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Transcription regulation of nuclear receptor PXR: Role of SUMO-1 modification and NDSM in receptor function

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ABSTRACT

Pregnane & Xenobiotic Receptor (PXR) is one of the 48 members of the nuclear receptor superfamily of ligand-modulated transcription factors. PXR plays an important role in metabolism and elimination of diverse noxious endobiotics and xenobiotics. Like in case of some nuclear receptors its function may also be differentially altered, positively or negatively, by various post-translational modifications. In this context, regulation of PXR function by SUMOylation is the subject of present investigation. Here, we report that human PXR is modified by SUMO-1 resulting in its enhanced transcriptional activity. RT-PCR analysis showed that PXR SUMOylation in presence of rifampicin also enhances the endogenous expression levels of key PXR-regulated genes like CYP3A4, CYP2C9, MDR1 and UGT1A1. In addition, mammalian two-hybrid assay exhibited enhanced interaction between PXR and co-activator SRC-1. EMSA results revealed that SUMOylation has no influence on the DNA binding ability of PXR. *In silico* analysis suggested that PXR protein contains four putative SUMOylation sites, centered at K108, K129, K160 and K170. In addition to this, we identified the presence of NDSM (Negative charge amino acid Dependent SUMOylation Motif) in PXR. Substitution of all its four putative lysine residues along with NDSM abolished the effect of SUMO-1-mediated transactivation function of PXR. Furthermore, we show that interaction between PXR and E2-conjugation enzyme UBCh9, an important step for implementation of SUMOylation event, was reduced in case of NDSM mutant PXRD115A. Overall, our results suggest that SUMOylation at specific sites on PXR protein are involved in enhancement of transcription function of this receptor.

1. Introduction

The Pregnane & Xenobiotic Receptor (PXR) is a ligand-activated intracellular transcription factor belonging to the nuclear receptor (NR) superfamily (Bertilsson et al., 1998; Kliewer et al., 1998). Human-PXR predominantly resides in the nuclear compartment (like PR, ERα etc.), while unliganded mouse PXR may exhibit cytoplasmic or nuclear localization in different cell types. Conceivably, under normal circumstances receptors from both the species are nuclear when bound to their ligands (Kumar et al., 2006; Saradhi et al., 2005a; Squires et al., 2004). As a transcription factor, PXR is primarily involved in the regulation of the components of phase I, II and III of xenobiotic metabolism and elimination machinery. In addition to different exogenous and endogenous factors, PXR activity is also reported to be regulated by different PTMs that further expand the functional output of receptor activity. Recent reports suggest that PXR undergoes different post-translational modifications (PTMs) like phosphorylation, ubiquitination, acetylation and SUMOylation. Eventually, these PTMs are suggested to modulate PXR activity to execute differential and distinct biological roles (Biswas et al., 2011; Staudinger et al., 2011; Hu et al., 2010; Lichti-Kaiser et al., 2009a, 2009b; Pondugula et al., 2009a, 2009b; Masuyama et al., 2002, 2000).

Post-translational modifications (PTM) of a protein by small ubiquitin-like modifier (SUMO) are being increasingly recognized as an important regulatory mechanism. In this context, SUMOylation is a dynamic and reversible process that involves a series of enzymes that are analogous to ubiquitinating enzymes or machinery. It consists of an E1-activating enzyme, an E2-conjugating enzyme (UBCh9 or Ubiquitin-Conjugating enzymes), three

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different groups of E3-ligases (PIAS1, PIASxα, PIASxβ, PIASγ and PIAS3) with differing target-protein specificities to conjugate, and several SENP1 isopeptidases to deconjugate SUMO from target proteins (Hay, 2007, 2005; Zhao, 2007; Desterro et al., 1997). While protein degradation is primarily accomplished by ubiquitination, SUMOylation is emerging as a regulatory mechanism that affects diverse cellular processes such as transcriptional activity, protein–protein interaction, chromatin structure, DNA binding activity, signal transduction, apoptosis, autophagy, cell-cycle control, cell fate, sub-cellular and intra-nuclear localization of its target protein (Ward et al., 2013; Seeler and Dejean, 2003; Muller et al., 2001; Melchior, 2000). In higher eukaryotes, four isoforms are reported namely, SUMO-1, SUMO-2, SUMO-3 and SUMO-4, which are encoded by different genes (Guo et al., 2004; Holmstrom et al., 2003; Melchior, 2000). Of these, SUMO-1, SUMO-2 and SUMO-3 are ubiquitously expressed, whereas SUMO-4 is expressed primarily in the kidney, lymph node and spleen (Guo et al., 2004). The mature forms of SUMO-2 and SUMO-3 have 90% sequence homology but only 20% sequence homology with SUMO-1. SUMO-1 covalently modifies a number of NR proteins including glucocorticoid receptor (GR) (Le et al., 2002; Tian et al., 2002), mineralocorticoid receptor (MR) (Tirard et al., 2007; Pascual-Le Tallec and Lombès, 2005), androgen receptor (AR) (Rytinki et al., 2011; Poukka et al., 2000), progesterone receptor (PR) (Abdel-Hafiz and Horwitz, 2014, 2012; Chauchereau et al., 2003), estrogen receptor (ER) (Picard et al., 2012; Sentis et al., 2005), farnesoid X receptor (FXR) (Balasubramaniyan et al., 2013), retinoid X receptor (RXR) (Choi et al., 2006) etc. From the existing literature it appears that multiple PTM events may affect NR functions by modulating their protein-protein interactions, sub-cellular localization and stability that ultimately fine-tune gene transcription. Therefore, a systematic investigation of these processes is essential for an in-depth understanding of the cellular implications of these modifications on transcription factors.

A recent study has shown that PXR, upon SUMOylation, represses the inflammatory response (Hu et al., 2010). Observations made by *in vitro* study suggested that PXR undergoes SUMOylation by SUMO-1, SUMO-2, and SUMO-3 isoforms. On the contrary, *in vivo* study suggested that PXR undergoes SUMOylation only by SUMO-3 isoform (Hu et al., 2010). In view of the contradictory and fragmented observations an in-depth and systematic study of PXR SUMOylation is warranted to gain insight into this post-translational modification. Most SUMO-modified target proteins contain tetra-peptide consensus motif ΨKXE, where Ψ is a large hydrophobic amino acid and K (lysine) is the site of SUMO conjugation. Some of the reports also suggest existence of non-consensus SUMO-acceptor sites (Rodriguez et al., 2001; Johnson and Blobel, 1999; Sternsdorf et al., 1999). Recent studies indicate that additional longer sequences exist on proteins that lie adjacent to SUMO consensus sites called i) PDSM (Phosphorylation Dependent SUMOylation Motif), and ii) NDSM (Negative charge amino acid Dependent SUMOylation Motif) (Hietakangas et al., 2006; Yang and Grégoire, 2006, Yang et al., 2003). The fundamental highlight of PDSM is a proline-directed phosphorylation site that cannot be SUMOylated unless phosphorylated (Hietakangas et al., 2006), whereas acidic amino acids present in NDSM are essential for SUMO conjugation and for binding to a positively charged patch on E2-conjugation enzyme UBCh9 (Yang and Grégoire, 2006). So, the question arises whether presence of such motifs also regulate the PXR SUMOylation and its function. By using conventional bioinformatics tool 'SUMOplot' (http://www.abgent.com/doc/sumoplot) four potential SUMOylation sites in human PXR are predicted, with one having a high probability SUMOylation motif in hinge region and three low probability SUMOylation motifs in the ligand binding domain. Subsequently, we show for the first time that PXR is modified under *in vivo* conditions by covalent attachment of SUMO-1 in ligand-independent manner. Following *in silico* and *in vivo* analyses, we attempted to explore the influence of putative SUMOylation sites on PXR-mediated transcriptional output. Overall, our data suggest that transcriptional activation by SUMO-1 conjugation and NDSM may play important roles in distinct transcriptional response of PXR.

2. Materials and methods

2.1. Reagents

All the plastic wares for mammalian cell culture were purchased from Corning Costar Corp. (Corning, NY, USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), dextran-charcoal-stripped serum and other mammalian cell culture reagents were procured from Hyclone (Logan, UT, USA). Transfection reagent Escort III & IV, HRP-conjugated anti-rabbit and anti-mouse secondary antibodies were procured from Sigma Chemicals Co. (St. Louis, MO, USA). Antibodies against SUMO-1 and PXR were generated in our laboratory (Priyanka, 2013; Saradhi et al., 2005b). Rifampicin and N-ethylmaleimide were purchased from Sigma, St. Louis, MO, USA. Protein A-Sepharose beads were purchased from Bangalore Genei, India. Luciferase assay kit was obtained from Promega (Madison, WI, USA). All other general chemicals (unless otherwise mentioned) were obtained from Sigma Chemicals Co. (St. Louis, MO, USA) or local commercial sources supplying molecular biology grade reagents.

2.2. Vectors

Mammalian expression GFP-SUMO-1, Flag-PIASXα and bacterial expression GST-SUMO-1 constructs for SUMOylation studies were provided by Jorma J. Palvimo, University of Kuopio, Finland. GST-SUMO-1GG plasmid was obtained from Ronald T. Hay, UK. RFP-SUMO-1 mammalian expression vector encoding full-length SUMO-1GG was sub-cloned at BamHI site of pDsRed-Express-C1 vector. Plasmids encoding Gal4-PXR was made by subcloning of full length PXR cDNA (derived from pSG5-hPXR) into pM vector at EcoR1 and BamH1 restriction sites. VP, VP-PXR and pM vectors for mammalian two-hybrid assay were a kind gift from Jeff Staudinger, Department of Pharmacology and Toxicology, University of Kansas, USA. VP16-UBCh9 was a kind gift from Marilyn Tirard (Max Planck Institute of Psychiatry, Munich, Germany). Gal-SRC-1 and Gal-NcoR1 plasmids were kind gift from Barry Forman. Flag-PIAS1 is a kind gift from Ke Shuai. Mammalian expression vector (pSG5) encoding human PXR-1(hPXR) plasmid construct was kindly provided by S.A. Kliewer, University of Texas Southwestern Medical Center, Dallas, USA. GFP-PXR construct has been previously described (Saradhi et al., 2005). XREM-Luc and FR-Luc promoter-reporter expression plasmid kindly provided by C. Liddle, University of Sydney at Westmead Hospital, Australia and S. Stoney Simons, Jr., NIDDK, NIH, Bethesda, USA respectively.

2.3. Maintenance of cell lines

Kidney cell line COS-1(from African green monkey), human liver cell line HepG2 were obtained from National Centre for Cell Science repository (Pune, India). All cells were cultured in DMEM supplemented with 10% FBS, 100 mg/ml penicillin and 100 mg/ml streptomycin. The cultures were routinely maintained in a humidified incubator at 5% CO_2 and 95% air atmosphere at 37 °C.

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