SUMOylation and Ubiquitylation Circuitry Controls Pregnane X Receptor Biology in Hepatocytes

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ABSTRACT

Several nuclear receptor (NR) superfamily members are known to be the molecular target of either the small ubiquitin-related modifier (SUMO) or ubiquitin-signaling pathways. However, little is currently known regarding how these two post-translational modifications interact to control NR biology. We show that SUMO and ubiquitin circuitry coordinately modifies the pregnane X receptor (PXR, NR1I2) to play a key role in regulating PXR protein stability, transactivation capacity, and transcriptional repression. The SUMOylation and ubiquitylation of PXR is increased in a ligand- and tumor necrosis factor α -dependent manner in hepatocytes. The SUMO-E3 ligase enzymes protein inhibitor of activated signal transducer and activator of transcription-1 (STAT1) STAT-1 (PIAS1) and protein inhibitor of activated STAT Y (PIASy) drive high levels of PXR SUMOylation. Expression of protein inhibitor of activated stat 1 selectively increases SUMO(3)ylation as well as PXR-mediated induction of cytochrome P450, family 3, subfamily A and the xenobiotic response.

The PIASy-mediated SUMO(1)ylation imparts a transcriptionally repressive function by ameliorating interaction of PXR with coactivator protein peroxisome proliferator-activated receptor gamma coactivator-1- α . The SUMO modification of PXR is effectively antagonized by the SUMO protease sentrin protease (SENP) 2, whereas SENP3 and SENP6 proteases are highly active in the removal of SUMO2/3 chains. The PIASy-mediated SUMO(1)ylation of PXR inhibits ubiquitin-mediated degradation of this important liver-enriched NR by the 26S proteasome. Our data reveal a working model that delineates the interactive role that these two post-translational modifications play in reconciling PXR-mediated gene activation of the xenobiotic response versus transcriptional repression of the proinflammatory response in hepatocytes. Taken together, our data reveal that the SUMOylation and ubiquitylation of the PXR interface in a fundamental manner directs its biologic function in the liver in response to xenobiotic or inflammatory stress.

Introduction

Ligand-dependent activation of the pregnane X receptor (PXR, NR1I2) is associated with increased metabolism and clearance of a myriad of potentially toxic compounds from the body and is thus thought of as a master regulator of the protective xenobiotic response. However, clinical treatment with PXR activators can also lead to the repression or attenuation of other biochemical pathways, including the inflammatory response in the liver and intestine (Moreau et al., 2008). It is now well accepted that activation of PXR is associated with general suppression of the inflammatory response in these tissues (Shah et al., 2007; Cheng et al., 2012; Dou et al., 2012, 2014; Sun et al., 2015).

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Post-translational modification with the small-ubiquitin related modifier (SUMO) plays a key role in determining the biologic fate and function of a myriad of transcription factors, including several liver-enriched nuclear receptor (NR) superfamily members, to alter inflammatory signaling pathways (Treuter and Venteclef, 2011). There are a number of different SUMO-E3 ligase enzymes, and the best characterized family is the protein inhibitors of activated STAT (PIAS) family (Shuai and Liu, 2005). SUMOylation is a reversible process through the action of a family of sentrin proteases (SENPs) that function as isopeptidases to deconjugate SUMO from substrates (Drag and Salvesen, 2008).

The SUMO- and ubiquitin-signaling pathways share a high degree of commonality (Glickman and Ciechanover, 2002). A recent thrust of research indicates that these two signaling pathways not only share structural similarity, but also share a multitude of functional interrelations. These interactions include two discreet and distinct modes. The first mode of interaction is characterized by a stress-dependent competition for shared target lysine residues on a given protein substrate, whereas the second mode of interaction is characterized by a stress-induced formation of SUMOylation-dependent ubiquitin chains on unique lysine residues in close proximity in a given target protein (Schimmel et al., 2008; Praefcke et al., 2012; Gibbs-Seymour

ABBREVIATIONS: Cyp, cytochrome P450; IL-6, interleukin 6; KO, knockout; LC, liquid chromatography; MS, mass spectrometry; NR, nuclear receptor; PCR, polymerase chain reaction; PGC-1 α , peroxisome proliferator-activated receptor gamma coactivator 1- α ; PIAS, protein inhibitor of activated stat; PXR, pregnane X receptor; qPCR, qualitative polymerase chain reaction; RT, real time; SENP, sentrin protease; SIM, small ubiquitin-related modifier-interacting motif; STAT, signal transducer and activator of transcription; SUMO, small ubiquitin-related modifier; TNF α , tumor necrosis factor α .

et al., 2015). The first mode of competitive interaction between SUMO and ubiquitin occurs on lysine residues within the nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor- α , as well as within the proliferating cell nuclear antigen protein (Desterro et al., 1998; Hoege et al., 2002). An example of the second mode of stress-induced SUMOylation-dependent ubiquitylation is exemplified by arsenic inducing promyelocytic leukemia-retinoic acid receptor- α oncoprotein SUMOylation and its subsequent ubiquitylation/K48-linked chain-mediated degradation by the proteasome (Lallemand-Breitenbach et al., 2008). Another example of a protein that undergoes SUMO-dependent ubiquitylation is nuclear factor kappa-B essential modulator, which is activated by consecutive modifications with SUMO and ubiquitin that initiate K48-linked degradation by the proteasome following genotoxic stress (Huang et al., 2003). In each case, the interaction between these two post-translational modifications determines the biologic function and molecular fate of the resulting modified protein.

Our laboratory has previously demonstrated that PXR is SUMOylated to suppress the expression of tumor necrosis factor α (TNF α)-inducible interleukin 1 β gene expression in hepatocytes (Hu et al., 2010). We and others have also previously shown PXR to be a target for the ubiquitin-signaling pathway (Masuyama et al., 2002; Staudinger et al., 2011; Rana et al., 2013), and it is well known that ubiquitylation is an integral part of canonical NR-mediated gene expression (Dennis and O'Malley, 2005). Several studies have shown that phosphorylation controls PXR biologic function as well (Lichti-Kaiser et al., 2009a,b; Pondugula et al., 2009; Sugatani et al., 2012, 2014; Smutny et al., 2013; Elias et al., 2014). Moreover, PXR has been shown to be a target for acetylation (Biswas et al., 2011). Although these respective post-translational modifications of PXR have been observed and characterized in isolation, there has been no examination of the potential biologic role of the interaction between these key signaling pathways at the level of the PXR protein. Here, we characterize the enzymatic reactions that promote SUMOylation and de-SUMOylation of PXR. Using primary cultures of hepatocytes and cell-based assays to demonstrate that the molecular consequence of the SUMO-ubiquitin interaction at the level of PXR defines its role in mediating canonical activation of the xenobiotic response contrasted with PXR-mediated repression of the proinflammatory response.

Materials and Methods

Plasmids and Chemicals. The full-length human PXR expression vectors were previously described (Kliwer et al., 1998; Lehmann et al., 1998). To construct the FLAG-tagged human PXR expression vector, the cDNA-encoding

human PXR was excised from the pSG5-PXR expression vector using EcoRI and SalI sites and inserted into pCMV-Tag 2B (Agilent, Santa Clara, CA) using EcoRI and SalI restriction sites. Expression vectors encoding (His) $_6$ -tagged SUMO1, SUMO2, and SUMO3 were a kind gift of Dr. Ronald T. Hay and were previously described (Tatham et al., 2009). Expression vectors encoding PIAS proteins were a kind gift from Dr. Ke Shuai (Liu et al., 1998) and obtained from Addgene (Cambridge, MA) (plasmid numbers: 15206, FLAG-PIAS1; 15209, FLAG-PIAS α ; 15210, FLAG-PIAS β ; 15207, FLAG-PIAS3; and 15208, FLAG-PIAS γ). The expression vectors encoding the respective SENPs and the corresponding catalytically deficient mutant SENPs were a kind gift from Dr. Ed Yeh (Cheng et al., 2007) and obtained from Addgene (plasmid numbers: 17357, FLAG-SENP1; 17358, FLAG-SENP1m; 18047, FLAG-SENP2; 18713, FLAG-SENP2m; 18048, RGS-SENP3; 18714, RGS-SENP3m; 18053, RGS-SENP5; 18715, RGS-SENP5m; 18065, FLAG-SENP6; 18716, FLAG-SENP6m; and 42886, 3XFLAG-SENP7). Expression vectors encoding hemagglutinin (HA)-tagged wild-type and K48R mutant ubiquitin constructs were previously described (Lim et al., 2005). Inserts encoding wild-type and K48R mutant ubiquitin were excised using EcoRI and NotI restriction enzyme sites and inserted into the EcoRI and NotI sites in pCDNA4-HisMax-A to create (His) $_6$ -tagged forms of wild-type and K48R (His) $_6$ -ubiquitin. The single mutant (His) $_6$ -K63R and double mutant (His) $_6$ -K48,63R expression vectors were created using the primers listed in Table 1 in a QuickChange site-directed mutagenesis reaction per the manufacturer's instructions (Agilent). The reporter plasmid (ER-6) $_3$ -tk-Luc was generated by the insertion of three copies of the double-stranded annealed oligonucleotide primers listed in Table 1 into the Bgl II site of pGL3-Basic. The FLAG-tagged PIAS1 adenoviral expression vector was constructed using the polymerase chain reaction (PCR) primers listed in Table 1 to introduce a Xho I site and was inserted into the pShuttle IRES-hrGFP expression vector (Agilent). The SUMO3 adenoviral expression vector was constructed using the PCR primers listed in Table 1 to insert Spe I and Xho I restriction sites into the 5' and 3' end of the open reading frame of (His) $_6$ -SUMO3, respectively, and was inserted into the pShuttle IRES-hrGFP expression vector (Agilent). The human PXR adenoviral expression vector was constructed using PCR primers to remove the STOP codon in PXR and introduce EcoRV and Xho I restriction sites into the open reading frame of PXR using (His) $_6$ -tagged PXR as a template. The resulting PCR amplicon was inserted into the multiple cloning site in the pShuttle IRES-hrGFP expression vector, and the adenovirus was generated as described (He et al., 1998).

Cell-Based Cobalt-Bead Affinity Pull-Down Assay. The Hepa-6 cell line was used due to its proven utility in studies of hepatic gene expression and liver biochemistry (Darlington et al., 1980). The general strategy for enrichment of SUMO- and ubiquitin-modified PXR was previously described (Staudinger et al., 2011).

Isolation and Culturing of Primary Hepatocytes. PXR knockout (PXR-KO) mice were generated as previously described (Staudinger et al., 2001). Hepatocytes were isolated from congenic (C57BL6) wild-type and PXR-KO mice aged 6–10 weeks using a standard collagenase perfusion method as

TABLE 1
Polymerase chain reaction primers used in cloning and site-directed mutagenesis

Ubiquitin K63R	Left primer	5' gCT gTC TgA TTA CAA CAT TCA gAg gGa gTC CAC CCT 3'
	Right primer	5' Agg gTg gAC TCC CTC TgA ATg TTg TAA TCA gAC AgC 3'
CYP3A4-ER6-PXRE	Sense primer	5' gAT CAA TAT gAA CTC AAA gGa ggT Cag Tg 3'
	Antisense primer	5' gAT CCA CTg ACC TCC TTT gAg TTC ATA TT 3'
FLAG-tagged PIAS1	Left primer	5' gAC ggC CTC gAg ACC ATG GAC TAC AAG GAC GAC 3'
	Right primer	5' gAC ggC CTC gAg TCA gTC CAA TgA gAT AAT gTC Tgg 3'
SUMO3	Left primer	5' gAC ggC ACT AgT Cgg ACg gCC TCC gAA ACC ATG g 3'
	Right primer	5' gAC ggC CTC gAg CTA ACC TCC CgT CTg CCg g 3'
(His)-tagged PXR	Left primer	5' gAC ggC gAT ATC TTA ATA CgA CTC ACT ATA ggg Ag 3'
	Right primer	5' gAC ggC CTC gAg GCT ACC TGT GAT GCC GAA CAA CTC C 3'
PXR-K108R	Left primer	5' CTg gAg AgC ggC ATg Agg AAg gAg ATg ATC ATg 3'
	Right primer	5' CAT gAT CAT CTC CTT CCT CAT gCC gCT CTC Cag 3'
PXR-K128R	Left primer	5' CTT gAT CAA gCg gAA gAg AAg TgA ACg gAC Agg gA 3'
	Right primer	5' TCC CTg TCC gTT CAC TTC TCT TCC gCT TgA TAC CA 3'
PXR-K160R	Left primer	5' gAT ggA CgC TCA gAT gAg AAC CTT TgA CAC TAC CT 3'
	Right primer	5' Agg TAg TgT CAA Agg TTC TCA TCT gAg CgT CCA TC 3'
PXR-K170R	Left primer	5' TAC CTT CTC CCA TTT Cag gAA TTT CCg gCT gCC Ag 3'
	Right primer	5' CTg gCA gCC gGa AAT TCC TgA AAT ggg AgA Agg TA 3'

described previously (Staudinger et al., 2003). Hepatocytes isolated from either male or female mice were used throughout this study to identify any potential sex difference. Identical results were obtained in both sexes. The results shown are from the male mice. The hepatocytes were allowed to attach to the plate for 4 hours, and the medium was then replaced with serum-free Williams E medium as described previously (Staudinger et al., 2003).

Total RNA Isolation, Reverse Transcription, and Real-Time Quantitative-Polymerase Chain Reaction Analysis. Real-time (RT) quantitative polymerase chain reaction (qPCR) was performed as described (Ding and Staudinger, 2005).

Western Blot Analysis. Western blot analysis was performed as described previously (Xu et al., 2009). The antibodies used include anti-PXR antibody (H-11; Santa Cruz, Dallas, TX), anti-SUMO1 antibody (C9H1; Cell Signaling, Danvers, MA), anti-SUMO2/3 antibody (18H8; Cell Signaling), anti-ubiquitin (P4D1; Cell Signaling), and anti- β -actin (monoclonal antibody 1501; Chemicon, Billerica, MA).

Liquid Chromatography-Tandem Mass Spectrometry Analysis. Liquid chromatography (LC)/mass spectrometry (MS) experiments were performed essentially as described (Rankin et al., 2014). Data were processed using Thermo Proteome Discoverer software (version 1.4; Thermo Fisher Scientific, Waltham, MA), whose workflow combined two complementary search engines, Sequest (Eng et al., 1994) and Mascot (version 2.5; Matrix Science, London, UK). The search parameters covered a fragment ion mass tolerance of 0.8 Da, parent ion tolerance of 20 ppm, and cysteine carbamidomethylation as a fixed modification. The modification of lysine by GlyGly (+114.04), a tryptic remainder of ubiquitin attachment to lysine, were included in the search. The protein FASTA database was composed of all murine entries of Uniprot (<http://www.uniprot.org/>). Search results were imported into the Scaffold software (version 4.4; Proteome Software Inc., Portland, OR) for further validation of MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than

90.0% probability, as specified by the Peptide Prophet algorithm (Keller et al., 2002).

Statistical Analysis. Where appropriate, the statistical differences among an experimental group were determined using a one-way analysis of variance followed by the Duncan's multiple range post hoc test. Statistical differences between experimental groups were determined using the Student's *t* test.

Results

PXR Is the Molecular Target of Both the SUMO- and Ubiquitin-Signaling Pathways in Primary Hepatocytes. Several type II liver-enriched NR superfamily members are SUMOylated to modify their transactivation capacity. In most cases, PIAS1 functions as an E3 SUMO ligase to enhance their modification (Pascual et al., 2005; Lee et al., 2009; Lu et al., 2013). Previous research from our laboratory indicated that endogenous hepatic PXR was the molecular target of the SUMO-signaling pathway (Hu et al., 2010). However, the specific SUMO-E3 ligase(s) that performed this function was not investigated. We therefore constructed several adenoviral expression vectors to examine whether PIAS1 could function as a SUMO-E3 ligase to enhance PXR-SUMO(3)ylation in primary hepatocytes (Fig. 1A). Indeed, coexpression of PXR together with PIAS1 and SUMO3 in hepatocytes produced robust SUMO3 modification of this NR family member, as detected using an anti-PXR antibody in western blot analysis following the metal affinity (cobalt beads) methods as described (Staudinger et al., 2011) (Fig. 1B).

A recent report indicated that hepatocyte nuclear factor 4- α is the simultaneous target of SUMO2/3 and ubiquitin to regulate its stability and biologic function (Zhou et al., 2012). We therefore sought

Fig. 1. Adenoviral-mediated approach to examine PXR post-translational modifications. (A) Depiction of adenoviral constructs, including Ad-PIAS1, Ad-PXR, and Ad-SUMO3. Note the use of FLAG epitope and (His)₆-affinity tags that increase the apparent molecular weight of the exogenously expressed proteins. (B) Primary hepatocytes isolated from wild-type (C57Bl6) mice were left nontransduced or were transduced as indicated in the figure. Hepatocytes were lysed using strong denaturing conditions as described in *Materials and Methods*. Cell lysates were subjected to enrichment using cobalt beads, and captured proteins were washed sequentially using guanidine-HCl and urea-based wash buffers. Proteins were eluted using 2X-Laemmli buffer and resolved using 10% SDS-PAGE. Western blot analysis was performed with an anti-PXR antibody that detects all modified forms of the protein (H-11 monoclonal antibody; Santa Cruz). (C) Primary hepatocytes isolated from wild-type (C57Bl6) mice were left nontransduced or were transduced with either blank virus (Ad-GFP) or Ad-PXR. Hepatocytes were lysed using strong denaturing conditions, and western blot analysis was performed with an anti-PXR antibody (H-11 monoclonal antibody; Santa Cruz), anti-SUMO1 antibody (C9H1; Cell Signaling), anti-SUMO2/3 antibody (18H8; Cell Signaling), anti-ubiquitin (P4D1; Cell Signaling), or anti- β -actin (monoclonal antibody 1501; Chemicon) as a loading control.

to determine the extent to which the endogenous SUMO- and ubiquitin-signaling pathways converge at the level of the PXR protein in hepatocytes. Wild-type mouse hepatocytes were transduced with the blank virus adenoviral-green fluorescent protein or the virus encoding a (His)₆-tagged form of adenoviral PXR. Twenty-four hours post-transduction, cells were treated with rifampicin (10 μ M), a potent and efficacious human PXR ligand, for 24 hours. Following rifampicin treatment, hepatocytes were treated for an additional 3 hours with TNF α (10 ng/ml) alone or were cotreated with rifampicin and TNF α together as indicated. Total PXR protein was enriched from whole cell lysates. Protein aliquots were resolved using SDS-PAGE, and a subsequent western blot analysis was performed to detect PXR, SUMO1-, SUMO2/3-, and ubiquitin-modified forms of PXR and β -actin as a loading control (Fig. 1C). The SUMO- and ubiquitin-signaling pathways modified the exogenously added PXR protein, with ligand and TNF α treatment both increasing the levels of detectable nonmodified PXR as well as the SUMO- and ubiquitin-modified forms of PXR. We note here that SUMO(1)ylation of PXR was observed with comparatively low stoichiometry when compared with SUMO(2/3) ylation and ubiquitylation, as judged by their respective sensitivity in the western blot analysis.

Expression of PIAS1 Modulates PXR Activity in Primary Mouse Hepatocytes. We next examined whether expression of PIAS1 altered PXR activity in liver cells. Primary hepatocytes were isolated from 8-week-old male *pdxr*-nullizygous (PXR-KO) mice and cultured overnight. The next day, hepatocytes were transduced with purified adenoviral vectors encoding human PXR, PIAS1, or were cotransduced with both adenoviral expression vectors together. Forty-eight hours post-transduction, hepatocytes were treated with rifampicin (10 μ M), a potent and efficacious human PXR ligand for an additional 24 hours. Following rifampicin treatment, hepatocytes were treated as indicated with TNF α (10 ng/ml) alone or were cotreated with rifampicin and TNF α together for an additional 3 hours as indicated. Total RNA was isolated, and an RT-qPCR analysis was performed to determine expression levels of PXR-target genes. As expected, induction of the well known PXR target gene, cytochrome P450 (Cyp), family 3, subfamily a, polypeptide 11 (*Cyp3a11*), was absent following rifampicin treatment in both nontransduced as well as PIAS1-transduced PXR-KO hepatocytes (Fig. 2A). In contrast, rifampicin treatment produced an approximately 14-fold induction of *Cyp3a11* gene expression levels in PXR-KO mouse hepatocytes expressing human PXR. Treatment of hepatocytes expressing both PXR and PIAS1 with rifampicin produced an approximately 30 5-fold increase in *Cyp3a11* gene expression levels, indicating that PIAS1 has a coactivator effect on PXR with respect to the *Cyp3a11* promoter. Cotreatment of PXR-transduced hepatocytes as well as PXR- and PIAS1-cotransduced hepatocytes, with rifampicin and TNF α , produced significant repression of *Cyp3a11* expression when compared with rifampicin treatment alone. There was no modulation of *Cyp3a11* expression in PXR-KO hepatocytes by any treatment when the addition of exogenous PXR was omitted.

Previous research from our laboratory and others indicates that PXR activation can suppress the cytokine-inducible expression of TNF α and interleukin 6 (IL-6) in the liver and intestine (Teng and Piquette-Miller, 2005; Shah et al., 2007; Hu et al., 2010; Dou et al., 2012; Koutsounas et al., 2013; Sun et al., 2015). We therefore examined the role of PIAS1 in promoting this effect in a PXR-dependent manner in the liver. Primary hepatocytes isolated from PXR-KO mice were transduced with PIAS1 alone, human PXR alone, or both PIAS1 and PXR together for 24 hours. Hepatocyte cultures were treated with either vehicle, rifampicin, TNF α , or rifampicin and TNF α together as indicated. The expression levels of proinflammatory cytokines TNF α

Fig. 2. PIAS1-mediated modulation of the xenobiotic response and inflammatory response in hepatocytes. Primary hepatocytes isolated from *pdxr* nullizygous (PXR-KO) mice were transduced as indicated. Following treatments, total RNA was isolated and the relative expression level of (A) *Cyp3a11*, (B) TNF α , and (C) IL-6 were determined. All data are normalized to β -actin levels and are presented as fold regulation. Asterisks indicate a statistical difference between treatment groups ($n = 3$; $P < 0.05$).

and IL-6 were subsequently examined (Fig. 2, B and C). As expected, in nontransduced PXR-KO hepatocytes, treatment with TNF α (10 ng/ml) for 3 hours increased the expression of TNF α messenger RNA approximately 16-fold. In contrast, cotreatment of nontransduced PXR-KO cells with rifampicin and TNF α together or treatment with rifampicin alone had no significant effect on TNF α messenger RNA levels. Similarly, expression of PIAS1 alone did not modify the TNF α -inducible expression of TNF α messenger RNA. Expression of exogenous PXR significantly increased the basal levels of TNF α messenger RNA by approximately 8-fold when compared with vehicle-treated nontransduced PXR-KO hepatocytes. This is consistent with our previous publication that indicates that hepatocytes lacking PXR exhibit a diminished capacity to mount a robust immune response following challenge with a lipopolysaccharide (Sun et al., 2015). Hepatocytes expressing exogenous PXR that were cotreated with TNF α and rifampicin together exhibited significant repression of TNF α -inducible TNF α messenger RNA expression. The coexpression of PIAS1 and PXR further suppressed TNF α -inducible TNF α expression in a PXR-dependent manner, indicating that PXR and PIAS1 collaborate to suppress cytokine-inducible TNF α expression in hepatocytes. A similar effect was noted when expression levels of IL-6 messenger RNA were examined as well (Fig. 2C).

Detection of the SUMOylation Machinery in Primary Hepatocytes.

To characterize the levels of SUMO and its associated enzymes in the mouse liver, we first examined the expression levels of SUMO1, SUMO2/3, Sae1, Sae2, Ubc9, PIAS1, PIAS2, PIAS3, PIASy, SENP1, SENP2, SENP3, SENP5, SENP6, and SENP7 using cDNA generated using RNA isolated from primary cultures of wild-type C57BL6 hepatocytes isolated from male animals aged 6–10 weeks. Initial studies using standard RT-PCR analysis revealed that all of the genes examined were expressed at detectable levels in mouse hepatocytes (Fig. 3), except for SENP3 (Fig. 3, arrow lane 12). Subsequent RT-qPCR analysis determined that 24-hour treatment with the PXR ligand pregnenolone 16 α -carbonitrile (10 μ M) or 3-hour treatment with TNF α (10 ng/ml) had no effect on expression levels of the genes encoding these enzymes (data not shown).

Fig. 3. Detection of SUMO1-, SUMO2/3-, and SUMO-associated enzymes at the level of gene expression in hepatocytes. Primary hepatocytes isolated from wild-type (C57BL6) mice were isolated and cultured as described in *Materials and Methods*. Total RNA was isolated, and the expression of the indicated genes was determined using standard nonquantitative reverse transcriptase-PCR and agarose gel (2%) electrophoresis methods. The arrow (lane 12) indicates equivocal detection of SENP3 in hepatocytes.

Identification of the SUMO-E3 Ligase Enzymes Important for SUMO Modification of PXR. To determine which PIAS family members could function as the most effective SUMO-E3 ligase toward PXR, the murine hepatoma-derived cell Hepal-6 cells was used (Darlington et al., 1980). Cultured cells were cotransfected with expression vectors encoding FLAG-tagged PXR together with either (His)₆-SUMO1 or (His)₆-SUMO3. An additional expression vector encoding a specific PIAS family member was added as indicated (Fig. 4A). The expression levels of all five PIAS proteins examined in this assay were roughly equivalent (data not shown). In the presence of PIASy, modification of PXR by SUMO1 was supported in at least two sites, as determined using western blot analysis with an anti-PXR antibody following enrichment with cobalt beads (Fig. 4A, asterisks in lane 7). The other four PIAS family members examined (PIAS1, PIASx α , PIASx β , and PIAS3) promoted more modest SUMO1 modification of PXR. When (His)₆-SUMO3 was used in the assay, a more robust SUMO-chain formation was observed, with PIASy promoting robust SUMO(3)ylation of PXR (Fig. 4A, bracket lanes 8, 11, and 12). Although both PIAS1 and PIAS3 promoted SUMO(3)ylation of PXR with high efficiency, PIASy was the most effective SUMO-E3 ligase examined, with respect to SUMO(3)ylation of PXR. Of note, the intensity of the primary nonmodified PXR band was increased in direct proportion to the level of SUMO-modified PXR (Fig. 4A, arrow in lanes 7, 8, 11, and 12).

Identification of the Sentrin Protease Enzymes Important for De-SUMOylation of PXR. There is increasing recognition that de-SUMOylation of SUMO substrates by SENPs represents a key regulatory step in the SUMO-signaling pathway (Mikolajczyk et al., 2007; Bawa-Khalfe and Yeh, 2010). Similar to SUMO-E3 ligase enzymes, the specific SENP(s) that remove SUMO from PXR are currently unknown. We therefore sought to identify the specific SENPs capable of de-SUMOylating PXR using a variation of our transient transfection cell-based assay. Expression vectors encoding FLAG-PXR, PIASy, and (His)₆-SUMO1 were introduced into Hepal-6 cells together with selected SENPs as indicated (Fig. 4B). Where available, the catalytically deficient mutant forms of each SENP (Δ SP1, Δ SP2, Δ SP3, Δ SP5, and Δ SP6) were used as negative controls as indicated. Expression of SENP2 completely abolished SUMOylation of PXR, whereas the catalytically deficient form of SENP2 (Δ SP2) was ineffective. Although expression of SENP1 and SENP6 promoted de-SUMOylation of PXR to some extent, the removal was incomplete. It is noteworthy that the 52-kDa immunoreactive band that corresponds to nonmodified PXR decreases in direct proportion to the level of PXR de-SUMOylation (Fig. 4B, arrow).

PGC-1 α -Mediated Transactivation of PXR Is Regulated by PIASy-Mediated SUMOylation. Interaction of peroxisome proliferator-activated receptor gamma coactivator 1- α (PGC-1 α) with liver X receptor-beta is attenuated by increased SUMO signaling (Ghisletti et al., 2007; Zhang et al., 2012). The PXR NR family member also strongly interacts with and is coactivated by PGC-1 α (Bhalla et al., 2004). We therefore sought to determine the extent to which PGC-1 α -mediated transactivation of PXR is modulated by the SUMO1- or SUMO2/3-signaling pathways. To accomplish this, we constructed a multimerized (3X) PXR-dependent (ER-6 PXR enhancer) luciferase reporter gene [(ER-6)₃-tk-Luc] as described in *Materials and Methods*. Treatment of PXR-transfected CV-1 cells with rifampicin induced the expression of this reporter gene approximately 2.7-fold (Fig. 5). The addition of PGC-1 α significantly increased reporter gene activity in a PXR-dependent manner, whereas the addition of SUMO1 and PIASy together significantly inhibited PGC-1 α -mediated transactivation of PXR. Importantly, the de-SUMOylating enzyme SENP2 significantly restored the SUMO1/PIASy-mediated suppression

Fig. 4. Characterization of SUMO-E3 ligases and SENPs associated with PXR SUMOylation and de-SUMOylation. (A) Indicated expression vectors were transfected into Hepa1-6 cells. Forty-eight hours post-transfection, cells were harvested and SUMOylated proteins were gathered. Proteins were subjected to SDS-PAGE and subsequent western blot analysis using an anti-PXR antibody. Asterisks (*) indicate modified forms of PXR, and the arrow (←) indicates nonmodified PXR protein. (B and C) Expression vectors encoding SENPs and control mutant catalytically deficient SENPs (ΔSPX) were transfected into Hepa1-6 cells in combination with either SUMO1 or SUMO3 together with PXR and PIASy as indicated. Forty-eight hours post-transfection, cells were harvested in denaturing buffer and SUMOylated proteins were captured. Proteins were subjected to SDS-PAGE and subsequent western blot analysis using the anti-PXR antibody. Asterisks (*) indicate SUMO-modified forms of PXR, and the arrow (←) indicates the nonmodified PXR protein. The brackets in (A) and (C) represent the formation of the SUMO chain on PXR.

of PXR/PGC-1α reporter gene activity when compared with cells expressing only PIASy and SUMO1, whereas SENP6 was less effective in this regard. When SUMO3 was used in place of SUMO1 in identical

Fig. 5. PIASy and SUMO1 abrogate association of PXR with PGC-1α to attenuate its transactivation capacity. CV-1 cells were transfected with a PXR-dependent luciferase reporter gene [(ER6)₃-tk-Luc] and expression vectors encoding PXR, PGC-1α, PIASy, SUMO1, SUMO3, SENP2, or SENP6 as indicated. Twenty-four hours post-transfection, cells were treated with vehicle (0.1% dimethylsulfoxide) or rifampicin (10 μM) for an additional 24 hours. Luciferase activity was normalized to β-gal controls, and data are presented as fold induction ± S.E.M. Asterisks indicate statistically significant differences between relevant treatment groups ($P \leq 0.05$).

experiments, the PIASy-mediated suppression of PXR activity was absent, suggesting a differential role for SUMO(1)ylation versus SUMO(3)ylation. However, the addition of SENP2 had a significant positive effect upon both basal- and rifampicin-dependent PGC-1α-mediated transactivation of PXR-dependent reporter gene activity in this case. It is interesting to note that SENP6, which has strong SUMO-chain editing activity and is ineffective at completely removing SUMO3 from PXR (Fig. 4C), had little to no effect on PGC-1α-mediated transactivation of PXR in the face of SUMO3 and PIASy.

SUMO- and Ubiquitin-Signaling Pathways Interface at the Level of PXR. Ubiquitylation of PXR has previously been demonstrated by our group and others (Masuyama et al., 2002; Staudinger et al., 2011; Rana et al., 2013), and pharmacological inhibition of the 26S proteasome in cells inhibits PXR function (Staudinger et al., 2011). However, the precise molecular nature of the ubiquitin chain formation, specific lysine residue on PXR that is the target of ubiquitin, and biologic significance of the ubiquitin-SUMO interaction at the level of PXR are not currently well defined. The novel ubiquitin expression vector we constructed adds approximately 17 kDa to the size of the PXR protein due to the presence of an extended N terminus (Fig. 6A). We engineered several key features into the N terminus of ubiquitin, including a (His)₆-metal-affinity tag for bead-based enrichment as well as both an Xpress-epitope tag and an HA-epitope antibody tag for enrichment and western blot strategies. We have termed this expression vector His-Ub. The His-Ub expression vector was used as a template to create expression constructs that contain mutations at key lysine residues, including 1) lysine 48 mutated to arginine (His-K48R), 2) lysine 63 mutated to arginine (His-K63R), and 3) both lysine 48 and lysine 63 mutated to arginine (His-K48R,K63R). Using His-Ub and the mutant ubiquitin expression vectors in our cell-based assay together with a plasmid encoding FLAG-PXR (52 kDa) in transfection-based experiments, we detect heavily mono-ubiquitinated PXR at the predicted 69-kDa molecular weight (Fig. 6B, lanes 3–6). When His-K48R was used

Fig. 6. K48-linked ubiquitin chain formation on a single lysine residue (K170) within PXR. (A) Single letter amino acid representation of the open reading frame of (His)₆-tagged wild-type ubiquitin. (B) Hepal-6 cells were transfected with expression vectors encoding PXR alone or the wild-type and indicated mutant forms of ubiquitin together with PXR as shown. Cells were lysed and total (His)₆-tagged ubiquitinated proteins were captured using cobalt beads. Captured proteins were subjected to SDS-PAGE, and subsequent western blot analysis was performed using an anti-PXR antibody. Asterisks (*) indicate ubiquitin-modified forms of PXR, and the arrow (←) indicates the nonmodified PXR protein. The bracket and asterisks indicate poly-ubiquitinated forms of PXR. (C) Lysine residue (K170) in PXR was identified based on assignment of multiple product ions (*b* and *y* ions) in the MS/MS scan of the precursor ion at *m/z* 587.95 to the PXR tryptic peptide sequence, with a mass addition of 114 at the lysine residue (ubiquitin di-glycine post-tryptic digestion). (D) MS/MS spectrum assigned to ubiquitin tryptic peptide sequence showed that the lysine residue (K48) carried a modification with a mass of 114 which indicates poly-ubiquitylation.

coexpressed with PXR, chain formation was dramatically reduced. In contrast, when the His-K63R mutant was coexpressed with PXR, chain formation was completely intact. When the double mutant His-K48,K63R construct was used in the assay, ubiquitin chain formation was completely lacking.

To directly examine the nature of ubiquitin chains and site of PXR ubiquitylation using LC-MS/MS-based methods, we took advantage of our adenoviral expression vector encoding (His)₆-tagged human PXR to achieve a high level of expression and relative ease of purification. Primary hepatocytes were transduced with an appropriate amount of PXR virus. Forty-eight hours post-transduction, cells were treated with either vehicle (0.1% dimethylsulfoxide) or rifampicin for an additional 24 hours. Hepatocytes were lysed using denaturing conditions, and total PXR was isolated using cobalt-bead affinity methods (Staudinger et al., 2011). Following SDS-PAGE of PXR-enriched protein lysates, LC-MS/MS methods were used to probe the site of PXR ubiquitylation (Fig. 6C) and the precise nature of observed ubiquitin chains (Fig. 6D). This analysis detected lysine 170 (K170) as the site of PXR ubiquitylation and also confirmed our previous analysis indicating a high level of K48-linked ubiquitin chains.

We next sought to determine whether promoting SUMO1ylation of PXR in cells can affect its modification by ubiquitin. Transfection of Hepal-6 cells with expression vectors encoding His-Ub and PXR produces detectable forms of ubiquitinated PXR, both in the absence and presence of the PXR ligand (Fig. 7, lanes 3 and 4). Coexpression of PIASy and PXR in the absence of His-Ub produces increased levels of unmodified PXR (Fig. 7, lanes 5 and 6). When His-Ub was coexpressed with PIASy and SUMO1 together, the modification of PXR by ubiquitin was dramatically increased (Fig. 7, lanes 3 and 4 versus lanes 7 and 8).

Identification of Site 1 and Site 2 as the Primary Sites of SUMO Modification. The PXR protein contains several lysine residues that are predicted to serve as acceptor sites for SUMOylation (Hu et al., 2010). Among the four sites (labeled Site 1–Site 4) is one high probability type I consensus site (Ψ -K-x-D/E, where Ψ is the hydrophobic residue) at lysine 108 (Site 1, K108) (Fig. 8A). The other three predicted sites have a lower probability to serve as SUMO-acceptor sites. We created a series of mutant PXR expression vectors as indicated at these four potential sites of SUMOylation shown in Fig. 8B. Cotransfection of Hepa1-6 cells with (His)₆-SUMO1, PIASy, and PXR together produced two clear sites of modification following enrichment and western blot with an anti-PXR antibody (Fig. 8B, asterisks). Consistently, wherever mutation of Site 1 appears (K108R, lanes 4, 5, 6, and 8), the upper band disappears. No other lysine to arginine mutation examined in Site 2, 3, or 4 appeared to support SUMO(1)ylation in this analysis. When (His)₆-SUMO3 was used in place of (His)₆-SUMO1, both Sites 1 and 2 in PXR appeared to support SUMO(3)ylation and SUMO-chain formation in a cooperative manner, with adjacent lysine residues 128 and 129 serving as likely sites of further SUMO(3)ylation. It is interesting to note that both Site 1 (MKKE) and Site 2 (KKSE) contain di-lysine residues (KK) embedded within the predicted SUMO-acceptor sites.

Discussion

Protein modification by SUMO was historically thought of as a post-translational modification that largely regulates the biologic function and subcellular localization of many cellular proteins (Yeh et al., 2000). Recent evidence indicates that SUMOylation is often

Fig. 7. PIASy increases levels of ubiquitinated PXR. Hepa1-6 cells were transfected with expression vectors as indicated. Ubiquitinated proteins were captured using cobalt-linked agarose beads. Captured proteins were subjected to SDS-PAGE, and the blots were probed for PXR immunoreactivity. Asterisks (*) indicate ubiquitin-modified proteins.

a prerequisite for or a competitor of shared substrate protein ubiquitylation (Praefcke et al., 2012). Hence, SUMOylation was proposed as a post-translational modification that can possibly influence the degradation of shared SUMO-ubiquitin substrate proteins. More

recently, SUMO2/3-dependent ubiquitin-proteasome proteolysis has been clearly demonstrated to play a critical role in the regulation of the biologic function and fate of key tumor suppressor proteins involved in the development of Fanconi's anemia and other yet to be identified proteins (Schimmel et al., 2008; Praefcke et al., 2012; Gibbs-Seymour et al., 2015).

Although the SUMOylation of liver-enriched NR family members is strongly associated with suppression of the acute phase response, there are important molecular differences governing their anti-inflammatory effect. One key distinction is evident with respect to ligand dependence of the SUMOylation reaction. On the one hand, SUMOylation of the farnesoid X receptor, liver receptor homolog-1, and liver X receptor-beta are all enhanced by the ligand (Venteclef et al., 2010; Balasubramanian et al., 2013), whereas the SUMOylation of the peroxisome proliferator activated receptor- α is decreased by the ligand (Pourcet et al., 2010). Additional differences exist in whether NRs are modified by SUMO1 or SUMO2/3. Most NRs are reported to be modified by either SUMO1 or SUMO2/3, whereas in this study, modification of PXR was observed with both SUMO1 and SUMO2/3. We also found that multiple PIAS family members are capable of promoting SUMO modification of PXR and may thus play distinct roles in modulating its function. Intriguingly, the SENP1, SENP3, and SENP6 de-SUMOylating enzymes selectively remove SUMO chains from PXR, whereas SENP2 is the most effective at removing all SUMO moieties from PXR. Hence, our data indicate that it is likely that SUMO1 and SUMO2/3 play differing roles in regulating PXR biologic function. Our studies also raise the fascinating possibility that PIASy and PIAS1 may play differing respective roles in regulating PXR biology, likely through the selective promotion of SUMO2/3 versus

Fig. 8. Site-directed mutagenesis study of potential sites of SUMOylation of PXR. (A) Four probable sites of SUMO modification were identified using the SUMOplot (<http://www.abgent.com/sumoplot>) and SUMPsp (<http://sumosp.biocuckoo.org/>) prediction analysis servers. (B) Site-directed mutagenesis was performed, and the indicated mutant PXR proteins were transfected together with PIASy and (C) SUMO1 or SUMO3. SUMOylated proteins were captured using cobalt-linked agarose beads. Captured proteins were subjected to SDS-PAGE, and the blots were probed for PXR immunoreactivity. Asterisks (*) and brackets indicate modified PXR proteins.

SUMO1 modification of this NR family member. Further support for this notion can be found in our observation that PIASy-mediated SUMO(1)ylation of PXR strongly increased the presence of both ubiquitylated and nonmodified forms of this NR, suggesting a key interaction between these two post-translational modifications at the level of PXR.

It is worth noting here that inflammatory mediators increase SUMOylation of retinoid X receptor α , a critical heterodimeric partner of PXR (Choi et al., 2006; Schneider Aguirre and Karpen, 2013). This is particularly interesting in light of the fact that not only can retinoid X receptor α function as a partner for the xenobiotic sensor PXR, it also functions as an obligate heterodimeric partner for many other NR family members, including those for retinoic acid, thyroid hormone, vitamin D, prostanoids, oxysterols, and bile acids. Four potential sites for SUMO and ubiquitin modification of PXR were identified using prediction software (Sites 1–4), and our studies identified Site 4 (K170) as a primary site of ubiquitylation of PXR. Furthermore, our studies revealed the formation of K48-linked ubiquitin chains on the PXR protein through Site 4. These data suggest a likely role for ubiquitylation of PXR in regulating its degradation by the 26S proteasome, as K48-linked ubiquitin chains on a single substrate lysine comprises the canonical signal for marking proteins for proteasome-mediated degradation (Chau et al., 1989). Mutation of the well conserved type I (Ψ K-x-D/E) SUMOylation consensus site at lysine 108 (Site 1 **MKKE**) abolished a discrete form of PXR SUMO(1)ylation. In contrast, modification of PXR by SUMO2/3 and subsequent chain formation on PXR required mutations at both Sites 1 and 2. Mutation of Site 2 alone had no effect on PXR SUMOylation, whereas mutation of all four sites strongly reduced the capacity of PXR to support chain formation. These data suggest that SUMO2/3 modification at Site 1 and Site 2 affect ubiquitylation at Site 4, likely through the formation of mixed SUMO-ubiquitin chains or SUMO-dependent ubiquitylation at Site 4 to promote proteasome-mediated degradation of the PXR protein (Fig. 9). Taken together, these results indicate that Sites 1, 2, and 4 serve as the principal attachment sites for SUMO1, SUMO2/3, and ubiquitin to regulate its degradation during the xenobiotic response and mediate PXR-dependent repression of the proinflammatory response.

An increasing number of proteins have been shown to bind SUMO or SUMOylated proteins noncovalently through SUMO-interacting motifs (SIMs). The PXR protein also contains a SIM consensus amino acid sequence, but how this contributes to SUMO-dependent PXR biologic function is yet to be determined. Regardless, there are multiple proteins for which SUMOylation is dependent on the presence of a SIM in the substrate (Song et al., 2004; Kerscher, 2007). The current thought is that SUMO binding to the SIM domain comprises the initial association with its target protein, which precedes SUMO conjugation to the SUMO consensus motif. Another possibility is that the SIM allows modified proteins to interact with new and novel protein partners or allows SUMO substrates to interact with themselves following SUMO modification. Alternatively, there are many transcription factors and coactivator proteins, such as PGC-1 α , involved with PXR transactivation, which are also subject to SUMOylation, thereby allowing the formation of SUMO-dependent multiprotein complexes. Our data indicate that it is likely that PXR interacts with PGC-1 α and that modification by SUMO1, but not SUMO2/3, prevents this protein-protein interaction. The functional significance of this and other complex regulatory networks will require additional studies.

In vivo, SUMOylation can influence single or multiple properties of a target protein, including its stability, localization, or activity. In most cases, SUMO1 modification inhibits transcriptional activity of

Fig. 9. Working model and hypothesis of the role of ubiquitin and SUMO signaling in the regulation of PXR biology. A schematic representation of the molecular basis of the interface between canonical PXR activation and the xenobiotic response (left) and the molecular basis of the role of SUMO PXR in suppression of the proinflammatory response (right).

an NR. Synergistic or antagonistic cross-talk among different types of post-translational modifications can occur, and our data clearly show that PXR modification by SUMO2/3 likely promotes its ubiquitylation to play a pivotal role in facilitating PXR protein degradation and thereby facilitates another round of messenger RNA production. These data are consistent with the ubiquitin-mediated promoter clearance hypothesis put forth by Dennis and O'Malley (2005). However, our data indicate that PXR is likely ubiquitinated in a SUMO2/3-dependent manner and that SUMO(1)ylated PXR is refractory to this phenomenon. More importantly, our data suggest that a very low stoichiometric amount of PXR is in fact modified by SUMO1 in ligand- and TNF α -stimulated primary cultures of hepatocytes, and furthermore, that this particular post-translational modification results in PXR-mediated repression of the proinflammatory response in a ligand-dependent manner. Unraveling the details of how phosphorylation and acetylation or other post-translational modifications on PXR and associated protein cofactors influence PXR biology in the context of SUMOylation and ubiquitylation will undoubtedly require further effort and represent interesting issues for the future.

Authorship Contributions

Participated in research design: Staudinger, Williams, Azuma.

Conducted experiments: Staudinger, Cui, Sun, Galeva.

Contributed new reagents or analytic tools: Staudinger, Galeva, Azuma.

Performed data analysis: Staudinger, Cui, Williams, Galeva.

Wrote or contributed to the writing of the manuscript: Staudinger.

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