Multiple Nuclear Localization Sequences Allow Modulation of 5-Lipoxygenase Nuclear Import

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The nuclear import of proteins typically requires the presence of a nuclear localization sequence (NLS). Some proteins have more than one NLS, but the significance of having multiple NLSs is unclear. The enzyme 5-lipoxygenase (5-LO) has three NLSs that, unlike the tight cluster of basic residues of the classical SV40 large T antigen NLS, contain dispersed basic residues. When attached to green fluorescent protein (GFP), individual 5-LO NLSs caused quantitatively and statistically less import than the SV40 NLS. Combined 5-LO NLSs produced nuclear import that was comparable to that of the SV40 NLS. As expected, GFP/NLS proteins displayed relatively uniform import in all cells. However, a fusion protein of GFP plus the 5-LO protein, modified to contain only one functional NLS, produced some cells with import and some cells without import. A GFP/5-LO fusion protein containing two functional NLSs produced four identifiable levels of nuclear import. Quantitative and visual analysis of a population of cells expressing the intact GFP/5-LO protein, with three intact NLSs, indicated five levels of nuclear import. This suggested that the subcellular distribution of 5-LO may vary widely in normal cells of the body. Consistent with this, immunohistochemical staining of lung sections found that individual macrophages, *in situ*, displayed cell-specific levels of import of 5-LO. Since nuclear accumulation is known to affect 5-LO activity, multiple NLSs may allow graded regulation of activity via controlled import. Multiple NLSs on other proteins may likewise allow fine control of protein action through modulation of the level of import.

Nuclear import is an important step in the regulation of many proteins, including transcription factors, signaling proteins, and various enzymes. The nuclear import of larger proteins requires the presence of a short cluster of amino acids comprising a nuclear localization sequence (NLS) (1). The NLS serves as a recognition site for binding proteins called importins or karyopherins (2). Binding to these proteins facilitates docking to the nuclear pore complex, allowing entry into the nucleus.

There is no consensus motif that defines an NLS. However, most NLSs are rich in basic amino acids. In the classical monopartite NLS, these arginines and/or lysines form a tight cluster of four to six residues, as typified by the SV40 large T antigen. A different pattern is found in the bipartite NLS, where the tight cluster of basic residues is preceded, with a 10-amino acid spacer, by a pair of basic residues, as in nucleoplasmin. For either type of NLS, binding to an importin protein with subsequent nuclear import is commonly regulated by phosphorylation (3). In the simplest scenario, the nuclear import process for a given protein can have two states: active or inactive. This model is commonly applied to transcription factors (4). Alternatively, phosphorylation can switch the nuclear import process from slow to fast (5). In either model, a protein with a single NLS and one phosphorylation regulatory mechanism is limited to two rates of nuclear import.

Recent studies have identified several proteins that contain more than one NLS, including nuclear factor 1-A (6), the cell division control protein mcm10 (7), herpes simplex virus gene product ICP22 (8), the HIV preintegration complex (9), Epstein–Barr virus DNase (10), papillomavirus oncoprotein E6 (11), BRCA1 (12) and BRCA2 (13). It isn't clear why some proteins contain multiple NLSs. One explanation is that multiple NLSs afford redundancy in proteins that require successful nuclear import, as in cell cycle proteins or viral integration proteins. Or, each NLS may utilize unique importin isoforms which, in turn, are differentially expressed (14). Finally, multiple NLSs may cooperate with one another and allow more efficient nuclear import (10).

The enzyme 5-lipoxygenase (5-LO), which initiates the synthesis of leukotrienes from arachidonic acid, shows regulated nuclear import (15). Initial evaluation of this enzyme identified two basic regions, BR1 and BR2, that resembled the tight clustered monopartite NLS, and a third, BR3, with a bipartite pattern (16). However, thorough evaluation demonstrated that these sites were not functional NLSs (16,17). Instead, three other sites, BR4-6, were shown to be both necessary and sufficient for nuclear import (17,18). These NLSs were characterized by dispersed basic residues on an accessible random coil structure, the secondary structure found to associate with importins in resolved crystal structures (19–21). Since 5-LO is not recognized as playing a role in cell cycle regulation or viral action, it is unclear why this enzyme should have multiple NLSs.

This study compares nuclear import as driven by the three 5-LO NLSs, as unregulated peptides attached to green fluorescent protein (GFP) and as regulated NLSs in 5-LO fused to GFP. Nuclear import is quantitated objectively by comparing nuclear fluorescence as a percentage of the nuclear plus cytoplasmic, or 'total', fluorescence. The quantitative analysis indicates that all three NLSs from 5-LO, which have dispersed basic amino acids, are statistically weaker import sequences than the classical monopartite NLS of SV40 large T antigen. More interestingly, evaluation of individual cells expressing 5-LO with one, two or three intact NLSs suggests that having multiple NLSs allows multiple degrees of nuclear accumulation of 5-LO. This mechanism of controlling protein redistribution within individual cells may represent a previously unrecognized way to regulate the impact of nuclear import on cell response.

Results and Discussion

Quantitative analysis of isolated individual NLSs

Because GFP is a small protein of $\sim$27 kDa, it is able to freely diffuse through the nuclear pore. Consistent with this, GFP was found in both the cytoplasm and the nucleoplasm of transfected 3T3 cells (Figure 1A). The GFP/SV40 NLS fusion protein would be only slightly larger than GFP itself, but it was accumulated strongly in the nucleus (Figure 1B). When the mean nuclear fluorescence of individual GFP-expressing cells was calculated as a percentage of the nuclear plus cytoplasmic ('total') fluorescence, most cells scored as having 50% nuclear fluorescence (Figure 1C). Quantitation of fluorescence of individual GFP/SV40 NLS-expressing cells indicated that most cells had 85–95% nuclear fluorescence. Qualitatively identical results were obtained when cells were imaged using a confocal microscope (data not shown). Thus, quantitation confirmed the visual impression that the SV40 NLS is a strong import sequence.

The three 'dispersed' NLSs of 5-LO differ from one another in number and distribution of basic residues, length of NLS and types of intervening residues. To determine whether they differ from one another in nuclear import strength, cells expressing GFP/5-LO NLSs were quantitated for fluorescence distribution. Cells expressing either GFP/BR5 or GFP/BR6 showed comparable, weak nuclear accumulation, with most cells scored at 55–65% nuclear fluorescence (Figure 2).
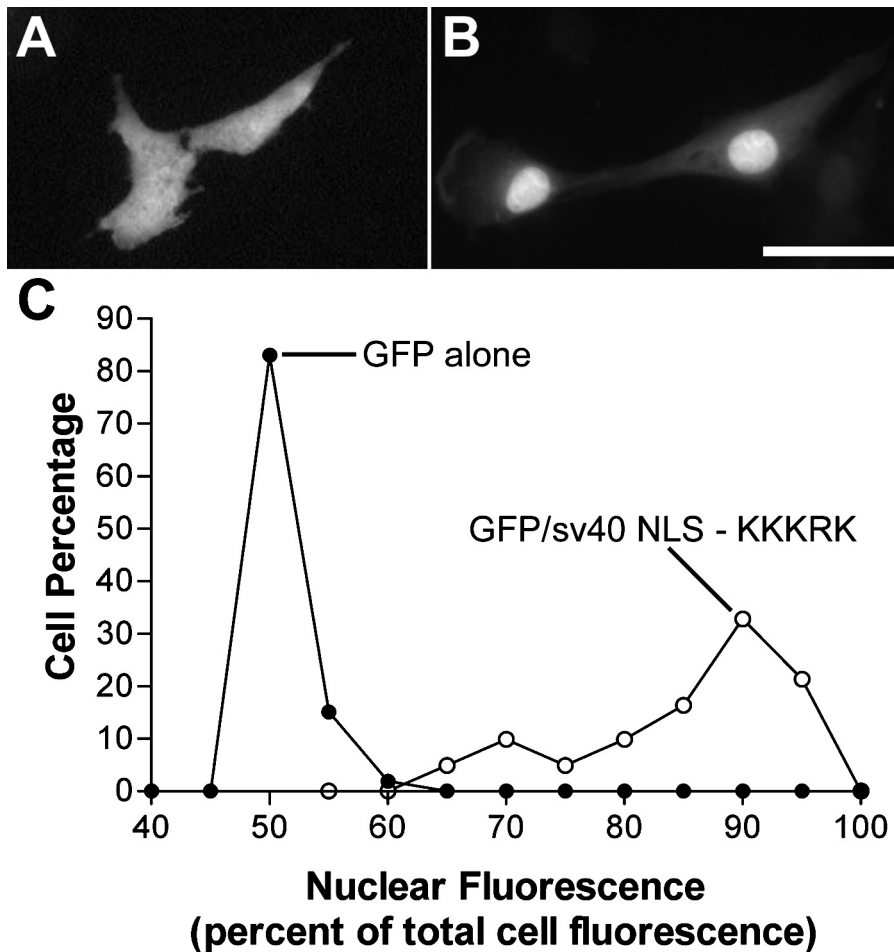

Figure 1: Nuclear import efficiency of the SV40 NLS. A, B) Visualization of GFP subcellular distribution without (A) or with (B) the SV40 NLS. Bar indicates 40 μm. C) Quantitation of GFP subcellular distribution without or with the SV40 NLS. 3T3 cells were transfected with plasmids encoding either GFP alone or GFP with the SV40 NLS attached and the subcellular fluorescence distribution was determined in expressing cells as described in Materials and Methods. Results are from one experiment and are representative of three independent experiments.
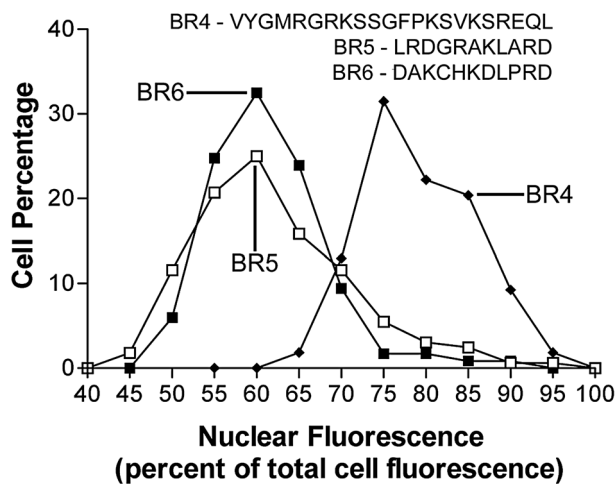
Figure 2: Nuclear import efficiency of the three different 5-LO NLSs. Quantitation of GFP subcellular distribution when fused to BR4, BR5 or BR6 from the 5-LO protein. Cells were transfected with plasmids encoding GFP with individual NLSs from the 5-LO protein attached and the subcellular fluorescence distribution was determined in expressing cells as described in Materials and Methods. Results are from one experiment and are representative of three independent experiments.

Cells expressing GFP/BR4 had stronger nuclear accumulation, with 75–85% of the total fluorescence localized to the nucleus (Figure 2). Immunoblot analysis of cell lysates indicated comparable levels of expression between different constructs (data not shown). These results indicated that two of the 5-LO NLSs, BR5 and BR6, were similarly weak import sequences, whereas the third was stronger. It should be noted that nuclear retention may play a role in determining the subcellular distribution of these constructs.

To evaluate the import strengths of the different NLSs statistically, the nuclear fluorescence values of all cells quantified for each construct were evaluated as a group (Figure 3). This indicated that nuclear fluorescence of GFP/SV40 NLS ($85.7 \pm 1.1\%$) was statistically greater than that of GFP/BR4 ($79.0 \pm 0.9\%$), GFP/BR5 ($62.4 \pm 0.72\%$) or GFP/BR6 ($63.6 \pm 0.64\%$). Similarly, the nuclear fluorescence of GFP/BR4 was statistically greater than that of either GFP/BR5 or GFP/BR6. The nuclear fluorescence of BR6 was not significantly different from that of BR5. More interestingly, the nuclear fluorescence of the BR4 + 5 construct, which contained the two NLSs in tandem, was greater than either alone and not significantly different from that of the SV40 NLS. These results demonstrated, quantitatively, that the tight cluster of the SV40 NLS is a stronger import sequence than any of the dispersed NLSs of 5-LO, but that multiple dispersed NLSs can match the import strength of a single tight cluster NLS.

Quantitative analysis of NLSs in situ

The import of the GFP protein with a single or dual NLS attached to it was relatively uniform across different cells,
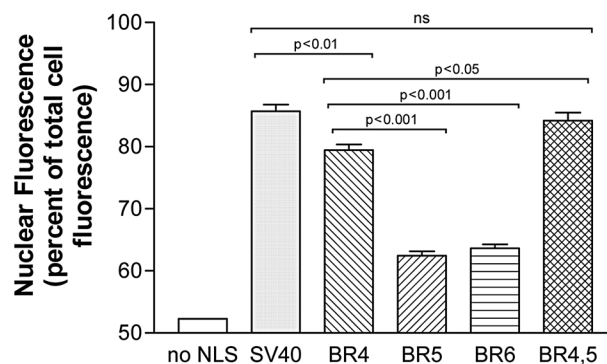

Figure 3: Statistical evaluation of the nuclear import efficiency of individual NLSs as well as the combined BR4+ 5 NLSs, when fused to GFP. Data are means ($\pm$ SE) of nuclear fluorescence values for all cells expressing GFP with the indicated NLSs attached. Results are from one experiment and are representative of three independent experiments. Standard deviations for no NLS, SV40, BR4, BR5, BR6 and BR4,5 were 4.40, 8.75, 6.55, 9.22, 6.94, and 10.73, respectively.

as indicated by the small standard errors for each construct presented in Figure 3. However, each of these NLSs was taken out of the context of the parent protein and thus was free from potential regulation. To test the import of 5-LO as directed by the weak BR6 NLS alone, import by BR4 and BR5 was prevented by site-directed replacement of their basic residues with glutamine. Visualization of positive transfectants revealed two distinct phenotypes, those without import and those with import (not shown). Quantitation of cells indicated a subpopulation with 25–35% nuclear fluorescence (Figure 4), which corresponded to the cells that appeared, visually, to lack
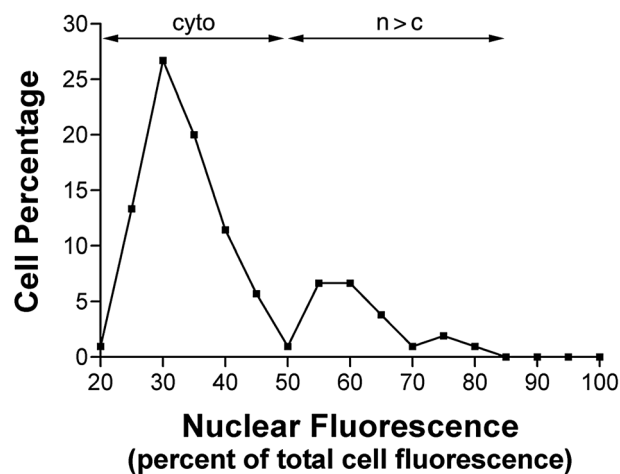

Figure 4: Nuclear import of GFP/5-LO with only BR6 intact. BR4 and BR5 of GFP/5-LO were inactivated by site-directed mutagenesis of selected basic residues. 3T3 cells were transfected with the single NLS construct and the subcellular fluorescence distribution was determined in 100 individual cells as described in Materials and Methods. The presented data are from one experiment and are representative of six independent experiments.

nuclear import. The second subpopulation had 55–65% nuclear fluorescence, a value similar to that observed when the peptide for BR6 was appended to GFP (Figure 2).

The import resulting from two NLSs, BR5 plus BR6, acting in the context of the 5-LO protein, was evaluated by site-directed inactivation of BR4. Surprisingly, a wide range of subcellular distributions of fluorescence, in cells expressing GFP/5-LO with active BR5 and BR6, were evident. These included cells with no import, balanced nuclear and cyto-plasmic fluorescence reminiscent of GFP alone, and weak and strong nuclear accumulation. Quantitative analysis indi-cated the presence of discrete subpopulations, rather than a gradient of variable import (Figure 5). Similar subpopulations were evident in multiple different experiments and ana-lyses, with the population at 60–65% nuclear fluorescence typically conspicuous (not shown). Also, in a separate study, site-directed mutagenesis of either BR5 (leaving BR4 plus BR6 intact) or BR6 (BR4 plus BR5 intact) produced a mixed population of cells, with some characterized by no 5-LO import, weak 5-LO import and others with strong nuclear 5-LO accumulation (18).

When all three NLSs were functional on 5-LO, as evalu-ated in WT 5-LO fused to GFP, import was again heterogeneous across cells, with selected fields pre-sented in Figure 6. Various cells in any given population of transfected cells showed no nuclear fluorescence (Figure 6A), nuclear fluorescence equal to cytosolic (Fig-ure 6B), nuclear fluorescence somewhat (Figure 6C) or much greater than cytosolic (Figure 6B), and all nuclear fluorescence (Figures 6A,C). Quantitation again indi-cated that there were distinct subpopulations that dif-fered in abundance (Figure 6D). Typical ratios of the five
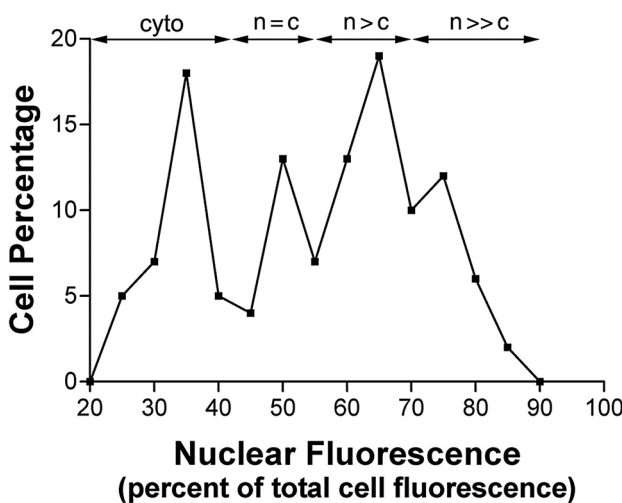

Figure 5: Nuclear import of GFP/5-LO with two NLSs, BR5 and BR6, intact. BR4 of GFP/5-LO was inactivated by site-directed mutagenesis, cells were transfected, and the subcellular fluorescence distribution was determined in 100 individual cells. The presented data are from one experiment and are representative of five independent experiments.

subpopulations (cyto:[n = c]:[n > c]:[n » c]:nuc) were 2 : 10 : 45 : 35 : 8. These ratios could be altered by chan-ging the culture conditions (e.g. in confluent cultures and after serum depletion, data not shown). Immuno-blot analysis of cell lysates indicated comparable levels of expression between different constructs (data not shown). It is possible that the higher level of nuclear fluorescence observed in some cells is related to an increase in nuclear volume, which doubles during the cell cycle. However, we have imaged transfected cells with live fluorescent microscopy before, during and after mitosis, as nuclear volume rapidly decreases. In these cells, the nuclear : cytoplasmic fluorescence ratio after reestablishment of the 5-LO gradient, within 1 h after mitosis, was remarkably similar to that before mitosis (data not shown). Thus, the localization of the GFP/5-LO fusion protein was not uniform across cells, but could reach the extremes of completely cytosolic or completely nuclear within a given population of cells.

Localization of 5-LO in macrophages in vivo

The heterogeneous import of 5-LO in 3T3 cells, which do not normally express 5-LO, brings into question the rele-vance of these results to in vivo 5-LO import. To address this, 5-LO subcellular distribution was evaluated by immu-nohistochemical staining of macrophages, which normally express 5-LO, in lung sections from untreated rats. Macro-phages are the predominant leukocyte in the alveolar space of normal lungs and, as a result, should be the primary cell type that stains positive for 5-LO. Positive brown staining for 5-LO was evident in numerous cells from normal rat lung (Figure 7A). However, in some of these cells, the blue nuclear counterstain clearly marked the nucleus as negative for 5-LO (Figure 7B), indicating a lack of nuclear import. In other cells, the brown staining of the nucleus was comparable to that of cytoplasm (Figure 7C), while in others the nucleus appeared more darkly brown than the cytoplasm (Figure 7D). Thus, as observed in 3T3 cells, the subcellular distribution of 5-LO was cell-specific in macrophages from normal rat lung. It should be noted that lung macrophages are recognized to be hetero-geneous: they may differ by location (i.e. interstitial vs. alveolar), age and prior exposure to stimuli. These differ-ences, as well as differences in microenvironments, may affect 5-LO import (22).

Our results show, for the first time, that the presence of multiple NLSs on the enzyme 5-LO allow multiple levels of import. We have demonstrated previously that different levels of import of 5-LO lead to different amounts of leukotriene synthesis by activated cells (23). This indicates that the pre-sence of multiple NLSs can allow more precise control of protein import, and thus protein function, than a single NLS. This type of mechanism may occur in other proteins to achieve graded import and action. For example, multiple NLSs on transcriptional regulating proteins may allow fine regulation of nuclear import and gene transcription.
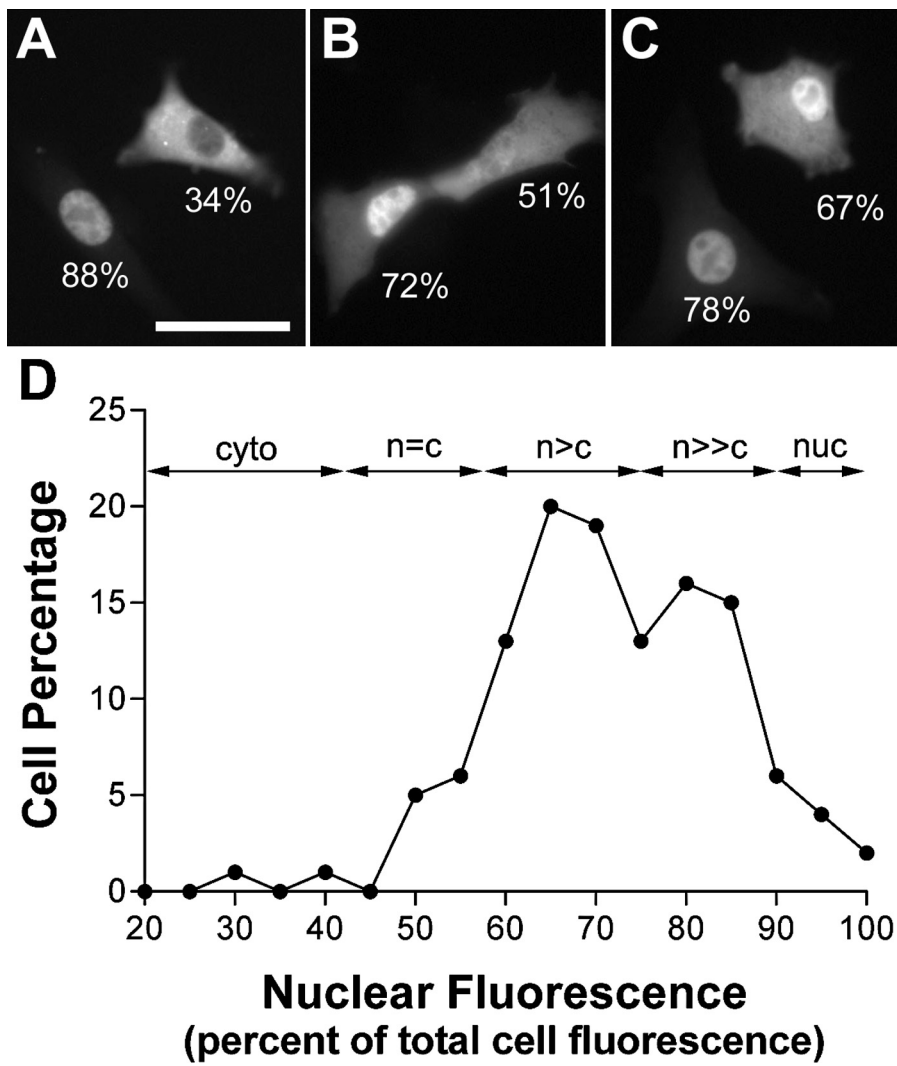
Figure 6: Localization of WT GFP/5-LO in transfected 3T3 cells. A–C) Visualization of different GFP/5-LO subcellular distributions in individual cells from a single transfection experiment. Bar indicates 40 μm. D) Quantitation of nuclear fluorescence in 100 individual cells from a population of 3T3 cells transfected with GFP/5-LO. The presented data are from one experiment and are representative of seven independent experiments.
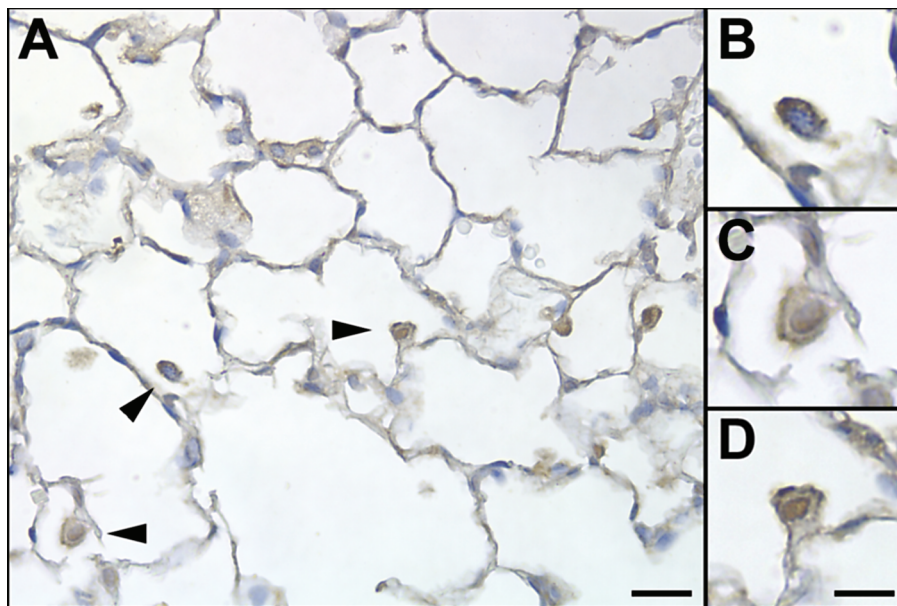

Figure 7: Localization of 5-LO in alveolar macrophages *in situ*. Sections of normal rat lung were stained for 5-LO (brown) and counterstained with hematoxylin (blue). Alveolar macrophages from a representative lower power field (A) are selected to indicate cells with cytoplasmic (B), nuclear approximates cytoplasmic (C) and nuclear greater than cytoplasmic (D) localization of 5-LO. Bars indicate 40 μm in (A) and 20 μm in (B–D).

An important feature of the three NLSs of 5-LO is their relative weak import efficiency. If all three NLSs were as strong as the SV40 NLS, then activation of a single NLS would lead to strong nuclear accumulation and activation of a second or third NLS would only enhance import a little. Taken another way, one strong regulated NLS allows two states (on, off), whereas three weak regulated NLSs allows multiple grades of import through multiple on/off combinations.

The strength of the import sequence may be determined by its primary structure. The classical monopartite and bipartite NLSs have a tight cluster of basic residues, whereas the three NLSs of 5-LO consist of dispersed residues. Presuming that the 5-LO NLSs are bound by importin isoforms to mediate import, it is possible that the arrangement of basic residues may affect the strength of this protein–protein interaction, as has been suggested (24). Alternatively, the residues between the basic amino acids may be important in reducing importin binding and import strength.

Of course, this model, by focusing solely on NLSs, over-simplifies the complex process of regulating the subcellular localization of proteins. Nuclear export may be similarly complex and important in determining the degree of nuclear localization of a protein. Multiple phosphorylation sites might regulate one NLS and achieve the same effects as multiple NLSs. Cytoplasmic and nuclear retention proteins may also be modulated in complex ways to achieve different levels of import or export. It seems likely that the regulation of 5-LO is similarly complex. In addition to multiple NLSs, 5-LO can be phosphorylated by multiple pathways (25–27), has modulated nuclear export (28), and can bind several proteins (29). The roles of these additional events in controlling the subcellular distribution of 5-LO and affecting leukotriene synthesis remain to be determined.

Materials and Methods

Cell culture, transfection
NIH 3T3 cells were obtained from American Type Culture Collection (Manassas, VA) and grown under 5% CO_2 in Dulbecco's Modified Eagle's Medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% calf serum, 100 units/mL penicillin, and 100 units/mL strepto-mycin. Cells were transfected using Polyfect (Qiagen, Inc., Valencia, CA) transfection reagent according to the manu-facturer's specifications. Transient transfectants were evaluated microscopically after fixation with 4% parafor-maldehyde, 20 h after transfection.

Animals
F1 male F-344xBN rats at 6 months of age were obtained from the National Institute on Aging (Bethesda, MD). The rats were housed individually in specific pathogen-free conditions for 2 weeks before experimentation. All proced-ures were performed in accordance with the Guide for the Care and Use of Laboratory Animals as approved by the Council of the American Physiological Society and the University of Michigan Committee on Use and Care of Animals.

Plasmids and constructs
The vector encoding enhanced GFP (pEGFP-C1, CLON-TECH, Palo Alto, CA) was used for all constructs. To con-struct GFP/SV40-NLS, oligonucleotides encoding the SV40 NLS (PKKKRK) (30) were subcloned into the EcoRI and SalI sites of the GFP vector. To construct a fusion peptide joining BR4 to GFP, complementary oligonucleotides encoding Val_{514}-Leu_{535} (VYGMRGRKSSGFPKSVKSREQL) were annealed and ligated to the BamHI and HindIII sites of pEGFP-C1. To construct a fusion peptide joining BR5 or BR6 to GFP, complementary oligonucleotides encoding the basic regions (indicated below) were annealed and ligated to the BamHI and HindIII sites of pEGFP-C1. BR5 peptide was Leu^{111}-Asp^{121} (LRDGRAKLARD). BR6 peptide was Asp^{156}-Asp^{166} (DAKCHKDLPRD). Specific amino acids within BR4 or BR5 were substituted in the pEGFP/ 5-LO template, using the QuikChange site-directed muta-genesis kit (Stratagene, La Jolla, CA). Briefly, two comple-mentary 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 poly-merase 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 methyl-ated template was removed by incubation with 10 units of Dpn I at 37 °C for 1 h. The mutation at BR4 was R518Q/ R520Q/K521Q/K527Q/K530Q. The mutation at BR5 was R115Q/K117Q/R120Q. All constructs were verified by DNA sequence analysis (DNA Sequencing Core, University of Michigan, Ann Arbor, MI). Oligonucleotides were synthesized by Integrated DNA Technologies Inc. (Coral-ville, IA) or Invitrogen Life Technologies, the sequences of which are available upon request.

Imaging and quantitation of subcellular distribution
Cells were fixed 20 h after transfection and random fields were imaged using a Nikon E600 microscope equipped for epifluorescence and digital image capture using a SPOT RT camera. In parallel experiments, cells were imaged by confocal microscopy using a Bio-Rad MRC-600 laser confocal microscope. For quantitation, grayscale digital images were adjusted to include the full black-to-white range, and mean densitometric gray values across linear fields were obtained for the cytoplasm and nucleoplasm of 100 individual cells, using Scion Image software (Scion Corp., Frederick, MD). Cytoplasmic and nuclear values for each cell were summed to define total cellular fluores-cence and the percent fluorescence values of this total fluorescence were calculated for the nuclear compart-ment. For graphical display, values were pooled in intervals

of $5 \pm 2.5\%$ for smoothing. For statistical analysis, all values from a given transfection were pooled. 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.

Immunohistochemical staining

Rat lungs were removed, inflated and fixed *en bloc* with 4% paraformaldehyde overnight at 4 °C and processed for paraffin sections. Tissue sections were dewaxed in Ameri-clear and rehydrated through decreasing concentrations of ethanol. All materials were then quenched of endogenous peroxidase activity by treatment with 0.3% hydrogen per-oxide for 30 min, washed, and blocked with Powerblock (InnoGenex, San Ramon, CA). Rabbit polyclonal antibody raised against human 5-LO was a generous gift from Dr. J. Evans (Merck Research Laboratories, Rahway, NJ) (31). Antibodies were prepared in phosphte-buffered saline (PBS) containing 0.1% bovine serum albumin (titer 1 : 750) and applied overnight at 4 °C. After washing with 0.1% bovine serum albumin in PBS, slides were probed with secondary antibody (biotinylated goat anti-rabbit, 1 : 250) for 30 min at 37 °C, washed again, then treated with avidin-biotinylated peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA) for 30 min at room temperature. 3,3′-Diaminobenzidine (DAB) was used as peroxidase substrate; preparations were counter-stained with Harris′ hematoxylin.

Acknowledgments

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