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Purification and characterization of a 32-kDa phospholipase A_2 inhibitory protein (lipocortin) from human peripheral blood mononuclear cells

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A 32-kDa protein was isolated from human monocytes after calcium precipitation and chromatography. The protein activity was assessed by the inhibition of soluble phospholipase A_2 (PLA_2). This in vitro inhibitory effect on phospholipases A_2 was found only with negatively charged phospholipids. The protein was also able to inhibit cellular PLA_2 in mouse thymocytes. The biochemical properties and amino acid composition strongly suggest that the protein shares similarities with endonexin. Using a neutralizing monoclonal antibody against rat lipocortin, we found a cross-reactivity with the 32-kDa protein. According to the biochemical and immunological properties, we propose to relate this PLA_2 inhibitory protein from human monocytes to lipocortin.

Lipocortin; Phospholipase A_2; Monocyte; Phospholipid

1. INTRODUCTION

Lipocortins are a family of anti-inflammatory proteins acting by inhibiting the activity of phospholipase A_2, and thus preventing the formation of inflammatory mediators, prostaglandins and leukotrienes. They have been described in several cell systems, rat macrophages [1], rabbit neutrophils [2], rat renomedullary interstitial cells [3], human fibroblasts [4] and mouse and bovine thymus [5]. The predominant form is a 35–40-kDa protein but smaller proteolytic active fragments were identified. All these proteins have been characterized by the capacity to be induced by glucocorticoids, released in the supernatant of treated cells and regulated by phosphorylation [6–9]. Recently a human lipocortin (lipocortin I) has been purified [10], cloned and expressed in *E. coli* [11]. The purified protein was identified as the 35-kDa substrate of the EGF-receptor kinase from A431 cells [12], and shown to exert all the biochemical and pharmacological properties of lipocortin. The same group [13] purified from human placenta another 35-kDa inhibitory protein which they referred to as lipocortin II. This protein is the human analogue of pp36, a major substrate for $pp60^{src}$.

In another field of investigations, a group of calcium and phospholipid binding proteins has been identified in different cells and tissues (calpactin I and II, calelectrin, endonexin, protein I and II, calcimedin, chromobindin) [14–25]. All these proteins share a common characteristic consisting of a conserved 17 amino acid consensus se-

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quence present in multiple copies [21]. They also have the capacity to bind phospholipids in a calcium dependent manner. These proteins have been identified in cells or tissues without glucocorticoid treatment, and were shown to be present in certain tissues at a high level up to 1% of the total protein content. In this communication, we bring the evidence that the 32-kDa anti-phospholipase A_2 protein from human mononuclear cells is a lipocortin.

2. MATERIALS AND METHODS

2.1. *Preparation of human cells for protein purification*

Mononuclear cells from healthy donors (70% lymphocytes and 30% monocytes) were purified by sequential Ficoll-Hypaque density sedimentation, and continuous Percoll gradient. Cells were cultured at 5×10^6 cells/ml in RPMI 1640 medium (Flow Laboratories, Puteaux, France), containing 20 mM Hepes, 2% Ultroser G, a serum substitute (IBF, Villeneuve la Garenne, France) and 1 μM dexamethasone and incubated in a Bellco spinner (Bellco, England) (25 rpm) overnight at 37°C. After this overnight incubation period, cells were reincubated in the same medium devoid of Ultroser for another 5 h. The cells were then pelleted and resuspended, after removal of red cells by hypotonic lysis, in buffer A (10 mM Hepes, 5 mM EDTA, 100 mM NaCl, 200 μM PMSF, 1000 U/ml aprotinin, pH 7.6) and frozen at −80°C until use.

2.2. *Assay of phospholipase A_2 inhibitory activity*

2.2.1. Using *E. coli* as substrate and porcine pancreatic phospholipase A_2

The phospholipase A_2 inhibitory assay was performed as described in [26] and in the accompanying paper.

2.2.2. Using a zwitterionic or a negatively charged substrate and PLA_2 from *Naja naja* according to Jordan, L. (personal communication)

The fluorescent zwitterionic 1-palmitoyl-2-(10-pyrenyldecanoyl) *sn*-glycerol-3-phosphorylcholine substrate and the negatively 1-palmitoyl-2-(10-pyrenyldecanoyl) *sn*-glycerol monomethyl phosphatidic acid substrate (KSV Company, Helsinki, Finland) were used at 2 μM. The assay was performed at room temperature by adding substrate to a buffer solution containing 50 mM Tris-HCl, 100 mM NaCl and 1 mM EDTA, pH 7.5. Then 0.1% bovine serum albumin (BSA) from Sigma was added and after a 2 min equilibration period, 0.8 ng of PLA_2 was added followed by the fractions to be tested and the reaction initiated by the addition of 5 mM $CaCl_2$ in a total volume of 1 ml. The excitation wavelength was set at 345 nm and the emission wavelength at 398 nm. The slit width on all lamps was set at 4 nm.

2.3. *Preparation of lipocortin by Ca^{2+} precipitation*

Cells in buffer A were thawed and homogenized in a teflon-glass homogenizer and sonicated 3 × 10 s at 4°C. The homogenate was then centrifuged at 20000 × *g* for 30 min (Sorvall RC-5B centrifuge, Du Pont Instruments). 6 mM of $CaCl_2$ (1 mM excess) was added to the supernatant and left for 30 min on ice. After centrifugation at 39000 × *g* for 30 min, the pellet was washed with a buffer containing 10 mM Hepes, 150 mM NaCl, 1 mM $CaCl_2$, pH 7.4, and centrifuged at 39000 × *g* for 30 min. The pellet was recovered and resuspended in 10 ml of a buffer containing 10 mM Hepes, 10 mM EGTA, pH 7.60, 1000 U/ml aprotinin and 200 μM PMSF. After centrifugation at 100000 × *g* for 30 min (TGA-65 ultracentrifuge, Kontron), the supernatant was used for further purification.

2.4. *Purification of phospholipase A_2 inhibitory proteins*

The purification by fast protein liquid chromatography (FPLC, Pharmacia, Uppsala, Sweden) was carried out at room temperature. The volume of the calcium precipitate was adjusted to 50 ml with buffer B containing 20 mM ethanolamine, 200 μM PMSF, pH 9.00, and injected onto a column of a strong anion-exchange resin Mono Q HR 5/5 (FPLC, Pharmacia), at a flow rate of 1 ml/min. After elution of unabsorbed material, a linear gradient of NaCl was generated from 0 to 0.35 M over 30 min and 0.35 to 1 M NaCl over 15 min. Protein fractions were collected according to the peaks detected at 280 nm and tested for their ability to inhibit PLA_2 activity as described.

2.5. *SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF)*

Gradient-SDS (10–15% acrylamide) gels were run using the PHAST-SYSTEM from Pharmacia and proteins visualized by silver staining. IEF was performed under native conditions (pH gradients 3–9) using the PHAST-SYSTEM according to the indications provided by the manufacturer.

2.6. *Monoclonal antibody recognition*

The chromatographic fractions were analysed by enzyme-linked immunosorbent assay (ELISA) using the monoclonal antibody (BF26) raised against lipocortin from rat renomedullary interstitial cells [27]. For immunoprecipitation, the monoclonal antibody (at different concentrations) was incubated with the protein for 16 h at 4°C. The fractions were incubated with 50 μl of CNBr-Sepharose coupled protein A (120 mg/ml) overnight at 4°C. The immune complex was then pelleted (5 min at 10000 × *g*) and the resulting supernatant was tested for anti-PLA_2 activity. In parallel, control experiments were done with a non-specific immunoglobulin (M460) of the same class and in the same concentrations. The antibody was also used in Western blot analysis by the method of Towbin et al. [28] using [^{125}I]protein A for the immunological detection.

2.7. *Inhibition of celllular PLA_2 using [^{3}H]arachidonic acid labeled mouse thymocytes*

Mouse thymocytes were labeled for 30 min at 37°C with 5 μCi of [^{3}H]arachidonic acid (128 Ci/mM, Amersham) and washed twice with PBS containing 0.5% of BSA fatty acid free (BSA-FAF). The labeled cells (25000–30000 dpm/0.4 ml) adjusted to 4–5 × 10^6 cells/ml with MEM-Hepes, were preincubated with increasing concentrations of lipocortin for 20 min. The reaction was started by the addition of 50 μl of the calcium ionophore A23187 at known concentrations and terminated 10 min later by the addition of 500 μl of 10 mM cold EGTA, 0.5% BSA-FAF. The samples were centrifuged for 5 min and 500 μl of the supernatant containing released arachidonic acid was counted by liquid scintillation spectrometry. The results are expressed in percent inhibition of the stimulation obtained in the presence or absence of lipocortin.

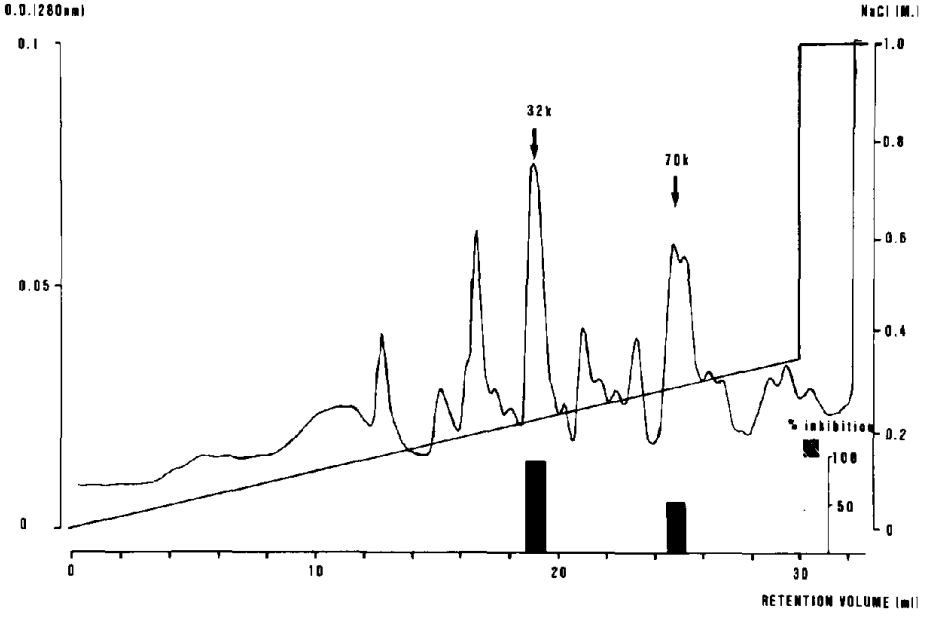

Fig.1. Chromatographic profile of the purified 32-kDa protein on Mono Q. The sample was applied to the column and the proteins eluting with a gradient of NaCl were detected by absorbance at 280 nm. Fractions were collected and tested for their ability to inhibit phospholipase A_2 activity. Hatched columns: % inhibition of PLA_2 activity, OD, NaCl gradient: 0–1 M.

2.8. *Miscellaneous*

Protein concentration was measured according to Lowry et al. [29], and the amino acid composition determined according to Lederer et al. [30].

3. RESULTS

3.1. *Purification of a 32-kDa protein from human mononuclear cells*

In the chromatographic conditions used, a PLA_2 inhibitory fraction eluted as a single peak at 0.23 M NaCl. Another inhibitory fraction eluted at 0.3 M NaCl (fig.1). The protein eluting at 0.23 M NaCl has an apparent molecular mass of 32 kDa and the other species of 73 kDa (fig.2a).

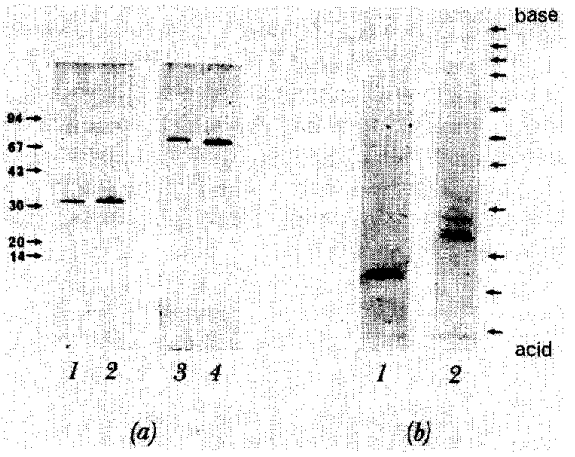

Fig.2. SDS-PAGE and IEF of the eluted proteins. Samples from Mono Q were analyzed by SDS-PAGE (10–15% gradient gels) and IEF (pH gradient 3–9) and visualized by silver staining. (a) SDS-PAGE: Arrows on the left indicate the molecular mass markers, kDa: phosphorylase *b*, 94; bovine serum albumin, 67; ovalbumin, 43; carbonic anhydrase, 30; trypsin inhibitor, 20.1; α-lactalbumin, 14.4. Lanes 1–2 represent the analysis of two different preparations of the 32-kDa protein and lanes 3–4, the 73-kDa protein. (b) IEF: The arrows on the right represent the p*I* markers (from basic to acid): trypsinogen, 9.3; lentil lectin-basic band, 8.65; lentil lectin-middle band, 8.45; lentil lectin-acidic band, 8.15; horse myoglobin-basic band, 7.35; horse myoglobin-acidic band, 6.85; human carbonic anhydrase B, 6.55; bovine carbonic anhydrase B, 5.85; β-lactoglobulin A, 5.20; soybean trypsin inhibitor, 4.55; amyloglucosidase, 3.50; Lane 1, p*I* of the 32-kDa protein; lane 2, p*I* values of the 73-kDa protein.

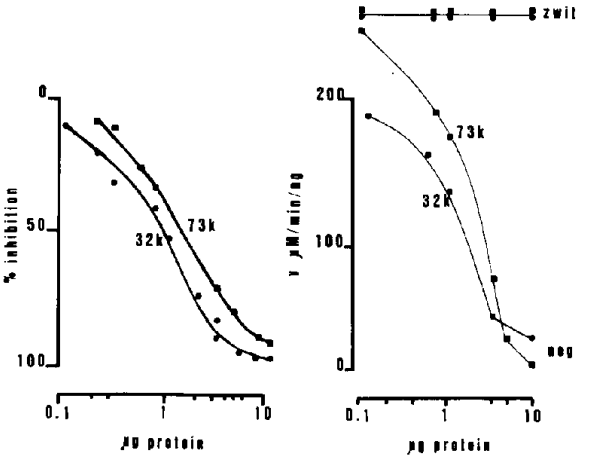

Fig.3. Dose-response curve of lipocortin on PLA_2 activity. (Left) Different concentrations of the purified inhibitors (32-kDa and 73-kDa) are incubated with 0.1 μg of porcine pancreatic phospholipase A_2 using *E. coli* membranes as substrate. Results are expressed in % inhibition of PLA_2 activity. (Right) Different concentrations of the proteins were tested on *Naja naja* PLA_2 using a negatively charged (neg) and a zwitterionic (zwit) substrate. The rate of substrate hydrolysis ($\mu M \cdot min^{-1} \cdot ng^{-1}$) is expressed vs lipocortin concentration (μg).

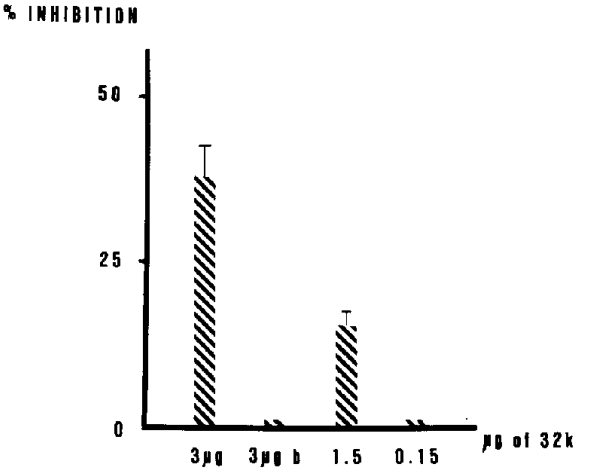

Fig.4. Inhibitory effect of the 32-kDa protein on [^{3}H]arachidonic acid release from prelabeled mouse thymocytes. Results are expressed in % inhibition of arachidonic acid release vs different concentrations of the 32-kDa protein (μg) and 3 μg of boiled lipocortin (3 μg b). Each point is the mean of triplicate determinations ± SD.

Using IEF, the 32-kDa protein has a p*I* of 4.80, whereas the 73-kDa protein shows three different closely migrating isoelectric forms, of 5.2, 5.3 and 5.5 (fig.2b).

3.2. *PLA_2 inhibitory properties*

1 μg of the pure 32-kDa protein gave a 50% inhibition when tested with 0.1 μg of porcine phospholipase A_2 on the *E. coli* substrate. The 73-kDa protein was slightly less effective in inhibiting PLA_2. A typical dose response curve is presented in fig.3 left. After boiling, the proteins were shown not to be any more able to inhibit the enzymatic activity.

Both proteins were inhibiting *Naja naja* phospholipase A_2 when a negatively charged substrate was used, whereas there was no inhibition when the substrate was zwitterionic. A typical dose response curve is presented in fig.3 right.

The pure protein was also tested in another assay in which the cellular phospholipase A_2 activity was monitored. The results in fig.4 show that the 32-kDa protein was able to block [^{3}H]arachidonic acid release from labeled mouse thymocytes in a dose dependent manner and that this activity was destroyed by boiling.

3.3. *Recognition by a monoclonal antibody against lipocortin*

Immunoreactive proteins were detected using the antibody in ELISA and by Western blotting (fig.5). The monoclonal antibody recognizes the 32-kDa protein and another species of 36 kDa from the calcium precipitated fraction. Lane 2 confirms that the pure 32-kDa inhibitory protein eluted from the column is effectively the one recognized by the antibody. In addition, the monoclonal antibody is able, in a dose dependent manner, to reverse the phospholipase A_2 inhibitory activity of the protein using *E. coli* substrate (fig.6). A control antibody was ineffective in the same conditions (not shown).

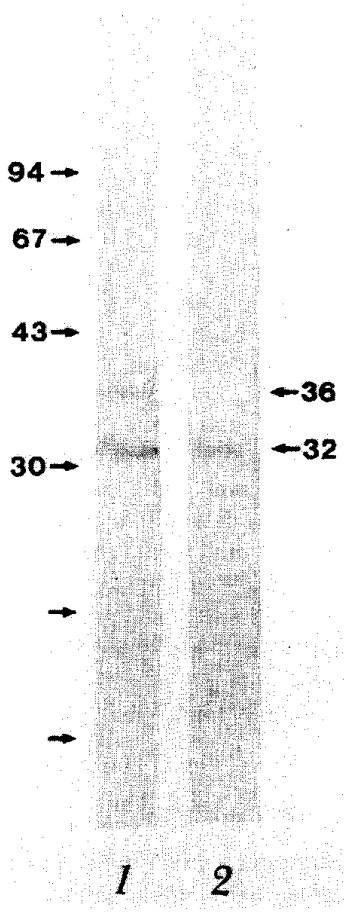

Fig.5. Western blot analysis of the proteins using the monoclonal antibody (BF26) raised against rat lipocortin. Lane 1: protein fraction of the calcium precipitate. Lane 2: Mono Q fraction of the 32-kDa inhibitory protein eluted at 0.23 M NaCl. The arrows on the left represent the migration of the molecular mass markers; on the right, the relative molecular masses of the immunoreactive proteins.

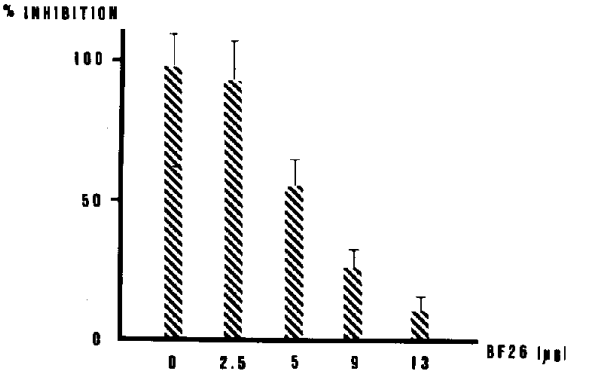

Fig.6. Effect of increasing concentrations of the monoclonal antibody (BF26) on the inhibitory activity (%) of the protein using *E. coli* membranes as substrate and porcine pancreatic PLA_2. Each point is the mean of triplicate determinations ± SD.

Table 1

Amino acid composition of the 32-kDa protein

Amino acid	mol%	± SD	*n*
Asx	9.9	1.0	(3)
Thr	6.0	0.3	(3)
Ser	8.3	1.6	(3)
Glx	13.8	0.9	(3)
Pro	2.2	0.2	(2)
Gly	9.3	1.2	(3)
Ala	8.2	0.5	(3)
Val	4.0	0.2	(3)
Met	1.2	0.6	(2)
Ile	4.5	0.1	(3)
Leu	10.7	0.9	(3)
Tyr	3.8	0.6	(3)
Phe	4.0	0.4	(3)
His	1.5	0.4	(3)
Lys	7.6	1.1	(3)
Arg	5.7	0.7	(3)
Cys	N.D.		

Tryptophan and cysteine were not determined (N.D.). Values are expressed as mol% ± SD. *n*, the number of preparations

3.4. *Amino acid composition*

Table 1 shows the amino acid analysis of the 32-kDa protein.

4. DISCUSSION

In this paper we describe the purification of two proteins of 32 and 73 kDa from human mononuclear cells based on their capacity to bind calcium. The identification of the proteins was followed by their ability to inhibit porcine pancreatic and *Naja naja* phospholipases A_2 using *E. coli* membranes and negatively charged or zwitterionic substrates, respectively. In these conditions, both proteins were inhibiting the two enzymes in a dose dependent manner but only when negatively charged phospholipids were used as substrates. In the accompanying paper [31], we show that this inhibitory effect can be reversed by increasing the amount of substrate. These results as those of Davidson et al. [32] suggest that the phospholipase A_2 inhibition could not be due to a direct enzyme-inhibitor interaction. In addition we have found an inhibitory effect of the 32-kDa protein on cellular phospholipase A_2, indicating either that the enzyme was accessible from the outside of the cell or that the protein interacted in some way with the membrane phospholipids of the cell surface leading to an inhibition of the cellular enzyme. Nevertheless we do not know how the inhibitor behaves when incubated with the whole cells. In the accompanying paper [31], we have shown that the 32 kDa protein was indeed able to inhibit intracellular phospholipase A_2 and this point is discussed in the other paper. Biochemical studies (molecular mass, p*I* and amino acid composition; table 1) show that the 32-kDa protein shares common properties with endonexin [33], but further experiments are necessary to prove they are identical. Nevertheless we could demonstrate that this protein was recognized by a monoclonal antibody raised against lipocortin indicating that this 32-kDa protein belongs to the lipocortin family. In the Western blot experiments, the antibody also recognized a 36-kDa protein, but did not reveal the 73-kDa PLA_2 inhibitory protein. Further experiments are necessary to try to correlate them with other lipocortin species.

In summary, we report that in human peripheral mononuclear cells, there exist proteins of the lipocortin family, able to bind calcium, and to inhibit phospholipase A_2. These cells are known to be targets for glucocorticosteroid action, as for the inhibition of prostaglandin secretion involving a phospholipase A_2 inhibition (unpublished results). The 32-kDa lipocortin-like protein could be a candidate for the glucocorticosteroid action in these cells. Presently, our data do not permit us to conclude that the 32-kDa protein is the right candidate, further experiments are required to test this hypothesis.

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