Molecular Basis of the Intracellular Localization of the Constitutive Androstane Receptor (CAR)

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Control of the intracellular localization of the constitutive androstane receptor (CAR, NR1I3) has more impact on the transcriptional activation of target genes by CAR ligands/activators, including medicines, environmental contaminants and endogenous metabolites, than the conformational change induced by ligand binding, because CAR, unlike other members of nuclear receptor superfamily, is constitutively active. Human CAR (hCAR) and rat CAR (rCAR) were comparatively studied, in primary hepatocytes and in immortal cells, to assess the functional domains controlling nucleo-cytoplasmic shuttling and/or intracellular localization. There are two nuclear localization signals (NLSs) and two nuclear export signals (NESs) in rCAR as well as a cytoplasmic retention region (CRR), whereas hCAR has a single NLS and two NESs. A xenocentral response signal (XRS) controls the neighboring NLS, in a phenobarbital-responsive manner, in both rCAR and hCAR in vivo, but not in immortal cells.

Key words —— constitutive androstane receptor, NR1I3, nuclear localization signal, nuclear export signal, nucleo-cytoplasmic shuttling, cytochrome P450

STRUCTURE AND FUNCTION OF NUCLEAR RECEPTORS

The nuclear receptor superfamily consists of ligand-dependent transcription factors that include receptors for steroid hormone, thyroid hormone, retinoic acid and vitamin D, and also numerous orphan receptors. Members of the nuclear receptor superfamily are structurally characterized by an N-terminal trans-activation domain (AF-1), a DNA binding domain (DBD), a hinge region and a ligand binding domain (LBD) that includes a C-terminal trans-activation domain (AF-2). Both constitutive androstane receptor (CAR, NR1I3) and pregnane X receptor (PXR, NR1I2) play pivotal roles in the transcriptional activation of partially overlapping genes that encode xenobiotic/steroid and drug metabolizing enzymes. Both CAR and PXR are activated by steroids and xenocentral chemicals. Phenobarbital (PB), a typical CAR activator, induces expression of numerous genes including phase I enzymes (cytochrome P450 2B, 2C and 3A),1–4 Phase II enzymes (UGT1A, SULTs)5,6 and phase III transporter proteins (MRP2, OATPs).7 Thus, CAR is enrolled in the detoxification of xenocentics, bilirubin (Bil) clearance and the control of steroid and thyroid hormone levels (Fig. 1A).

INTRACELLULAR LOCALIZATION AND NUCLEOCYTOPLASTIC SHUTTLING OF NUCLEAR RECEPTORS

It has become increasingly clear that nuclear receptors shuttle between the nucleus and the cytoplasm. Unlike other steroid receptors, such as estrogen receptor (ER) and progesterone receptor (PR) that are located in the nucleus in the absence of ligand, androgen receptor (AR) and glucocorticoid receptor (GR) are distributed predominantly in the cytoplasmic compartment. Following ligand binding, AR and GR translocate to the nucleus, while steroid withdrawal facilitates their export from the nucleus.8,9 Nucleo-cytoplasmic shuttling requires both a nuclear localization signal (NLS)10 and a
CAR is unique among nuclear receptors, having a constitutive activity in cell-based reporter assays and being activated by PB, without direct CAR/PB interaction. To date, PB-mediated activation of CAR is not clear. In HepG2 cells or other cell lines, exogenously expressed CAR is accumulated in nucleus without stimuli and regulate the expression of target genes. In contrast, CAR is primarily a cytoplasmic protein in the liver and in primary hepatocytes, in a complex with Hsp90 via cytoplasmic CAR retention protein (CCRP). CAR translocates into the nucleus after being stimulated with endogenous and exogenous activators, represented by Bil and PB, respectively (Fig. 1C). Following translocation to the nucleus, CAR binds to the response element on target gene promoters in a heterodimeric complex with retinoid X receptor (RXR). Thus, retention of CAR in the cytoplasmic compartment prevents the chronic activation of target genes by constitutively active CAR.

DIFFERENCES BETWEEN IN VIVO-MIMIC AND IN VITRO BEHAVIORS OF CAR

In the liver and in primary hepatocytes, CAR is normally sequestered in the cytoplasmic compartment. Upon treatment of cells with PB, CAR translocates to the nucleus and binds to response elements in the promoter regions of the target genes. Thus, primary hepatocyte culture can be used as a model for in vivo studies. In reporter gene assays using PB-responsive enhancer module (PBREM) from CYP2B, transcriptional activation is observed in the presence of PB irrespectively of the overexpression of CAR (Fig. 2A). Furthermore, the PB-responsive translocation of Green fluorescent protein (GFP)-tagged CAR from cytoplasm to nucleus is also observed (Fig. 2B right panel). In contrast, CAR ectopically expressed in immortalized cells accumulates spontaneously in nucleus (Fig. 2D) and causes constitutive activation of a reporter gene (Fig. 2C). Taken together, the PB-dependent nuclear translocation of CAR is not observed in immortalized cells.
SPECIES DIFFERENCES IN NUCLEOCYTOPLASMIC SHUTTLING AND IN INTRACELLULAR LOCALIZATION SIGNALS OF CAR

Steroid hormone receptors are known to shuttle between the nucleus and the cytoplasm. Although GFP-CAR is confined in the nuclear compartment in immortalized cells, CAR is expected to behave dynamically like steroid hormone receptors. In fact, fluorescence recovery after photobleaching (FRAP) in spontaneous multinuclear cells showed that both rat CAR (rCAR) and human CAR (hCAR) were shuttling proteins containing both NLS and NES. We identified two NLSs and two NESs in rCAR by expressing various truncated, deletion and substitution mutants in immortalized cells.23) The monopartite NLS, which is named NLS1, exists in the hinge region of rCAR and NLS2 is an assembly of noncontiguous amino acid residues within the LBD and widely spread between residues 111 and 320.24) Two NESs exist in the LBD of rCAR. One of them is an export receptor CRM1-dependent NES (NES2), which is inhibited by CRM1 inhibitor, leptomycin B (LMB), and is located in residues 244 to 253. The other is a CRM1-independent NES in the C-terminal end (317–358). Interestingly, these intracellular localization signals of CAR have species differences between rat and human. NLS1 is defective in hCAR, because Arg106, which is part of NLS1, is replaced by Glu. Similarly, it is suggested that mouse CAR (mCAR) lacks functional NLS1.23) Additionally, hCAR is devoid of functional NES2, because Ile in the NES2 of rCAR is replaced with Phe in the corresponding hCAR sequence. However, hCAR does contain a CRM1-dependent NES (named NES1) between residues 170 and 220.23) The schematic illustration of these intracellular localization signals is shown in Fig. 3.

It appears that CAR is nuclear localized in immortal cells in vitro, even in the absence of an activator, such as PB, whereas in the liver or in primary hepatocytes, the nuclear localization of CAR requires activation by PB or PB-like compounds. In liver and in primary hepatocytes, where NLS1 may not function, NLS2 may be masked by unknown mechanisms that involve the xenobiochemical response signal (XRS). XRS, a subdomain of the C-terminal leptomycin B-insensitive NES, may negatively regulate the function of the adjacent NLS2 in liver and in primary hepatocytes. In the presence of PB-like activators, following suppres-
Fig. 3. Schematic Illustration of the Difference in Intracellular Localization Signals between rCAR and hCAR.

A: Depiction of full length rCAR and hCAR (358 and 348 amino acids, respectively), with the positions of the DBD, hinge region (Hinge), and LBD. Figure shows the nuclear localization signals (rat NLS1 and common NLS2s), nuclear export signals (human NES1, rat NES2, and common C-terminal NESs), rat CRR, and common XRSs. B–D: Alignment and comparison of the amino acid sequences of NLS1 (B), NES2 (C), NES1 (D). Boxes indicate critical differences for the function of localization signals.

sion of this regulation, the nuclear transportation of CAR may be directed by the unmasked NLS2, whereas under normal conditions, NLS2 may be masked by an unidentified protein(s), which would be anchored by a naïve XRS. In the presence of PB-like activators, the XRS would release the masking protein. In contrast, in immortal cells, CAR would be nuclear localized even in the absence of PB-like substances, causing its residence in the nuclei.

In rat primary hepatocytes, rCAR is retained in the cytoplasmic compartment. Various deletion mutants were designed to search for the region(s) responsible for this cytoplasmic retention of CAR.24) GFP-rCAR(1–358), GFP-rCAR(1–320), GFP-rCAR(1–287), and GFP-rCAR(1–258) tended to remain in the cytoplasmic compartment. In contrast, GFP-rCAR(1–220), GFP-rCAR(1–176), and GFP-rCAR(1–110) were mostly localized in the nuclear compartment. In marked contrast to the cytoplasmic localization of GFP-rCAR(1–358), GFP-rCAR(1–358)Δ220–258 was mostly localized in the nuclear compartment in rat primary hepatocytes. These results suggest the sequence covering amino acids 220 through 258 is required for the cytoplasmic retention of rCAR. Therefore, we named it the cytoplasmic retention region (CRR), which might be functional in rat primary hepatocytes but not in RL34 cells. We expected that the NLSs in rCAR would be masked by chaperone proteins in the cytoplasmic complex, as occurs with the NLS of the aryl hydrocarbon receptor.26) In the latter case, in the presence of ligand, the receptor is liberated from its molecular chaperones, unmasking the NLS, allowing it to translocate into the nucleus.
The NLSs of the GR are also unmasked due to a conformational change induced by ligand binding. The NLS1 of GFP-rCAR(1–358) might be masked by the CRR binding protein(s). Truncation from the C-terminal end past the CRR unmasks NLS1, which, in turn, contributes to the nuclear localization of GFP-rCAR(1–220), GFP-rCAR(1–176), and GFP-rCAR(1–110). In addition, GFP-rCAR(72–320) and GFP-rCAR(111–320) were also localized in the cytoplasm, suggesting the simultaneous masking of NLS2 by the same set of chaperones interacting with the rCAR CRR, though the detailed mechanisms of CRR-mediated cytoplasmic retention remain to be elucidated.

HepG2 cells lack the capability of retaining CAR in the cytoplasm. Kobayashi et al. (2003) cloned and characterized a tetratricopeptide repeat (TPR) protein, designated CCRP, for its ability to accumulate mCAR in the cytoplasm of co-transfected HepG2 cells. mCAR forms a complex with CCRP, heat shock protein 90 (HSP90), and other accessory proteins. CCRP directly binds to the LBD of mCAR and mediates the indirect association between mCAR and HSP90. This might retain mCAR in the cytoplasmic compartment in hepatocytes in the absence of any ligands or activators. CCRP and HSP90 might be involved in CRR function.

The GR cycles between the cytoplasm, in a naive chaperone-complexed form, and the nucleus, in a transcriptionally active steroid-bound form. Nuclear import of GR occurs rapidly through the importin α/β-mediated pathway. Upon removal of steroidal agonist, however, nuclear export of GR occurs only slowly under most conditions. Carrigan et al. have defined the sequence in the hinge region that retards the nuclear export of GR as a nuclear retention signal (NRS). The existence of NRS-type function in CAR is indicated in FRAP assays using spontaneous multinuclear cells, because the rate of nuclear import is much faster than that of nuclear export.

The CRR-interacting chaperon protein(s), which might be responsible for the cytoplasmic localization of CAR but which remain to be elucidated, would enable systems to be designed that could screen for ligands and activators of CAR using immortal cells instead of whole animals.

REFERENCES


