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Pregnane X Receptor (PXR), Constitutive Androstane Receptor (CAR), and Benzoate X Receptor (BXR) Define Three Pharmacologically Distinct Classes of Nuclear Receptors

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The NR1I subfamily of nuclear receptors contains a phylogenetically diverse array of receptors related to the mammalian pregnane X receptor (PXR) (NR1I2) and constitutive androstane receptor (CAR) (NR1I3). We have carried out an extensive comparative analysis of this subgroup with representatives from fish, birds, amphibians, and mammals. Four novel receptors were isolated from fish, dog, pig, and monkey for this study and combined with a previously reported set of related receptors including human PXR, rabbit PXR, mouse PXR, chicken CXR, frog benzoate X receptors (BXRα, BXRβ), and human and mouse CAR. A broad range of xenobiotics, steroids, and bile acids were tested for their ability to activate the ligand binding domain of each receptor. Three distinct groups of receptors were identified based on their pharmacological profiles: 1) the PXRs were activated by a broad range of xenobiotics and, along with the mammalian PXRs, included the chicken and fish receptors; 2) the CARs were less promiscuous, had high basal activities, and were generally repressed rather than activated by those compounds that modulated their activity; and 3) the BXRs were selectively activated by a subset of benzoate analogs and are likely to be specialized receptors for this chemical class of ligands. The PXRs are differentiated from the other NR1I receptors by a stretch of amino acids between helices 1 and 3, which we designate the H1–3 insert. This insert was present in the mammalian, chicken, and fish PXRs but absent in the CARs and BXRs. Modeling studies suggest that the H1–3 insert contributes to the promiscuity of the PXRs by facilitating the unwinding of helices-6 and -7, thereby expanding the ligand binding pocket. (*Molecular Endocrinology* 16: 977–986, 2002)

THE NR1I SUBFAMILY includes the VDR, pregnane X receptors (PXRs), constitutive androstane receptors (CARs), and benzoate X receptors (BXRs) (1, 2). When the ligand binding domains (LBDs) from this subfamily are compared across species, there is an unusually low degree of sequence identity. This marked interspecies sequence variability prevents any obvious classification based on sequence data alone. We have therefore carried out a comprehensive comparative study of this diverse set of receptors utilizing both functional assays and structural analysis to better define their functional and structural relationships. The previously reported differences between the mammalian PXRs (NR1I2) and CARs (NR1I3) serve as the starting point for this comparative study. Though closely related, these two receptors exhibit distinct, but overlapping, pharmacological activation profiles by xenobiotics (3).

The chemical and structural diversity of known PXR activators is remarkable. PXRs have been shown to be activated by various xenobiotics (*e.g.* rifampicin, clotrimazole, the bisphosphonate ester SR12813, hyperforin), natural and synthetic steroids (*e.g.* 5β-pregnane-3, 20-dione, pregnenolone 16α-carbonitrile, dexamethasone), and bile acids (*e.g.* lithocholic acid and 6-keto lithocholic acid) (4–7). Scintillation proximity competition-type assays using human PXR demonstrate that these chemically unrelated compounds compete for binding within the ligand binding pocket, the majority with dissociation constant values in the micromolar range (8). Thus, in contrast to other nuclear receptors that bind one or few ligands with high affinity, PXR has evolved the ability to respond to a diverse set of low affinity ligands. Thus, PXR is a promiscuous xenobiotic receptor that protects the body from chemical insult.

The repertoire of genes activated by PXR is consistent with its role as a xenobiotic, steroid, and bile acid sensor. In response to a diverse array of compounds, PXR coordinately regulates a program of genes involved in the metabolism, transport, and ultimately, elimination of these molecules from the body. Among the genes regulated by PXR are the cytochrome P450 3A (*Cyp3a*) gene (5) whose gene product catalyzes

Abbreviations: BXR, Benzoate X receptor; CAR, constitutive androstane receptor; d(T), deoxythymidine; LBD, ligand binding domain; PXR, pregnane X receptor; TCPOBOP, 1,4-bis[2-(3, 5-dichloropyridyloxy)] benzene.

hydroxylation of a broad range of substrates, rendering these compounds more hydrophilic and hence subject to hepatic clearance (9). PXR also regulates expression of the organic anion transporter protein 2 (6), multidrug resistance protein 1 (10, 11), multidrug resistance related protein 2 (12), and CYP7A1 (6). The sum of these findings supports a general role of PXR in hepatoprotection in response to potentially harmful compounds, both endogenous and exogenous. This model is confirmed in PXR-null mice, which are more sensitive to treatment with xenobiotics and bile acids (6, 13).

The mammalian CAR has also been proposed to function as xenosensor (13). CAR represents the closest mammalian relative of PXR; is activated by some of the same ligands as PXR (3); regulates a subset of common genes, *e.g. CYP3a* and *CYP2b* (14, 15); and can signal through the same signaling pathways (16). Like PXR, CAR displays differences in ligand activation profiles across species (3, 8). Despite these similarities, studies using limited sets of compounds show clear differences between PXR and CAR (3). CAR has been shown to be less promiscuous than PXR (3). Furthermore, CAR displays a high basal level of activity relative to PXR that can be reduced by the binding of either naturally occurring androstanes or xenobiotics such as clotrimazole (3, 17). Finally, CAR displays fundamental differences from PXR with regard to its cellular regulation. In mouse primary hepatocytes and in mouse liver *in vivo*, CAR is cytoplasmic in the naive state and translocates to the nucleus upon activation (18), a process thought to be regulated in part by dephosphorylation of the receptor (19). Induction of CAR nuclear translocation does not necessarily depend upon ligand binding, as phenobarbital has been shown to be an activator of CAR *in vivo* and in hepatocytes, but does not appear to interact directly with the CAR ligand binding domain (3).

Recently, a receptor related to mammalian CAR and PXR was identified in chicken and named CXR (20). Sequence comparison showed that this receptor was roughly equally distant from both the mammalian PXR and CAR receptors. Also, two receptors from *Xenopus laevis*, BXRα (21) and BXRβ (22), have been identified which bear similarity to both the mammalian CARs and PXRs. BXRα was activated by an ethyl benzoate, while potent ligands had not yet been identified for BXRβ. Before the work presented in this study, the relationships between these various receptors and their functional relationship to the mammalian CARs and PXRs was unclear.

In this study, we have cloned and characterized the ligand binding domain (LBD) sequences of four novel PXR-related receptors from monkey, pig, dog, and fish. We have compared the pharmacological activation profiles of these novel receptors with previously characterized PXRs, CARs, and BXRs. Based on structural and functional data, we have separated this set of nuclear receptors into three distinct subfamilies.

RESULTS

Novel PXR LBDs

In an effort to understand the evolution and biological function of the NR1I family of nuclear receptors, we cloned the LBDs of novel members of this family from monkey, pig, dog, and zebrafish and added these to the collection of previously cloned receptors including the human, rabbit, rat, and mouse PXR, the human and mouse CAR, the chicken CXR, and BXRα and BXRβ from *Xenopus laevis*. Alignment of the LBD sequences of NR1I family members revealed that the combined set of LBD sequences have diverged through evolution (Table 1). Even among the mammalian PXR LBDs, the sequence identity is as low as 75%, unusually low for nuclear receptor orthologs. The chicken and fish sequences show only 49% and 52% sequence identity, respectively, with human PXR, and 54% and 44% identity with human CAR. This level of identity is comparable with that between the mammalian PXRs and the mammalian CAR sequences. Construction of a dendogram using the LBD sequences of these receptors revealed that the novel monkey, pig, and dog receptors segregated with the PXRs (Fig. 1). Notably, the novel fish receptor defined a new subgroup among the NR1I receptors in this analysis (Fig. 1). The presence of an extended stretch of amino acids between helices 1 and 3 (designated the helix 1–3 insert, see below) in the fish receptor implicates the progenitor receptor as possessing this sequence. CXR was situated nearly midway between CAR and PXR receptors, but grouped slightly closer to CAR in this analysis, consistent with previous studies (20).

To determine whether characteristics other than sequence homology could be used to subclassify these members of the NR1I family, they were compared in functional assays. Because we had LBD sequences for a large number of the receptors, we used the Gal4 chimera assay to systematically profile the receptors (see *Materials and Methods*). We evaluated this assay using human and mouse PXR, human and mouse CAR, and *Xenopus* BXRα, three receptors with established responses to selected ligands. In our laboratory, we have used both the Gal4 assay and full-length receptor assays extensively to evaluate mammalian PXR LBD sequences and have found that nearly identical results are obtained in both assays (data not shown). Likewise, the *Xenopus* BXRα displayed robust activation by the positive control benzoate ligands (Table 2), as was previously demonstrated in full-length assays (21). When CAR was tested in the Gal4 chimera assay, it had a considerably higher basal activity than in the CAR full-length assays. In the Gal4 chimera assay, the established transrepression effects of androstanol and other compounds shown in Table 2 on human and mouse CAR were observed, but not the robust agonist effects of 1,4-bis[2-(3, 5-dichloropyridyloxy)] benzene (TCPOBOP) on mouse CAR (data

Table 1. Percent Sequence Identity of PXR/CAR/BXR LBD

	hPXR	rhPXR	pgPXR	dgPXR	rbPXR	mPXR	rPXR	CXR	fPXR	xBXRα	xBXRβ	hCAR	hVDR
hPXR	***	96	87	83	82	77	76	49	52	51	51	45	41
rhPXR		***	89	83	83	78	77	49	52	51	51	45	40
pgPXR			***	84	83	78	76	49	51	52	52	43	39
dgPXR				***	78	76	75	49	50	48	49	44	39
rbPXR					***	79	78	49	51	50	51	43	41
mPXR						***	97	49	53	50	51	46	42
rPXR							***	49	53	50	51	46	41
CXR								***	47	42	43	54	39
fPXR									***	46	46	44	40
xBXRα										***	82	39	38
xBXRβ											***	40	39
hCAR												***	37
hVDR													***

Identity (%)

The LBD sequences of the 12 PXR/CAR/BXR-related receptors were compared using the Jotun Hein algorithm. The remaining NR1I family member (VDR) was also included in this analysis for comparison. Species are abbreviated as human (h), rhesus (rh), pig (pg), dog (dg), rabbit (rb), mouse (m), rat (r), fish (f), and *Xenopus* (x).

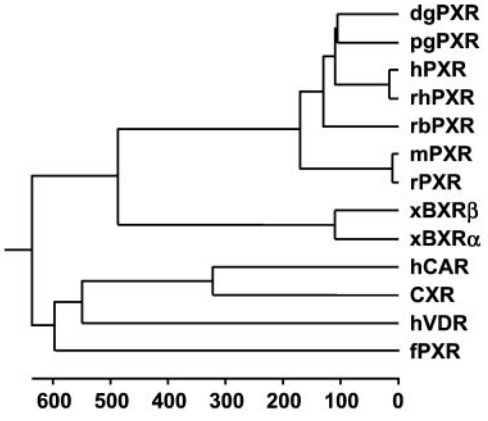

Fig. 1. Phylogenetic Tree

Based on the LBD sequence of each receptor, the phylogenetic relationships of the 12 PXR/CAR/BXR-related receptors were derived by the Juton Hein method. VDR was included for comparison. *Scale* indicates number of amino acid substitution events.

not shown). Because the robust agonist effects of TCPOBOP on mouse CAR was only seen in the full-length assay, the full-length assay was substituted for the Gal4 chimera assay for the CAR receptors.

Using appropriate transient transfection assay formats, expression constructs were tested against broad panels of relevant ligands including ten xenobiotics, eleven natural and synthetic steroids, nine bile acids, and five benzoates (Table 2). We defined activators as those compounds that modulate receptor activity at least 2.5-fold relative to vehicle treatment. Whereas only agonist activity was detected with most of the receptors, CAR could be either activated (transactivation) or deactivated (transrepression) because of its high constitutive activity. We defined transrepressors as those compounds that suppressed basal activity by 0.25-fold or more.

The activation profiles of the full panel of receptors is shown in Table 2. A general overview of these data reveals that the receptors fall into three pharmacologically distinct categories, which we term the PXRs, CARs, and BXRs. The PXRs were promiscuous because nearly all were activated by compounds in all four ligand classes. This subgroup included all of the mammalian PXRs, as well as the chicken and fish receptors. The second subgroup, the CARs, was clearly distinct when compared with the PXRs across a broad panel of compounds. Most CAR modulators were transrepressors, and several novel CAR transrepressors were observed among the bile acid and benzoate classes of molecules. The BXRs comprised a third category, distinct from either the CAR or PXR receptors, and were almost exclusively activated by the benzoate class of molecules. A more detailed analysis of these different subclasses of NR1I receptors follows.

The PXRs

Among all of the NR1I receptors, the mammalian PXRs were modulated by the widest range of compounds (Table 2). The human and rhesus PXRs had the most similar profiles, as expected from their high degree of

Table 2. PXR/CAR/BXR-Related Receptor Sequences

	xBXRα	xBXRβ	mCAR	hCAR	fPXR	CXR	mPXR	pgPXR	dgPXR	rbPXR	rhPXR	hPXR
Xenobiotics												
rifampicin	2.6					3.7		110	39	60	4.6	5.8
mevastatin						14	5.4	160	71	99	5.4	7.1
SR12813	11	7.7				17		250	800	130	8.2	10
nifedipine					3.3	16	2.8	12	5.6	26	5.2	4.5
reserpine						8.3			2.7	3.4		8.8
trans-nonaclor			0.32			8.8		92	110	28	2.5	3.6
TCPOBOP			3.5					2.8	5.4		3.2	2.8
hyperforin						3.2		160	440	52	5.2	5.4
phenobarbital			0.37	0.45	3.4	6.8		80	8.8	10	5.7	9.0
clotrimazole				0.37	7.8	8.6		100	170	36	3.8	3.5
Steroids												
dexamethasone						5.9	2.9			29		
dex-*t*-butyl acetate						4.0	6.0	5.9		61	3.2	4.6
17β-estradiol						4.4		2.8	2.6	12	3.1	3.8
RU486							5.6	4.6		40	4.7	6.3
5β-pregnane-3,20-dione					6.0	9.0	3.0	30	16	47	4.3	5.2
progesterone						6.8			3.6	18	3.8	
PCN						2.7	5.0			13	2.7	
6,16-dimethylpregnenolone						12	7.6	17	4.3	43	4.8	4.4
androstanol			0.03	0.64	6.1	8.5	2.8	9.7	12	31	3.1	4.2
DHT					3.0	5.8	2.6			6.8		
DHEA					6.6	7.0					3.6	
Bile Acids												
cholic acid			0.54	0.71						2.5		5.0
lithocholic acid								34	3.7	60		3.6
6-ketolithocholic acid			0.55	0.71			3.4			44		
12-ketolithocholic acid			0.70					20	3.8	22	4.9	4.3
dehydrolithocholic acid								79	9.4	46	2.6	5.5
taurocholanic acid			0.56	0.70				6.9	9.9	16		
3,7-diketocholanic acid			0.59					150	5.5	71	4.5	4.6
7-ketodeoxycholic acid										120	3.6	3.5
7-ketoDCA methyl ester			0.45	0.66		3.5		170	100	110	4.1	7.2
Benzoates												
3-aminoethylbenzoate	400					3.1						
ethyl 4-hydroxybenzoate	180	170				3.2						
ethyl 3-hydroxybenzoate	1200	39	0.69									
n-butyl *p*-aminobenzoate	1200	33	0.48	0.51		7.5		9.8	7.7	30	3.9	3.1
n-propyl *p*-hydroxybenzoate	650	180	0.41	0.47	6.1	4.1		3.1	3.5	26	3.2	2.8

Twelve PXR/CAR/BXR-related receptor sequences were tested in transient transfection assays against a panel of 10 xenobiotics, 11 natural and synthetic steroids, 9 bile acids, and 5 benzoates. Xenobiotics and steroids were tested at 10 μM with the exception of hyperforin (1 μM) and phenobarbital (1 mM). Bile acids and benzoates were tested at 100 μM. Effects of the ligands were expressed as fold-activation relative to a vehicle (dimethylsulfoxide) control. Values represent averages (n = 4) and SE were all less than 10%. The effects of the compounds were color coded as suppression by at least 0.5-fold (*dark blue*), suppression by 0.25- to 0.5-fold (*light blue*), activation by 2.5- to 9.9-fold (*yellow*), activation by 10- to 99-fold (*light orange*), activation by 100- to 999-fold (*dark orange*), and activation greater than 1000-fold (*red*). Effects that did not fall into any of these groups were indicated by a *white square*. Species are abbreviated as human (h), rhesus (rh), pig (pg), dog (dg), rabbit (rb), mouse (m), fish (f), and *Xenopus* (x).

sequence identity (96%). These receptors showed 3- to 10-fold activation by the majority of xenobiotics tested, including several known cytochrome P450 3A inducers such as rifampicin, SR12813, and hyperforin. The rabbit PXR activation profile with xenobiotics was very similar to the primate receptors, with the exception that TCPOBOP did not activate the rabbit receptor. The dog and the pig receptors displayed the same promiscuous character as the other mammalian PXR receptors, but were hyper-responsive to certain compounds, including SR12813 and mevastatin. This is likely due to the fact that the dog and pig PXRs had relatively low basal activities in this assay. The mouse PXR was activated to a lesser degree by this set of compounds, likely a reflection of its higher basal activity in this assay. Notably, the chicken CXR and the fish PXR, showed similar profiles in the functional assays to the other PXRs. The chicken CXR was among the most promiscuous with regard to the xenobiotics tested (Table 2). The fish receptor was not as promiscuous as the chicken PXR but was activated by many of the known PXR xenobiotic activators, including nifedipine, phenobarbital, and clotrimazole. Interestingly, the bisphosphonate ester SR12813 was the most efficacious activator of the chicken, pig, dog, rabbit, rhesus, and human PXRs, indicating that the LBDs of these receptors recognize similar pharmacophores.

As was seen with the xenobiotics, the PXRs were promiscuous when tested against various synthetic and natural steroids, including bile acids. With the exception of the fish PXR, these receptors were activated by many if not most of the steroids and bile acids tested. In contrast to an earlier study (23) where lithocholic acid was reported to induce human PXR by approximately 2-fold, we report induction by 3.6-fold (Table 2). It is possible that the increased robustness of lithocholic acid data might reflect the fact that our bile acid assays incorporated intestinal bile acid transporter to facilitate bile acid transport. Also, Xie *et al*. (15) found that androstanol induced human PXR by 1.5- to 2-fold in a transient transfection assay using full-length receptor whereas we found that androstanol induced human PXR by 4.2-fold in the Gal4 chimera assay (Table 2). This could indicate that androstanol is somewhat more robust in the Gal4 assay but more likely reflects variation between experiments. All of the PXRs were activated by the naturally occurring steroids 5β-pregnane-3,20-dione and androstanol. Nevertheless, each receptor had a distinct activation profile. For example, the chicken and fish PXRs were activated by fewer bile acids than most of the other PXRs, possibly indicating that PXR activation by bile acids does not serve a hepatoprotective function in these species. Conversely, the pig and dog PXRs were activated by more of the bile acids than the other steroids. The pig, dog, and rabbit PXRs were all activated very efficiently by bile acids, including the synthetic bile acid 7-ketodeoxycholic acid methyl ester. Whereas the fish PXR was activated efficiently by 5β-pregnane-3,20-dione, androstanol, and dehydroepiandrosterone, it was not activated by any of the bile acids tested. Taken together, these data suggest that the PXRs evolved to recognize different steroids in different species. The unique PXR activation profiles seen with the steroids and bile acids most likely reflect species-specific differences in endogenous steroid and bile acid composition and metabolism.

Interestingly, consistent with their promiscuous activation profile, most of the PXRs were activated by one or more of the benzoates (Table 2). The ability of benzoate ligands to activate PXRs likely reflects the common evolutionary history shared between these receptors and the BXRs (see below).

The CARs

As indicated above, the mouse and human CAR were characterized by a higher basal level of activity relative to either the PXR or BXR classes (Table 2). The high basal activity of CAR in cell lines reflects the fact that, in contrast to primary cell lines or *in vivo*, CAR is constitutively nuclear in immortalized cell lines. Some compounds that do not directly bind to CAR, like phenobarbital (3), can strongly activate CAR *in vivo* by inducing CAR translocation (18). In these experiments, only the effect of compounds on nuclear CAR transactivation was monitored. With the single exception of TCPOBOP activation of mouse CAR, the CARs were not activated above 2.5-fold by any of the xenobiotics tested. However, several compounds acted as transrepressors on the CAR receptors. Within the xenobiotic class, mouse CAR basal activity was suppressed by *trans*-nonachlor and phenobarbital. Additionally, human CAR activity was suppressed by clotrimazole, consistent with previous reports (3). Phenobarbital has been shown in other cell line studies to activate CAR (24), but these studies were carried out in a different manner. Sueyoshi *et al.* (24) carried out phenobarbital induction experiments in HepG2 cells stably transformed with mouse CAR. Because CAR is constitutively nuclear in this cell line and because CAR has a high basal activity, these authors lowered the high basal activity of CAR by including a CAR transrepressor (androstanol). Thus, this experiment demonstrated the ability of phenobarbital to overcome androstanol suppression. In the absence of androstanol, the results from the two systems would likely have agreed. The slight suppression we saw with phenobarbital in both mouse and human CAR (Table 2) is consistent with our earlier report (3).

Neither the human nor mouse CAR receptors were significantly activated by any of the steroids or bile acids, although androstanol showed dramatic suppression of mouse CAR constitutive activity as previously reported (17). Surprisingly, several bile acids were efficacious transrepressors on the CARs. Cholic acid, 6-ketolithocholic acid, and 7-ketodeoxycholic acid methyl ester were all transrepressors on both the mouse and human CARs. Modulation of CAR activity by bile acids has not previously been reported.

The BXRs

The BXRs were not activated by the majority of xenobiotics, though BXRα showed slight activation by rifampicin and both BXRs were activated by SR12813 (Table 2). However, the effects of SR12813 were minimal compared with those of the benzoates. Though both are strongly activated by benzoates, BXRα and BXRβ showed distinct preferences. BXRα was most strongly activated by ethyl 3-hydroxybenzoate and n-butyl p-aminobenzoate and BXRβ most strongly by ethyl-4 hydroxybenzoate and n-propyl p-hydroxybenzoate. None of the steroids or bile acids displayed activity when tested on the BXRs. Taken together, these data show that both of the BXRs are specific for benzoates and are not promiscuous xenobiotic receptors.

Molecular Modeling

Stuctural studies have shown that most nuclear receptors adopt a common three-dimensional fold with 11–13 α-helices, and one β-sheet with 2–4 strands (25). The ligand binding pocket is generally enclosed by helices-3, -4, -5, -6, -7, -10, and -AF2. The recent x-ray structure of human PXR (26) departs significantly from this standard model, with two additional strands in the β-sheet, partial unwinding of helix-7, and complete unwinding of helix-6. The expanded β-sheet may allow a novel mode of dimerization (26) or possibly other protein-protein interactions. Helices-6 and -7 normally provide one wall for the ligand binding pocket. The unwinding of helices-6 and -7 in PXR would open this wall, leaving a gaping hole to the external solvent. However, in PXR, this hole is closed by a "capping segment," residues 204–210 in the H1–3 insert (Fig. 2). This capping segment occupies less volume than helices-6 and -7, and effectively expands the volume available to ligands inside the pocket.

In most nuclear receptors, the ligand binding pocket has a distinctive shape, with 2–4 key polar groups positioned to make strong hydrogen bonds with a specific ligand. In PXR, by contrast, the ligand binding pocket is more nearly spherical, with the few polar groups clustered such that they can form hydrogen bonds among themselves rather than with a specific ligand (Fig. 2A). This, and the large size of the pocket, enable PXR to accommodate a wide range of different lipophilic molecules. The nondiscriminating nature of the pocket is highlighted by the finding that the bisphosphonate ester, SR12813, binds to PXR in three distinct orientations (26).

The novel β-strands in PXR, which are labeled as beta-a and beta-b in Fig. 3, were successfully predicted (8) from the amino acid sequence before the x-ray structure using the PRISM algorithm (27) as implemented in the MVP program (28). In contrast, VDR is not predicted to have this beta sheet structure. In fact, the region was absent in the deletion mutant (VDR-LBDΔ165–215) from which the x-ray crystal structure was determined (29) (Fig. 2B), and may be unstructured in the wild-type protein. Comparison with the VDR sequence shows no specific mutations in human PXR that would necessarily break either helix-6 or helix-7 (Fig. 3), and the PRISM algorithm failed to predict any unwinding in either of these helices. This suggests that the unwinding of helices-6 and -7 in PXR is not necessarily a direct consequence of their own amino acid sequences, but is perhaps induced by changes elsewhere in the protein. In particular, the presence of beta-a and beta-b sheet structure requires a connecting segment to pass near, or through, the volume of helix-6. The amino acids in the H1–3 insert that form the beta-strands and the capping segment might thus be responsible for the unwinding of helices-6 and -7, and the resultant enlargement of the ligand binding pocket.

The mammalian PXRs all have amino acid sequences compatible with the expanded b-sheet and capping segment, and are thus predicted to have a binding pocket similar to that of human PXR (Fig. 3). The chicken and fish PXR sequences have long H1–3 inserts, but the expanded b-sheet is not obvious within this sequence (Fig. 3). However, these sequences can be aligned so as to identify possible capping segments, as shown in Fig. 3. Capping segments of this sort could induce unwinding of helices-6 and -7, even in the absence of an expanded b-sheet, suggesting that chicken and fish PXRs have enlarged ligand binding pockets similar to that of human PXR. By contrast, in BXR and CAR, the H1–3 insert is too short to reach into the volume occupied by the capping segments in human PXR. Thus, it seems likely that helices-6 and -7 are intact in BXR and CAR, where they could serve their usual function, acting as walls to constrict the ligand binding pocket. In BXR, the constricted ligand binding pocket would still be more than adequate for binding to benzoate esters. Likewise, the CAR binding pocket should remain large enough for specific ligands with appropriate shape but not large enough to accommodate the wide range of ligands that fit the PXR pocket. This analysis does not offer any explanation for the high basal level of CAR, or the tendency of its ligands to act as transrepressors.

DISCUSSION

In this report, we describe the cloning of the LBDs of four additional members of the NR1I nuclear receptor subfamily from monkey, pig, dog, and fish. Based upon their pharmacological activation profiles in cell-based reporter assays, we have classified these and the other reported NR1I family members into three groups: PXRs, which are transactivated by a wide range of xenobiotics and steroids; CARs, which have high basal activity and can be transrepressed by a relatively limited set of compounds; and BXRs, which are activated very efficiently by benzoate derivatives and are much less promiscuous than the PXRs. The striking differences in their pharmacological profiles

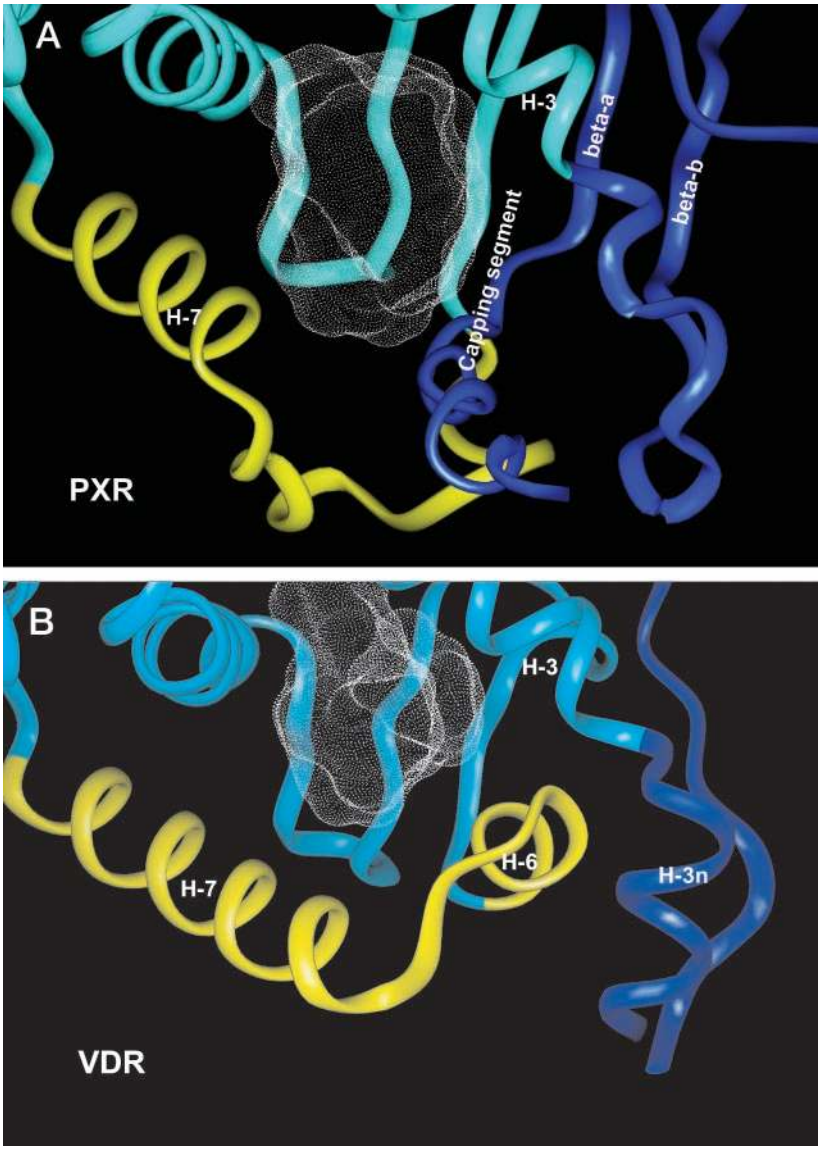

Fig. 2. Comparison of the Ligand Binding Pockets of the Human PXR (A) and the Human VDR (B)

The H1–3 insert is shown in *dark blue*. The helix-6/7 region is shown in *yellow*. The remainder of the chain is shown in *light blue*. The available volume in the ligand binding pocket is depicted by a Connolly dot surface (36).

suggested that each of these receptor subgroups may have evolved to subserve distinct physiological roles.

There is mounting evidence that the PXRs evolved to serve as promiscuous xenoreceptors for detecting potentially harmful compounds of both endogenous and exogenous origin. Why are the PXRs so much more promiscuous than their CAR and BXR relatives? Molecular modeling studies suggest that the H1–3 insert in PXR receptors acts to unwind helices-6 and -7, thereby expanding the ligand binding pocket. We propose that the H1–3 insert distinguishes the PXR subfamily from the CAR and BXR subfamilies. The VDR subfamily has a different H1–3 insert that probably does not enlarge the ligand binding pocket.

Based on the human PXR crystal structure, 28 residues were defined as lining the ligand binding pocket. Alignment of the PXR-like members of the NRI1 family shows a large amount of variation in these residues across species (26). It is possible that mutations in these residues provide a means for rapidly evolving new binding specificities in response to either xenobiotic challenges or differences in steroid and/or bile acid metabolism across species.

How did the PXRs, CARs, and BXRs evolve within the NRI1 subfamily? It is clear from their sequence similarities and overlapping ligand profiles that these receptors share a close evolutionary history. The fact that the receptors are distinguished by the presence or absence of the H1–3 insert region gives clues to the origin of these receptors. The H1-H3 deleted receptors are not likely to have originated by simple exon deletion (30) because the exon/intron boundaries from the

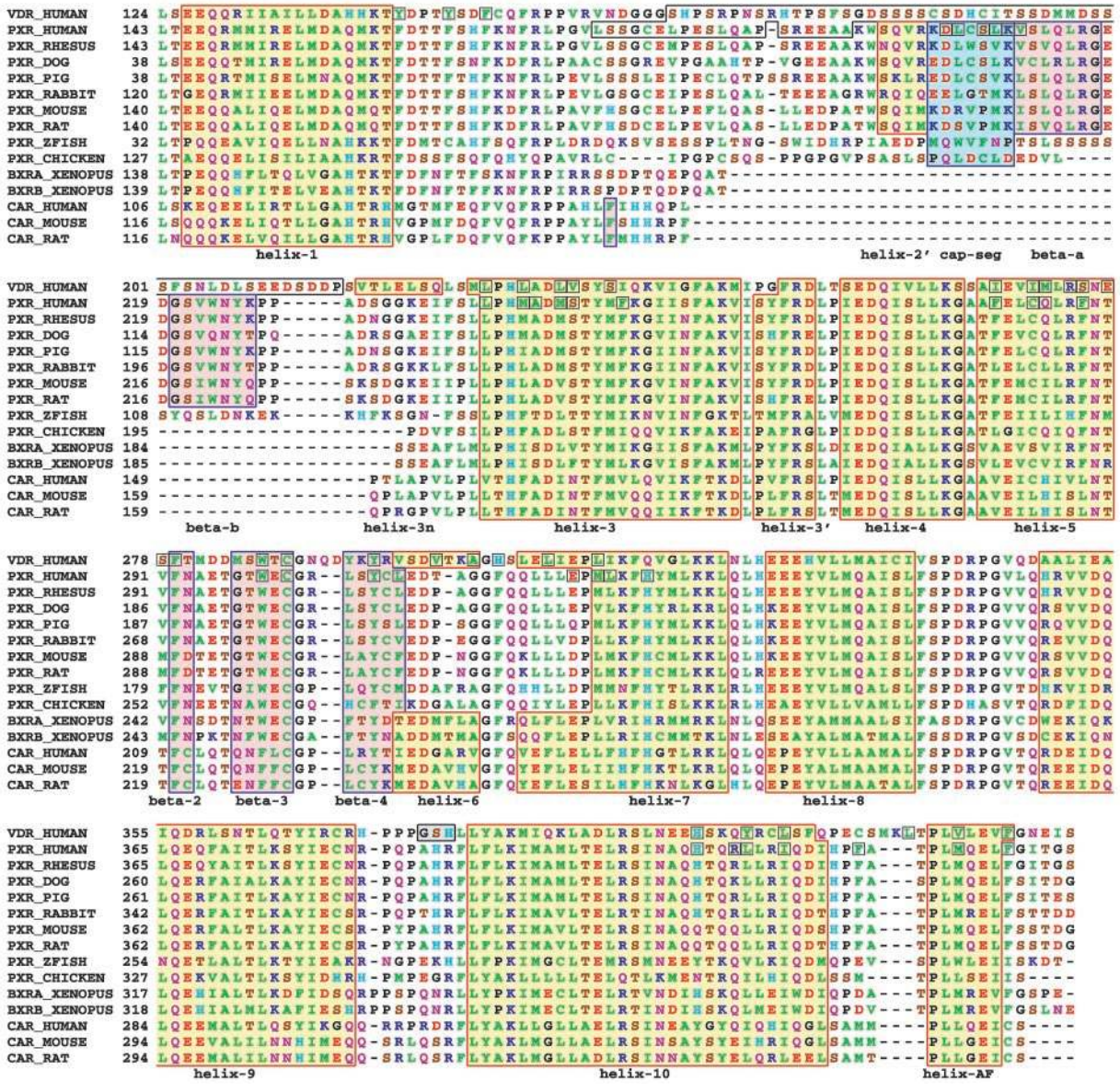

Fig. 3. Structure-Based Alignment of the PXR, CAR, BXR, and VDR Amino Acid Sequences

α-Helix and β-strand residues are identified with *yellow* and *red* background highlighting, respectively. The capping segment is highlighted in *blue*, and segments that were disordered in the x-ray structures are identified with a *gray* background color. The secondary structures of human PXR and VDR were determined by examination of the x-ray structures. For the remaining sequences, the secondary structure was predicted from the alignment according to the expected effects of their H1–3 inserts, *i.e.* PXR secondary structures were taken from human PXR, whereas BXR and CAR secondary structures were taken from VDR. Note that the alignment of the H1–3 insert is particularly uncertain in the fish and chicken PXR sequences.

mammalian CAR and PXR genes are not consistent with this scenario. Analysis of homologous genes from additional species is needed to provide additional insight into the mechanism by which a progenitor receptor (presumably similar to the fish PXR) gave rise to receptors with differentiated ligand activation properties. In the case of CAR, these differences extended to developing novel modes of regulation relative to the PXRs, as evidenced by its nuclear translocation properties. In the case of BXRs, these receptors may have taken on a nonxenobiotic sensing role. It has been suggested that the BXRs recognize endogenous benzoates. This is consistent with their expression in the hatching gland and nervous system in *Xenopus* during development and the notable absence of data supporting a role of the BXRs in the regulation of hepatic cytochrome P450 expression.

With nearly all of the human genome sequenced, it appears that humans have single PXR and CAR subtypes and no BXR ortholog (2). However, the complement of NRI1 receptors present in other species remains an open question. It is interesting that, since

neither BXRα nor BXRβ appear to be promiscuous xenobiotic receptors, a xenobiotic receptor has not yet been found in *Xenopus*. It would seem likely that *Xenopus* will have such a promiscuous receptor because the fish genome contains a PXR. Likewise, only a single PXR-like receptor has been identified in chickens, thus leaving open the question of whether chickens have a CAR-like receptor. Complete sequencing of the genomes of these different species will provide important insights into the evolution and function of these receptors.

In summary, our results differentiate four main subgroups in the NR1I subfamily: 1) VDRs well characterized mediators of vitamin D signaling; 2) BXRs: specialized for benzoate ligands and not subserving a promiscuous xenobiotic receptor function; 3) CARs: xenosensors with a higher degree of ligand selectivity than the PXRs and to date, only identified in mammals; and 4) PXRs: highly promiscuous xenosensors that we have found in species ranging from fish to man. The structural criteria we have defined that distinguish the PXRs from the less promiscuous receptors will be useful in defining the molecular characteristics that give rise to promiscuity and xenoprotection, as well as in categorizing orthologous receptors as they appear in other genomes.

MATERIALS AND METHODS

Reagents

The chemicals used and their suppliers are as follows: 5β-pregnane 3,20-dione, ethyl 3-hydroxybenzoate, dimethylsulfoxide (Aldrich Chemical Co., Inc., Milwaukee, WI); hyperforin (Apin Chemicals Ltd., Abingdon, Oxon, UK); RU 486 (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA); dex-t-butylacetate (Research Plus, Inc., Bayonne, NJ); 6,16-dimethylpregnenolone, β-E2, ethyl 4-hydroxybenzoate, clotrimazole, dexamethasone, dehydroepiandrosterone, dihydrotestosterone, n-butyl p-aminobenzoate, n-propyl p-hydroxybenzoate, mevastatin, nifedipine, pregnenolone-16α-carbonitrile, phenobarbital, progesterone, reserpine, rifampicin, cholic acid, lithocholic acid, 6-ketolithocholic acid, dehydrolithocholic acid (Sigma, St. Louis, MO); 12-ketolithocholic acid, taurocholanic acid, 3,7-diketocholanic acid, 7-ketodeoxycholic acid, 7-ketodeoxycholic acid methyl ester (Steraloids, Inc., Newport, RI); *trans*-nonachlor (Supelco, Inc., Bellefonte, PA); androstanol, SR 12813, and TCPOBOP were synthesized in house.

Isolation of PXR Sequences

Four novel PXR LBD sequences (from pig, dog, zebrafish, and rhesus) were cloned. The isolation of each sequence was achieved using essentially the same strategy for each. A small stretch of the LBD was obtained using either cross-hybridizing PCR primers from another species, or by finding some portion of the LBD sequence in the EST database. The remainder of the LBD was subsequently isolated by PCR amplification of flanking sequence using a primer from within the starting sequence combined with either 1) a degenerate oligo representing the canonical P-box of the DBD to isolate 5′ sequence, or 2) oligo deoxythymidine $[d(T)]_{20}$-G, $d(T)_{20}$-C, or $d(T)_{20}$-A to isolate 3′ sequence. After deriving the sequence to the poly (A) tail, the full-length LBD was produced using primers flanking the coding sequence. Wild-type sequence was determined through examination of at least three independent amplifications of each LBD.

To clone pig PXR LBD, total mRNA was prepared from frozen pig liver (1 g) using the FastTrack 2.0 RNA Preparation kit (Invitrogen, San Diego, CA). Oligo d(T)-primed cDNA synthesis was carried out by RT-PCR using a cDNA Cycle Kit (Invitrogen). An approximately 250-bp stretch of pig PXR LBD was amplified from this cDNA using homologous mouse PXR LBD primers. To clone dog PXR LBD, human PXR LBD primers were used to amplify an approximately 450-bp fragment from a dog liver 5′-stretch λgt11 cDNA library (CLONTECH Laboratories, Inc., Palo Alto, CA). To clone rhesus PXR LBD, human primers were used to amplify all but the termini of the rhesus PXR LBD from a rhesus liver cDNA library. To clone zebrafish (*Danio rerio*) PXR LBD, an initial fragment of the PXR LBD was identified as an EST sequence (accession no. AI943313). This sequence was used to design primers for amplification of the entire LBD from cDNA synthesized using zebrafish embryo (48 h) oligo d(T)-primed cDNA. The sequence of each novel PXR was deposited into GenBank (dog, rhesus, pig, zebrafish PXR sequences, AF454670–AF454673, respectively).

Isolation of the remainder of the PXR LBD sequences has previously been reported for mouse (5), human (4), and rabbit PXR (8). *Xenopus* BXRα (21), *Xenopus* BXRβ (22), chicken CXR (20) sequences were derived by PCR.

Cotransfection Assays

The transient transfection format that was chosen was either a Gal4 chimera assay (for the PXRs and BXRs) or full-length receptor assay (for the CARs). Gal4 expression constructs using the LBD of each PXR/BXR/CXR were prepared as described previously (31). A human SRC-1 construct (representing amino acids 1–1005) (32) was prepared in the pSG5 expression vector (Stratagene Corp., La Jolla, CA). The Gal4 chimera constructs were tested in combination with a reporter plasmid harboring the Gal4 enhancer region linked to a reporter gene. In the full-length CAR receptor assays, the reporter gene was linked to the XREM promoter element (33). CV-1 cells were maintained and transiently transfected as described (3, 34), except for inclusion of 7 ng/well of the SRC-1 expression plasmid in the transfections utilizing the Gal4 chimera constructs. In experiments involving bile acids, an expression plasmid containing the intestinal bile acid transporter (8 ng/well in a 96-well plate format) was included (35).

Sequence Alignment and Structural Modeling

The structure-based sequence alignment of Fig. 3 was carried out with the MVP program (28) using the human PXR (26) and VDR LBD crystal structures (29).

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