Subcellular Trafficking Signals of Constitutive Androstane Receptor: Evidence for a Nuclear Export Signal in the DNA-Binding Domain

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ABSTRACT:

Translocation of constitutive androstane receptor (CAR) from the cytoplasm to the nucleus is induced by phenobarbital-like drugs. Nuclear localization signals (NLSs) and a sequence [xenochemical response signal (XRS)] required for xenobiotic-induced nuclear translocation have been defined in rat and human CAR, but a nuclear export signal (NES) has not been identified. To identify cellular localization signals of CAR, the localization of fragments and mutants of mouse CAR expressed in mouse hepatocytes in vivo was examined. Consistent with other studies, an NLS in the hinge region, a diffuse NLS in the ligand-binding domain, and a cytoplasmic retention sequence were identified, and mutation of the XRS blocked nuclear accumulation both in phenobarbital-treated mice in vivo and in untreated HepG2 cells. Fusing the simian virus 40 NLS to the mutant proteins reversed the localization defect resulting from mutation of the hinge NLS but not that from mutation of the XRS, indicating that the XRS is not simply a novel phenobarbital-responsive NES. In the DNA-binding domain, a sequence in CAR is conserved with an NES identified in other nuclear receptors. Mutation of two conserved phenylalanines in this sequence resulted in increased nuclear localization of both full-length CAR and a CAR fragment containing the DNA-binding domain. The DNA-binding domain sequence, therefore, may contain an NES, which is consistent with nucleocytoplasmic shuttling of CAR. The results demonstrate that the cellular localization of CAR is complex, with multiple sequences mediating nuclear import and export and retention in the cytoplasm.

Constitutive androstane receptor (CAR) is an unusual nuclear receptor because of its constitutive activity (Baes et al., 1994) and its activation by phenobarbital (PB) without direct binding of PB to CAR (Moore et al., 2000; Tzameli et al., 2000). CAR is primarily a cytoplasmic protein in untreated animals in a complex with Hsp90 and CAR cytoplasmic retention protein (CCRP), a tetratricopeptide repeat protein that interacts with the ligand-binding domain (LBD) of CAR and retains CAR in the cytoplasm in cultured cells (Kobayashi et al., 2003; Yoshinari et al., 2003). After PB treatment, protein phosphatase 2A is recruited to the complex, and CAR dissociates and translocates to the nucleus by an unknown mechanism. Retention in the cytoplasm prevents the chronic activation of target genes by the constitutively active CAR.

We have shown that exogenous expression of GRIP1 in mouse liver mediated nuclear accumulation of CAR without PB treatment and have proposed that CAR shuttles between the cytoplasm and nucleus in untreated animals and that activation of CAR by GRIP1 results in its nuclear retention and accumulation (Xia and Kemper, 2005). It has become increasingly clear that nuclear receptors dynamically shuttle between the nucleus and cytoplasm and that regulation of cellular localization is an important mechanism for regulating the activity of these nuclear receptors (Kawata, 2001; Maruvada et al., 2003). Nucleocytoplasmic shuttling of nuclear receptors requires specific nuclear localization signals (NLSs) (Kaffman and O'Shea, 1999) and nuclear export signals (NESs) (Fischer et al., 1995; Wen et al., 1995). Two NLSs and a cytoplasmic retention region have been identified in rat CAR (Kanno et al., 2005). A specific NLS, 99LRRARQRAR-109 (critical residues underlined) in the hinge region was defined by mutational analysis. Deletion of the 110 amino acids in the N-terminal, including the hinge NLS, however, did not eliminate nuclear localization, and a second diffuse NLS was proposed within the sequence from amino acid residues 111 to 320. In addition to the NLSs, a cytoplasmic retention region (CCR), which is a potential binding site for CCRP, was defined in the LBD by deletional analysis (Kanno et al., 2005).

Deletion of a leucine-rich peptide, LXXLXXL, in the LBD of human CAR, or mutation of the Leu residues, blocked nuclear trans-
performed as described previously (Min et al., 2002). The average intensity of nuclear DNA with propidium iodide, and detection of fluorescence were injected intraperitoneally with either isotonic saline or 100

Delivery System (Mirus Bio Corporation, Madison, WI) as described previ-

BALB/c male mice (Harlan, Indianapolis, IN) using the TransIT In Vivo Gene

enzymes, ligated together, and then inserted into pEGFPC1. To construct CAR(

CRR), the CAR sequences encoding the relevant amino acids were amplified by polymerase chain reaction, and appropriate restriction enzyme sites were introduced by the

same method, the mutations F44A/F45A were introduced in CARDBD, and

introduced in pEGFPC1CAR by the QuikChange site-directed mutagenesis

in which Ala was substituted for Leu at positions 322, 326, and 329 in the XRS

values of 10

PB, and after an additional 4 h, the mice were sacrificed. The livers

were critical for the NES function. To determine whether CAR contains both NES and NLS sequences, we examined potential subcellular localization signals of mCAR in cultured HepG2 cells and mouse hepatocytes in vivo by analyzing chimeras of GFP and mCAR muta-

tants. The results provide strong evidence that an NES is present in the

mCAR DBD, an NLS is present in the hinge region of mCAR, and that the XRS does not function simply as a PB-dependent NLS.

Materials and Methods

Plasmid Constructions. The expression vector pEGFP-C1CAR has been described previously (Min et al., 2002). Expression vectors for mutant mCAR in which Ala was substituted for Leu at positions 322, 326, and 329 in the XRS and in which the SV40 NLS was fused to either wild-type mCAR or mutant mCAR with the three Ala substitutions in the XRS have been described previously (Xia and Kemper, 2005). The mutations in mCAR for the mutants, F44A/F45A, R107A/R108A, and 4RA(R100A/R101A/R107A/R108A) were introduced in pEGFP-C1CAR by the QuikChange site-directed mutagenesis as described by the manufacturer (Stratagene, La Jolla, CA). By the same method, the mutations F44A/F45A were introduced in CARDBD, and the mutation R107A/R108A was introduced into NLS-GFP-CAR. For the dele-
tion mutants, CAR1-328, CARDBD (residues 18–86), CARHinge (residues

87–172), CARDBD (residues 173–358), and CAR(1–220), the sequences en-
coding the relevant amino acids were amplified by polymerase chain reaction, and appropriate restriction digestion sites were introduced by the primers for insertion into pEGFP-C1. To construct CAR(ACCR), the CAR sequences encoding 1 to 219 and 259 to 358 were amplified by polymerase chain reaction, and appropriate restriction enzyme sites were introduced by the primers. The amplified fragments were digested with appropriate restriction enzymes, ligated together, and then inserted into pEGFP-C1CAR.

Localization of Chimeras of GFP and mCAR or the p160 Coactivators in Mouse Hepatocytes in Vivo. For in vivo transfections, plasmid DNA was isolated and injected into tail veins of six- to eight-week-old (20–25 g) BALB/c male mice (Harlan, Indianapolis, IN) using the TransIT In Vivo Gene Delivery System (Mirus Bio Corporation, Madison, WI) as described previ-

ously (Xia and Kemper, 2005). Two hours after injection of the DNA, the mice were injected intraperitoneally with either isotonic saline or 100 μg/g body weight of PB, and after an additional 4 h, the mice were sacrificed. The livers were cut into small pieces, placed in Tissue-Tek O.C.T. Compound (Sakura Finetek U.S.A., Inc., Torrance, CA), and frozen in liquid N₂. Frozen sections of 10 μm were prepared with a cryostatic microtome. Fixation, staining of nuclear DNA with propidium iodide, and detection of fluorescence were performed as described previously (Min et al., 2002). The average intensity of fluorescence in the nucleus and cytoplasm was determined using ImageJ software to measure the average intensity in the whole cell and nuclear compartment. Relative nuclear fluorescence (RNF) was calculated by dividing the average intensity of fluorescence in the nucleus by the average intensities in the whole cell (nucleus + cytoplasm) as before (Min et al., 2002). At least 40 cells from at least two mice were randomly selected for analysis for each GFP construction. Statistical differences between the means of the RNF values of two groups were tested by the Student’s t test with an assumption of unequal variances.

Results

Localization of Mouse CAR Fragments Containing the Hinge Domain or the LBD. To determine whether NLS sequences were present in mCAR, fragments of mCAR were fused to GFP (Fig. 1), and the cellular localizations of the chimeric proteins were determined in hepatocytes transfected in vivo. Representative images of cells that fall into each of the four categories of RNF (<0.25, 0.25–0.5, 0.5–0.75, and >0.75) are shown in Fig. 2. Staining with propidium iodide marks the nucleus, and the intensity of GFP fluorescence in the nucleus and whole cell was determined to calculate the RNF, the ratio of the green fluorescence intensity in the nucleus compared with that in the whole cell.

NLSs have been identified in the hinge and LBD domains of rat CAR based on experiments in cultured rat RL34 cells (Kanno et al., 2005), and mutation of the hinge motif blocked PB-induced nuclear translocation in rat primary hepatocytes. To determine whether these NLS activities were present in mCAR and functioned in vivo, hepatocytes of untreated or PB-treated mice were transfected with the expression vector of GFPCARHinge or GFPCARLBD by tail-vein injection of plasmid DNA. In untreated animals, GFPCARHinge was significantly smaller than observed with wild-type mCAR (Fig. 3A, a). After PB treatment, the percentage of the cells that seemed to translocate in untreated mice, GFPCARLBD, increased from 2 to 35%—again a significant shift than observed with wild-type mCAR (Fig. 3A, a).
These results indicate that both the hinge domain and the LBD retain partial NLS activity after PB treatment, but neither of the two domains alone is sufficient for maximal nuclear import of mCAR.

The XRS Motif Is Not Simply an NLS. The function of the XRS motif remains unclear. It is required for nuclear translocation after PB treatment (Zelko et al., 2001; Xia and Kemper, 2005), for nuclear accumulation in untreated cultured cells (Xia and Kemper, 2005), and for GRIP1-mediated nuclear translocation in vivo in untreated mice (Xia and Kemper, 2005). In cultured cells, the fusion of an NLS sequence to mCAR mutated in the XRS partially restores nuclear accumulation, suggesting the XRS is functioning as an NLS in these cells (Xia and Kemper, 2005). On the other hand, rat CAR XRS fragments did not exhibit NLS activity in cultured cells (Kanno et al., 2005). If the XRS motif functions as an NLS that is masked by some mechanism or blocked by a cytoplasmic retention signal in untreated animals but becomes functional after PB treatment, then fusion of a strong NLS to CAR mutated in the XRS should restore nuclear accumulation after PB treatment in vivo. As shown previously (Xia and Kemper, 2005), mutation of three Leu residues in the XRS has little effect on cellular distribution of mCAR in untreated animals but prevents nuclear localization after PB treatment (Fig. 3B, a). Fusion of the SV40 NLS sequence to wild-type GFPmCAR resulted in a moderate statistically significant shift of mCAR to the nucleus in untreated mice, with an increase from 5 to 40% of cells with mCAR predominantly in the nucleus (Fig. 3B, c), providing evidence that the fused NLS was functional. PB treatment resulted in modest but statistically significant additional nuclear localization of wild-type NLS/GFPmCAR (Fig. 3B, b) so that the final distribution was similar to that of wild-type GFPmCAR (Fig. 3A, a) after PB treatment. In contrast, PB treatment did not increase the nuclear localization of NLS/GFPmCAR222/6/9A (Fig. 3B, c), consistent with the lack of nuclear translocation of GFPmCAR222/6/9A after PB treatment. These results are not consistent with the XRS functioning solely as an NLS after activation by PB because, if this were the case, mutation of the XRS should not inhibit PB-mediated nuclear translocation if a strong NLS is present.

**99^LRRARQARRRA^109 in the mCAR Hinge Domain Is an NLS Signal.** The sequence **99^LRRARQARRRA^109** located in the hinge domain of rat CAR was reported to be an NLS signal (Kanno et al., 2005) and is highly conserved in mCAR. Arg residues critical for the NLS function are underlined. Deletion of the hinge domain, however, did not prevent nuclear translocation of human CAR (Sueyoshi et al., 2005). If the XRS motif functions as an NLS that is masked by some mechanism or blocked by a cytoplasmic retention signal in untreated animals but becomes functional after PB treatment, then fusion of a strong NLS to CAR mutated in the XRS should restore nuclear accumulation after PB treatment in vivo. As shown previously (Xia and Kemper, 2005), mutation of three Leu residues in the XRS has little effect on cellular distribution of mCAR in untreated animals but prevents nuclear localization after PB treatment (Fig. 3B, a). Fusion of the SV40 NLS sequence to wild-type GFPmCAR resulted in a moderate statistically significant shift of mCAR to the nucleus in untreated mice, with an increase from 5 to 40% of cells with mCAR predominantly in the nucleus (Fig. 3B, c), providing evidence that the fused NLS was functional. PB treatment resulted in modest but statistically significant additional nuclear localization of wild-type NLS/GFPmCAR (Fig. 3B, b) so that the final distribution was similar to that of wild-type GFPmCAR (Fig. 3A, a) after PB treatment. In contrast, PB treatment did not increase the nuclear localization of NLS/GFPmCAR222/6/9A (Fig. 3B, c), consistent with the lack of nuclear translocation of GFPmCAR222/6/9A after PB treatment. These results are not consistent with the XRS functioning solely as an NLS after activation by PB because, if this were the case, mutation of the XRS should not inhibit PB-mediated nuclear translocation if a strong NLS is present.

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Arg-108 or for all of the critical Arg residues in the NLS analogous to the mutations made in rat CAR. These two mutants were then transfected into mouse hepatocytes in vivo by tail-vein injection, and the mice were treated with PB. Compared with wild-type mCAR, the percentage of cells expressing the R107A/R108A mutant for which fluorescence was primarily nuclear (RNF >0.5) decreased from 63 to 30%, and the RNF mean value was significantly less than that of wild type (Fig. 3C, a). Similar results were obtained if all four Arg residues were mutated (Fig. 3C, b). These results are consistent with previous studies of rat CAR (Kanno et al., 2005). Interestingly, for the fraction of cells in which the mutant protein was primarily nuclear, nearly all were strongly nuclear (RNF >0.75). These data suggest that factors other than the hinge NLS predominantly mediate nuclear localization in a subset of cells.

If the hinge sequence is functioning as an NLS, then fusion of an NLS to the hinge mutants should restore the nuclear localization of mCAR. As shown in Fig. 3C, c, fusion of the SV40 NLS to R107A/R108A mCAR resulted in nuclear accumulation of fluorescence in untreated mice similar to that of wild-type mCAR fused to the NLS (Fig. 3, A, B, and C, c). After PB treatment, a modest but statistically significant additional nuclear accumulation of R107A/R108A mCAR was observed similar to that observed for wild-type NLS-mCAR. This contrasts with the impaired nuclear accumulation of R107A/R108A mCAR (Fig. 3C, a) compared with wild type (Fig. 3A, a) after PB treatment. The fused NLS, therefore, compensates for the mutations in the hinge region, which is consistent with an NLS function for this region.

Loss of Cytoplasmic Retention Is Not Sufficient for Maximal Nuclear Import of Mouse CAR. In rat CAR, a CRR was identified within the sequence from residues 220 to 258 (Kanno et al., 2005). To investigate the function of the CRR region in mCAR in hepatocytes in vivo, we constructed GFP chimeras of mCAR fragments truncated to residue 220, CAR(1-220), or with residues 200 to 258 deleted, CAR(ΔCRR) analogous to the rat CAR mutants that were analyzed. In untreated animals, primary nuclear localization of fluorescence was observed in 34 and 21% of cells for CAR(1-220) and CAR(ΔCRR), respectively, compared with only 5% for wild-type mCAR, and the RNF mean values were significantly increased (Fig. 3D, a and b). These results are qualitatively similar to the rat studies, although in rat studies the mutant CARs were predominantly nuclear in 79% of cells. The results are consistent with a cytoplasmic retention function for the 220- to 258-region.

The effect of PB treatment on the localization of CAR(ΔCRR) was examined since this construction retains the XRS and should be responsive to PB. However, there was no statistically significant difference in the nuclear localization of CAR(ΔCRR) after PB treatment (Fig. 3D, b). These data suggest that deletion of the CRR region not only results in a shift to the nucleus in untreated mice but also substantially reduces PB-mediated nuclear import.

Does the CAR DBD Contain an NES? The PB-independent nuclear translocation of mCAR in mouse hepatocytes in vivo mediated by exogenous expression of GRIP1 in untreated animals led to a proposal that CAR was shuttling between the nucleus and cytoplasm (Xia and Kemper, 2005). Furthermore, mutation of two Cys in the CAR DBD resulted in modest nuclear accumulation of mCAR in vivo. A conserved sequence, containing two critical Phe residues, in the DBD of several steroid and orphan nuclear receptors has been reported to be critical for receptor nuclear export (Black et al., 2001). To determine whether an NES was present in the mCAR DBD, Ala was substituted for the critical Phe residues 44 and 45 in a full-length or truncated mutant, CARDDBD (amino acids 18–86), that contains the DBD. In untreated animals, a substantial statistically significant shift to the nucleus was observed for the F44/F45A mutant with approximately 40% of the cells predominantly nuclear compared with 5% for wild type (Fig. 4, A and B). The truncated CARDDBD remained primarily cytoplasmic (RNF <0.5) but contained more cells with predominantly nuclear localization than the full-length wild-type mCAR (Fig. 4C). Since the hinge-region NLS, the CRR motif, and the XRS motif are all deleted in CARDDBD, translocation into the nucleus presumably represents passive diffusion of the relatively small GFP-CARDDBD (mol. wt., ~35 kDa) into the nucleus, in contrast to the larger full-length GFPCAR (mol. wt., ~68 kDa), which is also bound to other proteins in the cytoplasm (Yoshinari et al., 2003). Mutation of the two Phe residues in the CAR DBD fragment resulted in a significant additional shift to the nucleus, with the percentage of cells primarily nuclear (RNF >0.5) increasing from 35% (Fig. 4C) to 87% (Fig. 4D). The substantial shift from the nucleus to the cytoplasm for both the full-length mCAR and the CARDDBD when the two Phe residues critical for NES function in other nuclear receptors are mutated strongly indicates that the CAR DBD has an NES function in vivo.

Discussion

Our previous study on the GRIP1-mediated nuclear accumulation of mCAR led us to propose that GRIP1 mediates the PB-independent nuclear accumulation of mCAR by activating and retaining mCAR in the nucleus (Xia and Kemper, 2005). This suggests that even in untreated animals, mCAR is not simply sequestered in the cytoplasm but constantly shuttles between the nucleus and cytoplasm and implies that mCAR contains both NLSs and NESs. In the present work, nuclear import activity was found to be present in the hinge domain and the LBD of mCAR. These results from in vivo studies are consistent with the cell culture studies of rat CAR in which an NLS was identified in the hinge domain and a second NLS-like activity
The XRS motif was originally identified as a xenochemical response signal necessary for nuclear translocation, XRS blocks nuclear translocation, XRS probably functions as a positive signal for nuclear translocation that is masked in untreated animals rather than as a cytoplasmic retention signal. Supporting this conclusion, fusing an SV40 NLS sequence to the N terminus of full-length mCAR resulted in a modest increase in nuclear localization in untreated animals for both wild-type and XRS mutant mCAR. However, additional PB-induced nuclear accumulation of the mutant mCAR with the fused NLS was still blocked so that the XRS is not acting just as an NLS activated by PB treatment. Some constructions containing the XRS, i.e., hinge mutations and ΔCRR, are not PB-responsive, indicating that the XRS is not sufficient alone for PB-mediated nuclear translocation of mCAR. Furthermore, a chimera protein containing (1-116)mCAR and the LBD of PXR (i.e., without the mCAR XRS) was present in the cytoplasm in hepatocytes in vivo and translocated to the nucleus similarly to wild-type CAR after PB treatment (Hosseinpour et al., 2006). Therefore, the XRS is required for nuclear transport independent of PB treatment but is not sufficient or required in the CAR/PXR chimera for PB-mediated nuclear accumulation and, thus, is not likely a direct target of PB.

Cytoplasmic retention of CAR also contributes to the subcellular distribution of CAR. In untreated animals, CAR is retained in the cytoplasm as a complex with Hsp90 and CCRP (Kobayashi et al., 2003; Yoshinari et al., 2003). After PB treatment, protein phosphatase 2A is recruited to the complex, and CAR is presumably released and translocated into the nucleus. CCRP was shown to interact with the LBD of CAR, but the motif was not identified. A CRR was identified in the LBD of rat CAR (Kanno et al., 2005), which is conserved in mCAR. Mutation or deletion of the CRR in rat CAR resulted in increased nuclear localization in untreated rat primary hepatocytes. Analogous mutations of mCAR, GFPCAR(1-220), and GFPCAR(ΔCRR) also exhibited increased nuclear localization in mouse hepatocytes in vivo, although the shift was not as dramatic as that observed with the rat CAR. These results are consistent with a cytoplasmic retention function within the region 220 to 258, and its location in the LBD is consistent with it functioning as a binding site for CCRP, but this has not been shown directly. Interestingly, deletion of the CRR does not result in nuclear accumulation equivalent to that observed after PB treatment. In addition to the reversal of cytoplasmic retention, therefore, PB must also actively mediate increased nuclear translocation.

The mechanism that regulates the cellular localization of CAR, which is critical to the function of this constitutively active transcription factor, is clearly complex. In untreated animals, the NLSs and XRS must be either weak signals or masked since fusion of an SV40 NLS results in substantial nuclear accumulation. The PXR/CAR fusion study suggests that the PB-responsive target is within the 116-amino acid N-terminal (Hosseinpour et al., 2006), but CAR with truncations in the C terminus or mutation of the XRS that retain the first 116 amino acids are not PB-responsive, suggesting that there are C-terminal sequences that block the PB-mediated nuclear transport function of the N-terminal region. The CRR functions in untreated animals, possibly by binding CCRP. A reasonable mechanism is that CCRP binding blocks the NLS, and the XRS functions either directly or by altering the conformation of CAR. PB treatment results in a PP2A-mediated dephosphorylation that causes dissociation of CCRP and activation of the NLS and XRS functions. The recent demonstration of the importance of dephosphorylation of Ser-202 for nuclear accumulation, which is near the CRR, would be consistent with this mechanism (Hosseinpour et al., 2006). The XRS sequence resembles a protein-protein interaction motif so that an unknown protein that is necessary for nuclear translocation may bind to the XRS as has been
suggested (Zelko et al., 2001). The NES may function to reduce nuclear concentrations in untreated animals or to export CAR after PB treatment to terminate the activation. The effect of PB treatment on the NES function is not known. To clearly resolve the role of these different motifs in CAR cellular localization, the proteins binding to each motif must be identified, and the effects of PB treatment on the binding of the proteins and on post-translational modification of CAR will need to be elucidated.

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References


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