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Sumoylation of the Estrogen Receptor α Hinge Region Regulates Its Transcriptional Activity

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The steroid hormone 17β-estradiol (estrogen) plays a significant role in the normal physiology of the mammary gland and breast cancer development primarily through binding to its receptor, the estrogen receptor α (ERα). ERα is a nuclear transcription factor undergoing different types of posttranslational modifications, *i.e*. phosphorylation, acetylation, and ubiquitination, which regulate its transcriptional activation and/or stability. Here we identify ERα as a new target for small ubiquitin-like modifier (SUMO)-1 modification in intact cells and *in vitro*. Moreover, ERα sumoylation occurs strictly in the presence of hormone. SUMO-1 appears to regulate ERα-dependent transcription. Using a series of mutants, we demonstrated that ERα is sumoylated at conserved lysine residues within the hinge region. Mutations that prevented SUMO modification impaired ERα-induced transcription without influencing ERα cellular localization. In addition to identifying protein inhibitor of activated signal transducer and activator of transcription (PIAS)1 and PIAS3 as E3 ligases for ERα, we also found that PIAS1 and PIAS3, as well as Ubc9, modulated ERα-dependent transcription independently from their SUMO-1 conjugation activity. These findings identify sumoylation as a new mechanism modulating ERα-dependent cellular response and provide a link between the SUMO and estrogen pathways. (*Molecular Endocrinology* 19: 2671–2684, 2005)

ESTROGEN RECEPTOR α (ERα) is a member of a large conserved superfamily of steroid hormone nuclear receptors acting as ligand-regulated transcription factors (1, 2). ERα regulates many physiological pathways in response to its natural ligand, 17β-estradiol (E2). Hormone binding induces conformational changes that create a new interface for the recruitment of transcriptional auxiliary factors, the binding of ERα to estrogen response element (ERE) in gene promoters, and the regulation of the transcriptional activity of genes involved in proliferation, development, and differentiation. The cofactors bind directly to receptors and generally function as components of large, multimolecular complexes that mediate transcriptional activation or repression by recruiting the transcriptional machinery to the promoter or by remodeling chromatin, as well as exercising their own enzymatic activities, such as histone acetyltransferases and deacetylase, ATPase, protease, kinase, ubiquitin ligase, and histone methyl transferases (3–7). Moreover, different types of posttranslational modifications, namely phosphorylation, acetylation, and ubiquitination, also regulate the transcriptional activation and/or stability of nuclear receptors (8, 9).

Recently, a new covalent modification of proteins, especially of transcriptional regulators, has been described: SUMO (small ubiquitin-like modifier) modification. This is a covalent modification leading to the attachment of SUMO to specific lysine residues of target proteins, mainly nuclear proteins. SUMO represents a class of ubiquitin-like proteins conjugated, like ubiquitin, by a set of enzymes to cellular proteins. Even if mechanistically similar to ubiquitination, sumoylation does not promote protein degradation and the two processes involve distinct enzymes. Protein sumoylation involves SAE1/SAE2 heterodimer acting as E1 enzyme in mammals (Aos1/Uba2 in yeast) and Ubc9 acting as E2 SUMO-conjugating enzyme (10–13). In ubiquitination, E3 ubiquitin ligases, which promote ubiquitin transfer from E2 enzyme to the target lysine, are responsible for substrate specificity (14). Although target specificity remains unclear in the SUMO modification pathway, proteins of the mammalian protein inhibitor of activated signal transducer and activator of transcription (PIAS) family, Ran BP2 and the polycomb PC2 repressor, have recently been shown to function as E3-type SUMO ligases (15–19). The analysis of many SUMO substrates indicates that SUMO modification occurs at a particular sequence, ψKxE [where ψ represents L, I, V, or F, and x is any amino acid (aa)]; thus, specificity of SUMO conjugation might be conferred by recognition of this sequence by the thioester-linked Ubc9-SUMO conjugate. SUMO modification

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Abbreviations: aa, Amino acids; AF, activation function; AR, androgen receptor; DBD, DNA-binding domain; E2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen response element; GR, glucocorticoid receptor; GST, glutathione-*S*-transferase; NEM, *N*-ethylmaleimide; Ni-NTA, nickel-nitrilotriacetic acid; PIAS, protein inhibitor of activated signal transducer and activator of transcription; SUMO, small ubiquitin-like modifier.

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appears to regulate diverse cellular processes, including nuclear transport, signal transduction, apoptosis, autophagy, cell cycle control, ubiquitin-dependent degradation, as well as gene transcription (reviewed in Refs. 20–23). Interestingly, several studies have reported that SUMO-1 regulates the hormone-induced transactivation of some nuclear receptors. This regulation can be achieved by sumoylation of either receptors or coregulators (24–27), identifying sumoylation as an integral part of nuclear hormone receptor functions. In particular, a recent report indicated that ERα-mediated transcription is stimulated by SUMO-1 expression (28). Although it has been speculated that the enhancement of ERα transcription by SUMO-1 may be realized by the sumoylation of the coactivator steroid receptor coactivator 1, the molecular basis by which SUMO-1 regulates ERα transcriptional capacity remains to be determined.

In this study, we show, for the first time, that the hinge region of ERα is a specific target of SUMO-1, and that PIAS1 and PIAS3 proteins act as SUMO-E3 ligases for this receptor. These effects occur strictly in the presence of hormone and affect the transcriptional properties of ERα. We propose that ERα sumoylation is viewed as a new mechanism modulating ER-mediated processes in both normal and cancer cells.

RESULTS

ERα Is Modified by SUMO-1 *in Vivo*

To determine whether ERα undergoes sumoylation, we analyzed COS-7 cells that were transiently transfected with constructs encoding ERα and His-tagged SUMO-1. Transfected cells were lysed in the presence of *N*-ethylmaleimide (NEM), an inhibitor of SUMO-1 hydrolase, and transfection efficiency was evaluated by Western immunoblotting of total cellular proteins. As shown in Fig. 1A, anti-ERα antibody detected a major band of approximately 66 kDa (marked with an *arrowhead*) corresponding to ERα and two slow-migrating forms of ERα in the presence of SUMO-1 (marked with a *star*). ERα was then immunoprecipitated from these extracts and analyzed by Western immunoblotting with anti-SUMO1 and anti-ERα antibodies. Figure 1B shows that anti-SUMO-1 recognized two bands in the immunoprecipitate from cells transfected with both ERα and SUMO-1-encoding constructs. Anti-ERα monoclonal antibody was used to confirm that a similar amount of ERα was expressed in and immunoprecipitated from COS-7 transfected cells. Anti-ERα antibody also detected two slower ERα forms that comigrated with the two bands recognized by anti-SUMO-1 (Fig. 1B, *left panel*). None of these bands was detected in COS-7 cells transfected with constructs encoding ERα alone. To unambiguously establish that the high molecular weight bands corresponded to sumoylated ERα, we transfected COS-7 cells as described above, and then lysed them under denaturing conditions to protect sumoylated proteins from isopeptidases. We next purified the His-tagged proteins by chromatography on nickel-charged agarose beads [Ni-nitrilotriacetic acid (NTA)] and analyzed

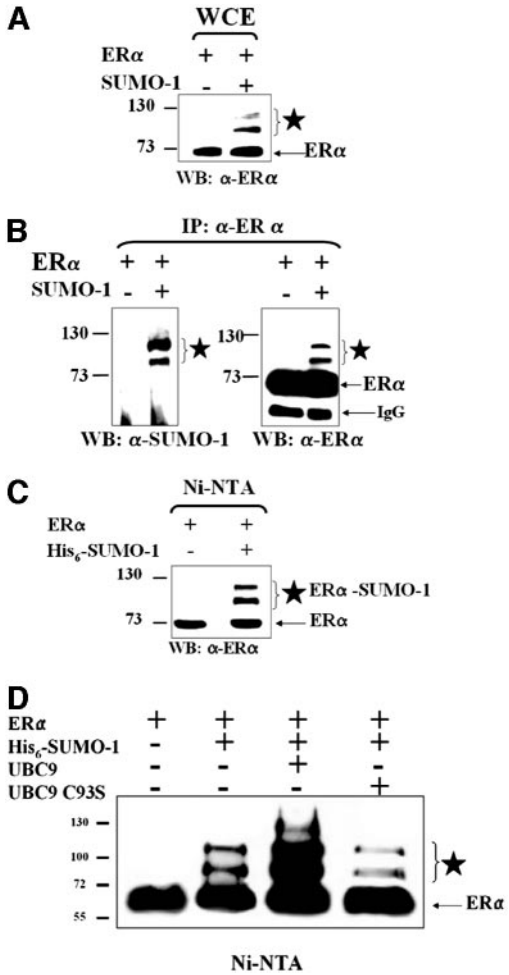

Fig. 1. ERα Is SUMO-1 Modified *in Vivo*

COS-7 cells were cotransfected with expression plasmids encoding ERα and His_6-SUMO-1 in the presence of 100 nM E2. Cells were harvested 24 h after transfection. A, Transfected cells were lysed in the presence of NEM [whole-cell extract (WCE)] and immunoblotted with the anti-ERα monoclonal antibody Ab15. B, The NEM cell extract was immunoprecipitated with the anti-ERα antibody Ab10. Immunoprecipitates were resolved by SDS-PAGE and then probed with anti-SUMO-1 (*left*) and anti-ERα antibody Ab15 (*right*). C, Cells were lysed in buffer containing guanidium-HCl, and SUMO-1 modified proteins were purified by chromatography on nickel-charged agarose beads (Ni-NTA) as described in *Material and Methods*, resolved by SDS-PAGE, and analyzed by immunoblotting using anti-ERα monoclonal antibody Ab15. Molecular mass markers are shown in kilodaltons. D, COS-7 cells were cotransfected with expression plasmids encoding ERα, His_6-SUMO-1, Ubc9, and Ubc9/C93S in the presence of 100 nM E2. His-tagged proteins were purified and analyzed as described in panel C. The *star* indicates the position of SUMO-1 conjugated ERα. WB, Western blot; IP, immunoprecipitation.

them by Western blotting using an anti-ERα monoclonal antibody (Fig. 1C). As can be seen from Fig. 1B, in the absence of SUMO-1 we detected a band corresponding to unmodified ERα, probably retained on Ni-NTA beads by the zinc finger motifs. When ERα was coexpressed with SUMO-1, two slower-migrating forms of ERα (marked with a *star*), absent in control cells, were revealed (Fig. 1C). These data provide strong evidence that slower-migrating forms of ERα originate from covalent linking to SUMO-1. We then tested whether the expression of Ubc9, the E2 SUMO-conjugating enzyme, affected the sumoylation of ERα. COS-7 cells were transiently transfected with expression plasmids for ERα, His_6-SUMO-1, and Ubc9. His-tagged proteins purified by chromatography on nickel-charged resin were subjected to immunoblotting with anti-ERα. As shown in Fig. 1D, we observed a clear increase of ERα sumoylation when Ubc9 was expressed in the presence of SUMO-1. To further support that Ubc9 directly affected the SUMO modification of ERα, we expressed under the same conditions the catalytically inactive form of Ubc9, Ubc9/C93S, with SUMO-1. Western blot analysis indicated that Ubc9/C93S expression resulted in a significant reduction of the SUMO-modified ERα (Fig. 1D). Ubc9/C93S probably behaves as a dominant-negative mutant suppressing the function of the SUMO-modification pathway.

Taken together, our experiments indicate that ERα is a new target for SUMO-1 modification *in vivo*.

Mapping of Sumoylation Regions in ERα

Because ERα lacks the described SUMO-1 acceptor consensus sequences, we have so far been unable to predict the modified lysines among the 28 ones present in ERα protein. To address this issue, we constructed deletion mutants containing different regions of ERα fused to the transactivation domain of VP16 (see Fig. 2A). The VP16-fused mutants were expressed in COS-7 cells with His_6-SUMO-1, and their sumoylation state was examined by chromatography on nickel-charged agarose beads and analyzed by Western blotting. As shown in Fig. 2B, only the mutant ER2 (aa 176–302) was strongly postmodified in this assay. Two slower migrating bands in the range of approximately 50 and 70 kDa were detected by anti-ERα only in the presence of SUMO-1 (Fig. 2B). Because SUMO-1 migrates at about 20 kDa, these bands represent the addition of one to two SUMO molecules to VP16-ER2 (~32 kDa apparent molecular mass). The two ERα regions, ER1 (aa 1–180) encompassing the activation function (AF)1 region, and ER3 (aa 297–595) containing the ligand-binding domain and the AF2 region, were not affected by cotransfection with SUMO-1 (Fig. 2B). The mutant ER2 contains the DNA-binding domain (DBD) consisting of two zinc finger motifs that, through the so-called P box, is responsible for ER binding to EREs and, in combination with a D box, controls the dimerization of ERα on EREs. The D

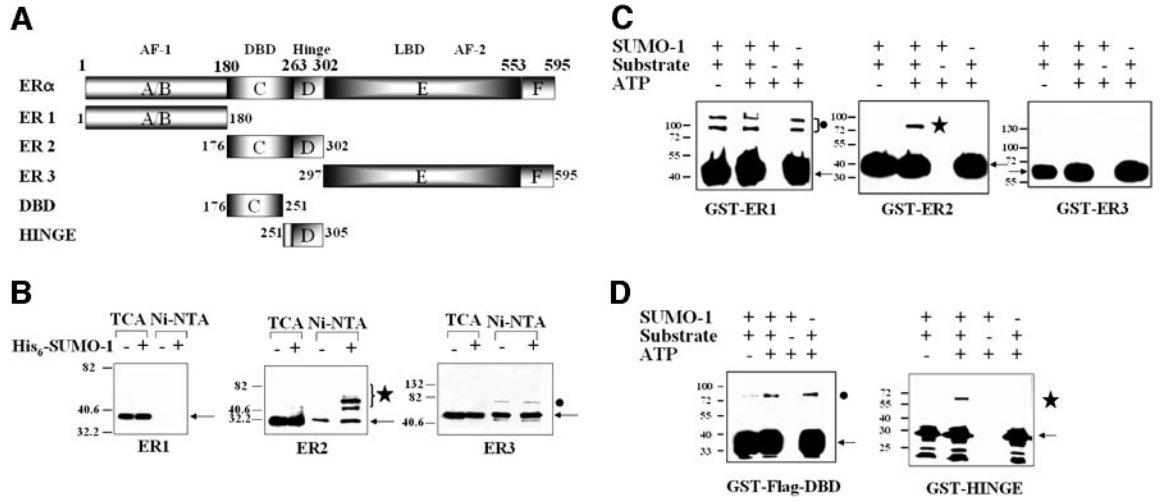

Fig. 2. SUMO-1 Modification at ERα Hinge Region

A, Schematic representation of ERα deletion mutants used in this study. B, *In vivo* sumoylation of ERα fragments. COS-7 cells were transfected with expression vectors encoding ERα deletion mutants in the presence or absence of His-tagged SUMO-1 with 100 nM E2. Cells were harvested 24 h after transfection. His-tagged proteins were purified as described in the legend of Fig. 1C and analyzed by immunoblotting using Ab15 antibody to detect ER1 and G-20 and HC-20 antibodies to detect ER2 and ER3, respectively. As Western control, 60 μg of total protein extracts were trichloroacetic acid (TCA) precipitated. C and D, *In vitro* sumoylation of ERα mutants. GST-tagged mutants were incubated with purified recombinant components required for SUMO-1 modification: GST-SUMO-1, His-Uba2/Aos1 dimer, and GST-Ubc9. Control reactions were also performed in the absence of ATP, substrate, and GST-SUMO-1, respectively. Reaction products were fractionated by SDS-PAGE and analyzed by Western blotting as described above. GST-Flag DBD and GST-Hinge were analyzed using M2 anti Flag and G-20 antibodies, respectively. Molecular mass markers are shown in kilodaltons. The unmodified and the modified forms of the mutants are marked by an *arrowhead* and by a *star,* respectively. *Solid circles* indicate aspecific cross-reactive bands.

domain, also called the hinge region, is implicated in coregulatory protein binding. Our results clearly indicate that ERα sumoylation sites are located in the central region of the receptor (residues 176–302) (see Fig. 2B). To confirm these results *in vitro*, we used the same ERα mutants fused to glutathione *S*-transferase (GST). Purified GST-tagged mutants were incubated with purified recombinant components required for SUMO-1 modification *in vitro*. Control reactions were also performed in the absence of substrate, ATP, and GST-SUMO-1, conditions that do not support SUMO modification. Under these conditions, a more slowly migrating band of approximately 80 kDa, marked with a *star*, was observed only for GST-ER2 incubated in the presence of GST-SUMO-1 and ATP (Fig. 2C). This band was absent in both control assays without ATP or SUMO-1. This finding is consistent with the predicted size of GST-ER2 (~40 kDa) modified by a single molecule of GST-SUMO-1 (~45 kDa) and confirms results obtained *in vivo*. The slowly migrating bands observed in GST-ER1 assay and marked with a *solid circle* are nonspecific cross-reacting signals because they are present also in the controls without ATP and SUMO-1. Then we constructed two new GST-tagged mutants encompassing the C region (aa 176–251) and the hinge region (aa 251–305) (see Fig. 2A). As shown in Fig. 2D, only the ERα hinge region was modified by SUMO-1 in this *in vitro* assay, indicating that the ERα domain from residues 251–305 was sufficient to function as a substrate for sumoylation, at least *in vitro*.

Identification of Major Sumoylation Sites in ERα

The ERα hinge region contains seven lysine residues (see Fig. 3A). To examine whether these lysine residues serve as SUMO-1 acceptors in this region, we constructed into pSG5-ERα vector, several ERα mutants (illustrated in Fig. 3A) in which lysines were either deleted or mutated to arginines. We next examined whether these mutations would affect the extent of ERα sumoylation *in vivo* by transfection in COS-7 cells. Transfected cells were lysed in the presence of NEM and ERα sumoylation evaluated by Western immunoblotting using equal amounts of total cellular proteins. Results are shown in Fig. 3B. Del1 mutant was found to be modified by SUMO-1 as efficiently as wild-type ERα, whereas Del2 appeared to be a stronger substrate for SUMO-1. One possible interpretation of this intriguing result could be that this deletion altering ERα conformation generates more efficient SUMO acceptor sites. However, double mutant K266R/K268R and triple mutant 3K/R exhibited a significant lower level of sumoylation compared with the wild type, indicating that ERα was mainly sumoylated at these two sites. The result obtained with the mutant 3K/R was unexpected because these lysines are included in the both the VP16- and GST-ER3, which were not sumoylated either *in vivo* or *in vitro* (see Fig. 2). The apparent contradiction between these two results can be explained by the ERα constructions used in these experiences. In the experiences illustrated in Fig. 2 we used VP16 or GST fusion versions of ERα regions, in which the lysines 299, 302, and 303 are in the boundary to VP16 or GST proteins. Our interpretation is that these fusion configurations alter the conformation of these potential SUMO acceptor sites. Moreover, when the five lysines located in the hinge region were mutated (5K/R), the sumoylation signal drastically diminished, even if a residual band was barely detectable. Thus, we cannot exclude the possibility that residual sumoylation may occur at the lysine residues outside of the hinge region. To begin to address the effects of SUMO-1 modification on ERα function, we examined the impact of SUMO-1 modification on ERα transcription activity. To this aim, we performed transient transfection assays using ERE-Luc as reporter construct. HeLa cells were cotransfected with the reporter plasmid together with wild-type ERα or mutants expressing plasmids in the presence of E2. All hinge region mutants analyzed showed impaired E2-induced transcription, compared with wild-type ERα. Expression levels of wild-type ERα and mutants were normalized based on the results of Western blot analysis (data not shown), and immunofluorescence experiments revealed no significant difference between the subcellular localizations of these mutants and of wild-type ERα transfected in HeLa cells (data not shown). Thus these data indicate that SUMO-1 modification positively regulates the ligand-dependent ERα activity. However, coexpression with SUMO-1 still enhanced the transcriptional activity of hinge region mutants in a dose-dependent manner (see Fig. 6, D–F). These results indicate that the effect of SUMO-1 on ERα transcriptional activation cannot be attributed only to the sumoylation of ERα itself. However, we cannot rule out that this effect results from the residual sumoylation present in the mutants.

Due to the importance of DNA binding affinity for ERα transcriptional function, we tested the capacity of each hinge region mutant to bind DNA. An EMSA was performed using nuclear extract prepared from COS-7 cells transfected with wild-type ERα or mutants. The results of this assay show that 3K/R and K266R/K268R mutants exhibited reduced DNA binding intensity compared with wild-type ERα (Fig. 3D), and that the affinity of 5K mutant for its binding site was substantially decreased. ERα binding specificity was further confirmed by the application of ERα-specific antibody (Fig. 3D). Immunoblot of nuclear extracts used in the band shift experiments shows that all proteins were equivalently expressed and that comparable amounts of proteins were used in these experiments (Fig. 3D, *right panel*).

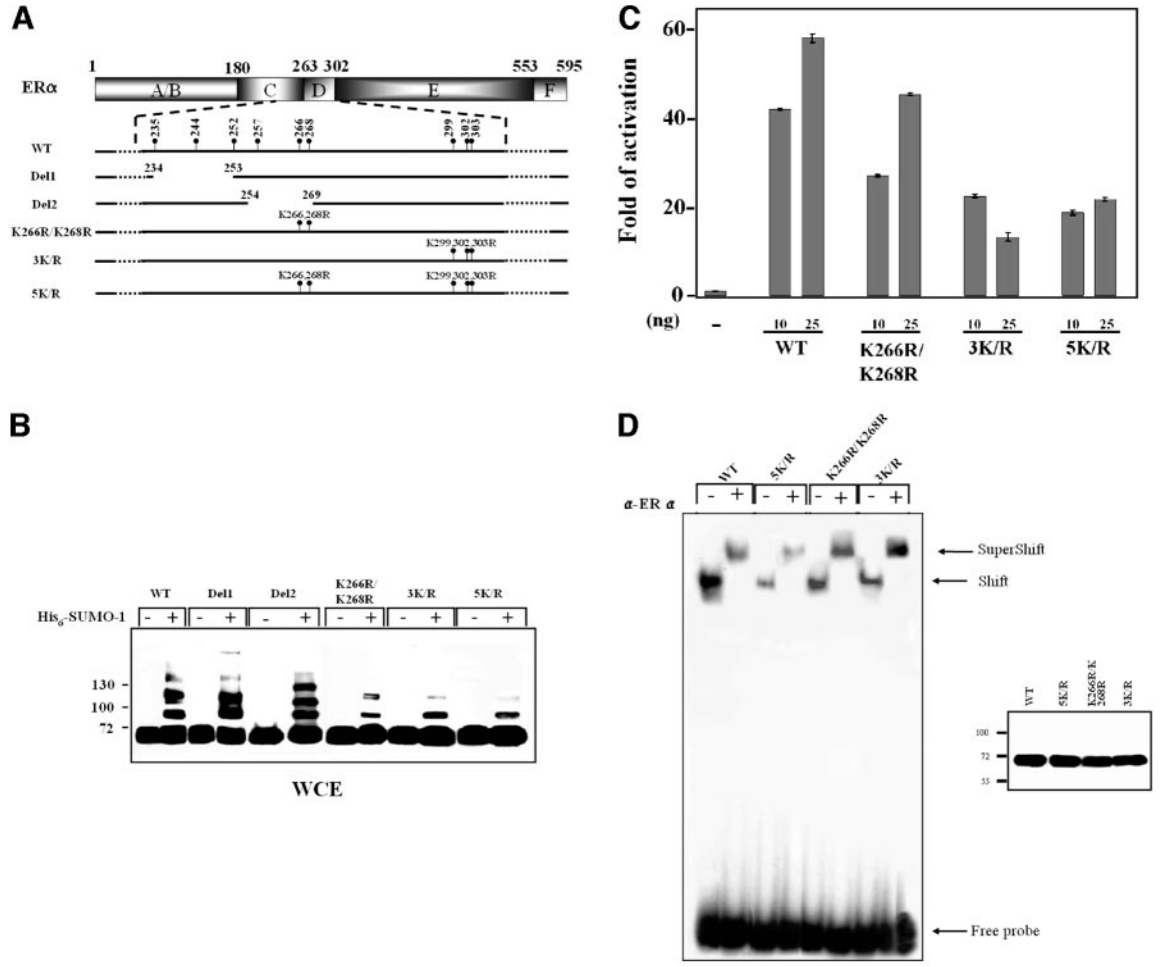

Fig. 3. Mutations That Prevent SUMO Modification Impaired ERα-Induced Transcription

A, Schematic representation of ERα mutants used in this study. B, *In vivo* sumoylation of ERα mutants. COS-7 cells were transfected with expression vectors encoding ERα mutants in the presence or absence of His-tagged SUMO-1 with 100 nM E2. Transfected cells were lysed in the presence of NEM [whole cell extract (WCE)] and analyzed by immunoblotting using Ab15 antibody. C, HeLa cells were transiently transfected with 50 ng of ERα-dependent reporter plasmid ERE-Luc and 25 ng of pRL-TK *Renilla* luciferase vector used as internal control, together with the indicated amounts of ERα or ERα mutant-expressing vectors. All transfections were normalized to equal amounts of DNA with addition of parental expression vectors. The cells were treated with 10 nM E2 24 h after transfection. After another 24 h, they were harvested and lysed, after which samples of the whole-cell extracts were assayed for luciferase activity. Results correspond to the average and standard deviation of at least three independent experiments. D, The ^{32}P-labeled ERE probe (14,000 cpm), containing the binding site for ERα, was incubated with 7.5 μg of nuclear extract of COS-7 cells transfected with wild-type ERα or mutants, alone or in the presence of 1 μg of anti-ERα antibody Ab10 as indicated at the *top* of the EMSA lanes. The expression levels of wild-type ERα and mutants in nuclear extracts used are shown by Western blot analysis using anti-ERα Ab15 antibody (*right panel*). WT, Wild type.

PIAS1 and PIAS3 Act as E3 Ligase for ERα Sumoylation

Recently, a family of PIAS (protein inhibitor of activated signal transducer and activator of transcription) proteins was described as SUMO-E3 ligases for critical target proteins such as p53, c-jun, LEF1 (32–35), and certain steroid receptors (36–38). Therefore, we investigated whether PIAS1 and PIAS3 proteins, which can directly interact with ERα *in vitro* (data not shown), were capable of enhancing SUMO-1 attachment to ERα in intact cells. COS-7 cells were cotransfected with ERα, His_6-SUMO-1, and PIAS1- or PIAS3-expressing vectors (see Fig. 4A). The coexpression of PIAS1 or PIAS3 with ERα and SUMO-1 strongly enhanced the intensity of the slower-migrating forms of ERα corresponding to SUMO-1-conjugate receptor (Fig. 4A, lanes 4 and 6), indicating that both PIAS1 and PIAS3 act as SUMO-1 E3 ligases for ERα sumoylation. Notably, PIAS1 expression induced additional bands of ERα that had shifted to a higher molecular weight than the two bands detected in the presence of SUMO alone. This result suggests that PIAS1 expression may

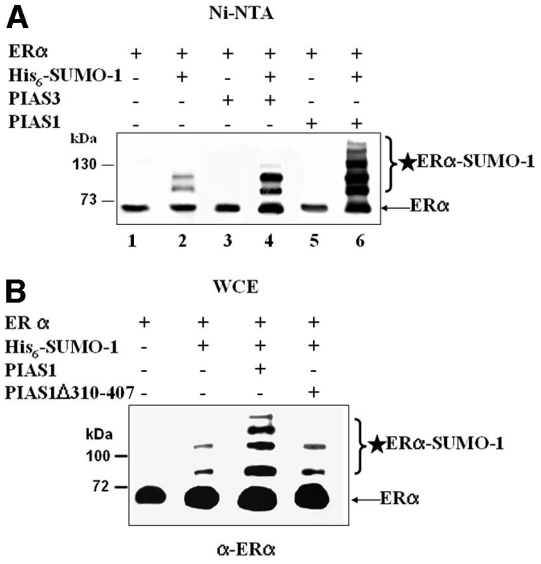

Fig. 4. PIAS1 and PIAS3 Stimulate SUMO-1 Conjugation to ERα

COS-7 cells were cotransfected, in the presence of 100 nM E2, with expression plasmids encoding A) ERα, His_6-SUMO-1, and the indicated PIAS proteins and B) ERα, His_6-SUMO-1, PIAS-1, and the PIAS1 mutant, PIAS1 Δ310–407, lacking the whole zinc-binding domain. Cells were harvested 24 h after transfection, and His-tagged proteins were purified as indicated, resolved by SDS-PAGE, and analyzed by immunoblotting using anti-ERα monoclonal antibody Ab15. Molecular mass markers are shown in kilodaltons. The position of SUMO-1-conjugated ERα is indicated by a *star*. WCE, Whole-cell extract.

induce sumoylation of other sites or can form polysumoylation chains.

The ability of PIAS1 to stimulate protein sumoylation is dependent on the conserved PIAS RING finger-like domain (36). The deletion of the whole zinc-binding domain (PIAS1 Δ310–407) abolished the ability of PIAS1 to enhance ERα sumoylation (Fig. 4B), confirming that PIAS1 functions as an E3 ligase toward ERα.

Ligand-Mediated Sumoylation of ERα

The binding of ligands to ERα, in addition to altering the conformation and activity of the receptor, influences its stability. For instance, estradiol binding accelerates receptor degradation, and tamoxifen treatment has been shown to stabilize ERα (39). We next addressed the issue whether ERα ligands 17β-estradiol (E2) and 4-OH-tamoxifen could affect the sumoylation of ERα. COS-7 cells were cultured under sterol-depleted conditions for 48 h, and then transfected with ERα and His_6-SUMO-1-expressing plasmids, and treated with E2, tamoxifen, or ethanol vehicle. The His-tagged proteins were purified by chromatography on nickel-charged agarose beads 18 hr after hormone treatment and analyzed by Western blotting using an anti-ERα monoclonal antibody. As seen in Fig. 5A, no

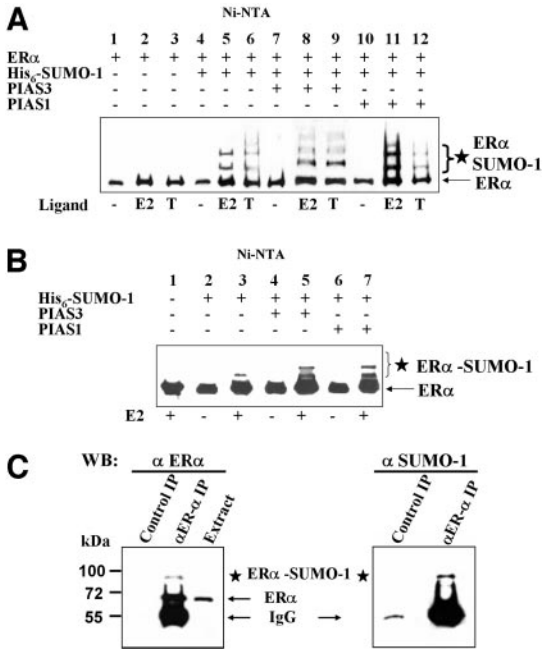

Fig. 5. ERα Sumoylation Is Ligand Dependent

A, ERα sumoylation is hormone dependent in COS-7 cells. COS-7 cells, maintained under sterol-depleted conditions for 48 h, were transiently transfected with ERα- and His_6-SUMO-1-expressing plasmids in the presence or absence of PIAS1 or PIAS3. Transfected cells were then induced with ethanol vehicle, 100 nM E2, or 5 μM tamoxifen. Cells were harvested 18 h after hormone treatments, and His-tagged proteins were analyzed as in Fig. 1C. B, Endogenous ERα is modified by SUMO-1 in intact MCF-7 cells. MCF-7 cells grown on 100-mm dishes were transfected with vectors encoding His_6-SUMO-1 in the presence or absence of PIAS1- or PIAS3-expressing vectors. Cell extracts were analyzed as in Fig. 1C. C, Endogenous sumoylated ERα is present in MCF-7 cells. ERα was immunoprecipitated from extracts of MCF-7 cells treated for 18 h with 100 nM E2 with anti-ERα D12 antibody and analyzed by Western immunoblotting with anti-SUMO1 and anti-ERα G20 antibodies. Anti-ERα antibody detected a major band of approximately 66 kDa (marked with an *arrowhead*) corresponding to ERα and one slow-migrating form (marked with a *star*) that comigrated with the major band recognized by anti-SUMO-1 in the *right panel* (marked with a *star*). Control lines represent immunoprecipitations without anti-ERα antibody. Total protein extract (40 μg) was loaded in extract line. Molecular mass markers are shown in kilodaltons. The *star* indicates the position of SUMO-1-conjugated ERα. T, Tamoxifen; WB, Western blot; IP, immunoprecipitation.

ERα slower-migrating bands were detected in the presence of SUMO-1 and in the absence of ligands (lanes 4, 7, and 10). However, ERα sumoylation was stimulated in the presence of E2 and, also, to a lesser extent, of 4-OH-tamoxifen (Fig. 5A, lanes 5–6). Reflecting our previous results, we found that PIAS1 or PIAS3 enhanced the sumoylation of ERα, but only in the presence of E2 (Fig. 5A, lanes 8 and 11) and tamoxifen (Fig. 5A, lanes 9 and 12). These data, obtained in intact cells, clearly indicate that ERα sumoy-

lation is strictly ligand dependent also in the presence of PIAS1 and PIAS3.

We then examined whether the sumoylation of overexpressed ERα reflects a property of endogenous proteins. Specifically, we attempted to detect sumoylated ERα in ERα-positive MCF-7 breast cancer cells. The His_6-SUMO-1-expressing plasmid was transiently expressed in MCF-7 cells cultured under sterol-depleted conditions for 48 h, and then treated with E2 or with the ethanol vehicle. His_6-SUMO-1 and its conjugates were then purified by nickel chromatography, and ERα was detected by immunoblotting analyses with anti-ERα antibodies. Slower-migrating bands corresponding to sumoylated ERα were clearly detected when His_6-SUMO-1 was expressed in MCF-7 cells treated with E2 (Fig. 5B, lane 3), thus confirming that the sumoylation of ERα is not merely a result of the overexpression of the protein. Analogous experiments carried out in the presence of PIAS1 and PIAS3 proteins showed an enhanced ERα sumoylation (Fig. 5B, lanes 5 and 7). In light of these observations, we investigated whether endogenous sumoylation of ERα occurs naturally in the cells in the absence of ectopically expressed SUMO-1 and SUMO-E3 ligases. To determine the sumoylation of endogenous proteins, ERα was immunoprecipitated from extracts of MCF7 cells treated with E2 for 18 h with anti-ERα antibody and analyzed by Western immunoblotting with anti-SUMO1 and anti-ERα antibodies. As shown in Fig. 5C, anti-ERα antibody detected a major band of approximately 66 kDa (marked with an *arrowhead*) corresponding to ERα and one slow-migrating form (marked with a *star*) that comigrated with the major band recognized by anti-SUMO-1 in the *right panel* (marked with a *star*). Taken together, these results strongly indicate that the *in vivo* sumoylation of endogenous, as well as exogenously expressed, ERα is hormone dependent and that members of the PIAS family, PIAS1 and PIAS3, function as E3 ligases for this nuclear receptor.

Regulation of ERα-Dependent Transactivation by SUMO-1 Modification Machinery

We then investigated whether important components of the sumoylation pathway, PIAS proteins, Ubc9 and SUMO-1 itself, affected the transcriptional activity of ERα. We performed transient transfection assay in HeLa cells using ERE-Luc as reporter construct. As shown in Fig. 6, A–C the coexpression of ERα with SUMO-1 increased the E2-dependent transcriptional activity of the reporter promoter in a dose-dependent manner. The expression of PIAS-3 with ERα strongly activated the E2-dependent transcription (Fig. 6A). Surprisingly, PIAS1, which has been shown to enhance ligand-dependent ERα transactivation (40), inhibited ERα function (Fig. 6B). However, we did not use the same ERα-sensitive reporter gene, and several studies indicate that PIAS proteins may play a role of activators or repressors depending on the cell and promoter contexts (41). In addition, we demonstrate that PIAS1 modulated ERα-dependent transcription independently from its SUMO-1 conjugation activity, because the PIAS1 Δ310–407 mutant, which lacks its E3 ligase activity, inhibited ERα function as well as the wild type (Fig. 6B). Using the same reporter gene assay, we tested the effect of Ubc9 on ERα-dependent transactivation. As shown in Fig. 6C, the expression of SUMO-1 and Ubc9 enhanced the transcriptional activity of ERα in a dose-dependent manner, pointing out Ubc9 as a new regulator of ERα. However, this behavior was not altered when the catalytically inactive form of Ubc9, Ubc9/C93S, was used. These experiments are consistent with the observations that Ubc9 or Ubc9/C93S still enhanced the transcriptional activity of sumoylation-deficient ERα mutants (see Fig. 6, D–F).

Taken together, these results indicate that, although Ubc9, PIAS1, and PIAS3 are members of the sumoylation machinery, they regulate ERα transcription via a sumoylation-independent mechanism.

SUMO Conjugation Regulates ERα-Mediated Transcription

To further examine the role of sumoylation on ERα transcription regulation, we used two different approaches. First, we analyzed the influence of SUMO-1 on ERα transcription mediated by PIAS proteins and Ubc9. When SUMO-1 was coexpressed with PIAS1, PIAS3, or Ubc9, the SUMO-1-mediated activation of ERα was strongly repressed (see Fig. 6, A–C). This repression was dose dependent, and was not due to a decrease of ERα expression (data not shown). One interpretation of these data might be that simultaneous expression of SUMO-1 and sumoylation enzymes resulted in an extensive sumoylation of ERα and/or its cofactors, which was responsible for the repression of reporter activity. To support this point, we used the catalytically inactive form of Ubc9, Ubc9/C93S, which acts as dominant-negative mutant. As shown in Fig. 6 C, coexpression of Ubc9/C93S and SUMO-1, instead of repressing the SUMO-1-mediated activation of ERα, enhanced the reporter activity.

Next we used a Gal4-ER3 fusion protein containing the ERα ligand-binding domain and the AF2 region (aa 297–595) and lacking the hinge region. This chimeric protein has full transactivation potential, compared with its wild-type counterpart, in the context of a reporter plasmid containing a Gal4-driven promoter. First, we tested the effect of PIAS3 and SUMO-1, alone or in combination, on the activity of GAL4-ER3 in the presence of E2 in HeLa cells. As shown in Fig. 7, the addition of E2 activated Gal4-ER3 transcription, and this activity was strongly enhanced by PIAS3, similarly to wild-type ERα when targeted to its own DBD (see Fig. 6A). In contrast, increased amounts of SUMO-1 barely affected the activity of Gal4-ER3, on which it had, rather, an inhibitory effect. Thus, in the absence of the hinge region, ERα is not responsive to

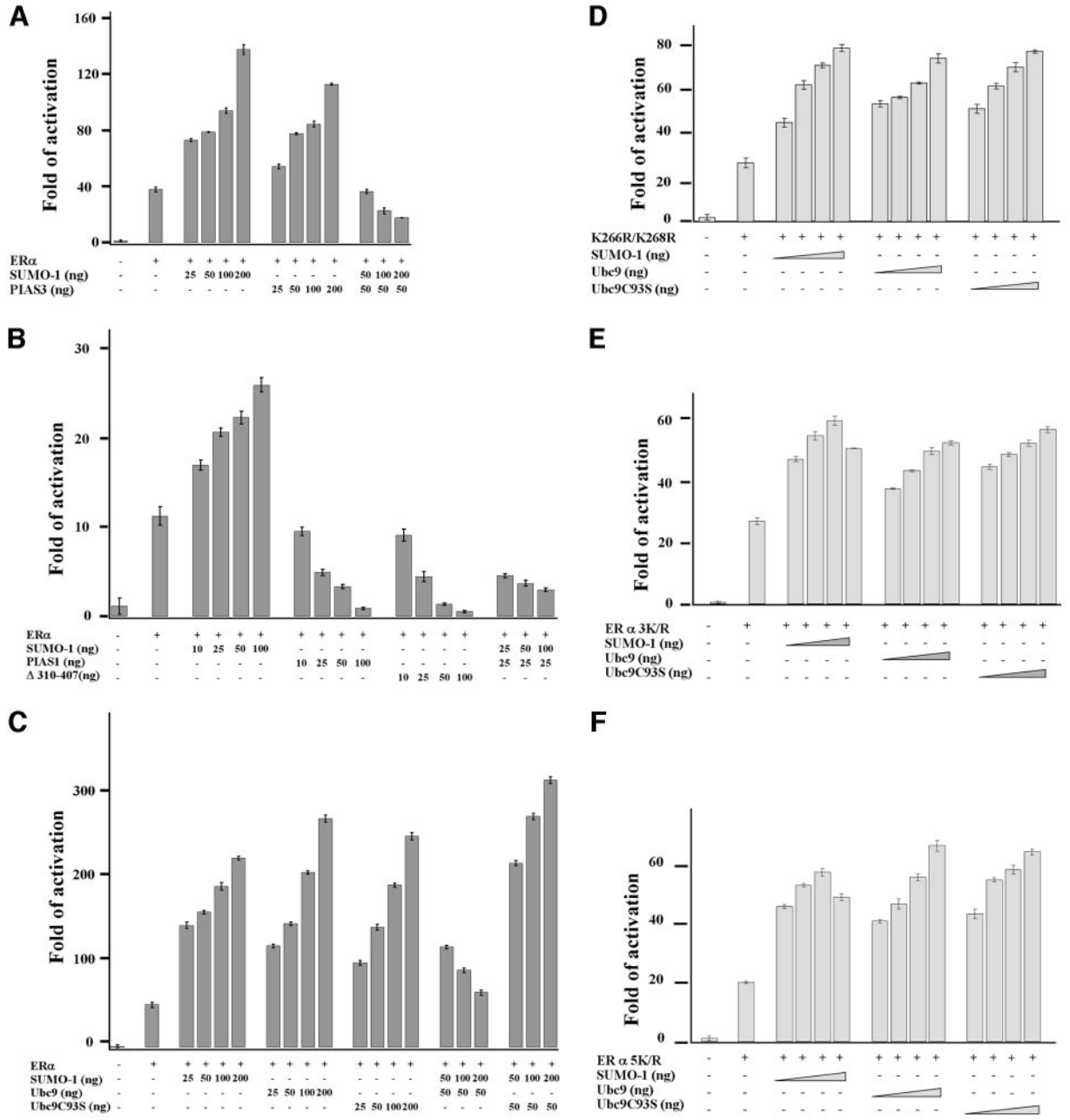

Fig. 6. Effect of SUMO-1 Modification Machinery on ERα Transcriptional Regulation

HeLa cells were transiently transfected with 50 ng of ERα-dependent reporter ERE-Luc and 25 ng of pRL-TK *Renilla* luciferase vector used as internal control, together with ERα wild-type or mutants expressing vectors alone or with the indicated amounts of His_6-SUMO-1-expressing construct and PIAS or Ubc9 constructs. All transfections were normalized to equal amounts of DNA with addition of parental expression vectors. The cells were treated with 10 nM E2 24 h after transfection, harvested, and lysed after another 24 h. Aliquots of the whole-cell extracts were assayed for luciferase activity. Results correspond to the average and standard deviation of at least three independent experiments. *Left panel*: Effect of A, PIAS3; B, PIAS1 or PIAS1 RING finger-like domain mutant, PIAS1 Δ310–407; and C, Ubc9 or its catalytically inactive form, Ubc9/C93S, on ERα-dependent transactivation. *Right panel*: Effect of SUMO-1, Ubc9, or Ubc9/C93S on transcriptional activation of ERα mutants described in Fig. 3A. D, K266R/K268R; E, 3K/R; and F, 5K/R. Mean ± SD values from three independent experiments are shown.

SUMO-1 activation. The coexpression of increased amounts of SUMO-1 with PIAS3 resulted in an almost complete inhibition of Gal4-ER3 transactivation. Because we demonstrated that ER3 region was not sumoylated either *in vivo* or *in vitro* (see Fig. 2), the inhibitory effect of SUMO-1 on PIAS3-induced Gal4-ER3 activity could be a consequence of an increased SUMO modification of ERα coregulators. Collectively, these data indicate that SUMO-1 regulates the transcriptional activity of ERα directly through ERα mod-

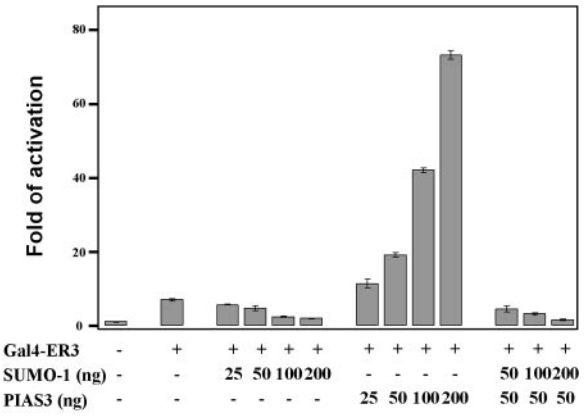

Fig. 7. Effect of SUMO-1 and PIAS3 on Gal4-ER3 Transcriptional Activation

HeLa cells were transiently transfected as described in Fig. 6 with Gal4-Luc as reporter plasmid and pRL-TK *Renilla* luciferase as internal control, together with Gal4-ER3-expressing plasmid alone or with the indicated amounts of His_6-SUMO-1 or PIAS3 constructs. Mean ± SD values from three independent experiments are shown.

ification and indirectly by affecting the activity of coregulators. Moreover, the coexpression of PIAS or Ubc9 proteins with SUMO-1 could lead to the formation of repressor complexe(s) or titrate away a coactivator.

DISCUSSION

In the present work, we demonstrated that ERα is a direct substrate of SUMO-1 posttranslational modification in intact cells and under cell-free conditions, even if ERα sequence lacks consensus SUMO-1 attachment motifs. Importantly, we show that sumoylated forms of endogenous receptor are present in the ERα-positive cell line, MCF-7 (Fig. 5C), signifying this posttranslational modification occurs in a natural cell environment. Mutagenesis analysis indicates that sumoylated lysines are located within the hinge region of ERα. Mutations inhibiting SUMO modification reduced the transcriptional activity of ERα, indicating that the sumoylation of this receptor may have a positive effect on ERα transcriptional activity. Previous studies have shown that different nuclear receptors, namely androgen receptor (AR), glucocorticoid receptor (GR), and progesterone receptor, undergo sumoylation within a region that functions as a transcriptional inhibitory domain (38, 42, 43). In particular, progesterone receptor sumoylation regulates its autoinhibition and transrepression activities (43). Substituting the SUMO-attachment lysines to arginines in this domain enhances the transactivation potential of the receptor, indicating an inhibitory role of SUMO in signaling of this nuclear receptor (28). The major sites of SUMO modification in the GR and AR map to the previously defined synergy control motif, suggesting SUMO-1 may regulate synergistic activation for these factors. According to this idea, suppression of SUMO acceptor lysines or overexpression of SUMO-1 was found to increase the activity of GR (42, 44) and AR (38, 45) on promoters containing multiple, but not single, binding sites. The picture emerging from different reports suggests that the effects of SUMO posttranslational modification on nuclear receptor activity are promoter specific, might depend on the cellular context, and reflect combinatorial effects of receptors and coactivators. These findings may explain some discrepancies concerning the effects of SUMO modification on specific transcription factors.

The mechanisms governing SUMO substrate specificity and the recognition of nonconsensus sites are not well understood at this time. Additional parameters should help determine SUMO target sites. In fact, in several verified SUMO-1 targets, modified lysines are not always present within the postulated consensus motif (K65 of PML, K1182 of HIPK2, K446 of Mdm2, K164 of PCNA. . .), and others are still sumoylated when all consensus sites are mutated (reviewed in Ref. 46). In addition, tandem mass spectrometry (MS)-based strategies recently permitted the identification, within CENP-C, SUMO-2, and Ubc9 proteins, of several sumoylated lysine residues that were not in the consensus sumoylation motif (47). It seems that even in the absence of a targeting motif, SUMO-E3 ligase can determine substrate specificity for *in vivo* sumoylation. Recently, several SUMO-E3 ligases that may promote the selection of target proteins have been identified (19, 32, 34, 41, 46). Our results show that both PIAS1 and PIAS3 strongly stimulate the sumoylation of ERα in the presence of ligands, E2 and tamoxifen (Figs. 4A and 5A); thus both proteins act as E3-SUMO ligases for ERα sumoylation. We have also shown that ERα sumoylation is strictly ligand dependent in living cells, suggesting that hormone binding, and perhaps the subsequent altering of receptor conformation, is essential for its interaction with components of the SUMO modification machinery. However, when diverse regions of ERα were conjugated to the transactivation region of VP16 or to GST, the sumoylation of fusion proteins was independent of the presence of ligand, both *in vivo* and *in vitro*. In our experiments, we observed another major difference between ERα and truncated fusion proteins. As Fig. 3B illustrates, lysines 299, 302, and 303 seem to be important SUMO-1 acceptors, because their mutation to arginines clearly reduces sumoylation. However, these lysines are included in both VP16- and GST-ER3, which were not sumoylated either *in vivo* or *in vitro* (see Fig. 2). One possibility might thus be that in the truncated fusion proteins, in which lysines 299, 302, and 303 are located in the boundary to VP16 and GST fragments (see Fig. 2A), these sumoylation sites are not accessible to the posttranslational modification machinery.

The ability of ligands to regulate ERα sumoylation *in vivo* may also be explained, at least in part, by receptor compartmentalization. The unliganded form of ERα is diffusely distributed throughout the nucleoplasm, and addition of estrogen or tamoxifen results in a redistribution of ERα in discrete punctuate structures, which can regulate or facilitate the interaction of ERα with the sumoylation machinery.

In our effort to understand the functional significance of ERα sumoylation, we tested whether key molecules of the SUMO-1 conjugation system, including SUMO-1, Ubc9, and PIAS, affected the transcriptional activity of ERα. As expected, the E2-dependent transcription of ERα was enhanced by SUMO-1 overexpression (Fig. 6, A–C). As shown in Fig. 6, A and B, whereas PIAS3 strongly increased the transcriptional activity of ERα, PIAS1 acted as an inhibitor. Mammalian PIAS proteins, originally described as specific inhibitors of signal transducer and activator of transcription signaling (48), can interact directly with several transcription factors, including various nuclear receptors, and regulate their transcriptional activity (40, 49–51). Several studies indicate that PIAS proteins have various activities, either dependent or independent from their E3 ligase function (36, 41). Our results show that the E3 ligase activity of PIAS1 is not required for ERα regulation; in fact, a PIAS1 mutant lacking the ring finger-like domain, essential for ERα sumoylation (see Fig. 4B), could still modulate ERα transcription (Fig. 6B). This was also the case for Ubc9, which activated ERα transcriptional activity independently from its sumoylation activity (Fig. 6C). In the same reporter assay, PIAS1, PIAS3, and Ubc9 almost completely repressed ERα-mediated transactivation when coexpressed with SUMO-1. One attractive possibility is that this repression could be a consequence of increased SUMO modification of ERα and its regulators. In fact, when ERα was modified to a low extent by SUMO-1 alone (Fig. 4A, lane 2), ERα-mediated transcription increased (Fig. 6, A–C). The addition of PIAS or Ubc9 proteins, which strongly increases sumoylation of ERα (see Fig. 4A; compare lane 2 with lanes 4 and 6 and Fig. 4B) as well as of ERα coactivators, drastically repressed SUMO-1-dependent ERα transactivation (Fig. 6, A–C). This is consistent with the observation that the coexpression of SUMO-1 with Ubc9/C93S, the expression of which resulted in a significant reduction of the SUMO-modified ERα (Fig. 1D), did not inhibit the SUMO-1-dependent ERα activation (Fig. 6C). This raises the possibility that sumoylation of ERα and coregulators occurs sequentially at different stages of the ERα transcription complex formation. Initially, sumoylation has a direct effect on ERα itself (modifying dimerization, interactions with coregulators, binding to estradiol or to DNA responsive elements. . .) and may promote its recruitment to the promoter to activate transcription. Subsequent modifications of the receptor and/or coregulators may later disassemble transcriptional complexes and turn down transcription. It is important to note that the function of ERα as a transcriptional regulator can be modulated through associations with a number of cofactors that are also sumoylated, *i.e*. GR-interacting protein 1, steroid receptor coactivator 1, histone deacetylase 1, histone deacetylase 4, p300, and PIAS proteins themselves (24–27), which further complicates the analysis of SUMO effects on ERα transcriptional activity. Several results in the present study supported the conclusion that SUMO-1 pathway regulates ERα-dependent transcription through the sumoylation of ERα itself and its coregulators. As shown in Fig. 7, no SUMO-1-mediated enhancement of reporter activity was observed when we used Gal4-ER3, which lacks the hinge region containing the SUMO sites. Coexpression of SUMO-1 with PIAS3 resulted in an almost complete inhibition of PIA3-mediated Gal4-ER3 transactivation. Thus the synergic effect of PIAS3 and SUMO-1 to repress Gal4-ER3 activation did not result from an increased sumoylation of ER3 region, which was neither sumoylated *in vivo* or *in vitro* (see Fig. 2). Finally, mutations that prevented SUMO modification significantly impaired E2-induced transcription (Fig. 3C).

Because lysine serves as the attachment site for several modifications, including ubiquitination, acetylation, and methylation, it is also reasonable to speculate that ERα sumoylation plays a role by antagonizing other posttranslational modifications. Importantly, ERα can be acetylated *in vitro* and *in vivo* by p300 at the same lysines as those required for SUMO modification (lysines 299, 302, and 303 within the hinge region) (52). Compared with wild-type ERα, arginine substitution at the ERα acetylation site enhanced the E2-dependent activity of ERα, suggesting that direct ERα acetylation normally suppresses ligand sensitivity (52). Of particular interest is the observation that a somatic mutation (K303R) within the ERα hinge region has been correlated to breast cancer development. This mutation is present in 34% of analyzed patients with atypical breast hyperplasia (53). These experiences indicate that the acetylation of ERα regulates the ligand sensitivity; hence, this mutation can be involved in early stages of breast cancer development. The findings that ERα can be efficiently acetylated *in vitro* by p300, and that acetylated ERα is present in MCF-7 cells (52), point out the intriguing possibility that the transcriptional activity of ERα could be dynamically regulated by competition between SUMO and other modifiers for common lysine(s).

Although the molecular mechanism underlying ERα sumoylation and its regulation remains unknown, our study has demonstrated the importance of SUMO modifications as a novel posttranslational step for the regulation of ERα function. Chromatin immunoprecipitation studies have shown that ERα and coactivators assemble on target promoters in a sequential and rapidly cycling manner. Because SUMO-1 modification is a specific, reversible, and highly dynamic process, it may provide an important means to regulate the assembly and disassembly of transcriptional complexes. Further studies are essential to better elucidate

the complexity of the molecular mechanism involved in ERα-mediated gene activation. In particular, it will be important to determine exactly when and where ERα sumoylation occurs, how it is regulated, and how it contributes to the physiological estrogen response. The functional analysis of lysines identified as targets for ERα sumoylation will help determine whether they play a role in the normal development of the mammary gland and whether they may be involved in breast cancer progression.

MATERIALS AND METHODS

Expression Constructs and Reporter Plasmid

The pSG5-ERα expression vector, which encodes the full-length human ERα, and the pERE-Luc reporter plasmid, which contains three copies of ERα consensus elements (ERE) upstream from the TATA box fused to the luciferase gene, have been described previously (29). The pGal4-Luc reporter plasmid contains six Gal4 consensus elements upstream from the thymidine kinase promoter region fused to the luciferase gene (30). ERα deletion mutants were constructed by PCR. Fragments encoding amino aa 1–180, 176–302, 297–595, 176–251, and 251–305 to create, respectively, ER1, ER2, ER3, DBD, and hinge domain were generated by amplification of ERα cDNA. The PCR products were cloned into the pSG-FNV vector downstream and in frame with the sequence coding for the VP16 activation domain to create VP16-ER1, VP16-ER2, and VP16-ER3, and into the pGEX-4T2 vector (Amersham Pharmacia Biotech) downstream and in frame with the GST tag to create GST-ER1, GST-ER2, GST-ER3, and GST-Hinge. To generate the GST-Flag-DBD vector, the DBD amplification product was first cloned into a pSG5-Flag vector (31) downstream and in frame with the Flag epitope. The Flag-DBD insert was then subcloned into the pGEX-4T2 vector downstream and in frame with the GST. Gal4-ER3 mammalian expression vector was obtained by subcloning the ER3 amplification products into the Gal4-poly II plasmids described previously (31). The His_6-tagged SUMO-1 (pSG5-His_6-SUMO-1) expression vector and the GST-tagged SUMO-1 (GST-SUMO-1) expression vector were kind gifts from Anne Dejean (Institut Pasteur, Paris, France) and David Wotton (University of Virginia, Charlottesville, VA), respectively. pSG5-Flag-PIAS3 and pSG5-Flag-PIAS1 were obtained by cloning the full-length cDNAs of PIAS3 and PIAS1 from pCMV-Flag-PIAS3 and pCMV-Flag PIAS1 [kindly provided by Tarik Möröy (Institut für Zellbiologie, Essen, Germany) and Ke Shuai (University of California, Los Angeles, CA)] in frame with the Flag epitope in pSG5-Flag. GST-tagged UBC9 and the pFLAG-PIAS1Δ310–407 expression vectors were gifts from Nadine Varinn-Blank (Institut Cochin, Paris, France) and Jorma J. Palvimo (Biomedicum Helsinki, Institute of Biomedicine, Helsinki, Finland), respectively. pDuet-Aos1/Uba2, a bacterial expression plasmid encoding both subunits of SUMO-E1, was a gift from Christopher D. Lima (Sloan Kettering Institute, New York, NY). pSG5-Ubc9 and pSG5-Ubc9/C93S were gifts from Helen Hurst (Cancer Research, Molecular Oncology Unit, Hammersmith Hospital, London, UK) and Ron Hay (Center for Biomolecular Sciences, University of St. Andrews, St. Andrews, Scotland, UK). The cloned products were verified by DNA sequencing, and protein expression was checked.

Site-Directed Mutagenesis

The plasmid used for expression and mutagenesis was pSG5-ERα. ERα del1 (deletion aa 234–253), ERα del2 (deletion aa 254–269) and all ERα K/R mutants (K266R/K268R in which Lys 266 and Lys 268 codons were mutated to an Arg codon, 3K/R in which Lys 299, Lys 302 and Lys 303 were mutated to an Arg codon and 5K/R in which Lys 266, Lys 268, Lys 299, Lys 302 and Lys 303 were mutated to an Arg codon) were obtained by using the QuikChange XL Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. All constructs were confirmed by DNA sequencing.

Cell Culture and Transient Transfection

HeLa, MCF-7, and COS-7 cells were maintained at 37 C in DMEM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies, Gaithersburg, MD) and 10% fetal calf serum (BioWhittaker, Inc., Walkersville, MD). For MCF-7 cells, the medium was further supplemented with 1 mM sodium pyruvate, nonessential aa, and glutamine. HeLa and MCF-7 cells were transfected using ExGen 500 (Euromedex, Mundolsheim, France). COS-7 cells were transfected using Fugene 6 (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's protocol. Briefly, for *in vivo* sumoylation, COS-7 and MCF-7 cells were plated, respectively, at a density of 1.5×10^6 cells and 3×10^6 cells per 100-mm tissue culture dishes, 24 h before transfection. Six hours after transfection, the cells were treated with 100 nM E2 (Sigma Chemical Co., St. Louis, MO), and then harvested 18 h later. To study the effect of hormones on ERα sumoylation, COS-7 and MCF7 cells were cultured under sterol-depleted conditions in fresh phenol red-free medium (Life Technologies) containing 10% charcoal-stripped fetal bovine serum (Life Technologies) for 48 h before transfection. Eight hours after transfection, the cells were supplied with 100 nM E2, 5 μM 4-OH-tamoxifen (Sigma), or ethanol vehicle as indicated, and then harvested 18 h later. Protein expression was analyzed by Western blotting.

Immunoblotting and Immunoprecipitation

For the preparation of whole cell extracts, transfected cells were washed with ice-cold PBS and harvested in modified RIPA buffer (50 mM Tris-HCl, pH 7.8; 150 mM NaCl; 5 mM EDTA; 0.5% Triton X-100; 0.5% Nonidet P-40; 0.1% sodium deoxycholate) and a cocktail of protease inhibitors in the presence, when indicated, of 20 mM NEM. Lysates were centrifuged to separate insoluble proteins. Concentrated (2×) sodium dodecyl sulfate (SDS) sample buffer was added to lysate samples; after 5 min heating at 95 C, proteins were resolved on SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, and then blocked by incubation at room temperature for 1 h in PBS containing 5% nonfat dry milk. ERα was detected with mouse monoclonal anti-ERα Ab15 antibody (NeoMarkers) and horseradish peroxidase-conjugated rabbit antimouse Igs (DAKO Corp., Carpinteria, CA). For ERα immunoprecipitation, Cos-7 cell extracts were precleared with protein G-agarose and incubated with the anti-ERα mouse monoclonal antibody Ab10 (NeoMarkers, Labvision Corp., Fremont, CA), after which immune complexes were collected with protein G-agarose. After washing, bound proteins were released in 2× SDS sample buffer and resolved on 8% SDS-PAGE gel, electroblotted onto a polyvinylidene difluoride membrane, and immunoblotted with rabbit polyclonal antibody against SUMO-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and horseradish peroxidase-conjugated goat antirabbit Igs (DAKO Corp.). To analyze the sumoylation of endogenous ERα, extracts from MCF7 cells, treated for 18 h with 100 nM E2, were immunoprecipitated with anti-ERα D12 antibody (Santa Cruz Biotechnology) and analyzed by Western immunoblotting with mouse monoclonal anti-SUMO1 (Zymed Laboratories, Inc., South San Francisco, CA) and anti-ERα antibody G20 (Santa Cruz Biotechnology).

The proteins were visualized by chemiluminescence (Roche Molecular Biochemicals) following the manufacturer's instructions.

Nickel Affinity Pull-Down Assay

COS-7 cells (70% confluent in 10-cm diameter Petri dishes) transfected as described above were washed in ice-cold PBS and harvested in 1ml of buffer A (6 M guanidinium-HCl; 10 mM Tris-HCl, pH 8; 100 mM NaH_2PO_4), briefly sonicated to reduce viscosity, and then centrifuged. The protein concentration of the supernatants was measured by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) and equal protein amounts were mixed with 40 μl of Ni-NTA-magnetic agarose beads (QIAGEN, Chatsworth, CA), prewashed with lysis buffer A, and then incubated overnight at 4 C. The beads were successively washed three times with 1 ml of buffer A plus 10 mM imidazole, and then twice with 1 ml of buffer B (8 M urea; 100 mM NaH_2PO_4; 10 mM Tris-HCl, pH 6.3), and once in cold PBS. After the last wash, the beads, eluted by boiling in the SDS-sample buffer, were subjected to SDS-PAGE, and the proteins were transferred to a PVDF membrane (Millipore Corp, Milford, MA). Western blotting was performed using the mouse monoclonal antibody Ab15 (NeoMarkers) to detect full-length ERα and ER1 (aa 1–180), the rabbit polyclonal antibody G-20 (Santa Cruz) was used to detect ER2 (aa 176–302), and the rabbit polyclonal antibody HC-20 (Santa Cruz) was used to detect ER3 (aa 297–595).

In Vitro SUMO-1 Conjugation Assay

GST-tagged Ubc9, -SUMO-1(GG), -ER1, -ER2, -ER3, Flag-DBD and -Hinge were isopropyl-β-D-thiogalactopyranoside induced, expressed in *Escherichia coli* BL21 (Invitrogen), and purified by affinity chromatography using glutathione-sepharose 4B (Amersham Pharmacia Biotech, Uppsala, Sweden) as recommended by the manufacturer. Bound proteins were eluted using 20 mM glutathione. The SUMO E1 (Aos1/Uba2 dimer) was expressed in Rosetta (Novagen, EMD Biosciences, Darmstadt, Germany) and purified via a His tag on Uba2 with a nickel chelation affinity column using NI-NTA agarose (QIAGEN) according to the manufacturer's recommended protocol. One microgram of purified GST-tagged ERα mutants was mixed with 250 ng Ubc9, 125 ng Aos1/Uba2 with or without 2 μg SUMO-1, and then incubated for 2 h at 30 C in the presence of 50 mM Tris (pH 7.5), 5 mM $MgCl_2$, with or without 2 mM ATP in a 20 μl volume. Reactions were stopped with SDS-PAGE sample buffer, and SUMO conjugates were separated on SDS-PAGE and analyzed by Western blotting using the mouse monoclonal antibody Ab15 (NeoMarkers) to detect ER1 (aa 1–180), the rabbit polyclonal antibody G-20 (Santa Cruz Biotechnology) to detect ER2 (aa 176–302) and hinge domain (aa 251–305), and the rabbit polyclonal antibody HC-20 (Santa Cruz Biotechnology) to detect ER3 (aa 297–595). M2 monoclonal antibody (Sigma) was used to detect the Flag-DBD domain (aa 176–251).

Luciferase Reporter Assay

HeLa cells (7.5×10^4) were plated in 24-well plates 24 h before transfection. Transfections were performed using ExGen 500 (Euromedex). The transfected DNA included 50 ng of reporter plasmid and 25 ng of pRL-TK *Renilla* luciferase vector (Promega) used as internal control, together with various amounts of expression vectors, as indicated. Total transfected DNA was kept constant by adding empty pSG5-Flag vectors. The cells were induced with 10 nM E2 24 h after transfection and then harvested after an additional 24 h and assayed for luciferase activity following the manufacturer's instructions. Luciferase activities were normalized to the activity of the internal control *Renilla* luciferase. Each set of experiments was performed in triplicate and repeated at least three times.

EMSA

EMSAs were carried out using the Gelshift ER Kit (Active Motif Europe, Rixensart, Belgium) according to the manufacturer's instructions. Briefly, 7.5 μg nuclear extracts, purified from COS-7 cells (using nuclear extract kit, active motif) transfected with vectors expressing wild-type or mutant ERα, were incubated for 20 min at 4 C in binding buffer B. In supershift assays, nuclear extracts were incubated for 20 min in the presence of specific ERα antibody. We then added 14,000 cpm of ^{32}P-labeled double-stranded templates containing the consensus binding site for ERα in buffer C and left the mixture at 4 C for 20 min. After incubation, the mixture was loaded onto a 5% (wt/vol) polyacrylamide gel (29:1 acrylamide to bisacrylamide ratio) in 0.5× TBE, and run at room temperature at 12.5 V/cm for 2 h. The gels were dried and the protein-DNA complexes were visualized by autoradiography. The probes were end-labeled using [γ-^{32}P]ATP (Amersham Pharmacia Biotech) and T4 polynucleotide kinase (Roche), and purified through MicroSpin TMG-25 columns (Amersham Biosciences, Piscataway, NJ).

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