Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 1796–1800, March 1989
Biochemistry

Molecular cloning of rat type 2C (IA) protein phosphatase mRNA

(cDNA/adenylate cyclase)

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Communicated by Oscar L. Miller, Jr., December 5, 1988 (received for review September 2, 1988)

ABSTRACT **A full-length cDNA encoding rat type 2C (IA) protein phosphatase was isolated from a kidney cDNA library. The cDNA was identified by screening the library with oligonucleotides based on a partial amino acid sequence determined from purified rat liver phosphatase. This clone is 2.35 kilobase pairs long and has a single extended translation reading frame that predicts a 382-amino acid protein of 42,416 daltons. The deduced amino acid sequence contains segments corresponding to three peptides from rat liver type 2C protein phosphatase and two peptides from rabbit skeletal muscle type 2C phosphatase. Rat kidney type 2C protein phosphatase is distantly related to yeast adenylate cyclase but is not related to the catalytic subunits of two other protein phosphatases (types 1 and 2A).**

Protein phosphorylation plays a key role in the regulation of cellular functions. Although most studies focus on protein kinases, the importance of protein phosphatases in the phosphorylation/dephosphorylation cycle has been well documented (1). In this context, we previously reported the purification and characterization of three types of cytosolic rat liver phosphatases, types IA, IB, and II (2–5). These enzymes were later independently identified and named types 2C, $2A_1$, and $2A_2$, respectively (6). Since this later nomenclature is more widely used, we have adopted it in this report. The type 2C enzyme (M_r 42,000–48,000) is monomeric and requires Mg^{2+} for activity (4, 5). Types $2A_1$ and $2A_2$ are heterooligomeric and have two common subunits termed α (M_r 35,000) and β (M_r 69,000); the α subunit is the catalytic moiety (2, 3). Type $2A_1$ has a third subunit, γ (M_r 58,000). The functions of the β and γ subunits are unknown. Although the type 2C enzyme was originally thought to be specific for glycogen synthase (7, 8), it was later shown that this enzyme is active with a wide variety of substrates, at least *in vitro* (5, 6). Neither the role of this enzyme in dephosphorylation *in vivo* nor the mechanism of its regulation has been described to date. To further understand the biology of the type 2C phosphatase, we undertook the molecular cloning of its mRNA.‡‡

MATERIALS AND METHODS

Isolation and Sequence Analysis of Peptides. Type 2C protein phosphatase was purified from rat liver as described (4, 5). The purified protein (100 μg) was digested with trypsin and the resulting peptides were purified by HPLC (9). The peptides were sequenced by automated Edman degradation (10).

Oligodeoxynucleotide Synthesis. Oligonucleotides encoding regions of amino acid sequence were designed using the consensus rules of Lathe (11) and synthesized on a BioSearch 8600 synthesizer.

Cloning. The rat liver and kidney libraries were constructed and screened as described (12, 13). The *Eco*RI inserts were subcloned into pGEM-series plasmids (Promega Biotec) and DNA sequence was determined by the dideoxy chain-termination technique of Sanger *et al.* (14) after construction of a series of nested deletions. In some cases, the dGTP analogue 7-deaza-dGTP (Toyoba, Osaka, Japan) was used to reduce band compression in G+C-rich sequences (15).

RESULTS

Amino Acid Sequencing. Attempts to analyze the amino terminus of the type 2C phosphatase failed, presumably because the amino terminus is blocked. Tryptic peptides were therefore generated and isolated by reverse-phase HPLC, and limited amino acid sequence was determined from several of these by automated Edman degradation. The most probable nucleotide sequences encoding two of these peptides (Op-1 and Op-2) were determined using the rules of Lathe (11) and their complements were synthesized. The amino acid sequences of the peptides and the sequences of the corresponding oligonucleotides (On-1 and On-2) are presented in Table 1.

cDNA Cloning. The two oligonucleotides were labeled with phosphorus-32, mixed together, and used to screen $\approx 10^6$ phage plaques from a rat liver cDNA library (12). Phage DNA was purified from 16 clones that yielded a signal; all of these hybridized to oligonucleotide On-2, whereas none hybridized to probe On-1, even under conditions of lowest stringency (data not shown). Cross-hybridization of the cDNAs reduced the 16 individual clones to four populations. The largest insert in each group was subcloned into the *Eco*RI site of pGEM-4Z and sequenced. One clone, pST-7, contained a 0.37-kilobase-pair (kbp) insert that encoded the peptide Op-2. This cDNA was used to probe RNA isolated from rat kidney and liver; a single-size RNA [estimated at 2.4 kilobases (kb)] from both tissues hybridized to this cDNA (data not shown). To obtain a full-length type 2C phosphatase cDNA, 10^6 clones from a rat kidney library (13) were probed with pST-7. This screening yielded an additional 5 clones, one of which, pST-11, had an insert of 2.35 kbp. DNA from clone pST-11 hybridized to a single 2.4-kb RNA species in rat liver and kidney (Fig. 1).

Analysis of pST-11. The 2.35-kbp insert was isolated by partial *Eco*RI digestion and subcloned into pGEM-7Zf(+) for sequence analysis. The sequence of 1.60 kbp of this cDNA is

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‡‡The sequence reported in this paper is being deposited in the EMBL/GenBank data base (accession no. J04503).

Table 1. Peptide and synthetic oligonucleotide sequences

Peptides		Oligonucleotides	
Name	Sequence	Name	Sequence (5′ → 3′)
Op-1	ALGDFDYK	On-1	CTTGTAGTTGAAGTTGCCCAGGGC
Op-2	VEMEDAHTAV	On-2	CACAGCTGTGTGGGCATTCTCCATCTCCAC
Op-3	TAVQVLIE	—	
Op-4	GFELLD	—	

Peptide sequences were determined from HPLC-purified tryptic fragments derived from 100 μg of purified rat liver type 2C protein phosphatase and are given in standard one-letter code. The On-1 and On-2 oligonucleotide sequences are the complements of the most probable sequences [as determined by the rules of Lathe (11)] encoding the peptides Op-1 and Op-2, respectively.

presented in Fig. 2. This sequence contains a single long open translation reading frame of 1146 bp that encodes a protein of 382 amino acids. This reading frame is preceded by an 87-nucleotide G+C-rich region, and the putative translation initiation codon is in the context of the translation start consensus sequence (A at −3, G at +4, where the A in ATG is +1) (17). The open reading frame is terminated by a stop codon (TAA) at nucleotide 1294 and predicts a protein of 42,416 daltons. The sequences of both Op-1 and Op-2 are found within the conceptualized protein; furthermore, a peptide similar to Op-3 (six of eight amino acids) is also found. However, the sequence of Op-4 is not found within the protein sequence. Finally, the amino acid composition of the predicted protein is compatible with that determined for the purified rat liver phosphatase (data not shown).

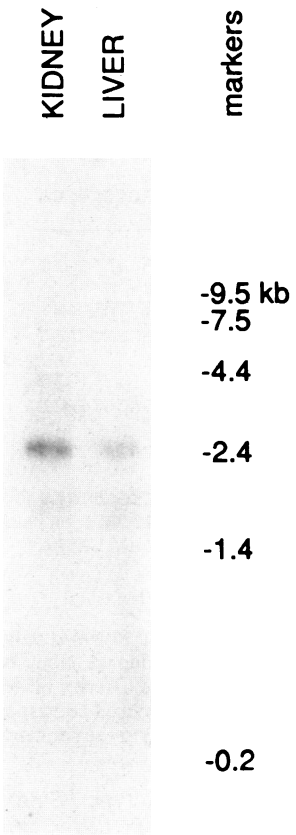

FIG. 1. Type 2C protein phosphatase RNA from rat liver and kidney. Two micrograms of poly(A)$^+$ RNA extracted from liver or kidney was electrophoresed through an agarose gel under denaturing conditions and transferred to a nylon membrane (Northern blot). The bound RNA was hybridized to 100 ng of an 800-bp 5′ *Eco*RI fragment of cDNA pST-11 radiolabeled with phosphorus-32 to a specific activity of 1.5×10^8 dpm/μg. Autoradiographic exposure time was 5 days in the presence of an image-intensifying screen. Methods were as described (12, 13).

Sequence Comparisons. The deduced amino acid sequence of the type 2C phosphatase was compared with other reported sequences by using the FASTP algorithm of Lipman and Pearson (18). This analysis demonstrated that the type 2C phosphatase is similar to the carboxyl-terminal region of yeast adenylate cyclase (19, 20); regions of similarity are shown in Fig. 3. The type 2C phosphate shares 22% identity with yeast adenylate cyclase (ref. 19 and Fig. 3); this similarity extends throughout 305 (of 382) amino acids of the type 2C phosphatase. To assess the significance of this similarity, the phosphatase and adenylate cyclase amino acid sequences were compared directly by using the RDF2 algorithm (21). In 200 shuffles of the adenylate cyclase sequence, the highest optimized similarity score was 84, while the similarity score of the type 2C phosphatase with adenylate cyclase was 140. The z value calculated from this RDF2 analysis is 11.7, indicating a high probability that the similarity between these two proteins represents an homology (21). Interestingly, the similar region overlaps the domain of yeast adenylate cyclase that reportedly (19, 20) contains its catalytic center. No other similarities were found among the sequences in two data bases (Genbank release no. 57 and the Protein Identification Resource release no. 17). Specifically, there is no significant similarity between protein phosphatase type 2C and the catalytic subunits of several other protein phosphatases (see below).

DISCUSSION

Our identification of cDNA clone pST-11 as encoding rat kidney type 2C protein phosphatase mRNA is based on the observation that two peptide sequences derived from the highly purified rat liver protein were identical to regions of the deduced protein, and a region similar to a third peptide sequence (75% identity) also was predicted. Furthermore, the size (382 amino acids, 42,416 daltons) of the predicted protein agrees well with the observed molecular weight (M_r 42,000–48,000) from SDS/polyacrylamide gel electrophoresis (4, 5, 22, 23). The 2.35-kbp cDNA pST-11 is likely to be full-length because it hybridizes to a single, 2.4-kb poly(A)$^+$ RNA species from rat liver and kidney.

Cohen and colleagues recently reported the purification of two isozymes of rabbit skeletal muscle type 2C protein phosphatase (23) and determined the amino acid sequences of two peptides derived from each isozyme (16). Both peptide sequences of one isozyme (subtype $2C_1$) are accurately predicted by our cDNA pST-11 (Fig. 2). This finding lends further credence to our identification of this cDNA as encoding a type 2C protein phosphatase and shows unequivocally that our type IA and Cohen's type 2C phosphatases are the same protein. We note that one of the peptide sequences that we determined from rat liver type 2C phosphatase (peptide Op-4) was not found in the predicted amino acid sequence of cDNA pST-11, nor was a second pair of peptides identified by Cohen's group (isolated from isozyme $2C_2$) found in our cDNA-derived sequence. These missing pep-

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        10                  30                  50                  70
TGCCCGGCCGCCGTCGCCACCGCCGCCCCGGCCGACCGAGGGACCCGCCCGCCCGCGGCTGCTCCGGATCTAGAG
              90                  110                 130                 150
GATCAAGTCATAATGGGAGCATTTTTAGACAAGCCAAAGATGGAAAAGCATAATGCCCAGGGCCAGGGGAACGGG
            M  G  A  F  L  D  K  P  K  M  E  K  H  N  A  Q  G  Q  G  N  G     21
                   170                 190                 210
TTGCGATACGGCCTAAGCAGCATGCAAGGTTGGCGAGTTGAAATGGAGGATGCACATACGGCTGTGATCGGTTTG
L  R  Y  G  L  S  S  M  Q  G  W  R  V  E  M  E  D  A  H  T  A  V  I  G  L     46
  230                 250                 270                 290
CCAAGTGGACTTGAGACATGGTCATTCTTTGCTGTATATGATGGGCATGCTGGTTCTCAGGTTGCCAAATACTGC
P  S  G  L  E  T  W  S  F  F  A  V  Y  D  G  H  A  G  S  Q  V  A  K  Y  C     71
       310                 330                 350                 370
TGTGAGCACTTGTTAGATCACATCACCAATAACCAGGATTTTAAAGGCTCTGCAGGAGCACCCTCTGTGGAAAAC
C  E  H  L  L  D  H  I  T  N  N  Q  D  F  K  G  S  A  G  A  P  S  V  E  N     96
              390                 410                 430                 450
GTAAAGAACGGAATCAGAACAGGTTTTCTGGAGATTGATGAACACATGAGAGTTATGTCAGAGAAGAAACACGGT
V  K  N  G  I  R  T  G  F  L  E  I  D  E  H  M  R  V  M  S  E  K  K  H  G    121
                   470                 490                 510
GCAGATAGAAGTGGGTCAACAGCTGTGGGTGTCTTAATTTCTCCCCAACATACTTATTTCATTAACTGTGGGGAC
A  D  R  S  G  S  T  A  V  G  V  L  I  S  P  Q  H  T  Y  F  I  N  C  G  D    146
  530                 550                 570                 590
TCAAGAGGTTTACTTTGTAGAAATAGAAAAGTTCACTTCTTCACTCAAGACCATAAACCAAGTAATCCTCTGGAA
S  R  G  L  L  C  R  N  R  K  V  H  F  F  T  Q  D  H  K  P  S  N  P  L  E    171
       610                 630                 650                 670
AAAGAACGAATTCAGAATGCAGGGGGCTCTGTGATGATTCAGCGTGTCAATGGCTCTCTGGCTGTATCAAGAGCT
K  E  R  I  Q  N  A  G  G  S  V  M  I  Q  R  V  N  G  S  L  A  V  S  R  A    196
              690                 710                 730                 750
CTTGGGGATTTTGATTACAAATGTGTCCATGGAAAAGGTCCCACAGAGCAGCTTGTCTCGCCAGAGCCTGAAGTC
L  G  D  F  D  Y  K  C  V  H  G  K  G  P  T  E  Q  L  V  S  P  E  P  E  V    221
                   770                 790                 810
CATGATATTGAAAGGTCTGAAGAAGATGACCAGTTCATCATTCTTGCATGTGATGGCATCTGGGACGTCATGGGC
H  D  I  E  R  S  E  E  D  D  Q  F  I  I  L  A  C  D  G  I  W  D  V  M  G    246
  830                 850                 870                 890
AACGAAGAGCTCTGTGACTTTGTGAGATCCAGACTTGAAGTCACTGATGACCTTGAGAAAGTTTGCAATGAAGTA
N  E  E  L  C  D  F  V  R  S  R  L  E  V  T  D  D  L  E  K  V  C  N  E  V    271
       910                 930                 950                 970
GTCGACACCTGTTTGTATAAGGGAAGTCGAGACAACATGAGTGTGATTTTGATCTGCTTTCCAAACGCGCCTAAA
V  D  T  C  L  Y  K  G  S  R  D  N  M  S  V  I  L  I  C  F  P  N  A  P  K    296
              990                1010                1030                1050
GTCTCAGCAGAGGCGGTGAAGAAGGAGGCAGAGCTGGACAAGTACCTGGAGAACAGAGTAGAAGAAATCATAAAG
V  S  A  E  A  V  K  K  E  A  E  L  D  K  Y  L  E  N  R  V  E  E  I  I  K    321
                  1070                1090                1110
AAGCAGGGGGAAGGCGTCCCTGACTTAGTCCATGTGATGCGCACGTTAGCCAGTGAGAACATCCCCAGCCTCCCA
K  Q  G  E  G  V  P  D  L  V  H  V  M  R  T  L  A  S  E  N  I  P  S  L  P    346
 1130                1150                1170                1190
CCAGGGGGTGAACTGGCAAGCAAGCGGAATGTAATTGAAGCCGTTTACAATAGACTGAACCCTTACAAAAACGAC
P  G  G  E  L  A  S  K  R  N  V  I  E  A  V  Y  N  R  L  N  P  Y  K  N  D    371
       1210                1230                1250                1270
GACACTGATTCTGCGTCAACTGATGATATGTGGTAAAGCTGCTCATCTAGCCATGGTTTCACCTTTGCCTGGAAG
D  T  D  S  A  S  T  D  D  M  W                                              382
              1290                1310                1330                1350
AGAGAGAGAGATCAGCGCATCCTTGCTGAGCCTTTAACATCCATCACCGACTTAAGGAAGGGGTCCGACATGGGT
                  1370                1390                1410
GAGGGACTACAGCTTCAGCGATGGAACAGCCAGCCCAGAATGGATTTTTTTTTTATTGTAAATTTGAGACTTAT
 1430                1450                1470                1490
GTAAGCGTGATTTCAAACCGTAATTCATGTTGTAAATCAGACTCCAGCAATTTTTGTTGTATGATTTTGGTTTTT
       1510                1530                1550                1570
TGTAAAGTGTAATTGTCCTTGTACAAAGTGCTCATATTTAATTATGAACTGCTTTAAAATCACTATCAATGATAA
              1590
AGGAAATTTGGCTTGTTGTGTGGCACA
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FIG. 2. Nucleotide and predicted protein sequence of cDNA pST-11. The 2.35-kbp rat cDNA insert of pST-11 was removed from λgt10 DNA by partial *Eco*RI digestion and subcloned into the same site in plasmid pGEM-7Zf(+). The nucleotide sequence along both strands of 1602 contiguous nucleotides was determined by the dideoxy chain-termination method (14) after construction of a series of nested deletions. The amino acids are given in one-letter code under the nucleotide character strings. The peptide sequences overscored correspond to peptides Op-2 (residues 34–43), Op-3 (residues 128–135), and Op-1 (residues 196–203). The underscored regions correspond to the sequences of peptides CB-1 (residues 116–145) and CB-2 (residues 335–370) (both from isozyme $2C_1$) described by another group (16).

tides are presumably parts of a type 2C phosphatase isozyme(s).

A surprising finding came from our comparison of the predicted type 2C phosphatase primary structure with those of other proteins, particularly other protein phosphatases. Type 2C protein phosphatase is unrelated to the catalytic subunit of bovine (24, 25), rabbit (26), porcine (27), or human (28) type 2A protein phosphatase as determined by an algorithm that detects local similarities (21). Furthermore, rabbit skeletal muscle type 1 protein phosphatase catalytic subunit, which is clearly related to type 2A protein phosphatase (29), is also not related to type 2C protein phosphatase. Ingebritsen and Cohen (30) have classified the four serine/threonine protein phosphatases into two groups (type 1 and type 2) on the basis of activity against the β or α subunit of phosphorylase kinase, respectively, and on differential sensitivity to two inhibitors. The type 2 protein phosphatases were further differentiated into types 2A, 2B, and 2C (our type IA) on the basis of divalent cation requirements. The sequence relatedness of the type 1 and type 2A phosphatases and the lack of relatedness between these phosphatases and the type 2C phosphatase belies this classification scheme;

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                          10        20        30        40
pST-11              MGAFLDKPKMEKHNAQGQGNGLRYGLSSMQGWRVEMEDAHTAVIGLPSG
                                                ::.:::...  : : .  ........  .:
YSCCDC VLGLMDVTLNTTKVPDENVNFRLRTTASIINGMRYGVADTLGQR-DYVSSRDVTFERFRG
            290       300       310       320       330       340

      50        60        70        80        90       100
pST-11 LETWSFFAVYDGHAGSQVAKYCCEHLLDHITNNQDFK--GSAGAPSVENVKNGIRTGFLE
        .. ... ..:... ..   .  .... .: ..  ..  .. :. . .:.:...: .::.
YSCCDC NDDECLLCLHDSKNQNADYGHNISRIVRDIYDKILIRQLERYGDDTDDNIKTALRFSFLQ
            350       360       370       380       390       400

       110       120                 130       140       150
pST-11 IDEHMR-VMSEKKHGADR---------SGSTAVGVLISPQHTYFINCGDSRGLLCRNR-K
       ..... .... ..:::           ::. .. . :. .. .  : ::....:..:. .
YSCCDC LNKEINGMLNSVDNGADVTNLSYADLLSGACSTVIYIRGKKLFAANLGDCMAILSKNNGD
            410       420       430       440       450       460

        160       170       180       190       200       210
pST-11 VHFFTQDHKPSNPLEKERIQNAGGSVMIQRVNGSLAVSRALGDFDYKCVHGKGPTEQLVS
        . .:..: :... : :::. .:: :   ...: ..:::::.: ::         :  . .:
YSCCDC YQTLTKQHLPTKREEYERIRISGGYVNNGKLDGVVDVSRAVGFFDLL------PHIH-AS
            470       480       490       500               510

        220       230       240       250       260       270
pST-11 PEPEVHDIERSEEDDQFIILACDGIWDVMGNEELCDFVRSRLEVTDDLEKVCNEVVDTCL
       :. .: ......:   ..:.: . .:. :. ...::..:.  . :: : .. .:. :  .
YSCCDC PDISVVTLTKADE---MLIVATHKLWEYMDVDTVCDIARE--NSTDPL-RAAAELKDHAM
          520       530       540       550       560

        280       290       300       310       320       330
pST-11 YKGSRDNMSVI-LICFPNAPKVSAEAVKKEAELDKYLENRVEEIIKKQGEGVPDLVHVMR
         :. .:.... :  . : .. .  ...:.. ...
YSCCDC AYGCTENITILCLALYENIQQQNRFTLNKNSLMTRRSTFEDTTLRRLQPEISPPTGNLAM
      570       580       590       600       610       620

         340       350       360       370       380
pST-11 TLASENIPSLPPGGELASKRNVIEAVYNRLNPYKNDDTDSASTDDMW

YSCCDC VFTDIKSSTFLWELFPNAMRTAIKTHNDIMRRQLRIYGGYEVKTEGDAFMVAFPTPTSGL
      630       640       650       660       670       680
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FIG. 3. Similarity between type 2C protein phosphatase (encoded by pST-11) and yeast adenylate cyclase (YSCCDS), as detected with the FASTP algorithm of Lipman and Pearson (18). Two dots denote amino acid identity; a single dot denotes a conservative amino acid substitution. This analysis revealed 22% identity in a 305-amino acid overlap. The numbering of the yeast adenylate cyclase amino acids is that of Masson *et al.* (19).

perhaps a more meaningful classification would be based on sequence homologies and thus awaits the molecular description of type 2B phosphatase.

A global search of protein phosphatase type 2C against all known protein sequences by the FASTP algorithm (18) detected a similarity with yeast adenylate cyclase (22% identity in a 305-amino acid overlap, Fig. 3). The similarity score, percent identity, and the results of the RDF2 analysis all suggest that the type 2C protein phosphatase and yeast adenylate cyclase share a common ancestor. It is intriguing that the region of similarity overlaps a portion of the yeast adenylate cyclase catalytic center; perhaps this homology reflects the Mg^{2+} requirement of these two enzymes.

We wish to express our gratitude to Dr. Atsushi Oikawa (Tohoku University) for his advice and support during the course of this work. We thank Drs. Peter C. Isakson and William R. Pearson for critically reviewing the manuscript. This research was supported by grants from the Ministries of Education, Science, and Culture and Public Welfare of Japan, from the National Institutes of Health, and from the March of Dimes Foundation.

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