Expression of human recombinant lipocortin I in a wheat-germ cell-free system and *Xenopus* oocytes

Lipocortin is not secreted

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Lipocortin I has been presumed to be synthesized and secreted in response to glucocorticoids yet the amino acid sequence of lipocortin I reveals no signal sequence typically necessary for proteins to enter the secretory pathway. The translocation of lipocortin I across membranes was analyzed in a cell-free system and in *Xenopus* oocytes. Based on the published sequence, the cDNA of human lipocortin I was cloned and expressed in *Escherichia coli*. Lipocortin I was purified and used to raise monoclonal antibodies. To test whether lipocortin I is secreted *in vitro*, transcribed lipocortin mRNA was translated in a wheat germ cell-free system in the absence and presence of microsomal membranes. Prolactin mRNA was used as a control for translocation of newly synthesized protein into membrane vesicles. Prolactin, but not lipocortin I, was translocated into the membranes. To test for secretion of lipocortin I *in vivo*, *Xenopus* oocytes were co-injected with transcripts encoding lipocortin I and prolactin, with and without the signal sequence. Prolactin with the signal sequence was released into the medium. However, neither prolactin without a signal sequence nor lipocortin I was released. Carbonate extraction, using an integral transmembrane protein as control, revealed no evidence for membrane integration of lipocortin I. Thus lipocortin I is not a secreted protein.

INTRODUCTION

Characterization of the biological properties of glucocorticoid-inducible phospholipase A_2 inhibitory proteins (lipocortins) has indicated that they (i) are synthesized and secreted in response to glucocorticoids, (ii) inhibit pancreatic phospholipase A_2 *in vitro*, (iii) inhibit eicosanoid synthesis when added to cells, (iv) have anti-inflammatory activity in animal model systems of acute inflammation, and (v) are regulated by phosphorylation (Flower & Blackwell, 1979; Hirata, 1981; DiRosa *et al.*, 1984; Flower, 1984; Bienkowski *et al.*, 1989; Cirino *et al.*, 1989). The gene encoding a member of the lipocortin family, human lipocortin I, has been cloned and sequenced (Wallner *et al.*, 1986). Human lipocortin I expressed in *Escherichia coli* (r-lipocortin I) displays anti-phospholipase A_2 activity *in vitro* (Wallner *et al.*, 1986), blocks the release of eicosanoids from guinea-pig perfused lung (Cirino *et al.*, 1987), inhibits the release of prostacyclin from human umbilical artery rings (Cirino & Flower, 1987), mimics the inhibitory effect on superoxide generation by glucocorticoids in alveolar macrophages and diminishes carrageenin-induced oedema in the rat paw assay (Wallner *et al.*, 1986; Cirino *et al.*, 1987; Cirino & Flower, 1987; Cirino *et al.*, 1989; Maridonneau-Parini *et al.*, 1989). Similar biological effects were also observed when non-recombinant lipocortin I was purified and applied extracellularly in various model systems (Hirata *et al.*, 1981; Blackwell *et al.*, 1983; Parente *et al.*, 1984; Pepinsky *et al.*, 1986; Fradin *et al.*, 1988; Maridonneau-Parini *et al.*, 1989).

That extracellular addition of r-lipocortin I yields biological effects suggests the site of action of lipocortin I is extracellular and therefore lipocortin I must be secreted. This assumption concords with abrogation of dexamethasone-induced inhibition of prostanoid release from human embryonic skin fibroblasts or polymorphonuclear neutrophils and dexamethasone-induced inhibition of glycoconjugate secretion in tracheal organ cultures

being reversed by addition to the culture medium of a monoclonal antibody against lipocortin (Errasfa *et al.*, 1985; Fradin *et al.*, 1988; Lundgren *et al.*, 1988; Solito & Parente, 1989). Further evidence for lipocortin I release comes from the findings that lipocortin I can be purified from cell-free peritoneal exudates of dexamethasone-stimulated rats (Pepinsky *et al.*, 1986; Wallner *et al.*, 1986) and from the partial purification and/or demonstration of lipocortin-like activity in the supernatants of glucocorticoid-stimulated lymphocytes, thymocytes, macrophages, fibroblasts, renal medullary or endometrial cells and in the perfusate of dexamethasone-treated lung preparations (Flower & Blackwell, 1979; Blackwell *et al.*, 1980; Russo-Marie & Duval, 1982; Hirata & Iwata, 1983; Gupta *et al.*, 1984; Errasfa *et al.*, 1985; Gurpide *et al.*, 1986).

Recently, the mode of regulation and action of lipocortin I has been questioned. Evidence has been presented that first glucocorticoids do not induce lipocortin I (Broennegard *et al.*, 1988; Bienkowski *et al.*, 1989) and second, that lipocortin I does inhibit neither phospholipase A_2 itself or the release of prostanoids (Davidson *et al.*, 1987; Hullin *et al.*, 1989; van Binsbergen *et al.*, 1989). Using translocation experiments across membranes in a cell-free system and in *Xenopus* oocytes, we demonstrate that lipocortin I is not secreted. Thus the conclusion that lipocortin I, the first clone isolated coding for a member of the lipocortin family, fulfils the operational definitions of a lipocortin, has to be revised.

MATERIALS AND METHODS

Materials

Restriction endonucleases, SP6 RNA polymerase, T4 DNA ligase, Klenow fragment of *E. coli* and proteinase K were from Boehringer Mannheim Diagnostics, Houston, TX, U.S.A. or from New England Biolabs, Beverly, MA, U.S.A. RNasin

Abbreviations used: HAT, hypoxanthine-aminopterin-thymidine; MBSH, modified Barth's saline solution; PMSF, phenylmethanesulphonyl fluoride; TCA, trichloroacetic acid; r-, recombinant.

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(RNAase inhibitor) was from Promega Biotec, Madison, WI, U.S.A. Rabbit anti-(ovine prolactin) serum was from United States Biochemical Corp., Cleveland, OH, U.S.A., anti-mouse IgG coupled to Sepharose 4B was from Cappel Laboratories, Cochranville, PA, U.S.A., and Protein A-Sepharose, pUC19 and M13 from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A. Frogs (*Xenopus laevis*) were obtained from Nasco, Fort Atkinson, WI, U.S.A., and [³⁵S]methionine was from New England Nuclear, Boston, MA, U.S.A., and [³²P]ATP from Amersham, Amersham, Bucks., U.K. The oligonucleotide was synthesized by Dr. L. Kuehn, ISREC, Lausanne, Switzerland, the *EcoRI*/*NdeI* linkers were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden, and the *NdeI* linkers were from Promega Biotec, Madison, WI, U.S.A. BCA protein assay reagent was purchased from Pierce Chemical Corp., Rockford, IL, U.S.A.

Methods

Cloning of human lipocortin I. A human placenta cDNA library (a gift from Dr. B. Wermuth, Bern, Switzerland) constructed in the vector λ gt11 was screened by hybridization with a ³²P-labelled oligonucleotide probe. This probe was a 50-mer complementary to the nucleotide sequence 1–50 of human lipocortin I (Wallner *et al.*, 1986). Phage from plaques hybridizing with the probe were isolated and amplified. The lipocortin I cDNA insert was obtained by digesting λ gt11 DNA with *EcoRI*. The lipocortin I cDNA insert was digested with *Sau3A*, sub-cloned into phage M13 and characterized by sequencing the entire coding region using the dideoxy-chain-termination method (Sanger *et al.*, 1977). The *EcoRI*-cleaved DNA fragment, starting at nucleotide 16 of the lipocortin I open reading frame and extending into the 3' untranslated region, was sub-cloned into pUC19 and amplified using *E. coli* JM 101 as a host. Gel-purified lipocortin I cDNA was then inserted either in the correct or in the reverse (control) orientation into *NdeI*-digested expression vector pET-3c (a gift from Dr. A. Rosenberg, Upton, NY, U.S.A.; Rosenberg *et al.*, 1987) after ligation to a synthetic *EcoRI*/*NdeI* linker (pET-3cLip). The *EcoRI*/*NdeI* linker:

restored the missing first five amino acids of the lipocortin I open reading frame.

Cloning of human lipocortin I into pSP64T. The vector pSP64T, generously given by Dr. D. Melton (Krieg & Melton, 1984), was cut with *Bgl*II and treated with Klenow fragment to generate blunt ends. Phosphorylated *NdeI* linkers (5'-d(CCATATGG)-3') were ligated to the Klenow-treated plasmid using T4 DNA ligase. The linker-bearing plasmid was transformed into *E. coli* δ H5- α . *NdeI* site-containing plasmid was isolated, digested with *NdeI*, re-ligated with T4 DNA ligase and re-cut with *NdeI*. This *NdeI*-digested plasmid was treated with calf intestinal phosphatase, added in a 1:16 molar ratio to a gel-purified lipocortin I cDNA insert. The lipocortin cDNA insert was obtained by digesting pET-3cLip with *NdeI*. The mixture was treated with T4 DNA ligase to generate pSP64TLip and transformed into *E. coli* δ H5- α .

In vitro synthesis of capped transcript. The plasmid pSP64TLip was linearized with *Pvu*II, extracted with phenol/chloroform, precipitated with ethanol and dissolved in water. Transcription was carried out in 10 μ l volumes containing 2.0 μ g of linearized DNA, 4 units of SP6 polymerase, 0.2 mg/ml of calf liver tRNA, 0.5 mM-ATP, 0.5 mM-CTP, 0.5 mM-UTP, 0.1 mM-GTP, 0.5 mM-m⁷GpppG, 10 mM-dithiothreitol, 2 mM-spermidine,

6 mM-MgCl₂, 40 mM-Tris/HCl, pH 7.5, and 8 units of RNasin. Reactions were carried out at 40 °C for 1 h and aliquots of total reaction mix were used directly for injection or *in vitro* translation.

Cell-free translation. Wheat germ extract was prepared according to Roman *et al.* (1976). Aliquots (2 μ l) of transcription reaction mix were added to translation reactions of 10 μ l volume containing 20 % wheat-germ extract, 1 mCi [³⁵S]methionine/ml, 0.2 mM each of the other 19 amino acids, 1 mM-ATP, 1 mM-GTP, 4 mM-phosphocreatine, 40 μ g of creatine phosphokinase/ml, 100 μ g of calf liver tRNA/ml, 20 mM-Tris/HCl, pH 7.5, 2 mM-MgCl₂, 0.8 mM-spermidine, 140 mM-potassium acetate, 2 mM-dithiothreitol and 8 units of RNasin. Reaction mixtures were incubated at 24 °C for 1 h in the absence or presence of dog pancreas rough microsomes (Walter & Blobel, 1983).

Post-translational proteinase protection. After incubation for 1 h at 24 °C in the presence of dog pancreas membranes, translation mixtures were chilled on ice, adjusted to 10 mM-CaCl₂, and divided into aliquots of 10 μ l. Some were treated with proteinase K [dissolved in 10 mM-Tris/HCl (pH 8)/10 mM-CaCl₂, and pre-incubated at 37 °C for 30 min] at a final concentration of 0.2 mg/ml in either the presence or absence of 0.5 % Triton X-100. Proteinase K digestion was stopped by transferring the entire sample into 10 vols. of 0.1 M-Tris/HCl (pH 8.0)/1 % SDS/2 mM-phenylmethanesulphonyl fluoride (PMSF) in a boiling-water bath and incubating at 100 °C for 10 min. Samples were diluted 20-fold in 1 % Triton X-100/0.1 M-Tris/HCl (pH 8.0)/10 mM-EDTA/100 mM-NaCl, subjected to immunoprecipitation with monoclonal anti-lipocortin antibody (see below) or polyclonal anti-prolactin antibody (Rothman *et al.*, 1988) and fractionated by SDS/PAGE. Fluorographed gels were subjected to autoradiography.

Microinjection of *Xenopus* oocytes. *Xenopus* oocytes were manually dissected and subsequently injected and labelled in modified Barth's saline solution (MBSH), containing [³⁵S]methionine, as described previously (Gurdon *et al.*, 1971). MBSH contains 88 mM-NaCl, 1 mM-KCl, 2.4 mM-NaHCO₃, 0.82 mM-MgSO₄, 0.33 mM-Ca(NO₃)₂, 0.41 mM-CaCl₂, 10 mM-Hepes, pH 7.6.

Immunoprecipitation of oocyte translation products. Medium and oocytes were separated. To 100 μ l of medium, 50 μ l of 1 % SDS/0.1 M-Tris/HCl (pH 8.0)/2 mM-PMSF was added and the mixture, boiled for 5 min; oocytes were homogenized in 20 μ l of 1 % SDS/0.1 M-Tris/HCl (pH 8.0)/2 mM-PMSF/oocyte and boiled for 5 min. Medium and oocyte homogenates were adjusted to 1 ml with buffer A [1 % Triton X-100/0.1 M-Tris/HCl (pH 8.0)/0.1 M-NaCl/0.01 M-EDTA/2 mM-PMSF] and incubated for at least 12–16 h at 4 °C in order to release proteins. After incubation, samples were centrifuged in an Eppendorf centrifuge at 10000 rev./min for 5 min to remove aggregates. The supernatants were transferred to fresh tubes. Purified monoclonal anti-lipocortin antibody or polyclonal anti-prolactin antibody (control) was added stepwise to each tube. The mixtures were incubated for 8–12 h at 4 °C, 15 μ l of a 50 % slurry of anti-mouse IgG coupled to Sepharose 4B (for lipocortin precipitation) or Protein A-Sepharose 4 CL4B (for prolactin precipitation) in buffer A added, and incubation continued overnight at 4 °C using a rotator. The Sepharose samples were washed three times with buffer A and subsequently twice with buffer containing 0.1 M-Tris/HCl, pH 7.5, 0.1 M-NaCl and 2 mM-PMSF to remove residual Triton X-100.

Carbonate extraction (Fujiki *et al.*, 1982). A crude vesicle

preparation was obtained from oocytes micro-injected with [^{35} S]methionine and transcripts encoding lipocortin, bovine pre-prolactin and a chimaeric transmembrane protein composed of the signal sequence of *E. coli* β -lactamase (S_L) fused sequentially to 109 amino acids of chimpanzee α -globin containing a glycosylation site (gG), the stop transfer sequence (ST) of mouse IgM heavy chain and 150 residues of bovine prolactin (P) (Simon *et al.*, 1987; Rothman *et al.*, 1988). The chimaeric protein was named pSPS $_L$ -gG-ST-P (Rothman *et al.*, 1988). [^{35} S]methionine and the three transcripts were micro-injected simultaneously (1.2:0.2:0.6, v/v). The oocytes were homogenized (20 μ l/oocyte) at 4 °C in an iso-osmotic buffer containing 10% (w/v) sucrose, 150 mM-NaCl, 10 mM-magnesium acetate and 20 mM-Tris/HCl, pH 7.6. A crude vesicle preparation was obtained by using a ground-glass homogenizer. Each sample was divided into two aliquots. One aliquot was treated with 5 ml of 0.1 M-Na $_2$ CO $_3$, pH 11.5, the other with 0.25 M-sucrose/0.1 M-Tris/HCl (pH 7.5), and both were incubated for 30 min on ice. The extracted samples were then centrifuged for 1 h at 55000 rev./min in an ultracentrifuge (Rotor Ti70.1, Beckman). The supernatants were removed, neutralized with acetic acid, 50 μ g of lysozyme added as carrier and the mixture precipitated with a final concn. of 15% trichloroacetic acid. Trichloroacetic acid precipitates were spun down and washed with diethyl ether/ethanol (1:1, v/v). Both precipitates and original pellets were dissolved in 100 μ l of 0.1 M-Tris/HCl (pH 8.9)/1% SDS and incubated at 37 °C for 30 min and at 100 °C for 2 min. Samples were then diluted with buffer A for immunoprecipitation.

Expression of lipocortin in *E. coli* HB101. The pET-3cLip plasmid and the control plasmid contained the entire coding sequence of lipocortin I either in correct or reverse orientation, the phage T7 promoter, the phage T7 gene 10 translation start and transcription terminator site (Rosenberg *et al.*, 1987). This vector directs the production of large amounts of proteins in *E. coli* HB101 after infection by CE6, a phage λ derivative that carries the gene for T7 RNA polymerase (Studier & Moffatt, 1986). Transformed *E. coli* HB101 cultures were grown in the presence of ampicillin (20 μ g/ml) and 0.2% maltose at 37 °C in a shaking water bath (Schwartz, 1967). When the A_{600} reached a value of 0.6–1.0, MgSO $_4$ was added to a final concentration of 10 mM and purified CE6 phage to a final concentration of 1×10^9 /ml. Cells were harvested 3 h after infection. The bacteria were centrifuged at 1000 g for 5 min, suspended in SDS/PAGE loading buffer (Maniatis *et al.*, 1982), boiled for 10 min and loaded on to a 12.5% polyacrylamide gel. Gels were stained with Coomassie Blue or silver.

Monoclonal antibodies. Human lipocortin I was prepared for transformed *E. coli* HB101. The bacteria were centrifuged, suspended in 150 mM-KCl/2 mM-MgCl $_2$ /20 mM-Hepes, pH 7.4, and passed twice through a Yeda press operated at a nitrogen pressure of 505 kPa. Immediately CaCl $_2$ was added to the lysate to a final concentration of 1 mM. The mixture was kept on ice for 30 min, and centrifuged for 15 min at 7200 g at 4 °C. The sediment was washed twice with 150 mM-KCl/2 mM-MgCl $_2$ /20 mM-Hepes, pH 7.4, containing 1 mM-CaCl $_2$. The sediment was re-suspended in the same buffer containing 2 mM-EGTA instead of CaCl $_2$ in order to abrogate the Ca $^{2+}$ -dependent binding of lipocortin I to phospholipids (Glenney & Zokas, 1988). Following centrifugation (7200 g , 15 min, 4 °C) the supernatant was fractionated on a 12.5% polyacrylamide gel. A small part of the gel was stained with Coomassie Blue and the rest of the gel was soaked in a solution containing 0.25 M-KCl/2 mM-dithiothreitol at 4 °C until protein bands became visible. The lipocortin I band was removed from the gel and washed with

water until clear. Lipocortin I was isolated from the gel slice by electro-elution (Electro-Eluter, model 422; Bio-Rad, Glattbrugg, Switzerland).

Lipocortin I (100 μ g), emulsified with 1 vol. of complete Freund's adjuvant, was injected into the peritoneal cavity of 6-week-old BALB/cJ mice. Mice were boosted twice with 100 μ g of lipocortin I in incomplete Freund's adjuvant and killed 4 days after the last booster injection. The spleen was excised and somatic-cell fusion was performed by the method initially described by Koehler & Milstein (1975) using PA1 myeloma cells (Stocker *et al.*, 1982). Suspensions of fused cells in selective medium containing hypoxanthine, aminopterin and thymidine (HAT) were divided into ten tissue-culture plates (Costar 3596; 96 wells/plate) at the rate of 200 μ l/well. Samples of medium from each well were screened for anti-(lipocortin I) antibody activity using the dot-blot procedure and Western blots. Cells from positive wells were sub-cloned by limiting dilution in 96-well tissue-culture plates. Selected clones were expanded *in vitro* using Iscove's modified Dulbecco's medium enriched with 2% fetal-calf serum. The monoclonal antibodies were isolated from the hybridoma supernatants by protein G Sepharose affinity purification using buffers as recommended by the manufacturer. Immunoglobulin classes of monoclonal antibodies were determined with the Mouse Typer Isotyping Kit (Bio-Rad). Protein concentration was measured by using the BCA protein assay.

RESULTS AND DISCUSSION

A cDNA encoding human lipocortin I was isolated from a human placenta library in the vector λ gt11 by screening with an oligonucleotide probe ('Materials and methods' section). The full-length lipocortin I cDNA was sequenced and revealed complete identity with the published sequence of lipocortin I

Fig. 1. Expression of lipocortin I in *E. coli* HB101

Total cell lysates were fractionated by SDS/PAGE and the protein bands revealed by Coomassie Blue staining. Lanes: 1, vector pET-3cLip in *E. coli* HB101, not induced; 2, pET-3cLip, protein production from vector induced by addition of 1×10^8 phages/ml; 3, pET-3cLip, induced with 1×10^8 phages/ml; 4, control (cDNA in the reverse orientation), not induced; 5, control, induced with 1×10^8 phages/ml; 6, control, induced with 1×10^8 phages/ml. The position in the gel of lipocortin I is indicated by the arrow.

Fig. 2. Enrichment of lipocortin I expressed in *E. coli* HB101

SDS/PAGE analysis of enriched lipocortin I. Lanes: 1 and 2, supernatant and sediment before enrichment; 3, lipocortin-enriched supernatant following sequential washing of the sediment with 1 mM- CaCl_2 and treating the sediment with 2 mM-EGTA.

(Wallner *et al.*, 1986). The lipocortin I cDNA was cloned downstream of the phage T7 promoter in the vector pET (Rosenberg *et al.*, 1987) and expressed in *E. coli* using CE6 phages for delivery of the gene encoding T7 polymerase (Fig. 1). The appearance of a strong band of the expected molecular mass (38.7 kDa) in lane 3 (plasmid carrying cDNA insert in the correct orientation), but not in lanes 5 and 6 (plasmid carrying cDNA insert in reverse orientation), shows that human lipocortin I is indeed produced. As suggested by Studier & Moffatt (1986), optimal protein synthesis was obtained after infection with 1.0×10^8 phages/ml (Fig. 1, lane 3).

Glenney & Zokas (1988) showed that calpactin II (which is identical with lipocortin I) extracted from tissue associates with phospholipids in the presence of Ca^{2+} . Since recombinant lipocortin I is expected to exhibit the same physico-chemical properties, bacterial lysates were precipitated in the presence of Ca^{2+} , and Ca^{2+} -binding proteins were re-solubilized by addition of EGTA. Lipocortin I was found in the supernatant (Fig. 2, lane 1) and in the sediment (Fig. 2, lane 2) of bacterial lysates. By washing the sediment sequentially with buffer containing 1 mM- CaCl_2 , non- Ca^{2+} -dependent binding proteins were removed. Upon re-solubilization of the sediment with EDTA, a strong band of lipocortin I was detected (Fig. 2, lane 3), indicating that the purification procedure used by Glenney & Zokas (1988) can be applied for the enrichment of recombinant lipocortin I. Lipocortin I was further purified by reversed-phase h.p.l.c. (results not shown) and its amino acid composition analysed. Amino acid analysis revealed a composition in accordance with the sequence published by Wallner *et al.* (1986) (results not shown). Therefore, on the basis of molecular mass, the amino acid composition and the physico-chemical properties, it can be concluded that the protein expressed in *E. coli* was human lipocortin I.

The concept of glucocorticoid-induced lipocortin I secretion is in apparent contradiction with the absence of a signal peptide in this protein (Wallner *et al.*, 1986; Verner & Schatz, 1988). It is conceivable that lipocortin I belongs to the rare family of

Fig. 3. Translocation assays for lipocortin I and prolactin

(a) Translocation assay for lipocortin I. The protein was synthesized in the wheat-germ system supplemented with dog pancreas membranes and immunoprecipitates were fractionated by PAGE. The autoradiogram of polyacrylamide gel is given. Lanes: 1, translation product in the absence of membranes (MB); 2, translation product in the presence of membranes; 3 and 4, post-translational proteolysis with proteinase K (PK) without (lane 3) and with (lane 4) solubilization of the membrane bilayer by Triton X-100 (DET). Molecular masses are given in kDa. (b) Translocation assay for prolactin. The procedure was performed as in (a). Lanes: 1, translation product in the absence of membranes; 2, translation product in the absence of membranes hydrolysed by proteinase K; 3, translation product in the presence of membranes (▼, unprocessed prolactin; ▲, translocated prolactin); 4 and 5, post-translational proteolysis with proteinase K without (lane 4) and with (lane 5) solubilization of the membrane bilayer.

secreted proteins which do not have a signal sequence (Price *et al.*, 1987; Silve *et al.*, 1987; Pfaffle *et al.*, 1988). In order to determine unambiguously that lipocortin I is a secreted protein, we investigated biosynthesis and translocation of human lipocortin I in *Xenopus* oocytes and in a cell-free translation

Fig. 4. Expression of lipocortin and prolactin

(a) Expression of lipocortin in *Xenopus* oocytes during the first 48 h after injection of lipocortin I transcript. For each time point, five oocytes were analysed. Lanes: 1, translation product of lipocortin I from the wheat-germ system; 2–9, analyses of oocytes (O); 10–17, analyses of medium (M). For each time point, the autoradiograms of the total product (1/10th of one oocyte or 1/33rd of the medium) without and with immunoprecipitation (IP) of lipocortin I are given. (b) Controls for (a). The same procedure as for (a) after the injection of control solution. Note that the translation product of lipocortin I is only given in (a). Lanes: 1–8, analyses of oocytes; 9–16, analyses of medium. (c) Expression of prolactin with (BPI) and without (PT) an *N*-terminal signal sequence in *Xenopus* oocytes during the first 48 h after injection of transcripts. Lanes: 1, unprocessed ‘*in vitro*’ translation product from the wheat-germ system; 2–5, analysis of medium; 6, analysis of oocytes.

system from wheat-germ in the presence of dog pancreas membranes. Therefore lipocortin I cDNA was transcribed from linearized pSP64TLip using SP6 polymerase. The resulting mRNA was translated in a wheat-germ cell-free system in the absence or presence of microsomal membranes prepared from dog pancreas (Fig. 3a, lanes 1 and 2). It has previously been shown that proteins translocated across microsomal membranes

are protected from proteinase K and that the protection is abrogated following solubilization of the lipid bilayer with the non-ionic detergent Triton X-100 (Rothman *et al.*, 1988). As shown in Fig. 3(a) (lanes 3 and 4) lipocortin I was not protected from proteinase K, indicating that it was not translocated into microsomes, and hence is unlikely to follow the conventional secretory pathway. In the same experiment, prolactin was used as

Fig. 5. Carbonate extraction of lipocortin (39 kDa), the transmembrane protein pSPS_L-gG-ST-P (39 kDa) and prolactin (30 kDa)

Homogenates from 10 oocytes were prepared and exposed to Na₂CO₃ (lanes 2, 3, 6, 7) or sucrose (lanes 4, 5, 8 and 9), then separated into supernatant (S) and pellet (P) fractions by centrifugation. Each fraction was precipitated with anti-lipocortin or anti-prolactin antibody. The precipitated products were revealed by SDS/PAGE and autoradiography. Lanes: 1, lipocortin I mRNA translated in the cell-free system and used as a marker; 2 and 3, pellet and supernatant following carbonate extraction of lipocortin; 4 and 5, pellet and supernatant following sucrose extraction of lipocortin; 6 and 7, pellet and supernatant following carbonate extraction of pSPS_L-gG-ST-P and prolactin; 8 and 9, pellet and supernatant following sucrose extraction of pSPS_L-gG-ST-P and prolactin.

a control in order to demonstrate that secreted proteins are translocated into microsomal membranes (Fig. 3*b*, lane 3) and that this translocated protein is protected from proteinase K (Fig. 3*b*, lane 4) in the cell-free system used.

Further evidence against lipocortin I secretion was obtained *in vivo* using the *Xenopus* oocyte system. Before *Xenopus* oocytes were micro-injected with human lipocortin I transcript, the absence of an endogenous protein synthesized by the oocytes that reacts with a monoclonal antibody (IgG1, κ) against human lipocortin I was demonstrated by Western-blot analysis (results not shown) and immunoprecipitation of extracts of oocytes previously micro-injected with control solution. RNA extracted from oocytes and hybridized on a Northern blot with a ³²P-labelled human lipocortin I probe revealed no signal, indicating that *Xenopus* oocytes do not express an endogenous lipocortin I gene. The RNA extracted for Northern blot analysis was translationally active in the wheat-germ cell-free system.

Xenopus oocytes were micro-injected with transcripts of

lipocortin I and the corresponding control solution (i.e. solution containing all the constituents but no nucleic acids). The expression of lipocortin I, as assessed by immunoprecipitation, increased steadily in the oocytes during the first 48 h (Fig. 4*a*, lanes 3, 5, 7, 9). No lipocortin I was found in the culture medium (Fig. 4*a*, lanes 11, 13, 15, 17). Oocytes injected with control solution did not express lipocortin I (Fig. 4*b*, lanes 2, 4, 6, 8). In order to demonstrate unambiguously that the oocytes were able to handle proteins according to the presence or absence of the signal sequence, *Xenopus* oocytes were co-injected with transcripts encoding lipocortin I, prolactin with an *N*-terminal signal sequence (BPI) and a prolactin fragment (amino acids 57–199) lacking a signal sequence (PT) (which are expressed as a cytoplasmic protein) (Simon *et al.*, 1987). Again these oocytes did not release either lipocortin I (results similar to Fig. 4*a*) or PT (Fig. 4*c*, lanes 2–5) into the medium, but did secrete authentic prolactin (Fig. 4*c*, lanes 2–5).

It is conceivable that lipocortin I, while not secreted, is integrated into membranes of *Xenopus* oocytes. Carbonate extraction was used to investigate this possibility (Fujiki *et al.*, 1982). With this procedure, vesicles of oocytes are converted into sheets and protein–protein interactions disrupted by denaturation, whereas protein–lipid interactions are retained. By that method, lipocortin I was extracted (Fig. 5, lane 2), whereas the transmembrane protein pSPS_L-gG-ST-P remained unextracted from the pellet (Fig. 5, lane 6). As a control, the same procedure was performed with sucrose instead of carbonate (Fig. 5, lanes 4, 5, 8, 9). Sucrose does not disrupt vesicles and protein–protein interactions (Fig. 5, lanes 8 and 9). Thus a stable insertion of lipocortin I into the bilayer of oocytes can be excluded on this basis.

The analysis of the expression of a protein in the wheat-germ cell-free system allows investigations of translocation processes which are of predictive value for a protein in intact cells (Katz & Lodish, 1979; Lingappa, 1989). For lipocortin I, the absence of translocation into pancreas membranes is in agreement with the absence of secretion of that protein by oocytes. *Xenopus* oocytes translate injected mRNA with great efficiency, process the resulting proteins and distribute them into the correct compartment (David & Sargent, 1988). The analyses of the results of the expression of lipocortin I in these oocytes make it unlikely that lipocortin I is either a constitutively or regulatedly secreted protein. It is more likely that lipocortin I is a member of the soluble pool of cytosolic proteins, as suggested by Zokas & Glenney (1987) on the basis of cell-fractionation studies using human fibroblasts. The reason why in many (Flower & Blackwell, 1979; Blackwell *et al.*, 1980; Russo-Marie & Duval, 1982; Hirata & Iwata, 1983; Gupta *et al.*, 1984; Errasfa *et al.*, 1985; Gurrpide *et al.*, 1986; Wallner *et al.*, 1986; Lundgren *et al.*, 1988; Solito & Parente, 1989), but not in all (Bienkowski *et al.*, 1989), of the previous investigations, lipocortin was found in the culture medium is unclear. Since lipocortin I is an abundant intracellular protein in some cell lines, it is likely that cell death accentuated by glucocorticoids or possibly an unorthodox secretion mechanism might have accounted for the extracellular detection of this protein. By using small numbers of *Xenopus* oocytes in the present investigation, it was possible to exclude cell death as a potential mechanism for lipocortin I release.

Broennegard *et al.* (1988) and Bienkovsky *et al.* (1989) have presented evidence that glucocorticoids do not induce lipocortin I and indeed several groups of investigators have suggested that lipocortin I inhibits neither phospholipase A₂ itself nor the release of prostanooids (Davidson *et al.*, 1987; Hullin *et al.*, 1989; van Binsbergen *et al.*, 1989). Furthermore, in contrast to what has previously been suggested, the present results clearly indicate that lipocortin I is not secreted. Thus the conclusion that

lipocortin I, the first clone isolated coding for a member of the lipocortin family, fulfils the operational definitions of a lipocortin, has to be revised.

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