Determinants of 5-Lipoxygenase Nuclear Localization Using Green Fluorescent Protein/5-Lipoxygenase Fusion Proteins*

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5-Lipoxygenase catalyzes the first two steps in the biosynthesis of leukotrienes, potent extracellular mediators of inflammation and allergic disorders. The unanticipated observation of 5-lipoxygenase in the nucleus of some cell types including bone marrow-derived mast cells (Chen, X. S., Naumann, T. A., Kurre, U., Jenkins, N. A., Copeland, N. G., and Funk, C. D. (1995) J. Biol. Chem. 270, 17993–17999) has raised speculation about intranuclear actions of leukotrienes or the enzyme itself. To explore the entry of 5-lipoxygenase into the nucleus we have transfected various cell types with expression vectors encoding native 5-lipoxygenase and green fluorescent protein/5-lipoxygenase (GFP-5LO) fusion proteins. 5-Lipoxygenase and green fluorescent protein/5-lipoxygenase co-localized with the nuclear DNA stain Hoechst 33258 in each cell type. The three main basic regions of 5-lipoxygenase were incapable of acting as "classical" nuclear localization signal sequences. Mutations that abolished enzyme activity/non-active iron resulted in proteins that would no longer enter the nucleus. An NH₂-terminal 5-lipoxygenase fragment of 80 residues was sufficient for directing nuclear localization of green fluorescent protein but not cytosolic pyruvate kinase. The combined data suggest that 5-lipoxygenase enters the nucleus not by a classical nuclear localization signal but by a non-conventional signal located in the predicted b-barrel domain that may be masked by structural alterations.

5-Lipoxygenase (arachidonate:oxygen 5-oxidoreductase, EC 1.13.11.34) is a non-heme enzyme found primarily in white blood cells, macrophages, and mast cells that converts arachidonic acid first to 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and then to leukotriene (LT)A₄ (5,6-oxido-7,9,11,14-eicosatetraenoic acid(1)). Subsequent conversion of leukotriene A₄ by leukotriene A₄ hydrolase yields the potent neutrophil chemoattractant leukotriene B₄. Alternatively, conjugation of LTA₄ with glutathione by leukotriene C₄ synthase plus downstream metabolism leads to the cysteinyl leukotrienes that influence airway reactivity and mucus secretion especially in asthmatics (1–3).

5-Lipoxygenase was isolated originally from the cytosol fraction of human and porcine neutrophils (4, 5). The enzyme was shown subsequently to undergo a calcium-dependent translocation to the nuclear envelope upon ionophore A23187 stimulation and was dependent on the 5-lipoxygenase-activating protein situated in this location for leukotriene biosynthesis (6, 7). More recent work based on immunofluorescence techniques and cellular fractionation demonstrated that certain cell types capable of leukotriene formation (alveolar macrophages, rat basophilic leukemia cells, and mouse bone marrow-derived mast cells) express 5-lipoxygenase completely or partially in the nucleus (8–11). Additionally, it was shown that cytosolic 5-lipoxygenase in rat neutrophils could enter the nucleus if they were first elicited in vivo with various inflammatory agents or subjected to adherence to glass in vitro (12).

The discovery of 5-lipoxygenase in the nucleus was a surprising observation since it is well known that leukotrienes must exit the cell once synthesized to act on cell surface G protein-coupled receptors to exert their actions on neutrophils or bronchiale smooth muscle (13, 14). The possibility of 5-lipoxygenase itself or leukotrienes acting in the nucleus was raised. The recent observation that LTB₄ could bind to the nuclear peroxisomal proliferator-activated receptor-α indicated that intranuclear actions of leukotrienes are feasible (15).

Nothing is known about control of 5-lipoxygenase entry into the nucleus in some cell types but not others. Proteins enter the nucleus by nuclear localization signal (NLS) sequences that are recognized by specific importins, prior to nuclear pore docking, translocation through the pore and release from the pore’s inner side (16, 17). The NLS is typically a short basic region or bipartite basic sequence (18, 19). Increasingly, however, novel NLS sequences are being recognized for import of particular classes of proteins; for example, the 38-amino acid M9 domain of heterogeneous nuclear ribonucleoprotein A1 (20, 21). Here, we demonstrate primarily with the use of green fluorescent protein (GFP)/5-lipoxygenase fusion proteins the complexity of events for 5-lipoxygenase nuclear entry.

EXPERIMENTAL PROCEDURES

Construction of Expression Vectors—The full-length cDNA encoding human 5-lipoxygenase from pT3–5LO (22) subsequently cloned into pCDNA3 (Invitrogen) or a version with deletion of sequence encoding six amino acids at the carboxyl terminus (22) was cloned into the EcoRI/ApaI sites of a GFP vector pEGFP-C2 (CLONTECH) to obtain pEGFP-C2/5LO (GFP-5LO) and pEGFP-C2/5LO(C6-deletion) (GFP-5LO(C6-deletion)). To construct GFP-SV40/NLS containing a sequence representing the NLS (PKKKRKV) from SV40 large T antigen (18) and GFP-5LO(128–135) in which basic amino acid region 2 (see Fig. 3 and “Results”) is included, two complementary pairs of oligonucleotides

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The abbreviations used are: 5-HPETE, 5-hydroperoxyeicosatetraenoic acid; 5LO, 5-lipoxygenase; GFP, green fluorescent protein; GFP-5LO, green fluorescent protein/5-lipoxygenase fusion protein; 5-HETE, 5-hydroxyeicosatetraenoic acid; 5LO, 5-lipoxygenase deficient; NLS, nuclear localization signal; PK, pyruvate kinase; BMCC, bone marrow-derived mast cells; HEK, human embryonic kidney; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatography.
with restriction enzyme overhangs were synthesized as follows: AAT
TCCCAAGAGAACGAAAGGTGGG for SV40/NLS and AAT
TCAAAGACACCGCTAAAGACACTGGG for the second basic region
of 5-lipoxygenase (bases for EcoRI and BamHI sites are underlined).
The oligonucleotides with 5′-ends phosphorylated by T4 polynucleotide
kinase were ligated and limited by the EcoRI and BamHI sites of
pEGFP-C2. Correct insertion of the oligonucleotides was verified by
automated DNA sequencing (Applied Biosystems BigDye Terminator
Ready reaction kit reagents; ABI 373 sequencer) using the facilities of
the Department of Genetics.

The plasmid pcDNA3/Myc pyruvate kinase (PK) was used for con-
struction of PK-5LO and PK-5LO mutants fused to a protein tag. Gen-
erally, cDNAs for 5-lipoxygenase and its mutants were cloned into the
KpnI site that corresponds to codon 443 of PK and different restriction
sites available in the downstream linker region.

In vitro mutagenesis was carried out using the QuikChange Site-
directed Mutagenesis kit (Stratagene) or by polymerase chain reaction
on pEGFP-C2/5LO template. Transcripts of human 5-lipoxygenase or
platelet-type 12-lipoxygenase cDNA were performed by subcloning and/or
polymerase chain reaction on pEGFP-C2/5LO, pcDNA3/5LO, and
pcDNA6/H6His-12LX (23). Details of the procedures and the se-
quences of mutagenic primers are available upon request. DNA se-
quencing as mentioned above was used to verify the introduced muta-
tions and polymerase chain reaction products.

Cell Transfection—Mouse bone marrow-derived mast cells
(BMMC) were isolated and cultured from wild-type (5LO+/+) and
5-lipoxygenase deficient (5LO−/−) mice as described previously (10, 24).
In order to transfet 5LO−/− BMMC, 5 × 106 cells were washed twice
with RPMI 1640 and resuspended in 0.8 ml of RPMI 1640 with 40 µg of
pcDNA3/5LO. Cells in the suspension were electroporated at 960 mi-
crofarads and 400 V (Bio-Rad Gene Pulser) and transferred immedi-
ately into fresh bMMC culture medium. Transfected cells were used for
immunocytochemistry 48 h after electroporation.

Human embryonic kidney (HEK) 293 cells and NIH-3T3 fibroblasts
were grown in Dulbecco's modified Eagle's medium (Life Technolo-
gies, Inc.) supplemented with 10% fetal bovine serum. Chinese hamster
ovary (CHO) cells were cultured in Ham's F-12 medium with 10% fetal
bovine serum. Cells growing in Petri dishes or 0.1% gelatin-coated glass
chamber slides were transfected with plasmid DNA by calcium phos-
phate co-precipitation techniques (10). The transfected cells were used
for GFP fluorescence microscopy (20 h post-transfection), protein prep-
uation, 5-lipoxygenase activity assay, and immunocytochemistry (40 h
post-transfection).

Fluorescence Microscopy—Indirect immunofluorescence analysis
was carried out as described (10) for mouse BMMC with the following
modifications: 5LO+/− BMMC, 5LO−/− transfected BMMC and HEK
293 cells transfected with pcDNA3/human or mouse 5LO plasmid
were fixed with 2% paraformaldehyde in PBS for 20 min at room tempera-
ture followed by a 10-min incubation in 0.3% Triton X-100 in PBS. Cells
were incubated with 3% bovine serum albumin for 30 min to block
nonspecific binding and then incubated with rabbit anti-human 5LO
antiserum (1:100 dilution; see below) or monoclonal anti-Myc tag anto-
bodies (9E10; 1:1,500; (20)) in PBS with 3% bovine serum albumin.
After three 10-min washes with PBS, cells were incubated with the DNA
stain Hoechst 33258 (Molecular Probes, 0.5 µg/ml in PBS) for 5 min and
mounted with Gel/Mount (Biomedica Corp.) after brief rinses with
PBS.

For initial assessment of time course of GFP-5LO expression, flu-
orescence was monitored in living cells with a Nikon inverted microscope
equipped with fluorescence capability. For data documentation, HEK
293, NIH-3T3 fibroblasts, and CHO cells transfected with pEGFP-C2/
human 5LO or its mutant plasmids were fixed with 2% paraformalde-
yde in PBS for 20 min followed by a 5-min incubation with Hoechst
33258 stain. Slides were mounted with Gel/Mount and kept at 4 °C.

Slides were examined with an Olympus AX-70 analytical microscope
incorporated with fluorescence capability. For data documentation, HEK
293 cells (Fig. 1B) the fusion protein co-localized with the nuclear stain
Hoechst 33258 as in native 5-lipoxygenase-transfected cells. Comparison
of the enzyme activity of native 5-lipoxygenase and GFP-5LO in HEK
293 sonicated cell supernatants indicated that arachidonic acid was conv-
tered to 5-HPETE and 5-HETE to approximately the same extent (Fig. 2). In
agreement with previous studies, GFP alone exhibited a nonspecific
acellular expression pattern (26).

Analysis of Basic Regions of 5-Lipoxygenase as NLS Sequences—The classical NLS is a short basic region of 3 or more
residues or a bipartite basic region separated by a variable
number of nonbasic residues (27). We chose the three most
likely candidate regions to represent a classical NLS that dif-
fminated to some extent from other non-nuclear localized lipo-
xygenases for mutational analysis (Fig. 3). Three double mutant
GFP-5LO constructs were prepared and transfected into HEK
293 cells (Fig. 4). Both the basic region 1 (R72Q/K73Q) and
basic region 3 (K653Q/K654Q) constructs yielded nuclear local-
ization patterns identical to GFP-5LO (Fig. 4). However, the
double mutant of basic region 2 (R131Q/R132Q) yielded a pre-
dominantly cytosolic localization pattern. All proteins were
expressed at similar levels in the transfected cells (Fig. 4C).

When we tested the enzymatic activity of the double mutants
the basic region 1 and 3 mutations did not influence 5-lipoxygen-
ase activity but the R131Q/R132Q mutations abolished en-
yme activity (Fig. 2, left panel). This result raised the possi-
bility that basic region 2 was not really a NLS but that the

RESULTS

Transfection of cDNA Encoding 5-Lipoxygenase into Various
Cell Types Results in Nuclear Localization—Since 5-lipoxy-
genase is situated in the cytosol of resting neutrophils, the nu-
cleus of BMMC and in both compartments in rat basophilic
leukemia-1 cells and macrophages, we sought to determine the
local cellular localization of 5-lipoxygenase in various cell types
transfected with 5-lipoxygenase expression vectors prior to de-
tailed analysis of NLS sequences. As previously determined
(10), 5-lipoxygenase was detected in the nucleus of BMMC
using indirect immunofluorescence studies (Fig. 1A). A similar
expression pattern was observed in transfected BMMC
obtained from 5LO−/− mice that had been generated by gene
targeting techniques (Fig. 1A). Nuclear localization was also
observed for either human or mouse 5-lipoxygenase in trans-
fected HEK 293 cells (Fig. 1A).

A GFP Tag Attached to the NH2 Terminus of 5-Lipoxygenase
Does Not Alter Enzyme Activity or Cellular Localization in
Transfected Cells—To simplify the localization studies we de-
veloped a construct encoding a GFP-5LO fusion protein. In
transfected HEK 293 cells, CHO cells, and NIH-3T3 fibroblasts
(Fig. 1B) the fusion protein co-localized with the nuclear stain
Hoechst 33258 as in native 5-lipoxygenase-transfected cells.

Comparison of the enzyme activity of native 5-lipoxygenase
and GFP-5LO in HEK 293 sonicated cell supernatants
indicated that arachidonic acid was converted to 5-HPETE
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Western Blot Analysis and Activity Assay of 5-Lipoxygenase—HEK
293 cells, 20 or 40 h post-transfection, were harvested to prepare soluble
proteins as described previously (10) for Western blot and 5-lipoxygen-
ase activity assay. Western blot analysis was carried out using poly-
clonal 5-lipoxygenase antiserum 1550 (25) (1:1000 dilution) or a mouse
monoclonal antibody against GFP (Berkeley Antibody Co., 1:1000 dilu-
tion) with enhanced chemiluminescence detection. Activity assay of
5-HPETE and 5-HETE were separated by reverse phase-HPLC in a
mobile phase consisting of acetonitrile/water:acetic acid (60:40:0.1) or
methanol:water:acetic acid (85:15:0.1) with UV detection at 235 nm and
a flow rate of 1 ml/min on a Hewlett-Packard Series 1050 system
equipped with a Columbus 5 µm C18 column (250 × 4.6 mm; Phenome-
nox, Torrance, CA).

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enzyme activity/folding of the protein might also influence nuclear localization.

**Analysis of 5-Lipoxygenase Structural Alterations on Nuclear Localization**—5-Lipoxygenase contains a non-heme iron atom essential for catalytic activity (28). Based on the crystal structures of soybean and rabbit reticulocyte lipoxygenases (29, 30) and mutational analyses of 5-lipoxygenase (22, 31, 32), there are three important histidine residues (His-367, -372, and -550) and the COOH-terminal isoleucine (Ile-673) which participate in binding the iron atom and presumably which are essential for maintaining proper structural integrity for enzyme activity. Five constructs with mutations to abolish enzyme activity and influence iron binding were prepared and introduced into HEK 293 cells (Fig. 5). Mutations known to completely abolish iron presence in 5-lipoxygenase (H372Q, H550Q, and C6-deletion (deletion of the COOH-terminal 6 amino acid residues including Ile-673); Refs. 31 and 32) led to nearly exclusive cytosolic-localized enzyme (Fig. 5B). Mutations known to abolish enzyme activity yet leave partial presence of iron in the protein (H367Q, H367N) showed graded distribution of 5-lipoxygenase in the nucleus and cytosol depending on the iron content (Fig. 5B; Ref. 31). All the mutated 5-lipoxygenase constructs were expressed in the transfected cells to a similar extent (Fig. 5C) but had lost enzyme activity by HPLC analysis.

**Are There Regions of 5-Lipoxygenase Sufficient for Directing Nuclear Localization?**—Various portions of 5-lipoxygenase were fused to GFP to see if a specific region of the protein could directly direct nuclear localization. Initially, four constructs were designed based on restriction enzyme sites suitable for cloning for maintaining proper structural integrity for enzyme activity. Five constructs with mutations to abolish enzyme activity and influence iron binding were prepared and introduced into HEK 293 cells (Fig. 5). Mutations known to completely abolish iron presence in 5-lipoxygenase (H372Q, H550Q, and C6-deletion (deletion of the COOH-terminal 6 amino acid residues including Ile-673); Refs. 31 and 32) led to nearly exclusive cytosolic-localized enzyme (Fig. 5B). Mutations known to abolish enzyme activity yet leave partial presence of iron in the protein (H367Q, H367N) showed graded distribution of 5-lipoxygenase in the nucleus and cytosol depending on the iron content (Fig. 5B; Ref. 31). All the mutated 5-lipoxygenase constructs were expressed in the transfected cells to a similar extent (Fig. 5C) but had lost enzyme activity by HPLC analysis.

**Fig. 2.** Representative reverse phase-HPLC chromatograms of 5-H(PE)TE formed from arachidonic acid (100 μM) by human 5LO (top right), GFP-5LO (middle right), GFP alone (bottom right), and GFP-basic region 5LO mutant (left panels) transfected cell supernatants. Samples were analyzed with a mobile phase consisting of acetonitrile:H2O:HOAc (60:40:0.1). n = 5–9.

| Amino acid residue | 71 | 72 | 73 | 83 |
|-------------------|----|----|----|----|
| Basic region 1     | K | R | K | xxxxxxxxx | K |
|                   | 128 | 129 | 130 | 131 | 132 | 133 |
| Basic region 2     | K | Q | H | R | R | K |
|                   | 651 | 652 | 653 | 654 | 655 | 666 |
| Basic region 3     | R | N | K | K | K | xxxxxxxxx | R |

**Fig. 3.** The three main basic regions of human 5-lipoxygenase targeted for analysis of potential NLS sequences. Amino acid numbering is based on (27) excluding the initiator methionine. Number of x’s indicates spacer amino acids between basic regions.
FIG. 4. Three basic region sequences in 5-lipoxygenase studied by mutational analysis for effects on nuclear localization. A, drawings that depict GFP-5LO fusion proteins and their resulting cellular localization and enzyme activity. GFP is shown as a dark oval and 5-lipoxygenase by the shaded rectangle. Asterisks indicate mutations. Relative 5-lipoxygenase activity is indicated by a scale of +++ (80–100% activity), ++ (low but detectable activity), and 0 (no enzyme activity detected). B, DNA stain (left panels), GFP fluorescence (middle panels), and combined DNA/GFP fluorescence overlays (right panels) for various GFP-5LO fusion proteins expressed in HEK 293 cells 20 h post-transfection, n = 3–5. C, immunoblot analysis with a 5-lipoxygenase antibody to detect relative expression levels of the various constructs. Approximately 15 μg of protein was loaded in each well. A 104-kDa fusion protein was detected at nearly equal levels for the three double mutant transfectants (see "Results"). For comparison purposes, native 5-lipoxygenase from transfected cells and purified recombinant enzyme (78 kDa) are included. Mock-transfected cells express no 5-lipoxygenase, n = 3–5.
FIG. 5. Effects of iron atom ligand mutations on nuclear localization of 5-lipoxygenase. A-C, GFP-5LO fusion drawings, fluorescence micrographs, and immunoblot analysis of expressed 5-lipoxygenase proteins (see Fig. 4 legend for details). A GFP monoclonal antibody was used for the immunoblot analysis. Asterisk, iron content of mutant proteins is based on Refs. 31 and 32 assuming no changes for the extra GFP addition. Mutants that retain partial structural characteristics (as estimated by iron content) can direct some nuclear localization whereas those proteins that have lost iron no longer enter the nucleus.
FIG. 6. Determination if 5-lipoxygenase fragments can direct GFP to the nucleus. A-C, GFP-5LO fusion drawings, fluorescence micrographs, and immunoblot analysis of expressed 5LO proteins (see Fig. 4 legend and "Results" for details). A GFP monoclonal antibody was used for the immunoblot analysis. An NLS peptide from SV40 was used as a control. A sequence in platelet 12-lipoxygenase (P-12LO(1–75)) corresponding to residues 1–80 of 5-lipoxygenase that directed nuclear localization was used as another control.
5-Lipoxygenase Nuclear Localization

Fig. 7. 5-Lipoxygenase/pyruvate kinase fusions are not nuclear localized. The black circle represents the Myc tag, the cross-hatched ovals the PK sequence, and the shaded box 5-lipoxygenase. PK-M1 is a fusion with a region containing the 38-residue NLS (M9) found in heterogeneous nuclear ribonucleoprotein A1 (20).

None of these constructs, with the exception of 5LO(1–80) could direct a nuclear localization pattern (Fig. 6). The pattern of nuclear localization gave a mottled appearance as previously determined for 5-lipoxygenase in alveolar macrophages (11). As a control, we fused the 7-residue SV40-NLS to GFP and found that it directed a predominant nuclear expression pattern (data not shown). In contrast, an 8-residue sequence (basic region 2, Fig. 3; 5LO(128–135)) did not direct nuclear localization although mutations at positions 131/132 in the GFP-5LO fusion altered cellular localization as mentioned above. Since the NH2-terminal region of 5-lipoxygenase appeared sufficient to direct nuclear localization we constructed a GFP fusion construct from the exact region of another lipoxygenase known not to enter the nucleus (“platelet-type” 12-lipoxygenase; P12LO(1–75)). This construct did not enter the nucleus (Fig. 6B). Mutation of basic region 1 located in the 5LO(1–80) construct also did not alter nuclear targeting. However, extending the region 5LO(1–127) and 5LO(1–166) resulted in a diminished ability to target to the nucleus. Once again all the various constructs were expressed well in the transfected HEK 293 cells as judged by immunoblot analysis (Fig. 6C).

5-Lipoxygenase Domains Are Incapable of Directing the Cytosolic Protein Pyruvate Kinase to the Nucleus—We tested the ability of various 5-lipoxygenase fusions to direct a commonly used cytosolic protein, pyruvate kinase (20, 21) to the nucleus (Fig. 7). Neither the full-length 5-lipoxygenase, which retained enzyme activity with the PK fusion, nor shortened fragments of 5-lipoxygenase could direct the enzyme to the nucleus, although the M9 NLS sequence of heterogeneous nuclear ribonucleoprotein A1 could (data not shown).

Discussion

We have carried out a study to investigate the nuclear targeting of 5-lipoxygenase. More than 25 fusion protein constructs were prepared and analyzed by immunofluorescence microscopy in various transfected cell lines. The combined data indicate that 5-lipoxygenase nuclear targeting is dependent on a number of complex factors. First, 5-lipoxygenase does not possess a “classical” basic region that functions as a NLS although the protein does contain at least three potential basic cluster regions that could act in this function. Second, the proper folding of the enzyme is critical for nuclear localization. This result is based on the fact that fusion proteins, which completely lack or partially retain the non-heme iron atom, show a predominantly cytosolic localization. Third, a short NH2-terminal region (first 80 amino acids) appears sufficient for directing the enzyme to the nucleus but when this segment is extended the recognition sequence(s) are lost. Fourth, any NLS sequence(s) in the NH2-terminal region of 5LO are relatively weak since only one of two proteins tested could be transported to the nucleus (e.g. GFP versus pyruvate kinase).

The nuclear import of proteins bearing the classical NLS such as those for the SV40 large T antigen and nucleoplasmin begins by binding to karyopherin-α which acts as the NLS receptor (17). Karyopherin-β1 interacts further with the NLS-bound karyopherin-α to form the ternary complex, which is targeted to the nucleoporins in the nuclear pore complex. Subsequent translocation into the nucleus through the nuclear pore complex depends on GTPase Ran and its modulators (16, 17). Novel import pathways have been identified recently. For instance, heterogeneous nuclear ribonucleoprotein A1 interacts directly with karyopherin-β2/transportin, which is one of the members in the β-karyopherin superfamily, through the substrate’s distinct NLS known as M9 or NLS2 to target to the nuclear pore complex (20, 21). Common features of the novel nuclear import pathways appear to be NLS sequences distinct from the classical pathway and direct interaction with an individual karyopherin-β form independent of interaction with the adapter karyopherin-α. Perhaps 5-lipoxygenase is using a novel means of nuclear entry in this respect. 5-Lipoxygenase does not contain an M9 domain and it is not yet known if it can interact with karyopherin-α.

5-Lipoxygenase is the only known mammalian lipoxygenase that resides in the nucleus. The NH2 termini of lipoxygenases differ to the greatest extent in this region (33, 34). Thus, this region appears to be the most important for 5LO nuclear targeting since there was one fragment (1–80) that could direct GFP to the nucleus. This was not a nonspecific result since both a COOH-terminal fragment from 5-lipoxygenase (5LO(574–673), see Fig. 6) of similar size and a fragment corresponding to the same residues of platelet 12-lipoxygenase (1–75; in the NH2-terminal sequence five residues are not present in this lipoxygenase and several other mammalian lipoxygenases) did not direct nuclear localization. Platelet 12-lipoxygenase is known to reside in the cytosol, either soluble or membrane-bound, of human erythroblastemia cells, A431 cells, and epidermal homogenates (23, 35, 36). The basic cluster within the 5LO(1–80) sequence was not essential for nuclear localization.

Based on the three-dimensional x-ray crystal structures of soybean lipoxygenases and rabbit reticulocyte 15-lipoxygenase (29, 30, 37), the lipoxygenase family members possess two domains; a short β-barrel NH2-terminal domain of unknown function and a major catalytic domain that includes the non-heme iron atom. Gilmore et al. (30) have hypothesized that the β-barrel NH2-terminal region, with homology to lipoprotein lipase, may participate in binding lipid membranes to gain access to the source of substrate. In the case of 5-lipoxygenase, they suggested a possible site of interaction with 5-lipoxygenase-activating protein, a co-accessory protein in leukotriene biosynthesis that may help to “transfer” arachidonic acid substrate to the enzyme (7, 38). The data here could extend the possible list of functions for this domain, in particular for 5-lipoxygenase, as aiding in nuclear localization. The putative β-barrel domain of 5-lipoxygenase is about 125 amino acid residues in length. A construct with this domain still directed nuclear localization of GFP. However, if it was extended to 166 amino acids the fusion protein remained cytosolic. This result suggests that the folding of the extra portion beyond the β-barrel may have masked any potential NLS. The context within which the NLS is situated is important for nuclear localization (39, 40).

Precedents for weak NLS sequences in proteins are prevalent in the literature and 5-lipoxygenase seems to fit in this class of proteins. For example, a 29-amino acid stretch of GAL4...
could direct cytosolic invertase to the nucleus but not β-galactosidase (40). Likewise, a NLS motif near the NH$_2$ terminus of fibroblast growth factor 3 conferred nuclear localization to cytoplasmic β-galactosidase but not pyruvate kinase (41). Signals in 5-lipoxygenase were capable of directing the GFP reporter protein to the nucleus but not pyruvate kinase. Complex patterns of nuclear targeting that may involve weak additive signals from opposite ends of the protein are known (e.g. fibroblast growth factor 3) (41). Examples of NLS “masking” by structural alterations and/or other cellular proteins, a prime example being the transcription factor NF-kB/Rel bound by its inhibitor IκB are known (42). The data herein could be consistent with some sort of unmasking of an NLS to gain nuclear entry and also do not rule out the possibility that 5-lipoxygenase is “piggybacked” to the nucleus by some other chaperone protein. A somewhat surprising result was that 5-lipoxygenase could be localized to the nucleus in four different transfected cell types. Perhaps, in human and rat neutrophils, 5-lipoxygenase is specifically bound by an inhibitor protein that prevents nuclear transport since the enzyme in these resting cells is exclusively cytosolic. Upon in vivo activation or adherence to glass in vitro (12), therefore, these cells would lose the capacity to inhibit the endocytosis of the nucleus. Alternatively, some sort of post-translational modification such as phosphorylation of 5-lipoxygenase, particularly in neutrophils, may influence NLS recognition. Phosphorylation of a nuclear 5-lipoxygenase fraction has been detected in HL-60 cells but the site of phosphorylation has not been identified (43). We are currently studying potential interactions of 5-lipoxygenase with other proteins using the yeast two-hybrid system with pcDNA3/myc-PK vector and anti-Myc antibody.

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