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Distinct functional modes of SUMOylation for retinoid X receptor alpha

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ABSTRACT

The present study investigated human retinoid X receptor alpha (hRXRα) as a substrate for modification with small ubiquitin like modifier (SUMO) and how members of the protein inhibitor of activated STAT (PIAS) family may impact upon this process. In agreement with a previous study, we validate Ubc9 to facilitate SUMOylation of hRXRα at lysine 108 but note this modification to occur for all isoforms rather than specifically with SUMO1 and to preferentially occur with the unliganded form of hRXRα. SUMOylation of hRXRα is significantly enhanced through PIAS4-mediated activity with lysine 245 identified as a specific SUMO2 acceptor site modified in a PIAS4-dependent fashion. While individual mutations at lysine 108 or 245 modestly increase receptor activity, the combined loss of SUMOylation at both sites significantly potentiates the transcriptional responsiveness of hRXRα suggesting both sites may cooperate in a DNA element-dependent context. Our findings highlight combinatorial effects of SUMOylation may regulate RXRα-directed signalling in a gene-specific fashion.

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1. Introduction

Retinoid X receptor (RXR) is a nuclear receptor (NR) represented by three isotypes (α, β and γ) for which 9-*cis* retinoic acid (9-*cis* RA), a stereoisomer of all-*trans* retinoic acid (ATRA), represents its most commonly noted ligand [1]. RXR plays a pivotal role within NR biology through its capacity to regulate transcription as a homodimer and also as a heteropartner for NRs such as the vitamin D receptor (VDR), retinoic acid receptor (RAR), peroxisome proliferator activated receptor (PPAR) and liver X receptor (LXR) [2]. Functional loss of the RXRα subtype has the most profound consequences which are lethal in homozygous knockout mice and presenting a heterozygous phenotype that closely reflects the biological functions of RXR-containing heterodimers [3]. Given its unique position within NR biology, RXR represents an attractive target of pharmaceutical interest with its most noted selective agonist Bexarotene (trade name Targretin) currently applied in the treatment of cutaneous T-cell lymphoma [4] and more recently implicated as a putative therapy for Alzheimer's disease [5]. A clear requirement exists for a detailed understanding of RXRα function and how its pleiotropic effects are impacted through metabolic challenges associated with disease, drug treatment or altered nutrient status, which can invoke changes in NR signalling through posttranslational modifications [6]. Human RXRα (hRXRα) is known to be phosphorylated at serine 260, an event that underpins resistance of *Ras*-transformed keratinocytes to the growth-regulatory effects of both ATRA and the active vitamin D metabolite 1,25$(OH)_2D_3$ (1,25D) [7,8]. hRXRα has also been reported to be modified with SUMO1 at lysine 108 (K108), an event which appears to diminish the transcriptional potency of hRXRα [9], although the impact of other SUMO isoforms and RXR ligand(s) were not considered in this study. Given its unique and critical role within NR-directed signalling, we further explored hRXRα as a substrate for SUMO-modification and evaluated novel factors that may modulate this process.

Abbreviations: STAT, Signal Transducers and Activators of Transcription; PIAS, Protein Inhibitor of Activated STAT; SUMO, Small Ubiquitin-Related Modifier; VDR, Vitamin D Receptor; RAR, Retinoic Acid Receptor; FXR, Farnesoid X Receptor; LXR, Liver X Receptor; PPAR, Peroxisome Proliferator Activated Receptor; RXRα, Retinoid X Receptor Alpha; Ubc9, Ubiquitin-Conjugation Enzyme 9; RING, Really Interesting Gene.

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2. Materials and Methods

2.1. Cell Culture and ligands

HEK293 cells were obtained from the European Collection of Cell Culture (ECACC) and maintained at 37 °C, 5% CO2 in presence of DMEM + 10% FBS, 2 mM L-glutamine, 50 units/ml penicillin G and 50 μg/ml streptomycin. Media and supplements were purchased from GIBCO (Invitrogen, Carlsbad, CA) and 9-*cis* retinoic acid (9-*cis* RA) from Sigma. Bexarotene was synthesized as detailed by Wagner and co-workers [10].

2.2. Plasmids

Expression constructs encoding hRXRα (pSG5hRXRα and pcDNAV5-hRXRα), mouse PIAS proteins (pcDNAV5-PIAS, pcDNAGFP-PIAS and pcDNAHA-PIAS) and the Gal4-based hybrid expression vector (pCMVBD-RXRα) have been previously detailed [11]. Plasmids expressing His-tagged SUMO isoforms have been described by Dorval and Fraser [12] and were a generous gift of Prof Paul Fraser, University of Toronto. The pcDNAV5-UBC9 expression construct and its C93S mutant variant were provided by Prof Ron Hay, University of Dundee and used as a template to generate the vector encoding 'untagged' Ubc9 as previously detailed [13]. The firefly luciferase-based reporter construct pLUC-RXRE contains a retinoid X receptor responsive element (RXRE) based on a naturally occurring double repeat responsive element from the rat cellular retinol binding protein II gene [14]. The sequence used was 5′-AAAATGAACTGTGACCTGTGACCTGTGACCTGTGAC-3'

One copy of this double RXRE sequence was synthesized with an additional four base overhang for cloning into the HindIII and BglII sites of the pLUC-MCS reporter plasmid (Invitrogen). The transcriptional responses of Gal4-RXRα hybrid proteins were monitored through the luciferase signal generated from the pFLUC reporter (Stratagene) that contains five copies of the Gal4 response element.

2.3. Western blotting

Protein lysates were separated on 4–12% NuPAGE Bis/Tris gels (Invitrogen), transferred onto Immobilon-P membranes (Millipore Corp) and then probed using the following antibodies: mouse monoclonal anti-V5 (Invitrogen) at a 1:5000 dilution; mouse monoclonal anti-GFP (Invitrogen) at a 1:1000 dilution. Target proteins were visualized using Supersignal®West Pico Chemiluminescent solution (Thermo Scientific) and development on autoradiographic film. All membranes were re-probed with a mouse monoclonal anti-β-actin antibody (Sigma) at 1:10,000 dilution. The secondary antibody was a rabbit anti-mouse IgG (whole molecule) peroxidase conjugate antibody (Sigma).

2.4. Cell-based SUMOylation assays

Modification of hRXRα with SUMO was monitored through a cell-based protocol we have previously described [13]. Briefly, HEK293 cells were seeded in 60 mm plates before transfection with the appropriate combination of constructs expressing hRXRα (2 μg), SUMO1/2/3 (2 μg), Ubc9 (1 μg), PIAS (1 μg) or mutated variant and appropriate parent vector control. Cells were incubated for a total of 48 h post-transfection including a 24 h period ± 9-*cis* RA (1 μM) when considering effects of ligand. Cell lysates were processed as previously detailed before overnight incubation with V5 agarose beads (Abcam) followed by elution and subsequent analysis through western blotting.

2.5. Site-directed mutagenesis

Synthesis of point mutations within the appropriate hRXRα encoding construct was achieved through the Quikchange XL site-directed mutagenesis system (Agilent Technologies) using the following mutagenic primer pairs;

RXRα K108R
5′-CAGCGAGGACATCAGGCCCCCCCTGGGC-3′
5′-GCCCAGGGGGGGCCTGATGTCCTCGCTG-3′
RXRα K245R
5′-GGCCGTGGAGCCCAGGACCGAGACCTACG-3′
5′-CGTAGGTCTCGGTCCTGGGCTCCACGGCC-3′

2.6. Transcriptional activation assays

HEK293 cells were seeded unto a 24-well plate (100,000 cells/well) and maintained for a period of 24 h before introduction of plasmid via calcium phosphate precipitation. At 16 h post-transfection, cells were incubated in media supplemented with 1 μM ligand (9-*cis* RA or bexarotene) or appropriate control for a further 24 h, before recording of chemiluminescent signal using the Dual-Glo Luciferase Reporter Assay System (Promega). Transfection data were normalized relative to the luciferase signal produced from the constitutively active renilla vector (pRL-TK) and expressed as the means ± SEM from triplicate assays.

3. Results

We employed cell-based assays to evaluate the potential for hRXRα to be modified with different SUMO isoforms and how binding of its 9-*cis* RA cognate ligand may impact upon this process. Fig. 1A details that under our specified conditions hRXRα can be conjugated with each tested SUMO isoform, with the major modified form of the receptor appearing as a distinct band migrating at ~90 KDa (Fig. 1A, upper arrow) and the presence of 9-*cis* RA appearing to significantly diminish this process. To confirm these bands as specific products of the SUMO-enzymatic pathway, comparative assays were performed employing Ubc9 or its C93S variant possessing a deficient E2-SUMO conjugation function. As depicted in Fig. 1B, hRXRα-SUMO2 could only be detected in the presence of the enzymatically intact Ubc9 (middle lane). Finally, as data detailed above were derived from transfected cell lysates in which hRXRα and Ubc9 were both expressed as V5-'tagged' proteins, we wished to verify that higher migrating bands were not related to SUMO-modified forms of V5-Ubc9 that may have been co-precipitated in our assay system. Fig. 1C illustrates that significantly increased levels of hRXRα-SUMO2 are detected when using a non-tagged version of Ubc9 which also elicited additional higher migrating bands (~110KDa/160 KDa) signifying formation of multiple forms of hRXRα-SUMO2. Subsequent assays described in this manuscript incorporate the use of the non-tagged form of Ubc9.

Fig. 2A describes the capacity of PIAS proteins to modulate hRXRα SUMOylation. These experiments involved co-expression of V5-hRXRα with the indicated combination of Ubc9, His-SUMO and HA-PIAS1/2/3/4 or the HA-PIAS4 (W356A) mutant that is defective in E3-SUMO ligase activity. The upper panel details the ability of PIAS1, 2, and 3 to increase the formation of SUMO1-hRXRα (~90 KDa), however such effects are modest when compared to those achieved through PIAS4 for which the appearance of an additional band (~110 KDa) suggest possible modification at more than one site. PIAS4 also enhanced hRXRα conjugation with SUMO2 (Fig. 2A, middle panel and Fig. 2B) and SUMO3 (Fig. 2A, lower panel) with no such activity exhibited by the other PIAS proteins. As

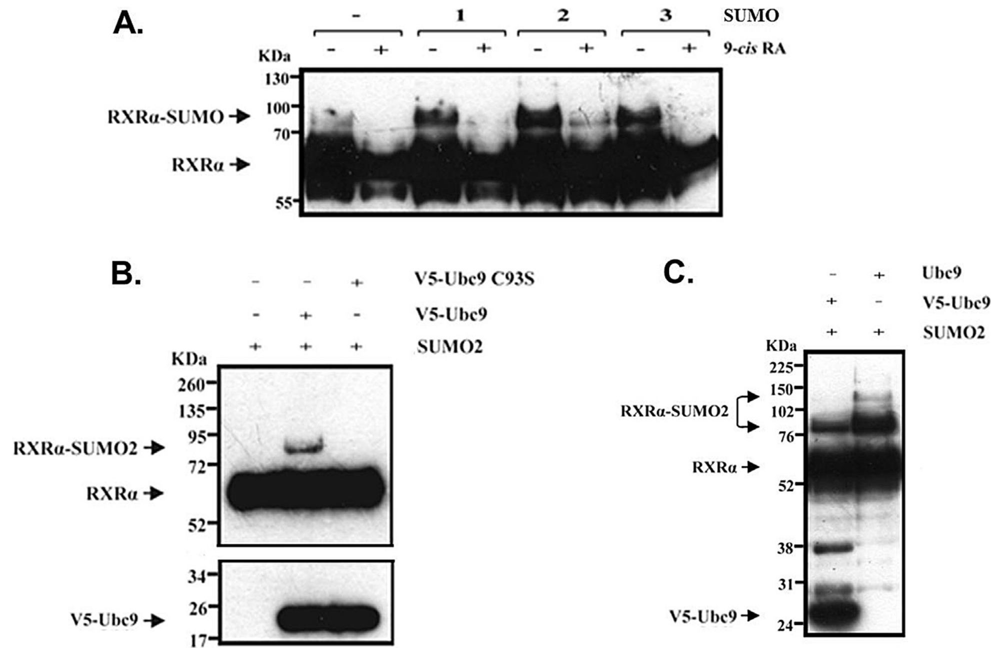

Fig. 1. **A**. The unliganded form of hRXRα can be covalently modified with SUMO1, 2 and 3. HEK293 cells were transfected with expression constructs for V5-hRXRαand V5-Ubc9 in combination with His-SUMO1, His-SUMO2, His-SUMO3 or empty vector control (−) where indicated. After a 24 h period of incubation ± 9-*cis* RA, lysates from each treatment group were processed as detailed in Materials and Methods and resulting immunoprecipitated material subjected to western blot analysis using an antibody specific for the V5 epitope tag. The arrows highlight the detected SUMO-modified (90 KDa) and unconjugated (62 KDa) forms of V5-RXRα. **B**. SUMOylation of hRXRα is dependent upon an enzymatically intact Ubc9. Cell-based assay was performed to compare hRXRα modification with SUMO2 when using constructs encoding V5-Ubc9 or its catalytically inactive C93S variant. The lower panel confirms equal expression within precipitated lysates of both V5-Ubc9 forms. **C**. Detection of SUMO-hRXRα is increased using the native (untagged) form of Ubc9. Cell-based assays compared levels of SUMO2-hRXRα detected using the V5-tagged (left lane) and native (right lane) forms of Ubc9. The arrows highlight the V5-Ubc9 (left lane only) and hRXRα detected through western blot analysis of precipitated lysates with enhanced levels of SUMO2-hRXRα noted when untagged Ubc9 was employed.

expected, the PIAS4 W356A mutant had no effect on modification with SUMO1 or SUMO2 but unexpectedly retained a capacity to enhance formation of SUMO2-hRXRα. We probed this observation further, as well as the impact of PIAS4 when expressed in the absence of exogenous SUMO protein. While Fig. 2C confirms co-expression of SUMO2 and Ubc9 to result in the modification of hRXRα (lane 2), we note also the presence of PIAS4 and Ubc9 are by themselves sufficient to generate a modified hRXRα (lane 3) likely conjugated with endogenous SUMO protein. The combined expressions of PIAS4, Ubc9 and SUMO2 (lane 4) dramatically enhanced receptor SUMOylation and invoked the appearance of multiple SUMO-hRXRα conjugates. In contrast, the W356A mutant exhibited no comparable effects when reliant upon endogenous SUMO as substrate (lane 5) but surprisingly when co-expressed in combination with SUMO2 generated a single dominant band at ~90 KDa (lane 6). Taken in combination, these data indicate PIAS4 to enhance RXRα modification with all three SUMO isoforms, an event which may involve formation of poly-SUMO chains on RXRα and/or modification at more than one acceptor site within the receptor. For one of these potential sites, PIAS4 may facilitate generation of hRXRα-SUMO2 independently of its RING finger ligase function.

To further investigate the potential for hRXRα to be modified at more than one site, we compared the patterns of SUMOylation exhibited by the wild type receptor and a variant possessing a lysine (K) to arginine (R) substitution at amino acid 108 (K108R) previously reported to serve as a SUMO1 acceptor site within hRXRα [9]. The experiments detailed in Fig. 3 included Ubc9 and PIAS4 within all tested parameters. Fig. 3A verifies modification of wild type hRXRα with each SUMO isoform but also demonstrates hRXRαK108R retains the capacity to form a SUMO2-conjugated species, while Fig. 3B details that this event is inhibited or reversed when assessed in the presence of 9-*cis* RA. No conjugates were detected to form between the K108R mutant and SUMO1 or SUMO3. These results implicate that hRXRα possesses at least one acceptor site additional to K108 that is specific for SUMO2. Analysis of the hRXRα protein sequence reveals SUMO consensus motifs at K201 and K245 which we then evaluated for their relevance to receptor modification through generating K to R mutations at each site, alone and in combination with K108R. In Fig. 3C highlights those experiments that focused on K245 and K108 as potential acceptor sites. While the expected pattern of modification is observed with wild type hRXRα, both the K245R and K108R variants exhibit a single band corresponding to SUMO2-hRXRα with a complete loss of receptor modification noted for the double (K108R/K245R) mutant RXRα. These data verify hRXRα to contain two acceptor sites represented by K108 and K245, with the latter apparently specific for modification with SUMO2.

We next determined the functional relevance of both acceptor sites in the context of hRXRα transactivation by its ligand, employing two distinct reporter assay systems. In Fig. 4A, HEK293 cells received the appropriate expression constructs for V5-RXRα or its mutant variants in combination with the pLUC-RXRE based reporter construct containing two copies of a retinoid x response element (RXRE) sequence. The data is expressed as the fold increase (induction) of reporter activity achieved through treatment with the synthetic RXR agonist bexarotene over that obtained with vehicle control. As illustrated, both mutant receptors exhibit fold inductions that are significantly higher than that of the wild type hRXRα and while their levels of activity are overall statistically equivalent, a clear trend is demonstrated in which the K108R/K245R variant exhibits induction levels approximately 240% greater that of the wild type hRXRα compared to 98% and 128% for K108R and K245R, respectively. Consideration of these transcriptional

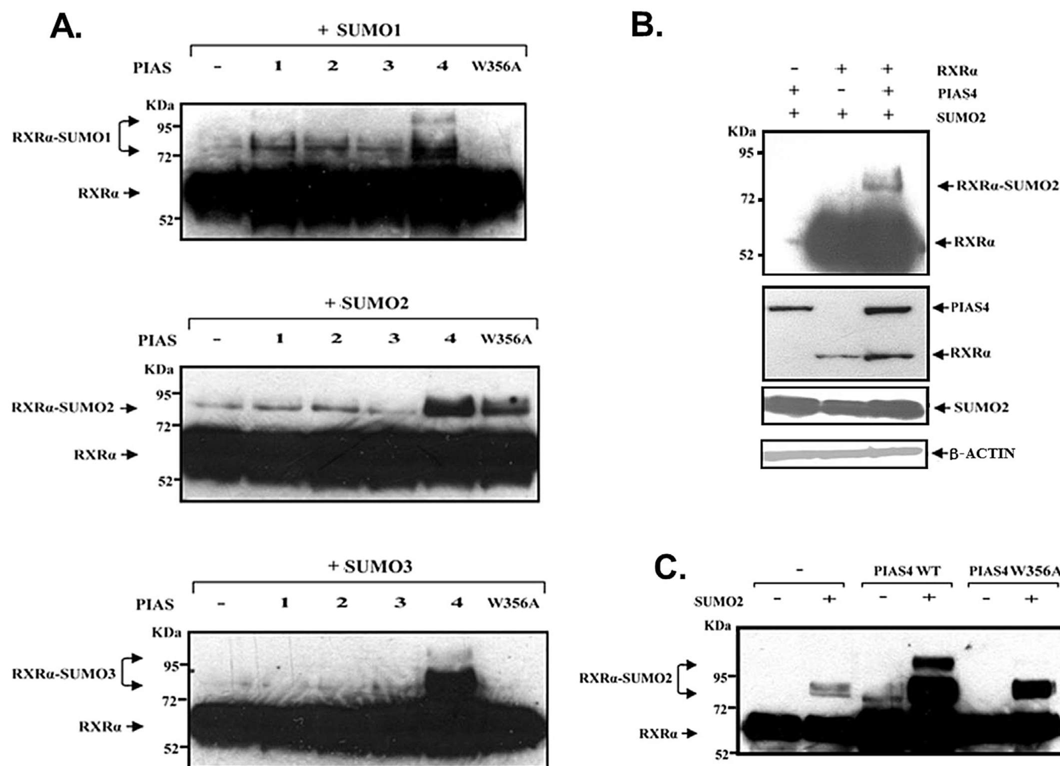

Fig. 2. A. PIAS4 facilitates SUMOylation of hRXRα. Depicted are cell-based SUMOylation assays in which HEK293 cells received expression constructs for His-SUMO1 (upper panel), His-SUMO2 (middle panel) or His-SUMO3 (lower panel) each in combination with V5-hRXRα and where indicated, Ubc9, HA-PIAS1, 2, 3, 4 or the catalytically inactive HA-PIAS4 W356A. Resulting immune-precipitated lysates were probed through western blot analysis using the V5-specific antibody. Arrows highlight detected V5-hRXRα and its slower migrating species conjugated with the different SUMO isoforms. **B**. Confirmation of PIAS4 as a catalyst in modification of hRXR with SUMO2. HEK293 cells were co-transfected with the indicated combination of plasmids encoding V5-hRXRα, GFP-PIAS4, His-SUMO-2 and Ubc9. The upper panel denotes western blot analysis of immune-precipitated material processed as described above highlighting V5-hRXRα and its SUMO-modified form. The lower panels confirm the expression status of GFP-PIAS4, V5-hRXRα and His-SUMO2 within cell lysates derived from each treatment group using the appropriate antibodies specific for each epitope tag. **C**. PIAS4 can promote SUMOylation of hRXRα independent of its E3 ligase function. The abilities of PIAS4 and its enzymatically inactive W356A variant were assessed for their abilities to enhance modification of hRXRα with SUMO2. HEK293 cells were transfected with the indicated combinations of expression constructs encoding V5-hRXRα, His-SUMO2, Ubc9, HA-PIAS4 (WT) or HA-PIAS4W356A with (−) representing inclusion of the appropriate parent vector control. Immune-precipitated lysates were subjected to the depicted western blot analysis that highlights the unconjugated and SUMO2-modified forms of V5-hRXRα.

effects should note that when compared to the wild type hRXRα, the protein levels for the double mutation are consistently observed to be lower in the presence of ligand suggesting the detected functional differences may be even more pronounced if normalized based on equivalency of protein expression.

In Fig. 4B, each hRXRα was expressed as a hybrid protein fused to the Gal4 DNA binding domain with transactivation monitored through the pFLUC reporter containing five tandem copies of the Gal4 response element. The data are depicted as overall reporter activity and reveal the K108R/K245R mutant to exhibit a level of activity in the presence of ligand that is approximately 907% greater than that achieved using the wild type-based construct. In contrast, the individual K108R or K245R mutants do not exhibit significantly different activities from the intact receptor, at least when tested in the context of this reporter system.

4. Discussion

In this current study we probe hRXRα as a substrate for SUMOylation and demonstrate Ubc9 to facilitate modification of this receptor with all three SUMO isoforms, an event that is apparently reversed or impeded in the presence of 9-*cis* RA. Ligand binding is known to invoke co-repressor release followed by ubiquitination and proteasome-mediated degradation as events integral to the RXRα transcriptional cycle [15,16]. While we note hRXRα protein levels to be slightly reduced within precipitates derived from ligand-treated samples compared to vehicle control counterparts, they are not sufficient to explain the striking loss of receptor modification in the presence of 9-*cis* RA. It is possible that ligand-binding may elicit changes in receptor conformation that limit the accessibility of acceptor sites or introduce alternate modifications at the same or neighbouring residues. Choi and co-workers identified the removal of SUMO1 from hRXRα to be mediated through sentrin/SUMO specific protease (SENP) activity, specifically that of SENP6, although the impact of ligand within this process was not defined [9]. We recently reported that ligand bound VDR will recruit members of the SENP family to reverse its modification with SUMO2 [11]. It will be interesting to probe if such a model of ligand-driven deSUMOylation is also a modulatory component within the RXR transcriptional response.

The data discussed above considers the SUMOylation of hRXRα when driven through Ubc9 activity alone. In this context, Choi and co-workers identified lysine 108 (K108) located within the AF1 region of RXRα as a single and atypical acceptor site [9]. While Ubc9 can effectively mediate SUMOylation, other sites may be ‘hidden’ and require the additional presence of an E3-SUMO ligase for their modification [17]. As such an example, we recently reported PIAS4 to serve as a SUMO ligase for the modification of VDR with SUMO2 [13] and in the present context of hRXRα we identify PIAS4 to also robustly enhance conjugation of this receptor with each co-

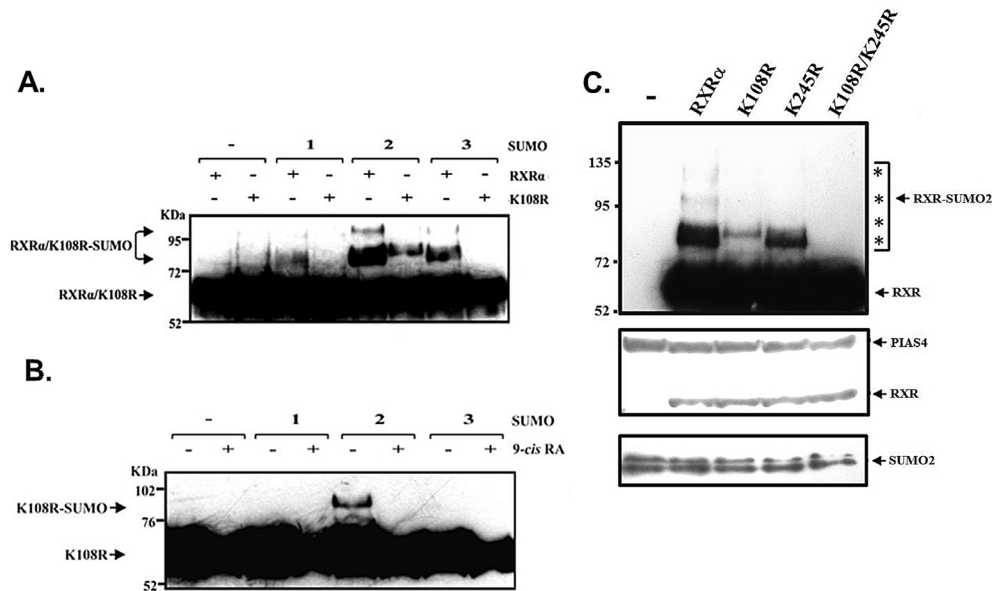

Fig. 3. **A**. Evidence for a second SUMO acceptor site within hRXRα. HEK293 cell-based SUMOylation assays were performed as previously described to compare modification profiles exhibited by V5-hRXRα and its K108R variant that harbours a mutation of a previously identified SUMO1 acceptor site. Depicted are resulting western blot analysis using the V5-specific antibody of immune-precipitated cell lysates containing either wild type or mutant forms of V5-hRXRα in combination with the indicated construct for SUMO1, 2, 3 or empty vector control. **B**. Confirmation that second acceptor site within hRXRα is specific for SUMO2. HEK293 cells received expression constructs for V5-K108R RXRα mutant and the indicated SUMO isoform and incubated ± 9-*cis* RA. All treatment groups were processed and subjected to analysis as described above and in Materials and Methods. Arrows highlight both SUMO-conjugated and unmodified forms of the RXRαK108R protein. **C**. Identification of K245 as a SUMO2 acceptor site within hRXRα Cell-based SUMOylation assays were performed to compare patterns of modification with SUMO2 exhibited by wild type V5-RXRα and its variants K108R, K245R and K108R/K245R. The upper panel depicts western blot analysis using the anti-V5 antibody of immune-precipitated lysates derived from each treatment group. The lower panels confirm the expression status of GFP-PIAS4, V5-RXRα and His-SUMO2 within lysates representing each treatment group.

expressed SUMO isoform in addition to increased modification with endogenous SUMO. Any effects noted for other PIAS proteins are considerably weaker and limited to a modest increase in receptor conjugated with SUMO1. Surprisingly we find the PIAS4 variant that is deficient in E3-ligase activity retains a significant capacity to enhance formation of hRXRα–SUMO2. Similar observations have been reported for YY1 in which PIAS4 is proposed to facilitate interactions between substrate, Ubc9 and SUMO that serve to increase the accessibility of acceptor sites for conjugation [18]. Our results also highlight the intriguing possibility that hRXRα may possess acceptor sites that are alternately modified through distinct PIAS4-directed pathways which we seek to verify and further characterize.

Our data identify K245, a residue located within the omega loop region of hRXRα as an additional SUMO acceptor site to K108. The pattern of modification exhibited by the K108R-RXRα mutant reveals it retains a capacity to be specifically modified with SUMO2 in the presence of PIAS4, an event that is again reversed through the presence of ligand. Taken in combination our data indicate K108 to be the predominant acceptor site within hRXRα that can be

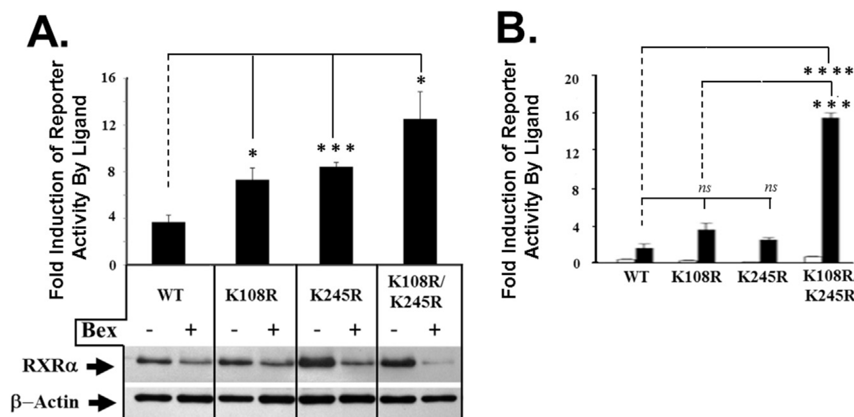

Fig. 4. **A & B**. Loss of the K108 and K245 SUMOylation sites within hRXRα increases its transactivation by ligand. HEK293 cells received: **A**. the pMCS-RXRE luciferase based reporter construct in combination with the appropriate expression constructs for V5-hRXRα or its K108R, K245R or K108R/K245R variants; **B**. the pFLUC reporter vector together with pCMVBD-based expression vector encoding hRXRα or the indicated mutant variants. Treated cells were dosed with bexarotene (10^{-6} M) ligand or vehicle control for a period of 24 h before measurement of luciferase activity. After normalization for transfection efficiency based on the activity of the pRL-TK control, results were expressed as fold-inductions (ratio of activity in the presence:absence of ligand) for **A**. or relative luciferase activity for **B**. All data within each figure represents means (±SE) of triplicate assays (n = 3) where ns $p \geq 0.05$, *p = 0.01–0.05, ***p = 0.0001–0.001, ****p < 0.0001.

conjugated with all three SUMO isoforms, while K245 is specifically modified by SUMO2 in a PIAS4-dependent fashion. SUMO-modified nuclear receptors typically exhibit decreased levels of transactivation and our data also signify SUMOylation to repress the transcriptional responsiveness of hRXRα to its ligand. Our analysis focused on hRXRα signalling in a homodimeric context with consideration for how SUMOylation impacts upon its function as a heteropartner beyond the scope of this current study. We confirm the findings of Choi and co-workers that loss of the K108 as an acceptor site will result in a modest but significant increase in the transcriptional potency of RXRα [9] but note similar effects with the K245R-RXRα variant signifying that although this site is not as extensively modified as K108, it is of at least equal importance in terms of functional relevance. The most significant changes in ligand response are displayed by the hRXRα variant that is completely deficient in SUMOylation. When assessed through the RXRE-based reporter system, the heightened levels of transactivation exhibited by the K108R/K245R RXRα variant are an additive combination of the effects achieved by each individual mutation. More striking effects are noted when activity is monitored through the Gal4-hybrid system that utilizes a reporter construct containing five tandem copies of the Gal4 response element. In this context, the levels of transactivation to ligand exhibited by the double mutant are profoundly increased and potentiated in a synergistic manner over those displayed by the intact receptor and its single mutant variants.

Taken in combination, our data suggest that SUMOylation and the involvement of PIAS4 activity may serve to modulate hRXRα-directed signalling in a promoter-specific context through functional interactions between its K108 and K245 acceptor sites. This represents a possible mechanism through which hRXRα may achieve gene-selective actions under varying physiological challenges.

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Transparency document

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