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The alternatively spliced murine pregnane X receptor isoform, $mPXR_{\Delta 171-211}$ exhibits a repressive action

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ABSTRACT

The orphan nuclear receptor pregnane X receptor regulates enzymes and transport proteins involved in the detoxification and clearance of numerous endobiotic and xenobiotic compounds, including pharmaceutical agents. Multiple alternatively spliced pregnane X receptor isoforms have been identified which are significantly expressed in humans and mice (up to 30% of the total pregnane X receptor transcript), however, little is known about their biological action. We explored functional differences between the major mouse pregnane X receptor isoforms $mPXR_{431}$ and $mPXR_{\Delta 171-211}$ that lacks 41 amino acids adjacent to the ligand-binding pocket. Transient transfection assays showed that $mPXR_{\Delta 171-211}$ reduced the basal transcription of cytochrome P450 3A4 and the drug transporter P-glycoprotein/Multi Drug Resistance Protein 1 and directly repressed the regulatory effects of $mPXR_{431}$ on these genes. Replacement of the $mPXR_{\Delta 171-211}$ DNA-binding domain with that of GAL4 showed $mPXR_{\Delta 171-211}$ retained its repressive role independent of binding to PXR responsive elements located within the cytochrome P450 3A4 and Multi Drug Resistance Protein 1 regulatory regions. Use of the histone deacetylase inhibitor, trichostatin A, demonstrated that the repressive function of $mPXR_{\Delta 171-211}$ acts independently of histone acetylation state. Protein interaction assays revealed $mPXR_{\Delta 171-211}$ and $mPXR_{431}$ differentially bind the obligatory heterodimer partner retinoid X receptor. Furthermore, $mPXR_{431}$ and $mPXR_{\Delta 171-211}$ proteins could heterodimerize. These studies demonstrate that the variant mouse PXR isoform, $mPXR_{\Delta 171-211}$, has a distinct repressive function from $mPXR_{431}$ in regulating genes encoding important drug metabolizing enzymes and transport proteins.

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1. Introduction

Pregnane X receptor (PXR; NR1I2 – also known as the steroid and xenobiotic (Blumberg et al., 1998) or pregnane activated receptor (Bertilsson et al., 1998)) is a member of the nuclear receptor (NR) family of ligand-activated transcription factors. Nuclear receptors regulate a wide range of physiological processes including development, cell differentiation and organ function. Classical nuclear receptors such as steroid hormone receptors have high ligand specificity and sensitivity, usually carrying out transcriptional regulation as a homodimer. However, PXR is a member of the orphan nuclear receptor sub-family for which a cognate physiological ligand has not yet been identified. Orphan receptors such as PXR are capable of binding a wide range of ligands with low specificity. In addition, rather than acting as homodimers, orphan nuclear receptors usually carry out their transcriptional regulation as heterodimers with the retinoid X receptor (RXR).

PXR plays an important role in coordinating clearance of xenobiotic and endobiotic compounds within the body. It regulates genes encoding enzymes involved in biotransformation as well as transporter proteins for uptake and efflux of foreign substances, including pharmaceutical compounds (Gibson et al., 2006; Tirona and Kim, 2005). One of the best characterized PXR targets is the cytochrome P450 3A4 (CYP3A4) gene, which encodes the most abundant cytochrome P450 enzyme in the human liver (Ingelman-Sundberg, 2004). As the CYP3A4 enzyme is involved in the metabolism of up to 60% of all currently marketed therapeutic

Abbreviations: PXR, pregnane X receptor; CYP, cytochrome P450; MDR1, Multi Drug Resistance Protein 1; P-gp, P-glycoprotein; TSA, trichostatin A; RXR, retinoid X receptor; NR, nuclear receptors; h, human; m, mouse; mRNA, messenger ribonucleic acid; mP1, $mPXR_{431}$; mP2, $mPXR_{\Delta 171-211}$; CAR, constitutive androstane receptor; DMEM, Dulbecco's Modified Eagle's Medium; FCS, fetal calf serum; GST, glutathione-S-transferase; VDR, vitamin D receptor; PR, progesterone receptor.

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agents (Hustert et al., 2001; Liu et al., 2008), it is quantitatively and qualitatively one of the most important enzymes in human drug metabolism. Many of the compounds known to induce CYP3A4 also activate PXR, consequently up-regulating drug clearance pathways, thereby increasing the rate of drug elimination. This forms the basis of many clinically important PXR-mediated drug interactions involving altered pharmacokinetics of therapeutic compounds.

An additional level of complexity to nuclear receptor action occurs through existence of multiple isoforms (Keightley, 1998). These can arise through alternative mRNA splicing events, transcriptional initiation sites or via different genomic loci. Such isoforms can have tissue-specific expression patterns, differential sub-cellular localization and interactions with proteins and/or DNA response elements. These properties can result in loss, gain or alteration of function.

Full-length PXR sequences have been characterized in mouse (Kliewer et al., 1998), human (Bertilsson et al., 1998; Blumberg et al., 1998; Lehmann et al., 1998), rabbit and rat (Jones et al., 2000; Zhang et al., 1999). Partial PXR sequences encompassing the ligand-binding domain (LBD) have been cloned from pig, dog, rhesus monkey, zebra and fugu fish (Maglich et al., 2003; Moore et al., 2002). Human PXR (hPXR) can exist as 15 isoforms, while two mouse PXR (mPXR) isoforms were described in the initial discovery of PXR (Kliewer et al., 1998) (Table 1). The expression level and composition of human PXR isoforms can vary dramatically between individuals (He et al., 2006; Lamba et al., 2004). Additional rabbit and rat PXR liver transcripts have been identified, however these have not been extensively studied (Jones et al., 2000; Zhang et al., 1999).

The majority of research has focused on full-length human PXR (i.e. $hPXR_{434}$), whose dominant expression and susceptibility to drug activation have established its potential to impact on clinical outcomes via inductive drug interactions. Beyond $hPXR_{434}$, significantly expressed hPXR isoforms include splice variants $hPXR_{\Delta174–210}$ and $hPXR_{\Delta174–214}$ [designated PXR.2 and PXR.3 (Lamba et al., 2004)] and another splice variant which generates a premature stop codon at residue 196 [designated SV3 (He et al., 2006)]. The combined expression of these isoforms can contribute up to 28% of the total hPXR mRNA transcripts (Lamba et al., 2004). $hPXR_{\Delta174–210}$ and $hPXR_{\Delta174–214}$ are virtually the same, possessing a deletion of 111 and 123 nucleotides, respectively in exon 5 of the hPXR gene. The precise 41 amino acids (aa) exclusion found in $hPXR_{\Delta174–214}$ is conserved in the only mouse PXR isoform identified to date, $mPXR_{\Delta171–211}$. Limited functional characterization of the mouse PXR isoforms comparing full-length $mPXR_{431}$ and $mPXR_{\Delta171–211}$ (herein designated mP1 and mP2, respectively) revealed that they are both capable of binding the same responsive elements within the *CYP3A* gene. However, mP2 has a reduced ligand activation profile compared to mP1 (Kliewer et al., 1998). Elucidation of the $hPXR_{434}$ LBD crystal structure revealed a flexible loop region at the ligand entry to the ligand-binding pocket (Watkins et al., 2001) which overlaps the amino acids corresponding to the region deleted in the variant isoforms mP2, $hPXR_{\Delta174–210}$ and $hPXR_{\Delta174–214}$ (Fig. 1). The lack of this flexible loop could explain the restricted activation profile of mP2 relative to the promiscuous mP1. Interestingly, alignment of PXR and its isoforms, with other nuclear receptors reveals the missing region of mP2, $hPXR_{\Delta174–210}$ and $hPXR_{\Delta174–214}$ is also absent in other nuclear receptors such as constitutive androstane receptor (CAR), farnesoid X receptor and liver X receptor (Ekins et al., 2002; Lamba et al., 2004; Matic et al., 2007) which play important roles in a range of metabolic processes. This, together with evidence of tissue-specific expression patterns of isoforms in a wide variety of organs (including liver, stomach, adrenal gland, bone marrow and brain) (Fukuen et al., 2002; Kliewer et al., 1998; Lamba et al., 2004) suggests these PXR isoforms may possess important biological functions.

In this study, we demonstrate that the variant mouse isoform mP2 is functionally distinct from full-length mP1. mP2 by itself reduces the basal transcription of CYP3A4 and MDR1 and directly represses the regulatory effects of mP1 on these genes.

2. Materials and methods

2.1. Plasmid constructs for in vitro translation and mammalian expression

A modified pSG5 vector backbone, $pSG5_{EX}$, was generated by ligating annealed $pSG5_{EX-}$ (F) 5′-AATTGCTCGAGAGCGGCCGCAGAATTCAA-3′ and $pSG5_{EX-}$ (R) 5′-GATCTTGAATTCTGCGGCCGCTCTCGAGC-3′ oligonucleotides into the EcoRI and BglII sites of the pSG5 vector (Stratagene, CA, U.S.A.). This abolished the pSG5 EcoRI and BamHI and introduced XhoI, NotI and EcoRI sites.

The $pSG5_{EX}mPXR_{431}$ and $pSG5_{EX}mPXR_{\Delta171–211}$ constructs were generated by PCR amplification of $mPXR_{431}$ and $mPXR_{\Delta171–211}$ cDNA fragments from cDNA derived from total mouse liver RNA (Nakhel, S. and Robertson, G.R., unpublished). The following primers were used: (F) 5′-AT*CTCGAG*CGCCACCATGAGACCTGAGGAGAGCTGG-3′ XhoI *restriction site*, Kozac translation sequence, start codon and (R) 5′-GA*GAATTC*TCAGCCATCTGTGCTGCTAAATAACTCTTGC-3′ EcoRI *restriction site*, stop codon). These primers incorporated an XhoI restriction site and a consensus Kozac translation sequence immediately upstream of the mPXR start codon, in addition to an EcoRI restriction site immediately downstream of the stop codon. The amplicon was ligated into the pGEM-T vector (Promega, New South Wales, Australia) then digested using EcoRI and XhoI followed by sub-cloning into the EcoRI and XhoI sites of $pSG5_{EX}$ to generate $pSG5_{EX}mPXR_{431}$ and $pSG5_{EX}mPXR_{\Delta171–211}$.

pSG5–GAL4–PXR.2LBD encoding $mPXR_{\Delta171–211}$ LBD fused to a GAL4 was a kind gift from Prof. Steve Kliewer (University of Texas Southwestern Medical Centre, Dallas, U.S.A. (Kliewer et al., 1998)). Prof. Carsten Carlberg (University of Luxemburg, Luxembourg) provided the human RXRα expression construct pSG5hRXRα.

2.2. Plasmid constructs for bacterial expression

The bacterial expression constructs pGEX-$mPXR_{431}$ and pGEX-$mPXR_{\Delta171–211}$ were generated by PCR amplification of cDNA encoding amino acids 1–431 of $mPXR_{431}$ or 1–390 of $mPXR_{\Delta171–211}$ followed by insertion into EcoRI (GAATTC) and XhoI (CTCGAGC) sites of a pGEX-4T vector. The PCR primer sequences corresponding to nucleotides 1–21 and 1275–1296 of the mPXR coding region were: (F) 5′-TCAGAATTCATGAGACCTGAGGAGAGCTGG-3′; (R) 5′-CATCTCGAGTCAGCCATCTGTGCTGCTAAA-3′.

2.3. Reporter gene plasmid constructs

The p3A4-13000, p3A4-362(7836/7208 ins) reporter gene constructs containing DNA response elements from the CYP3A4 upstream promoter were a kind gift from Prof. Chris Liddle (Westmead Millennium Institute, Westmead, Australia (Goodwin et al., 1999)). The p-7975(Δ7012–1804) reporter gene construct containing DNA response elements from the MDR1 upstream regulatory region was kindly provided by Prof. Oliver Burk (Dr. Margarete Fischer-Bosch-Institute of Clinical Pharmacology, Stuttgart, Germany (Geick et al., 2001)). All reporter constructs consist of segments of the respective gene's upstream regulatory region directly linked to the luciferase gene contained within the pGL3 basic vector (Promega, Madison, WI).

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