

Functional Characterization and Localization of Protein Phosphatase Type 2C from *Paramecium**

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We cloned a protein phosphatase 2C gene from *Paramecium* (PtPP2C), which codes for one of the smallest PP2C isoforms (Klumpp, S., Hanke, C., Donella-Deana, A., Beyer, A., Kellner, R., Pinna, L. A., and Schultz, J. E. (1994) *J. Biol. Chem.* 269, 32774–32780). After mutation of 9 ciliate Q codons (TAA) to CAA PtPP2C was expressed as an active protein in *Escherichia coli*. The catalytic core region contains 284 amino acids as defined by C- and N-terminal deletions. The C terminus from amino acid 200–300 of PP2C isoforms has only about 20% similarity. To demonstrate that the carboxy end is in fact needed for activity, we generated an enzymatically active PtPP2C containing a C-terminally located tobacco etch virus-protease site. Upon proteolytic truncation enzyme activity was lost, i.e. the C terminus of PP2C is indispensable for enzyme activity. During these experiments isoleucine 214 was fortuitously identified to be essential for PP2C catalysis. Mutation of the hydrophobic amino acid to glycine in the ciliate or bovine isoforms resulted in inactive protein. Because Ile²¹⁴ is in a loop region without defined secondary structure, our data clearly go beyond the x-ray structure. The functional equivalence of the 180 amino acid long C terminus from the bovine PP2C with the 100 amino acid long carboxy end of the PtPP2C was demonstrated by producing an active chimera, i.e. the PP2C from *Paramecium* has no obvious regions which may be specifically involved in subcellular localization or substrate recognition. Using antibodies against recombinant PtPP2C we localized the enzyme by immunogold labeling in the cytosol and nucleus and very distinctly on the ciliary microtubule/dynein complex. The data suggest a role for PtPP2C in the regulation of dyneins, i.e. in cellular cargo transport and ciliary motility.

family, have been crystallized (1–4). In addition, several physiological functions of PP1, 2A, and 2B have been tentatively identified using specific inhibitors (for a review, see Cohen (5)). No specific inhibitors are available for protein phosphatase 2C isozymes (PP2C) which belong to a separate family of Mg²⁺- or Mn²⁺-dependent phosphatases (PPM-family) with a low degree of conservation in their primary structure (6, 7). The molecular weights range from 48 kDa (from *Arabidopsis thaliana*; 433 amino acids) to the smallest versions of 33 kDa from *Paramecium tetraurelia* (300 amino acids) and 31 kDa from *Saccharomyces cerevisiae* (TPD1, 282 amino acids) (8). This large variability was suggested to indicate a functional diversity of PP2C enzymes although presently our knowledge about PP2C functions is rather limited (9). Lately, a deletion analysis with a human liver PP2C α isoform (382 amino acids) was reported (10). Two C-terminal truncations of 75 and 179 and one N-terminal deletion of 51 amino acids were constructed. Only the expression product of PP2C α 1–307 was active. This is in agreement with the recently published x-ray structure of a human PP2C α , which shows that the C terminus starting around amino acid position 300 contains three α helices forming an appendix to the catalytic core (9). Consequently, the C-terminal domain was speculated to affect substrate specificity. The 300-amino acid long protozoan PP2C completely lacks this C-terminal domain. How are the cellular localization and substrate specificity of PP2C defined in the ciliate? First, we determined the minimal core structure of the PtPP2C, which is essential for catalytic activity, i.e. we constructed N- and C-terminal deletion mutants at the DNA level and, additionally, C-terminal truncations via targeted proteolysis. Fortuitously, we identified an invariant isoleucine at position 214 in an unordered loop region as cardinal to enzyme activity in PP2C enzymes. Second, we assessed the functional equivalence of protozoan PP2C regions with their mammalian counterparts by generating an active bovine/ciliate chimera. Finally we localized PP2C in *Paramecium* by immunochemical and -cytochemical methods in the cytoplasm, nucleus and the cilia. Because of the targeting of PtPP2C to motor components in the cilia we propose that PtPP2C participates in the regulation of cytoplasmic, nuclear and ciliary dynein regulation.

Recently, substantial progress has been made in the area of serine/threonine protein phosphatases. Recombinant type 1 protein phosphatase (PP1)¹ α and β isoforms and PP2B, members of the same, highly conserved PPP protein phosphatase

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¹ The abbreviations used are: PP, protein phosphatase; PtPP2C, *P. tetraurelia* protein phosphatase 2C; PCR, polymerase chain reaction; pA-Au₆, 6-nm gold-protein A-conjugates; NiNTA, Ni²⁺-nitriloacetate-agarose; PIPES, 1,4-piperazinediethanesulfonic acid; TEV, tobacco etch virus.

EXPERIMENTAL PROCEDURES

Materials—pBluescriptII SK(+/-) and *E. coli* XL1 Blue were from Stratagene. All restriction enzymes and nucleases were purchased from either Boehringer Mannheim or New England Biolabs. Sequenase Version 2.0 from U. S. Biochemical Corp. was used for DNA sequencing. *E. coli* BL21(DE3)pLysS and the plasmid pET16b were from Novagen. Point mutations were made in *E. coli* BMH71-18mutS from CLONTECH. Ni²⁺-nitriloacetate-agarose (NiNTA) was from Qiagen, TEV-protease from Life Technologies, Inc., and factor Xa-protease from Boehringer Mannheim. Radioisotopes were from Amersham Corp.

Construction of a PtPP2C Expression Plasmid—The PtPP2C gene contains 9 TAA triplets coding for Q which were converted to CAA by

site-directed mutagenesis (11). *NdeI* and *XhoI* restriction sites were added by PCR to the 5' and 3' ends, respectively, to clone the mutated PtPP2C cDNA into the pET16b expression vector.

Construction of a Ciliate/Bovine PP2C Chimera—The cDNA for the bovine PP2C α isoform with 5'-*XhoI* and 3'-*BamHI* restriction sites was from Dr. D. Selke, University of Marburg. Using the PtPP2C as the N-terminal region a PCR fragment was generated which had an *MaeI* 3'-restriction site at base pairs 578, i.e. at G193 within the third conserved region (12) and an *NdeI* cutting site for ligation into pET16b at the 5'-end. The complementary C-terminal fragment of the bovine PP2C α had an *MaeI* site 5' and an *XhoI* site 3'. The appropriately digested and gel-purified PCR products and *NdeI/XhoI* cut pET16b were used in a triple ligation to obtain the full-length chimeric PP2C DNA construct in the expression vector.

Deletions—At the N terminus all deletions retained the methionine at position 1 as part of the *NdeI* restriction site. The N-terminal truncations were PtPP2C Δ 2–3, PtPP2C Δ 2–11, PtPP2C Δ 2–13, PtPP2C Δ 2–15, and PtPP2C Δ 2–19. Truncations at the C terminus were made by cutting off amino acids one by one up to a total of 10. All constructs were generated by PCR using respective oligonucleotide primers and plasmid pET16b/PtPP2C as a template. The PCR products were cloned into the *NdeI/XhoI* sites of the pET16b expression vector.

Insertion of Protease Digestion Sites—A TEV protease digestion site (ENLYFQG) was introduced into PtPP2C at positions 219 and 254. Because the DNA sequence encoding the TEV cleavage site can be made to contain an *EcoRII* restriction site (GAGAATCTTTATTTCCAGGGC) which is absent in the PtPP2C cDNA, placement of the TEV sites could be freely selected. We replaced amino acids 219–226, PtPP2C_{219TEV}, and 254–261, PtPP2C_{254TEV}HisN. To switch the His tag in PtPP2C_{254TEV}HisN to the C terminus creating PtPP2C_{254TEV}HisC, we recloned PtPP2C_{254TEV} into the *NcoI* site of pET16b in front of the His tag. A Factor Xa cleavage site (IEGRA), whose encoding cDNA contained an *AscI* restriction site (ATCGAAGGGCGCGCC), was introduced into the PtPP2C by replacement of the amino acids 212–216, PtPP2C_{212FXa}. Single amino acid exchanges at positions 212 to 215 are listed in Table I. The fidelity of all constructs was verified by DNA sequencing.

DNA Primers—A list of the primers used in this study is available from J.E.S.

Expression and Purification—Usually, single transformed colonies of *E. coli* BL21DE3plysS were inoculated into 5 ml of LB medium containing 50 μ g/ml ampicillin and 34 μ g/ml chloramphenicol and grown overnight at 37 °C and 260 rpm. 400 μ l were used to inoculate 20 ml of LB medium with antibiotics, grown to an A_{600} of 0.4–0.6 and induced with 1 mM isopropyl-1-thio- β -D-galactopyranoside. After 3 h, cells were harvested (3,200 \times g, 4 °C, 10 min) and resuspended in 800 μ l of binding buffer (20 mM Tris/HCl, 5 mM imidazole, 250 mM NaCl, pH 7.9). Bacteria were broken by freeze-thaw and sonication and centrifuged (10,000 \times g, 4 °C, 20 min). 100–300 μ l of the supernatant were applied to a 25- μ l NiNTA column equilibrated with binding buffer. The column was washed with 250 μ l of binding buffer and 150 μ l of wash buffer (20 mM Tris/HCl, 60 mM imidazole, 500 mM NaCl, pH 7.9). Bound protein was eluted with 150 μ l of elution buffer (20 mM Tris/HCl, 1 M imidazole, 500 mM NaCl, pH 7.9). Protein purity was monitored by SDS-polyacrylamide gel electrophoresis stained with Coomassie Brilliant Blue. Protein concentration was determined by the Bio-Rad method. The conditions for TEV digestion were as suggested by Life Technologies, Inc.

PP2C Assay—Bovine casein (Sigma C-4765) was phosphorylated with [γ -³²P]ATP by cAMP-dependent protein kinase and purified according to McGowan and Cohen (13). PP2C assays (30 μ l) contained 20 mM Mg²⁺ acetate, 50 mM Tris/HCl, pH 7.0, 0.1% 2-mercaptoethanol, 0.6 mg/ml bovine serum albumin, and 1 μ M [³²P]casein (about 30,000 cpm). After 10 min at 30 °C dephosphorylation was stopped by addition of 200 μ l of 20% trichloroacetic acid (w/v). After centrifugation radioactivity was determined in the supernatant. Dephosphorylation was restricted to 30% of the total ³²P activity to guarantee linearity. One unit of activity is the amount of enzyme which catalyzes the dephosphorylation of 1 μ mol of [³²P]casein/min (13).

Antibodies—Polyclonal antibodies were raised in a rabbit using 200 μ g of the recombinant PtPP2C as an immunogen together with Pam₃Cys-Ser-(Lys)₄x3HCl and Pam₃Cys-T_H* (250 μ g each) as adjuvants (14, 15). The animal was injected subcutaneously and boosted twice at 4-week intervals. The antiserum could be used without further purification.

Western Blot—Cilia and affinity-purified recombinant proteins were transferred from SDS-polyacrylamide gels onto nitrocellulose membranes (Optitrans, Schleicher & Schuell) by semidry blotting (75 min, 1.1 mA/cm²; blotting buffer 48 mM Tris/HCl, pH 9.2, 39 mM glycol, 0.0375%

SDS, 20% methanol). The membrane was blocked with TBS (50 mM Tris/HCl, pH 7.5, 0.9% NaCl) containing 5% non-fat milk powder (M-TBS) and probed with the antiserum diluted 1:5000 in M-TBS. The Amersham ECL detection system was used.

Cell Fractionation for Immunohistochemical Analysis—*Paramecium* 7S wild-type cells, grown to early stationary phase in sterile medium (16), were harvested and deciliated by a CaCl₂ shock (10 min on ice in 5 mM Pipes/HCl, pH 7.0, 3 mM KCl, and 40 mM CaCl₂). Cell fractionation, protein determination, and enzyme-linked immunosorbent assays were carried out as described earlier (17).

Immunocytochemistry—For immunofluorescence cells were fixed for 10 min at 0 °C in 4% formaldehyde in phosphate-buffered saline supplemented with either 0.02% digitonin, 1% saponin, or 0.05% Triton X-100 (with equal results), incubated with anti-PP2C-antiserum (diluted 1:30) and subsequently with goat anti-rabbit fluorescein isothiocyanate-IgGs. Similarly, pre-embedding electron microscopy immunolabeling studies were carried out with horseradish peroxidase- or 6-nm gold-protein A conjugates (pA-Au₆; for details, see Momayez *et al.* (17)).

For post-embedding labeling cells were fixed for 10 min at 22 °C in 4% formaldehyde + 0.25% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.1. After ethanol dehydration, cells were impregnated with Unicyrly® (British BioCell Int., London) according to the manufacturer's instructions. Ultrathin sections were prepared, labeled with pA-Au₆, stained, and quantitatively evaluated as described elsewhere (18).

RESULTS

Expression and Characterization—We cloned a PP2C gene from a *Paramecium* cDNA library (12). Its heterologous expression was impossible because in this protozoan the universal stops TAA and TAG code for Q. We now mutated all 9 TAA/Q triplets of the PP2C cDNA to universal CAA/Q triplets and inserted the gene into the expression vector pET16b, thereby attaching a deca His tag and a factor Xa protease site to the N terminus. PtPP2C was expressed in *E. coli* BL21(DE3)plysS. Approximately 8 mg of enzymatically active PtPP2C (about 30 milliunits/mg) were purified from a 1-liter culture, i.e. the high A/T content of the *Paramecium* gene (62%) and the resulting skewed codon usage did not affect the expression in *E. coli* which has an A/T content of only 50%. A comparison of the enzymatic properties of the native, ciliary and the recombinant PtPP2C showed identical temperature optima (46 °C), yet, the activation energies derived from linear Arrhenius plots were 69 and 30 kJ/mol for the ciliary and the recombinant enzyme, respectively. The most prominent difference was the effect of divalent cations. Substituting 20 mM Mg²⁺ with 20 mM Mn²⁺ decreased the activity of the native enzyme by 36%, whereas the activity of the recombinant enzyme was almost doubled. Posttranslational modifications of the ciliary enzyme may account for these differences.

Deletion Analysis—With only 300 amino acids, the PtPP2C is one of the shortest enzymes of the PPM family. To determine the amino acids essential for catalytic activity we constructed and expressed enzyme proteins which were truncated either at the 5'- or the 3'-end. At the N terminus the following amino acid deletion mutants were generated: PtPP2C Δ 2,3, PtPP2C Δ 2–11, PtPP2C Δ 2–13, PtPP2C Δ 2–15, and PtPP2C Δ 2–19. All constructs were expressed as soluble proteins with approximately equal efficiency (Fig. 1A). Removal of up to 10 N-terminal amino acids affected enzymatic activity only slightly. However, deletion of 12 N-terminal amino acids (PtPP2C Δ 2–13) diminished activity by 80%. Larger N-terminal truncations yielded soluble expression products, albeit without activity (Fig. 1A).

At the C terminus 4 amino acids (YLRR) were sequentially removed one by one without loss of activity, e.g. the specific activity of PtPP2C Δ 297–300 was comparable to the full-length enzyme (Fig. 1B). The expression of these constructs was about as good as the untruncated PtPP2C. Removal of 5 (VYLRR) or more amino acids (up to 10) from the carboxy end led to drastic changes. First, enzyme activity was lost; second, the yield of

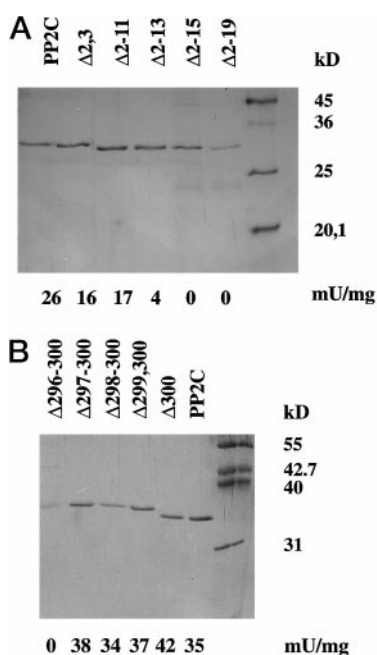


FIG. 1. SDS-polyacrylamide gel electrophoresis analysis and protein phosphatase activity of N- and C-terminally truncated protein phosphatase 2C from *Paramecium*. The mutated proteins were expressed in *E. coli* BL21(DE3)plysS and purified on NiNTA. Protein phosphatase activity was measured as described. The specific protein phosphatase activities are listed below each lane. The data are from single experiments repeated several times. Note the drop in enzyme activity upon deletion of 12 and more N-terminal amino acids (A) or 5 C-terminal amino acids (B). Also clearly visible is the anomalous electrophoretic mobility of the C-terminally truncated enzyme in B.

purifiable material was diminished, *i.e.* either the expression rates or the stability of the expressed proteins was reduced. Notably, all C-terminally shortened expression products displayed an anomalous electrophoretic mobility upon SDS-polyacrylamide gel electrophoresis, running at an apparent larger molecular mass than the untruncated version (Fig. 1B and data not shown). Therefore, in addition to the routine verification of all constructs by DNA sequencing we used amino acid analysis and ion-spray mass spectrometry to ascertain the identity of PtPP2C Δ 299–300. Data from both methods unequivocally established the correct identity (data not shown). The anomalous electrophoretic mobility of the shortened expression products may be related to the removal of the two terminal basic arginine residues.

C-terminal Truncation by Proteolysis—PP2C isoforms from various phyla share three reasonably long conserved regions within the 200 N-terminal amino acids and only very short stretches of conservation from 200 to 300 (6, 12). Therefore, we reasoned that the highly diverged C termini may partly be dispensable for catalysis but serve other roles, *e.g.* in protein folding, targeting to specific cellular locations, or substrate binding. To test this hypothesis we inserted at two different positions a TEV-protease recognition site, which should permit a C-terminal truncation by proteolysis after expression, folding, and purification. Amino acids 219–226 (PtPP2C_{219TEV}, replacement of the weakly conserved sequence DVKKTEL in the β 9 strand) and 254–261 (PtPP2C_{254TEV}HisN, replacement of the nonconserved TIGQAQV in the α 5 helix) were changed to the TEV cutting sequence ENLYFQG. The constructs were expressed as soluble proteins, purified by NiNTA chromatography, and were recognized by a PtPP2C-specific antibody in Western blots at their calculated molecular masses (data not shown).

PtPP2C_{219TEV} was inactive. To reduce the number of ex-

TABLE I
Enzymatic activities of *Paramecium* protein phosphatase 2C mutants
The data are from a single experimental series, repeated three times with similar results.

Enzyme variant	Specific activity milliunits/mg
PtPP2C wild type	15.7
PtPP2C _{FXa}	0
PtPP2C Q212I	8.3
PtPP2C L213E	12.3
PtPP2C I214G	0
PtPP2C I214A	9.7
PtPP2C I214L	19.2
PtPP2C I215R	11.2
PtPP2C I215G	5.4
Bovine PP2C α	2.5
Bovine PP2C α V215G	0

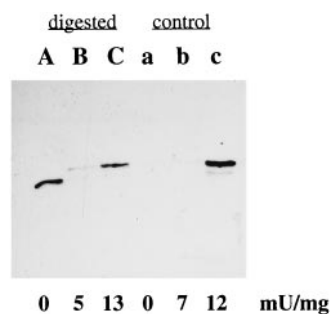


FIG. 2. Western blot of column fractions from a NiNTA chromatography of purified PtPP2C_{TEV254}HisC, which was digested for 5 h by TEV protease. Digested: A, flow-through (25 ng/lane); B, wash fraction (22.5 ng/lane); C, eluate (22 ng/lane). Control: a, flow-through (26 ng/lane); b, wash fraction (18 ng/lane); c, eluate (27 ng/lane). The specific protein phosphatase activity of the column fractions is listed below each lane.

changed amino acids as well as to replace less conserved amino acids we created PtPP2C_{212FXa} in which the sequence QLII (212–215) was changed to IEGR, thus generating, in conjunction with Ala²¹⁶, a factor Xa cleavage site (IEGRA). Somewhat surprisingly, PtPP2C_{212FXa} was also inactive (Table I). Individual amino acid exchanges at each of the changed positions showed that exclusively the I214G conversion was responsible for the loss of function (Table I). In an I214A PtPP2C expression product 62% of wild-type activity was retained, and an I214L PtPP2C was at least as active as the wild-type enzyme (Table I). Strikingly, the adjacent isoleucine at position 215 was not crucial for enzyme activity. Neither an I215R nor an I215G conversion abolished PtPP2C activity (Table I).

The highly specific knock-out of the enzymatic activity by the replacement of Ile²¹⁴ with G was unexpected because in the x-ray structure of the human PP2C α isoform the corresponding region is without a defined secondary structure (9). Therefore, we wanted to know whether the result with the PtPP2C was due to a structural peculiarity of the protozoan enzyme or was representative of an important, as yet unrecognized structural feature common to all members of this class of phosphatases. Alignment shows that in the bovine PP2C α isoform a valine at position 215 corresponds to Ile²¹⁴ of the PtPP2C (see Fig. 3 for an extended alignment of PP2C sequences). A bovine PP2C α construct in pPET16b, which contained a V215G conversion, was expressed in *E. coli*, yet it was enzymatically inactive, whereas the expressed Val²¹⁵ wild-type enzyme was active (Table I).

PtPP2C_{254TEV}HisN carries the TEV site in a linker region connecting the α 5 and α 6 helices. The expressed protein was active, *i.e.* the nonconservative exchange of 6 amino acids in

Pt.PP2C	193	SRALGD LEYKRDNKLRSNEQL	I	I	ALPDVKKTELTPQDK	FILMGCDG
hum. PP2C γ	397	SRAIGD HFFYKRKNLPPPEEQM	I	I	SALPDIKVLTLTDDHE	FMVIACDG
bov. PP2C β	199	SRALGD YDYKCVDGKGPTEQL	V	V	SPEPEVYFILRAEED	FIIILACDG
bov. PP2C α	194	SRALGD FDYKCVHGKGPTEQL	V	V	SPEPEVHDIERSEEDDQ	FIIILACDG
hum. PP2C α	194	SRALGD FDYKCVHGKGPTEQL	V	V	SPEPEVHDIERSEEDDQ	FIIILACDG

FIG. 3. Alignment of PtPP2C (EMBL accession no. z36985) with mammalian isozymes human PP2C α (EMBL accession no. p35813), human PP2C γ (EMBL accession no. y13936), bovine PP2C α and PP2C β (sequences from Dr. Selke, Marburg). The amino acids replaced in PtPP2C by IEGR, the factor Xa site, is *overlined*. The invariant hydrophobic amino acid at position 214 (*Paramecium* numbering) is *boxed*. Identical amino acids are in *boldface type*. The human PP2C γ has a long insert of unknown function at the N terminus, which is responsible for the skewed numbering compared with the other isoforms.

this region did not impair protein folding or phosphatase activity (Fig. 2). Upon TEV digestion enzymatic activity was strongly reduced. Yet, due to the incomplete proteolysis and an apparent association of the digested C-terminal peptide with the remaining protein we were unable to fully separate PtPP2C Δ 254–300 from the 40-amino acid proteolytic fragment as apparent in Western blots (data not shown). Therefore, we switched the His tag to the C terminus (PtPP2C254_{TEV}HisC). This did not affect phosphatase activity. Now, after TEV digestion we obtained pure PtPP2C Δ 254–300 in the flow-through of a NiNTA column which retained all undigested protein and the C-terminal peptide (Fig. 2). PtPP2C Δ 254–300 was devoid of phosphatase activity. We conclude that the complete C terminus of the PtPP2C with the exemption of the last 4 amino acids is required for catalytic activity. Taken together the truncation experiments define a PtPP2C of 284 (13–296) as the catalytic core.

PP2C Chimera—To prove the functional interchangeability of the C termini we constructed a hybrid PP2C enzyme in which the conservative N terminus (amino acids 1–193) from *Paramecium* was linked within the conserved region III (¹⁹³SRALGD¹⁹⁸) to the C-terminal end from the bovine PP2C α , *i.e.* 188 amino acids (195–382) of the mammalian enzyme replaced 105 amino acids (194–300) from the PtPP2C. This chimera was expressed as a soluble protein and had a specific activity of 14 milliunits/mg, roughly as the parent PP2C isoforms (35 and 8 milliunits/mg for the *Paramecium* and bovine PP2C, respectively). This demonstrated that the N and C termini of the bovine and protozoan PP2C are functionally interchangeable despite the low degree of sequence conservation at the amino acid level (22%). Furthermore, it showed that the 80-amino acid extension of the bovine PP2C is irrelevant as far as enzyme activity is considered.

Cellular Localization of PP2C in *Paramecium*—All PP2C proteins lack pre- or prosequences and nothing is known about secondary modifications which may serve to target the protein to a particular cytoplasmic compartment. Based on the above data, we must assume that in the PtPP2C only 12 N-terminal and possibly four C-terminal amino acids may be used for subcellular targeting or substrate recognition. It is the more important to determine whether the PtPP2C shows a distinct subcellular localization in the ciliate, which might lead to clues concerning physiological functions of this enzyme. PtPP2C-specific enzyme-linked immunosorbent assays of different cellular fractions showed that 98.6% of the immunoreactivity reside in the deciliated cell bodies, 0.8% in the isolated cilia and 0.6% in the supernatant of the deciliation procedure. Considering that the volume of the cell body is about 98% and of the cilia 2%, one can assume that the PP2C concentration in these compartments is similar. As seen in immunofluorescence analysis of permeabilized cells (Fig. 4A), cilia contained substantial amounts of PP2C.

A more detailed immunocytochemical analysis was carried out by post-embedding pA-Au₆ labeling which avoids loss of soluble proteins (Fig. 4 and Table II). Labeling density in the cytoplasm, including the endoplasmic reticulum, exceeded that

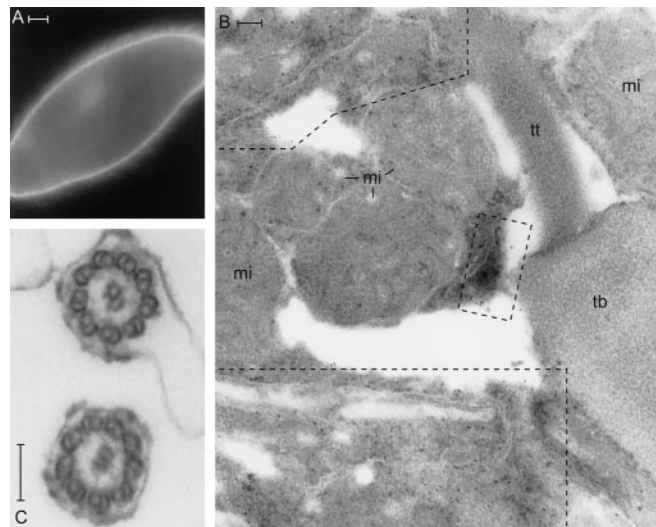


FIG. 4. Immunolocalization of PP2C in formaldehyde fixed *Paramecium* by fluorescein isothiocyanate (A), pA-Au₆ (B), or horseradish peroxidase (POX) labeling (C). A, fixation in presence of Triton X-100, goat anti rabbit fluorescein isothiocyanate-conjugates; note labeling of cilia. B, postembedding labeling of Unicryl[®] sections with pA-Au₆; note gold granula in the three boxed cytosolic areas (including the endoplasmic reticulum), but absence from mitochondria (*mi*), trichocyst tips (*tt*), and trichocyst bodies (*tb*). C, preparation as in A, but followed by goat anti-rabbit POX conjugates; note label on axonemal structures, but scarcity on ciliary membrane. Magnification bars, 10 μ m (A), 0.1 μ m (B and C).

TABLE II

Quantitative evaluation of anti-PP2C-antiserum / pA-Au₆ labeling on unicryl sections of nonpermeabilized cells (postembedding labeling), off-cell background corrected

Subcellular structure	Au grains	<i>n</i> ^a
	<i>ms</i> / μ m ²	
Cytoplasm (including endoplasmic reticulum)	53.9	10
Macronucleus	13.0	3
Cilia	9.4	29
Trichocysts	0.9	10
Alveolar sacs	0.3	11
Fat droplets	0.0	3
Mitochondria	0.0	33

^a *n* = individual structures analyzed from a total of 10 cells.

in any other organelles. Only the nuclei and the cilia were also labeled, whereas other identifiable structures were not significantly labeled (Fig. 4B). Within the cytoplasm a differentiation between cytosol and endoplasmic reticulum was not possible. Within the cilia, a more detailed structural analysis was made using pre-embedding labeling with pA-Au₆. In this method access of the antibody is facilitated by the presence of 0.05% Triton X-100 as a detergent during fixation. This method could be used because biochemical data have shown that in the cilia the PtPP2C is in the particulate fraction and requires 1% Brij 35 as a detergent for solubilization (12). We observed that 83% of the gold label was associated with axonemal structures, *i.e.* with the microtubules (42%) and dynein arms (26%) and only

TABLE III
Distribution of anti-PP2C-antiserum/pA-Au₆ labeling
in cilia of permeabilized cells (preembedding labeling),
off-cell background corrected

A total of 174 Au-grains (Au₆-grains on or within ~10 nm distance of any structure) in 86 strictly cross-sectioned cilia from 50 cells were evaluated.

Structure analyzed	Gold grains found	
		%
Ciliary membrane		3
Central microtubules		15
Peripheral microtubules		
A microtubule		10
B microtubule		32
Dynein arms		26
Free space		
Outer ^a		9
Inner ^b		5

^a Space between outer microtubule doublets and cell membrane.

^b Space between outer and inner microtubule doublets.

3% with the ciliary membrane (Table III). In agreement with this it was found that horseradish peroxidase labeling was restricted to the axonemes (Fig. 4C).

DISCUSSION

With a length of 300 amino acids the PtPP2C is short compared with most mammalian isoforms, which are between 380 and 410 amino acids long (6). The PtPP2C sequence mainly comprises those regions that form the catalytic core. In particular, it lacks the mammalian C-terminal domain of 80–100 amino acids, which is placed as an appendix apart from the catalytic site. This C-terminal extension is hypothesized to be involved in substrate recognition and specificity (9). Other PP2C catalytic domains occur in a variety of different structural contexts, which probably mediate interactions with particular substrates (6). In the short PtPP2C, substrate recognition and thus functional unambiguity must either reside within its 300 amino acids or be determined by a specific subcellular localization. We approached this question in three ways, (i) definition of regions within the PtPP2C not essential for enzyme activity, (ii) demonstration of the interchangeability of the highly diverged C-terminal domains, and (iii) immunocytochemistry.

At the N terminus the first inactive deletion mutant was PtPP2C Δ 2–15. This truncated enzyme lacks 6 (RDKTTT) of the 11 amino acids which assemble into the β 1 domain. Although the β 1 region does not itself participate in catalysis, the loss of it probably destabilizes the central β sandwich which serves as a scaffold for the active site (9). At the C terminus, removal of only five amino acids resulted in an inactive protein. This is most plausibly explained by the fact that the β 11 strand is affected, which connects into the active site and stabilizes the conserved Asp²⁸⁹, one of the amino acids of the metal-binding site. Thus, the minimal sequence required for formation of a catalytically active entity seems to be a 284-amino acid long protein, PtPP2C₁₃₋₂₉₆. The truncation data support the notion that the three-dimensional structure of the PtPP2C probably is more or less identical to that determined with the recombinant human PP2C α despite the overall poor amino acid conservation (31%).

To unequivocally demonstrate that the C-terminal end of PtPP2C is indispensable for activity, we created mutants that could be proteolytically truncated after expression and folding. A nonconservative 7-amino acid replacement at position 254 with a TEV-protease site resulted in formation of an active protein. The truncation by TEV showed that the α 5 helix on the surface of the enzyme is accessible to proteolysis. The digested protein was inactive. This means that the α 5, α 6-helices and the β 11 strand are cardinal structural elements for enzyme

activity. In PtPP2C_{219TEV} 7 amino acids in the β 9 strand were exchanged. Obviously, the β 9 strand was affected so profoundly that an inactive protein resulted. Therefore, a factor Xa cutting site was introduced into an unordered loop region in front of the predicted β 9 strand. Surprisingly, PtPP2C_{212FXa} was also inactive. This observation compellingly argued that the unordered loop region plays a completely unsuspected role in catalysis. Individual permutations at all positions involved identified Ile²¹⁴ to be most important for activity (see Table I). Indeed, a sequence comparison of eukaryotic PP2C isoforms showed that in the equivalent position there is always an I or V at an invariant distance of 15 amino acids from a conserved RXXGD sequence and four amino acids in front of the β 1 region (Fig. 3). The latter invariant amino acids are still in the loop region which has no secondary structure (9). Because the I214A mutant had 60% of the wild-type activity and an I214L mutant was fully active, we conclude that the steric bulk of Ile²¹⁴ (PtPP2C numbering) is needed to sustain a catalytically competent conformation when phosphocasein is used as a substrate. This point was further strengthened by the lack of activity of a corresponding mutation in the bovine PP2C α . In this respect, our data clearly go beyond the picture which has emerged from the x-ray structure of PP2C (9). It does not show why a mutation that replaces Ile²¹⁴ in PP2C results in inactivation. The loss of a hydrophobic side chain must alter the stability or a directed movability of this part of the protein such that the active site centers cannot stabilize an enzyme/substrate configuration involved in a stable transition state, *i.e.* Ile²¹⁴ may be involved in substrate recognition and specification. The sequence comparison (Fig. 3) identified position 215 (PtPP2C numbering) as variant (Fig. 3) thus explaining why amino acid replacements of Ile²¹⁵ were immaterial. On the other hand it is notable that the invariant Gln²¹² could be replaced by I without affecting enzyme activity.

The C-terminal domains of PP2C, which start around position 200, are characterized by a particularly high extent of divergence (up to 80%). This region might, therefore, participate in the definition of substrate specificity as a secondary function. We tested the functional equivalence of the C termini by generating a PP2C chimera which consisted of PtPP2C1–193/bovine PP2C194–382. The full functional replacement of the carboxyl-terminal 100 amino acids from the PtPP2C by the very poorly conserved 184 amino acids from of the bovine PP2C α demonstrated that quite extensive alterations in the primary amino acid sequence at the carboxy end are tolerated without dramatically affecting enzyme activity. This finding strengthens the assumption that the C-terminal end of mammalian PP2Cs is not even peripherally involved in catalysis and has other functions like subcellular targeting or substrate recognition.

In an attempt to reveal clues to a physiological function we established the subcellular localization of PtPP2C in the ciliate. Biochemically, the major fraction of the PtPP2C is cytosolic, a minor part is structurally associated with the cilia (12). Now we combined partial cell-fractionation and enzyme-linked immunosorbent assay with (ultra-)structural immunolabeling techniques. Considering the soluble cytosolic PtPP2C we used postembedding immunogold electron microscopy techniques and with the cilia additionally preembedding labeling.

The most distinct subcellular localization of PtPP2C was found in the cilia, where it was associated with microtubules and dynein, *i.e.* it was clearly targeted to the ciliary motor (Table III), implicating a motor component as a PtPP2C substrate. This peculiar ciliary localization may be helpful when discussing the cytoplasmic and nuclear localization of the PP2C reported here. A family of cytoplasmic dyneins exists, which in

association with dynactin and microtubules are major players in the transport of cellular cargo such as membrane organelles, possibly large protein complexes and mRNA. This dynein-motor complex can be regulated by phosphorylation and dephosphorylation (for a recent review, see Hirokawa (19)). Indeed, Schroeder *et al.* (20) have demonstrated the presence of a microtubule-based motor in the cell body of *Paramecium*, which in association with a cytoplasmic dynein is involved in vesicle transport. It seems reasonable to consider that the cytoplasmic and nuclear localization of PtPP2C may actually correspond to cellular transport complexes in the ciliate. In agreement with this view is the recent identification of the gene for a PP2C catalytic domain in the vicinity of a cytoplasmic dynein light chain in *Drosophila* (21). This possibly points to a functional coupling of the gene products. Also recently, PP2C was identified immunocytochemically in the bovine rod outer segment, which is separated from the inner segment by the connecting cilium (22). All metabolic traffic between both segments must run through this structural bottleneck. The PP2C identified in the outer segments may in association with microtubular structures and further motor constituents have a function in this process. The co-localization of dynein and PP2C in the cilia of *Paramecium* not only would support such a notion, but also identifies this interaction as a meaningful future experimental target. Finally, the structural features encoded in the small PP2C from *Paramecium* are sufficient to sustain activity and to specifically interact with motor components. The large C-terminal, which is present in so many mammalian isoforms and has no known function, is not required in the ciliate.

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