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Molecular cloning and primary structure of a protein phosphatase 2C isoform

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Complementary DNA encoding the isoform of protein phosphatase 2C, termed PP2C2, has been isolated. The cDNA predicts a protein of 390 amino acid residues with a molecular mass of 42,888 Da. The protein displays 76% identity to the PP2C1 isoform.

Protein phosphatase; Amino acid sequence; Isoenzyme

1. INTRODUCTION

Protein phosphatase 2C (PP2C), one of the four major classes of Ser/Thr-specific protein phosphatases in mammalian cells (for a review see [1]) is characterised by its complete dependence on divalent cations (Mg^{2+} or Mn^{2+}) for activity and insensitivity to the toxins okadaic acid [2] and microcystin [3] that potently inhibit protein phosphatases 1 and 2A (PP1, PP2A). PP2C has a broad substrate specificity and is capable of dephosphorylating a number of enzymes that regulate metabolic pathways [4], but its activity towards most of these substrates is low in vitro compared to PP1 and PP2A [5], even at the supraphysiological concentration of free Mg^{2+} (10–20 mM) required for maximal PP2C activity. Furthermore, in vivo studies using okadaic acid as a membrane permeable inhibitor, demonstrate that PP1 and/or PP2A are indeed the major phosphatases acting on many of these substrates [6]. The physiological roles of PP2C are therefore unclear although there is evidence that it may be the major protein phosphatase that dephosphorylates and inactivates the AMP-activated protein kinase, a key enzyme involved in the regulation of the hepatic fatty acid and cholesterol biosynthesis [7,8]. However, the equally high levels of PP2C in other tissues where these pathways are absent [5], such as the brain, imply additional functions for PP2C that have not yet been identified.

PP2C is a monomeric enzyme with an apparent molecular mass of about 40–45 kDa [9,10]. Two isoforms have been purified from rabbit skeletal muscle and rabbit liver [11] and amino acid sequencing of several peptides demonstrated that they were the products of distinct genes [12]. The isoform termed PP2C1 (apparent molecular mass 44 kDa) migrated slightly slower on SDS/polyacrylamide gels than the isoform termed PP2C (42 kDa) [11]. Tamura et al. [13] subsequently isolated and sequenced a cDNA encoding the complete sequence of PP2C1 from rat kidney. We have amplified a DNA fragment out of rat liver mRNA encoding for PP2C1 and cloned PP2C2 from a rat liver cDNA library in order to carry out a detailed molecular characterisation of these enzymes and to explore their structure and function using recombinant DNA techniques. In this paper we present the sequence of a full-length cDNA clone encoding rat liver PP2C2 and compare it with PP2C1.

Abbreviations: bp, base pairs; PCR, polymerase chain reaction; PP, protein phosphatase

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2. MATERIALS AND METHODS

2.1. *Materials*

M-MLV reverse transcriptase and restriction enzymes were obtained from Gibco BRL, Eggenstein, Germany. Taq-polymerase (Ampli-Taq) was from Perkin-Elmer Cetus, Norwalk, USA. T4-polynucleotide kinase, [α-^{32}P]dCTP, [γ-^{32}P]ATP, [α-^{35}S]dATP as well as Hybond N nylon membranes came from the Amersham Buchler Co., Braunschweig, Germany. RNAsin, the Random Priming DNA labelling kit were purchased from Boehringer, Mannheim, Germany. The Sequenase 2.0-Kit was from United States Biochemicals Co., Cleveland, OH, USA. Southern blots were performed using GeneScreenPlus from NEN-Dupont, Bad Homburg, Germany, and autoradiography using Kodak XAR-5 films. Oligo dT_{12-18}-DNA and M13mp18RF-DNA were purchased from Pharmacia, Freiburg, Germany. The Lambda ZAP II rat liver cDNA library was obtained from Stratagene, Heidelberg, Germany.

2.2. *Methods*

Oligonucleotides were synthesized on an Applied Biosystems 381A Oligonucleotide Synthesizer, deprotected with concentrated ammonia and desalted using Pharmacia NAP-10 columns.

Total RNA was isolated from rat liver according to [14]. The reverse transcriptase reaction was carried out for 60 min at 37°C with 40 μg of total RNA, 0.6 μg of oligo dT_{12-18} primers, 125 U RNAsin, 0.8 mM of each desoxynucleotide and 600 U of M-MLV reverse transcriptase in 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$ in a total volume of 160 μl. 5 μl of this reaction were used directly in a polymerase chain reaction (PCR) using 3 μg of each oligonucleotide SN1 and ASC1 as primers. The reaction conditions were: 0.2 mM of each desoxynucleotide, 2.5 U Taq-polymerase, 10 mM Tris-HCl, pH 8.3, 50 mM KCl and 2.5 mM $MgCl_2$. The reaction was performed in a Thermocycler 60 (Biomed/Theres, Germany) under the following conditions: denaturing at 92°C for 2 min, extension at 72°C for 1.5 min and annealing for 1.5 min at 45°C for the first five cycles and 55°C for the following 20 cycles.

PCR products were analysed on 1.0% agarose gels, eluted from the gels, digested with both *Hin*dIII and *Sst*I and subcloned into M13mp18. Single-stranded DNA from recombinant M13-clones was sequenced using Sequenase 2.0 according to the manufacturer's instructions. Parallel sequenase reactions were run with dGTP and dITP respectively; the sequencing strategy followed the 'primer hopping' method as described in [15].

A probe for library screening was prepared as follows: M13 inserts were PCR-amplified out of the bacteriophage stocks using primers corresponding to the 3′ and 5′ ends of the insert at an annealing temperature of 60°C and 30 cycles. All other conditions were as described above. The amplified inserts were eluted out of an agarose gel and labelled with [α-^{32}P]dCTP using the random priming method [16].

Bacteriophage plaques from the cDNA library were transferred onto Hybond N membranes according to [17] and hybridization with the labelled 723-bp probe was performed as in [18]. Positive plaques were picked and single positive plaques obtained by two rescreening cycles using the same probe. Phage DNA was prepared from plate lysates, digested with *Eco*RI and separated by agarose gel electrophoresis. The DNA was alkali-blotted onto GeneScreenPlus membranes and examined for insert size and cross hybridization (performed as in [19]) with oligonucleotide SN2, which had been 5′-end-labelled using [γ-^{32}P]ATP and T4-polynucleotide kinase. *Eco*RI-cut cDNA fragments chosen for sequencing were subcloned into M13mp18 and sequenced as described above.

3. RESULTS

Two degenerate oligonucleotides (SN1 and ASC1) constructed to represent two peptides derived from the N-terminus (SN1) and the C-terminus (ASC1) of PP2C2 with additional *Hin*dIII/*Kpn*I- and *Sst*I/*Bam*HI-restriction sites at their 5′ ends are shown in Fig. 1. The sequence of C-terminal peptide [12], but not the N-terminal peptide has been published previously. SN1 and ASC1 served as specific primers to amplify a PP2C2-fragment from cDNA, that had been synthesized with reverse transcriptase using rat liver total RNA as template. This PCR reaction should provide a highly specific probe for screening a cDNA library. A 1.1-kb DNA fragment was expected from the apparent molecular masses reported for the two isoforms [11], and DNA of this size was indeed found as the most intense band when analysing the PCR products by agarose gel electrophoresis. The fragment thought to encode PP2C2 was eluted from the gel, digested with *Hin*dIII and *Sst*I and subcloned into M13mp18. One positive M13-subclone was found to carry a 723-bp fragment with a single open reading frame and strong homology to PP2C1. The reduced insert length was thought to be due to an internal *Sst*I site in the 1.1 kb fragment.

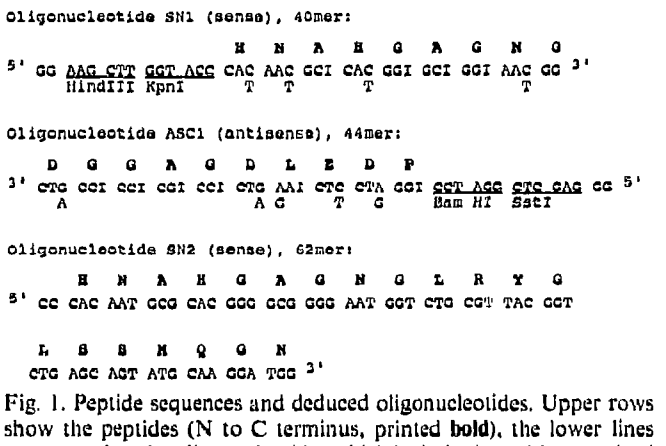

Fig. 1. Peptide sequences and deduced oligonucleotides. Upper rows show the peptides (N to C terminus, printed **bold**), the lower lines correspond to the oligonucleotides which include sites with two mixed bases. The underlined sequences are the additional restriction sites. Note that the mixed bases and inosins in SN1 and ASC1 are responsible for the sequence differences between the 723 bp probe and JW5.

In order to determine the complete primary structure of PP2C2, the isolated 723 bp M13-insert was labelled with ^{32}P and used as a probe to screen 2×10^7 clones of a Lambda ZAP II rat liver cDNA library. Eleven positive signals survived two rescreening cycles, and phage DNA was prepared from these clones. Following *Eco*RI digestion and Southern blotting of the resulting fragments, the cDNA inserts were examined for cross hybridization with the 723-bp probe and the oligonucleotide SN2 (Fig. 1), which had been derived from the 5′-end of the 723-bp sequence. Four clones hybridized with both probes, suggesting that they carried the N-terminal sequences, and the longest (clone JW5) was chosen for further sequencing. Insert DNA of JW5 was about 2.0 kb and contained an internal *Eco*RI site yielding a 0.7 kb fragment that hybridized with oligonucleotide SN2 and a 1.3 kb fragment. Both fragments hybridized with the 723-bp probe and were subsequently cloned into M13mp18 and sequenced.

Clone JW5 contained the complete translated region for PP2C2, 690 bp of the 5′ non-translated region and 87 bp of the 3′ non-translated region. The 723-bp probe was identical to the overlapping part of JW5 (including the boundary between the two *Eco*RI fragments of JW5), except for a few changes due to the degenerate PCR-primers at the 5′ and the 3′ end of the 723-bp DNA and one additional base exchange. JW5 encoded a protein of 390 amino acid residues with a molecular mass of 42,888 Da. The complete sequence and the deduced primary structure are shown in Fig. 2.

4. DISCUSSION

Cohen and co-workers reported the amino acid sequence of a peptide (**DGGAGDLEDP**) corresponding

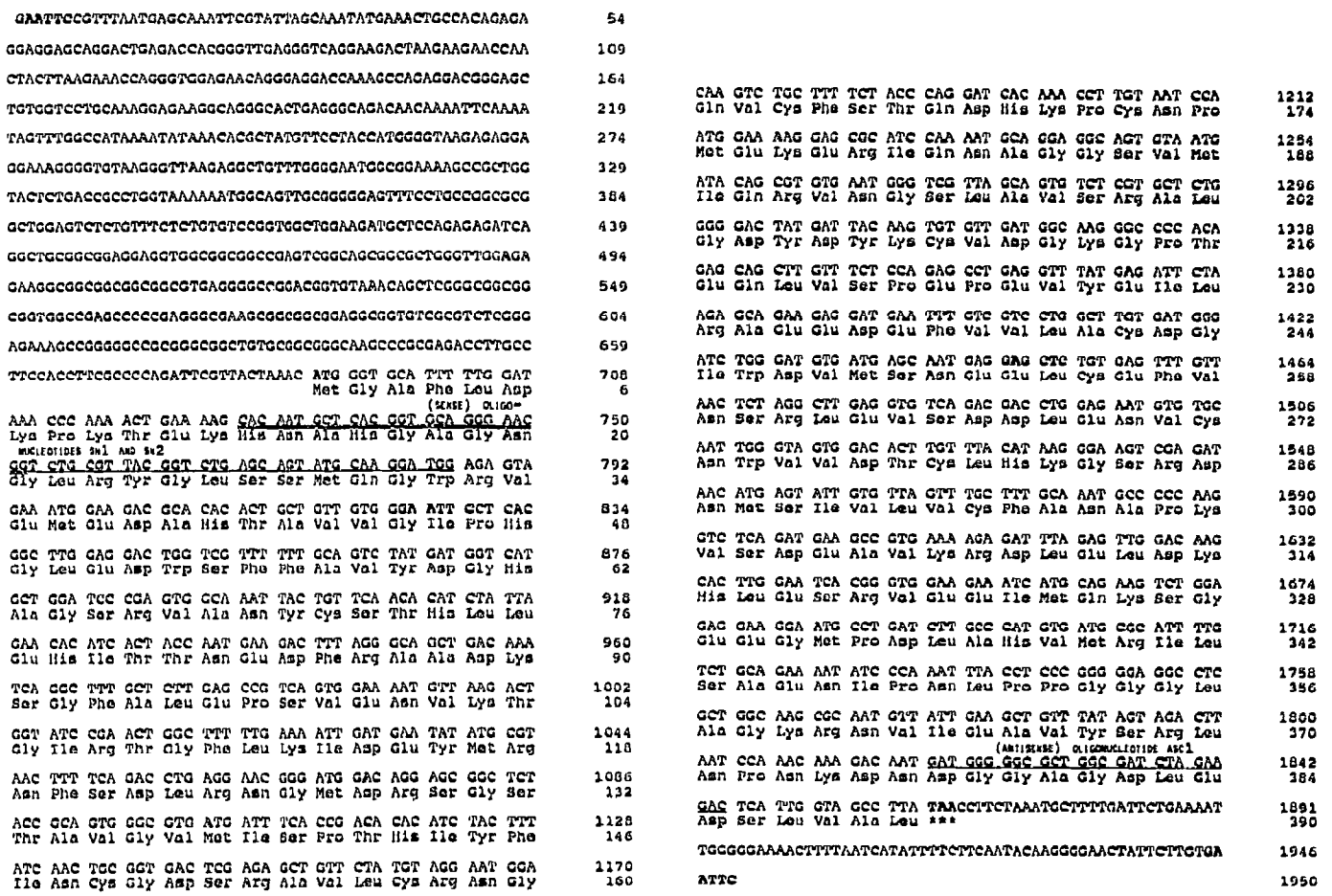

Fig. 2. Complete DNA sequence of the *Eco*RI insert of clone JW5 and the deduced primary structure of PP2C2. *Eco*RI, *Sst*I sites, start and termination codons are printed **bold**. The termination codon is marked by three asterisks. The underlined sequences mark the positions of the three oligonucleotides SN1, SN2 and ASC1.

to the C terminus of rabbit PP2C2 [12], while one peptide the sequence of which was determined subsequently (**HNAHGAGNG**) showed high homology to an N-terminal sequence of rat PP2C1 [13]. The positions of these two peptides in the sequence suggested that they would be suitable for constructing oligonucleotides to serve as primers which could then be used to amplify PP2C2-specific sequences from rat mRNA. Using these probes a 1.1-kb DNA fragment was found, exactly the length expected from the known apparent molecular mass of rabbit PP2C2. The reduced length of 723 bp of the fragment cut with *Hin*dIII/*Sst*I and subcloned into M13mp18 was obviously due to an internal *Sst*I site, which later was found at base 1449 in clone JW5.

The 723 bp fragment was used successfully to isolate JW5 from a rat liver cDNA library, and apart from the errors in the regions corresponding to SN1 and ASC1, only one base exchange was found in the overlapping parts of these two DNAs. This change at base 1118 would cause an exchange of arginine for tyrosine, but we believe the JW5 sequence to be correct, since the PCR reaction is known to be error-sensitive and amino acid sequencing has shown that this residue is tyrosine in rabbit PP2C2 [20].

Both peptides used to construct the original oligonucleotide probes (Fig. 1) were found in clone JW5 at the expected N-terminal and C-terminal positions (printed **bold** in Fig. 3), apart from the last residue of the C-terminal peptide which was serine in JW5 and proline in rabbit PP2C2. This presumably reflects a species difference between the rat and rabbit proteins. A peptide sequencing error is unlikely, since the amino acid composition of the C-terminal tryptic peptide of rabbit PP2C2 was identical to the primary structure determined by amino acid sequencing (ESDGGAGDLEDP) [12]. Other previously sequenced peptides [12] were also found in JW5.

We have isolated and sequenced a cDNA fragment amplified out of rat liver total RNA whose nucleotide and deduced amino acid sequence are identical to the rat kidney sequence of PP2C1 reported by Tamura et al. [13] (G. Mieskes, unpublished results). This indicates

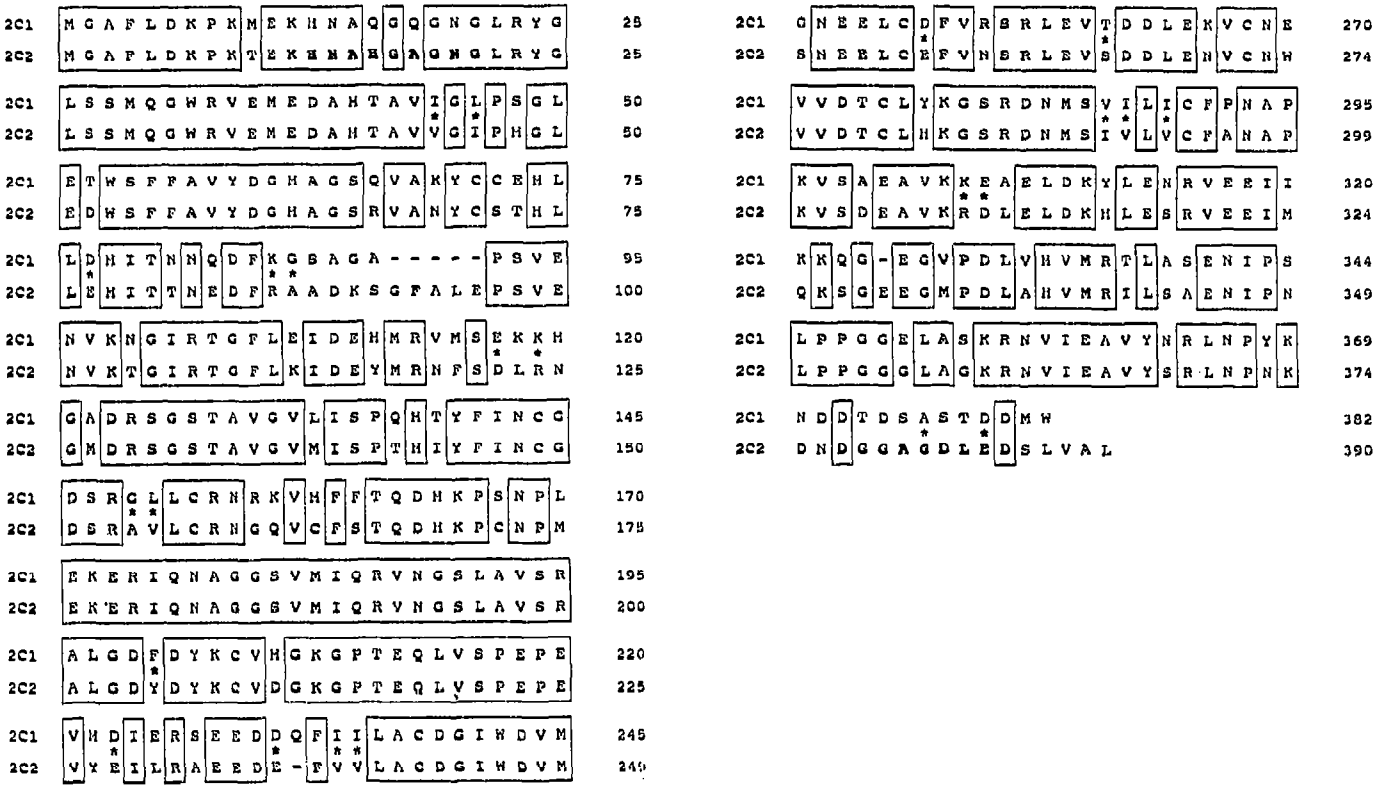

Fig. 3. Sequence comparison between rat PP2C1 and rat PP2C2. The boxes mark regions of identity between the isoforms, the asterisks show conservative amino acid exchanges. Previously known peptides are printed **bold**.

that the liver and kidney enzymes are products of the same gene. The amino acid sequences of rat PP2C1 [13] and rat PP2C2 are compared in Fig. 3. The two isoforms display an overall homology of 76% and 23 of the differences between the isoforms are conservative replacements.

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