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Nuclear Localization of Liver X Receptor α and β is Differentially Regulated

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Abstract Activity of nuclear receptors is regulated by their nuclear localization. Liver X receptors (LXR) α and β are nuclear receptors that regulate transcription of genes for cholesterol metabolism, cholesterol transport, and lipogenesis. While LXR α and β are very similar in structure and exhibit similar ligand binding properties, their physiological roles are quite different. Since the LXRs fall into a class of receptors that move between the nucleus and cytoplasm, experiments were conducted to determine whether LXR α and LXR β show differences in their nuclear localization pattern. To determine the location of each receptor, cell lines stably expressing yellow fluorescent protein (YFP) chimeras with either LXR α or LXR β were examined. Retention in the nucleus of the chimeric proteins in the presence or absence of ligands was assessed using fluorescence microscopy coupled with digitonin permeabilization assays. Surprisingly, differences were found between LXR α and LXR β. Whereas unliganded LXR α was retained in the nucleus, unliganded LXR β was partially exported. Mutations were then introduced into putative nuclear localization sequences (NLS) to determine which sequences are important for nuclear localization and function. Mutation in one such sequence abolished nuclear localization of LXR α, whereas the analogous change in LXR β had a much less dramatic effect. Mutations in analogous putative NLS also differentially affected transcriptional activation by LXR α and LXR β. These data demonstrate for the first time that nuclear retention and localization as well as function of LXR α and LXR β are differentially regulated. J. Cell. Biochem. 100: 69–85, 2007. © 2006 Wiley-Liss, Inc.

Key words: nuclear localization; nuclear retention; liver X receptor; retinoid X receptor

Liver X receptors (LXR) α and LXR β are members of the superfamily of ligand-induced transcription factors. Both LXR α and LXR β regulate expression of gene products for transport, de novo synthesis, and catabolism of cholesterol [Peet et al., 1998b; Alberti et al., 2001; Stulnig et al., 2002], and for lipogenesis [Laffitte et al., 2003]. LXR α and LXR β belong to the type II family of nuclear receptors. Receptors of this family require heterodimerization with retinoid X receptor (RXR) for high affinity binding to DNA. LXR α and LXR β share 78% of their amino acid sequences and mostly bind similar ligands with similar affinities. Natural ligands for both LXR α and LXR β are oxysterols including 22-(R) hydroxycholesterol [Janowski et al., 1996; Peet et al., 1998a]. Whereas these ligands as well as the RXR ligand 9-*cis* retinoic acid activate transcription [Willy et al., 1995], geranyl geranyl pyrophosphate is an antagonist that inhibits transcription. This antagonistic effect is partially due to inhibition of co-activator binding to LXR [Gan et al., 2001] and/or inhibition of binding of LXR-RXR heterodimers to DNA [Forman et al., 1997]. Knockout studies in mice showed that LXR α plays a greater role than LXR β in sensing cholesterol levels and for lipogenesis.

For pharmacological purposes the effect of LXR on cholesterol metabolism and transport would be desirable whereas the effect of LXR on lipogenesis would be undesirable. Data show that such an effect could be achieved by selective activation of LXR β. Whereas knockout of LXR α in mice inhibited expression of lipogenic genes encoding steroyl coenzyme A desaturase 1 and fatty acid synthase, knockout of LXR β in mice did not affect expression of these genes [Alberti et al., 2001]. Instead, knockout of LXR β increased expression of another lipogenic gene,

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encoding acetyl CoA carboxylase [Alberti et al., 2001] and of the key regulator of lipogenic genes, sterol regulatory element binding protein-1 (SREBP-1) [Schuster et al., 2002]. Such increases in gene expression indicate a repressive effect of LXR β on these genes. Data also indicate that lipogenesis is regulated primarily by LXR α and that LXR β might even have an inhibitory effect on lipogenesis. Selective tissue expression of LXR α and LXR β and/or selective activation by ligands can not fully explain such differences in function. Since the control of subcellular localization often regulates the activity of these receptors, selective regulation of nucleo-cytoplasmic trafficking might contribute to selective activation of gene expression by LXR α and LXR β.

Nuclear receptors must be present in the nucleus to regulate the transcription of genes. The intranuclear concentration of nuclear proteins is maintained by a balance between nuclear import, nuclear export, and nuclear retention. Regulation of this balance provides important regulatory mechanisms for transcription. Unliganded androgen receptor (AR) [Tyagi et al., 2000], glucocorticoid receptor (GR) [Picard and Yamamoto, 1987], and vitamin D receptor (VDR) [Barsony et al., 1997] localize at least partially in the cytoplasm but translocate into the nucleus after ligand binding. In contrast, progesterone receptor [Guiochon-Mantel et al., 1991], thyroid hormone receptor (TR) [Bunn et al., 2001], estrogen receptor (ER) [Htun et al., 1999], RXR [Prufer et al., 2000], LXR α [Watanabe et al., 2003], and LXR β [Mo et al., 2002] are primarily located in the nuclei with or without bound ligand. However, nucleo-cytoplasmic trafficking has been shown for both types of nuclear receptors [Dauvois et al., 1993; Guiochon-Mantel et al., 1994; Hache et al., 1999; Baumann et al., 2001; Bunn et al., 2001; Prufer and Barsony, 2002]. We and others showed recently that nuclear receptors such as the GR [Hache et al., 1999], the progesterone receptor (PR) [Guiochon-Mantel et al., 1994], TR [Baumann et al., 2001; Bunn et al., 2001], ER [Dauvois et al., 1993], VDR [Prufer and Barsony, 2002], and RXR [Prufer and Barsony, 2002] are continuously imported into and exported out of the nucleus.

Most nucleo-cytoplasmic transport pathways are mediated by members of the large evolutionary conserved karyopherin family. Export from the nucleus occurs through receptor-mediated mechanisms. Studies using heterokaryon and imaging experiments showed that exported nuclear receptors are reimported into the nucleus [Guiochon-Mantel et al., 1991; Hache et al., 1999; Prufer and Barsony, 2002]. It is well established that nuclear receptors are imported into the nucleus by karyopherins. Binding to karyopherin β and adapter protein karyopherin α requires basic amino acids in the cargo protein [Conti et al., 1998]; and regions rich in basic amino acids arginine and lysine have been identified as nuclear localization sequences (NLS) in many nuclear proteins. These NLSs bind to karyopherin α or directly to karyopherin β which carries the cargo protein into the nucleus [Powers and Forbes, 1994; Kohler et al., 1999].

Individual NLSs have been identified in many nuclear receptors studied thus far. The typical nuclear receptor consists of a DNA binding domain (DBD), a hinge region, and a multifunctional ligand binding domain (LBD). Binding sites for nuclear import receptors are present in all three domains. An NLS has been identified by site-directed mutagenesis and live cell imaging of GFP-tagged receptors in the DNA binding domain of VDR and RXR. This NLS (NLS1) is in a conserved region between the two Zinc fingers [Hsieh et al., 1998; Prufer and Barsony, 2002]. One amino acid within NLS1 is conserved among all type II nuclear receptors as either arginine or lysine. Basic amino acids in the second Zinc finger in the DBD of orphan nuclear receptor TR2 [Yu et al., 1998] and the PR [Guiochon-Mantel et al., 1991; Ylikomi et al., 1992] have also been shown to act as NLS (NLS2). Analogous amino acids in the second Zinc finger exist in other nuclear receptors such as RXR, LXR α, and LXR β. Another NLS (NLS3) has been identified in the hinge region of many nuclear receptors including PR [Guiochon-Mantel et al., 1991], ER [Ylikomi et al., 1992], GR [Picard and Yamamoto, 1987], AR [Zhou et al., 1994], the steroid and xenobiotic receptor (SXR) [Kawana et al., 2003], the mineralocorticoid receptor (MR) [Pearce et al., 2002], and the VDR [Luo et al., 1994]. Analogous regions exist in other nuclear receptors such as LXR α and LXR β, but not in RXR. Finally, NLS have been identified in the LBD of GR [Picard and Yamamoto, 1987], PR, and ER [Ylikomi et al., 1992]. Conserved amino acid sequences among members of the nuclear receptor family usually interact with similar

proteins. Such interactions result in common mechanisms of DNA and cofactor binding as well as receptor-mediated nuclear transport.

The selectivity in regulation of nuclear retention and localization may contribute to selectivity in function of LXR α and LXR β. The goal of this article was to identify whether there are differences in nuclear retention of LXR α and LXR β and to identify amino acids in LXR α and LXR β that are important for nuclear localization. We show for the first time that both nuclear retention and nuclear localization of LXR α and LXR β are differentially regulated.

MATERIALS AND METHODS

Cells and Expression Constructs

Transcriptionally active YFP-RXR (RXR α) was generated as described earlier [Prufer et al., 2000]. We received LXR α and LXR β in the pDNR vector from the Harvard Institute of Proteomics. Donor vectors allow cloning into desired expression plasmids using the Creator system (BD Biosciences Clontech, Palo Alto, CA). We cloned both LXR α and LXR β into CMV driven mammalian expression vectors (BD Biosciences Clontech) with an N-terminal Yellow Fluorescent Protein (YFP) or Cyan Fluorescent Protein (CFP) fusion (YFP-LXR α, YFP-LXR β, CFP-LXR β). These reactions were performed using the Cre-recombinase reaction according to the manufacturer's instructions (BD Biosciences Clontech). Functionality of expression constructs was confirmed using transactivation assays as described below.

YFP, YFP-LXR α, and YFP-LXR β were stably expressed in HEK293 cells (293YFP, 293LXRa, and 293LXRb cells, respectively) and selected using geneticin as described previously [Prufer et al., 2000]. Expression of YFP-LXR α and YFP-LXR β at the expected molecular weight in each three clones was confirmed using Western blot analysis (not shown). Cells were plated in six-well plates and after 24 h lysed in SDS sample buffer with 5% mercaptoethanol. Extracts were separated on an 8% SDS gel and transferred to a membrane. After blocking unspecific binding using 5% milk in PBS-T, membranes were incubated over night at 4°C in anti LXR antibody (1:1,000, H144, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After several washing steps, membranes were incubated in secondary anti rabbit antibody (1:7,000, Santa Cruz Biotechnology, Inc.) for 30 min at RT. Membranes were washed again and bands were visualized using ECL (Pierce, Rockford, IL).

HEK293, and F9 cells were obtained from ATCC (Manassas, VA) and grown as described previously [Racz and Barsony, 1999; Prufer et al., 2000]. Briefly, cell cultures were maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Hyclone, Logan, UT), 2 mM glutamine (Invitrogen), and 0.1 mg/ml gentamicin (Invitrogen). F9RXR−/− cells lacking RXR [Rochette-Egly and Chambon, 2001] were a gift from Dr. P. Chambon (Institute of Genetic and Cellular and Molecular Biology, CNRS/INSERM/ULP/College of France, Illkirch).

Permeabilization Experiments

Nuclear export assays were performed as described previously [Prufer and Barsony, 2002]. 293LXRa and 293LXRb cells were plated onto chamber slides coated with gelatin. Before permeabilization, cells were treated with either vehicle (0.2% DMSO), agonist (0.1 μM 9-*cis* retinoic acid combined with 10 μM 22(R) hydroxycholesterol, Sigma, St. Louis, MO), or antagonist (1 μM geranyl geranyl pyrophosphate, Sigma) for 1 h at 37°C. These treatments were present both during permeabilization, during washing steps, and for incubation for 1 h at 37°C after permeabilization. Transport buffer (TB) contained both protease inhibitors and cycloheximide. Reticulocyte lysate addition provided all necessary components for nuclear transport. After fixation, slides were evaluated using a confocal scanning microscope (Leica) as described below. Brightness intensities were measured using IPLab software (Scanalytics, Inc., Fairfax, VA) in approximately 500–1,000 cells for each treatment. Ratio of intensities of permeabilized versus unpermeabilized cells was calculated based on brightness/cell. All experiments were repeated three times using each two different clones of 293LXRa and 293 LXRb cells. Values are averages of all six experiments. Average from data points is presented as the mean ± 1 SD. *P*-values were determined using Student's *t*-test.

Mutational Analysis of Putative Nuclear Localization Sequences

Point mutations were introduced into the coding sequences of cDNAs encoding YFP-RXR, YFP-LXR α, and CFP-LXR β using the QuikChange mutagenesis kit (Stratagene, La

Jolla, CA). Up- and downstream oligonucleotides were designed according to the manufacturer's instructions. Point mutations in the NLS1-2 of the YFP-RXR (*K165G*YFP-RXR, *K165E*YFP-RXR, and *R182E/R184E*YFP-RXR) were introduced at amino acid 165 (K165G, lysine to glycine, and K165E, lysine to glutamic acid), and amino acids 182 and 184 (R182E and R184E, arginines to glutamic acids). Point mutations in the putative NLS1, 2, 3, and 4 of the YFP-LXR α (*nls1G*YFP-LXR α, *nls1E*YFP-LXR α, *nls2*YFP-LXR α, *nls3*YFP-LXR α, *nls4*YFP-LXR α) were introduced at amino acid 128 (K128G, lysine to glycine, K128E, lysine to glutamic acid), at amino acids 147 and 148 (R147E and R148E, arginines to glutamic acid), at amino acids 175–181 (R175E, K177E, K178E, K180E, R181E, arginines and lysines to glutamic acid), and at amino acids 83–86 (K83E, R84E, K85E, K86E, arginines, and lysines to glutamic acid). Single, double, and triple point mutations in the putative NLS1, 2, 3, and 4 of the CFP-LXR β (*nls1G*CFP-LXR α, *nls1E*CFP-LXR α, *nls2*CFP-LXR β, *nls3*CFP-LXR β, and *nls4*CFP-LXR β) were introduced at amino acid 117 (R117G, arginine to glycine, R117E, arginine to glutamic acid), amino acids 138 and 139 (R138E and R139E, arginines to glutamic acids), amino acids 166–172 (R166E, K167E, K168E, K169E, R171E, K172E, arginines/lysines to glutamic acids), amino acids 147–150 as a potential part of a possible bipartite NLS3 (R147E, R149E, and K150E, arginines/lysines to glutamic acids) and amino acids 72–75 (K72E, R73E, K74E, K75E, arginines/lysines to glutamic acids), respectively.

In all constructs, including the mutants, the coding sequences for the fusion proteins were confirmed using the ABIPrism or BigDye terminator sequencing kits (Perkin Elmer, Norwalk, CT).

Microscopy

HEK293, F9, or F9RXR−/− cells were plated on chambered cover slips (Nalge Nunc Int., Naperville, IL) and transfected with either wild-type or mutant YFP-RXR, YFP-LXR α, and CFP-LXR β (0.25 μg/slide for HEK293 cells, 2 μg/slide for F9 and F9RXR−/− cells) using Lipofectamine 2000 according to the manufacturers instructions (Invitrogen). After transient transfection, cells were used for microscopy within 48 h. Cells were treated with 9-*cis* retinoic acid (Sigma) as indicated or with vehicle (0.1% ethanol). For imaging experiments, 293LXRa, 293LXRb, and cells expressing wild-type or mutant YFP-LXR α and CFP-LXR β were cultured for 18 h in media containing 5% FBS without lipoproteins (Intracel, Frederick, MD) prior to the experiment to deplete cells of the ligands for LXR. Then, cells were treated with vehicle (0.2% DMSO), antagonist (1 μM geranyl geranyl pyrophosphate), or a combination of 100 nM 9-*cis* retinoic acid (Sigma) and 10 μM 22(R)-hydroxycholesterol (Sigma). Images were collected from a Leica TCS SP2 confocal system (Leica Microsystems, Mannheim, Germany) using a 63 × 1.2 NA objective. The 458 nm line of a krypton-argon laser and a spectral detector of emission between 465 and 600 nm were used to detect CFP; the 488 nm line of a krypton-argon laser and a spectral detector of emission between 510 and 525 nm were used to detect YFP. Nuclear and cytoplasmic fluorescence intensities were measured using IPLab software (Scanalytics, Inc.) in each 50–100 cells expressing either YFP-LXR α, *nls3*YFP-LXR α, *nls4*YFP-LXR α, CFP-LXR β, *nls4*CFP-LXR β, or double mutants *nls1E/nls4*CFP-LXR β, *nls2/nls4*CFP-LXR β, and *nls3/nls4*CFP-LXR β. Cells were randomly chosen by imaging several areas of fluorescing cells. Data were collected from all cells within these areas. Average from 50 to 100 data points is presented as the mean ± 1 SD. *P*-values were determined using Student's *t*-test.

Transactivation Assays

HEK293 cells were subcultured into 96-well plates (Nalge Nunc Int.). After 24 h, cells were transfected using Lipofectamine 2000 reagents. Transcriptional activities of either wild-type or mutant YFP-RXR were tested by co-transfection experiments. Cells were transfected with expression plasmids (0.3 μg/8 wells) together with DR1-Luciferase reporter plasmid (0.4 μg/8 wells; gift from Dr. S. Minucci, EIO, Italy) and the Renilla-luciferase control plasmid (0.015 μg/8 wells; Promega, Madison, WI). Cells were treated with either vehicle (0.1% ethanol) or 100 nM 9-*cis* retinoic acid 24 h after transfection.

Transcriptional activities of either wild-type or mutant YFP-LXR α and CFP-LXR β were tested by co-transfection experiments. Cells were transfected with expression plasmids (0.25 μg/8 wells) together with LXRE luciferase

reporter (0.08 μg/8 wells; gift from Dr. Auwerx, CNRS/LGME-INSERM, Illkirch, France, [Brendel et al., 2002]) and the Renilla-luciferase control plasmid (0.015 μg/8 wells). Cells expressing wild-type or mutant YFP-LXR α and CFP-LXR β were cultured and transfected in media containing 5% FBS without lipoproteins (Intracel) prior to the experiment. Cells were treated with either vehicle (0.2% DMSO) or 0.1 μM 9-*cis* retinoic acid combined with 10 μM 22(R) hydroxycholesterol 24 h after transfection.

Twenty-four hours after treatment, cells were lysed on the culture plate using lysis buffer (Promega), after which the samples were frozen and thawed for better lysis. Luciferase activities were measured using Dual-Luciferase assay reagents (Promega) in a Victor II plate reader (Perkin-Elmer). Luminescence data were normalized to Renilla luciferase values and expressed as fold induction relative to vehicle-treated controls. Data are average from eight data points and are presented as the mean ± 1 SD.

RESULTS

LXR α and LXR β Are Nuclear With and Without Ligand

First, we evaluated the locations of LXR α and LXR β in the presence and absence of ligand. We used HEK293 cells stably expressing YFP-LXR α and YFP-LXR β (293LXRa and 293LXRb cells, respectively). Using confocal scanning microscopy we found that steady-state localization of these receptors is in the nucleus. Next, we tested the effect of ligands on intranuclear localization of LXR α and LXR β. We and others showed previously that fluorescent chimeras of nuclear receptors bound to ligand accumulate in nuclear foci [Htun et al., 1996, 1999; Lim et al., 1999; Prufer et al., 2000; Stenoien et al., 2000; Baumann et al., 2001; Prufer and Barsony, 2002]. We treated 293LXRa and 293LXRb cells with either vehicle (DMSO), agonist (a combination of 10 μM 22-(R) hydroxycholesterol and 100 nM 9-*cis* retinoic acid), or antagonist (1 μM geranyl geranyl pyrophosphate). Whereas treatment with vehicle (Fig. 1, left panel) or antagonist (Fig. 1, right panel) did not cause accumulation of either YFP-LXR α or YFP-LXR β in nuclear foci, treatment with agonist resulted in accumulation of both YFP-LXR α and YFP-LXR β in nuclear foci (Fig. 1, center panel). These data confirm that steady-state localization of both LXR α and LXR β is nuclear and that agonist but not antagonist treatment causes accumulation of both receptors in nuclear subcompartments.

Nuclear Retention of LXR α and LXR β

Next, we assessed if agonist and/or antagonist treatment affects nuclear retention of LXR α and LXR β. We used 293LXRa and 293LXRb

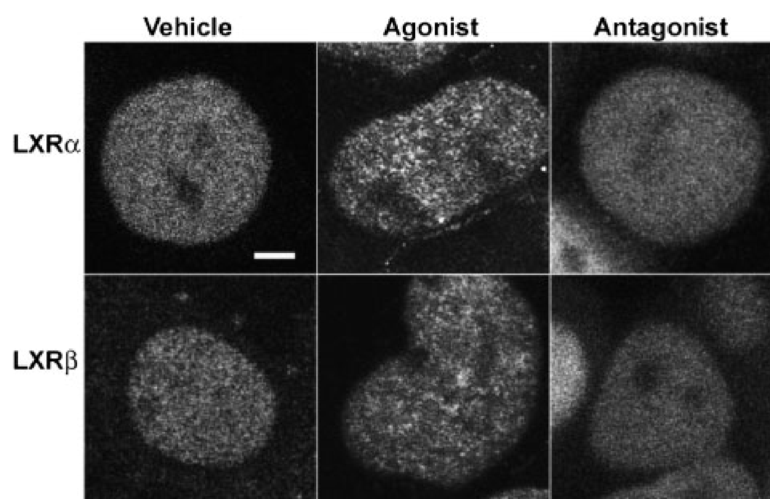

Fig. 1. Agonist-bound YFP-LXR α and YFP-LXR β accumulate in nuclear subcompartments (foci). 293LXRa and 293LXRb cells were subcultured for 24 h in sterile covered chamber glasses. Media supplemented with lipoprotein-free fetal bovine serum was used to deplete cells of natural ligands for LXR present in normal serum. Then, 293LXRa cells and 293LXRb cells were treated with DMSO (Vehicle, left panel), a combination of 10 μM 22-(R) hydroxycholesterol and 100 nM 9-*cis* retinoic acid (Agonist, center panel) or 1 μM geranyl geranyl pyrophosphate (Antagonist, right panels). Bar: 5 μm.

cells to perform permeabilization assays [Liu et al., 1999]. Cell membranes were selectively permeabilized using digitonin to determine how much receptor was retained in cell nuclei after 1 h. Due to the permeabilization of cell membranes, exported receptors are lost from the cell and are not reimported into the nucleus. Residual nuclear fluorescence intensities in permeabilized cells were then compared to nuclear fluorescence intensities in unpermeabilized cells. Fluorescence intensities of YFP-LXR α and YFP-LXR β indicated the portion of the receptors that was retained in the nucleus. Figure 2 shows a representative low magnification image of nuclear fluorescence in both unpermeabilized and permeabilized 293LXRa and 293LXRb cells. After treatment with agonist (a combination of 10 μM 22-(R) hydroxycholesterol and 100 nM 9-*cis* retinoic acid), YFP-LXR α fluorescence intensities differed between permeabilized and unpermeabilized 293LXRa cells, whereas YFP-LXR β fluorescence intensities were similar between permeabilized and unpermeabilized 293LXRb cells. Nuclear retention of YFP-LXR α and YFP-LXR β was evaluated in the presence or absence of ligands. Table I summarizes brightness values of permeabilized versus unpermeabilized 293LXRa and 293LXRb cells after incubation with either vehicle (DMSO) or agonist (a combination of 10 μM 22-(R) hydroxycholesterol and 100 nM 9-*cis* retinoic acid). Similar data are presented for 293LXRa and 293LXRb cells treated with antagonist (1 μM geranyl geranyl pyrophosphate). As a control, a similar permeabilization assay was performed with HEK 293 cells stably expressing YFP (293YFP cells). YFP diffuses freely between nucleus and cytoplasm, and the permeabilization assay showed that no fluorescence was detectable in

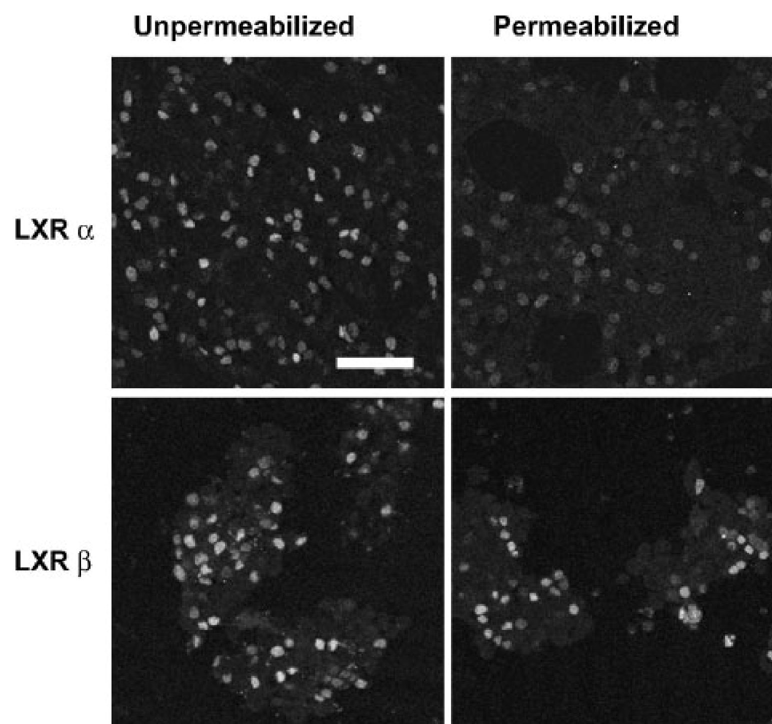

Fig. 2. Digitonin permeabilization assays show that agonist-bound LXR α is exported from the nucleus (upper panels) whereas agonist-bound LXR β is retained in the nucleus (lower panels). 293LXRa and 293LXRb cells were subcultured for 24 h in sterile covered chamber glasses coated with gelatin. Media supplemented with lipoprotein-free fetal bovine serum was used to deplete cells of natural ligands for LXR present in normal serum. Then, 293LXRa cells and 293LXRb cells were pretreated with a combination of 10 μM 22-(R) hydroxycholesterol and 100 nM 9-*cis* retinoic acid for 1 h, followed by treatment with either transport buffer (TB) with digitonin (permeabilized) or TB without digitonin (unpermeabilized) for 10 min. Cell membranes were permeabilized selectively by digitonin and export of receptors was determined in the presence of reticulocyte lysate. Reticulocyte lysate provides all necessary components for nuclear export. Exported receptors cannot be reimported into the nucleus since the cell membrane is not intact. After 1 h incubation in TB with a combination of 10 μM 22-(R) hydroxycholesterol and 100 nM 9-*cis* retinoic acid, cells were fixed in 4% formaldehyde in PBS. Residual fluorescence was determined using confocal laser scanning microscopy in nuclei of permeabilized and unpermeabilized cells. Bar: 100 μm.

TABLE I. Nuclear Retention of LXR α and LXR β

Treatment	LXR α	LXR β
Vehicle (DMSO)	98% ± 21%*	57% ± 27%
Agonist (10 μM 22-(R) hydroxycholesterol and 100 nM 9-*cis* retinoic acid)	65% ± 10%*,**	100% ± 29%**
Antagonist (1 μM geranyl geranyl pyrophosphate)	19% ± 19%**	32 ± 17%**

Permeabilization experiments were performed using 293LXRa and 293LXRb cells treated as described in the table. Data are presented as percent residual fluorescence in permeabilized cells (50 μg/ml digitonin) versus unpermeabilized cells 1 h after permeabilization. Fluorescence brightness was measured for each 3,000–6,000 cells selected by imaging randomly chosen areas of cells. A value of 100% means that all receptors were retained in the nucleus.
*Indicates that the LXR α value is significantly different from the LXR β value ($P < 0.05$).
**Indicates that the values for liganded receptors are significantly different from the values for unliganded receptors ($P < 0.05$).

the nuclei of these cells 1 h after digitonin permeabilization.

These data show that unliganded LXR α is retained in the nucleus, whereas agonist-bound LXR α is partially exported (Table I). In contrast, unliganded LXR β is partially exported from the nucleus, whereas agonist-bound LXR β is retained in the nucleus. After antagonist treatment, both LXR α and LXR β are exported from the nucleus.

Mutations in NLS1 and NLS2 Abolish Nuclear Localization of RXR

Exported receptors likely are reimported into the nucleus. Such import is mediated through binding of importins to NLS. NLS are characterized by the presence of basic amino acids. Amino acid changes in such sequences often abolish nuclear localization of nuclear receptors. Such NLS have not been identified in LXR α and LXR β. We compared NLS among nuclear receptors and found analogous sequences in the DBD of RXR, LXR α, and LXR β. We previously identified NLS1 in RXR [Prufer and Barsony, 2002] (Fig. 3, bold). Among type II nuclear receptors including LXR α and LXR β a basic amino acid is conserved within this NLS1 (Fig. 3, underlined). Among type I nuclear receptors such as GR, AR, and ER, a glycine is conserved at the same position. To determine if change of this conserved amino acid is sufficient to affect nuclear localization of RXR, we decided to introduce mutations changing the lysine into either glycine or glutamic acid (Fig. 3, underlined).

As shown previously [Prufer and Barsony, 2002], YFP-RXR is nuclear both in the presence and in the absence of its ligand, 100 nM 9-*cis* retinoic acid (Fig. 4A, YFP-RXR). We previously identified NLS1 in the DNA binding domain (DBD) of RXR between the two Zinc fingers (Fig. 3, bold, amino acids K160, R161, R164, K165) [Prufer and Barsony, 2002]. We found that mutation of only one amino acid, K165, into glutamic acid (E) in YFP-RXR resulted in predominantly cytoplasmic localization of mutant receptors in HEK293 cells (Fig. 4A), whereas mutation K165 into glycine (G) in YFP-RXR did not affect nuclear localization (Fig. 4A). This result shows that K165 is important for nuclear localization of RXR. A basic amino acid located at an analogous position in both LXR α and LXR β might also be important for nuclear localization.

Another region that is important for nuclear localization (NLS2) is located in the second Zinc finger in the DNA binding domain of nuclear receptors such as orphan nuclear receptor TR2 [Yu et al., 1998] and PR [Guiochon-Mantel et al., 1991; Ylikomi et al., 1992]. Analogous

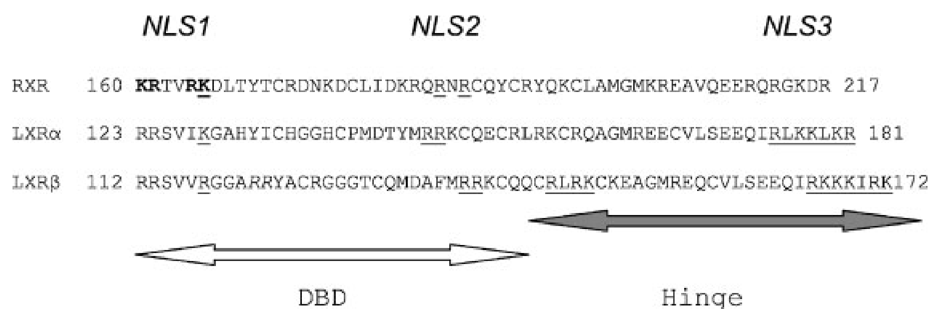

Fig. 3. Schematic representation of putative nuclear localization sequences (NLS1-3) in DNA binding domain (DBD) and hinge region of RXR, LXR α, and LXR β. The amino acids evaluated previously are in bold and the amino acids evaluated in this article are underlined.

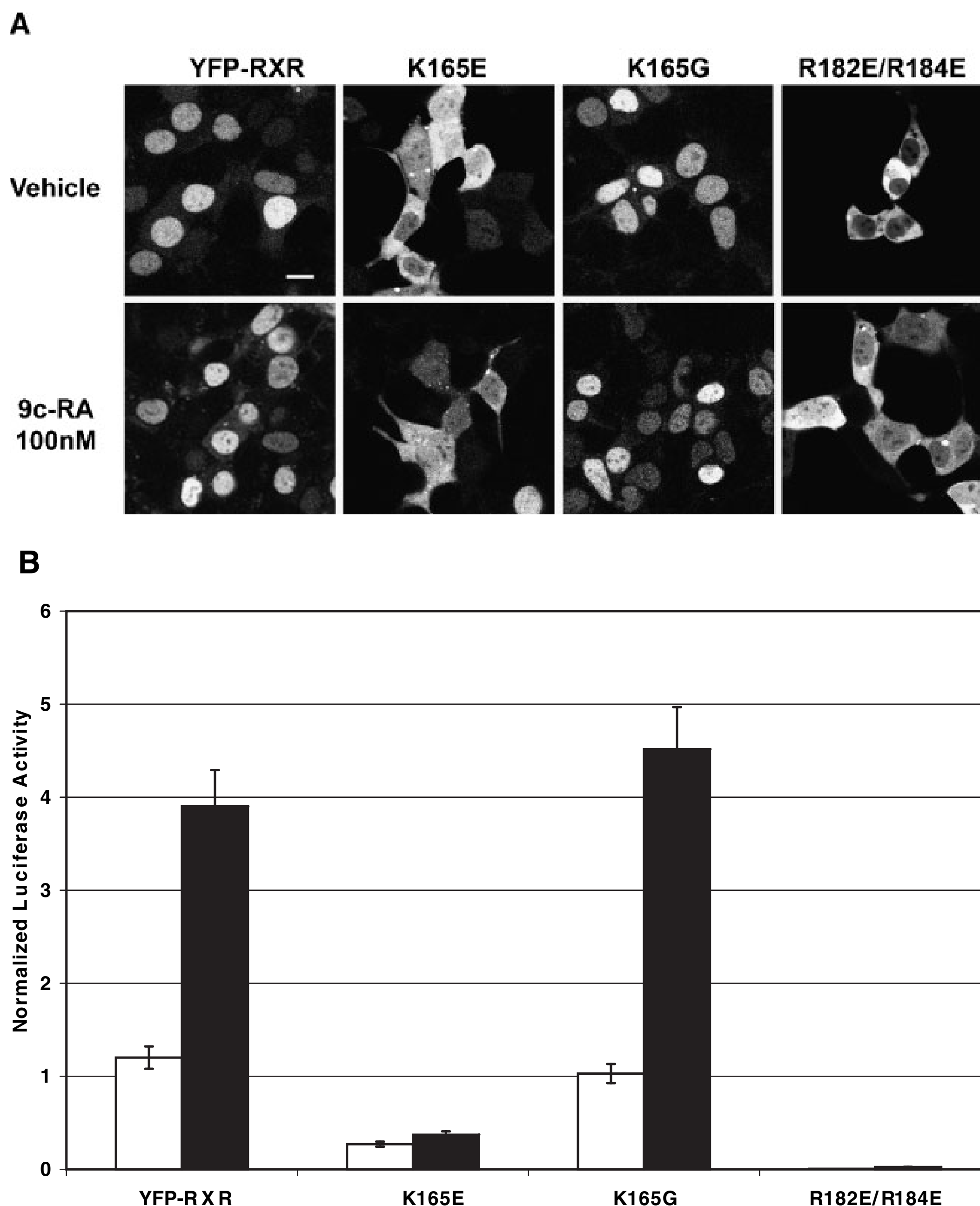

Fig. 4. Conserved arginines in the second Zinc finger serve as NLS2 in RXR. **A**: HEK293 cells were transiently transfected with YFP-RXR, *K165G*YFP-RXR, *K165E*YFP-RXR, and *R182E/R184E*YFP-RXR. Both in the absence (upper panel) and in the presence (lower panel) of 100 nM 9-*cis* retinoic acid (9c-RA), wild-type YFP-RXR and *K165G*YFP-RXR are predominantly nuclear, whereas *K165E*YFP-RXR and *R182E/R184E*YFP-RXR are predominantly cytoplasmic. Bar, 10 μm. **B**: HEK293 cells were transiently transfected with wild-type and mutant YFP-RXR, DR-1 luciferase reporter plasmids and luciferase control plasmids as described. Normalized luciferase activities from vehicle (ethanol) treated cells are shown as open bars, and from ligand (100 nM 9-*cis* retinoic acid) treated cells are shown as shaded bars. Luminescence data were normalized with Renilla luciferase activities, and data are expressed as the mean ± 1 SD.

sequences exist in the RXR as well as in LXR α and LXR β. To determine if this sequence in the RXR is also important for nuclear localization, we mutated R182 and R184 in NLS2 of YFP-RXR to glutamic acid (E) (Fig. 3, underlined). As shown in Figure 4A, mutation R182E/R184E in YFP-RXR resulted in predominantly cytoplasmic localization of mutant receptors.

We next tested the transcriptional activities of mutant YFP-RXRs. We found that mutation K165G did not significantly inhibit transcriptional activity, whereas mutations K165E

and R182E/R184E significantly inhibited transcriptional activity (Fig. 4B). Taken together, we revealed that amino acids in NLS1 and NLS2 in RXR are important for both nuclear localization and transcriptional activity.

Mutations in NLS3 Affect Nuclear Localization of LXR α but not of LXR β

In contrast to the situation found for RXR, where NLS1 and NLS2 play a key role for nuclear localization, mutations in both NLS1 and NLS2 of LXR α and LXR β did not affect nuclear localization. We compared location of wild-type and mutant YFP-LXR α and CFP-LXR β. Mutations were introduced into sequences coding for amino acids in the DBD of both LXR α and LXR β that are analogous to amino acids in NLS1 and NLS2 in RXR. Both wild-type YFP-LXR α and wild-type CFP-LXR β were nuclear in living HEK293 cells (Fig. 5A,B). First, we analyzed the importance for nuclear localization of amino acids K128 in LXR α and R117 in LXR β (Fig. 3, underlined). These amino acids are analogous to K165 in NLS1 of RXR. In contrast to K165 in RXR, mutation of K128 in YFP-LXR α and R117 in CFP-LXR β to either glutamic acid (E) (*nls1E*YFP-LXR α or *nls1E*CFP-LXR β, respectively) or glycine (G) (*nls1G*YFP-LXR α or *nls1G*CFP-LXR β, respectively) did not abolish nuclear localization (Fig. 5A,B). We then analyzed the importance for nuclear localization of amino acids R147/R148 in LXR α and R138/R139 in LXR β. These amino acids are analogous to R182/R184 in NLS2 of RXR (Fig. 3, underlined). In contrast to analogous amino acids in RXR, mutation of R147E/R148E in YFP-LXR α (*nls2*YFP-LXR α) and R138E/R139E in CFP-LXR β (*nls2*CFP-LXR β) did not abolish nuclear localization (Fig. 5A,B).

Another nuclear localization sequence (NLS3) has been identified in the hinge region of nuclear receptors including the GR [Picard and Yamamoto, 1987], the AR [Zhou et al., 1994], the SXR [Kawana et al., 2003], and the VDR [Luo et al., 1994]. Analogous regions exist in both LXR α and LXR β (Fig. 3, underlined). LXR α possesses a putative NLS3 at amino acids 175-181 (R175, K177, K178, K180, R181), while LXR β possesses a putative NLS3 at amino acids 166–172 (R166, K167, K168, K169, K170, R171, and K172). We mutated R175, K177, K178, K180, R181 in YFP-LXR α and R166, K167, K168, K169, R171, and K172 in CFP-LXR β into glutamic acids (E) (*nls3*YFP-LXR α and *nls3*CFP-LXR β, respectively), and expressed wild-type and mutant receptors in HEK293 cells. Surprisingly, we found that mutations of these amino acids in LXR α affected nuclear localization, whereas mutations of these amino acids in LXR β did not affect nuclear localization. As shown in Figure 5A,B, respectively, mutation of NLS3 caused a partitioning of YFP-LXR α between nucleus and cytoplasm whereas mutation of NLS3 did not affect nuclear localization of CFP-LXR β. We measured fluorescence intensities of *nls3*YFP-LXR α in cytoplasm and nucleus, and found that one quarter of the fluorescence was in the cytoplasm and three quarters were in the nucleus. Treatment of cells expressing *nls3*YFP-LXR α with its ligands, a combination of 100 nM 9-*cis* retinoic acid and 10 μM 22 (R) hydroxycholesterol, did not affect this ratio (Table II).

Next, we explored the possibility that NLS1, 2, and 3 act together for nuclear localization of LXR β. We determined whether mutation of both NLS1 and NLS2 abolishes nuclear localization of CFP-LXR β. Steady-state localization of the NLS1/NLS2 double-mutant (R117E, R138E/R139E) CFP-LXR β was nuclear (not shown). We then mutated NLS3 in addition to NLS1 and NLS2. As shown in Figure 5B, the steady-state localization of NLS1/NLS2/NLS3 triple mutant (R117E, R138E, R139E, R166E, K167E, K168E, K169E, K170E, R171E, and K172E) CFP-LXR β was also nuclear. Mutation of amino acids possibly contributing to a bipartite NLS3 (Fig. 3, underlined, R147, R149, and K150 to glutamic acid) in addition to mutations NLS1, 2, and 3 also did not abolish nuclear localization of CFP-LXR β (not shown).

We found previously that a mutated VDR with amino acid changes in NLS1 can be transported into the nucleus via dimerization with RXR [Prufer et al., 2000]. To test whether endogenous RXR is contributing to nuclear localization of LXR, we expressed wild-type and all mutant LXRs (*nls1E*YFP-LXR α, *nls2*YFP-LXR α, *nls3*YFP-LXR α, *nls1E*CFP-LXR β, *nls2*CFP-LXR β, *nls3*CFP-LXR β, as well as double and triple mutants) in wild-type F9 cells and in F9 RXR−/− cells. F9 RXR−/− cells are deficient of RXR [Rochette-Egly and Chambon, 2001] and if RXR plays a role in nuclear localization of LXR we expect a more cytoplasmic localization of wild-type and/or mutant

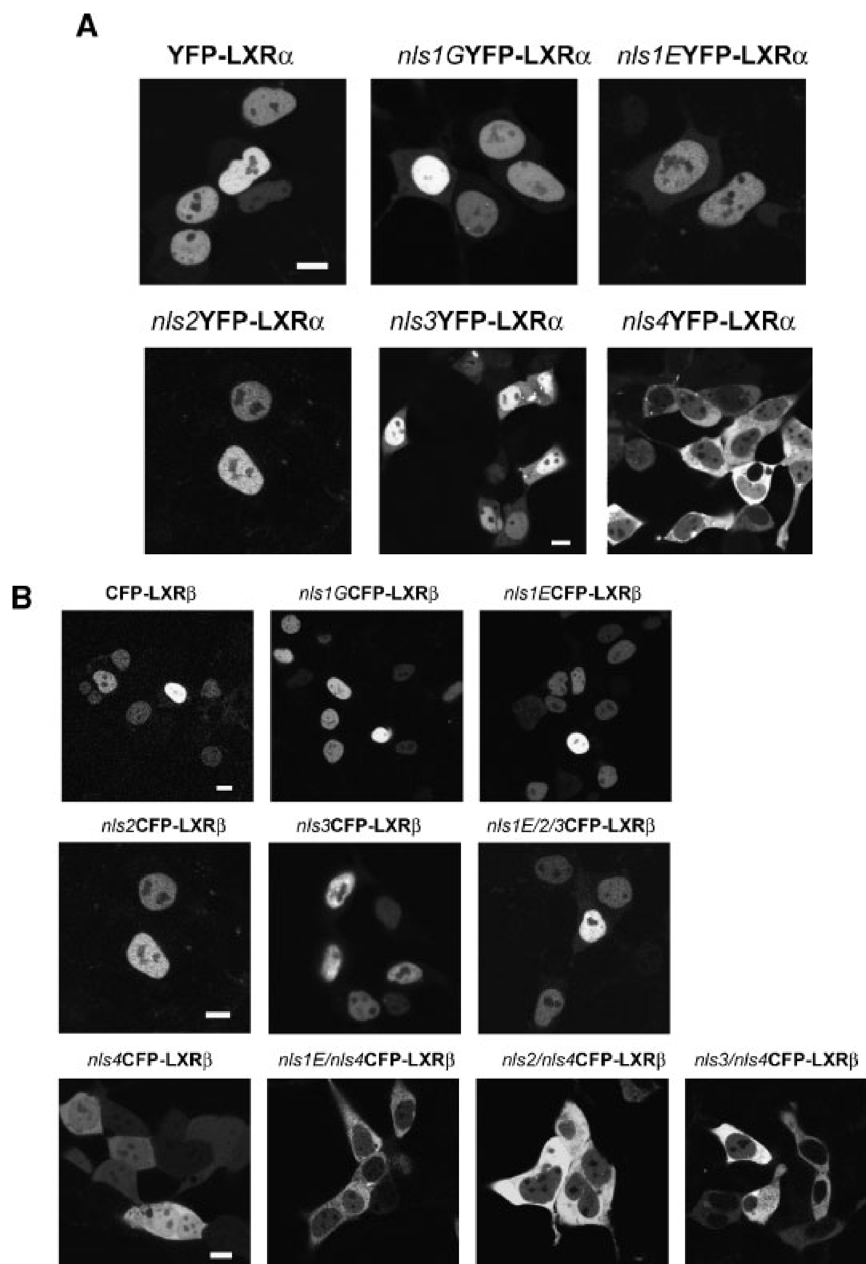

Fig. 5. NLSs in YFP-LXR α and CFP-LXR β. **A**: Both NLS3 and NLS4 are important for nuclear localization of YFP-LXR α. HEK293 cells were transiently transfected with YFP-LXR α, *nls1G*YFP-LXR α, and *nls1E*YFP-LXR α (upper panel), as well as *nls2*YFP-LXR α, *nls3*YFP-LXR α, and *nls4*YFP-LXR α (lower panel). **B**: NLS4 acts together with either NLS1, 2, or 3 for nuclear localization of CFP-LXR β. HEK293 cells were transiently transfected with CFP-LXR β, *nls1G*CFP-LXR β, *nls1E*CFP-LXR β (upper panel), *nls2*CFP-LXR β, and *nls3*CFP-LXR β, as well as triple mutant *nls1/2/3*CFP-LXR β (center panel), *nls4*CFP-LXR β, *nls4/nls1E*CFP-LXR β, *nls4/nls2*CFP-LXR β, and *nls4/nls3*CFP-LXR β (lower panel) Bar, 10 μm.

YFP-LXR α and CFP-LXR β in these cells compared to wild-type F9 cells. Our experiments showed that wild-type and all mutant LXR had similar distribution patterns in HEK293, F9, and F9 RXR−/− cells (not shown).

Taken together, we demonstrated that mutations in NLS1 and NLS2, which are functional in RXR and other nuclear receptors, do not affect localization of either LXR α or LXR β, while mutations in NLS3 affect nuclear localization of LXR α but not of LXR β.

LXR α and LXR β Have a Putative NLS Upstream of the DNA Binding Domain

Our data indicated that there might be an additional amino acid sequence important for

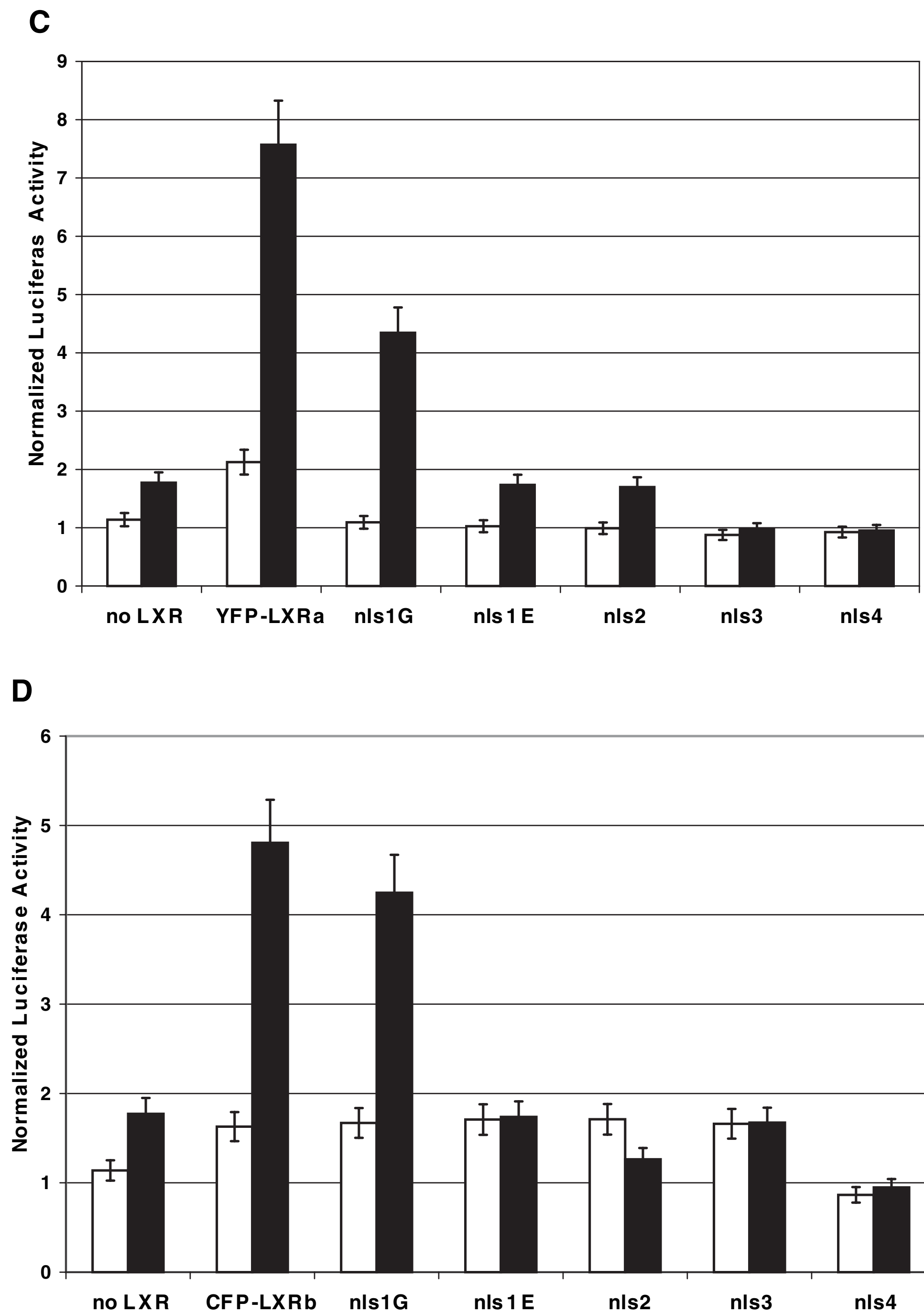

Fig. 5. (*Continued*) Transcriptional activities of wild-type and mutant YFP-LXR α (**C**) and CFP-LXR β (**D**). HEK293 cells were transiently transfected with wild-type and mutant YFP-LXR α and CFP-LXR β, LXRE-luciferase reporter, and luciferase control plasmids as described. Normalized luciferase activities from vehicle treated cells are shown as open bars, and from ligand (100 nM 9-*cis* retinoic acid combined with 10 μM 22-(R) hydroxycholesterol) treated cells are shown as shaded bars. Luminescence data were normalized with Renilla luciferase activities, and data are expressed as mean ± 1 SD.

nuclear localization of LXR α and LXR β. We performed deletion analysis from the C-terminus of CFP-LXR β in 50 amino acid increments and found that only a truncated CFP-LXR β consisting of the N-terminal 55 amino acids localized to the cytoplasm. Cytoplasmic localization could be due to the smaller size of this truncated protein that might not require

TABLE II. Nuclear Localization of Wild-type and Mutant YFP-LXR α and CFP-LXR β is not Affected by Agonist Treatment

	DMSO	100 nM 9-*cis* retinoic acid + 10 μM 22(R) hydroxycholesterol
YFP-LXR α	10 ± 7	11 ± 9
*nls3*YFP-LXR α	3.1 ± 1.6	2.9 ± 2.2
*nls4*YFP-LXR α	0.34 ± 0.13	0.38 ± 0.13
CFP-LXRβ	27 ± 17	26 ± 18
*nls4*CFP-LXR β	1.3 ± 0.8	1.1 ± 0.5
*nls4/1E*CFP-LXR β	0.27 ± 0.1	0.34 ± 0.1
*nls4/2*CFP-LXR β	0.37 ± 0.2	0.6 ± 0.3
*nls4/3*CFP-LXR β	0.53 ± 0.3	0.33 ± 0.2

Wild-type and mutant YFP-LXR α and CFP-LXR β were expressed in HEK293 cells. Then, cells were treated with either vehicle (DMSO) or with agonist (100 nM 9-*cis* retinoic acid combined with 10 μM 22 (R) hydroxycholesterol). Fluorescence brightness was measured both in the nucleus and in the cytoplasm of each 50–100 cells selected by imaging randomly chosen areas of fluorescing cells. Data are presented as ratio between nuclear and cytoplasmic brightness. Ratios for wild-type YFP-LXR α and CFP-LXR β were significantly different from ratios of all mutants including ratio between YFP-LXR α and *nls3*YFP-LXR α ($P < 0.001$). Ratios of nuclear versus cytoplasmic fluorescence of *nls4*CFP-LXR β were also significantly different from ratios of *nls4/1E*CFP-LXR β, *nls4/2*CFP-LXR β, and *nls4/3*CFP-LXR β double mutants ($P < 0.001$). Ratios between treated or untreated wild-type and mutant YFP-LXR α, and CFP-LXR β were not significantly different from each other.

active transport into and out of the nucleus. Alternatively, cytoplasmic localization could be due to an amino acid sequence at the N-terminus between amino acids 1 and 105 of LXR β that is important for nuclear localization. We searched for a region rich in basic amino acids in both LXR α and LXR β and found a putative NLS (NLS4) at a similar position in both proteins (amino acids 83–86 [K83, R84, K85, K86] in LXR α and amino acids 72–75 [K72, R73, K74, K75] in LXR β). We introduced point mutations into these regions, changing all of these amino acids into glutamic acid residues. We then expressed these mutants (*nls4*YFP-LXR α and *nls4*CFP-LXR β) in HEK293 cells. Surprisingly, we again found differences between LXR α and LXR β. As shown in Figure 5A,B, respectively, *nls4*YFP-LXR α was predominantly cytoplasmic whereas *nls4*CFP-LXR β was both nuclear and cytoplasmic. To study the contribution of NLS1, NLS2, and NLS3 to the nuclear localization of *nls4*LXR β, we created double mutants (*nls4/nls1E*CFP-LXR β, *nls4/nls2*CFP-LXR β, *nls4/nls3*CFP-LXR β) and expressed them in HEK293 cells. Figure 5B shows that NLS1, NLS 2, and NLS 3 each contributed to nuclear localization of CFP-LXR β; nuclear localization was abolished for all double mutants.

Next, we analyzed the fluorescence brightness in nuclei and cytoplasm of wild-type, and mutant YFP-LXR α and CFP-LXR β. As shown in Table II, the majority of fluorescence from wild-type YFP-LXR α and CFP-LXR β was within the nucleus, whereas *nls4*CFP-LXR β fluorescence distributed evenly between nucleus and cytoplasm. In contrast, fluorescence of *nls4*YFP-LXR α and *nls4/nls1E*CFP-LXR β, *nls4/nls2*CFP-LXR β, and *nls4/nls3*CFP-LXR β double mutants is predominantly in the cytoplasm.

Then, we evaluated the effect of agonist on localization of these mutants. We treated the cells expressing *nls4*YFP-LXR α, *nls4*CFP-LXR β, *nls4/nls1E*CFP-LXR β, *nls4/nls2*CFP-LXR β, and *nls4/nls3*CFP-LXR β with a combination of 100 nM 9-*cis* retinoic acid and 10 μM 22(R) hydroxycholesterol. Analysis of fluorescence brightness in nuclei and cytoplasm of these cells showed that ligand treatment did not affect localization of these mutants (Table II).

Because *nls4*CFP-LXR β is still partially nuclear, we next expressed *nls4*CFP-LXR β in both wild-type F9 and F9RXR−/− cells to explore the contribution of endogenous RXR to nuclear localization of LXR β. We found that the distribution of *nls4*CFP-LXR β in these cells did not differ from the distribution of the same mutant receptors in HEK293 cells (not shown).

Taken together, we identified a new putative NLS in both LXR α and LXR β, and demonstrated differences between LXR α and LXR β in the function of analogous amino acids for nuclear localization. Mutations in NLS4 alone abolished nuclear localization of LXR α, whereas mutations in either NLSs1, 2, or 3 in cooperation with NLS4 abolished nuclear localization of LXR β.

Mutations Inhibit Transcriptional Activities of Both YFP-LXR α and CFP-LXR β

Subsequently, we evaluated the transcriptional activities of both wild-type and mutant LXR α and LXR β. We found that the mutation K128G in YFP-LXR α inhibited both basal and ligand-induced transcriptional activity, whereas it did not affect fold induction by ligand. Mutation R117G in CFP-LXR β (*nls1G*CFP-LXR β) did not significantly inhibit transcriptional activity. Mutations K128E in

YFP-LXR α (*nls1E*YFP-LXR α) and R117E in CFP-LXR β (*nls1E*CFP-LXR β), as well as the mutations in *nls2*YFP-LXR α, *nls2*CFP-LXR β, *nls3*YFP-LXR α, *nls3*CFP-LXR β, *nls4*YFP-LXR α, and *nls4*CFP-LXR β inhibited transcriptional activity (Fig. 5C,D). Basal transcriptional activities of all mutant YFP-LXR α was significantly lower than basal transcriptional activity of wild-type YFP-LXR α. Whereas basal transcriptional activities of wild-type and mutant *nls1*CFP-LXR β, *nls2*CFP-LXR β, and *nls3*CFP-LXR β were indistinguishable, basal transcriptional activity of *nls4*CFP-LXR β was significantly lower than that of wild-type CFP-LXR β. Taken together, these results show that, as expected, defective nuclear localization results in impaired function. In addition, we show that amino acids in these regions are important for LXR function even if they are not important for nuclear localization. These results also show selective effects of mutations on basal transcriptional activities of LXR α and LXR β.

We identified a new putative NLS upstream of the DNA binding domain of both LXR α and LXR β that is necessary for their nuclear localization. NLS3 is important for nuclear localization of LXR α, but not of LXR β. In addition, we show that in contrast to similar regions in other receptors NLS1, 2, and 3 by themselves are not important for nuclear localization of LXR β but that each of them act together with NLS4 for nuclear localization. All data together indicate that regulation of nuclear localization of LXR α and LXR β is different.

DISCUSSION

In this study, we showed for the first time differences in regulation of nuclear localization of two very closely related nuclear receptors, LXR α and LXR β. First, we showed using confocal scanning microscopy that both LXR α and LXR β are nuclear both with and without ligand. We also showed that both receptors accumulate in nuclear foci after agonist treatment but not after antagonist treatment. Permeabilization experiments demonstrated that unliganded LXR β is exported from the nucleus whereas unliganded LXR α is retained in the nucleus. Agonist binding causes nuclear export of LXR α and retention of LXR β. Antagonist binding causes nuclear export of both LXR α and LXR β. Next, we evaluated the importance of analogous regions in RXR, LXR α, and LXR β for nuclear localization and receptor function using mutational analysis, imaging, and transactivation experiments. Detailed analysis of putative NLS revealed another sequence in RXR that is important for nuclear localization. In addition, we identified a novel putative NLS in both LXR α and LXR β upstream of their DNA binding domains as well as differences between LXR α and LXR β in the importance of analogous amino acid sequences for nuclear localization.

We found that LXR α and LXR β accumulated in nuclear foci after agonist treatment. The accumulation of agonist-bound fluorescent chimeras of nuclear receptors in nuclear foci appears to be a common phenomenon [Htun et al., 1996, 1999; Lim et al., 1999; Prufer et al., 2000; Stenoien et al., 2000; Baumann et al., 2001; Prufer and Barsony, 2002]. DNA binding mutants of TR also accumulated in such foci [Baumann et al., 2001] thus suggesting they are not receptors bound to DNA. In the cases of AR [Tyagi et al., 2000] and ER [Stenoien et al., 2000] such foci were identified as receptors bound to nuclear matrix. In fact, ligand-bound ER colocalize with co-activator, steroid receptor coactivator (SRC) −1 at the nuclear matrix [Stenoien et al., 2000]. Antagonist treatment did not cause such nuclear LXR α and LXR β foci, whereas antagonist treatment caused nuclear ER foci that were identified as immobilized ER at the nuclear matrix [Stenoien et al., 2001]. Interactions with ligands and co-factors likely determine the intranuclear distribution of LXR α and LXR β. Co-factors, specifically co-repressors and co-activators, play a major role in switching nuclear receptors between their repressed and activated states. Such interactions likely also play a role in intranuclear regulation of LXR α and LXR β function.

We found that no unliganded LXR α was exported whereas unliganded LXR β was partially exported within 1 h incubation after digitonin permeabilization. Nuclear export is important for function of many nuclear proteins. However, the ability of a protein to leave the nucleus might be determined predominantly by the strength of its interactions with other nuclear components [Schmidt-Zachmann et al., 1993]. Within the nucleus, nuclear proteins can be inhibited from export via masking their nuclear export sequence [Liu et al., 2006] or via interactions with proteins that are themselves retained in the nucleus.

Of particular relevance are nuclear proteins that can act as retention factors due to their interactions with LXR α and LXR β. Such nuclear proteins include co-activators and co-repressors. Unliganded LXR α and LXR β bind to co-repressors [Hu et al., 2003] and inhibit gene transcription. One of the described differences between LXR α and LXR β is their affinity to co-repressors. Whereas LXR α binds co-repressors NCo-R and silencing mediator for retinoid and thyroid receptor (SMRT) strongly, LXR β interacts only weakly [Hu et al., 2003]. If co-repressors act as retention factors, these differences in affinity to co-repressors likely contribute to stronger nuclear retention of LXR α than of LXR β. Similarly, overexpression of co-repressor NCo-R retains TRβ in the nucleus [Baumann et al., 2001].

Agonist binding resulted in nuclear export of LXR α and nuclear retention of LXR β, whereas antagonist binding caused nuclear export of the majority of LXR α and LXR β. Ligand binding causes a conformational change in the receptor that results in co-repressor release and co-activator binding. Co-activator SRC-1 binds to conserved LXXLL motifs at the C-terminus of nuclear receptors including LXR α and LXR β [Huuskonen et al., 2004]. Subsequently, activated receptors bind to response elements in the promoters of target genes. Nuclear receptors then attract the mediator complex including co-activators such as p300. Such binding to p300 is associated with the retention in the nucleus of another transcription factor, p53 [Kawai et al., 2001]. If such co-activator binding to LXR α and LXR β serves as retention factor, accelerated export after antagonist binding can be explained by inhibition of co-activator binding to LXR [Gan et al., 2001]. Antagonist binding likely causes co-repressor dissociation. If both co-repressor and co-activator serve as retention factors for LXR α and LXR β, lack of both might initiate accelerated nuclear export. The role of these co-factors and other protein–protein interactions necessary for nuclear retention and/or nuclear export of LXR α and LXR β are poorly understood. Future studies will identify the mechanisms by which LXR α and LXR β are retained in the nucleus.

Exported nuclear receptors are reimported into the nucleus after exposing NLSs that bind to importins. Multiple basic amino acid rich sequences are important for nuclear localization of LXR α and LXR β. We found that a basic amino acid rich sequence in the hinge region of LXR α (NLS3) is important for nuclear localization. An analogous sequence in MR [Tanaka et al., 2005], GR [Freedman and Yamamoto, 2004], and SXR [Kawana et al., 2003] binds to importin α. In addition, we identified a basic amino acid rich sequence (NLS4) −12 to −9 amino acids N-terminal of the DBD in both LXR α and LXR β that is important for nuclear localization. A truncated LXR β consisting only of the N-terminal first 105 amino acids transports CFP into the nucleus, whereas a truncated LXR β consisting only of the N-terminal first 55 amino acids does not transport CFP into the nucleus. This result strongly indicates that the putative NLS4, located between amino acids 55 and 105, is indeed an NLS. Further studies will determine the nuclear import receptors that bind to NLS3 and NLS4 in LXR α and LXR β.

Whereas mutation of NLS4 in LXR α completely abolished nuclear localization, NLS4 in LXR β is possibly part of a variable bipartite NLS involving either NLS1, 2, or 3. It is unknown which importin binds to NLS2 in other nuclear receptors; importin β binds to NLS1 in RXR [Yasmin et al., 2005]. We found that a conserved basic amino acid in NLS1 of both RXR (K165) and LXR β (R117) can be substituted with the neutral amino acid, glycine, without loss of nuclear localization or transcriptional activity. Interestingly, the amino acid analogous to K165 in RXR and R117 in LXR β is conserved as a glycine in type I nuclear receptors such as GR, MR, ER, and AR. Indeed, mutation of this conserved G451 to glutamic acid abolished ligand-induced nuclear import and transcriptional activity of GR whereas mutation to arginine did not (unpublished observation). Further studies will identify the importins that bind to NLS1 and NLS2 in LXR β.

Other nuclear proteins also have multiple NLSs. Multiple NLSs can cooperate or individual NLSs can be sufficient for nuclear import. The reason for this diversity is largely unknown. In ER and PR, a cooperation between two or three NLSs are needed for nuclear accumulation of receptors [Ylikomi et al., 1992]. Two of these NLSs are located in regions analogous to putative NLS3 and NLS2 of LXR α and LXR β. Multiple NLSs might afford redundancy in proteins that require nuclear import, or each NLS may utilize unique importin

isoforms, or multiple NLSs may cooperate with one another and allow more efficient import. Finally, possible variations of cooperation of multiple NLSs may allow fine control of nuclear import under various conditions. Such fine control might depend on protein conformations as shown for 5-lipoxygenase nuclear import [Luo et al., 2004]. The three NLSs in this protein produced five identifiable levels of nuclear import. In LXR α and LXR β protein–protein interactions might selectively mask NLS. Thus, multiple NLSs can provide an important check point for function of nuclear proteins through modulation of the level of import by various protein–protein interactions.

Regulation of nuclear localization of nuclear receptors represents a general mechanism for modulating nuclear receptor function that is likely to be controlled by signal transduction pathways within the cell. Our data show that multiple basic amino acid rich sequences contribute in different ways to nuclear localization of LXR α and LXR β. The three different functional combinations of NLS 4 with either NLS1, 2, or 3 in LXR β for nuclear localization might allow for various protein–protein interactions. Those protein–protein interactions might consequently modulate the function of nuclear receptors. Such mechanisms to regulate nuclear localization through protein–protein interactions have been shown. For example, the transforming growth factor β receptor, ALK-1, interacts with LXR β but not with LXR α. This interaction not only results in localization of LXR β in the cytoplasm but also in modulation of ALK-1 signaling [Mo et al., 2002]. These data also show that subcellular localization and regulation of nuclear import are important regulatory checkpoints of LXR β function.

Our data show that mutations in analogous amino acid sequences have differential effects on basal transcriptional activities of LXR α and LXR β. Both LXR α and LXR β are expressed in tissues such as the liver but have selective functions [Peet et al., 1998b; Alberti et al., 2001]. LXR α and LXR β share 78% amino acid sequence similarity in their DNA and LBDs, both bind DNA as heterodimer with the receptor RXR, and both bind preferentially to DR-4 response elements (LXRE) [Willy et al., 1995]. In addition, both LXR α and LXR β are activated by specific oxysterols [Peet et al., 1998a]. Despite these common properties, LXR α and LXR β have distinct functions in the same tissue in vivo [Lund et al., 2006] highlighting the need to identify their selective signaling pathways. Our data suggest that similar amino acids not only selectively contribute to nuclear localization but also to transcriptional activation by the two receptors. Effects of mutations are seen on basal transcriptional activities and also in mutants with no change in nuclear localization. These effects might be due to changed DNA binding affinity or due to changed heterodimerization with RXR. Also, yet unknown selective protein–protein interactions might contribute to these differences. Identification of such selective protein–protein interactions and putative selective receptor–DNA interactions will be the subject of further research.

One of the potential functions of nucleocytoplasmic trafficking is the interaction of receptors with proteins in cellular compartments such as nucleus and cytoplasm. Unveiling protein–protein interactions that regulate nuclear and subnuclear localization of nuclear receptors will give us further insight into the signaling pathways that regulate nuclear receptor functions.

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