0026-895X/03/6303-524–531$7.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 63:524–531, 2003

Vol. 63, No. 3
2148/1047487
Printed in U.S.A.

Molecular Mechanism of Nuclear Translocation of an Orphan Nuclear Receptor, SXR

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Received September 16, 2002; accepted November 27, 2002

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

The steroid and xenobiotic receptor (SXR) is an orphan nuclear receptor that plays a key role in the regulation of xenobiotic response by controlling the expression of drug metabolizing and clearance enzymes. We observed that pregnane X receptor (PXR), the mouse ortholog of SXR, was retained in the cytoplasm of hepatic cells of untreated mice, whereas PXR was translocated to the nucleus after administration of a ligand, pregnenolone 16α-carbonitrile. To understand the molecular mechanisms underlying the xenochemical-dependent nuclear translocation of SXR, we identified the signal sequence of SXR that regulates its nuclear translocation; using an in vitro expression system, we allocated the nuclear localization signal (NLS) to amino acid residues 66 to 92 within the DNA binding domain of SXR. The NLS of SXR is characterized as the bipartite type, and is recognized by the three molecular species of importin α: Rch1 (PTAC58), NPI1, and Qip1, in the presence of PTAC97 of importin β to target the nuclear pore. The nuclear translocation of SXR was observed as an essential regulatory event for transcription of its target genes such as *CYP3A4*. These results strongly suggest that the molecular mechanism of the nuclear import of SXR was different from that of another xenosensor, the constitutively active receptor, whose translocation into the nucleus is mediated by a leucine-rich xenochemical response signal in its ligand binding domain.

The human steroid and xenobiotic receptor (SXR) (Blumberg et al., 1998) encoded by *NR1I2* (Nuclear Receptors Nomenclature Committee, 1999), also known as the pregnane X receptor (PXR) (Lehmann et al., 1998) or human pregnenolone-activated receptor (Bertilsson et al., 1998), is a member of the nuclear orphan receptor family of ligand-activated transcription factors and is activated by many prescription drugs, environmental contaminants, steroids, and St John's wort (Bertilsson et al., 1998; Blumberg et al., 1998; Lehmann et al., 1998; Masuyama et al., 2000; Moore et al., 2000a). SXR has a key role not only as a xenosensor in the regulation of both drug metabolism and efflux (Blumberg and Evans, 1998; Waxman, 1999; Xie et al., 2000a; Schuetz and Strom, 2001; Synold et al., 2001) but also as a physiological sensor of the secondary bile acid derivatives, including lithocholic acid (Chawla et al., 2001; Schuetz et al., 2001; Staudinger et al., 2001; Xie et al., 2001). SXR activates the expression of such gene-encoding proteins as CYP3A4 and the drug efflux transporter ABCB1 (P-glycoprotein), which operate on reducing the concentrations of these xenochemicals and toxic bile acids. SXR is expressed in the same tissues as CYP3A4 and ABCB1, and they all possess the same spectrum of drugs as ligands including rifampicin (RIF) (Schuetz et al., 1996a,b). CYP3A4 is responsible for metabolizing more than 50% of all drugs, and its inducible expression through SXR activation plays a pivotal role in the clearance of hepatotoxic bile acids (Staudinger et al., 2001; Xie et al., 2001). Induction of transporter ATP-binding cassette subfamilies B and C by SXR activators (Geick et al., 2001; Kast et al., 2002), suggesting that SXR-mediated gene regulation also plays an important role in multidrug resistance to chemotherapeutic reagents.

The constitutively active receptor (CAR) (Forman et al., 1998) encoded by *NR1I3* (Nuclear Receptors Nomenclature

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, Health Sciences Research Grants from the Ministry of Health, Labor and Welfare.

ABBREVIATIONS: SXR, steroid and xenobiotic receptor; PXR, pregnane X receptor; RIF, rifampicin; CAR, constitutively active receptor; ABC, ATP-binding cassette; NLS, nuclear localization signal; AhR, aryl hydrocarbon receptor; MC, 3-methylcholanthrene; XRS, xenochemical response signal; PCN, pregnenolone 16α-carbonitrile; PCR, polymerase chain reaction; β-Gal, β-galactosidase; CMV, cytomegalovirus; PBS, phosphate-buffered saline; BSA, bovine serum albumin; GST, gluthathione *S*-transferase; GFP, green fluorescent protein; DMSO, dimethyl sulfoxide; DBD, DNA binding domain; LBD, ligand binding domain; SV40, simian virus 40; NES, nuclear export signal; PKC, protein kinase C; VDR, vitamin D receptor; AF2, activation function 2.

Committee, 1999) also works in the metabolic cascade to regulate the detoxification and elimination of xenobiotics (Blumberg and Evans, 1998; Waxman, 1999; Chawla et al., 2001). CAR *trans*-activates the *CYP2B* promoter in response to a narrow range of phenobarbital-like inducers such as 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene and chlorpromazine (Honkakoski et al., 1998). CAR also may be responsible for the induction of the ABCC3 transporter (Kikuchi et al., 1998), a member of the multidrug resistance-related protein subfamily. Therefore, the xenobiotic activation of SXR or CAR may constitute essentially an independent xenobiotic response circuit, although a cross-regulatory response between the two sensor systems has recently been reported (Moore et al., 2000b; Xie et al., 2000b).

In the case of transcription factors including some nuclear receptors, it has been demonstrated that nuclear entry is regulated by a variety of distinct mechanisms that facilitate diverse gene expression (Kaffman and O'Shea, 1999). In general, the nucleocytoplasmic transport of proteins larger than 40 to 60 kDa is specifically regulated by an energy-dependent reaction (Nigg, 1997; Mattaj and Engelmeier, 1998). The facilitated active nuclear import of proteins is dependent on the presence of a specific targeting sequence, designated as the nuclear localization signal (NLS), which is characterized by a single segment or a bipartite sequence of basic amino acids. The particular NLS sequence is specifically recognized by cargoes of importin α such as Rch1(PTAC58), NPI1, and Qip1; thereafter, the complex is transported into nuclei across a nuclear pore complex (Tsuji et al., 1997). CAR (Kawamoto et al., 1999) and aryl hydrocarbon receptor (AhR), which is a ligand-dependent transcription factor and binds various polycyclic aromatic hydrocarbons, including 3-methylcholanthrene (MC), and certain halogenated aromatic hydrocarbons, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, are retained in the cytoplasm and translocate to the nucleus after ligand treatment. Thus, regulation of nuclear translocation of these xenoreceptors is the first step in the induction of target genes by various xenobiotics. In fact, the typical bipartite type of NLS (Ikuta et al., 1998) and the leucine-rich xenochemical response signal (XRS) (Zelko et al., 2001) have been identified as the signals responsible for the nuclear import of AhR and CAR, respectively, although the details of nuclear import activity of the XRS are not yet understood.

In contrast to AhR and CAR, almost nothing is known about the molecular mechanisms underlying SXR translocation into the nucleus. The purpose of this study is to reveal the molecular mechanisms for better understanding of the SXR-dependent regulation of genes concerned with important medical and therapeutic treatments. Our results provide strong evidence that the nuclear import of SXR is mediated by bipartite type of NLS, which is recognized by three groups of importin α adaptors for targeting the nuclear rim, indicating that different molecular mechanisms are involved in SXR and CAR for the nuclear import of these two xenosensors.

Materials and Methods

Immunohistochemistry of Mouse Livers. Male C57BL/6 mice received a single intraperitoneal injection of pregnenolone 16α-carbonitorile (PCN; 400 mg/kg of body weight) (Sigma Chemical Co., St. Louis, MO) dissolved in corn oil each day for 2 successive days and were sacrificed 3 h after the second injection of PCN. Sections of frozen liver from PCN-treated mice or corn oil-treated control mice were fixed with 4% paraformaldehyde for 10 min and were blocked with goat serum. Colorimetric detection was performed by the protocol of the streptavidin method, using anti-PXR.1 antibody (A-20) (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-goat IgG antibody (Nichirei Co, Tokyo, Japan) as primary and secondary antibodies, respectively. A blocking peptide of PXR (sc-7737 P; Santa Cruz) was used to show antibody specificity. The liver of the mouse was also stained with hematoxylin and eosin.

Cell Cultures. Cell lines used for this study were HeLa, Madin-Darby bovine kidney, and HepG2 cells. They were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37°C with 5% CO_2 atmosphere.

Plasmid Construction. Human SXR cDNA was prepared by polymerase chain reaction of human liver QUICK-Clone cDNA (BD Biosciences Clontech, Palo Alto, CA) using specific primers and LA-*Taq* polymerase (Takara, Tokyo, Japan). Full-length cDNA and various cDNA segments produced by polymerase chain reaction (PCR) were subcloned into adequate vectors such as pCMX. A modified pSV–β-galactosidase (β-Gal) (Eguchi et al., 1997) vector and pCMV-Myc expression vector (BD Biosciences Clontech) were used to yield the in-frame fusion genes, βGal/SXR, and Myc/SXR. The QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to produce mutant forms of SXR according to the manufacturer's instructions. Each mutant was confirmed by sequencing.

Electroporation of DNA into HeLa Cells. Electroporation was carried out using 15 μg each of β-Gal fusion protein expression vectors and HeLa cells (3.5×10^6) in 400 μl of potassium phosphate buffer solution buffer at 960 mF/450 V with Gene Pulser (Bio-Rad, Hercules, CA). The electroporated cells were seeded onto a 10-cm plastic dish and incubated at 37°C with 5% CO_2 atmosphere for 48 h. In situ staining of expressed β-Gal fusion proteins was performed as described previously (Eguchi et al., 1997).

DNA Transfection by Lipofectin, Immunoblotting, and Immunofluorescence. HeLa cells were transfected with Myc/SXR or pCMX/SXR expression plasmid by the Lipofectin method. The cells transfected with Myc/SXR were lysed in electrophoresis sample buffer 48 h after transfection and run on 10% SDS-polyacrylamide gel electrophoresis. The proteins separated in the polyacrylamide gel electrophoresis were transferred to a nitrocellulose membrane and probed with anti-cMyc mouse IgG (BD Biosciences Clontech) coupled to alkaline phosphatase. For immunofluoresence (Ikuta et al., 2002), transfected cells cultured on coverslips were washed three times with phosphate buffer solution (PBS) and then fixed with 4% formaldehyde for 10 min at room temperature. After washing with PBS, the cells were immersed in methanol for 5 min at −20°C. The coverslips were washed three times with PBS, then incubated for 30 min in 4% bovine serum albumin (BSA) in PBS. Cells were incubated with anti-cMyc mouse IgG or anti-PXR goat IgG (Santa Cruz Biotechnology) at a dilution of 1:100 or 1:50 with 4% BSA for 1 h at room temperature, and probed with anti-mouse IgG or anti-goat IgG coupled with fluorescein isothiocyanate. Coverslips were mounted onto glass slides and visualized under a Leica DMR microscope (Leica, Wetzlar, Germany).

Preparation and Microinjection of GST-SXR-GFP Fusion Proteins. SXR(66–92) was amplified by means of PCR using the β-Gal/SXR(1–434) vector as a template and specific primers to generate artificial *Bam*HI sites at both ends. After cleavage with *Bam*HI, the fragment was ligated to the *Bam*HI site of the glutathione *S*-transferase (GST)-green fluorescent protein (GFP) 2 vector (Eguchi et al., 1997) to produce an in-frame fusion gene. The GST-SXR(66–92)-GFP vector was introduced into the *Escherichia coli* strain BL21. Purification of the expressed fusion protein was carried out as described previously (Eguchi et al., 1997). Mutants having mutations in the SXR(66–92) were obtained by site-directed mutagenesis using GST-SXR(66–92)-GFP vector as a template and ad-

equate specific primers. The construction of the GST-NLSc-GFP vector was described previously (Eguchi et al., 1997). The purified preparations of fusion proteins were microinjected into the cytoplasm of HeLa cells along with Texas Red-labeled BSA, which was coinjected at the site of injection. After microinjection, the cells were incubated at 37°C for 30 min before fixation with 3.7% formaldehyde. The localization of injected GST-SXR-GFP fusion proteins was examined by fluorescent microscopy.

In Vitro Nuclear Transport Assay. Digitonin-permeabilized Madin-Darby bovine kidney cells and Ehrlich ascites tumor-cell cytosol were prepared as described previously (Eguchi et al., 1997). Recombinant expression and purification of importins α and β were performed as described previously. In vitro nuclear transport and nuclear-rim binding assays were performed at 37°C and 4°C, respectively, as described previously (Eguchi et al., 1997).

Luciferase Reporter Assay. Transient transfection of HepG2 cells was performed in 12-well plates using Lipofectin (Invitrogen, Carlsbad, CA). Cells were transfected with 1.0 μg of human CYP3A4-luciferase reporter plasmid p3A4–362(7836/7208ins) (Goodwin et al., 1999), which was generated by PCR and constructed, 100 ng of pCH110 (Amersham Biosciences, Piscataway, NJ) and 100 ng of pCMX-SXR expression vector in the presence of RIF (5 μM) or vehicle, dimethyl sulfoxide (DMSO). Cells were collected 48 h after transfection, and luciferase assays were performed according to the protocol for the luciferase assay system (Promega, Madison, WI). The luciferase activity was normalized by β-Gal activity.

Results

Subcellular Localization of PXR (SXR) in Mouse Liver. To confirm the intracellular localization of SXR in liver, immunohistochemical staining of PXR (Kliewer et al., 1998), the mouse ortholog of SXR, was carried out on frozen liver sections prepared from control and PCN-treated mice (Fig. 1). In PCN-treated mouse livers, PXR was clearly observed in the nuclei (Fig. 1a), whereas no positively immunostained nucleus was observed in the case of control corn oil-treated mouse livers (Fig. 1d). In addition, a nuclear staining of PXR was eradicated by the presence of a blocking peptide of PXR (Fig. 1b). These results led us to conclude that PXR was normally retained in the cytoplasm of hepatocytes and was translocated into the nuclei by administration of the ligand to the animals.

Identification of NLS of SXR by Transient Expression Assay. To clarify the molecular mechanisms underlying the nuclear translocation of the orphan nuclear receptor SXR, we first carried out the transient expression of native SXR in HeLa cells and then stained the cells with anti-PXR antibody. As shown in Fig. 2A, a, transiently expressed SXR was localized in the nucleus regardless of whether charcoal-treated or untreated calf serum was used. The nuclear localization of SXR was also confirmed when a fusion protein containing SXR linked to Myc-Tag (Myc/SXR) at its N terminus was expressed and stained with anti-cMyc antibody (Fig.

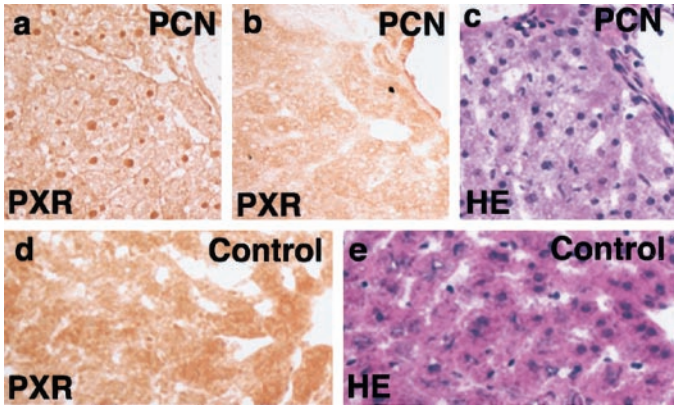

Fig. 1. Nuclear localization of PXR after PCN treatment. Sections of frozen liver tissue from PCN-treated (a, b) or corn oil-treated control (d) mice were incubated with anti-PXR goat antibody in the absence (a, d) or presence (b) of a blocking peptide of PXR (sc-7737 P, Santa Cruz). Colorimetric detection was performed by the streptavidin method. The liver sections were also stained with hematoxylin and eosin (c, e).

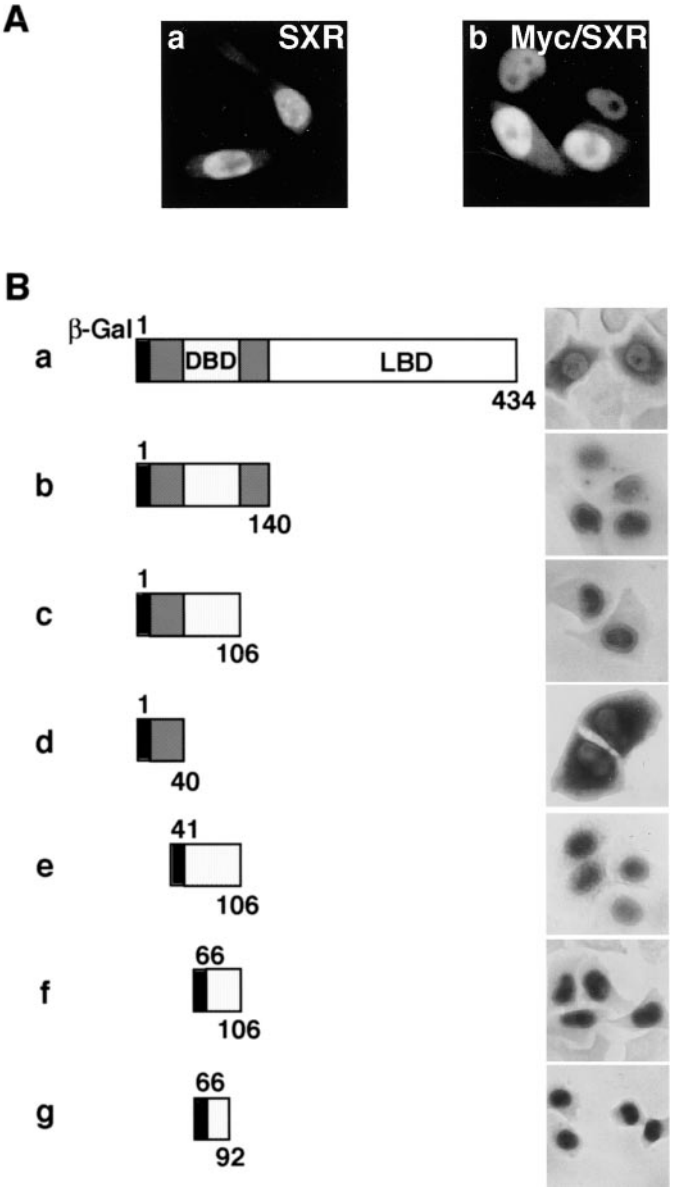

Fig. 2. A, subcellular localization of expressed native full-length SXR (a) or its fusion protein fused with Myc-tag (b) in HeLa cells. HeLa cells were transfected with pCMX/SXR or Myc/SXR expression vector as described under *Materials and Methods*. After fixation with 4% formaldehyde followed by immersion in methanol, cells on the coverslips were incubated with anti-PXR goat antibody or anti-cMyc mouse antibody as the primary antibodies and fluorescein isothiocyanate-conjugated anti-goat IgG or anti-mouse IgG as the secondary antibodies. B, identification of the region responsible for the nuclear localization of SXR. Various SXR segments were synthesized using PCR, and the resulting fragments were fused to the modified β-Gal control vector. Subcellular localization was examined as described under *Materials and Methods*. a, β-Gal/SXR(1–434); b, β-Gal/SXR(1–140); c, β-Gal/SXR(1–106); d, β-Gal/SXR(1–40); e, β-Gal/SXR(41–106); f, β-Gal/SXR(66–106); g, β-Gal/SXR(66–92).

2A, b). Myc-Tag is so small that it does not seem to affect nuclear translocation via the nuclear pore. These results show that SXR overexpressed in cultured HeLa cells translocates spontaneously to the nucleus without exposing the cells to exogenous xenochemicals. Thus, these in vitro systems provide a convenient and practical tool for the identification of SXR signal sequences which is necessary for nuclear translocation of the protein.

To identify the region of SXR required for nuclear localization, various portions of cDNA for SXR were synthesized by PCR and ligated to the modified β-gal vector to produce fusion proteins, which were large enough to prevent passage through the nuclear pore by diffusion. The chimeric constructs were introduced into HeLa cells and their localization was examined (Fig. 2B). When a fusion protein containing full-length SXR(1–434) linked to β-Gal at its N terminus was expressed, no nuclear localization of the fusion protein was observed (Fig. 2B, a). This is probably attributable to the steric hindrance caused by the large Tag β-Gal (120 kDa) at the N terminus, which may mask the NLS of SXR. Then, we divided SXR(1–434) into two fragments, SXR(1–140) and SXR(141–434). A fusion protein containing the DNA binding domain (DBD) of SXR(1–140) showed strong nuclear staining (Fig. 2B, b), whereas that containing the ligand binding domain (LBD) of the C-terminal portion of SXR(141–434) showed cytoplasmic localization (data not shown). These results strongly suggest that the NLS of SXR may exist in the N-terminal half of the molecule. Using a series of experiments, we finally narrowed the NLS of SXR to be located in the region between the 66th and 92nd amino acid residues in the DBD (Fig. 2B, g).

Microinjection of GST-SXR(66–92)-GFP Protein into Cytoplasm of HeLa Cells. To confirm the ability of SXR(66–92) to translocate to the nucleus, we next examined the fate of purified recombinant proteins microinjected into the cytoplasm of HeLa cells (Fig. 3). The cDNA of GFP was inserted into the region downstream of the *GST* gene to give a fusion gene of GST-GFP, and the gene product showed no nuclear localization (Eguchi et al., 1997). We constructed a plasmid by insertion of the SV40 NLSc fragment into the junction of the fusion gene (GST-NLSc-GFP), and the gene product was prepared as a positive control. Microinjected GST-NLSc-GFP protein, coinjected with Texas Red-conjugated BSA, revealed efficient nuclear translocation within 30 min of incubation at 37°C (Fig. 3B, g). As was seen for the transient expression of β-Gal fusions (Fig. 2B, g), the GST-GFP fusion protein, which contains wild-type SXR(66–92), showed efficient nuclear import activity, confirming that this fragment serves as an NLS (Fig. 3B, a). As shown in Fig. 3A, the NLS of SXR seems to have a bipartite nature composed of two basic amino acid segments, SXR(66–71: *RR*AM*KR*) and SXR(88–92: *RK*T*RR*). To confirm this, we next considered possible roles of R66 and R67 in the N-terminal side of the segment by replacing them with Ala (R66A/R67A). Notably, an R66A/R67A mutant of SXR(66–92) lost efficient nuclear translocation activity in the microinjection assay (Fig. 3B, b). An R91A/R92A mutant in the C-terminal side segment also drastically reduced the NLS activity; the fluorescence intensity was almost equal in the cytoplasm and nucleus (Fig. 3B, c). The simultaneous mutations in the two segments containing R66A/R67A and R91A/R92A resulted in the complete loss of translocation activity in the microinjection assay (Fig. 3B, d). In addition, a mutant SXR(66–92) having a single mutation of Lys70 to serine (K70S), which mimics NLS of CAR, also did not show any nuclear import activity (Fig. 3B, e). SXR(66–81), which does not contain the C-terminal side of the basic amino acid segment of SXR(88–92), also showed no

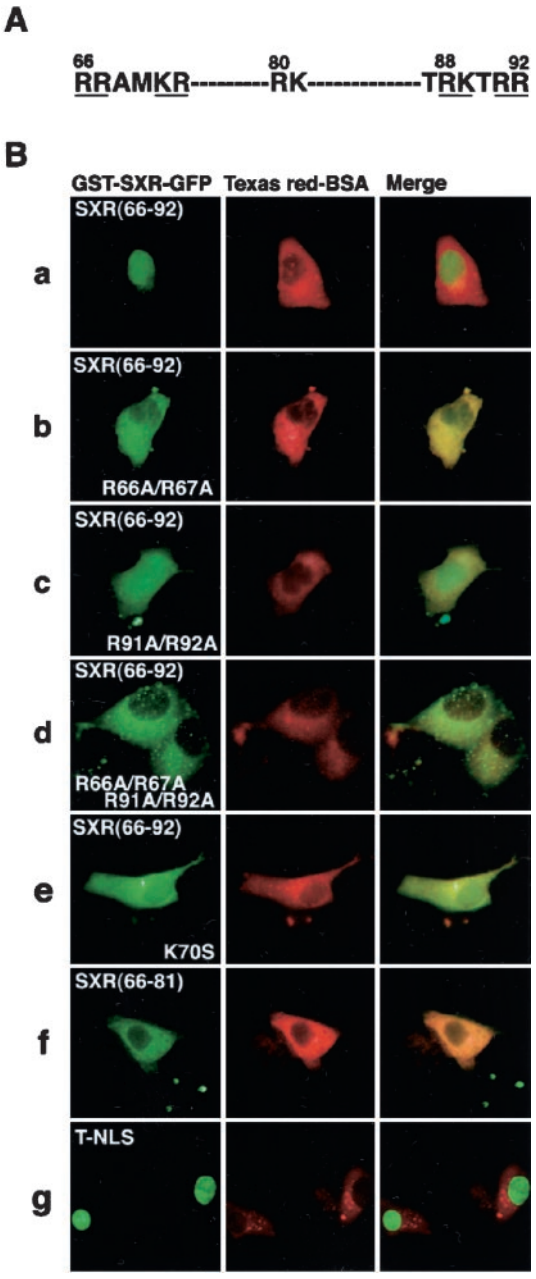

Fig. 3. A, amino acid sequence of bipartite segments of SXR(66–92). Either the N- or C-terminal segment of basic amino acid clusters is shown. B, microinjection of recombinant GST-SXR-GFP proteins into the cytoplasm of HeLa cells. The affinity-purified recombinant proteins were microinjected into the cytoplasm of HeLa cells coinjected with Texas Red-conjugated BSA. After incubation at 37°C for 30 min, the cells were fixed and the localization of the microinjected proteins was examined by fluorescent microscopy. a, GST-SXR(66–92)-GFP; b, a mutant GST-SXR(66–92)-GFP having mutations at R66A/R67A; c, a mutant GST-SXR(66–92)-GFP having mutations at R91A/R92A; d, a mutant GST-SXR(66–92)-GFP having mutations at R66A/R67A/R91A/R92A; e, a mutant GST-SXR(66–92)-GFP having mutations at K70S; f, a deletion mutant of GST-SXR(66–81)-GFP; g, GST-NLSc-GFP having an NLS of SV40 large T antigen. Amino acid sequence of NLSc used was PKKKRKV (Eguchi et al., 1997).

nuclear translocation activity (Fig. 3B, f). These findings indicate that both segments of basic amino acid residues are necessary for efficient nuclear translocation activity of SXR.

Amino Acid Residues 66 to 92 of SXR Function As a Bipartite NLS of SXR. To confirm the functional role of SXR(66–92) as the NLS of the whole SXR molecule, we carried out the transient expression of Myc/SXR with mutations or deletions in the region of the putative NLS (Fig. 4A). Unlike the microinjection analysis using a short piece of the mutated NLS of SXR, mutants of full-length SXR containing R66A/R67A (Fig. 4A, b) or R91A/R92A (Fig. 4A, e) did not show a reduction in their nuclear translocation activity. However, no nuclear translocation activity was observed in SXR mutants containing mutations in R66A/R67A/R91A/R92A (Fig. 4A, g). Furthermore, one mutant, SXR(Δ66–92), with the deleted amino acid sequence of SXR(66–92), has no nuclear import activity (Fig. 4A, h). An adequate molecular size of the expressed fusion proteins of SXR was confirmed by Western blotting with anti-cMyc antibody, as shown in Fig. 4B. Taken together, these results led us to conclude that SXR(66–92) does function as a bipartite NLS of SXR.

SXR(66–92) Interacts with Various Adaptors of Importin α to Target the Nuclear Pore. We next investigated the nuclear import activity of the SXR-NLS using an in vitro nuclear transport assay (Fig. 5). We used GST-NLSc-GFP fusion protein as the control substrate and observed a clear nuclear accumulation when this protein was incubated with cell extracts of Ehrlich tumor cytosol in the presence of ATP at 37°C (Fig. 5A, f). When GST-SXR(66–92)-GFP was incubated in the absence of cytosol, a clear cytoplasmic localization profile was obtained (Fig. 5A, c). When GST-SXR(66–92)-GFP was incubated with cytosol in the presence of ATP at 37°C, it was localized in the nucleus, confirming that the prepared recombinant protein can be a good substrate for this assay as well as the positive control (Fig. 5A, g). On the contrary, a fusion protein containing a mutant (Mt) NLS of SXR, which has the mutation R66A/R67A/R91A/R92A in SXR(66–92), has no nuclear import activity at all, even when incubation was carried out in the presence of cytosol (Fig. 5A, h). Thus, it is concluded that SXR(66–92) functions as a bipartite type of NLS and that mutations in this region may lead to a loss of interaction between the NLS and adaptors of importin α, resulting in the abolishment of nuclear translocation.

To evaluate the specificity between multiple types of importin α and the SXR-NLS, we performed an in vitro nuclear-rim targeting assay at 4°C (Fig. 5B) using three recombinant fusion proteins of GST-importin α (PTAC58, NPI1, and Qip1)

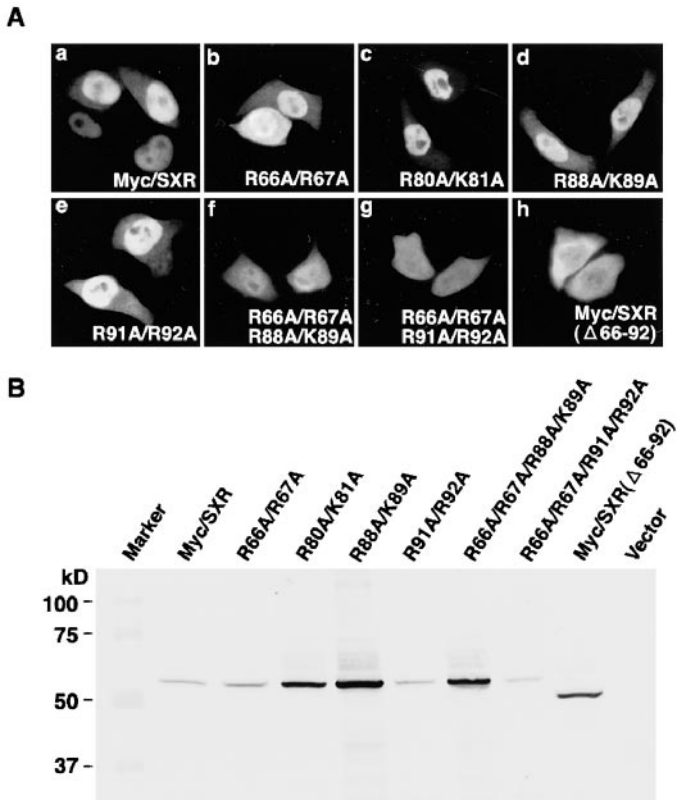

Fig. 4. A, subcellular localization of various SXR constructs fused to Myc-Tag. HeLa cells were transfected with various SXR constructs fused with Myc-tag, and localization was observed as described in the legend of Fig. 2. a, Myc/SXR; b, a mutant Myc/SXR having R66A/R67A; c, a mutant Myc/SXR having R80A/K81A; d, a mutant Myc/SXR having R88A/K89A; e, a mutant Myc/SXR having R91A/R92A; *f*, a mutant Myc/SXR having R66A/R67A/R88A/K89A; g, a mutant Myc/SXR having R66A/R67A/R91A/R92A; h, a deletion mutant Myc/SXR (Δ66–92). B, Western blot analysis of expressed SXR constructs fused with Myc-tag in HeLa cells. Various SXR constructs, as indicated in A, were expressed in HeLa cells, and immunoblotting was carried out as described under *Materials and Methods*.

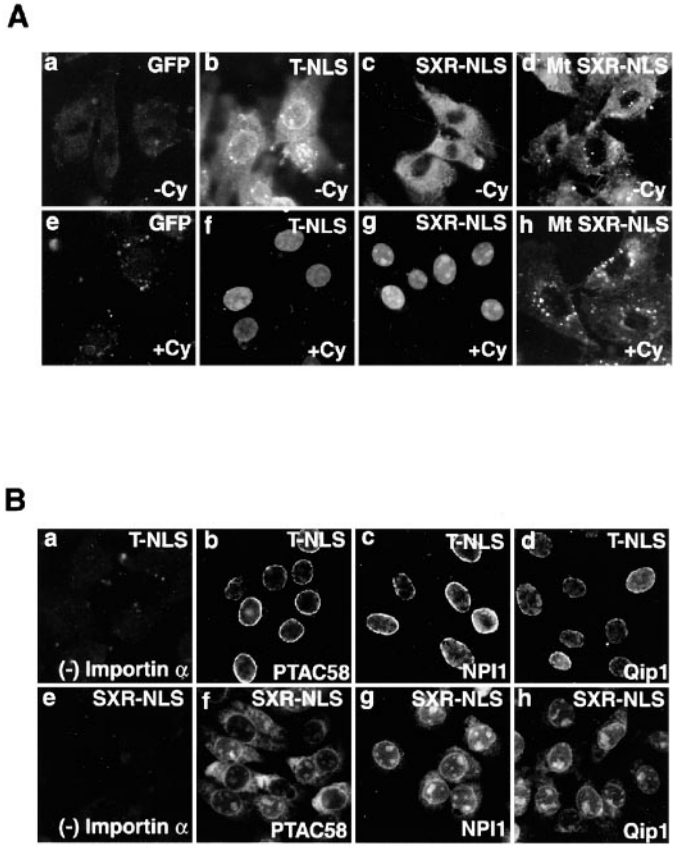

Fig. 5. Analysis of SXR(66–92) using in vitro nuclear transport assay. A, GST-GFP (a and e), GST-NLSc-GFP (b and f), GST-SXR(66–92)-GFP (c and g), and a mutant GST-SXR(66–92)-GFP having mutations at R66A/R67A/R91A/R92A (d and h) were incubated with buffer and ATP (a–d), or with cytosol and ATP (e–h) at 37°C as described under *Materials and Methods*. Amino acid sequence of NLSc used was described in the legend of Fig. 3. B, GST-NLSc-GFP (a–d) and GST-SXR(66–92)-GFP (e–h) were incubated without importin α (a and e) or with PTAC58 (b and f), with NPI1 (c and g), or with Qip1 (d and h) at 4°C. Localization of GFP-fused proteins was observed by fluorescence microscopy.

in combination with importin β (PTAC97). Incubation of GST-SXR(66–92)-GFP with the three groups of importin α enabled targeting of the recombinant protein to the nuclear rim (Fig. 5B, f to h), suggesting that the inserted fragment SXR(66–92) is recognized by these three molecules of importin α, as is the case of the classic SV40-like NLS (Fig. 5B, b to d).

Effect of Molecular Modulation on the Subcellular Localization of SXR in Vitro. It is known that molecular modulation by phosphorylation or dephosphorylation near the NLS or nuclear export signal (NES) affects the nucleocytoplasmic trafficking of some proteins. From this point of view, we carried out a computer-search for consensus sequences of phosphorylation and found several possible phosphorylation sites of SXR. An alanine substitution mimics a dephosphorylated form of SXR, whereas an aspartic acid substitution mimics the negative charge of a phosphorylated side chain. Using Myc/SXR as a template, Thr87 or Thr90 of a possible protein kinase C (PKC) site was mutated and expressed in HeLa cells (Fig. 6A). However, these molecular modulations did not show any influence on the nuclear localization of SXR (Fig. 6A). We also introduced a mutation at Ser208 of a possible PKC site, which belongs to a leucine-rich NES-like sequence of SXR, but no effect was observed on the subcellular localization of SXR (Fig. 6B).

Furthermore, because the AF2 domain in the LBD of some nuclear receptors, including vitamin D3 receptor (VDR), has been known to participate in its nuclear translocation (Racz and Barsony, 1999), we studied the effect of both a mutation of the conserved Glu residue in AF2 and a deletion of the AF2 domain on the nuclear transport of SXR. However, both the mutation of the conserved Glu 427 and the deletion of the AF2 domain [SXR(423–434)] did not participate in the nuclear import of SXR (Fig. 6C). In addition, we investigated the effect of okadaic acid, a potent inhibitor of Ser/Thr phosphatases, on the nuclear localization of SXR, but again no effect was observed (Fig. 6D). Thus, none of the possible molecular modulation studied here seems to be involved in regulation of the subcellular localization of SXR in cultured cells.

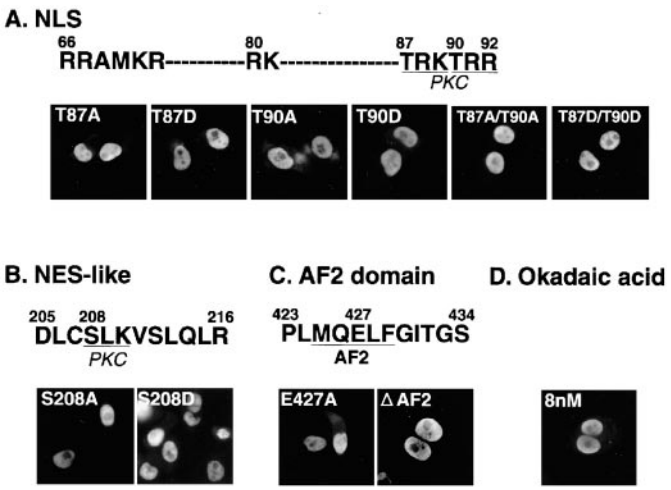

Fig. 6. A, the potential PKC sites adjacent to the NLS of SXR do not regulate the nuclear import. In the SXR-NLS sequence determined, two consensus PKC sites were observed. Full-length SXR fused with Myc-tag with the indicated mutations were expressed in HeLa cells and stained with anti-cMyc antibody as described under *Materials and Methods*. B, in the amino acid sequence of SXR, a possible PKC site exists in an NES-like consensus. Expression and localization were carried out as described in A. C, effect of mutation of the conserved glutamic acid (E427) in the AF2 domain of nuclear receptors or deletion of the AF2 domain [SXR(423–434)] on the nuclear import of SXR. D, effect of okadaic acid, a protein phosphatase inhibitor, on the nuclear import of SXR. SXR fused with Myc-tag was expressed in HeLa cells in the presence of okadaic acid (8 nM). Expression and localization were carried out as described in *A*.

Essential Role of SXR(66–92) in Transcriptional Activation of a Target Gene CYP3A4. We next investigated a functional role of SXR(66–92) in transcriptional activation of the target gene CYP3A4 using a CYP3A4-luciferase reporter p3A4–362(7836/7208ins) construct, which has been shown to be a good monitor of the transcriptional induction of CYP3A4 mediated by activation of SXR (Goodwin et al., 1999). When a reporter p3A4–362(7836/7208ins) construct was transiently cotransfected into HepG2 cells with wild-type SXR expression vector (WT-SXR) in the presence or absence of RIF, a 35-fold ligand-dependent induction of the reporter activity was observed (Fig. 7). However, ligand treatment of nonliver-derived HeLa cells resulted in a modest 2- to 3-fold induction of the reporter activity (data not shown). By contrast, when SXR, having mutations at R66A/R67A/R91A/R92A in the NLS (Mt-SXR) or a deletion of the NLS region of SXR(66–92) [ΔNLS-SXR], was transfected into HepG2 cells, the RIF-dependent induction of the CYP3A4 reporter activity was drastically decreased compared with that of wild-type SXR (Fig. 7). These observations strongly suggest that the nuclear translocation of SXR is the first step of regulatory gene expression mediated by the receptor, and

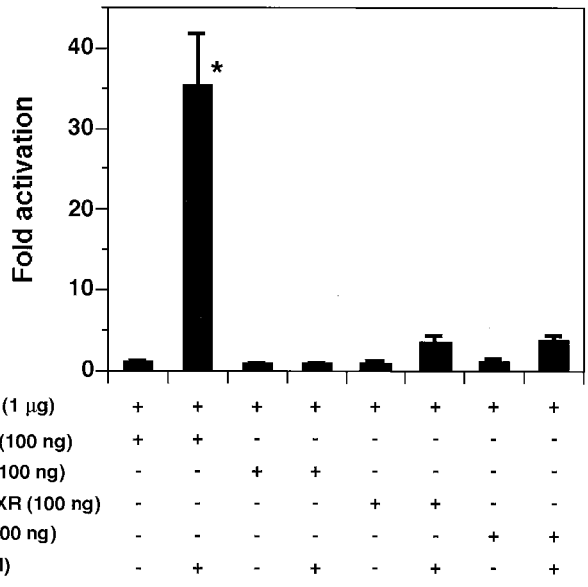

Fig. 7. Effect of mutations or deletion of the NLS of SXR(66–92) on the ligand-dependent activation of the *CYP3A4* gene in HepG2 cells. HepG2 cells were cotransfected with the CYP3A4-luciferase reporter plasmid p3A4–362(7836/7208ins) (Goodwin et al., 1999) and a wild-type SXR expression plasmid (WT-SXR), a mutant SXR having mutations at R66A/R67A/R91A/R92A (Mt-SXR), an SXR expression plasmid with NLS deletion (ΔNLS-SXR), or with pCMX vector only (vector). The cells were treated with the ligand RIF (5 μM) or with DMSO. After 48 h of transfection, the cells were harvested, and the luciferase assay was carried out as described under *Materials and Methods*. The luciferase activity, which was normalized for transfection efficiency with β-gal activity, was calculated as the ratio of the activity with the WT-SXR treated with DMSO to the activity with various experimental conditions used, and the results are given as the mean ± S.D. value of three experiments. *, $p < 0.05$; significantly different from cells transfected with WT-SXR treated with DMSO.

NLS of SXR(66–92) actually participates in the transcription activation through its nuclear translocation.

Discussion

SXR (PXR) is structurally and functionally related to VDR and CAR, and these proteins constitute a nuclear receptor subfamily *NR1I*, as shown in Fig. 8A. SXR cDNA encodes a predicted protein of 434 amino acids that is 68 and 66% identical to hVDR and hCAR, respectively, in the DBD (Blumberg et al., 1998). Domains that regulate the nuclear import and DNA binding have been found to overlap in many transcription factors and may represent the outcome of functional coevolution (La Casse and Lefebvre, 1995; Cokol et al., 2000). In this context, it is interesting to note that molecular mechanisms underlying the nuclear translocation of SXR and CAR are quite different. The nuclear translocation of SXR is mediated by a typical bipartite NLS of SXR(66–92), which overlaps completely with its DBD of amino acid residues 41 to 106, whereas the nuclear translocation of hCAR has been shown to be mediated by a leucine-rich XRS near its C terminus in the LBD (Zelko et al., 2001). Figure 8B shows a comparison of consensus for the NLS between SXR and the corresponding region of proteins in the *NR1I* subfamily. The N-terminal side of the basic amino acid cluster is completely conserved among nine species of VDR proteins and seven SXR (PXR) proteins, including two BXRs as shown in *RR*xx*KR*. Among the group of CARs, however, one basic amino acid, lysine, which is conserved among all of the VDR and SXR (PXR, benzoate X receptor) proteins, was substituted by serine (human, rat, and mouse CAR) or by leucine (chicken xenobiotic receptor) resulting in *RR*xxS(L)*K*. On the contrary, the C-terminal side of the bipartite NLS is completely conserved among all 20 proteins of the *NR1I* subfamily, as represented by the consensus, *R(K)*xx*RR*. Thus, it is likely that one basic amino acid substitution of the conserved lysine by serine or by leucine observed in CARs may lead to decrease in a nuclear import activity. In fact, microinjection analysis showed a loss of nuclear translocation activity of a mutant SXR(66–92) having one amino acid substitution at K70S (Fig. 3B, e), indicating the reason why the NLS activity of CAR is weaker than that of SXR.

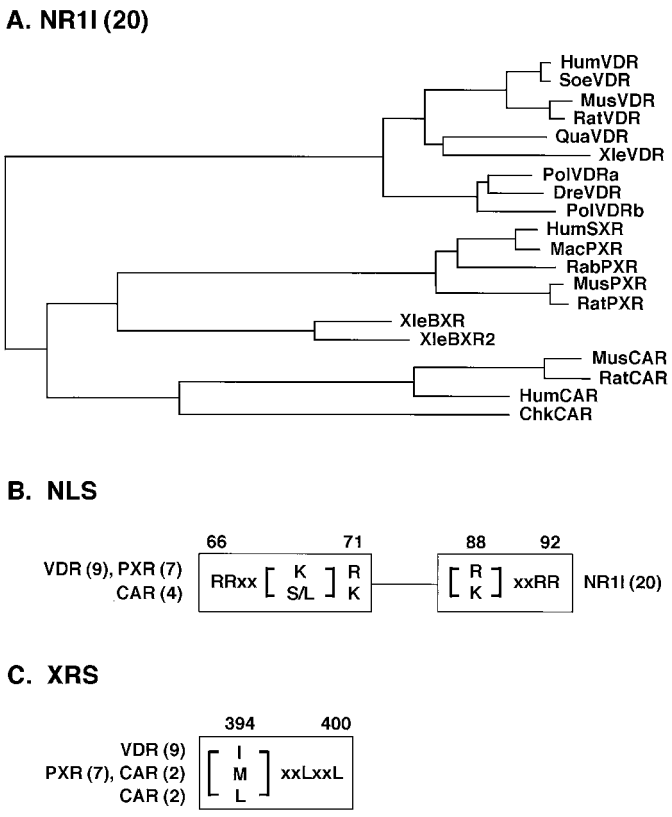

Fig. 8. A, the phylogenetic tree of the *NR1I* subfamily. Protein sequences of nine VDRs, seven PXRs (SXR, BXRs), and four CARs were multiply aligned and used to construct a tree by the neighbor-joining method (Saitou and Nei, 1987). B, comparison of a consensus bipartite NLS of human SXR with the corresponding region of proteins in the *NR1I* subfamily from twenty animal species. Both the N- and C-terminal sides of basic amino acid segments are boxed, with the number indicating amino acid residues in SXR. C, comparison of a consensus XRS motif for the proteins in the NR1I subfamily in twenty animal species with the number indicating amino acid residues in SXR.

We also compared a consensus of leucine-rich XRS in the LBD of hCAR with the corresponding region of twenty proteins in the *NR1I* subfamily (Fig. 8C). As shown in the figure, nine VDR proteins have a common sequence of IxxLxxL, seven PXR (SXR, benzoate X receptor) species and two CAR species (mouse and rat) have MxxLxxL, and hCAR and chicken xenobiotic receptor have LxxLxxL sequences. Thus, we can summarize the XRS motif determined by Zelko et al. (2001) as a consensus, @xxLxxL (@ indicates an aliphatic amino acid, such as Ile, Met, or Leu). Although the XRS motif may participate in the ligand-dependent nuclear translocation of hCAR, it seems unlikely that XRS functions as the direct signal for nuclear localization, because the fusion protein SXR(141–434), which contains XRS, fused with β-Gal show no nuclear import activity whatsoever (data not shown). Alternatively, XRS may be responsible for the interior intramolecular interaction with some protein factors through helix-to-helix hydrophobic interaction. These factors may anchor the xenosensors to the cytoplasm in liver cells under ligand-free conditions. The leucine-rich XRS motif reminds us of the LxxLL motif that participates in the subcellular localization of AhR (Ikuta et al., 2002). The LxxLL motif is also known as the NR box and was originally identified as an element important for protein-protein interaction between nuclear receptors and their coactivators (Heery et al., 1997).

Accumulated evidence has shown that both CAR (Kawamoto et al., 1999) and PXR (SXR) (Fig. 1) are localized in the cytoplasm of mouse liver and are translocated into the nuclei after administration of their respective ligands. However, when these two xenoreceptors were overexpressed in cultured cells, ligand-independent nuclear localization was observed (Zelko et al., 2001; Fig. 2). Considering that two motifs, NLS in the DBD and XRS in the LBD, participate in the subcellular localization of these two xenosensors, we may be able to ascribe the contradictory result of ligand dependence between the in vivo and in vitro conditions in their nuclear import to a different balance of NLS and XRS activities in the two experimental systems. When ligands bind to the LBD of xenoreceptors in liver cells, the nuclear translocation of these receptors may be promoted through a change of interaction between receptors and some anchoring proteins or complex. Then, SXR translocates into the nucleus by a facilitated active nuclear import mechanism using its bipartite NLS, whereas CAR may move into the nuclei by a

passive diffusion mechanism because of its low molecular mass of about 40 kDa and its weakened NLS activity. The individual roles of NLS and XRS and their interplay in the nuclear translocation of xenosensors need further investigation.

Acknowledgments

We thank Dr. Y. Yoneda of Osaka University for providing the plasmid of importins.

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