (including a human brain library) yielded only sequence coding for KVHF, we presume that a mutation may have arisen during the preparation or cloning of SARP3 cDNA. Methionine has never been reported or predicted at position 2 [11,12]. (**B**) Schematic comparison of the protein domain structures of SARP1, SARP2 and SARP3.

prior to some assays was performed by incubating each pellet with 14 units of trypsin (Sigma) for 5 min at 30 °C, followed by addition of 1 mg of soybean trypsin inhibitor (Type 1-S). One unit of phosphatase activity was the amount of enzyme that catalysed the release of 1 μmol [^{32}P]phosphate/min from [^{32}P]-labelled phosphorylase *a* in the assay.

DNA-binding assays

SARP2(331–847) protein was generated by IVT (*in vitro* translation) from the pET-SARP2(331–847) expression vector using the TnT-coupled transcription/translation kit (Promega) according to the manufacturer's instructions. For radioactive detection of proteins, reactions were carried out in the presence of [^{35}S]methionine. For the DNA-binding assays, aliquots of labelled protein were mixed with immobilized DNA [ss (single-stranded) DNA cellulose; Sigma] in 150 μl TBS (tris-buffered saline) [31]. The specificity of binding was tested in the absence or presence of bacterial and human competitor DNA. After gently mixing for 3 h at 4 °C, the cellulose was pelleted in a microcentrifuge at 10 000 ***g*** for 1 min and washed three times with 500 μl TBS before bound proteins were eluted in SDS/PAGE sample buffer. The eluted fractions were separated by electrophoresis on SDS/PAGE (10 % gels) that were subsequently dried and exposed to X-ray films at − 70 °C.

RESULTS

Identification of a novel ankyrin repeat-containing PP1-binding subunit

In order to identify proteins capable of interacting with PP1, PP1γ_1 was used as bait to screen human B-lymphocyte and human testis cDNA libraries by the yeast two-hybrid approach [20,32]. One of ten positive clones (H2) identified from the human B-lymphocyte library and one of 120 clones (40Q3) isolated from the human testis library encoded part of a novel protein, termed SARP. H2 comprised an open reading frame of 1779 nt that encoded 593 amino acids followed by a stop codon and a short 3′-untranslated region, whereas 40Q3 encoded 782 amino acids in an open reading frame of 2346 nt, followed by a stop codon and a different 3′-untranslated sequence (see Figure 1 of supplementary material at http://www.BiochemJ.org/bj/402/bj4020187add.htm). The H2-encoded protein sequence (SARP1) contained eight ankyrin

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repeats preceded by a potential PP1-binding motif (KVHF), and the 40Q3-encoded protein (SARP2) represented a splice variant differing in its C-terminal sequence (Figure 1A). The absence of a consensus start codon (specifying an initiating methionine residue) indicated that both clones were incomplete. Screening of a human universal cDNA library (Stratagene) using a 300 bp fragment from the 5′-end of H2 resulted in the identification of clone 1G07 with overlapping sequence but a different 3′-end. 1G07 contained an open reading frame encoding SARP3 (591 amino acids), but still no initiating methionine codon. Several PCR-based cDNA cloning strategies were followed to attempt to find the 5′-end of the clone, including 5′-RACE (rapid amplification of cDNA ends) carried out on total RNA from MCF7 cells and the Marathon-Ready human testis cDNA system (Clontech). Although these methods technically worked and confirmed the known sequence (including the KVHF putative PP1-binding domain), none were able to add to the existing sequence information generated from the 1G07 cDNA. Consequently, a 475 bp fragment from the 5′-end of this clone was sent to Genome Systems (St. Louis, U.S.A.) and an identified genomic clone (BAC 23122) was sequenced 3′ to 5′ starting from the known sequence. Analysis of the genomic sequence combined with the cDNA sequence using the Sanger Centre FGENESH gene-finder programme predicted the exon 1–intron 1 structure (see Figure 1 of supplementary material at http://www.BiochemJ.org/bj/402/bj4020187add.htm). Exon 1, encoding the initiating methionine and five additional amino acids, was located 728 base pairs 5′ to the start of exon 2 that contained previously determined cDNA sequence (see Table 1 of supplementary material at http://www.BiochemJ.org/bj/402/bj4020187add.htm). The intron/exon structure for exon 2–exon 14 of the SARP gene was determined by comparison of the cDNA sequences with information in the NCBI (National Center for Biotechnology Information) human genomic sequence database (see Figure 1 and Table 1 of supplementary material; GenBank® DQ508815, EF041819, DQ508934 and AP000873). The chromosomal location for the entire SARP coding sequence was identified at chr11q14.

The amino acid sequence for SARP3 predicted from the cDNA sequence is depicted in Figure 1(A) and the calculated molecular mass was 66 kDa, whereas those for SARP1 and SARP2 assuming the same N-terminal section are 92.5 kDa and 94.3 kDa respectively. These molecular masses for SARP1, SARP2 and SARP3 are consistent with the sizes of endogenous SARP determined by SDS/PAGE (see below). The domain structures of the SARP proteins are depicted in Figure 1(B). Six ankyrin repeats are identified in SARP3, whereas SARP1 and SARP2, besides containing eight ankyrin repeats, also possess a putative Ca^{2+}-binding EF hand and a putative leucine-zipper domain. A possible nuclear localization sequence was predicted at amino acids 14–18 in the N-terminus of SARP, suggesting that the protein isoforms may be localized in the nucleus (Figure 1A).

Interaction of SARP with PP1 *in vitro* and *in vivo*

The binding of SARP1 to PP1γ_1 was confirmed by immunoadsorption analysis of bacterially expressed MBP–SARP1 in the presence of bacterially expressed PP1γ_1 using anti-PP1γ antibodies non-covalently coupled to Sepharose. Detection of proteins present in the pellets and supernatants by Coomassie Blue staining showed that all of the PP1γ_1 was in the pellets and that, as the MBP–SARP concentration was increased, higher levels of MBP–SARP were present in the pellets. In the absence of PP1γ_1, the MBP–SARP1 remained in the supernatant (Figure 2A, lane 4).

PP1γ_2 interaction with SARP *in vitro* was demonstrated by blot overlay. His$_6$-SARP2(331–847) expression was induced in bacteria and aliquots taken at several time points after induction. After SDS/PAGE analysis, samples were transferred on to a nitrocellulose membrane that was incubated successively with recombinant PP1γ_2, rabbit anti-PP1γ_2 antibody and HRP-conjugated anti-rabbit secondary antibody followed by ECL detection. Increased expression of His$_6$-SARP2(331–847) with time (Figure 2B), resulted in proportionately increased binding of PP1γ_2, thus demonstrating its interaction with SARP2.

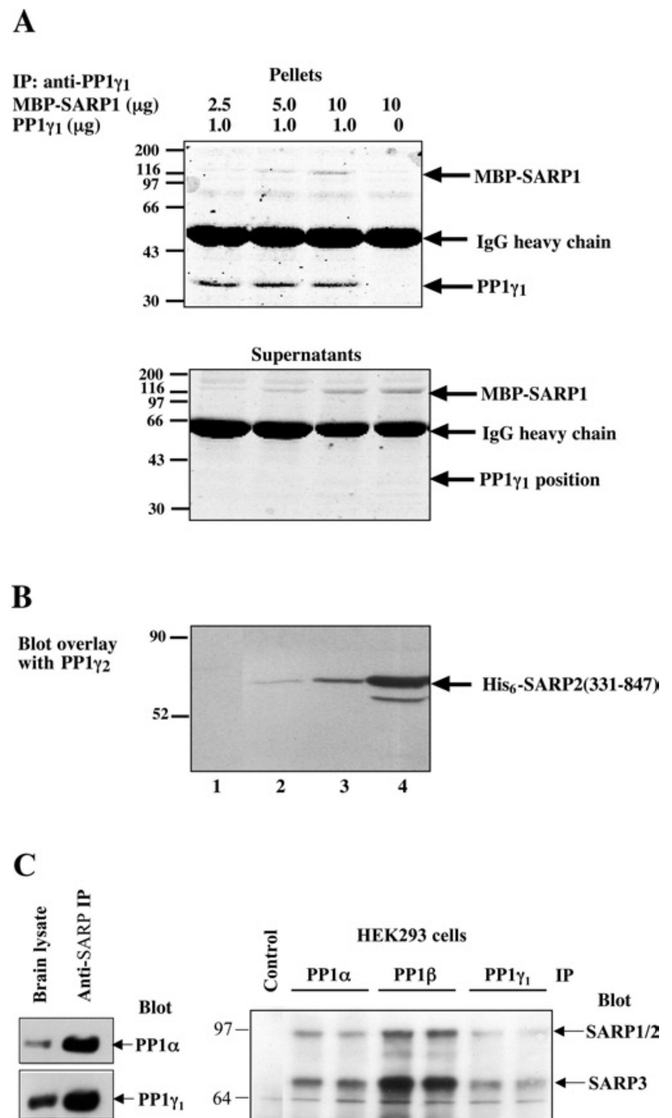

Figure 2 Interaction of SARP with PP1γ_1, PP1γ_2, PP1α and PP1β

(**A**) Co-immunoadsorption of bacterially expressed MBP–SARP1 with bacterially expressed PP1γ_1 using anti-PP1γ_1 antibodies bound to Sepharose. The quantities of SARP1 and PP1γ_1 in each immune reaction are indicated. Washed anti-PP1γ_1 immune pellets (upper panel) and the supernatants after removal of the immune pellets (lower panel) were subjected to SDS/PAGE. Protein bands were detected with Coomassie Blue. Molecular-mass markers in kDa are indicated. (**B**) Overlay of bacterially expressed His-tagged SARP2(331–847) with recombinant PP1γ_2. Bacterial cultures were collected at different times after induction of recombinant protein expression and equal amounts of protein were separated by SDS/PAGE and immunoblotted. Interaction was demonstrated as described in the Materials and methods section. Lane 1, control; lane 2, 1 h induced expression; lane 3, 2 h induced expression; lane 4, 3 h induced expression. (**C**) Left-hand panel: co-immunoadsorption of PP1α and PP1γ_1 from lysates of rat brain cortex with anti-SARP(708–832) antibodies coupled to Protein G–Sepharose. Right-hand panel: co-immunoadsorption of SARP from HEK-293 cell lysates with anti-PP1α, anti-PP1β and anti-PP1γ_1 antibodies coupled to Protein G–Sepharose.

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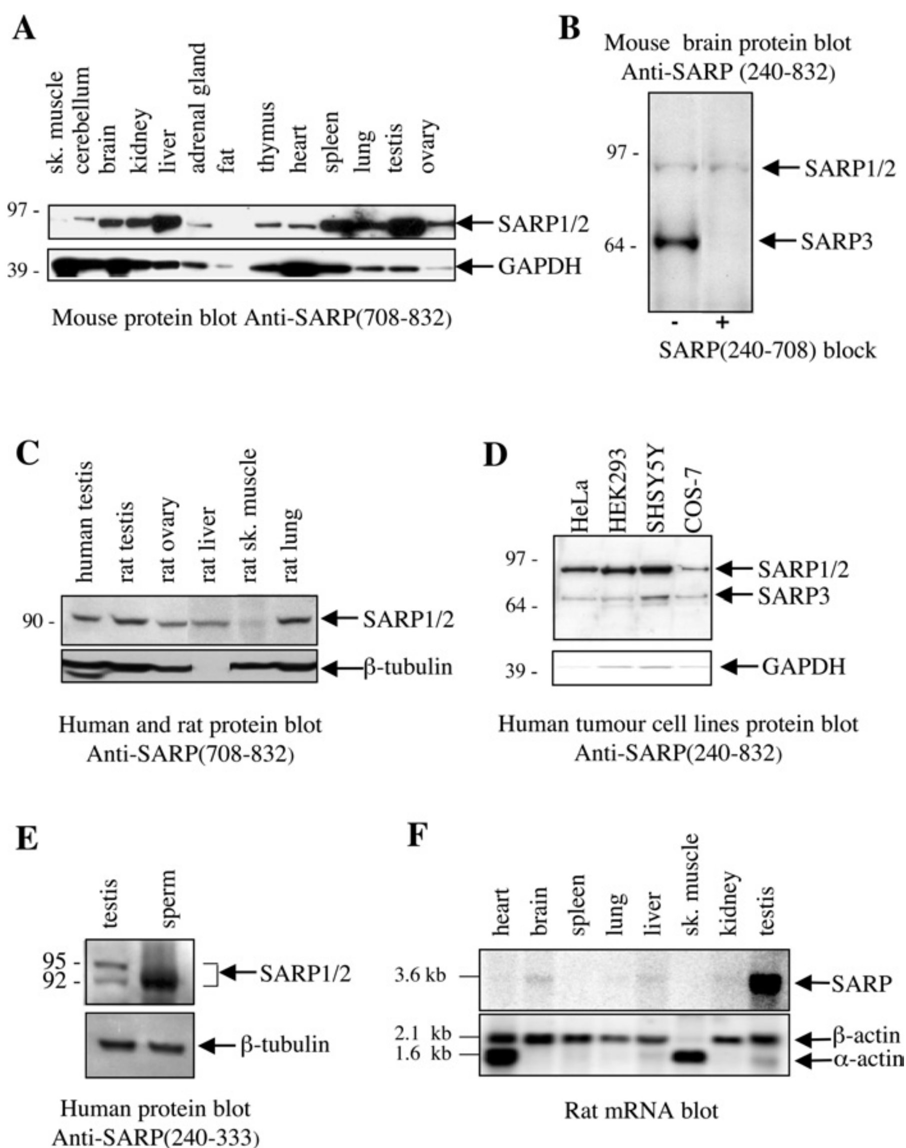

Figure 3 Detection of SARP in cell lines and tissue lysates

(**A**) SDS/PAGE separation and immunoblot analysis of SARP1/2 in mouse tissues using an anti-SARP(708–832) antibody. In order to prevent proteolytic degradation, ground tissue samples were heated directly at 100 °C in gel sample buffer and equal volumes were loaded on to the gel. The levels of SARP are therefore presented relative to the levels of mouse GAPDH in the same samples. (**B**) Lysates (20 μg of protein) from mouse brain were separated by SDS/PAGE and analysed by immunoblotting using an anti-SARP(240–832) antibody in the absence (−) and presence (+) of SARP (240–708) protein. (**C**) Immunoblot analysis of SARP1/2 in human testis and rat tissues (100 μg) using an anti-SARP(708–832) antibody. β-Tubulin was used as a control. (**D**) Lysates (20 μg of protein) from tumour cell lines were analysed by SDS/PAGE and immunoblotting using an anti-SARP(708–832) antibody. HeLa, human cervical carcinoma cell line; HEK-293, human embryonic kidney cell line; SHSY5Y, human neuroblastoma SHSY5Y epithelial cell line; COS-7, monkey kidney COS-7 fibroblast cell line. Human GAPDH was employed as a control. (**E**) SDS/PAGE separation and immunoblot analysis of SARP1/2 in human testis and sperm (50 μg). Molecular-mass markers in kDa are indicated in panels (**A**–**E**). (**F**) Northern blot analysis of SARP mRNA from rat tissues. A single mRNA of approx. 3.6 kb was particularly abundant in rat testis. Actin mRNA was employed as a control.

Although SARP was identified by interaction with PP1γ isoforms, SARP was found to bind PP1α and PP1β in tissues or cell lines where these PP1 isoforms were more abundant. Figure 2C (left-hand panel) shows that anti-SARP(708–832) immune complexes from rat cortex lysates contained both PP1α and PP1γ_1. Figure 2C (right-hand panel) shows that in HEK-293 (human embryonic kidney 293) cells anti-PP1α, anti-PP1β and anti-PP1γ_1 immune complexes contained both SARP1/2 and SARP3.

Immunodetection of endogenous SARP

Immunoblotting of lysates from rodent and human tissues with anti-SARP (708–832) antibodies identified a protein band of approximately 95 kDa, consistent with the predicted sizes of 92.5 kDa and 94.3 kDa for full length SARP1 and SARP2 (Figures 3A and 3C). SARP1/2 was present in all mouse tissues, with appreciable levels being present in testis, liver, spleen, lung and ovary compared with the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) or tubulin controls. High levels of SARP1/2 detected in testis (Figure 3A) are consistent with the mRNA analysis (Figure 3F). However, the high sensitivity of SARP isoforms to proteolytic degradation may lower detection in some tissues samples. SARP1/2 appeared extremely abundant in human sperm (Figure 3E). An immunoreactive protein of similar size was present in several human tumour cell lines (Figure 3D) and also in mammary gland carcinoma epithelial MCF7, epidermis

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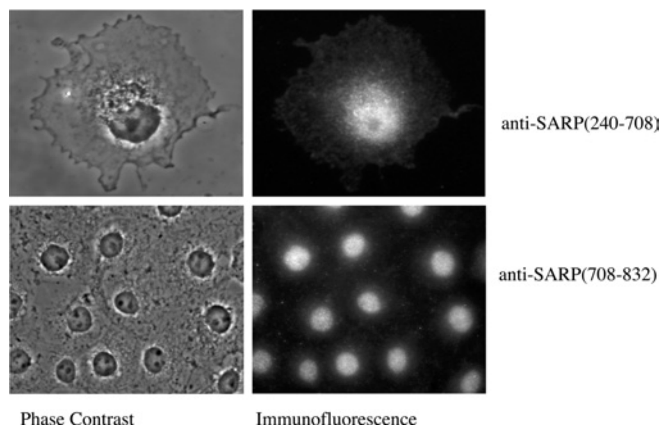

Figure 4 Immunocytological localization of endogenous SARP in COS-7 cells

COS-7 cells were cultured as described in the Materials and methods section and fixed in paraformaldehyde. The left-hand panels show the phase-contrast image of the same cells analysed for endogenous SARP in the right-hand panels. For the detection of SARP, anti-SARP(240–708) and anti-SARP(708–832) antibodies were used, followed by a FITC-conjugated secondary antibody. Microscopic visualization and image acquisition were performed using an F-View digital monochromatic camera coupled to an Olympus IX-81 motorized inverted microscope. All images are at 60× magnification.

carcinoma epithelial A431 and fibroblast MRC5 cell lines (results not shown). The highest levels of SARP3 were found in mouse brain (Figure 3B). Lower levels of SARP3 in other tissues were often difficult to differentiate from non-specific bands (results not shown). Immuno cross-reactivity of brain SARP3 could be specifically blocked by the antigen SARP(240–708), which did block detection of SARP1/2 (Figure 3B). Immunocytological localization of SARP using two different anti-SARP antibodies showed that endogenous SARP isoforms are highly enriched in the nucleus of COS-7 cells (Figure 4), although SARP also appears to be present at low levels in the cytoplasm. Since the anti-SARP (708–832) antibodies recognize SARP1/2 but not SARP3, the nuclear localization indicates that SARP1/2 are likely to possess the same (or similar) N-terminal sequence as SARP3 containing the nuclear localization motif.

SARP is associated with and modulates the phosphatase activity of PP1

In order to ascertain whether SARP was associated with PP1 activity *in vivo*, anti-SARP(240–333) antibodies coupled to Sepharose beads were mixed with HeLa cell lysates. After centrifugation, the immunoadsorbed proteins on the Sepharose were analysed for phosphatase activity using phosphorylase as a substrate. A small but significant amount of phosphatase activity is found associated with SARP (Figure 5A). The phosphatase activity increased approx. 10-fold either upon mild trypsin digestion of the immune complex or on incubation with a specific PP1-complex 'dissociating' peptide [derived from the PP1-binding domain of 53BP2 (p53 binding protein 2), containing an RVxF motif] prior to the assay, indicating that the $K^{354}VHF^{357}$ motif is a major site of interaction of SARP with PP1. Pre-incubation with a control peptide, where the phenylalanine residue of the PP1-binding domain of the peptide was substituted with an alanine residue, had no stimulatory effect on the phosphatase activity. Dissociation of PP1 from its regulatory subunit by the dissociating peptide and digestion of the regulatory subunit with trypsin have been used previously to determine the effects of glycogen and

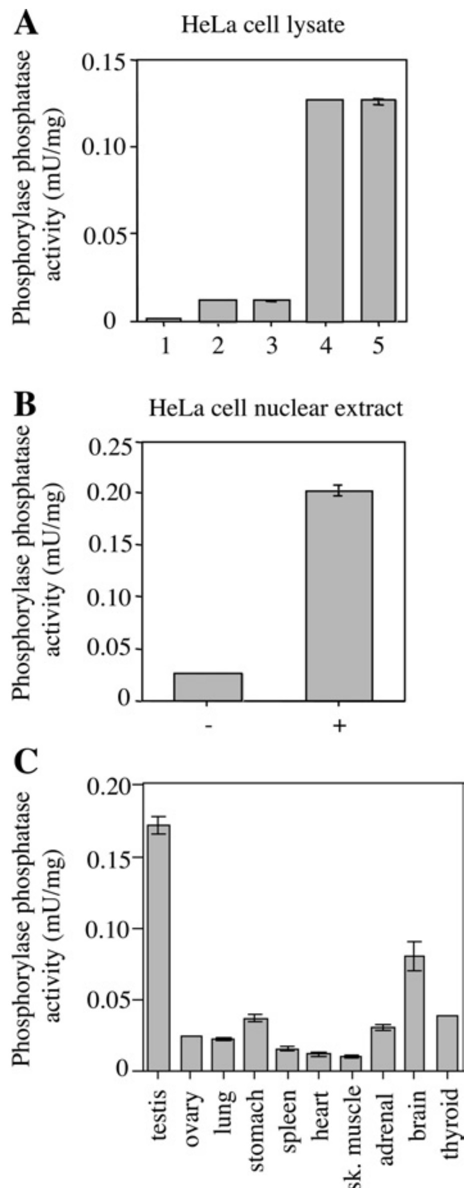

Figure 5 Analysis of SARP–PP1 phosphatase activity

(**A**) Protein phosphatase activity of endogenous SARP–PP1 complexes in HeLa cell lysates. Anti-SARP(240–333) antibody coupled to Protein G–Sepharose beads was mixed with HeLa cell lysates. Immune pellets were assayed for phosphorylase phosphatase activity either in the presence or absence of trypsin or in the presence of PP1-complex dissociating peptide or control peptide. The immunopelleted activity is expressed as m-units per mg of total protein in the HeLa cell lysate. Error bars indicate the S.D. of the mean of triplicate immuno adsorptions. Assays: (1) pre-immune IgG pellet + trypsin treatment, (2) anti-SARP(240–333) antibody pellet, (3) anti-SARP(240–333) antibody pellet + control peptide, (4) anti-SARP(240–333) antibody pellet + dissociating peptide, (5) anti-SARP(240–333) antibody pellet + trypsin treatment. (**B**) Protein phosphatase activity of endogenous SARP–PP1 complexes in HeLa cell nuclei. Anti-SARP(240–333) antibody coupled to Protein G–Sepharose beads was mixed with HeLa cell nuclear lysates. Immune pellets were assayed for phosphorylase phosphatase activity either in the presence (+) or absence (−) of trypsin. (**C**) Endogenous trypsin-revealed SARP–PP1 phosphatase activity in different rat tissues as indicated. Anti-SARP(240–333) antibody immune pellets were prepared from rat tissue lysates and assayed for trypsin-revealed phosphorylase phosphatase activity. The activity is expressed as m-units/mg of lysate protein. Error bars indicate the S.D. of the mean of triplicate immunoprecipitations. The assays were carried out in the presence of 2 nM okadaic acid.

other regulatory subunits on PP1 [25,29]. The results described in the present study indicate that the phosphorylase phosphatase activity in the anti-SARP(240–333) immune complex is due to

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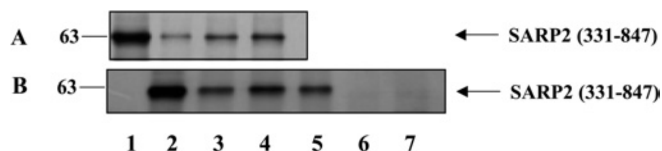

Figure 6 Analysis of SARP DNA binding

SARP DNA-binding assay. (**A**) Lane 1, 1 μl IVT SARP2(331–847); lanes 2–4, DNA-binding assay with 1 mg ssDNA–cellulose with increasing amounts of IVT SARP2(331–847): 1 μl (lane 2), 3 μl (lane 3) or 5 μl (lane 4). (**B**) DNA-binding assay using competitor DNA. Lane 1, IVT negative control; lane 2, 1 μl IVT SARP2(331–847); lanes 3–7, DNA-binding assay with 1 mg ssDNA–cellulose plus 3 μl of SARP2(331–847) in the absence (lane 3) or presence of 10-fold or 100-fold excess of competitor bacterial DNA (lanes 4 and 5) and human placental DNA (lanes 6 and 7).

PP1 and that the PP1 in the immune complex is inhibited by SARP towards the assay substrate phosphorylase *a*. To examine whether the $K^{354}VHF^{357}$ motif was solely responsible for maintaining binding of SARP with PP1 we expressed GST–SARP1 and GST–SARP1(F357A), in which the phenylalanine of the $K^{354}VHF^{357}$ motif was mutated to alanine, in HEK-293 cells. However, mutation of the Phe^{357} was insufficient to disrupt the interaction of SARP1 with PP1. Furthermore, addition of the 'dissociating' peptide, although clearly modulating the activity of endogenous SARP–PP1 complexes (Figure 5A), did not disrupt the binding of PP1 to GST–SARP1 or GST–SARP1(F357A), indicating that there is more than one motif that maintains the SARP1 interaction with PP1 (results not shown).

Immunoprecipitation of SARP complexes was also carried out from a HeLa cell nuclear extract using an anti-SARP(240–333) antibody coupled to Sepharose beads, and the phosphorylase phosphatase activity was measured after mild trypsin digestion (Figure 5B). The trypsin revealed phosphorylase phosphatase activity of this nuclear fraction was found to be $\sim$0.2 m-units/mg of lysate protein, approximately twice that observed in the whole HeLa cell lysate, consistent with enrichment of SARP in the nucleus.

A range of rat tissues was examined for anti-SARP(240–333) immunoadsorbed phosphatase activity that could be revealed by subsequent trypsin treatment (Figure 5C). The highest levels of SARP-associated phosphorylase phosphatase activity was found in rat testis while the brain contained the next highest level. These studies were consistent with the immunoblotting, which indicated that high levels of the SARP protein were present in testis.

SARP is highly abundant in the nucleus and binds to mammalian DNA

The presence of a nuclear localization signal in the SARP sequences, and the observed enrichment of SARP in the nucleus suggest that SARP functions within the nucleus. The identification of a leucine-zipper domain near the C-terminus of SARP1/2 (Figure 1A), a domain found in many transcription factors, raised the question of whether SARP1/2 is a transcription factor or a cofactor. We therefore examined whether SARP1/2 was able to bind to DNA. Figure 6 shows that [^{35}S]-labelled SARP2(331–847) synthesized by *in vitro* transcription/translation binds to mammalian ssDNA immobilized on cellulose in the presence of *E. coli* competitor DNA, but not in the presence of human competitor DNA. These studies indicate that SARP2(331–847) binds specifically to mammalian DNA.

DISCUSSION

PP1 participates in many distinct cellular processes by virtue of interaction with numerous regulatory subunits. In the present paper we describe a novel protein in which a PP1-binding motif precedes an ankyrin-repeat domain. At least three isoforms have been identified, which are derived by alternative splicing from a single gene on chromosome 11 of at least 65 kb and 14 exons. One isoform has six ankyrin repeats, while the other two possess eight ankyrin repeats and, therefore, we have termed the protein SARP (several ankyrin repeat protein). Binding of SARP to PP1 was demonstrated by a number of techniques. Not only was SARP identified in two different yeast two-hybrid screens using PP1γ_1 as bait, but bacterially expressed SARP was found to bind bacterially expressed PP1γ_1 and PP1γ_2 by immunoadsorption and blot overlay experiments respectively, and endogenous PP1γ_2 was found in complex with SARP in human sperm. In addition, endogenous SARP was also found associated with PP1α and PP1β in immune complexes. A putative PP1-binding motif, $K^{354}VHF^{357}$, was identified in SARP by sequence comparison with the consensus PP1-interaction motif $(R/K)X_{A(0-1)}(V/I)X_B$ (F/W). The interaction of SARP with PP1 via this canonical motif was supported by modulation of SARP–PP1 activity with a peptide covering this region, the PP1 activity increase being virtually identical with that obtained by trypsin digestion. However, neither the mutation of the SARP Phe^{357} to alanine within the $K^{354}VHF^{357}$motif, nor the addition of a peptide covering this region, resulted in the complete disruption of the binding of SARP to PP1, indicating the existence of one or more further SARP interaction sites with PP1. Other PP1-regulator complexes with more than one interaction site have been reported previously [1,15].

Although most regulatory subunits of PP1 share little or no obvious sequence similarities unless they are isoforms, the coupling of a canonical PP1-binding motif and an ankyrin-repeat domain in the manner found in SARP is observed in three other known PP1-associated proteins: 53BP2 [32], TIMAP (transforming growth factor β inhibited, membrane-associated protein), which also binds to a laminin receptor [33,34], both with four ankyrin repeats, and MYPT1/M_{110}/MBS (myosin-binding protein of PP1) with seven ankyrin repeats [35,36] (Figure 7A). The PP1-binding motif immediately precedes the start of the first ankyrin repeat in 53BP2, TIMAP and MYPT1, but in SARP the $K^{354}VHF^{357}$ PP1-binding motif lies partially within the first ankyrin repeat. Thus the precise location of the PP1 interaction with respect to the ankyrin repeat appears to be flexible, suggesting that the ankyrin-repeat domain folds favourably to present an exposed RVXF motif that can initiate the binding to PP1, whether it lies immediately preceding or at the start of the first ankyrin repeat. Secondary interactions similar to those observed in PP1–MYPT1 may stabilize the complex [37–39]. The crystal structure of PP1 bound to the N-terminal domain of MYPT1 shows that ankyrin-repeat domains possess many secondary interactions with PP1 [40]. Since the ankyrin repeats are degenerate sequences and there is considerable variation between those in SARP and MYPT1, and TIMAP and 53BP2, there exists a real potential for differential binding and/or modulation of secondary interactions. In addition, localization of the canonical PP1-binding motif partially within the first ankyrin repeat of SARP may aid stabilization of the SARP–PP1 complex and perhaps allow an ankyrin-repeat interaction with PP1 that is stable to disruption of the canonical PP1-binding site.

SARP is widely distributed in different tissues and PP1 activity was observed in endogenous SARP immunocomplexes from a variety of tissues. When phosphorylase was used as a substrate, SARP inhibited the bound PP1 catalytic activity, but one might expect PP1 to be less inhibited or even activated towards an *in vivo*

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A

SARP	348	NPCSRK**K**		354
53BP2	795	HGM**RVKF**		801
MYPT1	30	QKT**KVKF**		38
TIMAP	60	RRK**KVSF**		66
SARP	355	**VHF**GSIHDAVRAGDVKQLSEIVVRGASINE	LDVL	388
53BP2	802	NPLALLLDSSLEGEFDLVQRIIYEVDDPSL	PND	834
MYPT1	39	DDGAVFLAACSSGDTDEVLKLLHRGADINY	ANV	71
TIMAP	67	EASVALLEASLRNDAEEVRYFLKNKVSPDL	CNE	99
SARP	389	HKFTPLHWAAHSGSLECLHWLLWHGADITH	VTT	421
53BP2	835	EGITALHNAVCAGHTEIVKFLVQFGVNVNA	ADS	867
MYPT1	72	DGLTALHQACIDDNVDMVKFLVENGANINQ	PDN	104
TIMAP	100	DGLTALHQCCIDNFEEIVKLLLSHGANVNA	KDN	132
SARP	422	RDWTASHIAAIRGQDACVQALIMNGANLTA	QDD	454
53BP2	868	DGWTPLHCAASCNNVQVCKFLVESGAAVFA	MTY	900
MYPT1	105	EGWIPLHAAASCGYLDIAEFLIGQGAHVGA	VNS	137
TIMAP	133	ELWTPLHAAATCGHINLVKILVQYGADLLA	VNS	165
SARP	455	RGCTPLHLAATHGHSFTLQIMLR-SGVDPS		483
53BP2	901	SDMQTAADKCEEMEEGYTQCSQFLYGVQEK		930
MYPT1	138	EGDTPLDIAEEEAMEELLQNEVNRQGVDIE		167

B

SARP1/2	780	LEYERLRREK**L**ECQLDE**Y**RAEVDQ**L**RETLEK**I**QVPNFV**A**	818
KE2 (PFD)	66	KQELGEARAT**V**GKRLDY**I**TAEIKR**Y**ESQLRD**L**ERQSEQQ	104
bZIP Maf (NRL)	177	RSKRLQQRRG**L**EAERAR**L**AAQLDA**L**RAEVAR**L**ARERDL**Y**	215
bZIP1 (JUN-B)	286	RKRKLERIAR**L**EDKVKT**L**KAENAG**L**SSTAGL**L**REQVAQ**L**	324
bZIP2 (HLF)	244	RDARRLKENQ**I**AIRASF**L**EKENSA**L**RQEVAD**L**RKLGKCK	282
MYC	403	ILSVQAEEQK**L**ISEEDL**L**RKRREQ**L**KHKLEQ**L**RNSCA	439

Figure 7 Sequence comparison of the ankyrin repeat and leucine-zipper motifs of SARP to other family members

(**A**) Alignment of the first four ankyrin repeats of human SARP with those present in other human PP1-binding proteins (53BP2, MYPT1 and TIMAP). Only the three contiguous ankyrin repeats of TIMAP are shown. Note that the PP1-binding motif of SARP (indicated in bold) lies partially within the first ankyrin repeat, while the PP1-binding motif of 53BP2, MYPT1 and TIMAP (indicated in bold) precede the start of the first ankyrin repeat. Isoforms of these proteins have been identified: ASPP2 is an isoform of 53BP2 [50], MYPT2 is an isoform of MYPT1, whereas MYPT3 is probably an isoform of TIMAP [33]. (**B**) Alignment of the SARP C-terminal putative leucine-zipper domain with the leucine-zipper domains of representative human members of different families: PFD (prefoldin-6) in the KE2 leucine-zipper family, NRL (neural retina leucine zipper) in the bZIP Maf transcription factor family, JUN-B in the bZIP1, and HLF (hepatic leukemia factor) in the bZIP2 transcription factor families, and the MYC transcription factor.

substrate of the PP1–SARP complex, as observed with PP1–Nek2 (NIMA-related kinase 2) [25]. Overall, the highest levels of SARP mRNA, protein and PP1 activity bound to SARP were observed in testis. Previous studies have shown that mice homozygous for a targeted mutation of the PP1γ gene, which disrupts expression of both PP1γ_1 and PP1γ_2 isoforms, exhibit a defect in spermiogenesis but are otherwise healthy, suggesting that the function of PP1γ in testis cannot be substituted by other PP1 isoforms, but the functions of PP1γ_1 present in all other tissues can be compensated for by PP1α [41]. Therefore one would not necessarily expect the function(s) of SARP, which interacts with PP1γ_1, PP1γ_2, PP1α and PP1β, to be compromised in these PP1γ null mice, unless the interaction of SARP1/2 with PP1γ2 serves a unique function in testis.

Endogenous SARP appears to be highly enriched in the nucleus, consistent with the putative nuclear localization signal at the N-terminus. A variety of PP1-binding proteins with predominantly nuclear localization and/or nuclear functions have been identified [1,17,42,43]. The demonstration in the present study that SARP2 specifically binds to mammalian DNA suggests a role for SARP2, and therefore probably also SARP1, in the regulation of transcription. Several studies have indicated that PP1 may interact with proteins that modulate transcription. PP1 interacts with the homeodomain transcription factor Hox11 [44], host cell factor HCF [45], the retinoblastoma protein p110Rb, which regulates the transcription factor EF2 [46,47] and 53BP2, which associates with the p53 transcription factor [32,48].

SARP1 and SARP2 possess a putative leucine-zipper motif near the C-terminus suggesting that they may function as transcription factors or cofactors. An alignment of the SARP1/2 C-terminal region with the leucine-zipper regions of representative members of transcription factor families is shown in Figure 7(B). However, leucine-zipper domains may also serve as protein–protein interaction domains, and the SARP1/2 C-terminus is also related to the KE2 family of leucine-zipper proteins, which encompass proteins involved in tubulin folding [49]. Nevertheless, the specific binding of SARP2 to mammalian DNA supports a role for SARP1/2 as transcription factors or cofactors and suggests that PP1 may participate in the regulation of transcription in a wide variety of tissues via SARP1/2. The shorter isoform SARP3, which may be particularly important in brain where it is most abundant, terminates before the leucine-zipper motif and therefore may perform a different function from SARP1 and 2.

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