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Internal amino acid sequence analysis of the 80 kDa protein kinase C substrate from rat brain: relationship to the 87 kDa substrate from bovine brain

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We have obtained sequence data from five proteolytic peptides totalling 102 amino acid residues of the 80 kDa protein kinase C substrate purified from rat brain. The amino acid sequences of these five peptides were compared with that deduced from a cDNA encoding the 87 kDa protein kinase C substrate from bovine brain. The overall amino acid sequence identity within the regions covered by our peptides is 54%. Two peptides aligned at the C- and N-termini of the bovine protein kinase C substrate with a very high degree of homology (more than 80% identity). Two other peptides exhibited 62% and 46% identity with two regions located in the C-terminal half of the bovine protein. The fifth peptide which contains the sequence PEQPEQPEQ did not reveal any similarity with the bovine protein. Based on the homologies of our experimentally determined sequences, which represent about 30% of the deduced sequence of the bovine protein, we suggest that although these protein kinase C substrates are not identical, they may belong to a family of related proteins.

Protein kinase C substrate; Amino acid sequence; Cellular signalling

1. INTRODUCTION

The activation of protein kinase C (PKC) constitutes one of the signal transduction pathways leading to fibroblast proliferation [1]. Thus, the molecular characterization of physiological substrates for this kinase may prove crucial to understand the mechanism(s) by which PKC-mediated signals elicit mitogenesis. An acidic protein of 80 kDa has been identified as a major and specific substrate for PKC in quiescent mouse 3T3 cells [2–7] and other cultured cells [2,5,8,9]. The phosphorylation of this protein is stimulated by phorbol esters [2,5,8], diacylglycerols [3,4,8], platelet-derived growth factor [2,8,10], fibroblast growth factor [4,8], bombesin [7,10] and vasopressin [11], all of which stimulate reinitiation of DNA synthesis in quiescent 3T3 cells [1].

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Since the rapid phosphorylation of the 80 kDa protein may be a significant event in the initiation of mitogenesis, it is important to characterize fully this PKC substrate. Initially, we have approached this question by purifying the 80 kDa protein from rat brain [12]. Rat brain expresses an 80 kDa protein kinase C substrate closely related to the phosphoprotein found in cultured fibroblasts [12,13], and therefore represents a convenient source for large scale purification.

Other laboratories have also reported the purification of 80–87 kDa substrates of PKC from bovine [14] and rat [15] brain. The protein from bovine brain differs from the heat stable substrate found in rat brain in its behaviour in SDS-PAGE [14], immunological cross-reactivity [13], elution from anion-exchange columns [14] and amino acid composition (28.6% alanine was reported) [14]. Thus, the precise relationship between these PKC substrates remains poorly understood.

In the present paper, we report the amino acid sequences of peptides generated by proteolytic

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cleavage of the 80 kDa PKC substrate purified from rat brain and compare these experimentally determined sequences with that deduced from a recently published cDNA encoding the bovine 87 kDa PKC substrate [16]. The sequences are clearly related but not identical raising the possibility that these proteins are members of a family of PKC substrates.

2. MATERIALS AND METHODS

2.1. *Materials*

Frozen rat brains were purchased from Charles River UK Ltd. L-1-Tosyl-amido-2-phenylethylchloromethyl ketone-treated trypsin and *S. aureus* V-8 protease were obtained from Sigma. Other items were from standard supplier sources or as listed in the text.

2.2. *Methods*

2.2.1. Procedures for the purification of the 80 kDa protein from rat brain

Two alternative methods were used for the purification of the 80 kDa protein. Method I was described before [12]. Method II was modified from method I as follows: heat-treated extracts were prepared as in method I [12] but the homogenization step was performed in the presence of 1 mM phenylmethylsulfonyl fluoride. The DEAE-cellulose step described in method I [12] was scaled up by mixing heat-treated extracts from 1 kg of frozen brains with 500 ml of DEAE-cellulose (Whatman). Proteins were eluted from the DEAE-cellulose column with a 2 l linear gradient of 0–1 M NaCl in 20 mM Tris-HCl, pH 7.5, and 1 mM EDTA. The gel filtration step described in method I [12] was modified using a Sephadex G-150 superfine column (Pharmacia Biotechnology, Inc., 2.5 × 82 cm) pre-equilibrated and eluted with 1 M NaCl in 20 mM HCl. This column was loaded with half the amount of material obtained from the previous step. The reverse-phase FPLC step described in method I [12] was replaced by preparative SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electroelution as follows: the pooled material from the previous step was concentrated with Aquacide II (Calbiochem) and separated by SDS-PAGE according to Laemmli [17], using extensively polymerized 7.5% polyacrylamide separating gels (1 mm thick). The 80 kDa protein band was visualized by staining for 15 min with 0.2% Coomassie Blue R-250 and was excised from the gel. Gel slices were washed with water and then soaked for 30 min in diluted electroelution buffer which contained 4 mM Tris-acetate, pH 8.4, 2 mM EDTA, 4 mM dithiothreitol and 0.02% SDS. Ten gel slices, each containing about 5 μg of 80 kDa protein, were then placed in a sample cup of an Isco Model 1750 electrophoretic sample concentrator in which the inner buffer compartments were filled with diluted electroelution buffer and the electrode compartments were filled with electroelution buffer in which the concentration of Tris-acetate was raised to 40 mM. Electroelution was performed at 3 W for 3 h followed by 1 W overnight at 4°C. The eluted 80 kDa protein was collected from the sample cup in a volume of 0.2–0.3 ml and stored frozen at −20°C until required for the analyses described below.

2.2.2. Amino acid analysis

The purified 80 kDa protein was hydrolysed for 24 h at 110°C in 6 N HCl containing 5% (v/v) 1,4-butanedithiol. The hydrolysate was analyzed on a Beckman 6300 amino acid analyser using post-column ninhydrin detection.

2.2.3. Enzymatic cleavage of the 80 kDa protein and proteolytic peptide fractionation

Purified 80 kDa protein samples were adjusted to pH 7.8 with 50 mM ammonium bicarbonate and digested with trypsin or V-8 protease at 37°C for 24 h. The enzyme-to-substrate ratio was kept at 1:30 (w/w). Alternatively, prior to V-8 protease digestion, the 80 kDa protein was alkylated by vapour phase *S*-pyridylethylation as described by Amons [18]. The resulting peptides were resolved on a reverse-phase HPLC system using a Brownlee Aquapore RP-300 column (2.1 × 100 mm). The following buffer system was used. Buffer A: 0.08% trifluoroacetic acid in acetonitrile/water (double glass distilled), 1:99 (v/v). Buffer B: 0.06% trifluoroacetic acid in acetonitrile/water, 90:10 (v/v). The column was equilibrated with buffer A and developed at a flow rate of 0.4 ml/min with a gradient of 0–60% buffer B for 70 min followed by 60–100% buffer B for 20 min. Poorly resolved peaks were further purified using the same column with the following buffer system. Buffer C: 45 mM ammonium acetate in acetonitrile/water, 1:99 (v/v). Buffer D: 40 mM ammonium acetate in acetonitrile/water, 80:20 (v/v). The column was equilibrated with buffer C and developed with a gradient of 0–50% buffer D for 30 min followed by 50–100% buffer D for 10 min. Peptides were detected by simultaneous monitoring at 214 nm and 280 nm.

2.2.4. Peptide sequence analysis

Proteolytic peptides were subjected to gas/liquid phase microsequencing on Applied Biosystems models 475 and 477A peptide sequencers with on-line PTH amino acid analysis (Applied Biosystems model 120A PTH analyzer) using the reagents and solvents supplied by the manufacturer.

3. RESULTS AND DISCUSSION

Previous results have indicated that rat brain expresses a PKC substrate closely related to the 80 kDa protein identified in quiescent 3T3 cells [12]. Therefore, we used rat brain as a source for the purification of this phosphoprotein. The purification was based on DEAE-cellulose chromatography, Sephadex G 150 gel filtration followed by either reverse-phase FPLC, as previously described [12], or by electroelution from preparative SDS-PAGE (see method II in section 2.2.1). Method II of purification yielded about 0.5 mg of homogeneous 80 kDa protein from 1 kg of frozen brains. This represents an increase of 3-fold over the yield obtained by method I [12]. The protein purified by method II had the same

Table 1

Amino acid composition of the 80 kDa protein

Amino acid	mol%	
	I	II
Asx	7.3	5.9
Thr	4.0	3.6
Ser	8.0	8.3
Glx	19.0	17.5
Pro	10.2	11.6
Gly	9.9	9.7
Ala	20.0	22.4
Val	2.0	2.3
Met	0.5	0.3
Ile	1.1	0.9
Leu	3.3	2.4
Tyr	0.9	0.9
Phe	2.1	1.9
His	0.7	0.7
Lys	7.7	8.3
Arg	2.1	2.9
Cys	ND	ND
Trp	ND	ND

The 80 kDa protein was purified according to method I or II as described in section 2. Samples containing about 20 pmol purified protein were subjected to acid hydrolysis and amino acid analysis. ND, not determined

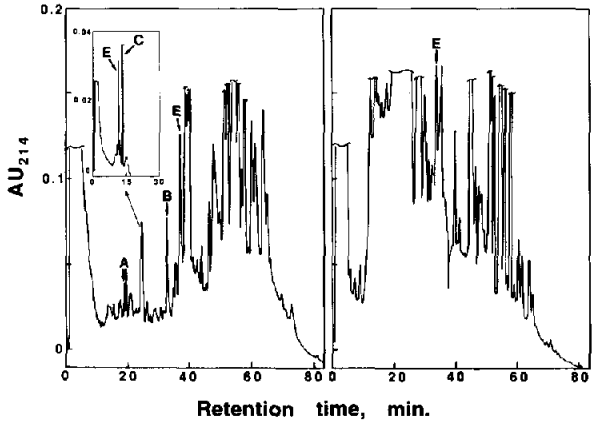

Fig.1. Purification of V-8 proteolytic peptides from the rat brain 80 kDa protein by reverse-phase HPLC. (Left panel) 30 μg of 80 kDa protein purified from preparative polyacrylamide gels was digested with V-8 protease and separated by HPLC as described in section 2. (Inset) The indicated peak, representing a peptide mixture, was further purified using the same column with a different solvent system as described in section 2. (Right panel) As for left panel, except that the protein sample was pyridylethylated before carrying out the proteolytic digestion. Letters over selected peaks correspond to peptides in table 2.

mobility on polyacrylamide gels as that prepared by reverse-phase FPLC (data not shown). Amino acid analysis of the 80 kDa PKC substrate, purified by either of these procedures gave virtually identical results (table 1). The protein has a high proportion of alanine, acidic amino acids and proline.

Attempts to determine the N-terminal amino acid sequence of the 80 kDa protein failed to give any discernible sequence. This suggests that the α-amino group may be blocked and therefore is not available for Edman degradation. This possibility agrees with a recent report indicating that a related PKC substrate is myristoylated [19].

To enable internal amino acid sequence information of the rat brain 80 kDa protein to be obtained, purified samples of this protein were digested with either *S. aureus* V-8 protease or trypsin and the resulting mixtures of peptide fragments were separated by reverse-phase HPLC (fig.1). Six independent digests made from 80 kDa protein purified either by reverse-phase FPLC (method I) or by electroelution from polyacrylamide gels (method II) were analysed by this procedure. Selected peaks were applied to a gas/liquid-phase sequenator and amino acid sequences were determined at the 50–100 pmol level. A total of 18 peaks contained substantially homogeneous peptide species. The sequences obtained from these peaks are presented in table 2. Each peptide sequence was confirmed in various circumstances resulting from different combinations of protein purification and digestion procedures (see legend to table 2). In all, the sequence of 102 amino acid residues was obtained from this analysis. Computer-assisted searches of protein sequence databases revealed that peptides A–E did not show significant homology to any known protein. These include two previously cloned protein kinase C substrates, namely P47 from platelets [21] and the neuronal protein GAP-43 [22].

Recently, Stumpo et al. [16] isolated a cDNA from a bovine caudate library encoding the 87 kDa PKC substrate. This cDNA contains an open reading frame which predicts a protein of only 335 amino acids, interrupted by a large intron [16]. The amount of amino acid sequence information obtained in the present study represents about 30% of the sequence of the bovine protein predicted from the work of Stumpo et al. Thus, our data provide

Table 2

Experimentally determined peptide sequences of the 80 kDa phosphoprotein from rat brain

Peptide	Sequence
A	A A AA A GG D AAA A P G EQAGGAGAEGAEGEE
B	K A EE P AP G ATA D D A PSAAGPEQEA
C	X S PE A PP A PVA(E)
D	G E AA A ER P GEA A V A SSP
D_1	A A V A SSPGXA
E	A(E)EP E QP E QP E QPA A A(A)E (A) (E)
E_1	XAXX A EP(E)QP(E)QP(E)QPA (A) (G) (S)

Residues are indicated by single letter amino acid codes. X indicates that no residue could be identified. Residues that could not be unequivocally identified are enclosed by parentheses. Peptides A and B were each isolated from two V-8 digests, one made from 80 kDa protein purified by method I and the other made from 80 kDa protein purified by method II. Sequences of peptide A were observed in four peaks of one digest and two from the other (fig.1A). These multiple peaks containing the same sequence (or a shorter version) are likely to originate from alternative cleavage at different glutamic acid residues [20]. Peptide C was isolated from three V-8 digests, one made from 80 kDa protein purified by method I and two others made from 80 kDa protein purified by method II. In this case, the signal of the PTH derivative dropped sharply in the 12th cycle of sequencing, and after this cycle no further PTH derivatives were detected. Therefore, it is likely that peptide C was sequenced to the end. Peptide D was obtained after trypsin digestion and was found to overlap with peptide D_1 which was generated in a V-8 digest. Peptide E was isolated from three V-8 protease digests. One of the digests was made from 80 kDa protein purified according to method I (not shown). The two others were made from 80 kDa protein purified according to method II, and of these, one protein sample was *S*-pyridylethylated before digestion. Sequences of peptide E were observed in two peaks of one separation. The separation of these digests by reverse-phase HPLC, is shown in fig.1. Peptide E_1 was isolated from a tryptic digest. Although its sequence shows ambiguity, it overlaps clearly with V-8 protease-generated peptide E

an excellent opportunity to define the relationship between these PKC substrates. Fig.2 shows the amino acid sequences of the experimentally determined peptides derived from the rat brain PKC substrate aligned with the predicted sequence of the bovine protein. The overall amino acid sequence identity within the regions covered by our peptides is 54%. However, in each of these regions the degree of homology varied substantially. Peptides C and D which align at the C- and N-terminal ends of the molecule, respectively, exhibit the highest degree of identity (82% and 89%, respectively). Peptides A and B contain clusters of conserved amino acids which enable their alignment against two regions located on the C-terminal half of the bovine protein. However, the number of non-matching residues (38% and 54%, respectively) in these peptides denote a considerable degree of divergence between the internal sequences of the two PKC substrates. About half of the non-matching residues of peptide A were conservative substitutions whereas in peptide B only 2 out of 13 scored in this category. A striking feature of the results presented here is that peptide E which was sequenced 5 times (see legend to table 2) does not reveal any similarity with the bovine sequence. It is therefore conceivable that this peptide represents a variable region between the two PKC substrates.

On the basis of these findings, we suggest that the 80 kDa PKC substrate purified from rat brain is related but not identical to the 87 kDa PKC substrate from bovine brain. This interpretation is in line with differences noted previously in immunoreactivity [13] and elution from ion-exchange col-

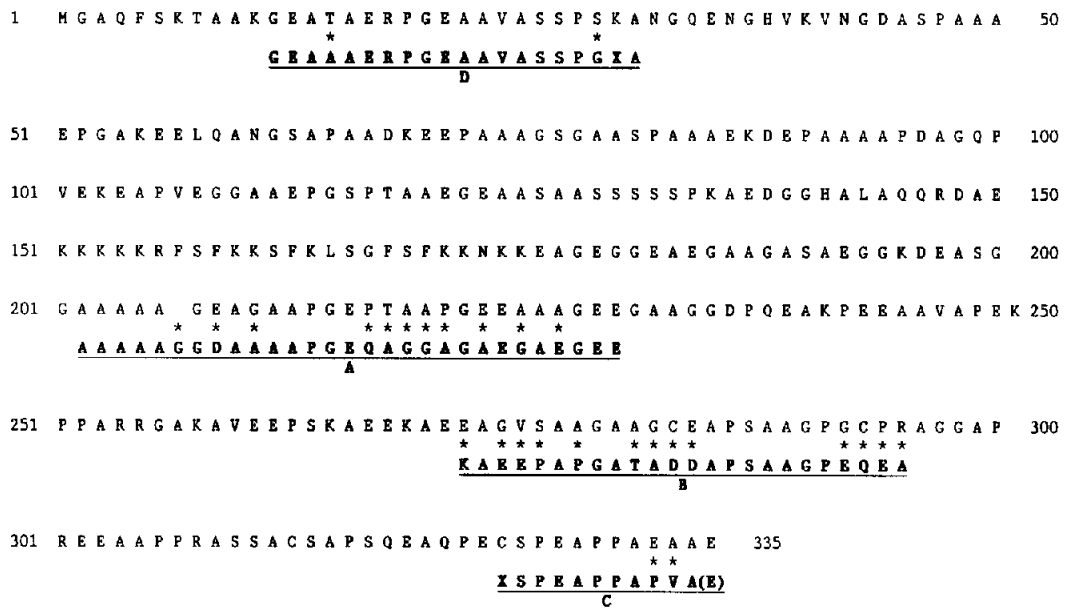

Fig.2. Alignment of experimentally determined amino acid sequences from rat brain 80 kDa PKC substrate with the predicted sequence of the bovine 87 kDa PKC substrate. The sequence of the bovine PKC substrate was deduced from the DNA sequence published by Stumpo et al. [16]. A gap after amino acid residue 206 was introduced for optimal alignment. Asterisks denote non-matching residues. The PEQ repeated motif of peptide E could not be aligned against any part of the bovine sequence.

umns [12]. It is likely that these protein kinase C substrates are part of a family of related proteins, a proposition that warrants considerable further work.

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