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The novel human protein serine/threonine phosphatase 6 is a functional homologue of budding yeast Sit4p and fission yeast ppe1, which are involved in cell cycle regulation

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SUMMARY

We identified a novel human protein serine/threonine phosphatase cDNA, designated protein phosphatase 6 (PP6) by using a homology-based polymerase chain reaction. The predicted amino acid sequence indicates a 35 kDa protein showing high homology to other protein phosphatases including human PP2A (57%), human PP4 (59%), rat PPV (98%), *Drosophila* PPV (74%), *Schizosaccharomyces pombe* ppe1 (68%) and *Saccharomyces cerevisiae* Sit4p (61%). In human cells, three forms of PP6 mRNA were found with highest levels of expression in testis, heart and skeletal muscle. The PP6 protein was detected in lysates of human heart muscle and in bull testis. Complementation studies using a temperature sensitive mutant strain of *S. cerevisiae SIT4*, which is required for the G_1 to S transition of the cell cycle, showed that PP6 can rescue the mutant growth arrest. In addition, a loss of function mutant of *S. pombe ppe1*, described as a gene interacting with the pim1/spi1 mitotic checkpoint and involved in cell shape control, can be complemented by expression of human PP6. These data indicate that human PP6 is a functional homologue of budding yeast Sit4p and fission yeast ppe1, implying a function of PP6 in cell cycle regulation.

Key words: dis3, PPV, Ran GTPase, SSD1

INTRODUCTION

Reversible phosphorylation of proteins on serine and threonine residues is an important biochemical event that regulates a broad variety of intracellular processes. The phosphorylation state is determined by the well controlled balance of activities of serine/threonine-specific protein kinases and protein phosphatases. This latter class of enzymes is subdivided into four broad categories: type 1 (PP1), type 2A (PP2A), type 2B (PP2B) and and type 2C (PP2C), according to their differential action on in vitro substrates, their sensitivity to specific inhibitors like okadaic acid and microcystin LR, and their different requirements for metal ions (for reviews see Cohen, 1989; Shenolikar, 1994). In addition to isoforms of PP1, PP2A and PP2B, cDNA cloning has revealed a number of novel catalytic subunits of protein phosphatases. Among them are PP4 (also termed PPX; Brewis and Cohen, 1992) and PP5 (Chen et al., 1994) from man, PPV from *Drosophila* (Mann et al., 1993) and its structural homologue from rat (Becker et al., 1994), ppe1 from *S. pombe* (Matsumoto and Beach, 1993; Shimanuki et al., 1993) and *S. cerevisiae* Sit4p (Arndt et al., 1989).

Protein serine/threonine phosphatases play a crucial role in cell cycle progression. Studies employing yeasts, *Drosophila*, starfish and *Xenopus* demonstrated that PP2A represents a positive as well as a negative regulator for entry into mitosis (Ohkura et al., 1989; Picard et al., 1989; Kinoshita et al., 1990; Clarke et al., 1993; Mayer-Jaeckel et al., 1993; Lee et al., 1994; Lin and Arndt, 1995) by activating or inhibiting the $p34^{cdc2}$ activation. Furthermore, PP1 is essential for entry into as well as for exit from M phase in fungi, *Drosophila* and mammalian cells (Doonan and Morris, 1989; Ohkura et al., 1989; Axton et al., 1990; Fernandez et al., 1992; Hisamoto et al., 1994).

Novel protein serine/threonine phosphatases have also been implicated in cell cycle regulation. The budding yeast protein phosphatase Sit4p was shown to be essential for progression into S phase by its requirement for the normal accumulation of *CLN1*, *CLN2* and *HCS26* RNAs during late G_1 (Fernandez-Sarabia et al., 1992). A mutant allele of *SIT4*, *sit4*-102, in a certain genetic background, results in growth arrest in late G_1 with unbudded cells displaying a 1n DNA content (Sutton et al., 1991). Recent studies have shown that Sit4p is the catalytic subunit of a ceramide-activated protein phosphatase in budding yeast indicating a function of Sit4p in the ceramide-induced transduction pathway (Nickels and Broach, 1996).

In fission yeast, the $ppe1^+$ gene encodes a protein phosphatase with homology to mammalian PP2A and high homology to budding yeast Sit4p (72% identity). It was isolated as an extragenic suppressor of two different mutant alleles of *pim1* (Matsumoto and Beach, 1993; Sazer and Nurse, 1994), the fission yeast homologue of mammalian RCC1 (Ohtsubo et al., 1987). RCC1 acts as a nucleotide exchange

factor for the small nuclear GTPase Ran (Bischoff and Ponstingl, 1991). The Ran GTPase cycle has been shown to be involved in nucleo-cytoplasmic transport (Melchior et al., 1993; Moore and Blobel, 1993) and cell cycle regulation (reviewed by Dasso, 1993). Independently, the *ppe1*$^+$ gene was isolated by hybridization, and its involvement in cell shape control and mitotic division was demonstrated (Shimanuki et al., 1993). Cells carrying a mutant allele or a deletion of the *ppe1*$^+$ gene, show strong abnormalities in cell shape and cold-sensitive growth with cells accumulating in G_2 at the restrictive temperature. A cDNA encoding a protein phosphatase from *Drosophila*, termed PPV, is highly homologous to Sit4p (63% identity) and ppe1 (68% identity). PPV can complement a *sit4*-102 mutation in *S. cerevisiae* (Mann et al., 1993), but its actual function in *Drosophila* is still unknown.

In human cells, to date two novel protein serine/threonine phosphatase genes are cloned. Protein phosphatase 4 represents a member of the PP1/PP2A/PP2B family and localizes to centrosomes during mitosis, suggesting a possible role in microtubule nucleation (Brewis and Cohen, 1992; Brewis et al., 1993). This phosphatase is highly homologous to budding yeast Sit4p (60% amino acid sequence identity) and fission yeast ppe1 (61% identity), and therefore PP4 could represent their functional human homologue. Recently, the cDNA sequence of human PP5 was reported (Chen et al., 1994). It encodes a protein containing four tetratricopeptide repeats (TPR) in its N-terminal domain, a motif previously found in proteins involved in cell cycle regulation, mRNA splicing, transcriptional regulation and protein transport. Such a sequence motif is unique for protein phosphatases, and thus PP5 represents a distinct group within the PP1/PP2A/PP2B family.

Here, we report the identification of a cDNA encoding a novel human protein serine/threonine phosphatase, termed protein phosphatase 6 (PP6), which is highly homologous to budding yeast Sit4p, fission yeast ppe1, *Drosophila* and rat PPV. We show that PP6 is a functional homologue of *S. cerevisiae* Sit4p as well of *S. pombe* ppe1, suggesting a function in cell cycle progression.

MATERIALS AND METHODS

Reverse transcription-PCR

Total RNA was prepared from exponentially growing HeLa S3 cells by using the RNA-Clean™ Kit (AGS, Heidelberg, Germany). A 1 μg sample of total RNA was reverse transcribed into single stranded cDNA (SUPERSCRIPT™ reverse transcriptase, Gibco BRL, Eggenstein, Germany), which was used as a template for polymerase chain reaction (PCR). Amplifications were performed (1 minute 94°C, 0.5 minute 60°C, 0.5 minute 72°C) for 30 cycles using Taq polymerase (AGS) and the following set of degenerate oligonucleotides (see also Fig. 1; *Cla*I site underlined, *Pst*I site double underlined): (1) [5′-CCATCGATG(C/T)GA(A/G)ATGGTIAA(A/G)G-3′], corresponding to amino acid residues -CEMVK/C-, and (2) [5′-AACTGCAGIAC(C/T)T(G/T)(A/G)CA(A/G)CA(A/G)TA (C/T)TT-3′], corresponding to amino acid residues -KYCCQ/KV-; or (A) [5′-AACTGCACCCIGTIACIGTITG(C/T)GGIGA-3′], corresponding to amino acid residues -PVTVCGD-, and (B) [5′-CCATCGATC-CIC(G/T)(A/G)TCIAC(A/G)(A/T)A(A/G)TCICC-3′], corresponding to amino acid residues -GDF/YVDRG-. The amplification using the primer pair 1/2 yielded multiple cDNA fragments which served as templates for a nested PCR amplification using the primer pair A/B. This second amplification yielded a single 120 bp band, which was excised and eluted from agarose gels and subsequently subcloned into the *Pst*I-*Cla*I sites of pBluescript®II-KS$^+$ (Stratagene, La Jolla, CA). Sequencing was performed using the T7-Sequencing™ mixes (Pharmacia, Uppsala, Sweden). In this way partial sequences of human PP5 and PP6 were obtained.

Isolation of human cDNA clones

Using the 120 bp cDNA fragment encoding a partial sequence of human PP6 as a probe, a HeLa S3 λgt11 cDNA library (Clontech, Palo Alto, CA) was screened on duplicate nylon filters (Hybond™-N$^+$; Amersham Buchler, Braunschweig, Germany). The library filters were pre-hybridized in 0.5 M sodium phosphate, pH 7.2, 7% SDS, 1 mM EDTA and 0.1 mg/ml salmon sperm DNA (Church and Gilbert, 1984) at 60°C for 4-6 hours. Hybridization was performed for 14 hours at 60°C in the same buffer containing the labelled probe at a concentration of 1.5-3×10^6 cpm/ml. The probe was labelled with [α-^{32}P]dCTP using the DECAprimeII™ DNA labelling kit (Ambion, Austin, TX). The filters were washed twice for 10 minutes at 60°C in 40 mM sodium phosphate, pH 7.2, 0.1% SDS. Cross-hybridizing bacteriophage clones were isolated and DNA was prepared as described by Sambrook et al. (1989). Twelve PP6 cross-hybridizing cDNA inserts were isolated, ranging from 540 bp to 1,225 bp in size, subcloned into the *Eco*RI site of pBluescript®II-KS$^+$ (Stratagene) and their sequences were determined. None of the isolated cDNAs extended beyond the 'T' of the translation initiation 'ATG' codon of PP6 cDNA.

Rapid amplification of 5′-cDNA ends (5′-RACE)

In order to obtain the 5′-end of the PP6 cDNA, rapid amplification of 5′-cDNA ends (5′-RACE) was performed. HeLa poly(A)$^+$RNA was prepared by affinity chromatography on oligo(dT) matrix (Oligotex-dT™; DIAGEN, Hilden, Germany) from isolated total RNA. The RACE procedure was performed according to the manufacturer's instructions using the Marathon™ cDNA Amplification Kit (Clontech) and an internal 3′-PP6 primer (5′-CCTGCTC-CTCGGGGACTGATAGCCCAGG-3′), corresponding to nucleotides 682-709. A single band of 700 bp was obtained, which was cloned into the pCR™II vector (Invitrogen®, Leek, Netherlands) and sequenced.

Northern blot analysis

Samples (2 μg and 4 μg) of poly(A)$^+$ RNA prepared from HeLa cells were separated on a denaturating agarose gel and transferred onto nylon membranes (Hybond™-N$^+$, Amersham Buchler). Northern blots displaying 2 μg of poly(A)$^+$ RNA from 16 different human tissues were purchased from Clontech. Hybridization was performed at 65°C in the same solution as described for bacteriophage screening. A 1.27 kb *Eco*RI phage insert of PP6, corresponding to nucleotides 2-1,342, was used as hybridization probe, labelled as described above and used in a final concentration of 3-5×10^6 cpm/ml. To confirm an equal loading of mRNA in each lane the blots were stripped in 5 mM sodium phosphate, pH 7.2, 0.1% SDS for 20 minutes. at 90°C and hybridized with a labelled 2 kb human β-actin cDNA probe as a control.

Yeast strains and methodology

The *S. cerevisiae sit4* mutant strain used was CY249 [*MAT*α *ade2-1 his3-11,15 leu2-3,112 can1-100 ura3-1 trp1-1 ssd1-d2 sit4::HIS3 (sit4-102* on Ycp50)] (Sutton et al., 1991; kindly provided by K. Arndt, Cold Spring Harbor, NY). This strain was grown in YPAD medium (Rose et al., 1990). The entire open reading frame of *SIT4* (wt) was PCR-amplified from genomic DNA isolated from the *S. cerevisiae* strain Y190 [*MAT*a, *leu2-3.-112, ura3-52, trp1-901, his3-Δ200, ade2-101, gal4 Δgal80Δ URA3 GAL-lacZ, LYS GAL-HIS3, cyh*^{r}] (Harper et al., 1993; kindly provided by S. Elledge, Houston, TX). Template DNA was prepared according to the method of Hoffman and Winston (1987), using a sense-primer (5′GCG-CAAGCTTATGGTATCTAGAGGCCCCG-3′) and an antisense-

primer (5′-GCGCAAGCTTATAAGAAATAGCCGGCTC-3′, *Hin*dIII sites underlined). The complete open reading frame of *ppe1*$^+$ was PCR-amplified from chromosomal DNA prepared from wild-type *S. pombe* (strain 972 h$^-$) following standard procedures (Alfa et al., 1993). As 5′ primer: 5′-CCCAAGCTTATGTTTGACTTGGATGAA-3′ and as 3′ primer: 5′-CCAAGCTTTTAGATGAAATACTCGCT-3′ (*Hin*dIII sites underlined) were used. These fragments together with the coding sequences of human PP4 and PP6 were cloned into the *Hin*dIII site of pADH (a gift from K. Arndt, Cold Spring Harbor, NY), where expression is driven by the *ADH1* promoter. Transformation of *S. cerevisiae* was performed as previously described (Schiestl and Gietz, 1989). Growth of transformants was monitored at 28°C and 37°C in drop-out medium lacking leucine (SC-leu) or tryptophan (SC-trp), respectively (Rose et al., 1990).

The *S. pombe* haploid strain used was HM123 (*h*$^-$ *leu1*), *ppe1*-374 (kindly provided by X. He and S. Sazer, Houston, TX). This strain was grown in yeast extract medium (YE) supplemented with adenine, uracil and leucine (Alfa et al., 1993). The coding sequences of *S. pombe ppe1*$^+$, *S. cerevisiae SIT4* and human PP4 and PP6 were PCR-amplified as described above using primers that included a *Xho*I site at the 5′ end and a *Bam*HI site at the 3′ end. The fragments obtained were cloned into the *Xho*I/*Bam*HI sites of pREP3X (Forsburg, 1993; a gift from S. Sazer, Houston, TX), where expression is driven by the thiamine repressible *nmt* promoter. *S. pombe* was transformed using the lithium acetate method (Okazaki et al., 1990) and transformants were grown on synthetic EMM (Moreno et al., 1991) at 20°C, 28°C and 36°C.

Preparation of tissue lysates

Tissue lysates from human heart muscle were purchased from Clontech. Total lysates from bull testes were prepared by homogenizing decapsulated testes in buffer A (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 2 μg/ml aprotinin) using a Teflon glass homogenizer (Kwon and Hecht, 1993). Following centrifugation at 5,000 ***g***, the supernatant was mixed with 0.11 volume of buffer B (100 mM Hepes, pH 7.9, 30 mM $MgCl_2$, 250 mM KCl) and re-centrifuged for 1.5 hours at 100,000 ***g***. The high speed supernatant was used for immunoblotting.

Immunological methods and transfection

Antibodies were raised in rabbits against the purified 6×His-tagged PP6 protein. The entire coding sequence of PP6 was cloned into the *Nde*I and *Bam*HI sites of the bacterial expression vector pET14b (Invitrogen®). An expected 36 kDa protein was expressed as an insoluble His-tagged fusion protein in *E. coli* BL21 after induction with 1 mM IPTG. The recombinant protein was purified on Ni-NTA columns (Diagen) under denaturing conditions following the manufacturer's instructions. Immunization of rabbits and serum preparation was perfomed according to standard procedures (Eurogentec, Seraing, Belgium).

For affinity purification the antisera were incubated for 14 hours with PP6 protein blotted onto nitrocellulose. The PP6-specific antibodies were eluted from the immobilized protein with 100 mM glycine (pH 2.5) followed by a titration with 1 M Tris to neutralize the eluate.

For immunoblotting 30 ng of recombinant PP6 protein solubilized in SDS-sample buffer, 50 μg of total protein of a lysate derived from human heart muscle tissue (Clontech) and 50 μg of total protein of a lysate prepared from bull testis were separated by SDS gel electrophoresis and transferred onto nitrocellulose membranes. The PP6 protein in tissue lysates was detected using affinity-purified rabbit anti-PP6 antibodies. Horseradish peroxidase-conjugated goat anti-rabbit IgG (Dianova) was used as secondary antibody and the enhanced chemoluminiscence system (ECL™; Amersham) was utilized for visualization.

To overexpress the PP6 protein, HeLa cells were transfected with a cDNA encoding full-length PP6 fused to an N-terminal or C-terminal HA-tag cloned into pcDNA3 (Invitrogen, Leek, Netherland). Then 1.6×10^6 exponentially growing HeLa cells were resuspended in MEM followed by the addition of 10 μg pcDNA3-PP6-HA and electroporation in a Bio-Rad electroporator at 240 V and 960 μF.

RESULTS

Identification of cDNA fragments encoding novel protein serine/threonine phosphatases

We used a homology-based RT-PCR approach to identify novel human protein serine/threonine phosphatases, which could contribute to cell cycle regulation. By multiple sequence alignment (Fig. 1) we identified highly conserved stretches of amino acid sequences within a subgroup of protein serine/threonine phosphatases including *Drosophila* PPV, *S. pombe* ppe1 and *S. cerevisiae* Sit4p, which are implicated in

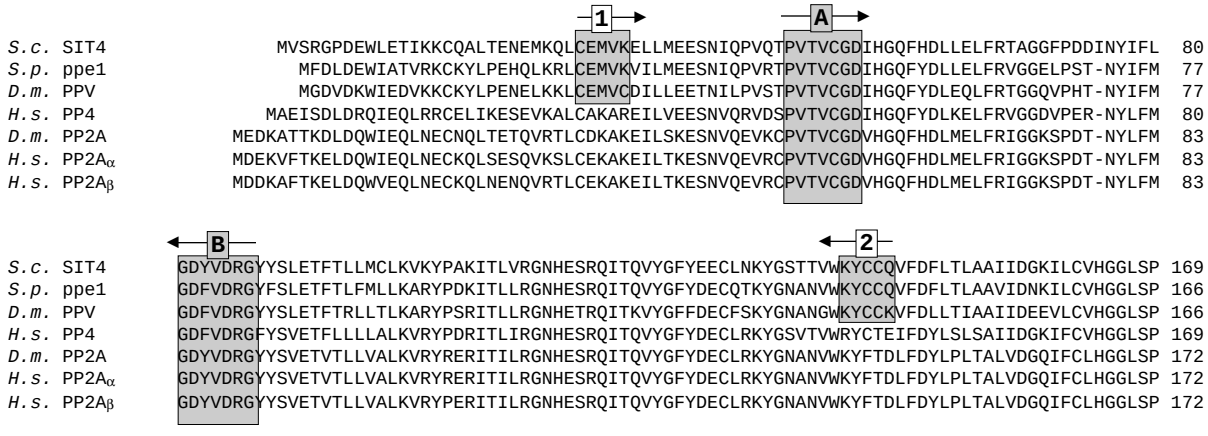

Fig. 1. Design of degenerate oligonucleotides used for homology-based PCR. Amino acid sequences of the amino-terminal region of *S. cerevisiae* Sit4p (Arndt et al., 1989), *S. pombe* ppe1 (Matsumoto and Beach, 1993; Shimanuki et al., 1993), *Drosophila* PPV (Mann et al., 1993), human PP4 (PPX; Brewis and Cohen, 1992), *Drosophila* PP2A (Orgad et al., 1990) and the two isoforms (α and β) of human PP2A (Arino et al., 1988; Stone et al., 1988) were aligned. Highly degenerate oligonucleotides were designed corresponding to the boxed homology regions. Primers 1 and 2 reflect sequences conserved in the Sit4p/ppe1/PPV subgroup, while primer A corresponds to a sequence motif conserved in all PP2A-like protein phosphatases and primer B is based on a amino acid sequence identified in all protein phosphatases of the PP1/PP2A/PP2B family (Barton et al., 1994).

cell cycle regulation. We designed highly degenerate oligonucleotides corresponding to these sequences (Fig. 1, primers 1 and 2), which are not present in PP4 and type 2A protein phosphatases. Single stranded cDNA was synthesized by reverse transcription from total RNA isolated from HeLa cells. PCR amplifications using this cDNA as a template and the primer pair 1/2 yielded multiple bands. To increase the specificity, we designed a second set of degenerate primers corresponding to the highly conserved sequence motifs present in all PP2A and PP2A-like protein phosphatases (Fig. 1, primers A and B). A nested PCR amplification using the products of the first amplification as a template and the primers A and B was performed and electrophoretic analysis of the resulting PCR products showed the expected single 120 bp band. This band was excised, eluted from agarose gels and cloned into pBluescript®II-KS$^+$. Sequence analysis of about hundred clones revealed two cDNA fragments encoding novel protein serine/threonine phosphatases not present in the data base at this time. One fragment showed significant homology to *Drosophila* and rat PPV and was termed human PP6.

While this work was in progress it was shown that the second cDNA fragment obtained was derived from the recently identified human PP5 (Chen et al., 1994). The presence of several partial clones of PP2A isoforms as well as of PP4 and PP5 during the sequencing procedure indicated that residual single stranded cDNA from HeLa was present in the nested PCR reaction, since primers 1 and 2 did not anneal to PP2A, PP4 and PP5 sequences.

cDNA sequence of human PP6

Screening of 1×10^6 plaque forming units of a HeLa S3 λgt11 cDNA library at high stringency identified twelve clones

A

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   1 ccatcctaatacgactcactatagggctcgagcggccgcccgggcaggtgccgcggcttgttcttcttaaa ATG GCG  77
   1                                                                        M   A    2

  78 CCG CTA GAC CTG GAC AAG TAT GTG GAA ATA GCG CGG CTG TGC AAG TAC CTG CCA GAG AAC  137
   3 P   L   D   L   D   K   Y   V   E   I   A   R   L   C   K   Y   L   P   E   N    22

 138 GAC CTG AAG CGG CTA TGT GAC TAC GTT TGT GAC CTC CTC TTA GAA GAG TCA AAT GTT CAG  197
  23 D   L   K   R   L   C   D   Y   V   C   D   L   L   L   E   E   S   N   V   Q    42

 198 CCA GTA TCA ACA CCA GTA ACA GTG TGT GGA GAT ATC CAT GGA CAG TTT TAT GAC CTT TGT  257
  43 P   V   S   T   P   V   T   V   C   G   D   I   H   G   Q   F   Y   D   L   C    62

 258 GAA CTG TTC AGA ACT GGA GGT CAG GTT CCT GAC ACA AAC TAC ATA TTT ATG GGT GAT TTT  317
  63 E   L   F   R   T   G   G   Q   V   P   D   T   N   Y   I   F   M   G   D   F    82

 318 GTA GAC AGA GGT TAC TAT AGT TTG GAG ACC TTC ACT TAC CTT CTT GCA TTA AAG GCT AAA  377
  83 V   D   R   G   Y   Y   S   L   E   T   F   T   Y   L   L   A   L   K   A   K   102

 378 TGG CCT GAT CGT ATT ACA CTT TTG CGA GGA AAT CAT GAG AGT AGA CAG ATA ACA CAG GTC  437
 103 W   P   D   R   I   T   L   L   R   G   N   H   E   S   R   Q   I   T   Q   V   122

 438 TAT GGA TTT TAT GAT GAG TGC CAA ACC AAA TAT GGA AAT GCT AAT GCC TGG AGA TAC TGT  497
 123 Y   G   F   Y   D   E   C   Q   T   K   Y   G   N   A   N   A   W   R   Y   C   142

 498 ACC AAA GTT TTT GAC ATG CTC ACA GTA GCA GCT TTA ATA GAT GAG CAG ATT TTG TGT GTC  557
 143 T   K   V   F   D   M   L   T   V   A   A   L   I   D   E   Q   I   L   C   V   162

 558 CAT GGT GGT TTA TCT CCT GAT ATC AAA ACA CTG GAT CAA ATT CGA ACC ATC GAA CGG AAT  617
 163 H   G   G   L   S   P   D   I   K   T   L   D   Q   I   R   T   I   E   R   N   182

 618 CAG GAA ATT CCT CAT AAA GGA GCA TTT TGT GAT CTG GTT TGG TCA GAT CCT GAA GAT GTG  677
 183 Q   E   I   P   H   K   G   A   F   C   D   L   V   W   S   D   P   E   D   V   202

 678 GAT ACC TGG GCT ATC AGT CCC CGA GGA GCA GGT TGG CTT TTT GGA GCA AAG GTC ACA AAT  737
 203 D   T   W   A   I   S   P   R   G   A   G   W   L   F   G   A   K   V   T   N   222

 738 GAG TTT GTT CAT ATC AAC AAC TTA AAA CTC ATC TGC AGA GCA CAT CAA CTA GTG CAC GAA  797
 223 E   F   V   H   I   N   N   L   K   L   I   C   R   A   H   Q   L   V   H   E   242

 798 GGC TAT AAA TTT ATG TTT GAT GAG AAG CTG GTG ACA GTA TGG TCT GCT CCT AAT TAC TGC  857
 243 G   Y   K   F   M   F   D   E   K   L   V   T   V   W   S   A   P   N   Y   C   262

 858 TAT CGT TGT GGA AAT ATT GCT TCG ATC ATG GTC TTC AAA GAT GTA AAT ACA AGA GAA CCA  917
 263 Y   R   C   G   N   I   A   S   I   M   V   F   K   D   V   N   T   R   E   P   282

 918 AAG TTA TTC CGG GCA GTT CCA GAT TCA GAA CGT GTT ATT CCT CCC AGA ACG ACA ACG CCA  977
 283 K   L   F   R   A   V   P   D   S   E   R   V   I   P   P   R   T   T   T   P   302

 978 TAT TTC CTT TGA ggccttcgcccatcctgctgacccatttttctgccctcttcttaccccaatttttcttgtattac 1053
 303 Y   F   L   *                                                                    305

1054 cctctacaatatacttttttattgagcactttgctgctgaaatgctgcctcttgccttttttttttttttaaatttttaaat 1133

1134 tatctaaatttattgtttgttgtggtgtctatagcaaagtttttctatcaattttccccccatcccatccccaccctggac 1213

1214 tcatttgagaagacttgagaaatgtcttaatactcacactgctgcatgtagctcttgcttatttactggtctgggaaaca 1293

1294 ggatgtgtttccttttttttaaaagccaattgacagattacacctaaatc                              1342
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hybridizing with the 120 bp probe of human PP6. The sequences of two overlapping clones were completely determined (nucleotides 73-1,065 and 117-1,342, Fig. 2A). The deduced amino acid sequence showed high homology to *Drosophila* PPV, but the cDNA of PP6 appeared to be truncated at the 5′-end of the coding region, starting with the 'T' of the initiation 'ATG'. To verify the translation initiation 'ATG' in the PP6 cDNA and to obtain sequence information on the 5′-UTR we performed 5′-RACE using an internal antisense primer (see Materials and Methods), which resulted in a sequence extension of 72 nucleotides at the 5′-end. The first methionine is surrounded by an adenine at position –3 and a guanine at position +4 (Kozak, 1991) and was further verified as the translational start methionine by sequence comparison to *Drosophila* and rat PPV. The 3′ noncoding region is 352 nucleotides in length, but a consensus polyadenylation signal is lacking. The nucleotide and deduced amino acid sequence of human PP6 is shown in Fig. 2A. The PP6 cDNA contained a single open reading frame of 915 nucleotides encoding a protein of 305 amino acid residues with a calculated molecular mass of 35.1 kDa. The amino acid sequence revealed highest homology to a particular subgroup of protein serine/threonine phosphatases including *S. cerevisiae* Sit4p (61% sequence identity), *S. pombe* ppe1 (68%; Fig. 2B), *Drosophila* PPV (74%) and its homologue from rat (98%).

In addition to the identification of PP6, we used the 120 bp fragment encoding the corresponding sequence of human PP5 to screen a HeLa λgt11 cDNA library as the cDNA sequence reported for human PP5 appears to be incomplete (Chen et al., 1994). We were able to isolate the complete coding sequence of human PP5, which we deposited in the EMBL Nucleotide Sequence Database (H. Bastians and H. Ponstingl, accession number X84415).

Expression of PP6 mRNA in human tissues

To analyze the distribution of mRNA of human PP6 in different human tissues and in HeLa cells, northern blot analyses were carried out. Using a 1.2 kb cDNA fragment of the PP6 cDNA as a probe we found three different transcripts of 1.8 kb, 4.2 kb and 4.7 kb in all 16 human tissues examined (Fig. 3). Expression levels were highest in testis, heart and skeletal muscle, whereas very low levels were present in placenta, lung and kidney. The highest expression of the two larger transcripts was found in human skeletal muscle. Using mRNA prepared from HeLa cells we detected lower levels of the 1.8 kb transcript compared to human testis, heart and skeletal muscle and only very low levels of the 4.2 kb and 4.7 kb forms. Similiarly, the two larger transcripts are almost absent in human colon, peripheral blood leukocytes and liver. This resembles the situation in mouse tissues, where three different transcripts of 2.2 kb, 4.4 kb and 4.9 kb have been identified which seem to be derived from the same gene by alternative polyadenylation (Becker et al., 1994). However, when the expression pattern in mouse tissues was examined using rat PPV as a probe, the highest level of expression was found in brain, differing from what we found in human tissues.

Detection of the PP6 protein

To demonstrate the presence of the PP6 protein in mammalian cells, we raised antibodies against the recombinant protein expressed in *Escherichia coli* BL21 under the control of the T7 promoter. The recombinant PP6 protein was found exclusively as insoluble particles in inclusion bodies. Attempts to refold the protein solubilized in guanidine chloride and to restore the catalytic activity were unsuccessful, but we used the purified protein to raise antisera in rabbits. The antisera specifically recognized as little as 10 ng of recombinant PP6 protein and no

B

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S.p. ppe1  M--FDLDEWIATVRKCKYLPEHQLKRLCEMVKVILMEESNIQPVRTPVTVCGDIHGQFYDLL   60
human PP6  MAPLDLDKYVEIARLCKYLPENDLERLCDYVCDLLLEESNVQPVSTPVTVCGDIHGQFYDLC   62
S.c. SIT4  MVSRGPDEWLETIKKCQALTENEMKQLCEMVKELLMEESNIQPVQTPVTVCGDIHGQFHDLL   62

S.p. ppe1  ELFRVGGELP-STNYIFMGDFVDRGYFSLETFTLFMLLKARYPDKITLLRGNHESRQITQVY  121
human PP6  ELFRTGGQVPD-TNYIFMGDFVDRGYYSLETFTYLLALKAKWPDRITLLRGNHESRQITQVY  123
S.c. SIT4  ELFRTAGGFPDDINYIFLGDYVDRGYYSLETFTLLMCLKVKYPAKITLVRGNHESRQITQVY  124

S.p. ppe1  GFYDECQTKYGNANVWKYCCQVFDFLTLAAVIDNKILCVHGGLSPEVRTLDQIRILARAQEI  183
human PP6  GFYDECQTKYGNANAWRYCTKVFDMLTVAALIDEQILCVHGGLYPDIKTLDQIRTIERNQEI  185
S.c. SIT4  GFYEECLNKYGSTTVWKYCCQVFDFLTLAAIIDGKILCVHGGLSPEIRMLDQIRVLSRAQEV  186

S.p. ppe1  PHEGSFCDLMWSDPEDIESWTVSPRGAGWLFGSKVTTEFSQINDLTLIARAHQLVQEGYKYH  245
human PP6  PHKGAFCDLVWSDPEDVDTWAISPRGAGWLFGAKVTNEFVHINNLKLICRAHQLVHEGYKFM  247
S.c. SIT4  PHEGGFSDLLWSDPDNVEAWQVSPRGAGWLFGSKVAREFNHVNGLNLIARAHQLVMEGFKYH  248

S.p. ppe1  FADKNLVTVWSAPNYCYRCGNVASVMKVDESLEPEFRIFSAVADEDRTVPPSRKRSE---YFI 305
human PP6  FDEK-LVTVWSAPNYCYRCGNIASIMVFKDVNTREPKLFRAVPDSERVIPPRTTTP----YFL 305
S.c. SIT4  FPEKDVVTVWSAPNYCYRCGNVASVMKVDEDLEPTFKIFSAVPDDYIRESTANHNNQRAGYFL 311
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Fig. 2. (A) cDNA and predicted amino acid sequence of human PP6. The 42 amino acid residues conserved in all protein serine/threonine phosphatases of the PP1/PP2A/PP2B family are given in bold face and underlined. (B) Comparison of the amino acid sequences of *S. pombe* ppe1, human PP6 and *S. cerevisiae* Sit4p. Residues that are identical between two sequences are indicated by vertical lines, residues conserved among all PP2A-like protein phosphatases are marked by asterisks. The nucleotide sequence of human PP6 is available from the EMBL data base under the accession number X92972.

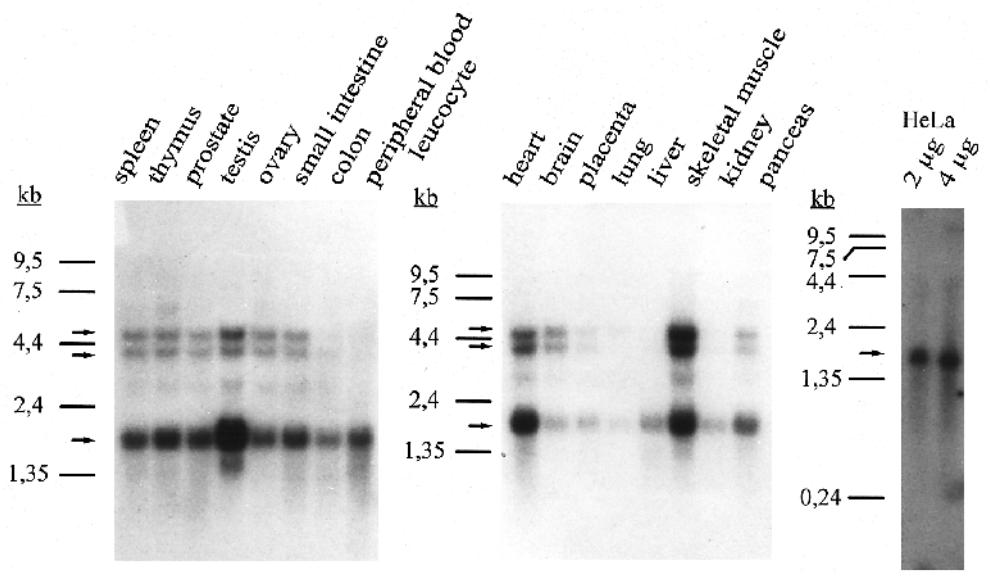

Fig. 3. Distribution of PP6 poly(A)$^+$ RNA in human tissues and HeLa cells. Northern blots displaying 2 µg mRNA each from 16 different human tissues and a blot containing 2 µg and 4 µg mRNA prepared from exponentially growing HeLa cells were hybridized with a 1.2 kb PP6 cDNA fragment. The three PP6 transcripts of 1.8 kb, 4.2 kb and 4.7 kb are indicated by arrows. After stripping, the same blots were probed with a human β-actin cDNA, to ensure that equal amounts of mRNA were loaded in each lane (data not shown).

cross-reaction with several other human protein serine/threonine phosphatases was observed (data not shown). However, in immunoblot analyses using HeLa cell lysates we could not detect any polypeptide, also in immunofluorescence experiments with HeLa cells we did not obtain a specific signal. Since the PP6 poly(A)$^+$ RNA showed elevated transcription levels in heart muscle and in testis, we used a lysate derived from human heart muscle tissue as well as lysate prepared from bull testes for immunoblots. As shown in Fig. 4, the affinity-purified antiserum specifically recognized a polypeptide of 35 kDa in both the human heart muscle lysate and the high speed supernatant of a bull testis lysate. The PP6 protein detected in human and bull tissues showed the same electrophoretic mobility during SDS-PAGE as the recombinant protein expressed in *E. coli* (Fig. 4). We concluded that the PP6 protein is present in human heart muscle tissues and the homologous protein in bull testis as a 35 kDa protein. Consistent with the higher level of mRNA expression the protein levels also appear to be elevated in these tissues.

To characterize the PP6 protein further we attempted to overexpress PP6 in HeLa cells. We cloned the coding sequence of PP6 fused to an HA-tag either at the N- or at the C terminus under the control of the CMV promoter and transfected these constructs by an electroporation protocol into exponentially growing HeLa cells (data not shown). In each experiment we observed an efficiency of transfection of about 70-80%. Upon transfection a dramatic loss of viability was observed, while in control experiments cells transfected with the same fusion constructs of human PP5, this protein phosphatase was expressed without a significant phenotype (H. Bastians and H. Ponstingl, unpublished data). This deleterious effect was observed already a few hours after transfection and we were not able to detect the overexpressed PP6 fusion protein at early time points.

Human PP6 rescues the growth defect of a *S. cerevisiae sit4*-102 mutant

To investigate the possibility of PP6 being a functional homologue of *S. cerevisiae* Sit4p, we performed complementation studies in a *S. cerevisiae sit4-102* mutant (kindly donated by K. Arndt). The budding yeast strain CY249 carries a chromosomal deletion of the *SIT4* gene and a *ssd1-d2* allele. The lethal phenotype of a *sit4* null mutant depends on the latter allele (Sutton et al., 1991). In addition, this strain carries the *sit4-102* allele on the YCp50 plasmid, responsible for a temperature-sensitive growth phenotype. At the permissive temperature of 28°C growth is comparable to wild-type cells, while at the restrictive temperature of 37°C unbudded cells arrest in G_1 (Sutton et al., 1991).

To complement the *sit4-102* mutation, we cloned the coding sequence of human PP6 into pADH under the control of the *ADH1* promoter. As controls we used the empty vector, wild-type *SIT4* as well as the coding sequences of human PP4 and S. *pombe ppe1*$^+$, which are also highly homologous to Sit4p (60% and 72% identity, respectively), cloned into the same vector. CY249 was transformed with these constructs and growth rates were determined at the permissive temperature of 28°C (Fig. 5A) as well as at the restrictive temperature of 37°C (Fig. 5B). All transformants grew well at the permissive temperature, but at the restrictive temperature only human PP6 and wild-type Sit4p were able to suppress the growth defect of the *sit4-102* mutant completely. In contrast, human PP4 was unable to complement this mutation, whereas the homologous *S. pombe* ppe1 showed a weak suppression of the growth arrest, indicating a partial complementation of the *sit4-102* mutation.

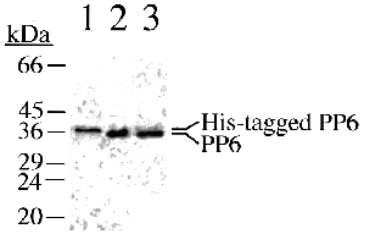

Fig. 4. Detection of the PP6 polypeptide in mammalian tissues: 30 ng of recombinant His-tagged PP6 protein expressed in *E. coli* (lane 1), 50 µg of total protein of a heart muscle tissue lysate (lane 2) and 50 µg of total protein of a high speed supernatant derived from a bull testes lysate (lane 3) were run on an SDS-PAGE gel and blotted onto nitrocellulose. For immunodetection affinity-purified anti-PP6 antibodies were used.

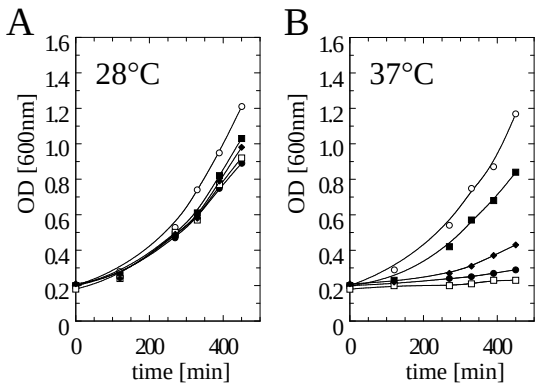

Fig. 5. Human PP6 suppresses the temperature-sensitive growth phenotype of *sit4*-102. Growth rates in synthetic drop-out medium lacking leucine were monitored (A) at the permissive temperature of 28°C and (B) at the restrictive temperature of 37°C after transformation of *S. cerevisiae* CY249 with pADH (vector; ●), pADH-*SIT4* (○), pADH-*ppe1*$^+$ (◆), pADH-PP4 (□) and pADH-PP6 (■).

To support this result, we also cloned the complete coding sequences of human PP4, PP5 and PP6 into pAS1-CYH2. This vector allows expression of the protein phosphatases as fusion proteins including a HA-tag and the GAL4 DNA-binding domain (amino acid residues 1-147 of GAL4). We expressed PP4, PP5 and PP6 as such fusion constructs in CY249 and monitored the growth rates at the restrictive temperature. We observed again that the PP6 hybrid but not the homologous protein phosphatases PP4 and PP5 can suppress the growth defect of the mutant (data not shown). Taken together, these results suggest that human PP6 represents a specific functional human homologue of *S. cerevisiae* Sit4p, as other highly homologous protein phosphatases failed to complement.

Human PP6 suppresses the morphological and growth phenotypes of a *S. pombe ppe1* mutant

Considering the 68% amino acid identity of human PP6 to *S. pombe* ppe1, we were interested to investigate whether PP6 could provide a functional link between PP6, Sit4p and ppe1, by its ability to suppress the phenotypes of a *S. pombe ppe1* mutant in addition to its ability to complement a *sit4* mutation. We used the mutant *ppe1*-374 strain (kindly donated by X. He and S. Sazer), isolated as a suppressor of the *pim1*-d1^{ts} mutant (Sazer and Nurse, 1994), which is defective at the mitosis to interphase transition. A *ppe1* mutant, *ppe1*-68, was isolated as a suppressor of another *pim1* mutant allele, *pim1*-46^{ts} (Matsumoto and Beach, 1993). The two *ppe1* mutant strains, as well as a null mutant, show a strongly altered cell shape and a cold-sensitive lethality at 20°C (Matsumoto and Beach, 1993; Shimanuki et al., 1993; X. He and S. Sazer, personal communication). We cloned the same set of genes into pREP3X, in which expression of human PP6, human PP4, *S. cerevisiae SIT4* and *S. pombe ppe1*$^+$ was driven by the thiamine repressible *nmt* promoter (Forsburg, 1993). After introduction of these constructs into the *ppe1* mutant strain, we monitored the morphological phenotype (Fig. 6) and cold sensitive growth on plates in the absence of thiamine (Fig. 7).

Mutant cells show strong abnormalities in cell shape at all temperatures examined (20°C, 28°C and 36°C). They appear very small, compared to wild-type cells, and are often round or pear-shaped. Cells transformed with vector alone are indistinguishable from mutants (Fig. 6, vector), but cells after transformation with *ppe1*$^+$ are capable of suppressing the morphological phenotype resulting in rod-shaped, cylindrical cells (Fig. 6, *ppe1*$^+$). Cells harbouring the *SIT4* gene containing plasmid show a partial suppression of this mutant phenotype (Fig. 6, *SIT4*). The majority of cells still show abnormalities in their cell shape, but they appear to be clearly distinct from the mutant cells, a phenotype also observed previously by Shimanuki et al. (1993). No complementation of the *ppe1* mutation was seen after transformation with human PP4 (Fig. 6, PP4), a phosphatase 61% identical to *S. pombe* ppe1. The cells still show the shape defect of the mutant. In contrast, human PP6 is able to completely suppress the mutant phenotype (Fig. 6, PP6). We also examined the phenotypes in the presence of thiamine, when the *nmt* promoter should be repressed. Surprisingly, we observed phenotypes very similiar to those described above, indicating an incomplete repression/derepression of the *nmt* promoter by thiamine which has been reported previously (Forsburg, 1993).

Next, we investigated the ability of the various transformants to grow at the restrictive temperature (Fig. 7). We tested the growth at 36°C, 28°C and 20°C on EMM plates lacking thiamine. Mutant cells grow very slowly even at permissive temperatures (doubling time approximately 3.5 hours in supplemented YE at 36°C; Shimanuki et al., 1993), but they show a growth arrest at 20°C. All transformants examined formed colonies at the permissive temperature of 36°C. A slightly reduced growth was observed for cells transformed with pREP3X (vector control), with pREP-*SIT4* and with pREP-PP4 at 28°C, while no difference in growth was seen for mutant cells transformed with pREP-*ppe1*$^+$ and with pREP-PP6. After

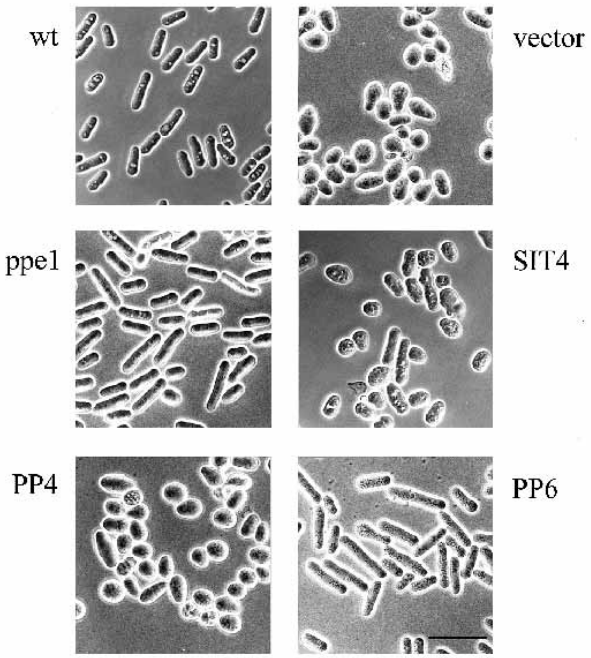

Fig. 6. Human PP6 suppresses the morphological phenotype of *S. pombe ppe1*-374. Mutant cells were transformed with either pREP3X (vector), pREP-*ppe1*$^+$ (ppe1), pREP-*SIT4* (SIT4), pREP-PP4 (PP4) or pREP-PP6 (PP6). Transformants were grown in EMM in the absence of thiamine at 36°C. Wild-type cells (wt) are shown as a control. Bar, 20 μm.

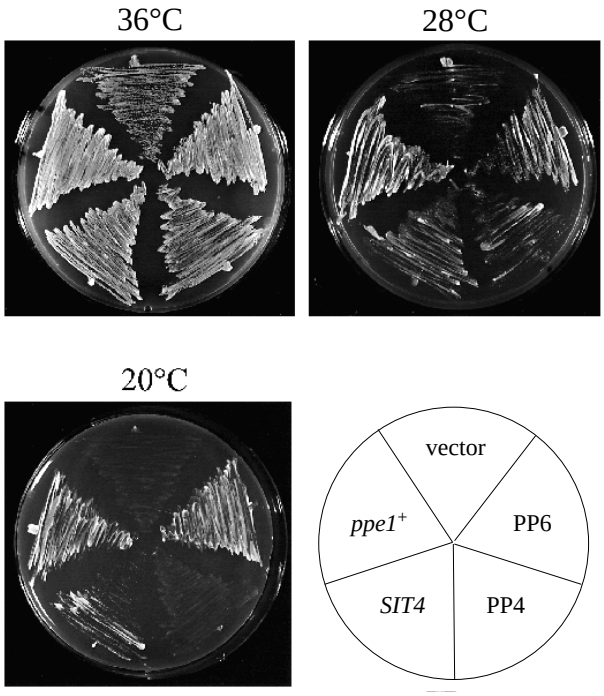

Fig. 7. Cold sensitive growth of *ppe1-374* after transformation with the indicated vectors. *S. pombe ppe1* mutant cells were transformed with either pREP3X (vector), pREP3X-*ppe1*$^+$, pREP3X-*SIT4*, pREP3X-PP4 or pREP3X-PP6. Transformants were grown on EMM plates at 36°C, 28°C and 20°C for 3, 5 and 8 days, respectively.

incubation at the restrictive temperature (20°C) only *ppe1*$^+$ and human PP6 expressing cells produced colonies on EMM plates. The expression of *S. cerevisiae SIT4* resulted in a reduced growth at the restrictive temperature, but formation of colonies. This result is in good agreement with the observation that the expression of *SIT4* leads to a partial suppression of the cell shape phenotype. Hence, the introduction of the wild-type *ppe1*$^+$ gene and of human PP6 gene results in a complete suppression of the cs lethality of the *ppe1*-374 mutant, whereas *SIT4* produces a semi-complementation phenotype. Therefore, we suggest that PP6 is the functional human homologue of fission yeast ppe1 and PP6 provides a functional link between fission yeast ppe1 and budding yeast Sit4p.

DISCUSSION

Human PP6 is a novel member of a distinct subgroup of protein phosphatases

We identified a cDNA encoding a novel human protein serine/threonine phosphatase, termed protein phosphatase 6 (PP6). The deduced amino acid sequence of human PP6 revealed homology to isoforms of human PP1 (40-41% identity), isoforms of human PP2A (56-57%), and to a lesser extent to human PP2B (34%). Homology is highest to rat PPV (98%; Becker et al., 1994), *Drosophila* PPV (74%; Mann et al., 1993), *S. pombe* ppe1 (68%; Matsumoto and Beach, 1993; Shimanuki et al., 1993) and *S. cerevisiae* Sit4p (61%; Arndt et al., 1989). These homologies of PP6 place it into a distinct subgroup of the PP1/PP2A/PP2B family including *S. cerevisiae* Sit4p, *S. pombe* ppe1 and *Drosophila* and rat PPV.

All 42 amino acid residues that are invariant among protein serine/threonine phosphatases (Barton et al., 1994) and essential for catalytic activity (Goldberg et al., 1995) are also present in PP6, suggesting that it is a functional human protein phosphatase. In addition, PP6 contains a carboxy-terminal sequence (-YFL) highly conserved among PP2A-like protein phosphatases, which was shown to be subject to tyrosine phosphorylation (Chen et al., 1992) and carboxymethylation (Xie and Clarke, 1994) in PP2A, suggesting that these posttranslational modifications might be also present in PP6.

In northern blot analyses we found three different mRNA transcripts of human PP6, of 1.8 kb, 4.2 kb, and 4.7 kb, with varying ratios in the tissues examined, indicating additional isoforms or alternatively processed forms of this protein phosphatase in human cells. Highest levels of expression were found in human testis, heart and skeletal muscle. Interestingly, in mouse tissues the presence of three transcripts of the homologous PPV with similiar sizes was reported previously (Becker et al., 1994). These three transcripts seem to be derived from a single gene due to alternative polyadenylation, which could also be true for human PP6. In contrast to human tissues, the highest level of expression in the mouse was found in brain, suggesting a brain specific function of PPV in mouse. Consistent with an elevated level of transcription in heart muscle and testis, we detected the PP6 protein in lysates derived from human heart muscle and in lysates prepared from bull testes as a polypeptide with the expected molecular mass of 35 kDa.

Human PP6 can complement mutations in *SIT4* and *ppe1*

We showed that human PP6 is a functional homologue of budding yeast Sit4p by its ability to complement a *sit4*-102 mutation. Previously it had been demonstrated that *Drosophila* PPV can complement the same mutation in budding yeast *SIT4*, indicating that PPV is the functional *Drosophila* homologue of Sit4p (Mann et al., 1993). The amino-terminal domain of PPV is sufficient for this complementation, when fused to PP1. Most of the residues conserved between PPV and Sit4p in the amino-terminal region are also present in fission yeast ppe1 and human PP6, indicating the possibility of an interaction with regulatory subunits or specific substrates via this subdomain. In addition, we established that PP6 can complement a loss of function mutation of *S. pombe ppe1*, indicating PP6 being also a functional homologue of ppe1. These functional interchangeabilities suggest a common function for Sit4p, ppe1, PPV and PP6 protein phosphatases (for a proposed model see Fig. 8).

At a first glance, the pathways in which Sit4p and ppe1 protein phosphatases are involved appear to be quite different, but there are remarkable similiarities. Sit4p protein phosphatase was originally identified by suppressor mutations that result in enhanced transcription of the *HIS4* gene in the absence of the transcription factors Gcn4p, Bas1p and Bas2p (Arndt et al., 1989). Later, it was shown that Sit4p functions in late G_1 and is required for the normal accumulation of G_1 cyclin mRNAs in budding yeast (Fernandez-Sarabia et al., 1992). The lethality of a *SIT4* deletion is dependent on a second gene, termed *SSD1* (suppressor of *SIT4* deletion). This gene is polymorphic, judging from the fact that one allele, *ssd1-d* (dead), produces a lethal phenotype in *SIT4* null mutants, whereas deletion mutants containing another allele, *ssd1-v*, are viable (Sutton et al., 1991).

This genetic interaction is reminiscent of another one found

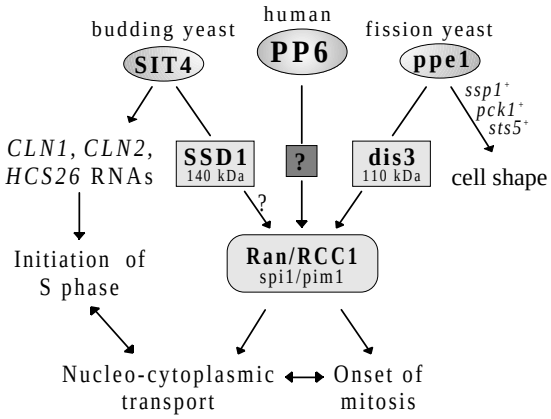

Fig. 8. Proposed roles of human PP6 in analogy to *S. cerevisiae* Sit4p and *S. pombe* ppe1. The Ran/RCC1 pathway is involved in nucleo-cytoplasmic transport, initiation of S-phase, and progression through mitosis. Fission yeast ppe1 has at least one additional function in the regulation of cell shape and actin organization, and functions overlapping those of *sts5*$^+$, *pck1*$^+$ and *ssp1*$^+$ have been suggested (Shimanuki et al., 1993; Matsusaka et al., 1995).

in *S. pombe* between *ppe1*$^+$ and *dis3*$^+$. A deletion mutant of *ppe1* can be rescued by overexpression of *dis3*$^+$, suggesting an interaction of these components (Shimanuki et al., 1993). The *dis3*$^+$ gene encodes a 110 kDa nuclear protein essential for exit from mitosis (Kinoshita et al., 1991), and shows regions of significant homology to the 140 kDa SSD1 protein (Sutton et al., 1991). From the analogy of these pathways in two evolutionary divergent yeasts we infer that a dis3/SSD1-like protein may exist in human cells as well as in *Drosophila*, interacting with the functional equivalents PP6 and PPV, respectively.

In addition, in *S. cerevisiae* Sit4p recently was identified as the catalytic subunit of the heterotrimeric protein phosphatase CAPP, which is greatly stimulated by the lipid second messenger ceramide (Nickels and Broach, 1996). In human cells, activation of CAPP in response to certain growth modulators, such as tumor necrosis factor α or interleukin-1β induces an antiproliferative response. This finding raises the possibility that the catalytic activity of human PP6 is also stimulated by ceramide, suggesting a possible function of PP6 in a novel signal transduction pathway mediated by ceramide.

In spite of the similiarities between Sit4p, ppe1, PPV and PP6, some findings are at variance with the assumption of completely overlapping functions. Mutants of *SIT4* arrest in late G_1, whereas mutants of *ppe1* arrest in G_2. The expression of *Drosophila* PPV peaks at the fourteenth nuclear division cycle in the embryo, when the G_2 phase occurs for the first time, rather indicating a function in G_2 (Mann et al., 1993). We showed that *SIT4* can partially complement a *ppe1* mutation, and a *sit4-102* phenotype is weakly suppressed by *ppe1*$^+$. This phenomenon could be due to different regulatory pathways of cell cycle progression and to additional functions of Sit4p in budding yeast and ppe1 in fission yeast. Ppe1 protein phosphatase probably has more than one function in *S. pombe*, since the *ppe1* mutant shows altered cell shapes at all temperatures examined and a growth arrest restricted to low temperatures. Matsusaka et al. (1995) reported the identification of *ssp1*$^+$, encoding a novel protein kinase, as a suppressor of a *ppe1* mutant. Ssp1 kinase and ppe1 phosphatase are essential for alteration of growth polarity and actin localization (Matsusaka et al., 1995), a regulatory process producing rod-like cells in fission yeast. To date, there is no evidence that Sit4p or PP6 are also involved in similiar processes.

Human PP6 could provide a functional linkage to the Ran/RCC1 pathway

An interesting functional linkage arises from the fact that a *ppe1* mutation was identified as a suppressor of a *S. pombe* *pim1*-d1^{ts} mutant, which is defective in chromatin decondensation and exhibits nuclear envelope fragmentation at the restrictive temperature (Sazer and Nurse, 1994; Demeter et al., 1995). From another allelic mutant, *pim1*-46^{ts} (Matsumoto and Beach, 1991), *ppe1* was also isolated as a suppressor mutant (Matsumoto and Beach, 1993). The pim1 protein is a structural homologue of mammalian RCC1, previously identified in a golden hamster cell line (tsBN2) as a regulator of chromosome condensation (Ohtsubo et al., 1987). Biochemical studies revealed that RCC1 functions as a nucleotide exchange factor on Ran, a small Ras-related nuclear GTPase (Bischoff and Ponstingl, 1991). This GTPase cycle has been shown to be involved in nucleo-cytoplasmic transport (Melchior et al., 1993; Moore and Blobel, 1993) and regulation of the p34^{cdc2} kinase required for entry into mitosis (Ren et al., 1994; Clarke et al., 1995). Interestingly, *dis3*$^+$ has been implicated in pathways leading to the activation of the cdc2 kinase in fission yeast (Kinoshita et al., 1991). To date it is unclear, whether the regulation of cell cycle progression is dependent on nucleo-cytoplasmic transport regulated by the Ran/RCC1 pathway. To analyze the putative interaction of PP6 with components of the Ran/RCC1 pathway, we have embarked on identifying PP6 interacting proteins by using the yeast two-hybrid system.

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