Identification and Characterization of an Unusual Double Serine/Threonine Protein Phosphatase 2C in the Malaria Parasite Plasmodium falciparum

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We have cloned a gene from Plasmodium falciparum with homology to the Mg$^{2+}$-dependent serine/threonine protein phosphatase 2C (PP2C) family. The predicted coding region is 920 amino acids long, twice the size of other members of this family. We show that this predicted protein can be divided into two halves (PF2C-1 and PF2C-2), each a complete phosphatase unit with homology to other phosphatases of this class. To study the function of this PP2C, we have tested the ability of different constructs to complement conditional null mutants of yeast. Our results show that expression of the full-length protein, the first half alone, the second half alone, or a hybrid with the N terminus of the first half and the C terminus of the second half was able to complement the heat shock response defect of a Schizosaccharomyces pombe strain with a PP2C (PTC1) deletion. Recombinant P. falciparum PP2C expressed in Escherichia coli was active in dephosphorylating $^{32}$P-labeled casein in an Mg$^{2+}$- or Mn$^{2+}$-dependent reaction. Each half alone was also active in recombinant form. Using the two-hybrid system, we have shown that the two halves can interact. Gel filtration assay of P. falciparum protein extracts suggests that full-length PFP2C is a dimer, and phosphatase activity competition experiments indicate that dimerization of PFP2C is required for its optimal activity. This unusual phosphatase molecule appears to be composed of four catalytic units on two polypeptide chains.

Two major families of protein phosphatases (phosphoprotein phosphatase or PPP and Mg$^{2+}$-dependent protein phosphatase or PPM) catalyze the dephosphorylation of serine/threonine residues (1–3). In both eucaryotes and procaryotes, the Mg$^{2+}$-dependent phosphatase or PPP of large and varied family whose defining member is PP2C. PP2C is present in cells at lower levels than other members of the serine/threonine phosphatase family (4). From bacteria to mammals, PP2C is a monomeric protein with a molecular mass range of 40–50 kDa and requires divalent metal ions (Mg$^{2+}$ or Mn$^{2+}$) for its activity (5).

It is becoming clear that the PP2C proteins are involved in regulating stress response pathways in both procaryotes and eucaryotes. In Saccharomyces cerevisiae and Schizosaccharomyces pombe, the PP2C genes PTC1 and PTC3 were shown to be involved in the regulation of protein kinase cascades that are induced in response to heat shock or osmotic changes (6, 7). In mammalian hepatocytes, PP2C reverses, by dephosphorylating the AMP-activated protein kinase, the inhibition of cholesterol and fatty acid biosynthesis (8). Similarly, in Bacillus subtilis, the protein phosphatase SpoIIE regulates the steady-state level of phosphorylation of SpoIIAA, an anti-transcription factor, to control sporation in response to ADP/ATP ratios (9, 10). WIP1, a human PP2C-like gene, has been isolated as a transcript that is highly expressed in response to ionizing radiation (11).

Some proteins in the Mg$^{2+}$-dependent protein phosphatase family contain, in addition to a catalytic region, a domain involved in other physiological functions. Thus, the N-terminal domain of ABI1 in Arabidopsis thaliana contains a consensus sequence for a calcium-binding EF-hand (12, 13). This domain may gauge the level of calcium in the cytoplasm in response to abscisic acid hormone and determine the level of phosphorylated proteins required for the maintenance of the cell cycle or stomatal aperture (13). SpoIE of B. subtilis contains a region with 10 membrane-spanning segments (9, 10), and the S. cerevisiae adenylyl cyclase contains an upstream region of 300 amino acids that shares sequence similarity with PP2C (14).

This work describes the isolation of a PP2C-like protein, PIP2C, from the malaria parasite Plasmodium falciparum. Each year, P. falciparum is estimated to kill between 1.5 and 2.7 million people, the majority of whom are children under five (15). Because the parasite and mosquitoes have developed resistance to drugs and pesticides, the identification of new targets for prophylaxis and treatment is urgently needed. The P. falciparum PP2C is composed of two active phosphatases fused together. The two units of PIP2C complement a yeast deletion mutant, suggesting that this protein, like its homologues, may be active in response to osmolarity changes or other stress. PIP2C may play a role in protecting the parasite against changes in the erythrocyte environment during infection.

EXPERIMENTAL PROCEDURES

Strains, Growth Conditions, and Materials—The strains and plasmids used in this study are listed in Table I. The Edinburgh minimal medium contained 0.3% potassium hydroxym phthalate, 0.22% sodium phosphate, 0.5% ammonium chloride, 2% dextrose, 0.21% minimal salts, 0.02 g/liter vitamians, and 3 mg/liter trace elements. Parasite Culture—Clone HB3 of P. falciparum (a gift of Dr. W. Trager, Rockefeller University) was grown using the method developed by Trager and Jensen (16). Albumax replaced serum in the culture medium.

Molecular Biology—All enzyme reactions, DNA and RNA extrac-
| Strains/plasmids | Description | Source or Ref. |
|------------------|-------------|----------------|
| S. cerevisiae | MATα, gal4, gal80, his3, trp1-901, ade2-101, ada3-52, leu2-3, -112 | Durfee et al. (22) |
| Y190 | (URA3::GAL-LacZ, LYS2::GAL-HIS3 cyh') | |
| S. pombe | Wild-type strain | Mitchinson (31) |
| h72h-1 | h, leu1-32, ura4-D18, ptc1::LEU2 | Schiozaki et al. (24) |
| K584 | Plasmid vector suitable for fusing genes to the ADH promoter | Vernet et al. (32) |
| pVT-U2 | Plasmid vector suitable for fusing genes to the GAL1, GAL7, or GAL10 promoter | Johnston and Davis (33) |
| pBM272 | | |
| pSF172 | nmt expression vectors for fusing genes to be fused to C terminus | Forsburg and Sherman (34) |
| pGPPPP-2C | Derivative of pBM272 expressing PIPP2C under GAL1 promoter | This work |
| pGP2C-1 | Derivative of pBM272 expressing P2C-1 under GAL1 promoter | This work |
| pGP2C-2 | Derivative of pBM272 expressing P2C-2 under GAL1 promoter | This work |
| pGP2C-Δ | Derivative of pBM272 expressing P2C-Δ under GAL1 promoter | This work |
| pAPPPP-2C | Derivative of pVT-U2 expressing PIPP2C under ADH promoter | This work |
| pAP2C-1 | Derivative of pVT-U2 expressing P2C-1 under ADH promoter | This work |
| pAP2C-2 | Derivative of pVT-U2 expressing P2C-2 under ADH promoter | This work |
| pAP2C-Δ | Derivative of pVT-U2 expressing P2C-Δ under ADH promoter | This work |
| pSF172-PIPP2C | Derivative of pSF172 expressing PIPP2C under nmt promoter | This work |
| pSF172-P2C-1 | Derivative of pSF172 expressing P2C-1 under nmt promoter | This work |
| pSF172-P2C-2 | Derivative of pSF172 expressing P2C-2 under nmt promoter | This work |
| pSF172-P2C-Δ | Derivative of pSF172 expressing P2C-Δ under nmt promoter | This work |
| pACTII | Gal4 DNA activation domain under ADH promoter | Durfee et al. (22) |
| pAS-CYH2 | Gal4 DNA-binding domain under ADH promoter | Durfee et al. (22) |
| pACTII-P2C-1 | Derivative of pACTII expressing P2C-1 fused to Gal4 DNA activation domain | This work |
| pAS-CYH2-2 | Derivative of pAS-CYH2 expressing P2C-2 fused to Gal4 DNA-binding domain | This work |
| pAS-CYH2-P2C-2 | | |
| pAS-CYH2-P2C-Δ | Derivative of pAS-CYH2 expressing P2C-Δ fused to Gal4 DNA-binding domain | This work |

**Isolation of a New PP2C Homologue in P. falciparum**—Using a cDNA library made from asexual stages of the red blood cell parasite *P. falciparum*, we isolated a full-length cDNA clone encoding a protein with homology to PP2C enzymes. The 3.7-kilobase pair cDNA (GenBank™ accession number AF023665) has an open reading frame (PIPP2C) starting at position 391. The first ATG codon has a standard nucleotide sequence for translation initiation. PIPP2C is predicted to encode a protein of 920 amino acids with a molecular mass of 103 kDa, which is twice the size of other PP2C proteins. Homology searches conducted for the PIPP2C protein sequence revealed a significant similarity to PP2C proteins from different organisms. Computer alignments revealed that certain PP2C sequences in the data base matched best with the N-terminal part of PIPP2C (P2C-1), whereas others matched best the C terminus of PIPP2C (P2C-2). Fig. 1 shows the alignment of both P2C-1 and P2C-2 with PP2C proteins from different organisms. Conserved motifs needed for phosphatase function in other organisms (20) are conserved in the *Plasmodium* sequence, but otherwise, homology is remote. The two halves share distant homology, suggesting that PIPP2C may have resulted from the fusion of two ancestral phosphatases during evolution.

**PIPP2C Expression during the Intracytoplasmic Cycle of the Parasite**—To examine PIPP2C expression in *P. falciparum*, we generated polyclonal antibodies against an internal 100-amino acid portion of PIPP2C fused to GST. On a Western blot of parasite cell extract (Fig. 2), the immune serum (second through fourth lanes), but not the preimmune serum (first lane), recognized a ~103-kDa protein in all intraerythrocytic stages of the parasite.

**PIPP2C Is a Double Serine/Threonine Protein Phosphatase**—To test whether the full-length protein has a cation-dependent phosphatase activity, PIPP2C was fused to the C-terminal region of GST, and the fusion protein was purified and
tested for phosphatase activity (Fig. 3A). Recombinant PfPP2C is active in the dephosphorylation of casein and is highly activated in the presence of Mg\(^{2+}\) or Mn\(^{2+}\). The activity was minimally inhibited by 5 μM okadaic acid, a compound effective in the blockage of type 1 and 2A phosphatases.

Because PfPP2C is twice the size of normal PP2C proteins, and each half has homology to other PP2C proteins, we were interested in knowing if both halves can function independently. To test this, the first half alone (Pf2C-1), the second half alone (Pf2C-2), and a hybrid form with the N-terminal part of the first half and the C-terminal part of the second half (Pf2C-D) were expressed as GST fusion proteins in E. coli (Fig. 3B). The purified proteins were then assayed for phosphatase activity. The results in Fig. 3C show that each construct had phosphatase activity that was stimulated by Mg\(^{2+}\) or Mn\(^{2+}\), suggesting that both halves and the hybrid form possess PP2C activities. The activity obtained with each half alone (Pf2C-1 or Pf2C-2) was substantially lower than that obtained with full-length PfPP2C (Fig. 3A), suggesting that the presence of both phosphatases in the same molecule is necessary for optimal activity.

The Two Units (Pf2C-1 and Pf2C-2) Interact with Each
Other—The fact that PIPP2C is composed of two fused phosphatases raised the question of whether these two units interact with each other. We used the two-hybrid system in *S. cerevisiae*, expressing Pf2C-1 downstream of the Gal4 DNA activation domain (pACTII-Pf2C-1) and Pf2C-2 or the hybrid form downstream of the Gal4-binding domain (pAS-CYH2-Pf2C-2 or pAS-CYH2-Pf2C-Δ, respectively) (21). The plasmids obtained were used to transform the yeast strain Y190, and the Leu+,Trp+ transformants carrying both plasmids were tested for β-galactosidase expression using the X-gal filter lift assay (22, 23). In transformants expressing each vector alone (data not shown) or coexpressing pACTII-Pf2C-1 and pAS-CYH2-Pf2C-Δ (Fig. 4), no β-galactosidase expression was detected. In contrast, the transformants carrying pACTII-Pf2C-1 and pAS-CYH2-Pf2C-2 became blue in the presence of X-gal, indicating that the β-galactosidase gene was being transcribed and translated. This suggests that the two halves (PTC-1 and PTC-2) interact with each other.

**The Dimerization of PIPP2C Is Necessary for Full Activity**—Results from the two-hybrid system experiments suggest two hypotheses about the interaction between the two units (PTC-1 and PTC-2) in vivo. First, there could be an intramolecular association. Alternatively, PIPP2C may form dimers in which the PTC-1 of one polypeptide can interact with the PTC-2 of another. In an attempt to distinguish between these two possibilities, proteins from *P. falciparum* extracts were separated on a Superose 12 gel filtration column, and the fractions collected were then tested for phosphatase activity in the presence of Mg2+ and okadaic acid. The results in Fig. 5 show a single peak of activity at an approximate molecular mass of 240 ± 30 kDa, indicative of a dimer structure of PIPP2C. To confirm that the phosphatase activity of this peak corresponds to PIPP2C activity, all fractions (fractions 1–47) collected by gel filtration were tested by Western blotting using the anti-PP2C antibodies. The results in Fig. 5 show complete agreement between the Western blot and the phosphatase activity peaks, suggesting that the unique Mg2+-dependent phosphatase activity detected in this fraction corresponds to PIPP2C.

We have further explored subunit and domain interactions by mixing purified components and assessing activity (Fig. 6). PIPP2C has higher specific activity than either half alone. When the two halves are mixed together, there is no stimulation of activity (data not shown). When PIPP2C is mixed with P2C-1 or P2C-2, the activity decreases 5–6-fold from the level of PIPP2C alone, approaching the activity of the isolated halves.

**PIPP2C Can Function in a Heterologous Stress Response**—To test whether PIPP2C is a functional homologue of yeast PP2C, we tested the effect of PIPP2C expression in a PTC1-deleted strain of *S. pombe*. The PTC1 gene encodes a PP2C that is proposed to play an important role in the yeast osmolarity response. The deletion strain has a greatly reduced ability to survive exposure to elevated temperature; the cells die more quickly after shifting from 25 to 48 °C than do wild-type cells (24). We made a construct in which PIPP2C is expressed under the control of the *S. cerevisiae* GAL1 or ADH promoter or under the control of the *S. pombe nmt* promoter. Both *S. cerevisiae* promoters are constitutive in *S. pombe*. The plasmids obtained were then used to transform the KS834 strain deleted for the PTC1 gene. The transformants were first tested by Western blotting and showed expression of PIPP2C (data not shown). They were then tested for growth at 37 °C and for their ability to survive after shifting from 25 to 48 °C with or without...
pretreatment at 37 °C. Similar results were obtained with all three promoters; data are shown only for the GAL1 promoter construct. The growth rate of the ΔPTC1 strain expressing PIPP2C at 37 °C was faster than that of the ΔPTC1 strain carrying the vector alone, and its ability to form colonies after shifting directly from 25 to 48 °C was similar to that of the wild-type strain (Fig. 7). This suggests that the malaria PP2C is able to play the same role as its homologue PTC1 in the stress response.

Pf2C-1, Pf2C-2, and Pf2C-Δ constructs were also active in the yeast system. Each was able to partially correct the heat response defect of the ΔPTC1 strain (Fig. 7). These results correlate with the in vitro activity data, indicating that PIPP2C is composed of two active phosphatases fused together and that their fusion is necessary for optimal activity within the cell.

**DISCUSSION**

We have isolated a new PP2C gene from *P. falciparum* called PfPP2C. In yeast, PP2C has been implicated in the response to extracellular stimuli such as osmolarity changes or heat shock.
Such stresses are signaled through the MAPK cascade, consisting of Wik1, a MAPK kinase that phosphorylates Wis1, a MAPK kinase, which in turn phosphorylates Spc1, a MAPK. Data in *S. cerevisiae* and *S. pombe* indicate that tyrosine-specific and serine/threonine phosphatases negatively regulate the stress-activated kinase cascades (6, 25–27). In both yeasts, genetic and biochemical studies are in agreement concerning the direct involvement of tyrosine-specific phosphatases in de-phosphorylation of MAPK (Spc1 in *S. pombe* and Hog1p in *S. cerevisiae*). Genetic data also suggest that serine/threonine phosphatases negatively regulate the stress-activated MAPK cascades. 1) In *S. cerevisiae*, two PP2C genes (PTC1 and PTC3) were isolated as multicopy suppressors of mutations that caused hyperactivation of the Hog1p kinase cascade (6). 2) Mutations in the *HOG1* gene suppress the synthetic lethality interaction involving *ptp2* and *ptc1* mutations (28). 3) In *S. pombe*, mutations in *Spcl* (MAPK) or *Wis1* (MAPK kinase) suppress the calcium hypersensitivity of mutants carrying deletions of two or three serine/threonine phosphatase genes (26). Biochemical data from Gaits *et al.* (7), however, indicate that in *S. pombe*, PTC1 does not negatively regulate Wis1 or Spc1. These authors suggest that PTC1 may act downstream of Spc1 in the stress-activated signal transduction pathway by dephosphorylating an Atf1 cofactor involved in transcriptional induction (7).

In this study, we have shown by heterologous complementation that PP2PC2 can play the same role as PTC1 in *S. pombe* and perhaps functions similarly in *P. falciparum*. We have also shown that PP2PC2 is composed of two protein phosphatases (P2C-1 and P2C-2) fused together in the same polypeptide. Both P2C-1 and P2C-2 are active in *vitro* and are able to complement the heat shock stress response defect of a PTC1 deletion in *S. pombe*.

Most organisms have at least two PP2C genes. Often, different phosphatase 2C proteins are involved in the same signal transduction pathway. For example, in both *S. cerevisiae* and *S. pombe*, three genes encoding PP2C-like proteins are involved in the regulation of the MAPK cascade (6, 26, 28, 29). Other genes have been isolated during the *S. cerevisiae* genome sequencing project encoding proteins that show homology to PP2C sequences, but as of yet have no function or activity assigned. In *A. thaliana*, both ABI1 and ABI2 are PP2C-like proteins involved in an abscisic acid-dependent stress response pathway (12, 13, 30).

In *P. falciparum*, we have evidence for only a single PP2C gene. Low stringency Southern and Northern blot analyses detect a single gene and transcript (data not shown), whereas gel filtration analysis reveals only a single activity peak eluting at a position expected of a molecule four times the size of a standard PP2C (Fig. 6). Our data are consistent with the notion that PP2PC2 is a double phosphatase, wherein both halves possess a phosphatase fold. Purified PP2PC2, P2C-1, P2C-2, and a hybrid form composed of the first half of P2C-1 and the second half of P2C-2 are each active in dephosphorylating casein in *vitro*. A complete dimer appears to be necessary for maximal activity since the two halves alone or in combination possess considerably lower activity than the whole molecule, and either half interferes with the ability of the whole to achieve maximal activity upon mixing.

Taken together, these results suggest the model presented in Fig. 8, in which PP2PC2 acts as dimer, and the P2C-1 of one polypeptide interacts with the P2C-2 of the second polypeptide. The whole dimer is, in effect, a tetrmeric phosphatase.

Perhaps the unusual structure of PP2PC2 can overcome the need in other organisms for additional genes, offering a novel way to regulate the signal transduction pathway. Study of the expression and activity of PP2PC2 during stress conditions and analysis of its crystal structure could better our understanding of the mechanisms that monitor the stress response of the malaria parasite. Its unusual configuration is different enough from PP2C proteins of human cells to be considered a good target for new antimalarial drugs.

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