Volume 255, number 2, 231–236 FEB 07612 September 1989

Expression of the cDNA encoding lipocortin-like 39 kDa protein of guinea pig neutrophils in yeast

Purification and biological characterization

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Received 19 July 1989

The cDNA encoding lipocortin-like 39 kDa protein in guinea pig neutrophils was cloned into a yeast expression vector and the constructed plasmid was introduced into a yeast. The gene was expressed in an eukaryotic cell, yeast *Saccharomyces cerevisiae* and the recombinant protein was purified and characterized. The purified protein was identical with the native one with respect to the antigenicity and several biochemical properties, such as inhibitory action against phospholipase A_2, Ca^{2+}-dependent binding to acidic-phospholipids and F-actin and availability as a substrate for tyrosine kinase (EGF receptor/kinase) and protein kinase C.

Lipocortin; Recombinant protein; Neutrophil; Protein kinase

1. INTRODUCTION

Lipocortins are a family of calcium-dependent phospholipid-binding proteins that inhibit PLA_2 [1–3]. The 39K in guinea pig neutrophils, which was previously designated as p33 [4–7], is also a family of lipocortins: the 39K specifically localizes in the cytoplasm of the resting neutrophils, translocates from cytoplasm to the inner surface of plasma membranes of activated neutrophils [5], inhibits degradation of membranous phospholipids by PLA_2, binds to muscle actin under certain conditions [6], and is phosphorylated by PKC [8].

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Abbreviations: cDNA, complementary DNA; 39K, 39 kDa protein; PLA_2, phospholipase A_2; PKC, Ca^{2+}- and phospholipid-dependent protein kinase; EGF, epidermal growth factor; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

Analysis of the cDNA for 39K revealed an extremely high homology with the cDNA for lipocortin I from human U937 cells [9] and from the lung of rodents [10,11].

Wallner et al. [9] reported that human recombinant lipocortin I inhibited the catalytic activity of PLA_2. Moreover, Cirino et al. [12–14] reported that the recombinant lipocortin I inhibited the production of prostacycline and thromboxane A_2 by human endothelial cells and guinea pig lung, respectively, and suppressed the carrageenin-induced paw edema of the rat. Since the recombinant protein is the product from procaryotic, *Escherichia coli*, its N-terminal residue, glycosylation and the mode of processing may not be identical with those of native lipocortin I. The present work describes the construction of a high expression vector of recombinant 39K protein in an eukaryotic cell, yeast *Saccharomyces cerevisiae*, and a simple and efficient method for purification of the recombinant 39K. We also demonstrate that

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00145793/89/$3.50

the biological properties of the recombinant protein are identical with those of native one.

2. MATERIALS AND METHODS

2.1. *Materials*

Conventional enzymes were obtained from Toyobo Co., Ltd (Osaka) and Takara Shuzo Co., Ltd (Kyoto). Mono P column, polybuffer 96 and polybuffer 74 were obtained from Pharmacia-LKB (Uppsala); TSK gel G-3000SW column from Toyo Soda; [^{3}H]oleic acid (8.9 Ci/mmol) from New England Nuclear (Dreieich); EGF from Funakoshi Co., Ltd (Tokyo); PLA_2 from Boehringer Mannheim (Mannheim). All other chemicals used were of analytical grade.

2.2. *Construction of expression plasmid*

As previously reported [7], we obtained λgpL8 and λgpL7 cDNA clones, each containing the 5′-terminal and 3′-terminal region of 39K cDNA. In this study, we also used λgpL9, which contains the 5′-terminal region (fig.1A). These cDNA clones were subcloned into the *Eco*RI site of pUC9 and designated respectively pGPL8, pGPL7 and pGPL9. Each of the 0.4 kb *Eco*RI-*Hha*I fragment from pGPL8, the 0.2 kb *Hha*I-*Bgl*II fragment from pGPL9 and the 0.8 kb *Bgl*II-*Sal*I fragment from pGPL7 were ligated with the 8 kb *Eco*RI-*Sal*I fragment of pYHCC101, which was a yeast expression vector described in [15] and the resultant plasmid was designated as pYGPL100 (fig.1B). In plasmid pYGPL100, guinea pig neutrophil 39K cDNA was regulated by a glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter, which is known to be a strong promoter in yeast [16].

2.3. *Yeast strains and media for yeast*

The strain of *Saccharomyces cerevisiae* used in this experiment was EH-13-15 (MAT, α, trp1) [17]. Transformation of the yeast was carried out by the method of Ito et al. [18]. Yeast strains containing pYGPL100 were grown at 30°C for 40 h in Burkholder's medium [19].

2.4. *Purification of recombinant 39K*

The strain EH-13-15 containing pYGPL100 was cultivated at

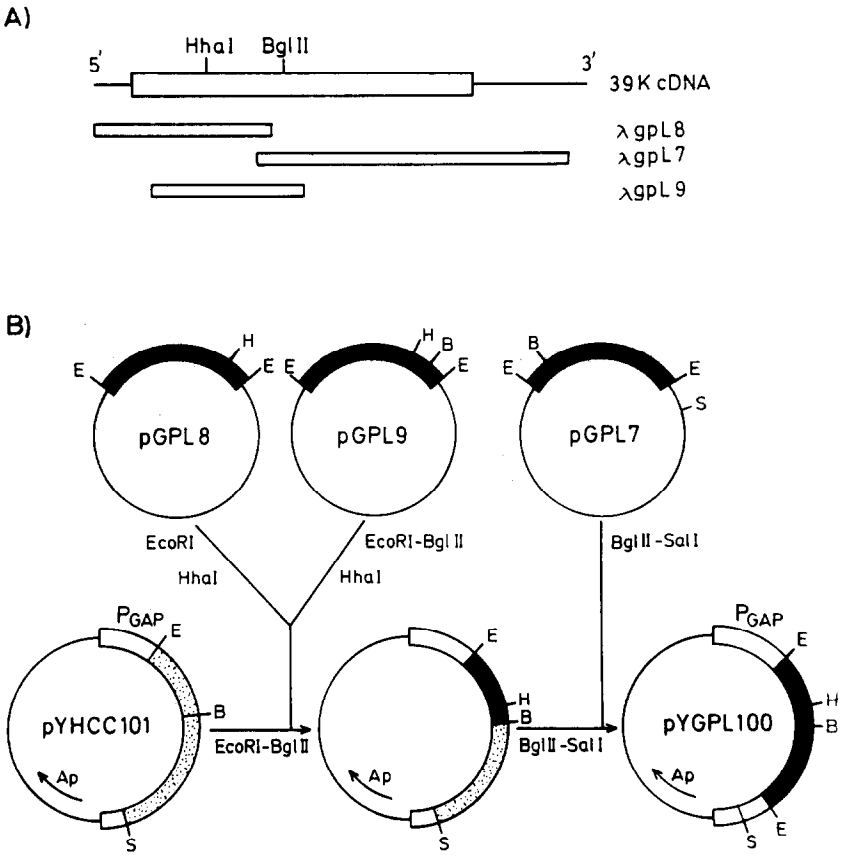

Fig.1. Construction of a vector for expressing 39K cDNA in yeast. (A) Restriction map of 39K cDNA's overlapping cDNA clones. The coding sequence is indicated by thick box. (B) pYHCC101 was described in [15]. The solid boxes denote 39K cDNA. P_{GAP}, promoter of glyceraldehyde-3-phosphate dehydrogenase; Ap, ampicillin resistance; E, *Eco*RI; B, *Bgl*II; H, *Hha*I; S, *Sal*I.

30°C in Burkholder's medium. About 40 g of cells were obtained from a 7 l broth. The yeast cells, which were suspended in 50 ml of 50 mM Tris-HCl (pH 7.5) containing 5 mM EGTA, 1 mM PMSF, and 0.01% leupeptin, were disrupted with bead beader (Biospeck products) at 4°C. The resulting lysate was centrifuged at 5000 × *g* at 4°C for 30 min. This supernatant was further incubated with 6 mM $CaCl_2$ at 4°C for 30 min and centrifuged at 100000 × *g* at 4°C for 60 min. The supernatant was removed and the pellet was suspended in 20 mM Hepes-KOH (pH 7.4) containing 0.1 M KCl, 25 mM EGTA, 1 mM PMSF, 0.01% leupeptin at 4°C for 30 min. This suspension was centrifuged at 100000 × *g* at 4°C for 60 min. The following procedure is the same as that described previously [6]. Briefly, 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. The pellet was dissolved in a minimum volume (less than 10 ml) of 25 mM triethanolamine-iminodiacetic acid (pH 8.3) (buffer A) and dialyzed overnight vs two 1-l volumes of buffer A. After dialysis, insoluble material was precipitated by centrifugation at 10000 × *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 the elution buffer B (10% polybuffer-iminodiacetic acid, pH 5.0) at a flow rate of 1.0 ml/min. The resulting fractions were analyzed by 12% SDS-PAGE. The 39K-rich fraction was loaded onto a column (300 × 5 mm) of TSK gel G-3000SW previously equilibrated with 20 mM Hepes-KOH, 0.1 M KCl buffer (pH 7.4) and proteins were eluted with the same buffer at a flow rate of 0.5 ml/min.

2.5. *Determination of PLA_2 activity*

Samples were tested for PLA_2 inhibitory activity by an in vitro assay using [^{3}H]oleic acid-labeled *E. coli* membranes as described by Davidson et al. [20].

2.6. *Binding of F-actin*

The binding activity of 39K to F-actin was determined by the method of Hayashi et al. [21].

2.7. *Phosphorylation of 39K by PKC*

Phosphorylation of 39K by PKC was routinely assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into 39K at 30°C for 3 min by the method of Boni and Rondo [22]. The reaction mixture was subjected to SDS-PAGE and the gels were stained with Coomassie brilliant blue R250 (CBB). Then, autoradiograms of the ^{32}P-labeled proteins were obtained as described previously [23].

2.8. *EGF-dependent phosphorylation*

A-431 cells were grown in 75 cm^2, culture flask containing Dulbecco's modified Eagle's medium (Flow Laboratories) supplemented with 10% fetal bovine serum, streptomycin and penicillin. The membrane fraction of the culture cells was obtained as described by De et al. [24]. Briefly, the reaction mixtures contained in a final volume of 60 μl, 20 mM Hepes (pH 7.4), 2 mM $MgCl_2$, 10 μM sodium vanadate, 15 μCi [γ-^{32}P]ATP and aliquots of the A-431 total particulate fraction. The reaction tubes containing all components except the ATP were placed on ice and preincubated for 10 min at 0°C in the absence or the presence of EGF (2 μg/ml). The reaction was initiated by adding ^{32}P-labeled ATP. After 10 min of incubation at 0°C, the reaction was terminated by adding 30 μl of the stop solution containing 10% of SDS and heating for 3 min at 100°C.

3. RESULTS AND DISCUSSION

3.1. *Purification of recombinant 39K*

A yeast transformant containing pYGPL100 was cultivated in Burkholder's medium for 40 h at 30°C. The organisms were collected by centrifugation and disrupted by bead beader. The crude samples were extracted by Ca^{2+}-dependent precipitation. When these extracts were applied to the Mono P column, most of the 39K was eluted at pH 7.8 (fig.2, lane 5). The 39K-enriched fractions were pooled and subjected to TSK gel G-3000SW column chromatography. As shown in fig.2, lane 6, 39K was highly purified by this method: the purified protein migrated as a single band in SDS-PAGE with a molecular weight of 35000. Immunological identity of native and the recombinant 39K was confirmed by immunoblot-

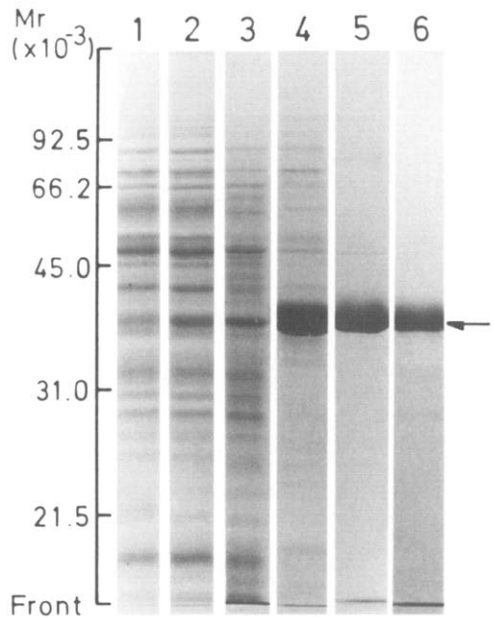

Fig.2. SDS-PAGE analysis of recombinant 39K at different steps of purification. Samples were analyzed by 12% SDS-PAGE and stained by Coomassie brilliant blue. Lanes: 1, total lysate of yeast (control); 2, total lysate of yeast containing pYGPL100; 3, post-nuclear fraction; 4, a protein fraction which bound to membranes Ca^{2+}-dependently; 5, Mono P fraction; 6, TSK gel G-3000SW fraction.

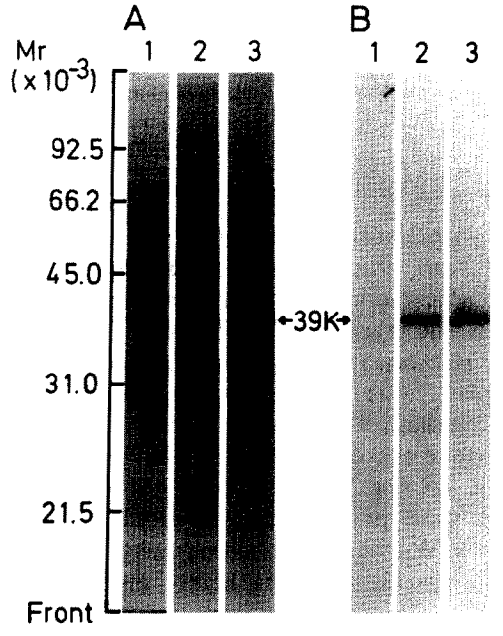

Fig.3. SDS-PAGE and Western blotting of total lysate of yeast and purified recombinant 39K. (A) Coomassie brilliant blue stain; (B) electroblotted onto Durapore filter and immunostained with mouse antiserum against native 39K. Lanes: 1, total lysate of yeast (control); 2, total lysate of yeast containing pYGPL100; 3, purified recombinant 39K.

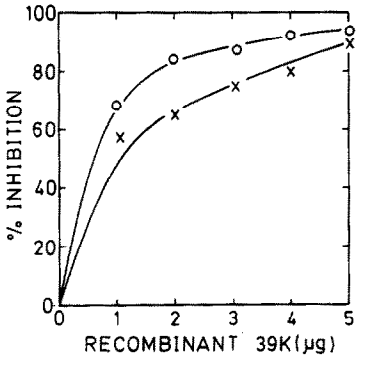

Fig.4. Inhibition of PLA_2 by recombinant 39K. Aliquots of the purified recombinant 39K were preincubated with 50 ng porcine pancreatic PLA_2 (○) or ^{3}H-labeled *E. coli* membranes (×) at 4°C for 10 min. The reaction was initiated by adding ^{3}H-labeled *E. coli* membranes (○) or 50 ng porcine pancreatic PLA_2 (×) at 4°C and terminated 5 min later by adding 100 μl of 2 N HCl and 100 mg/ml of bovine serum albumin. After centrifugation to remove *E. coli* membranes, the radioactivity in the supernatant fraction was determined in a liquid scintillation spectrometer.

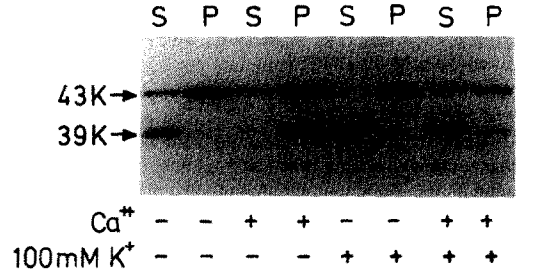

Fig.5. Binding of F-actin to recombinant 39K. The purified recombinant 39K was incubated with muscle F-actin for 1 h at 25°C in the buffer solutions containing 1 mM $CaCl_2$ (+) or 1 mM EGTA (−) in the presence (+) or absence (−) of 100 mM KCl. Then, the incubated samples were centrifuged and the supernatant (S) and the precipitates (P) were analyzed by SDS-PAGE and CBB staining.

ting with antibody against native 39K (fig.3). The N-terminal amino acid sequence of purified recombinant 39K was blocked as well as native one [6].

3.2. *Inhibition of PLA_2 activity by recombinant 39K*

Previous studies in this laboratory [6] revealed

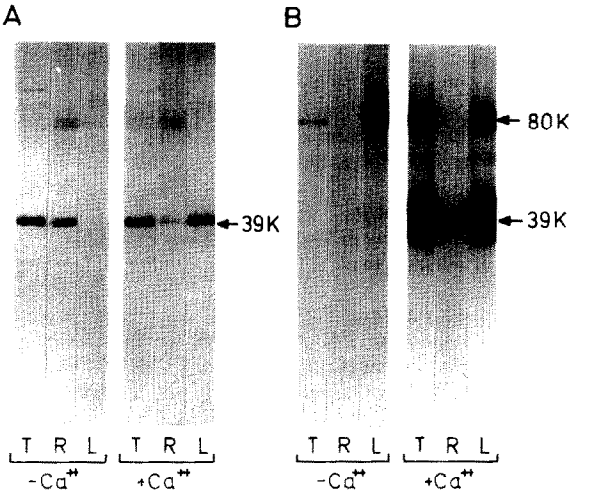

Fig.6. Autoradiograms of the phosphorylated recombinant 39K by PKC. Recombinant 39K was incubated in the medium containing dipalmitoylphosphatidylcholine/cholesterol/phosphatidylserine liposome (2:1:1 in molar ratio), 100 nM PMA and [γ-^{32}P]ATP in the presence or absence of Ca^{2+} for 30 min at 30°C. Free and membrane bound proteins were separated by Ficoll density gradient centrifugation (flotation method) [4]. Proteins were analyzed by 12% SDS-PAGE and silver staining (A). Phosphorylated proteins were analyzed by autoradiography (B). T, total protein fraction; R, unbound protein fraction; L, membrane-bound protein fraction. $+Ca^{++}$, 0.3 mM Ca^{2+}; $-Ca^{++}$, 1 mM EGTA.

that native 39K inhibited PLA_2 as did lipocortin I [2,3]. Thus, the PLA_2 inhibitory activity of the purified recombinant 39K was tested. Fig.4 shows that recombinant 39K inhibited the PLA_2 activity as measured by [^{3}H]oleic acid release from bacterial membranes. The inhibitory activity of recombinant 39K was slightly higher when preincubated with bacterial membranes than when preincubated with PLA_2. These results suggested that 39K might interact with substrate phospholipids rather than with the enzyme. Davidson et al. [18] recently proposed that lipocortins might inhibit PLA_2 activity by masking the surface of phospholipid membranes. This concept is also supported by the study using sedimentation equilibrium analysis [25]. The results obtained in the present experiments are also consistent with this proposal.

3.3. *Binding of F-actin*

Since the naturally occurring 39K binds to F-actin, the binding activity of the recombinant 39K to F-actin was also measured. In the presence of Ca^{2+} the recombinant 39K bound to F-actin a K^+-free medium. However, EGTA inhibited the binding either in the presence or absence of K^+ (fig.5). Thus, recombinant 39K also binds to F-actin by a Ca^{2+}-dependent mechanism as did native 39K.

3.4. *Substrate for PKC or tyrosine kinase*

Lipocortin I has been shown to be phosphorylated either by PKC [26,27] or EGF receptor/kinase (tyrosine kinase) [28,29]. Previous studies revealed that the native 39K was also phosphorylated by PKC [8]. Thus, we tested whether the recombinant 39K also serves as a substrate for

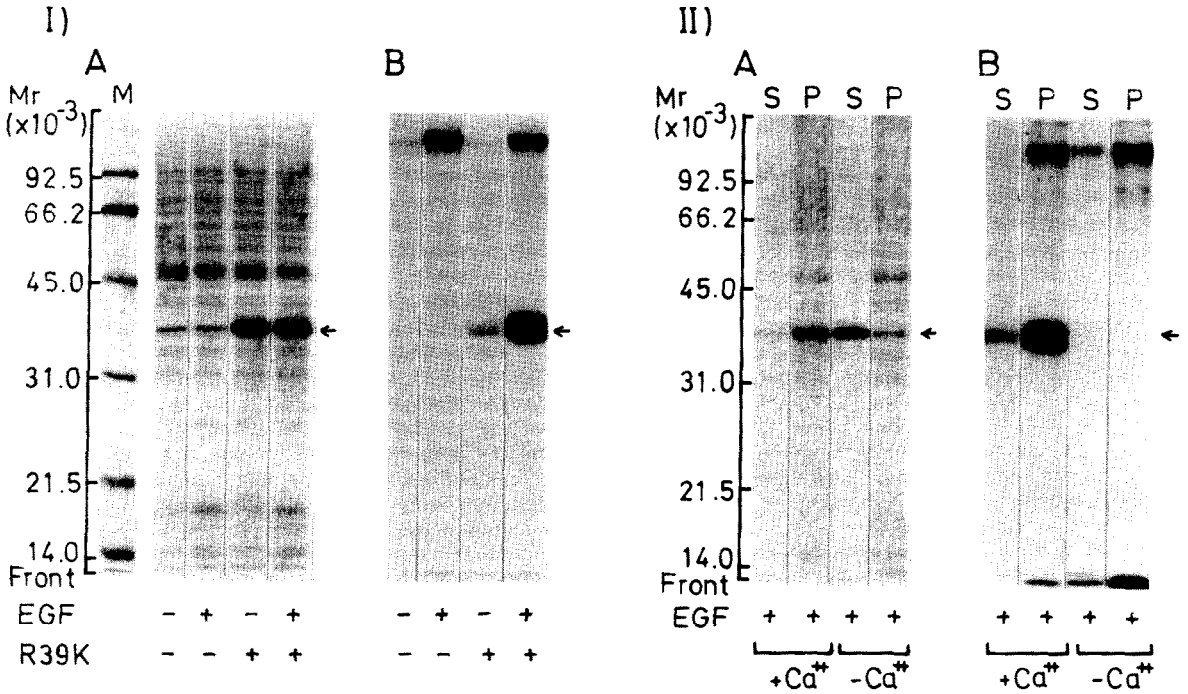

Fig.7. EGF-dependent phosphorylation of 39K. (I) A-431 cells were grown at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. In vitro phosphorylation reactions using isolated A-431 cell membranes were analyzed by SDS-PAGE (A) and the radioactive products were visualized by autoradiography (B). EGF, epidermal growth factor; R39K, recombinant 39K (200 μg/ml); M, molecular marker. (II) After phosphorylation by EGF receptor kinase with or without Ca^{2+}, the recombinant samples were centrifuged at 10000 × *g* for 10 min at 4°C. Each of the supernatant (S) or precipitant (P) fraction was analyzed by 12% SDS-PAGE (A) and the radioactive products were visualized by autoradiography (B). + Ca^{++}, 0.5 mM $CaCl_2$; − Ca^{++}, 1 mM EGTA.

PKC or EGF receptor/kinase. As shown in figs 6 and 7, in the presence of Ca^{2+}, the recombinant 39K bound to liposomal and native membranes. Thus, only when associated with the membranes, recombinant 39K was phosphorylated by PKC and tyrosine kinase (fig.6, fig.7II). Fava et al. [28] and Cohen et al. [30] have also reported that Ca^{2+} is required for the binding and phosphorylation of the 35 kDa protein (lipocortin I) isolated from A-431 cells by membrane or endosome preparation derived from the same cells. The membrane-binding activity of recombinant 39K remained unchanged after phosphorylation by PKC (fig.6L) or EGF receptor/kinase (fig.7II).

The present work clearly demonstrates that the recombinant 39K is expressed in *Saccharomyces cerevisiae* and the protein has identical properties to those of native 39K from guinea pig neutrophils. Lipocortins have recently been shown to have a potent anti-inflammatory action, such as in carrageenin-induced paw edema [14,31]. Preliminary experiments revealed that the carrageenin-induced paw edema of the rat was also inhibited significantly by a low dose (2 nmol/kg) of recombinant 39K. Thus, like other lipocortin families, the neutrophil-specific 39K might have an anti-inflammatory action. We are currently investigating the pathological role of 39K in various inflammatory processes.

Acknowledgements: This work was supported by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan. The authors wish to thank Dr K. Owada for the gift of A-431 cells and Miss Yumi Kitaoka for preparing the manuscript.

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