Monoclonal antibodies to lipocortin-1 as probes for biological function

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We have developed two monoclonal antibodies to human lipocortin-1 (103 and 105) as reagents for quantitating the protein in biological systems and neutralizing its activity. Lipo 105 is a high affinity antibody that is functional in ELISA and Western blot formats. The antibody recognizes a site between amino acids 30 and 55 in the lipocortin-1 sequence and can be used on native or denatured protein. Lipo 103 is an antibody that neutralizes the phospholipase A_2 inhibitory activity of lipocortin-1 by blocking binding of the protein to phospholipid surfaces. The antibody is specific for native human lipocortin-1. Lipo 103 was recently shown to block lipocortin-1-dependent differentiation of a squamous carcinoma cell line, demonstrating its usefulness as a probe for function.

1. INTRODUCTION

The lipocortins or annexins are a family of calcium and phospholipid binding proteins that have been implicated in diverse processes, ranging from intracellular events where they have been postulated to play roles in membrane-cytoskeleton interactions and signal transduction to extracellular events where they affect inflammation, blood coagulation, the immune response, growth and differentiation (see [1] for refs). Seven distinct proteins have been characterized to date, all sharing approx. 50% sequence identity (see [2,3] for refs). Most conserved is a 70 amino acid repeat unit that is responsible for calcium and phospholipid binding. The 35–45 kDa lipocortins contain 4 copies of the unit and the 70 kDa lipocortins, 8 copies. Distinct from the repeat units is a short amino-terminal segment that is unique to each protein. Although the repeat units bind calcium and phospholipid, the amino-terminal segment regulates the binding affinities and thus is likely to provide specificity to the different family members [4–6]. Lipocortin-like proteins have been purified from a variety of systems. Other names for these related proteins include chromobindins, calpactins, calelectrins, calcimedins, endonexins, placental anticoagulant proteins, and proteins I–III (see [7,8] for reviews).

Lipocortin-1, as the first member of the family to have been cloned and expressed [9], has become the prototype of the group. The recombinant protein has been tested in a variety of systems and shown to display diverse activities [10–16]; however, it remains unclear which of these activities are physiologically relevant.

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Furthermore the mechanism of action is unknown. To better evaluate the biological functions of lipocortin-1 we have produced a panel of monoclonal antibodies, screening in particular for antibodies that could block activity. Here we describe the properties of a neutralizing antibody, which functions by blocking the association of lipocortin with membranes. Because of its specificity, the antibody should serve as a valuable reagent for studying the function of lipocortin-1.

2. MATERIALS AND METHODS

2.1. Purification of lipocortin-like proteins

Recombinant human lipocortin-1 was produced in *E. coli* [9]. ^{35}S-labeled lipocortin-1 was produced in the same strain, using cells that were adapted to grow in low sulphate medium. A 50 ml culture was grown in the presence of 10 mCi [^{35}S]sulphate and labeled lipocortin purified as previously described [17,18]. Human, rat, and bovine lipocortins 1–6 were purified as described [1,2].

2.2. Production of monoclonal antibodies

BALB/c mice (female, 4–6 weeks) were immunized with an intraperitoneal injection (10 µg/mouse) of recombinant lipocortin-1 in complete Freund's adjuvant. The animals were boosted on days 14 and 28 with lipocortin-1 (10 µg/mouse) in incomplete Freund's adjuvant. After 6 weeks, test bleeds were taken and screened for antibodies that blocked lipocortin-1 activity in the phospholipase A_2 inhibition assay. Antisera from 2 of 6 mice displayed anti-lipocortin activity. Four days prior to the fusion, both animals were boosted with intravenous injections containing 20 µg of lipocortin. The animals were sacrificed and the spleen cells fused with SP3 × Ag8 cells for hybridoma production [19].

Hybridoma culture supernatants were assayed for antibodies that could precipitate radiolabeled lipocortin-1. 20000 cpm of ^{35}S-labeled lipocortin was mixed with 50 µl of conditioned medium from each clone and incubated for 1 h at 4°C. Mouse IgG and goat anti-mouse IgG preparations were added and samples further incubated for 2 h at 4°C. The immune complexes were pelleted, washed with PBS, dissolved in 0.1 N NaOH and quantitated by scintillation counting. Hybridomas producing antibodies that precipitated over 50% of the input counts were subcloned by limiting dilution. The 5 most promis-

ing lines (lipo 101–105) were grown as ascites in pristane-primed mice. The monoclonal antibodies were affinity-purified on protein A Sepharose, using the Pierce binding and elution buffer systems. Lipo 101–104 were isotyped as IgG_1 and lipo 105, as IgG_{2a}.

2.3. Western blotting

Lipocortin-1 and its cleavage products were analyzed by SDS-PAGE [1]. Proteins were either stained directly with Coomassie brilliant blue R-250 or subjected to Western analysis. For Western blots, proteins were transferred to nitrocellulose and probed with monoclonal antibodies at 10 μg/ml or with rabbit antisera to recombinant lipocortin (no.646) diluted at 1:1000. Immunoreactive bands were visualized with goat anti-mouse or goat anti-rabbit IgG-HRP conjugates as appropriate.

CNBr fragments of lipocortin-1 were generated under limiting conditions. Immunoreactive fragments were either directly probed with lipo 101–105 on Western blots [1] or precipitated with the monoclonal antibodies and the complexes blotted and probed with the 646 antiserum. In the latter case, lipocortin was treated with CNBr in 70% formic acid for 1 h at 23°C, lyophilized, and the digestion products renatured [18]. Fragments were diluted with 10 vols of lysis buffer [17] and incubated for 1 h at 4°C with a 5-fold molar excess of monoclonal antibody. The immune complexes were collected on goat anti-mouse IgG Sepharose, disrupted with electrophoresis sample buffer, and subjected to Western analysis.

2.4. Phospholipase A_2 inhibition assay

Antibodies were tested for their effect on lipocortin activity in a phospholipase A_2 assay, using [^{3}H]oleic acid-labeled E. coli as substrate [1]. Lipocortins were incubated with antibody for 1 h at 4°C, then mixed with phospholipase A_2 and with labeled E. coli and incubated at 8°C for 6 min. Reactions were quenched with HCl and activity assessed by monitoring released [^{3}H]oleic acid from the labeled substrate by scintillation counting.

2.5. Binding of lipocortin-1 to formalin-fixed bacteria

Recombinant human lipocortin-1 (5 μg/tube) was mixed with 0–100 μg of the 103 antibody in 100 μl of 25 mM Tris-HCl, pH 7.7, 0.1 mg/ml BSA, 8 mM $CaCl_2$ and incubated at 4°C for 1 h. 8 μl of formalin-fixed E. coli (see below) was added and the samples incubated on ice for 1 h. The bacteria were pelleted in an Eppendorf centrifuge (1 min, 10000 rpm) and the supernatants transferred to fresh tubes. Supernatants were treated with 25 μl of 5 × electrophoresis sample buffer and the pellets, with 125 μl of 1 × sample buffer. Both sets of samples were heated at 65°C for 10 min and 15 μl aliquots analyzed by SDS-PAGE. Formalin-fixed E. coli [20] were stored at −20°C as a 20% solution in PBS, 0.02% NaN_3. Immediately prior to their use, the bacteria were washed with 25 mM Tris-HCl, pH 7.7, and then diluted 6-fold with the same buffer.

2.6. ELISA analysis

Immulon-2 plates (Dynatech) were coated overnight at 4°C with 10 μg/ml antibody in 50 mM Na_2CO_3, pH 9.6. Wells were blocked with 1% fetal bovine serum in the same buffer. Plates were treated with serial dilutions of lipocortin in 50 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 1 mM EDTA, 0.05% Tween-20, 1% fetal bovine serum for 3 h at 23°C, then with a 1:1000 dilution of anti-human lipocortin-1 antiserum (rabbit no.842) for 2 h at 23°C, and finally with a 1:10000 of goat anti-rabbit IgG-HRP conjugate (Cappel) for 1 h at 23°C. Plates were developed with tetramethyl benzidine color reagent. 100 μl vols were used for all additions. Between steps, plates were washed 3 times with PBS containing 0.05% Tween-20.

3. RESULTS

Recombinant human lipocortin-1 was used as an immunogen in the production of monoclonal antibodies in BALB/c mice. Hybridoma supernatants were screen-

ed for antibodies that recognized radiolabeled lipocortin-1 by immune precipitation, insuring that all of the antibodies selected recognized protein in a native conformation. Five monoclonal antibodies were generated and designated lipo 101–105. Subsequently, the antibodies were checked by ELISA, Western blotting, and for their ability to block lipocortin activity in a phospholipase A_2 assay. Although no single antibody covered all the desired applications, two of the antibodies together were useful in this respect, providing a set of reagents for quantitating lipocortin-1 and for neutralizing its activity.

Fig.1 shows results from Western blots where recombinant and natural preparations of lipocortin-1 were electrophoretically transferred to nitrocellulose and then probed with culture medium from the various hybridoma lines. 2 of the 5 antibodies – lipo 102 and 105 – detected the denatured lipocortin. In the natural preparation a small portion of the product was proteolytically clipped near its N-terminus forming a des-30 adduct. Both antibodies recognized the truncated lipocortin; however, the 102 antibody was less
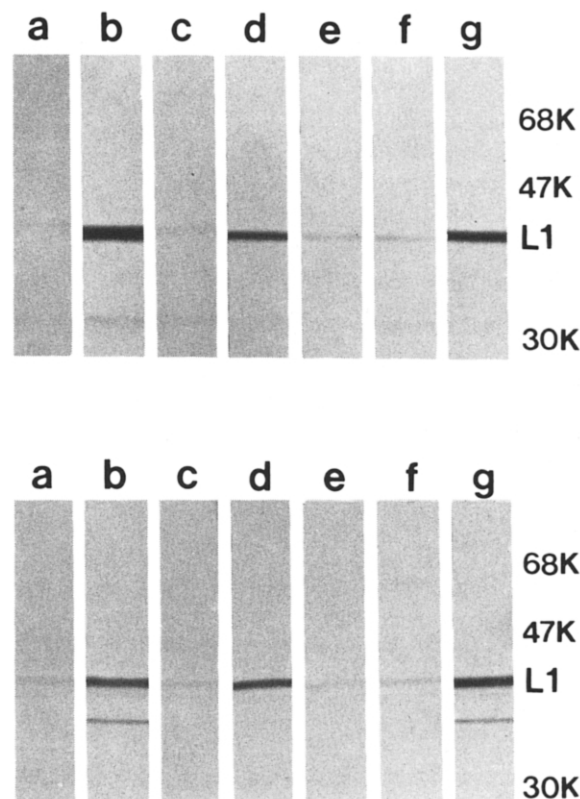

Fig.1. Western blot analysis of lipocortin-1 monoclonal antibodies. Recombinant (top panel) and natural (bottom panel) preparations of human lipocortin-1 (100 ng/track) were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with conditioned medium from the lipo 101–105 hybridoma cell lines (lanes c–g, respectively). Control medium (lane a) and immune serum from one of the mice used for antibody production (lane b) are presented as negative and positive controls. L1 denotes the position of lipocortin-1.
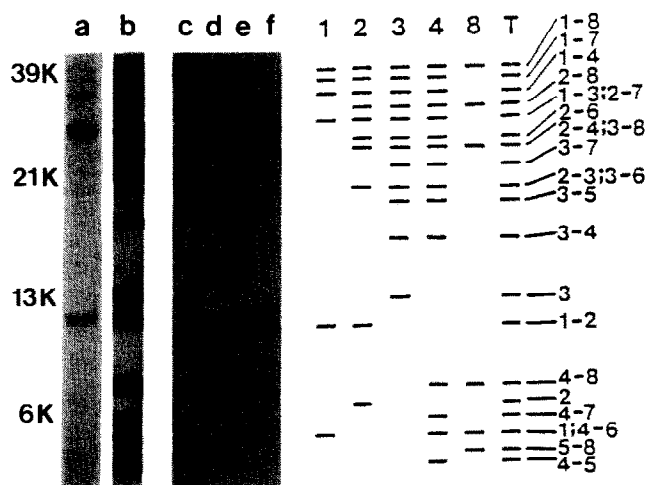
Fig.2. CNBr mapping of immunoreactive fragments. CNBr fragments of lipocortin-1 were separated on SDS-gels, transferred to nitrocellulose, and probed either with lipo 105 antibody (lane a), with rabbit antiserum to lipocortin-1 (lane b), or immune-precipitated with lipo 105 antibody, and these fragments detected on Western blots with the anti-lipocortin-1 rabbit antiserum (lanes c–f). Lipocortin in lanes c–f was treated with 0, 7, 14 and 21 mg/ml CNBr. Lanes 1–4, 8 and T show schematic diagrams summarizing the CNBr mapping data [21] where 1–4, and 8 represent profiles for specific fragments and T represents the total digest. Numbers at the right denote fragment compositions where 1 refers to the N-terminal CNBr fragment and 8 to the C-terminal fragment.

reactive than lipo 105. In fig.1, the 35 kDa fragment is barely detected with the 102 antibody.

The reactive epitopes for the 102 and 105 antibodies were further localized by CNBr mapping (see fig.2). CNBr cleaves lipocortin-1 into 8 fragments with masses of: 6.2, 7.8, 13.3, 4.2, 1.5, 0.9, 1.1 and 3.1 kDa from N- to C-terminus, respectively. By analyzing partial CNBr digests in a nearest neighbor-type analysis, each complete CNBr fragment is represented by a distinct subset of the cleavage products. When CNBr cleavage products were probed with lipo 102 and 105, a series of 5 fragments were detected that were identical for the two monoclonals (only the lipo-105 map is presented). Of the 23 known cleavage products in the cleavage map of lipocortin-1 [21], only the 5 fragment-1-containing cleavage products were observed, indicating that the reactive epitope is associated with CNBr fragment-1. In conjunction with results for the des-30 variant, the mapping analysis localized the reactive epitope between amino acids 30–55 in the lipocortin-1 sequence.

Attempts to define the binding sites for the 101, 103 and 104 antibodies were unsuccessful. In addition to direct and indirect analyses of fragments by CNBr mapping (fig.2), we tried to localize the epitope by cleaving lipocortin with elastase under limiting conditions [18] and then using the antibodies to immune-precipitate functional fragments. None of the fragments were detected. We also attempted various cross-linking approaches where lipocortin-antibody

complexes were fixed with chemical cross-linkers, SDS-gel purified, and analyzed by CNBr mapping [22]. Extensive cross-linking was observed with N-5-azido-2-nitrobenzoyloxysuccimide and with p-azidophenyl glyoxal and minimal cross-linking, with dimethyl suberimidate, difluorodinitrobenzene and ethylene glycol-bis(succimidyl-succinate). Peptide maps of the covalent lipocortin-antibody complexes failed to localize the binding sites (data not shown).

Since the primary goal for producing monoclonals was to obtain antibodies that could be used as probes for investigating the biology of lipocortin-1, lipo 101–105 were screened for their effects on lipocortin activity towards phospholipase A_2. While the mechanism of phospholipase inhibition does not involve a direct enzyme-inhibitor interaction, the role of this pathway has been supported in various recent studies [10–12,16]. When conditioned medium from lipo 101–105 hybridomas were tested for anti-lipocortin-1 activity, lipo 103 and 105 showed blocking activity. In a more quantitative analysis of the inhibition using affinity-purified antibody, only the lipo 103 antibody showed significant blocking activity (see fig.3) and thus most subsequent studies focused on characterizing lipo 103.

To better understand the mechanism of inhibition, a simple membrane binding system was used where binding of lipocortin-1 to bacterial membranes could be monitored in the same format as binding occurs in the phospholipase assay. When lipocortin was incubated with varying concentrations of lipo 103 antibody and
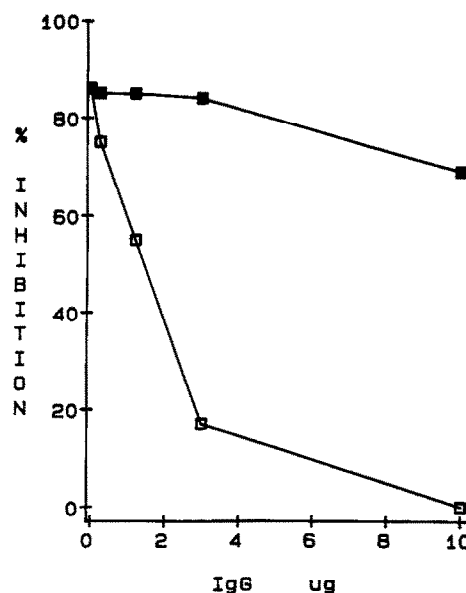

Fig.3. Dose-response curves of anti-lipocortin activity. Affinity purified lipo 103 and 105 antibodies were incubated with lipocortin-1 and then assayed for phospholipase inhibitory activity. Numbers presented represent residual phospholipase inhibitory activity remaining after antibody treatment. (■---■) Lipo 105; (□---□) lipo 103.

the membrane binding of lipocortin assessed on SDS-gels, we obtained the results shown in fig.4. In the absence of antibody, all of the lipocortin was associated with the bacteria whereas the presence of antibody led to a dose-dependent inhibition of lipocortin binding. Near stoichiometric concentrations of antibody blocked membrane binding. Since membrane binding is a prerequisite for phospholipase inhibition, this result suggests that the inhibition of lipocortin activity results from blocking its binding to membranes. Interestingly, in the lipocortin-1 preparation used in the membrane binding assay, about 20% of the protein existed as a des-30 fragment. The ability of lipo 103 to recognize the truncated variant indicates that the epitope for the antibody lies within the core structure of lipocortin.

Since lipocortins are a family of proteins that share extensive sequence homology, ranging from 40–60% identity among the various members, the specificity of the antibody was an important consideration. Table 1 summarizes results where human lipocortins 1–6 were assayed for cross-reactivity with the lipo 103 antibody by testing for blocking activity in the phospholipase A_2 assay. Only lipocortin-1 activity was affected by the antibody, indicating a high degree of specificity for the antibody. No cross-reactivity was seen towards bovine lipocortins 2–6 as well, supporting its specificity (data not shown). Finally, we tested the lipo 103 antibody for potential cross-reactivity with rat lipocortin-1, which shares 85% identity with the human protein. The rat lipocortin was not inhibited by the 103 antibody.

Although the association of lipo 103 with lipocortin is readily detected in solution, there was no apparent binding when lipocortin was coated on plastic or bound to cell surfaces and then probed for binding. When lipocortin-coated immulon-2 plates were incubated with lipo 103 and 105 antibodies and binding assessed using a goat anti-mouse IgG-HRP reporter system, we observed a typical dose-response with an ID_{50} for lipo 105 of 15 ng antibody/ml. In contrast, the apparent
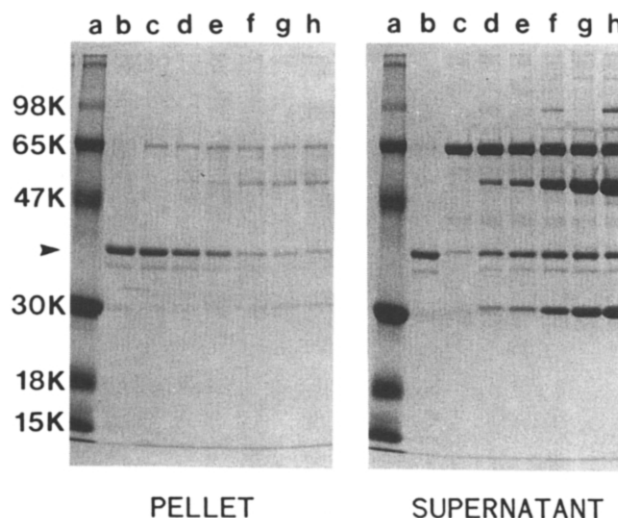

Fig.4. The 103 antibody blocks lipocortin-1 from binding to membranes. The binding of lipocortin to formalin-fixed bacteria was evaluated in the presence and absence of the lipo 103 antibody. Each sample was split into bound (pellet) and unbound (supernatant) fractions and then subjected to SDS-PAGE. Proteins were stained with Coomassie blue. (Lane a) BRL prestained high molecular weight markers; (lane b) 0.5 μg of recombinant lipocortin-1; (lanes c–h) supernatant and pellet fractions from samples that had received 0, 0.5, 1, 2.5, 10 and 15 μg of antibody, respectively.

titer for the lipo 103 antibody was less than one-thousandth of what the 105 antibody produced in the same format. Similar differences were obtained when recombinant lipocortin was bound to cell surfaces and quantitated using fluorescence activated cell sorting. Surface-bound lipocortin was readily detected with the lipo 105 antibody, but absent in a parallel analysis run with lipo 103 antibody (Browning, J., unpublished). Fig.5 shows a direct comparison of the two antibodies in a sandwich ELISA. While both antibodies detect lipocortin in this format, lipo 105 is 10 times as sensitive as lipo 103 and provides a simple method for quantitating ng amounts of the protein.

4. DISCUSSION

We have characterized two monoclonal antibodies, lipo 103 and 105, as reagents that can be used to study the biology of lipocortin-1. Lipo 103 (which we now refer to as **1A**) is of interest as a neutralizing antibody. In addition to blocking lipocortin-1 activity in phospholipase A_2 assays, the antibody recently was shown to block activity in a lipocortin-1-dependent cell differentiation assay [15] and thus should be a useful tool for functional studies involving lipocortin-1. The 105 antibody (which we now refer to as **1B**) provides a simple method for quantitating lipocortin-1. The antibody has proven to be useful for evaluating lipocortin-1 in human clinical samples in a sandwich ELISA format and for detecting surface-bound

Table 1

Specificity of the lipo 103 antibody towards human lipocortins

Lipocortin species	Percent inhibition	
	−103	+103
1	88	25
2	89	90
3	87	91
4	98	96
5	97	98
6	84	82

Human lipocortins 1–6 (1 μg each) were incubated with or without 10 μg of affinity-purified 103 antibody and then assayed for phospholipase A_2 inhibitory activity. Numbers presented represent residual phospholipase inhibitory activity remaining after antibody treatment
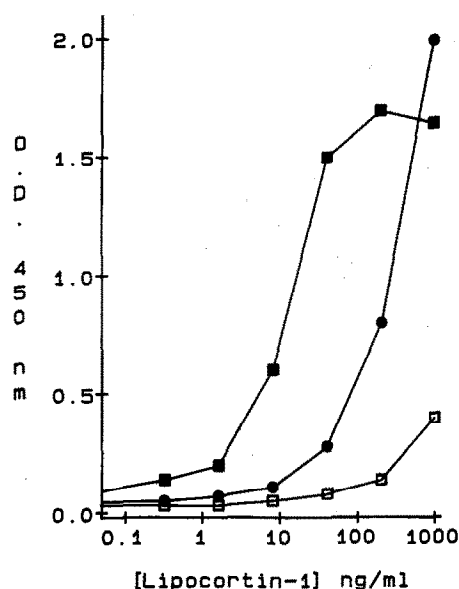
Fig.5. A sandwich ELISA for quantitating lipocortin-1. Immulon-2 plates coated with lipo 103, 105, or control mouse IgG were treated with serial dilutions of lipocortin, then with rabbit anti-lipocortin-1 antiserum, and finally with goat anti-rabbit IgG-HRP conjugate. Signals were quantitated spectrophotometrically at 450 nm with a Dynatech microplate reader. (■---■) Lipo 105; (●---●) lipo 103; (□---□) control IgG$_{2a}$.

lipocortin. The epitope for the 105 antibody is localized within a segment between amino acids 30 and 55 in the lipocortin-1 sequence. The lipo 105 antibody recognized rat lipocortin-1 on Western blots and thus should be useful for evaluating lipocortin-1 levels in animal models.

The specificity and mode of action of the 103 antibody are of special interest because of the potential applications of the antibody as a probe for function. Out of the 6 lipocortins that we have characterized [2], only lipocortin-1 was neutralized by lipo 103, indicating that the antibody is a specific reagent. Rat lipocortin-1, which shares 85% identity with the human protein, was not blocked by the antibody further supporting this notion. Although we were unable to precisely define the reactive epitope, the antibody recognized the truncated des-30 variant and thus interacts with the core structure of lipocortin, which contains the calcium and phospholipid binding domain. Since many of the activities of lipocortin-like proteins result from membrane binding, we tested whether the antisera might affect this property. In a simple assay using formalin-fixed bacteria as a surface for monitoring binding, we have demonstrated that the antibody blocks lipocortin binding to membrane, indicating its likely site of action.

The complexity of the lipocortins in the number of proteins alone has created confusion in understanding the biological significance of any one family member.

Interestingly, in those cases where the proteins have been localized by immunohistochemical techniques, they exhibit distinctive cellular distributions [23,24], indicating specialized roles. Neutralizing antibodies provide a simple method for probing function, by allowing one to inactivate existing protein and test the effect of the perturbation on the system. Historically 3 different neutralizing antibodies to lipocortin-like proteins have been used in various systems. All were prepared with ill-defined immunogens. Furthermore, none of these antibodies were characterized for specificity or mode of action and thus they have not been widely used [25–27]. The 1A antibody is unique in that it represents the first reagent for which specificity and mechanism are known.

Recently several groups have produced monoclonal antibodies against specific lipocortins [5,28]. To date little is known about their properties or applications. Furthermore, none of the antibodies were developed as neutralizing reagents. In the extensive study by Glenney and Zokas [5], 13 monoclonals to lipocortin-1 were produced and characterized with respect to potential uses for studying actin binding, phosphorylation by the EGF receptor, and binding to phosphatidyl serine vesicles. All 13 antibodies worked on Western blots and 9 were found to recognize the amino-terminal tail region. In our panel of antibodies, only two of the antibodies work on Western blots and none recognized the tail region, indicating that the screen we adopted yielded a distinctive set of antibodies. While further studies are needed to better define the applications of the 103 and 105 antibodies, initial studies [15,29] indicate that they should serve as valuable tools for quantitating the protein and for probing biological function.

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