Dictyostelium discoideum protein phosphatase-1 catalytic subunit exhibits distinct biochemical properties

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Protein phosphatase-1 (PP1) is expressed ubiquitously and is involved in many eukaryotic cellular functions, although PP1 enzyme activity could not be detected in the social amoeba *Dictyostelium discoideum* cell extracts. In the present paper, we show that *D. discoideum* has a single copy gene that codes for the catalytic subunit of PP1 (DdPP1c). DdPP1c is expressed throughout the *D. discoideum* life cycle with constant levels of mRNA, and its protein and amino acid sequence show a mean identity of 80 % with other PP1c enzymes. However, it has a distinctive difference: the substitution of a phenylalanine residue (Phe²⁶⁹ in the DdPP1c) for a highly conserved cysteine residue (Cys²⁷³ in rabbit PP1c) in a region that was shown to have a critical role in the interaction of rabbit PP1c with toxin inhibitors. Wild-type

DdPP1c and an engineered mutant form in which Phe²⁶⁹ was replaced by a cysteine residue were expressed in *Escherichia coli*. Both recombinant activities were similarly inhibited by okadaic acid, tautomycin and microcystin. However, the Phe²⁶⁹ → Cys mutation resulted in a large increase in enzyme activity towards phosphorylase *a* and a higher sensitivity to calyculin A. These results, together with the molecular modelling of DdPP1c structure, indicate that the Phe²⁶⁹ residue, which occurs naturally in *D. discoideum*, confers distinct biochemical properties on this enzyme.

Key words: calyculin A, phosphatase inhibitor, protein phosphatase-1 (PP1).

INTRODUCTION

Protein phosphatase-1 (PP1) is one of the major types of eukaryotic protein phosphatases. It consists of a catalytic subunit (PP1c) that interacts with a number of different regulatory subunits in a mutually exclusive manner, generating holoenzymes with unique substrate affinities and restricted subcellular localization [1–5].

PP1c belongs to the PPP (protein phosphatase P) family of serine/threonine protein phosphatases that share a conserved catalytic domain of approx. 280 amino acids [6,7]. This characteristic is reflected in their conserved spatial structures and similar catalytic mechanisms [8–10]. An important feature of the PPP enzymes is that they are targets for several drugs and toxins. For example, PP1, PP2A and other members of this family are differentially inhibited by naturally occurring toxins such as okadaic acid, which is produced by marine dinoflagellates, and microcystins, which are produced by cyanobacteria [11–13]. Indeed, the three-dimensional structure of rabbit PP1c was also determined by analysing stable complexes between the PP1c and these inhibitors [9,10,14,15].

PP1c is a protein of approx. 37 kDa with a primary structure that is highly conserved from protists to plants and mammals [16]. There is a single PP1c gene (*GLC7*) in *Saccharomyces cerevisiae*, four genes in *Drosophila melanogaster*, three genes in humans and at least eight genes in *Arabidopsis thaliana* [17–20]. *GLC7* is an essential gene and different conditional alleles in budding yeast cause defects in glycogen metabolism, mitosis, meiosis and cell-wall integrity [21,22]. In flies and mammals, individual PP1c isoforms have been implicated in distinct cellular processes, and also exhibit different tissue or subcellular distributions [23–27]. In

spite of this, PP1c isoforms are 90 % identical at the amino-acid level and exhibit indistinguishable *in vitro* activity. The current belief is that the regulation of PP1 functional diversity is mostly due to the regulatory subunits with which the PP1c isoforms interact [3–5].

Previous work demonstrated that cell extracts of the social amoeba *Dictyostelium discoideum* exhibit PP2A, PP2B, and PP2C catalytic activities, with biochemical properties similar to those of their mammalian and yeast counterparts [28,29]. The cloning and characterization of PP2B catalytic and regulatory subunits have been reported, but their roles in *Dictyostelium* development remain unclear [30]. A PP2C-like enzyme, a magnesium-dependent enzyme of the PPM (protein phosphatase M) family of serine/threonine protein phosphatases, was also cloned and was demonstrated to be essential for *D. discoideum* cell type differentiation [31]. *D. discoideum* PP2A was shown to be involved in the dephosphorylation of myosin heavy chains, promoting filament assembly [32]. In contrast, typical PP1 activity was never detected at any point in the *D. discoideum* life cycle, despite its critical importance in all other organisms analysed [28].

In the present work, we describe the cloning of *D. discoideum* PP1c (DdPP1c). DdPP1c is a single copy gene expressed ubiquitously throughout the *D. discoideum* life cycle. We expressed recombinant forms of DdPP1c in bacterial cells and tested their phosphatase activities in the presence and absence of inhibitors. We concluded that DdPP1c possesses distinct biochemical properties compared with other known PP1 enzymes, due to a single amino-acid substitution. Molecular modelling of DdPP1c confirmed this possibility. Thus it is possible that DdPP1c distinct properties make its activity undetectable in *Dictyostelium* cellular extracts.

Abbreviations used: DdCKII, casein kinase II of *Dictyostelium discoideum*; DdPP1c, PP1c of *Dictyostelium discoideum*; DdPP1cF269C, Phe²⁶⁹ → Cys mutant of DdPP1c; IPTG, isopropyl β-thiogalactoside; PP1 (etc.), protein phosphatase-1 (etc.); PP1c (etc.), catalytic subunit of PP1 (etc.); PPP family, protein phosphatase P family; RT, reverse transcriptase.

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The nucleotide sequence for the cDNA of *Dictyostelium discoideum* PP1c appears in the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AF020537.

EXPERIMENTAL

Materials

Phosphorylase *b* and phosphorylase kinase were purchased from Sigma. [γ - ^{32} P]ATP, [α - ^{32} P]dATP and [α - ^{32} P]dCTP were obtained from PerkinElmer Life Sciences. Tautomycin, calyculin A and microcystin-LR were purchased from Calbiochem and okadaic acid was from Boehringer Ingelheim. We used antibodies against both intact human PP1c (FL-18) and the C-terminus (C-19) of the human PP1c α -isoform, obtained from Santa Cruz Biotechnology, and an anti-pentahistidine antibody came from Qiagen. Oligonucleotide primers for sequencing and PCR were synthesized by Bio-Synthesis, Lewisville, TX, U.S.A.

D. discoideum culture conditions

D. discoideum AX2 or AX4 strains were grown in axenic HL-5 medium [1 % (w/v) peptone, 1 % (w/v) glucose, 0.5 % (w/v) yeast extract and 20 mM KH₂PO₄, pH 6.8] to densities of (2–6) $\times$ 10⁶ cells/ml, and the complete developmental programme was undertaken by washing cells with 20 mM phosphate buffer (20 mM KH₂PO₄, pH 6.4) before spreading them on to nitrocellulose filters as described in [33]. Transfection of cells with DNA was performed by electroporation and transformants were selected in HL-5 supplemented with 4 μ g/ml blasticidin S [34].

Isolation of DdPP1c cDNA

Genomic DNA of *D. discoideum* AX2 strain was used as a template to amplify segments of genes potentially encoding members of the PPP gene family. The pair of degenerate primers (forward primer, 5'-AGTACTGCAGGGNGACGTICACGGNCAA-3', and reverse primer, 5'-GTAGGATCCIGACTCATGATTNCCNC-3') was based respectively on the amino-acid motifs GD{V/I}HGQ and RGNHE{S/C} conserved in the catalytic core of the PPP family [6]. PCR products were subjected to electrophoresis on a 1 % (w/v) agarose gel and DNA fragments of the expected size (approx. 200 bp) were isolated from the gel and cloned into a TA-plasmid vector (Invitrogen Life Technologies). The inserts of 50 clones were sequenced and correlated with different members of the PPP family. One of the clones with high similarity to PP1c was used to screen a Lambda-ZAP (Stratagene) cDNA library derived from AX4 *D. discoideum* vegetative cells (kindly provided by Dr Hudson Freeze, The Burnham Institute, La Jolla, CA, U.S.A.). Screening of 200 000 plaques under high stringency conditions yielded eight positive clones that were subjected to *in vivo* excision from a phagemid by transformation of *Escherichia coli* XL-1 blue MRF' (Stratagene). Two pBluescript SK clones with the largest cDNA inserts were completely sequenced on both strands and the sequence of the longer one was deposited in GenBank® (accession number AF020537).

DNA sequencing was performed using a Sequenase™ Version 2.0 DNA Sequencing Kit (Amersham Biosciences) or a Taq Dye Terminator Cycle Sequencing Kit on a PerkinElmer Biosystems automated 377 DNA sequencer.

Preparation of recombinant DdPP1c

For bacterial recombinant expression of DdPP1c, the complete coding region of DdPP1 cDNA was subcloned into pTrcHis vector (Invitrogen) which allows expression of an N-terminal hexahistidine-tagged protein. The original full-length cDNA clone was used in a PCR as template with primers designed to introduce the restriction sites *Bam*HI/*Nde*I followed by the

DdPP1c start methionine, and *Xho*I/*Kpn*I after the stop codon (forward primer, 5'-GCGCGGATCCCATATGGAAATTGATT-TAGATTCA-3', and reverse primer, 5'-CGCGGTACCTCGAG-TTATTTTTTTGGTTTTTGTGTTTA-3'). The PCR fragment was digested using the newly introduced *Bam*HI and *Kpn*I restriction sites and ligated into a *Bam*HI/*Kpn*I-digested yeast expression vector pYeDP. After confirmation of the insert sequence it was subcloned into a *Bam*HI/*Xho*I-digested pTrcHis vector. The expression construct pTrcHis-DdPP1c was then used to transform *E. coli* BL21(DE3) competent cells and the expression of recombinant DdPP1c was induced with 0.3 mM isopropyl β -D-thiogalactoside (IPTG) for 20 h at 26 °C in growth medium [1 % (w/v) bacto-tryptone, 0.5 % (w/v) yeast extract, 170 mM NaCl, pH 7.5], supplemented with 50 μ g/ml carbenicillin and 0.5 mM MnCl₂, as described in [35].

Site-directed mutagenesis to change Phe²⁶⁹ into a cysteine residue in the DdPP1c was performed using the QuikChange Site-Directed Mutagenesis kit (Stratagene). The pTrcHis-DdPP1c was used as a template in a reaction with a pair of primers including a mismatched base pair as underlined (5'-CGCTCCAAATTACTG-TGGTGAATTTGATA-3' and 5'-TATCAAATTCACCA^{AGT}-AATTTGGAGCG-3'). The entire sequence of the mutant DdPP1c cDNA insert was determined and no additional mutation was found. The recombinant DdPP1cF269C protein was obtained exactly as described above.

Protein phosphatase assay

The substrate [^{32} P]phosphorylase *a* (1 $\times$ 10⁶ c.p.m./nmol) was prepared essentially as described in [36]. Phosphorylase *a* phosphatase activity in bacterial extracts was determined according to Zhang et al. [35]. Bacterial cells expressing recombinant DdPP1c were harvested by centrifugation at 4000 *g* for 25 min at 4 °C, resuspended in ice-cold lysis buffer [20 mM Tris/HCl (pH 8.0), 2 mM MnCl₂, 10 % (v/v) glycerol, 2 mM 2-mercaptoethanol and 1 mM PMSF] and lysed by sonication or by using a French press. The supernatant was obtained by centrifugation of the lysate at 20 000 *g* for 20 min at 4 °C. The cleared bacterial extracts were diluted in buffer A [50 mM imidazole (pH 7.4), 1 mM EDTA, 2 mM dithiothreitol, 2 mM MnCl₂ and 0.2 mg/ml BSA] and incubated with 30 000 c.p.m. of phosphorylase *a* in buffer B [50 mM imidazole (pH 7.2), 2 mM dithiothreitol, 0.5 mg/ml BSA, 5 mM caffeine and 0.2 mM MnCl₂] at 30 °C for 10 min in a total reaction volume of 100 μ l. The assay was terminated by the addition of 100 μ l of 10 % (w/v) trichloroacetic acid and incubation on ice for 3 min. The protein suspension was centrifuged at 16 000 *g* for 4 min at room temperature (24 °C) and [^{32} P]phosphate released into the supernatant was measured by liquid scintillation counting. Total substrate consumption was kept below 20 % to ensure linear reaction rates. Toxins were diluted in 10 mM Tris/HCl (pH 7.0) and were pre-incubated with the enzyme at 30 °C for 10 min.

Immunoblotting

D. discoideum whole-cell lysates or supernatants from bacterial extracts were separated by SDS/PAGE (10 % gel) [37] and transferred on to nitrocellulose membranes using the semi-dry method as described in [38]. Immunoblots were probed with each primary antibody diluted 1:100 (anti-PP1c) or 1:2000 (anti-pentahistidine) in blocking solution [10 mM Tris/HCl (pH 7.4), 150 mM NaCl and 5 % (w/v) non-fat powdered milk] overnight at 20–22 °C. The membranes were rinsed for 1 h with 10 mM Tris/HCl (pH 7.4), 150 mM NaCl and 0.1 % (w/v) Tween 20

before immunodetection with horseradish-peroxidase-conjugated secondary antibodies using the Enhanced Chemiluminescence (ECL®) Western Blotting System (Amersham Biosciences).

RNA and DNA analysis

Total RNA was prepared from various *D. discoideum* developmental stages using the TRIzol® reagent (Invitrogen), as described by the manufacturer. The RNA samples were quantified by UV absorption and were checked for integrity by formaldehyde-agarose gel electrophoresis [38]. Reverse transcriptase (RT)-PCRs were performed using the 3' RACE System (Invitrogen) with 1 µg of total RNA for each oligo-dT-primed reverse transcription. An aliquot of the reaction was then subjected to amplification using DdPP1c-specific primers (5'-CACCCAT-CATAGCAGCAGCG-3' and 5'-ACATTCAAGGTTGGGA-AG-3') as well as DdCKII (*D. discoideum* casein kinase II [39])-specific primers (5'-GAATATTGGAAGTATGAA-3' and 5'-ATGATCAATCATAACGTT-3'). The reactions were limited to 18 cycles to keep a linear range and therefore produce semi-quantitative data. RT-PCR products were analysed by Southern blotting using the DdPP1c cDNA or DdCKII gene fragment as probes.

Southern blots were performed as previously described in [38] and were probed with gel-purified DNA fragments radiolabelled with [α -³²P]dATP and [α -³²P]dCTP by random hexanucleotide priming method (Random Primers DNA Labeling System; Invitrogen).

Molecular modelling of DdPP1c

Three-dimensional modelling of wild-type DdPP1c and the DdPP1cF269C mutant was performed by homology building using, as a reference, the atomic co-ordinates from rabbit PP1c α -isoform bound to microcystin-LR (code 1FJM) available from the Protein Data Bank (PDB) [9]. The calyculin A and okadaic acid were docked on to the *D. discoideum* PP1c model structures using the atomic co-ordinates of PP1-okaadaic acid or PP1-calyculin A crystal structure complexes (PDB codes 1JK7 and 1IT6, respectively) as references for the inhibitors' relative orientation [12,14]. Then, the structures were fully relaxed.

Models were refined by successive steps of geometry optimization and simulated annealing. Simulated annealing refinement consisted of 20 ps steps at each of the descending temperatures, 300 K, 200 K and 100 K, after 15 ps of equilibration. Energy minimizations were carried out by successive use of Steepest Descent and Conjugate Gradient minimizers until the final energy derivative was 0.05 kcal/mol per Å (1 kcal $\approx$ 4.184 kJ and 1 Å = 0.1 nm). All potential-energy evaluations and minimizations were performed using the CFF91 forcefield and the dielectric constant $\epsilon = 80$ in order to simulate the aqueous environment of the protein. Both calculations, as well as model building, were performed on an IBM-RS6000 model 3AT workstation using the INSIGHT-II package (MSI/Accelrys, San Diego, CA, U.S.A.).

RESULTS

Sequence analysis of the cDNA encoding *D. discoideum* PP1c

Previous attempts to detect a PP1-like enzymic activity in *D. discoideum* cell extracts were unsuccessful [28]. Given the ubiquitous presence of this enzyme in all other organisms examined, we reasoned that this failure might be due to some unusual biochemical property of *D. discoideum* PP1c that prevents its

Figure 1 Comparison of DdPP1c with representative members of the PP1c family

The deduced amino-acid sequence of the cDNA for *D. discoideum* PP1c (GenBank® accession number 2443826, DdPP1) was aligned to PP1c from *S. cerevisiae* (GenBank® accession number 603372, ScGlc7), PP1c α -isoform (GenBank® accession number 130704, HsPP1a) and PP1c γ -isoform (GenBank® accession number 548573, HsPP1g) from *Homo sapiens*, PP1c α -isoform from *Oryctolagus cuniculus* (GenBank® accession number 67255, OcPP1a) and from *Drosophila melanogaster* (GenBank® accession number 1346751, DmPP1a) using CLUSTAL W [41]. Identical and conserved amino acids are denoted with * and ·, respectively. Semi-conservative changes are indicated with a single dot. Black circles mark residues previously detected to be involved in catalysis, or binding to metal ions, substrates or regulatory subunits. The arrows mark amino-acid substitutions unique to DdPP1c.

detection by established protein phosphatase enzymic assays. To search for a PP1c-like enzyme as well as other unknown members of the PPP family in *D. discoideum*, we designed a two-step cloning strategy. First, a pair of degenerate primers corresponding to conserved regions in the catalytic core of the PPP enzymes were used to amplify fragments containing approx. 192 bp from genomic DNA of *D. discoideum*. Then, PCR products encoding PPP-like enzymes were used to probe a cDNA library of *D. discoideum*.

Using a PCR product highly similar to known PP1c (results not shown), we isolated a 1182 bp cDNA (DdPP1c, GenBank® accession number AF020537). This cDNA has an open reading frame of 963 bp that encodes a putative protein of 321 amino acids. This open reading frame starts at an ATG initiator codon, surrounded by a sequence (AAAAAGAATGG) that matches very well with the consensus for *D. discoideum* translation initiation [40]. The predicted protein encoded by DdPP1c cDNA displays high similarity (85–90%) to PP1c from human, rabbit, *Drosophila* and yeast (Figure 1). Most residues proposed to be involved in catalysis, or binding to metal ions, substrates or regulatory subunits [6,8,9,16] are conserved in DdPP1c as indicated in the alignment shown in Figure 1. Despite this overall

Figure 2 Expression of DdPP1c during growth and development

Levels of DdPP1c (A) and casein kinase II (CKII) (B) transcripts were detected by semi-quantitative RT-PCR followed by Southern blot analysis using DdPP1c- and CKII-specific probes respectively. Lane 1, growth phase cells; lane 2, 30-min-starved cells; lane 3, 2-h-starved cells; lane 4, 6-h-starved cells (streaming); lane 5, mounds; lane 6, tipped mounds; lane 7, fingers; lane 8, early culminants; lane 9, culminants; lane 10, fruiting bodies. Cells were harvested at identical stages for analysis of DdPP1c protein levels by immunoblotting with anti-PP1c (C). Size markers in bp (A) and (B) or in kDa (C) are indicated on the right. (D) Identical Western blots from vegetative (lanes 1 and 3) and aggregation phase cell extracts (lanes 2 and 4) were probed with anti-PP1c and with anti-PP2Ac antibodies. The relative migration of PP1c and PP2Ac polypeptides is indicated.

high conservation, we detected few amino-acid changes in DdPP1c that are conserved between other PP1c sequences. Among them, a well-conserved cysteine residue present in all other eukaryotic PP1c enzymes (position 273 of rabbit PP1c α) is replaced by a phenylalanine residue in DdPP1c (position 269 of *D. discoideum* sequence) (Figure 1).

The amino-acid substitutions in DdPP1c were further confirmed by a number of approaches including sequencing another PP1c cDNA clone isolated from the same library. We also sequenced PCR products of genomic fragments amplified with DdPP1c-specific primers from at least two different cell strains (results not shown). Moreover, sequences of ESTs and of genomic clones from *D. discoideum* databases [42,43] were used to confirm the nucleotide sequence for the DdPP1c cDNAs we have isolated.

We concluded that DdPP1c gene appears to be present as a single copy and intronless gene, based on PCR with DdPP1c-specific primers and Southern blot analysis of *D. discoideum* genomic DNA cleaved with several restriction endonucleases (results not shown). A single perfect match of DdPP1 cDNA nucleotide sequence on *D. discoideum* chromosome 2 further supports these conclusions [43].

Regulation of the DdPP1c expression

The temporal expression pattern of DdPP1c was monitored by RT-PCR with sequence-specific primers and by immunoblots using anti-PP1c antibodies. As shown in Figure 2(A), DdPP1c mRNA is constitutively expressed throughout the life cycle without significant variation. As a control for these experiments, we used DdCKII-sequence-specific primers in parallel reactions (Figure 2B). Casein kinase II is expressed constitutively at similar levels throughout the *D. discoideum* life cycle [39].

The level of DdPP1c protein was also examined at *D. discoideum* growth and different developmental stages. As seen in

Figure 2(C), the protein is detected throughout all phases analysed correlating well to the mRNA expression data (Figure 2A). The anti-PP1c antibody detected a polypeptide of approx. 39 kDa according to its migration by SDS/PAGE, whereas the calculated molecular mass for the deduced amino acid sequence of DdPP1c is 36.9 kDa. To assure the specificity of detection, growing-cell extracts were probed in parallel with anti-PP1c and with a polyclonal antibody prepared against recombinant DdPP2Ac (kindly provided by R. Dottin, Hunter College, New York, NY, U.S.A.), which has a molecular mass of 37 kDa [30,33]. Figure 2(D) shows that both antibodies detect polypeptides with different migration rates. We also tested the anti-PP1c antibody against purified DdPP2Ac and did not detect cross reaction (results not shown).

To test the role of DdPP1c in normal growth and development, we attempted to knock out the gene by the gene-replacement technique [44]. For this, we used a cDNA DdPP1c construct where the coding region of PP1c was interrupted by insertion of a cassette that confers resistance to blasticidin S [34]. More than 1000 resistant clones of *D. discoideum* were selected in medium containing this blasticidin S after four independent transfection rounds. Southern blot analysis of restricted genomic DNA of 40 randomly selected clones revealed integration of the truncated PP1c cDNA, but not homologous recombination (results not shown). These observations suggest that PP1c knockout impairs the growth of *D. discoideum*. Inactivation of the PP1c gene in *S. cerevisiae* was also shown to be lethal [17], and, indeed, it has been demonstrated that PP1c has essential roles in the regulation of the cell-cycle progression in several species [21,23,25,27].

Biochemical properties of DdPP1c

With the results presented above, we showed that *D. discoideum* has a PP1-like gene that is expressed in all phases of *Dic-tyostelium*'s life cycle, and it is probably an essential gene. It is not clear, though, why the PP1c activity in *D. discoideum* cellular extracts was not detected in previous experiments ([28], and A.M. da Silva, unpublished work). To examine the enzymic properties of the wild-type DdPP1c, we expressed it as a recombinant protein in bacteria.

Despite the difficulties that have been reported to obtain soluble recombinant PP1c in bacteria [35], we were able to express soluble and active enzymes in *E. coli*. The amount of the recombinant protein was not enough to identify the DdPP1c after gel protein staining, but, as shown in Figures 3(A) and 3(B), it could be detected with the anti-PP1c antibody in immunoblots. Using the soluble fraction of bacterial lysates, we were able to detect phosphorylase phosphatase activity from cultures that expressed DdPP1c, but not from non-induced ones (Figure 3C). We chose to use soluble bacterial extracts in the assays because DdPP1c activity is quite unstable during the purification steps (L.P.M. Andrioli, unpublished work).

As mentioned above, DdPP1c cDNA has an amino-acid substitution of a phenylalanine residue for a well conserved cysteine residue. It is worth noting that this substitution is observed only in *D. discoideum*, after examining all other known PP1 catalytic subunits ([6], and A.M. da Silva, unpublished work). Furthermore, the region that contains this cysteine residue has been investigated for crucial enzymic properties of PP1c, including its binding to toxin inhibitors [13,45–48]. Thus we decided to investigate if the presence of Phe²⁶⁹ affects the properties of DdPP1c by engineering a mutation of Phe²⁶⁹→Cys and comparing the biochemical properties of both enzymes expressed in bacteria.

Figure 3 Phosphorylase phosphatase activity of recombinant wild-type and mutated DdPP1c

(A) Equal amounts of total protein from the soluble fraction of lysates from bacteria transformed with pTrcHis empty vector (lane 1) or with the constructions pTrcHisDdPP1c induced with IPTG (lane 3) or not induced (lane 2) were analysed by immunoblotting with anti-PP1c antibody. The arrow points to the relative migration of recombinant DdPP1c and the molecular mass markers in kDa are indicated. Note the leaky expression of the pTrcHisDdPP1c construct (lane 2). (B) The indicated protein amounts (1, 5 and 10 µg) of soluble lysates prepared from bacterial cultures expressing wild-type (DdPP1c) and mutant PP1c (DdPP1cF269C) were analysed by immunoblotting with anti-PP1c followed by densitometric scanning. The upper panel shows a representative immunoblot and the lower panel demonstrates the means $\pm$ S.D. ($n=3$) from densitometric analysis of immunoblots. (C) Phosphorylase phosphatase activity of the soluble fraction of bacterial lysates prepared from cultures expressing DdPP1c or DdPP1cF269C induced (grey bars) or not (black bars) with IPTG was assayed for 10 min at 30 °C with equal amounts of total bacterial soluble protein. (D) Different amounts of total protein from soluble fraction of bacterial lysates expressing DdPP1c (□) or the DdPP1cF269C (■) were assayed for phosphorylase phosphatase activity for 10 min at 30 °C. The substrate consumption is expressed as a percentage of total labelled phosphorylase *a* added to the reaction. Results are presented as means $\pm$ S.D. from three independent experiments with duplicate measurements.

The levels of expression of the wild-type DdPP1c and the mutated enzyme (DdPP1cF269C) were similar upon IPTG induction of bacterial cultures as shown in Figure 3(B). However,

Figure 4 Effect of toxin inhibitors on phosphorylase phosphatase activity of DdPP1c and DdPP1cF269C recombinant enzymes

Soluble fractions of bacterial lysates prepared from cultures expressing wild-type (DdPP1c; open symbols) and mutant PP1c (DdPP1cF269C; closed symbols) were assayed for phosphorylase phosphatase activity for 10 min at 30 °C in the presence of increasing concentrations of okadaic acid and microcystin-LR (A, squares and diamonds respectively), tautomycin and calyculin (B, triangles and circles respectively). PP1c activity is expressed as the percentage of activity in the absence of inhibitors. Results are presented as means $\pm$ S.D. from 3–5 independent experiments each carried out in duplicate.

the phosphorylase-phosphatase-specific activity of recombinant DdPP1c is approx. 6–18-fold lower when compared with DdPP1cF269C (Figure 3C). The differences between wild-type DdPP1c and DdPP1cF269C enzymic activities can be better observed in assays where their rate of substrate consumption was measured (Figure 3D). The wild-type DdPP1c does not exceed 20% of substrate consumption, in contrast with DdPP1cF269C, which can hydrolyse > 80% of labelled phosphorylase *a*.

To compare further wild-type DdPP1c with DdPP1cF269C, we checked their sensitivities to specific protein phosphatase inhibitors. We found that okadaic acid (Figure 4A), as well as tautomycin (Figure 4B), inhibited both recombinant forms in a similar manner, but calyculin A did not (Figure 4B). For okadaic acid, there was only a modest difference between the determined IC_{50} values: 150 nM for DdPP1c and 80 nM for DdPP1cF269C, and for tautomycin, the calculated IC_{50} values were almost identical (approx. 0.50 nM). On the other hand, calyculin A inhibited DdPP1cF269C more efficiently than wild-type DdPP1c (Figure 4B). The calculated IC_{50} values were 2.07 nM for DdPP1c and 0.13 nM for DdPP1cF269C. For other PP1c enzymes,

Figure 5 Superposition of carbon backbone from model structures of rabbit PP1c α , wild-type and mutant DdPP1c

The carbon backbone from model structures of rabbit PP1c α (blue), DdPP1c (red) and DdPP1cF269C (yellow) were superimposed. Lateral chain of Phe²⁶⁹ and Cys²⁶⁹ are shown in stick representation in red and yellow respectively. The orientation of the hydrophobic groove within the Y-shaped groove and of the β 12- β 13 loop previously reported on the surface of PP1c is indicated. The models were built based on their similarity to rabbit PP1c α complexed with microcystin-LR (PDB code 1FJM).

the IC₅₀ of calyculin A was 0.3–0.7 nM, consistent with that obtained for DdPP1cF269C [49].

The Cys²⁷³ in the rabbit PP1c is known to bind covalently to microcystin-LR, another potent inhibitor of protein phosphatases from the PPP family [13]. Thus we tested the ability of microcystin-LR to inhibit the wild-type DdPP1c and the mutated enzyme DdPP1cF269C. As shown in Figure 4(A), only a slight decreased sensitivity to microcystin-LR was detected for the wild-type in comparison with the mutated enzyme. However, the IC₅₀ for both forms are in the nanomolar range, typical of other PP1c enzymes. Therefore, despite the covalent linkage of microcystin to the cysteine residue, this appears to be inessential for the inhibition of the enzyme by this toxin [45].

Molecular modelling of DdPP1c

We then investigated if the unusual biochemical properties exhibited by the wild-type version of the recombinant DdPP1c could be correlated with alterations in the three-dimensional structure of the enzyme. Molecular models for both wild-type and mutated versions of DdPP1c were generated based on the known three-dimensional structure of the rabbit PP1c α complexed with microcystin-LR [9]. As can be seen in Figure 5, the carbon backbone of both models of DdPP1c superimpose quite well with rabbit PP1c α and are remarkably similar to the four PP1c structures determined previously [8,9,14,15]. This indicates that the presence of Phe²⁶⁹ in the β 12- β 13 loop does not affect the overall structure of DdPP1c. Moreover, the Y-shaped groove previously reported on the surface of PP1c [8,9], which consists of a hydrophobic groove, a C-terminal groove and an acidic groove, also appears to maintain its conformation in both models.

However, local changes in the Y-shaped groove are evident if we compare the solvent accessible surface models of DdPP1c and

DdPP1cF269C bound to calyculin A or okadaic acid (Figure 6). A spatial rearrangement in the β 12- β 13 loop in the vicinity of the bifurcation of the Y-shaped groove begins with the establishment of aromatic interactions of Phe²⁶⁹ with the Tyr²⁶⁸ side chain. Previous studies with other PP1c enzymes proposed that the conformation of the β 12- β 13 loop is essential for the association of PP1c with inhibitors [13,45–48] and the tyrosine residue at position 272, which corresponds to position 268 in DdPP1c, has been pointed to have a key role in the binding of these toxins [9,14,15,47]. Indeed, Tyr²⁶⁸ is located on the threshold of the catalytic site, and when its side chain is drawn to the vicinity of Phe²⁶⁹ (rings face-to-side distance of 4.02 Å, where 1 Å = 0.1 nm), the distance of its hydroxyl oxygen from the nearest metal ion atom increases to 7.13 Å. In the absence of an aromatic interaction, e.g. for the DdPP1cF269C mutant, this value would be 2.32 Å. This may interfere with enzyme activity as well as the binding of PP1c inhibitors. As shown before, recombinant wild-type DdPP1c is considerably less active than recombinant DdPP1cF269C mutated form.

The conformation changes we have observed extend to the hydrophobic groove responsible for the inhibitors' predominant interactions with the protein. The hydrophobic groove appears to be narrower in DdPP1c than in DdPP1cF269C when comparing the models (results not shown). The sum of both Tyr²⁶⁸–Phe²⁶⁹ reorientation and hydrophobic groove deformation effects may account for the differential behaviour we observed in our modelling studies of calyculin A docking to DdPP1c and DdPP1cF269C structures. Calyculin A appears to have little tendency to induce the mutual fitting when positioned in the wild-type DdPP1c active site and prefers to quit the pocket. Such difficulty was not observed for the DdPP1cF269C–calyculin A complex formation (Figure 6B), which underwent a successful relaxation upon docking of the inhibitor. Only by means of a very careful stepwise and rigorous ligand positioning into the active site would the wild-type DdPP1c lead to a relaxed stable complex (Figure 6A). This indicates that there might be a difference in the ligand recognition by the protein, more precisely in the complex activation energy, since the Tyr²⁶⁸–Phe²⁶⁹ tandem hinders the calyculin A entrance into the site. However, once the ligand is bound, the complex is as stable as in the DdPP1cF269C–calyculin A complex. These observations are consistent with the relative sensitivity of wild-type and mutant enzymes to inhibition (Figure 4B), where the wild-type DdPP1c recombinant enzyme appears to be more resistant to calyculin A inhibition relative to the mutated DdPP1cF269C. It is interesting to note that the recently resolved crystal structure of PP1c bound to calyculin has shown that Tyr²⁷² (Tyr²⁶⁸ in DdPP1c) is the only residue that interacts with calyculin A within the β 12- β 13 loop [15].

On the other hand, according to our modelling experiments, the hydrophobic tail of okadaic acid binds to the hydrophobic groove of both forms of PP1c without major conformational differences (Figures 6C and 6D), despite the distortion in the acidic portion of okadaic acid seen in its complex to DdPP1c. It has been reported that Tyr²⁷² interacts through a hydrogen bond to the acid group of okadaic acid and that this interaction together with the hydrophobic and hydrogen bonding to Arg²²¹ accounts for its inhibitory activity [14,47]. However, our observations suggest that, in the case of DdPP1c, the interaction of Tyr²⁷² (Tyr²⁶⁸ in DdPP1c) with okadaic acid seems to be of minor importance as both wild-type and DdPP1cF269C recombinant enzymes show a similar sensitivity to okadaic acid in their expected IC₅₀ values (Figure 4A). Our results also show that tautomycin sensitivity is not affected by the replacement of Cys²⁶⁹ with a phenylalanine residue (Figure 4B).

Figure 6 Docking of calyculin A and okadaic acid into wild-type and mutant DdPP1c three-dimensional models

Molecular model of wild-type DdPP1c (**A**) and (**C**) and mutant DdPP1cF269C (**B**) and (**D**) complexed with calyculin A (**A**) and (**B**) or with okadaic acid (**C**) and (**D**). Solvent accessible surfaces are shown in blue with Phe²⁶⁹ in red, Cys²⁶⁹ in yellow and Tyr²⁶⁸ in white. The inhibitors are shown in stick representation. The models were based on their similarity to rabbit PP1 α complexed with microcystin-LR (PDB code 1FJM). Calyculin A and okadaic acid were docked on to DdPP1c model structures using the crystal structures of PP1–okadaic acid or PP1–calyculin A complexes (PDB codes 1JK7 and 1IT6 respectively) as a reference for the inhibitors' relative orientation.

DISCUSSION

The results of the present study show that the *D. discoideum* genome has a single copy gene encoding a PP1c-like enzyme that is expressed throughout its life cycle. The DdPP1c cDNA encodes an active enzyme as shown by expression of a recombinant form in bacterial cells. Taken together, these observations lead us to conclude that *D. discoideum* expresses an active PP1 catalytic subunit, despite unsuccessful attempts to detect a PP1-like activity in cellular extracts [28], and A.M. da Silva, unpublished work). It was suggested that DdPP1c may be expressed at levels that are undetectable by the methodology used [28]. This seems unlikely as we were able to detect DdPP1c protein present in *D. discoideum* cellular extracts by immunoblotting. Nevertheless, wild-type DdPP1c recombinant protein showed lower phosphorylase phosphatase activity levels when compared with a mutated recombinant form that has an amino-acid sequence that resembles a more typical PP1c (see below). Thus it is possible that DdPP1c is undetected because of its intrinsic low activity.

It is intriguing that, despite the high degree of conservation of the DdPP1c amino-acid sequence, a well-conserved cysteine residue in other PP1c enzymes is replaced by a phenylalanine in the DdPP1c sequence (position 269 in DdPP1c, which

corresponds to position 273 of rabbit PP1c). It was even more surprising that this substitution apparently renders *D. discoideum* a less active enzyme. Although Phe²⁶⁹ does not cause significant alterations in the overall three-dimensional structure of the DdPP1c protein, it clearly affects the local structure surrounding this residue. According to our modelling, the local alteration interferes not only with the enzymic activity, but also with the binding kinetics of the specific inhibitor calyculin A, but does not appear to interfere with the binding of okadaic acid, microcystin-LR or tautomycin. Indeed, our enzymic assays confirmed these observations.

The resistance of DdPP1c to calyculin A could be a clue to why the *D. discoideum* enzyme contains this unexpected amino-acid substitution. Resistance to inhibitors was observed for PP1-like enzymes in cyanobacterial strains [50]. In these organisms, their PP1-like enzymes are protected against microcystin-LR, which they produce as a secondary metabolite and accumulate within their cells. In this context, it is tempting to speculate that Phe²⁶⁹ in the DdPP1c sequence could provide resistance to a natural toxin that mimics calyculin A present in *D. discoideum*'s niche.

Alternatively, the substitution of phenylalanine for Cys²⁶⁹ in DdPP1c may reflect a requirement for *D. discoideum* substrates or regulatory proteins. There are several known examples of

inhibitory proteins interacting with PP1c in other species. It has been suggested that the interaction of PP1c with inhibitory proteins is a mechanism that keeps the enzyme off, but still available for recruitment by other regulatory subunits [3–5]. However, no regulatory or inhibitory subunits for PP1c have yet been described in *D. discoideum*. With the DdPP1c cDNA available, it now becomes feasible to search for proteins that might interact and regulate this enzyme during *D. discoideum* growth and development. This would then lead to an explanation of the problems in detecting DdPP1c activity in *D. discoideum* cell extracts.

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