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COMMENTARY

LIPOCORTIN-DERIVED PEPTIDES

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The anti-inflammatory activity of GC† hormones is the result of several modulating effects exerted on cells and mediators involved in the inflammatory process. In the late 1970s, an important step in understanding the molecular mechanism of action of GCs was made by identifying a protein involved in the GC-induced inhibition of the release of active metabolites of AA [1, 2]. Although it is now accepted that GCs act at multiple levels to affect AA metabolism (i.e. suppression of the synthesis of metabolic enzymes) [3–5], the induction of a specific protein(s) with an inhibitory action on AA release is still an important concept. One of these proteins has now been characterised, cloned and shown to belong to a new superfamily, termed *lipocortins* (**LC**s) or *annexins* [6–8]. All members of this family possess a 70 amino acid motif which is repeated 4 times (in one case, 8 times). Within each repeat there is a sequence responsible for the phospholipid- and calcium-binding property which is characteristic of all LCs [8, 9]. In contrast to the 4-repeat portion (or *core*), the N-terminus region is highly variable among the various proteins [8, 9] not only for the amino acid sequence but also for its length; for instance, LC1 and LC2 have a long N-terminus of 33 and 24 amino acids, respectively, whereas LC5 has only a truncated form of 6 amino acids [8, 9]. Several disparate biological roles have been attributed to LCs, including interference with elements of signal transduction, inhibition of PLA_2, and involvement in exocytosis, in coagulation and in the cytoskeletal structure [10]. It is not likely that all LCs have a similar biological function. From the point of view of this article it is important to note that three members of this family, namely LC1, LC2 and LC5, exert a potent anti-inflammatory effect when injected into rats and mice [11–14].

Anti-inflammatory effects of human recombinant lipocortins

The cloning of hrLC1 allowed not only the identification of other proteins related in their sequences, as mentioned above, but also a better investigation of the anti-inflammatory profile of this protein. After the first original paper which showed hrLC1 to inhibit the release of thromboxane A_2 from perfused lungs *in vitro* [15], it was reported that local injection of microgram doses of LC1 into the rat paw potently inhibits the edema measured in response to carrageenin [11]. These data have been confirmed [16] and extended further by the report of an inhibitory effect on paw edema following systemic treatment with this protein [17, 18].

The carrageenin edema model, widely used as a screening method for anti-inflammatory agents, is a multi-step inflammatory response where virtually all soluble and cellular mediators are involved [19]. Although inhibition of prostanoid generation could explain the anti-edema action of LC1, other studies have focused on the cellular component of the inflammatory process. We have reported that systemic administration of hrLC1 potently reduced the infiltration of PMN into the pleural cavity following challenge with zymosan in the rat [18]. An effect on inflammatory cells was apparent even in the first of this type of studies [11], although not fully appreciated at the time, as hrLC1 exerted a smaller inhibition on carrageenin edema when leukopenic animals were used [11].

More recently, this aspect of LC1 action has been investigated using a murine air-pouch model [12]. Systemic treatment with hrLC1 caused a potent and dose-dependent inhibition of PMN migration in response to the pro-inflammatory cytokine interleukin-1 (IL-1) [12]. It is noteworthy that in this experimental model the inhibition of AA metabolism, achieved by using selective inhibitors of cyclooxygenase and lipoxygenase, did not modify the cellular response to the cytokine, which, on the contrary, was exquisitely sensitive to the GC dexamethasone as well as to LC1. Taken together, these observations indicated that hrLC1 is a potent inhibitor of acute inflammation not only as a result of a reduced release of AA metabolites, as originally proposed [1, 2], but also by potently inhibiting PMN trafficking. Table 1 summarizes the various experimental models in which hrLC1 has shown anti-inflammatory activity and also reports the few studies that have investigated the effect of hrLC2 and hrLC5. At systemic doses comparable to those of LC1, hrLC2 and hrLC5 inhibited the inflammatory

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† Abbreviations: GC, glucocorticoid; AA, arachidonic acid; LC, lipocortin; PLA_2, phospholipase A_2; hr, human recombinant; PMN, polymorphonuclear leukocytes; AF, antiflammin; and fMLP, formyl-Met-Leu-Phe.

Table 1. Inhibitory effects of human recombinant lipocortins (LCs) *in vivo*

Protein	Effective dose	Parameter evaluated	Experimental model	Reference
LC1	10–50 μg local	Paw swelling	Rat carrageenin edema	[11]
	25–50 μg local			[16]
	75–100 μg, i.v.			[17]
	1 mg/kg, i.v.			[18]
	1 mg/kg, i.v.	PMN migration	Rat zymosan pleurisy	[18]
	15 μg, i.v.	PMN migration	Mouse gel granuloma	[14]
	5–10 μg, i.v.	PMN migration	Mouse air-pouch	[12]
	1 mg/kg, i.v.	Mø migration	Rat zymosan pleurisy	[18]
	10 μg, i.v.	Circulating PMN	Mouse fMLP-neutropenia	[20]
	50 μg/kg, i.v.	Body temperature	Rabbit poly I:C fever	[21]
LC2	2 mg/kg, s.c.	Paw swelling	Rat carrageenin edema	[13]
LC5	1 mg/kg, i.v.	PMN migration	Rat zymosan pleurisy	[18]
	1 mg/kg, i.v.	Mø migration	Rat zymosan pleurisy	[18]

Abbreviations: PMN, polymorphonuclear leukocyte; Mø, macrophage; and fMLP, formyl-Met-Leu-Phe.

Table 2. Comparison of lipocortin (LC)-derived nonapeptide sequences

Amino acid	Parent protein	Sequence*	Potency ratio†
39-47 (AF‡-1)	Uteroglobin	M Q M K K V L D S	Not done
246-254 (AF-2)	LC1	H D M N K V L D L	1.0
204-212	LC5	S H L R K V F D K	1.0
223-231	LC2	P H L G K V F D R	0.7
231-239	LC1	P Q L R R V F Q K	3.5

* Underlined letters indicate amino acid identity with peptide 204-212 of LC5 sequence (the single letter code for amino acid is used).

† Potency ratio is calculated on the basis of two screening assays (ADP-induced platelet aggregation and PLA_2-induced stomach strip contractions) [23].

‡ AF = antiflammin.

response elicited by carrageenin and zymosan in the rat [13, 18]. The identification of small portions which may mimic, at least in part, the effect of these full length proteins represents an attractive and practical way to identify a potential "drug" with the biological activities of hrLCs. Active peptides may represent a step forward in solving the problems of stability (particularly evident in the case of hrLC1 [17]) as well as the perennial problem of protein delivery.

Peptides identified on the basis of a sequence similarity between lipocortins and uteroglobin

The first successful attempt to find active LC1-derived peptides came with the identification of a region of homology between LC1 and uteroglobin (another protein with anti-PLA_2 activity) [22]. The corresponding nonapeptides (Table 2) have been termed *antiflammins* (**AF**s), with AF-1 being drawn from the uteroglobin sequence (amino acids 39–47), and AF-2 corresponding to amino acids 246–254 of LC1. Both AFs inhibited in a concentration-dependent manner PLA_2 activity *in vitro* and reduced the edema response to carrageenin when injected locally into the rat paw *in vivo* [22]. However, the inhibitory effect on PLA_2 activity has only been reproduced with difficulty, raising doubts on the real effectiveness of AFs [24, 25]. Despite another study claiming that there is a direct interaction between AFs and PLA_2 [26], the observation that a LC1 mutant lacking AF-2 sequence can still inhibit PLA_2 *in vitro* to a similar degree as the naive protein [27] suggests that the portion of the molecule that AF-2 represents is not important for either interaction with, or inhibition of this enzyme.

Irrespective of the difference in data concerning the inhibitory action upon PLA_2 *in vitro*, several independent investigators have confirmed the anti-inflammatory nature of AF-1 and AF-2 not only in the rat paw edema test but also in other experimental models, thus broadening the interest for their potential applications. AF-1 and AF-2 inhibited the synthesis of PAF-acether from human neutrophils [28], human platelet aggregation induced by ADP [29], ocular inflammation in rats [30], and ear edema in mice [31, 32]. The latter study demonstrated that both peptides have potent anti-inflammatory actions in the absence of any effect on PLA_2 activity *in vitro*, and it was suggested that AFs could interfere with the process of PLA_2 activation in whole cell systems [31, 32]. In addition, a recent investigation has shown that AF-1 and, more potently, AF-2

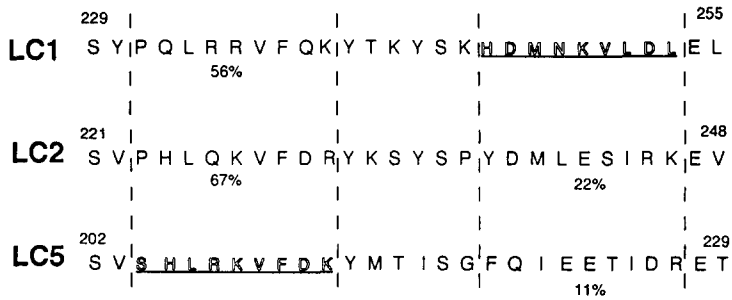

Fig. 1. Alignment of a portion of the third repeat of human LC1, LC2 and LC5. Sequence identities (%) with peptide 204-212 of LC5 (outlined and underlined in the first pair of broken lines) on LC1 and LC2 are reported. Similarly, the AF-2 region on LC1 is outlined and underlined in the last pair of broken lines, and sequence identities (%) on LC2 and LC5 are shown. The single amino acid code letter is used.

inhibited the conversion of AA to eicosanoids in rabbit colonic mucosa, this being indicative of an effect upon cyclo-oxygenase activity [33]. Since AFs affected neither AA-induced edema nor AA-induced platelet aggregation [29, 31, 32], it is possible that distinct mechanisms are responsible for the effect observed on different target cells.

The alignment of the uteroglobin sequence with that of LC5 enabled us to identify an LC5-derived nonapeptide with a good sequence similarity to AF-1 (Table 2). This peptide (**peptide 204-212** of LC5) potently reduced the release of AA metabolites from cells in culture and from arterial preparations with an approximate IC_{50} of 10 μg/mL (9 μM) [34, 35]. Though not affecting PLA_2 activity *in vitro*, peptide 204–212 was able to block PLA_2-induced contractions of rat stomach strips, which are largely due to prostaglandin generation [34]. This effect was specific because peptide 204-212 did not modify either AA-induced contractions or those elicited by serotonin or prostaglandin E_2 [34]. Similar to AFs, peptide 204-212 inhibited platelet aggregation [23] and also inhibited neutrophil adhesion to plastic surfaces [36]. Finally, the nonapeptide 204-212 reduced the edema response to carrageenin in a dose-dependent manner with a maximal inhibition of 30–40% when given locally [34]. The effectiveness in all these biological systems points to peptide 204-212 as a good candidate for a structure–activity-relationship investigation. On a structural basis there are at least three reasons which justify the choice of 204-212 instead of AF-1 or AF-2:

(a) Peptide 204-212 differs from AF-1 and AF-2 in that it has no methionine in its sequence, thus reducing the risk of inactivation by oxidation. Methionine oxidation has been proposed to account for the instability of AFs [37] and possibly for the conflicting enzymological data described above.

(b) Both peptide 204-212 and AF-2 fall within the third repeat of LC5 and LC1, respectively (see Fig. 1). However, the 204-212 sequence is highly conserved on LC2 and LC1 with a 67 and 56% identity, respectively. On the contrary, the AF-2 sequence is much less well conserved either on LC2 (22% identity) or on LC5 (11% identity) (Fig. 1 and [9]). Since both LC1, LC2 and LC5 possess anti-inflammatory activity *in vivo*, it is therefore possible that the conserved common region, corresponding to the sequence of nonapeptide 204-212, is responsible for this activity of the parent proteins.

(c) The crystal structure of human LC5 is available [38]. The four repeats are organized to form a slightly curved flat structure with two faces, one concave and one convex [38–40]. The five putative calcium binding sites are located on the convex face and protrude for binding to vesicle membranes [39, 40]. The third repeat is organised in five α-helix segments: segment III contains amino acids 204-212 and it is exposed on the convex surface of the molecule [38]; therefore, this region may potentially interact with biological membranes. The description of the LC5 crystal structure appears to confirm the suggestion that it is actually the third repeat that contains pharmacophore sites, with repeats 1, 2 and 4 contributing to the tertiary structure [9].

Structure–activity-relationship studies on peptide 204-212 sequence have been performed using two simple models: human platelet aggregation and rat stomach strip contractions. Confirming initial observations [34], the heptapeptide 206-212 was found to be equiactive to the nonapeptide in both systems [23]. Of the two pentapeptides SHLRK and KVFDK, the biological activity was retained by the second one, corresponding to region 208-212 (Table 2). This sequence has a high homology with the core of the AF sequence (KVLDL). The nonapeptides drawn from the alignment of the 204-212 sequence on LC1 and LC2 (Table 2 and Fig. 1) also possess biological activity. However, the LC2-derived peptide was as active as 204-212, while the LC1 nonapeptide (amino acid 231-239) was 3–4 times less active [23]. By analysing these sequences (Table 2), it is possible to identify two crucial residues, **K** 208 and **D** 211, in the nonapeptide 204-212 of human LC5. These two charged amino acids are separated by two nonpolar amino acids in 204-212 sequence as well as in the AF sequences. Keeping in mind that 204-212 falls into an α-helix structure within the surface-exposed third repeat of LC5 [38], and that one turn of the α-helix structure requires 2.6 amino acids, it is evident that the two charged amino acids K and D would be exposed almost on the same side

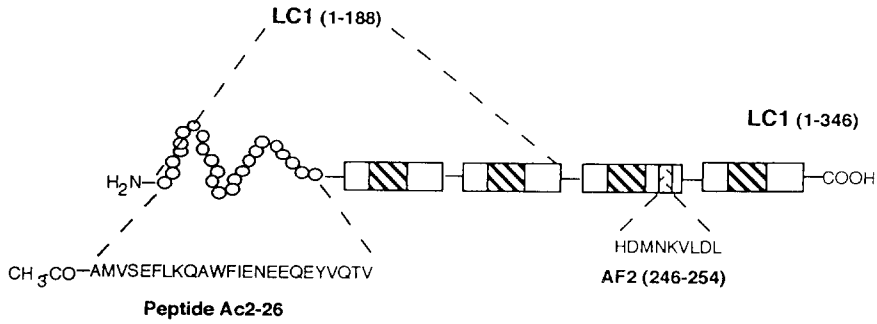

Fig. 2. LC1-derived active fragments. The LC1 structure (1-346) is highlighted with the N-terminus and 4-similar repeat units (≈70 amino acids each). Each unit contains the 17 amino acid consensus sequence (hatched box) which characterises LC families.

in a three-dimensional structure. A molecule containing lysine and aspartic acid spaced by two non-polar residues may represent a useful backbone for developing novel anti-inflammatory compounds endowed with the biological activity of the nonapeptide 204-212 of LC5.

N-Terminus peptide of lipocortin 1

LCs are grouped because of their common 4-fold repeat structure [8, 9]. The N-terminus region, however, is poorly conserved between family members, and it has been proposed to account for the selectivity of action of the different members of the family [9]. The anti-inflammatory property of LC1 has been studied extensively [11, 12, 16, 17] with some of its effects being mimicked by a long fragment (amino acids 1-188), which contains the N-terminus but lacks the AF-2 sequence as shown in Fig. 2 [41]. It is of interest that an hrLC1 preparation deprived of an intact N-terminus is without biological activity [17, *]. For all these reasons a peptide corresponding to a portion of the LC1 N-terminus (amino acids 2-26) has been tested for its potential anti-inflammatory effects *in vivo*. This peptide (hereafter referred to as **Ac2-26**) mimicked the LC1 effect in the carrageenin edema model when given locally [42]. Again similar to LC1, though ≈ 200 times less potently, Ac2-26 inhibited the *in vivo* cell trafficking in response to IL-1, with a calculated ED_{50} of 90 μg, i.v., per mouse [42]. Ac2-26 action was not restricted solely to IL-1-induced accumulation, and indeed it inhibited the neutrophil accumulation into the mouse air-pouch in response to other stimuli such as IL-8 and substance P (Table 3). This lack of specificity with regard to the stimulus applied implicates neutrophils as the site of action of Ac2-26. Systemic treatment with AC2-26 greatly reduced the intense neutrophil influx in the more complex inflammatory condition produced in the zymosan peritonitis model [20]. Ac2-26 (200 μg, i.v.) selectively attenuated edema formation in the skin in response to a neutrophil-dependent stimulus (zymosan-activated serum) with no effect on the edema induced by an agent that directly increases vasopermeability, such as histamine. Finally, this peptide, as well as hrLC1 itself, prevented the fall in the number of circulating PMN induced by intravenous treatment with the chemoattractant fMLP [20]. All these observations highlight an action of Ac2-26 on the neutrophil rather than on other cells involved in the migration process (e.g. endothelial cells). In support of an effect at the cellular level, Ac2-26 has been found to inhibit enzyme release from human neutrophils following stimulation with fMLP or leukotriene B_4 (Perretti M, Wheller SK and Flower RJ, unpublished observations). In line with these findings, previous studies have demonstrated hrLC1 to selectively inhibit the release of reactive oxygen species from alveolar macrophages [43] and peripheral neutrophils [44]. Such an effect could easily explain the protection exerted by the long fragment 1-188 in a rat model of cerebral ischemia [45]. Indeed, the fragment 1-188 has been found to inhibit IL-1-induced PMN infiltration into the mouse air-pouch [20]. All these findings lend strong support to the idea that LC1 and its active peptides, like Ac2-26, may be useful therapeutically in pathophysiological conditions in which PMN activation is implicated.

Table 3. Effect of peptide Ac2-26 on neutrophil migration *in vivo*

Treatment	Stimulus	Neutrophil migration (million per mouse)
PBS	Murine IL-1β	12.20 ± 1.1 (9)
Ac2-26 (200 μg)	(5 ng)	5.48 ± 1.5* (9)
PBS	Human IL-8	6.20 ± 0.25 (5)
Ac2-26 (200 μg)	(1 μg)	3.02 ± 0.42* (6)
PBS	Substance P	4.84 ± 0.74 (5)
Ac2-26 (200 μg)	(10 μg)	1.76 ± 0.42* (6)

Peptide Ac2-26 was given i.v. 10 min prior to challenge with the specific stimulus in 6-day-old murine air-pouches and neutrophil accumulation evaluated at a 4-hr time point. Values are means ± SEM (number of mice).
* $P < 0.05$.

* Cirino G, Browning JL and Flower RJ, unpublished data. Cited with permission.

Ac2-26 also maintains some similarity to LC1 with regards to *in vitro* biological effect, and, for instance, it reduces both basal and EGF-induced proliferation of a tumor cell line [46]. Again the peptide is ≈ 50 times less potent than hrLC1. In this particular study, smaller fragments of the 24 amino acid sequence have also been tested, finding that the full biological activity was retained by the second portion of the molecule (amino acids 13-25). This smaller peptide was also effective *in vivo* in the murine air-pouch model with a potency 3–4 times lower than Ac2-26. There is, however, the possibility of developing novel anti-inflammatory molecules based on this LC1 region, though further studies are necessary to identify the minimum number of amino acids essential for a fully active peptide.

A "unified model" for lipocortin peptide action

Many pharmacological investigations have been carried out since the original papers described LCs as PLA_2 inhibitors. Using a variety of techniques for measuring PLA_2 activity, it has been proposed that LC-induced inhibition is the result of a substrate depletion rather than a direct interaction with the enzyme [47]. However, a more specific mechanism cannot be excluded completely, in view of the recent observation that four out of the six calcium binding sites of LC1 are similar to the phospholipid binding sites present in PLA_2 [48]. Apart from understanding the molecular mechanism, it is important to underline the fact that the anti-inflammatory hrLCs reduce AA-metabolite formation from cells in culture or organ preparations [15, 18, 49, 50]; undoubtedly, this may have a potential application.

The conflicting reports on AF activity in PLA_2 assays have also contributed to the confusion surrounding the identification of active LC-derived peptides. Again, most of the problems were raised in reference to their anti-PLA_2 activity *in vitro*, and this is not surprising when we consider that it is not yet clear how the full length LCs inhibit this enzyme. However, AFs consistently showed anti-inflammatory activity in various models of experimental inflammation. The recent identification of active peptides from the N-terminus of LC1 may appear, at first glance, to further complicate the LC story. However, Ac2-26 and AFs mimic some of the biological properties of the parent protein with a different profile of effects. For instance, the inhibition of prostacyclin generation from aorta rings is inhibited by LC1 [49] and also by AF-2 and peptide 204-212 [35], but not by peptide Ac2-26. Similarly, AF-1 and 204-212 inhibited neutrophil adhesion to a plastic surface [36], but Ac2-26 was found inactive. LC1 has been reported to affect neutrophil accumulation *in vivo* [12], and this effect is mimicked by Ac2-26 [20, 42]. An identical pattern of behaviour was obtained in the neutropenia experiments where the fall in circulating neutrophils induced by fMLP was prevented by LC1 and Ac2-26 [20], but not by systemic treatment with AF-1 or peptide 204-212. It is clear from these data that different regions of LC1 may mediate different actions and that the N-terminus and the AF-2 regions represent two distinct active sites of the protein. Different active sites may exist also for other LCs, and, for example, within the 204-212 region of LC5 we have shown that the inhibitory action on AA metabolism is fully retained by the heptapeptide 206-212 and that it is due to the core portion 208-212, whereas other investigators have shown that the anti-coagulant action is attributable to histidine 204 [51].

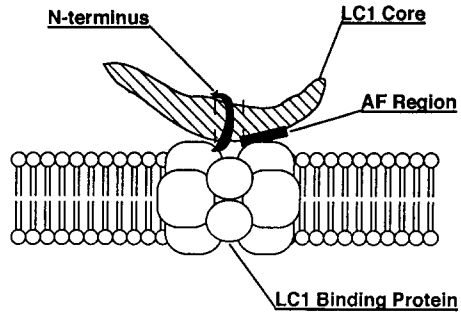

Fig. 3. Model for the interaction between LC1 and its putative receptor (LC1 binding protein). The LC1 core (315 amino acids) has a convex face which exposes the AF region and a concave face where is attached to the N-terminus (33 amino acids). The latter starts from the exit of the polar pore (dotted lines) and, if long enough, may interact with the receptor.

Having accepted that there may be at least two active sites for LC1, it remains to draw conclusions about the potential mechanism of action not only for the entire protein but also for its short peptides. A new aspect of LC1 pharmacology has arisen recently from the description of a specific and saturable binding to both human and murine leukocytes [52–54]. These binding sites are likely to represent an LC1 "receptor." It is therefore possible that the active peptides correspond to the regions involved in the interaction between LC1 and its "receptor," and each peptide may be able to transduce a second messenger signal. Alternatively, there could be different LC1 "receptors" on different target cells, and Ac2-26 and AF-2 may represent the regions involved in the activation of each distinct "receptor." This is, at the moment, mainly a matter of speculation, and further experiments are necessary to clarify this important aspect of LC1 biology. Congruent with this mechanism is the observation that blocking of LC1 binding sites on PMN *in vivo* abrogated the anti-inflammatory action of Ac2-26 [20]. A full understanding of the LC1 "receptor" pharmacology will eventually help to integrate all these data and to explain the different activities found for the various peptides. Interestingly, preliminary observations indicate that LC1 binding to human leukocytes (monocytes and granulocytes) has different characteristics from the binding to epithelial and endothelial cells.* The existence of specific binding sites on target cells is not only a feature for LC1 biology inasmuch as a recent paper has shown specific and saturating binding for LC5 to a human ovarian carcinoma cell line [55]. Since

* Goulding N and Guyre PM, unpublished data. Cited with permission.

LC1 and LC5 are both released from target cells by treatment with dexamethasone [56, 57], the existence of selective "receptors" that mediate their actions reinforces the idea of these proteins as endogenous mediators of some actions of GC hormones.

Some structural propositions arise from the recent elucidation of the crystal structures of human recombinant LC1 and LC5: both proteins have a similar crystal structure, at least for that concerning the four-repeat core region [38, 48]. Both have a concave and a convex face, the latter exposing the calcium binding sites and therefore being involved for the binding to biomembranes [39, 40]. Inside the crystal there is a structural organisation characteristic of a polar pore. The short (six amino acids) LC5 N-terminus is close to the exit of this central pore and has been proposed to have a modulatory action [39]. In the case of LC1 the N-terminus is longer (33 amino acids), and it may well go inside the pore and interact with specific regions on the "receptor." On the basis of these characteristics arising from a consideration of the crystal structure, we propose a model where the AF-2 region of LC1, or 204-212 in the case of LC5, present on the convex face of the protein is ready to interact with the "receptor," whereas the N-terminus has to go through the pore and therefore must be sufficiently long, as for LC1, to reach the "receptor" and exert its action. This model is shown in Fig. 3.

Conclusion

Some members of the LC family possess potent anti-inflammatory activity *in vivo*. This important observation in itself justifies the attempts to find small active peptides derived from their sequences. Active peptides from LC1 and LC5 are now available and structure–activity-relationship studies on their sequence may lead to the identification of novel anti-inflammatory molecules. In view of the fact that PLA_2 inhibition apparently no longer represents the major target of hrLCs, and that a specific binding to cells pivotal during the inflammatory process has been identified, the identification of small peptides interacting with these binding sites may lead to the discovery of a completely new class of anti-inflammatory agents.

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