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Presence of lipocortins I and IV, but not II and VI, in human platelets

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The present investigation revealed the presence of lipocortins I and IV, but not lipocortins II and VI, in human platelets. Lipocortin I was found in the Triton-soluble fraction of both resting and thrombin-activated platelets and was not covalently bound to skeletal components. Without detergents, when resting platelets were lysed and fractionated in the absence of Ca^{2+}, lipocortin I was found only in the cytosolic fraction, whereas, in the presence of Ca^{2+}, lipocortin I was associated only with the crude particulate and not with the membrane nor the cytosolic fractions.

Lipocortin; Annexin; Platelet

1. INTRODUCTION

Lipocortins (annexins) are widely distributed calcium-dependent phospholipid- and actin-binding proteins exhibiting both phospholipase- and coagulation-inhibitory activity. They have been suggested to play important roles in the regulation of membrane- and cytoskeleton-associated events [1,2]. In platelets, upon thrombin activation, a series of such events take place including Ca^{2+} mobilization, protein phosphorylation, phospholipase activation and exocytosis [3]. Because exogenously added LCs have been found to bind to activated-platelet phospholipid membranes [4,5], to inhibit platelet phospholipases [6,7] and to be tyrosine-phosphorylated [8] all in a Ca^{2+}-dependent manner, it has been proposed that LCs might play a key role in platelet physiology. However, their presence in platelets has not been established, except for a recent report briefly describing LC V in platelets [9].

The present study is the first to identify the presence of LCs I and IV, and the absence of detectable amounts of LCs II and VI, in human platelets. In addition, the subcellular distribution of LC I with respect to Ca^{2+} was further characterized.

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Abbreviations: LC, lipocortin; rLC, recombinant lipocortin; mAB, monoclonal antibody; PMSF, phenylmethylsulfonyl fluoride; NEM, *N*-ethylmaleimide; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; IEF, isoelectric focusing; Tris, trishydroxymethylaminomethane.

2. MATERIALS AND METHODS

The purified human rLC I and the antiLC I mAB, 6G6, were prepared as previously described [10]. The placental LC II and the mABs specific for LCs I, II, IV and VI were obtained from Zymed Laboratory Inc. (CA, USA). Prestained SDS-PAGE protein standards were purchased from Bio-Rad Inc. (CA, USA).

2.1. *Platelet isolation*

Human platelets were isolated from citrate-treated blood as previously described [11] with some modifications. Briefly, buffy coats were obtained from the Central Laboratory of the Swiss Red Cross Transfusion Service, approximately 15 h after blood collection, and diluted with citrate buffer (30 mM sodium citrate, 100 mM NaCl, 3 mM KCl, 9.6 mM glucose, 2 mM EDTA, pH 6.5) then centrifuged to yield platelet-rich plasma. The platelets were washed twice in 60 mM sodium citrate, 30 mM glucose, 120 mM NaCl, 10 mM EDTA, 0.5 ng/ml Iloprost (Schering Inc.), pH 6.5, and once in incubation buffer (10 mM Tris, 154 mM NaCl, 5 mM EDTA, pH 6.5). EDTA was replaced by EGTA in the case of experiments which examined specifically the effects of Ca^{2+}. In addition, when Ca^{2+} was to be included, the platelet pellet was resuspended in the incubation buffer without any chelator followed by the addition of Ca^{2+} (1 mM) and incubated for 30 min at 37°C in order to replenish intracellular stores of Ca^{2+}. When a lower concentration of Ca^{2+} was to be used in subsequent experiments, the Ca^{2+}-replenished platelets were washed twice in incubation buffer, in the absence of any chelator, followed by the addition of Ca^{2+} (10 μM). Prior to all experiments, aliquots of platelets (3×10^9/ml) were equilibrated for at least 30 min at 37°C.

2.2. *Separation of Triton-soluble and -insoluble platelet components*

Aliquots of resting platelets containing EDTA (5 mM) were incubated without or with bovine thrombin (1 U/ml, Merck Inc.) for 3 min at 37°C, then immediately solubilized in 1% Triton X-100 containing NEM (2 mM) and PMSF (2 mM). The Triton-insoluble components were separated into two fractions [12], the cytoskeleton ($15{,}600 \times g$, 4 min, 4°C) and the membrane-associated cytoskeleton ($150{,}000 \times g$, 15 min, 4°C) and then solubilized in 1% SDS.

2.3. *Lysis and separation of platelet components in the absence of detergent*

Resting platelets, containing either EGTA (5 mM) or Ca^{2+} (10 μM

or 1 mM), underwent four rapid freeze/thaw cycles in the presence of PMSF (2 mM) and calpain inhibitors I and II (100 μg/ml; Boehringer Mannheim). The resulting lysates were then separated into three fractions [13]; the crude particulate containing cytoskeleton (13,100 × *g*, 20 min, 4°C), membranes (100,000 × *g*, 1 h, 4°C) and the cytosol. The pellets were solubilized by sonication in 1% SDS.

2.4. *Western blot analysis*

Platelet proteins were reduced (100 mM DTT) and denatured (100°C, 5 min) before being resolved by 12.5% SDS-PAGE or 2-dimensional IEF/SDS-PAGE [14]. Platelet proteins were electrophoretically transferred to PVDF Immobilon membrane (Millipore Inc.). The LC proteins were detected by treating the blots with the respective mABs, followed by treatment with peroxidase-conjugated anti-mouse IgG (Bio-Rad Inc.), and then visualized on X-ray film (Fuji Inc.) using an enhanced chemiluminescence detection system (Amersham Inc.).

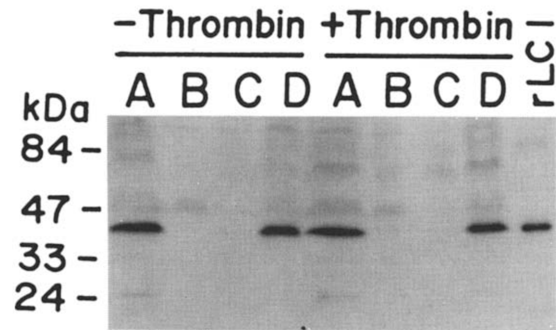

Fig. 2. LC I is present in the Triton-soluble fraction of resting and thrombin-stimulated (1 U/ml) platelets. Western blot using antiLC I mAB, 6G6. Each lane contains the equivalent of 5×10^7 platelets: whole platelets solubilized in SDS (lanes A); Triton-insoluble cytoskeleton (lanes B); Triton-insoluble membrane skeleton (lanes C); Triton-soluble components (lanes D). rLC I (5 ng).

3. RESULTS AND DISCUSSION

In order to determine the presence or absence of LCs I, II, IV and VI in human platelets, Western blots of platelet proteins were prepared and each individual lane was treated with a distinct mAB specific for a single LC. Fig. 1 demonstrates the presence of LC I (lane A) at 36–38 kDa and LC IV (lane D) at 33–35 kDa, whereas LC II (lanes B, heavy chain, and C, light chain) and LC VI (lane E) appear to be absent from human platelets. rLC I was recognized only by anti-LC I mAB (Fig. 1) and not by the other antiLC mABs (data not shown).

Glenney [15] previously detected two 38 kDa proteins, LC I and LC II, in human carcinoma cells. These proteins share similar structural and functional features making their separate analysis difficult. Because the 38 kDa LC proteins contain conserved domains and might exhibit crossreactivity with select ABs, the specificity of

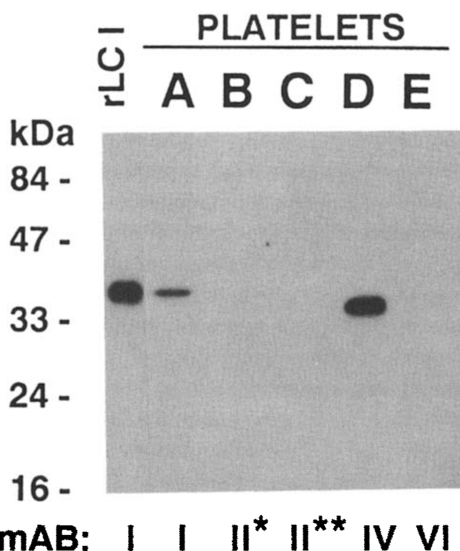

Fig. 1. LC proteins identified in human platelets (lanes A–E; 45 μg protein/lane) and rLC I (5 ng) detected by Western blot using mABs: antiLC I (rLC I and lane A); antiLC II* heavy chain (lane B); antiLC II** light chain (lane C); antiLC IV (lane D); antiLC VI (lane E).

the antiLC I mAB, 6G6, used in the subsequent experiments, had to be established. Therefore, rLC I and placental LC II protein preparations were subjected to Western blot analysis. The mAB, 6G6, recognized a 38 kDa protein corresponding to LC I but not LC II, whereas an antiLC II mAB detected only LC II but not LC I (data not shown). A 68 kDa band was recognized by 6G6 in the placental preparation of LC II and may represent a crosslinked dimeric form of LC I known to exist in placenta [16,17]. Furthermore, when platelet proteins were subjected to 2-dimensional IEF/SDS-PAGE followed by Western blot analysis, only a single protein was recognized by the mAB, 6G6, at the appropriate pI (6.5) for LC I (data not shown), confirming the presence of LC I but not LC II in human platelets.

LC I is known to associate with both actin filaments in vitro [15] and in intact human carcinoma cells, with the inner plasma membrane [16]. To examine the subcellular distribution of LC I in platelets, resting and thrombin-stimulated platelets were solubilized in 1% Triton X-100 then separated into three fractions. The results in Fig. 2 show that all of LC I, in whole unfractionated platelets (lane A), is entirely in the Triton-soluble fraction (lane D) and is not covalently bound to the cytoskeletal (lane B) nor membrane-associated cytoskeletal (lane C) components. LC I remained in the Triton-soluble fraction and did not shift to the cytoskeletal fractions even after thrombin-stimulation (Fig. 2) indicating that LC I is not covalently bound to skeletal components upon platelet activation.

Since Ca^{2+} is a second messenger for platelet activation [18] and can also influence the binding affinity of LC I for many cellular components [15,16], the impact of Ca^{2+} on the subcellular distribution of LC I in platelets was investigated in the absence of detergents. Fig. 3 shows the distribution of LC I in whole platelets (lanes A) which have been lysed and separated into three subcellular fractions (crude particulate, lanes B; crude

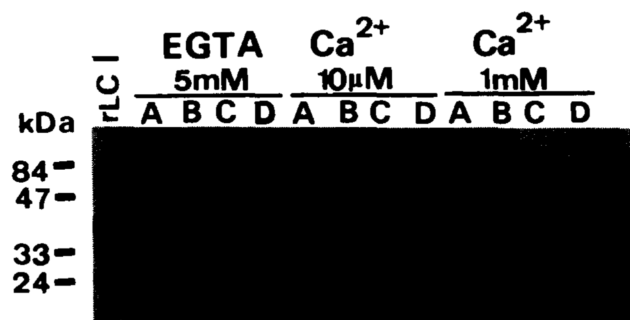

Fig. 3. Platelet subcellular localization of LC I changes with the absence (EGTA 5 mM) or presence of Ca^{2+} (10 μM or 1 mM). Western blot using antiLC I mAB, 6G6. Each lane contains the equivalent of 3×10^7 whole (lanes A) or fractionated platelets: crude particulate (lanes B); crude membrane (lanes C); cytosol (lanes D). rLC I (5 ng).

membrane, lanes C; and cytosol, lanes D) in the absence (EGTA 5 mM) or presence of 10 μM or 1 mM Ca^{2+}. The results in Fig. 3 demonstrate that Ca^{2+}, even within the physiological μM range sufficient to stimulate secretory exocytosis [18], causes a shift in the subcellular distribution of LC I from the cytosol (lanes D) to the crude particulate (lanes B) in human platelets. The crude particulate contains cytoskeletal filaments and granules to which LCs have been reported to associate with in a Ca^{2+}-dependent manner in sheep anterior pituitary cells [19], human neutrophils [20] and cell-free systems [15]. The results of the present study suggest that LC I, potentially regulated by Ca^{2+}, may have a functional role during platelet activation.

Interestingly, when platelets were lysed in the presence of Ca^{2+} (1 mM), LC I was completely degraded and this degradation was blocked specifically by calpain inhibitors I and II, and not by NEM nor PMSF (data not shown). Calpain is a Ca^{2+}-dependent protease, abundant in platelets, without an established function [21]. It is also worthy of mention that LC I was inconsistently found in the crude particulate fraction, in addition to the cytosolic fraction, in the absence of Ca^{2+} (EDTA 5 mM), only when platelets were lysed by sonication (data not shown) rather than the freeze/thaw method used in the present study.

Table I presents a summary of the LC proteins with their possible biological functions and their presence in platelets. Previously, a 40 kDa protein in platelets has been claimed to be immunologically similar to LC [22] and there are proteins in this mass range that are tyrosine-phosphorylated upon platelet activation [23,24]. Because both LCs I and II fulfill these criteria [8] either protein may be the proposed LC in platelets. The results in the present study indicate that this aforementioned 40 kDa protein in platelets is LC I and not LC II. Further investigations are necessary to establish the biological relevance of LCs during platelet activation.

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Table I

Characteristics and functions of lipocortins

Lipocortin	M.W.	Isoelectric point	Presence in platelets	Phosphorylated	Possible biological activities: Inhibition of PLA_2	Inhibition of PLC	Anti-coagulant	Others
I	38 kDa [32,10]	6.5 pI (see results, 8)	Yes (Fig. 1)	Yes [34,8]	Yes [6,32]	Yes [7]	Yes [30]	Endogenous substrate for tyrosine kinase [34,8]
II*	38 kDa [33]	7.9 pI [34]	No (Fig. 1)	Yes [34]	Yes [25]	Yes [7]	Yes [30]	Substrate for tyrosine kinase [34]
III	35 kDa [29,30]	5.9 pI [30]	N.D.	N.D.	Yes [29]	N.D.	Yes [30]	–
IV	35 kDa [28]	6.0 pI [28]	Yes (Fig. 1)	N.D.	Yes [28]	N.D.	Yes [28]	–
V	35 kDa [26,28,29]	4.8 pI [28]	Yes [14]	N.D.	Yes [28,29]	N.D.	Yes [26,28]	–
VI	68 kDa [29,30]	6.1 pI [30]	No (Fig. 1)	Yes [27]	Yes [29]	N.D.	Yes [30]	Modifier of Ca^{2+} channels [31]

* Heavy chain only; N.D., not determined.

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