Differential Localization of 5- and 15-Lipoxygenases to the Nuclear Envelope in RAW Macrophages*

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Leukotriene formation is initiated in myeloid cells by an increase in intracellular calcium and translocation of 5-lipoxygenase from the cytoplasm to the nuclear envelope where it can utilize arachidonic acid. Monocyte-macrophages and eosinophils also express 15-lipoxygenase, which converts arachidonic acid to 15(S)-hydroxyeicosatetraenoic acid. Enhanced green fluorescent 5-lipoxygenase (5-LO) and 15-lipoxygenase (15-LO) fusion proteins were expressed in the cytoplasm of RAW 264.7 macrophages. Only 5-lipoxygenase translocated to the nuclear envelope after cell stimulation, suggesting that differential subcellular compartmentalization can regulate the generation of leukotrienes versus 15(S)-hydroxyeicosatetraenoic acid in cells that possess both lipoxygenases. A series of truncation mutants of 5-LO were created to identify putative targeting domains; none of these mutants localized to the nuclear envelope. The lack of targeting of 15-LO was then exploited to search for specific targeting motifs in 5-LO, by creating 5-LO/15-LO chimeric molecules. The only chimera that could sustain nuclear envelope translocation was one which involved replacement of the N-terminal 237 amino acids with the corresponding segment of 15-LO. Significantly, no discrete targeting domain could be identified in 5-LO, suggesting that sequences throughout the molecule are required for nuclear envelope localization.

Macrophages and eosinophils express two related lipoxygenase (LO) enzymes capable of utilizing arachidonic acid as a substrate (1–7). 5-LO initiates the generation of biologically active leukotrienes (7–9), whereas 15-LO generates 15-hydroxyeicosatetraenoic acid. Enhanced green fluorescent 5-lipoxygenase (5-LO) and 15-lipoxygenase (15-LO) fusion proteins were expressed in the cytoplasm of RAW 264.7 macrophages. Only 5-lipoxygenase translocated to the nuclear envelope after cell stimulation, suggesting that differential subcellular compartmentalization can regulate the generation of leukotrienes versus 15(S)-hydroxyeicosatetraenoic acid in cells that possess both lipoxygenases. A series of truncation mutants of 5-LO were created to identify putative targeting domains; none of these mutants localized to the nuclear envelope. The lack of targeting of 15-LO was then exploited to search for specific targeting motifs in 5-LO, by creating 5-LO/15-LO chimeric molecules. The only chimera that could sustain nuclear envelope translocation was one which involved replacement of the N-terminal 237 amino acids with the corresponding segment of 15-LO. Significantly, no discrete targeting domain could be identified in 5-LO, suggesting that sequences throughout the molecule are required for nuclear envelope localization.

* This work was supported by National Institutes of Health Grants R01ES-50859 (to R. J. S.), PO1DK-38452, and P30DK-43351, and a gift from the Jewish Communal Fund (to R. J. S.). The costs of publication of this article were defrayed in part by the payment of page charges. The functions of 15-LO are still being elucidated. 15-LO has the capacity to utilize phospholipids as a substrate, oxidizing arachidonic acid and linoleic acid esterified at the SN2 position (27, 28). The direct oxidation of intracellular membranes may be an intermediate step in membrane degradation and turnover in the differentiation of certain cells (27–29). It has also been suggested that 15-LO plays a role in the initiation of atherosclerosis by oxidizing fatty acids esterified in cholesterol esters (30–32). The production of 15-hydroxyeicosatetraenoic acid in hematopoietic cells might require the liberation of arachidonic acid from phospholipids prior to oxidation. 15-LO has been reported to associate with membranes after stimulation of hematopoietic cells (5), but it is not known whether it can be co-localized with the leukotriene forming enzymes on the nuclear envelope.

The assembly of multiprotein complexes on specific membrane boundaries is now recognized as a process of general importance. It is a feature of signal transduction systems such as cytokine receptor complexes on the plasma membrane and enzyme systems such as the NADPH oxidase complex of activated phagocytes, and it may regulate leukotriene formation at the nuclear envelope. A growing number of peptide motifs are being identified which mediate precise targeting of the protein components (33). These include motifs which mediate direct binding to phospholipids such as PH domains, or motifs which mediate protein-protein interactions such as SH2 and SH3 domains. Whereas enzyme activity is critically dependent on the tertiary structure of a protein, targeting motifs are modular in nature. Both 5-LO and 15-LO have a proline-rich sequence which resembles an SH3 binding domain located 95 aa from the
C-terminus (11, 12, 34). 15-LO has a second proline-rich sequence between aa 326–338, and it is possible that the two enzymes have other targeting motifs which are currently unrecognized.

We have studied the localization of 5-LO and 15-LO in stimulated RAW macrophages and demonstrate that 5-LO, but not 15-LO, translocates to the nuclear envelope in these cells. Because 5-LO and 15-LO are related enzymes and share common structural features, we considered the possibility that selective targeting of 5-LO is mediated by a relatively small domain with a distinct targeting motif. 5-LO has three putative nuclear localization signals (NLS) that are not observed in 15-LO (35–37). NLS domains target proteins to the nuclear pore for transport into the nucleus, a distinct process from the targeting of proteins to membrane locations. 5-LO was not imported into the nucleus in RAW macrophages, enabling translocation to the nuclear membrane to be studied independently of NLS-mediated transport through the nuclear pore. We created chimeras of 5-LO and 15-LO to determine which regions of 5-LO are essential for translocation to the nuclear envelope. Our results provide evidence that a complex interaction of domains throughout the molecule is required for membrane targeting and suggest that no small linear sequence by itself can sustain nuclear envelope localization.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—The cDNAs coding for human 5-LO (11) and 15-LO (12) were ligated in-frame into the EcoRI site of EGFP-C2 (CLONTECH). Deletion mutants of 5-LO were made by digestion of the coding region with BamHI (C↓A↓110 and N↓A↓564), NotI (C↓A↓79), or XhoI (C↓A↓33) prior to ligation. EGFP-C2 does not contain a NotI cloning site and C↓A↓79 was ligated into pcDNA3 (Invitrogen) and excised with EcoRI and ApoI for cloning into EGFP-C2. Chimeric 5-LO/15-LO molecules were made from the EGFP-C2 plasmid constructs. Chimera A was made by cutting EGFP-C2/5-LO with BglII (cuts in cloning site of EGFP-C2 upstream of 5-LO) and XcmI (cuts at cDNA residue 749 in 5-LO) and replacing the excised fragment with the corresponding fragment derived from EGFP-C2/15-LO with BglII and XcmI (cuts at residue 693 in 15-LO). Chimera B was made by cutting EGFP-C2/5-LO with BamHI (cuts in cloning site of EGFP-C2 downstream of 5-LO) and PpuMI (cuts at residue 1369 in 5-LO) and replacing the excised fragment. The corresponding segment of 15-LO was amplified by polymerase chain reaction using a forward primer corresponding to bp 1309–1332 with a BpuMI site added at the 5′-end (5′-AAGGACCTAACCTA-CAGCTCCTCTGTGCCC-3′), and a reverse primer corresponding to bp 1992–1969 with a BamHI site added at the 5′-end (5′-GGATCTTTCATGTCGCCCCAGGTTTTCCAC-3′). Chimera C was made by cutting EGFP-C2/5-LO with XcmI and replacing the excised fragment. The corresponding segment of 5-LO was amplified by polymerase chain reaction using a forward primer corresponding to bp 744–770 (5′-GGGTAGACGTTCGTTAATGCTG-CAAC-3′), which contains the endogenous XcmI site, and a reverse primer corresponding to bp 1286–1260 with an additional 4 bp at the 5′-end to generate an XcmI site (5′-CCAGCTGCTCTGCTCCTCGCTTG-GTTGATG-3′). The cycling conditions used for polymerase chain reactions were 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min: 25 cycles were followed by 1 cycle with a 10-min extension time. All constructs were sequenced at the Massachusetts General Hospital Molecular Biology Core Facility to confirm their identity.

**Cell Culture and Transfections**—RAW 267.4 mouse macrophages were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. The cells were grown to 50% confluency on double chamber slides (Falcon) and transfected with 2 μg of plasmid DNA/chamber using DEAE-dextran or SuperFect Reagent (Qiagen). Transfection efficiencies were determined to be 5–10% by EGFP fluorescence or by parallel transfections with pcDNA3.1(+)-myc-His-c-lacZ (Invitrogen) followed by staining with X-gal solution. HL-60 cells were maintained in RPMI supplemented with 10% fetal bovine serum and were differentiated by incubating 5 × 10^6 cells/ml with 1.3% MeSO for 5 days.

**Cell Activation and Fluorescence Analysis**—Cells were washed three times in PBS 48 h after transfection and were then incubated for 10 min at 37 °C in PBS containing 1 mM CaCl_2_ and 5 μM A23187. The cells were fixed for 30 min in 4% paraformaldehyde in PBS, washed twice in PBS, and mounted under coverslips in Gel/Mount (Biomeda). EGFP fusion proteins were localized by direct fluorescence. Representative results from multiple transfection/cell activation experiments are shown. In control experiments, cells were incubated for 10 min at 37 °C in PBS without A23187 or were preincubated in PBS containing 1 μM MK-886 for 5 min at 37 °C prior to the addition of A23187. As an alternative to A23187 stimulation, some cells were incubated with PMA (100 ng/ml) or dibutyryl cAMP (1 μM) for 20 min at 37 °C prior to fixation.

For indirect immunofluorescence of lamin B, nontransfected cells were fixed as before and permeabilized with 0.1% Triton X-100 in PBS for 4 min. They were blocked in 10% goat serum for 30 min and then incubated with rabbit anti-lamin B (Dr. J. Casanova, Massachusetts General Hospital) diluted 1:1000 for 1 h, followed by fluorescein isothiocyanate goat anti-rabbit IgG (Molecular Probes) diluted 1:200 for 40 min. Fluorescence was analyzed under fluorescent microscopes (490/525 nm) using a Nikon FXA photomicroscope, and images were processed using IP Spectrum acquisition analysis software (Scandianics, Vienna, VA).

**Immunoprecipitations and Western Blotting**—The expression of the constructs was confirmed by Western blotting using a mouse monoclonal antibody to EGFP (CLONTECH). 48 h after transfection, the cells were extracted in 50 mM Tris HCl, pH 7.5, 250 mM NaCl, 0.1% Nonidet P-40, 10% glycerol, and centrifuged at 12,000 × g for 20 min. The cell lysate was incubated with anti-EGFP diluted 1:500 for 1 h and then with protein G-agarose (Boehringer) for 3 h. The agrose beads were washed, and bound proteins were solubilized in SDS sample buffer, fractionated by SDS-PAGE, and electrophoretically transferred to nitrocellulose Trans-Blot membranes (Bio-Rad). The membranes were incubated with anti-EGFP diluted 1:500 and sheep anti-mouse peroxidase (Amersham Pharmacia Biotech) diluted 1:5000 and were processed for enhanced chemiluminescence (Amersham Pharmacia Biotech). For analysis of FLAP, RAW macrophages or HL-60 cells were suspended in 50 mM Tris HCl, pH 7.5, 5 mM EDTA, 1 mM benzamidine, 2 mM PMSF, and disrupted by sonication at 4 °C using a Vibramec probe sonicator (3 × 1 min, setting 4, 50% output, 10^6 cells/ml). The sonicate was fractionated on a 15% gel (84 μg of protein/well), transferred to nitrocellulose, and analyzed with anti-FLAP antibody (Dr. F. Fitzpatrick, Huntsman Cancer Institute) diluted 1:200.

**RESULTS AND DISCUSSION**

EGFP-15-LO and EGFP-5-LO were detected as immunoreactive bands with the expected molecular mass of 100 and 105 kDa, respectively, following immunoprecipitation analysis of transfected RAW cells (Fig. 1). EGFP-5-LO was distributed throughout the cytoplasm of unstimulated RAW cells (Fig. 2A). When cells were stimulated with the calcium ionophore A23187, EGFP-5-LO translocated to the nuclear envelope (Fig. 2D). The distribution of fluorescence around the nucleus appeared the same as that observed following staining for lamin B (Fig. 2C). RAW macrophages therefore provide a convenient model system for studying nuclear envelope translocation, and demonstrate that the machinery for translocation (19, 20) is conserved between humans and mice.

No enrichment of fluorescence was observed on the nuclear envelope in unstimulated RAW cells, indicating that overexpression of 5-LO is insufficient to drive equilibrium binding to the membrane in the absence of a calcium signal. Stimulation...
EGFP-5-LO and EGFP-15-LO. RAW 264.7 macrophages were transfected with EGFP-5-LO (A and D) or EGFP-15-LO (B and E). The transfected cells were incubated for 10 min at 37 °C in PBS containing 1 mM Ca\(^2+\) and either 5 μM A23187 (D and E) or no A23187 (A and B). The cells were fixed, and EGFP fusion proteins were localized by direct fluorescence. The distribution of a chimeric 5-LO/15-LO molecule (chimera A) expressed as a fusion protein with EGFP is shown in A23187-stimulated cells (F). The nuclear envelope was stained by indirect immunofluorescence of nontransfected cells using rabbit anti-lamin B followed by fluoresceinated goat anti-rabbit IgG (C).

EGFP-15-LO was expressed in unstimulated RAW macrophages with the same distribution as EGFP-5-LO (Fig. 2B) but did not change its distribution with the addition of calcium ionophore (Fig. 2E). It can be concluded that 15-LO does not translocate to membranes in RAW macrophages by the same mechanism as 5-LO. This difference may prevent the two lipoxygenases competing for the same pool of arachidonic acid in cells that express both enzymes. Previous studies suggest that 15-LO can associate with nonnuclear membranes in a calcium-dependent manner (5, 29), and the resolution of our assay system does not rule out this possibility. The results obtained here do not necessarily extend to the movement of 15-LO in epithelial cells but are consistent with observations in eosinophils and monocytes (5).

We created a series of deletion mutants of 5-LO to search for domains required for translocation to the nuclear envelope (Fig. 3). The mutants were expressed as EGFP fusion proteins and were all localized to the cytoplasm of unstimulated RAW cells. Deletion of the C-terminal 110 aa (CΔ:110) abolished the ability of the fusion protein to localize to the nuclear envelope. A number of domains located in this deleted region have been suggested to play a role in translocation. A proline-rich sequence between amino acids 565–577 resembles an SH3 binding domain and has been implicated in mediating protein-protein interactions (34, 38). Pharmacological evidence suggests a role for MAP kinase mediated phosphorylation as a requisite for both nuclear envelope localization and activity (38, 39), presumably by phosphorylation of a MAP kinase/Cdc2 kinase site at aa 662–667. The ability of these domains to disrupt the molecule over a more extensive region. Deletion of the C-terminal 110 aa as expressed as an EGFP fusion protein. The protein could not translocate to the nuclear envelope, indicating that although this C-terminal region is required for translocation it is not by itself sufficient.

Smaller deletions of the C-terminal (the CΔ:79 truncation which includes the proline-rich domain but not the MAP kinase site, and the CΔ:33 truncation which lacks the kinase site but contains an extended portion of the C-terminal) do not localize to the nuclear envelope on cell stimulation. However, these deletion mutants had a low level of expression, and we cannot rule out the possibility that the deletions affect folding and disrupt the molecule over a more extensive region. Deletion of the C-terminal isoelucine is sufficient to prevent co-ordination with iron and probably results in improper folding which blocks enzyme activity (14). To circumvent this problem, we created a series of chimeras between 5-LO and 15-LO (Fig. 4). These were chosen to allow the chimeric molecule to retain common LO structural properties in an intact configuration while replacing domains of 5-LO with those of the homologous nontargeted 15-LO molecule. For example, 15-LO contains a C-terminal isoelucine that can participate in binding iron so as to maintain structural integrity. It was not considered essential to retain enzyme activity in the chimeras because targeting domains are often modular and generally less sensitive to small variations in folding. The chimeras, that were expressed as
The junctions of chimeras take into account alignment of 5-LO and 15-LO. The location of each junction is numbered according to the corresponding amino acids in 5-LO (bold type) and 15-LO (normal type). The junctions occur at restriction enzyme sites for XcmI (chimeras A and C) or PpuMI (chimera B). Abbreviations and symbols are explained in the legend for Fig. 3. The sequences of the proline-rich domains (P1–P3) contain two prolines (boxed) in appropriate positions for binding an SH3 domain.

EGFP fusion proteins, all localized to the cytoplasm of resting RAW cells.

When aa 1–237 at the N-terminal of 5-LO were replaced with the corresponding aa 1–232 of 15-LO, the resulting molecule (chimera A) retained the ability to translocate after addition of A23187 (Fig. 2F). The fluorescence observed on the nuclear envelope, however, was of lower intensity than that seen with the native 5-LO fusion protein. This might indicate a reduced efficiency of translocation, but nuclear envelope localization was clearly discernible and distinct from unstimulated cells. In contrast, replacement of the C-terminal 231 aa of 5-LO with the corresponding aa of 15-LO (chimera B) abolished translocation. This chimeric molecule lacks the C-terminal MAP kinase site but contains a proline-rich region the same distance from the C-terminal as in native 5-LO. The C-terminal proline-rich regions of 5-LO and 15-LO are closely related (10 aa are identical in the 13 aa sequence). Both contain two prolines in critical positions for binding the polyproline helix to an SH3 domain. Exchange of the proline-rich domains is therefore unlikely to account for the loss of targeting of chimera B. This domain might mediate interactions with cytoskeletal proteins in the cytoplasm (34, 38) rather than with the nuclear envelope.

15-LO contains a second proline-rich region between aa 326–338. This also resembles an SH3 binding domain but has a different context and might mediate different interactions. It is centrally located in a region which contains the most hydrophobic portions of the molecule. Hydropathy plots derived by the method of Kyte and Doolittle (40) are very similar for both 15-LO and 5-LO, and the moderately hydrophobic domains shown in Figs. 3 and 4 (boxed) correspond to aa 278–326 and 352–424 in 5-LO (41). The latter segment contains hydrophobic amino acids that form a binding pocket for arachidonic acid and two histidines that coordinate iron at the active site. 5-LO and 15-LO appear structurally similar throughout this region, but 5-LO does not contain a central proline-rich domain. We considered the possibility that this domain might be sufficient to prevent translocation of 15-LO to the nuclear envelope by mediating inhibitory interactions or alternative targeting. We replaced the central core of 15-LO (aa 233–403) with the corresponding region (aa 238–411) of 5-LO. The resulting molecule (chimera C) localized to the cytoplasm but remained unable to translocate to the nuclear envelope after cell stimulation with A23187, ruling out this possibility.

The lack of targeting of chimera C and the CA:110 deletion mutant indicate that the hydrophobic regions in the core of 5-LO are insufficient for membrane association. These mutant molecules remained exclusively in the cytoplasm and did not show any detectable localization to membrane boundaries. The C-terminal portion of the molecule is also insufficient for targeting (deletion mutant NA:564) but the results using chimera B confirm its importance. The critical part of the C-terminal cannot be localized to the proline-rich domain because this is substituted with a homologous domain in chimera B. Collectively our results demonstrate that no small linear domain by itself can sustain nuclear envelope localization of 5-LO, and targeting must depend on cooperative interactions between different parts of the molecule. The N-terminal 237 aa of 5-LO do not appear to be essential for membrane interaction but may still participate. Chimera A was able to translocate to the nuclear envelope on cell stimulation but with reduced efficiency. The N-terminal of 15-LO contains a lipase-like β-barrel of 115 amino acids (16) which might serve a permissive role in mediating membrane localization of chimera A. The N-terminal portions of 5-LO and 15-LO (aa 1–237) share 56% amino acid identity and might exhibit similar secondary structures.

Recent studies describe localization of 5-LO in the nucleus of certain cells (42–46). Import of proteins into the nucleus is mediated by NLS sequences, and the 5-LO molecule has three putative NLS domains that may contribute to this transport step (35). However, transport of 5-LO through the nuclear pore probably represents a distinct event from targeting to the membranes of the nuclear envelope. For example, a nuclear pool of 5-LO is associated with alveolar macrophages and recruited neutrophils, but this is distributed throughout the nucleoplasm and still requires a calcium stimulus to translocate to the nuclear membrane (45, 46). In the present study no fluorescence was detected in the nucleoplasm of RAW macrophages in resting cells or at any observation time up to 20 min after the addition of A23187. This suggests that translocation occurs from the cytoplasm to the outer nuclear membrane and is independent of the NLS domains in the 5-LO molecule in these cells.

15-LO lacks putative NLS domains, but this may not be a sufficient explanation for its failure to target to the outer nuclear membrane in RAW macrophages which do not appear to import 5-LO into the nucleus. The role of NLS domains in 5-LO has been investigated with EGFP fusion proteins (36). This work is distinct from our study because it analyzes movement from the cytoplasm to the nucleoplasm rather than translocation to the nuclear envelope. Taken together the two studies suggest considerable complexity in both nuclear transport and nuclear envelope localization. It has recently been suggested that there may in fact be mechanistic links between the two events (37), for example docking of 5-LO at the nuclear pore might be an intermediate step which enables 5-LO to engage other binding partners in the plane of the membrane. A putative NLS at aa 652–656 was implicated in participating in nuclear envelope localization (37). Our observations do not rule out this possibility because NLS[652–656] is located within the critical C-terminal domain required for translocation of 5-LO in RAW macrophages.

Unlike cPLA2 and conventional isoforms of protein kinase C, 5-LO does not contain an obvious calcium-binding domain.
5-LO co-localizes with cPLA₂ and FLAP in the nuclear envelope, but no direct interactions with these proteins have yet been demonstrated. Despite recent analyses of putative nuclear localization signals (36, 37), 5-LO still has no clearly identifiable targeting motif to direct translocation to the nuclear envelope. Our comparison of 5-LO with a homologous nontargeted enzyme (15-LO) in RAW macrophages suggest that multiple domains participate in mediating translocation to the nuclear envelope and demonstrate that the two enzymes use separate mechanisms to access a common substrate.

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