The Nuclear Receptor Interaction Domain of GRIP1 Is Modulated by Covalent Attachment of SUMO-1*

The steroid receptor coactivator (SRC) proteins comprise a well-characterized family of nuclear receptor (NR) coactivators that increase transcriptional activation by NRs via covalent modification of chromatin proteins and recruitment of other coactivators. We have recently shown that the SRC family member GRIP1 interacts with a class of SUMO-1 (small ubiquitin-like modifier 1) E3 ligases, the PIAS proteins, and that the coactivator is subjected to SUMO-1 modifications (sumoylation). In this work, we demonstrate that lysine residues 239, 731, and 788 of GRIP1 serve as principal attachment sites for SUMO-1. Lys-731 and Lys-788 are located in the NR interaction domain (NID), and their substitution by arginines impairs the ability of GRIP1 to colocalize with androgen receptor (AR) in nuclei. Likewise, Lys-731 and Lys-788 mutants of GRIP1 have attenuated ability to enhance AR-dependent transcription and fail to synergize with PIASx-mediated activation of AR function, indicating that sumoylation modifies the ability of GRIP1 to function as a steroid receptor coactivator. The Lys-731 sumoylation site is conserved in SRC-3 and SRC-1, and the NIDs of the latter coactivators harbor one or two additional sites matching with the consensus sites for SUMO-1 attachment, respectively, suggesting a more general role for the modification in the regulation of SRC protein activity.

The steroid, retinoic acid, and thyroid hormone receptors belong to a large family of nuclear receptors (NR). The NRs are ligand-regulated transcription factors that recognize specific DNA elements and thereby regulate transcription of their target genes (1). Transcriptional activity of NRs relies on coregulator complexes (2). The SRC family comprises a well-established and important group of NR coactivator proteins (3), including SRC-1/NcoA-1, GRIP1/TIF2/NcoA-2 (SRC-2), and pCIP/ACTR/AIB1 (SRC-3) (4–9). The SRC proteins interact with the NRs in a ligand-dependent manner and enhance transcriptional activity of the receptors via recruitment of additional cofactors such as CBP/p300 and proteins with acetyltransferase or methyltransferase activity. The activation domain 1 (AD1) of SRC proteins (amino acids 1040–1120 in GRIP1, Fig. 1A) is responsible for binding to the cofactor CBP/p300 (5, 6). The NR interaction domain (NID) harboring three LXXLL motifs is located in the central part of SRC proteins and interacts with the NR activation function 2 (AF-2) in the ligand-binding domain (LBD) (5, 10–12). The C terminus of SRC proteins that contains the activation domain 2 (AD2) binds other coactivator proteins, such as the arginine methyltransferase CARM1 and the Zac1 protein (13, 14).

Even though many mechanistic aspects of NR and coregulator function have become more clear, the ways by which these proteins compartmentalize in cell nuclei together with factors regulating dynamics of coactivator-receptor complex formation and exchange have remained elusive. Post-translational modifications allow fast alterations in protein-protein interactions in protein assemblies or subcellular structures and thus potentially control the dynamics and exchange of steroid receptor coactivator complexes. Recent evidence indicates that phosphorylation indeed regulates the activity of SRC proteins. Elimination of two mitogen-activated protein kinase (MAPK) sites of SRC-1 attenuated its ability to coactivate progesterone receptor-dependent transcription (15). Furthermore, MAP kinase-mediated phosphorylation of GRIP1 and AIB1 have been implicated in their regulation (16, 17), and in the case of AIB1 phosphorylation stimulates recruitment of p300 (17). Acetylation of ACTR by CBP/p300 in turn disrupts the association of this coactivator with NRs (18).

SUMO (small ubiquitin-like modifier) modification (sumoylation) is a recently characterized covalent modification leading to attachment of SUMO to specific lysine residues of target proteins, especially nuclear proteins (19–21). Even though sumoylation is mechanistically similar to ubiquitination, sumoylation does not promote protein degradation. Instead, the modification appears to regulate protein-protein interactions and protein targeting. SUMO proteins can be divided into two families, SUMO-1 and SUMO-2/SUMO-3 proteins (19–21). In ubiquitination, E3 ubiquitin ligases that promote ubiquitin transfer from the E2 enzyme to the target lysine are responsible for substrate specificity (22). Interestingly, PIAS proteins that interact with steroid receptors and GRIP1 and modulate their transcriptional activity (23, 24) have recently been shown to function as E3-type SUMO ligases (25–27). In this work, we have identified the principal SUMO-1 acceptor sites in GRIP1 and show that the sites located in the NID regulate the function of this SRC protein. Conversion of the SUMO-1 acceptor

* This work was supported by grants from the Academy of Finland, the Finnish Foundation for Cancer Research, the Sigrid Juselius Foundation, Biocentrum Helsinki, and the Helsinki University Central Hospital. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ The abbreviations used are: NR, nuclear receptor; AR, androgen receptor; ARE, androgen response element; EGFP, enhanced green fluorescent protein; GRIP1, glucocorticoid receptor-interacting protein 1; LUC, luciferase; NID, nuclear receptor interaction domain; STAT, signal transducer and activator of transcription; PIAS, protein inhibitor of activated STAT; SRC, steroid receptor coactivator; SUMO, small ubiquitin-like modifier; CREB, cAMP-response element-binding protein; CBP, CREB-binding protein; LBD, ligand-binding domain; FBS, fetal bovine serum; CS, charcoal stripped; PML, promyelocytic leukemia protein.
lysines to arginines in the NID impairs nuclear colocalization of GRIP1 with AR and compromises the activity of GRIP1 on AR-dependent transcription. Our results suggest that SUMO-1 modification can act as a novel determinant in NR-SRC interactions.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—pARE-TATA-LUC and pB8-285′-32′-LUC have been described previously (28). pSG5-LUC was from Promega. pSG5-AR, pSG5-K239R, pSG5-K1452R, pSG5-His-GRIP1, and pFLAG-Miz1 (full-length PIASxI) were constructed as described (23, 28–30). pSG5-GRIP1, pSG5-His-SUMO-1, and pFLAG-PIAS1 were from Drs. M. R. Stallcup, A. Dejean, and K. Shuai, respectively. pGST-U1p was obtained from Dr. M. Hochstrasser. pSG5-GRIP1 Lys→Arg mutants (K239R, K1452R, K731R/K788R, and K239R/K731R/K788R) were generated using the site-directed mutagenesis system according to the manufacturer’s instructions (Stratagene).

**Cell Culture and Transfections**—COS-1 cells and HeLa cells (American Type Culture Collection) were maintained in Dulbecco’s minimal essential medium containing penicillin (25 units/ml), streptomycin (25 units/ml) and 10% (v/v) fetal bovine serum (FBS). HeLa cells were supplemented with non-essential amino acids. Cells were seeded for reporter assays and immunoprecipitations onto 12-well plates (3 × 10⁴ cells) or 6-cm dishes (3 × 10⁵ cells), respectively, and transfected 24 h later using FuGENE reagent (Roche Molecular Biochemicals) and 10 mM [35S]-ethylmaleimide. Concentrated (2 ×) SDS sample buffer (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 5 mM EDTA, 15 mM MgCl₂, 0.5% Nonidet P-40, 0.3% Triton X-100, 1 mM dithiothreitol, 1:200 protease inhibitor mixture (Sigma), and 10 mM N-ethylmaleimide). Concentrated (2 ×) SDS sample buffer was added to lysate samples, and after heating at 95 °C for 5 min proteins were resolved on SDS-PAGE and transferred onto Hybond membranes (Amersham Biosciences). GRIP1 was detected with mouse monoclonal anti-GRIP1 antibody (NeoMarkers) and horseradish peroxidase-conjugated anti-mouse immunoglobulin G (Zymed Laboratories Inc.). Immunocomplexes were visualized with ECL Western blotting detection reagents from Amersham Biosciences. Immunoprecipitation of GRIP1 from cell extracts was performed with mouse monoclonal anti-GRIP1 antibody. Bound proteins were released in 2× SDS sample buffer and immunoblotted with monoclonal antibody against GMP-1 (SUMO-1) (Zymed Laboratories Inc.) and horseradish peroxidase-conjugated secondary antibody.

**Microscopic Inspection of AR and GRIP1 Distribution**—COS-1 cells grown on glass coverslips (1.5 × 10⁵ cells/well) on six-well plates were transfected with FuGENE with 0.75 µg of pEGFP-AR with or without pSG5-GRIP1, and the total amount of DNA was balanced to 1.5 µg with empty pSG5. 24 h after transfection the cells received fresh medium with 2% CS FBS containing vehicle or testosterone after which they were fixed with paraformaldehyde (4% in phosphate-buffered saline) and permeabilized with Triton X-100 (30). Immunofluorescence labeling was performed with Rhodamine-Red-X-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories) recognizing monoclonal anti-GRIP1 (a gift from Dr. Myles Brown). Fluorescence analysis was carried out with the Bio-Rad MRC-1024 confocal laser system (488 nm excitation for EGFP and 568 nm for rhodamine) connected to the Zeiss Axiovert 135M microscope using a ×63, 1.4 numerical aperture oil immersion objective. Both channels were excited, collected separately, and merged for the inspection of colocalization.

**RESULTS**

**Identification of SUMO-1 Attachment Sites in GRIP1**—Four lysine residues (Lys-239, Lys-731, Lys-788, and Lys-1452) in the GRIP1 sequence fulfill the consensus SUMO-1 attachment site (31). To examine whether these lysine residues serve as SUMO-1 acceptors in mammalian cells, GRIP1 was expressed in COS-1 cells without or with SUMO-1, and the cell lysates were immunoblotted with the anti-GRIP1 antibody. Expression of wild-type GRIP1 with or without 100 nm testosterone or dexamethasone. 48 h after transfection the cells were harvested, and luciferase and β-galactosidase activities were determined (29).

**Immunoblotting and Immunoprecipitation**—Cells were harvested, and luciferase and β-galactosidase activities were determined (29). To examine whether these lysine residues serve as SUMO-1 acceptors in mammalian cells, COS-1 cells were transfected with 1 µg of pSG5-GRIP1 or pSG5-GRIP1 mutants and 1.5 µg of empty pSG5 or pSG5-His-SUMO-1 as depicted. 48 h after transfection the cells were collected, and cell lysates were immunoblotted with anti-GRIP1 antibody. C, 5% of lysates derived from COS-1 cells transfected with GRIP1 or GRIP1(K239R/K731R/K788R) in the presence and absence of SUMO-1 were immunoblotted with anti-GRIP1 antibody, and the remaining lysates were subjected to immunoprecipitation (IP) with anti-GRIP1 antibody followed by immunoblotting (WB) with anti-SUMO-1 antibody.

**Identification of the major sumoylation sites in GRIP1**—A, schematic structure of GRIP1 showing potential SUMO-1 acceptor lysine residues 239, 731, 788, and 1452. B, sumoylation of wild-type and mutated GRIP1 in mammalian cells. COS-1 cells were transfected with 1 µg of pSG5-GRIP1 or pSG5-GRIP1 mutants and 1.5 µg of empty pSG5 or pSG5-His-SUMO-1 as depicted. 48 h after transfection the cells were collected, and cell lysates were immunoblotted with anti-GRIP1 antibody. C, 5% of lysates derived from COS-1 cells transfected with GRIP1 or GRIP1(K239R/K731R/K788R) in the presence and absence of SUMO-1 were immunoblotted with anti-GRIP1 antibody, and the remaining lysates were subjected to immunoprecipitation (IP) with anti-GRIP1 antibody followed by immunoblotting (WB) with anti-SUMO-1 antibody.

**SUMO-1** was thought to be incapable of forming polymers (32), but SUMO-1 chain formation has recently been demon-
strated on nucleoporin RanBP2 (33). However, mechanistic aspects of (poly)SUMO formation and characteristics of the SUMO-1-SUMO-1 linkage are unknown. Treatment of immunopurified GRIP1 with Ulp1, a SUMO-specific protease (34), yielded monomers rather than species indicative of SUMO-1 multimers, implying that sumoylation of GRIP1 corresponds to attachment of SUMO-1 monomers to specific lysine acceptors.

Substitutions of the Sumoylation Sites in GRIP1 Impair Its Colocalization with AR in Nuclei—Sumoylation plays a role in protein targeting (19, 21). To elucidate whether substitutions of SUMO-1 acceptor sites in GRIP1 alter subcellular distribution or localization of GRIP1 in relation to AR, full-length AR fused to enhanced green fluorescent protein (EGFP-AR) and wild-type GRIP1 or GRIP1 mutants were coexpressed in COS-1 cells. GRIP1 was visualized by monoclonal antibody against GRIP1 along with a rhodamine-conjugated secondary antibody. When expressed alone, wild-type GRIP1 typically resided in round nuclear granules that were considerably larger in size and fewer in number than the holoAR foci (Fig. 2A). Interestingly, coexpression of holoAR with wild-type GRIP1 elicited coactivator redistribution in nuclei, resulting in a complete colocalization with AR (Fig. 2B). The overall nuclear distribution of GRIP1(K731R/K788R) or other GRIP1 mutants alone did not differ from that of the wild-type protein (Fig. 2, C and H). However, the K731R/K788R mutant showed significantly diminished colocalization with AR in that one-half of the cells expressing both proteins displayed the GRIP1 mutant in nuclear foci that were distinct from AR granules (Fig. 2F). The colocalization pattern of GRIP1(K239R/K731R/K788R) was comparable to that of GRIP1(K731R/K788R), whereas that of GRIP1(K239R) was only slightly impaired (see Fig. 2, G and H). Distribution of the GRIP1(K1452R) was indistinguishable from that of wild-type GRIP1 with respect to colocalization with AR. These results indicate that sumoylation sites in the NID of GRIP play a role in targeting or recruitment of the coactivator to AR-containing subnuclear sites.

Disruption of SUMO-1 Acceptor Sites Blunts the Transcriptional Activity of GRIP1 on AR-dependent Transcription—To study the importance of GRIP1’s sumoylation sites on AR-dependent transcription, COS-1 cells were transfected with ARE2TATA-LUC containing two androgen response elements in front of a TATA-box together with AR and wild-type GRIP1 or GRIP1 mutants. Wild-type GRIP1 activated AR-dependent transcription in a dose-dependent manner yielding a 40-fold enhancement yielding a 7-fold increase in transcription (Fig. 2B). Therefore, we also tested whether mutations of these sites attenuate the function of GRIP1 on glucocorticoid receptor-dependent transcription; both GRIP1(K731R/K788R) and GRIP1(K239R/K731R/K788R) displayed only ~50% of the activity of the wild-type protein.

Lysines 731 and 788 of GRIP1 are located in the NID region. Therefore, we also tested whether mutations of these sites attenuate the function of GRIP1 on AR LBD in isolation. To this end expression vectors encoding AR LBD fused to the Gal4 DNA-binding domain, and wild-type GRIP1 or GRIP1 mutants were expressed in COS-1 cells. The activity of AR AF-2 was measured by using a reporter driven by Gal4-binding sites (G5-LUC). In agreement with our previous results (29), the holoAR LBD alone was transcriptionally silent in COS-1 cells, but ectopic expression of wild-type GRIP1 yielded a 40-fold activation of AR LBD-dependent transcription (Fig. 4A). Mutation of Lys-239 or Lys-1452 failed to influence the activity of GRIP1 on AR LBD in isolation, but GRIP1(K731R/K788R) had less than half of wild-type GRIP1 activity. Thus, the sumoylation sites located in the NID influence the interaction of the coactivator with the AR LBD. In keeping with these results the NID mutations did not alter the function of the coactivator on an AR mutant devoid of the whole LBD (ARΔ641–902, Ref. 29) (Fig. 4B).
Sumoylation Sites of GRIP1 Are Critical for the Transcriptional Cooperation with PIAS Proteins—To study the importance of SUMO-1 acceptor sites of GRIP1 for its transcriptional synergy with PIAS proteins (24), wild-type GRIP1 or GRIP1(K239R/K731R/K788R) was expressed with and without PIAS1 or Miz1 (PIASx/H9252), and their effects on AR-dependent transcription were assayed. A small amount of PIASx/H9252 or PIAS1 alone caused only a modest 1.6- or 1.2-fold transcriptional enhancement, respectively (Fig. 5). When wild-type GRIP1 was expressed together with PIASx/H9252 or PIAS1, their effects were clearly more than additive in that AR-dependent transcription was activated by 15.1- or 10.1-fold, versus 5.3-fold activation by GRIP1 alone (Fig. 5). Elimination of the three sumoylation sites in GRIP1 blunted the synergy with PIAS proteins, as only 5.6- or 3.2-fold activation of AR-dependent transcription was seen when the GRIP1(K239R/K731R/K788R) was coexpressed with PIASx/H9252 or PIAS1, respectively. These results imply that the SUMO-1 modifications of GRIP1 are involved in the transcriptional cooperation between GRIP1 and PIAS proteins.

DISCUSSION

In this work, we have characterized SUMO-1 modification of the nuclear receptor coactivator protein GRIP1 and shown that residues Lys-239, Lys-731, and Lys-788 of GRIP1 act as potential SUMO-1 acceptors. Lys-731 and Lys-788 are located in the NID, and their concomitant mutation abolishes most of GRIP1 sumoylation. The NID harbors three LXXLL motifs (NR-boxes), and Lys-731 is located between NR-boxes II and III.
whereas Lys-788 resides C-terminally to NR-box III. The latter two NR-boxes are crucial for the ability of GRIP1 to bind to NRs, with the NR-box III being essential for the interaction with AR LBD (11). Interestingly, GRIP1(K731R/K788R) that carries two substitutions in the NID exhibited markedly compromised ability to enhance transcription dependent on holoAR and also showed attenuated activity on AR LBD in isolation. It is worth pointing out that GRIP1 function was blunted by the K731R/K788R mutation to an extent comparable to that elicited by a compound mutation disrupting NR-boxes II and III (35). Moreover, the effects of these mutations are not restricted to AR, as they also influence the activity of GRIP1 on glucocorticoid receptor-dependent transcription. Intriguingly, the Lys-731 sumoylation site is conserved in the two other members of the SRC family, SRC-1 and SRC-3, and the NIDs of these coactivators harbor one (Lys-786 in SRC-3) or two (Lys-780 and Lys-806 in SRC-1) additional sites matching the consensus motif for SUMO-1 attachment.

SUMO-1 modifications are involved in the regulation of subcellular and subnuclear targeting and localization of proteins (19, 21). The best characterized examples include RanGAP1 and promyelocytic leukemia protein (PML). Attachment of SUMO-1 to RanGAP1 targets it to the nuclear pore complex and during mitosis to the kinetochores and mitotic spindles (36, 37). Modification of PML by SUMO-1 directs it to subnuclear domains termed PML nuclear bodies (38). Sumoylation also regulates protein-protein complex formation. Attachment of SUMO-1 to PML is needed for PML-mediated recruitment of other proteins (39). In view of these data, one can envision that SUMO-1 modifications also modulate transcription factor-co-regulator targeting. In agreement with this notion, the reduced transcriptional activity of the GRIP1 NID mutant was accompanied by its impaired colocalization with AR in nuclei, suggesting that sumoylation of GRIP1 at specific sites is involved in targeting the coactivator to AR-containing domains. By and large, the factors influencing subnuclear localization and targeting of steroid receptors and their coactivators are poorly understood. Therefore, it is not currently clear why the localization pattern of GRIP1(K731R/K788R) in relation to AR varied among the cells; about one-half of the unsynchronized cells displayed a colocalization pattern indistinguishable from that of wild-type GRIP1. Potential explanations include cell cycle-related factors that influence nuclear architecture. GRIP1 has recently been shown to localize in nuclear foci, a subset of which associates with the PML bodies (40), and it is plausible that the sumoylation of GRIP1 influences the tethering of the coactivator to these bodies. Interestingly, association of certain proteins with the PML bodies is indeed regulated with the cell cycle (41, 42).

Many DNA-binding transcription factors, such as AR, p53, c-Myb and c-Jun are subjected to SUMO-1 modification (43–46). Elimination of sumoylation sites from these proteins increases their transcriptional activity in most instances. In view of this it was somewhat unexpected that mutations of the SUMO-1 acceptor sites in GRIP1 attenuated their activity on AR-dependent transcription. However, it is possible that SUMO-1 modifications of AR and GRIP1 occur at different stages of AR-transcription complex formation to serve distinct roles in gene activation.

Chromatin immunoprecipitation studies have shown that estrogen receptor α and coactivators assemble on target promoters in a fashion that is sequential and rapidly cycling (47). Because SUMO-1 modification is a specific, reversible, and highly dynamic process, it may provide an important means to regulate assembly and disassembly of transcriptional complexes. We hypothesize that PIAS protein-promoted sumoylation of GRIP1 (25) enables the recruitment of the coactivator along with a PIAS protein from subnuclear domains to AR-containing sites. Subsequently, AR-bound GRIP1 recruits CBP/p300 and other coactivators to the promoter, which in turn leads to activation of target gene transcription. Sumoylation of AR may take place after the first cycle of transcription has commenced, leading to displacement of the receptor from DNA. The involvement of PIAS proteins in this assembly is supported by our findings that these proteins act in a synergistic fashion with GRIP1 in steroid receptor-dependent transcription (24). As shown in the current study, this synergism is abolished when the SUMO-1 acceptor sites of GRIP1 are mutated. Sumoylation may also regulate CBP/p300 function, since the two proteins possess three potential SUMO-1 acceptor sites at their bromo domain adjacent to their acetyltransferase domain. In view of this it is interesting that SUMO-1 modification of histone deacetylase 1 has recently been shown to potentiate its activity (48). Taken together, SUMO-1 modification is emerging as an important control mechanism in subnuclear targeting and protein-protein interactions. Its role in the regulation of steroid receptor-coactivator assemblies warrants further studies.
GRIP1 Is Modulated by Covalent Attachment of SUMO-1

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