Sumoylation of CCAAT/Enhancer-binding Protein α and Its Functional Roles in Hepatocyte Differentiation*

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The sumoylation of CCAAT/enhancer-binding proteins (C/EBPs) by small ubiquitin-related modifier-1 (SUMO-1) has been reported recently. In this study, we investigated the functional role of the sumoylation of C/EBPα in the differentiation of hepatocytes. The amount of sumoylated C/EBPα gradually decreased during the differentiation, which suggests that the sumoylation is important for the control of growth/differentiation especially in the fetal liver. To analyze the function of the sumoylation of C/EBPα in liver-specific gene expression, we studied its effects on the expression of the albumin gene. The C/EBPα-mediated transactivation of the albumin gene was reduced by sumoylation of C/EBPα in primary fetal hepatocytes. The enhancement of C/EBPα-mediated transactivation by BRG1, a core subunit of the SWI/SNF chromatin remodeling complex, was hampered by sumoylation in a luciferase reporter assay. In addition, we discovered that sumoylation of C/EBPα blocked its inhibitory effect on cell proliferation by leading to the disruption of a proliferation-inhibitory complex because of a failure of the sumoylated C/EBPα to interact with BRG1. BRG1 was recruited to the dihydrofolate reductase promoter in nonproliferating C33a cells but was not detected in proliferating cells where C/EBPα, BRG1, and SUMO-1 were overexpressed. This result suggests that BRG1 down-regulates the expression of the dihydrofolate reductase gene. These findings provide the insight that SUMO acts as a space regulator, which affects protein-protein interactions.

The post-translational modification of proteins by small ubiquitin-related modifiers (SUMOs)3 is an important regulatory mechanism that impinges on many cellular processes (1–4) because of the ability of SUMOs to cause rapid changes in the function and distribution of pre-existing proteins, subcellular structures, and multiprotein complexes (3). For example, the attachment of SUMO-1 to promyelocytic leukemia protein, Sp100, and Daxx is involved in the formation of nuclear subdomains such as the promyelocytic leukemia protein oncogenic domain (5). Moreover, the modification of SUMO-1 prevents the degradation of IκBα by competing with ubiquitination (6).

SUMO is a member of a family of ubiquitin-like proteins that can be covalently attached to a large number of proteins. The pathway of sumoylation resembles that of ubiquitination, although the conjugation of SUMO involves a different set of enzymes. SUMO is synthesized as a precursor protein and is processed at the C terminus by a class of cysteine proteases (7). Subsequently, the conjugation of SUMO to proteins involves the ATP-dependent heterodimeric SUMO-activating E1 enzyme (Aos1/Uba2). Once activated, SUMO is transferred to Ubc9, the E2-conjugating enzyme for SUMO, and attached to the ε-amino group of a specific lysine residue of a target protein that contains the consensus sequence ψKXE (ψ, large hydrophobic residue) recognized by Ubc9 (8). Recently, SUMO E3 ligases have been identified in yeast and mammalian cells. An E3 ligase enhances transfer of the SUMO molecule from E2 to a specific substrate, although E3 ligase is not required for sumoylation in vitro (8). To date, three unrelated proteins have been identified as SUMO E3 ligases: RanBP2, PIAS (protein inhibitor of activated STAT proteins), and the polycomb group protein Pc2 (4). Subramanian et al. (9) reported that PIASy acts as an E3 ligase to enhance the modification of SUMO by a transcription factor, CCAAT/enhancer-binding protein α (C/EBPα).

Six members of the C/EBP family (C/EBPα to C/EBPγ) have been isolated and characterized to date (10). They all have a highly conserved basic leucine zipper dimerization domain and a DNA-binding domain at the C terminus. Their divergent N-terminal regions contain the transcriptional regulatory domains and specify their activities (3, 11). C/EBPα is a central regulator of energy homeostasis as it directly activates the transcription of many metabolically important genes (12) and also plays central roles in the growth and differentiation of hepatocytes (13) and adipocytes (14, 15).

C/EBPα was reported recently to be sumoylated at the lysine residue of a consensus sequence called synergy control motif or regulatory domain motif. The motif resides in a region that causes an inhibitory effect on its transcriptional activity (9, 11). Subramanian et al. (9) reported that the synergetic response resulting from the recruitment of C/EBPα to multiple
roles of the binding sites was observed only when the synergy control motif was disrupted and that the motif plays important roles in inhibiting the synergistic activation. On the other hand, Kim et al. (11) reported that the regulatory domain motif corresponding to the synergy control motif had a negative regulatory effect on transcription and that the disruption of the domain motif caused release from the repression. Although they speculated that sumoylation of the consensus motif can affect the inhibitory function by influencing protein-protein interactions, the molecular mechanism by which sumoylation regulates the activity of the transcription factor is poorly understood.

In this study, we investigated the level and functional roles of sumoylated C/EBPα during the differentiation of hepatocytes. Furthermore, we showed that the BRG1-binding site of C/EBPα was masked by SUMO-1, and sumoylation dramatically decreased the stimulation of C/EBPα-mediated transcription of the liver-specific albumin gene by BRG1, the core subunit of an ATP-dependent chromatin remodeling complex (16, 17). This result indicates that modification with SUMO can change the activity of the transcription factor by affecting protein-protein interactions.

In addition, C/EBPα is a strong inhibitor of cell proliferation. It is reported that the molecular mechanisms of the C/EBPα-mediated proliferation arrest depend on several pathways (18–22). The action of C/EBPα fails into the following two categories: direct or p21-dependent inhibition of Cdk activity (nontranscriptional process) and repression of S-phase genes (transcriptional process). Müller et al. (21) recently demonstrated that a functional SWI/SNF complex is required for C/EBPα-mediated proliferation arrest. Here we also report that sumoylated C/EBPα failed to induce proliferation arrest because its interaction with BRG1 was inhibited.

EXPERIMENTAL PROCEDURES

Isolation and Culture of Rat Hepatocytes—Adult rat hepatocytes were obtained from male Sprague-Dawley rats (6–7 weeks old; Nihon SLC) by collagenase perfusion, and fetal hepatocytes were isolated from fetuses (embryonic days 13–15, 17 and 18) by collagenase perfusion, and fetal hepatocytes were cultured to semiconfluence and transfected with siRNA (100 pmol/well in a 24-well plate) by using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s instructions using a luminometer (Luminescencer JNR II; Atto).

Inhibition of PIASy Expression by siRNA—For the knockdown of PIASy, siRNAs (siPIASy-1, 5′-AACCTTACCCACCTCATGTATCT-3′, and siPIASy-2, 5′-ACCTACAAAATGAGAGAAGGAG-3′) were synthesized. Cells cultured to semiconfluence were transfected with siRNA (100 pmol/well in a 24-well plate) by using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s instructions. Cells were washed twice with phosphate-buffered saline 6 h post-transfection and grown in fresh medium for an additional 42 h.

RT-PCR Analysis—Total RNA was prepared from rat hepatocytes and C33a cells using an EASYPrep RNA kit (Takara), and 1 μg of total RNA was then reverse-transcribed with a reverse transcriptase (ReverTra Ace®; Toyoobo) to generate cDNAs. The detection of albumin, GAPDH, PIASy, human dehydrofolate reductase (DHFR), and human proliferating cell nuclear antigen (PCNA) cDNAs was performed by PCR using the following as primers: 5′-GCCATTTCTCAAGACAGT-3′ and 3′-TTTCTTCTGCTGTCGTC-3′.
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CGTGA-3' and 5'-CGGGGTTTGGGAATCCATA-CG-3' for rat albumin; 5'-ACCACAGTCCATGCACTCAGC-3' and 5'-TCCACACCTGTGACTGTA-3' for GAPDH; 5'-ATGAGACTCGTATGTCCAA-3' and 5'-GCCCGGCAGTGCAGC-3' for PIASy; 5'-ATGAGACTCGTATGTCCAA-3' and 5'-GCCCGGCAGTGCAGC-3' for human PCNA; and 5'-AGGTTGGAGGCACCTC-3' and 5'-CATTTCCAAGTTTCTCCAC-3' for human PCNA. Albumin, GAPDH, PIASy, human DHFR, and human PCNA were amplified for 16, 24, 22, 28, and 28 cycles, respectively. These PCRs did not result in saturation. The human PCNA were amplified for 16, 24, 22, 28, and 28 cycles, respectively. These PCRs did not result in saturation. The GAPDH gene served as an internal control in the calculation of the densitometric results.

Immunoprecipitation and GST Pulldown Assay—Immunoprecipitation and the GST pulldown assay were performed as described previously (25). The GST-C/EBPα TEI-III, sumoylated GST-C/EBPα TEI-III, or GST-SUMO-1 fusion protein was expressed in *Escherichia coli* BL21 (DE3) and purified using a glutathione-Sepharose column (Amersham Biosciences). Sumoylated C/EBPα was obtained by introducing both pGEX-C/EBPα TEI-III and pTS1 into *E. coli* BL21.

ChIP Assay and Re-precipitation (Re-ChIP)—Immunoprecipitation of formaldehyde-cross-linked chromatin from the transfected SW13, C33a cells, and primary hepatocytes was performed using the ChIP assay kit (Upstate Biotechnology, Inc.) according to the manufacturer's instructions with some modifications (27, 28). The immunoprecipitated DNAs and input DNA were analyzed by PCR using the following as primers: 5'-CATAACTTATGAAATTGACAAAGC-3' and 5'-GAGAAACTCGCTCTAATATACTTCTC-3' for the rat albumin promoter; 5'-TTTTGGCAAGGATGCATGC-3' and GLprimer2 (Promega) for the promoter region of the luciferase reporter plasmid (albumin promoter); 5'-GCAGACTGTCGAGAGACGTGGG-3' and 5'-CTGCAGCCGGAGGATGAGC-3' for the rat DHFR promoter; and 5'-AGGTTGGCCGGAGAGGATGAGC-3' and 5'-AGGTTGGCCGGAGGATGAGC-3' for the human DHFR promoter. For negative controls, the following were used: 5'-TGTGTGTTTCAAGGC-

TACCCTGA-3' and 5'-GATGAAGGGAGAACCAG-3' for the rat albumin coding region; 5'-CTGGGGAATCAGCAGC-3' and 5'-GATACACCTCTCAGTTATTTT-3' for the rat DHFR-coding region; and 5'-TAAAGGCTGAAAAGGCAGC-3' and 5'-CCCAGCGCTGAAATTATCT-3' for the human DHFR-coding region.

Proliferation Assay—The transfected cells were seeded at a density of 2×10^4 cells per well. Every 24 h, cells were harvested, and the number of living cells was determined by the trypan blue dye exclusion method.

Two-dimensional Gel-Western Blotting—The cell lysates were separated by two-dimensional gel electrophoresis with immobiline IEF (Amersham Biosciences) as described previously (29), and proteins were transferred to the membrane and detected with anti-C/EBPα rabbit antibody.

RESULTS

Sumoylated C/EBPα Levels Decrease during Hepatocyte Differentiation—Primary fetal and adult hepatocytes were used to study the sumoylation of C/EBPα because C/EBPα is known to play important roles in the liver development (13). As shown
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in Fig. 1A, a shifted band attributable to modified C/EBPα was detected at an early stage in the differentiation of hepatocytes (E13–15), and the intensity of the band decreased in the late stages of the differentiation process. The shifted band seemed to be sumoylated C/EBPα, because the mobility of the band detected in SW13 cells cotransfected with the C/EBPα and SUMO-1 expression vectors was consistent with that of the putative sumoylated C/EBPα in the primary hepatocytes. We then performed an immunoprecipitation assay to confirm whether this shifted band was indeed SUMO-1-modified C/EBPα. As shown in Fig. 1B, the putative sumoylated C/EBPα precipitated with the anti-C/EBPα antibody reacted with the anti-SUMO-1 antibody. We therefore concluded that the shifted C/EBPα band was generated by sumoylation.

Modification of C/EBPα by SUMO-1 Has a Repressive Effect on the Transactivation of the Albumin Promoter—As indicated above, we demonstrated that the C/EBPα protein was sumoylated in vivo. Thus, a question is raised regarding the function of the sumoylated C/EBPα protein. It is well recognized that the liver-specific expression of several genes such as those for albumin, α-fetoprotein, and tyrosine aminotransferase at least partly depends on C/EBPα (30–32). Because the level of sumoylated C/EBPα protein present in primary hepatocytes decreased during differentiation, we hypothesized that liver-specific gene expression is controlled partly by the sumoylation of C/EBPα. The expression of the albumin gene increased during hepatocyte differentiation (Fig. 2A), and the expression showed a pattern roughly opposite that of sumoylated C/EBPα protein. Therefore, we analyzed the effect of sumoylation of C/EBPα on albumin gene expression by conducting luciferase reporter assays in 293 cells that do not contain endogenous C/EBPα, with the luciferase vector and the expression vectors for C/EBPα and SUMO-1. C/EBPα-mediated expression from the albumin promoter was repressed by SUMO-1 (Fig. 2B). The transcription from the albumin promoter was not affected by the expression of SUMO-1 alone in the absence of C/EBPα.

The expression of albumin is facilitated by both C/EBPα and hepatocyte nuclear factor-1 (HNF-1), and these transcription factors have additive effects on the expression (23). Therefore, to confirm that the modification of C/EBPα by SUMO-1 directly affects C/EBPα-mediated transcription, lysine 159 at the SUMO attachment site was replaced with either alanine or arginine. As can be seen in Fig. 2B, these mutations relieved the repression. Thus, it seems likely that SUMO-1 expression directly repressed C/EBPα-mediated transactivation of the albumin gene, and we can rule out the possibility that other transcription factors such as HNF-1 are involved in the SUMO-1-mediated repression of the albumin gene.

Attachment of SUMO-1 to C/EBPα Represses Liver-specific Albumin Expression in Primary Hepatocytes—To show that the sumoylation represses the transactivation by C/EBPα in primary hepatocytes, the C/EBPα-mediated expression of the albumin gene was monitored by RT-PCR in primary fetal hepatocytes transfected with expression vectors for SUMO-1, E3 ligase PIASy (9), E1 enzyme Aos1/Uba2, and E2 enzyme Ubc9 (8). The simultaneous transfection of SUMO-1 and E1–E3 ligases reduced the expression of the albumin gene in fetal hepatocytes (Fig. 3A, lanes 3–5). A detailed analysis of the expression of albumin following the transfection of several combinations of SUMO E1–E3 ligases showed that exogenously expressed E1 and E2 enzymes had little effect on albumin expression without PIASy (supplemental Fig. S1, A and B). On the other hand, exogenously expressed PIASy alone decreased the albumin expression to 70% of the control level, suggesting that the E3 ligase may be a key for sumoylation in fetal liver, although E1–E3 ligases act cooperatively for sufficient sumoylation. In addition to the overexpression of SUMO-1 and its ligases, we also performed siRNA experiments for blocking the expression of PIASy. With siRNA for PIASy (siPIASy-1), albumin expression...
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FIGURE 4. Sumoylation of C/EBPα dramatically decreases the cooperation between BRG1 and C/EBPα in albumin expression. A, schematic representation of the domain structure of C/EBPα protein. The transactivation elements (TE) I, II, and III, basic leucine zipper DNA-binding domain (bZip), SUMO-1-conjugation site, and BRG1 (BRM)-binding site are indicated. B, ChIP assays were performed using anti-BRG1 antibody with SW13 cells that were transfected with 0.3 μg of pREP4-albumin promoter luciferase reporter plasmid and various combinations of expression vectors. The albumin promoter was detected by PCR amplification. C, sumoylated C/EBPα does not interact with BRG1. 293 cells were cotransfected with C/EBPα and SUMO-1 or with C/EBPα alone, and immunoprecipitation (IP) was performed with anti-C/EBPα antibody followed by Western blotting using anti-BRG1 antibody. D, production of GST-fused proteins and sumoylation in E. coli. Total lysates from E. coli cells harboring pGEX-C/EBPα TE-I alone (lane 1), or plus pTS-1 (lane 2) and pGEX-SUMO-1 (lane 3) were incubated with glutathione-Sepharose 4B beads, and the bound proteins were analyzed by SDS-PAGE followed by Coomassie Blue staining. E, GST pulldown assay using purified BRG1 protein. First, 3 μg of purified HSV-tagged BRG1 helicase domain in 500 μl of binding buffer and glutathione-Sepharose 4B beads containing GST-fused proteins were incubated. The beads were then washed, and absorbed proteins were analyzed by Western blotting with anti-BRG1 antibody. F, pulldown assay with BRG1 as the bait. His-tagged BRG1 (amino acids 719–1308) was attached to TALON beads (Clontech). GST-sumoylated C/EBPα TE-I protein (10 μg) in 500 μl of PBS, and TALON beads containing purified His-tagged-BRG1 were incubated. The beads were then washed and spun down. Proteins absorbed to the beads were analyzed by SDS-PAGE followed by Coomassie Blue staining. Asterisk, GST-sumoylated C/EBPα TE-I protein. Arrowhead, GST-C/EBPα TE-I protein. Ppt, precipitated resin fraction. G, luciferase reporter assay shows that sumoylation of C/EBPα hampers activation of albumin gene expression by BRG1. SW13 cells were transfected with the pREP4-albumin promoter luciferase reporter plasmid together with pG3-ER-fenilla and various combinations of expression plasmids. Lysates of luciferase samples were analyzed by Western blotting with anti-C/EBPα and anti-BRG1 antibodies. Asterisk, sumoylated C/EBPα. P.C., the extract of SW13 cells overexpressing both C/EBPα and SUMO-1 as a positive control. H, C/EBPα mutants interact with BRG1. Lysates from C33a cells overexpressing BRG1, and C/EBPα and its mutants were immunoprecipitated with anti-C/EBPα antibody and analyzed by Western blotting using anti-BRG1 antibody.

increased 2-fold compared with that in control E14-hepatocytes (Fig. 3A, compare lanes 1 and 2). To exclude the off-target effect of siRNA, another siPIASy was designed (siPIASy-2). RT-PCR assay showed that this siPIASy also increased the expression of albumin (supplemental Fig. S1C). Because some C/EBPα was sumoylated in E13–15 hepatocytes, it is possible that the decrease in sumoylation of C/EBPα caused the increase in the expression of albumin.

Furthermore, SUMO-1GA, a dominant negative form of SUMO-1, was also tested in the presence of PIASy. Repression of the albumin gene was not observed in the primary fetal hepatocytes cotransfected with the SUMO-1GA and PIASy expression vectors. Furthermore, the level of albumin was higher than that in the nontransfected control (Fig. 3A, lanes 3 and 6). This result indicated that PIASy alone did not directly repress the transcription, although it was recently reported that PIASy represses androgen receptor target genes independent of sumoylation (33). Moreover, the above results suggest that the sumoylation of unidentified factors that affect albumin expression as well as the endogenous sumoylation of C/EBPα in fetal hepatocytes may be inhibited because PIASy has relatively broad substrate specificity for sumoylation (34–37), and SUMO-1GA can affect all cellular sumoylation.

To confirm that sumoylation of C/EBPα is indeed important, C/EBPα expression was blocked by siRNA. siRNA for C/EBPα reduced albumin expression in fetal hepatocytes (supplemental Fig. S2). Under conditions where C/EBPα expression was reduced by the siRNA, SUMO-1 and SUMO E1–3 ligases had no effect on albumin expression as well as the endogenous sumoylation of C/EBPα, suggesting that the sumoylation of unknown factors that affect albumin expression as well as the endogenous sumoylation of C/EBPα in fetal hepatocytes may be inhibited because PIASy has relatively broad substrate specificity for sumoylation (34–37), and SUMO-1GA can affect all cellular sumoylation.
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The siRNA for PIASy indeed reduced the sumoylation of C/EBPα, and the overexpression of the ligase increased the modification in hepatocytes in vivo as shown in Fig. 3B. This observation indicates the importance of the E3 ligase in the sumoylation. To clarify this point, we transfected the SUMO-1 expression vector alone into primary fetal hepatocytes. Contrary to the experiments performed in Fig. 3A, the transfection of SUMO-1 had no effect on the expression of albumin (Fig. 3C). These results imply that the SUMO E3 ligase is essential for controlling the sumoylation of C/EBPα in hepatocytes. We therefore determined the relative expression levels of PIASy mRNA in hepatocytes by using RT-PCR (Fig. 3D). The expression of PIASy decreased in the late stages of differentiation and became undetectable in adult hepatocytes. This result is clearly consistent with the finding that the amount of sumoylated C/EBPα decreased with the development of the liver as shown in Fig. 1A. In 293, SW13, and C33a cells, considerable levels of PIASy were detected (data not shown).

Modification of C/EBPα by SUMO-1 Decreases the Cooperation between BRG1 and C/EBPα—We tried to elucidate the molecular mechanism by which sumoylated C/EBPα repressed albumin expression. We and other groups have shown that C/EBPα interacts with BRG1, an ATPase subunit of SWI/SNF chromatin remodeling complex (14) and C/EBPα-mediated transactivation of the albumin gene is enhanced by the SWI/SNF complex in hepatocytes (25). Because the binding site for BRG1 in C/EBPα overlaps with the SUMO-1 conjugation site (Fig. 4A), it is possible that the sumoylated C/EBPα protein is unable to bind to BRG1. Thus, the recruitment of BRG1 to the albumin promoter by free and sumoylated C/EBPα was analyzed by conducting a ChIP assay using SW13 cells cotransfected with different combinations of the expression vectors for C/EBPα, SUMO-1, SUMO ligases, and BRG1. SW13 cells are BRG1- and C/EBPα-negative. In the cells transfected with both BRG1 and C/EBPα, BRG1 bound to the albumin promoter through the C/EBPα protein. However, in the cells cotransfected with expression vectors for C/EBPα, SUMO-1, and SUMO ligases, BRG1 did not bind to the promoter (Fig. 4B). Immunoprecipitation assays using the lysates of the 293 cells that were transfected with C/EBPα alone and C/EBPα plus SUMO-1 expression vectors were performed to show that sumoylation of C/EBPα blocks the binding of C/EBPα to BRG1. Because more than half of the C/EBPα was unsumoylated in this experiment, the BRG1 band could be detected with sumoylated C/EBPα, but the decrease in intensity was obvious (Fig. 4C). Thus, we speculated that the sumoylated C/EBPα failed to form this complex. To confirm the result of the immunoprecipitation experiments with a GST pulldown assay, GST-C/EBPα TEI-III or sumoylated GST-C/EBPα TEI-III (Fig. 4D), and the HVS-tagged BRG1 helicase domain were purified. Since we showed previously that C/EBPα binds to the helicase domain of BRG1 (25), the HVS-tagged helicase domain was used in this experiment. It is likely that sumoylated GST-C/EBPα TEI-III could not interact with the helicase domain of BRG1 (Fig. 4E). The weak band in the lane of sumoylated GST-C/EBPα TEI-III was probably caused by the unsumoylated protein. To clarify this point, an additional pulldown assay of GST-sumoylated C/EBPα TEI-III with His-tagged BRG1 (amino acids 714–1308) containing the helicase domain as bait was performed. In this experiment, TALON His tag affinity resin was used to pull down His-tagged BRG1. As shown in Fig. 4F, only unsumoylated C/EBPα was found to bind the His-tagged BRG1 protein. This experiment clearly revealed that BRG1 did not interact with sumoylated C/EBPα. Together with the results of the ChIP assay, the results of the immunoprecipitation and GST and His-tag pulldown experiments suggest that the attachment of SUMO-1 to C/EBPα inhibits the recruitment of BRG1 to the albumin promoter.

We performed a luciferase reporter assay to test the hypothesis that the attachment of SUMO-1 prevents the activity of C/EBPα by inhibiting the recruitment of SWI/SNF. Coexpression of C/EBPα and BRG1 proteins resulted in an enhancement of C/EBPα-mediated transcription of 7-fold in SW13 cells. In contrast, the cotransfection of C/EBPα with both BRG1 and SUMO-1 expression vectors significantly decreased the transactivation by C/EBPα (Fig. 4G). As judged by Western blotting, about 30% of C/EBPα was sumoylated under these conditions. Contrary to wild-type C/EBPα, mutant C/EBPα relieved the repression. These findings clearly indicate that the sumoylation of C/EBPα reduced its transactivation by inhibiting the cooperation with SWI/SNF. We also confirmed that C/EBPα mutants interacted with BRG1 (Fig. 4H).

Sumoylated Proteins Occupy the Albumin Promoter of Fetal Hepatocytes—To determine whether the sumoylated proteins can associate with the albumin promoter, we performed a ChIP assay using an antibody against SUMO-1 with fetal and adult hepatocytes. As shown in Fig. 5A, sumoylated proteins were found to be present at the albumin promoter of fetal hepa-
cytes but not at the adult hepatocytes. This result was consistent with the expression pattern of sumoylated C/EBPα in the developing liver. The sumoylated C/EBPα protein probably still has DNA binding activity, considering that the DNA-binding domain is located at the C terminus of the protein. To assess whether the sumoylated C/EBPα protein binds to the albumin promoter, re-ChIP was performed with serial precipitation using anti-C/EBPα and anti-SUMO-1 antibodies. The result shown in Fig. 5 revealed that C/EBPα and SUMO-1 were recruited to the albumin promoter in fetal hepatocytes. Although the possibility that other sumoylated proteins bind to C/EBPα on the albumin promoter cannot be excluded, it is possible that sumoylated C/EBPα exists on the albumin promoter in fetal hepatocytes.

**Sumoylation of C/EBPα Leads to the Disruption of the Proliferation-Inhibitory Complex**—It has been demonstrated by several groups that C/EBPα induces an inhibition of cell proliferation (18–22). To examine the effect of sumoylation of C/EBPα on cell proliferation, first the thymidine incorporation was measured with the 293 cells transfected with the C/EBPα expression vector alone. The transfection efficiency of the plasmid was more than 80% with the GFP expression vector under the same conditions. C/EBPα seemed to partly inhibit cell proliferation, but the cells transfected with both the C/EBPα and SUMO-1 expression vectors were released from the inhibition (Fig. 6A). A similar result was obtained by directly counting the cells (Fig. 6B). These results suggested that sumoylation blocks the C/EBPα-mediated inhibition of cell proliferation. To address the molecular mechanism of this phenomenon, we analyzed the effects of BRG1 on C/EBPα-mediated proliferation arrest, because a recent study demonstrated that C/EBPα required an SWI/SNF complex for proliferation arrest (21). The BRG1/BRM-defective
C33a cells did not show C/EBPα/Hi9251-induced proliferation arrest, and the sumoylation of C/EBPα/Hi9251 did not influence the cell proliferation as shown in Fig. 6C (upper panel). In contrast, the overexpression of BRG1 rendered these cells sensitive to C/EBPα-mediated arrest as a consequence of the reconstitution of a functional SWI/SNF complex. Moreover, upon the sumoylation of C/EBPα, the proliferative activity was restored to the control level (Fig. 6C, lower panel). Furthermore, the cells

FIGURE 7. Sumoylation of C/EBPα regulates recruitment of BRG1 to the DHFR promoter. A, repression of the DHFR promoter by C/EBPα and BRG1 is relieved by sumoylation. RT-PCR assays were carried out on RNAs extracted from C33a cells transfected with various combinations of expression vectors (0.3 μg of C/EBPα, 0.4 μg of BRG1, and 0.6 μg of SUMO-1) 48 h post-transfection. B, ATPase activity of BRG1 is required for the repression of the DHFR promoter. RT-PCR assays were carried out on RNAs extracted from C33a cells transfected with combinations of expression vectors (0.3 μg of C/EBPα and 0.4 μg of BRG1 or mutBRG1) 48 h post-transfection. The cell lysates were analyzed by immunoblotting with anti-BRG1 and anti-C/EBPα antibodies. C, ATPase activity of BRG1 is required for C/EBPα-dependent proliferation arrest. C33a cells were transfected with C/EBPα, and BRG1 or mutBRG1 expression vectors. Cells were directly counted under a microscope. The data shown were acquired from at least three independent experiments and represent the mean ± S.E. D and E, ChIP assays were performed with C33a cells overexpressing C/EBPα and BRG1 with or without SUMO-1 (D) and fetal hepatocytes (E). IP, immunoprecipitation. The DHFR promoter and coding region were detected by PCR amplification. F, fetal hepatocytes express Ser-193-phosphorylated C/EBPα. C/EBPα from E14 and E17 fetal hepatocytes was analyzed by two-dimensional gel-Western blotting. Positions of Ser-193-phosphorylated isoforms (spots a and b) are shown with arrowheads (22, 40).
transfected with Lys-159 mutants did not resume proliferating when SUMO-1 was expressed. We confirmed these results using another BRG1/BRM-defective cell line, SW13, by performing direct cell counting (Fig. 6D) and a thymidine incorporation assay (data not shown). We also confirmed that BRM, another core subunit of the SWI/SNF complex, caused C/EBPα-mediated proliferation arrest with C33a cells and that the arrest was released in the presence of SUMO-1 by conducting the thymidine incorporation assay (Fig. 6E). The results of the proliferation assay of the transfected C33a cells raised the possibility that C/EBPα cooperates with the SWI/SNF complex to arrest the proliferation of cells, and the modification of C/EBPα by SUMO-1 inhibits the recruitment of SWI/SNF, which is indispensable for forming the functional complex for proliferation arrest. To test this, we further performed an immunoprecipitation assay using the lysate of C33a cells transfected with C/EBPα, BRG1, and SUMO-1. BRG1 was precipitated with unsumoylated C/EBPα, whereas a smaller amount of BRG1 was precipitated with the sumoylated C/EBPα sample (Fig. 6F). Together, these results indicate that sumoylation hampers C/EBPα-mediated proliferation arrest by preventing the recruitment of SWI/SNF.

**Sumoylation of C/EBPα Regulates the Recruitment of BRG1 to the DHFR Promoter**—To analyze the molecular basis of C/EBPα-mediated proliferation arrest and its control by sumoylation, we first analyzed the repression of typical cell cycle regulator genes by C/EBPα as it was proposed that C/EBPα physically associates with E2F and hampers E2F-dependent transcription (38, 39). RT-PCR analysis showed that the expression of DHFR but not PCNA was inhibited by C/EBPα with the cooperation of BRG1, and the repression was released by the sumoylation of C/EBPα (Fig. 7A). This is consistent with the recent observation that a class of E2F-dependent promoters of S-phase genes, including the DHFR, E2F-1, and cyclin A genes, contain a C/EBPα-binding site but other E2F-dependent promoters do not (22). We then studied whether a functional BRG1 is essential for the repression of DHFR. RT-PCR analysis was performed with a mutant BRG1 (mutBRG1) that lacks ATPase activity. As shown in Fig. 7B, wild-type BRG1 down-regulated the gene expression from the DHFR promoter, but mutBRG1 did not affect the promoter activity indicating that the ATPase activity and possibly the remodeling activity are essential for the suppression. Consistent with the RT-PCR analysis, mutBRG1 did not hamper the proliferation of C33a cells following a transient transfection (Fig. 7C). We then investigated the binding of BRG1 to the DHFR promoter in vivo using a ChIP assay. As shown in Fig. 7D, BRG1 was recruited to the DHFR promoter with C/EBPα and E2Fs in the nonproliferating C33a cells. However, in the proliferating cells in which C/EBPα, BRG1, and SUMO-1 were over-expressed, BRG1 was not detected at the promoter site.

We also studied the formation of a proliferation-inhibitory complex on the DHFR promoter of fetal hepatocytes from E14 and E18 rats by ChIP assay. In our experience, E14 hepatocytes have the potential to grow in culture, but E18 cells have all but lost this ability. As can be seen in Fig. 7E, although C/EBPα was also present on the DHFR promoter in these hepatocytes, the binding of SUMO-1 and BRG1 was dramatically different between E14 and E18 hepatocytes; BRG1 was not found at the DHFR promoter in E14 proliferating hepatocytes but could be detected in E18 hepatocytes. Furthermore, anti-SUMO-1 antibody precipitated with the promoter region in E14 but not E18 hepatocytes. To date, a sumoylated protein bound to the promoter in E14 hepatocytes has not been identified, but it is possible that sumoylated C/EBPα bound to the promoter. These results were consistent with the finding that the sumoylation of C/EBPα disrupted the proliferation-inhibitory complex.

Recently, it was reported that the C/EBPα-mediated arrest of cell proliferation is regulated by the dephosphorylation of C/EBPα at Ser-193, which blocks interaction with Cdk2 and BRM in hepatocytes after a partial hepatectomy (22, 40). To analyze the phosphorylation of C/EBPα in fetal hepatocytes, we performed two-dimensional gel-Western blotting. As shown in Fig. 7F, the spots caused by the phosphorylation of Ser-193 appeared in both E14 and E17 hepatocytes (spots a and b) as reported by Wang et al. (22, 40). This suggests that C/EBPα was phosphorylated, and the binding of BRG1 to C/EBPα is mainly controlled by sumoylation but not phosphorylation during hepatic development. Therefore, it is suggested that the sumoylation of C/EBPα might act as a switch from growth arrest to cell proliferation.

**DISCUSSION**

We demonstrated that the level of sumoylated C/EBPα decreased during liver development, and inversely, the level of albumin increased. These findings suggest that the sumoylated C/EBPα repressed the expression of the albumin gene. We discovered that the attachment of SUMO-1 to C/EBPα inhibited the recruitment of the SWI/SNF complex that enhanced C/EBPα-mediated transactivation of the albumin gene in hepatocytes. This finding suggests that SUMO-1 acts as a spatial regulator and that sumoylation affects protein-protein interactions. Our observations are in general consistent with recently published results on the regulation of the human TfuD complex assembly on the promoter via sumoylation (41). Furthermore, Ledl et al. (42) showed that the sumoylation of Rb is inhibited by viral oncoproteins, and cellular inhibitors of Rb function by binding to the Rb pocket domains. Sumoylation regulates the activity of proteins by altering certain protein-protein interactions. It is therefore possible that the sumoylation of a transcription factor inhibits the recruitment of other protein factors and hampers the formation of a pre-initiation complex at a certain promoter. This model allows for SUMO-induced changes in the activity of transcription factors without novel regulators.

In several transcription factors, a synergetic control region has been identified, and putative synergetic control factors have been proposed (9, 43, 44). Recently, SUMO was found to take part in the control of transcriptional synergy (45, 46). The attachment of SUMO-1 to C/EBPα prevents the recruitment of the SWI/SNF complex to the promoter. In addition, it is possible that sumoylated C/EBPα recruits a repressor complex such as a histone deacetylase (HDAC). Indeed, there have been several reports of the SUMO-mediated recruitment of HDAC. Girdwood et al. (47) demonstrated that the transcriptional coactivator p300 has two tandem SUMO modification sites
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within the cell cycle regulatory domain 1 (CRD1), and SUMO-dependent transcriptional repression of p300 is mediated by the recruitment of HDAC6 to CRD1. The histone H4 was recently reported to be modified by SUMO-1 and SUMO-3, and sumoylated H4 mediates gene silencing through the recruitment of heterochromatin protein 1 and HDAC1 (48). Furthermore, the ETS domain transcription factor Elk-1 is negatively regulated by sumoylation, and the modification of Elk-1 by SUMO-1 results in the recruitment of HDAC2 to promoters. Sumoylation of Elk-1 is reversed by ERK-MAPK (extracellular signal-regulated kinase-mitogen-activated protein kinase) (49).

Although HDAC2 does not seem to be involved in the activity of sumoylated C/EBPα (49), it is still possible that the sumoylated C/EBPα recruits other HDACs. In fact, we currently observed that the HDAC inhibitor tricostatin A derepressed the sumoylated C/EBPα-dependent down-regulation of albumin expression (data not shown). Therefore, we cannot rule out the possibility that the down-regulation of albumin gene expression by sumoylated C/EBPα was an integral effect of HDACs and the removal of BRG1. To clarify this point, further studies are required.

Our results show that levels of sumoylated C/EBPα were low in developing hepatocytes as reported for other sumoylated proteins (43, 50–52). Furthermore, in almost all of our experiments, the biological effects of sumoylation were profound, although the level of sumoylated C/EBPα was less than half of the total level of C/EBPα. The sumoylation of proteins is considered to be transient (53). Therefore, sumoylation is a rapid and reversible process, and target proteins are conjugated and deconjugated over short periods of time, which is nevertheless sufficient to affect their biological activities. In fact, sumoylation of the glucocorticoid receptor (43) and sterol regulatory element-binding protein (50) has profound effects on transcriptional activity, although the levels of the sumoylated transcription factors in cells are very low.

C/EBPα also shows a major inhibitory effect on cell proliferation by using several mechanisms involving cooperation with the SWI/SNF complex, Cdks, or p21 depending on the cell type and experimental systems (reviewed in Ref. 54), and it is possible that more than one mechanism works under physiological conditions. With C33a and SW13 cells, Müller et al. (21) reported that the C/EBPα-BRM complex seems to be responsible for proliferation arrest. Our observation is clearly consistent with these results. In addition to BRM/BRG1, Cdks and p21 are known to associate with the TEFII region of C/EBPα (18, 19).

It is accepted that the binding of p21 to C/EBPα increases its stability and activity (19, 55). The Cdk2, Cdk4, and p21-binding sites on C/EBPα are amino acids 119–160 and 175–188, 175–188, and 119–226, respectively. These findings suggest that the sumoylation of C/EBPα hampers the binding of these factors. As far as we have found, the sumoylation of C/EBPα blocked the binding of p21 and Cdk4.4 Thus, it is reasonably assumed that sumoylation has the potential to inhibit the formation of other types of inhibitory complexes.

Our experiments showed that the overexpression of C/EBPα

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