Transcriptional Activity of CCAAT/Enhancer-binding Proteins Is Controlled by a Conserved Inhibitory Domain That Is a Target for Sumoylation*

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CCAAT/enhancer-binding proteins (C/EBPs) are basic region/leucine zipper transcription factors that function as regulators of cell growth and differentiation in numerous cell types. We previously localized transcriptional activation and inhibitory regions in one family member, C/EBPα. Here we describe the further characterization of a C/EBPα inhibitory domain termed regulatory domain I. We show that functionally related domains are present in C/EBPα, C/EBPβ, and C/EBPδ. These domains contain an evolutionarily conserved five-amino acid motif (the regulatory domain motif (RDM)) that conforms to the consensus sequence (I/V/L)XXE. Mutagenesis studies revealed that the residues at positions 1, 2, and 4 of the RDM are critical for inhibitory domain function. Data base searches identified RDM-like sequences in a number of nuclear proteins. We found that small regions from c-Jun, JunB, and JunD containing this sequence also function as transcriptional inhibitory domains. Importantly, the RDM is similar to the recognition sequence for attachment of the ubiquitin-like protein, small ubiquitin-like modifier-1 (SUMO-1), and the conserved lysine residue of each C/EBP RDM served as an attachment site for SUMO-1. SUMO-1 attachment decreased the inhibitory effect of the C/EBPα regulatory domain, suggesting that sumoylation may play an important role in modulating C/EBPα activity as well as that of the other C/EBP family members.

The CCAAT/enhancer binding proteins (C/EBPs) form a subgroup of the basic region/leucine zipper superfamily of transcription factors (1, 2). Six members of this family have been identified in mammalian cells, and four of these (C/EBPα, C/EBPβ, C/EBPδ, and C/EBPε) possess similar arrangements of functional domains (3). Each protein possesses a bipartite carboxyl-terminal DNA binding domain that mediates homodimerization and sequence-specific binding to a common DNA recognition motif (4, 5). The C/EBP family members have been implicated in the regulation of numerous cell processes, including differentiation, proliferation, and apoptosis. For example, mice bearing a targeted mutation in the C/EBPε gene exhibit immune defects that result from defective neutrophil function (6). The phenotype of C/EBPε-deficient mice resembles that seen in humans with neutrophil-specific granule deficiency, and mutations in the C/EBPε gene have been detected in patients with neutrophil-specific granule deficiency. Likewise, studies of mice carrying mutations in other C/EBP genes have shown that members of this family are critical for the development or function of numerous cell types, including hepatocytes, adipocytes, and macrophages (1, 2).

Although the C/EBPs possess similar DNA binding specificities and dimerization properties, each protein exhibits unique functional properties in vivo. The appearance of specific phenotypes in each C/EBP-deficient mouse supports the contention that these proteins do not have fully redundant functions. Although this observation could be partially explained by nonoverlapping expression patterns of the different proteins, direct evidence for specific functions has come from experiments where the coding sequence for one C/EBP has been replaced with that encoding a different family member. For example, the insertion of the C/EBPβ coding sequence into the C/EBPα locus rescued hepatic-specific defects in mice but could not rescue defects in adipose tissue (8).

Several studies have focused on the identification of functional domains within each C/EBP protein. As mentioned above, four family members (C/EBPα, C/EBPβ, C/EBPδ, and C/EBPε) possess very similar structures. A potent multimodule transcriptional activation domain (AD) is located at the amino terminus of each protein (3, 9, 10), and domains that negatively modulate transcriptional activity have been identified in C/EBPα, C/EBPβ, and C/EBPε (3, 11–13). In C/EBPα, we defined two inhibitory domains (RDI and RDII) and identified a short amino acid motif that was critical for RDI function (14). Interestingly, negative regulatory domains have been identified in a number of other nuclear transcription factors, including c-Fos (15), c-Myb (16, 17), Sp1/3 (18), and members of the nuclear hormone receptor superfamily (19, 20). The inhibitory functions of these various domains within their cognate proteins have been variously attributed to inhibition of DNA binding, transcriptional activation, or synergy with factors bound to adjacent promoter elements (3, 19). However, the relationship between these domains is currently unknown. A common feature of these domains is
that many have been suggested to be binding sites for currently uncharacterized regulatory proteins (14, 19).

Here, we have extended our previous studies on the RDI inhibitory domain of C/EBPα (14). We show that a conserved five-amino acid motif within the inhibitory domains of C/EBPα, C/EBPβ, and C/EBPδ is critical for regulatory domain function. Further, we demonstrate that a lysine residue within this motif is a site for covalent attachment of the small ubiquitin-like modifier-1 (SUMO-1) protein (21) and that attachment of SUMO-1 to this residue decreases the transcriptional inhibitory function of the regulatory domain. We propose that sumoylation of C/EBP proteins is a conserved modification that modulates the activity of each family member.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Expression plasmids for each full-length C/EBP were constructed using the pMEX expression vector (5). The coding sequence for the FLAG epitope tag (Asp-Tyr-Lys-Asp-Asp-Asp-Lys) was inserted immediately following the translation initiation codon, and the coding sequence of each C/EBP was inserted as NeuI/HindIII fragments. In these and all subsequent plasmids, mutations were introduced using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) using conditions recommended by the manufacturer. A restriction enzyme recognition sequence was introduced wherever possible to aid in the detection of plasmids carrying the desired mutation. All constructs were sequenced at the Texas Tech University Biotechnology Core Laboratory to ensure that unintended changes were not introduced during the mutagenesis procedure. Gal4 fusion genes were constructed in the Gal0 plasmid (14). The generation of each of the wild-type Gal4 fusion plasmids bearing N-terminal regions of C/EBPα, C/EBPβ, and C/EBPδ has been described previously (3, 14). Gal4 fusions bearing amino acids 1–92 and 1–142 of C/EBPδ were generated using EagI and BssHII restriction enzyme sites present in the mouse cDNA sequence. The coding regions for individual domains were generated as BamHI/BglII fragments and assembled into Gal4 fusion constructs by standard subcloning procedures. The VP16 AD segment inserted into Gal4 fusions consisted of amino acids 429–456 (14). The FLAG-SUMO-1 expression vector (22) was a kind gift of Dr. Giuseppina Nucifora (University of Illinois at Chicago). A vector expressing a non-processable version of SUMO-1 was generated by site-directed mutagenesis on a Gal4 construct containing amino acids 1–27 of the mouse C/EBPα, C/EBPβ, and C/EBPδ expression plasmid. The total DNA concentration of the appropriate expression plasmid. A control plasmid containing the thymidine kinase promoter, was described previously (26). The anti-Gal4 DNA binding domain (DBD) monoclonal antiserum (R3531) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and the anti-FLAG polyclonal antiserum was obtained from Sigma. The C/EBPα-specific rabbit polyclonal antiserum was raised against a specific epitope in the C-terminal portion of rat C/EBPα.

RESULTS

Identification of an Evolutionarily Conserved Sequence within the C/EBPα RDI—In a previous study, we performed a structure/function analysis of the C/EBPα polypeptide and identified several autonomous domains, including two activation and two inhibitory domains (14). One inhibitory domain, termed RDI, contained several potential sites for phosphorylation by members of the MAP kinase superfamily; thus, RDI activity is probably dependent on phosphorylation of C/EBPα in response to upstream signaling pathways. However, a second inhibitory domain, termed RDII, did not contain obvious signatures for post-translational modifications, and thus the mechanism of action of this domain remained unclear. Initial comparisons of the sequence of RDI with inhibitory domains from other transcription factors revealed weak sequence similarities, suggesting the existence of conserved inhibitory domains within multiple transcriptional regulatory proteins (14). We therefore performed extensive sequence comparisons of the RDI sequence with protein sequences in public databases. These searches revealed the existence of a five-amino acid motif that is conserved in three other members of the C/EBP family, C/EBPα, C/EBPβ, and C/EBPδ (see Table I), which represents the only conserved sequence within the experimentally defined minimal RDI domain (see sequence comparison in Ref. 2). This motif is defined by the consensus sequence (I/V/I)XEMP and will be referred to hereafter as the regulatory domain motif (RDM). The availability of peptide sequences for each C/EBP protein from multiple species permitted exploration of the evolutionary conservation of this sequence. In all vertebrate species in which orthologous relationships to the mammalian C/EBP proteins could be established, the RDM sequence was conserved, in some cases exactly (Table I). For example, in C/EBPα, the RDM sequence IKQEP was perfectly conserved from humans to fish. Significantly, C/EBP proteins from nonvertebrates (two Drosophila species (27) and two Aplysia species (28, 29)) also contain sequences that conform to the RDM consensus. These comparisons suggest that the RDM might be an important component of an evolutionarily conserved transcriptional inhibitory domain in C/EBP proteins.

Functional Analysis of C/EBP RDMs—To test the contribution of the RDM to RDI function in C/EBP proteins, we performed mutagenesis studies using Gal4-C/EBPα fusion proteins that either contained or lacked RDI. Our earlier studies mapped the minimal RDI element to the region between amino acids 97 and 128 of the mouse C/EBPα protein, with the RDM located between amino acids 120 and 124 (14). Therefore, we performed mutagenesis on a Gal4 construct containing amino acids 1–128 of C/EBPα and compared the activity of the wild type and mutant proteins with a fully active Gal4-C/EBPα fusion protein that only contained activation domain sequences located between amino acids 1 and 64 (Fig. 1A). Each construct was co-transfected into COS-1 cells along with a Gal4-responsive luciferase reporter plasmid. In agreement with previous data, the inclusion of sequences encompassing amino acids 65–128 were prepared from transfected COS-1 cells as described previously (24) except when isopeptidase inhibitors were used. In these samples, the cells were washed twice in phosphate-buffered saline containing 1 μg/ml E-64 (Roche Molecular Biochemicals) and lysed in Dignam Buffer A (25) supplemented with 10 μg/ml E-64. Samples were separated on 12 or 15% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes, and immune detection was performed using the Supersignal chemiluminescence detection kit (Pierce) as described previously (26). The anti-Gal4 DNA binding domain (DBD) monoclonal antiserum (R3531) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and the anti-FLAG polyclonal antiserum was obtained from Sigma. The C/EBPα-specific rabbit polyclonal antiserum was raised against a specific epitope in the C-terminal portion of rat C/EBPα.

Cell Culture and Transfections—The monkey kidney COS-1 cell line was cultured in Dulbecco’s modified Eagle’s medium (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (HyClone, Logan, UT). All luciferase-based transfections were performed in 3.5-cm dishes using 2 × 105 cells/well and transfections for preparing extracts for Western blotting were performed in 10-cm dishes using 1.5 × 106 cells/dish. Transfections were performed with the Effectene transfection reagent under conditions recommended by the manufacturer (Qiagen, Valencia, CA). The optimal concentration of each plasmid was determined empirically for each type of transfection experiment. Transfections that examined the activity of Gal4 fusion proteins contained 150 ng of the Gal4 expression plasmid and 150 ng of the G51bLUC reporter. Transfections that examined the ability of the C/EBPα RD to inhibit the activity of the Tet-VP16 activator in trans contained 250 ng of the TRE-UAS-TATA luciferase reporter, 200 ng of the Tet-VP16 expression plasmid, and 250 ng of the Gal4-C/EBPα expression plasmid. Transfections that tested the effect of co-expression of wild type or mutant SUMO-1 on RD function contained 150 ng of G51bLUC, 250 ng of the Gal4-C/EBPα expression plasmid, and 20 or 40 ng of the SUMO-1 expression plasmid. The total DNA concentration was normalized in this last experiment using empty pcMV expression plasmid. Transfections for Western extract preparation contained 3 μg of the appropriate expression plasmid. A control plasmid containing the Renilla luciferase gene under the control of the thymidine kinase promoter was used for all experiments for normalization of transfection efficiencies. All transfections were performed in triplicate at least three times and in some cases up to seven times. Statistical analyses were carried out using a two-tailed Student’s t test. Luciferase assays were performed using the Dual-Luciferase Reporter Assay kit (Promega, Madison, WI).

Nuclear Extract Preparation and Western Blotting—Nuclear extracts were prepared from transfected COS-1 cells as described previously (24) except when isopeptidase inhibitors were used. In these samples, the cells were washed twice in phosphate-buffered saline containing 1 μg/ml E-64 (Roche Molecular Biochemicals) and lysed in Dignam Buffer A (25) supplemented with 10 μg/ml E-64. Samples were separated on 12 or 15% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes, and immune detection was performed using the Supersignal chemiluminescence detection kit (Pierce) as described previously (26). The anti-Gal4 DNA binding domain (DBD) monoclonal antiserum (R3531) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and the anti-FLAG polyclonal antiserum was obtained from Sigma. The C/EBPα-specific rabbit polyclonal antiserum was raised against a specific epitope in the C-terminal portion of rat C/EBPα.
The peptide sequences of four C/EBP family members (C/EBPa, C/EBPβ, C/EBPδ, and C/EBPε) from a variety of species were compared to identify residues conserved with the region encompassing the minimal negative regulatory domain of C/EBPs (amino acids 97–128 of the mouse protein). A five-amino acid motif was identified that was highly conserved in each C/EBP in a variety of vertebrate species and also in nonvertebrate C/EBPs. This sequence has been named the regulatory domain motif (RDM), and the derived consensus is shown at the bottom right. The species from which each sequence was derived, the 15 amino acids that encompass the RDM, and the accession number for that sequence are listed for each entry. Hs, Homo sapiens; Bt, Bos taurus; Oa, Ovis aries; Mm, Mus musculus; Rn, Rattus norvegicus; Gg, Gallus gallus; XP, Xenopus laevis; Rc, Rana catesbeiana; Dr, Danio rerio; Dv, Dro sophila melanogaster; Ac, Aplysia californica; Ak, Aplysia kurodai.

Two potential RDMs were identified in zebrafish C/EBPδ. Although the introduction of mutations into the RDM of C/EBPβ resulted in significant increases in the activity of the resultant proteins, these proteins were still significantly less active than the protein containing the AD alone (Gal4-β-(1–83)). Further analysis of the inhibitory domain of C/EBPβ indicated the existence of additional inhibitory sequences in this protein,2 and further studies are under way to define these sequences. Nevertheless, these studies clearly demonstrated the critical importance of the RDM for inhibitory domain function in each C/EBP.

The C/EBPε RDI Inhibits AD Function Both in cis and in trans—We previously provided evidence that the RDI element of C/EBPε functioned as a site for protein/protein interactions (14). To further investigate the mechanism of action of this domain, we next tested the ability of this domain to inhibit the action of a heterologous activation domain in both cis and trans arrangements. Single or multiple copies of the C/EBPε RDI domain (amino acids 65–128) were attached to a fusion protein containing the Gal4 DBD and the VP16 AD, and the activity of the resulting proteins was tested as before (Fig. 3A). A Gal4-VP16 protein carrying a single RDI element possessed less than 10% of the activity of the parental protein, and attachment of additional RDI elements yielded further inhibition that was proportional to the number of RDI elements. We next tested whether the RDI element functioned only when located adjacent to an AD by inserting a mutant form of the RDI element between the VP16 AD and one or two copies of the wild type RDI element. Attachment of a mutant RDI element resulted in a modest decrease in the activity of the Gal4-VP16 fusion protein; however, the further attachment of one or two

| Species | Sequence | Accession no. | Species | Sequence | Accession no. |
|---------|----------|---------------|---------|----------|---------------|
| C/EBPa  | LRPLV 1KQEP REEEE P49715 | C/EBPβ | Hs PPFPA LKAE PGEPA NP 001796 |
| Hs      | LRPLV 1KQEP REEEE 002754 |          | Bt PPFPA LKAE PGEPA O02755 |
| Bt      | LRPLV 1KQEP REEEE AAH28890 |         | Mm PPFPA LKAE PGEPA NP 034013 |
| Mm      | LRPLV 1KQEP REEEE NP 056656 |        | Rn PPFPA LKAE PGEPA NP 077039 |
| Gn      | LRPLV 1KQEP REEEE S26295 |              | Gn PKIQ VKVEP VFTEL Q05826 |
| X1      | LRPLA 1KQEP REEEA A56554 |                | X1 PQLGE TKVEP VFESL CA67809 |
| Rc      | LRHLV 1KQEP RGEEE AAA52223 |               | Rc RAPA LRGE REPEA RAPA |
| Dr      | MRFAA 1KQEP REEEE NP 571960 |             | Dr SAPA LRGE REPEA SAPA |
| C/EBPδ  | AAPRL LKREP DWDGG NP 005186 | C/EBPε | Hs PRAVA VKEEP RGEPEP NP 001796 |
| Hs      | AAPRL LKREP DWDGG BAA20097 |         | Oa PRAVA VKEEP RGEPEP CAA10944 |
| Ft      | AAPRL LKREP DWDGG CAB29273 |           | Mm PRAVA VKEEP RGEPEP |
| Mm      | VARGP LKREP DWDGG NP 031700 |          | Rn PRAVA VKEEP RGEPEP P5621 |
| Rn      | TARGP LKREP DWDGG Q03484 |             | Rn PRAVA VKEEP RGEPEP P5621 |
| Ac      | QWYXS 1KQEP DWDSS AAS22224 |            | Ac RAPA LRGE REPEA RAPA |
| Ac      | LFNRT VKEEK PDFYM NP 571962 |          | Ac RAPA LRGE REPEA RAPA |
| Dr1     | SFCAP 1KKEA DWSIS NP 571962 |      | Dr2 SFCAP 1KKEA DWSIS NP 571962 |
| Nonvertebrate C/EBPs | Consensus RDM (1/V/L) KEKP |  |
| Dm      | SDDSST 1KEEK IDPEY A44541 |      | Dm PRAVA VKEEP RGEPEP |
| Dv      | SDDSST 1KEEK IDPYY Q02638 |        | Dv PRAVA VKEEP RGEPEP |
| Ac      | QWNLQ VKRED FGVRT A53066 |          | Ac PRAVA VKEEP RGEPEP |
| Ak      | PFNLQQ VKRED FGVRT AAG61258 |      | Ak PRAVA VKEEP RGEPEP |

*J. Kim and S. C. Williams, unpublished observations.*
functional RDI elements in this more distal arrangement led to dramatic transcriptional repression (Fig. 3A).

To examine RDI function in trans, Gal4 fusion proteins carrying single or triple copies of wild type or mutant RDI elements, without any AD sequences, were generated. These proteins were tested for their ability to inhibit the activity of an activator protein consisting of the DNA binding domain from the Tet repressor fused to the VP16 AD (14). The effect of each RDI-containing fusion protein was compared with the Gal4 DBD alone on a reporter construct containing seven copies of the Tet response element (TRE) in a distal position and five Gal4 binding sites located in a proximal position (TRE-UAS-TATA; Fig. 3B). Coexpression of Gal4 fusion proteins containing single or triple RDI elements decreased the measured activity of the Tet-VP16 activator protein to ∼10 and 1% of the control value, respectively. This effect was dependent on the integrity of the RDM, since fusion proteins containing mutant RDI elements had no inhibitory effects in this assay. These data suggest that the RDM can recruit proteins to a promoter that inhibit transcription in a position-independent and dose-dependent manner.

Identification of Functional RDM-containing Transcription Inhibitory Domains in Jun Family Members—Data base searches revealed the existence of several hundred proteins that contained sequences similar to the RDM sequences found in the C/EBPs. The majority of these proteins were nuclear proteins with known or predicted functions in transcription, replication, or DNA repair. Therefore, we were interested in determining whether the proteins identified in these searches contained transcriptional inhibitory domains similar to the domains described above in the C/EBPs. We selected the three members of the Jun family of bZIP transcription factors for these studies, since each contains an RDM-like sequence located adjacent to the N-terminal end of their DNA binding domains (Fig. 4A). Short segments of each Jun protein were attached to a Gal4-VP16 fusion protein as described above. The effect of single or, in some cases, multiple copies of each Jun module on the transcriptional activity of the resulting fusion proteins was assessed in transfection assays in COS-1 cells. Single copies of the c-Jun and JunD modules efficiently inhibited the activity of the Gal4-VP16 protein, whereas the JunB module had less of an effect (Fig. 4B). Inhibition was dependent

3 S. C. Williams, unpublished observations.
Modification of C/EBPs by SUMO-1

on the integrity of the RDM, since changing the lysine residue at position 2 of the c-Jun RDM to alanine nearly abolished its inhibitory effect (compare G4V-c-Jun with G4V-(c-Jun)m). To further test whether a functional inhibitory domain exists in JunB, a protein bearing two copies of the RDM-containing region was placed adjacent to the VP16 AD in Gal4 fusion proteins as diagrammed. B, the activity of a series of Gal4-VP16 fusion proteins carrying the single, double, or multiple copies of the Jun sequences was tested by co-transfection with a Gal4-responsive luciferase reporter plasmid in COS-1 cells. In the case of c-Jun, one construct carried a lysine to alanine substitution in the c-Jun segment. The activity of each protein is listed as described before. *, p < 0.05. BR, basic region; LZ, leucine zipper.

by covalent attachment of SUMO-1. Initially, expression vectors encoding two isoforms of C/EBPα (p29 and p32) were co-introduced into COS-1 cells either with or without an expression vector for SUMO-1, and cell extracts were prepared and analyzed by Western blotting. The C/EBPα proteins and SUMO-1 both carried FLAG epitope tags, and thus duplicate Western blots were probed with antisera directed against either FLAG or C/EBPα to detect these proteins. When expressed alone, the C/EBPα proteins migrated at the expected positions, with multiple closely spaced bands perhaps representing differentially phosphorylated isoforms (Fig. 5, lanes 1 and 2). In the presence of SUMO-1 and isopeptidase inhibitors, slower migrating complexes were observed whose positions were dependent on the mass of the C/EBPα isoform (lanes 4 and 5). These slower migrating complexes were detected using both anti-C/EBPα and anti-FLAG antisera. However, the slower migrating complexes were not detected following transfection of an expression plasmid encoding a C/EBPα protein bearing alanine substitutions in the KEE core of the RDM (lanes 3 and 6).

To confirm that the slower migrating complexes represented

![Image](Figure 3)  The C/EBP RDM Sequences Are Attachment Sites for SUMO-1

![Image](Figure 4)  The RDM is a component of a conserved negative regulatory domain in three members of the Jun family.

![Image](Figure 5)  The RDM is a component of a conserved negative regulatory domain in three members of the Jun family.
SUMO-1-modified C/EBPα, additional co-transfection experiments were performed using a C/EBPα protein (K121A) bearing a specific mutation in the lysine presumed to be the SUMO-1 attachment site and a mutant SUMO-1 protein that cannot be processed for attachment (SUMOGA). As before, the slower migrating complex (which resolved into three distinct species in this gel) was observed using the wild type but not the K121A mutant protein (Fig. 5B, compare lanes 2 and 4). The appearance of the slower migrating complex(es) was dependent on the presence of a functional SUMO-1 protein, since complex formation was not observed when the SUMOGA mutant protein was co-expressed at levels equivalent to the wild type protein (lane 3).

We next tested whether the other C/EBP proteins could also be modified by SUMO-1 attachment within their RDM sequences. FLAG-tagged wild type C/EBPα or C/EBPβ or proteins in which the conserved lysine in the RDM was mutated were co-expressed with SUMOGG or SUMOGA in COS-1 cells. Nuclear extracts were prepared and analyzed by Western blotting using an anti-FLAG antiserum as described above. We found that sumoylation appears to be a modification common to each of the C/EBPs examined here and may represent an important regulatory modification of C/EBP transcriptional activity.

**Sumoylation Decreases the Inhibitory Function of the C/EBPα RDI Element**—Having shown that the RDM is a target for
SUMO-1 attachment, we next tested the consequence of this modification on the function of the RDI element of C/EBP. Increasing amounts of the wild type and mutant SUMO proteins were co-expressed with Gal4-C/EBP fusion proteins bearing either wild type or mutant RDM sequences (Fig. 7). Co-expression of wild type SUMO-1 resulted in a consistent increase in the activity of the wild type Gal4-C/EBP fusion protein, with a maximal 7-fold increase observed with co-transfection of 40 ng of the SUMO-1 expression vector. By contrast, co-transfection of an expression vector encoding the SUMOGA mutant protein did not result in a significant change in Gal4-C/EBP activity. Furthermore, the activity of the RDM mutant Gal4-C/EBP protein was essentially unaffected by co-expression of either the wild type or mutant SUMO-1 protein. Therefore, attachment of SUMO-1 appears to relieve the inhibitory effect of the C/EBP RDM.

**DISCUSSION**

These studies have identified an evolutionarily conserved transcriptional regulatory domain within four members of the C/EBP family that is capable of inhibiting the activity of an adjacent transcriptional activation domain both in cis and in trans. These domains are characterized by the presence of a conserved five amino acid motif ((I/V/L)KKXP) that we named the RDM. Similar functional domains containing RDM-like sequences are also present in another family within the bZIP superfamily, the Jun proteins. The RDM is similar to the consensus sequence for covalent attachment of the SUMO-1 protein, and we demonstrated that C/EBPα, C/EBPβ, C/EBPδ, and C/EBPε can all be modified by attachment of SUMO-1 to the lysine at position 2 of the RDM. Attachment of SUMO-1 to the RDM of C/EBPε decreased the inhibitory function of the regulatory domain of C/EBPε, suggesting that sumoylation may be a common mechanism for regulating the activity of C/EBP and perhaps other bZIP proteins.

The existence of sequences within C/EBP proteins with the capacity to inhibit transcription was first indicated by early structure/function studies on C/EBPα (12). These studies identified a domain, originally named the "attenuator," that was located between amino acids 108 and 170 of the rat C/EBPα protein (12). Subsequent studies identified inhibitory domains in mammalian (3) and avian (13) C/EBPβ proteins and in mammalian C/EBPε (11, 14). Our data now show that the RDM is a functional component of the inhibitory domains of each of these proteins. Furthermore, the conservation of RDM-like sequences in each C/EBP throughout evolution supports the potential importance of this motif for C/EBP function. This is perhaps best exemplified by the existence of RDM-like sequences in nonvertebrate C/EBPs from *Drosophila* and *Aplysia*, although functional studies on these potential RDMs have not yet been performed.

The demonstration that the RDM of C/EBPs is a site for covalent attachment of the SUMO-1 protein may have significant implications for understanding the mechanisms by which this domain might control the activity of this family of transcription factors. SUMO-1 is a 102-amino acid protein that displays ~18% sequence identity to ubiquitin (21, 31). SUMO-1 is attached to the ε amino group of lysine residues in target proteins using a mechanism that is functionally analogous to ubiquitination. SUMO-1 is synthesized as a precursor protein that is first cleaved by C-terminal hydrolases at the C-terminal side of a diglycine sequence. It is then attached through a thioester bond to a heterodimeric E1 activating enzyme (SAE1/SAE2 in mammalian cells) and transferred to the Ubc9 E2 enzyme (21). Although sumoylation can occur *in vitro* in the absence of an E3 ligation activity, recent studies have identified a number of proteins that possess SUMO E3 ligase activity that may increase the efficiency and/or selectivity of the sumoylation reaction (32, 33). Sumoylation is a reversible reaction, and a number of isopeptidases have been identified that possess desumoylation activities (21). The list of proteins that can be targets for sumoylation continues to grow and consists primarily of nuclear proteins in mammalian cells, consistent with our observation that most RDM-containing proteins are likely to function in the nucleus. Known targets include RanGAP1 (34), PML (35), IkBα (36), p53 (30) and androgen receptor (37). Significantly, negative regulatory domains in other transcription factors, including c-Myb and c-Jun have also been identified as sumoylation targets, and the lysine residue within the c-Jun RDM sequence identified in our studies is the demonstrated site for covalent attachment of SUMO-1 (30, 38). Therefore, sumoylation of negative regulatory domains in transcription factors appears to be a common mechanism for regulating their activity.

Although our data indicate that sumoylation may modulate the activity of C/EBP proteins, the mechanism underlying this effect is not yet clear. Sumoylation does not appear to target proteins for degradation, an important role for ubiquitination (39). Instead, sumoylation may modify the activity of target proteins via a number of different mechanisms (21, 31). First, sumoylation may block alternative lysine-targeted modifications such as acetylation or ubiquitination. For example, sumoylation of IkBα occurs on a lysine residue that is also targeted for ubiquitination, and thus attachment of SUMO stabilizes IkBα by blocking ubiquitination and subsequent degradation (36). Stabilization of IkBα results in prolonged inhibition of NF-xB activity and thus may have strong significance for regulation of NF-xB activation in inflammatory and other reactions. Although both *Drosophila* and *Aplysia* C/EBPs have been shown to be targets for ubiquitination (40, 41), ubiquitination of mammalian C/EBPs has not yet been reported. In addition, we did not detect any significant differences in the steady state level of Gal4 fusion proteins carrying wild type or mutant RDM sequences, suggesting that sumoylation is unlikely to modulate the stability of C/EBP proteins. Likewise, although transcription factor acetylation is emerging as a powerful regulatory mechanism (42), there is no evidence as yet that C/EBPs are acetylated.
Sumoylation may also affect the subcellular, or more specifically the subnuclear localization of target proteins as, for example, appears to be the case for PML (43). Both C/EBPα and C/EBPβ are known to associate with the nuclear matrix (44) and to localize to specific dotlike structures within the nucleus (45, 46), although whether these domains represent sites where C/EBP proteins are active or inactive remains uncertain. C/EBPβ is also localized to specific nuclear subdomains4; however, this localization appears to be unaffected by the integrity of the RDM. Nevertheless, further definition of the specific subnuclear location of C/EBP proteins may provide important insights into the role of sumoylation in controlling C/EBPβ activity.

Finally, sumoylation modulates the transcriptional activity of a growing number of target proteins, including androgen receptor (37), p53 and c-Jun (30), c-Myc (38), and two members of the histone deacetylase family (47, 48). Again, the exact mechanisms underlying SUMO-dependent regulation of transcriptional activity are poorly understood, but they may involve modulation of critical protein/protein interactions. Previous models to explain the action of the inhibitory domains of C/EBPβ envisioned the formation of intermolecular interactions that prevented AD access to the transcriptional machinery (3, 13). These interactions may be disrupted by sumoylation, thereby leading to activation of the modified protein. Alternatively, the inhibitory domain may be a site for interactions with an as yet unknown inhibitory protein, and sumoylation results in dissociation of this protein. This latter model is more consistent with our current data, particularly the ability of the C/EBPβ RDM to inhibit the activity of an adjacent AD in trans. One attractive set of candidates for this function are the protein inhibitor of activated STAT (PIAS) proteins (49). PIAS proteins were first identified as inhibitors of the STAT family of transcription factors (50) and possess SUMO E3 ligase activity (32). Experiments are currently under way to test whether PIAS proteins interact with the inhibitory domain of the C/EBP proteins and to determine the consequence of such interactions on AD function and sumoylation. Furthermore, there are two other SUMO proteins in mammalian cells, SUMO-2 and SUMO-3 (21), and we are interested in determining whether these two proteins can also be attached to C/EBP RDM sequences. Clearly, elucidation of the full complement of RD-interacting factors will be critical for our understanding of the contribution of inhibitory domains to C/EBP function in vivo.

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Transcriptional Activity of CCAAT/Enhancer-binding Proteins Is Controlled by a Conserved Inhibitory Domain That Is a Target for Sumoylation

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