Biochem. J. (1990) 269, 709–715 (Printed in Great Britain)

Monoclonal antibodies specific to a Ca^{2+}-bound form of lipocortin I distinguish its Ca^{2+}-dependent phospholipid-binding ability from its ability to inhibit phospholipase A_2

Hideki HAYASHI,*‖ M. Koji OWADA,†¶ Seiji SONOBE,*†† Kiyoko DOMAE,‡ Takahisa YAMANOUCHI,‡ Takeo KAKUNAGA,*‡‡ Yasuo KITAJIMA§ and Hideo YAOITA§
Departments of *Oncogene Research, †Tumor Virology, and ‡Science for Laboratory Animal Experimentation Research, Institute for Microbial Diseases, Osaka University, Suita, Osaka 565, and §Department of Dermatology, Jichi Medical School, Tochigi-ken 329–40, Japan

Lipocortin I, a Ca^{2+}- and phospholipid-binding protein without EF-hand structures, has many biological effects *in vitro*. Its actual role *in vivo*, however is unknown. We obtained and characterized five monoclonal antibodies to lipocortin I. Two of these monoclonal antibodies (L2 and L4-MAbs) reacted with the Ca^{2+}-bound form of lipocortin I, but not with the Ca^{2+}-free form, both *in vivo* and *in vitro*. Lipocortin I required $\geqslant 10$ μM-Ca^{2+} to bind the two antibodies, and this Ca^{2+} requirement was not affected by phosphatidylserine. L2-MAb abolished the phospholipase A_2 inhibitory activity of lipocortin I and inhibited its binding to *Escherichia coli* membranes and to phosphatidylserine *in vitro*. L4-MAb abolished the phospholipase A_2 inhibitory activity of lipocortin I, but did not affect its binding to *E. coli* membranes or to phosphatidylserine. These findings indicated that the inhibition of phospholipase A_2 by lipocortin I was not simply due to removal or capping of the substrates in *E. coli* membranes. Furthermore, an immunofluorescence study using L2-MAb showed the actual existence of a Ca^{2+}-bound form of lipocortin I *in vivo*.

INTRODUCTION

A family of a Ca^{2+}- and membrane-binding proteins, known as lipocortins or calpactins, are thought to be important in signal transduction, inflammation and exocytosis in cells (for reviews, see [1] and [2]). Lipocortin was first demonstrated as a phospholipase A_2 inhibitor [3,4]. Lipocortin I (p35) was found to be phosphorylated at tyrosine residues by A-431 cell membranes [5,6]. When a cDNA encoding lipocortin I was cloned, lipocortin I was found to be identical with calpactin II, a protein which binds Ca^{2+}, phospholipid and F-actin [7,8]. Lipocortin I also causes aggregation of chromaffin granules in a Ca^{2+}-dependent manner and may be involved in exocytosis [9–12]. These biological properties seem to be regulated mainly by Ca^{2+}, although lipocortin I requires rather a high concentration of Ca^{2+} for its actions [13,14]. The Ca^{2+}-dependency of lipocortin I is lowered by phosphatidylserine and by its phosphorylation with A-431 membranes [14]. However, as phosphatidylserine binds Ca^{2+}, it is difficult to determine whether lipocortin I has a binding site for Ca^{2+} or for Ca^{2+}-bound phosphatidylserine by the equilibrium dialysis method. U.v. spectroscopy is also unsuitable for this purpose. Thus, despite extensive studies, little is known about the functions of lipocortin I *in vivo* [15].

To examine the functions of lipocortin I, we have prepared several monoclonal antibodies (MAbs) that inhibit different functions of lipocortin I. We have named five of these MAbs L1, L2, L3, L4 and L5, and have characterized them. Interestingly, L2- and L4-MAbs reacted with Ca^{2+}-bound lipocortin I, but not with the non-Ca^{2+}-bound form. This indicates that L2- and L4-MAbs recognize conformational changes in lipocortin I that are induced by Ca^{2+}. This characteristic of these MAbs is very useful in the study of the Ca^{2+}-dependent mechanism of action of lipocortin I *in vivo* and *in vitro*.

Using these MAbs, we determined the Ca^{2+} concentration at which the conformation of lipocortin I changes from a Ca^{2+}-free form to a Ca^{2+}-bound form. We found that the Ca^{2+}-dependency of lipocortin I was not affected by phospholipids. We also show that the two MAbs could differentiate the Ca^{2+}-dependent phospholipid-binding ability of lipocortin I from its phospholipase A_2 inhibitory activity. Finally, we demonstrate the actual existence and cellular localization of a Ca^{2+}-bound form of lipocortin I in human skin cells.

EXPERIMENTAL

Purification of lipocortin I, calpactin I and other Ca^{2+}- and phospholipid-binding proteins

Lipocortin I, calpactin I, and other Ca^{2+}- and phospholipid-binding proteins were purified from human placenta as described previously [16].

Preparation of MAbs

We obtained nine stable hybridoma clones against lipocortin I as described previously [16]. To prepare monoclonal antibodies specific to Ca^{2+}-bound lipocortin I, we used 5% non-fat dried milk containing 1 mM-Ca^{2+} as the blocking and dilution buffer in e.l.i.s.a. selection. Five out of nine specific antibody-producing hybridomas were injected into pristane-primed nude mice, and the resulting ascites were collected after 2 weeks. Each antibody was purified from ascites on ion-exchange and hydroxyapatite columns. The purified MAbs were composed mainly of immuno-

Abbreviations used: MAb, monoclonal antibody; PBS, phosphate-buffered saline; FCS, fetal calf serum; EGF, epidermal growth factor.

‖ Present address: Department of Enzyme Genetics, Institute for Enzyme Research, The University of Tokushima, 3-18-15 Kuramoto-cho, Tokushima 770, Japan.
¶ To whom correspondence should be sent, at present address: Department of Molecular Bioregulation, Institute of Molecular and Cellular Biology for Pharmaceutical Sciences, Kyoto Pharmaceutical University, 1 Shichonocho, Misasagi, Yamashina, Kyoto 607, Japan.
†† Present address: Department of Biology, Faculty of Science, Osaka University, 1-1 Machikaneyama, Toyonaka, Osaka 565, Japan.
‡‡ Died 21 September, 1988.

globulin heavy and light chains (results not shown). For simplicity, we named the five MAbs L1, L2, L3, L4 and L5. L1- and L2-MAbs were identified as the G2b subclass and L3- and L4- and L5-MAbs as the G1 subclass using an MAb subtyping kit (Bio-Rad).

Western blotting

Purified Ca^{2+}- and phospholipid-binding proteins (1 μg) were separated by SDS/PAGE and transferred to nitrocellulose paper. The paper was incubated overnight with purified MAbs (1:200) in 5 % non-fat dried milk, containing Ca^{2+}, and then with ^{125}I-labelled Protein A (Amersham) in 5 % non-fat dried milk. It was then washed extensively, and protein bands were located by autoradiography as described previously [16].

Growth and labelling of A-431 cells

A-431 cells were grown in 100 mm dishes containing Dulbecco's modified Eagle's medium (Gibco) supplemented with 10 % fetal calf serum (FCS) and kanamycin (Meiji). Cells were labelled with 1 mCi of [^{32}P]P_i/ml in phosphate-free medium containing dialysed FCS for 4 h in the presence of epidermal growth factor (EGF) (200 ng/ml) at 37 °C. For labelling of lipocortin I with [^{35}S]methionine, cells were grown in 100 μCi of [^{35}S]methionine/ml of normal medium for 4 h in the absence of EGF at 37 °C with minor modifications as previously described [17].

Immunoprecipitation

A-431 cells labelled with either [^{32}P]P_i or [^{35}S]methionine were washed three times with Dulbecco's phosphate-buffered saline (PBS) (137 mM-NaCl, 2.7 mM-KCl, 8.10 mM-Na_2HPO_4, 1.47 mM-KH_2PO_4). Cells were lysed in RIPA buffer (50 mM-Tris, pH 8.2, 150 mM-NaCl, 1 % Triton X-100, 1 % deoxycholate, 0.1 % SDS) containing 5 μg of leupeptin/ml and 50 μg of (*p*-amidinophenyl)-methanesulphonyl fluoride hydrochloride (Wako Pure Chemicals) in the presence of 1 mM-Ca^{2+} or -EGTA, and scraped off the dish [18]. After centrifugation at 15000 ***g*** for 30 min at 4 °C, an appropriate volume of the supernatant was incubated with normal mouse serum (5 μl) or purified MAbs (20 μg of each) for 1 h at 4 °C, and then immune complexes were precipitated with Protein A–Sepharose CL-4B (Pharmacia LKB Biotechnology). The immune complexes were washed extensively with RIPA buffer containing 1 mM-Ca^{2+} or -EGTA, eluted with SDS sample buffer and subjected to SDS/PAGE. Protein bands were located by autoradiography.

In some assays, unlabelled purified lipocortin I (5 μg per assay) was used in place of ^{35}S- or ^{32}P-labelled lysates.

Dot-blot assay

Purified lipocortin I (1 μg) was preincubated for 30 min at room temperature with various concentrations of Ca^{2+} (20 mM-Hepes, pH 7.4, Ca^{2+}/EDTA buffer) [19] in the presence or absence of phospholipids (200 μg/ml), and dotted on to square nitrocellulose paper (3 mm × 3 mm). The dotted paper was air dried and placed in a Falcon microtest 96-well dish. The paper was blocked with 200 μl of Ca^{2+}/EGTA buffer containing 1 % BSA, and then incubated with 200 μl of diluted antibodies (1:2000) in the same Ca^{2+}/EGTA buffer containing 1 % BSA. The location of antibodies was then determined with ^{125}I-labelled Protein A by autoradiography.

Phospholipase A_2 assay

Phospholipase A_2 was assayed as previously described [16] with minor modifications. Briefly, purified lipocortin I or calpactin I (3 μg of each) was preincubated in the buffer containing 1 mM-Ca^{2+} with various amounts of MAbs for 30 min at room temperature, and then with 50 ng of bee venom phospholipase A_2 (Sigma) for 10 min at 4 °C. The reaction was initiated at 30 °C by addition of [^{3}H]oleic-acid-labelled *Escherichia coli* to the sample. The released radioactivity was measured as phospholipase A_2 activity.

Assays of binding to *E. coli* membranes, phospholipid and F-actin

E. coli membranes, phospholipid liposomes and F-actin were prepared as described previously [16]. Lipocortin I (3 μg) was preincubated with MAbs (20 μg) for 30 min at room temperature in binding buffer (10 mM-imidazole/HCl, pH 7.4, 1 mM-$CaCl_2$, 100 mM-KCl, 2 mM-$MgCl_2$ and 0.1 mM-ATP), and then the test material was added to the immune complexes. The mixture was centrifuged at 400000 ***g*** for 10 min in a Beckman TL-100 ultracentrifuge, and unbound lipocortin I and MAbs were recovered in the supernatant. The supernatant was analysed by SDS/PAGE and Coomassie Blue staining. The inhibitory activities of the MAbs were determined by densitometry, and compared with control mixture without MAbs.

Assay of phosphorylation *in vitro*

Lipocortin I (3 μg) was preincubated with MAbs (20 μg) for 30 min at room temperature in the phosphorylation buffer containing 1 mM-Ca^{2+}, and then subjected to phosphorylation with A-431 membranes [16].

Immunofluorescence

For human skin, biopsy specimens were taken from perilesional sites of benign skin tumours and frozen in optimal cutting temperature compound. Sections (4 μm) were fixed with cold (−20 °C) acetone and stained for Mabs.

Explant cultures of human epidermal keratinocytes were used. Several small pieces of human skin were placed on coverslips and fed with Dulbecco's modified Eagle's medium supplemented with 20 % FCS, 0.4 μg of hydrocortisone/ml, 10 ng of EGF/ml, 84 ng of cholera toxin/ml, 100 μg of streptomycin/ml and 100 units of penicillin/ml. Cultures were grown in a humidified atmosphere under 5 % CO_2, and the medium was changed at 2-day intervals. The cells, on the coverslips, were rinsed with PBS at room temperature and placed into methanol at −20 °C for 7 min. The fixed cells were soaked in a solution of 0.5 % Triton X-100, 2 mM-phenylmethane sulphonyl fluoride and 2 mM-*N*-tosyl-L-phenylalanine chloromethyl ketone (1-chloro-4-phenyl-3-L-tosylamidobuta-2-one) for 10 min at room temperature. The specimens were then stained with monoclonal antibodies.

The specimens for immunostaining were incubated with the first antibodies (L1-MAb and L2-MAb) for 45 min at room temperature, followed by several washes in PBS, and then stained with the second antibodies [rabbit anti-(mouse IgG) labelled with fluorescein isothiocyanate] for 45 min at room temperature.

Other procedures

SDS/PAGE was carried out by the method of Laemmli [20]. Gels contained 12.5 % acrylamide unless otherwise noted. Protein concentrations were determined by the dye-binding method [21].

RESULTS

Monoclonal antibodies specific for Ca^{2+}-binding forms of lipocortin I

The effects of Ca^{2+} on the reactivities of monoclonal antibodies (L1–L5) were examined by immunoprecipitation using purified lipocortin I. L1-, L3- and L5-MAbs precipitated lipocortin I irrespective of the Ca^{2+} concentration. However L2- and L4-

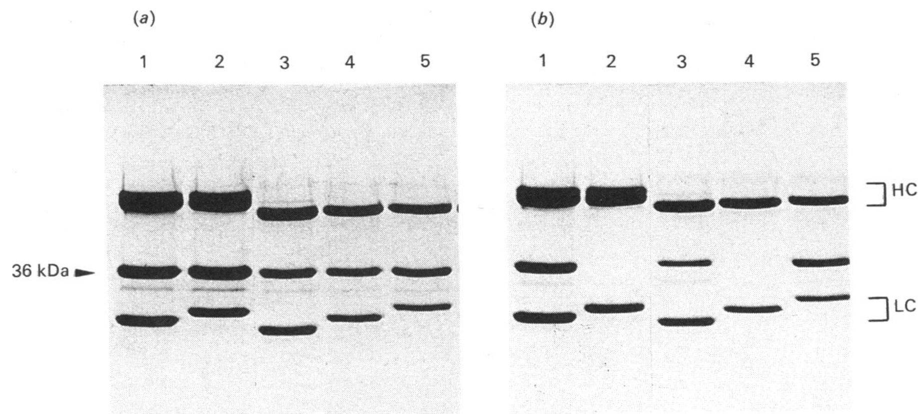

Fig. 1. Immunoprecipitation of purified lipocortin I in the presence (*a*) and absence (*b*) of Ca^{2+}

Lipocortin I (5 μg) was incubated with the MAbs (L1–L5, 20 μg of each) in the presence (*a*) and absence (*b*) of Ca^{2+}, and treated with Protein A–Sepharose CL-4B. After extensive washing and elution, samples were analysed by SDS/PAGE and Coomassie Blue staining as described in the Experimental section. Lane 1, L1; lane 2, L2; lane 3, L3; lane 4, L4; lane 5, L5. The arrowhead indicates the position of 36 kDa lipocortin I. Immunoglobulin heavy chains (HC) and light chains (LC) are shown. The extra band under lipocortin I was a degraded product of lipocortin I.

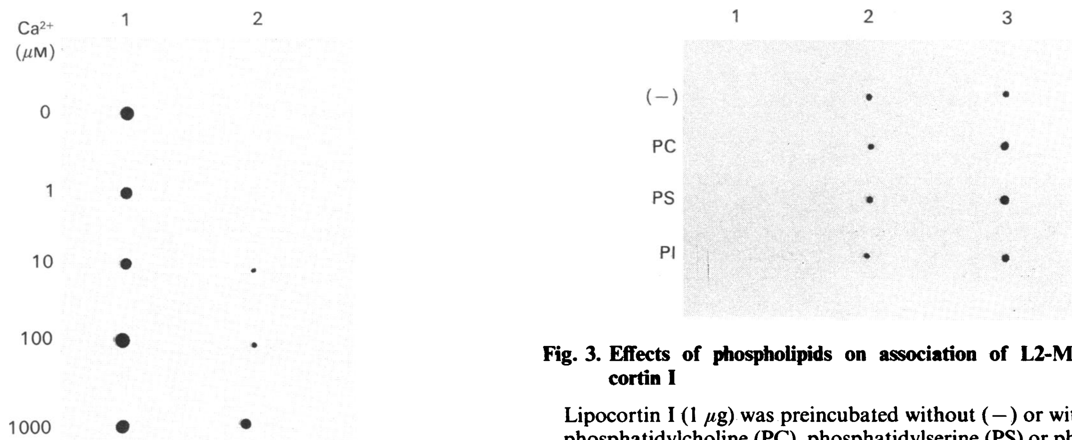

Fig. 2. Effect of Ca^{2+} concentration on the association of the MAbs with lipocortin I

After preincubation in buffer containing various concentrations of Ca^{2+}, lipocortin I (1 μg) was dotted on to nitrocellulose paper. Incubation was carried out with the MAbs (lane 1, L1; lane 2, L2) and then with ^{125}I-Protein A. Dotted proteins were located by autoradiography as described in the Experimental section.

Fig. 3. Effects of phospholipids on association of L2-MAb with lipocortin I

Lipocortin I (1 μg) was preincubated without (−) or with 200 μg of phosphatidylcholine (PC), phosphatidylserine (PS) or phosphatidylinositol (PI)/ml with various concentrations of Ca^{2+} (lane 1, 1 μM; lane 2, 10 μM; lane 3, 100 μM) for 30 min at room temperature, and then dotted on to nitrocellulose paper. Incubation was carried out with L2-MAb and then with ^{125}I-Protein A. Dotted proteins were located by autoradiography as described in the Experimental section.

MAbs precipitated lipocortin I only in the presence of Ca^{2+} (Fig. 1). This indicates that L2- and L4-MAbs recognize a Ca^{2+}-induced conformational change in lipocortin I. We investigated the cross-reactivities among six Ca^{2+}- and phospholipid-binding proteins purified from human placenta by Western blotting analysis using these MAbs. None of the MAbs cross-reacted with 32K-I (PP4-X), 32K-II (PP4 or endonexin II), 36 kDa calpactin I, or 68K-II (p68/67 kDa calelectrin) [16, 22–26].

We determined the concentration of Ca^{2+} causing this conformational change in lipocortin I by dot-blot assay with these MAbs. L2-MAb reacted with lipocortin I at Ca^{2+} concentration of $\geqslant 10$ μM, whereas L1-MAb reacted with lipocortin I at any concentration of Ca^{2+} (Fig. 2). The results of dot-blot assay in Fig. 3 show that none of the phospholipids tested affected the Ca^{2+}-dependent association of lipocortin I with L2-MAb.

The five MAbs reacted with ^{35}S- and ^{32}P-labelled lipocortin I from A-431 cells in the same way: L1-MAb precipitated both ^{35}S- and ^{32}P-labelled lipocortin I at any concentration of Ca^{2+}, whereas L2-MAb precipitated both ^{35}S- and ^{32}P-labelled lipocortin I in the presence of Ca^{2+}, but not in its absence (Fig. 4).

Effects of the MAbs on biological effects of lipocortin I *in vitro*

We examined the Ca^{2+}-mediated effects of lipocortin I using the MAbs *in vitro*. Lipocortin I (3 μg) inhibited the activity of bee venom phospholipase A_2 (50 ng) by about 50%, and the activity was restored completely by preincubation with 10–20 μg of any of the MAbs. The inhibition of phospholipase A_2 by calpactin I (3 μg) was not affected by up to 20 μg of any of the MAbs (Fig. 5 and Table 1).

On the other hand, the binding of lipocortin I to *E. coli* membranes, phosphatidylserine liposomes and F-actin was partially inhibited by certain MAbs (Fig. 6 and Table 1). L1- and L3-MAbs, which react with both Ca^{2+}-bound and Ca^{2+}-free forms of lipocortin I, did not inhibit binding of lipocortin to *E. coli* membranes, phosphatidylserine or F-actin. L2-MAb, which reacts specifically with a Ca^{2+}-bound form of lipocortin I, partially inhibited all three Ca^{2+}-mediated bindings of lipocortin I ($\geqslant 20$%, determined by densitometry). L4-MAb, which also

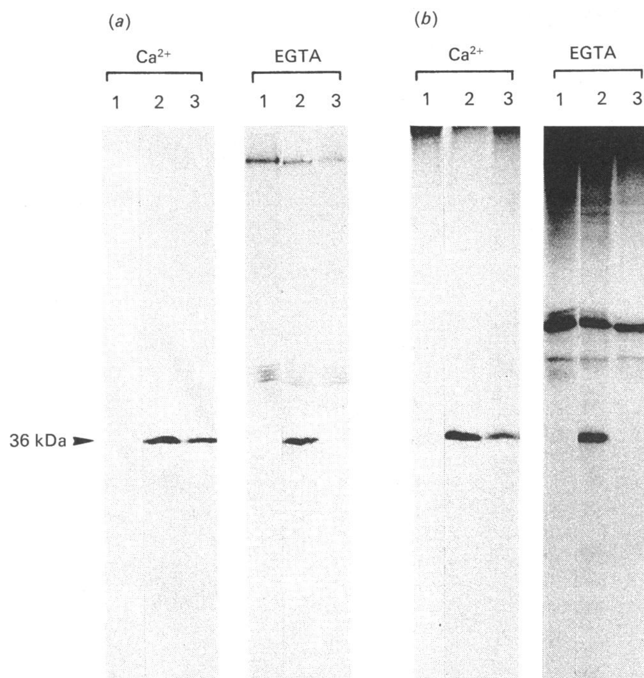

Fig. 4. Immunoprecipitation of lipocortin I from A-431 cells labelled with [^{35}S]methionine (*a*) or [^{32}P]P$_i$ (*b*) in the presence or absence of Ca^{2+}

A-431 cells were labelled with [^{35}S]methionine (*a*) or [^{32}P]Pi (*b*) after preincubation with EGF. Cells were lysed in RIPA buffer in the presence or absence of Ca^{2+}, incubated with normal mouse serum (lane 1) or the MAbs (lane 2, L1; lane 3, L2), and precipitated with Protein A–Sepharose CL-4B. After extensive washing and elution, samples were subjected to SDS/PAGE. Protein bands were located by autoradiography as described in the Experimental section. The arrowhead indicates the position of 36 kDa lipocortin I.

reacts with a Ca^{2+}-bound form of lipocortin I, but not the Ca^{2+}-free form, did not inhibit the binding of lipocortin I to *E. coli* membranes, phosphatidylserine or F-actin. L5-MAb, which reacts with both Ca^{2+}-bound and Ca^{2+}-free forms, inhibited the binding of lipocortin I to *E. coli* membranes, but had no effect on its binding to phosphatidylserine or F-actin.

The phosphorylation of lipocortin I *in vitro* with A-431 membranes was completely inhibited by L2-MAb, but was not affected by the other four MAbs (Fig. 7 and Table 1).

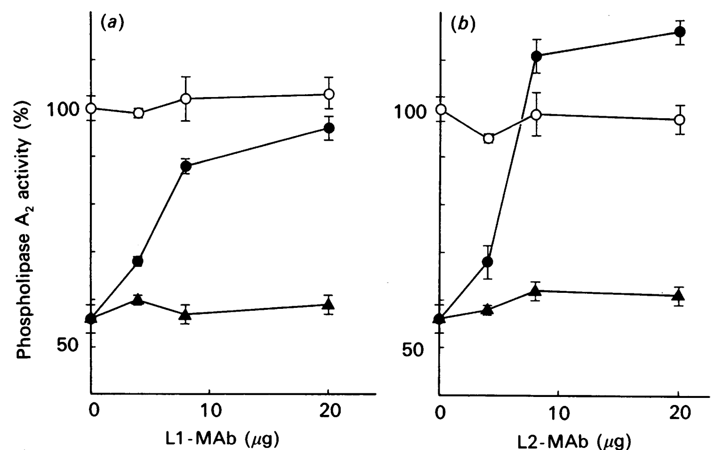

Fig. 5. Effects of MAbs on inhibition of phospholipase A_2 by lipocortin I

Phospholipase A_2 activity was measured with the indicated amounts of the MAbs (*a*, L1; *b*, L2) in the absence (○) or presence of 3 μg of lipocortin I (●) or 3 μg of calpactin I (▲) using bee venom phospholipase A_2 (50 ng/assay) as described in the Experimental section. Values are means ± S.E.M. ($n = 3$).

Table 1. Summary of the effects of monoclonal antibodies on activities of lipocortin I *in vitro*

Phospholipase A_2 inhibitory activities, binding to *E. coli* membranes, phosphatidylserine liposomes and F-actin, and phosphorylation of lipocortin I *in vitro* were assayed as described in the Experimental section. Each experiment was repeated at least three times. For inhibition of phospholipase A_2, + indicates a complete block by MAbs of inhibition of phospholipase A_2 by lipocortin I (see Fig. 5). For binding experiments, + indicates partial inhibition (⩾ 20%) and − indicates no inhibition by MAbs of the binding of lipocortin I (see Fig. 6). For phosphorylation experiments. + indicates complete inhibition and − indicates no inhibition by MAbs of the phosphorylation of lipocortin I (see Fig. 7).

Activity of lipocortin I	Monoclonal antibody				
	L1	L2	L3	L4	L5
Phospholipase A_2 inhibition	+	+	+	+	+
Binding to:					
E. coli membranes	−	+	−	−	+
Phosphatidylserine	−	+	−	−	−
F-actin	−	+	−	−	−
Phosphorylation *in vitro* with A-431 membranes	−	+	−	−	−

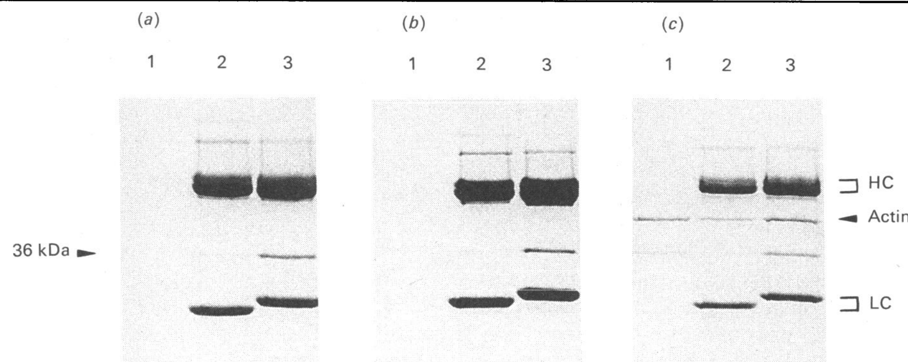

Fig. 6. Effects of MAbs on binding of lipocortin I to *E. coli* membranes (*a*), phosphatidylserine (*b*) and F-actin (*c*)

The binding *in vitro* of lipocortin I (3 μg) to *E. coli* membranes (*a*), phosphatidylserine (*b*) and F-actin (*c*) in the absence (lane 1) and presence of the MAbs (20 μg) (lane 2, L1; lane 3, L2) were examined as described in the Experimental section. Unbound lipocortin I was recovered in the assay supernatant and analysed by SDS/PAGE and Coomassie Blue staining. The arrowheads indicate the positions of unbound 36 kDa lipocortin I and actin. Immunoglobulin heavy chains (HC) and light chains (LC) are indicated.

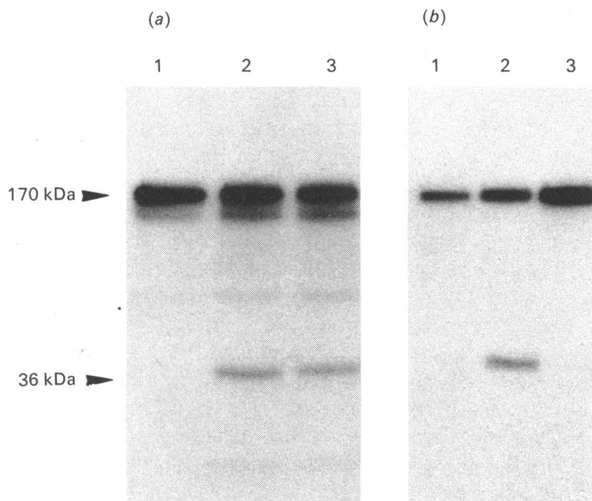

Fig. 7. Effects of MAbs on phosphorylation of lipocortin I with A-431 membranes *in vitro*

Phosphorylation of lipocortin I (3 μg) *in vitro* was carried out in the presence of Ca^{2+} (1 mM) and EGF (200 ng) for 20 min on ice. Samples were applied to SDS/PAGE and bands of phosphorylated protein were located by autoradiography as described in the Experimental section. Lane 1, A-431 membranes; lane 2, A-431 membranes with lipocortin I; lane 3, A-431 membranes with lipocortin I and 20 μg of MAb (*a*, L1; *b*, L2). Arrowheads show the positions of phosphorylated EGF receptor (170 kDa) and lipocortin I (36 kDa).

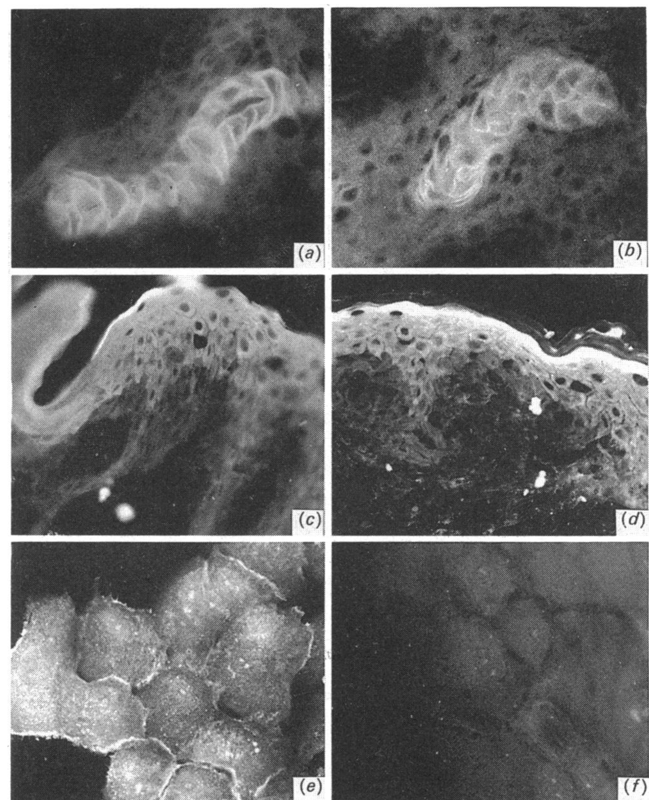

Fig. 8. Immunofluorescence of the Ca^{2+}-bound form of lipocortin I in human skin and keratinocytes

(*a*), (*c*) and (*e*) were stained with antibody L1-MAb, which reacts with both the Ca^{2+}-free and Ca^{2+}-bound forms. (*b*), (*d*) and (*f*) were stained with antibody L2-MAb, which reacts only with a Ca^{2+}-bound form. (*a*), (*b*) Ductal epithelial cells, which are in the epidermis of eccrine sweat glands were stained with both antibodies on the cell membrane, showing a honeycomb pattern. (*c*), (*d*) Suprabasal cells of human epidermis showed a positive reaction in the cytoplasm with both antibodies. (*e*), (*f*) Cultured human epidermal keratinocytes were stained on the cell membrane at cell–cell contact areas and in the cytoplasm with L1-MAb (*e*), but they were not stained with L2-MAb, the antibody against the Ca^{2+}-bound form only (*f*).

Localization of a Ca^{2+}-bound form of lipocortin I in tissues and cells

Localization of a Ca^{2+}-free form and a Ca^{2+}-bound form of lipocortin I was studied by immunofluorescence microscopy with L1-MAb and L2-MAb. With both antibodies, strong fluorescence was seen along the cell membrane, presenting the appearance of honeycomb pattern in the ductal epithelium of sweat glands of human skin (Figs. 8*a* and 8*b*). Treatment of cryosections of the sample on the coverslips with 10 mM-EGTA for 1 h before fixation greatly reduced or abolished the reactivity against L2-MAb but not that against L1-MAb or polyclonal anti-(lipocortin I) antibody. This result indicates that L2-MAb recognized specifically the Ca^{2+}-bound form of lipocortin I. Both antibodies were also present in the cytoplasm of suprabasal cells of the human epidermis (Figs. 8*c* and 8*d*). However, in cultured human epidermal keratinocytes, only L1-MAb showed a positive reaction with the cell membrane at cell–cell contact areas and cytoplasm, whereas L2-MAb was not reactive (Figs. 8*e* and 8*f*). L2-MAb also detected a Ca^{2+}-bound form of lipocortin I in A-431 and human fibroblast cells (results not shown).

DISCUSSION

Ca^{2+}-bound form of lipocortin I

Lipocortin I has been investigated in relation to many of its functions, e.g. as an anti-inflammatory protein, as a substrate for EGF receptor/kinase and as an exocytosis-mediating protein. Most functions of lipocortin I *in vitro* are mediated by Ca^{2+}. Thus in studies on the roles of lipocortin I *in vivo* and *in vitro*, it is important to investigate its Ca^{2+}-binding domains. Many investigators have deduced consensus amino acid sequences of Ca^{2+}- and phospholipid-binding domains of the Ca^{2+}- and phospholipid-binding proteins which lack EF-hand structures [27–30]. Lipocortin I has four consensus repeats and binds four Ca^{2+} ions per mol in the presence of phosphatidylserine [14]. In the absence of phosphatidylserine, however, the Ca^{2+}-binding characteristics of lipocortin are barely detectable by equilibrium dialysis assay.

In this study, we obtained two MAbs (L2 and L4) specific to a Ca^{2+}-bound form of lipocortin I. L2-MAb inhibited the binding of phosphatidylserine to lipocortin I, whereas L4-MAb did not. L2-MAb bound to lipocortin I at Ca^{2+} concentrations of $\geqslant 10$ μM. No phospholipids, including phosphatidylserine, affected the Ca^{2+}-dependency of lipocortin I. These findings indicate that L2- and L4-MAbs recognized different epitopes of the conformational changes of lipocortin I induced by Ca^{2+}. We speculate that the Ca^{2+}-binding site and the phospholipid-binding site of lipocortin I are located close together, but are different.

Although all five MAbs reacted with lipocortin I and 68K-I (a novel Ca^{2+}- and phospholipid-binding protein specific to human placenta) in the presence of Ca^{2+}, they did not cross-react with other Ca^{2+}- and phospholipid-binding proteins [16]. We have also obtained an MAb that reacts with the Ca^{2+}-bound forms of both calpactin I and lipocortin I (H. Hayashi, unpublished work). This MAb may be useful in the study of common structures of the Ca^{2+}-binding domain of this family.

Mechanism of phospholipase A_2 inhibition by lipocortin I

Lipocortin was first described as an anti-inflammatory mediator which was induced by steroids, which inhibited phospholipase A_2 [3] and which consequently inhibited the formation of arachidonate and lysophosphatide from phospholipid. Many investigators have discussed the mechanism of phospholipase A_2 inhibition by lipocortin I in various assays [31–36], and three main models have been proposed. (1) Substrate depletion model [31]. Lipocortin I inhibits phospholipase A_2 by removing the substrate (especially phosphatidylserine) from *E. coli* membranes. This model was based on the fact that a large molar excess of lipocortin I was required to inhibit phospholipase A_2 activity in an assay with ^{3}H-labelled *E. coli* membranes as substrate. Furthermore, the inhibitory activity of lipocortin I was blocked by preincubation with phosphatidylserine. (2) Substrate coating model [32]. Lipocortin I inhibits phospholipase A_2 activity by coating *E. coli* membranes (its binding is not necessarily with phosphatidylserine). In this model, the percentage inhibition of phospholipase A_2 activity is approximately proportional to the percentage binding of lipocortin I to *E. coli* membranes. (3) Direct association with phospholipase A_2 [33,34]. Lipocortin I inhibits phospholipase A_2 activity by direct association with it at a nonamer amino acid sequence (HDMNKVLDL).

To test these three models, we examined the effects of the five MAbs on the ability of lipocortin I to inhibit phospholipase A_2 activity and on its binding to *E. coli* membranes, phosphatidylserine and F-actin. If the first model were correct, the MAbs that abolish the inhibition of phospholipase A_2 should also inhibit the binding of lipocortin I to phosphatidylserine. The recoveries of phospholipase A_2 activity after preincubation with the respective MAbs were not, however, proportional to their inhibition of binding of lipocortin I to phosphatidylserine (Fig. 6 and Table 1). If the second model were correct, the MAbs that abolish the phospholipase A_2 inhibitory activity of lipocortin I should inhibit its binding to *E. coli* membranes. The recoveries of phospholipase A_2 after preincubation with the respective MAbs were not, however, proportional to their inhibition of binding of lipocortin I to *E. coli* membranes (Fig. 6 and Table 1). Although the third model can explain results in an assay using deoxycholate-solubilized 1-stearoyl,2-[1-^{14}C]arachidonyl phosphatidylcholine as substrate, it cannot explain why a large molar excess of lipocortin I is required to inhibit phospholipase A_2 activity in an assay using [^{3}H]oleic-acid-labelled *E. coli* membranes as substrate. Furthermore, under our assay conditions we could not detect any direct association between phospholipase A_2 and lipocortin I using our MAbs that apparently recognize different epitopes (results not shown). Thus we conclude that the mechanism of phospholipase A_2 inhibition by lipocortin I cannot be explained simply by any of the models described above.

Inhibition by L2-MAb of phosphorylation of lipocortin I by A-431 membranes

Lipocortin I has also been purified as substrate *in vivo* of EGF receptor kinase, which seems to mediate an external growth signal by tyrosine phosphorylation [5,6]. The *N*-terminus of lipocortin I affects Ca^{2+}- binding and phosphorylation [14,32,37,38]. Only L2-MAb inhibited the phosphorylation of lipocortin I by A-431 membranes as well as inhibiting its binding to phosphatidylserine. Therefore, L2-MAbs may recognize a tertiary structure including a Ca^{2+}- and phospholipid-binding domain as well as the *N*-terminus of lipocortin I.

Cellular localization of Ca^{2+}-bound form of lipocortin I

Lipocortin I requires rather high concentrations of Ca^{2+} for its actions. Thus it was questionable whether a Ca^{2+}-bound form of lipocortin I actually exists *in vivo*, even in the presence of phospholipids. However, using L2-MAb, we have obtained direct evidence that a Ca^{2+}-bound form is actually present in the ductal epithelium of sweat gland of human skin. Most of this form was detected in a membrane-bound state (Fig. 8*b*). This distribution pattern was almost identical with that of lipocortin I detected by L1-MAb, which reacted with both Ca^{2+}-bound and Ca^{2+}-free forms (Fig. 8*a*). Both L1-MAb and L2-MAb stained lipocortin I diffusely in the cytoplasm of suprabasal cells in the epidermis (Figs. 8*c* and 8*d*). However, in cultured human keratinocytes, the Ca^{2+}-bound form was not detected whereas the Ca^{2+}-free form was present in a membrane-bound state at cell–cell contact areas as well as in the cytoplasm (Figs. 8*e* and 8*f*). These observations indicate that there are at least two forms of membrane-bound lipocortin I, the Ca^{2+}-bound and Ca^{2+}-free forms. We speculate that the Ca^{2+}-bound form of lipocortin I may be associated with the differentiation of epidermal keratinocytes.

Our results suggest that L2- and L4-MAbs should be useful for studying the Ca^{2+}-mediated biological effects of lipocortin I *in vivo*.

We thank Mrs. Hiroko Kawamata for typing the manuscript. This investigation was supported by a Grant-in-Aid for Special Project Research on Cancer-Bioscience (No. 63614518) to T. K. from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1. Geisow, M. J. & Walker, J. H. (1986) Trends Biochem. Sci. **11**, 420–423
2. Crompton, M. R., Moss, S. E. & Crumpton, M. J. (1988) Cell **55**, 1–3
3. Hirata, F., Schiffmann, E., Venkatasubramanian, K., Salomon, D. & Axelrod, J. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 2533–2536
4. Hirata, F., Matsuda, K., Notsu, Y., Hattori, T. & del Carmine, R. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 4717–4721
5. Fava, R. A. & Cohen, S. (1984) J. Biol. Chem. **259**, 2636–2645
6. De, B. K., Misono, K. S., Lukas, T. J., Mroczkowski, B. & Cohen, S. (1986) J. Biol. Chem. **261**, 13784–13792
7. Huang, K.-S., Wallner, B. P., Mattaliano, R. J., Tizard, R., Burne, C., Frey, A., Hession, C., MacGray, P., Sinclair, L. K., Chow, E. P., Browning, J. L., Ramachandran, K. L., Tang, J., Smart, J. E. & Pepinsky, R. B. (1986) Cell **46**, 191–199
8. Saris, C. J. M., Tack, B. F., Kristensen, T., Glenney, J. R., Jr. & Hunter, T. (1986) Cell **46**, 201–212
9. Creutz, C. E., Dowling, L. G., Sando, J. J., Villar-Palasi, C., Whipple, J. H. & Zaks, W. J. (1983) J. Biol. Chem. **258**, 14664–14674
10. Kretsinger, R. H. & Creutz, C. E. (1986) Nature (London) **320**, 573
11. Burgoyne, R. D. (1988) Nature (London) **331**, 20
12. Drust, D. S. & Creutz, C. E. (1988) Nature (London) **331**, 88–91
13. Glenney, J. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 4258–4262
14. Schlaepfer, D. D. & Haigler, H. T. (1987) J. Biol. Chem. **262**, 6931–6937
15. Cirino, G., Flower, R. J., Browning, J. L., Sinclar, L. K. & Pepinsky, R. B. (1987) Nature (London) **328**, 270–272
16. Hayashi, H., Owada, M. K., Sonobe, S. & Kakunaga, T. (1989) J. Biol. Chem. **264**, 17222–17230
17. Seki, J., Owada, M. K., Sakato, N. & Fujio., H. (1986) Cancer Res. **46**, 907–916
18. Owada, M. K., Hakura, A., Iida, K., Yahara, I., Sobue, K. & Kakiuchi, S. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 3133–3137
19. Hellewell, S. B. & Taylor, D. L. (1979) J. Cell Biol. **83**, 633–648
20. Laemmli, U. K. (1970) Nature (London) **227**, 680–685
21. Duhamel, R. C., Meezan, E. & Brendel, K. (1981) J. Biochem. Biophys. Methods **5**, 67–74
22. Crundmann, U., Abel, K.-J., Bohn, H., Lobermann, H., Lottspeich, F. & Küpper, H. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 3708–3712
23. Schlaepfer, D. D., Mehlman, T., Burgess, W. H. & Haigler, H. T. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 6078–6082
24. Crompton, M. R., Owens R. J., Totty, N. F., Moss, S. E., Waterfield, M. D. & Crumpton, M. J. (1988) EMBO J. **7**, 21–27

25. Südhof, T. C., Slaughter, C. A., Leznicki, I., Barjon, P. & Reynolds, G. A. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 664–668
26. Glenney, J. R., Jr., Tack, B. & Powell, M. A. (1987) J. Cell Biol. **104**, 503–511
27. Geisow, M. J., Fritshe, U., Hexham, J. M., Dash, B. & Johnson, T. (1986) Nature (London) **320**, 636–638
28. Geisow, M. J. (1986) FEBS Lett. **203**, 99–103
29. Kristensen, T., Saris, C. M., Hunter, T., Hicks, L. J., Noonan, D. J., Glenney, J. R., Jr. & Tack, B. F. (1986) Biochemistry **25**, 4497–4503
30. Weber, K., Johnsson, N., Plessmann, U., Van, P. N., Söling, H.-D., Ampe, C. & Vandekerckhove, J. (1987) EMBO J. **6**, 1599–1604
31. Davidson, F. F., Dennis, E. A., Powell, M. & Glenney, J. R., Jr. (1987) J. Biol. Chem. **262**, 1698–1705
32. Haighler, H. T., Schlaepfer, D. D. & Burgess, W. H. (1987) J. Biol. Chem. **262**, 6921–6930
33. Miele, L., Cordella-Miele, E., Facchiano, A. & Mukherjee, A. B. (1988) Nature (London) **335**, 726–730
34. Hirata, F. (1981) J. Biol. Chem. **256**, 7730–7733
35. Touqui, L., Rothhut, B., Shaw, A. M., Fradin, A., Vargaftig, B. B. & Russo-Marie, F. (1986) Nature (London) **321**, 177–180
36. Huang, K.-S., McGray, P., Mattaliano, R. J., Burne, C., Chow, E. P., Sinclair, L. K. & Pepinsky, R. B. (1987) J. Biol. Chem. **262**, 7639–7645
37. Glenney, J. & Zokas, L. (1988) Biochemistry **27**, 2069–2076
38. Ando, Y., Imamura, S., Hong, Y.-M., Owada, M. K., Kakunaga, T. & Kannagi, R. (1989) J. Biol. Chem. **264**, 6948–6955

Received 15 December 1989/9 April 1990; accepted 18 April 1990