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The Primary Structure of a Membrane-associated Phospholipase A_2 from Human Spleen

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SUMMARY : The complete primary structure of membrane-associated phospholipase A_2 purified from a human splenic membrane fraction was determined by sequence analysis of the peptides generated by lysyl endopeptidase and Staphylococcus aureus V8 protease cleavage. The enzyme consists of 124 amino acid residues corresponding to a molecular weight of 13,904. The primary structure reveals the characteristics of Group II phospholipases A_2 and a large ratio of basic amino acid residues to acidic ones, that ratio being 3.4 : 1.

Membrane-associated phospholipases A_2 (PLA_2s) are present in nearly all cells (1) and thought to participate in the regulation of the phospholipid metabolism in biomembranes including eicosanoid biosynthesis (2). The enzymes are also thought to be involved in the process of inflammation and cellular injury through direct action or through its metabolites such as lysophospholipids, leukotrienes, platelet-activating factor and lipid peroxides (3). In fact, increased levels of secretory PLA_2 are found in rheumatoid arthritic synovial fluid (4) and serum from septic shock patients (5), and a PLA_2 in rheumatoid arthritic synovial fluid has been partially sequenced (6). We were interested in the possible relationship of cellular PLA_2 to the pathogenesis of various inflammatory diseases and decided to investigate the spleen, as the enzyme source, which includes lymphoid tissue and macrophage cells. We have already purified a membrane-

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Abbreviations : PLA_2, phospholipase A_2 (EC 3.1.1.4.); PLA_2 M, human splenic membrane-associated PLA_2; Lys-C, Lysyl endopeptidase; Glu-C, Staphylococcus aureus V8 protease; HPLC, high performance liquid chromatography; RCm-, reduced and S-carboxymethylated; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol.

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associated PLA_2 from rat spleen (7) and determined its primary structure by sequence analysis of both the protein and its cDNA (8). Also, it has been reported that a PLA_2 which does not react with anti-human pancreatic PLA_2 antibody exists in human spleen (9), and the further investigation of the enzyme is needed.

Here we report the purification and primary structure of a human splenic membrane-associated PLA_2, named PLA_2 M.

MATERIALS AND METHODS

Materials : Human spleen tissue was supplied from autopsy materials and from patients who suffered from gastric cancer when they underwent radical operation including splenectomy for lymph node resection. Lysyl endopeptidase (Lys-C) and Staphylococcus aureus V8 protease (Glu-C) were purchased from Wako Pure Chemical Industries, Ltd. and Boehringer Mannheim Biochemica, respectively. All other chemicals and reagents were of the highest purity available from commercial source.

Assay for PLA_2 activity : The PLA_2 activity was determined by the method of Tojo et al. (10), in which the derivatives of released fatty acid with 9-anthryldiazomethane were assayed quantitatively with high performance liquid chromatography (HPLC), using 1mM 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (PG) as substrate and 5mM sodium deoxycholate as detergent.

Purification of PLA_2 M : Human spleen tissue was homogenized in 10mM Tris-HCl buffer (pH7.0) and the homogenate was centrifuged at 108,000×g for 1h at 4°C. The PLA_2 M was extracted from the pellet with 1M KBr and purified by the consecutive use of S-Sepharose, Octyl-Sepharose, and second S-Sepharose chromatographies and HPLC (column, Aquapore RP-300 2.1×30mm; elution, linear gradient from 15% to 60% acetonitrile in 0.1% trifluoroacetic acid).

Enzymatic digestion of PLA_2 M : Purified PLA_2 M was reduced and S-carboxymethylated (RCm-) by the method of Crestfield (11). RCm-PLA_2 M (2nmol) was digested with Lys-C (10% of PLA_2 M, w/w) in 65µl of 0.1M Tris-HCl (pH9.0) containing 2.7M urea at 37°C for 18h. In the case of Glu-C digestion, RCm-PLA_2 (1nmol) was dissolved in 12.5µl of 7M urea and was incubated at 37°C for 8h. 28µl of 0.1M ammonium bicarbonate (pH7.8), 5µl of 20mM EDTA and 1.5µg of Glu-C was added to the sample solution, and the mixture was incubated at 25°C for 38h. Subsequently, another 1.5µg of Glu-C was added and the mixture was further incubated at 25°C for 32h.

Separation of peptide mixture : Peptides obtained by proteolytic digestion were separated by HPLC on a VYDAC C_{18} column (2.1×250mm) in a Waters HPLC system. Peptides were eluted with a linear gradient from 0% to 50% acetonitrile in 0.1% trifluoroacetic acid. A flow rate was 0.4ml/min. The peak of L-9 obtained by Lys-C digestion was re-separated with a linear gradient from 0% to 30% acetonitrile in 10mM ammonium acetate buffer (pH6.0) at a flow rate of 0.3ml/min. Peaks were detected at 210nm and 280nm.

Sequence determination : Automated Edman degradation was performed with a model 477A Applied Biosystems gas phase sequencer equipped with a 120A PTH amino acid analyzer.

Amino acid analysis : Purified PLA_2 M was hydrolyzed in 4N methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole at 110°C for 24h and amino acid analysis was performed with a Hitachi amino acid analyzer (model 835).

RESULTS

Human spleen tissue was homogenized and centrifuged at 108,000×g. While PLA_2 activity was not detected from the supernatant, from the pellet, PLA_2 M was purified to homogeneity, giving a single peak on HPLC and a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Its molecular weight was estimated to be 14,000 on SDS-PAGE. The total activity yield of the final product was 30% for the splenic homogenate and the specific activity was 1.0 mmol/min/mg when mixed micelles of 1mM PG and 5mM sodium deoxycholate were used. The specific activity was 5 times the value of that of human pancreatic PLA_2.

The amino acid composition of PLA_2 M is presented in Table 1 and the sequence of 45 amino acid residues from the amino-terminus of the RCm-PLA_2 M was determined as shown in Fig. 1.

The majority of the sequence of PLA_2 M was determined from peptides generated from Lys-C cleavage. The Lys-C-digested peptides were separated by HPLC into thirteen peaks (Fig. 2A) followed by amino acid sequence analysis. The amino acid sequences of eleven peptides, except for L-1 and L-9, were determined. Amino acid analysis of the fraction that passed through the HPLC, L-1, showed that it contained 1.3nmol of lysine (65% for used RCm-PLA_2). Because of an ambiguous result for L-

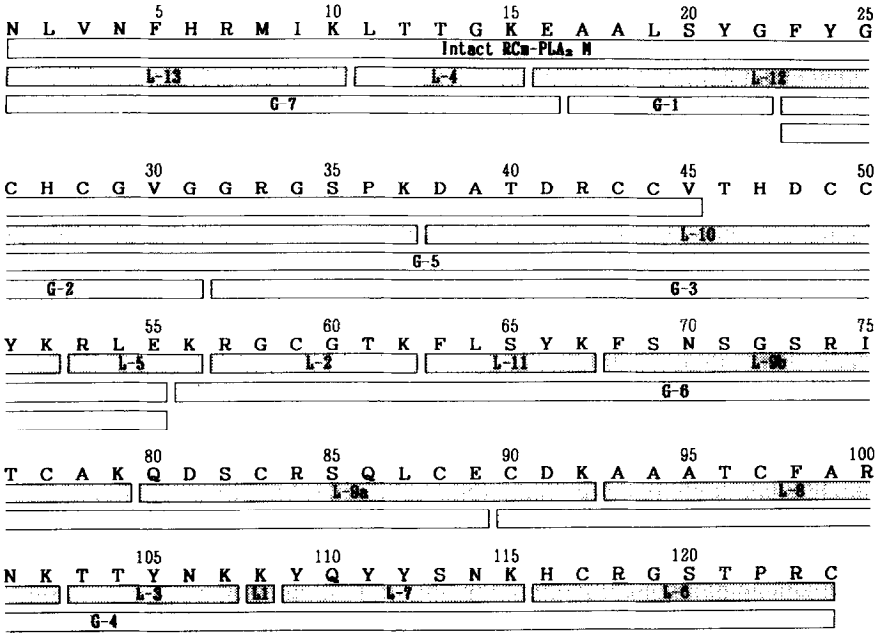

Fig. 1. Summary of the proof of the sequence of human splenic PLA_2 M. Intact RCm-PLA_2 M has been sequenced to the 45th residue from the amino-terminus. The designations [L] and [G] represent peptides derived by Lys-C and Glu-C digestions, respectively.

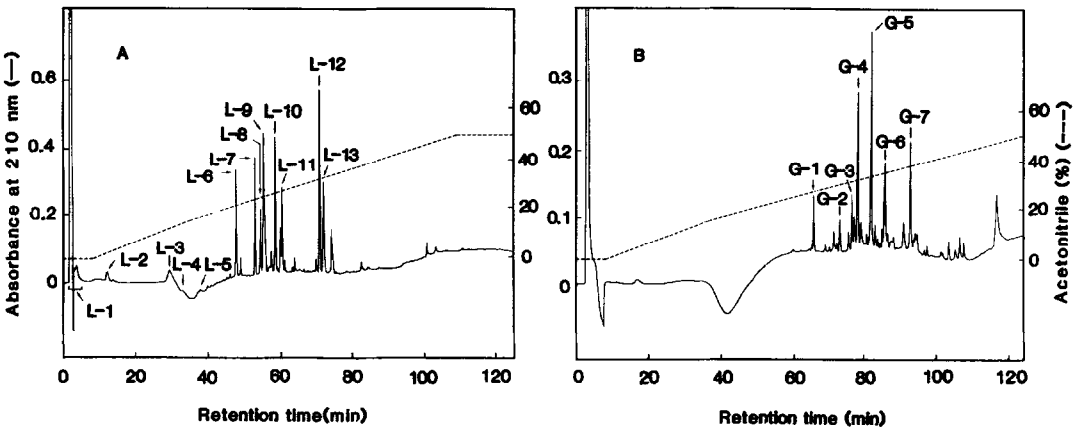

Fig. 2. Separation by HPLC of proteases digests of human splenic PLA_2 M. A, Lys-C digest ; B, Glu-C digest.

9, the fraction was further separated by HPLC using another condition, which revealed two peaks, L-9a and L-9b, followed by amino acid sequence analysis. The sum of the number of each amino acid residue obtained from the fourteen Lys-C-digested peptides agreed well with that of the amino acid composition of PLA_2 M determined experimentally (Table I). This confirmed that the whole primary structure of PLA_2 M could be constructed with the fourteen peptides obtained by Lys-C cleavage.

Table I. Amino acid composition of PLA_2 M

Amino acid	Amino acid composition (mol/mol protein)	
	Found[a]	Sequence
Asx	10.8 (11)	11 [5[b]]
Thr	8.9 (9)	10
Ser	8.2 (8)	10
Glx	6.3 (6)	6 [3[c]]
Gly	11.3 (11)	11
Ala	8.2 (8)	8
Val	3.0 (3)	3
Met	0.9 (1)	1
Ile	2.0 (2)	2
Leu	6.0 (6)	6
Tyr	7.9 (8)	8
Phe	5.1 (5)	5
Lys	12.8 (13)	13
His	3.8 (4)	4
Arg	10.1 (10)	10
Pro	2.2 (2)	2
1/2Cys	13.8 (14)	14

a. The values are uncorrected for partial destruction by 24h hydrolysis.
b. The number of Aspartic acid residue.
c. The number of Glutamic acid residue.
The values in parentheses are the nearest integers

To obtain the overlapping peptides for the Lys-C-digested peptides, RCm-PLA_2 M was digested with Glu-C and seven peptides were purified by HPLC as shown in Fig. 2B. Their sequences were determined. Although it is reported that Glu-C specifically cleaves the glutamyl bond in ammonium bicarbonate (pH7.8) (12), in RCm-PLA_2 M, the protease also cleaved two glycyl peptide bonds, the carboxyl-terminal site of Gly22 and Gly31, besides glutamyl bonds.

The alignment of Lys-C-digested peptides was determined to be (L-13)-(L-4)-(L-12)-(L-10)-(L-5)-(L-2)-(L-11)-(L-9b)-(L-9a)-(L-8)-(L-3)-(L-1)-(L-7)-(L-6) based on the amino acid sequences of four Glu-C digested peptides, G-7, G-5, G-6 and G-4, together with the result of the direct sequencing of intact RCm-PLA_2 M (Asn1-Val45). Thus, the complete amino acid sequence of PLA_2 M was established to be that shown in Fig. 1. PLA_2 M consists of 124 amino acid residues and has a molecular weight of 13,904, which agrees with the result estimated by SDS-PAGE.

DISCUSSION

Much has been reported on mammalian extracellular PLA_2s, whereas little information is available concerning mammalian intracellular PLA_2s (7,13). In the present study, we described the complete primary structure of PLA_2 M isolated from human splenic membrane fraction, of which the sequence was determined by sequence analysis based on Edman degradation of the intact RCm-protein and of the proteolytically digested peptides. This is the first report on the primary structure of cellular PLA_2 from a human organ.

The primary structures of PLA_2 M and the PLA_2s from rat spleen (7), basic Agkistrodon halys (A.h.) blomhoffii (14) and human pancreas (15) are shown in Fig. 3. The numbering system and the spacing for these PLA_2s are based on the method of Renetseder et al. (16). PLA_2 M does not have Cys11 and Cys77, but has Cys50, Cys133 and the carboxyl terminal short extension. These characteristics show that PLA_2 M

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          10         20         30         40         50         60
a. NLVNFHRMIK LTTG-KEAAL SYGFYGCHCG VGGRGSPKDA TDRCCVTHDC CYKRLEKRG- C-----G
b. SLLEFGQMIL FKTG-KRADV SYGFYGCHCG VGGRGSPKDA TDWCCVTHDC CYNRLEKRG- C-----G
c. HLLQFRKMIK KMTG-KEPVI SYAFYGCYCG SGGRGKPKDA TDRCCFVHDC CYEKVT--G- C-----K
d. AVWQFRKMIK CVIPGSDPFL EYNNYGCYCG LGGSGTPVDE LDKCCQTHDN CYDQAKKLDS CKFLLDN

    70         80         90         100        110        120        130
a. TKF LSYKFSNSGS RITCAK-QDS CRSQLCECDK AAATCFARNK TTYNKKYQYY SN-KHCRGST PRC
b. TKF LTYKFSYRGG QISCSTNQDS CRKQLCQCDK AAAECFARNK KSYSLKYQFY PN-KFCKGKT PSC
c. PKW DDYTYSWKNG DIVCGG-DDP CKKEICECDR AAAICFRDNL KTYKKRYMAY PD-ILCSSKS EKC
d. PYT HTYSYSCSGS AITCSSKNKE CEAFICNCDR NAAICFSKA- P-YNKAHKNL DTKYSCQ
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Fig. 3. Comparison of the primary structures of human splenic PLA_2 M (a) and rat splenic (b) (7), basic A.h.blomhoffii(c) (14) and human pancreatic (d) (15) PLA_2s. Position 121 of rat splenic PLA_2 displays either proline or leucine.

belongs to Group II, one of the two groups into which exocrine PLA_2s were classified by Heinrikson et al. (17).

The primary structure of PLA_2 M is very similar to that of rat splenic PLA_2. PLA_2 M has 72% homology with rat splenic PLA_2, 49% with basic A.h.blomhoffii PLA_2, and 39% with human pancreatic PLA_2. Well-conserved amino acid residues including the catalytic network, a hydrophobic wall surrounding the active center and a calcium binding region in the investigated PLA_2s (16) are retained in PLA_2 M except at position 28 (His in PLA_2 M) in a calcium binding region. This homology shows that the catalytic mechanism of PLA_2 M is fundamentally similar to that of exocrine PLA_2s.

PLA_2 M is characterized by a larger ratio of basic amino acid residues to acidic ones, in comparison with the other PLA_2s. The ratios are 3.4, 2.6, 1.6 and 1.4 for PLA_2 M, rat spleen PLA_2, basic A.h.blomhoffii PLA_2 and human pancreatic PLA_2, respectively. Based on the analogy of the available crystallographic structure of PLA_2 (16), it appears that the surface of PLA_2 M is occupied by more basic amino acid residues as compared with the other PLA_2s. Especially, the clusters of basic amino acid residues are observed in the amino-terminal region of residues 1-16 and the region of residues 53-70, which form part of the so-called "interface recognition site" (18,19). Furthermore, the carboxyl-terminal region of residues 127-133 also has a positive charge. The distribution of positively charged regions in the PLA_2 M molecule seems to be similar to that in basic A.h.blomhoffii PLA_2 which can hydrolyze the phospholipids of E.coli killed by the bactericidal protein of neutrophils (14). Such cationic sites are thought to be required for the interaction with the specific targets and be important for a variety of physiological reactions (14,20). We would like to investigate the significance of these characteristics by site-directed mutagenesis.

While we have obtained PLA_2 M that belongs to Group II from KBr extract, the extraction with Triton X-100 has yielded another type PLA_2 which was confirmed to be pancreatic type PLA_2 based on the analysis of its amino acid composition and peptide map. Since a pancreatic type PLA_2 has also been prepared from rat spleen (10), an equivalent type can be expected to occur in the human splenic membrane fraction. The fact that both Group I (pancreatic type) and Group II PLA_2s have been isolated from human spleen is noteworthy. What needs to be examined is the physiological role that they play.

We have purified the PLA_2 from rheumatoid arthritic synovial fluid, using a method similar to that for purifying of PLA_2 M, to compare it with splenic PLA_2 M. Two peaks (peak I and II) having PLA_2 activity were detected on HPLC. The retarded fraction, peak II, had the same retention time on HPLC as that of PLA_2 M. After we had completed our study on the primary structure of PLA_2 M, two reports came to our

attention concerning the nucleotide sequence of PLA_2 in rheumatoid arthritic synovial fluid (21,22). The primary structure deduced from the cDNA sequence was identical with that of PLA_2 M. Therefore, the PLA_2 in peak II may be the same enzyme as the rheumatoid arthritic synovial fluid PLA_2 for which the primary structure was determined from the cDNA sequence. Study of the PLA_2 in peak I is currently in progress.

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