Identification of a Bipartite Nuclear Localization Sequence Necessary for Nuclear Import of 5-Lipoxygenase*

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5-Lipoxygenase catalyzes the synthesis of leukotrienes from arachidonic acid. This enzyme can reside either in the cytoplasm or the nucleus; its subcellular distribution is influenced by extracellular factors, and its nuclear import correlates with changes in leukotriene synthetic capacity. To identify sequences responsible for the nuclear import of 5-lipoxygenase, we transfected NIH 3T3 cells and RAW 264.7 macrophages with expression vectors encoding various 5-lipoxygenase constructs fused to green fluorescent protein. Overexpression of wild type 5-lipoxygenase with or without fusion to green fluorescent protein resulted in a predominantly intranuclear pattern of fluorescence, similar to the distribution of native 5-lipoxygenase in primary alveolar macrophages. Within the 5-lipoxygenase protein is a sequence (Arg638–Lys655) that closely resembles a bipartite nuclear localization signal. Studies using deletion mutants indicated that this region was necessary for nuclear import of 5-lipoxygenase. Analysis of mutants containing specific amino acid substitutions within this sequence confirmed that it was this sequence that was necessary for nuclear import of 5-lipoxygenase and that a specific arginine residue was critical for this function. As nuclear import of 5-lipoxygenase may regulate leukotriene production, natural or induced mutations in this bipartite nuclear localization sequence may also be important in affecting leukotriene synthesis.

Leukotrienes (LTs) are lipid mediators with important roles in immune responsiveness and antimicrobial host defense. However, overproduction of LTs can contribute to a variety of pathophysiological inflammatory processes, including asthma and allergic responses (1). Understanding the regulation of LT synthesis is critical to understanding both normal immune responses and the underlying pathophysiology of a variety of diseases.

The synthesis of LTs from arachidonic acid (AA) is initiated by the enzyme 5-lipoxygenase (5-LO). Activation of 5-LO, which is characterized by its translocation to the nuclear envelope (2–5), positions 5-LO close to its substrate and 5-LO activating protein (6–8) and is essential for LT synthesis. Changes in substrate availability or in the level of expression of proteins essential for AA metabolism (5-LO and/or 5-LO activating protein) have been correlated with the modulation of LT synthetic capacity (9–14). However, changes in these variables do not account for all observed changes in LT synthetic capacity (15–17).

Evidence is accumulating that, even in resting or unstimulated cells, the subcellular distribution of 5-LO is dynamic and may influence LT synthetic capacity. In resting cells, the subcellular distribution of 5-LO is cell type-dependent; 5-LO is predominantly cytoplasmic in peripheral blood neutrophils (18–20), peripheral blood monocytes (4), and peritoneal macrophages (3), whereas it is found in both the nucleus and cytoplasm of alveolar macrophages (4, 5), mast cells (21), and the mast cell–like rat basophilic leukemia cell line (20). However, these subcellular distributions are not necessarily fixed. For example, differentiation of monocytes to alveolar macrophages (22), but not peritoneal macrophages (3), is associated with nuclear accumulation of 5-LO. Conversely, removal of macrophages from the alveolar milieu results in subsequent accumulation of 5-LO to the cytoplasm (22), suggesting that factors in the alveolar drive nuclear import of 5-LO. Furthermore, adherence or recruitment of peripheral blood neutrophils (23) or eosinophils (24) induces nuclear import of 5-LO from the cytoplasm. In each case, a change in the subcellular distribution of 5-LO is associated with a change in LT synthetic capacity.

All transport into the nucleus occurs through the nuclear pore complex (25–27). The nuclear pore allows small molecules (<45 kDa) to diffuse freely into and out of the nucleus (25–27). Targeting of larger proteins to the nucleus usually requires the presence of a nuclear localization signal (NLS) (25). NLSs are defined as sequences sufficient and necessary for nuclear import of their respective proteins. Two major types of NLSs are recognized: 1) a single cluster of basic amino acids exemplified by the SV40 large T antigen NLS; and 2) a bipartite NLS composed of two basic amino acids, a spacer region of 10–12 amino acids, and a basic cluster in which three of five amino acids must be basic as found in nucleoplasmin (27, 28). For NLS-mediated nuclear import to occur, the NLS must be recognized by and associate with specific importins, which allows docking at the nuclear pore and translocation through the pore via an energy-dependent process (25–27).

A recent study of nuclear import of 5-LO failed to identify a classical NLS as responsible for targeting this enzyme to the nucleus (29). Rather, that study concluded that an as yet unidentified unconventional signal located in the amino terminus targets 5-LO to the nucleus. In the current study, we have addressed this same question and have reached a completely
different conclusion. Our studies have identified a basic region in the carboxy terminus that resembles a classical bipartite NLS, have demonstrated that specific basic residues within this region are necessary for targeting 5-LO to the nucleus, and have provided evidence that the amino terminus is not sufficient to direct nuclear import.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection—**RAW 264.7 cells and NIH 3T3 cells were obtained from the American Type Culture Collection (Rockville, MD). RAW cells were incubated with rhodamine-conjugated goat anti-rabbit antibody (SigmA, titer, 1:200), and washed again. Rhodamine fluorescence was visualized as described above using a 565-nm band-pass filter. DNA was stained with diamidino-2-phenylindole and visualized using ultraviolet excitation. Differential interference contrast microscopy was also performed with the Zeiss Axioskop microscope equipped for epifluorescence using a 495-nm bandpass filter. Indirect immunofluorescent microscopy was carried out as described previously (20). Briefly, adherent cells were fixed in methanol at 20 °C for 30 min, permeabilized in acetone at −20 °C for 5 min, and air-dried. Cells were rehydrated and blocked with 1% BSA in phosphate-buffered saline containing nonimmune goat serum. Rabbit polyclonal antibody raised against purified human leukocyte 5-LO (a generous gift from Dr. J. Evans, Merck Frosst Center for Therapeutic Research) (2) was prepared in 0.1% bovine serum albumin in phosphate-buffered saline (titer, 1:200) and applied for 1 h at 37 °C. Mounts were also performed with the Zeiss Aristoplan microscope.

**Nuclear Import** of 5-Lipoxygenase

**RESULTS**

**5-LO Nuclear Import in NIH 3T3 and RAW 264.7 Cells**—We analyzed 5-LO nuclear import in the mouse cell line NIH 3T3 and in the mouse macrophage cell line RAW 264.7. Because 5-LO is predominantly expressed in cells of leukocytic origin, we preferred to analyze 5-LO nuclear import in RAW cells. However, the low transfection efficiency in these cells precluded their use in techniques requiring large numbers of transfected cells (e.g. metabolic studies); the higher transfection efficiency of 3T3 cells permitted these analyses. Western blot analysis of cell lysates revealed little or no detectable 5-LO expression in untransfected RAW and 3T3 cells (Fig. 1A). However, substantial 5-LO expression was detected in 3T3 cells transiently transfected with pcDNA/5LO, a plasmid that supports constitutive expression of human 5-LO (Fig. 1A). Immunofluorescent microscopy revealed that 5-LO colocalized with DNA and thus was localized predominantly in the nucleus in both 3T3 (Fig. 1B) and RAW (Fig. 1C) cells transfected with pcDNA/5LO. Nontransfected cells were used by their lack of staining for 5-LO and positive staining for DNA. After stimulation of transfected 3T3 cells with the calcium ionophore A23187, appreciable LTB₄ synthesis (1556 ± 96 ng/ml, n = 3) was detected by enzyme immunoassay. LT synthesis could not be detected in untransfected 3T3 cells. These data indicate that these cell lines are useful models for the analysis of 5-LO nuclear import.

**Use of GFP Fusions to Study Nuclear Import**—Next, we determined whether a GFP tag would facilitate the study of 5-LO nuclear import. Transiently transfected RAW or 3T3 cells expressing only the ~27-kDa GFP exhibited fluorescence distributed evenly between nucleus and cytoplasm (Fig. 2A), in agreement with results observed in other cell lines (e.g. Ref. 32). This lack of a gradient in distribution between nucleus and cytoplasm was presumably because of the small size of GFP, which would allow it to move freely through the nuclear pore.
To determine the subcellular localization of a GFP/5LO fusion protein, we inserted the full-length human 5-LO cDNA into pEGFP-C1 (fusing the GFP to the carboxyl terminus of 5-LO) was not attempted as the carboxyl terminus is known to be necessary for binding the iron in 5-LO. The resulting GFP/5LO construct would express an ~105-kDa fusion protein, which would be too large to diffuse through the nuclear pore. Upon transient transfection with this construct, a strong intranuclear fluorescence pattern was observed (Fig. 2B) in both cell lines. Thus, both 5-LO and GFP/5LO accumulate within the nucleus in RAW and 3T3 cells, suggesting the presence of a functional NLS in 5-LO. Upon stimulation with A23187, the GFP/5LO fusion protein translocated to the nuclear envelope and endoplasmic reticulum in both cell types (Fig. 2C). In addition, significant LTB4 production (3667 ± 1365 pg/ml, n = 4) could be detected in transfected 3T3 cells, but not in untransfected cells, after stimulation with A23187. Thus, the GFP tag does not interfere with subcellular localization, translocation, or catalytic activity of 5-LO. Taken together, these results indicate that the use of a GFP tag is an appropriate approach for analysis of 5-LO nuclear import.

**Analysis of the Nuclear Import of GFP/5LO Constructs**—As reported previously (29), 5-LO contains three regions of basic amino acids that are candidate NLSs (Fig. 3). The first 80 amino acids of 5-LO, which contains the first basic region (BR1), have been reported to be sufficient to target GFP to the nucleus (29). However, images supporting that conclusion were unconvincing; the nuclei were many times larger than those in any other image, and the staining pattern for both DNA and the GFP/1–80 construct was much more clumped than the patterns of DNA or GFP/5-LO in other images. To re-examine these results, we also constructed GFP/5LO (1–80) in which the first 80 amino acids of 5-LO were fused to GFP. Sequence analysis indicated that this construct placed the 1–80 sequence in frame with GFP (not shown); immunoblot analysis confirmed that the produced protein was of the expected size (~36 kDa) (Fig. 3). Upon transfection, fluorescence was observed to be evenly distributed between the nucleus and cytoplasm in both cell types (Fig. 3B) and was distinctly different from the pattern produced by the intact 5-LO protein (Fig. 3A). This pattern of fluorescence is likely because of the passive diffusion of this ~36-kDa fusion protein and indicates that the first 80 amino acids of 5-LO are not sufficient to direct nuclear import of GFP in these cell lines. A larger segment of 5-LO, encoding amino acids 1–562 and including both BR1 and BR2, was also fused to GFP (5LO-N, Fig. 3C). This truncated ~92-kDa protein was completely excluded from the nucleus in both cell types (Fig. 3C), indicating that the putative BR1 and BR2 were insufficient to drive nuclear import of this size-excluded protein. These results also pointed to a possible role for the carboxyl terminus in nuclear import. Analysis of the predicted amino acid sequence of 5-LO revealed an excellent candidate bipartite NLS at amino acids 638–655 (BR3, Fig. 3). This region contains two basic amino acids, an 11-residue spacer region, and a basic cluster in which four of the five amino acids are basic. To determine if the carboxyl terminus of 5-LO could direct nuclear import, we fused the region containing amino acids 332–673 to GFP (GFP/5LO-C; Fig. 3D). In cells transfected with this construct, which encoded an ~67-kDa fusion protein, fluorescence was observed to be distributed evenly between nucleus and cytoplasm (Fig. 3D). Because this fusion protein is unlikely to freely diffuse through the nuclear pore because of its size, these results suggest that this construct is being targeted, albeit inefficiently, to the nucleus. Immunoblot analysis confirmed that each fusion protein was of the expected size (Fig. 3). Nonspecific bands at approximately 50, 54, 68, and 105 kDa were found in all lanes when this commercial antibody to GFP was used.

Many NLSs are able to direct nuclear import of a heterolo-
gous protein. GFP alone, again, is a small protein which, like GFP/5LO (1–80), distributed equally between the nucleus and cytoplasm (Fig. 4A). The fusion of the SV40 large T antigen NLS to GFP, which results in a protein of 28 kDa, produced strong intranuclear fluorescence and weak cytoplasmic fluorescence in 3T3 cells (GFP/SV40-NLS, Fig. 4B) as well as RAW cells (data not shown). These results are in agreement with those observed in other cell lines (e.g. Ref. 29) and reflect that the GFP/SV40 NLS fusion protein is strongly targeted to the nucleus, despite being small enough (28 kDa) to move freely through the nuclear pore. To determine if BR3 (amino acids 638–655) alone could direct nuclear import of an unrelated protein, we fused the sequence encoding BR3 to GFP (GFP/BR3). In cells expressing this fusion, slightly more intranuclear fluorescence than cytoplasmic fluorescence was observed (Fig. 4C). In multiple experiments in both 3T3 cells and RAW cells, nuclear accumulation of GFP/BR3 was greater than GFP alone but less than either GFP/SV40-NLS or GFP/5LO (Fig. 4D). These results suggest that this sequence is able to weakly target a heterologous protein to the nucleus.

Identification of Amino Acids Necessary for 5-LO Nuclear Import—To determine if the 5-LO BR3 is necessary for nuclear import, we performed site-directed mutagenesis on specific residues of this sequence, changing positively charged arginine and/or lysine residues to alanine, which has a small uncharged side chain. The effects of these mutations were evaluated in the context of the entire GFP/5-LO fusion protein. As described above, the 5-LO BR3 possesses two clusters of basic amino acids separated by a spacer region. Mutagenesis of two basic residues in either the first (AA...RNKKK, Fig. 5A) or second basic cluster (RK...RNAAK, Fig. 5B) did not disrupt nuclear import in either cell type. Mutations in both basic clusters (AA...RNAAK, Fig. 5C) also did not disrupt nuclear import. However, mutagenesis of a single residue, the arginine at 651 (RK...ANKKK), resulted in an equal distribution of fluorescence between nucleus and cytoplasm (Fig. 5D), suggesting a partial disruption of nuclear import. Combining all the mutations in both clusters (AA...RKAAM, Fig. 5F) resulted in an extranuclear pattern of fluorescence, indicating a complete loss of nuclear import. Combining all the mutations in both clusters (AA...RKAAM, Fig. 5G) also resulted in a failure to import. Immunoblot analysis confirmed that each mutant fusion protein was of comparable size and expression as GFP/5LO (Fig. 5); no breakdown products were found in any sample.

These results indicated a critical role for Arg651 in nuclear import of 5-Lipoxygenase.
import. However, the side chain of the substituted amino acid, alanine, differs both in size and charge from that of arginine. To eliminate size differences, Arg651 was also substituted with glutamine. Surprisingly, the R651Q substitution alone was sufficient to completely eliminate nuclear import in 3T3 cells (Fig. 6A). In contrast, substituting Lys 653 and Lys 654 with glutamine had no effect on nuclear import (Fig. 6B), as previously reported (29).

To determine whether these NLS mutations resulted in similar localization patterns when expressed in the context of 5-LO alone rather than as a GFP fusion protein, mutant 5-LO constructs were subcloned into pcDNA3.1. After transfection of 3T3 cells with these pcDNA constructs, immunoblot analysis (Fig. 7) confirmed that each construct was of comparable size to pcDNA/5LO (lane A). Immunofluorescent microscopy revealed subcellular distributions identical to those observed with the GFP fusion constructs (Fig. 7). These results showed that GFP did not influence 5-LO subcellular localization, confirmed the central role of Arg651, and supported the additive effects of deletions at basic residues other than Arg651.

**DISCUSSION**

The nuclear import of 5-LO appears to be a critical regulatory component of its function. The current study identifies a key group of amino acids at positions 638–655 in the human protein, which are essential for nuclear import. Substitution of basic residues within this bipartite NLS completely block nuclear import of 5-LO, and the NLS alone can direct nuclear import of a heterologous protein (GFP) albeit poorly. We further demonstrate that these features are shared by both a

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**FIG. 5.** Subcellular localization of GFP/5LO fusion proteins containing specific amino acid substitutions. The 5-LO bipartite NLS (RKNEAIVSVAERKNKKK) was abbreviated RK...RNKKK. Seven different mutants (A–G), containing substitutions of alanine (A) for arginine (R) or lysine (K) at the indicated sites within the putative NLS, were transiently expressed in 3T3 and RAW cells. For 3T3 cells, 5-LO and DNA localization are shown. For RAW cells, 5-LO localization and differential interference contrast (DIC) images are shown. Immunoblot analysis of these constructs expressed in 3T3 cells and probed with an antibody against GFP is also shown. Results are representative of five independent experiments. WT, wild type.

**FIG. 6.** Subcellular localization of GFP/5LO fusion proteins containing glutamine substitutions. 3T3 cells were transfected with R651Q (A) or K653Q,K654Q (B). Results are representative of three independent experiments.

**FIG. 7.** Immunofluorescent localization of 5-LO and 5-LO constructs containing specific amino acid substitutions. 3T3 cells were transfected with pcDNA/5LO (A) or the indicated mutant subcloned into pcDNA3.1 (B–E). Immunoblot analysis of each construct expressed in 3T3 cells and probed with an antibody against 5-LO is also shown. Results are representative of three independent experiments.
myeloid cell line (RAW 264.7 macrophages) and a nonmyeloid cell line (NIH 3T3 cells), which is significant because 5-LO is largely limited in distribution to myeloid cells. This study also presents evidence that alternative candidate NLSs within the amino terminus are not sufficient to direct nuclear import. Together, these results provide a foundation for understanding the molecular regulation of nuclear import of 5-LO.

The altered production of LTs can contribute to many inflammatory processes. Recent evidence correlates altered nuclear import of 5-LO with altered leukotriene synthetic capacity (22–24). Our site-directed mutagenesis experiments point to Arg651 as being critical for nuclear import of 5-LO with partial or complete loss of import when replaced with alanine or glutamine, respectively. Substitution of basic residues around Arg651 could enhance, but not mimic, this effect. This arginine is conserved across species, including human, rat, mouse, and hamster 5-LO (Fig. 8). These results may have significant clinical implications: they predict a site within the 5-LO gene where a mutation could alter localization of the 5-LO enzyme and, conceivably, alter leukotriene synthetic capacity. Studies are currently underway to determine the effect of these mutations on leukotriene synthetic capacity in relevant cells.

Both the bipartite NLS alone as well as the much larger carboxyl terminus of 5-LO could target GFP to the nucleus; the nuclear import of the carboxyl fragment, which should be excluded because of its size, supports the presence of an NLS. However, import was less efficient than that driven by the complete 5-LO protein fused to GFP. The results obtained using these fragments must be interpreted with caution, foremost because fragments may exclude key elements or include conflicting elements. For example, the weakness of import observed with the carboxyl fragment may indicate that a deleted sequence might augment the action of BR3. Similarly, the amino-terminal fragments may fail to be imported either because they lack an NLS or because they contain a dominant nuclear export sequence. These possibilities will require further study.

Our findings also indicate that nuclear import of 5-LO is constitutive, but incomplete, in both cell lines, that is, 5-LO accumulates within the nucleus without cell stimulation, but a significant pool of 5-LO remains in the cytoplasm. Such a pattern of distribution may arise simply by protein overexpression to the extent that the capacity for import becomes saturated. Alternatively, the distribution may indicate that import is faster than export or that import is slower than synthesis. In any case, this pattern of a large nuclear pool with a lesser cytoplasmic pool is characteristic of many cell types; it is also reported for primary alveolar macrophages (4), recruited neutrophils (23), adherent eosinophils (24), and the mast cell-like rat basophilic cell (20). However, 5-LO is found largely in the cytoplasm of several cell types, including monocytes (4), peripheral blood neutrophils (20) and eosinophils (24, 33), and peritoneal macrophages (3). These results suggest that the nuclear import of 5-LO can be regulated. For many proteins, nuclear import is modulated by phosphorylation (25). 5-LO reportedly can be phosphorylated (34), and a comparison of the 5-LO sequence with a Prosite data base found at least 16 consensus phosphorylation sites scattered through the length of the protein. Phosphorylation at a site distinct from the bipartite NLS site may affect 5-LO localization and may be important in explaining some of the current findings.

A previous study (29) was the first to examine the molecular regulation of the nuclear import of 5-LO, and those results, combined with the current study, give a more complete picture of our current understanding. The region BR1 (AA 68–73) was analyzed in the previous work (29) by site-directed mutagenesis of two of four basic residues and found not to be necessary for import. As we have shown for BR3, a more complete mutagenesis study is necessary to ascertain that BR1 is not necessary for import. Chen et al. (29) also found that 1–80 fragment was sufficient for import. As noted above, the images presented to support import were unusual; the nuclei were much larger than those presented in any other image, and the clumped patterns of nuclear and GFP fluorescence were also unlike other images. Furthermore, the conclusions from the previous study were based on preparations that were fixed prior to examination. Fixation can result in a disproportionate loss of soluble cytosolic proteins relative to soluble nuclear proteins (23), and this may have contributed to the impression that the 1–80 fragment had accumulated in the nucleus. Our findings, using live cells, contradict this conclusion, because neither the smaller 1–80 fragment nor the larger amino terminus fragment promoted nuclear import in either cell line; this site apparently requires further study. Previous mutagenesis of region BR2 (AA 128–133) blocked import, suggesting that this element contains an NLS (29). However, in both this study and the previous work (29), fragments that included this region do not direct import of size-excluded proteins, indicating that this region is not sufficient for import. Finally, Chen et al. (29) found that limited mutagenesis of BR3 (AA 638–655) did not block import. We performed the same mutations and obtained the same results. However, our more complete mutational strategy revealed that replacement of Arg651 with alanine diminished nuclear import and that additional replacements of other basic residues augmented this effect. Chen et al. (29) also reported that fragments that included BR3 were localized to the cytoplasm. However, the only image presented for the short carboxyl terminus (AA 564–673) showed an equal nuclear and cytoplasmic distribution as would be expected for a small protein, although they reported it to be exclusively cytoplasmic. In our hands, larger fragments, which should be size-excluded from the nucleus, are able to enter the nucleus if they contain BR3. However, BR3 is insufficient to drive import comparable to the intact 5-LO protein. Together, these results indicate that, although this basic region may have an essential role in the nuclear import of 5-LO, other parts of the protein must also contribute to the regulation of import.

Understanding the molecular determinants regulating the subcellular localization of 5-LO and the consequences of its compartmentalization will be critical to understanding the metabolic function of this important component of the LT synthetic pathway. In addition, because the subcellular distribution of 5-LO may affect LT synthetic capacity, the 5-LO NLS is a potential site for mutations having clinical implications. Finally, the 5-LO protein may modulate cellular functions independent of its catalytic capacity by e.g. protein-protein interactions (35, 36). Therefore, the potential nonmetabolic consequences of nuclear localization of 5-LO will need to be addressed. Stable cell lines overexpressing 5-LO exclusively in the nucleus or in the cytoplasm will be invaluable for dissecting the mechanisms regulating the subcellular distribution of 5-LO.
and determining its impact on LT synthesis as well as other cellular properties.

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