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A dimeric form of lipocortin-1 in human placenta

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We have characterized a 68 kDa lipocortin from human placenta that was identified as a covalently linked homodimer of lipocortin-1 by peptide mapping and sequence analysis. The site of cross-linking was localized within the 3 kDa *N*-terminal tail region, an exposed domain that contains the phosphorylation sites for protein tyrosine kinases and protein kinase C and is sensitive to proteolysis. Sequence analysis of the corresponding peptide revealed that glutamine-18 was modified, suggesting that the cross-link may be generated by a transglutaminase. By incubating lipocortin-1 with placental membranes and with labelled glycine ethyl ester we observed a Ca^{2+}-dependent labelling of lipocortin-1 within the tail region, supporting this notion. Like lipocortin-1, the dimer inhibits phospholipase A_2 activity, is a substrate for the epidermal-growth-factor (EGF) receptor/kinase, and displays Ca^{2+}-dependent binding to phosphatidylserine-containing vesicles. In preparations from human placenta the dimer is particularly abundant, accounting for approx. 20% of the lipocortin-1.

INTRODUCTION

Lipocortins are a family of Ca^{2+}- and phospholipid-binding proteins that have been implicated in the regulation of various aspects of inflammation, the immune response, blood coagulation, growth, differentiation, cell adhesion, exocytosis, and in the structural role of linking cytoskeletal elements to membranes (see [1–5] for references). Six distinct proteins have been characterized to date (see [5] for references). All share approx. 50% sequence identity and contain multiple copies of a 70-amino-acid repeat unit. The 30–40 kDa lipocortins contain four copies of the unit, and the 70 kDa lipocortins eight copies. Distinct from the repeat units is a short *N*-terminal segment (5–30 amino acids) that is unique to each protein. Although the repeat units are responsible for Ca^{2+} and phospholipid binding, the *N*-terminal segment regulates the binding affinities and thus is likely to provide specificity to the different family members.

Lipocortins differ from conventional Ca^{2+}-binding proteins in that they lack an EF-hand-type Ca^{2+}-binding site [6,7] and they inhibit phospholipase A_2 activity [8]. Although the mechanism of phospholipase inhibition is controversial [5], the inhibition assay has provided a simple biochemical tool for their purification. Similar proteins have been purified by other groups, using Ca^{2+} binding as an affinity step. Other names for these related proteins include chromobindins [9], calpactins [10], calelectrins [11], calcimedins [12], endonexins [7], placental anticoagulant proteins [2] and proteins I–III [13].

Of the six proteins, lipocortins-1 and -2 have been of particular interest as substrates for oncogene and receptor protein kinases, and have been implicated in the transduction pathways of various mitogenic signals. In intact cells they are substrates for the EGF [14–17] and insulin [18] receptor kinases and for $pp60^{v\text{-}src}$ (see [10,19] for references), and have been shown to translocate from cytoplasm to the membrane upon Ca^{2+} treatment [20]; yet the key biological functions of the proteins are unclear. In assays *in vitro*, phosphorylation decreases their Ca^{2+} affinity, decreases their ability to bind membranes, and neutralizes the phospholipase-inhibitory activity [21–25]. Because alterations in the *N*-terminal segment affect these processes [26,27], an important step in understanding the roles of these two proteins is to understand the state of the *N*-terminal segment and of its interactions. With lipocortin-2, a large fraction of the protein occurs as a multimeric complex where the *N*-terminus is tightly associated with a 7 kDa S100-like protein, forming a tetrameric actin-binding complex termed protein I [28] or calpactin-I [29]; however, no complex has been observed for lipocortin-1. In studying lipocortin-1 from placenta we observed that about 20% of the protein was dimeric, and localized the cross-link within the *N*-terminal segment. The abundance of the dimer in placenta suggests that a head-to-head association of adjacent lipocortin-1 molecules is a common state of the protein, which may be relevant for its activity.

MATERIALS AND METHODS

Isolation of the 68 kDa lipocortin-1 variant from placenta

A fresh term human placenta was quick frozen on solid CO_2 and stored at −70 °C. Tissue (150 g) was thawed on ice in 50 mM-Tris/HCl (pH 7.7), containing 1 mM-$CaCl_2$, 5 mM-benzamidine, 1 mM-phenylmethanesulphonyl fluoride and 100 μg of soybean trypsin inhibitor/ml, skinned, and cut into chunks. Then 250 ml of the buffer was added and the tissue disrupted with a Polytron. The homogenate was centrifuged for 1 h at 4 °C in a SA600 rotor (14000 rev./min). Pellets were resuspended with a Polytron into 250 ml of the same buffer and again centrifuged. After an additional wash, the Ca^{2+}-binding proteins were extracted with 240 ml of 15 mM-Hepes (pH 7.5)/4 mM-EDTA/1 mM-EGTA. Debris was removed by centrifugation and the extract loaded on to a 20 ml DEAE-cellulose column

Abbreviations used: EGF, epidermal growth factor; PAGE, polyacrylamide-gel electrophoresis.

equilibrated with 25 mM-Tris/HCl, pH 7.7. The flow-through fraction was diluted 1:1 with 50 mM-Mes, pH 6.0, and loaded on to a 4 ml Fast S Sepharose column that was equilibrated with 50 mM-Mes (pH 6.0)/0.1 mM-EDTA. The column was subjected to sequential salt steps (50, 100, 150, 200, 250, 300, 400, 500, 1000 mM-NaCl) in the same buffer, each step consisting of two 3 ml collections. Peak fractions were subjected to gel filtration on a P150 column (1 cm × 40 cm) in 25 mM-Tris/HCl (pH 7.7)/1 mM-EDTA at a flow rate of 2 ml/h. Fractions were analysed for A_{280} and for phospholipase-A_2-inhibitory activity [5]. The 68 kDa protein inhibited the phospholipase A_2 with a specific activity of 10000 units (1 unit inhibits 15 ng of phospholipase A_2)/mg, displaying the same inhibition as lipocortin-1.

For peptide mapping with trypsin, the 68 kDa protein and lipocortin-1 (1 nmol each) first were subjected to reverse-phase h.p.l.c. on a C_4 column (Vydac; 0.46 cm × 25 cm). Polypeptides were eluted at 1 ml/min with a 45 min gradient of acetonitrile (0–75%) in 0.1% trifluoroacetic acid (0.5 min fractions were collected). Peak fractions were dried in a Savant Speed Vac Concentrator and suspended in 400 μl of 0.1 M-NH_4HCO_3/0.1 mM-$CaCl_2$. Trypsin was added in three equal portions of 1 μg each per sample: the first at zero time, the second after 3 h, and the third after 12 h. After a total of 16 h at 37 °C, the samples were acidified with formic acid to 20% (v/v) and subjected to reverse-phase h.p.l.c. on an Aquapore RP300 column (Brownlee Labs, Santa Clara, CA, U.S.A.; 0.21 cm × 10 cm). Peptides were eluted with a 95 min gradient of acetonitrile (0–75%) in 0.1% trifluoroacetic acid at 0.3 ml/min (0.5 min fractions were collected).

Preparation of transglutaminase-containing placental membranes

Transglutaminase preparations were generated from human placenta by the keratinocyte procedure [30]. Tissue (2 g) was suspended in 20 ml of 50 mM-Hepes (pH 7.5)/150 mM-NaCl containing 0.4 mg of bovine serum albumin/ml, 0.2 mM-phenylmethanesulphonyl fluoride, 10 mM-ε-aminohexanoic acid, 10 mM-benzamidine, 1 unit of aprotinin/ml, 2.5 mM-EDTA and 1 mM-$MgCl_2$. The tissue was dispersed with a Polytron, and sonicated. Membranes were pelleted by centrifugation at 100000 *g* for 30 min at 4 °C and washed twice with the same buffer, each time being followed by centrifugation at 30000 *g* for 15 min. The final pellet was suspended in 3 ml of the buffer, divided into batches, and stored at −70 °C.

For cross-linking assays, placental membrane preparations were washed three times with 60 mM-Hepes (pH 7.5)/100 mM-NaCl/2.5 mM-EDTA, each followed by a 5 min centrifugation step in a refrigerated Eppendorf centrifuge. The membranes were suspended at their initial concentration in the same buffer and subjected to cross-linking as described by Simon & Green [31]. Lipocortin (20 μl) was mixed with 20 μl of membranes, 10 μCi of [^{14}C]glycine ethyl ester (52 mCi/mmol) and 10 μl of 50 mM-$CaCl_2$/150 mM-Hepes, pH 7.5, and incubated at 37 °C for 15 min. The membranes were pelleted by centrifugation, and the lipocortin was extracted from the pellet with 50 μl of the EDTA-wash buffer. Particulates were again pelleted, and the EDTA extracts were either analysed directly by SDS/PAGE or quick-frozen for subsequent work. Plasmin digestion of cross-linked lipocortin-1 was performed as described in ref. [32].

Other procedures

Samples were analysed by SDS/PAGE in the Laemmli system [33]. Intact proteins were analysed on 12% polyacrylamide gels, and CNBr fragments on 15% gels. Gel profiles were made visible by staining with Coomassie Brilliant Blue R-250, by silver staining or by Western blotting [5] as indicated. Anti-lipocortin-1 antisera was produced in rabbits with recombinant human protein as immunogen [15]. Monoclonal antibodies also were developed by using recombinant lipocortin as immunogen (R. B. Pepinsky, L. K. Sinclair, I. Dougas & J. L. Browning, unpublished work). For protein blots, immunoreactive bands were detected with horseradish-peroxidase-conjugated second antibodies.

RESULTS AND DISCUSSION

Identification of a dimeric form of lipocortin-1 in placenta

Ca^{2+}/phospholipid-binding proteins from human placenta were isolated by disrupting the tissue with a Polytron in the presence of Ca^{2+} and then releasing the binding proteins with EDTA. The EDTA extract was fractionated by DEAE-cellulose chromatography as described previously [19]. Fig. 1 (lane a) shows an SDS/PAGE profile of the flow-through fraction. Three prominent bands with masses of 68, 35, and 32 kDa were detected. By Western-blot analysis the 35 kDa species was immunoreactive with polyclonal antibodies from human lipocortins-1 and -2, consistent with its being a mixture of both proteins [19]. The 32 kDa band was

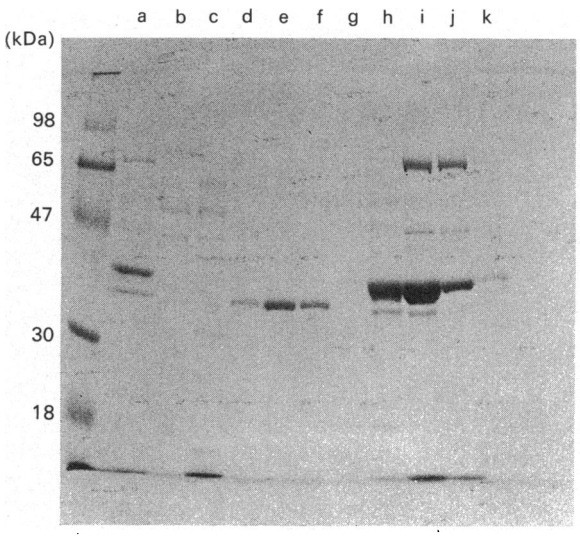

Fig. 1. Fractionation of placental Ca^{2+}-binding proteins on Fast S Sepharose

The DEAE-cellulose flow-through containing Ca^{2+} phospholipid-binding proteins from human placenta was fractionated on Fast S Sepharose. Bound proteins were eluted with sequential salt steps and analysed by SDS/PAGE. Proteins were detected with Coomassie Blue. Molecular-mass standards (kDa) at the left are BRL prestained markers. Before electrophoresis, samples were heated at 65 °C for 10 min in sample buffer (2% SDS, 0.05 M-Tris/HCl, pH 6.8, 12.5% glycerol, 1.5% 2-mercaptoethanol). Lane a, DEAE-cellulose flow-through. Lanes b–k, Fast S fractions corresponding to 100–1, 100–2, 200–1, 200–2, 250–1, 400–1, 400–2, 500–1, 500–2 and 1000 mM-NaCl.

recognized by antiserum raised against human lipocortin-4, and the 68 kDa band with the lipocortin-1 antibody. The 68 kDa protein was further purified by concentrating the preparation on Fast S Sepharose and then using gel filtration to resolve the protein from the 35–40 kDa lipocortins. Lanes b–k show fractions from the Fast S step. Lipocortin-4 was eluted with 200 mM-NaCl, whereas lipocortins 1 and 2 and the 68 kDa form were eluted with 500 mM-NaCl. Fraction 500–2 was subjected to gel filtration on a P150 column (see Fig. 2). The 68 kDa protein (fractions 31–34) was eluted with an apparent mass of 70 kDa, in agreement with its predicted size of 70 kDa by SDS/PAGE, and was well resolved from lipocortins 1 and 2 (fractions 39–40). From 150 g of washed placenta we recovered 6 mg of lipocortin-1, 3 mg of lipocortin-2, 1 mg of lipocortin-4 and 1.5 mg of the 68 kDa protein.

To test whether the immunoreactive component of the 68 kDa band was related to lipocortin-1 or a unique protein, lipocortin-1 and the 68 kDa form were subjected to CNBr mapping where cleavage products were detected by Western blotting using anti-lipocortin-1 antisera. The two cleavage profiles when analysed either directly by silver staining (Fig. 3, lanes a and b) or by Western blotting (lanes c and d) were quite similar, with 14 of the 18 bands from lipocortin-1 being identical in the two profiles. All of the cleavage products were immunoreactive, except for those under 8 kDa, which stain poorly on blotting, indicating that the 68 kDa protein is a form of lipocortin-1. Although a 68 kDa protein immunoreactive with anti-lipocortin-1 antisera has been observed previously [16,34,35], the identity of the protein was not established. The 68 kDa band differs from the acidic 68 kDa protein called lipocortin-6 [5], p68 [36], or 67 kDa calelectrin [11], which is not recognized by anti-lipocortin-1 antisera and has a distinct CNBr map [5,34].

We infer that the 68 kDa band is a dimer of lipocortin-1, based on its size and lack of additional cleavage products in the CNBr cleavage map. This inference was further substantiated by cleaving the protein with plasmin under conditions that produce a single cleavage [32] and subjecting these products to CNBr mapping. Plasmin digestion of the dimer produced a 32 and a 38 kDa fragment. CNBr-cleavage profiles for both fragments, like the CNBr profile for the intact 68 kDa moiety, were similar to the profile of lipocortin-1, sharing identity over the same 14 out of 18 CNBr fragments. Finally, this result was borne out in subsequent analyses where the site of cross-linking was localized to within the *N*-terminal tail of two adjacent lipocortin-1 molecules (see below).

Although lipocortin-1 is abundant in various tissues [16,19], the dimer is only observed as a prominent form in placenta. Why such a disparity exists in its distribution is unknown; however, several observations support the dimer being a physiological form in placenta. (i) The protein is prominent in crude placental fractions, and thus is not created artificially during purification [37]. (ii) In characterizing placental lipocortin-1, -2, -4, and -6 by Western blotting, Webb & Mahadevan [37] found that, of the four proteins, only lipocortin-1 was affected by the cross-linking. (iii) The protein has been observed in-

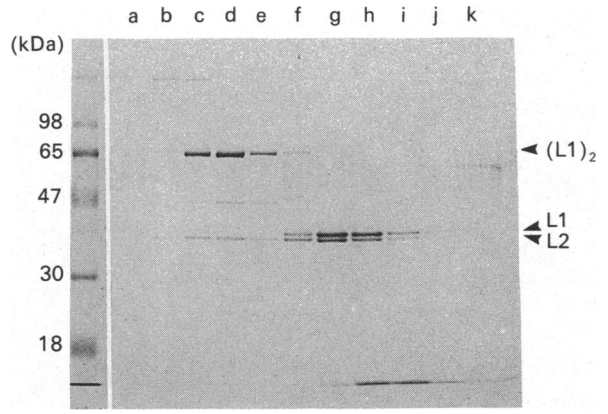

Fig. 2. Gel-filtration analysis of the Fast S 500–2 fraction

Ca^{2+}-binding proteins in the Fast S 500–2 fraction were subjected to gel filtration on a P150 column. Fractions were dissolved in reducing electrophoresis sample buffer and subjected to SDS/PAGE. Proteins were detected by staining with Coomassie Blue. Arrowheads denote the positions of lipocortin-1 (L1), lipocortin-2 (L2) and the lipocortin-1 dimer $(L1)_2$. Standards at the left of the panel are BRL prestained high-molecular-mass markers (kDa). Lanes a–k correspond to fractions 27, 29, 31, 33, 35, 37, 39, 41, 43, 45 and 47 respectively.

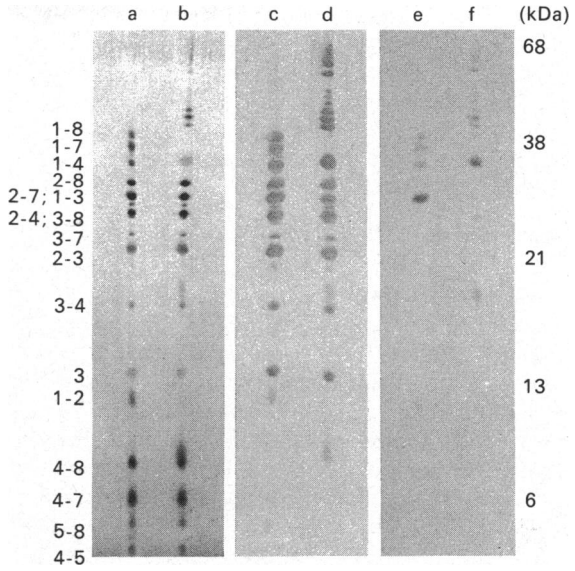

Fig. 3. Localization of intermolecular cross-link in dimeric lipocortin-1 by CNBr mapping

Preparations of lipocortin-1 and dimeric lipocortin-1 were subjected to SDS/PAGE. Bands were detected by copper staining [40], and the appropriate regions were excised. Gel slices were incubated for 1 h with 21 mg of CNBr/ml in 0.1 M-HCl/1% 2-mercaptoethanol, washed, and the fragments were subjected to SDS/PAGE analysis as previously described [15]. Cleavage products either were detected directly by silver staining (lanes a and b) or by Western blotting (lanes c–f). For protein blots, immunoreactive bands were stained with a polyclonal antibody to lipocortin-1 (lanes c and d) or with a monoclonal antibody that is specific for CNBr fragment-1 (lanes e and f). Lanes a, c and e are digests of lipocortin-1, and lanes b, d and f are digests of dimeric lipocortin-1. Fragment designations at the left are based on previously described data for lipocortin-1 [39]. Apparent molecular masses (kDa) of specific fragments are indicated at the right.

dependently in placental preparations from five different laboratories using very different purification strategies [16,19,21,34,37]. Although the role of the dimer is unknown, the sheer amount of the form argues that lipocortin-1–lipocortin-1 interactions must be of sufficient magnitude and affinity that the protein can be specifically cross-linked with itself.

Localization of the site of cross-linking by peptide mapping

The site of cross-linking within the lipocortin-1 dimer was localized by a two-step process, first using CNBr mapping to identify the region in lipocortin that was modified and then using tryptic mapping to focus in on peptides of interest within the larger CNBr fragment. Digestion with CNBr cleaves lipocortin-1 into eight 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 analysing partial CNBr digests in a nearest-neighbour-type analysis, each complete CNBr fragment is represented by a distinct subset of the cleavage products, which can be used to map structural differences [38]. This approach has been applied previously to lipocortin-1 for identifying phosphorylation sites [39]. Although most CNBr-cleavage products of lipocortin-1 and of the 68 kDa variant are identical, those that are different in each instance correspond to fragment-1-containing partial cleavage products. This result is most apparent by analysing fragment-1-containing cleavage products directly by Western blotting using a monoclonal antibody specific for fragment 1 of lipocortin-1. In the digest of lipocortin-1 (Fig. 3, lane e), fragments 1-2, 1-3, 1-4, 1-7 and 1-8 are specifically recognized. In the digest of the 68 kDa variant (lane f) all of the corresponding fragments are shifted by about 4 kDa in their apparent mass. The differences in the two profiles highlighted by the monoclonal antibody correspond to all of the differences in the cleavage profiles observed by silver staining and by Western blotting. The disappearance of fragment-1-containing cleavage products in the digest of the 68 kDa form of lipocortin-1 indicates that the cross-link is homotypic, linking fragment 1 from one molecule to fragment 1 in a second, since this is the only type of linkage that will completely eliminate a CNBr fragment.

The site of cross-linking was further localized by tryptic-peptide mapping, using h.p.l.c. to separate cleavage products. Published sequences for the 36 major peaks from the tryptic map of lipocortin-1 [32] identify about 85% of its primary structure. Of these, the elution positions for all of the tryptic fragments from CNBr fragment 1 are known, and thus the profile can be used directly to identify the modified fragment. Fig. 4 shows tryptic-peptide maps for lipocortin-1 (panels *a* and *b*) and for the 68 kDa variant (panels *c* and *d*). Like CNBr maps, the tryptic-peptide maps of the two proteins are quite similar. Fractions 81, 82 and 75 (panel *a*) correspond to peptides derived from CNBr fragment 1 and represent amino acid residues 1–8, 9–25 and 29–52 respectively. The short peptide encoded by residues 26–28 is in the column flow-through, and fraction 88 contains a partial cleavage product that corresponds to residues 1–25. In the dimer profile, the peak corresponding to residues 9–25 (fraction 82) is missing from the map, whereas other peptides from this region are unchanged, indicating that peptide 9–25 is involved in the linkage. This result is particularly apparent in the A_{280} profiles (compare panels *b* and *d*), since peptide 9–25 is the only tryptophan-containing peptide in lipocortin-1. The corresponding peak is prominent in the lipocortin-1 map (panel *b*, fractions 82 and 88) and missing from the map of dimeric

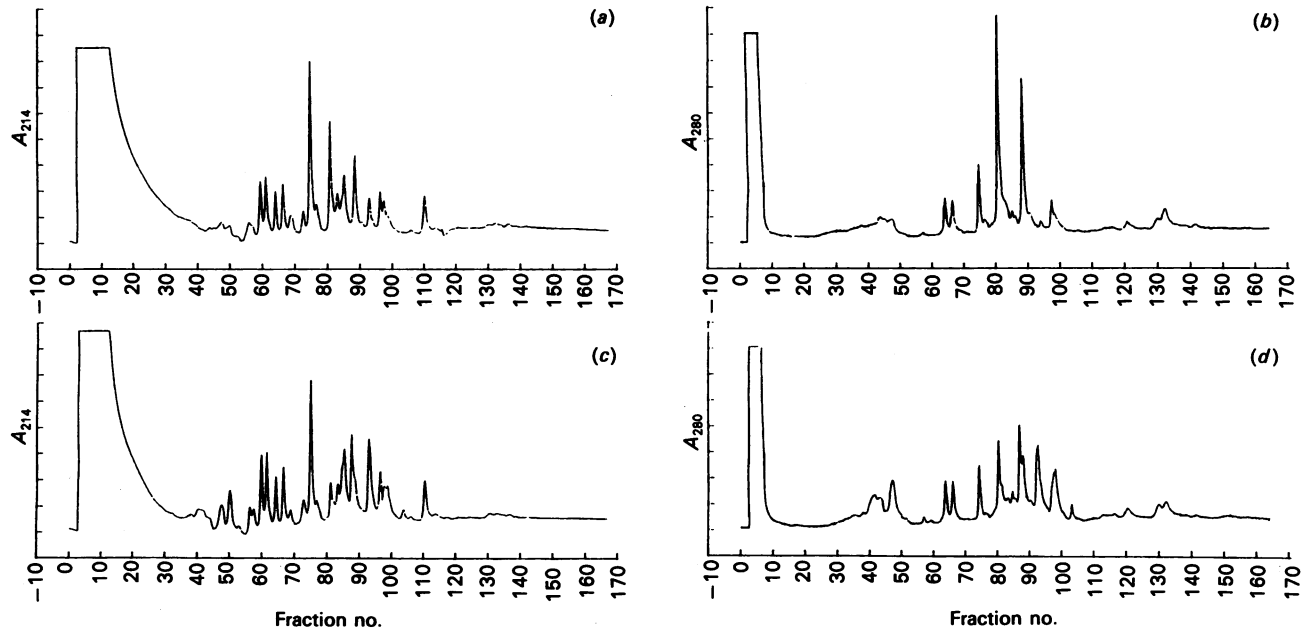

Fig. 4. Tryptic-peptide maps of lipocortin-1 and of dimeric lipocortin-1

H.p.l.c.-purified preparations of lipocortin-1 and of the dimeric protein were digested with trypsin, and the digests were analysed by reverse-phase h.p.l.c. on a C_8 column. Column eluates were monitored for protein by absorbance at 214 nm and at 280 nm: (*a*) lipocortin-1, 214 nm; (*b*) lipocortin-1, 280 nm; (*c*) dimeric lipocortin-1, 214 nm; (*d*) dimeric lipocortin-1, 280 nm.

Table 1. Sequence analysis of selective tryptic peptides from the lipocortin-1 dimer

Tryptic fragments from the human lipocortin-1 dimer were subjected to *N*-terminal protein sequence analysis in an Applied Biosystems 470A gas-phase sequencer in the presence of Polybrene [41]. Phenylthiohydantoin (PTH) derivatives were identified on-line with an Applied Biosystems 120A PTH analyser. Fragment designations correspond to peak fractions from peptide maps. Each sequence was compared with the corresponding amino acid sequences from human lipocortin-1 [42], and their positions with respect to the sequence are indicated.

Human sequences	Alignments
Lipocortin-1 dimer	
T75: G G P G S A V S P Y P T F N P S S D V A	29–48
T88a: G T D V N V F N T I L T T R	214–227
T88b: M Y G I S L – Q A I L D E T K	317–331
T89: Q A W F I E N E E – E Y V Q T V K	9–25
T93: G V D E A T I I D I L T K	58–70
T98: G L G T D E D T L I E I L A S R	128–143

lipocortin-1 (panel *d*). In panel (*d*), new peaks containing A_{280} occur at fractions 89, 93 and 98, and thus are likely to have been generated by the cross-linking.

Sequence information for selective peaks from the tryptic map of the lipocortin-1 dimer are presented in Table 1. In particular the sequence corresponding to fragment 29–52 (T75) confirms that it is not affected by the cross-link. Fractions T89, T93 and T98 also were analysed, but of these only fraction T89 produced a sequence that was derived from the *N*-terminal region of lipocortin-1. Although the other fractions yielded major sequences, neither contained A_{280}-producing amino acids, suggesting that the fractions both contain components that were missed by sequencing. Since the lipocortin sequence starts with acetylated alanine, one possible explanation for this result is that the missing fragments are derived from amino acid residues 1–25 and thus blocked to sequencing. Interestingly, in the T89 sequence, glutamine-18 from cycle 10 was missing, whereas the appropriate amino acids from all other cycles were normal. Throughout the run, sequences that carry over from peptides found in the adjacent fraction T88 were readily scored, suggesting that the specific loss of glutamine-18 may be a result of the dimer linkage. In previous studies, where we sequenced the corresponding fragment from lipocortin-1, glutamine-18 was observed [32], supporting this notion. Although further work is needed to characterize the linkage better, the specific loss of glutamine suggests that the cross-linking may result from a transglutaminase reaction.

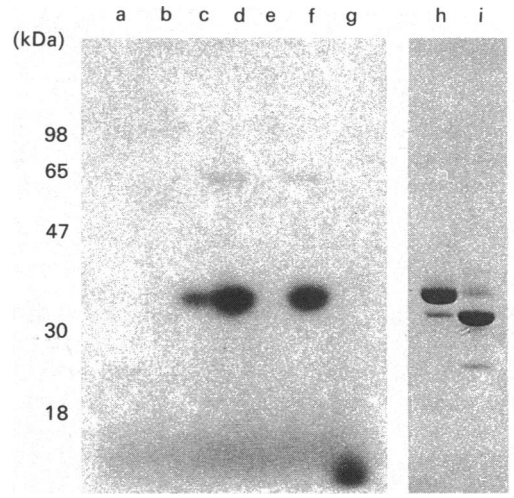

Fig. 5. Cross-linking of labelled glycine to lipocortin-1

Placental membranes were incubated with lipocortin-1 and with [^{14}C]glycine ethyl ester, and the extent of cross-linking was assessed by SDS/PAGE (lanes a–e). Labelled lipocortin was detected by fluorography. Lane a, no lipocortin; lane b, 5 μM-lipocortin 1; lane c, 13 μM-lipocortin 1; lane d, 40 μM-lipocortin-1; lane e, 40 μM-lipocortin-1 in the absence of added Ca^{2+}. Samples of cross-linked extracts from lane d were subjected to limited digestion with plasmin for 1 h at 23 °C at a lipocortin/plasmin ratio of 10:1 (w/w). Samples were subjected to gel analysis and detected by fluorography (lanes f and g) or by staining with Coomassie Blue (lanes h and i). Lanes f and h, no added plasmin; lanes g and i, with plasmin.

Transglutaminase-induced cross-linking of lipocortin-1

To test if lipocortin-1 is a transglutaminase substrate, we followed procedures developed for studying cross-linking of involucrin *in vitro* by using sonicated cell membranes as a source for the Ca^{2+}-dependent transglutaminase and labelled glycine ethyl ester as a co-substrate for the enzyme [30,31]. When placental membranes were incubated with lipocortin-1 and with [^{14}C]glycine ethyl ester and then analysed by SDS/PAGE, the lipocortin was labelled (see lanes a–d, Fig. 5). The incorporation of label into lipocortin varied as a function of added protein. At the highest protein concentration (lane d), about 10% of the protein was labelled, and thus the efficiency of cross-linking for glycine to lipocortin is similar to that reported for cross-linking glycine to involucrin. No label was incorporated into lipocortin in the absence of added Ca^{2+} (lane e).

The site of cross-linking was localized within the *N*-terminal tail region of lipocortin-1 by limited proteolysis with plasmin. Digestion with plasmin provides a simple method for dissecting the *N*-terminal region from the remainder of lipocortin-1, and has been used previously to localize sites of phosphorylation [39]. Lanes f and g (radioactivity) and h and i (stained profiles) show results for digestion of lipocortin-1 cross-linked to the labelled glycine. After digestion, the label was selectively released, as evident by the label migrating near the front of the gel

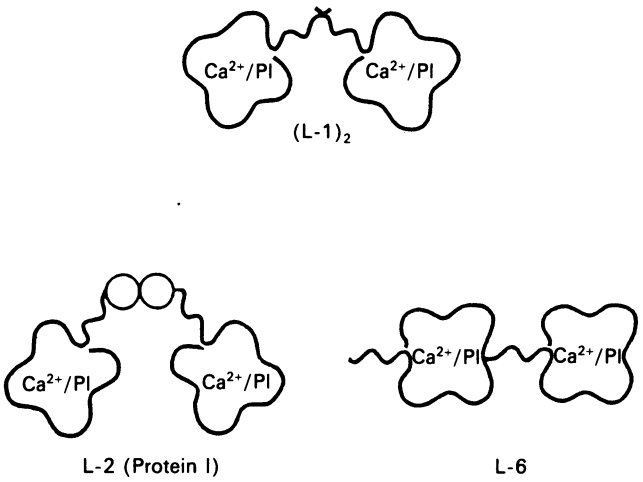

Fig. 6. Schematic drawings of structural variants of 70 kDa lipocortins

Schematic drawings of the subunit structures of lipocortin-1 dimer $(L-1)_2$ and protein I (L–2) and for lipocortin-6 (L–6; p68) are presented, and highlight contact points among the various tail regions. Ca^{2+}/Pl denotes the fourfold 70-amino-acid repeat units that serve as the Ca^{2+}/phospholipid-binding domain, and crooked lines denote the exposed *N*-terminal tail region. Closed circles in L–2 represent p10 molecules.

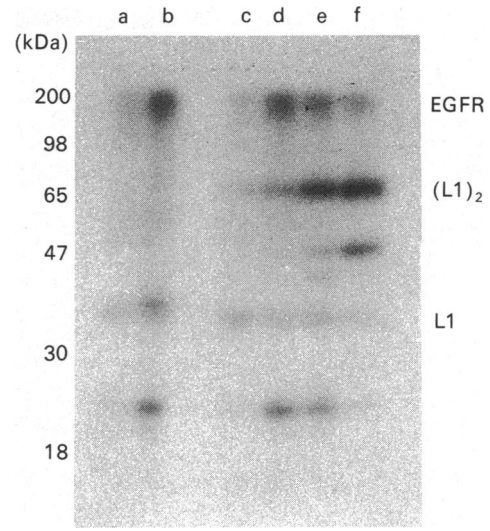

Fig. 7. EGF-dependent phosphorylation of dimeric lipocortin-1

A431 cells were grown at 37 °C in Dulbecco's modified Eagle medium with 10% (v/v) fetal-calf serum. Cell membranes were prepared as previously described [15] in either the absence (lanes a and c) or the presence (lanes b, d–f) of EGF. Membranes were incubated at 0 °C for 5 min with 0.2 μg of lipocortin-1 (lanes a and b) or dimer (lanes c–f), and the reactions were quenched with electrophoresis sample buffer. Samples were subjected to SDS/PAGE on 12.5%-polyacrylamide gels. Gels were treated with 1 M-KOH for 1 h at 55 °C, washed, dried, and subjected to autoradiography. Regions corresponding to the 68 kDa band were excised and counted for radioactivity: lane c, 0.6 μg of dimer (970 c.p.m); lane d, 0.2 μg of dimer (2650 c.p.m.); lane e, 0.6 μg of dimer (5920 c.p.m.); lane f, 1.8 μg of dimer (7310 c.p.m.). Positions of lipocortin-1 (L1), dimer $[(L1)_2]$ and the EGF receptor/kinase (EGFR) are indicated.

(lane g). In contrast, most of the protein (lane i) only shifted from a molecular mass of 35 kDa to 32 kDa, confirming that the cross-linking is specific and selective for the tail region.

Fig. 6 shows a schematic summary of the various 70 kDa lipocortin-like proteins, contrasting the structural features of the lipocortin-1 dimer with the protein I complex of lipocortin-2 (L-2) and with lipocortin-6 (L-6). Like protein I, the lipocortin-1 dimer is organized in a head-to-head association with the subunits tied together at their *N*-terminus. In lipocortin-2 this is mediated through a non-covalent association of the tail region of each subunit with a p10 molecule forming a tetrameric complex (see [28] for references). With lipocortin-1, the dimer is covalently fixed in this conformation. Lipocortin-6 is a single polypeptide and thus is fixed in a head-to-tail arrangement of the two 35 kDa units.

Properties of dimeric lipocortin-1

The Ca^{2+}/phospholipid binding property of the dimeric lipocortin-1 was investigated by monitoring the extent of binding to sonicated phosphatidylcholine/phosphatidylserine (1:1) vesicles at a constant concentration of lipid and increasing amounts of Ca^{2+} as previously described [17]. To facilitate the comparison with lipocortin-1, we mixed the two proteins and then simultaneously analysed their binding to vesicles by SDS/PAGE. Lipocortin-1 binding was first detected at 80 μM-Ca^{2+} and was complete with 120 μM, whereas binding with the dimeric form was detected at 40 μM-Ca^{2+} and was complete at 80 μM, thus indicating that dimerization does not contribute significantly to the binding affinity (results not shown).

Lipocortin-1 is a major substrate of the EGF receptor/kinase [14–16]. To test if dimer formation affects this property, we evaluated the phosphorylation of the dimer using A431 cell membranes as a source of the kinase (see Fig. 7). Whereas in the absence of EGF the protein was barely detected (lane c), the dimer is the major phosphorylated protein in the EGF-treated samples. Lanes d–f show a concentration-dependent phosphorylation of the dimer. EGF treatment resulted in about a 6-fold increase in phosphorylation of the 68 kDa band, which is similar to the enhancement previously seen with lipocortin-1. Using placental preparations, Haigler and co-workers also observed EGF-dependent phosphorylation of a 68 kDa band that was recognized by an anti-lipocortin-1 antiserum; however, the protein was not further characterized [35]. From our studies, we infer that this form is dimeric lipocortin-1.

In addition to the dimer, we also observed a 45 kDa protein in the placental preparation that showed EGF-dependent phosphorylation (see Fig. 7, lane f). Although at present we have not characterized the protein, it is recognized by the anti-lipocortin-1 monoclonal antibody, and thus is likely to be another modified form of lipocortin-1. The presence of other immunoreactive species such as the 45 kDa band suggest that other proteins also become cross-linked to lipocortin-1, which may allow us to identify potential targets of lipocortin action. Although the role of the transglutaminase-induced cross-linking of lipocortin-1 is unknown, it

defines another post-translational modification of lipocortin-1 which may be functionally significant.

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