Repression and Coactivation of CCAAT/Enhancer-binding Protein ε by Sumoylation and Protein Inhibitor of Activated STATx Proteins*

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The CCAAT/enhancer-binding protein ε (C/EBPε) is a neutrophil-specific transcription factor whose activity is controlled by juxtaposed activating and regulatory domains. We previously determined that the function of the major regulatory domain (RD1) in C/EBPε was dependent on the integrity of a five-amino acid motif that was identical to the recognition site for members of the small ubiquitin-like modifier (SUMO) family of ubiquitin-related proteins. We show here that the SUMO attachment site (the regulatory domain motif) is necessary and sufficient both for the intrinsic inhibitory function of RD1 and for coactivation by PIASxα and PIASxβ, two members of the protein inhibitor of activated STAT (PIAS) family of SUMO E3 ligases. PIASxα was a more potent coactivator than PIASxα of both full-length C/EBPε and fusion proteins containing the N-terminal portion of C/EBPε, whereas PIASxα was more active on fusion proteins containing a heterologous activation domain. Two modes of coactivation were observed, one that was dependent on the integrity of the RING finger (RF) domain and was shared by both PIASxα and PIASxβ and a second mode that was independent of the RF and was only observed with PIASxβ. Sumoylation of C/EBPε was enhanced by coexpression of PIASxα, suggesting that this modification is associated with the enhanced activity of the target protein. These results suggest that a complex interplay of accessory factors, including SUMO and PIAS proteins, modulates the activity of C/EBPε.

The CCAAT/enhancer-binding proteins form a subgroup within the basic region/leucine zipper superfamily of transcriptional regulatory proteins (1, 2). There are six members of the C/EBP family, four of which (C/EBPα, C/EBPβ, C/EBPδ, and C/EBPε) are transcriptional activators that control the differentiation and function of cells in a large number of tissues. C/EBPε was first identified based on sequence similarity to C/EBPα (3) and is primarily expressed in myeloid cells within the hematopoietic system (4–6). C/EBPε expression increases during neutrophil differentiation (7), and it is also expressed in macrophages (6, 8). C/EBPε is implicated in the regulation of a number of neutrophil- and macrophage-specific genes (6, 8–13). Targeted disruption of the C/EBPε gene in mice caused a block in neutrophil differentiation that resulted in the production of morphologically and functionally abnormal neutrophils (14). In addition, mutations in the human C/EBPε gene have been detected in patients with the rare congenital disease, neutrophil-specific granule deficiency (15–17). The similar consequences of C/EBPε mutations in mice and humans suggest that disruption of C/EBPε activity is a major causative event in neutrophil-specific granule deficiency.

The C/EBPs are characterized by a highly conserved carboxy-terminal DNA binding domain that directs sequence-specific binding to the palindromic sequence 5′-ATTGCGCAAT-3′ and variants thereof (1, 2). Each C/EBP can form homo- and heterodimers in all combinations (3) and can heterodimerize with other members of the basic region/leucine zipper superfamily. The amino-terminal portions of C/EBPα, C/EBPβ, C/EBPδ, and C/EBPε contain juxtaposed activation and inhibitory domains that control their transcriptional regulatory activities (18–22). We recently identified two inhibitory domains in the C/EBPε polypeptide that we named regulatory domains 1 and 2 (RD1 and -2). The RD1 domain functions as an autonomous transcriptional inhibitory domain when attached to heterologous transcriptional activation domains and is functionally similar to the attenuator domain of C/EBPα (18) and the RD1 domain of C/EBPβ (20). Sequence comparisons of RD1 domains from each C/EBP revealed a conserved five-amino acid motif with the consensus sequence (IVL/K)XXE. We named this sequence the regulatory domain motif (RDM) and noted its similarity both to the synergy control element initially found in members of the nuclear hormone receptor superfamily (23, 24) and to the consensus sequence for attachment of SUMO proteins (25).

The SUMO family consists of four members, SUMO-1 to -4 (25–27). SUMO proteins can be covalently attached to target proteins via an enzymatic mechanism analogous to, but using distinct enzymes from, ubiquitilation. SUMO proteins are synthesized as precursors that must first undergo proteolytic processing to produce the mature polypeptide. The mature protein can then be attached to lysine residues in the target protein, usually within the consensus sequence ϕKXE (where ϕ and X represent a hydrophobic and any amino acid, respectively), via a two-step process involving an E1 activating enzyme complex and an E2 conjugating enzyme. Although sumoylation in vitro...
requires only E1 and E2 enzymes, three classes of proteins with E3 ligase activity have recently been identified (28). These proteins, which include members of the protein inhibitor of activated STAT (PIAS) family, the nuclear pore-associated RanBP2 protein, and Pc2, a member of the polycomb family, may enhance the efficiency or selectivity of the sumoylation process in vivo. We showed previously that the RDM of C/EBPβ was an attachment site for SUMO-1 and that the three other C/EBPs that contain RDM-like sequences (C/EBPa, C/EBPβ, and C/EBPδ) were also sumoylation targets (22). This modification has subsequently been confirmed for both C/EBPa (29) and C/EBPβ (30). A growing number of proteins have been identified as SUMO targets and include numerous proteins that function in transcriptional regulation and other nuclear processes. The consequences of sumoylation include modification of subcellular or subnuclear location, protein stability, or activity and appear to be dependent on the identity of the target protein. Since sumoylation of C/EBPs occurs within a transcriptional inhibitory domain, it has been proposed that sumoylation inhibits the activity of C/EBPs. However, we previously demonstrated that enhancing sumoylation of C/EBPβ, at least in the context of a Gal4 fusion protein, actually increased its activity, and thus the ultimate consequence of sumoylation of C/EBPα (and presumably other C/EBPs) remains to be clearly determined.

Two models have been proposed to explain the mechanism by which the RD1 domain of C/EBP proteins inhibits their activity. An intramolecular interaction model was proposed based on studies on C/EBPβ in which interactions between the RD1 domain and the N-terminal activation domain prevented access of the AD to the transcriptional coactivators and/or components of the general transcriptional machinery (19, 20). More recently, we proposed an intermolecular interaction model based on our studies on C/EBPβ and suggested that SUMO attachment may play a critical role in controlling interactions between the RD1 domain of C/EBPβ and transcriptional coregulators (22). In this report, we have extended our earlier studies on the regulation of C/EBPβ by sumoylation to attempt to define the mechanism by which C/EBPβ activity is controlled.

EXPERIMENTAL PROCEDURES

Plasmid Construction—All Gal4-based expression plasmids were constructed in the Gal0 parental plasmid (20). The construction of C/EBPα-(1–64), C/EBPδ-(1–128), C/EBPδ-(1–129/K121A), G4-V, G4-V-RD1, G4-V-RD1/K121A, G4-α-(1–108), and G4-α-(1–170) was described previously (22). Gal4 fusion constructs bearing the wild type and mutated activity is controlled.

Phosphorylation of C/EBPα by sumoylation inhibits the activity of C/EBPs. However, we previously mapped the RD1 domain of C/EBPβ and suggested that SUMO attachment may play a critical role in controlling interactions between the RD1 domain of C/EBPβ and transcriptional coregulators (22). In this report, we have extended our earlier studies on the regulation of C/EBPβ by sumoylation to attempt to define the mechanism by which C/EBPβ activity is controlled. same enzymes. pCMV-FLAG-SUMO-1 was a generous gift from Dr. Zelig Grossman (The Hebrew University, Jerusalem), pCMV-FLAG-Pi3Kα (15, 34.5 μg of each) and whole cell extracts were prepared after 48 h as described previously (22). SUMO-modified and unmodified forms of C/EBPβ were detected by immunoblotting using a Myc-specific monoclonal antibody (9E10; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The proportion of total C/EBPβ protein that was sumoylated was calculated by densitometric analysis using the Fluorchem program (Alpha Innotech Co., San Leandro, CA).

Pull-down Assays—Nuclear extracts were prepared from COS-1 cells transfected with various combinations of pDNA3.1 MHB/C/EBPα-Myc (2.0 μg), pCMV-FLAG-SUMO-1 (0.5 μg), and pCMV-FLAG-Pi3Kα (0.5 μg), and whole cell extracts were prepared after 48 h as described previously (22). SUMO-modified and unmodified forms of C/EBPβ were detected by immunoblotting using a Myc-specific monoclonal antibody (9E10; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The proportion of total C/EBPβ protein that was sumoylated was determined by densitometric analysis of the autoradiographs on a Metaquant (Ambion, Austin, TX) using the Fluorchem program (Alpha Innotech Co., San Leandro, CA).

RESULTS

The RDM Is Necessary and Sufficient for Transcriptional Repression in Gal4 Fusion Proteins—We previously mapped the location of transcriptional activation and repression domains in the mouse C/EBPα protein using Gal4 fusion proteins (21, 22). As shown in Fig. 1A, Gal4 fusion proteins bearing the first 64 amino acids of C/EBPα (referred to here as AD1) were potent activators in COS-1 cells of a Gal4-responsive luciferase reporter plasmid, whereas the inclusion of amino acids 64–128 of C/EBPα resulted in a significant decrease in transcriptional activity. Based on these data, we previously mapped the RD1 region between amino acids 64 and 128 of C/EBPδ and further...
joined to a short peptide containing either the wild type RDM sequence (VKEEP) or a variant sequence in which the lysine that serves as the attachment site for SUMO proteins was changed to arginine (VREEP). We previously showed that alanine and arginine substitutions at this position have equivalent effects, and arginine was used in this instance to conserve the positive charge at this position (22). The activity of the fusion protein containing the wild type RDM sequence was similar to that of the fusion protein containing the complete RD1 domain (compare G4-ε(1–128) with G4-ε(1–64)-RDM in Fig. 1). The fusion protein containing the VREEP sequence (G4-ε(1–64)-RDMm) was significantly more active than the protein containing the wild type RDM sequence, confirming the importance of the wild type sequence for the repression activity. The difference in activities between the two proteins bearing mutations in the RDM is due to the presence of additional sequences with transcriptional stimulatory activity between amino acids 65 and 97 (see Fig. 3A in Ref. 21).

To test the AD specificity of this inhibitory function, we constructed a similar set of expression plasmids containing the AD for the herpes simplex virus VP16 protein, since we previously showed that the RD1 region of C/EBPβ could repress the activity of this heterologous AD (21). As in Gal4-C/EBPα fusion proteins, inclusion of either the wild type RD1 or RDM efficiently repressed the activity of the VP16 AD (compare G4-V-εRD1 with G4-V-RDM in Fig. 1B), and alteration of the lysine residues in either context relieved this repression (compare G4-V-εRD1(K121A) with G4-V-RDMm in Fig. 1B). We had previously proposed that the ability of the RD1 domain to repress a heterologous AD was inconsistent with an intramolecular inhibition model due to a lack of sequence conservation between the C/EBPα and VP16 ADs. However, we noted that the C/EBPα and VP16 ADs are both classified as acidic activators. Based on this observation, we decided to test whether that C/EBPα RD1 region could also inhibit the activity of ADs from other classes. Therefore, we constructed expression vectors for Gal4 fusion proteins containing AD sequences from the Sp3 (amino acids 6–426, classified as a glutamine-rich AD) and CTF-1 (amino acids 354–499, classified as a proline-rich AD) transcription factors, joined to the wild type and RDM mutant RD1 domains from C/EBPα. Although the ADs from Sp3 and CTF-1 differed in their intrinsic activity compared with each other and the C/EBPα and VP16 ADs, the activities of both ADs were efficiently inhibited in proteins containing the wild type RD1 (compare G4-Sp3 with G4-Sp3-εRD1 and G4-CTF1 with G4-CTF1-εRD1 in Fig. 1C). Similarly, the repressive effect of RD1 was relieved in both cases when the lysine within the RDM was changed to alanine (G4-Sp3-εRD1(K121A) and G4-CTF1-εRD1(K121A) in Fig. 1C). Collectively, these data suggest that the repressive activity of the RD1 domain is likely to involve intermolecular interactions between the RD and a protein with co-repressor activity.

C/EBPα Is Coactivated by the SUMO E3 Ligases, PIASxα and PIASxβ—To date, the only proteins known to interact with the RDM are members of the SUMO family (22). However, as mentioned above, it is not clear whether sumoylation of C/EBPα is associated with repression or derepression of its activity. To address the specific consequences of sumoylation on C/EBPα activity, we tested whether coexpression of two members of the PIAS family of SUMO E3 ligases, PIASxα and PIASxβ (Fig. 2A), affected the transcriptional activity of C/EBPα. These two PIAS family members were chosen because they are coexpressed with C/EBPα during neutrophil differentiation and were previously shown to function as coregulators of proteins.

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*H. Youn and S. C. Williams, unpublished results.*
such as the androgen receptor, that contain regulatory domains similar to the RD1 domain of C/EBPβ (33). Expression vectors encoding either PIASxα or PIASxβ, two members of the protein inhibitor of activated STAT family, SAP (SAF, genius, PIAS), may be involved in DNA binding; PINIT, domain containing the sequence PINIT that is associated with nuclear localization (44); RF (RING finger), associated with SUMO E3 ligase activity and protein-protein interactions; NLS, putative nuclear localization signals; D/E-rich and S/T-rich, domains rich either in Asp/Glu or Ser/Thr; IISLD, a five-amino acid motif conserved in PIASxα, PIAS1, and PIASxβ. The RING finger deletion mutant forms of the two proteins (referred to throughout as PIASxαΔRF and PIASxβΔRF) are also schematically depicted. B, C/EBPα was coexpressed with the indicated PIASx proteins in COS-1 cells, and activity was measured on a C/EBP-dependent luciferase reporter plasmid. Two different amounts of each PIASx expression plasmid (80 and 160 ng) were used, and data are depicted as luciferase units. This experiment was performed in triplicate and repeated at least six times, and data are represented as mean ± S.D.

FIG. 2. PIASx proteins function as coactivators of C/EBPα.

A, domain structure of PIASxα and PIASxβ, two members of the protein inhibitor of activated STAT family. SAP (SAF, genius, PIAS), may be involved in DNA binding; PINIT, domain containing the sequence PINIT that is associated with nuclear localization (44); RF (RING finger), associated with SUMO E3 ligase activity and protein-protein interactions; NLS, putative nuclear localization signals; D/E-rich and S/T-rich, domains rich either in Asp/Glu or Ser/Thr; IISLD, a five-amino acid motif conserved in PIASxα, PIAS1, and PIASxβ. The RING finger deletion mutant forms of the two proteins (referred to throughout as PIASxαΔRF and PIASxβΔRF) are also schematically depicted. B, C/EBPα was coexpressed with the indicated PIASx proteins in COS-1 cells, and activity was measured on a C/EBP-dependent luciferase reporter plasmid. Two different amounts of each PIASx expression plasmid (80 and 160 ng) were used, and data are depicted as luciferase units. This experiment was performed in triplicate and repeated at least six times, and data are represented as mean ± S.D.

We next determined whether specific regions of C/EBPβ were required for coactivation by PIASxα and PIASxβ. Differential usage of two in-frame translation initiation codons in the mouse C/EBPβ mRNA directed the synthesis of two isoforms (named C/EBPβ p32 and C/EBPβ p29) of 281 and 252 amino acids, respectively (Fig. 3A). The shorter isoform lacks a portion of the tripartite N-terminal activation domain and is a slightly weaker activator than the p32 isoform. The two C/EBPβ isoforms responded similarly to coexpression of each PIASx isoform, with PIASxβ functioning as a more potent coactivator in each case (Fig. 3B).

Cotransfection experiments were performed in COS-1 cells to determine whether either or both regulatory domains within C/EBPβ (Fig. 3A) were required for PIASx-dependent coactivation. As reported earlier (21), deletion of RD1 or RD2 resulted in ~7- and 1.5-fold increases in the activity of C/EBPβ on the C/EBP-dependent reporter plasmid (Fig. 3C). Deletion of RD2 (ΔRD2) did not dramatically affect coactivation by either PIASxα or PIASxβ. However, the C/EBPβ protein lacking the RD1 domain (ΔRD1) was essentially unresponsive to PIASxα or PIASxβ.
and only weakly responsive to PIASxβ (Fig. 3C). Thus, maximal PIASx-dependent coactivation was dependent on the integrity of the RD1 within the C/EBPε polypeptide.

PIASx Proteins Interact with C/EBPε and Enhance Its Sumoylation—PIASxα and PIASxβ physically interact with several transcription factors, including members of the steroid hormone receptor superfamily (33). We therefore next tested whether C/EBPε and PIASx proteins physically interact by performing co-immunoprecipitation and Ni²⁺-NTA pull-down assays. Nuclear extracts were prepared from COS-1 cells transfected with combinations of expression vectors encoding C/EBPε-Myc-His₆, FLAG-PIASxα, FLAG-JunD, or G4-V. JunD is a member of the AP-1 family, and Jun and C/EBP proteins are known to physically interact (34). G4-V was used as a negative control for these experiments. PIASxα and JunD efficiently coprecipitated with C/EBPε (Fig. 4A, compare lanes 2 and 3 with lanes 4 and 5). Likewise, C/EBPε efficiently coprecipitated with both PIASxα and JunD (Fig. 4A, lanes 7 and 8), and interactions were dependent on the presence of both protein partners in all cases (see lanes 1 and 6 and lower panels showing input levels of each protein). These interactions were specific, since G4-V did not coprecipitate with either C/EBPε or PIASxα (Fig. 4B). C/EBPε also coprecipitated with PIASxβ (data not shown).

The RING finger domain of PIAS proteins is required for their SUMO E3 ligase function and can also function as a protein–protein interaction interface (35). Since PIASxα coactivation of C/EBPε was entirely dependent on the integrity of the RING finger domain, and coactivation by PIASxβ was partially dependent on this domain, we next tested whether PIASx proteins could enhance C/EBPε sumoylation in COS-1 cells. PIASxα was consistently expressed at significantly higher levels than PIASxβ in the large scale transfections necessary for performing these assays; therefore, we have only tested the activity of PIASxα in these experiments. Myc-tagged C/EBPε was expressed in COS-1 cells in the presence or absence of SUMO-1 and PIASxα, and unmodified and modified forms of C/EBPε were detected by immunoblotting with either Myc- or C/EBPε-specific antisera (Fig. 4C; data not shown). Unmodified C/EBPε migrated at its expected molecular weight (~34,000), and the SUMO-C/EBPε complex was only detected in the presence of excess SUMO-1 (Fig. 4B, lanes 1 and 2). The identity of this band as SUMO-C/EBPε was confirmed by expressing a mutant form of C/EBPε in which the lysine acceptor site was changed to alanine. In this case, the SUMO-C/EBPε band was absent (Fig. 4B, lane 5). The intensity of the SUMO-C/EBPε band increased (from 1.7 to 9.6% of total C/EBPε protein) in the presence of PIASxα; however, the increase in the intensity of the SUMO-C/EBPε band was dependent on the integrity of the RING finger domain of PIASxα. Therefore, the coactivation of C/EBPε by PIASxα (and presumably PIASxβ) correlates with enhanced sumoylation within the RD1.

PIASxα and PIASxβ-dependent Coactivation of Gal4 Fusion Proteins—As shown above, the RD1 domain of C/EBPε functions as an autonomous inhibitory domain in Gal4 fusion proteins. As deletion of RD1 abolished coactivation by PIASx proteins in the context of the full-length C/EBPε polypeptide, we next examined whether Gal4 fusion proteins containing RD1 were also targets for PIASx coactivation. Two different RD1-containing Gal4 fusion proteins introduced earlier (Gal4-c1–128) and G4-V-RD1) were utilized in these experiments (Fig. 5A). These proteins differ in the origin of AD sequences, and we

C/EBPε proteins. The positions of the unmodified C/EBPε protein and the SUMO-C/EBPε complex are shown with arrowheads. The relative intensities of each band were measured by densitometry, and the proportion of C/EBPε represented as SUMO-C/EBPε complexes was calculated.
PIASx Proteins Are Also Differential Coactivators of C/EBPα—We previously demonstrated that each of the four members of the C/EBP family contain inhibitory domains located adjacent to their major ADs that were dependent for function on the integrity of RDM-like sequences (22). To extend this intrafamily comparison, we tested whether PIASx proteins could function as coactivators of a Gal4 fusion protein carrying the N-terminal segment of C/EBPα. As shown in Fig. 7, a Gal4 fusion protein containing just the N-terminal AD of C/EBPα (G4-α-(1–108)) was ~30-fold more active than G4-α-(1–170), which contains an RD1-like domain and a RDM-like element (22). However, G4-α-(1–170) was a target for differential coactivation by both PIASxα and PIASxβ, and the pattern of coactivation was similar to that observed for Gal4 fusion proteins containing the equivalent region of C/EBPα (amino acids 1–128). Specifically, G4-α-(1–170) was weakly coactivated by PIASxα, and this activity was dependent on the RF of PIASxα, whereas it was more strongly coactivated by PIASxβ, and some activity (about 25% of maximal) was retained by the RF-deletion mutant of PIASxβ (Fig. 7). Thus differential coactivation by PIASx isoforms appears to be shared within the C/EBP family.
In this study, we report that two members of the PIAS family of RING finger domain proteins function as differential coactivators of the neutrophil-specific transcription factor C/EBP. This study was prompted by our recent demonstration that C/EBP is one of a growing number of nuclear proteins that are targets for modification with the ubiquitin-related protein SUMO-1 and its relatives. SUMO modification of C/EBP occurs on a consensus SUMO attachment site (the RDM) within a domain (RD1) that inhibits the activity of C/EBP, and we show here that the RDM can functionally replace RD1 in Gal4 fusion proteins. Remarkably, PIASx-dependent coactivation also required RD1, and the RDM can also functionally substitute for RD1 as a target for PIASx coactivation. The ability of a short motif to participate in both inhibitory and stimulatory regulation of transcription suggests that this motif is a site for complex regulatory interactions.

**RDM-dependent Inhibition of C/EBP**—We initially addressed two potential models that explain the inhibitory function of the RD1 domain, the intra- and intermolecular inhibition models. The intramolecular inhibition model, originally proposed based on studies of the domain structure and regulation of C/EBPβ, suggests that physical interactions between RD1 and the N-terminal AD prevent access to the transcriptional machinery. However, we show here that the RD1 is capable of inhibiting the activity of examples of three different classes of AD, the acidic ADs of C/EBP and VP16, the proline-rich AD of CTF-1, and the glutamine-rich AD of Sp3. Since these ADs are unrelated at the sequence level, we propose that it is unlikely that direct physical interactions would occur between the RD1 domain and each AD. Instead, we propose that an intermolecular inhibition model, involving proteins that interact with specific sequences within RD1, is more likely. The best candidate for a relevant RD1-interacting protein is a member of the SUMO family, and this candidacy was strengthened by the observation that attachment of an intact RDM, but not a nonsumoylatable form of the RDM, was sufficient to replicate the inhibitory function of the entire RD1 domain. These data are consistent with observations in several other transcriptional regulatory proteins, where SUMO attachment sites have been shown to be critical functional components of transcriptional inhibitory domains. A model has recently been proposed that explains how transient SUMO attachment may mediate transcriptional repression and that takes into account the generally low steady-state level of the sumoylated form of most SUMO targets. In this model, initial transient SUMO attachment promotes association of the SUMO target with corepressor proteins or complexes. Although
we do not yet know the identity of these putative corepressors, this model is consistent with the inhibitory function of the RDM in C/EBPε.

PIASx-dependent Coactivation: RF-dependent and -independent Functions—There are four PIAS genes in mammals that encode several different proteins due to the use of alternative splicing patterns (35). The PIAS proteins can function as transcriptional coregulators in at least three ways. First, they can inhibit DNA binding by the target protein, a function ascribed to PIAS1 in inhibiting STAT1 activity (36). Second, they can function as coregulators by modulating SUMO attachment to a target protein, and most examples of PIAS-dependent coactivation have detected enhancement of SUMO attachment (see, for example, Refs. 37–39). Third, PIAS proteins can function as SUMO-independent transcriptional coregulators (40–42), and this latter function may involve recruitment of accessory proteins or localization of target proteins to subnuclear domains. Previous studies indicated that PIASγ functions as a corepressor of C/EBPε (29), but the present report provides the first evidence that PIAS proteins can function as coactivators of C/EBP family members.

PIASα and PIASβ are the products of alternatively spliced mRNAs from the same gene (35). These two mRNAs only differ at the 3'-end, where a single exon utilized in PIASα is replaced by two alternative exons in PIASβ. Consequently, the two proteins only differ at their C termini and are identical over the first 550 amino acids (33, 43). However, despite their overall similarity, these two proteins exhibited both shared and unique C/EBPε coactivation properties. The shared function was dependent on the integrity of the RING finger domain of both PIASα and PIASβ, a domain that is required for the E3 ligase function of PIAS proteins but also participates in protein-protein interactions (35). Since PIASα can enhance sumoylation of C/EBPε, we postulate that this modification may play a role in increasing C/EBPε activity. However, we will refer to this mode of coactivation as RF-dependent coactivation (Fig. 8) until a direct connection between SUMO attachment and C/EBPε coactivation can be established. The second mode of coactivation was RF-independent and was only observed with PIASβ (Fig. 8). Maximal coactivation by PIASβ required RD1 and the N-terminal AD of C/EBPε (or C/EBPα), and deletion of RD1 or replacement of the AD with the VP16 AD significantly decreased PIASβ-dependent coactivation. Since coactivation by PIASβα was relatively insensitive to the identity of the AD in the target protein, we conclude that specific functional interactions between PIASβ and the C/EBP AD are required for maximal activity. We hypothesize that these interactions involve the C-terminal domain of PIASβ that is distinct from PIASα. Interestingly, when tested as Gal4 fusions, PIASβ possessed an inherent transcriptional activating function that was lacking in PIASα (33), and this function may contribute to the enhanced activity of PIASβ as a C/EBPε coactivator. The C-terminal domain of PIASβ contains two notable features, a Ser/Thr-rich domain and a five-amino acid motif (ISLD) that is shared with PIAS1 and PIAS3. The contribution of these features to PIASβ coactivation is currently under investigation. Based on the fact that PIASβ was also a more potent coactivator of other C/EBP family members, including C/EBPα, we propose that PIASβ is a specific coactivator for C/EBPε, whereas PIASα is a more general coactivator of proteins with RD-1-like inhibitory domains. These differential activities may be important in cell types that express both isoforms of PIASx.

**Regulation of C/EBPε Activity: The Role of Sumoylation—** Our data indicate that two opposing regulatory mechanisms affecting the activity of C/EBPε, repression through the RD1 domain and coactivation by PIAS proteins, appear to act through the same sequence in C/EBPε, the RDM. As mentioned above, the only protein known to directly interact with the RDM are members of the SUMO family, and both repression and coactivation require sequences within the RDM that are part of the consensus SUMO attachment site. These observations raise the intriguing question of how the same modification could be associated with two functionally opposite effects. There are several possible explanations that will need to be addressed by further experimentation. First, repression and coactivation could be associated with attachment of different members of the SUMO family. There are now four members of this family identified in mammals, and the different proteins are likely to have different functions (25–27). We have deter-
tained that C/EBPβ can be modified by three members of the family, SUMO-1 to -3; however, we have not detected any differences in the effects of attaching different family members. Second, the difference in SUMO-dependent effects could be due to the recruitment of accessory proteins with different coregulatory properties that lead to repression or coactivation. In light of our current data, we would propose that in the absence of high levels of PIASx proteins, SUMO attachment would lead to the recruitment of coactivators, leading to elevated C/EBPβ activity.4 Clearly, the identification of coregulators recruited to C/EBPβ in a SUMO/PIASx-dependent manner should elucidate the mechanisms underlying the complex regulation of this activity of this protein.

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REFERENCES

1. Johnson, P. F., and Williams, S. C. (1994) in Liver Gene Expression (Trench, F., and Yaniv, M., eds) pp. 231–258, R. G. Landes Co., Austin, TX
2. Lekstrom-Himes, J., and Xanthopoulos, K. G. (1998) J. Biol. Chem. 273, 28545–28548
3. Williams, S. C., Cantwell, C. A., and Johnson, P. F. (1991) Genes Dev. 5, 1553–1567
4. Antonson, P., Stellan, B., Yamanaka, R., and Xanthopoulos, K. G. (1996) Genomics 35, 30–36
5. Chumakov, A. M., Grillier, I., Chumakova, E., Chih, D., Slater, J., and Koeffler, H. P. (1997) Mol. Cell. Biol. 17, 1375–1386
6. Williams, S. C., Du, Y., Schwartz, R. C., Weiler, S. R., Ortiz, M., Keller, J. R., and Johnson, P. F. (1998) J. Biol. Chem. 273, 13493–13501
7. Chih, D. Y., Chumakov, A. M., Park, D. J., Silia, A. G., and Koeffler, H. P. (1997) Blood 90, 2967–2994
8. Tave, S., Vuong, P. T., Park, D. J., Gombart, A. F., Cohen, A. H., and Koeffler, H. P. (2002) Blood 99, 1794–1801
9. Khanna-Gupta, A., Zibello, T., Sun, H., Lekstrom-Himes, J., and Berliner, N. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 8900–8905
10. Khanna-Gupta, A., Zibello, T., Sun, H., Gombart, A. F., Cohen, A. H., and Koeffler, H. P. (2003) Blood 101, 3460–3468
11. Bebeek, W., Gombart, A. F., Chumakov, A. M., Muller, C., Friedman, A. D., and Koeffler, H. P. (1999) Blood 93, 3327–3337
12. Verbeek, W., Lekstrom-Himes, J., Park, D. J., Dang, P. M., Vuong, P. T., Kawano, S., Babior, B. M., Xanthopoulos, K., and Koeffler, H. P. (1999) Blood 94, 3141–3150
13. Kuhota, T., Kawano, S., Chih, D. Y., Hisatake, Y., Chumakov, A. M., Taguchi, H., and Koeffler, H. P. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3953–3957
14. Yamanaka, R., Barlow, C., Lekstrom-Hines, J., Castilla, L. H., Liu, P. P., Eckhaus, M., Decker, T., Wynshaw-Boris, A., and Xanthopoulos, K. G. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 13187–13192
15. Gombart, A. F., and Koeffler, H. P. (2002) Curr. Opin. Hematol. 9, 36–42
16. Gombart, A. F., Shiohara, M., Kwock, S. H., Agamatsu, K., Komiyama, A., and Koeffler, H. P. (2001) Blood 97, 2561–2567
17. Lekstrom-Hines, J. A., Derman, S. F., Kopar, P., Holland, S. M., and Gallin, J. I. (1999) J. Exp. Med. 189, 1847–1852
18. Pei, D. Q., and Shih, C. H. (1991) Mol. Cell. Biol. 11, 1480–1487
19. Kowenz-Leutz, E., Twamley, G., Anseui, S., and Leutz, A. (1994) Genes Dev. 8, 2781–2791
20. Williams, S. C., Baer, M., Dillner, A. J., and Johnson, P. F. (1995) EMBO J. 14, 3170–3183
21. Angerer, N. D., Du, Y., Nalbant, D., and Williams, S. C. (1999) J. Biol. Chem. 274, 4147–4154
22. Kim, J., Cantwell, C. A., Johnson, P. F., Pfarr, C. M., and Williams, S. C. (2002) J. Biol. Chem. 277, 38037–38044
23. Iniguez-Lluhi, J. A., Lou, D. Y., and Yamamoto, K. R. (1997) J. Biol. Chem. 272, 4149–4156
24. Iniguez-Lluhi, J. A., and Pearse, D. (2000) Mol. Cell. Biol. 20, 6040–6050
25. Melchior, F. (2000) Annu. Rev. Cell Dev. Biol. 16, 591–626
26. Guo, D., Li, M., Zhang, Y., Yang, P., Eckenrode, S., Hopkins, D., Zheng, W., Purdhit, S., Podoisky, R. L., Mair, A., Wang, J., Dong, Z., Brusko, T., Atkinson, M., Pozzilli, P., Zeidler, A., Raffel, L. J., Jacob, C. O., Park, Y., Serrano-Rios, M., Larrad, M. T. M., Zhang, Z., Garchen, H.-J., Bach, J.-F., Rotter, J. I., She, J.-X., and Wang, C.-Y. (2004) Nat. Genet. 36, 837–841
27. Ehren, K. M., Nadkarni, V., Song, J. H., Gabbay, K. H., and Overbach, D. (2004) J. Biol. Chem. 279, 27233–27238
28. Girdwood, D. W. H., and Koeffler, H. P. (1999) Blood 93, 36–42
29. Tussie-Luna, M. I., Michel, B., Hakre, S., and Roy, A. L. (2002) Annu. Rev. Cell Dev. Biol. 18, 111–118
30. Eaton, E. M., and Sealy, L. (2003) J. Biol. Chem. 278, 27723–27728
31. Prado, F., Vicent, G., Cardalda, C., and Beato, M. (2002) J. Biol. Chem. 277, 16388–16390
32. Schmidt, D., and Muller, S. (2003) EMBO J. 22, 3059–3066
33. Kotaja, N., Aittomaki, S., Silvennoinen, O., Puhlwo, J. J., and Janne, O. A. (2000) Mol. Endocrinol. 14, 1886–2000
34. Hsu, W., Kerpola, T. K., Chen, P.-L., Curran, T., and Chen-Kiang, S. (1994) Mol. Cell. Biol. 14, 268–276
35. Schmidt, D., and Muller, S. (2003) Cell Mol. Life Sci. 60, 2561–2574
36. Liu, B., Liao, J., Kushner, S. A., Chung, C. D., Chang, D. D., and Purohit, S., Podolsky, R. K., Dang, P. M., Vuong, P. T., Buchner, A. F., Cohen, A. H., and Koeffler, H. P. (1999) Blood 93, 36–42
37. Megidish, T., Xu, J. H., and Xu, C. W. (2002) J. Biol. Chem. 277, 8255–8259
38. Gross, M., Yang, R., Top, I., Gasper, C., and Shuai, K. (2004) Oncogene 23, 3059–3066
39. Tussie-Luna, M. I., Michel, B., Hakre, S., and Roy, A. L. (2002) J. Biol. Chem. 277, 43185–43193
40. Duval, D., Duval, G., Kedinger, C., Pech, O., and Bocu, H. (2003) FEBS Lett. 554, 111–118