The EMBO Journal vol.13 no.18 pp.4278–4290, 1994

A novel human protein serine/threonine phosphatase, which possesses four tetratricopeptide repeat motifs and localizes to the nucleus

Mao Xiang Chen, Alison E.McPartlin, Linda Brown, Yu Hua Chen, Hazel M.Barker and Patricia T.W.Cohen[1]

Medical Research Council Protein Phosphorylation Unit, Department of Biochemistry, The University, Dundee DD1 4HN, UK
[1]Corresponding author

Communicated by P.Cohen

A novel human protein serine/threonine phosphatase, PP5, and a structurally related phosphatase in *Saccharomyces cerevisiae*, PPT1, have been identified from their cDNA and gene respectively. Their predicted molecular mass is 58 kDa and they comprise a C-terminal phosphatase catalytic domain and an N-terminal domain, which has four repeats of 34 amino acids, three of which are tandemly arranged. The phosphatase domain possesses all the invariant motifs of the PP1/PP2A/PP2B gene family, but is not closely related to any other known member (≤40% identity). Thus PP5 and PPT1 comprise a new subfamily. The repeats in the N-terminal domain are similar to the tetratricopeptide repeat (TPR) motifs which have been found in several proteins that are required for mitosis, transcription and RNA splicing. Bacterially expressed PP5 is able to dephosphorylate serine residues in proteins and is more sensitive than PP1 to the tumour promoter okadaic acid. A 2.3 kb mRNA encoding PP5 is present in all human tissues examined. Investigation of the intracellular distribution of PP5 by immunofluorescence, using two different antibodies raised against the TPR and phosphatase domains, localizes PP5 predominantly to the nucleus. This suggests that, like other nuclear TPR-containing proteins, it may play a role in the regulation of RNA biogenesis and/or mitosis.

Key words: mitosis/nucleus/protein phosphatase/RNA synthesis/tetratricopeptide repeat

Introduction

A wide variety of cellular functions, including cell signalling, gene expression and mitosis, are regulated by the reversible phosphorylation of proteins on serine and threonine residues (Fischer and Krebs, 1990). The phosphatases that catalyse the dephosphorylation of these amino acids are a crucial component of this regulation. Four types of protein serine/threonine phosphatase activity (PP1, PP2A, PP2B and PP2C) were distinguished in the cell cytosol (Cohen, 1989) and molecular cloning has revealed that they fall into two structurally distinct families, the PP1/PP2A/PP2B family and the PP2C family (Cohen *et al.*, 1990). Recent studies have uncovered an increasing number and structural variety of enzymes in the PP1/PP2A/PP2B family (Chen *et al.*, 1992; Cohen, 1993), including mammalian PP4 (also termed PPX; Brewis *et al.*, 1993), *Drosophila* PPV (Mann *et al.*, 1993), PPY (Dombrádi *et al.*, 1989) and rdgC (Steele *et al.*, 1992), *Saccharomyces cerevisiae* PPZ1 and PPZ2 (Hughes *et al.*, 1993; Lee *et al.*, 1993), PPQ (Chen *et al.*, 1993), PPG (Posas *et al.*, 1993), SIT4 (Arndt *et al.*, 1989) and *Schizosaccharomyces pombe* ppe$^+$ (Shimanuki *et al.*, 1993), which is probably the homologue of SIT4 and PPV.

PP1 and PP2A have broad and overlapping specificities *in vitro* and their activities are believed to be controlled *in vivo* by regulatory subunits which may modify their substrate specificity and/or target them to particular subcellular locations, thus restricting their actions to substrates in that locality. In skeletal muscle, the glycogen binding subunit targets PP1 catalytic subunit to glycogen and the sarcoplasmic reticulum, while the myosin binding subunits localize PP1 to myofibrils and enhance its activity towards myosin (Hubbard and Cohen, 1993). In the cytosol PP1 interacts with two small thermostable proteins, inhibitors 1 and 2, which inhibit its activity, thus preventing illicit dephosphorylation events; inhibitor 2 can also act like a chaperone to fold PP1 into its native conformation (Alessi *et al.*, 1993). As yet no other protein phosphatases are known to be sensitive to inhibitors 1 and 2. PP2A, like PP1, is active in the absence of metal ions, while PP2B and PP2C are dependent on Ca^{2+} and Mg^{2+} ions, respectively. *In vivo* PP2A forms a complex with a 65 kDa subunit and the dimer then interacts with a second regulatory subunit which may vary in structure and size (52–107 kDa). Both regulatory subunits can alter the specificity of the catalytic subunit (Ferrigno *et al.*, 1993). The localization of PP4 to centrosomes may enhance its activity towards substrates in this location (Brewis *et al.*, 1993). PP1, PP2A and PP4 are potently inhibited by the tumour promoters okadaic acid and microcystin, PP2A and PP4 being the most sensitive. PP2B is almost insensitive to these inhibitors and PP2C is unaffected by them, but the sensitivity of other novel phosphatases has not been determined.

Although initially identified in the cell cytosol, PP1 and PP2A are also found in the nucleus (Jakes *et al.*, 1986; Kuret *et al.*, 1986). More recent studies have shown that a 16–18 kDa protein, termed NIPP-1, forms an inhibitory complex with PP1 in the nucleus of mammalian cells, the inhibition being relieved by the phosphorylation of NIPP-1 (Bollen *et al.*, 1993). In fission yeast the 30 kDa protein product of the *sds22* gene is localized in the nucleus. Sds22 forms a complex with PP1 and may modulate its activity towards nuclear substrates (Stone *et al.*, 1993). In addition to PP1 and PP2A, PP4 is also found in the nucleus (Brewis *et al.*, 1993). However, since several nuclear functions are regulated by phosphorylation, such

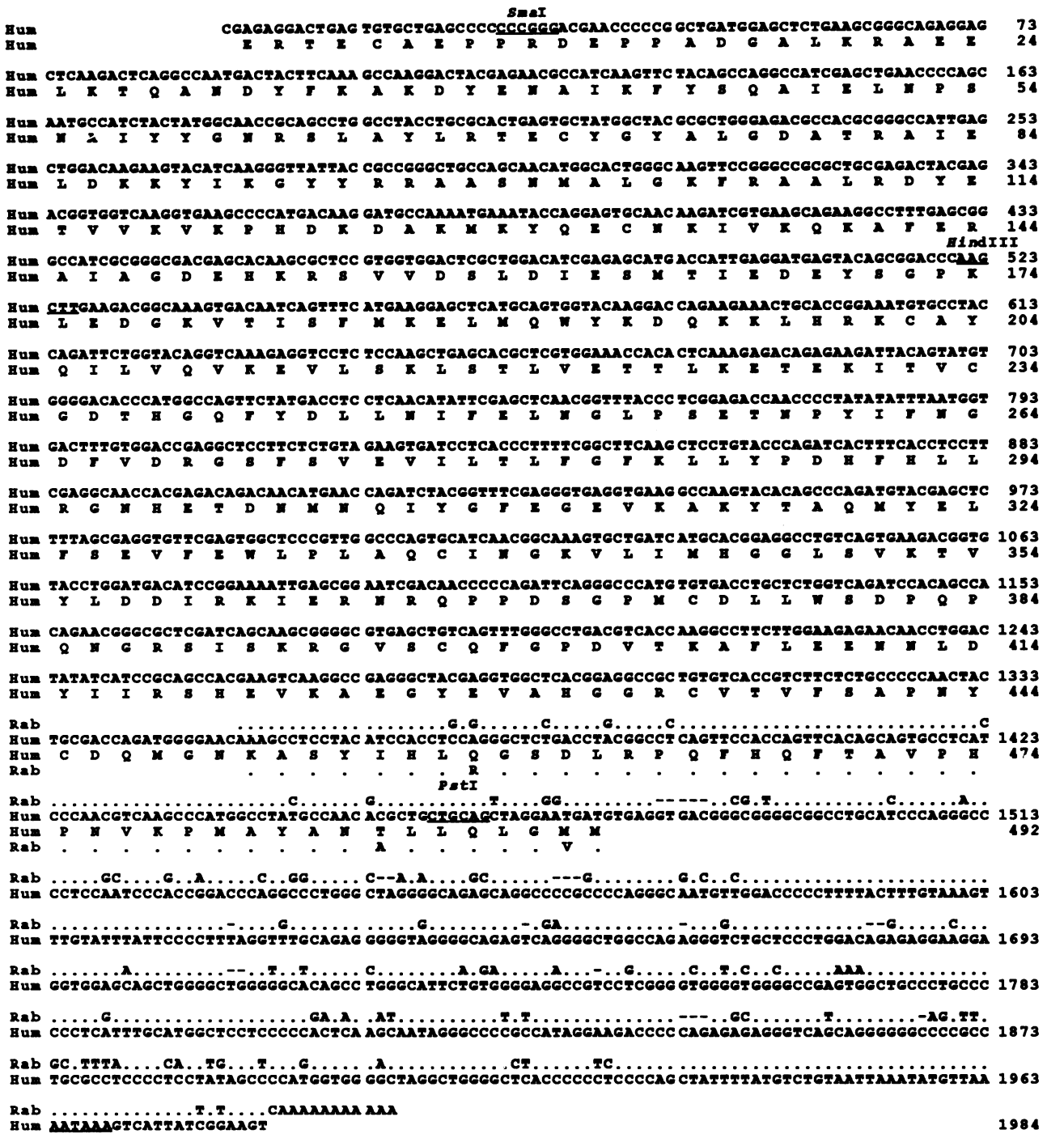

Fig. 1. Nucleotide sequence and predicted amino acid sequence of human PP5 cDNA. The C-terminal coding region and 3′ untranslated region of rabbit PP5 are shown. Dots indicate identity and dashes signify deletions with respect to the human sequence.

as cell division, DNA replication, gene transcription and probably RNA splicing, other nuclear protein serine/threonine phosphatases may exist. In this paper, we identify a novel human protein phosphatase that localizes to the nucleus, and a structurally related enzyme in yeast. Both of these enzymes comprise a phosphatase domain preceded by a long N-terminal domain containing four tetratricopeptide repeats (TPRs), similar to those found in several nuclear proteins involved in mitosis and RNA biogenesis.

Results

Isolation and sequence of human PP5 cDNA

Screening of 2×10^5 p.f.u. of a human teratocarcinoma cDNA library at low stringency identified one clone that hybridized only weakly with a PP2B probe and did not hybridize with a PP1- or a PP2A-related probe. The clone contains a 2.0 kb insert, with the sequence shown in Figure 1. The DNA encodes a protein of >492 amino

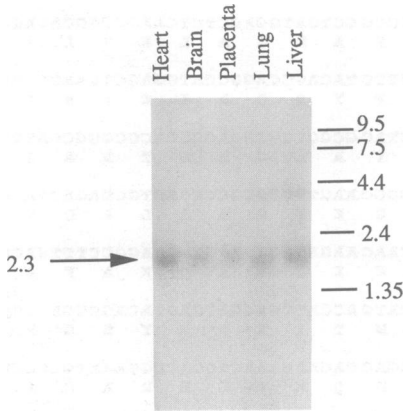

Fig. 2. Distribution of PP5 mRNA in human tissues. The 2.3 kb PP5 mRNA is indicated by an arrow. This mRNA species was also present in skeletal muscle, kidney and pancreas. The sizes of marker RNA fragments are indicated in kb on the right. The same blot was hybridized with a PP1β probe (Barker *et al.*, 1994), which indicated that, although the Northern blot (Clontech) is said to contain 2 μg of poly(A) RNA, stronger signals seen in skeletal muscle and kidney (not shown) are likely to be the result of unequal loading of the RNA samples.

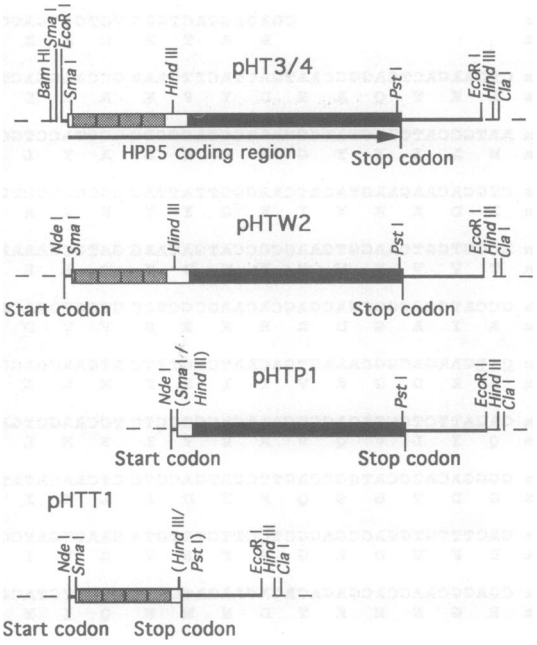

Fig. 3. Partial restriction map of human PP5 cDNA in Bluescript (clone HT 3/4) and constructs for the expression of the whole PP5 (clone pHTW2), the phosphatase domain of PP5 (clone pHTP1) and the TPR domain of PP5 (clone pHTT1) in *E.coli* from the pT7.7 vector and pCW vector. The PP5 coding region is indicated by a bar, the phosphatase domain by a black bar and the four TPRs by stippled bars. The non-coding regions are denoted by lines and the vector sequences by broken lines.

acids, and possesses a 3′ non-coding region of 507 nt, with a putative polyadenylation signal 15 nt before the 3′ end. At the 5′ end, the open reading frame is incomplete, but the predicted molecular mass of the encoded protein (56 kDa) is only slightly smaller than that determined for the native protein in a cell extract (58 kDa, see below). Subsequent rescreening of the human teratocarcinoma library and screening of an oligo(dT) and random primed human skeletal muscle cDNA library (Clontech) with a probe corresponding to nt 1–521 of the human cDNA yielded clones with the same sequence, but none that were longer at the 5′ end.

The deduced amino acid sequence of human PP5 comprises distinct N- and C-terminal domains. The C-terminal half contains all of the regions that are conserved among other members of the PP1/PP2A/PP2B family of protein phosphatases (Cohen, 1991) although it shows only 37–40%, 38% and 36–38% amino acid identity to human PP1, PP2A and PP2B isoforms, respectively.

Screening of a rabbit liver cDNA library with the human cDNA yielded a 623 bp cDNA, which comprised the region encoding the C-terminal 42 amino acids and the 3′ non-coding region with a polyadenylation signal and a poly(A) tail (Figure 1). The rabbit and human C-terminal amino acid sequences are 93% identical and the 3′ non-coding regions show 84% identity.

Distribution of PP5 mRNA in human tissues

Expression of PP5 in poly(A)$^+$ RNA from different human tissues is shown in Figure 2. A major 2.3 kb PP5 mRNA was observed in all tissues examined. The 2.3 kb mRNA is 0.3 kb larger than the cDNA sequence in Figure 1, a difference which can be accounted for by the presence of a poly(A) tail and a short sequence at the 5′ end encoding the extreme N-terminus of the protein. The PP5 mRNA is present in heart, brain, placenta, lung and liver (Figure 2) and also in skeletal muscle, kidney and pancreas (data not shown). The ratio of the PP5 mRNA:PP1β mRNA hybridization signals [the latter RNA is present at similar levels in many tissues (Barker *et al.*, 1994)] was lowest in skeletal muscle (1.0) and highest in liver (3.7).

Properties of bacterially expressed human PP5

Constructs encoding virtually the whole of PP5, its C-terminal domain and its N-terminal domain (Figure 3) were expressed from the pT7.7 vector in *Escherichia coli*. All three proteins were expressed at high levels (~15 mg per litre of culture) but largely as insoluble forms in inclusion bodies in *E.coli*. Assay of the soluble fractions for protein phosphatase activity using ^{32}P-labelled phosphorylase as substrate showed that, while no activity was detected in *E.coli* expressing the N- or C-terminal domains of PP5, or the vector alone, *E.coli* expressing the whole PP5 possessed a low level of activity (0.2 mU/ml supernatant). Expression of PP5 in the pCW vector (Muchmore *et al.*, 1989) which proved useful for the expression of PP1 (Alessi *et al.*, 1993) also only led to low level expression of PP5 activity (0.8 mU/ml). The activity of PP5 with histone H1 as substrate was 3-fold higher than with phosphorylase, but since the specific activity with phosphorylase as substrate was only 30 mU/mg (see below), the specific activity with histone H1 as substrate was still only 90 mU/mg, >100-fold lower than that of the PP2A catalytic subunit towards this substrate.

PP5 and its separate domains expressed in the pT7.7 vector could be isolated with >90% purity from inclusion bodies as described for PP4 (Brewis *et al.*, 1993). They

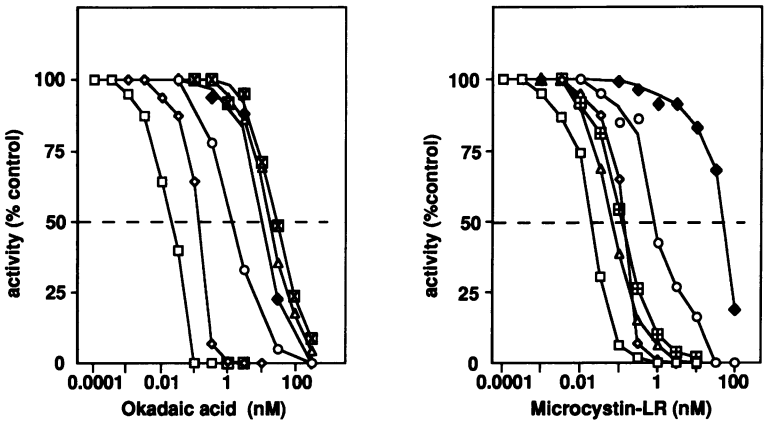

Fig. 4. Inhibition of PP5, PP1 and PP2A by okadaic acid and microcystin-LR. Protein phosphatases were assayed at the following concentrations using ^{32}P-labelled phosphorylase as substrate. PP5, 0.01 mU/ml (○); PP5, 0.2 mU/ml (⊕); PP1, 0.02 mU/ml (△); PP1, 0.1 mU/ml (okadaic acid) (⊠), 0.2 mU/ml (microcystin) (⊞); PP2A, 0.02 mU/ml (□); PP2A, 0.2 mU/ml (◇).

were solubilized and refolded and, as described for PP1, Mn^{2+} was essential for production of the active enzyme (Cohen and Berndt, 1991). The following protocol gave optimal reactivation: 0.2 mg of insoluble PP5 was dissolved in 0.1 ml 6 M guanidinium hydrochloride over a period of 30 min at room temperature. The denatured PP5 was diluted 200-fold in 50 mM Tris−HCl pH 7.5, 1 mg/ml bovine serum albumin, 5 mM dithiothreitol, 0.1% 2-mercaptoethanol, 0.4 M NaCl, 0.03% Brij-35, 0.1% Tween 20 and 1 mM $MnCl_2$ and left for 1 h at room temperature. After filtration though a 0.45 μm filter, measurement of the soluble protein by A_{280} or Coomassie protein assay reagent showed that 30–40% of the PP5 was solubilized using this protocol. The specific activity of the soluble PP5 using ^{32}P-labelled phosphorylase as a substrate was ~30 mU/mg, 100- to 1000-fold lower than that of PP1 and PP2A purified from mammalian tissues or refolded from expressed PP1.

When the same refolding procedure was applied to the C-terminal catalytic domain no phosphatase activity was generated. Similarly, when equimolar amounts of the N- and C-terminal domains of PP5 were refolded, no activity was detected. It appears, therefore, that covalent linkage of the N-terminal domain aids the folding of the phosphatase domain into an active conformation.

The virtually pure and enzymatically active PP5, obtained after refolding the insoluble PP5, was concentrated using a Centricon-10 unit and then tested for sensitivity to several protein phosphatase inhibitors using ^{32}P-labelled phosphorylase as substrate. As expected, PP5 was insensitive to inhibitors 1 and 2 at 200 nM, at which concentration PP1 was completely inhibited (data not shown). However, PP5 was sensitive to both okadaic acid and microcystin-LR (Figure 4). When the phosphatases under examination were diluted to 0.01–0.02 mU/ml in the assay, the apparent IC_{50} values for okadaic acid were 0.06 nM, 1.4 nM and 18 nM for PP2A, PP5 and PP1 respectively, while at 0.1–0.2 mU/ml, they were 0.2 nM, 10 nM and 28 nM respectively. Since apparent IC_{50} values for PP5, like PP2A, depend on the phosphatase concentration, the real K_i of PP5 for okadaic acid is likely to be <1 nM. In contrast, PP5 is less sensitive to microcystin than PP1 and PP2A (apparent IC_{50} 15 nM compared with 0.12 nM and 0.14 nM for PP1 and PP2A at a substrate concentration of 0.2 mU/ml). An *E.coli* supernatant fraction of the soluble active PP5 expressed from the pCW vector showed similar inhibition characteristics to the pure refolded PP5.

Sequence and chromosomal localization of S.cerevisiae PPT1 gene

We previously reported the identification of a novel protein phosphatase in *S.cerevisiae* from a 0.1 kb polymerase chain reaction (PCR) fragment, Y58. This sequence was not closely related to any known protein phosphatase, showing only 40–52% amino acid identity to all members of the PP1/PP2A/PP2B family. Using the PCR fragment, Y58, as a probe to screen an *S.cerevisiae* genomic library constructed in λDASH, several hybridizing clones were isolated. A 7.5 kb *Eco*RI fragment of one clone, λY58A, was subcloned into the *Eco*RI site of Bluescript. A single open reading frame was identified encoding a protein (PPT1) of 513 amino acids, with a molecular mass of 58 kDa (Figure 5). The first ATG codon in the open reading frame is most likely to be the translational start, since it is preceded by a sequence that closely resembles the consensus sequence for translational initiation (Hamilton *et al.*, 1987) and by an in-frame stop codon. The C-terminal half of PPT1 shows 35%, 31–33% and 30–34% identity respectively to *S.cerevisiae* DIS2 (PP1), PPH2 (PP2A) and CMP (PP2B). Comparison of *S.cerevisiae* PPT1 with human PP5 shows that they are 42% identical and 63% similar if conservative substitutions are taken into account (Figure 6A) and sequence similarities to PP5 are found in the N-terminal non-catalytic domain (which shows 36% identity to PP5) as well as in the C-terminal domain (which is 46% identical to PP5) (Figure 6B).

In order to localize the *PPT1* gene in the *S.cerevisiae* genome, a nitrocellulose blot of separated yeast chromosomes was hybridized with ^{32}P-labelled pYKS581 under high stringency. The hybridization signal was located to either chromosome VII or XV which migrate close together during electrophoresis. Hybridization of grid filters containing cosmid clones, which comprise ~95% of the yeast genome, with the same probe identified two overlapping clones on chromosome VII. One of these clones also hybridizes to the *spt6* gene. Therefore, the *PPT1* gene is located close to the *spt6* locus on chromosome VII.

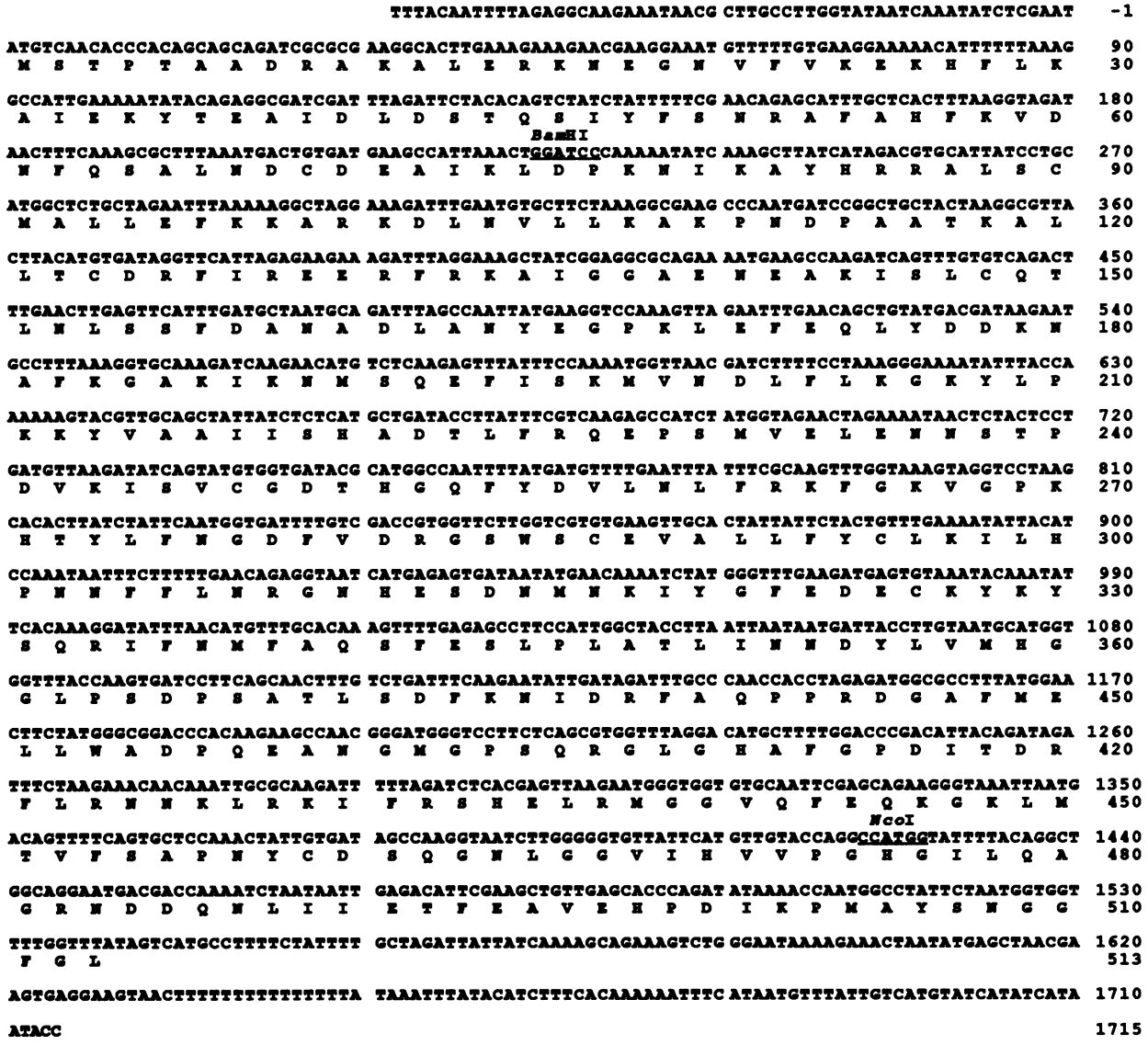

Fig. 5. Nucleotide sequence and predicted amino acid sequence of the *Saccharomyces cerevisiae PPT1* gene.

S.cerevisiae PPT1 null mutants are viable

To ascertain whether *PPT1* encodes an essential function, we created deletion mutants of the *PPT1* gene. The *ppt1::LEU2* construct used for this experiment is shown in Figure 7A. A linear DNA fragment carrying *ppt1::LEU2* was introduced into the yeast genome by transformation of the diploid strain AY927 to leucine prototrophy. It replaced the *PPT1* gene by recombination and this resulted in the production of a null allele deleted in the region encoding amino acids 77–475. Since the inserted DNA fragment, containing the *LEU2* gene, also contained stop codons in all three frames, the formation of a fusion protein involving the N-terminal remnant of the *PPT1* gene was prevented. The haploid spores of three individual diploid transformants were examined for the presence of a null allele of *PPT1* (AY925*ppt1* and AY926*ppt1*). PCR using genomic DNA demonstrated that one of the three transformants had been successfully disrupted (Figure 7B). Diploid cells homozygous for the null allele of PPT1 (AY927*ppt1*) were formed by mating *MAT***a** and *MAT*α haploid cells, each carrying a null allele for *PPT1*, on YPD agar and selecting for cells that were able to sporulate.

Both *ppt1* haploid and diploid cells were found to be viable. Growth was identical to wild type in rich medium (YPD) and in synthetic medium (S) containing either glucose, galactose, glycerol or acetate. Cell size was normal in both log phase and stationary phase, when analysed either visually under the microscope or in a flow cytometer. Mating and sporulation were normal, although *MAT***a** *ppt1* cells did show a very slightly lower sensitivity to α-pheromone than AY925 haploid cells. The diameter of the growth inhibition zone observed with 20 μg α-pheromone was 13.7 ± 0.7 (n = 27) for *MAT***a** *ppt1* cells as compared with 14.0 ± 0.8 (n = 28) for AY925 cells which is on the borderline of being significantly different $\{P(t = 1.8\ \phi = 53) < 0.05\}$. Repeated experiments at different concentrations of α-pheromone gave similar results.

The *PPT1* null mutants did not show increased sensitivity to high or low temperatures or high concentrations of salts including NaCl, $CaCl_2$ and $MgCl_2$, or to low pH,

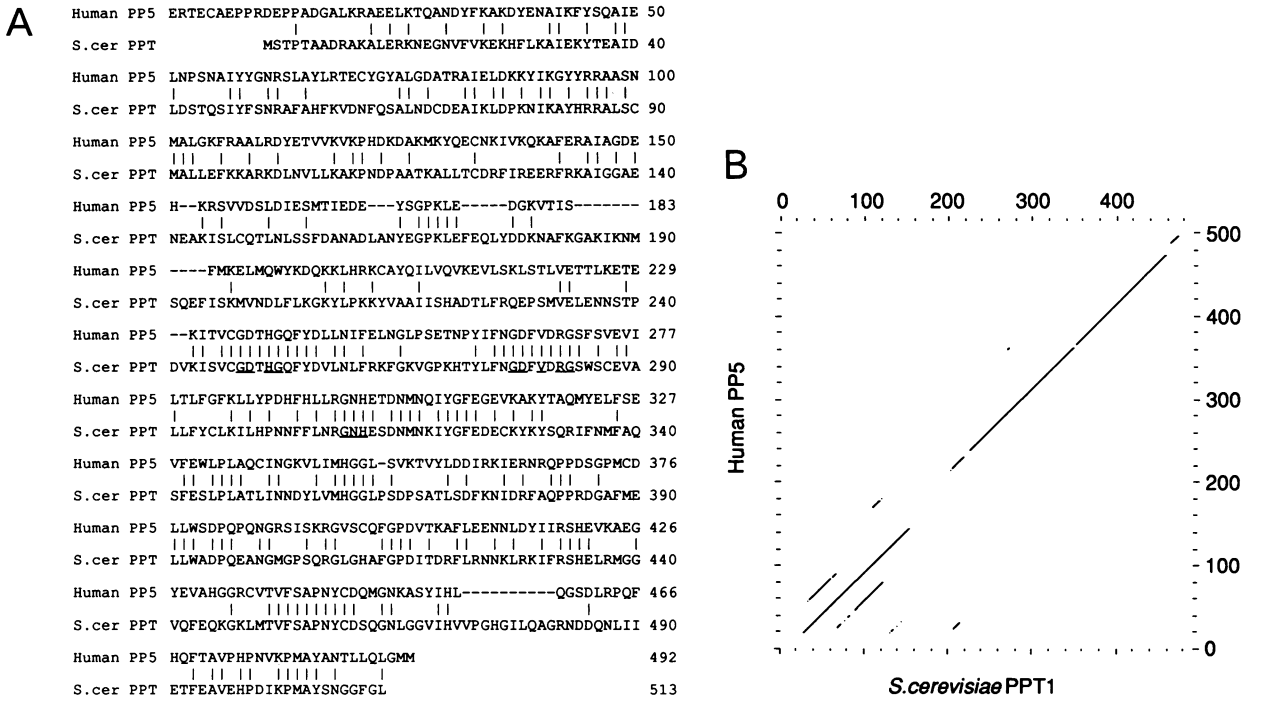

Fig. 6. (**A**) Comparison of human PP5 and *S.cerevisiae* PPT1. Both proteins contain a C-terminal phosphatase domain (amino acids 184–492 in PP5 and 188–513 in PPT1) and an N-terminal TPR domain (amino acids 1–183 in PP5 and 1–187 in PPT1). Residues which are identical in all protein serine/threonine phosphatases in PP1/PP2A/PP2B/ORF221 gene family are underlined. (**B**) Dot plot of *S.cerevisiae* PPT1 compared with human PP5. The plot was constructed by inserting the output from the University of Wisconsin GCG programme COMPARE (with a window of 34 amino acids and a stringency of 17) into the programme DOTPLOT.

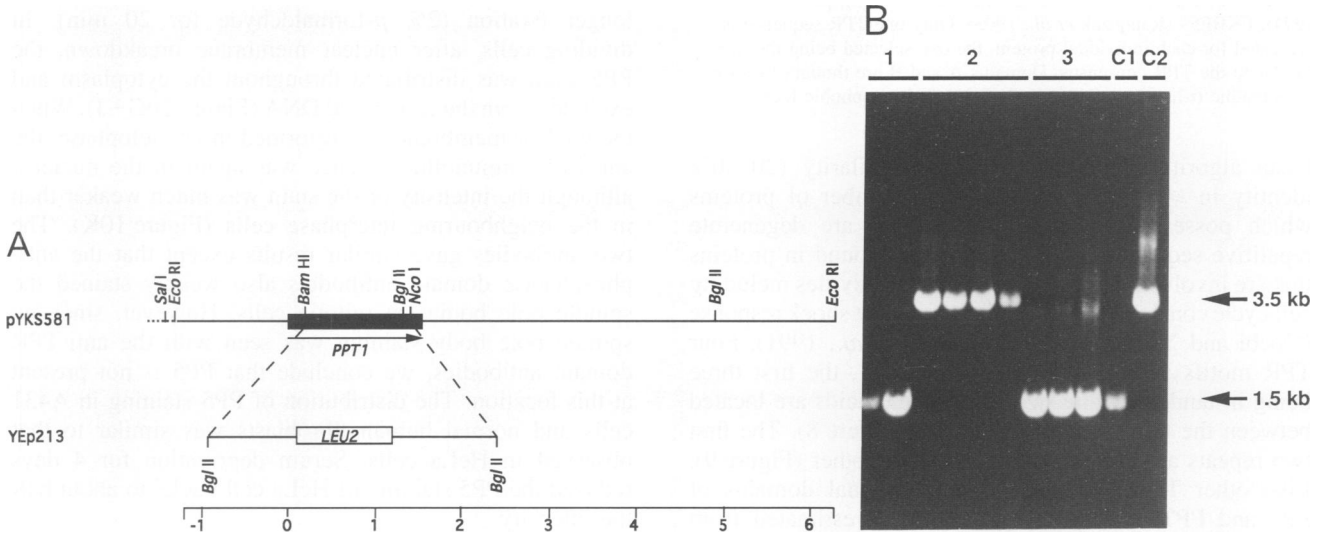

Fig. 7. (**A**) Production of a null allele of *PPT1*. A partial restriction map of the *PPT1* locus depicts the production of the construct used to create yeast strain *ppt1::LEU2*. The coding region of *PPT1* is denoted by a black bar, non-coding regions by lines and vector sequences by dotted lines. The *LEU2* marker fragment from YEP213 is labelled. The scale below is in kilobases. (**B**) Detection of a transformant carrying a null allele of *PPT1*. *LEU2* colonies from the spores of each putative transformant (1, 2 and 3) were analysed by PCR using primers 1 and 2 (see Materials and methods). A control plasmid containing wild type genomic DNA (C1) yields a 1.5 kb fragment. A second control plasmid carrying the *PPT1* gene disrupted by the LEU2 marker (C2) yields a fragment of 3.5 kb. The *PPT1* gene was only disrupted in transformant 2.

EGTA, caffeine, hydroxyurea ± polymyxin B, cycloheximide, the neomycin analogue G418, rifampicin and actinomycin D. Glycogen levels were normal on YPD, SD or sporulation agar. Invertase activity was similar to that in wild type cells in media containing glucose, galactose or glycerol. In addition, overexpression of both the complete *PPT1* gene and the C-terminal phosphatase domain from the GAL1 promoter in YCp-Gal did not affect growth of *ppt1* cells compared with wild type cells carrying the vector alone.

Human PP5 and S.cerevisiae PPT possess N-terminal domains with four tetratricopeptide repeat (TPR) motifs

A search of the SwissProt and NBRF protein databases with the N-terminal domains of PP5 and PPT1 by the

```
                          DOMAIN A      DOMAIN B
Human  PP5          AEELKTQANDYFKAKDYENAIKFYSQAIELNPSN      55
S.cer  PPT          ALERKNEGNVLVKEKHFLKAIEKYTEAIDLDSTQ      45

Human  PP5          AIYYGNRSLAYLRTECYGYALGDATRAIELDKKY      89
S.cer  PPT          SIYFSNRAFAHFKVDNFQSALNDCDEAIKLDPKN      79

Human  PP5          IKGYYRRAASNMALGKFRAALRDYETVVKVKPHD KD 125
S.cer  PPT          IKAYHRRALSCMALLEFKKARKDLNVLLKAKPND PA 115

Human  PP5          AKMKYQECNKYVKQKAFERAIAGDEHKRSVVDSL     159
S.cer  PPT          ATKALLTCDRFIREERFRKAIGGAENEAKISLCQ     149

PP5+PPT  consensus  A..Ø..RA..ØØK...Ø..AØ.DØ..AØKØ.P..
                                R

TPR consensus for
other  proteins     ...Ø..ØG.ØØ.....Ø..A...Ø..AØ...P..
                          A       S       S
                          S               G

nuc2+               YNAWYGLGMVYLKTGRNDQADFHFQRAAEINPNW
CDC23               TNAWTLMGHEFVELSNSHAAIECYRRAVDICPRD
CDC16               AITWFSVATYYMSLDRISEAQKYYSKSSILDPSF
crn                 VSHWIKYAQWEEQQQEIQRARSIWERALDWEHRN
SSN6                PLKWHGIGILYDRYGSLDYAEEAFAKVLELDPHF
SKI3                VESWVGLGQAYHACGRIEASIKVKDKAIQLRPSH
TFIIIC              TDVFKPLARCYKEIESYETAKEFYELAIKSEPDD
PRP6                HKFFLQLGQIYESMGNIEMSRETYLSGTRLVPNC
STI1                SKGYWRLGAAHLGLGDLDEAESNYKKALELDASN
MAS70               SSVYYERGQMHFILQNYDQAGKDFDKAKELDPEN
PAS10               ELMWWRLGASLANSNRSEEAIQAYHRALQLKPSF
p58                 LWKLIESAEELIKEGRYTDAISKYESVMKTEPGV
FKBP59              KAILFRRGEAHLAVNDFDLARADFQKVLQLYPSN
```

Fig. 8. Comparison of the N-terminal TPR domains of human PP5 and *S.cerevisiae* PPT1 with the TPR consensus sequence derived from other TPR-containing proteins. The single letter code is used for amino acids. ϕ indicates any amino acid with a large hydrophobic side chain. Residues doubly underlined in PP5 and PPT1 conform to the PP5/PPT1 consensus sequence. The TPR consensus for other proteins is derived from all TPR motifs in the following sequences: nuc2$^+$ (Hirano *et al.*, 1990), CDC23 (Sikorski *et al.*, 1990), CDC16 (Icho and Wickner, 1987), crn (Zhang *et al.*, 1992), SSN6 (Schultz and Carlson, 1987), SKI3 (Rhee *et al.*, 1989), TFIIIC (Rameau *et al.*, 1994), PRP6 (Legrain and Choulika, 1990), STI1 (Schultz *et al.*, 1990), MAS70 (Hase *et al.*, 1983), PAS10 (Van der Leij *et al.*, 1993), p58 (Lee *et al.*, 1994), FKBP59 (Ratajczak *et al.*, 1993). Only one TPR sequence is presented for each individual protein, the one selected being the most similar to the TPR consensus. Domains A and B are thought to form amphipathic α-helices, which interact at their hydrophobic faces.

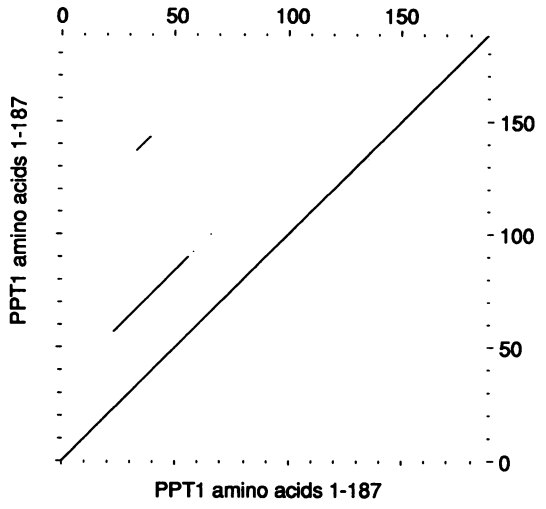

Fig. 9. Dot plot of N-terminal domain of *S.cerevisiae* PPT1, showing that the second TPR motif is more similar to the first, than are the third repeat (not visible at this stringency) and the fourth repeat (partially visible). The plot was constructed by inserting the output from the UWGCG programme COMPARE (with a window of 34 amino acids and a stringency of 17) into the programme DOTPLOT.

Fasta algorithm revealed sequence similarity (20–30% identity in ≥100 amino acids) to a number of proteins which possess TPR motifs. The latter are degenerate repetitive sequences of 34 amino acids found in proteins that are involved in a variety of cellular activities including cell cycle control, RNA biogenesis and heat shock response (Goebl and Yanagida, 1991; Sikorski *et al.*, 1991). Four TPR motifs are found in PP5 and PPT1, the first three being in tandem, while two extra amino acids are located between the third and fourth repeats (Figure 8). The first two repeats are the most similar to each other (Figure 9). Like other TPR sequences, the N-terminal domains of PP5 and PPT1 are quite hydrophilic as estimated from hydrophilicity/hydrophobicity plots (Hopp and Woods, 1981; Kyte and Doolittle, 1982). However, in contrast to other TPR sequences, position 12 is always a hydrophobic amino acid, while position 13 is predominantly Lys or Arg.

Cytological localization of PP5 in human cells and immunological analysis of subcellular fractions

The subcellular localization of PP5 in HeLa cells was examined by immunofluorescence using polyclonal affinity purified anti-PP5 TPR domain and anti-PP5 phosphatase domain antibodies. Both anti-PP5 antibodies stained the nuclei of HeLa cells very strongly, while being excluded from the nucleolus (Figure 10A, B, D and F). The nuclear stain was uneven and in some regions of the nucleoplasm punctate staining was observed. A weak stain was observed in the cytoplasm, particularly when a minimal fixation process was used (2% *p*-formaldehyde for 5 min or 90% methanol for 5 min). However, this was less evident with longer fixation (2% *p*-formaldehyde for 20 min). In dividing cells, after nuclear membrane breakdown, the PP5 stain was distributed throughout the cytoplasm and excluded from the condensed DNA (Figure 10G–J). When the nuclear membrane was reformed in late telophase, the anti-PP5 immunofluorescence was again in the nucleus, although the intensity of the stain was much weaker than in the neighbouring interphase cells (Figure 10K). The two antibodies gave similar results except that the anti-phosphatase domain antibodies also weakly stained the spindle pole bodies in mitotic cells. However, since no spindle pole body staining was seen with the anti-TPR domain antibodies, we conclude that PP5 is not present at this location. The distribution of PP5 staining in A431 cells and normal human fibroblasts was similar to that observed in HeLa cells. Serum deprivation for 4 days reduced the PP5 staining of HeLa cell nuclei to about half the intensity.

In order to confirm the nuclear localization of PP5, cytoplasmic and nuclear fractions of HeLa cells were prepared and analysed by immunoblotting using both anti-phosphatase domain and anti-TPR domain antibodies. As shown in Figure 11, PP5 is present at a much higher concentration (7- to 10-fold) in the nuclear fraction than in the cytoplasmic fraction. Estimation of the amount of PP5 in the cell by immunoblotting showed that 1 ng protein was extracted from 24 000 cells, indicating that there are ~400 000 molecules of PP5 per cell. This indicates that PP5 is 5- to 10-fold less abundant than PP1 and PP2A. The concentration of PP5 in the nucleus drops significantly in cells that are confluent as judged by immunofluorescence (data not shown), suggesting a requirement for PP5 in cell growth.

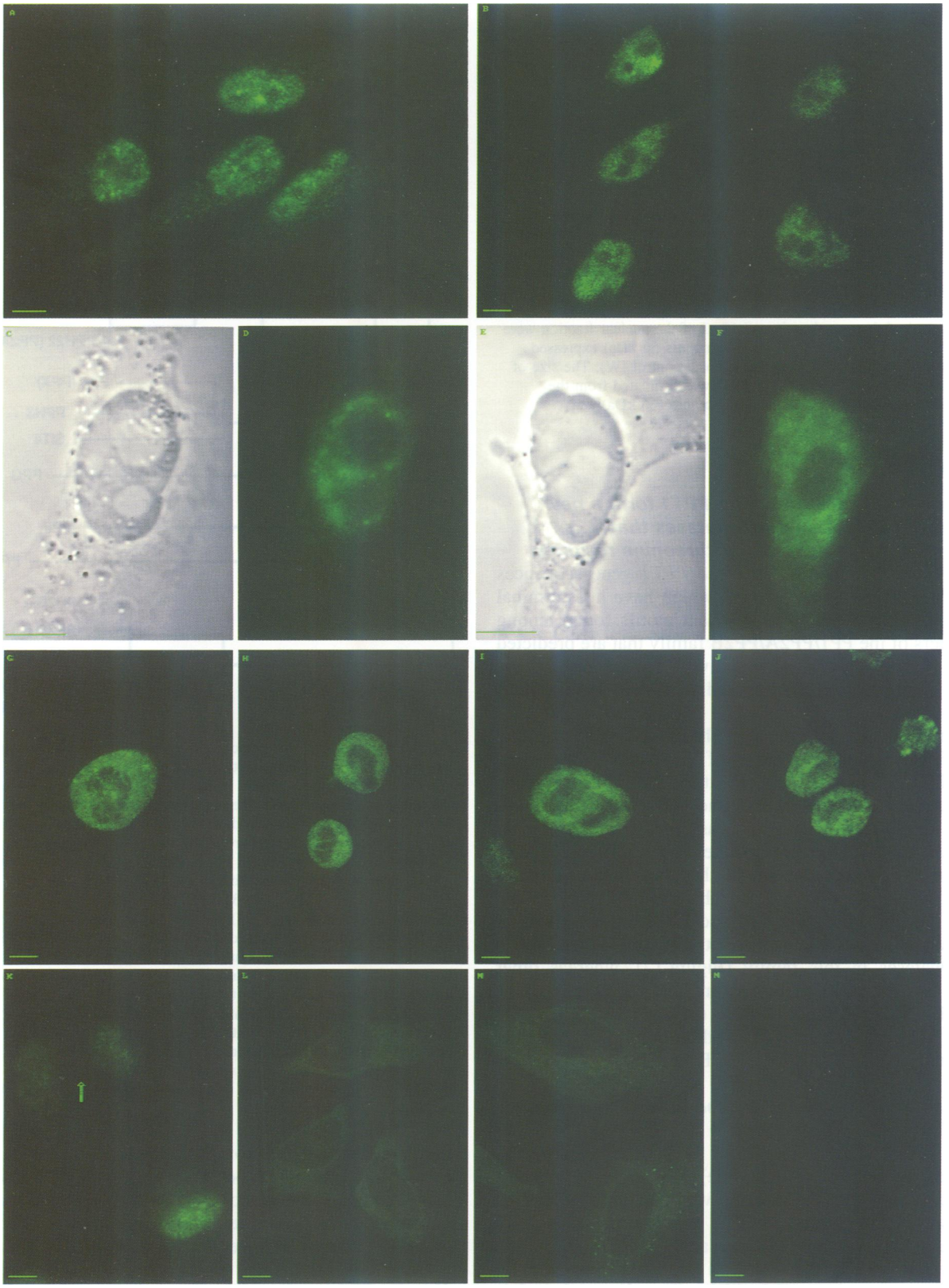

Fig. 10. Immunofluorescent localization of PP5 in HeLa cells fixed with *p*-formaldehyde. PP5 anti-phosphatase domain and anti-TPR domain antibodies were detected with fluorescein-labelled donkey anti-rabbit IgG antibodies (green stain). Fluorescence micrographs were taken with a confocal microscope. (**A** and **D**) Staining of the nucleus with PP5 anti-phosphatase domain antibodies; (**B** and **F**) staining of the nucleus with PP5 anti-TPR domain antibodies; (**C** and **E**) phase image of nuclei from same cells as in (D) and (F) respectively; (**G**–**K**) mitotic cells stained with PP5 anti-TPR domain antibodies, (G) prophase, (H) metaphase, (I) anaphase, (J) telophase and (K) late telophase, where the arrow shows the position of the midbody between two daughter cells; the cell at the lower right hand corner is in interphase. (**L**) Cells stained with PP5 anti-phosphatase domain antibodies which had been incubated for 3 h with excess phosphatase domain polypeptide; (**M**) cells stained with PP5 anti-TPR domain antibodies which had been incubated with excess TPR domain polypeptide for 3 h; (**N**) cells stained with fluorescein-labelled donkey anti-rabbit IgG antibodies. The scale bars denote 10 μm.

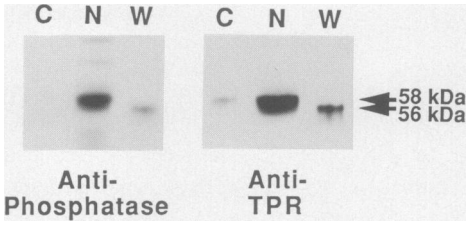

Fig. 11. Subcellular localization of PP5. Cytoplasmic (C) and crude nuclear (N) fractions of HeLa cells were subjected to electrophoresis in an SDS−polyacrylamide (10%) gel, blotted onto nitrocellulose and visualized by cross-reaction with (**A**) PP5 anti-phosphatase domain antibodies and (**B**) PP5 anti-TPR domain antibodies. In order to correct for the volume of the nucleus being 20% of the total cell volume, 2 μl of the cytoplasmic and 10 μl of the nuclear fraction were loaded. 5 ng of PP5 (calculated molecular mass 56 kDa) expressed from the construct pHTW2 were loaded as a control (W). The size of PP5 seen in HeLa cell extracts (58 kDa) was estimated from the molecular mass markers bovine serum albumin (66 kDa) and ovalbumin (43 kDa).

Discussion

Human PP5 and S.cerevisiae PPT1 form a new subfamily of protein phosphatases that dephosphorylate serine and threonine residues

The human PP5 and *S.cerevisiae PPT1* gene sequences presented here encode proteins which have a C-terminal domain possessing all the invariant motifs found among members of the PP1/PP2A/PP2B family that are predicted to be essential for catalytic activity (Cohen and Cohen, 1989). Assay of PP5 expressed in *E.coli* demonstrates that it acts as a protein serine phosphatase capable of dephosphorylating ^{32}P-labelled phosphorylase and histone H1. The N-terminal domains of PP5 and PPT1 contain four TPR or 34 amino acid repeat motifs and the presence of sequence identities in PP5 and PPT1 throughout both the N-terminal domain as well as the C-terminal domain suggests that PPT1 is likely to be the yeast homologue of human PP5. A comparison of the phosphatase domains with those of other protein phosphatases in the PP1/PP2A/PP2B family shows that PP5 and PPT1 are most similar to each other and quite distinct from the other known phosphatases in this family (Figure 12), whereas other novel phosphatases identified are related either to PP1 (PPZ1, PPZ2, PPQ), to PP2A (PP4, PPV, PPG) or to PP2B (rdgC). PP5 and PPT1 thus comprise a distinct subfamily of the PP1/PP2A/PP2B family.

Human PP5 is inhibited by the tumour promoters okadaic acid and microcystin

Examination of the properties of the pure refolded PP5 as well as the soluble (but impure) PP5 in *E.coli* extracts showed that while the enzyme was not inhibited by the PP1-specific inhibitors 1 and 2, it was very sensitive to the tumour promoter okadaic acid with an inhibition constant lower than that observed for PP1 and only slightly higher than that observed for PP2A. The effects of okadaic acid on cellular functions are frequently taken as evidence that PP1 and PP2A are involved in regulating these processes. However, it is now clear that this toxin may exert its effect by inhibiting other phosphatases, such as PP4 (Brewis *et al.*, 1993) and PP5. The sensitivity of PP5 to microcystin is lower than that of PP1 and PP2A and therefore it is possible that this inhibitor might be useful

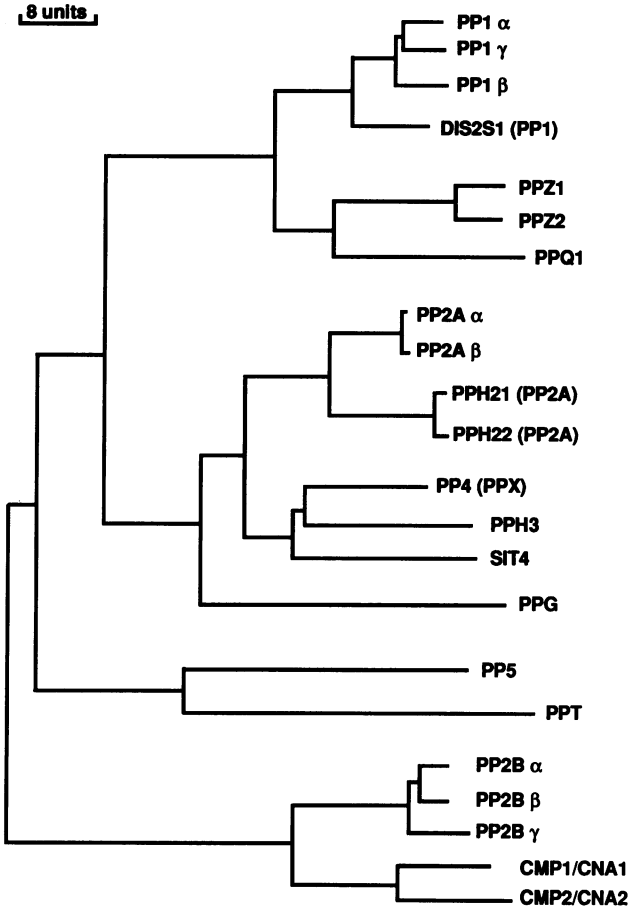

Fig. 12. Phylogenetic tree depicting the relationship between human and *S.cerevisiae* protein serine/threonine phosphatases. The tree is derived from a distance matrix after progressive multiple alignment (Doolittle and Feng, 1990). The scale indicates evolutionary distance units. The distance D is derived according to the Poisson relationship $D = -\ln S$ where S is a measure of the similarity between sequences. The sequences used are human PP1α, PP1β and PP1γ (Barker *et al.*, 1994), PP2Aα and PP2Aβ (Arino *et al.*, 1988; Hemmings *et al.*, 1988), PP4 (Brewis *et al.*, 1993), PP5 (this paper), PP2Bα$_1$ (Muramatsu and Kincaid, 1993), PP2Bβ$_3$ (McPartlin *et al.*, 1991), PP2Bγ (Muramatsu and Kincaid, 1992) and *S.cerevisiae* PP1(DIS2S1) (Ohkura *et al.*, 1989), PPH21 and PPH22 (Sneddon *et al.*, 1989), SIT4 (Arndt *et al.*, 1989), PPH3 (Ronne *et al.*, 1991), PPG (Posas *et al.*, 1993), CMP1 and CMP2, also termed CNA1 and CNA2 (Cyert *et al.*, 1991; Liu *et al.*, 1991), PPZ1 and PPZ2 (Hughes *et al.*, 1993), PPQ (Chen *et al.*, 1993) and PPT1 (this paper). Only the phosphatase domains (starting 60 amino acids prior to the conserved GDXHG motif and terminating 50 amino acids following the conserved SAPNY motif) were employed in order to prevent spurious alignment of the additional dissimilar domains.

for distinguishing PP5 from PP2A and PP4 in cell extracts. However, it should be borne in mind that refolded PP5 may contain some inactive protein capable of binding microcystin and, therefore, that the true K_i for microcystin of native PP5 may be lower than that observed in Figure 4.

Human PP5 localizes to the nucleus and possesses four tetratricopeptide repeat motifs

About 20 different proteins have been shown to contain TPR motifs and most of these are located in the nucleus or have been shown to possess nuclear functions (Boguski *et al.*, 1990; Sikorski *et al.*, 1991). *Schizosaccharomyces pombe* nuc2$^+$ may be a component of the nuclear scaffold (Hirano *et al.*, 1990) and is essential for mitosis, as

are its homologues *Saccharomyces cerevisiae* CDC27 (Sikorski *et al.*, 1991) and *Aspergillus nidulans* bimA$^+$ (Mirabito and Morris, 1993). *S.cerevisiae* CDC23 (Sikorski *et al.*, 1990), CDC16 (Icho and Wickner, 1987) and its homologue *S.pombe* cut9$^+$ (Samejima *et al.*, 1993) are essential for passage through the G_2-M phase of the cell cycle, while the crooked neck (crn) protein in *Drosophila* (Zhang *et al.*, 1991) is required for the proliferation of brain neuroblasts. Other fungal genes encoding TPR-containing proteins are involved in the regulation of RNA biogenesis. *S.cerevisiae* SSN6 (Schultz and Carlson, 1987), SKI3 (Rhee *et al.*, 1989) and TFIIIC (Marck *et al.*, 1993; Rameau *et al.*, 1994) regulate transcription. *S.cerevisiae* STI1 (Nicolet and Craig, 1989) is a heat inducible protein which increases the level of mRNA for stress 70-related subfamily A (SSA) proteins, suggesting that it may regulate transcription. *S.cerevisiae* PRP6 (Legrain and Choulika, 1990) is involved in RNA splicing. However, although most TPR-containing proteins function in the nucleus, they are not exclusively found in this location. *S.cerevisiae* MAS70 (Hase *et al.*, 1983) and *Neurospora crassa* MOM72 (Steger *et al.*, 1990), are bound to the outer mitochondrial membrane and are required for protein import into mitochondria, while *Pichia pastoris* PAS8 and *S.cerevisiae* PAS10 are essential for the import of proteins into peroxisomes (McCollum *et al.*, 1993; Van der Leij *et al.*, 1993). A 58 kDa inhibitor (p58) of the interferon-induced, double-stranded RNA-activated protein kinase which regulates protein synthesis contains a TPR motif (Lee *et al.*, 1994) as do the oestrogen receptor-binding cyclophilin and the FK506 binding protein FKBP59 (Ratajczak *et al.*, 1993).

The detection of PP5 in the nucleus, but only weakly in the cytoplasm, using two different affinity-purified antibodies raised against the TPR and phosphatase domains, demonstrates that PP5 is likely to be predominantly located in this organelle. Like other nuclear TPR proteins, PP5 may therefore play a role in the regulation of mitotic function or RNA biogenesis. The lower level of PP5 in late telophase cells relative to interphase cells, in stationary phase cells relative to log phase cells and in serum-deprived cells shows a requirement for PP5 in cell growth, which would be compatible with a role for PP5 in RNA biogenesis. The punctate staining of anti-PP5 antibodies indicates that PP5 is bound to discrete structures rather than being uniformly dispersed throughout the nucleus. Disruption of the *S.cerevisiae PPT1* gene, which encodes the probable homologue of PP5, did not yield an obvious alteration in phenotype, demonstrating that *PPT1* by itself does not serve an essential function. The very slightly lower sensitivity of *ppt1* cells to α-pheromone compared with wild type is unlikely to be a primary effect of the *PPT1* disruption. The lack of an obvious effect on phenotype may occur if PPT1 merely increases or decreases the efficiency of a process which it regulates by dephosphorylation. Alternatively, there may be another PPT isoform that has overlapping function. Although no evidence for an isoform was obtained from low stringency hybridization, such searches have been shown to be unreliable for other proteins, such as protein kinase C: only one isoform of protein kinase C was thought to be present in *S.cerevisiae*, but later work revealed further isoforms (Levin *et al.*, 1990; Simon *et al.*, 1993).

Mutagenesis studies show that select TPR motifs are absolutely essential for biological function of the proteins that contain them (Schultz *et al.*, 1990; Sikorski *et al.*, 1993). Structural considerations suggest that each TPR motif may form two amphipathic α-helical regions (subdomains A and B) while a proline residue at position 32 in many repeats may induce a turn (Figure 8). One repeat is thought to interact with the next repeat via hydrophobic protein−protein interactions, bulky side chains from one repeat interlocking with small side chains of the next repeat (Goebl and Yanagida, 1991). Using the yeast two-hybrid system, the TPR proteins encoded by *CDC23* and *CDC27* have been shown to form homodimers and heterodimers *in vivo* (Sikorski *et al.*, 1991), supporting the tenet that TPR-containing proteins physically interact with themselves and with each other via their TPR domains. It is possible, therefore, that PP5 may interact with other TPR-containing proteins. Since the number of TPR motifs in different proteins is variable, ranging from three to 16 in most proteins, this could affect the strength of such interactions. PP5 and PPT1 have a low number of repeats (four), and consequently they may interact only weakly with other TPR-containing protein(s), consistent with a transient regulation of nuclear TPR-containing protein(s) by dephosphorylation. It is of interest that FKBP59, the target of the immunosuppressant drug FK506, possesses only three TPR motifs and is known to interact with protein phosphatase 2B (Schreiber, 1992).

In addition to forming complexes with heterologous TPR-containing proteins, genetic and biochemical studies in *S.cerevisiae* indicate that some TPR-containing proteins interact functionally with proteins which possess repeat motifs consisting of 43 amino acids, similar to the β-subunits of G proteins (Goebl and Yanagida, 1991). Examples of such interactions include SSN6/TUP1, PRP6/PRP4, SKI3/MAK11, CDC23/CDC20 and CDC16/CDC20 (Goebl and Yanagida, 1991). It is possible, therefore, that linking of PP5 and PPT1 to a Gβ-related protein could form part of a signalling pathway that might influence nuclear functions, such as RNA biogenesis, DNA replication and/or cell division.

Materials and methods

Materials

Oligonucleotides were synthesized by Alastair I.Murchie on an Applied Biosystems 394 DNA synthesizer (Foster City, CA). Nitrocellulose filters (Hybond C), nylon membranes (Hybond N$^+$) and the Enhanced Chemiluminescence system were obtained from Amersham International plc (Amersham, UK). Centricon-10 units were from Amicon (Beverly, MA). Okadaic acid was a gift from Dr Y.Tsukitani, Fujisawa Pharmaceutical Company, Tokyo. Microcystin-LR was purified by Dr C.MacKintosh (MacKintosh *et al.*, 1990). The catalytic subunit of protein phosphatase 2A (Cohen *et al.*, 1988a) and inhibitor 2 (Cohen *et al.*, 1988b) were purified to homogeneity from rabbit skeletal muscle by Dr D.L.Schelling. Bacterially expressed PP1γ was purified by Drs D.R.Alessi and A.J.Street (Alessi *et al.*, 1993).

HeLa cells, A431 cells and human skin fibroblasts were kindly provided by Dr A.R.Prescott, Dr E.Smythe (Department of Biochemistry, Dundee University) and Dr M.J.W.Faed (Ninewells Hospital Medical School, Dundee) respectively. Tissue culture media, trypsin−EDTA solution and other tissue culture reagents were obtained from ICN Biomedicals (Costa Mesa, CA). Biotinylated goat anti-rabbit IgG and

Coomassie protein assay reagent were obtained from Pierce (Rockford, IL). Fluorescein-labelled donkey anti-rabbit IgG was obtained from Jackson Immunoresearch (West Baltimore Pike, PA) and donkey serum from Sigma. Vectorshield H100 mountant and Vectashield ABC kit were purchased from Vector Laboratories, Burlingame, CA.

Yeast strains, plasmids and methodology

S.cerevisiae haploid strains AY925 (*MAT***a**, *ade2*-1, *his3*-11, *leu2*-3, *trp1*-1, *ura3*-1, *can1*-100) and AY926 (*MAT*α, *ade2*-1, *his3*-11, *leu2*-3, *trp1*-1, *ura3*-1) were a gift from Dr K.Arndt, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY. AY927 is a diploid strain of AY925 and AY926. The *LEU2* gene used as a selective marker for the *PPT1* gene disruption was obtained from the plasmid YEp213. The plasmid employed for inducible expression of *PPT1* was YCp-Gal (*TRP1*) (kindly provided by Dr M.J.R.Stark, University of Dundee).

Yeast media and general methods were as described in Chen *et al.* (1993).

Isolation of mammalian cDNAs

A cDNA library constructed in λgt10 from human teratocarcinoma cell line NTERA2-cloneD1 (kindly provided by Dr J.Skowronski) was screened with cDNA probes for rabbit PP1α [1.2 kb *Sma*I–*Eco*RI fragment encoding amino acids 43–330 and containing 289 bases of 3′ non-coding region (Berndt *et al.*, 1987)], rabbit PPX (PP4) [0.57 kb *Eco*RI–*Sma*I fragment encoding amino acids 105–293 (da Cruz e Silva *et al.*, 1988)] and $PP2B_w$ [1.1 kb *Eco*RI–*Kpn*I fragment encoding the first 368 amino acids (da Cruz e Silva and Cohen, 1989)] in *E.coli* C600 Hfl on duplicate nitrocellulose filters. Hybridization was at 55°C and filters were washed at low stringency in 30 mM NaCl, 3 mM sodium citrate pH 7.0, 0.1% SDS at 55°C. One recombinant phage that hybridized to the $PP2B_w$, but not the PP1α and PP4 probes, was purified by CsCl density centrifugation and phage DNA was purified by formamide extraction. Screening of a rabbit liver cDNA library in λgt10 (Clontech, Palo Alto, CA) as described above with the cDNA insert from the human clone yielded a hybridization positive recombinant phage carrying a rabbit cDNA. Recombinant human and rabbit DNA was digested with *Eco*RI to release 2 kb and 0.7 kb inserts respectively, which were subcloned into Bluescript pKS M13+ vector (Stratagene, La Jolla, CA).

Isolation of S.cerevisiae PPT1 genomic DNA

An *S.cerevisiae* S288C genomic library containing DNA fragments of 10–20 kb in the vector in EMBL4 λDASH (Stratagene, La Jolla, CA) was screened in *E.coli* P2PLK17 on duplicate nitrocellulose filters. The PCR fragment Y58 was used as a probe and labelled with [α-^{32}P]dATP according to Feinberg and Vogelstein (1984) using the degenerate primers previously described (Chen *et al.*, 1992). High stringency hybridization was performed according to the Amersham protocol, with two 15 min washes in 15 mM NaCl, 1.5 mM sodium citrate pH 7.0, 0.1% SDS at 60°C. One phage clone, termed λY58A, that hybridized with the probe was grown up in *E.coli* C600Hfl. DNA restriction fragments of the phage, which were positive with the Y58 probe were subcloned into the Bluescript pKS M13+ (Stratagene, La Jolla, CA).

Sequence analysis

DNA sequencing was performed on double-stranded plasmid DNA either using an Applied Biosystems automatic DNA sequencer model 373A or manually using [α-^{35}S]dATP and Sequenase Version 2.0 (Cambridge Bioscience, Cambridge, UK) and Bluescript or specific oligonucleotide primers. Deletion subclones were generated in order to obtain some parts of the sequences. Both strands of human PP5 cDNA, rabbit PP5 coding region and yeast PPT1 gene were sequenced.

Disruption of the PPT1 gene in S.cerevisiae

The *PPT1* gene was disrupted by the one-step gene replacement method (Rothstein, 1983). This procedure utilized the plasmid pYKS581, which contained an *Eco*RI fragment of the phage clone, λY58A. A *Bam*HI–*Nco*I restriction fragment of pYKS581, corresponding to amino acids 77–475 of the *PPT1* gene, was excised and replaced with a 3 kb *Bgl*II restriction fragment of YEp213 plasmid which contains the *LEU2* gene. This was achieved by filling the *Nco*I, *Bam*HI and *Bgl*II protruding ends with the Klenow fragment of DNA polymerase and excess dNTPs (Sambrook *et al.*, 1989), followed by blunt end ligation. The resulting *ppt1::LEU2* construct in Bluescript was cleaved with *Sal*I and *Bgl*II and the fragment containing the *ppt1::LEU2* allele was used to transform the diploid isogenic strain, AY927, to leucine prototrophy. PCRs using primers 1 (nt −48 to −25) and 2 (nt 1506–1529) with genomic DNA was employed to confirm disruption of a single allele of *PPT1* in the transformed diploid strain and in haploid cells obtained after sporulation.

Analyses of the PPT1 null mutant phenotype in S.cerevisiae

α-pheromone sensitivity, glycogen levels, invertase assays, heat shock and UV sensitivity were carried out as described previously (Chen *et al.*, 1993). Cell size and DNA content were analysed using a fluorescence activated cell sorter as described by Hughes *et al.* (1993). Sensitivity to high salt, low pH, metal ions and other chemicals was tested by spreading exponentially growing wild type and mutant cells on YPD plates containing one of the following: 0.5–2 M NaCl, 0.1–0.8 M $MgCl_2$, 0.1–0.8 M $CaCl_2$, 12.5–100 mM EGTA, 0.5% HCl and 2.25–15 mM caffeine, 80–120 mM hydroxyurea ± 0.3 mg/ml polymyxin B, 0.06–0.1 μg/ml cycloheximide, 0.6–1 μg/ml of the neomycin analogue G418, 0.4–0.6 mg/ml rifampicin and 10–20 μg/ml actinomycin D. The size and number of colonies were analysed after 2–3 days' incubation.

Constructs for the expression of PPT1 and its TPR domain were produced by PCR (five cycles) of the appropriate fragment using primers 3/5 and 3/4, followed by insertion of the product into the expression vector YCpGal under the *gal1* promoter, using the restriction sites present in the primers. Primer 3 was at the start of the PPT1 coding region and contained a *Kpn*I site, primer 4 was at the end of the PPT1 coding region and contains an *Eco*RI site and a stop codon, and primer 5 was at the end of the PPT1 coding region and also contains an *Eco*RI site. The YCpGal PPT1 and TPR constructs were transformed into *PPT1* and *ppt1* diploid cells. Three individual transformants for each construct were analysed for phenotype.

Localization of PPT1 gene in S.cerevisiae genome

Chromo-blots of *S.cerevisiae* chromosomal DNA separated by pulsed field gel electrophoresis were purchased from Clontech, Palo Alto, CA. Prime λ clone grid filters containing ordered arrays of *S.cerevisiae* genomic fragments in bacteriophage λ were kindly provided by Dr Linda Ryles (Washington University School of Medicine, St Louis, USA). The probe used was the whole plasmid pYKS581 labelled with [α-^{32}P]dATP as described by Feinberg and Vogelstein (1984) using several internal sequencing primers in place of random primers. Hybridizations were performed at high stringency as described in Hughes *et al.* (1993).

Analysis of human mRNA

A blot of 2 μg poly(A) RNA from several different human tissues (Clontech, Palo Alto, CA) was hybridized with the 2.0 kb *Eco*RI human PP5 cDNA according to the manufacturer's instructions.

Expression of human PP5 in E.coli

Using restriction sites in the Bluescript vector, a *Bam*HI–*Cla*I fragment of pHT3/4 (Figure 3) encoding the 513 amino acids of PP5 (Figure 1) was inserted into the same sites in the pT7.7 expression vector (Studier *et al.*, 1990). The construct contained a sequence encoding 13 additional amino acids (MARIRARGSPGLQ) before the start of PP5. However, since this construct was expressed at very low levels (<5% of the total protein was PP5), a further construct was made by deleting a *Sma*I fragment, which removed a short section encoding 16 amino acids near the N-terminus. The resulting construct, designated pHTW2 (Figure 3), contained a sequence encoding six amino acids (MARIRA) prior to amino acid 10 of PP5. This construct expressed PP5 as 30% of the total protein after 3 h induction with isopropyl-thio-β-D-galactopyranoside. In order to express the catalytic domain of PP5 a *Sma*I–*Hin*dIII fragment (nt 30–521 in Figure 1) was deleted from pHTW2, resulting in the expression of the catalytic domain of PP5 from amino acid 175 to amino acid 513 with a leader sequence of MARIRAQ. The N-terminal (TPR) domain (amino acids 10–174) was expressed with the same leader sequence as the whole protein and with amino acids 510–513 at the C-terminus by deleting a *Hin*dIII–*Pst*I fragment (nt 522–1464 in Figure 1) of pHTW2. PP5 and its N- and C-terminal domains were predominantly insoluble and were purified from inclusion bodies to >90% purity as described for PP4 (Brewis *et al.*, 1993).

In order to express PP5 in the pCW vector (a generous gift from Drs A.Roth and R.W.Dalquist, University of Oregon, USA), a restriction fragment from the *Nde*I site to the second *Hin*dIII site was excised from a partial digest of pHTW2 and inserted into the same sites of pCW. No major band was visible in the *E.coli* extracts after induction with isopropyl-thio-β-D-galactopyranoside, but a low phosphatase activity using phosphorylase as substrate could be detected in the soluble fraction.

Protein phosphatase assays

^{32}P-labelled rabbit skeletal muscle phosphorylase was prepared by phosphorylation with phosphorylase kinase to a stoichiometry of 1 mol phosphate per mol subunit (Cohen *et al.*, 1988a). The specific activity of the [γ-^{32}P]ATP used for the phosphorylation was 10^6 c.p.m./nmol. Phosphatase assays were performed in the presence of 0.3 mM Mn^{2+} using the above substrate at 10 μM. Alternatively, ^{32}P-labelled calf thymus histone H1 phosphorylated by cdc2 to a stoichiometry of 4 mol phosphate per mol subunit was used as substrate (prepared by Paul Ferrigno). One unit of phosphatase activity is that amount of enzyme which catalyses the release of 1 μmol of [^{32}P]phosphate/min from ^{32}P-labelled substrate.

Production and affinity purification of antibodies

Anti-PP5 phosphatase domain and anti PP5-TPR domain antibodies were raised in rabbits by four injections of 0.1 mg protein at 10–15 day intervals. Antibodies were affinity purified from the serum using CNBr-activated Sepharose 4B (Pharmacia) as described in Brewis *et al.* (1993). 2 mg gel-purified PP5-phosphatase domain and PP5-TPR domain polypeptides were separately linked to the Sepharose beads, and ~0.1 mg antibodies were obtained from 8 ml serum. The anti-phosphatase domain antibodies did not cross-react with PP1, PPX, PPV and PPY, but strongly reacted with PP2A. They were therefore further purified by two passages through a Sepharose 4B column to which PP2A had been linked. This removed 40% of total protein. The remaining 60% did not cross-react with PP2A, and was used for localization and immunoblotting of PPT1.

Cell fractionation and immunoblotting

Non-confluent HeLa cells were washed three times in PBS, digested with trypsin–EDTA solution for 10 min at 37°C, washed with serum to inhibit the trypsin and harvested by centrifugation at 2000 *g* for 10 min at 4°C. The pellet was resuspended in five packed cell volumes of 10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 2 mM PMSC and 0.5 mM dithiothreitol. After incubating on ice for 10 min, cells were harvested by centrifugation at 2000 *g* for 10 min at 4°C. The pellet was resuspended in twice the packed cell volume of the same buffer and homogenized using a Potter homogenizer with a loose fitting pestle. Examination of the homogenate under the microscope showed that the majority of cells lysed with nuclei intact after 15 stokes. The homogenate was centrifuged at 2000 *g* for 10 min at 4°C and the supernatant was collected as the cytoplasmic fraction. The pellet was resuspended in an equal volume of the same buffer and termed the nuclear fraction.

The subcellular fractions were subjected to electrophoresis in a 10% SDS–polyacrylamide gel and the separated proteins were transferred to nitrocellulose membranes using a Transblot apparatus (Bio-Rad) at 45 V/h/cm overnight in a buffer containing 2.9 g Tris and 14.5 g glycine per litre methanol/water (1:4 v/v). Non-specific binding was reduced by incubating the blot for 2 h in 4% fat-free milk powder ('Marvel', Premier Brands, Birmingham) in a buffer comprising 1 M NaCl, 0.1 M Tris–Cl pH 7.5, 0.5% Triton X-100. All subsequent steps were carried out in the same buffer. After washing the membrane for 45 min with at least three changes of buffer, it was incubated overnight with 0.5 μg/ml rabbit anti-PP5 antibodies in the presence of 3% bovine serum albumin and 1% goat serum. Following three washes as described above, it was incubated with 80 ng/ml biotinylated goat anti-rabbit IgG, also in the presence of albumin and serum, for 2 h and then washed again as above. PP5 antibody binding was detected by incubation with avidin/horseradish peroxidase using a Vectastain ABC kit and the same buffer containing albumin and serum for 30 min, followed by washing as described above. Peroxidase activity was detected by fluorography using an enhanced chemiluminescence system.

Immunofluorescence staining of cells

HeLa and A431 cells were cultured at 37°C in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin, plated onto 12 mm glass cover slips and allowed to grow for a further 24–48 h, but not to confluency. After a brief wash at 37°C in PBS, the cells were fixed with 2% freshly prepared *p*-formaldehyde or 90% methanol in PBS for 5–20 min at ambient temperature, and washed several times with PBS. All subsequent steps were carried out in a buffer comprising 1 M NaCl, 0.1 M Tris–HCl pH 7.5 and 0.5% Triton X-100 at room temperature. Cells were incubated in the buffer in the presence of 3% BSA and 1% donkey serum for 10 min. They were then incubated in 2–4 μg/ml rabbit anti-PP5 antibodies in the presence of BSA and serum for 4 h, followed by three 15 min washes with buffer, and incubation for 1 h with 10 μg/ml fluorescein-labelled donkey anti-rabbit IgG in the presence of BSA and serum, followed by three 15 min washes. The cover slips were mounted using Vectorshield H100 mountant and the cells examined using a Bio-Rad 600 laser scanning confocal microscope.

Acknowledgements

We thank Dario Alessi for histone phosphatase assays, and Alan Prescott and Michael Stark for helpful advice; we are grateful to Birgitte and David Lane for use of their confocal microscope. This work was supported by the Medical Research Council, London, and by a Human Frontier Science Programme Grant. A.E.M. and L.B. were recipients of Medical Research Council Studentships.

References

Alessi,D.R., Street,A.J., Cohen,P. and Cohen,P.T.W. (1993) *Eur. J. Biochem.*, **213**, 1055–1066.
Arino,J., Woon,C.W., Brautigan,D., Miller,T.B. and Johnson,G.L. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 4252–4256.
Arndt,K.T., Styles,C.A. and Fink,G.R. (1989) *Cell*, **56**, 527–537.
Barker,H.M., Brewis,N.D., Street,A.J., Spurr,N.K. and Cohen,P.T.W. (1994) *Biochim. Biophys. Acta*, **1220**, 212–218.
Berndt,N., Campbell,D.G., Caudwell,F.B., Cohen,P., da Cruz e Silva,E.F., da Cruz e Silva,O.B. and Cohen,P.T.W. (1987) *FEBS Lett.*, **223**, 340–346.
Boguski,M.S., Sikorski,R.S., Hieter,P. and Goebl,M. (1990) *Nature*, **346**, 114.
Bollen,M., Beullens,M., van Eynde,A. and Stalmans,W. (1993) In W.Merlevede (ed.), *Advances in Protein Phosphatases*. Leuven University Press, Vol. 7, pp. 31–47,
Brewis,N.D., Street,A.J., Prescott,A.R. and Cohen,P.T.W. (1993) *EMBO J.*, **12**, 987–996.
Chen,M.X., Chen,Y.H. and Cohen,P.T.W. (1992) *FEBS Lett.*, **306**, 54–58.
Chen,M.X., Chen,Y.H. and Cohen,P.T.W. (1993) *Eur. J. Biochem.*, **218**, 689–699.
Cohen,P. (1989) *Annu. Rev. Biochem.*, **58**, 453–508.
Cohen,P., Alemany,S., Hemmings,B.A., Resink,T.J., Stralfors,P. and Tung,H.Y.L. (1988a) *Methods Enzymol.*, **159**, 390–408.
Cohen,P., Foulkes,J.G., Holmes,C.F., Nimmo,G.A. and Tonks,N.K. (1988b) *Methods Enzymol*, **159**, 427–437.
Cohen,P.T.W. (1991) *Methods Enzymol.*, **201**, 398–408.
Cohen,P.T.W. (1993) *Biochem. Soc. Trans.*, **21**, 884–888.
Cohen,P.T.W. and Berndt,N.D. (1991) *Methods Enzymol.*, **201**, 408–414.
Cohen,P.T.W. and Cohen,P. (1989) *Biochem. J.*, **260**, 931–934.
Cohen,P.T.W., Brewis,N.D., Hughes,V. and Mann,D.J. (1990) *FEBS Lett.*, **268**, 355–359.
Cyert,M.S., Kunisawa,R., Kaim,D. and Thorner,J. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 7376–7380.
da Cruz e Silva,O.B., da Cruz e Silva,E.F. and Cohen,P.T.W. (1988) *FEBS Lett.*, **242**, 106–110.
da Cruz e Silva,E.F. and Cohen,P.T.W. (1989) *Biochim. Biophys. Acta*, **1009**, 293–296.
Dombrádi,V., Axton,J.M., Glover,D.M. and Cohen,P.T.W. (1989) *FEBS Lett.*, **247**, 391–395.
Doolittle,R.F. and Feng,D.-F. (1990) *Methods Enzymol.*, **183**, 659–669.
Feinberg,A.P. and Vogelstein,B. (1984) *Anal. Biochem.*, **137**, 266–267.
Ferrigno,P., Langan,T.A. and Cohen,P. (1993) *Mol. Biol. Cell*, **4**, 669–677.
Fischer,E.H. and Krebs,E.G. (1990) *Biochim. Biophys. Acta*, **1000**, 297–301.
Goebl,M. and Yanagida,M. (1991) *Trends Biochem. Sci.*, **16**, 173–177.
Hamilton,R., Watanabe,C.K. and de Boer,H.A. (1987) *Nucleic Acids Res.*, **15**, 3581–3593
Hase,T., Reizman,H., Suda,K. and Schatz,G. (1983) *EMBO J.*, **2**, 2169–2172.
Hemmings,B.A., Wernet,W., Mayer,R., Maurer,F., Hofsteenge,J. and Stone,S.R. (1988) *Nucleic Acids Res.*, **16**, 11366.
Hirano,T., Kinoshita,N., Morikawa,K. and Tanagida,M. (1990) *Cell*, **60**, 319–328.
Hopp,T.P. and Woods,K.R. (1981) *Proc. Natl Acad. Sci. USA*, **78**, 3824–3828.
Hubbard,M.J. and Cohen,P. (1993) *Trends Biochem. Sci.*, **18**, 172–177.
Hughes,V., Müller,A., Stark,M.J.R. and Cohen,P.T.W. (1993) *Eur. J. Biochem.*, **216**, 269–279.
Icho,T. and Wickner,R.B. (1987) *Nucleic Acids Res.*, **15**, 8439–8450.
Jakes,S., Mellgren,R.L. and Schlender,K.K. (1986) *Biochim. Biophys. Acta*, **888**, 138–142.
Kuret,J., Bell,H. and Cohen,P. (1986) *FEBS Lett.*, **203**, 197–202.

Kyte,J. and Doolittle,R.F. (1982) *J. Mol. Biol.*, **157**, 105–132.
Lee,K.S., Hines,L.K. and Levin,D.E. (1993) *Mol. Cell. Biol.*, **13**, 5843–5853.
Lee,T.G., Tang,N., Thompson,S., Miller,J. and Katze,M.G. (1994) *Mol. Cell. Biol.*, **14**, 2331–2342.
Legrain,P. and Choulika,A. (1990) *EMBO J.*, **9**, 2775–2781.
Levin,D.E., Fields,F.O., Kunisawa,R., Bishop,J.M. and Thorner,J. (1990) *Cell*, **62**, 213–224.
Liu,Y., Ishii,S., Tokai,M., Tsutsumi,H., Ohki,O., Akada,R., Tanaka,K., Tsuchiya,E., Fukui,S. and Miyakawa,T. (1991) *Mol. Gen. Genet.*, **227**, 52–59.
MacKintosh,C., Beattie,K.A., Klumpp,S., Cohen,P. and Codd,G.A. (1990) *FEBS Lett.*, **264**, 187–192.
Mann,D.J., Dombrádi,V. and Cohen,P.T.W. (1993) *EMBO J.*, **12**, 4833–4842.
Marck,C., Lefebvre,O., Carles,C., Riva,M., Chaussivert,N., Ruet,A. and Sentenac,A. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 4027–4031.
McCollum,D., Monosov,E. and Subramani,S. (1993) *J. Cell Biol.*, **121**, 761–774.
McPartlin,A.E., Barker,H.M. and Cohen,P.T.W. (1991) *Biochim. Biophys. Acta*, **1088**, 308–310.
Mirabito,P.M. and Morris,N.R. (1993) *J. Cell Biol.*, **120**, 959–968.
Muchmore,D.C., McIntsoh,L.P., Russel,C.B., Anderson,D.E. and Dahlquist,F.W. (1989) *Methods Enzymol.*, **177**, 44–73.
Muramatsu,T. and Kincaid,R.L. (1992) *Biochem. Biophys. Res. Commun.*, **188**, 265–271.
Muramatsu,T. and Kincaid,R.L. (1993) *Biochim. Biophys. Acta*, **1178**, 117–120.
Nicolet,C.M. and Craig,E.A. (1989) *Mol. Cell. Biol.*, **9**, 3638–3646.
Ohkura,H., Kinoshita,N., Miyatani,S., Toda,T. and Yanagida,M. (1989) *Cell*, **57**, 997–1007.
Posas,F., Clotet,J., Muns,M.T., Corominas,J.A., Casamayor,A. and Ariño,J. (1993) *J. Biol. Chem.*, **268**, 1349–1354.
Rameau,G., Puglia,K., Crowe,A., Sethy,I. and Willis,I. (1994) *Mol. Cell. Biol.*, **14**, 822–830.
Ratajczak,T., Carrello,A., Mark,P.J., Warner,B.J., Simpson,R.J., Moritz,R.L. and House,A.K. (1993) *J. Biol. Chem.*, **268**, 13187–13192.
Rhee,S.-K., Icho,T. and Wickner,R.B. (1989) *Yeast*, **5**, 149–158.
Ronne,H., Carlberg,M., Hu,G.-Z. and Nehlin,J.O. (1991) *Mol. Cell. Biol.*, **11**, 4876–4884.
Rothstein,R.J. (1983) *Methods Enzymol.*, **101**, 202–211.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual.* 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Samejima,I., Matsumoto,T., Nakaseko,Y., Beach,D. and Yanagida,M. (1993) *J. Cell Sci.*, **105**, 135–143.
Schreiber,S.L. (1992) *Cell*, **70**, 365–368.
Schultz,J., Marshall-Carlson,L. and Carlson,M. (1990) *Mol. Cell. Biol.*, **10**, 4744–4756.
Shimanuki,M., Kinoshita,N., Ohkura,H., Yoshida,T., Toda,T. and Yanagida,M. (1993) *Mol. Biol. Cell*, **4**, 303–313.
Sikorski,R.S., Boguski,M.S., Goebl,M. and Hieter,P. (1990) *Cell*, **60**, 307–317.
Sikorski,R.S., Michaud,W.A., Wootton,J.C., Boguski,M.S., Connelly,C. and Hieter,P. (1991) *Cold Spring Harbor Symp. Quant. Biol.*, **56**, 663–673.
Sikorski,R.S., Michaud,W.A. and Hieter,P. (1993) *Mol. Cell. Biol.*, **13**, 1212–1221.
Simon,A.J., Saville,S.P., Jamieson,L., Pocklington,M.J., Donnelly,S.F.H., Ron,D., Milner,Y., Mochly-Rosen,D. and Orr-Sternlicht,E. (1993) *Curr. Biol.*, **3**, 813–821.
Sneddon,A.A., Cohen,P.T. and Stark,M.J. (1990) *EMBO J.*, **9**, 4339–4346.
Steele,F.R., Washburn,T., Rieger,R. and O'Tousa,J.E. (1992) *Cell*, **69**, 669–676.
Steger,H.F., Sollner,T., Kiebler,M., Dietmeier,K.A., Pfaller,R., Trulzsch,K.S., Tropshug,M., Neupert,W. and Pfanner,N. (1990) *J. Cell. Biol.*, **111**, 2353–2363.
Stone,E.M., Yamano,H., Kinoshita,N. and Yanagida,M. (1993) *Curr. Biol.*, **3**, 13–26.
Studier,F.W., Rosenberg,A.H., Dunn,J.J. and Dubendorff,J.W. (1990) *Methods Enzymol.*, **185**, 60–89.
Van der Leij,I., Franse,M.M., Elgersma,Y., Distel,B. and Tabak,H.F. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 11782–11786.
Zhang,K., Smouse,D. and Perrimon,N. (1991) *Genes Dev.*, **5**, 1080–1091.

Received on May 2, 1994; revised on July 4, 1994