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Inhibitory effect of peptides derived from the N-terminus of lipocortin 1 on arachidonic acid release and proliferation in the A549 cell line: identification of E-Q-E-Y-V as a crucial component

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1 The ability of the glucocorticoid-induced protein lipocortin 1 (LC1) to inhibit arachidonic acid release and cell proliferation in A549 cells may be mimicked by a sequence taken from the N-terminal, $LC1_{13-25}$ (FIENEEQEYVQTV). We have now synthesized and tested for biological activity a library of 25 smaller peptides derived from this sequence.

2 Peptides were tested in two assays: A549 cells were prelabelled with tritiated arachidonic acid and thapsigargin (50 nM) and EGF (10 nM) used to stimulate the release of this fatty acid. Cell proliferation was determined by counting cell numbers following 3 day incubation with these peptides, or controls.

3 Many of the peptides were highly insoluble but could be more readily dissolved in aqueous solution in the presence of commercial liposomes or phosphatidyl serine (5 μM). Since neither of these agents alone had any effect on arachidonic acid release or cell proliferation, all peptides were tested in the presence of 5 μM phosphatidyl serine. Under these conditions $LC1_{13-25}$ was active in both assay systems with an IC_{40} of 40.7 and 57.0 μM respectively.

4 Deletion of amino acids from the C-terminus of the peptide progressively diminished (2–3 fold) the molar potency of $LC1_{13-25}$ in both assays: after the removal of Val^{22} biological activity was virtually undetectable or very weak (<30% of $LC1_{13-25}$).

5 Removal of amino acids from the N-terminus also lead to a progressive reduction (3–5 fold) in the molar potency of the peptides and biological activity became undetectable, or very weak, after the removal of Glu^{18}.

6 All active peptides contained the core sequence EQEYV(Glu-Gln-Glu-Tyr-Val) which seems to represent a crucial component of the pharmacophore, although this sequence on its own was inactive and the shortest peptide with significant activity was $LC1_{18-25}$ (EQEYVQTV).

7 Methoxylation of Tyr^{21} abolished the ability of $LC1_{18-25}$ to inhibit cell proliferation and arachidonic acid release. A cyclized version of $LC1_{18-25}$ was also tested and found to be inactive.

8 $LC1_{18-25}$ (178 μM) inhibits $cPLA_2$ activation in A549 cells as judged by a band-shift assay, whereas equimolar concentrations of an inactive peptide $LC1_{19-25}$ were without effect in this assay system.

9 Several possible mechanisms whereby these peptides act are discussed in the light of LC1 biology and of the effect of glucocorticoids on cell function.

Keywords: Annexins; $cPLA_2$; glucocorticoids; cell proliferation; tyrosine kinase; SH2 domains

Introduction

Experimental evidence reviewed by us (Flower & Rothwell, 1994) supports the concept that lipocortin (LC) 1 is a key mediator of many effects of glucocorticoids including the suppression of lipid mediator release (Cirino *et al*., 1987) the inhibition of fever, (Carey *et al*., 1990; Davidson *et al*., 1991), paw oedema (Cirino *et al*., 1989) and polymorphonuclear leukocyte (PMN) migration (Perretti & Flower 1993), the inhibition of the release of adrenocorticotrophic hormone (ACTH) (Taylor *et al*., 1993) and other anterior pituitary hormones (e.g. Taylor *et al*., 1993, 1995) and the inhibition of the induction by endotoxin of nitric oxide synthase (Wu *et al*., 1995).

Lipocortin 1 is a member of a super-family of proteins termed the annexins (for a recent review see Raynal & Pollard, 1994). Members of this protein group are identified by a common structural motif comprising four repeating subunits (in some members of the family, eight repeating subunits). Whilst this core domain is highly conserved amongst members of the annexin family each of the individual proteins has a distinct N-terminal domain of variable length and it has been suggested that since this is a distinguishing feature, it probably contributes to the biological activity specifically associated with each member. Indeed, previous work from our group has demonstrated that LC1 lacking the N-terminal domain is without activity in some assays of inflammation and mediator release, whereas the full length N-terminus N-acetyl $LC1_{2-26}$ is biologically active in several systems (Cirino *et al*., 1993; Perretti, 1994).

The A549 cell line is a useful model for studying LC1 biology. The inhibitory action of glucocorticoids on cell proliferation in this model seems to be mediated by the induction and externalization of LC1, which subsequently impairs arachidonic acid release and therefore the release of eicosanoids which function as autocrine growth stimulators in this cell system (Croxtall & Flower, 1992). The glucocorticoid block of arachidonic acid release and cell growth may be neutralized by anti-LC1 neutralizing monoclonal antibodies (Croxtall & Flower, 1992; Croxtall *et al*., 1995) or antisense deoxynucleotides (Croxtall & Flower, 1994), thus confirming the central role for this protein in glucocorticoid action.

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In previous publications (Croxtall *et al*., 1993), we have demonstrated that the N-terminal domain of LC1 is crucial in exerting this inhibitory effect on A549 cell function and that this biological property seems to reside in the downstream portion of the N-terminal domain ($LC1_{13-25}$) as $LC1_{1-12}$ is inactive in this model.

To define further the region necessary for the biological activity of the lipocortin N-terminal domain, we have now synthesized a family of 25 peptides in which systematic deletions have been made from the N- and C- terminii and this has enabled us to search with more precision for the biological active region of the molecule. We now present the results of these studies which highlight the importance of the domain EQEYV, as a highly conserved sequence present in all active peptides. However the shortest peptide which produced significant inhibitory activity was $LC1_{18-25}$ (EQEYVQTV), implying that the domain EQEYV, whilst essential, is not sufficient for biological activity.

Methods

Cell culture

A549 cells (Flow) were maintained in continuous log phase growth in Dulbecco's modified Eagle's medium/F-12 (DMEM/F-12) containing phenol red and 10% foetal calf serum (FCS) in T-150 flasks (Greiner). The cells were not allowed to reach confluence at any time as this diminishes their response to growth factors (Croxtall & Flower, 1992).

Use of EGF

Epidermal growth factor (EGF) is a powerful growth factor which stimulates the proliferation of A549 cells. In our previous work we have used EGF extensively and have shown that its stimulating action upon growth is dependent at least in part upon the ability of this growth factor to release eicosanoids (Croxtall & Flower, 1992). Glucocorticoids such as dexamethasone, as well as lipocortin 1 and some of its N-terminal peptides, block the release of eicosanoids caused by EGF thus preventing this growth factor from producing its proliferative action.

EGF stimulates the release of eicosanoids apparently by activating the enzyme phospholipase A_2 ($cPLA_2$) which results in arachidonic acid release (Croxtall *et al*., 1995, 1996). The effect is greatly enhanced in the presence of agents such as thapsigargin which concomitantly liberate calcium from internal stores. Thus, we have used EGF alone, or in combination with thapsigargin as a technique for eliciting the release of this fatty acid from A549 cells.

Measurement of arachidonic acid release

Subconfluent cells were seeded into 12-place multi-well plates (Falcon) at 3×10^5 cells/ml/well in DMEM/F-12, 10% FCS and incubated overnight. [^{3}H]-arachidonic acid ([^{3}H]-AA) in ethanol was evaporated to dryness under N_2 and resuspended in an appropriate volume of DMEM/F-12 (without phenol red) and after vortex mixing left at 37°C for 1 h. After the cells had been washed with PBS, 9.25 KBq of [^{3}H]-AA in 0.5 ml DMEM/F-12 (without phenol red) was added to each well and incubated overnight. The media containing free [^{3}H]-AA was then removed and the cells washed three times with 1 ml DMEM/F-12 containing 1 mg ml BSA. The cells thus labelled with [^{3}H]-AA were then treated for 3 h with test peptides, vehicle controls (DMSO), phosphatidylserine or liposome preparation diluted into DMEM/F-12 (without phenol red). Then 10 nM EGF was added for 30 min and 50 nM thapsigargin added for a further 30 min. After incubation, 0.4 ml of medium was removed from each well for scintillation counting.

Cell proliferation experiments

Sub-confluent cells were seeded into 12-place multi-well plates at 5×10^4 cells ml^{-1} well in DMEM/F-12, 10% FCS, and incubated overnight. The medium was replaced with DMEM/F-12 (without phenol red or FCS) containing dilutions of test peptide, vehicle control (DMSO) or phosphatidylserine. The cells were incubated for a further 3 days with one media change on day 2 providing fresh reagents. At the end of this period each well was incubated with 1 ml of 0.05% trypsin, 0.02% EDTA, PBS and the dispersed cells counted with a Coulter Multisizer II. The % inhibition of cell proliferation for each peptide treated culture was calculated compared to control wells.

$cPLA_2$ band shifts

A549 cells release arachidonic acid largely through the action of the cytosolic form of (c)PLA_2 (Croxtall *et al*., 1995, 1996). EGF activation of $cPLA_2$ is accomplished by a MAPK-dependent phosphorylation of the protein which results in an electrophoretic 'band shift'. The activation of $cPLA_2$ revealed by this technique is inhibited by pretreatment with dexamethasone or N-terminal peptide fragments of lipocortin 1 (Croxtall *et al*., 1996). The smallest active peptide identified in this study was screened in this assay in the following manner. 3×10^6 sub-confluent cells were seeded into T-75 flasks (Greiner) in DMEM/F-12, 10% FCS and incubated overnight. The medium was replaced with DMEM/F-12 (without phenol red or FCS) and incubated for a further 24 h. The cells were then incubated with peptides at 200 μg ml^{-1} for 3 h before treatment with 10 nM EGF for 30 min. The medium was aspirated and the cell monolayer lyzed in 1.5 ml PBS containing 10 mM EDTA, 1% Triton T-X100, 1 mM PMSF, 1 mM sodium orthovanadate and 0.01% leupeptin. The protein concentration of each sample was determined by Bradford assay and total protein equivalents loaded in each lane. SDS–PAGE was performed according to the method of Laemmli (1970), but using 10×8 cm gels with 12% acrylamide, overun by 2 h after the dye front reached the bottom. Colour markers (Sigma) were used as a guide to cut out the appropriate portion of the gel for transfer to Immobilon PVDF membrane (Millipore, Watford, UK). Membranes were blocked with 5% dried milk fat powder in PBS, 0.1% Tween-20 for 1 h at 4°C. The primary antibody to $cPLA_2$ was generously provided by Clive Jackson of Astra, (U.K.) and used at 1 : 4,000 in an overnight incubation. After the membrane had been washed, goat anti-rabbit HRP conjugate (Sigma) was used at 1 : 2,000 for 2 h, then washed again and the signal was detected with DAB.

Peptide preparation

All peptides were synthesized by Dr Stefan Peters (Boehringer Ingelheim KG, D6507 Ingelheim am Rhein, Germany) by conventional solid phase techniques, and purified by high performance liquid chromatography (h.p.l.c.). The sequences were validated by FAB-MS. All peptides and growth factors were stored and dispensed in siliconized plasticware.

Materials

EGF, phosphatidylserine, liposome preparations, DMEM/F-12, trypsin and FCS were from Sigma (Poole, U.K.). [5,6,8,9,11,12,14,15-^{3}H-(N)]-arachidonic acid was from NEN Du Pont (Belgium).

Statistical analysis

All experiments were performed at least in triplicate and each experiment presented is a typical example of at least 3 such experiments. Results are expressed as the mean $\pm$ 1 s.d. and presented as % changes. All statistical calculations were performed on the raw numerical data of the experiments presented. Student's t test (unpaired) with the Bonferonni correction to allow for multiple testing within each group was used to determine statistical significance with $P<0.05$ as the minimal value for significance.

Results

Effect of liposomes and phosphatidyl serine on peptide action

Many peptides seemed insoluble in tissue culture medium and so the actual potencies of such peptides would be underestimated. In an attempt to improve the solubility we first tested a variety of commercially available liposome preparations (Sigma). Each preparation which contained varying proportions of L-α-phospatidylcholine (PC), β-oleoyl-γ-palmitoyl (POPC) and cholesterol gave remarkably similar results. We first checked the activity of known bioactive peptides in the arachidonic acid release system using each liposome preparation. In the presence of 100 μg ml^{-1} $LC1_{13\text{-}25}$, which we had previously demonstrated to be biological active in this cell line (Croxtall *et al*., 1995), we obtained 24% ($P<0.01$) inhibition of arachidonic acid release, but by adding a preparation containing 0.97 μmol PC and POPC and 0.69 μmol cholesterol, which was without effect on its own, we were able to observe greater than 90% inhibition at the same concentration of peptide ($P<0.01$). Other liposome preparations gave very similar results. However, for routine use these liposome preparations were expensive and difficult to handle. Therefore, a more simple lipid vehicle was required and we subsequently used phosphatidyl serine for our experiments.

Again to validate our use of phosphatidyl serine we first checked the effect of this phospholipid on the activity of known bioactive peptides in the arachidonic acid release system. In the presence of 100 μg ml^{-1} $LC1_{13\text{-}25}$, we obtained 24% ($P<0.01$) inhibition of arachidonic acid release, but by adding 5 μM phosphatidyl serine, which again was without effect on its own, we were able to observe 47% inhibition at the same concentration of peptide ($P<0.01$). We have previously observed that the the N-terminus portion of LC1, $LC1_{1\text{-}12}$, was inactive in this assay (Croxtall *et al*., 1995) and when we checked the activity of this peptide in 5 μM phosphatidyl serine, we found that it was still without activity.

Similarly, treatment of A549 cells with 100 μg ml^{-1} $LC1_{13\text{-}25}$ significantly inhibited cell growth (12.4%; $P<0.01$),

Table 1 Inhibition of A549 cell arachidonic acid release by peptides from the N-terminus of LC1

Peptide	*25 μg ml^{-1}*	*50 μg ml^{-1}*	*100 μg ml^{-1}*	*200 μg ml^{-1}*
13–25	15±2	23±4	38±8*	55±3*
13–24	3±3.2	12.6±1.5	21.9±3.5	39.8±13*-
13–23	4±1.5	11±0.9	19±0.9	37±5*
13–22	0±2.1	8±3.1	15±5.4	29±4*
13–21	4±4.1	8±2.1	21±4.4	32±9*
13–20	3.9±3	7±4	8±3	14±4
13–19	7±4	8±2	8±1	14±5
13–18	0±6	0±6	6±3	18±6.7
13–17	0±11	0±5	11±3	21±5
13–16	0±2	8±3	11±6	18±8
13–15	0±1	0±7	0±3	3±1
14–25	0±6	3±2	6±5	33±4*
15–25	9±4	10±6	19±9	23±6
16–25	3±1	13±4	26±4*	48±7*
17–25	0±4	7±11	11±1	20±3
18–25	12±4	20±3	30±8*	37±5*
19–25	7±2	13±3	19±1	8±2
20–25	5±2	8±1	13±3	16±7
21–25	7±4	3±4	9±3	13±5
18–24	1±2	1.5±1	0±5	0±11
18–23	0±5	0±3	2.3±9	9.5±4
18–22	1.7±2	0±5	1±6	14±5
18–21	2.4±8	3±6	10.4±3	13±4
19–23	1.3±3	1±5	0±13	2.7±7
20–22	0±13	0±6	0±14	0±7

The raw data presented were obtained when the various peptides derived from the LC1 N-terminus were tested as inhibitors of EGF/thapsigargin-stimulated arachidonic acid release from A549 cells. Each peptide was tested at four concentrations, 25, 50, 100 and 200 μg ml^{-1} and the data expressed as percentage inhibition of control release. Figures which were significantly (minimum of $P<0.01$) different from controls are indicated within an asterisk. Each data point is the mean of three experiments ($\pm$s.e.mean) in which each value itself was determined in triplicate.

Table 2 Inhibition of A549 cell proliferation by peptides derived from the N-terminus of LC1

Peptide	*50 μg ml^{-1}*	*100 μg ml^{-1}*	*200 μg ml^{-1}*
13–25	21.1±3.2	45±2.5*	65±3.2*
13–24	3.4±4.4	18.7±6.6	44±7*
13–23	0±3.6	8.3±1.9	42.2±14.3*
13–22	10.8±10.2	19.7±1.3*	38.5±3.2*
13–21	5±1.3	12.2±5.2	26±1.2*
13–20	9.7±2.7	14.2±3	18.8±1.4
13–19	0±9.3	11.5±15.1	13.2±2
13–18	8±1.8	12±2.1	13.2±1.2
13–17	1±4.2	0.3±3.7	17.2±3.3
13–16	0±3	09.2±4	20±12
13–15	0±3	0±2.2	19.1±6.8
14–25	4±6.4	13±3.3	39±8*
15–25	0±2.6	0±2.3	7±1.7
16–25	2.8±0.7	18.6±1.8	50.1±12.9*
17–25	0±6.7	18.6±1.8	43.6±15.1
18–25	3±3.3	12±2.1	33±4.9*
19–25	1.4±2.9	3.6±6.4	15.1±3.1
20–25	0±3.7	0±8.7	5.8±5
21–25	3.2±4.6	6.8±1.2	14±4.9
18–24	0±0.9	0±2	5.1±1.5
18–23	0±2.2	0±1.3	0±1
18–22	0±1.2	7.1±2.7	8.3±2.1
18–21	0±2.8	0±1.5	0.8±2.1
19–23	0±4	0.9±2.1	0±1.1
20–22	0±1.3	6.1±1.8	12.1±1.2

The raw data presented were obtained when the various peptides derived from the LC1 N-terminus were tested in the three day cell proliferation assay. Each peptide was tested at four concentrations, 25, 50, 100 and 200 μg ml^{-1} and the data expressed as percentage inhibition of control release. Figures which were significantly (minimum of $P<0.01$) different from controls are indicated within an asterisk. Each data point is the mean of three experiments ($\pm$s.e.mean) in which each value was itself determined in triplicate.

but in the presence of phosphatidyl serine at 5 μM the inhibitory activity was increased to 60.5% ($P<0.01$). Again $LC1_{1-12}$ was inactive at either concentration of the phospholipid. These results demonstrate that phosphatidyl serine enhances the activity of the active peptides but that inactive peptides remain unaffected. In all subsequent experiments described here we dissolved peptides in 5 μM phosphatidyl serine routinely.

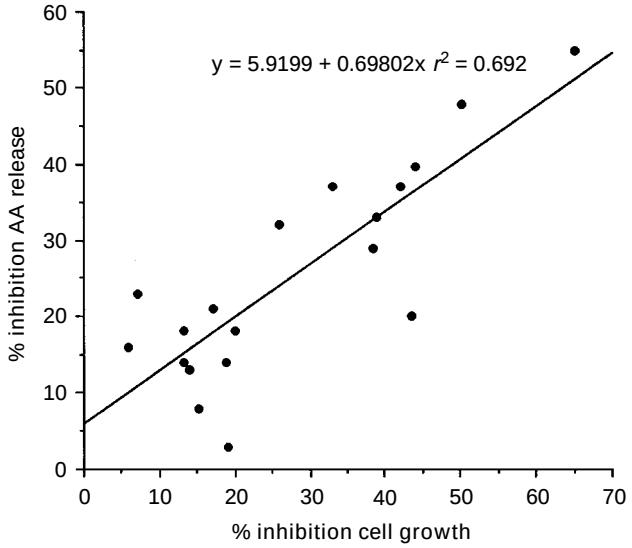

Figure 1 Correlation between the ability of peptides derived from the LC1 N-terminus to inhibit arachidonic acid release and to inhibit cellular proliferation. Data in the two models taken from single dose studies (200 μg ml^{-1}) was calculated as percentage inhibition relative to controls and analysed by use of a commercially available statistical package (Cricket-Graph for Macintosh).

Effect of peptides on arachidonic acid release and cell proliferation

A family of peptides was synthesized based around the core sequence of $LC1_{13-25}$ by systematically shortening the peptide by single amino acids sequentially from either the N- or C-terminus. A549 cells were treated with four concentrations of each peptide (25, 50, 100 and 200 μg ml) of peptide dissolved in 5 μM phosphatidyl serine as described above, for 3 h before treatment with the EGF-thapsigargin mixture in the arachidonic acid release assay. Table 1 shows these data expressed as percentage inhibition by the peptide of arachidonic acid release when compared to EGF-thapsigargin treatment alone. All statistical analyses were performed on the raw data.

Inhibition of arachidonic acid release was detectable with many of the peptides and was dose-dependent, although full dose-response curves could not be constructed in many cases, often because of problems with solubility despite the improvements achieved with PS as a solvent. However, those peptides which had lost residues 13–18 or 21–25 displayed only very weak activity, if any at all.

A549 cells were also treated with the same peptides at three concentrations (50, 100 and 200 μg ml^{-1}, dissolved in PS) for three days in the proliferation assay. Table 2 shows the results of this assay and again, the results are presented as % inhibition compared to the control cells treated with vehicle alone, with statistical analyses performed on the raw data. In a pattern of inhibition which was very similar to that seen in the

Table 3 Relative molar potencies of LC1 N-terminus peptides

Peptide (RMP)	*Sequence*	*IC_{40} Cell proliferation (RMP)*	*AA release*
13–15	F I E	NC	UD
13–16	F I E N	NC	NC
13–17	F I E N E	NC	NC
13–18	F I E N E E	NC	NC
13–19	F I E N E E Q	NC	NC
13–20	F I E N E E Q E	NC	NC
13–21	F I E N E E Q E Y	NC	≃185 (0.31)
13–22	F I E N E E Q E Y V	≃140.3 (0.29)	170.3 (0.33)
13–23	F I E N E E Q E Y V Q	120.2 (0.34)	133.8 (0.42)
13–24	F I E N E E Q E Y V Q T	106.1 (0.38)	115.9 (0.49)
13–25	F I E N E E Q E Y V Q T V	40.7 (1)	57.0 (1)
14–25	I E N E E Q E Y V Q T V	≃122.2 (0.33)	≃140.1 (0.41)
15–25	E N E E Q E Y V Q T V	UD	NC
16–25	N E E Q E Y V Q T V	120.1 (0.34)	111.5 (0.51)
17–25	E E Q E Y V Q T V	130.2 (0.31)	157.9 (0.36)
18–25	E Q E Y V Q T V	≃90.8 (0.45)	≃76.4 (0.75)
18–24	E Q E Y V Q T	UD	UD
18–23	E Q E Y V Q	UD	NC
18–22	E Q E Y V	NC	NC
18–21	E Q E Y	UD	NC
19–25	Q E Y V Q T V	NC	UD
19–23	Q E Y V Q	UD	NC
20–25	E Y V Q T V	UD	NC
20–22	E Y V	NC	UD
21–25	Y V Q T V	NC	NC

Inhibitory data relating to peptides derived from the LC1 N-terminus expressed as IC_{40} values and as molar potencies relative to $LC1_{13-25}$. The raw data were taken from Tables 1 and 2 and conventional molar log-dose responses curves constructed. Because of solubility and other factors only partial dose-response curves could sometimes be obtained and in many cases 50% inhibition was not observed even at the highest concentration achieved. For this reason IC_{40}s have been used for comparison purposes. UD: undetectable biological activity. NC: The IC_{40} was greater than 350 μg ml^{-1} and was not calculated. Because of poor solubility some IC_{40} values (designated thus; ≃) were estimated by graphical curve fitting to give approximate values. RMP: relative molar potency (compared to $LC1_{13-25}$).

previous assay, many peptides produced inhibitory effects on cell growth but those peptides which lacked residues 19–22 were inactive or only very weakly active. There was a good correlation ($r^2 = 0.692$) between the ability of the peptides to inhibit arachidonic acid release and their ability to inhibit cell proliferation at the highest concentration tested (200 μg ml; see Figure 1).

As sequential removal of amino acids alters the molecular weight of the peptide the values for inhibition in the two assays have been replotted in molar form in Table 3. Many peptides, whilst exhibiting dose-dependent inhibition, did not achieve concentrations high enough to bring about 50% inhibition so we have chosen an IC_{40} for comparison purposes. Even with this technique the potencies of several peptides could only be estimated following graphical curve fitting. $LC1_{13\text{-}25}$ was chosen as the standard compound and Table 3 shows that removal of residues from either end of $LC1_{13\text{-}25}$ inevitably results in the loss of some activity. For example removal of the terminal Val from $LC1_{13\text{-}25}$ diminishes the biological activity between 2–3 fold in both assays. Likewise removal of this N-terminal Phe also diminished the inhibitory action by approximately the same amount. Once again, a common pattern emerged in that the residues EQEYV seem crucially important, being present in all the active peptides, although the sequence by itself was inactive.

Sequential removal of further amino acids from either end of the molecule resulted in a progressive and dramatic fall off in activity relative to $LC1_{13\text{-}25}$. The only exception to this sequence was $LC1_{15\text{-}25}$ which unlike its neighbours, $LC1_{14\text{-}25}$ and $LC1_{16\text{-}25}$, displayed no activity in any test. We were unable to explain this anomalous observation.

Figure 2 shows partial log dose-response curves for $LC1_{13\text{-}25}$, $LC1_{18\text{-}25}$, $LC1_{1\text{-}188}$ and $LC1_{1\text{-}346}$ (full length human recombinant LC1) in the AA release assay for comparison purposes. From the graph it can be seen that the curves are approximately parallel, although the peptides were approximately 3 orders of magnitude less active than the full length protein.

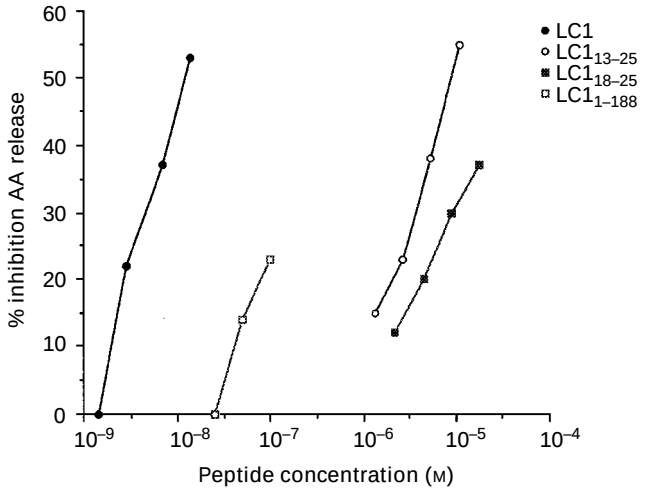

Figure 2 A comparison between full length lipocortin ($LC1_{1\text{-}346}$), the truncated fragment $LC1_{1\text{-}188}$ and the peptides $LC1_{13\text{-}25}$ and $LC1_{18\text{-}25}$ in the arachidonic acid release assay. Mean data (taken from Tables 1–3) was used to calculate percentage inhibition by the peptides and data were plotted on a conventional log-dose response curve. Error bars were omitted for clarity (see Tables 1–2). The data indicate that the truncated fragment $LC1_{1\text{-}188}$ is approximately 150 fold less potent on a molar basis than full length LC1 and that the two peptides, $LC1_{13\text{-}25}$ and $LC1_{18\text{-}25}$ are approximately 1000 times less potent on a molar basis.

Modification of $LC_{18\text{-}25}$

The core sequence EQEYV is interesting as Tyr^{21} is an important phosphorylation site on LC1 (Pepinsky & Sinclair, 1986). The presence of a phosphate group at this site has been linked to changes in behaviour of the molecule, such as its solubility, calcium binding properties and other biological actions. We tested the importance of Tyr^{21} by preparing a substituted version where $LC1_{18\text{-}25}$ contained a methoxy Tyr (which cannot be phosphorylated). It can be seen from Figure 3a that this peptide was completely without activity. We also synthesized a cyclized version of $LC1_{18\text{-}25}$ in the hope that this would retain biological activity, but be more stable than the linear peptide. Figure 3b demonstrates that this peptide was

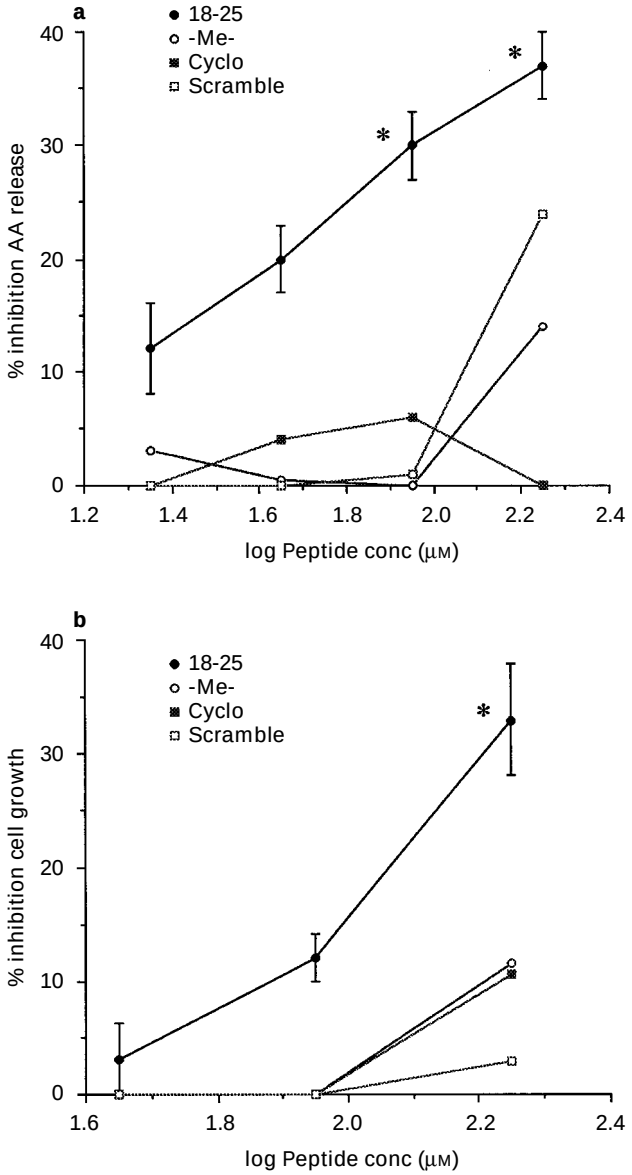

Figure 3 Lack of inhibition by the methoxy Tyr^{21} substituted $LC1_{18\text{-}25}$, a cyclized $LC1_{18\text{-}25}$ or a scrambled control, in the arachidonic acid and cell proliferation assays. Equimolar amounts of these peptides were compared with the biologically active $LC1_{18\text{-}25}$ in four concentrations and the results converted to percentage inhibition. $LC1_{18\text{-}25}$ displayed activity in both assays (IC_{40}s approximately 76.4 μM and 90.8 μM in the arachidonic acid release and cell proliferation assays, respectively), but the other peptides were inactive and the inhibition seen at the highest concentrations used were not significant.

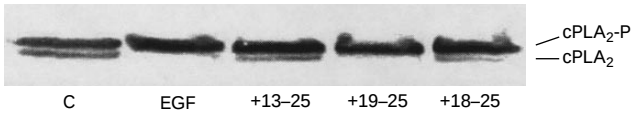

Figure 4 The effect of peptides derived from the LC1 N-terminus on EGF induced activation (phosphorylation) of cytosolic PLA_2 in A549 cells. The figure illustrates lanes taken from a Western blot comparing the relative abundance of the phosphorylated (upper) and non-phosphorylated (lower) bands of $cPLA_2$ as detected with a specific polyclonal antibody. The control (**C**) cells displayed a mixture of both species but 30 min after EGF (10 μM) all the enzyme was present in the phosphorylated form. In the presence of 200 μg ml^{-1} $LC1_{13\text{-}25}$ or $LC1_{18\text{-}25}$ significant amounts of the enzyme remain unactivated and are retarded on the gel. However, $LC1_{19\text{-}25}$, which was biologically inactive in the cell proliferation and arachidonic acid release assays, was also inactive in this respect at the highest dose used (200 μg ml^{-1}).

also inactive at the highest concentration (200 μg ml^{-1}: 178 μM) tested in both assays. We also tested a scrambled sequence of $LC1_{18\text{-}25}$ (VEQVEYQT) and this was found to be inactive in either test at the highest concentration used (200 μg ml^{-1}: 178 μM).

Effect of peptides on $cPLA_2$ activity

EGF treatment of A549 cells resulted in phosphorylation and activation of $cPLA_2$. This was revealed as an increase in the apparent molecular weight ('band shift') of the protein by Western blotting. Dexamethasone inhibited activation of $cPLA_2$ and this was also blocked by $LC1_{13\text{-}25}$, whereas $LC1_{1\text{-}12}$ was inactive. Figure 4 shows that peptide $LC1_{18\text{-}25}$ (200 μg ml^{-1}: 178 μm for 3h) inhibited the EGF-induced band shift ofM $cPLA_2$ in a similar fashion to $LC1_{13\text{-}25}$ whereas the inactive peptide $LC1_{19\text{-}25}$ was without effect.

Discussion

Previous research has demonstrated that the N-terminal domain ($LC1_{2\text{-}26}$) is the most likely site of the growth-inhibitory activity exhibited by the full length protein in A549 cells. For example, human recombinant $LC1_{1\text{-}188}$, which lacks the two terminal repeats exhibits biological activity similar to that of LC1 in this and several other systems (Carey *et al*., 1990; Relton *et al*., 1991; Perretti & Flower, 1993; Taylor *et al*., 1993), although it is somewhat (5–10 fold) less potent. The N-terminal peptide itself N-acetyl $LC1_{2\text{-}26}$ is also biologically active in a number of models including inhibition of leukocyte migration and other inflammatory processes (Cirino *et al*., 1993; Perretti *et al*., 1993). Conversely, removal of the N-terminus from full length ($LC1_{1\text{-}346}$) protein results in a loss of activity in models of inflammation and mediator release.

However, there have been studies showing that the core domain of the molecule can also have important biological effects. For example some authors (Gold *et al*., 1996) have demonstrated inhibition of lymphocyte proliferation by full length LC1 but also by the truncated *des* 1-26 species. In addition it may be noted in passing that the 'antiflammin' peptides which exhibit a spectrum of inhibitory properties in several systems are drawn from an area located in the third repeat of the LC1 core domain (Camussi *et al*., 1990).

Using the A549 cell system as a model we have previously found that whilst the full length $LC1_{1\text{-}346}$ was active (Croxtall & Flower, 1992), of the N-terminal peptides, $LC1_{1\text{-}12}$ was inactive whereas $LC1_{13\text{-}25}$ retained much of the activity of the intact molecule (Croxtall *et al*., 1993), although it was much less potent (approx 100 fold on a molar basis). In the present study, we have further developed our hypothesis that the pharmacophore of LC1 resides within the N-terminal domain and have demonstrated that a core domain EQEYV seems to be crucial (but not sufficient) for activity in this model, although it is evident from inspection of the data that removal of amino acids from any part of this peptide results in a reduction in overall potency.

The localization of this particular domain in the protein is interesting as it contains Tyr^{21} which is an important site of phosphorylation (Pepinsky, 1991) and is thought to be the residue phosphorylated by EGF receptor, and other tyrosine kinases. This also contains the putative PKC site, Thr^{24} (Browning *et al*., 1990). It is also noteworthy that this portion of the molecule is also found in some other peptides which display similar biological activity, for example the amphibian peptide sauvagine (a member of the CRF superfamily) contains a motif with a homology of 7 out of 9 amino acids taken around this core sequence (see Table 4) and MT peptide derived from middle T antigen of polyoma virus also has >50% homology. Interestingly, both sauvagine and MT peptide have biological activity in models of inflammation and neutrophil chemotaxis which are reminiscent of LC1 (Wei & Kiang, 1989; Hirata *et al*., 1984).

Table 5 presents a summary of the physico-chemical properties of the LC1 N-terminus as determined by conventional techniques, which calculate the hydrophobicity/hydrophilicity and predict the likelihood of helices, β-pleated sheets, coils or turns. The sequence $LC1_{13\text{-}25}$ contains a strongly hydrophobic N-terminus with further hydrophobic regions toward the tail end of this molecule, perhaps explaining difficulties in solubility encountered with the full-length peptides. However, the core domain, which includes the putative pharmacophore, is located mainly within a highly hydrophilic region of the peptide which includes a motif with a strongly-predicted turn. It is possible that the hydrophobic ends of the peptide $LC1_{13\text{-}25}$ interact providing a looping structure at which the central domain is exposed for interaction with putative binding proteins or other targets.

Table 4 Homologies between LC1 N-terminus and other biologically significant peptide sequences

Human LC1 14–25	I E N E E Q E Y V Q T V
Rat LC1	I E K Q E Q E Y V Q T V
Mouse LC1	L E N Q E Q E Y V Q A V
Guinea-pig	I D N Q E Q D Y V K T V
Pigeon	M E H Q E Q E Y I K S V
Chicken	M D N Q E Q E C I K S S
Sponge	L E K Q E Q E Y I E I V
Sauvagine 20–31	I E K Q E K E K Q Q A A
MT peptide	E E E E Y P M E
EGF phosphorylation sequences	E E E E Y F E L V
PDGF phosphorylation sequences	E E E E Y V F I E

Homologies between LC1 N-terminus and some other biologically significant peptide sequences. The sequence of human $LC1_{14-25}$ and the matching sequences from rat, mouse, guinea-pig, pigeon, chicken and sponge LC1 are compared. Also included is a sequence taken from sauvagine, an amphibian peptide with pronounced anti-inflammatory properties, MT peptide a synthetic peptide derived from middle T antigen which has anti-inflammatory properties and the phosphorylation sequences which are targets for the EGF and PDGF tyrosine kinases.

In A549 cells the effect of lipocortin 1 and the peptide $LC1_{13-25}$ is to suppress the release of arachidonic acid (Croxtall *et al.*, 1995). Our studies have demonstrated that this is mediated through an inhibition of activation of $cPLA_2$: i.e. $cPLA_2$ is not phosphorylated in the presence of LC1 or its peptides (Croxtall *et al.*, 1996). Precisely how this is accomplished is not clear. Undoubtably the effect occurs downstream of the G-protein signalling step as by-passing the G-protein receptor by (e.g.) direct addition of GTPγ S can also be blocked by dexamethasone, LC1 and $LC1_{13-25}$. We must therefore pose the question of how these peptides are exerting their effect.

We have formulated the hypothesis that LC1 must bind to sensitive protein binding structures on the external surface of the cell in order to function as an inhibitor (Goulding *et al.*, 1996). Elsewhere (Croxtall *et al.*, 1997), we describe the identification of proteins which bind $LC1_{13-25}$ within A549 cells and their possible role in mediating this effect, but other possibilities must also be considered. For example the minimum sequence EQEYV is strikingly similar to some SH2 recognition domains and Table 6 shows a comparison of this peptide with the well-characterized 'preferred' sequences for protein tyrosine kinase substrates of a variety of kinases (Songyang *et al.*, 1995). Whilst there is not a great deal of homology (except with the EGF-PDGF family substrates), there remains the possibility that these peptides could be taken up by A549 cells and that they are acting either as pseudo-substrates for important protein tyrosine kinases within the cell which are involved in signal transduction, or actually block the activity of kinases or compete with endogenous substrates at SH2 binding domains. Interestingly, Hirata *et al.* (1984) demonstrated that the sequence of short peptides derived from middle T antigen and *src* also contains similarities to the LC1 N-terminus domain. These were observed to have anti-neutrophil properties and appeared to posses very similar effects to those of LC1 and its peptides in various models, as we have described. Other authors have noted that peptides derived from *src* (412–421) which contain Tyr and which also have some degree of homology with $LC1_{13-25}$ can prevent cell differentiation and division in transformed cells (Goldberg *et al.*, 1980). Interestingly, and in accord with our own findings, was the observation by these authors that replacement of Tyr^{416} with a Phe produced a peptide that was without activity. Other workers too have observed that short peptides containing Tyr residues based upon *src* (Wong & Goldberg, 1981) or middle T antigen (Schauffhausen *et al.*, 1982) structures inhibit protein kinase activity and indeed may even become phosphorylated during such reactions e.g. in A431 membrane preparations containing EGF and TGF kinases (Pike *et al.*, 1982). However, against this explanation for the action of the peptides described here is the fact that these peptides have very low penetration into the cell (unpublished data) and that a well known tyrosine kinase inhibitor, genistein, has no effect on cell division in our system. In any case, the potency of the peptides formed here is about ten fold greater than with many of these other peptides (eg. middle T peptide; Hirata *et al.*, 1984).

Another possibility is that these peptides prevent the phosphorylation of endogenous LC1, perhaps by acting as pseudo-substrates as described for the *src* peptides referred to above (Pike *et al.*, 1982). Some authors have equated phosphorylation of LC1 to biological inactivation and proteolysis and therefore this is a viable possibility. However, gross visual inspection of Western blots of A549 cell extracts did not seem to support the notion of a dramatic change in the disposition or proteolytic status of LC1 in the presence of these peptides, although this possibility cannot be definitively ruled out. Indeed, one other peptide not described here that was not active in this system, $LC1_{21-33}$, has been clearly shown by us to decrease the LC1 proteolytic activity of A549 cells, perhaps because it spans a region of the molecule which is very sensitive to proteolysis, including the well-defined plasmin and elastase clipping site at Lys^{26}-Lys^{29} (Huang *et al.*, 1987) .

It is interesting to compare the region which we have identified as being important for the biological action of LC1 with other data which may have a bearing on the biological activity of this molecule. It is known that LC1 (and indeed other members of the annexin super family) can bind and associate with members of the intermediate filament family of proteins. For example, various members of the family have been shown to be associated with actin, spectrin, tropomyosin and calspectrin, although in many cases these associations may occur only in the presence of unphysiologically high calcium concentrations (Raynal & Pollard, 1994). Elsewhere, we have described a tight, calcium independent association of LC1 with cytokeratin 8, a member of the intermediate filament family

Table 6 Homologies between LC1 N-terminus and preferred tyrosine kinase substrates

Human LC1 17–25	E E Q E Y V Q T V
PTK: c-FPS/Fes	E E E I Y E E I E
MT/c-SRC	E E I Y G E F F
v-SRC	E E E I Y G E F D
Lck	X E X I Y G V L F
c-Abl	A E V I Y A A P F
Insulin	X E E E Y M M M M

Homologies between the LC1 N-terminus and 'preferred' substrates of various tyrosine kinases. The structure of $LC1_{17-25}$ is compared here with the preferred tyrosine kinase substrates of a variety of kinases including protein tyrosine kinase c-Fps/Fes, c-Src coexpressed with polyoma middle T, v-Src, Lck, c-Abl and insulin receptor kinase. The data were modified from Songyang *et al.* (1995).

Table 5 Physico-chemical properties of human LC1 N-terminal peptide

				5					10					15					20					25		
	A	M	V	S	E	F	L	K	Q	A	W	F	I	E	N	E	E	Q	E	Y	V	Q	T	V	K	
Helix	H	H	H	H	H	H	H	H	H	H	H	H	H	h	h	h	h	h	h	h	h	h	H			
Sheet	s	s	s	s	s	s	s	s	s	s	s	s						s	S	S	S	S	S	s		
Turn									t					T	T	T	T									
Coil																										C
Hydrophilic	–	–	–	–	–	1	4	2	–	–	–	–	–	–	8	12	20	11	9	4	–	–	–	–	2	
Hydrophobic	–	–	2	4	6	–	–	–	8	8	8	8	8	2	–	–	–	–	–	–	1	4	4	–	–	

Physico-chemical properties of the human LC1 N-terminus N-acetyl $LC1_{2-26}$. The propensity of the polypeptide to form helices, sheets, turns or coils was calculated by conventional computer-calculated algorithms and is expressed in lower case/upper case letters to indicate the approximate likelihood of this event occurring. Also shown are the relative hydrophilic or hydrophobic nature of the residues scored on a scale ranging from 1–20 where 1 represents the least and 20 the most hydrophylic/hydrophobic nature of the peptide.

(Croxtall *et al*., 1997). We have further shown that binding to this protein is mediated through the same $LC1_{13-25}$ sequence which we find to be biologically active in our system. This mirrors another study in which the association of LC1 with early endosomes was shown to require an intact N-terminus and that removal of the first 13 N-terminal residues did not effect this association, further confirming the importance of the second part of the N-terminal domain for protein interaction. However, other important interactions have been identified. Members of the annexin family have been shown to bind with members of the S100 protein family (Seeman *et al*., 1996). The most well studied of these is the association between lipocortin 2 and 'p11' where a hetrotetramer is formed. Other members of the S100 protein family have been shown to bind LC1 through an interaction with the domain present within peptides $LC1_{1-18}$ (Seeman *et al*., 1996). Interestingly, members of this S100 protein family, which have been shown to be involved in many cellular functions including cell proliferation, secretion and leucocyte chemotaxis, also bind to the cytoskeleton.

Whilst it is possible to state that such strong interactions occur between $LC1_{13-25}$ and cytoskeletal components, it is less easy to explain how binding to an intermediate filament protein such as cytokeratin 8 produces the biological effects which are reminiscent of LC1 or glucocorticoids. Elsewhere (Croxtall *et al*., 1997) we have speculated that this maybe through an effect on the binding or action of signal transduction proteins which may require cytoskeletal assemblies for full activation. One possible mode of peptide action might be through competition with native LC1 for cytoskeletal or other binding sites within the cell.

Whilst we cannot yet pinpoint the precise step at which these peptides act, it is clear that they are biologically effective molecules which, if their potency could be increased by (e.g.) substitution would potentially have a number of uses, including inhibition of cell growth, migration, mediator release and inflammation. Although care must be taken in extrapolating from these results in a single cell line, the fact that these peptides are active *in vivo* as well as *in vitro* indicates that they could prove useful therapeutic agents for a variety of conditions.

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