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Purification and characterization of a lipocortin-like 33 kDa protein from guinea pig neutrophils

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A lipocortin-like, phospholipase A_2 inhibitory 33 kDa protein was purified from guinea pig neutrophils. From amino acid composition and sequence data, this protein was found to have a high degree of homology to human lipocortin I. This protein inhibited porcine pancreatic phospholipase A_2 activity in the presence of [^{3}H]oleic acid-labeled *Escherichia coli* membranes as substrate. Maximal inhibition amounted to 65% whereas 50% inhibition occurred at 83.5 nM. This protein showed F-actin-binding ability in a Ca^{2+}-dependent manner.

Amino acid composition; Amino acid sequence; 33 kDa protein; Lipocortin; Phospholipase A_2

1. INTRODUCTION

The anti-inflammatory action of glucocorticoids has been attributed to the induction of a group of phospholipase A_2 (PLA_2) inhibitory proteins, collectively called lipocortin [1–6]. Lipocortin-like proteins have also been isolated as Ca^{2+}-dependent phospholipid-binding proteins [7,8] from various cells such as human monocytes [9], rat macrophages [10], rabbit neutrophils [3], rat renal medullary cell [6,11], human fibroblasts [12], splenic lymphocytes [13], and mouse and bovine thymocytes [14]. Recent work has revealed that there are two types of lipocortin (I and II): type I is monomeric and type II tetrameric, forms which are identical to calpactin II and I, respectively [15–17]. In addition, a 17 amino acid residue consensus sequence has been identified not only in lipocortins, but also in a group of other Ca^{2+}-dependent phospholipid-binding proteins including 34 and 67 kDa calelectrins as well as endonexin [18–21]. However, the biochemical relationship among these proteins remains obscure.

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In the previous paper, we reported the identification of the 33 kDa protein (p33) from guinea pig neutrophils as a Ca^{2+}-dependent phospholipid-binding protein [22]. Moreover, we demonstrated using a monoclonal anti-p33 antibody that this protein is neutrophil-specific and is translocated from the cytoplasm to the plasma membrane on treatment with various stimuli [23]. However, its physiological role remains obscure. Here, we report that p33 is closely related to human lipocortin I through the analysis of the functions, amino acid composition, and partial amino acid sequence of the protein.

2. MATERIALS AND METHODS

2.1. *Materials*

Hartley guinea pigs were obtained from the Shizuoka Laboratory Animal Center (Shizuoka, Japan). Mono P, polybuffer 96 and polybuffer 74 were obtained from Pharmacia (Uppsala); C_4 ($5C_4$-300) and CNBr from Nakarai (Kyoto); TSK G3000SW from LKB (Bromma, Sweden); μBondapak C_{18} from Waters Division of Millipore (Milford, MA); [^{3}H]oleic acid (8.9 Ci/mmol) from New England Nuclear (Dreieich); phosphatidylserine (PS, bovine brain), cardiolipin (CL, bovine heart), cholesterol (C), dipalmitoylphosphatidylcholine (DPPC) and bovine serum albumin (BSA) from Sigma (St. Louis, MO); trypsin from Worthington; and PLA_2 from Boehringer Mannheim. All other chemicals were of reagent grade.

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2.2. *Purification of p33*

A cytoplasmic fraction was prepared from guinea pig polymorphonuclear leukocytes (PMNs) as in [22,23]. This fraction was incubated with DPPC/C/PS or DPPC/C/CL (1:0.5:0.5 molar ratio) liposomes in the presence of 1 mM $CaCl_2$ for 10 min at 4°C as described [24,25]. The liposome-associated proteins were separated from non-associated proteins by centrifugation at 100000 × *g* at 4°C for 30 min. They were then released from liposomes by treatment with 5 mM EGTA, followed by centrifugation at 100000 × *g* at 4°C for 20 min. To concentrate the protein in the supernatant, solid $(NH_4)_2SO_4$ was added with constant stirring to a final saturation level of 100%. The mixture was allowed to stand overnight at 4°C, and centrifuged at 100000 × *g* for 60 min at 4°C. The pellet was redissolved in a minimum volume (less than 10 ml) of 25 mM triethanolamine-iminodiacetic acid (pH 8.3) (buffer A) and dialysed overnight vs two 1-l volumes of buffer A. After dialysis, insoluble material was precipitated by centrifugation at 100000 × *g* for 30 min. The supernatant was then loaded onto a Mono P column (200 × 5 mm) previously equilibrated with buffer A. Proteins were eluted with elution buffer B (10% polybuffer-iminodiacetic acid, pH 5.0), the flow rate being 1.0 ml/min. The resultant fractions were analyzed by 15% SDS-PAGE. The p33-rich fraction was loaded onto a column (300 × 5 mm) of TSK G3000SW previously equilibrated with 50 mM sodium phosphate buffer (pH 7.4) and eluted with the same buffer at a flow rate of 0.7 ml/min.

2.3. *Determination of PLA_2 activity*

Samples were tested for PLA_2 inhibitory activity by an in vitro assay described by Davidson et al. [26], using [^{3}H]oleic acid-labeled *Escherichia coli* membranes isolated after autoclaving [27] with the following modifications: p33 was preincubated with PLA_2 (50 ng) at 4°C for 10 min, followed by addition of *E. coli* membranes and further incubation at 4°C for 5 min. Radioactivity of released [^{3}H]oleic acid was determined as in [28].

2.4. *F-Actin binding*

Rabbit skeletal muscle F-actin was prepared from acetone powder [29]. Proteins and F-actin were incubated for 1 h at room temperature in binding buffer (10 mM imidazole-HCl, pH 7.4, 2 mM $MgCl_2$, 1 mM NaN_3, 0.5 mM DTT, 0.1 mM ATP) containing 1 mM $CaCl_2$ or 1 mM EGTA with or without 100 mM KCl. After centrifugation at 95000 rpm for 10 min in a Beckman TL-100 centrifuge, supernatants and pellets were subjected to SDS-PAGE and Coomassie blue staining.

2.5. *Amino acid analysis*

Amino acid hydrolysis of purified p33 was performed at 110°C under vacuum in 6 M HCl for 24, 48 or 72 h. Hydrolysates were analyzed using a JLC-200A analyzer (Jeol, Akishima, Japan).

2.6. *Amino acid sequence analysis*

The NH_2-terminal sequence of tryptic peptides and CNBr-cleavage peptides of p33 was determined by automated Edman degradation using an Applied Biosystems model 470A gas-phase protein sequencer (Foster City, USA). Phenylthiohydantoin (PTH)-amino acid from each cycle was analyzed on-line using an Applied Biosystems 120A PTH amino acid analyzer.

3. RESULTS AND DISCUSSION

3.1. *Purification of p33*

Most of the Ca^{2+}-dependent liposome-associated proteins were separated from non-associated proteins by centrifugation. These pro-

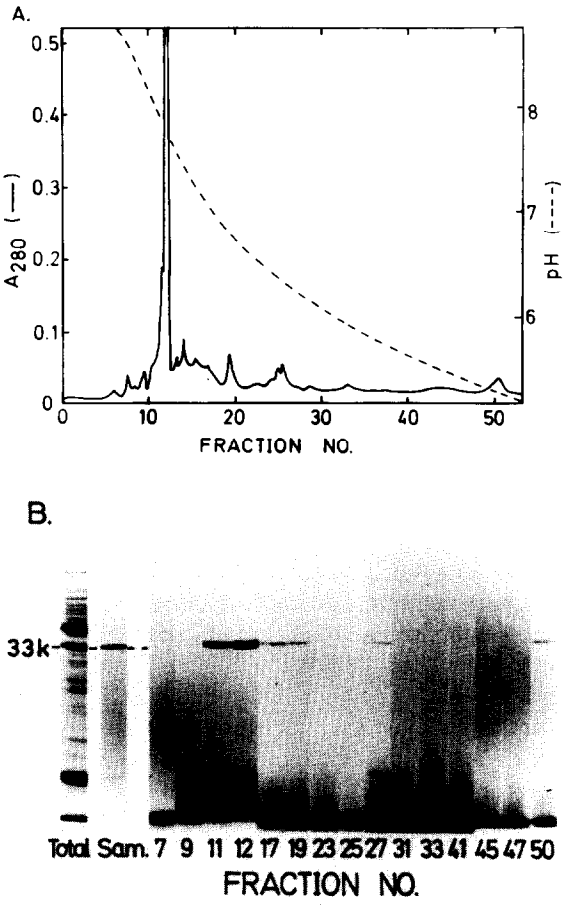

Fig.1. Elution profile of protein from isoelectric chromatofocussing column (Mono P) chromatography. The Ca^{2+}-dependent phospholipid-binding protein in the supernatant of guinea pig neutrophils was associated with CL-containing DPPC liposomes in the presence of 1 mM Ca^{2+} and dissociated from liposomes by treatment with 5 mM EGTA. The dissociated sample was concentrated with $(NH_4)_2SO_4$ and dialysed overnight vs buffer A (25 mM triethanolamine-iminodiacetic acid, pH 8.3). The sample was applied to a column (200 × 5 mm) of Mono P previously equilibrated with buffer A. After washing with buffer A, proteins were eluted with buffer B (3.0 ml polybuffer 96, 7.0 ml polybuffer 74 diluted to a volume of 100 ml iminoacetic acid, pH 5.0). Flow rate, 1 ml/min; fraction volume, 1 ml. (A) Elution profile of the proteins (A_{280}); (B) Coomassie blue-stained 15% SDS-PAGE of eluted proteins; Total, total cytoplasmic protein; Sam., phospholipid liposome-associated proteins.

teins were dissociated from the phospholipid liposomes by treatment with EGTA, and then concentrated with $(NH_4)_2SO_4$. After dialysis against buffer A, the sample was applied to a Mono P column equipped for high-performance liquid chromatography (HPLC) and elution carried out with buffer B. Fig.1A shows the elution profile of Ca^{2+}-dependent phospholipid-binding proteins from Mono P column chromatography. Most of the p33 was recovered in fractions 11 and 12, the pH of fraction 12 being 7.8 (fig.1B). Fraction 12 was further purified by gel filtration on TSK G3000SW. As shown in fig.2, a high degree of purification was achieved for p33 retained in fraction 11, no other proteins being detected in this fraction by SDS-PAGE analysis (fig.2, inset).

3.2. *Inhibition of PLA_2 by p33*

As shown in fig.3, increasing amounts of p33 lead to a progressive decrease in the quantity of [^{3}H]oleic acid released into the assay supernatant by PLA_2. Maximal inhibition was 65% whereas 50% inhibition occurred at 82.5 nM. Pepinsky et al. [10] reported that 72 nM lipocortin I (37 kDa) was required for 50% inhibition of PLA_2. Khanna et al. [30] observed 50% inhibition of PLA_2 by 233 nM lipocortin II but did not find any anti-PLA_2 activity in various calcium-binding proteins such as parvalbumin, calmodulin, calregulin, troponin C, CAB-48 or S-100. Faunel et al. [31] reported 50% inhibition of PLA_2 by 481 nM endonexin. The inhibitory activity of p33 is therefore quite similar to that of lipocortin I.

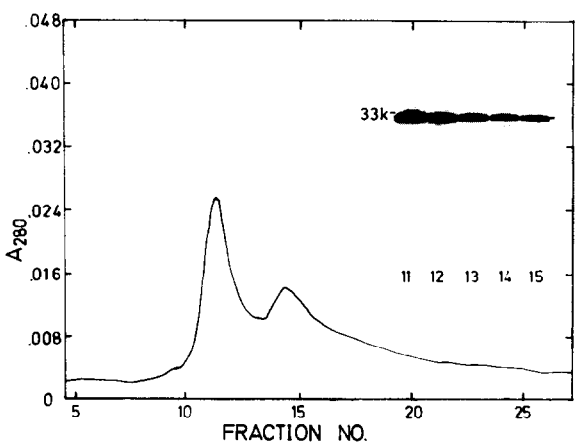

Fig.2. Elution profile of protein from TSK G3000SW column chromatography. An aliquot (0.2 ml) of the pool from a p33-rich fraction from a Mono P column was loaded onto a TSK G3000SW column, which had been equilibrated with 50 mM phosphate buffer (pH 7.4). Flow rate, 0.7 ml/min; fraction volume, 1 ml. (Inset) 15% SDS-PAGE of fractions detected by silver staining.

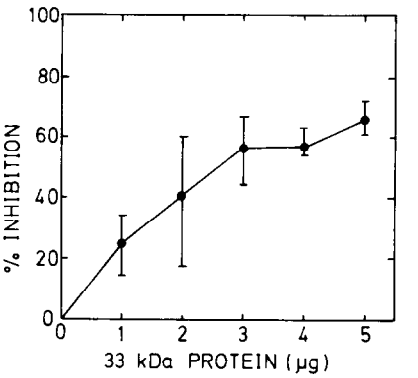

Fig.3. Phospholipase A_2 inhibitory activity of p33. Aliquots of purified p33 were preincubated with 50 ng porcine pancreatic PLA_2 at 4°C for 10 min. The reaction was initiated by adding ^{3}H-labeled *E. coli* membranes as substrate at 4°C and terminated 5 min later by addition of 2 N HCl and 100 mg/ml of BSA. After centrifugation to remove *E. coli* membranes, the radioactivity of the supernatant was counted in a liquid scintillation counter. In all analyses, samples were assayed in triplicate and adjusted for nonspecific release by subtracting the control value. Results are given as means ± SE from 3 experiments.

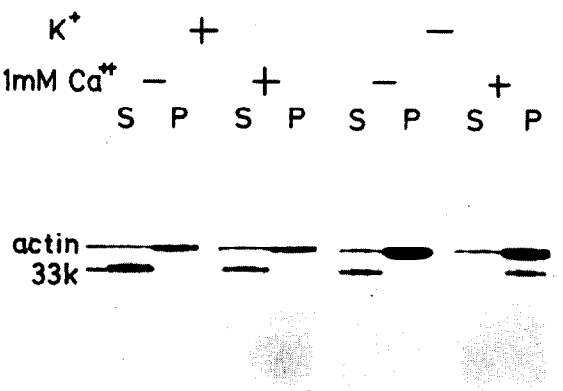

Fig.4. F-Actin binding of p33. Purified p33 was incubated with F-actin for 1 h at room temperature in the binding buffer containing 1 mM $CaCl_2$ or 1 mM EGTA with or without 100 mM KCl. After centrifugation, the supernatant (S) and pellet (P) were analyzed by SDS-PAGE and Coomassie blue staining.

3.3. *Binding to F-actin*

The p33 bound F-actin in the presence of Ca^{2+} in medium containing no K^+, however no binding was observed after addition of 1 mM EGTA in either the presence or absence of K^+ (fig.4). Therefore, the binding of p33 to F-actin occurred only under certain conditions.

3.4. *Amino acid composition of p33*

The amino acid analysis of purified p33 is detailed in table 1 together with the amino acid composition of human lipocortin I [32]. Both the guinea pig and human polypeptides are very polar molecules, with appprox. 30% of the residues being charged amino acids: these two proteins showed similar compositions.

3.5. *Partial sequence analysis of p33*

Three preliminary attempts to sequence untreated intact p33 were unsuccessful, indicating that the N-terminal end of the protein may be blocked. Therefore, p33 was digested with trypsin or cleaved with CNBr, and the cleavage products separated by reverse-phase HPLC on a C_{18} or C_4 column. Fig.5 (panel I) shows peptide maps generated from p33, which were monitored by their absorbance at 214 nm. Some of the major peaks were subjected to sequence analysis. Fig.5 (panel II) summarizes the sequences derived from the peak fractions. Recently, Wallner et al. [32] have cloned and expressed human lipocortin I. For each of the guinea pig polypeptides described in fig.5 (panel II), we have identified the corresponding region within the human sequence, based on the high degree of sequence homology between the two proteins. Out of 53 amino acids that we compared, only 14 residues differed (fig.5, panel II). From the partial analysis presented here, we estimate that the sequences of these guinea pig and human proteins are approx. 70% homologous.

Table 1

Amino acid composition (in mol%) of p33 and human lipocortin I

Amino acid	p33	Human lipocortin I[a]
Asx	12.4	11.6
Thr	6.3	6.4
Ser	7.4	5.5
Glx	11.0	11.4
Pro	1.1	2.3
Gly	6.4	5.5
Ala	11.3	9.5
Val	4.3	5.5
Met	1.9	2.6
Ile	6.7	6.1
Leu	10.9	11.6
Tyr	1.9	3.2
Phe	1.3	3.5
His	1.2	1.5
Lys	10.6	6.8
Arg	5.3	5.5
Cys	ND	1.2
Trp	ND	0.3

[a] From Wallner et al. [32]

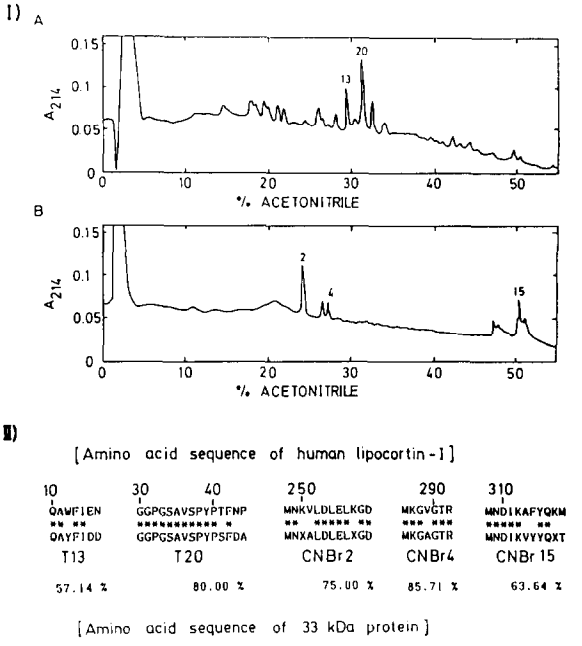

Fig.5. Partial amino acid sequence of p33. (I) Tryptic and CNBr-cleaved peptide maps of cleavage fragments derived from p33. (A) Preparations containing 100 μg HPLC-purified p33 were digested with trypsin for 24 h at 37°C and analyzed by reverse-phase HPLC on a C_{18} column. Fractions were monitored at 214 nm. (B) These preparations were cleaved with CNBr for 15 h at room temperature and analyzed by reverse-phase HPLC on a C_4 column. Fractions were monitored at 214 nm. Numbered peaks signify the polypeptides that have been sequenced. (II) Comparison of guinea pig neutrophil p33 and human lipocortin I sequences. Tryptic and CNBr fragments were subjected to amino-terminal protein sequence analysis in a gas-phase sequenator. Designations for tryptic and CNBr fragments correspond to the column peaks in panel I. The amino acid sequences of p33 fragments were compared with that of human lipocortin I [32]. * Same sequence; % homology between human lipocortin I and peptide fragments of p33 is indicated; numbers, sequence number of human lipocortin I.

Previously, we reported the identification of the p33 isolated from guinea pig neutrophils as being a Ca^{2+}-dependent phospholipid-binding protein [22]. In this study, it was found that p33 binds F-actin in the absence of K^+ in a Ca^{2+}-dependent manner, and that the protein shares functional homology with lipocortin I from data on the inhibitory activity for PLA_2, amino acid composition, and the partial amino acid sequence. Together with these data, it is clear that guinea pig p33 displays anti-PLA_2 activity similar to that of lipocortin-like protein in other cells. However, the isoelectric point of p33 is 7.8 and the protein is distributed only in neutrophils [23]: the molecular mass, isoelectric point, and cell specificity differ from those of human lipocortin-I. Furthermore, the content of p33 distributed in neutrophils is very high as described in previous papers [22,23]. From these facts, the biological roles and relationship between p33 in neutrophils and other lipocortin-like proteins remain to be elucidated. In connection with this, we are now attempting cDNA cloning of this protein.

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