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Difference in nucleocytoplasmic shuttling sequences of rat and human constitutive active/androstane receptor

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Abstract

Fluorescence recovery after photobleaching (FRAP) in spontaneous multinuclear cells shows that both rat and human constitutive active/androstane receptors (CARs) are shuttling proteins with both nuclear localization signals (NLSs) and nuclear export signals (NESs). We previously identified two NLSs in rat CAR: NLS1 in the hinge region (residues 100–108) and NLS2 in the ligand-binding domain (residues 111–320). In the present study, we compared the intracellular localization signals between rat and human CARs. There was a marked difference in their intracellular localization in COS-7 cells because, unlike rat CAR, human CAR does not contain NLS1 due to an amino acid change at position 106. A CRM1-dependent leucine-rich NES, which is sensitive to an inhibitory effect of leptomycin B, was found in the cytoplasmic retention region previously identified within the ligand-binding domain of rat CAR (residues 220–258). We found that human CAR instead has a NES in the ligand-binding domain between residues 170 and 220. Also, we detected CRM1-independent C-terminal NESs between residues 317–358 of rat and human CARs. Removal of NLS1 by N-terminal truncation and mutation of xenochemical response signal caused rat CAR to localize in the cytoplasm of COS-7 cells, which we suspect is due to the masking of NLS2.

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1. Introduction

Constitutive active/androstane receptor (CAR), a member of the nuclear hormone receptor superfamily, is a transcription factor that regulates gene expression in response to structurally divergent compounds such as environmental pollutants and endogenous metabolites. CAR regulates a variety of genes including those involved in the hepatic metabolism of xenobiotics, especially the cytochrome P450 2B [1], 2C [2], and 3A [3] subfamilies, various conjugation enzymes [4], and membrane transporters.

CAR is normally sequestered in the cytoplasmic compartment of untreated liver cells and translocated to the nucleus after exposure to phenobarbital (PB) and PB-like chemicals [5]. The cytoplasmic CAR retention protein (CCRP) has been reported to maintain CAR in the cytoplasm by forming a complex with CAR and heat shock protein 90 [6,7]. Following nuclear translocation, CAR binds to response elements in the promoter regions of the target genes, forming a heterodimer with retinoid X receptor alpha.

Inducer-mediated nuclear translocation of this type is not observed in transformed cell lines such as HepG2 and HEK293 because exogenously expressed CAR translocates spontaneously to the nucleus in the absence of PB-type inducers [8]. This provides a tool for identifying the signal sequences in CAR that direct its nuclear translocation. In this way, a leucine-rich sequence near the C-terminal region of human CAR (hCAR), called the xenochemical response signal (XRS), was identified as a motif essential for the PB-mediated nuclear translocation of CAR in mouse liver. The XRS motif, however, does a not contain a classical nuclear localization signal (NLS) [9]. By

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transiently transfecting rat liver RL-34 cells with various mutated rat CAR (rCAR) segments, we identified two nuclear localization signals: NLS1, a basic amino acid-rich sequence (RRARQARRR) within the hinge region between amino acid residues 100 to 108; and NLS2, an assembly of noncontiguous amino acid residues widely spread between residues 111 and 320 within the ligand-binding domain (LBD) [10]. Simultaneously, using rat primary hepatocytes, we also identified a sequence required for cytoplasmic retention between positions 220 and 258 in the middle of the LBD, which we termed the cytoplasmic retention region (CRR). The leucine-rich segment corresponding to the previously reported human XRS in the proximal C-terminal end, however, did not act as a functional NLS in RL-34 cells.

The transcriptional activity of nuclear receptors can be regulated by ligand binding and protein–protein interaction as well as by the modulation of their intracellular localization. The subcellular distribution of nuclear receptors results from the dynamic balance between nuclear import and export; in other words, shuttling between the cytoplasm and nucleus. Although significant progress has been made in understanding the mechanisms underlying nuclear import of NLS-bearing proteins, a general understanding of the nuclear export processes has just begun to emerge. Recently, nuclear export signals (NESs) were reported for several nuclear receptors, including the glucocorticoid, progesterone, and androgen receptors [11–14]; however, the role of NESs in CAR has not been investigated.

The fluorescence recovery after photobleaching (FRAP) assay in multinuclear stage cells is an efficient tool for observing the nucleocytoplasmic shuttling of green fluorescent protein (GFP)-tagged fusion proteins. This method also allows examination of the function of the NES in immortal cells. In the current study, we therefore used FRAP to examine the role of NES in GFP-tagged hCAR and rCAR.

2. Materials and methods

2.1. Cell culture

COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin (50 U/ml)-streptomycin (50 μg/ml) at 37 °C in a humidified atmosphere containing 5% CO_2. Primary hepatocytes were separated from the livers of 7-week-old male Wistar rats (Clea) using the collagenase perfusion method. After enumeration of the cells by Trypan blue dye exclusion, the hepatocytes were plated in collagen gel-coated 4-well Lab-Tek chambered coverglasses (Nunc). The medium was exchanged with HepatoZYME-SFM (GIBCO) 4 h after plating.

2.2. Plasmids

Various GFP-fused CAR deletion mutants were subcloned into the pEGFP-C1 vector (Clontech) by anchor PCR using pcDNA-rCAR and pcDNA-hCAR as templates, as described previously [15,16]. Site-directed mutagenesis was carried out using a QuickChange Site-directed Mutagenesis Kit (Stratagene) and specific primers.

2.3. Transfection and fluoresce visualization by confocal laser scanning microscopy

Transfection of cells was performed on 4-well Lab-Tek chambered coverglasses (Nunc) using LipofectAMINE™ 2000 (Invitrogen Corp) and 500 ng of the indicated expression construct. Transfection of the primary hepatocytes was also performed the next day after plating. After an overnight incubation, the expression profile of individual proteins was observed using a Zeiss LSM 510 (Carl Zeiss) confocal laser scanning microscope. Cytoplasmic fluorescence was quantified using Scion Image (Scion Corporation).

2.4. FRAP assay

FRAP assays were carried out according to published procedures [17] with some modifications. Briefly, COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were seeded on 4-well Lab-Tek chambered coverglasses and transfected with 500 ng of the indicated expression construct using LipofectAMINE™ 2000 (Invitrogen Corp). Following overnight incubation in Opti-MEM™ low serum medium (Invitrogen Corp), 20 μg/ml cycloheximide and 15 μg/ml leptomycin B were then added. After 1 h, the FRAP assay was performed, using confocal laser scanning microscopy to visualize the cells. Except for in the donor nuclei region, the fluorescence was photobleached using 30–40 rapid laser pulses at full power.

3. Results

3.1. Nucleocytoplasmic shuttling of rCAR and hCAR

Typical nucleocytoplasmic shuttling proteins have both a NLS and a NES. Recent results reveal that nuclear receptors are not statically bound to DNA at promoter sites of target genes but rather are highly mobile proteins that continuously shuttle between the cytoplasm and nucleus. Therefore, we used FRAP to determine whether hCAR and rCAR behave as shuttling proteins in COS-7 cells. We used a laser to irreversibly photobleach one nucleus in fluorescently stained, multinucleated COS-7 cells that had been transfected with either GFP-rCAR or GFP-hCAR. Because polyethylene glycol (PEG)-mediated cell fusion causes artificial nuclear export by accelerating calreticulin-mediated nuclear export of the glucocorticoid receptor [17], we applied the FRAP assay to naturally occurring rather than PEG-fused multinuclear cells. Images were captured at several time points to follow the appearance of fluorescence in the bleached (acceptor) nucleus. Recovery of the fluorescence in acceptor nuclei was observed in GFP-rCAR- and GFP-hCAR-transfected cells after 10 and 30 min, respectively, irrespective of the presence of leptomycin B, which is a specific inhibitor of CRM1 nuclear export receptor for leucine-rich NESs [18,19] (Fig. 1). Furthermore, the recovery of fluorescence in bleached nuclei was accompanied by a simultaneous decrease in the unbleached (donor) nuclei even in the presence of leptomycin B, suggesting nucleocytoplasmic shuttling in the presence of NLSs and at least one CRM1-independent NES for both rCAR and hCAR.

3.2. Difference in the function of NLSs between hCAR and rCAR

We identified two NLSs in rCAR: NLS1, which lies in the hinge region, and NSL2, which lies in the LBD. Additionally, the C-terminal leucine-rich amino acid sequence corresponding to the xenochemical response signal (XRS) was identified in both hCAR and mouse CAR (mCAR) [5,20]. We have also

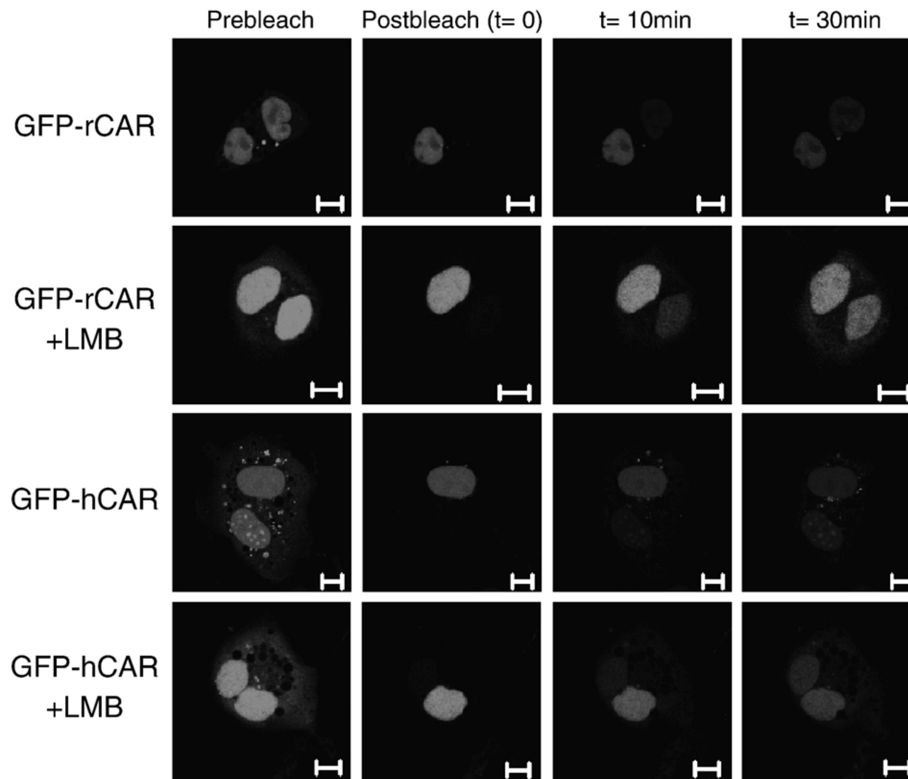

Fig. 1. Internuclear transfer of GFP-tagged rCAR and hCAR in COS-7 cells. Migration of GFP-tagged rat and human CARs from donor (untreated) to acceptor (photobleached) nuclei was observed by FRAP using confocal laser scanning microscopy in multinucleated COS-7 cells transfected with plasmids for expressing individual GFP-CARs. GFP-CAR-expressing multinucleated cells were treated with cycloheximide in the absence (−) or presence (+) of leptomycin B (LMB) for 1 h prior to the FRAP assay. Internuclear transfer of GFP-CAR was observed over a 30-min time course. Bar: 10 μm.

found that deletion of NLS1 or mutation of the XRS blocks the PB-dependent nuclear transport in rat primary hepatocytes [10]. Also, in the absence of PB, GFP-rCAR(1–358) and its deletion mutant GFP-rCAR(1–258) localize in the cytoplasm of rat primary hepatocytes, whereas GFP-rCAR(1–220) and GFP-rCAR(1–110) are mostly localized in the nucleus. In contrast, in RL-34 cells, all of the deletion mutants were confined to the nucleus.

To compare the intracellular localization of hCAR and rCAR, we designed plasmids for expressing GFP fusions of various hCAR (348 amino acids) truncation mutants. We then compared their localization with the corresponding GFP fusions of rCAR (358 amino acids) mutants in primary rat hepatocytes and COS-7 cells (Fig. 2A). Because rat CAR (rCAR; 358 amino acids) has 10 extra residues at its N-terminus compared with human CAR (hCAR; 348 residues), the amino acid residues in hCAR were renumbered 11 to 358 to facilitate comparison with those in rCAR. GFP-fused hCAR and its truncated mutants were intracellularly localized in both rat primary hepatocytes and COS-7 cells. Notably, all of the C-terminally truncated mutants were located in the cytoplasm in rat primary hepatocytes (Fig. 2B). This is in marked contrast with our previous finding that GFP-rCAR(1–220) and GFP-rCAR(1–110) localize in the nucleus [10].

Because the results in COS-7 cells indicate that both rCAR and hCAR are nucleocytoplasmic shuttling proteins, we next determined the location of the NLS and NES sequences in hCAR. For comparison, the NLS, XRS, and CRR sequences in rCAR are summarized in Fig. 2A. We previously identified two NLSs in rCAR by transfecting immortal cells with various GFP-tagged variants, but we failed to prove that the XRS is a NLS [10]. We first examined the subcellular localization of GFP-hCAR and GFP-rCAR and their truncated variants in COS-7 cells (Fig. 2C). GFP-wild-type hCAR and GFP-wild-type rCAR were both localized in the nuclei of COS-7 cells. GFP-rCAR(1–110) was also found in the nuclei, whereas GFP-hCAR(11–110) was found throughout the cytoplasm and nuclei, suggesting that hCAR lacks a homologue of NLS1. GFP-tagged hCAR(110–320), which corresponds to NLS2 in rCAR, was localized in the nucleus, but this was not the case for the other GFP-tagged C-terminally truncated variants, namely, GFP-hCAR(11–110), GFP-hCAR(11–220), and GFP-hCAR(11–258), demonstrating that hCAR contains a functional NLS2 between residues 110 and 320 (Fig. 2C). The distribution of these truncated variants was similar in RL-34 cells (data not shown).

Next, we analyzed which amino acid changes are responsible for the loss of a functional NLS1 in hCAR. The amino acid sequences for the region corresponding to NLS1 in rCAR, hCAR, and mCAR (358 amino acids) are compared in Fig. 3A. Arginine (Arg^{106}), which is part of the monopartite basic amino acid-rich NLS1 in rCAR, is replaced by glutamine (Gln^{106}) in hCAR and mCAR. Therefore, we designed plasmids encoding for GFP-hCAR(11–358) and GFP-hCAR(11–110), wherein Gln^{106} was replaced by arginine (Q106R) and GFP-rCAR

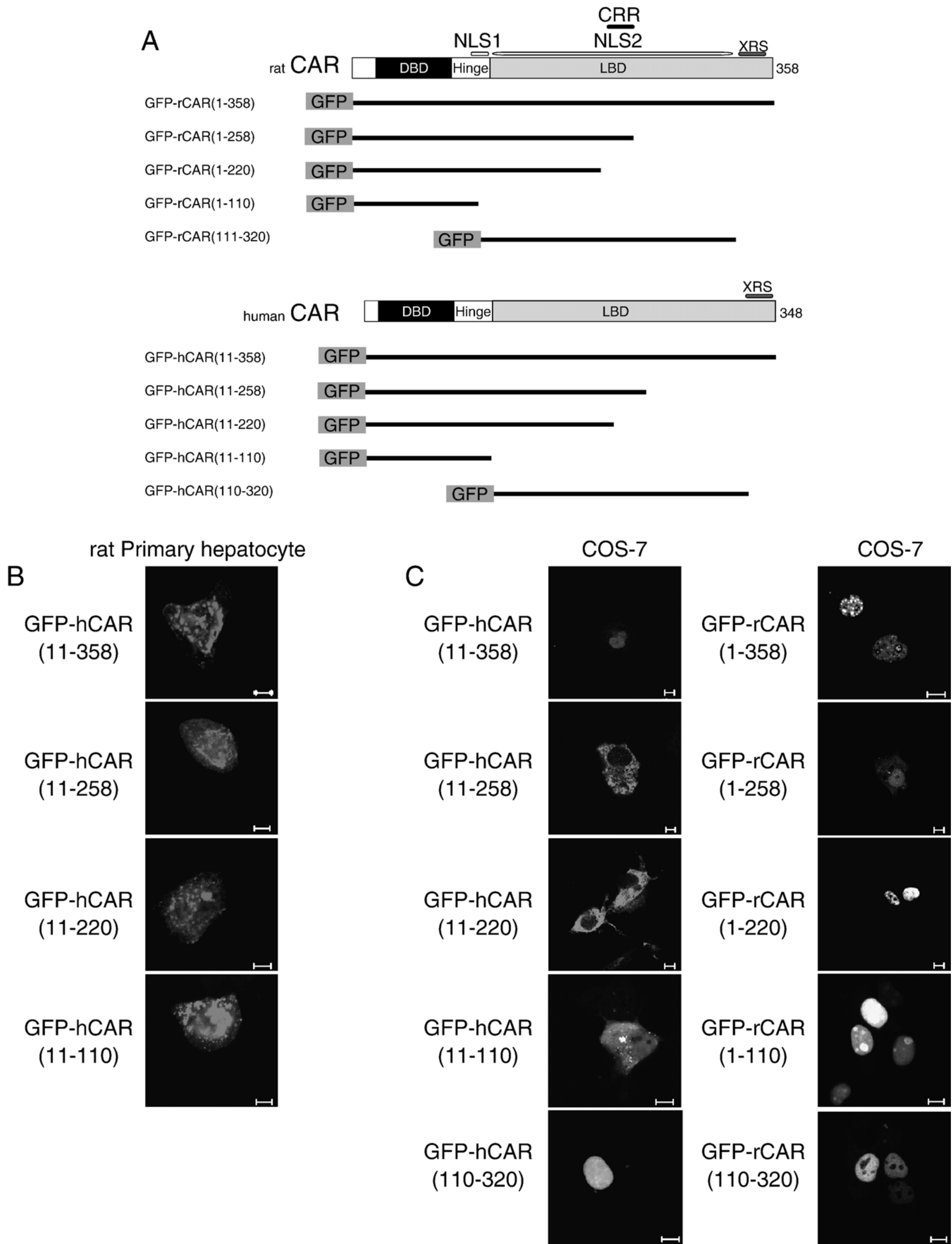

Fig. 2. Subcellular distribution of GFP-CAR deletion mutants. (A) Schematic illustrations of the hCAR deletion mutants. (B) Rat primary hepatocytes were transfected with expression plasmids for individual GFP-hCAR deletion mutants. Twenty-four hours after transfection, cells were observed with a confocal laser-scanning microscope. Bar: 10 μm. (C) COS-7 cells transfected with expression plasmids for individual GFP-tagged CAR deletion mutants were observed by confocal laser scanning microscopy 24 h after transfection.

(1–110) in which Arg[106] was replaced by glutamine (R106Q) (Fig. 3A). GFP-hCAR(11–110; Q106R) but not GFP-rCAR (1–110, R106Q) was localized in the nuclear compartment in transfected COS-7 cells (Fig. 3B), indicating that the single amino acid change causes the difference in the function of the NLS1 between rCAR and both hCAR and mCAR.

3.3. A novel species-specific CRM1-dependent NES in hCAR

We found that GFP-hCAR(11–220) localized in the cytoplasm, whereas GFP-hCAR(11–110) was spread throughout the cell (Fig. 2C). Thus, we suspected that an amino acid cluster between residues 110 and 220 harbors a functional NES. To narrow the functional region, we designed several plasmids encoding fusions of GFP and hCAR segments between residues 110 and 220. GFP-hCAR(110–220) and GFP-hCAR(170–220) were present in the cytoplasm, but GFP-hCAR(200–220) and GFP-hCAR(170–205) were distributed evenly in the cytoplasm and nuclei (Fig. 4A), suggesting that the NES function in hCAR is mediated by an amino acid cluster between residues 170 and 220. In contrast, GFP-rCAR(170–220) did not appear to have a functional NES (Fig. 4A).

We found that the nuclear-to-cytoplasmic trafficking of GFP-hCAR(170–220) is hampered in the presence of leptomycin B (Fig. 4B), suggesting that the NES in hCAR is CRM1-dependent. We compared this sequence, which we named NES1, with the corresponding sequence in rCAR. There were seven amino acid differences between the two sequences (asterisks in Fig. 4C). The leptomycin B-sensitive leucine-rich NES consensus sequence is generally considered to be L-x(2,3)-[L/I/V/F/M]-x(2,3)-L-x-[L/I], where x is any amino acid [21]. Therefore, of these seven amino acids, we focused on the three hydrophobic amino acids in the hCAR NES (closed circles in Fig. 4C). These amino acids in GFP-hCAR(170–220) were individually mutated to those in rCAR. Of the three mutations, L180Q, I197M, and V215S, only

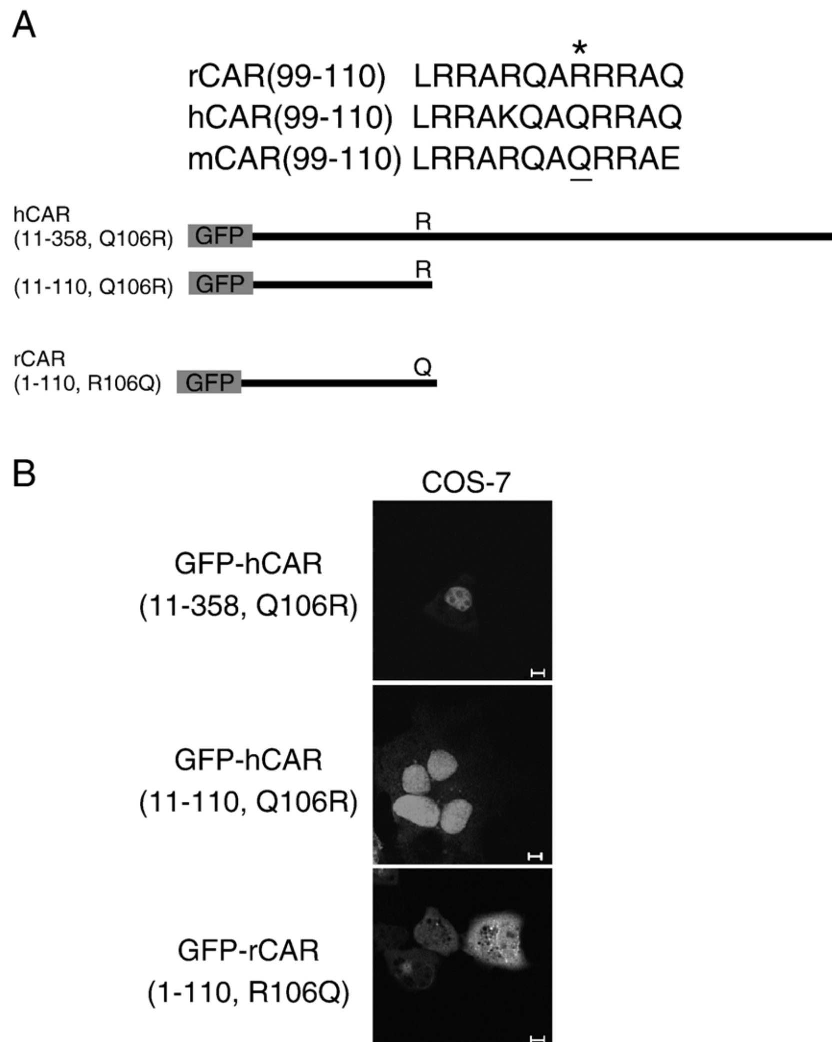

Fig. 3. Gln[106] is responsible for the lack of a functional NLS1 in hCAR. (A) Alignment and comparison of the amino acid sequences of rCAR NLS1 and the corresponding regions of hCAR and mCAR (top). Schematic illustrations for GFP-hCAR(Q106R) and its deletion mutant GFP-hCAR(11–110; Q106R) as well as GFP-rCAR(1–110; R106Q) (bottom). Amino acids that differ between hCAR and rCAR are indicated with asterisks. (B) COS-7 cells were transfected with plasmids for expressing individual GFP-CAR mutants. Twenty-four hours after transfection, cells were observed by confocal laser scanning microscopy. Fluorescence micrographs are shown. Bar: 10 μm.

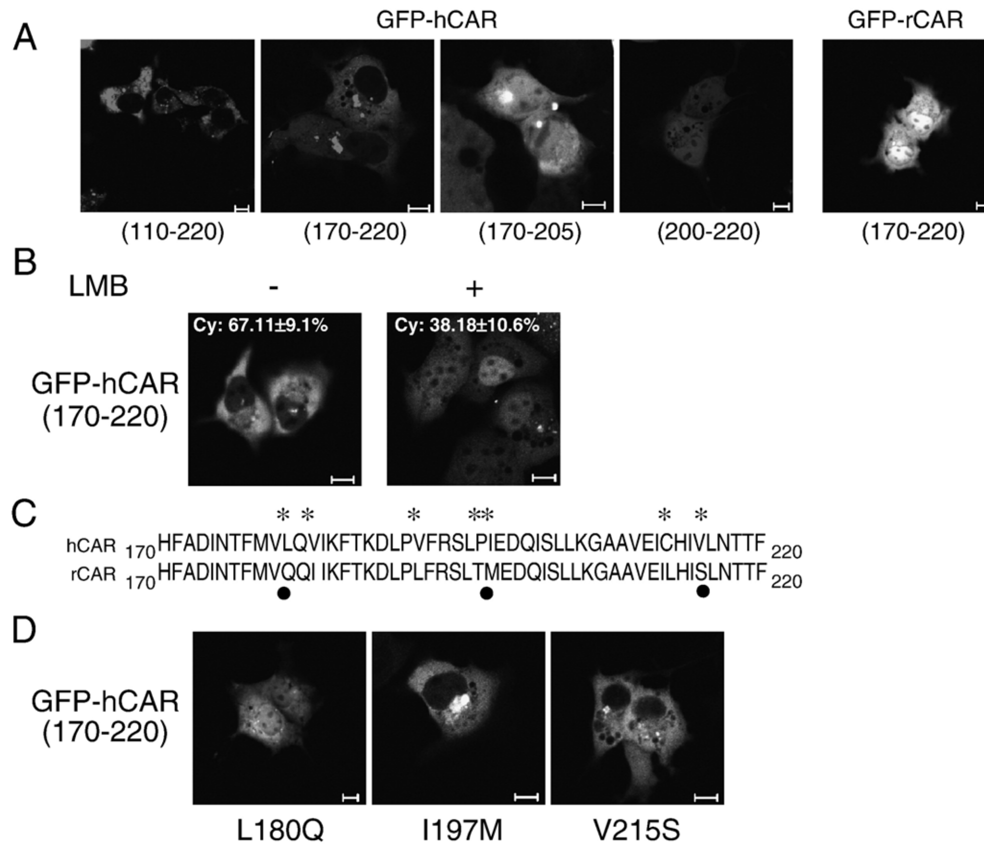

Fig. 4. Identification of a CRM1-dependent NES in the hCAR LBD. (A) COS-7 cells were transfected with plasmids for expressing individual GFP-tagged CAR segments. Twenty-four hours after transfection, cells were observed by confocal laser scanning microscopy. Fluorescence micrographs are shown. Bar: 10 μm. (B) COS-7 cells were transfected with plasmids for expressing GFP-tagged hCAR(170–220). Twenty hours after transfection, cells were incubated for 4 h in the absence (−) or presence (+) of leptomycin B (LMB) prior to observation by confocal laser scanning microscopy. The percent cytoplasmic fluorescence intensity was calculated as follows: cytoplasmic fluorescence intensity / (nuclear fluorescence intensity + cytoplasmic fluorescence intensity) × 100. (C) The amino acid sequence alignments of the putative NES of hCAR (positions 170–220) and the corresponding segment of rCAR(170–220) are compared, and amino acid differences are indicated with asterisks. Replacements of hydrophobic amino acid residues by other amino acid types are indicated with closed circles. (D) COS-7 cells were transfected with plasmids for expressing individual GFP-hCAR(170-220) amino acid-substituted mutants.

L180Q caused a change in the intracellular distribution of hCAR (Fig. 4D). In addition, the subcellular distribution of GFP-hCAR (170–220) was not altered by V182I, V190L, P196T, and C21 2L mutations (data not shown). Based on these observations, it appears that Leu^{180} in hCAR, which is replaced by Gln^{180} in rCAR, is responsible for the species-specific acquisition of CRM1-dependent NES1.

3.4. The CRR in rCAR contains the species-specific CRM1-dependent NES

We previously identified the CRR in rCAR as a sequence required for the cytoplasmic retention of CAR in primary hepatocytes but not in immortal cells [10]. The CRR in rCAR (residues 220–258) contained a leucine-rich NES consensus sequence (Fig. 5A). The last amino acid of the putative NES in rCAR (Ile) was replaced with Phe in the corresponding sequence of hCAR. Therefore, we examined the ability of these leucine-rich sequences in rat and human CARs to act as NESs. In COS-7 cells transfected with plasmids encoding CRR-containing GFP-rCAR(220–258), the fluorescence was confined to the cytoplasm in the absence of leptomycin B and moderately accumulated in the nuclei in its presence (Fig. 5B). In contrast, both GFP-rCAR(220–258; I253A) and GFP-rCAR (220–258; I253F) were distributed throughout the cell in the absence of leptomycin B (Fig. 5C). These results suggest that a CRM1-dependent NES, which we named NES2, lies within the previously reported CRR of rCAR but not in hCAR.

3.5. CRM1-independent NES in the C-terminal region of human and rat CARs

We previously reported that GFP-CAR(317–358), which contains the C-terminal region of rCAR including the XRS, is localized in the cytoplasm of rat hepatocyte-derived RL-34 cells [10]. We therefore examined the role of the 42 C-terminal amino acids of hCAR and rCAR in the intracellular localization in COS-7 cells. Both GFP-rCAR(317–358) and GFP-hCAR(317–358) were predominantly localized in the cytoplasm (Fig. 6A). To characterize this in more detail, we generated plasmids for expressing GFP-rCAR(317–358) and GFP-hCAR(317–358) truncated from both the C- and N-terminal ends. Unlike GFP-rCAR(317–358), all truncated variants of rCAR failed to localize in the cytoplasm of COS-7 cells (Fig. 6B), suggesting that

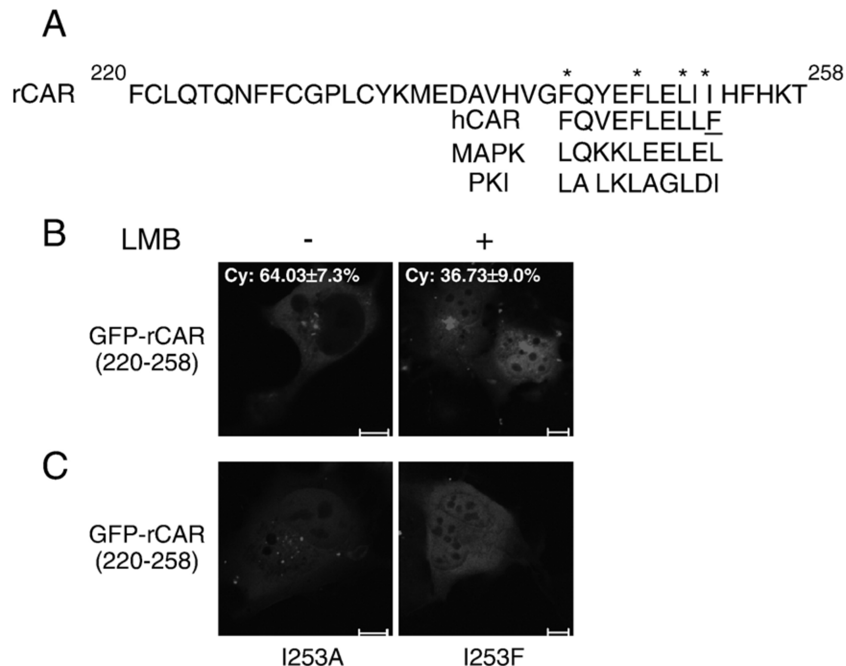

Fig. 5. Identification of the CRM1-dependent NES within the CRR of the rCAR LBD. (A) Comparison of the amino acid sequence alignments of CRR in rCAR, the corresponding region of hCAR, and known leucine-rich NESs of mitogen activated protein kinase (MAPK) and protein kinase inhibitor (PKI). (B) COS-7 cells were transfected with plasmids for expressing individual GFP-tagged rCAR (220–258). Twenty hours after transfection, cells were incubated for 4 h in the absence (−) or presence (+) of leptomycin B (LMB). Cells were observed by confocal laser scanning microscopy. Fluorescence micrographs are shown. Bar: 10 μm. The percent cytoplasmic fluorescence intensity was calculated as described in Fig. 4. (C) COS-7 cells were transfected with plasmids for expressing individual GFP-rCAR(220–258) amino acid-substituted mutants.

the entire sequence between residues 317 and 358 is required for the function of the NES.

We further investigated the contribution of the activation function (AF)-2 and XRS subdomains in the NES function of the C-terminal region. Variants of GFP-rCAR(317–358) in which three Leu/Ile residues in AF-2 were individually replaced with Ala remained in the cytoplasm, whereas a double mutation in the XRS, M323A/L326A, eliminated the NES function of the C-terminal region (Fig. 6C). In addition, the subcellular localization of GFP-rCAR(317–358) was unaffected by leptomycin B, indicating that rCAR(317–358) contains a CRM1-independent NES (Fig. 6D). Similarly, the NES function of the C-terminal region of hCAR, hCAR(317–358), was CRM1-independent (data not shown).

To further characterize the NESs that we identified in rCAR, we designed additional GFP-rCAR deletion mutants and analyzed their internuclear transport by FRAP in the presence or absence of leptomycin B. Both GFP-rCAR(1–320) and GFP-rCAR(111–320) showed internuclear trafficking only in the absence of leptomycin B, reflecting the CRM1 dependence of the NES within the CRR (Fig. 7). Therefore, the internuclear trafficking of GFP-rCAR and GFP-rCAR(111–358) in the presence of leptomycin B can be attributed to the function of the C-terminal NES (Figs. 1 and 7).

3.6. The XRS motif controls NLS2 activity

Mutation of the XRS motif has been shown to eliminate the PB-induced nuclear translocation of hCAR and mCAR in mouse livers in vivo and rCAR in rat primary hepatocytes in vitro [9,10,20]. In contrast, both hCAR and rCAR spontaneously translocate into the nuclei of various immortal cells. When the XRS was altered by site-directed mutagenesis, however, GFP-hCAR but not GFP-rCAR becomes confined to the cytoplasm. In immortal cells, the NLS1 of rCAR may be responsible for difference between the species because the intracellular behavior of GFP-rCAR(111–358) resembled that of GFP-hCAR (Fig. 8A). Because GFP-rCAR(140–358), another N-terminally truncated mutant lacking both NLS1 and NLS2, was localized in the cytoplasm (Fig. 8B), the spontaneous nuclear translocation of GFP-rCAR(111–358) and GFP-hCAR appears to be due to the function of NLS2 in the LBD. Thus, it seems that the XRS must be intact for the function of NLS2 in COS-7 cells. GFP-rCAR containing a mutated XRS motif did not undergo internuclear translocation in the presence of leptomycin B in COS-7 cells (Fig. 8C), indicating that mutagenesis of the XRS must eliminate the function of not only NLS2 but also the C-terminal CRM1-independent NES.

4. Discussion

Previously, we reported that rCAR has two NLSs. The first, NSL1, is a monopartite NLS consisting of a basic amino acid-rich sequence between residues 100 and 108 in the hinge region. The second, NSL2, is made up of noncontiguous residues widely spread between residues 111 to 320 in the LBD, the CRR, which is responsible for the cytoplasmic retention of

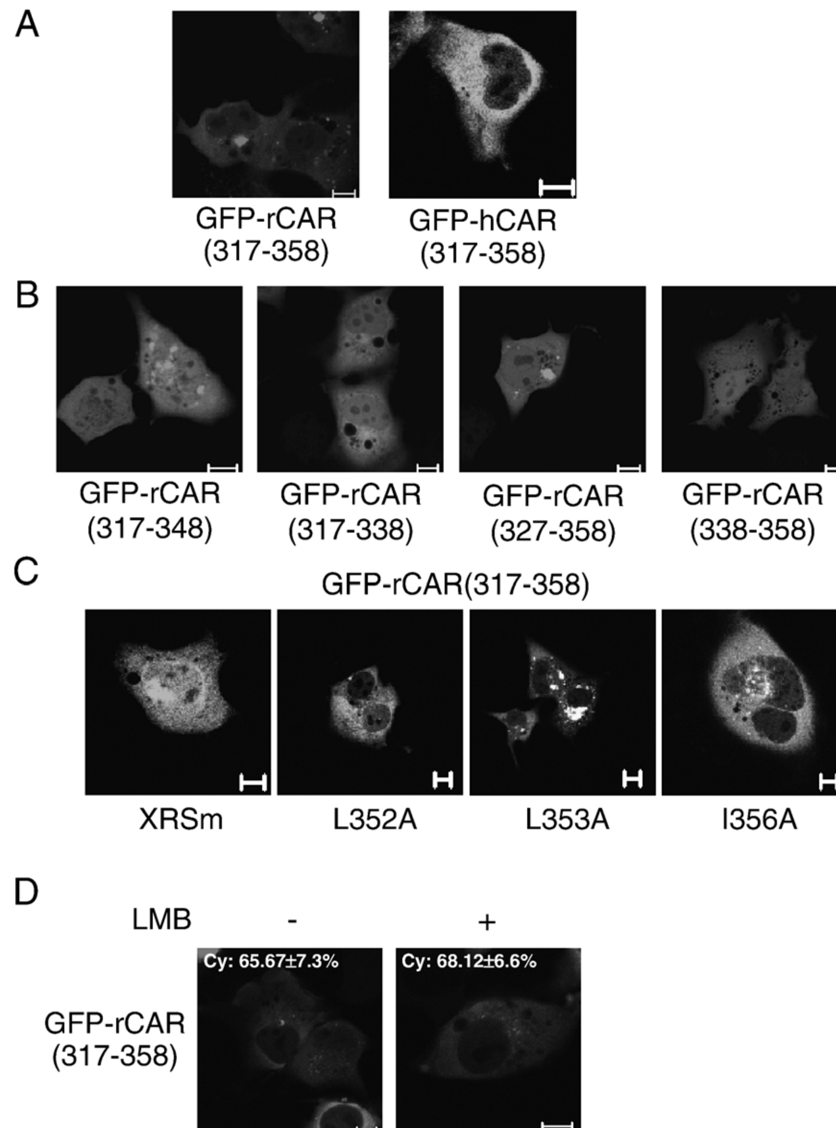

Fig. 6. Identification of shared C-terminal NES encompassing individual XRS motifs of both hCAR and rCAR. (A and B) COS-7 cells were transfected with expression plasmids encoding individual GFP-tagged CAR C-terminal segments. Twenty-four hours after transfection, cells were observed by confocal laser scanning microscopy. Fluorescence micrographs are shown. Bar: 10 μm. (C) COS-7 cells were transfected with plasmids for expressing individual amino acid-substituted mutants of GFP-rCAR(317–358). Twenty-four hours after transfection, cells were observed by confocal laser scanning microscopy. (D) COS-7 cells were transfected with expression plasmids encoding GFP-tagged rCAR(317–358). Twenty hours after transfection, cells were incubated for 4 h in the absence (−) or presence (+) of leptomycin B (LMB) prior to observation by confocal laser scanning microscopy. The percent cytoplasmic fluorescence intensity was calculated as described in Fig. 4.

rCAR in RL-34 cells, and the XRS in the C-terminal end of hCAR [9,10].

In the present study, we showed that there is a difference in the intracellular localization of hCAR and rCAR in COS-7 cells. This difference was due to the presence of a functional NLS1 in rCAR but not hCAR because the latter has an arginine at residue 106. An N-terminal portion of hCAR covering the DNA-binding domain and hinge region, hCAR(11–110), acted as a NLS similar to rCAR(1–110) when Gln^{106} was replaced with arginine. This arginine is replaced by other amino acids in mCAR and hCAR as well as in CARs from monkey and northern fur seal. Thus, NLS1 might be exclusive to rCAR (Fig. 9).

We identified a CRM1-dependent NES1 between residues 170 and 220 in hCAR but not in the corresponding sequence of rCAR (Fig. 4A, B). There are seven amino acid replacements between hCAR and rCAR. Of these, only the L180Q mutation was found to be involved in the lack of NES function in hCAR (170–220). We found another CRM1-dependent NES, which we named NES2, in rCAR at residues 244–253 in the CRR but not in the corresponding region of hCAR. Ile^{253} in rCAR NES2 was replaced by phenylalanine in hCAR, and changing Ile^{253} to phenylalanine in rCAR eliminates its NES function (Fig. 5B).

Mutation of the XRS prevents PB-dependent nuclear transport of hCAR in livers in vivo [9] and PB-independent nuclear localization of mCAR via p160 coactivator glucocorti-

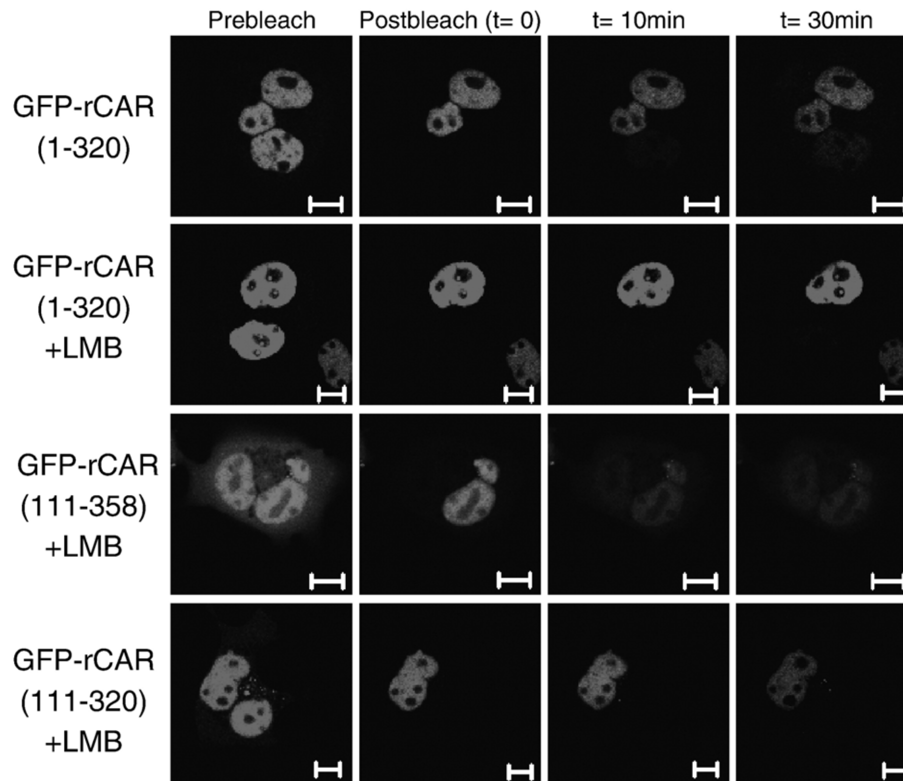

Fig. 7. Analysis of internuclear transfer of rCAR deletion mutants by FRAP. Migration of GFP-tagged rat CAR deletion mutants from donor to acceptor nuclei was observed using FRAP in transfected multinucleated COS-7 cells. Transfected cells were treated with cycloheximide in the absence (−) or presence (+) of leptomycin B for 1 h prior to FRAP assay. Transfer was observed over a 30-min period in COS-7 cells. Fluorescence micrographs are shown. Bar: 10 μm.

coid receptor interacting protein-1 in livers and in immortal cells [20]. Based on these observations, XRS appears to be a novel type of NLS; however, we also found a functional NES in the very C-terminal portion (residues 317–358) of rCAR in immortal cells.

Black et al. identified a calreticulin-dependent NES within the DNA-binding domain of divergent nuclear receptors [14] that was responsive to transient disruption of the endoplasmic reticulum that occurs during PEG-mediated cell fusion [17]. In the current study, however, nucleocytoplasmic shuttling of a rCAR mutant in which both the NES2 and terminal NES were inactivated was not detected after 30 min in the FRAP assay, implying that the calreticulin-dependent NES is artificial [14,17]. On the basis of the available information, we suggest the following hypothesis. It appears that CAR is active in immortal cells in vitro, even in the absence of an activator such as PB, whereas in the liver in vivo or in primary hepatocytes in vitro, the nuclear localization of CAR requires activation by PB or PB-like compounds. In livers and primary hepatocytes, where NLS1 may not function, NLS2 may be masked by unknown mechanisms that involve the XRS. XRS, a subdomain of the C-terminal leptomycin B-insensitive NES, may negatively regulate the function of the adjacent NLS2 in livers and primary hepatocytes. In the presence of PB-like activators, following suppression 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 in an active form even in the absence of PB-like substances, causing its residence in the nuclei. As indicated by the nuclear localization of the C-terminally truncated mutant GFP-rCAR(111–320) in COS-7 cells (Fig. 7), CAR with a mutated XRS would be in a state similar to inactive CAR in livers and primary hepatocytes and therefore remain in the cytoplasm in both in vivo and in vitro systems due to the anchoring by the mutated protein. Thus, when truncated, the C-terminal region behaved as if it were a NES in immortal cells [10], whereas in livers, it acted as a PB-responsive NLS [9], negatively regulating NLS2 function.

Identification of a masking protein in immortal cells would support our hypothesis. CCRP is a candidate for this NLS2-masking protein. In the presence of PB in vivo or in its absence in vitro, p160 coactivator glucocorticoid receptor interacting protein-1 (GRIP1) might replace the masking protein on the XRS, releasing the masking protein from CAR or acting as a NLS only when bound to CAR.

The pregnane and xenobiotic receptor (PXR), which is closely related to CAR, is retained in the cytoplasm of hepatic cells of untreated mice, whereas it is translocated to the nucleus after administration of PCN [21]. Furthermore, PXR is reported to undergo spontaneous nuclear translocation in HepG2, COS-1, and HeLa cells [22,23]. Immunoprecipitation studies with an antibody to heat shock protein 90 have shown that PXR forms a complex with endogenous CCRP in HepG2 cells. Also, fluore-

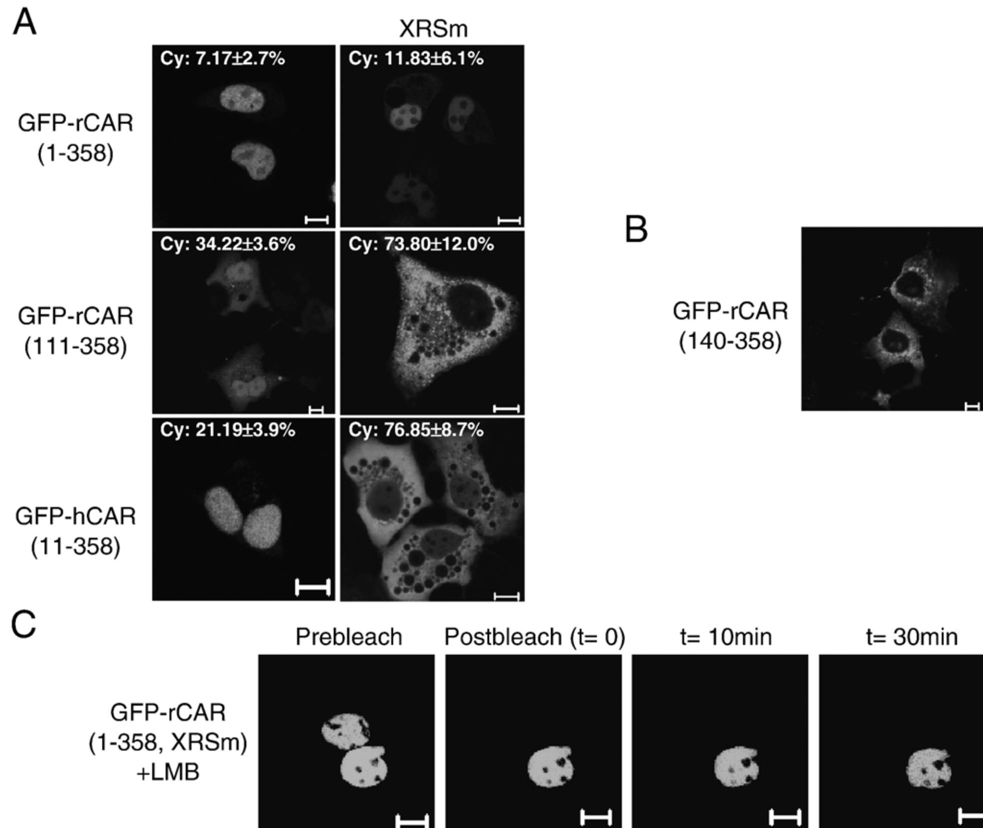

Fig. 8. Functional regulation of NLS2 by XRS motifs of human and rat CARs. (A) COS-7 cells were transfected with plasmids encoding for expressing XRS-mutated GFP-CARs and N-terminally truncated rCAR segments lacking NLS1 function. Twenty-four hours after transfection, cells were observed by confocal laser scanning microscopy. Fluorescence micrographs are shown. Bar: 10 μm. The percent cytoplasmic fluorescence intensity was calculated as described in Fig. 4. (B) Also, COS-7 cells transfected with plasmids for expressing GFP-tagged rCAR deletion mutants lacking both NLS1 and NLS2 functions were observed. (C) Migration from donor to acceptor nuclei by a GFP-rCAR XRS mutant was observed in transfected multinucleated COS-7 cells using FRAP. Transfected cells were treated with cycloheximide in the absence (−) or presence (+) of leptomycin B for 1 h prior to the FRAP assay. Transfer was observed over a 30-min period in COS-7 cells. XRSm, mutation of xenochemical response signal (M323A/L326A).

scence resonance transfer studies show that cyan fluorescence protein-tagged CCRP and yellow fluorescence protein-tagged PXR closely associate in mouse liver [24]; however, the co-expression of CCRP and PXR does not cause obvious nuclear localization of GFP-PXR in Cos-7, HepG2, or NIH/3T3 cells [22]. Furthermore, we have found that both rCAR and hCAR

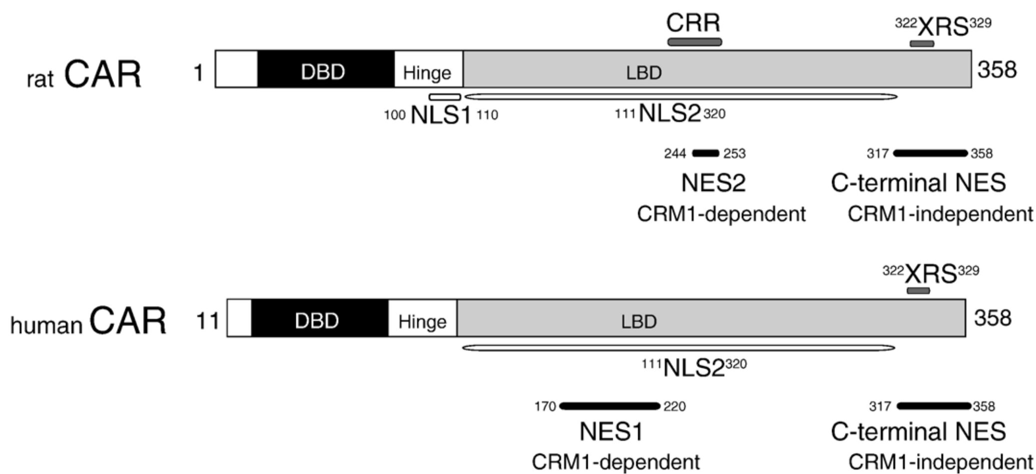

Fig. 9. Schematic illustration of the difference in intracellular localization signals between rCAR and hCAR. Diagrams show the full length of rCAR and hCAR (358 and 348 amino acids, respectively), with the positions of the DNA-binding domain (DBD), hinge region (Hinge), and LBD. Bars indicate the nuclear localization signals (rat NLS1 and common NLS2s) (ref. [10] and present study), nuclear export signals (human NES1, rat NES2, and common C-terminal NESs) (present study), rat CRR (10), and common XRSs (9, 10).

interact with rat CCRP in cotransfected COS-7 cells without changing the nuclear localization profile of CAR (our unpublished observations). On the basis of these results, we propose that CCRP does not play a key role in a spontaneous nuclear translocation of CAR and PXR. Because PXR is not responsive to PB [25], the mechanisms mediating spontaneous nuclear translocation of CAR and PXR in immortalized cells might be different than those mediating its PB-induced nuclear translocation in mouse liver.

Sueyoshi et al. have reported that GFP-tagged truncated hCAR, GFP-hCAR(181–348), is retained in the cytoplasm and accumulates in the nucleus after chlorpromazine treatment [26], indicating that NLS2 is not required for chlorpromazine-dependent nuclear transportation in mouse liver in vivo. Upon activation, coactivators with a functional NLS, which are lacking in immortal cells, might be recruited to the XRS.

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