A Membrane-bound Protein Phosphatase Type 2C from Paramecium tetraurelia

PURIFICATION, CHARACTERIZATION, AND CLONING*

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We isolated the first membrane-bound type 2C serine/threonine protein phosphatase from the ciliated protozoan Paramecium tetraurelia (PtPP2C). Three isozymes of 33, 32, and 31 kDa with a specific activity of 1 μmol·min⁻¹·mg⁻¹ were purified from the ciliary membrane. All enzymatic properties including (a) insensitivity toward inhibitors of other protein phosphatase families such as okadaic acid and microcystin, (b) absolute requirement for divalent cations, and (c) substrate specificity tested with synthetic phosphopeptides were identical to mammalian PP2C enzymes and identified the PtPP2C as a canonical PP2C in Paramecium. Sequence comparisons with PP2C enzymes from rat, rabbit, humans, and yeast are very similar in amino acids 45-303 and 78-349, respectively. The PP2C gene was obtained using degenerate oligonucleotide primers and the polymerase chain reaction. The gene coded for a 33-kDa protein with 300 amino acids and had an (A+T) content of 62%, typical for this protozoan. Nine of 15 Gln residues are encoded by TAA, a universal stop codon which codes for Gln in Paramecium. A large truncation at the COOH-terminal is responsible for the smaller size of the PtPP2C. Only a single transcript of 1 kilobase was detected with a Northern blot indicating that the 32- and 31-kDa proteins are proteolytic products of the 33-kDa enzyme. Sequence comparisons with PP2C enzymes from rat, rabbit, yeast, Arabidopsis, and Leishmania defined a highly diverged enzyme family which shares three conserved domains, I, II, and III, accounting for about 25% of the primary structure. We demonstrated further that the distances between domains I/II and II/III are very similar in all PP2C enzymes (9-13 and 74-80 amino acids, respectively). However, the amino acid sequences of the spacer regions are unrelated. In addition, the COOH-terminal ends of 100-200 amino acids which comprise 30-50% of the enzyme, display no identity. A dendrogram shows that PtPP2C surprisingly is most closely related to the mammalian PP2C, and enzymes from Leishmania, Arabidopsis, and yeast are more distant relatives.

The majority of all serine/threonine-specific protein phosphatases have been divided into four major groups (1). This classification is based solely on enzymological criteria using specific inhibitors and activators and, to a lesser extent, on preferential dephosphorylation of certain substrate proteins in vitro (1). For example, type 1 protein phosphatases (PP1) are preferentially inhibited by the thermostable proteins inhibitor-1 and inhibitor-2 while type 2 enzymes are much less affected (see Ref. 2 for data with inhibitor-2). The type 2 phosphatases consist of three distinct subclasses, 2A, 2B, and 2C, that are distinguished by their cation requirements. PP2A is independent of metal cations whereas PP2B depends on Ca²⁺ and PP2C requires Mg²⁺ for activity (1).

In the last decade many protein phosphatases have been cloned from complementary DNA and sequenced. The data revealed that PP1 enzymes which contain about 320 amino acids are extremely conserved throughout evolution with greater than 90% identity over 1 billion years (3, 4). Amino acids 48-263 of PP2A are 50% identical to amino acids 36-271 of PP1. PP2B belongs to this gene family since the catalytic domains of PP2A and 2B, amino acids 45-303 and 78-349, respectively, show 43% identity (3).

Molecular cloning has defined PP2C as a distinct and separate gene family (5-7). However, the extent of overall conservation among PP2C enzymes from different phyla for which sequence data are available is smaller than within the other protein phosphatase families. The considerable sequence disparities of PP2C enzymes from various organisms may also indicate a functional diversity of this subclass. Indeed, the list of potential physiological functions proposed for PP2C spans an astounding wide range (8). We report the biochemical isolation, enzymatic characterization, and cloning from cDNA of a novel, smaller PP2C localized to a substantial extent to the cilia, the highly specialized motile organelle of the protozoan Paramecium tetraurelia. Evaluation of the data sheds light on a group of proteins which has identical enzymatic properties in vitro, yet displays pronounced sequence similarity in only 25% of its primary structure.

EXPERIMENTAL PROCEDURES

Materials—Radioisotopes were from Amersham, chromatography materials from Pharmacia, protein, DNA, and RNA standards from Boehringer Mannheim. pBSK, XZapII vector, Escherichia coli XL1-Blue, and Gigapack packaging extract were obtained from Stratagene and...
Sequenator 2.0 from U. S. Biochemical Corp. The DECAprime DNA labeling kit was purchased from Ambion, the cDNA synthesis kit from Life Technologies, Inc., and the mRNA isolation kit from Invitrogen. The mRNA isolation kit was purchased from Ambion, the cDNA synthesis kit from Sequenase 2.0 from U. S. Biochemical Corp. The DECAprime DNA synthesis kit from Invitrogen. Restriction endonucleases and other enzymes were from either Boehringer Mannheim or New England Biolabs. Oligonucleotides were synthesized on an Applied Biosystems model 381A DNA synthesizer.

**Purification of PtPP2C**—Cilia from axenically mass-cultured *P. tetraurelia* (wild-type 51a nd) were isolated as described (9). Enzyme purification was carried out at 4°C with 0.92% Na2SO4 and 0.1% 2-mercaptoethanol in all buffers. PtPP2C was solubilized with 1% Brij 35 (50 mM Tris-HCl, pH 7.5, 0.1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride) at a protein concentration of 10 mg/ml by stirring for 10 min. The suspension was centrifuged (48,000 g, 30 min) and the supernatant applied to a heparin-Sepharose CL-4B column (2.5 x 5 cm) equilibrated with 20 mM Tris-HCl, pH 7.1, 1 mM EDTA, 5% glycerol at 1:20–100 mM. PtPP2C activity in the flow-through fractions was applied at 40 mM to a DEAE-Sepharose column (2 x 5 cm) equilibrated with 20 mM Tris-HCl, pH 7.1, 1 mM EDTA, 5% glycerol. The enzyme was eluted with buffer containing 0.2 M KCl at 60 h. Solid KCl was added to the active fractions to give 1 x final. This sample was chromatographed on phenyl-Sepharose CL-4B (2 x 5 cm) equilibrated with 20 mM Tris-HCl, pH 7.1, 1 mM EDTA, 1 M KCl at 30 ml/h. After washing, PtPP2C was released at 50 ml/h with 20% glycerol instead of 1 M KCl. Pooled fractions (10 ml) were diluted to 15 ml and applied to a Mono Q HR 5/5 (1 ml/min, 20 mM Tris-HCl, pH 7.1, 1 mM EDTA, 10% glycerol). PtPP2C was eluted at 4 ml/h to a step of 0.6 M KCl and was immediately applied to a Superose 75 preparation grade 26/690 column eluted in Mono Q buffer with 20 mM KCl at 1 ml/min. Active fractions were loaded on a second Mono Q column operated at 1 ml/min in 20 mM imidazole-HCl, pH 6.1, 1 mM EDTA, 10% glycerol, 5% magnesium acetate. PtPP2C was eluted with a linear 70-ml KCl gradient (0–175 mM). PtPP2C Assays—Bovine casein was phosphorylated by cAMP-dependent protein kinase (from Sigma) in the presence of 50 µM cAMP with 1-P32ATP to 3 nmol phosphatidylmg (10). Dephosphorylation reactions (30 µl) contained 20 mM magnesium acetate or 0.1 M EGTA, 50 mM Tris-HCl, pH 7, 0.1% 2-mercaptoethanol, 0.6 mg/ml BSA, and 1 µM [32P]cAMP (about 60,000 cpm/µl). Assays were terminated after 10 min at 30°C with 200 µl 20% trichloroacetic acid (w/v), and radioactivity of the supernatant was determined after centrifugation (10). Peptide substrates were prepared by a CBR peptide synthesizer and purified by reversed-phase HPLC. Synthet and the ribosomal 86 peptide (peptides 10 and 11 in Table II) were a gift of P. Cohen, Dundee, United Kingdom. Peptides 1–11 (Table II) were phosphorylated with [γ-32P]ATP and cAMP-dependent protein kinase, peptide 12 with casein kinase-2, and peptides 13 and 14 with the tyrosine kinase encoded by the proline-rich protein (11). 165% of PtPP2C was solubilized together with 19% of contaminating protein  were removed by chromatography on a Superose 75 column which was calibrated with blue-stained bands.

**Amino Acid Sequencing**—Proteins were treated by chromatography on SMART Mono Q (20 mM Tris-HCl, pH 7.1, 1 mM EDTA, 10% glycerol, 0.15 M NaCl, 0.6 x KCl). After separation on SDS-polyacrylamide gels (4–16% PAGE), the proteins were blotted onto nitrocellulose filters. The filters were washed twice for 20 min at 65°C in 1 x SSC, 0.1% SDS. Two clones were cut through three rounds of screening, obtained in pBSK, and sequenced. The insert of 962 bp displayed an open reading frame with a short poly(A) tail. The 5' end of the gene was obtained using the RACE method. Freshly prepared mRNA and a gene-specific primer 300 bp downstream of the available 5'-end (primer 4, 5'-CCCTTCG-CGCTTT-GGAGGT-CACCC-3') were used for cDNA synthesis. A PCR was carried out using this cDNA fragment and a nested primer 150 bp upstream of the cDNA primer (primer 5, 5'-CCCGGAA- TTCACTC-TCT- TAACCT-GTC-3') in combination with the anchor primer supplied with the kit (35 cycles at 94°C for 45 s, 65°C for 45 s, and 72°C for 1 min). A 450 bp fragment (primers 1 and 3) was subcloned into pBSK, sequenced, and identified as PtPP2C.

**cDNA Library Screening**—Nylon filter lifts from seven plates (30,000 plaques/plate) were hybridized with a probe generated from the 535-bp PCR product of 277 bp was subcloned by blunt-end ligation into pBSK and sequenced with universal primers.

**RESULTS**

**Purification and Characterization of PtPP2C**—Mg2+-dependent dephosphorylation of casein was observed in homogenates of *Paramecium* cilia and cell bodies. The enzyme activity was labile but could be stabilized by addition of 0.1% 2-mercaptoethanol and 5% glycerol. Total activity was 10-fold higher in homogenates of cell bodies compared to cilia, however, the specific activities were comparable. The apparent high casein phosphatase activity in cell body homogenates was to a considerable extent due to the presence of proteases which could not be completely inhibited by addition of the usual protease inhibitor mixtures. In some subcellular fractions protease activity was responsible for up to 90% of the apparent protein phosphatase activity as determined by organic extraction of the phosphomolybdate complex (9). Because of the dephosphorylation in cell body homogenates was Mg2+-dependent, the ratio of partition of PtPP2C between cilia and cell bodies could not be established exactly. We started PtPP2C purification from isolated cilia which constitute only 2% of total cellular protein because protease activity in purified cilia is negligible. The PtPP2C activity was mostly attached to the ciliary membrane. Axonemal fractions prepared by a sucrose density gradient (21) did not contain enzyme activity. Stimulation of casein dephosphorylation in ciliary membranes by 20 mM Mg2+ was 11-fold resulting in a specific activity of 0.13 milliunits/mg (Table I). 165% of PtPP2C was solubilized together with 19% of the ciliary protein by treatment with 1% 2-mercaptoethanol. The increase in total activity was due to a dose-dependent activation of PtPP2C by Brij-55 which occurred at all stages of the purification and amounted up to 200%.

**Characterization of a Paramecium cDNA Library**—A cDNA library containing 2 x 108 independent clones was constructed using the Not-I site of the Zap vector. The first cDNA strand was synthesized using 77 µg of size-selected (0.5–5 kb) poly(A) RNA as a template and oligo(dT) as a primer. After in vitro packaging, the library was stored by transfection of F. coli Blue cells and amplified to a titel of 7.5 x 1012 plaque-forming units/ml.

**Amplification and Subcloning of PtPP2C**—Oligonucleotides derived from PtPP2C peptide fragments were designed using the peculiar codon usage of the ciliate (18). Primers were used for: primer 1 (sense): 5'-ATCACGAGGTCATATGCTAGTCT-3' (277 bp) corresponding to the peptide sequence AGFGVSDG; primer 3 (antisense): 5'-CT-
The enzyme was isolated from purified cilia from 80 liters of culture as described under "Experimental Procedures." Phosphatase activity was assayed in the presence of 0.1 mM EGTA or 20 mM Mg"" with 1 μm casein as a substrate. Note that the removal of the detergent resulted in a drop in total and specific activity due to an unspecified activation by Brij-35. The yield and specific activity in the final step is related to the second peak of activity (see Fig. 1).

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Total activity (μmol)</th>
<th>Specific activity (nmol/mg)</th>
<th>Yield</th>
<th>Activation by Mg&quot;&quot;</th>
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<td>207</td>
<td>1.09</td>
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<td>152</td>
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<td>21</td>
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<td>100</td>
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<td>1.4</td>
<td>1100</td>
<td>1.2</td>
<td></td>
</tr>
</tbody>
</table>

* No activity without Mg""

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**Fig. 1.** Final purification step of PP2C from Paramecium. A, separation of PtPP2C on Mono Q anion-exchange chromatography at pH 6.0 (see "Experimental Procedures" for details). Numbers indicate those fractions analyzed by SDS-PAGE in B. Molecular mass standards are indicated. The gel was silver-stained.

The molecular mass standards BSA (68 kDa), ovalbumin (45 kDa), chymotrypsinogen (25 kDa), and cytochrome c (12.5 kDa). PtPP2C eluted as a single peak at 29 kDa. Examination of the active fractions by SDS-urea-PAGE showed the presence of three major polypeptides of 31, 32, and 33 kDa. This suggests a monomeric catalyst as is the case for all other known PP2C enzymes. PtPP2C eluted as a single peak at 29 kDa. Examination of the molecular mass standards BSA on the SDS-PAGE. From 160 liters of culture of Paramecium, 1–2 μg (30–60 pmol) of PtPP2C were obtained. The ratio of PtPP2C activities of the 33-, 32-, and 31-kDa isozymes was estimated to be about 10:8:3. No differences in enzyme properties were detectable when the PtPP2C activities of the homogenous 32- and 33-kDa isozymes were assayed. Because of the limited amount available, the Superdex eluate was used for further characterization. PtPP2C preferentially used casein as a substrate. Dephosphorylation of phosphorylase a was 20-fold slower compared to casein. The enzyme was insensitive toward low concentrations of inhibitor proteins 1 and 2 (2) and unaffected by okadaic acid, tautomycin, or microcystin LR which are potent inhibitors of PP1, 2A, and 2B (23). Heparin and protamin were inhibitory (IC50 = 1 mg/ml and 50 μg/ml, respectively). The enzyme had an absolute requirement for Mg"" for activity (A0.5 = 3 μM, maximum at 60 mM). Mn"" and Co"", both at 20 μM, supported only 64 and 28% activity, respectively, when compared to 20 mM Mg"". Ca"", Ni"", Cu"", and Zn"" were inactive. The optima for temperature and pH of PtPP2C were 45 °C and pH 7.25, respectively. An activation energy of 69 kJ/mol was deduced from a linear Arrhenius plot.

These properties indicate that the isolated protozoan enzymes belong to the type 2C protein phosphatases in spite of their considerably smaller size compared to other PP2C isoforms which are between 40 and 45 kDa (5–7, 24–28). This was further ascertained by investigating the substrate specificity with synthetic phosphopeptides which have been used to discriminate between various protein phosphatase families (Table II (11–14)). Using Kemptide analogs the PtPP2C showed a 26-fold preference for P-Thr over P-Ser (compare peptides 1 and 2 in Table II) which is characteristic for 2A and 2C phosphatases. PP1 and 2B do not dephosphorylate these peptides. The Kp of 1 μM for the phosphotheorenylpeptide was identical to that observed with PP2C from skeletal muscle (11). An unequivocal demonstration that the phosphotheorenyl residue is preferentially dephosphorylated by the PtPP2C, at least when
located in a short sequence, was provided by using synthetic phosphopeptides by FDRRVSpVAEE (Fig. 2). The P-Thr derivative inhibited the release of P, with much higher potency than the P-Ser homologue. Expectedly, the P-Tyr compound was ineffective. An acidic residue at position +1 next to serine (peptide 3) or 2 prolines at positions +1 and −1 next to threonine (peptide 7) further reduced dephosphorylation (compare peptides 1, 2, and 7 in Table II). Peptide 7 is almost exclusively dephosphorylated by PP2B, but not by PP1, 2A, and 2C (12). Additional arginines at the NH₂ terminus (peptides 1, 5, and 6) progressively enhanced acceptance of the Ser-containing substrates by PtPP2C. Again, this is highly characteristic for PP2C. Another phosphopeptide (peptide 12 in Table II) which is known to be only dephosphorylated by type 2A phosphatases was not dephosphorylated by the protozoan 2C enzyme. Phosphatase activity was moderate with an RII derived peptide (peptide 9 in Table II) which is preferentially dephosphorylated by PP2B (12), and very high with the 32 residues long ribosomal S6 peptide (peptide 11 in Table II) which is poorly dephosphorylated by PP2B. Expectedly, it does not dephosphorylate phosphotyrosyl peptides (peptides 13 and 14). For peptides 1, 2, 10, and 11 Kₐ and Vₘₐₓ values were determined (Table III). The data indicate that the threonine kemptide analog has 2.5-fold higher affinity to the enzyme (lower Kₐ) and a 6-fold higher Vₘₐₓ compared to the serine analogue (Table III). The difference is particularly striking when looking at the efficiency coefficient (Kₐ/Vₘₐₓ). The peptide analogue of the ribosomal S6 protein is a particularly good substrate for PtPP2C. This is due mostly to the very high Vₘₐₓ, the concomitant increase in Kₐ leaves the efficiency coefficient almost identical to the threonine kemptide analogue (Table III). In summary, all properties and substrate specificities of the PtPP2C identify it as a canonical PP2C enzyme.

**Sequencing and Cloning—** Purification of PtPP2C isoforms for amino acid sequencing purposes started from 480 liters of *Paramecium* culture. 11.5 μg of the 33 kDa, 5 μg of the 32 kDa, and 14 μg of an equal mixture of the 33- and 31-kDa proteins were available. The latter proteins could be separated by a 12.5% SDS-urea-PAGE. Attempts to directly sequence the purified proteins which were blotted onto polyvinyldiene difluoride membranes by Edman degradation failed indicating that all three had blocked NH₂-terminal ends. Microsequencing of trypsin-generated and HPLC-purified fragments yielded six peptide sequences: 1) VIFASEMQ (from 33- and 31-kDa proteins); 2) AGGFVSDGR (from 33 kDa); 3) ALGDLEY (from 33 and 32 kDa); 4) TITTGGGQ (from 33 kDa); 5) FKPEEQITAL-PDVK (from 32 kDa); 6) NEQLILALPXIK (from 31 kDa; X is uncertain; compare also to peptide 5). The marked sequence identities among peptides generated from the three isoforms strongly indicated that the 32 and 31 kDa entities were either proteolytic products of the 33-kDa enzyme or corresponded to closely related isoforms encoded by different genes. Further, peptides 1–3 were similar to amino acid positions 25–31, 177–181, and 196–203 contained in rat PP2C (see Fig. 3; (5)), and peptides 4–6 were novel.

**Degenerate primers** were designed according to peptide sequences 1–3. With primers 1 and 3, a 535-bp fragment was amplified by PCR from a *Paramecium* cDNA library. The reaction product served as a template for another PCR with primers for amino acid sequencing purposes started from 480 liters of *Paramecium* culture. 11.5 μg of the 33 kDa, 5 μg of the 32 kDa, and 14 μg of an equal mixture of the 33- and 31-kDa proteins were available. The latter proteins could be separated by a 12.5% SDS-urea-PAGE. Attempts to directly sequence the purified proteins which were blotted onto polyvinyldiene difluoride membranes by Edman degradation failed indicating that all three had blocked NH₂-terminal ends. Microsequencing of amplified by PCR from a *Paramecium* cDNA library. The reaction product served as a template for another PCR with primers for amino acid sequencing purposes started from 480 liters of *Paramecium* culture. 11.5 μg of the 33 kDa, 5 μg of the 32 kDa, and 14 μg of an equal mixture of the 33- and 31-kDa proteins were available. The latter proteins could be separated by a 12.5% SDS-urea-PAGE. Attempts to directly sequence the purified proteins which were blotted onto polyvinyldiene difluoride membranes by Edman degradation failed indicating that all three had blocked NH₂-terminal ends. Microsequencing of
Protein Phosphatase Type 2C

5'-TGAATTCGAGAATTTAACATATG

Table III

Kinetic constants of the protozan PP2C for phosphopeptides

<table>
<thead>
<tr>
<th>Peptides</th>
<th>V_{max}</th>
<th>K_m</th>
<th>Efficiency</th>
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<tr>
<td>RRATVA</td>
<td>2,400</td>
<td>1.0</td>
<td>2,400</td>
</tr>
<tr>
<td>RRASVA</td>
<td>416</td>
<td>2.5</td>
<td>166</td>
</tr>
<tr>
<td>KEAKKRRQEQIAKRRRLSSLRASTKSSEQK</td>
<td>34,367</td>
<td>15.0</td>
<td>2,298</td>
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</table>

2 and 3 and yielded a 78-bp fragment which was expected concerning the location of these primers in the mammalian PP2C (5–7). Sequencing of the subcloned 535-bp DNA fragment revealed an open reading frame which was 35% (39%) identical to PP2Cα (β) from rat liver. Screening of 2 x 10^6 recombinants from the amplified Paramecium cDNA library with the 535-bp fragment as a probe at high stringency yielded two positive clones both of which were sequenced. Each contained a single and identical open reading frame of 888 nucleotides and 63 bp of the 3'-untranslated region including a poly(A) tail. The missing 5'-end with the ATG start codon and 28 bp of the 5'-untranslated region was obtained with a single run of 5'-RACE (Fig. 3). The full-length PtPP2C nucleotide sequence showed an open reading frame for a protein of 300 amino acids with a calculated molecular mass of 33 kDa and a calculated pI of 5.2. It contained the amino acid sequences which were obtained by microsequencing. The heterogeneity observed in the sequenced peptides 5 and 6 from the 32- and 31-kDa proteins, respectively, could not be accounted for. The 3'-untranslated region consisted of 38 nucleotides including a non-consensus polyadenylation site (ATATA) followed by 24 nucleotides downstream by a run of 25 A residues. A Northern blot with poly(A)^+ RNA isolated from Paramecium showed a single band at 1 kb (Fig. 4) indicating (a) that the isolated and sequenced gene most likely represents the entire mRNA transcript, and (b) that probably only a single form of PtPP2C is expressed in Paramecium.

DISCUSSION

PP2C enzymes are the least characterized of the four types of serine/threonine-specific protein phosphatases. This family of protein phosphatases has enzymatic properties which clearly set it apart from the other three classes PP1, 2A, and 2B (1). The reduction in size of PtPP2C by almost 5-kb is mainly due to what looks like a major truncation at the COOH-terminal of at least 40 (compared to the yeast enzyme) to 80 (rabbit liver).

Fig. 4. Northern blot analysis for PP2C from Paramecium. Autoradiography of a blot of 20 μg of total RNA (A) and 5 μg of poly(A)^+ RNA (B) from P. tetraurelia probed with a radiolabeled DNA fragment corresponding to nucleotides 1–974. The size of the RNA markers (RNA length standards I from Boehringer) is indicated on the right.
Amino acids (Fig. 5). The unaltered enzymatic and biochemical properties of PtPP2C indicate that the structural features which are required for the common catalytic mechanism, have been retained and that the extended COOH terminus may not directly be involved in enzyme activity.

So far, all known members of the PP2C enzyme family are cytosolic, soluble proteins, e.g. from rat (5), rabbit (7), the plant Arabidopsis (25, 26), yeast (27), and the parasite Leishmania (28). In contrast, the enzyme from Paramecium is the first membrane-bound PP2C and needs detergent for solubilization. We do not know how the PtPP2C is attached to the membrane. Since in all three forms the NH₂-terminal is blocked, one may speculate that it carries a hydrophobic substituent such as a fatty acid or a prenyl group which plugs the protein into the membrane. The membrane anchor may specifically enhance interaction with a membranous substrate phosphoprotein in its neighborhood.

A major fraction of PtPP2C activity is localized to the cilium, an organelle specialized for motility. This is important when contemplating a physiological function for PP2C enzymes. To date, this area is rather speculative. In several plants, PP2C is supposed to serve as a quinate dehydrogenase phosphatase (29). In contrast, the enzyme from animals, proposals for a function for PP2C involve, among others the action of the plant hormone abscissic acid (25, 26), which are required for the common catalytic mechanism, have been retained and that the extended COOH terminus may not directly be involved in enzyme activity.

To date, five PP2C enzymes have been sequenced from rabbit and rat (PP2Ca and β), Arabidopsis, yeast, and Leishmania (5–7, 25–28, 35). In a multiple alignment with the PtPP2C, the overall sequence identity is only 6.3%. Taking conservative replacements into account, the similarity increases to 8.4%, barely indicating any relationship. However, if the PtPP2C sequence is compared separately to the other sequences, the extent of identity increases to 20–31%. Closer inspection of all PP2C enzymes sequenced so far. Because of the near identity between the PP2Ca and β forms from rabbit and rat, only the 2a isozyme from rabbit liver is listed. The references from which the sequences were taken are rabbit liver (7), Arabidopsis thaliana (25, 26), Schizosaccharomyces pombe (27), and Leishmania chagasi (28). Although not depicted here, the primary structure of PP2C from Saccharomyces cerevisiae (34) fully fits the above scheme.

**Fig. 5. Sequence comparisons of the Paramecium PP2C clone with other PP2C sequences.** The molecular masses of the respective enzymes are indicated at the right margin. The percent identity of the conserved regions I–III correspond to individual comparisons with the PP2C from Paramecium. On the bottom, the consensus sequences are depicted which are applicable to all PP2C enzymes sequenced so far. Because of the near identity between the PP2Ca and β forms from rabbit and rat, only the 2a isozyme from rabbit liver is listed. The references from which the sequences were taken are rabbit liver (7), Arabidopsis thaliana (25, 26), Schizosaccharomyces pombe (27), and Leishmania chagasi (28). Although not depicted here, the primary structure of PP2C from Saccharomyces cerevisiae (34) fully fits the above scheme.

<table>
<thead>
<tr>
<th>Organism</th>
<th>I (%)</th>
<th>II (%)</th>
<th>III (%)</th>
<th>Mass (kDa)</th>
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<tr>
<td>Paramecium</td>
<td>1</td>
<td>69%</td>
<td>69%</td>
<td>33</td>
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<tr>
<td>Rabbit liver</td>
<td>68%</td>
<td>14%</td>
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The unaltered enzymatic and biochemical properties of PtPP2C indicate that the structural features which are required for the common catalytic mechanism, have been retained and that the extended COOH terminus may not directly be involved in enzyme activity.

Since a single band of 1 kb was observed on a Northern blot we assume that the cloned gene represents the only and complete transcript for PtPP2C. This would classify the 32- and 31-kDa isozymes as either proteolytic or processing products of the 33-kDa enzyme. The existence of only a single transcript for PtPP2C should facilitate experiments to identify its function by microinjection of antisense RNA oligonucleotides (34).
acids with an identity of 38% in a multiple alignment. Again, separate comparisons yield higher levels of conservation (54–77%; Fig. 5). The next segment of 80 amino acids shows identities between 50% and 98% when compared individually to the PtPP2C sequence, whereas in a multiple alignment the extent of identity is negligible. This demonstrates that this region is probably not directly implicated in catalysis. A third, large region of sequence identity is found in a stretch comprising 60 amino acids around positions 120–200 (253–316 in Arabidopsis) with identities from 44 to 54% when compared separately with the ciliate PtPP2C (Fig. 5). The consensus sequence of this domain for all PP2C genes displays an overall identity of 30% (Fig. 5). The regions of 100–200 amino acids at the COOH-terminal ends which represent at least one-third of the protein, do not exhibit any relationship. Undoubtedly, the PP2C sequences delineate a highly divergent enzyme family which shares three conserved sites. It is reasonable to propose that only the conserved segments are responsible for the peculiar enzymatic properties of the PP2C. The rather uniform distances between region I/II (9–13 amino acids) and II/III (74–80 amino acids) could be important determinants for the architecture of the enzyme and be involved in building the spatial arrangement of the conserved domains which form the catalytic center and to all PP2C enzyme. The question of which remains which physiological functions are carried by the large COOH-terminal sections of the PP2C. They may represent built-in targeting subunits which specifically enhance interaction with particular phosphoprotein substrates. Considering the lack of any relationship in the extended COOH-terminal domains this would explain that such a wide range of functions has been suggested for PP2C in different systems.

A surprising finding is that the PP2C from Paramecium and rabbit/rat share a fourth domain of 39% identity within the 32 amino acids at the NH2 terminus. This similarity does not exist with PP2C from yeast, Arabidopsis, and Leishmania. Accordingly, a dendrogram of the alignment shows that the PP2C from Paramecium and mammals are most closely related while PP2Cs from the protozoan Leishmania, the plant Arabidopsis, and yeast, in that order, are more distant relatives within this gene family.

Finally, pyruvate dehydrogenase phosphatase has 16.3% identity to PtPP2C (20% to PP2Ca from rat (38)). A comparison along the lines developed here demonstrates that domain I is missing and that domain II exists in its entirety. Domain III is basically retained but has additionally 2 × 5 amino acids spaced in between. Pyruvate dehydrogenase phosphatase would, therefore, be a good candidate to investigate whether the COOH terminus is indeed involved in substrate binding.

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