Cloning of rat brain protein kinase C complementary DNA

Yoshitaka Ono, Tsutomu Kurokawa, Kenji Kawahara, Osamu Nishimura, Ryuji Marumoto, Koichi Igarashi, Yukio Sugino, Ushio Kikkawa*, Kouji Ogita* and Yasutomi Nishizuka*

Biotechnology Laboratories of Central Research Division, Takeda Chemical Industries, Ltd., Yodogawa-ku, Osaka 532 and **Department of Biochemistry, Kobe University School of Medicine, Chuo-ku, Kobe 650, Japan*

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Four peptides derived from rat brain protein kinase C were partially sequenced. Using synthetic oligonucleotides deduced from the amino acid sequences as probes, a clone of complementary DNA (cDNA) was isolated from a cDNA library prepared from the same tissue. The nucleotide sequence of this cDNA clone revealed the primary structure of the carboxyl-terminal region as having 224 amino acids, with significant sequence homology with cyclic AMP-dependent and cyclic GMP-dependent protein kinases.

Protein kinase C *Lysylendopeptidase* *complementary DNA* *Nucleotide sequence*

1. INTRODUCTION

Protein kinase C consists of a single polypeptide chain with an M_r of approx. 77000 [1]. This enzyme appears to play a crucial role in signal transduction for a variety of biologically active substances which activate cellular functions and proliferation [2,3]. Since the protein kinase C has been generally regarded as the receptor for tumour-promoting phorbol esters, elucidation of the structure of this enzyme may obviously provide clues for understanding the molecular mechanism of cell growth and differentiation. Here we wish to describe cloning of cDNA and its nucleotide sequence, that encodes the carboxyl-terminal region of rat brain protein kinase C.

2. MATERIALS AND METHODS

2.1. *Preparation of the peptide fragments with lysylendopeptidase*

Rat brain protein kinase C was purified by the procedure described in [4], and the enzyme employed for the present studies was practically pure as judged by silver staining after SDS-polyacrylamide gel electrophoresis. The purified enzyme (1.9 mg protein) was digested with lysylendopeptidase (47.5 μg) for 21 h at 37°C in 1.9 ml of 50 mM Tris-HCl (pH 9.0) containing 6 M urea. The resultant peptide mixture was separated by preparative reverse-phase HPLC equipped with a Micropak protein C18-10 column (0.4 × 30 cm, Varian associates) at a flow rate of 1.3 ml/min as described in [5].

Abbreviation: HPLC, high-performance liquid chromatography

2.2. *Amino acid sequencing*

Automated Edman degradation was carried out with a gas-phase protein sequencer, model 470A (Applied Biosystems). Phenylthiohydantoin derivatives of amino acids were determined by HPLC equipped with a Micropak SP C18-3 column (Varian Associates).

2.3. *Construction of cDNA library from rat brain poly(A) RNA*

Poly(A) RNA was isolated from rat brain by the guanidine isothiocyanate-CsCl procedure [6], followed by oligo(dT)-cellulose column chromatography [7]. The cDNA library was constructed by using the pcDV1 vector-primer and pL1 linker fragment according to Okayama and Berg [8,9].

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The cyclized vector-cDNA preparation was used to transform competent *E. coli* DH1 cells. About 5 μg poly(A) RNA yielded about 5×10^5 independent transformants.

2.4. *Screening of rat protein kinase C cDNA*

Colony screening was performed by the high-density colony hybridization method [10]. The probes were labelled by phosphorylation with [γ-^{32}P]ATP and T_4 polynucleotide kinase. Two replica nitrocellulose filters were prepared from the master filters, and each was probed with the radioactive synthetic oligonucleotides (probe no.2 or probe no.3 in fig.1). Hybridization was carried out for 16 h at 42°C in a solution containing 5 × SSPE [11], 5 × Denhardt's solution [11], 100 μg/ml sonicated heat-denatured salmon sperm DNA, 0.1% SDS, and the probes. After hybridization, the filters were washed by immersing them twice each time with a sufficient volume of 6 × SSC [11] containing 0.1% SDS at room temperature for 60 min, and further twice with the same solution at 47°C (for probe no.2) or at 43°C (for probe no.3) for 60 min. The filters were then dried and autoradiographed with intensifying screens.

2.5. *Nucleotide sequencing*

Nucleotide sequence analysis was carried out by subcloning suitable restriction endonuclease fragments into M13-based cloning vectors (M13mp10 and M13mp11) followed by primed DNA synthesis on single-stranded DNA templates in the presence of dideoxynucleoside triphosphates.

3. RESULTS

3.1. *Partial amino acid sequence of protein kinase C*

Treatment of protein kinase C with lysylendopeptidase resulted in the formation of approx. 50 peptides, which were fractionated by preparative reverse-phase HPLC. Four major peptides (nos 24, 37, 49 and 51) were selected, and their amino acid sequences were partially determined. The results are summarized in table 1. For screening of cDNA clones that contain the protein

Table 1

Amino acid sequences of the four major peptides selected

Peptide no.	Sequences	Probe no.
	5 10	
24	Glu-His-Ala-Phe-Phe-Arg-Tyr-Ile-Asp-Trp-	1
	Glu-Lys	
	5 10	
37	Ile-His-Thr-Tyr-Gly- X -Pro-Thr-Phe- X -	
	15	
	Asp-His- X -Gly-Gly-	
	5 10	
49	Ser-Val-Asp-Trp-Trp-Ala-Tyr-Gly-Val-Leu-	
	15 20	
	Leu-Tyr-Glu-Met-Leu-Ala-Gly-Gln-Pro-Pro-	
	5 10	
51	Ser-Val-Asp-Trp-Trp-Ala-Phe-Gly-Val-Leu-	2
	15 20	
	Leu-Tyr-Glu-Met-Leu-Ala-Gly-Gln-Ala-Pro-	
	25 30	
	Phe-Glu-Gly-Glu-Asp-Glu-Asp-Glu-Leu-Phe-	3
	Gln-	

The hexapeptide regions underlined were employed for preparing the oligonucleotides to be used as probes. X, unidentified amino acid

kinase C sequence, three batches of mixed oligonucleotides, each 17 nucleotides long, were prepared as probes, which are shown in fig.1. The first, probe no.1 (24 mixtures), encoded amino acids 7–12 of the peptide no.24. The second, probe no.2 (16 mixtures), encoded amino acids 3–8 of the peptide no.51. The third, probe no.3 (64 mixtures), corresponded to the region of amino acids 24–29 of the peptide no.51.

3.2. *cDNA cloning of protein kinase C*

A cDNA library complementary to poly(A) RNA of rat brain was constructed by the procedure of Okayama and Berg [8,9]. For the first screening of the cDNA library, a mixture of [^{32}P]oligonucleotide probes (nos 2 and 3) was employed. Two replica filters were prepared from the master filters, and examined with each oligonucleotide probe. Out of 3×10^5 colonies, only one clone was obtained which obviously hybridized to both probes. When plasmid DNA from this clone, designated to pCKR9, was subjected to Southern blot analysis, the pCKR9 insert was hybridized not only to probes no.2 and no.3, but also to probe no.1. To confirm that pCKR9 contained the protein kinase C sequence, the nucleotide sequence of the pCKR9 insert was determined by the chain termination method. Restriction endonuclease cleavage map and the strategy of nucleotide sequence determination are summarized in fig.2a. Fig.2b shows the partial nucleotide sequence of the pCKR9 insert, together with the amino acid sequence predicted therefrom. The nucleotide sequences of residues 445–480 and 220–312 corresponded exactly to the amino acid sequences of peptides no.24 and no.51, respective-

a) Tyr-Ile-Asp-Trp-Glu-Lys

5'-TT$^{T}_{C}$TCCCA$^{A}_{G}$TC$^{A}_{T}$GAT$^{A}_{G}$TA-3'

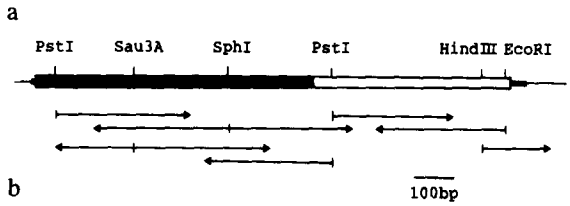
b) Asp-Trp-Trp-Ala-Phe-Gly

5'-CC$^{A}_{G}$TA$^{A}_{C}_{T}$GCCCACCA$^{A}_{G}$TC-3'

c) Glu-Asp-Glu-Asp-Glu-Leu

5'-A$^{AT}_{GC}$TC$^{A}_{G}$TC$^{T}_{C}$TC$^{A}_{G}$TC$^{T}_{C}$TC-3'

Fig.1. Synthetic oligonucleotides employed for screening cDNA clones for rat brain protein kinase C. Mixed 17-mer oligonucleotides are complementary to all possible coding sequences corresponding to the three hexapeptides shown in table 1. (a) Probe no.1, residues 7–12 of peptide no.24; (b) probe no.2, residues 3–8 of peptide no.51; (c) probe no.3, residues 24–29 of peptide no.51.

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GCAGAGATTGCCATCGGTCTTTTCTTCTTGCAGAGCAAGGGCATCATTTACCGTGACCTG    60
AlaGluIleAlaIleGlyLeuPhePheLeuGlnSerLysGlyIleIleTyrArgAspLeu    20

AAACTTGACAACGTGATGCTGGATTCCGAGGGGCACATCAAAATCGCTGACTTTGGCATG   120
LysLeuAspAsnValMetLeuAspSerGluGlyHisIleLysIleAlaAspPheGlyMet    40

TGTAAAGAGAATATCTGGGATGGGGTGACAACCAAGACATTCTGTGGCACTCCAGACTAC   180
CysLysGluAsnIleTrpAspGlyValThrThrLysThrPheCysGlyThrProAspTyr    60

ATTGCCCCAGAGATCATTGCTTATCAGCCCTACGGGAAGTCTGTGGACTGGTGGGCGTTT   240
IleAlaProGluIleIleAlaTyrGlnProTyrGlyLysSerValAspTrpTrpAlaPhe    80

GGAGTCCTGCTGTATGAAATGTTGGCTGGCCAGGCACCTTTTGAAGGGGAGGATGAGGAT   300
GlyValLeuLeuTyrGluMetLeuAlaGlyGlnAlaProPheGluGlyGluAspGluAsp   100

GAACTCTTCCAGTCAATCATGGAGCACAACGTGGCGTATCCCAAGTCCATGTCTAAGGAA   360
GluLeuPheGlnSerIleMetGluHisAsnValAlaTyrProLysSerMetSerLysGlu   120

GCTGTGGCAATCTGCAAAGGGCTAATGACCAAACACCCAGGCAAGCGCCTGGGTTGTGGG   420
AlaValAlaIleCysLysGlyLeuMetThrLysHisProGlyLysArgLeuGlyCysGly   140

CCTGAAGGGGAACGAGACATTAAGGAGCATGCATTTTTCCGGTATATCGACTGGGAGAAA   480
ProGluGlyGluArgAspIleLysGluHisAlaPhePheArgTyrIleAspTrpGluLys   160

CTCGAACGCAAGGAGATTCAGCCACCTTATAAACCAAAAGCTAGAGACAAGCGAGACACC   540
LeuGluArgLysGluIleGlnProProTyrLysProLysAlaArgAspLysArgAspThr   180

TCCAACTTCGACAAAGAGTTCACCAGGCAGCCTGTGGAACTGACTCCCACTGACAAACTC   600
SerAsnPheAspLysGluPheThrArgGlnProValGluLeuThrProThrAspLysLeu   200

TTCATCATGAACTTGGACCAAAATGAATTTGCTGGCTTCTCGTATACTAACCCAGAGTTT   660
PheIleMetAsnLeuAspGlnAsnGluPheAlaGlyPheSerTyrThrAsnProGluPhe   220

GTCATTAATGTGTAGGTGAATGCAGATTCCATCGCTGAGCCTGTGTGTAAGGCTGCAGGC   720
ValIleAsnVal -

TGAATGTCTATTATCAATTCCAGTCTTCCAGGATTCATGGTGCCTCTGTTGGCATCCGTC   780

ATGTGGAGAGCTTGTCTTAGAGGGCTTTTCTTTGTATGTATAGCTTGCTAGTTTGTTTTC   840

TACATTTCAAAATGTTTAGTTTAGAATAAGTGCATTGCCCACTGATAGAGGTACAATTTT   900

CCAGACTTCCAGAAACTCATCCAATGAACCAACAGTGTCAAAACTTAACTGTGTCCGATA   960

CCAAAATGCTTCAGTATTTGTAATTTTTAAAGTCAGATGCTGATGTTCCTGGTCAAAGTT  1020

TTTACAGTTACTCTCGAATATCTCCTTTGAATGCTACCTAAGCATGACCGGTATTTTTAA  1080

AAGTTGTGAGTAAGCTTTGCAGTTACTGTGAACTCTTGTCTCTTGGAGGAAACTTTTTGT  1140

TTAAGAATTGGTATGATTAAATGAATTCATATATGC(A)n
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Fig.2. Nucleotide sequence and predicted amino acid sequence of the pCKR9 insert. (a) Restriction endonuclease cleavage map of pCKR9 and strategy for nucleotide sequence determination. Thin lines and bold lines indicate vector and oligo (dG-dC) (or dA-dT) tails, respectively. Closed and open boxes indicate coding and untranslated region, respectively. Horizontal arrows indicate regions and directions of sequencing. (b) Nucleotide sequence and predicted amino acid sequence of the pCKR9 insert. Numbering of nucleotides and amino acids is tentative. The poly(A) additional signal is underlined.

ly. The amino acid composition of the predicted peptide of residues 74–114 was almost identical to that of peptide no.51 (not shown). A sequence of ATTAAA, an analogous sequence of the poly(A) additional signal AATAAA [12], was found 16 nucleotides upstream of the poly(A) homopolymer stretch.

From these results, it may be concluded that pCKR9 is the cDNA that encodes the carboxyl-terminal region of rat brain protein kinase C, and contains the complete 3′-untranslated region.

4. DISCUSSION

Based on the results presented above, it is possible to predict the sequence of the carboxyl-terminal 224 amino acid residues of protein kinase C. The cDNA clone thus obtained perhaps covers about one-third of the entire cDNA sequence, assuming that protein kinase C consists of 700–750 amino acid residues. Fig.3 shows an alignment of the predicted carboxyl-terminal amino acids of rat brain protein kinase C with those of the catalytic subunit of bovine heart cyclic AMP-dependent protein kinase [13] and bovine lung cyclic GMP-dependent protein kinase [14]. A significant amino acid homology may be seen among these three protein kinases. This sequence conservation over different species seems to indicate that this region is one of the essential domains for the protein kinase activity.

Comparing the amino acid sequence of peptide no.49 with that of peptide no.51, only two residues

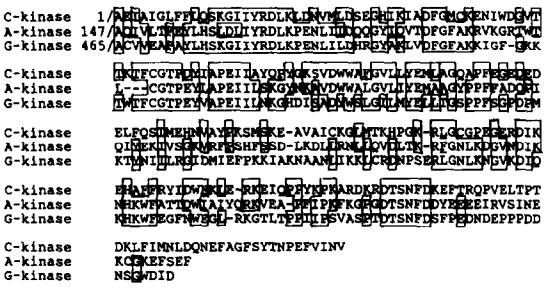

Fig.3. Comparison of the predicted carboxyl-terminal region of rat brain protein kinase C with other protein kinases. Hyphens have been introduced for optimal alignment. C-kinase, rat brain protein kinase C; A-kinase, catalytic subunit of bovine heart cyclic AMP-dependent protein kinase [13]; G-kinase, bovine lung cyclic GMP-dependent protein kinase [14].

differ (table 1, residues 7 and 19), suggesting that the purified protein kinase C is a mixture of two closely related polypeptides, although the existence of repeat of the almost same sequences in a single polypeptide chain of protein kinase C may not be ruled out. As described earlier [4], the enzyme purified from rat brain frequently reveals a doublet upon SDS-polyacrylamide gel electrophoresis, and both protein bands are reactive with three different monoclonal antibodies against protein kinase C. It is most likely that there exist at least two types of the enzyme which may be derived from closely similar but distinctly different genes. In fact, several cDNA clones were isolated which could hybridize to the pCKR9 insert but showed restriction maps different from that of the pCKR9 insert (not shown). Further analysis of these cDNA clones will provide more information on the heterogeneity of protein kinase C. Recently, Uhler et al. [15] have cloned the cDNA encoding the catalytic subunit of cyclic AMP-dependent protein kinase, and noted some heterogeneity of the enzyme. The heterogeneity might be a common feature among protein kinases.

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