The proteasome-mediated pathway involves the degradation of several nuclear receptors. Previously we demonstrated that the interaction between the suppressor for Gal 1 (SUG1) and nuclear receptors, the vitamin D receptor, or the pregnane X receptor was involved in proteasome-mediated degradation. In our recent experiments, we examined the potential role of SUG1 in the proteasome-mediated degradation of estrogen receptors (ERα and -β). Both ERs interacted with SUG1 in a ligand-dependent manner. Functionally, the overexpression of SUG1 inhibited both ERα and ERβ-mediated transcription in the presence of ligands. Transient expression studies demonstrated that the overexpression of wild-type SUG1 generated proteolytic fragments of both ERs and that these products were blocked by a proteasome inhibitor. The overexpression of SUG1 also enhanced the formation of ubiquitinated proteins of both ERs in the presence of ligand. On the other hand, bisphenol A (BSA), which activated ER-mediated transcription, did not enhance the interaction between ERβ and SUG1. Furthermore, the degradation of ERβ was much slower in the presence of BSA than in the presence of estradiol or phthalate, which is another endocrine-disrupting chemical. Also, BSA had no effect on the formation of proteolytic fragments of ERβ, and neither did it have any effect on the ubiquitination of ERβ. These findings indicate that the ubiquitin/proteasome-mediated degradation of both ER proteins may involve the interaction of SUG1 with both ERs. Moreover, BSA strongly blocked the ubiquitination and degradation of ERβ compared with estradiol, suggesting that BSA may affect the ERβ-mediated transcription of target genes by inhibiting ERβ degradation.

Steroid hormones, including estrogen and progesterone, as well as non-steroid hormones, vitamin D, retinoids, thyroid hormone, and prostanoids regulate their specific genes by binding to their specific receptors, which comprises the nuclear receptor superfamily. These receptors form homodimers or heterodimers with retinoid X receptor and are associated directly with specific DNA sequences, known as hormone-responsive elements, at the upstream regions of specific genes (1, 2). The DNA receptor complex interacts with basal transcriptional machinery and nuclear receptor coactivator proteins resulting in the ligand-dependent induction of transcription (2, 3, 4). Ligand binding, in addition to altering the conformational change of the receptor to interact with coactivators, has been shown to influence the stability of the nuclear receptors. The half-life of estrogen receptor (ER)α particularly is approximately 5 h in the absence of estradiol, whereas estradiol binding reduces the half-life to 3–4 h (5, 6), suggesting that receptor degradation may be an important event in regulating the response duration of transactivation to the ligand binding.

We have demonstrated that several putative cofactor proteins, including steroid hormone receptor coactivator-1 (7), receptor interacting protein 140 (8), and suppressor for Gal 1 (SUG1) (9), interacted with vitamin D receptor (VDR) and pregnane X receptor (PXR) in a natural steroid-dependent manner (10, 11). Expression of steroid hormone receptor coactivator-1 and receptor interacting protein 140 augments ligand-activated transcription by a variety of nuclear receptors, indicating that these proteins act as transcriptional coactivators (7, 8). Although yeast SUG1 was originally identified as a transcription factor (12) and interacts with several nuclear receptors, such as VDR, retinoid X receptor, and thyroid hormone receptor in a ligand-dependent manner (4, 9, 11, 13), more recent evidence indicates that this protein is actually a component of the 26 S proteasome complex (14). Moreover, overexpression of this protein enhanced the degradation of VDR (10) and PXR (15). Together, these findings suggested that SUG1 might be involved in the degradation of nuclear receptors by proteasome.

Endocrine-disrupting chemicals (EDCs) have been defined as exogenous agents that interfere with the synthesis, secretion, transport, binding, action, or elimination of the body’s natural hormones, which are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior (16). These chemicals can alter endocrine functions through a variety of mechanisms, including steroid hormone receptor-mediated changes in protein synthesis, interference with membrane receptor binding, steriodogenesis, or the synthesis of other hormones (17). Although major chemicals, such as phthalates, alkylphenols, bisphenol A (BSA), and 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane, have been shown to disrupt estrogenic actions mainly through their binding to estrogen or androgen receptors (17), it is not clear whether EDCs directly affect endocrine functions in vivo.

We have examined the potential role of SUG1 in the proteasome-mediated degradation of ERs and EDRs. In this aspect, we checked whether or not SUG1 interacts with ERs in the pres-
ence of several ligands, including estradiol and EDCs, which activate ER-mediated transcription. Next, the effects of ligands for ERs on the steady states of both ERs were examined because we observed a different effect of BSA, an EDC, on the interaction between SUG1 and ER\textsubscript{H9251} compared with the interaction between SUG1 and ER\textsubscript{H9252}. We also examined the effect of SUG1 on ER-mediated transcription in the presence of these ligands. Furthermore, we used a transient transfection assay to determine whether or not the interaction between SUG1 and either of the ERs is involved in this degradation system. Finally, we checked whether or not SUG1 plays a role in the ubiquitination of ER proteins. The results of these inquiries.
Involvement of SUG1 in Ubiquitin/Proteasome-mediated Degradation of ER

FIG. 2. Effect of proteasome inhibitors on ER protein levels in endometrial cancer cell lines. A, subconfluent HEC-1 and Ishikawa cells were treated with MeSO or protease inhibitors for 6 h, and nuclear extracts were prepared as described under “Experimental Procedures.” An equivalent amount of each extract was resolved by 10% SDS-PAGE. ER protein levels were determined by Western blotting using anti-ERα and -ERβ antibody. B, subconfluent Ishikawa cells were treated with cycloheximide (10 mg/ml media) for 10 min prior to the addition of ligands. Cells were then treated in the presence of 10−6 m estradiol, EDCs, or vehicle. Nuclear extracts were prepared, and ER protein levels were examined as described above. This level of cycloheximide inhibited >95% of 35S-labeled methionine incorporation into trichloroacetic acid-precipitated protein (data not shown).

indicate that the interactions between ERs and SUG1 may be involved in the ubiquitin/proteasome-mediated degradation of ER proteins. Moreover, BSA may affect ERβ-mediated transcription of target genes by inhibiting ERβ degradation.

EXPERIMENTAL PROCEDURES

Materials—BSA, phthalic acid bis(2-ethylhexyl ester) (phthalate), 4-pregnen-3,20-dione (progesterone), and 17β-estradiol (1,3,5(10)-estratriene-3,17(β-diol) (estradiol) were purchased from Sigma. 1,25-Dihydroxyvitamin D3 (1,25(OH)2D3) was kindly provided by Dr. M. R. Uskokovic (Hoffmann-La Roche). 1,1,1-Trichloro-2,2-bis(p-chlorophenyl)ethane was obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). All endocrine-disrupting chemicals and ligands were added simultaneously to the culture media at the indicated concentrations. Primary rabbit polyclonal antibody for ERα (MC-20), which reacts with mouse, rat, and human ERα but does not cross-react with ERβ, and for ERβ (H-150), which reacts with mouse, rat, and human ERβ but does not cross-react with ERα, were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal antibody for SUG1, which reacts with mouse and human SUG1, was generated against the N-terminal amino acid sequence of SUG1 (36-NDKSQNLRRL-45) (Sigma Genosys Japan K.K., Sapporo, Japan). Two plasmids, pSG5-ERα and pSG5-ERβ (ERE-ERα and -ERβ constructs) have been described previously (10). Ishikawa cells were kindly provided by Dr. M. Nishida (Teikyo University, Tochigi, Japan). HEC-1 cells and COS-7 cells were provided by the Health Science Research Resources Bank (Osaka, Japan).

Cell Culture and Transient Transfection Studies—COS-7, HEC-1, and Ishikawa cells were cultured in Dulbecco’s modified Eagle’s medium without phenol red. The medium was supplemented with 10% charcoal-stripped fetal bovine serum. COS-7 cells were cotransfected with 1 μg of a reporter gene construct (EREp-G-CAT) and 0.5 μg of a receptor expression vector (pSG5-ERα or ERβ) or of an empty vector (pSG5). Ishikawa cells were transfected with 1 μg of a reporter gene construct (EREp-G-CAT) or of an empty vector (G-CAT) without the ER expression vector. For SUG1 expression, pcDNA3-SUG1 (wild-type or K196H) or pcDNA3 alone was transfected into the cells. All transfections were liposome-mediated by the use of lipofectAMINE (Invitrogen) according to the manufacturer’s protocol. Transfected cells were treated for 36 h, either with the vehicle alone or with the indicated concentrations of estradiol or EDCs. Cell extracts were prepared and assayed for CAT activity. The amount of CAT was determined using a CAT enzyme-linked immunosorbent assay kit (Roche Diagnostics) according to the manufacturer’s protocol.

Preparation of Two-hybrid Expression Vectors and β-Gal Assay—All two-hybrid plasmid constructs used the pAS1 (18) and pAD-GALA yeast expression vectors (Stratagene, La Jolla, CA). The AS1-VDR, -PXR, -ERα, and -ERβ constructs have been previously described (11). The pAD-GALA-SUG1 (wild-type or K196H) was cotransformed with pAS1-ERα or ERβ into yeast strain H77c. Transformants were plated on synthetic complete medium lacking leucine and tryptophan (SC-Leu-Trp) and were grown for 4 days at 30 °C to yield yeast that had acquired both plasmids. Triplicate independent colonies from each plate were grown overnight in 2 ml of SC-Leu-Trp with or without the indicated concentrations of estradiol, progesterone, 1,25(OH)2D3, or EDCs. Cells were harvested and assayed for β-Gal activity as described previously (4).

Nuclear Extracts and Western Analysis—Nuclear extracts were obtained from Ishikawa cells and HEC-1 cells using nuclear and cytoplasmic extraction reagents (Pierce) according to the manufacturer’s protocol and were stored at −80 °C until analysis. Equivalent amounts of nuclear protein (25 μg/sample) from each extract were determined by bicinchoninic acid protein assay (Pierce), solubilized in SDS buffer (0.05 M Tris-HCl, 2% SDS, 6% mercaptoethanol, 10% glycerol, pH 6.8), and were analyzed by Western blot analysis as previously described (10) using rabbit polyclonal antibody for ERα (1:1000 dilution), ERβ (1:1000 dilution), and SUG1 (1:1000 dilution).
RESULTS

Differential Effects of EDCs on the Interaction between ER and SUG1—As illustrated in Fig. 1A, SUG1 interacted with VDR, PXR, ERα, and ERβ in the presence of endogenous ligands for each receptor in the two-hybrid system. ERα interacted with SUG1 in the presence of EDCs, phthalate and BSA, both of which have been demonstrated to activate ERα—mediated transcription (19). On the other hand, 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane, which had no transcriptional activity on ERβ (10, 13). The presence of any of the ligands had no effect on the interaction between ERα and the SUG1 mutant (Fig. 1E).

Effect of Proteasome Inhibitors on ER Protein Levels in Endometrial Cancer Cell Lines—Because the turnover of ERα has been demonstrated to be involved in the proteasome-mediated degradation system (20, 21), we examined whether or not ERβ as well as ERα is also a target for proteasome-mediated degradation in endometrial cancer cell lines, Ishikawa cells, and HEC-1 cells. The protein levels of ERα and ERβ were examined in Ishikawa cells and HEC-1 cells that had been exposed to proteasome inhibitors. The protein levels of both ERα and ERβ were markedly increased in the presence of 5 mM MG132 or 0.05 mM β-lactone, both of which strongly inhibit proteasome activities (22, 23) with or without estradiol, in HEC-1 cells and Ishikawa cells (Fig. 2A). Furthermore, Ishikawa cells were treated with cycloheximide (10 mg/ml), and the effect of estradiol or either of two EDCs, phthalate and BSA, on ER turnover was determined. In the presence of estradiol, the protein levels between ERα and SUG1 (K196H), which in previous studies did not interact with VDR or ERα (10, 13). The presence of any of the ligands had no effect on the interaction between ERα and the SUG1 mutant (Fig. 1E).

Involvement of SUG1 in Ubiquitin/Proteasome-mediated Degradation of ER

Suppression of ER-mediated transcription by overexpressed wild-type SUG1. A, COS-7 cells were transfected with 1 μg of a reporter gene construct ((ERE)2-G-CAT), 0.5 μg of a receptor expression vector (pSG5-ERα or -ERβ) or an empty vector (pSG5), and 0.5 μg of pcDNA3-SUG1 (wild-type (WT) or K196H) or pcDNA3 expression vector together. The cells were treated with an ethanol vehicle or 10−6 M estradiol for 36 h. The amount of CAT was determined with a CAT enzyme-linked immunosorbent assay kit (Roche Diagnostics) according to the manufacturer’s protocol. Results represent mean ± S.D. of triplicate determinations. B, COS-7 cells were transfected with 1 μg of a reporter gene construct ((ERE)2-G-CAT), 0.5 μg of a receptor expression vector (pSG5-ERα or -ERβ), and 0.5 μg of pcDNA3-SUG1 (wild-type (WT) or K196H) or pcDNA3 expression vector together. The cells were treated with an ethanol vehicle, 10−8 M estradiol, or 10−6 M EDCs for 36 h. The amount of CAT was determined as described above. Results represent mean ± S.D. of triplicate determinations. C, Ishikawa cells were transfected with 1 μg of a reporter gene construct ((ERE)2-G-CAT) and 0.5 μg of pcDNA3-SUG1 (wild-type (WT) or K196H) or pcDNA3 expression vector together. The cells were treated with an ethanol vehicle, 10−6 M estradiol, or 10−8 M EDCs for 36 h in the presence or absence of 5 mM MG132. The amount of CAT was determined as described above. Results represent mean ± S.D. of triplicate determinations.
of both ERs were rapidly degraded compared with ethanol-treated cells (Fig. 2B, lanes 2 and 6). The effects of BSA and phthalate on ERα turnover were similar to those in the presence of estradiol (Fig. 2B, lanes 3 and 4), but the degradation of ERβ was blocked in the presence of BSA (Fig. 2B, lane 7) compared with that in the presence of estradiol or phthalate.

**Suppression of ERα-mediated Transcription by Overexpressed Wild-type SUG1**—We used transient reporter expression assays in COS-7 cells and Ishikawa cells to examine whether or not SUG1 affected ERα- and ERβ-mediated transcription. Expression of SUG1 suppressed estradiol-mediated transactivation of both overexpressed ERαs and overexpressed ERβ, but SUG1 overexpression had no effect on basal transcription using COS-7 cells (Fig. 3A). The suppressive effect on estradiol-dependent transcription was the result of an interaction between ERs and SUG1 because the expression of mutant SUG1 (K196H), which interacted with neither of the ERs (Fig. 1E), had no significant effect on ER-mediated transactivation in this system (Fig. 3A). And, as described in Fig. 3B, SUG1 suppressed the ERα-mediated transcription in the presence of BSA or phthalate, both of which enhanced the interaction between ERαs and SUG1 (Fig. 1B). In contrast, SUG1 had no suppressive effect on ERβ-mediated transcription in the presence of BSA, which had no effect on the interaction between SUG1 and ERβ (Fig. 1B). Mutant SUG1 had no effect on ER-mediated transcription in any ligands. Moreover, SUG1 had a negative effect on endogenous ER-mediated transcription in the presence of phthalate or estradiol, but there was no change in the presence of BSA in Ishikawa cells (Fig. 3C). We also examined the effect of a proteasome inhibitor, MG132, on ER-mediated transcription using Ishikawa cells. MG132 weakly suppressed basal transcription and ER-mediated transcription in the presence of estradiol or BSA and completely blocked the suppressive effect of SUG1 on ER-mediated transcription in the presence of estradiol or phthalate (Fig. 3D), suggesting that SUG1 affected the transcription through the proteasome-mediated degradation.

**SUG1 Overexpression Enhances ER Proteolysis in Ishikawa Cells**—To test whether or not SUG1-ER interactions are involved in ER degradation in proteasome-mediated systems, wild-type and mutant SUG1 were transiently overexpressed in Ishikawa cells, and their respective effects on ER protein levels were examined by Western immunoblotting (Fig. 4A). In the absence of estradiol, overexpression of wild-type or mutant SUG1 did not significantly affect the protein levels of ERαs (Fig. 4A, lanes 1–3) or ERβ (Fig. 4A, lanes 7–9). However, in the presence of estradiol, novel proteolytic fragments of both ERs were observed when Ishikawa cells were transfected with the wild-type SUG1 expression vector (Fig. 4A, lane 5 and 11). The ER proteolytic fragments were not observed in the cells transfected with mutant SUG1 (Fig. 4A, lanes 6 and 12). The overexpression of SUG1 was confirmed using anti-SUG1 antibody. Moreover, proteolytic fragments of ERαs were also observed in the presence of BSA or phthalate (Fig. 4B, lanes 2 and 3). However, we observed proteolytic fragments of ERβ only in the presence of phthalate and not in the presence of BSA (Fig. 4B, lanes 8 and 9), suggesting that this phenomenon requires interaction between ERs and SUG1, whereas MG132 completely blocked the formation of SUG1-dependent fragments of both ERs (Fig. 4B, lanes 4–6 and 10–12). Then, we checked the dominant-negative effect of SUG1 (K196H) on wild-type SUG1-induced proteolysis of both ERs. As described in Fig. 4C, mutant SUG1 inhibited the proteolytic fragments of both ERs by overexpressed wild-type SUG1 in a dose-dependent manner.

**Wild-type SUG1 Enhanced the Ubiquitination of ER**—Because the ubiquitination of the target protein has been demonstrated to be important in the proteasome-mediated degradation system (24, 25), we checked whether or not SUG1 is involved in the process of ER ubiquitination. We used polyubiquitin affinity beads to pull polyubiquitinated proteins down...
from whole cell extracts of Ishikawa cells (Fig. 5A). We detected ubiquitinated proteins of both ERα and ERβ with overexpressed wild-type SUG1 alone; none were detected with the pcDNA3 expression vector alone or with SUG1 (K196H) (Fig. 5B). No nonspecific interaction with control beads was observed. Moreover, ubiquitinated ERα protein was observed in the presence of BSA or phthalate as well as estradiol, but the ubiquitination of ERβ was detected in the presence of phthalate or estradiol (Fig. 5C), suggesting that ubiquitination of ER proteins requires interaction between SUG1 and the ER. Then, the dominant-negative effect of mutant SUG1 on the ubiquitination of both ERs was examined. This mutant SUG1 inhibited the ubiquitination of both ERs in a dose-dependent manner (Fig. 5D).

**Discussion**

Proteasome is a major cytosolic and nuclear protease complex that is responsible for an ATP-dependent, extra-lysosomal proteolytic pathway. This complex is responsible for the degradation of most cellular proteins, and proteasome activity is necessary for cell viability (24, 25). Proteasome is highly conserved throughout eukaryotic evolution, and it exists as two major complexes; 20 S proteasome, which contains multiple peptidase activities, and 26 S proteasome, which contains the 20 S subunit as well as a 19 S regulatory complex composed of multiple ATPases and components necessary for binding protein substrates (24, 25). To date, a wide variety of substrates for proteasome have been identified, including rate-limiting enzymes such as ornithine decarboxylase, transcriptional regulators such as c-Jun, p53, and NF-κB, and critical regulatory proteins such as cyclins and tyrosine kinase receptors (24, 25). In addition, we and others (10, 15, 20, 21) have demonstrated that the proteasome system might be involved in the degradation of nuclear receptors, including vitamin D receptor, pregnane X receptor, progesterone receptor, and estrogen receptor. A common feature of proteasome-mediated degradation is the covalent attachment of ubiquitin to lysine residues of target proteins followed by polyubiquitin chains attached covalently to the proteins. A polyubiquitin chain of a target protein is responsible for an ATP-dependent, extra-lysosomal proteolytic pathway, MG-132 and 19-nor-lactone, dramatically increased the steady state levels of native ERα and ERβ proteins in nuclear extracts obtained from Ishikawa cells. Second, overexpression of wild-type SUG1 in Ishikawa cells resulted in the appearance of proteolytic derivatives of both ERs in the presence of estradiol. ER-SUG1 interaction was also required for the formation of these proteolytic fragments, because overexpression of mutant SUG1 did not produce a similar effect. Moreover, the ubiquitinated proteins of both ERs were observed in the presence of overexpressed wild-type SUG1 in estradiol-treated cells. Furthermore, MG132 abolished the formation of proteolytic fragments of both ERs. Taken together,
these results suggested that SUG1 interacted with both ERs and targeted these ERs for degradation by proteasome machinery through ubiquitination.

Deletion analysis of SUG1 demonstrated that mutant SUG1 (which abolished ATPase activity) as well as the N- or C-terminal domain of SUG1 acted as a dominant-negative in the proteasome-dependent degradation of transcription factor Sp1 (27). It has also been determined that the coiled coils in the N-terminal region of proteasomal ATPases including SUG1 direct the placement of these proteins within the proteasome (28). In our experiments, mutant SUG1, which eliminated ATPase activity, abolished the interaction between SUG1 and ERs and exhibited a dominant-negative effect on the ubiquitination and proteasome-mediated degradation of ERs. This suggested that mutant SUG1 might inhibit the recruitment of wild-type SUG1 to proteasome by occupying the available docking sites. Further analysis will be required to resolve in detail the question of how SUG1 participates in ubiquitination and targeting to proteasome machinery.

A variety of putative pathways by which EDCs affect the endocrine system have been reported (16, 17). We have already presented one such potential pathway, the PXR-mediated changes of steroidogenesis (11). We have also demonstrated the existence of a general mechanism for receptor down-regulation that may involve proteasome-mediated proteolysis with the interaction between PXR and SUG1 (15). In the present experiments, we demonstrated that SUG1 interacted differently with ERs than with ERβ in the presence of EDC, especially BSA. BSA had a positive effect on the interaction between ER and SUG1, but did not affect the interaction with ERβ. And, in the presence of BSA, the degradation of ERβ was much slower than that in the presence of estradiol or phthalate, another EDC. In addition, the formation of proteolytic fragments and the ubiquitination of ERβ by overexpressed SUG1 were not observed in the presence of BSA. This suggested that BSA blocked the turnover of ERβ by inhibiting the ubiquitin/proteasome-mediated degradation of ERβ, resulting in changes in ERβ protein levels in a turn may have affected ERβ-mediated gene regulation. If so, this chain of events may be a potential mechanism by which EDCs affect endocrine function.

In summary, we examined whether or not the interactions between SUG1 and ERs were involved in the ubiquitin/proteasome-mediated degradation. Both estrogen receptors α and β interacted with SUG1 in an estradiol-dependent manner, and the protein levels of both ERs were markedly increased in the presence of proteasome inhibitors. Functionally, the expression of SUG1 inhibited both ERα- and ERβ-mediated transcription in the presence of ligands. The transient expression studies demonstrated that overexpression of wild-type SUG1 generated proteolytic fragments of both ERs, and these products were blocked by a proteasome inhibitor. We also found that overexpression of SUG1 enhanced the formation of ubiquitinated proteins of both ERs in the presence of ligands. On the other hand, BSA, which activated ER-mediated transcription, did not enhance the interaction between ERβ and SUG1. In the presence of BSA, ERβ was degraded much more slowly than it is in the presence of estradiol and phthalate, another EDC. Also, BSA had no effect on the formation of proteolytic fragments of ERβ or on ubiquitination of ERβ in the presence of overexpressed wild-type SUG1. These findings indicate that the interactions between the ERs and SUG1 may be involved in the ubiquitin/proteasome-mediated degradation of both ER proteins. Moreover, compared with estradiol, BSA strongly blocked the ubiquitination and degradation of ERβ, suggesting that BSA may affect the ERβ-mediated transcription of target genes by inhibiting ERβ degradation.

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