Identification of Two Novel Nuclear Import Sequences on the 5-Lipoxygenase Protein*

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The nuclear import of 5-lipoxygenase modulates its capacity to produce leukotrienes from arachidonic acid. However, the molecular determinants of its nuclear import are unknown. Recently, we used structural and functional criteria to identify a novel import sequence at Arg518 on human 5-lipoxygenase (Jones, S. M., Luo, M., Healy, A. M., Peters-Golden, M., and Brock, T. G. (2002) J. Biol. Chem. 277, 38550–38556). However, this analysis also indicated that other import sequences must exist. Here, we identify two additional sites, at Arg112 and Lys734, as nuclear import sequences. Both sites were found to be common to 5-lipoxygenases from different species but not found on other lipoxygenases. Both sites also appeared to be a part of structures that were predominantly random loops. Peptide sequences at these sites were sufficient to direct nuclear import of green fluorescent protein. Mutation of basic residues in these sites impaired nuclear import and combinations of mutations at different sites were additive in effect. Mutations in all three sites were required to disable nuclear accumulation of 5-lipoxygenase in all cells. Significantly, mutation in these sites did not inhibit catalytic function. Taken together, these results indicate that nuclear import of 5-lipoxygenase may reflect the combined functional effects of three discrete import sequences. Mutation of individual sites can, by itself, impair nuclear import, which in turn could impact arachidonic acid metabolism.

Leukotrienes (LTs)1 are lipid mediators derived from arachidonic acid. They are synthesized primarily by leukocytes and orchestrate a variety of physiological responses in both host defense and inflammatory disease states (reviewed in Ref. 1). The enzyme 5-lipoxygenase (5-LO) catalyzes the rate-limiting first two steps of LT synthesis. Therefore, the regulation of 5-LO action and how it might be modulated in disease have been a focus of interest.

The cellular locale of 5-LO differs in different cell types. 5-LO is localized in the cytoplasm of peripheral blood neutrophils (2,3), eosinophils (4,5), and peritoneal macrophages (6). However, it is found predominantly in the nucleoplasm of rat basophilic leukemia cells (7), alveolar macrophages (8), mouse bone marrow-derived mast cells (9) and monocyte-derived dendritic cells (10). Subsequent observations have further indicated that nuclear import of 5-LO is a regulated process. Nuclear import can be triggered by adherence (2,4,11), by recruitment (2,3), or by cytokines (5,12). Conditions that cause nuclear import of 5-LO can enhance (2,5,12) or suppress (4) LT production. Thus, the nuclear import of the 5-LO enzyme is linked to its ability to synthesize LTs.

The molecular components that regulate nuclear import of 5-LO remain to be fully elucidated. Commonly, a nuclear import sequence (NIS), rich in the basic amino acids Arg and Lys, mediates nuclear import of proteins, and three such basic regions (BR) have been identified (13). Truncation of 5-LO suggested the presence of an NIS in the amino-terminal region of 5-LO (13). However, limited mutagenesis of a BR at Lys726 did not prevent nuclear import, suggesting that a non-conventional NIS may exist in the amino terminus of 5-LO. Another site, at Arg654, resembles a bipartite NIS. Mutational analysis of this region demonstrated that most basic residues could be replaced without affecting nuclear import (13–15), although Arg651 was replaced (14,15). However, replacement of Arg654 also caused loss of catalytic activity (15), suggesting that mutagenesis caused protein misfolding, which can also impair import (13). Consistent with this, analysis of 5-LO secondary structure indicated that Arg657 serves a critical structural role, through its association with Asp571 (16).

Recently, we developed novel structural and functional criteria to identify functional NIS on 5-LO (16). First, we sought basic residues that were common to 5-LO from different species but not shared by other LO, since nuclear import has been observed in 5-LO from all species but not in 15-LO and 12-LO. Second, we sought BRs having a predominantly random coil/loop secondary structure, which appears to be necessary for binding to importin-α proteins (17–19). Finally, mutations that altered nuclear import should not also inactivate the enzyme, since failed import may result from mutation-induced changes in protein structure (13); loss of activity, then, would be used as an indirect indication of such a false positive result. Application of these rigorous criteria to 5-LO revealed a novel site at Arg518, designated as BR518 (16). This BR alone was sufficient to drive nuclear import, and replacement of basic residues impaired import without inactivating the enzyme, indicating that BR518 is a functional NIS. Interestingly, however, mutations in BR518 could not totally abolish nuclear import in all cells, suggesting that additional NIS(s) must exist on 5-LO.

This study applies the same structural and functional criteria to search for the unidentified NIS(s) on 5-LO. Our results support the conclusion that BR518, the only basic region in the β-barrel region of 5-LO, is unlikely to be a functional NIS. However, a novel site at Arg112, which links the β-barrel region

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1 The abbreviations used are: LT, leukotriene; LO, lipoxygenase; NIS, nuclear import sequence; BR, basic region; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; WT, wild type; mut, mutant; PDB, protein data bank.

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3 To whom correspondence should be addressed: Dept. of Internal Medicine, Division of Pulmonary and Critical Care Medicine, University of Michigan, Ann Arbor, MI 48109-0642. Tel.: 734-763-9077; Fax: 734-764-4556; E-mail: brocko@umich.edu.
Sequence and Structural Analysis—Amino acid sequences were obtained from Swiss-Prot from the ExPaSy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics. Primary accession numbers for proteins are: 5-LOs, human P09917, mouse P48999, rat P12527, hamster P51399; 15-LOs, rabbit P12630, and human P16050; human platelet-type 12-LO P18054; *Clostridium perfringens* α-toxin P15310. Alignment of protein sequences was performed using ClustalW (20). Structural analysis utilized the resolved structures of rabbit 15-LO (PDB: 1lxo) and *C. perfringens* α-toxin (PDB: 1qmd), as well as published theoretical models of the 5-LO β-barrel region (21) and the 5-LO catalytic region (22).

Plasmids and Mutagenesis—To construct a fusion peptide joining BR region to a green fluorescent protein (GFP), complementary oligonucleotides encoding the basic regions (indicated below) were annealed and ligated to the BamHI and HindIII sites of pEGFP-C1. BR peptide was Leu<sub>111</sub>-Asp<sub>112</sub>-LRDGRAKLARD; BR<sub>518</sub> peptide was Asp<sub>112</sub>-Asp<sub>113</sub>-DACHKDLPRD. Specific amino acids within the putative 5-LO NISs were substituted in the pEGFP-C1-5-LO template (14) using the QuikChange site-directed mutagenesis kit (Stratagene). Briefly, two complementary primers (125 ng each) containing the desired mutation and 20 ng of template in 1× reaction buffer were denatured at 95 °C for 30 s and annealed at 55 °C for 30 s, and DNA synthesis was carried out by Pfu polymerase at 68 °C for 14 min. This cycle was repeated 12–18 times, depending on the number of bases substituted, according to the manufacturer’s directions. The methylated template was removed by incubation with 10 units of DpnI at 37 °C for 1 h. The mutation BR<sub>518</sub> was R518Q/R520Q/K158N; mutation BR<sub>158</sub> was K158N/H160Q/K161N. All substitutions and constructs were verified by DNA sequence analysis (DNA Sequencing Core, University of Michigan). Oligonucleotides (sequences available upon request) were synthesized and PAGE-purified by Integrated DNA Technologies Inc. (Corvalle, IA).

Cell Culture, Transfection, and Imaging—NIH 3T3 cells were obtained from American Type Culture Collection (Manassas, VA) and grown under 5% CO<sub>2</sub> in Dulbecco’s Modified Eagle Medium (Invitrogen). Cells were maintained with 10% fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin. Cells were transfected using Polyfect (Qiagen, Inc.) transfection reagents according to the manufacturer’s specifications. Transient transfectants were evaluated microscopically, live, or after fixation with 4% paraformaldehyde, 16–20 h after transfection. Comparable results were obtained when cells were examined as early as 9 h after transfection.

Immunoblotting—As described previously (14), cells were disrupted by sonication on ice, and protein concentrations were determined by a modified Coomassie Blue dye binding assay (Pierce). Samples containing 10 μg of protein were separated by SDS-polyacrylamide gel electrophoresis under reducing conditions and transferred to nitrocellulose. Membranes were probed with a rabbit polyclonal antibody raised against purified human leukocyte 5-LO (a generous gift from Dr. J. Evans, Merck Research Laboratories, Rahway, NJ) (23) or with rabbit polyclonal anti-GFP (Santa Cruz Biotechnology, Inc.; titer 1:500) followed by peroxidase-conjugated secondary antibody and enhanced chemiluminescence detection (Amersham Biosciences).

The ratio of conversion of radiolabeled AA to 5-HPETE/5-HETE was used to calculate activity. One peak, clearly separated from unmetabolized AA, was observed. A single peak, clearly separated from unmetabolized AA, was observed. The ratio of conversion of radiolabeled 5-LO was determined in 0.25 ml reaction mixtures containing 50 mM MAA, 2 mM MgCl<sub>2</sub>, 100 μg protein, and 500 μM radiolabeled AA (Avanti Polar Lipids, Alabaster, AL) using a mobile phase of acetonitrile/trifluoroacetic acid (50:1 v/v). After a 30-min incubation at 37 °C, the reaction was stopped by adding 1 ml of ether/methanol (30:4:1, v/v). The reaction was vortexed thoroughly, the reaction was stopped by adding 1 ml of ether/methanol (30:4:1, v/v). The reaction was vortexed thoroughly, and the supernatant was quantitated by on-line radiochromatography (HPLC) on a 5-μm Bondapak C<sub>18</sub> column (30 × 0.4 cm; Waters Associates, Milford, MA) using a mobile phase of acetonitrile/trifluoroacetic acid at a flow rate of 2 ml/min. 5-LO metabolites were eluted during a single run, linear gradient increase of acetonitrile from initial solvent of 50:50 (v/v) to 73:27 (v/v) at 7 min, then to 85:15 (v/v) at 9 min, and finally to 100:0 (v/v) at 15 min. Radioactivity in 1-ml eluted fractions was quantitated by off-line radiochromatography. There were no LTB<sub>4</sub> or LTB<sub>4</sub>-isomers detected in cell lysates; 5-HETE and 5-HETE co-eluted as a single peak, clearly separated from unmetabolized AA. The 5-LO specific activity of different mutants was calculated and compared based on the ratio of conversion of radiolabeled AA to 5-HPETE/5-HETE.

Quantitation of Subcellular Distribution—As an initial approach to quantitation, slides were fixed 16 h after transfection, and 100 positive cells were scored as to whether nuclear fluorescence was greater than, equal to or less than cytosolic fluorescence. Care was taken to avoid damaged, dead or autofluorescent cells. Results from at least three independent transfections per construct were used for statistical analysis. As a second approach, 100 individual cells per construct were scored for cytosolic and nuclear fluorescence intensity: using Adobe Photoshop 5.5, grayscale digital images were adjusted to include the full black-to-white range, and representative intensity values from 0 (white) to 100 (black), were obtained for the cytoplasm and nucleus. Cytoplasmic and nuclear values for each cell were summed to give total cellular fluorescence, and the percent fluorescence values for the nuclear compartment were calculated.

Statistical Analysis—Statistical significance was evaluated by one-way analysis of variance, using p < 0.05 as indicative of statistical significance. Pairs of group means were analyzed using the Tukey-Kramer post-test.

**RESULTS**

**Reassessment of BR<sub>518</sub> as a Functional NIS**—As outlined above, cells expressing GFP-5-LO with mutated BR<sub>518</sub> displayed either no import or significant import, indicating the existence of at least one other functional NIS on 5-LO. Since the BR<sub>518</sub> NIS was identified using structural criteria noted above, these criteria were applied to other candidate sites. Previous work indicated that an NIS in the amino-terminal region of 5-LO, potentially at BR<sub>518</sub>, might function as an NIS (13). This site was a good candidate because its primary structure, RXXKKK, fulfills the criteria of a monopartite NIS, being a cluster of 4 of 6 basic residues. Using the structural and functional criteria, BR<sub>518</sub> was evaluated further. Comparison with the primary sequences of other lipoxygenases, however, indicated that this BR was not unique to 5-LO (Fig. 1A). Thus, if it was a functional NIS on 5-LO, it might also be expected to direct the import of 15-LO and, perhaps, 12-LO. Regarding the...
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Fig. 1. Primary and secondary structural evaluation of the amino-terminal BR112. A, sequence alignments performed using ClustalW, with asterisks and colons indicating identical and similar residues, respectively. Shaded areas on rabbit 15-LO and α-toxin indicate residues involved in β-sheets from resolved structures. Region on 5-LO corresponds to amino acids 68–73. B, secondary structure of BR68 (shaded black) in the β-barrel region of human 5-LO, using the theoretical model developed using α-toxin as template (21). C, secondary structure of the corresponding region on the rabbit 15-LO protein.

The secondary structure of BR68, no resolved structure for 5-LO is available. However, predicted structures for the β-barrel domain have been published using C. perfringens α-toxin (21) or 15-LO (24) as templates, and the structure of the 15-LO β-barrel domain has been published (25). The majority of the amino acids in BR68 were found to be involved in the fifth β-sheet, in the predicted structure of 5-LO patterned after α-toxin (Fig. 1B) and in the resolved structure of 15-LO (Fig. 1C), as well as in the 5-LO structure patterned after 15-LO (Ref. 24 and not shown here). This suggests that this region serves a critical structural role in the amino-terminal β-barrel and is not available for binding importin. These results indicate that BR68 is unlikely to be a functional NIS.

Evaluation of BR112 as a Functional NIS—Alignment of LO primary sequences revealed a novel basic region, beginning at R112 on human 5-LO, which was conserved across 5-LOs and not found in 12- or 15-LOs (Fig. 2A). Correct alignment was suggested by high levels of amino acid similarity on both sides of the region as well as alignment of the α-helix in the catalytic domain. This region, designated BR112, contained 4 basic amino acids over a stretch of 9 residues. The region was located on a random coil between the β-barrel and catalytic domains of 5-LO (Fig. 2B). The presence of a conserved glycine, which can serve as a “helix breaker,” also indicated that this region would retain a random coil structure. Since the region was conserved across different 5-LOs, not found on other LOs and was on a coiled structural element, it met our primary and secondary structural criteria for a good candidate NIS.

To test whether BR112 was sufficient to cause nuclear import, oligonucleotides were synthesized and inserted into the GFP vector to produce GFP with the peptide LRDGRALKARD fused to the carboxyl terminus. As has been frequently described (e.g. (13)), GFP alone distributed evenly between nuclear and cytoplasmic compartments in transfected cells (Fig. 3), because it is small enough to diffuse freely through the nuclear pore. However, the GFP-BR112 fusion protein showed distinct nuclear accumulation (Fig. 3), indicating that this peptide alone is sufficient to drive nuclear import against a diffusion gradient.

To determine whether BR112 was necessary for nuclear import, the effect of basic residue replacement in BR112, in the context of GFP-5-LO, was evaluated. Three residues were replaced by site-directed mutagenesis: R115Q/K117Q/R120Q. As described previously (13, 14), the wild type (WT) GFP-5-LO fusion protein showed strong nuclear accumulation in most cells (Fig. 4, A and B). Cells expressing GFP-5-LO with mutation of BR112 included two distinct phenotypes: some cells had nuclear accumulation, some did not. This result was first quantitated by scoring individual cells as having nuclear fluorescence greater than, equal to or less than the cytosolic fluorescence. Representative images and numbers for one experiment are given in Fig. 5. While the majority (65%) of cells expressing WT GFP-5-LO had nuclear fluorescence greater than, equal to or less than the cytosolic fluorescence, Representative images and numbers for one experiment are given in Fig. 5. While the majority (65%) of cells expressing WT GFP-5-LO had nuclear accumulation, some cells did not.
served with WT or mutBR112 GFP/H18528 5-LO that was unmodified (WT with GFP/H18528) or mutated at BR112. After 10260 when expressed in 3T3 cells (Fig. 6A).

The finding that mutation in BR112 produced discrete import competent and non-importing populations was very similar to results previously found with mutations in BR518 (16). This suggested the possibility that these sites might overlap in function. To address this possibility, mutations in both sites were performed. The changes in BR518 were R518Q/R520Q/K521Q/K527Q/K530Q. Sample subcellular distributions and frequencies, for mutation in BR518 alone or in BR112 plus BR518, are given in Fig. 7A. Mutation of the BR518 site alone resulted in ~20% of the cells having impaired import, with about half of the cells still having significant nuclear accumulation of GFP-5-LO, as reported previously (16). The combined mutations of BR112 plus BR518 had an additive effect, with over half of the cells showing a failure to import GFP-5-LO.

Statistical analysis of results from multiple transfections showed that, for each of the two mutants, both the reduction of cells with nuclear import and the increase in cells with cytoso-
lic fluorescence were statistically significant (Table I). Moreover, mutation of both basic regions produced statistically greater changes in the two distribution groups than did either mutation alone. No statistically significant change in the group showing balanced distribution was found for any mutation. The additive nature of the mutations indicated that these sites represent distinct NISs.

Significantly, all mutants were functionally active, producing LTB₄ when stimulated with the calcium ionophore A23187 in the presence of 10 µM arachidonic acid (Table I). The double mutant produced marginally less LTB₄ than WT GFP-5-LO. Furthermore, mutated proteins of the appropriate size were expressed in similar amounts as wild type GFP-5-LO in transfected 3T3 cells (Fig. 7B). This result was obtained using antibodies to either GFP (Fig. 7B) or 5-LO (data not shown). Thus, the changes in nuclear import were unlikely to result from altered protein expression, conformational folding, or protein degradation.

**Evaluation of BR₁⁵⁸ as a Functional NIS**—Because mutations of both the BR₁⁵⁸ and BR₁₁² did not completely impair nuclear import of 5-LO, the protein sequence was further evaluated using the structural and functional criteria described previously. Another novel basic region was identified, beginning at Lys₁⁵⁸ on human 5-LO. Correct alignment was supported by amino acid similarity on both sides of the region as well as alignment of the DLP core (Fig. 8A). This region, BR₁⁵⁸ (KCHKDLPR), contained 3 basic residues, which were conserved across 5-LOs and not found in 12- or 15-LOs. This region was predicted to form a random coil on the catalytic domain of 5-LO (22), although a helix-like turn involving Leu-Thr on 15-LO (replaced by His-Lys on 5-LO) was evident (Fig. 8B). The presence of the conserved proline within the region, which can serve as helix breaker, also indicates that this region would retain a random coil structure. Since the region was conserved across different 5-LOs, not found on other LOs and was on a largely coiled structural element, it met our primary and secondary structural criteria for a good candidate NIS.

A vector was constructed to express the GFP-BR₁⁵⁸ fusion protein, with DAKCHKDLPRD representing BR₁⁵⁸. This fusion protein showed nuclear accumulation (Fig. 8, C and D). Quantitative analysis of nuclear/cytosolic fluorescence ratios for 100 cells revealed that nuclear accumulation of the GFP-BR₁⁵⁸ fusion protein was greater than that for the GFP-BR₁₁² fusion protein (data not shown). Thus, the BR₁⁵⁸ peptide is sufficient to drive nuclear import.

To determine whether the BR₁⁵⁸ region was necessary for nuclear import, site-directed replacement of basic residues on GFP-5-LO was performed. The mutation mutBR₁⁵⁸ was K₁⁵⁸N/H₁⁶⁰Q/K₁⁶¹N. Mutated proteins of the appropriate size were expressed in similar amounts as wild type GFP-5-LO in transfected 3T3 cells (data not shown). When the mutBR₁⁵⁸ protein showed nuclear accumulation (Fig. 8A), sequence alignments performed using ClustalW, with asterisks indicating identical residues, B, secondary structure of the corresponding region on rabbit 15-LO (black); C, effect of the BR₁⁵⁸ peptide on the subcellular distribution of GFP. 3T3 cells were transfected with GFP fused to the BR₁⁵⁸ peptide (DAKCHKDLPRD) and imaged after 16 h. D, DNA staining, using 4,6-diamidino-2-phenylindole, of field shown in C.

**Table I**

Effect of mutations in the BR₁₁² region on the subcellular distribution of GFP-5-LO and GFP-5-LO with mutBR₁⁵⁸ in 3T3 cells

Cells were transfected, incubated for 16 h and fixed. 100 cells/transfection were scored. Results are reported as percent of cells with the given distribution of green fluorescence. Data are means (S.E.) of n = 7 experiments. *p < 0.05 versus WT; **p < 0.05 versus mutBR₁⁵⁸. Activity was assessed as amount of LTB₄ produced by transfected cells, adjusted for protein expression, as measured by enzyme immunoassay.

| Plasmid | Nuclear > cytosolic | Nuclear = cytosolic | Nuclear < cytosolic | Activity |
|---------|---------------------|---------------------|---------------------|----------|
| Wild type | 67.3 (2.6) | 29.9 (3.0) | 3.07 (0.67) | +++ |
| mutBR₁² | 52.0 (3.0)* | 24.1 (3.4) | 23.9 (0.74)* | +++ |
| mutBR₁⁵⁸ | 42.3 (2.7)* | 33.1 (2.6) | 24.6 (1.5)* | ++ |
| mutBR₁² + mutBR₁⁵⁸ | 19.9 (3.2)* | 26.3 (2.7) | 53.8 (2.4)* | ++ |

Fig. 7. Evaluation of the distribution phenotypes observed with mutBR₁⁵⁸ with or without mutBR₁₁² GFP-5-LO. A, 3T3 cells were transfected with GFP-5-LO with mutBR₁² alone or with mutBR₁¹² plus mutBR₁⁵⁸. After 16 h, 100 positive cells were scored as in Fig. 5. Numbers indicate results from one experiment. B, immunoblot showing protein expression in 3T3 cells transfected with WT GFP-5-LO or GFP-5-LO with mutations at BR₁¹² or BR₁⁵⁸ or both sites. All lanes contain 10 µg of total cellular protein.
construct was expressed in 3T3 cells, the majority of cells showed nuclear accumulation, with only 10% of the cells clearly indicating a failure to import (Fig. 9). However, when this mutation was combined with mutations at the other two NIS sites, no cells showed nuclear accumulation (Fig. 9).

Statistical analysis of results from multiple transfections showed that mutation of BR\textsuperscript{158} produced a small but statistically significant increase in cells with impaired nuclear import (Table II). As described above in Table I, the combination of mutBR\textsuperscript{112} + mutBR\textsuperscript{518} significantly but incompletely decreased nuclear import. When all three BRs were altered, no cells showed nuclear accumulation of GFP-5-LO. Significantly, all mutants were functionally active, producing LTB\textsubscript{4} when stimulated with the calcium ionophore A23187 in the presence of 10 \mu M arachidonic acid (Table II). The double and triple mutants produced marginally less LTB\textsubscript{4} than the single mutants or WT GFP/H11001 when stimulated with the calcium ionophore A23187 in the presence of 10 \mu M arachidonic acid (Table II). The double and triple mutants produced marginally less LTB\textsubscript{4} than the single mutants or WT GFP/5-LO. Further analysis of mutants by cell-free assay confirmed that, although the multiple amino acid substitutions did indeed reduce activity relative to wild type GFP-5-LO, even the mutation of all three BRs did not abolish activity (Table III). These results indicated that all three BRs were necessary for nuclear accumulation of 5-LO in all cells within a population.

**DISCUSSION**

Previously, we used novel structural and functional criteria to identify an NIS, designated BR\textsuperscript{518}, on 5-LO, but predicted that at least one other NIS must also exist (16). In the present study, we continued to search for potential NISs on 5-LO using the same criteria. This work revealed two novel basic regions, BR\textsuperscript{112} and BR\textsuperscript{518}. Subsequent analysis showed that these sites were both sufficient and necessary for normal import. The body of work presented in this and the previous study confirms that three NISs exist on 5-LO and that all three are functional in determining the subcellular localization of 5-LO in 3T3 cells. Moreover, these studies indicate that the different NISs act independently from one another and that they can be activated and inactivated. Finally, these results demonstrate how mutations at different NISs will differ in their impact on the subcellular distribution of 5-LO.

Our mutagenesis data showed that the first basic region, BR\textsuperscript{112}, serves a relatively strong import role, since mutation of the basic residues in this region impaired 5-LO nuclear import to at least the same extent as the mutation of BR\textsuperscript{518}. Although mutation of BR\textsuperscript{112} significantly impaired 5-LO nuclear import, enzyme immunoassay showed that the catalytic activity of 5-LO was not compromised. Thus, the mutation specifically impaired nuclear import without changing the general enzyme secondary structure. This further substantiates that BR\textsuperscript{112} is a functional NIS. Mutations of both BR\textsuperscript{112} and BR\textsuperscript{518} had additive effects in reducing nuclear import (Table I), indicating that these sites act independently from one another.

The fact that mutations on both BR\textsuperscript{112} and BR\textsuperscript{518} could not totally eliminate nuclear import implied the existence of an additional import sequence. The third site, BR\textsuperscript{518}, was found to be unique to 5-LO, structurally appropriate for binding importin, and sufficient to import GFP. Because mutation of BR\textsuperscript{518} alone only slightly impaired nuclear import, we speculate that this region may act as a weak import sequence that has low affinity to the receptor protein importin. Alternatively, BR\textsuperscript{518} alone may not function as an independent import sequence; it may coordinate other NISs to mediate 5-LO nuclear import. Supporting this idea, when BR\textsuperscript{112}, BR\textsuperscript{518} and BR\textsuperscript{518} were all mutated at once, nuclear import of 5-LO was totally eliminated. Combined with the finding that the multiple mutant was still active, these studies strongly indicate that BR\textsuperscript{112}, BR\textsuperscript{518}, and BR\textsuperscript{518} are functional NISs and that multiple NISs orchestrate 5-LO nuclear import.

Previously, it was reported that a peptide containing the first 80 amino acids of 5-LO could drive import, leading to the suggestion that this region contains an NIS (13). The role of secondary structure in determining import capacity, as stressed in this study, may help explain this result. The region BR\textsuperscript{518}, as shown in Fig. 1 and in Ref. 21, normally forms the fifth sheet of the \beta-barrel domain. However, removal of residues

![Representative images of the effects of mutBR\textsuperscript{158}, alone or combined with mutBR\textsuperscript{112} plus mutBR\textsuperscript{518}, on the subcellular distribution of GFP-5-LO.](image)

**TABLE II**

| Table II | Comparison of the cell-free activity of GFP-5-LO fusion proteins |
|----------------|---------------------------------------------------------------|
| Plasmid | Relative 5-LO activity % |
| Wild type | 100 |
| mutBR\textsuperscript{518} | 79 |
| mutBR\textsuperscript{112} | 86 |
| mutBR\textsuperscript{518} | 93 |
| mutBR\textsuperscript{112} + mutBR\textsuperscript{518} | 64 |
| mutBR\textsuperscript{518} + mutBR\textsuperscript{112} + mutBR\textsuperscript{518} | 47 |

**TABLE III**

| Table III | Effect of mutations in the BR\textsuperscript{158} region on the subcellular distribution of GFP-5-LO and GFP-5-LO with mutBR\textsuperscript{112} and mutBR\textsuperscript{518} in 3T3 cells |
|----------------|-------------------------------------------------------------------------------------------------|
| Plasmid | Nuc > cytosolic | Nuc = cytosolic | Nuc < cytosolic | Activity |
| Wild type | 74.1 (4.8) | 22.3 (5.3) | 3.6 (0.85) | ++ + |
| mutBR\textsuperscript{518} | 70.3 (3.7) | 20.1 (3.6) | 9.6 (0.30)* | ++ + |
| mutBR\textsuperscript{112} + mutBR\textsuperscript{518} | 24.1 (1.9)* | 25.7 (1.2) | 50.2 (1.1)* | ++ + |
| mutBR\textsuperscript{112} + mutBR\textsuperscript{518} + mutBR\textsuperscript{518} | 0.0 (0.00)* | 33.6 (1.4) | 66.4 (1.4)* | ++ + |

Cells were transfected, incubated for 16 h and fixed. 100 cells/transfection were scored. Results are reported as percent of cells with the given distribution of green fluorescence. Data are means (S.E.) of n = 3 experiments. * p < 0.05 versus WT, ** p < 0.05 versus mutBR\textsuperscript{112} + mutBR\textsuperscript{518}. Activity was assessed as amount of LTB\textsubscript{4} produced by transfected cells, adjusted for protein expression, as measured by enzyme immunoassay.
81–111 will also remove the last three β-sheets of the barrel. In the LOs, sheet 5 is positioned between sheets 2 and 8, across from sheets 6 and 7; loss of sheets 6–8 might allow the fifth sheet, and BR10, to reform as a random coil. In this conformation, BR6 would be able to bind importin α, and misleadingly, act as an NIS.

As we have found for 5-LO, an increasing number of proteins have been described as having multiple NISs. These include BRCA1 (26), Epstein-Barr virus Dnase (27), herpes simplex virus proteins ICP22 (28), ICP27 (29), and XPG nuclease (30). The importance of having multiple NISs is unclear. In some cases, the individual NISs are weak, and the actions of multiple NISs can be additive or synergistic, as appears to be the case for 5-LO (this study) and XPG nuclease (30). Alternatively, the isoforms of importin-α are differentially expressed in different cell types and may bind each NIS with varied specificity (31). Thus, the NIS that is actually functional in a given cell type may depend on the isoform(s) of importin-α that is present.

Finally, each NIS may be regulated independently from the others. The observation of two distinct populations, one with import and one without, in cells expressing GFP-5-LO with either mutant BR112 (Fig. 4) or mutant BR154 (16) suggests that the remaining NISs may be subject to regulation. Protein phosphorylation in the vicinity of NISs has been repeatedly shown to play a role in regulating nuclear import (e.g., Refs. 32 and 33). There is evidence that 5-LO can be modulated by different virus products ICP22 (28), ICP27 (29), and XPG nuclease (30). Alternatively, the isoforms of importin-α might drive even greater accumulation.

Activation of a single NIS might be sufficient for significant accumulation of 5-LO in the nucleus, whereas activation of multiple NISs might drive even greater accumulation. Each of these cues might activate recruitments, no NIS is activated. As noted above, nuclear import of 5-LO with MAPKAP kinase 2 (41). It is not known which, if any, of these phosphorylation events regulate the three NISs of 5-LO.

In certain leukocytes, such as neutrophils and eosinophils in circulating blood, 5-LO is found exclusively in the cytoplasm (40) and by MAPKAP kinase 2 (41). It is not known which, if any, of these phosphorylation events regulate the three NISs of 5-LO.

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