AIB1, a member of the steroid receptor coactivator (SRC) family that participates in gene transcriptional activation by nuclear receptors and other transcription factors, is required for animal growth and reproductive development and implicated in breast carcinogenesis. The mechanisms underlying the AIB1 pleiotropic functions are not fully understood and neither is the regulation of its activity. Here, we showed that AIB1 was a sumoylated protein and the sumoylation attenuated the transactivation activity of AIB1, which is in contrast to the sumoylation of its paralogs, GRIP1 and SRC-1. The transactivation activity of AIB1 is enhanced by its phosphorylation by several kinases, including mitogen-activated protein kinase. We demonstrated in this report that estrogen treatment led to an increased phosphorylation and decreased sumoylation of AIB1 and that the sumoylation coordinated with phosphorylation in regulating the transcriptional activity of AIB1, providing a mechanism for post-translational modifications in regulating the transcriptional output of AIB1.

Nuclear receptors play critical roles in animal development and homeostasis through their bimodal function as repressors or activators of gene transcription (1). The repression and activation activity of nuclear receptors are determined by their association with corepressors and coactivators, respectively. Among the coactivators, the steroid receptor coactivator (SRC) family is both necessary and sufficient for nuclear receptor-mediated gene activation as we previously demonstrated (2). The SRC family of coactivators includes three distinct but related p160 members: SRC-1, GRIP1 (or SRC-2), and AIB1 (also named RAC3, ACTR, SRC-3, or p/CIP in mice) (3–6). They share 40% overall sequence similarity, and they all contain, in the N terminus, a basic helix-loop-helix domain that is known for protein dimerization-DNA interaction as well as a Per-Arnt-Sim domain, which is known to be involved in protein-protein interaction. Three LXXLL motifs, also known as nuclear receptor boxes (for nuclear receptor binding), are centrally located in all three proteins (7–9). In the C terminus, the SRC proteins contain two activation domains, AD1 and AD2, which are associated with histone acetyltransferases (CREB-binding protein/p300 and pCAF) and coactivator-associated arginine methyltransferase 1, respectively, and function to modify the configuration of the chromatin structure (10, 11).

Despite the structural similarities among the members of the SRC family of coactivators, evidence has been accumulated to suggest different physiological functions for SRC-1, GRIP1, and AIB1. Genetic studies with gene ablation showed that, although both male and female SRC-1 null mice are viable and fertile, they exhibit partial resistance to several hormones, including estrogen, progesterin, androgen, and thyroid hormones (12, 13). Elimination of GRIP1 impairs fertility in both male and female mice (14), and GRIP null mice are protected against obesity and display enhanced adaptive thermogenesis, whereas SRC-1 null mice are prone to obesity due to reduced energy expenditure (15). It has been documented that, unlike SRC-1 and GRIP1, AIB1 is more promiscuous; it can enhance the transcriptional activity of a number of different activators (16, 17). Indeed, AIB1 null mice are not only abnormal in reproductive function but also small in animal size (18, 19). At the molecular level, we recently demonstrated SRC-1, GRIP1, and AIB1 are differentially implicated in steroid receptor-mediated gene transcription (20–22).

The differential activity for SRC-1, GRIP1, and AIB1 is probably determined primarily by their intrinsic protein structures. Ultimately, the function of a protein is also regulated by additional mechanisms particularly post-translational modifications. It has been reported that the activity of the SRC proteins is regulated by phosphorylation. For example, SRC-1 has been shown to be phosphorylated by mitogen-activated protein kinase (MAPK) at Thr-1179 and Ser-1185. Mutations of these sites attenuated SRC-1 activity to coactivate progesterone receptor-dependent transcription (23); MAPK also phosphorylates GRIP1 but at Ser-736, the integrity of which is required for a full GRIP1 transcriptional activation function (24). Interestingly, these phosphorylation sites are not conserved in AIB1. AIB1 was shown to be phosphorylated by several different kinases, including MAPK, IκB kinase, and receptor tyrosine kinase HER2/neu (16, 25, 26), with Ser-505, Ser-543, Ser-860, and Ser-867 being identified.
as potential MAPK sites (16). Sequence alignments for the SRC coactivators revealed that, whereas Ser-505 and Ser-543 of AIB1 are conserved in SRC-1 and GRIP1, Ser-860 and Ser-867 are unique for AIB1. Thus, different phosphorylation profiles may contribute, in part, to the differential activity of AIB1, GRIP1, and SRC-1.

In addition to phosphorylation, recent studies have identified sumoylation as another important post-translational modification that regulates the biological functions of a protein. In sumoylation, SUMO (small ubiquitin-like modifier) is covalently attached to a lysine residue in a ψKXE sequence (where ψ is a large hydrophobic residue and X represents any amino acid) on the substrate protein, especially nuclear proteins, via a pathway similar to ubiquitination in which a battery of activating (E1), conjugating (E2), and ligating (E3) enzymes participate (27). Similar to phosphorylation, sumoylation is a reversible process, and desumoylation is the primary function of four SUMO-specific proteases: SENP1 is a nuclear protease that appears to deconjugate a large number of sumoylated proteins (28–31); SENP2 is a nuclear envelope-associated protease that has an activity similar to that of SENP1 when overexpressed (32, 33); SENP3 localizes in the nucleolus (28, 34), and SENP6 was found primarily in the cytoplasm (34, 35). SUMO modification correlates with either an enhanced or attenuated activity of target proteins, for example, as has been shown for nuclear receptors such as androgen receptor (36), glucocorticoid receptor (37), progesterone receptor (38, 39), and estrogen receptor (40) and their cofactors, including SRC-1 (38), GRIP1 (41), and p300 (42).

In this report, we demonstrated that SUMO-1 is conjugated to AIB1 protein, and in contrast to GRIP1 and SRC-1, SUMO modification attenuates, rather than enhances, the transactivation activity of AIB1. In addition, sumoylation of AIB1 coincided with a decreased level of AIB1 phosphorylation by MAPK, whereas AIB1 phosphorylation by MAPK was concomitant with a loss of the SUMO moiety. Therefore, phosphorylation and sumoylation appear to coordinately regulate the transcriptional activity of AIB1.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Antibodies—**AIB1 mutants (K723R, K786R, K1194R, K723R/K786R/K1194R, and S505A/S543A/S860A/S867A) were generated using site-directed mutagenesis according to the manufacturer’s instructions (Strategene, La Jolla, CA). Full-length AIB1 and the mutants were PCR-amplified and subcloned into pcDNA3.1-Gal4-DNA binding domain (DBD) to generate Gal4-DBD-AIB1 fusion constructs. pcDNA3.1-HA-SUMO-1 and pcDNA3.1-Ubc9-SV5 and dominant negative mutant DN-Ubc9 (C93S) were from Dr. Ronald Hay (University of St. Andrews, St. Andrews, UK). FLAG-SENP1, FLAG-SENP2, and Hs-SENP3 were provided by Dr. Edward Yeh (The University of Texas, M.D. Anderson Cancer Center). Polyclonal anti-extracellular signal-regulated kinase (ERK) 1/2 and anti-phosphor-ERK1/2, polyclonal antibodies against Gal4-DBD, mouse monoclonal anti-HA antibody, and anti-SUMO-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-FLAG was from Sigma. Rabbit polyclonal anti-phosphoserine was from Zymed Laboratories (South San Francisco, CA).

**Cell Culture and Reporter Assays—**MCF-7 human breast cancer cells and COS-7 African green monkey kidney cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Transient transfections were performed with Lipofectamine (Invitrogen) according to the company’s specification. For luciferase assays, MCF-7 or COS-7 cells were seeded onto 24-well plates for 18 h and transfected with appropriate plasmid constructs. Five hours after transfection, cells were switched to phenol red-free medium containing 10% charcoal-dextran-treated fetal bovine serum for 48 h followed by treatment with or without 10 nM 17β-estradiol (E2) or 5 μM ICI-182,780 for 16 h. The cells then were harvested, and luciferase and Renilla activities were measured using a dual luciferase kit (Promega, Madison, WI).

**Immunoprecipitation and Immunoblotting—**MCF-7 or COS-7 cells were collected in ice-cold phosphate-buffered saline, and cell extracts were prepared in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, and 0.5% sodium deoxycholate) with 1:100 diluted protease inhibitor mixture (Roche Applied Science, Indianapolis, IN). The cytosolic and nuclear protein extracts were prepared as previously described (43). The cell extracts was pre-cleared using protein-A-Sepharose at 4 °C for 2 h. Following centrifugation at 14,000 × g for 10 min, the supernatant was incubated with appropriate antibodies for 16 h at 4 °C. Immune complexes were then captured using protein-A-Sepharose. Following centrifugation at 6,000 × g for 10 min, the protein-A-Sepharose was washed four times in washing buffer (20 mM Tris-HCl, pH 7.5, 500 or 800 mM NaCl, 0.5% Nonidet P-40, and 0.05% sodium deoxycholate). Bound proteins were released from the protein-A-Sepharose by boiling for 5 min in 2 × SDS loading buffer, resolved on 6–8% SDS-PAGE, and transferred onto Pure Nitrocellulose Blotting Membrane (Pall Corp.). The membrane was then probed using a secondary antibody and visualized using the enhanced chemiluminescence detection reagents (Amersham Biosciences) according to the manufacturer’s instructions.

**RESULTS**

**AIB1 Is Modified by SUMO-1—**Sumoylation has recently been identified as an important mechanism that regulates protein functions in essential biological processes, such as gene transcription, subnuclear structure organization, and cell cycle progression. Within the SRC family of coactivators, both GRIP1 (41) and SRC-1 (38) have been reported to be sumoylated. To understand the basis for diverse activities of AIB1 and the mechanism of the regulation of AIB1 activity, we wanted to know if AIB1 is also a sumoylated protein in vivo. For this purpose, we performed immunoprecipitation analysis of cellular extracts from breast cancer cells MCF-7 using anti-AIB1 antibodies, followed by immunoblotting with anti-SUMO-1. As shown in Fig. 1A, immunoprecipitated AIB1 could also be detected with an anti-SUMO-1 antibody, indicating that AIB1 is a sumoylated protein. Interestingly, AIB1 sumoylation quickly disappeared upon treatment of MCF-7 cells with 17β-estradiol (E2); 5 min after E2 treatment, sumoylated AIB1 was...
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AIB1 is modified by SUMO-1 in the nucleus. A, MCF-7 cells were treated with E₂ at the indicated periods of time. Cellular protein extracts were prepared and immunoprecipitated (IP) with anti-AIB1 antibodies followed by Western blotting (WB) analyses with anti-SUMO-1 or anti-AIB1 antibodies. B, the cytosolic and nuclear protein extracts from MCF-7 cells treated or untreated with E₂ for 15 min were first immunoprecipitated with anti-AIB1 antibodies followed by Western blotting with the anti-SUMO-1 antibody. Fibrillarin and β-actin were measured by Western blotting to monitor the efficiency in preparation of nuclear and cytosolic protein extracts, respectively.

FIGURE 1. AIB1 is modified by SUMO-1 in the nucleus. A, MCF-7 cells were treated with E₂ at the indicated periods of time. Cellular protein extracts were prepared and immunoprecipitated (IP) with anti-AIB1 antibodies followed by Western blotting (WB) analyses with anti-SUMO-1 or anti-AIB1 antibodies. B, the cytosolic and nuclear protein extracts from MCF-7 cells treated or untreated with E₂ for 15 min were first immunoprecipitated with anti-AIB1 antibodies followed by Western blotting with the anti-SUMO-1 antibody. Fibrillarin and β-actin were measured by Western blotting to monitor the efficiency in preparation of nuclear and cytosolic protein extracts, respectively.

no longer detectable, although the total AIB1 level was unchanged (Fig. 1A, lower panel).

Most SUMO conjugates are nuclear proteins, and it is likely that most of the major functions of SUMO take place in the nucleus. In addition, AIB1 functions as a cofactor that coactivates estrogen receptor-mediated transcription in the nucleus under the treatment of E₂. In light of the observation that sumoylated AIB1 was only detected in the absence of E₂ stimulation, we wished to know whether the sumoylation of AIB1 occurred in cytoplasm or in the nucleus. For this purpose, we prepared cytosolic and nuclear protein extracts separately from MCF-7 cells cultured in the absence of E₂. Immunoprecipitation was performed with these two cellular protein fractions using the anti-AIB1 antibodies, and immunoprecipitates were then probed with the anti-SUMO-1 by Western blotting. As shown in Fig. 1B, sumoylated AIB1 was only detected in nuclear fraction of the cellular protein extracts, indicating that sumoylated AIB1 localizes in the nucleus. The efficiency for preparation of nuclear extracts and cytosolic extracts was monitored by measuring a nuclear protein marker, fibrillarin, and a cytosolic protein marker, β-actin (Fig. 1B).

Identification of Sumoylation Sites in AIB1 Sequence—SUMO attachment to target proteins occurs at specific lysine residues, which are in most cases embedded in a consensus sequence ψKXE. In searching the amino acid stretch for the consensus SUMO modification motifs, we found three potential SUMO-1 attachment sites, lysines 723, 786, and 1194, in the AIB1 sequence. The lysines 723 and 786 locate in the first nuclear receptor interaction domain. The Lys-1194 locates in the Q-rich region downstream to the CREB-binding protein/p300 interaction domain. Interestingly, the Lys-723 and Lys-786 sites are conserved residues that are sumoylated in the two other members of the SRC family, SRC-1 (Lys-732 and Lys-774) and GRIP1 (Lys-731 and Lys-788) (38, 41). To examine whether these sites indeed serve as SUMO-1 acceptors in AIB1 as well as to test the effect of AIB1 sumoylation on its transactivation activity, we first generated AIB1 mutants in which these lysine residues were individually (K723R, K786R, or K1194R) or collectively (K723R/K786R/K1194R) changed to arginine residues. Wild-type (wt) and the mutated AIB1 were then fused to Gal4-DNA binding domain (Gal4-DBD). The Gal4-DBD AIB1 fusions were then expressed in COS-7 cells with or without cotransfection of a HA-tagged SUMO-1 plasmid. The cell lysates were immunoprecipitated with the antibody against Gal4-DBD, and the immunoprecipitates were probed with an anti-HA antibody. As shown in Fig. 2A, HA tag could be detected in all three single lysine-mutated AIB1, K723R, K786R, or K1194R, whereas in AIB1 carrying K723R/K786R/K1194R, HA tag was no longer detected, suggesting that lysines 723, 786, and 1194 could all be the acceptors for SUMO conjugation in AIB1. The level of Gal4-DBD expression was similar in all experimental conditions (Fig. 2A, lower panel).

To further define the sumoylation site(s) in AIB1, we next constructed the following Gal4-DBD-fused AIB1 fragments: N-terminal fragments (aa 1–740) containing Lys-723 or K723R, N-terminal fragments (aa 1–792) containing K723R and Lys-786 or K723R and K786R, and C-terminal fragments (aa 793–1424) containing Lys-1194 or K1194R (Fig. 2B). These constructs were transfected into COS-7 cells together with the HA-tagged SUMO-1 plasmid. The cell lysates were immunoprecipitated with an anti-Gal4-DBD antibody followed by immunoblotting with an anti-HA antibody. As shown in Fig. 2A, HA tag could be detected in all three single lysine-mutated AIB1, K723R, K786R, or K1194R, whereas in AIB1 carrying K723R/K786R/K1194R, HA tag was no longer detected, suggesting that lysines 723, 786, and 1194 could all be the acceptors for SUMO conjugation in AIB1. The level of Gal4-DBD expression was similar in all experimental conditions (Fig. 2A, lower panel).
immunoblotted using the antibody against HA. As shown in Fig. 2B, SUMO conjugation was detected in the N-terminal fragments containing Lys-723 or containing K723R and Lys-786 but not in the N-terminal fragments containing K723R or containing K723R and K786R. In the C-terminal fragments, SUMO conjugation was detected in the AIB1 fragment containing Lys-1194 but not that containing K1194R. In addition, corresponding C-terminal fragments of SRC-1 (aa 780–1441) or GRIP1 (aa 790–1462) did not show any SUMO conjugations. These experiments clearly showed that Lys-723, Lys-786, and Lys-1194 are all sumoylation sites in AIB1 and an additional sumoylation site exists in AIB1 compared with SRC-1 and GRIP1.

Sumoylation of AIB1 Attenuates Its Transcriptional Activity—To examine the effect of AIB1 sumoylation on the transactivation activity of AIB1, we next transfected COS-7 cells with a Gal4 promoter-driven luciferase reporter. Forty-eight hours after transfection, cells were harvested and luciferase activity was determined. Each bar represents mean ± S.D. from three independent experiments. COS-7 cells grown in the presence of E2 were transfected with wild-type (WT) or mutated AIB1 fused to Gal4-DBD together with a Gal4-luciferase reporter. Fourty-eight hours after transfection, cells were harvested and luciferase activity was determined. Each bar represents mean ± S.D. from three independent experiments.

SUMO conjugation was detected in the AIB1 fragment containing Lys-1194 but not that containing K1194R. In addition, corresponding C-terminal fragments of SRC-1 (aa 780–1441) or GRIP1 (aa 790–1462) did not show any SUMO conjugations. These experiments clearly showed that Lys-723, Lys-786, and Lys-1194 are all sumoylation sites in AIB1 and an additional sumoylation site exists in AIB1 compared with SRC-1 and GRIP1.

**FIGURE 2. Identification of sumoylation sites in AIB1.** A, COS-7 cells were transfected with a SUMO-1 expression plasmid (HA-SUMO-1) along with Gal4-DBD-fused wild-type or mutated AIB1 carrying K723R, K786R, K1194R, or K723R/K786R/K1194R (3K/3R). Cells were harvested at 48 h after the transfection, and the cellular protein extracts were immunoprecipitated (IP) with anti-Gal4-DBD antibodies and subsequently immunoblotted (WB) with an anti-HA antibody. B, Gal4-DBD-fused constructs with AIB1 fragments: N-terminal (aa 1–740) containing Lys-723 or K723R, N-terminal (aa 1–792) containing K723R and Lys-786 or K723R and K786R, and C-terminal (aa 793–1424) containing Lys-1194 or K1194R; SRC-1 fragments: N-terminal (aa 1–779) and C-terminal (aa 780–1441); and GRIP1 fragments: N-terminal (aa 1–789) and C-terminal (aa 790–1462). These constructs were transfected into COS-7 cells together with the HA-tagged SUMO-1 plasmid. Forty-eight hours after transfection, cellular extracts were immunoprecipitated with the antibody against Gal4-DBD, and the immunoprecipitates were immunoblotted using the antibody against HA.

**FIGURE 3. Sumoylation attenuated AIB1 transcriptional activity.** A, COS-7 cells grown in the presence of E2 were transfected with increased amounts of wild-type (WT) or mutated AIB1 fused to Gal4-DBD together with a Gal4-luciferase reporter. Forty-eight hours after transfection, cells were harvested and luciferase activity was determined. Each bar represents mean ± S.D. from three independent experiments. B, COS-7 cells grown in the presence of E2 were transfected with wild-type (WT) or mutated AIB1 together with an estrogen receptor-responsive element-luciferase reporter. Forty-eight hours after transfection, cells were harvested and luciferase activity was determined. Each bar represents mean ± S.D. from three independent experiments.

Sumoylation of AIB1 Attenuates Its Transcriptional Activity—To examine the effect of AIB1 sumoylation on the transactivation activity of AIB1, we next transfected COS-7 cells with a Gal4 promoter-driven luciferase (Gal4-luc) construct together with the Gal4-DBD-AIB1 (wt or mutants), and the reporter activity was measured. As shown in Fig. 3, whereas AIB1 carrying K1194R had a similar effect as wt AIB1 on Gal4-luc activity, Gal4-luc activity was significantly increased in cells that were transfected with AIB1 carrying either K723R, K786R, or K723R/K786R/K1194R mutations (Fig. 3, upper panel). The maximal Gal4-luc activity was observed in cells that were transfected with AIB1 carrying K723R/K786R/K1194R mutations. In addition, the enhancement effect of these transfections on the Gal4-luc activity was dose-dependent. Similar results were obtained in COS-7 cells transfected with a luciferase expression vector.
construct driven by estrogen receptor-responsive element (Fig. 3, lower panel). These data suggest that the sumoylation of AIB1 attenuated its transactivation potential.

Sumoylation is a reversible chemical modification, and deconjugation of SUMO is believed to be a function of one of four SUMO-specific proteases: SENP1, SENP2, SENP3, or SENP6. Of these, SENP1, SENP2, and SENP3 function in the nucleus, whereas SENP6 is a cytoplasmic enzyme. To further support the notion that sumoylation of AIB1 attenuated its transactivation activity and to investigate which de-sumoylating enzyme might be involved in regulating AIB1 activity, we next cotransfected de-sumoylating enzyme SENP1, SENP2, or SENP3 with Gal4-DBD-fused wt AIB1 and AIB1 mutant 3K/3R. As shown in Fig. 4, while cotransfection of SENP2 or SENP3 had minimal effects on Gal4-luc activity in COS-7 cells, cotransfection of SENP1 resulted in dramatic increases in the Gal4-luc activity in cells transfected with wt AIB1 (Fig. 4A). In AIB1 mutant 3K/3R-transfected cells, although the base level of the Gal4-luc was higher than that of wt AIB1-transfected cells, transfection of SENP1 did not increase the Gal4-luc activity. In addition, the effect of SENP1 on Gal4-luc in wt AIB1-transfected cells was dose-dependent (Fig. 4B). Moreover, cotransfection of an SENP1 mutant, which carries mutated residues (R630L/K631M) that are crucial to its catalytic activity, did not significantly alter Gal4-DBD-AIB1-mediated transcription. Collectively, these data indicated that SENP1 functions to de-sumoylate AIB1. The expression of the Gal4-DBD-fused wt AIB1 and SENP1 was confirmed by Western blotting (Fig. 4C). Moreover, cotransfection of a mutant of the E2 SUMO-conjugating enzyme Ubc9, Ubc9/C93S, which had a dominant negative effect on its wild type (29), enhanced Gal4-DNA-AIB1-mediated transcription (data not shown). All these data strongly supported the notion that sumoylation of AIB1 attenuates its transcriptional activity, and SENP1 is responsible for deconjugating the SUMO mark from AIB1 protein.

**AIB1 Modifications by Sumoylation and Phosphorylation**—It has been documented that AIB1 is regulated by phosphorylation, and the transcriptional activity of AIB1 is enhanced by MAPK activation (16, 25). MAPK can specifically phosphorylate AIB1 on Ser-505, Ser-543, Ser-860, and Ser-867 (16). To investigate the mechanism of sumoylation and phosphorylation in relation to AIB1 transcriptional activity, we first confirmed MAPK activation and AIB1 phosphorylation in our system. MCF-7 cells were treated with E2 for different periods of time, and cellular pro-
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AIB1 sumoylation, whereas both ICI-182,780 and U0126 induced increased AIB1 sumoylation but decreased AIB1 phosphorylation, indicating that the sumoylation and phosphorylation of AIB1 may occur in a coordinated fashion.

Coordinated Regulation of AIB1 Activity by Sumoylation and Phosphorylation—To explore a functional correlation between the coordinated sumoylation and phosphorylation of AIB1, we next examined the effects of SUMO and MAPK pathways on the transactivation potential of AIB1. The SUMO target-mutated AIB1 (K723R, K786R, K1194R, and K723R/K786R/K1194R) as well as phosphorylation target-mutated AIB1 (S505A/S543A/S860A/S867A) were used in experiments to analyze the effect of these AIB1 mutants on the coactivation of the Gal4-luc activity under various experimental conditions, including treatment with E2, ICI-182,780, or U0126 or cotransfection with SENP1 in COS-7 cells. As shown in Fig. 6, treatment with ICI-182,780 or U0126, which was associated with an increased sumoylation and a decreased phosphorylation of AIB1 as demonstrated above, resulted in decreased Gal4-luc activities. Meanwhile, treatment with E2 or cotransfection of SENP1 led to an increased Gal4-luc activity. The highest Gal4-luc activity was detected with AIB1 mutant carrying all sumoylation site mutations (K723R/K786R/K1194R), whereas the lowest Gal4-luc activity was detected with AIB1 mutant carrying all phosphorylation site mutations (S505A/S543A/S860A/S867A). All these data suggest a coordinated pattern of sumoylation and phosphorylation in regulating AIB1 transcriptional activity.

To further support the coordinated pattern of sumoylation and phosphorylation of AIB1, MCF-7 cells were transfected with the Gal4-DBD-fused wt AIB1 or the AIB1 mutant carrying all sumoylation site mutations (K723R/K786R/K1194R) under no treatment or treatment with E2, and coinmunoprecipitation experiments were performed with the antibody against Gal4-DBD and then immunoblotted with anti-phospho-serine. The results of the experiments indicated that phosphorylation was detected in sumoylation site-mutated AIB1 even under no treatment of E2 (Fig. 6D, left panel). Similar experiments were performed with Gal4-DBD-fused wt AIB1 or the AIB1 mutant carrying all phosphorylation site mutations (S505A/S543A/S860A/S867A). The results of these experiments revealed the retention of sumoylation in phosphorylation site-mutated AIB1 even after the treatment of E2 (Fig. 6D, right panel). These data strongly support a coordinated pattern between phosphorylation and sumoylation in regulating AIB1 activity. Moreover, these data may also help to explain the higher base levels of Gal4-luc activity in 3K/3R transfections and the overall lower activation of the Gal4-luc activity in 4S/4A transfections described in Figs. 4A, 6A, 6B, and 6C.

**DISCUSSION**

AIB1 is highly expressed in the mammary gland, uterus, testis, pituitary gland, and muscle (44) and is strongly amplified/overexpressed in 64% of primary breast cancers as well as in some of primary ovarian tumors (3, 45); thus, AIB1 has important physiological and pathological roles. At the molecular level, AIB1, together with the other two members of the SRC family of coactivators, GRIP1 and SRC-1, coactivates gene tran-
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AIB1 exhibit differential functions that are manifested in animal phenotypes as well as in transcriptional outputs. Recently, we demonstrated that SRC coactivator proteins function in heterodimers in which AIB1 is the common dimerization partner for GRIP1 and SRC-1 (20).

The basic mechanisms underlying ligand-dependent transcription initiation by nuclear receptors indicate a sequential recruitment of an assortment of coactivators. Increasing numbers of coactivators have been identified in recent years, and both biochemical and genetic studies have demonstrated that these coactivators are differentially used by transcription factors, including nuclear receptors, in a cell/tissue type- and promoter-specific manner. However, the molecular basis underlying this specificity remains largely unknown. In the past years, both nuclear receptors and cofactors have been shown to be targeted for post-translational modifications by diverse cellular signaling pathways. It is proposed that post-translational modifications of these cofactor proteins provide the basis for a combinatorial code required for specific gene activation by nuclear receptors and coactivators and that this code also enables coactivators to efficiently stimulate the activity of other classes of transcription factors (46).

Phosphorylation is a fundamental mechanism of post-translational modifications implicated in signal transduction. Phosphorylation of AIB1 has been shown to involve different kinases, such as MAPK, IkB kinase, and receptor tyrosine kinase HER2/neu (16, 19, 24–26, 48). It has been shown that different signals differentially phosphorylate AIB1 at different combinations of sites and that these phosphorylated profiles of AIB1 encode for distinct physiological functions (16). In addition, studies in recent years have identified another important post-translational modification, sumoylation. SUMO has been shown to covalently modify a large number of proteins with important roles in many cellular processes, including gene expression, chromatin structure, signal transduction, and maintenance of the genome. Relevant to this report, two members of the SRC family of coactivators, GRIP1 and SRC-1, have been shown to be modified by SUMO (38, 41). In addition, sumoylated GRIP1 and SRC-1 are associated with their enhanced transactivation activity. In this report, we showed that the AIB1 is also a sumoylated protein. But surprisingly, sumoylation attenuated AIB1 transactivation activity.

Currently, there are no clear molecular explanations for the mechanism by which SUMO addition regulates transcription factor activity. Clearly, modification with SUMO alters the surface of the target protein and might cause either general conformational changes or specific changes at critical interfaces, thereby influencing the ability of the protein to interact with its partners. One possibility is that sumoylation promotes or inhibits protein-protein interactions and thereby regulates the assembly of transcriptional complexes. Alternatively, sumoylation might block other lysine-targeted modifications such as acetylation, methylation, or ubiquitination. Other possibilities include subnuclear redistribution of sumoylated transcription regulators. Sumoylation of transcription regulators could serve to organize specific protein complexes into nuclear matrix sites within nuclear bodies, thus regulating their activities either

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**FIGURE 6. SUMO and MAPK pathways coordinated AIB1 transcriptional activities.** COS-7 cells were transfected with Gal4-luc and Gal4-DBD-AIB1 (wt or mutant) (3K/3R: K723R/K786R/K1194R; 4S/4A: S505A/S543A/S860A/S867A) in the absence or presence of SENP1 plasmids. Twenty hours after transfections, cells were treated with E2 (A), ICI-182,780 (ICI) (B), or U0126 (C) for an additional 12 h, and the luciferase activity was measured. Each bar represents mean ± S.D. from three independent experiments. D, MCF-7 cells were transfected with the Gal4-DBD-fused wt AIB1 or the AIB1 mutant carrying all sumoylation site mutations (K723R/K786R/K1194R or 3K/3R) or the AIB1 mutant carrying all phosphorylation site mutations (SS505A/SS543A/SS600A/SS876A) under no treatment or treatment with E2, and coimmunoprecipitation experiments were performed with the antibody against Gal4-DBD and then immunoblotted with anti-phospho-serine or with anti-SUMO-1.
negatively or positively. In any event, how sumoylation affects the transcription activity of its target protein remains an intriguing question for further investigation. Our observation that SUMOylation renders AIB1 functionally opposite to its close cousins GRIP1 and SRC-1 is even more intriguing; after all, these proteins are so much alike. We reported a sumoylation site in AIB1 (Lys-1194) outside of the nuclear receptor box, which has not been reported for GRIP1 and SRC-1. Interestingly, AIB1 was also shown to harbor unique MAPK phosphorylation sites that are not conserved in SRC-1 and GRIP1; among MAPK-targeted Ser-505, Ser-543, Ser-860, and Ser-867 of AIB1, Ser-505 and Ser-543 are conserved in SRC-1 and GRIP1, and Ser-860 and Ser-867 are unique for AIB1 (16). Whether or not these unique modifications in AIB1 contribute in any way to specific AIB1 activities will be a subject of future investigations. Nevertheless, it is clear that functional specificity of a protein is partly determined by the specificity of the regulation/modification of the protein, and, as mentioned above, AIB1, GRIP1, and SRC-1 exhibit differential activities that may be consequences of differential post-translational modifications.

Dynamic interplays by different post-translational modifications are important for functional dynamics of proteins. The antagonistic relationship between phosphorylation and sumoylation has also been reported for transcription factor Elk-1 (49). Our results indicated that a dynamic phosphorylation and sumoylation may play important roles in regulating AIB1 activity. The overall transactivation potential of AIB1 could be enhanced by activation of the MAPK pathway accompanying a loss of SUMO mark in AIB1. On the other hand, the transcription activation potential of AIB1 is dampened upon its sumoylation, which is concomitant with its dephosphorylation. Thus, it is logical to assume that a balance between phosphorylation and sumoylation is critical in the final readout of the AIB1 activity. In addition, it is not clear how E2 treatment leads to de-sumoylation of AIB1. Clearly, the fast effect of E2 treatment on the sumoylation status of AIB1 suggests a non-genomic action for estrogen. It is conceivable that, similar to the activation of MAPK pathway, estrogen treatment emanates stimuli that activate signaling pathways responsible for SUMO de-conjugation. Future studies will focus on the delineation of the molecular details that influence the switch between phosphorylation and sumoylation.

REFERENCES

Regulation of AIB1 by Sumoylation and Phosphorylation