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Characterization of the LxxLL Motif in the Aryl Hydrocarbon Receptor: Effects on Subcellular Localization and Transcriptional Activity[1]

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The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that acts in concert with the AhR nuclear translocator (ARNT). Subcellular localization and transcriptional activation of target genes are mainly regulated by ligands such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). We have previously reported that AhR migrates in cells as a nucleocytoplasmic shuttling protein mediated by its nuclear localization and export signals. A short sequence motif LxxLL (L is leucine and x is any amino acid) found in transcriptional co-activators has been reported to mediate the binding to liganded nuclear receptors. The role of the two LxxLL motifs, AhR[50–54] and [224–228], has now been analyzed by determining the localization of AhR and its transcriptional activity with Leu to Ala mutations in full-length AhR. Immunocytostaining revealed that mutation of the motif at AhR[50–54] promotes the efficiency of nuclear localization in the absence of ligand without altering HSP90 and ARA9 binding or nuclear export activity. Furthermore, this mutation decreases the transcriptional activity of the AhR/ARNT system, which is likely due to the suppression of AhR/ARNT/XRE complex formation. Another LxxLL motif at AhR[224–227] affects neither the subcellular localization nor transcriptional activity. These results indicate that the LxxLL motif at AhR[50–54] is important for the regulation of AhR activity.

Key words: aryl hydrocarbon (TCDD) receptor, CYP1A1, HSP90, LxxLL motif.

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor belonging to the bHLH/PAS family of transcription factors. Environmental pollutants such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds are reported to bind to AhR, while candidates for the endogenous ligand have been reported by several groups (*1, 2*). This transcription factor plays an important role in mediating a broad range of toxic responses including teratogenesis (*3, 4*), carcinogenesis (*5, 6*), immune suppression (*7*), and reproductive toxicity (*8*). Unliganded AhR is a cytoplasmic protein that is a component of a macromolecule associated with HSP90, p23 (*9, 10*), and ARA9 (*11–13*). While the bHLH and PAS B regions have been indicated to be necessary for binding to HSP90 (*14, 15*), the C-terminal portion of the PAS region, including the PAS B domain, mediates the interaction with ARA9 (*16*). The macromolecule has an important role in keeping AhR in the cytoplasm (*17, 18*) and protecting it from degradation by the ubiquitin system (*19, 20*). When a ligand binds to AhR, the pair translocates to the nucleus and forms a heterodimer with the AhR nuclear translocator (ARNT) protein (*21*). The AhR/ARNT complex binds to XRE and transactivates target genes containing CYP1A1. We have shown that AhR has a nuclear localization signal (NLS) and a nuclear export signal (NES), and is a nucleocytoplasmic shuttling protein (*22, 23*). NLS is recognized by importins α and β and then transported to the nucleus, while NES is recognized by CRM1 and exported to the cytoplasm. Since the biological activity of AhR is controlled by its associated factors in each cell compartment, the regulation of the subcellular localization of AhR is essential to its function.

The transcriptional activity of nuclear receptors is regulated by transcriptional cofactors. In the case of AhR, it is reported to be associated with coactivator RIP140 (*24*) and corepressor SMRT (*25*). It has been shown that the LxxLL consensus motif in coactivators is necessary and sufficient for the interaction with nuclear receptors (*26, 27*). However, the LxxLL motif is also found in some nuclear receptors such as human estrogen receptor α and androgen receptor.

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Abbreviations: AhR, aryl hydrocarbon receptor; ARNT, AhR nuclear translocator; bHLH, basic helix-loop-helix; CRM1, chromosome region maintenance 1; GFP, green fluorescent protein; GST, glutathione S-transferase; HSP90, heat shock protein 90; LMB, leptomycin B; MC, 3-methylcholanthrene; NES, nuclear export signal; NLS, nuclear localization signal; PAS, Per-ARNT-Sim homology region; RT-PCR, reverse transcription–polymerase chain reaction; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XRE, xenobiotic responsive element.

In the case of AhR, there are two motifs (50–54 and 224–228) in its structure. Since this motif is involved in controlling the function of nuclear receptors through protein–protein interaction, it may be essential for AhR function.

In this report, we investigated the role of the LxxLL motif of the aryl hydrocarbon receptor relative to its subcellular localization and transactivation of CYP1A1 as a target gene. Various point mutations of this motif were prepared by site-directed mutagenesis and transiently expressed in COS cells to assess their effects. These studies show that the LxxLL motif at amino acids 50–54 is important for regulating the localization and transcriptional activation of AhR.

MATERIALS AND METHODS

Cell Culture—COS7 and MDBK cells were maintained in Dulbecco's modified minimum essential medium supplemented with 10% fetal calf serum. All cultures were maintained at 37°C in a 5% CO_2 atmosphere.

Generation of AhR Mutants—Wild-type human AhR cDNA was ligated to the SRHis expression vector (*28*). Leucine residues in the two LxxLL motifs in AhR at 50–54 and 224–228 were replaced with alanine using a Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer's instructions. Each mutant was confirmed by sequencing.

Luciferase Assay—Transient transfection of COS7 cells was performed in 12-well plates using Lipofectin (Gibco-BRL, Rockville, MD). Cells were transfected with 400 ng of rat CYP1A1-luciferase plasmid derived from pMC6.3K (*29*), 100 ng of pCH110 (Amersham Pharmacia, Buckinghamshire, England) and 400 ng of SRHis-AhR expression vector. 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-galactosidase activity.

Immunoblotting and Immunofluorescence—COS7 cells were transfected with SRHis-AhR expression plasmid by the Lipofectin method. The cells were lysed in electrophoresis sample buffer 48 h after transfection and run on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins separated in the polyacrylamide gel were transferred to a nitrocellulose membrane, probed with anti-6 × His rabbit IgG (Santa Cruz, Santa Cruz, CA) and developed using anti-rabbit IgG coupled to alkaline phosphatase. For immunofluorescence, transfected cells cultured on coverslips were washed with phosphate-buffered saline (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-6 × His antibody at a dilution of 1:500 with 4% BSA for 1 h at room temperature, and then anti-rabbit antibodies coupled with fluorescein isothiocyanate (FITC). Coverslips were mounted onto glass slides and visualized under a Leica DMR microscope. Cell images were captured and processed using Adobe Photoshop software.

Microinjection—cDNA encoding AhR[45–75] harbouring NR1 and the nuclear export signal was synthesized by PCR to produce a fusion gene, GST-AhR[45–75]-GFP. The fused protein was purified as described previously (*22, 23*), and injected into the nuclei of MDBK cells along with BSA labeled with Texas-red. After a 30 min incubation at 37°C, the cells were fixed with 4% formaldehyde and observed under a fluorescent microscope.

Electrophoretic Mobility Shift Assay—AhR and ARNT proteins were generated by the TNT T7 Quick Coupled Transcription/Translation System (Promega). A fragment of double-stranded xenobiotic responsive element was labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. Conditions for DNA binding were described previously (*23, 30*).

Immunoprecipitation—The cells were washed with PBS and collected in microtubes, and homogenized in MENG buffer (25 mM MOPS, 1 mM EDTA, 0.025% NaN_3, 10% glycerol) supplemented with 2% NP-40, 20 mM sodium molybdate, and 2 mM dithiothreitol. The lysates were centrifuged and the supernatants were precleared with protein A Sepharose (Amersham Pharmacia), incubated with primary antibodies for 3 h at 4°C, followed by incubation with protein A Sepharose for 1 h at 4°C. The Sepharose beads were washed five times with MENG buffer, then boiled for 3 min in SDS-PAGE sample buffer. Samples were subjected

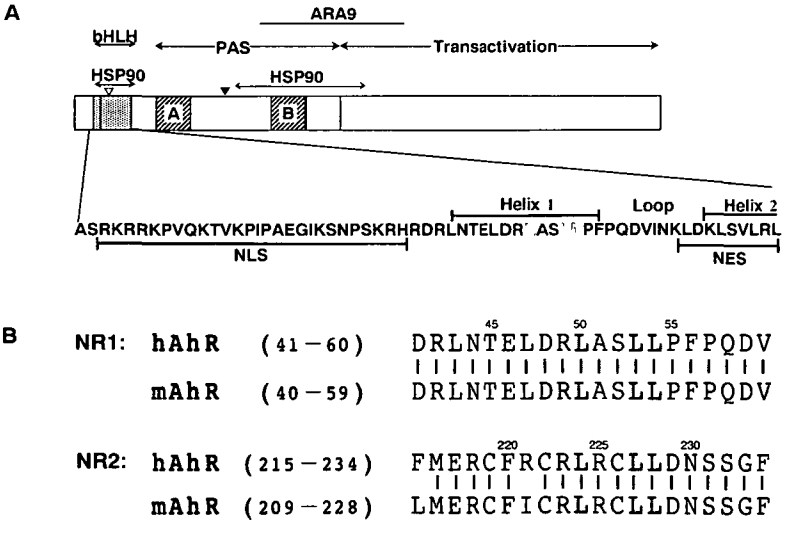

Fig. 1. **Schematic representation of the amino acid sequence around the LxxLL motif in the bHLH region of AhR.** (A) Domain structure of AhR. Location of the NR1 (▽) and NR2 (▼) is indicated. (B) Sequence alignment of human and murine AhR around NR1.

to SDS-PAGE and immunoblotting. In order to examine AhR-ARA9 binding, cDNA encoding ARA9 was produced by RT-PCR using cDNAs derived from the human leukemic cell line HL-60 as a template and subcloned into SRHis expression vector.

AhR/ARNT Heterodimerization—AhR and ARNT proteins were translated and transcribed *in vitro*. AhR was labeled with [^{35}S]methionine while ARNT was produced in the presence of unlaveled methionine. ARNT protein (4 μl) was incubated with 4 μl of AhR in the presence or absence of 1 μM 3-methylcholanthrene (MC) for 2 h at 25°C. Then, 250 μl of RIPA buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.1% Triton X-100) and 10 μl of protein A Sepharose were added for preclearing. After a 30 min incubation, the supernatant was immunoreacted with 2 μl of anti-ARNT antibody (NOVUS, Littleton, CO) for 1 h at room temperature. Normal rabbit serum was used as a negative control. The immunocomplex was collected by incubation with protein A Sepharose for 1 h followed by five washes with RIPA buffer, and resuspended in SDS-PAGE lysis buffer. The immunoprecipitates were separated by 8% SDS-PAGE followed by autoradiography.

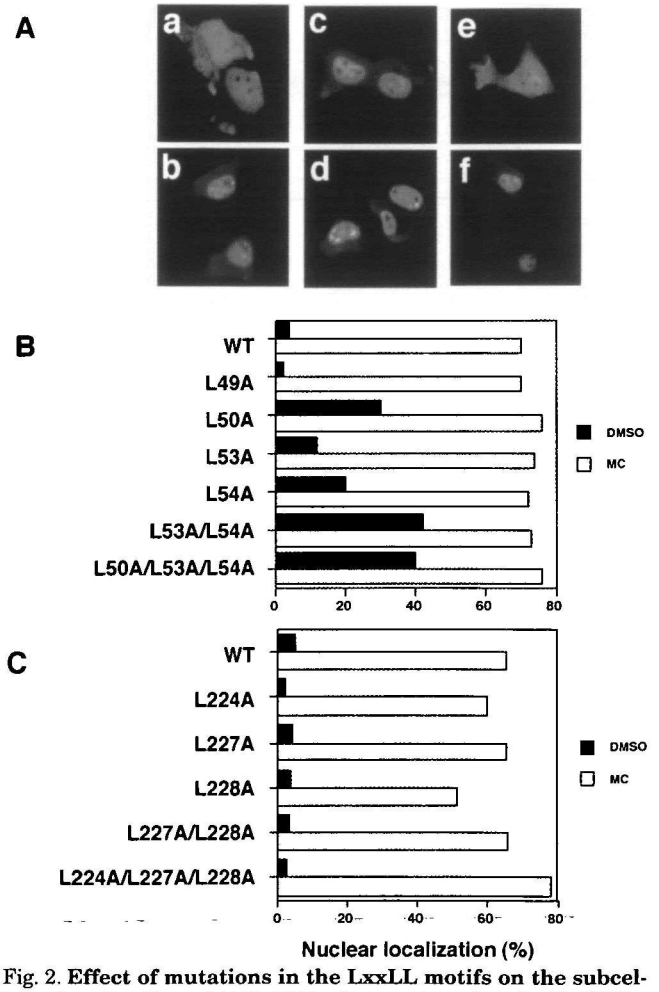

Fig. 2. **Effect of mutations in the LxxLL motifs on the subcellular localization of AhR.** COS cells were transfected with SRHis-AhR expression vector, and seeded on coverslips. Two days after transfection, the cells were treated with 1 μM MC for 2 h (A: b, d, f) or were left untreated (A: a, c, e). After fixation with 4% formaldehyde followed by immersion in methanol, the coverslips were incubated with anti-6 × His antibodies as the primary antibody and FITC-conjugated anti-rabbit IgG as the secondary antibody. (A) Representative localization profiles of wild-type (a, b), L53A/L54A (c, d) and L227A/L228A (e, f). A quantitative analysis of the NR1 mutant (B) and the NR2 mutant (C) was performed. About 200 cells were counted for each mutant. The percentage of cells showing exclusive nuclear localization is indicated.

RESULTS

The domain structure of the AhR protein is shown in Fig. 1. There are two LxxLL motifs in AhR at amino acids 50–54 (NR1) and at 224–228 (NR2). NR1 is located between NLS and NES, in the region of helix 1 of the bHLH domain. NR2 is in the region between the PAS A and PAS B domains, which mediate protein–protein interactions (Fig. 1A). The amino acid sequence around the motif is well conserved between humans and mice (Fig. 1B).

Role of the LxxLL Motif in the Localization of AhR—In order to study the biological significance of the LxxLL motifs in AhR, we prepared various AhR mutants in which leucine residues in the motif were replaced with alanine by site-directed mutagenesis. Each mutated protein was transiently expressed in COS cells to examine the subcellular localization by immunofluorescence (Fig. 2). When wild-type AhR was expressed, it appeared distributed almost equally in the cytoplasm and nucleus (Fig. 2A, a). Treatment with 1 μM MC as an exogenous ligand for 2 h stimulated nuclear translocation (Fig. 2A, b). When Leu^{50} was replaced by Ala (L50A), the number of cells showing nuclear localization increased even in the absence of MC (Fig. 2A, c). For quantitative analysis, cells showing exclusive nu-

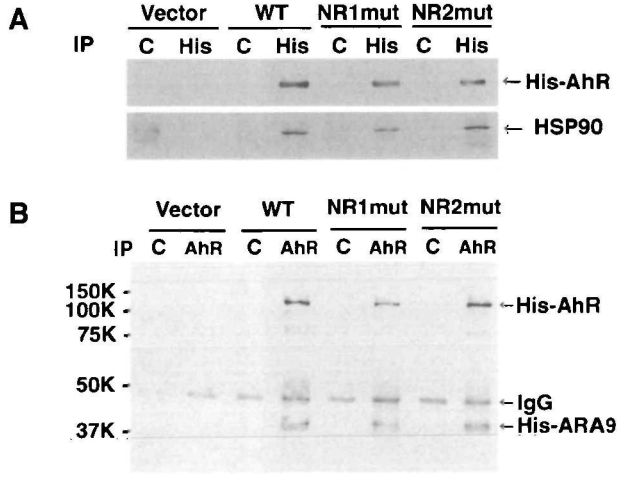

Fig. 3. **Interaction of AhR with HSP90 and ARA9.** (A) Detection of the interaction of AhR with HSP90 by immunoblot analysis. Immunoprecipitates obtained from COS cells transfected with vector alone (Vector) and with wild-type (WT), L53A/L54A (NR1mut) or L227A/L228A (NR2mut) type of His-AhR using anti-6 × His antibodies or normal rabbit IgG were separated by SDS-PAGE (8% gel), transferred to nitrocellulose membranes, and immunoblotted with anti-HSP90 antibodies. (B) Detection of the interaction of AhR with ARA9. COS cells were co-transfected with SRHis-AhR and SRHis-ARA9 expression vectors. Two days after transfection, immunoprecipitates using anti-AhR antibodies or normal mouse IgG were separated by SDS-PAGE (8% gel) transferred to nitrocellulose membranes, and probed with anti-6 × His antibodies.

clear localization were counted (Fig. 2B). While 3% (6/171) of the wild-type AhR-expressing cells showed nuclear localization in the absence of MC, this percentage increased to 30% (61/261) of the L50A-expressing cells. L53A/L54A (42%, 85/203) and L50A/L53A/L54A (40%, 141/350) also showed increased nuclear localization without an exogenous ligand. In a control experiment, we tested the distribution of the R49A mutated protein, which showed nuclear localization (2%, 4/197) similar to that of wild-type AhR. We next examined the localization of a series of NR2-mutated AhR proteins. In contrast to NR1-mutated AhR, we could not detect any change in localization in comparison with wild-type AhR (Fig. 2A, e and f, and 2C). These results indicate that the LxxLL motif in AhR[50–54] is important in regulating the localization of the AhR protein. Each recombinant protein, including wild and mutated types of AhR, was stimulated to translocate to the nucleus in the presence of MC (Fig. 2A, b, d, and f), showing that these motifs do not affect ligand dependency.

One model for explaining the stimulation of ligand-independent nuclear localization by NR1-mutated AhR is that HSP90, known to be a cytoplasmic anchoring protein (*18*) that binds to AhR, has become more labile, and only a fraction of the AhR remains associated with HSP90. The bHLH domain in AhR, which contains NR1, has been shown to be the HSP90-binding site as well as the PAS B region (*14, 15*). In order to address this hypothesis, we performed immunoprecipitation analysis to detect HSP90 bound to AhR. HSP90 was detected by immunoblot in the immunoprecipitates containing wild-type as well as mutant AhR proteins (Fig. 3A). The intensity of the detected bands was almost

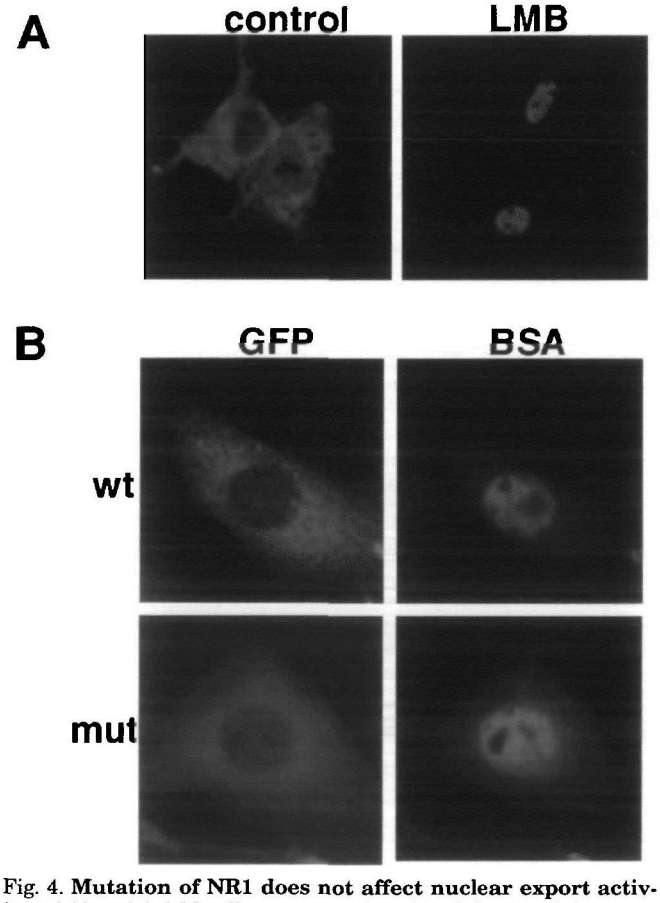

Fig. 4. **Mutation of NR1 does not affect nuclear export activity of AhR.** (A) COS cells were transfected with SRHis-AhR expression vector with Lipofectin reagent. The cells were seeded on coverslips 24 h after transfection and further incubated for an additional 24 h. The cells were treated with 10 ng/ml of leptomycin B for 2 h before fixation with formaldehyde. The subcellular localization of AhR was determined using rabbit anti-6 × His antibodies and FITC-conjugated anti-rabbit IgG. (B) A mixture of Texas red-labeled BSA and GST-AhR[45–75]-GFP was microinjected into the nuclei of MDBK cells. Representative localization profiles of fused protein coupled to wild-type and L53A/L54A mutant AhR are shown.

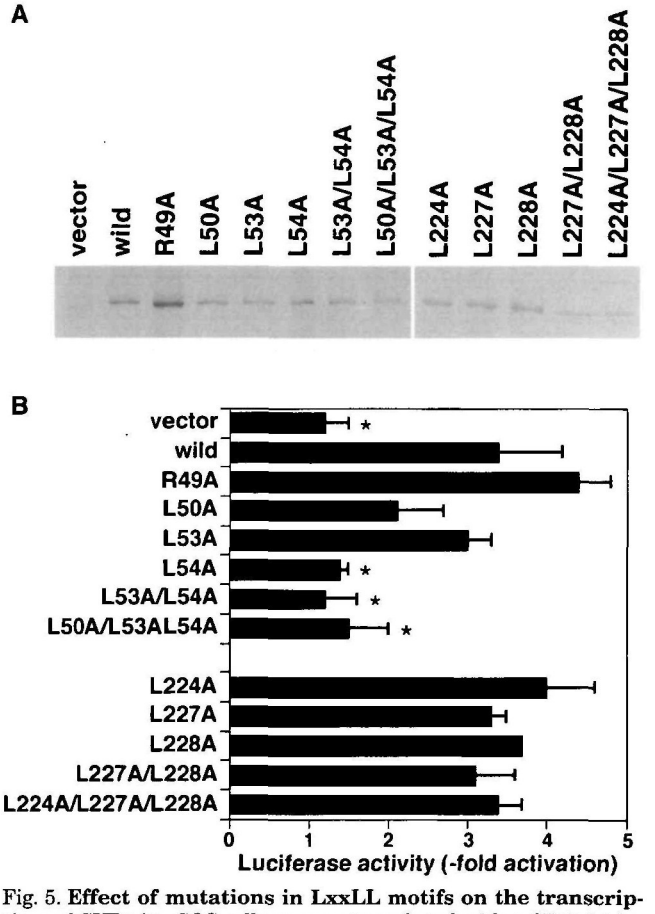

Fig. 5. **Effect of mutations in LxxLL motifs on the transcription of CYP1A1.** COS cells were co-transfected with a CYP1A1-luciferase construct and the SRHis-AhR expression vector by the Lipofectin method. Twenty-four hours after transfection, 1 μM MC was added to the culture medium. The cells were collected 48 h after transfection. (A) Expression level of wild-type and mutant His-AhR proteins. COS cell extracts were separated by SDS-PAGE and subjected to immunoblotting with anti-6 × His antibodies. The same amount of protein (20 μg) was loaded in each lane. (B) COS cells were seeded in 12-well plates and transfected using Lipofectin reagent with 100 ng of pCH110, 400 ng of CYP1A1-luciferase, and 400 ng of SRHis-AhR expression vectors encoding wild-type or mutant AhR proteins, as indicated. The cells received 1 μM MC or 0.1% DMSO 24 h after transfection, and were further incubated for 24 h. The luciferase activity, which was normalized for transfection efficiency with β-galactosidase activity, was calculated as the ratio of the activity with the control vector treated with DMSO to the activity with His-AhR. The data indicated here were obtained using cells incubated with MC, and the results are given as the mean ± SD value of three experiments. *Significantly different from cells transfected with wild-type AhR ($p < 0.05$).

the same for the wild-type and mutated AhR proteins. In addition, another factor shown to be a cytoplasmic anchor of AhR is ARA9. It has been indicated that the C-terminal portion of the PAS region containing PAS B is important for interaction with ARA9 (*16*). Actually, the mutation of either NR1 or NR2 had no effect on the binding with ARA9 (Fig. 3B). These results indicate that the stimulation of ligand-independent nuclear localization of the NR1 mutant is unlikely due to weaker binding of the cytoplasmic anchoring proteins, HSP90 and ARA9.

Another possibility is that mutation of NR1 results in a suppression of the nuclear export of AhR. In order to estimate the contribution of nuclear export to the localization of AhR, we investigated the distribution of AhR in the presence of leptomycin B (LMB), an inhibitor of nuclear export. Treatment with 10 nM LMB for 2 h resulted in an accumulation of AhR in the nucleus, showing that AhR is exported from the nucleus to the cytoplasm (Fig. 4A). We next examined whether the mutation of NR1 inhibits nuclear export by microinjection analysis. A GST-GFP fused protein connected to AhR[45–75] including NR1 and NES was injected into the nuclei of MDBK cells along with Texas red–labeled BSA to confirm the site of injection. As shown in Fig. 4B, the fused protein connected to mutant AhR (L53A/L54A) showed export activity similar to the wild-type AhR, indicating that the mutation of NR1 does not inhibit nuclear export. Taken together, it appears that nuclear transport rather than export is affected by the mutation at NR1.

Activation of CYP1A1-Luciferase by Mutated AhR—We next investigated the functional significance of the motifs on the transcriptional activity with an AhR/ARNT system using a CYP1A1-luciferase construct. Figure 5A shows the expression pattern of wild-type and mutant AhR proteins tagged with 6 × His. Every mutant and wild-type AhR was expressed efficiently. To further define the role of the LxxLL motifs, we co-expressed wild-type or mutant AhR expression vector with the CYP1A1-luciferase reporter gene. When wild-type AhR was co-transfected with the luciferase construct and treated with MC for 24 h, a 3.4-fold activation in comparison to the vector control was seen (Fig. 5B). Next, we examined whether the expression of mutant AhR results in an alteration in the luciferase activity. Co-transfection of L50A reduced the amount of stimulation to 2.1 times. While the L53A mutant AhR showed a 3.0-fold activation, L54A showed only a 1.4-fold activation. Furthermore, L53A/L54A and L50A/L53A/L54A showed almost no stimulation of activity (1.2-fold and 1.5-fold, respectively). We also examined the effect of the R49A mutant as a control, which showed a slightly higher activity (4.4 times) that might be due to more efficient expression (Fig. 5A). On the other hand, the expression of each NR2-mutant protein as well as wild-type AhR induced luciferase activity. These results suggest NR1 is necessary for the transcription of CYP1A1 by the AhR/ARNT system, but

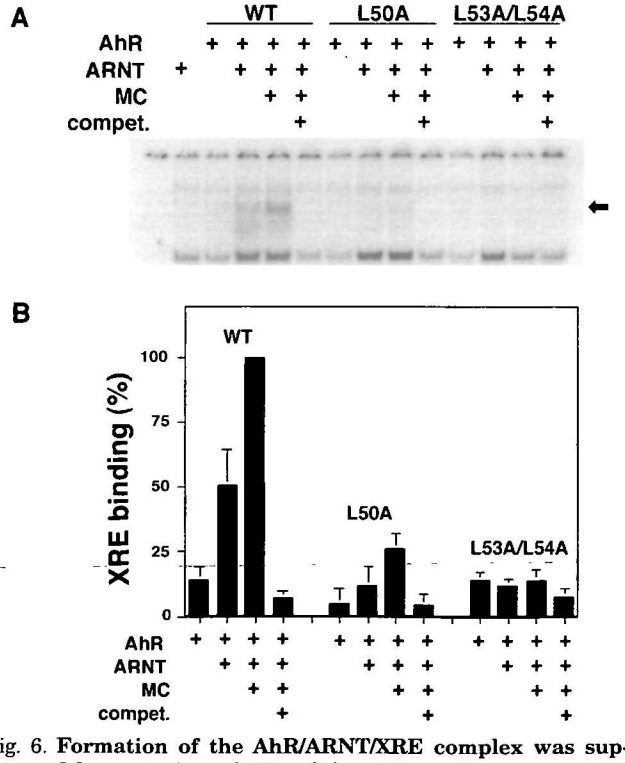

Fig. 6. **Formation of the AhR/ARNT/XRE complex was suppressed by mutation of NR1.** Gel mobility shift assays with *in vitro* transcribed and translated AhR and ARNT protein. Components of the reaction mixture as indicated by (+) were incubated with a ^{32}P-labeled XRE probe and separated by nondenaturing PAGE (4.5% gel). (A) Representative profile, (B) Densitometric analysis. Mean values and standard deviations of four experiments are indicated.

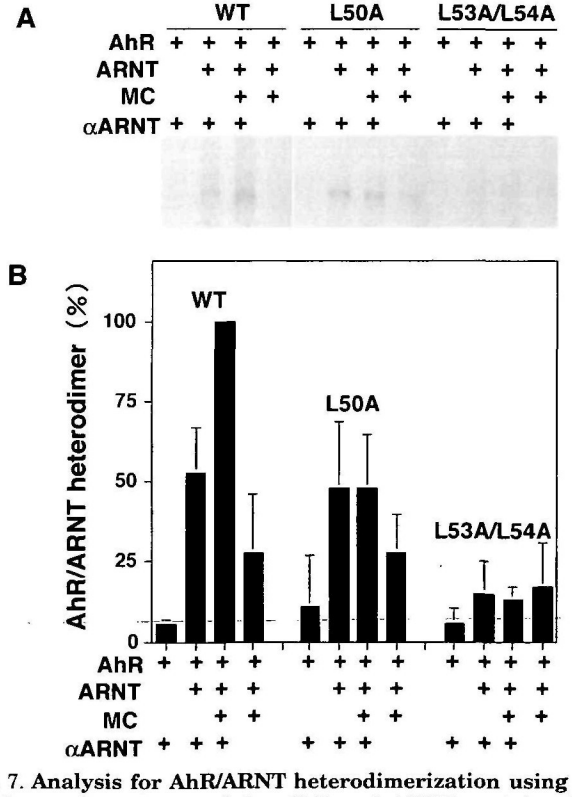

Fig. 7. **Analysis for AhR/ARNT heterodimerization using reticulocyte lysates containing AhR and ARNT proteins.** The reaction mixture indicated by (+) was incubated with vehicle alone (DMSO) or with 1 μM MC for 2 h at 25°C, and was immunoprecipitated with anti-ARNT antibodies or rabbit IgG, and separated by SDS-PAGE (8% gel), followed by autoradiography to detect ^{35}S-labeled AhR protein. (A) Representative profile, (B) Densitometric analysis. Mean values and standard deviations of three experiments are indicated.

that NR2 is not.

Since NR1 is located in the bHLH region, the reduced luciferase activity induced by the NR1 mutant might be attributed to a decrease in XRE binding by the AhR/ARNT heterodimer. To test this possibility, a gel shift assay was performed using *in vitro* transcribed and translated AhR and ARNT. As indicated in Fig. 6, wild-type AhR showed clear XRE binding in the presence of MC. In contrast, L50A exhibited lower binding even in the presence of MC (31% of the level of the wild-type AhR). Furthermore, L53A/L54A showed only basal binding levels. These results led us to check AhR/ARNT heterodimerization by immunoprecipitation analysis. Compared to the wild-type (indicated as 100%), mutant AhR, L50A and L53A/L54A showed only 48 and 13% activity, respectively (Fig. 7). These results suggest that the mutation in NR1 inactivates the AhR/ARNT heterodimer, leading to a loss of XRE binding, finally resulting in the abolishment of the transcriptional activity of AhR/ARNT.

DISCUSSION

The LxxLL motif was recently described as mediating the protein–protein interactions of transcriptional cofactors with nuclear receptors (*26, 27*). The presence of this motif in AhR suggested that it might be crucial for the regulation of AhR activity. Actually, we did find that mutations in this motif alter the subcellular localization and transcriptional activity of AhR.

We showed that a mutation in the LxxLL motif at AhR[50–54] promotes nuclear localization in the absence of ligand. Since nuclear export was not affected by the NR1 mutant, it is reasonable to consider that the nuclear transport system of AhR is stimulated by the mutation. Recently, Kazlauskas *et al.* showed the role of the HSP90 chaperone complex in the regulation of the intracellular localization of AhR (*31*). They identified the bHLH domain of AhR as the main target for the chaperone-dependent regulation of the nuclear translocation process. According to their model, in the absence of ligand, HSP90 occludes NLS by binding to the bHLH region of AhR. When the ligand binds, a conformational change in AhR allows the release of HSP90 from bHLH, and NLS is unmasked. Although we postulated that the bHLH region, especially NR1, is critical as a binding site for HSP90, our experiments were unable to confirm this assumption (Fig. 3A). This might be due to the presence of PAS B as another binding site for HSP90. In order to test this hypothesis, we further examined whether AhR[1–90] encoding bHLH including NR1 interacts with HSP90. However, we could not detect binding activity with this short fragment. Further analysis is needed to confirm how NR1 is involved in the regulation of AhR localization.

Another possible mechanism to explain the nuclear localization of the NR1-mutant AhR without a ligand is that this motif is critical for intramolecular interactions. Giannoukos *et al.* reported that a mutation in the LxxLL motif in rat glucocorticoid receptor (GR) resulted in a reduction in steroid binding capacity that might be due to a loss of important intramolecular interactions (*32, 33*). As shown in Fig. 2B, the ligand-independent nuclear localization induced by the mutation of NR1 was as much as 42%. In contrast, ligand-dependent nuclear localization was found up to approximately 80%. The incomplete nuclear localization of NR1 mutants in the absence of ligand may imply a partial loss of intramolecular interactions that allow the unmasking of NLS, normally triggerd by ligand binding.

We also investigated the biological significance of the LxxLL motif in the transcriptional activity of the AhR/ARNT system using a CYP1A1-luciferase construct. Experiments revealed that NR1 is necessary for the transactivation of CYP1A1, but NR2 is not. The decreased transcription was considered to be due to a loss of ARNT binding with AhR. However, we could not conclude that NR1 affects AhR transcriptional activiy since AhR[50–54] overlaps with the bHLH domain. It has been reported that the deletion of the helix 1 region containing the LxxLL motif abolishes dimerization with ARNT and XRE binding using murine AhR (*14*). Replacement of Leu at NR1 by Ala might inactivate the function of the LxxLL motif as well as the bHLH region. The most plausible explanation for our observation is that the mutation causes a disruption of the tertiary structure and the production of transcriptionally inactive AhR.

In conclusion, our results show that the LxxLL motif at AhR[50–54] is responsible for the regulation of the subcellular localization and transcriptional activity of AhR. As shown for the constitutive active receptor (CAR), a Leu-rich sequence, LxxLxxL, is important for nuclear localization (*34*). The LxxLL motif in nuclear receptors may also contribute to the control of their localization or transcriptional activity.

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