Nuclear-localization-signal-dependent and nuclear-export-signal-dependent mechanisms determine the localization of 5-lipoxygenase

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5-Lipoxygenase (5-LO) metabolizes arachidonic acid to leukotriene A_4, a key intermediate in leukotriene biosynthesis. To explore the molecular mechanisms of its cell-specific localization, a fusion protein between green fluorescent protein (GFP) and human 5-LO (GFP–5LO) was expressed in various cells. GFP–5LO was localized in the cytosol in HL-60 cells and in both the nucleus and the cytosol in RBL (rat basophilic leukaemia) cells, similarly to the native enzyme in these cells. The localization of GFP fusion proteins for mutant 5-LOs in a putative bipartite nuclear localization signal (NLS), amino acids 638–655, in Chinese hamster ovary (CHO)-K1 and Swiss3T3 cells revealed that this motif is important for the nuclear localization of 5-LO. A GFP fusion protein of this short peptide localized consistently in the nucleus. Leptomycin B, a specific inhibitor of nuclear export signal (NES)-dependent transport, diminished the cytosolic localization of 5-LO in HL-60 cells and that of GFP–5LO in CHO-K1 cells, suggesting that an NES-system might also function in determining 5-LO localization. Analysis of the localization of 5-LO during the cell cycle points to a controlled movement of this enzyme. Thus we conclude that a balance of NLS- and NES-dependent mechanisms determines the cell-type-specific localization of 5-LO, suggesting a nuclear function for this enzyme.

Key words: enzyme transport, laser scanning cytometry, leptomycin B, leukotriene.

INTRODUCTION

5-Lipoxygenase (5-LO) is the initial enzyme in the biosynthesis of leukotrienes (LTs) from arachidonic acid. LTs are potent lipid mediators involved in normal cell function and occur in excess in pathological processes. 5-LO inhibitors are now in therapeutic use, yet little is known of how 5-LO is regulated inside the cell. 5-LO was first isolated from cytosolic fractions of various cells [1–3]. It was reported that 5-LO translocates from the cytosol to phospholipid membranes when cells are activated [4]. Somewhat surprisingly, however, it was found by immunohistochemical analysis that the subcellular localization of 5-LO differs between cell types. 5-LO is predominantly localized in the cytosol of peripheral blood polymorphonuclear cells [5] and peritoneal macrophages [6], whereas it is found in both the nucleus and the cytosol of alveolar macrophages [7], mast cells [8] and RBL (rat basophilic leukaemia) cells [5]. Recent studies have also shown that most of the enzymes involved in LT biosynthesis seem to be localized to perinuclear regions in the cell [7,9,10]. Further, 5-LO has been reported to translocate primarily to the nuclear membrane rather than the plasma membrane in response to various stimuli [11,12]. The localization and translocation of 5-LO are the determining factors in the production of LTs, and they must therefore be tightly controlled [13–15].

Nuclear localization signal (NLS) and nuclear export signal (NES) have been proved to determine the localization of several important proteins. Proteins entering the nucleus require importin molecules to recognize NLS sequences, allowing nuclear pore docking. Recognition permits transport through the nuclear pore, followed by release inside the nucleus [16,17]. NLS is typically a short basic region or a bipartite basic sequence [18,19]. The existence of NLS-like motifs in 5-LO has been noted [20,21]; after our studies had been initiated a functional bipartite sequence (amino acid residues 638–655) was described [12]. An NES, a short sequence rich in leucine, which is needed to mediate the nuclear export of some proteins, would probably also be present on 5-LO.

To explore the molecular mechanisms of cell-specific localization of 5-LO, green fluorescent proteins (GFPs) fused with wild-type and mutant 5-LOs were expressed in Chinese hamster ovary (CHO)-K1 cells and Swiss3T3 cells, and their intracellular localization was analysed. In addition, a cytometric analysis was performed to determine the localization of 5-LO, which changes during the cell cycle. We describe the role of the bipartite NLS (B-NLS) in 5-LO localization and in enzymic function, and provide evidence for an NES-dependent mechanism in the movement of 5-LO between compartments within the cell.

EXPERIMENTAL

Construction of expression vectors

The cDNA of human 5-LO was cloned by PCR and ligated with *Eco*RI-cut pEGFP–C1 (ClonTech, Palo Alto, CA, U.S.A.). The PCR product was obtained with the primers 5′-CGGAATTCCC-CCTCCTACACGGTCACC-3′ (sense) and 5′-CGGAATTCC-GGTCAGATGGCCACACTGTTC-3′ (anti-sense) by using

Abbreviations used: B-NLS, bipartite NLS; CHO, Chinese hamster ovary; FBS, fetal bovine serum; GFP, green fluorescent protein; 5-HETE, 5-(*S*)-hydroxy-6,8,11,14-eicosatetraenoic acid; 5-HPETE, 5-(*S*)-hydroperoxy-6,8,11,14-eicosatetraenoic acid; LMB, leptomycin B; 5-LO, 5-lipoxygenase; LSC, laser scanning cytometry; LT, leukotriene; NES, nuclear export signal; NLS, nuclear localization signal; RBL, rat basophilic leukaemia.

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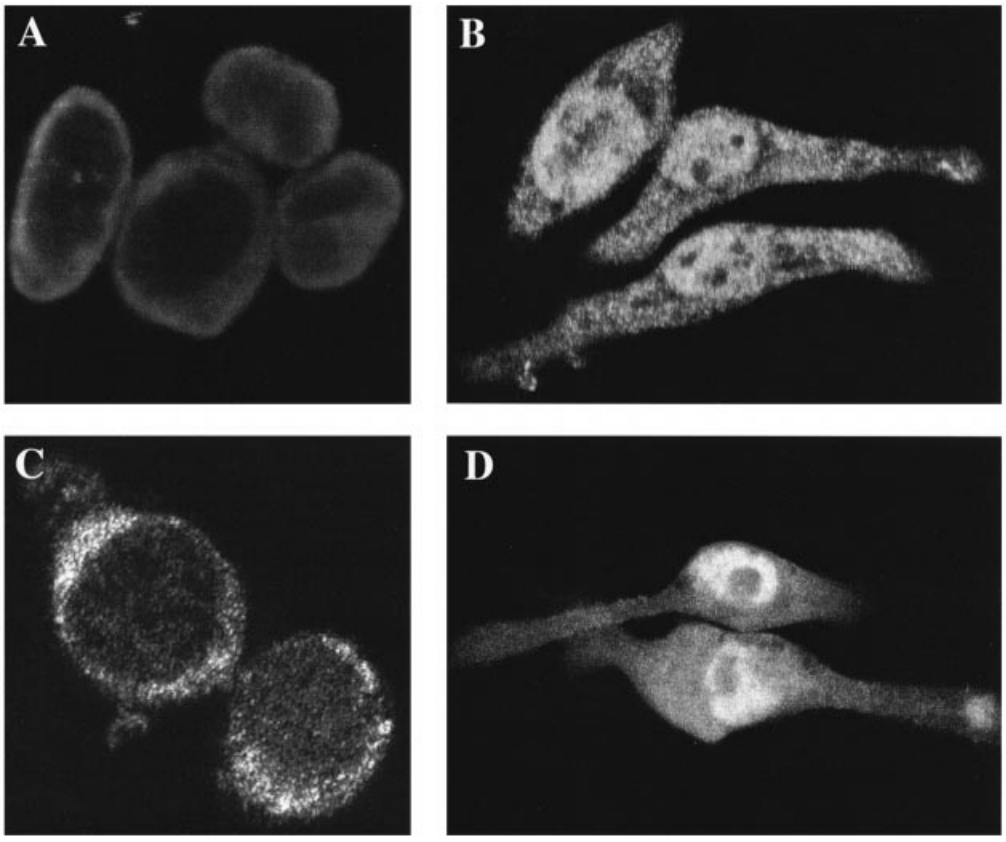

Figure 1 Subcellular localization of 5-LO and GFP–5LO in HL-60 cells and RBL cells

HL-60 cells (**A**) and RBL cells (**B**) were treated with an anti-(5-LO) antiserum and an FITC-conjugated second antibody as described in the Experimental section. HL-60 cells (**C**) and RBL cells (**D**) on chamber slides were transfected with pEGFP-5LO and the signals were observed 16 h after transfection. Fluorescence was observed with a confocal microscope.

AmpliTaq Gold (Perkin Elmer Biosystems, Foster City, CA, U.S.A.). A cDNA (pEGFP-5LO) encoding 5-LO fused with GFP at the N-terminus of 5-LO was obtained with pEGFP–C1. Mutagenesis was performed *in vitro* with a QuikChange Site-directed Mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) or a PCR-based protocol on pEGFP-5LO as a template. Details of the procedures and the sequences of mutagenic primers are available from T. I. upon request. A vector for GFP–B-NLS encoding the B-NLS (R^{638}KNLEAIVSVIAERNKKK^{655}) fused with GFP was constructed. The corresponding cDNA for B-NLS was cloned into *Eco*RI-cut pEGFP–C1 as a PCR product amplified with the primers 5′-CGGAATTCCCGCAAGAA-CCTCGAGGCC-3′ (sense) and 5′-CGGAATTCCCTTCTT-CTTGTTGCGCTC-3′ (anti-sense). DNA sequencing was performed to verify the correct insertion and the introduced mutations with an ABI 373 sequencer by using a Big Dye Terminator Ready Reaction Kit (Perkin Elmer Biosystems).

Cell culture and transfection

HL-60 cells and RBL cells were grown in RPMI-1640 medium (Nissui, Tokyo, Japan) supplemented with 10 % (w/v) FBS (fetal bovine serum) (Sigma, St Louis, MO, U.S.A.). HEK-293 cells and Swiss3T3 cells were grown in Dulbecco's modified Eagle's medium (Nissui) supplemented with 10 % (v/v) FBS. CHO cells were cultured in Ham's F-12 medium with 10 % (v/v) FBS. Cells growing in 60 mm-diameter tissue culture dishes (Corning Inc., Corning, NY, U.S.A.) were transfected with plasmid DNA species by LIPOFECTAMINE™ Plus Reagent (Life Technologies, Rockville, MA, U.S.A.). For microscopic analyses of GFP, the cells were observed 16 h after transfection and the 5-LO activity of the cell lysates was measured 48 h after transfection. In some experiments, cells were treated for 14 h with leptomycin B (LMB) (a gift from Dr M. Yoshida, University of Tokyo, Tokyo, Japan) at 10 ng/ml, and the localization of 5-LO and GFP–5LO was observed.

Laser scanning cytometry (LSC) and cell preparation

pEGFP or pEGFP-5LO was transfected into CHO-K1 cells. From more than 20 clones for each plasmid DNA resistant to geneticin (1 mg/ml), we selected two cell lines for each DNA expressing pEGFP–C1 (CHO-GFP cells) or pEGFP-5LO (CHO-GFP–5LO cells). No apparent difference was observed between two cell lines for each DNA in the following experiments. These cells were maintained in the presence of 0.3 mg/ml geneticin. CHO-GFP–5LO cells were grown on Lab-Tek Chamber slides (Nalge Nunc International, Rochester, NY, U.S.A.). After washes with PBS, cells were fixed in ethanol at −25 °C for 15 min, then incubated for 15 min with 200 μg/ml RNase in PBS. Their nuclear DNA species were counterstained with 5 μg/ml propidium iodide (Sigma). Stained cells were examined with an LSC system (Olympus), which provides GFP fluorescence, DNA content and morphological information in individual cells. Thus a chain of GFP–5LO expression profiles

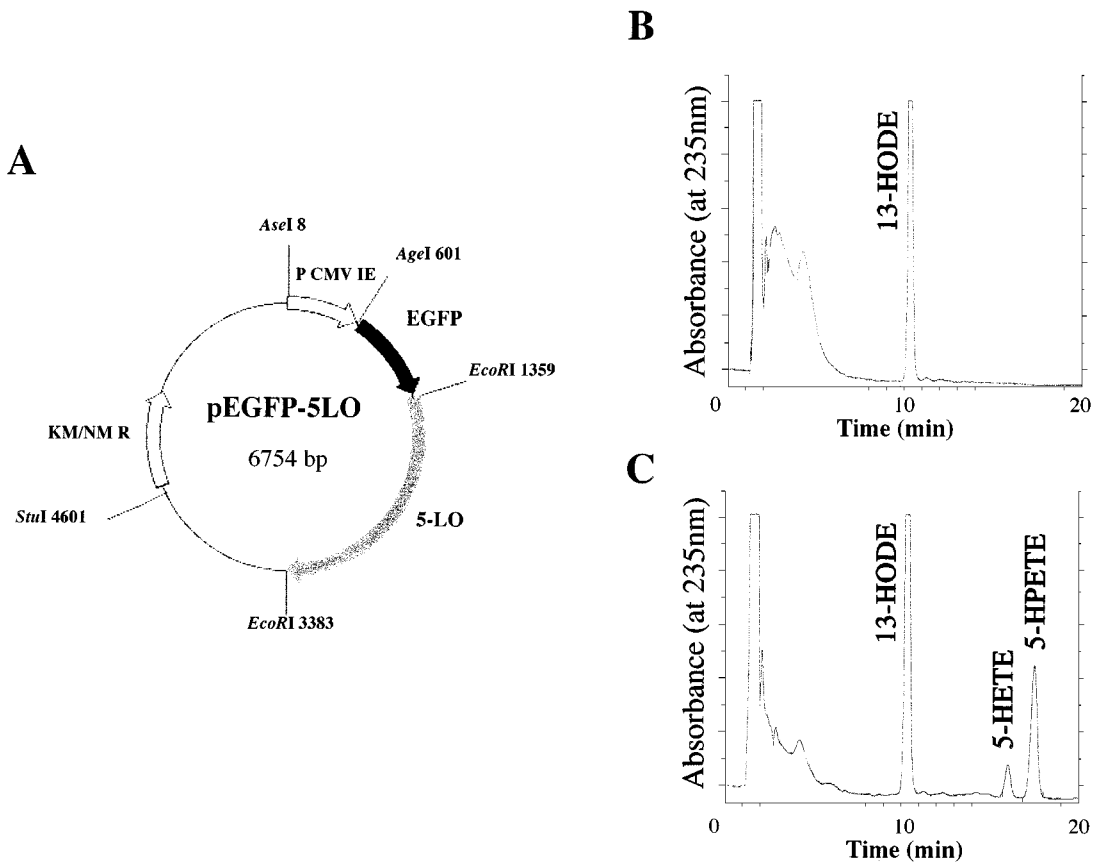

Figure 2 Construction of plasmid DNA for GFP–5LO and representative reverse-phase HPLC chromatograms

(**A**) A diagram of the GFP–5LO construct. (**B**, **C**) Arachidonic acid (160 μM) was incubated with cell lysates of HEK-293 cells transfected with pEGFP (**B**) or pEGFP-5LO (**C**) as described in the Experimental section. Products were analysed by HPLC with a mobile phase consisting of acetonitrile/methanol/water/acetic acid (350:150:250:1, by vol.). 13-Hydroxyoctadecadienoic acid (13-HODE) was used as an internal standard. Chromatograms representative of more than three independent experiments are shown.

during the cell cycle was constructed for each cell on the basis of DNA content.

Fluorescence microscopy

RBL cells and HL-60 cells were fixed with methanol for 15 min at −25 °C, then for 15 min with 3% (w/v) paraformaldehyde in PBS containing 1% (w/v) BSA at room temperature (25 °C). Cells were incubated with an anti-(human 5-LO) antiserum (dilution 1:200) and then with a goat FITC-conjugated anti-rabbit IgG (Zymed, San Francisco, CA, U.S.A.) (dilution 1:200) for 1 h at 37 °C. After three washes with PBS containing 1% BSA, cells were treated with SlowFade Antifade Kit (Molecular Probes, Eugene, OR, U.S.A.). The fluorescent signal was observed with an AX-80 analytical microscope system (Olympus, Tokyo, Japan) or with an LSM 510 Laser Scanning Microscope System (Carl Zeiss, Operkochen-Jena, Germany). Anti-(human 5-LO) antiserum was a gift from Dr J. Evans (Merck Frosst Centre for Therapeutic Research, Pointe Claire-Dorval, Québec, Canada).

Assay of 5-LO

At 48 h after transfection, HEK-293 cells were harvested and cell lysates were obtained by nitrogen cavitation at 2.8 MPa for 10 min at 4 °C. 5-LO activity was measured as reported previously [21], with minor modification. Cell lysates were incubated for 10 min in a buffer containing 1 mM ATP, 2 mM $CaCl_2$, 160 μM arachidonic acid and 50 mM Tris/HCl, pH 8.0, at 37 °C. The reactions were terminated with 2 vol. of an ice-cold stop solution consisting of acetonitrile/methanol/acetic acid (350:150:3, by vol.) and the mixtures were centrifuged at 10000 ***g*** for 10 min. Metabolites of arachidonic acid [5-(*S*)-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HPETE) and 5-(*S*)-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE)] in the supernatants were analysed by reverse-phase HPLC in a mobile phase consisting of acetonitrile/methanol/water/acetic acid (350:150:250:1, by vol.), with UV detection at 235 nm and at a flow rate of 1 ml/min on a Beckman System Gold equipped with a Cosmosil, 5C18-AR packed column (150 mm × 4.6 mm; Nacalai Tesque, Kyoto, Japan). In some cases, two all-*trans* isomers of LTB_4, non-enzymic products of LTA_4, were measured at 270 nm. However, their amount was always less than 10% of the combined amount of 5-HPETE and 5-HETE, and was not integrated with the 5-LO activity.

Immunoblot analysis

Cells were dissolved in PBS/1% (v/v) Triton X-100/1 mM EDTA/4% (v/v) Complete™ (Boehringer Manheim, Mann-

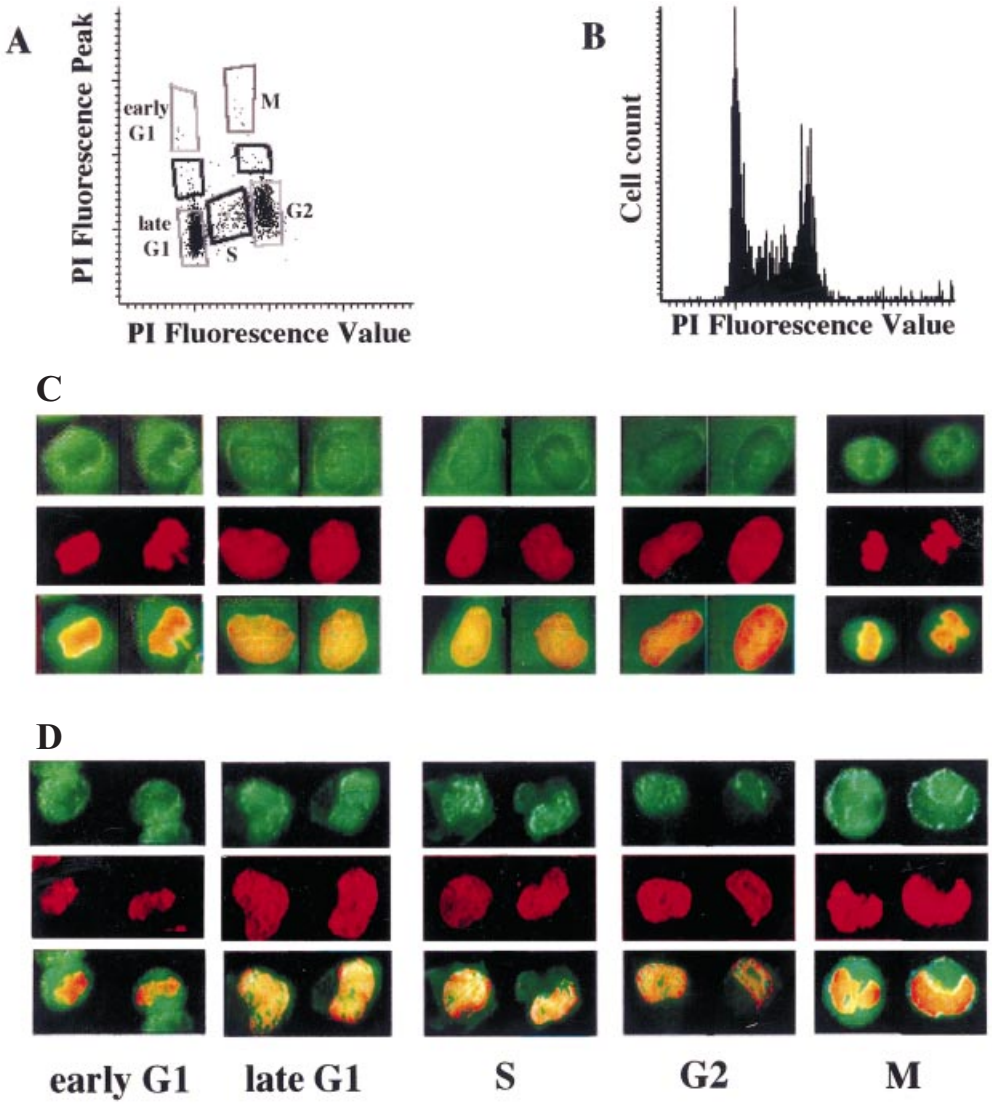

Figure 3 Distribution of GFP and GFP–5LO during the cell cycle

(**A**, **B**) Cell cycle (**A**) and distribution of DNA content (**B**) for each cell were analysed by LSC in CHO-GFP cells stained with propidium iodide (PI). (**C**, **D**) GFP fluorescence (top panels), nuclear DNA stain (middle panels) and overlapped images (bottom panels) for each cell are shown for CHO-GFP cells (**C**) and CHO-GFP–5LO cells (**D**).

heim, Germany); protein concentrations of the homogenates were determined with a Protein Assay Kit II (Bio-Rad, Hercules, CA, U.S.A.). Samples containing 40 μg of protein were separated by SDS/PAGE under reducing conditions and transferred to PVDF membranes (Bio-Rad). Membranes were probed with an anti-GFP polyclonal antibody (ClonTech) (dilution 1:2000), followed by incubation with a peroxidase-conjugated goat anti-rabbit antibody (Zymed) (dilution 1:2000) and treated with the ECL® (enhanced chemiluminescence detection system; Amersham Biosciences, Little Chalfont, Bucks., U.K.). In some cases, cell homogenates were fractionated to cytosolic and nuclear fractions as reported previously [11]. In brief, cells were disrupted by nitrogen cavitation at 2.8 MPa for 5 min at 4 °C. After removal of non-disrupted particles, the mixtures were centrifuged at 100000 ***g*** for 1 h at 4 °C to generate soluble (cytosolic) and insoluble (membrane) fractions.

RESULTS

Cellular localization and enzymic activity of a GFP-tagged 5-LO

Initially, the distribution of 5-LO was examined by immunohistochemistry in HL-60 cells and RBL cells. 5-LO was localized predominantly in the cytosol of HL-60 cells (Figure 1A), whereas it was distributed in both the nucleus and the cytosol of RBL cells (Figure 1B). To observe the localization of 5-LO more precisely in living cells, we developed a construct, pEGFP-5LO, encoding a GFP–5LO fusion protein (Figure 2A) and transfected it into various types of cell. GFP–5LO was distributed similarly to the native protein predominantly in the cytosol of HL-60 cells (Figure 1C) and was localized in both the nucleus and the cytosol of RBL cells (Figure 1D). Whereas the CHO-K1 cells expressing GFP had no 5-LO enzymic activity (Figure 2B), the cells expressing GFP–5LO displayed an enzymic activity (Figure 2C)

Table 1 Cargo proteins and signals for nuclear import

Amino acid residues of representative NLSs are indicated with the single-letter code. Bold letters in the top three proteins indicate residues that have been shown to be particularly important for the signal function [23], and those in 5-LO indicate basic amino acid residues.

Cargo protein	NLS
SV40 T antigen	P**KKKRK**V (monopartite NLS)
c-Myc	**PAAKR**V**KLD**
Nucleoplasmin	**KR**PAATKKAGQA**KKKK** (bipartite NLS)
5-LO	$\mathbf{R}^{638}$**K**NLEAIVSVIAE**R**N**KKK**^{655}

comparable with that of the native enzyme expressed in CHO-K1 cells, as reported previously [21] (results not shown) in the presence of a relatively high concentration of arachidonic acid (160 μM), although the precise kinetic properties of GFP–5LO were not analysed. Thus the GFP tag seems not to interfere with the subcellular localization and catalytic activity of 5-LO.

Distribution of GFP–5LO during cell cycle

The intracellular distribution of fluorescent signal in CHO-GFP–5LO cells was varied and depended on the cell cycle. To examine the localization of GFP–5LO in each phase of the cell cycle, we analysed stable transformants of CHO-GFP and CHO-GFP–5LO cells by LSC, which provides information on morphology and cellular properties such as the nuclear DNA content of each cell (Figure 3) [22]. In late G_1, S and G_2, GFP–5LO was localized mainly in the nucleus (Figure 3D), whereas GFP was localized evenly in both the nucleus and the cytoplasm (Figure 3C). In other phases (M and early G_1), the GFP signals were excluded from the condensed DNA species in both CHO-GFP and CHO-GFP–5LO cells. These results indicate that an NLS of 5-LO might operate in late G_1, S and G_2.

Localization of GFP–5LO in cells expressing it transiently

The classical NLS is a short basic region of three or more residues, or a bipartite basic region separated by a variable-length spacer sequence (Table 1) [23]. A likely candidate for a B-NLS region, residues 638–655 in 5-LO, which differs to some extent from other cytosolic lipoxygenases, was the focus for mutational analyses. Five mutant GFP–5LO constructs were prepared (Table 2) and transfected into CHO-K1 cells and Swiss3T3 cells. These cell lines were selected for their high efficiency of expression. GFP alone was distributed throughout the cell (Figures 4A and 4E), whereas GFP–5LO localized predominantly in the nucleus of CHO-K1 cells and Swiss3T3 cells (Figures 4B and 4F).

M1 (R638S/K639N; mutations shown with the single-letter amino acid code) was localized in the nucleus (Figures 4C and 4G). However, M2 (R651S/K653N/K654N/K655N) (Figures 4D and 4H), M3 (R638S/K639N/R651S/K653N/K654N/K655N) (Figures 4I and 4M) and M4 (R638S/K639N/R651S) (Figures 4J and 4N) were observed predominantly in the cytosol. The subcellular distribution of M5 (R651S) was not uniform in all cells. In most CHO-K1 cells M5 was distributed in the cytosol (Figure 4K) but in a subset of the population (less than 20 %) of CHO-K1 cells M5 was found in both compartments (Figure 4L). However, in most Swiss3T3 cells (more than 80 %) M5 was observed in both the cytosol and the nucleus (Figure 4O) and in other cells predominantly in the nucleus (Figure 4P). Immunoblot analysis of cell lysates confirmed that all mutant fusion proteins were of comparable sizes, and no breakdown products were found in any samples (Figure 5). The expression levels of all proteins were similar except for M3. The 5-LO activity of M5 was approx. 10 % of that of the wild-type fusion protein (GFP–5LO) but enzymic activity was not detected in M2 and M3 (Table 2). These results suggest that the mutated amino acids seem to be vital to localization and function. Next, to exclude the possibility that structural changes in the protein accounted for both these effects, the NLS motif alone was used in localization studies. We constructed pEGFP-B-NLS encoding this motif and transfected it into CHO-K1 cells. As expected, GFP–B-NLS was located mainly in the nucleus (Figure 6B), whereas GFP was localized evenly in the nucleus and the cytosol (Figure 6A). Immunoblot analysis also showed that GFP–B-NLS was present predominantly in the nuclear fraction (Figure 6C). These results provide direct evidence that a motif of residues 638–655 acts as an NLS in 5-LO. The region was also required to preserve enzymic activity (Table 2).

Effects of LMB on the localization of 5-LO in HL-60 cells and CHO-GFP–5LO cells

To examine a possibility that an NES-dependent transport system also determines the intracellular localization of 5-LO, a mutation study and a pharmacological approach were performed. NES is usually a leucine-rich sequence, of which we found two in 5-LO. Two mutant GFP–5LO constructs (L266A/L270A/L272A and L316A/I320A/I323A/I325A) in two putative NES motifs (L^{266}ERQLSL^{272} and L^{316}ANKIVPIAI^{325}) were prepared and transfected into CHO-K1 cells and Swiss3T3 cells. However, no difference in localization was observed compared with wild-type GFP–5LO. Next we analysed the effects of LMB on the localization of 5-LO in HL-60 cells and of GFP–5LO in CHO-

Table 2 Mutagenesis of the bipartite NLS

The putative NLS is given in the single-letter code (residues 638–655). Dashes (–) represent the amino acids not mutated. The subcellular location of the GFP fusion proteins bearing each mutant sequence is given at the right: N, nucleus; C, cytoplasm. Relative 5-LO activity is indicated by a scale of ++ (80–100% activity), + (approx. 10%), ± (trace, less than 5%) and n.d. (no enzyme activity detected), where the activity of wild-type 5-LO is defined as 100%.

Mutant	Position … / Wild-type …	638 R	639 K	– – –	651 R	652 N	653 K	654 K	655 K	Cellular location	5-LO activity
M1		S	N		–	–	–	–	–	N > C	±
M2		–	–		S	–	N	N	N	C	n.d.
M3		S	N		S	–	N	N	N	C	n.d.
M4		S	N		S	–	–	–	–	C	±
M5		–	–		S	–	–	–	–	N = C or C	+
GFP										N = C	n.d.
GFP–5LO		–	–		–	–	–	–	–	N ≫ C	++

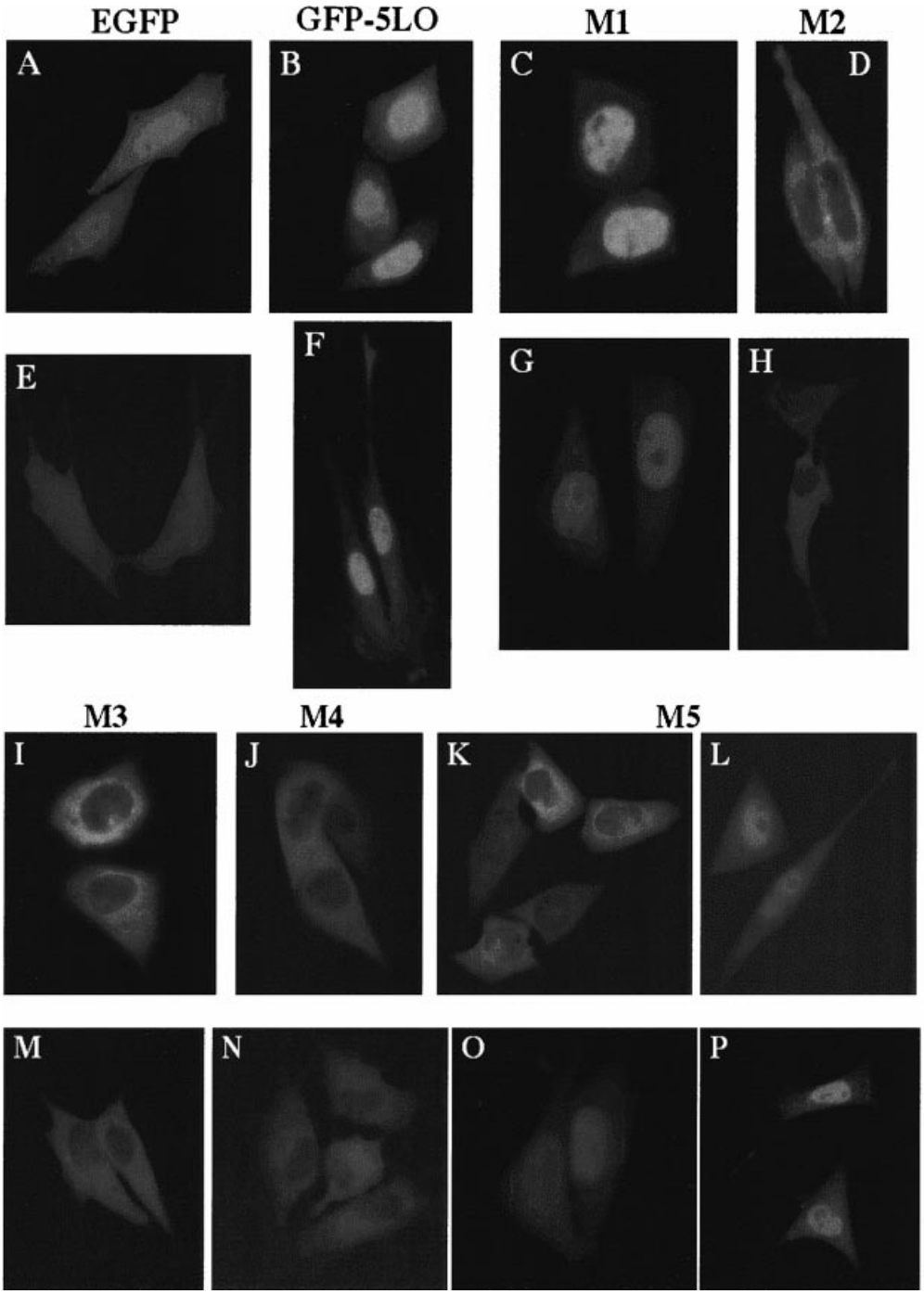

Figure 4 Localization of putative B-NLS mutants in CHO-K1 cells and Swiss3T3 cells

Fluorescent signals of GFP and GFP–5LO fusion proteins expressed in CHO-K1 cells (**A**–**D**, **I**–**L**) and Swiss3T3 cells (**E**–**H**, **M**–**P**) 16 h after transfection were observed with a fluorescent microscope. M1 to M5 are mutant GFP–5LO proteins in a putative B-NLS, R^{638}KNLEAIVSVIAERNKKK^{655}. The mutations were as follows: M1, SN ... RNKKK; M2, RK ... SNNNN; M3, SN ... SNNNN; M4, SN ... SNKKK; M5, RK ... SNKKK. (**A**, **E**) GFP; (**B**, **F**) GFP–5LO; (**C**, **G**) M1; (**D**, **H**) M2; (**I**, **M**) M3; (**J**, **N**) M4; (**K**, **L**, **O**, **P**) M5.

GFP–5LO cells. LMB is a specific inhibitor of nuclear export [24,25] that interferes with the binding of the leucine-rich Rev-type NES to exportin 1, CRM1 [26–28]. After the addition of LMB for 14 h, the localization of 5-LO in HL-60 cells was increased in the nucleus (Figure 7B), whereas 5-LO stayed in the cytoplasm without LMB (Figure 7A). However, in CHO-GFP–5LO cells the localization of GFP–5LO became exclusively to the nucleus with LMB (Figure 7D), whereas some GFP–5LO stayed in the cytoplasm without LMB (Figure 7C). The profile analyses of fluorescence intensity in the cross-sections clearly showed these effects of LMB. These results suggest the presence of an LMB-sensitive nuclear export of 5-LO, through an NES within 5-LO (but not at the sites that we mutated) or on one or more NES-containing adapter molecules associated with 5-LO.

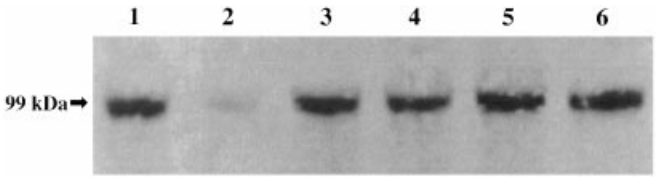

Figure 5 Immunoblot analysis of GFP–5LO fusion proteins containing specific amino acid substitutions

An immunoblot analysis of mutant GFP–5LO proteins transiently expressed in CHO-K1 cells is shown with an antibody against GFP. Lane 1, wild-type GFP–5LO; lane 2, M3; lane 3, M1; lane 4, M2; lane 5, M4; lane 6, M5. M1 to M5 are described in the legend to Figure 4.

DISCUSSION

The intracellular localization of 5-LO varies between cell types and might depend on how the nuclear import and export systems work on 5-LO. By using a database search we found that human 5-LO carries a putative monopartite NLS (residues 130–133) and a putative B-NLS (residues 638–655). Two previous studies addressed these motifs. One study on the nuclear import of 5-LO failed to identify a classical NLS [21]; instead, it indicated that an as yet unidentified unconventional signal located in the N-terminus targets 5-LO to the nucleus. In more recent study, the sequence at residues 638–655 was proved to be necessary for targeting 5-LO to the nucleus [12]. In the present study we extended the investigation of this motif at residues 638–655, showing that it acts as an NLS and that it is important for enzymic activity. Our study indicates that the intracellular localization of 5-LO also depends on an LMB-sensitive nuclear export system. Furthermore, the localization and movement of 5-LO during the cell cycle suggest a possible nuclear role for this enzyme.

Generally, NLSs are characterized by basic residues in either one (monopartite) or two (bipartite) clusters [29] (Table 1). In a previous study, the putative monopartite NLS (residues 130–133) was studied with the use of a mutant (R131Q/R132Q) that changed the location of 5-LO in NIH-3T3 cells but lost 5-LO activity [21]. In that study it was concluded that this portion might not act as an NLS. We also made a mutant GFP–5LO (R131S/R132S/K133N) in this portion. The localization of this mutant did not change in either CHO-K1 cells or Swiss3T3 cells (results not shown). This apparent discrepancy from the previous work might be due to differences in amino acid substitution or to the use of different cell lines, or both. There have been two previous studies on the putative B-NLS (residues 638–655). In one, a mutant (K653Q/K654Q) was made but its intracellular localization did not change [21]. In contrast, more recently a series of mutants were made in which basic amino acids of this portion were changed to alanine [12]. These authors concluded that this motif might act as an NLS. In the present study we conducted more extensive mutagenesis with substitutions of Arg to Ser and Lys to Asn. Such substitutions might be preferable to Ala substitution, as reported in [19]. In mutagenesis experiments,

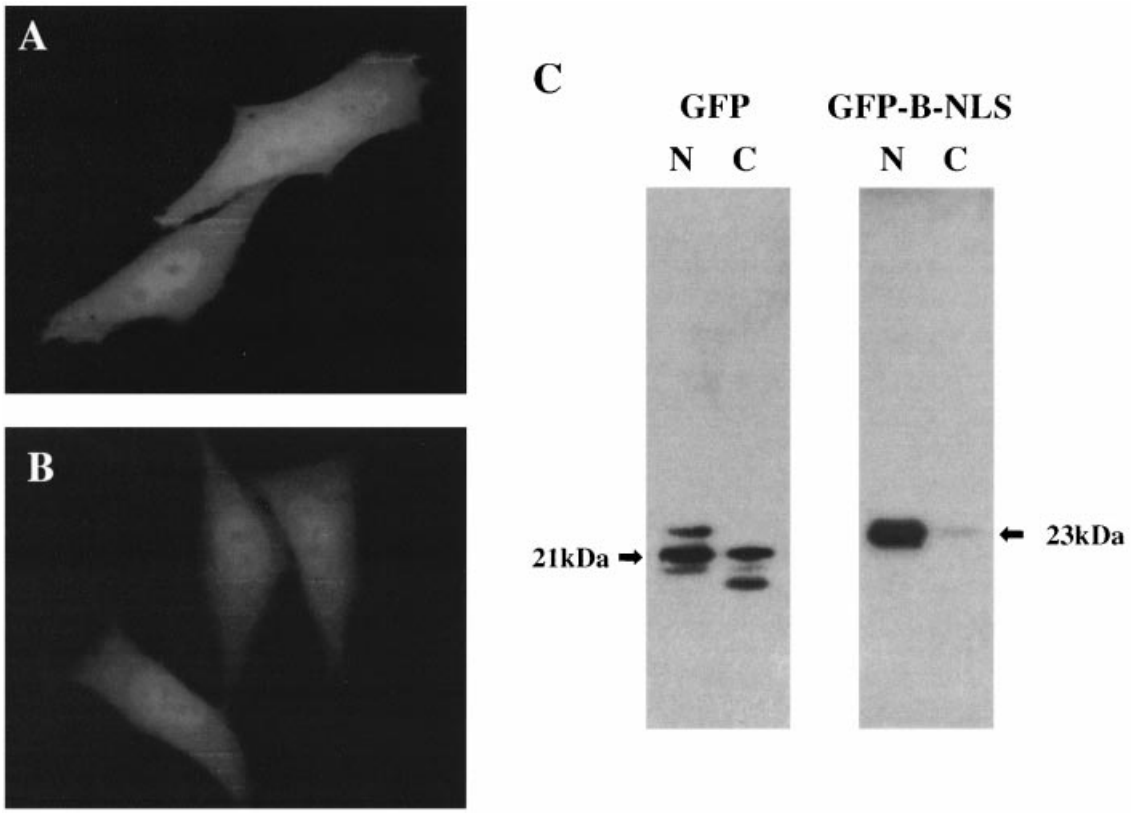

Figure 6 Localization of GFP–B-NLS in CHO-K1 cells

GFP fluorescent signals of GFP (**A**) and GFP–B-NLS (**B**) expressed in CHO-K1 cells 16 h after transfection observed by fluorescence microscopy. (**C**) Representative immunoblots for cytosolic (C) and nuclear (N) fractions of GFP and GFP–B-NLS expressed in CHO-K1 cells probed with an antibody against GFP.

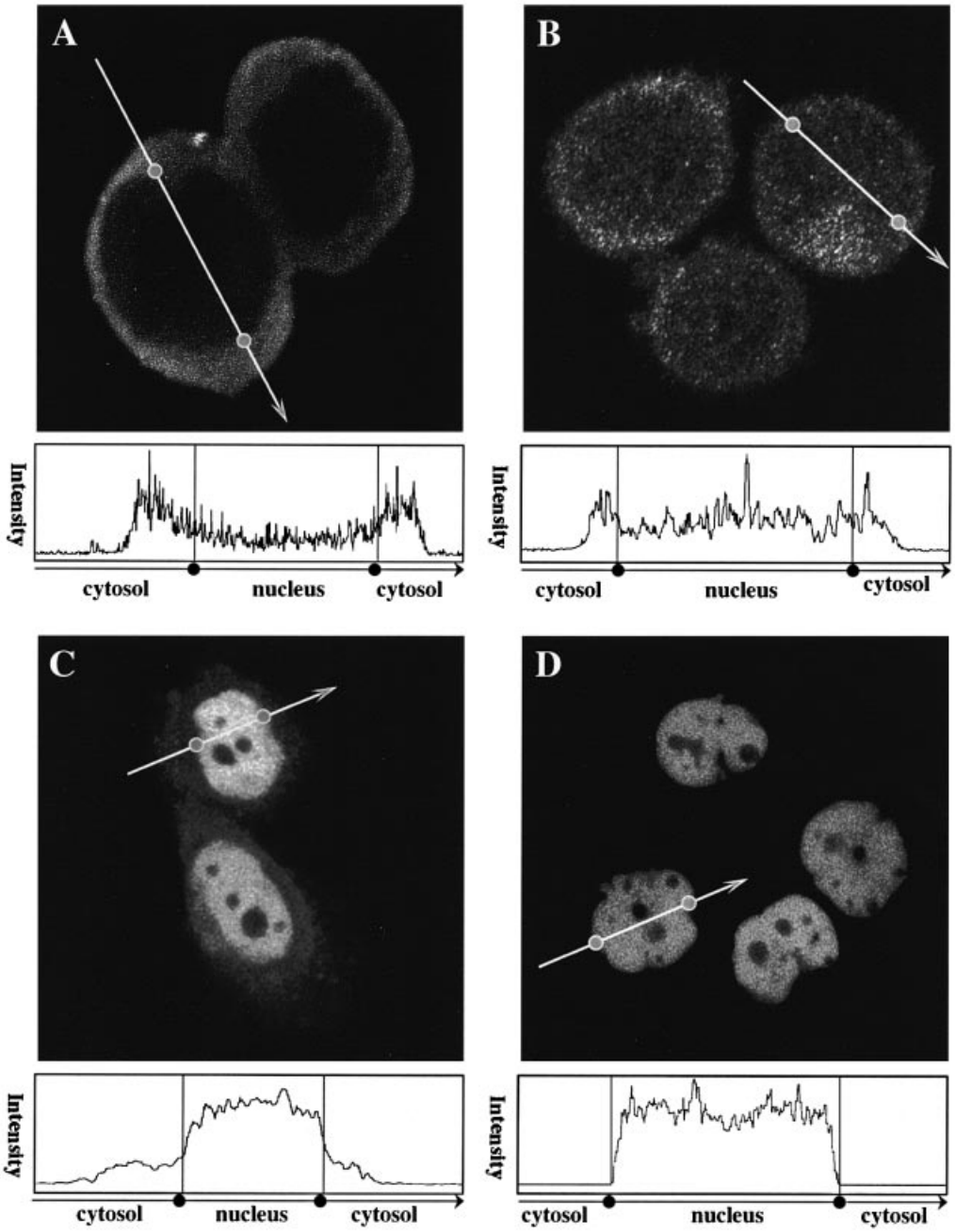

Figure 7 Effects of LMB on subcellular distribution of 5-LO

HL-60 cells (**A**, **B**) and CHO-GFP–5LO cells (**C**, **D**) were cultured on 35 mm-diameter dishes and treated without (**A**, **C**) or with (**B**, **D**) LMB (10 ng/ml) for 14 h, and they were then observed by confocal microscopy (upper panels). Bottom panels: fluorescent profiles of the cross sections indicated by white lines. Small circles indicate the edge of the nucleus. The experiments were repeated more than three times with essentially identical results.

M2 (R651S/K653N/K654N/K655N) and M3 (R638S/K639N/R651S/K653N/K654N/K655N) were observed in the cytosol. These results suggest that the second basic cluster ($R^{651}NKKK^{655}$) of this motif is needed for this putative NLS. Alternatively, this region might be important to the tertiary structure of 5-LO because no enzymic activity was detected in these mutants (Table 2). M1 (R638S/K639N) did not change its localization, whereas that of M4 (R638S/K639N/R651S) changed completely. One amino acid (R651) substitution of M5 (R651S) changed the localization of GFP–5LO but retained some enzymic activity. We also made a fusion protein of GFP and just the B-NLS (GFP–B-NLS) and showed that the motif alone could act as a NLS. Thus the whole motif from Arg^{638} to Lys^{655} might act as a B-NLS, with Arg^{651} having a central role in nuclear localization. Previous reports have pointed to a role in modulation of the nuclear import of 5-LO by phosphorylation [30,31]. Our study indicates that an NLS-dependent transport might be all that is needed but does not exclude an effect of phosphorylation.

The localization of various proteins is specifically controlled by their NES, including HIV-Rev [32], protein kinase inhibitor

[33], RanBP1 [34] and mitogen-activated protein kinase kinase [35]. We could not identify any NES sequence in 5-LO. However, the effects of LMB were clear. After the addition of LMB, 5-LO was found in the nucleus in significant amounts in HL-60 cells (Figure 7B) and exclusively in CHO-GFP–5LO cells (Figure 7D), indicating that an NES-dependent transport system is active in determining the localization of 5-LO in these cells. In HL-60 cells 5-LO localized predominantly in the cytosol, but in RBL cells 5-LO was in both the nucleus and the cytosol. Such different localizations of 5-LO are possibly due to differences in NES-dependent transport systems in these cells. In CHO-K1 cells and Swiss3T3 cells, GFP–5LO was localized predominantly in the nucleus, indicating that the NES system might act weakly and NLS strongly in these cells. Furthermore, most NLS mutants of GFP–5LO were localized in the cytosol, suggesting that the NES system becomes active when NLS does not work. We therefore conclude that 5-LO has both NLS and NES. Such a model of regulation by NLS and NES in one molecule has been suggested for MAP kinase-activated protein kinase 2 ('MAPKAP kinase 2') [36] and protein kinase Cλ [37].

Questions that remain are why and when the NLS of 5-LO works, and what the functions of nuclear 5-LO are. Possible roles for 5-LO in the nucleus are the following. (1) LTs are produced in the nucleus. LTB_4 has also been reported to activate PPARα and induce the genes that suppress inflammation [38]. (2) The nucleus is a shelter for 5-LO. 5-LO is evacuated from the cytosol to the nucleus, where cells do not need LT, but is exported to the cytosol by NES when needed. (3) 5-LO has function other than lipoxygenase activity in the nucleus. 5-LO is located to the euchromatin region, the transcriptionally active chromatin in rat alveolar macrophages [7], indicating a possible role for 5-LO in regulating gene expression. A human protein named ΔK12H4.8 homologue has been isolated as a protein partner of 5-LO by using a yeast two-hybrid system [39]. It contains an RNase III motif and a double-stranded-RNA-binding domain, indicative of a protein of nuclear origin. Further studies are needed to determine the nuclear role of 5-LO.

In conclusion, a motif at residues 638–655 acts alone as an NLS in 5-LO, and the region is also vital for enzymic activity. Furthermore, the nuclear export of 5-LO depends on an LMB-sensitive nuclear export system and the localization of 5-LO seems to depend on a balance between the NLS and NES transport systems. The regulation of balance between them has not been clarified, and its relationship to phosphorylation is now being studied in our laboratory.

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