Identification of a Co-repressor That Inhibits the Transcriptional and Growth-Arrest Activities of CCAAT/Enhancer-binding Protein α*

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We used a yeast two-hybrid screening approach to identify novel interactors of CCAAT/enhancer-binding protein α (C/EBPα) that may offer insight into its mechanism of action and regulation. One clone obtained was that for CA150, a nuclear protein previously characterized as a transcriptional elongation factor. In this report, we show that CA150 is a widely expressed co-repressor of C/EBP proteins. Two-hybrid and co-immunoprecipitation analyses indicated that CA150 interacts with C/EBPα. Overexpression of CA150 inhibited the transactivation produced by C/EBPα and was also able to reverse the enhancing effect of the co-activator p300 on C/EBPβ-mediated transactivation. Analysis of C/EBPα mutants indicated that CA150 interacts with C/EBPα primarily through a domain spanning amino acids 135–150. Chromatin immunoprecipitation assays showed that CA150 was present on a promoter that is repressed by CA150 but not present on a promoter that is activated by C/EBPα. Finally, we showed that in cells in which growth arrest had been induced by ectopic expression of C/EBPα, CA150 was able to release them from growth arrest. Interestingly, CA150 could not reverse the growth arrest produced by the minimal growth-repress domain of C/EBPα (amino acids 175–217), suggesting that the effect of CA150 was directed at a region of C/EBPα outside of this minimal domain, consistent with our two-hybrid analysis. Taken together, these data indicate that CA150 is a co-repressor of C/EBP proteins and provides a possible mechanism for how C/EBPα can repress transcription of specific genes.

CCAAT/enhancer binding proteins (C/EBPs)2 are eukaryotic transcription factors that regulate a large number of genes. There are eight members in the C/EBP protein family, and the first two members that were identified, C/EBPα and C/EBPβ, are the most extensively examined and characterized (1). Although they are expressed in a variety of cell types, both family members are enriched in liver and have been demonstrated to regulate the expression of a number of genes that are associated with energy metabolism (2).

C/EBPα and -β have been generally observed to be activators of transcription. The transactivation domains of these proteins reside in the N terminus of each protein, and can function independently of the basic region-leucine zipper DNA-binding domain if linked to a heterologous DNA-binding domain (1). There appear to be several sub-domains within the transactivation domains that mediate transactivation (3–8), although the precise boundaries of these regions are difficult to determine from the available studies because they are assigned based on the arbitrary design of the mutants used. For C/EBPα, studies indicate that the majority of the transactivation potential lies within amino acid residues 1–150 (5), whereas for C/EBPβ, the transactivation domain appears to lie within amino acid residues 1–108 (9).

In addition to its transactivation role, C/EBPβ is also a strong inhibitor of cell proliferation. It inhibits proliferation of cultured cells when overexpressed, and it inhibits proliferation in newborn liver (10) and in liver of adult animals (11). C/EBPα exerts its growth-arrest activity by several different mechanisms, depending on the tissue. In liver, C/EBPα binds Cdk 2 and prevents its interaction with cyclins (12). In adipose, C/EBPα represses E2F-dependent transcription of several genes, including S phase and mitosis-specific genes (13).

Precisely how the transactivation domains of C/EBPs confer their effects onto the preinitiation complex is poorly understood. For C/EBPα, part of the basis for the constitutive activity of this domain may be explained by its ability to physically interact with the TATA-binding protein and with TFII B (3). Moreover, the transactivation potential of this C/EBP isoform can be enhanced by CBP/p300 and Retinoblastoma protein (14–16). C/EBPβ, upon binding to a promoter, can recruit CBP/p300, which leads to enhanced transactivation (15, 17). In addition, both C/EBPα and -β can recruit SWI/SNF, a chromatin remodeling complex, to gene promoters (18). C/EBPα specifically has been shown to enhance histone H3 acetylation by recruiting a co-regulator containing histone acetylase activity (19). Thus, the evidence to date suggests that there are several mechanisms for C/EBP-dependent transactivation of gene promoters.

Herein, we report the identification and characterization of a novel co-repressor for C/EBPs that was uncovered during a yeast two-hybrid screen. Not only is CA150 a transcriptional co-repressor, but it also inhibits the growth-arrest activity of C/EBPα.

EXPERIMENTAL PROCEDURES

Materials—DNA-modifying enzymes were purchased from New England Biolabs (Mississauga, Ontario, Canada) and Promega (Nepean, Ontario, Canada). Acetyl-[3H]CoA (10 Ci/mmol) was purchased from PerkinElmer Life Sciences. Tissue culture supplies were from Invitro-
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gen. HepG2 cells were acquired from American Type Culture Collection. Yeast media were from Difco (Detroit, MI), whereas the amino acids and 3-amino-1,2,4-triazole were from Sigma-Aldrich. Biotinylated oligonucleotides were purchased from Invitrogen.

Yeast Two-hybrid Assay—A human liver cDNA MATCHMAKER library, containing 3 × 10^6 independent clones, and screening kit were purchased from Clontech-BD Biosciences. The transactivation domain of C/EBPα with three residues mutated (Y67A, F77A, and L78A) (5) was cloned in-frame into the bait vector pGBT9, which resulted in the expression of a GAL4-C/EBPα bait protein. Approximately 12 × 10^6 clones were screened in yeast strain CG-1945 as per the supplier’s instructions and were doubly selected based on their ability to grow in the absence of histidine/presence of 3-amino-1,2,4-triazole (through activation of the His3 reporter gene) and intensity of blue colony formation (through activation of the lacZ reporter gene). Selected clones were verified for the requirement for both bait and prey plasmids for reporter gene activation. The plasmids containing the prey cDNA were isolated and the insert sequenced.

Quantitative β-Galactosidase Assay—A quantitative β-galactosidase assay was performed as described in the manual provided with the Clontech MATCHMAKER kit, which can be used as an indirect assessment of the strength of interaction (20). Briefly, yeast were transformed with either bait plasmid, prey plasmid, or both, and plated onto the appropriate media. Pooled or individual colonies were inoculated into 5 ml of appropriate liquid media, and cultured overnight. 2 ml of this overnight culture were then used to inoculate 8 ml of YPD liquid media and cultured overnight at 30°C with shaking. 2 ml of this overnight culture were then used to inoculate 8 ml of YPD liquid media (1% yeast extract, 2% bactopeptone, 2% dextrose), and further shaken at 30°C for 4 h. Aliquots were removed, and cell extracts were prepared and assayed for β-galactosidase activity. Units are expressed in terms of the ΔA_420 per minute per equivalent number of yeast cells. The data shown was obtained from screening of 6–10 colonies from at least two independent transformations.

The C/EBPα bait protein used in this assay was the same as that used in the two-hybrid library described above. The various GAL4-C/EBPα mutants used to identify the interaction domain have been reported previously (5). All of the deletion mutants that contained amino acid residues 67, 77, and 78 had these three residues mutated to alanines to reduce the background activity of the bait proteins, as described above. The C/EBPβ bait protein consisted of the transactivation domain of C/EBPβ (amino acids 1–108) linked to the GAL4 DNA-binding domain, whereas the CREB bait contained the transactivation domain (amino acids 3-203) linked to the GAL4 DNA binding domain. The CA150 prey protein consisted of amino acid residues 89–480 of CA150 (representing the region contained in the clone pulled out in the yeast two-hybrid screen) fused to the GAL4 transactivation domain.mutCA150 contained amino acid residues 643–1098 of CA150, linked to the GAL4 transactivation domain.

Mammalian Reporter Gene Experiments—Transfections were performed in HepG2 cells by the calcium phosphate precipitation method as described previously (21). RSV-βgal was included in all transfections as a control for transfection efficiency. The amount of each plasmid used is indicated in the appropriate figure legend. Reporter genes −68FX4 (22) and −68GX4 (5), expression plasmids for C/EBPα (23), C/EBPβ (5), CA150 (24), p300 (14), and the catalytic subunit of protein kinase A (25), as well as expression plasmids for GAL4 fusions of C/EBPα (Ge) and related mutants (5), C/EBPβ (Gβ) (9), GAL4-Sp1 (26), and CREB (G-CREB) (27) have been described previously. (The GAL4-C/EBPα mutant N150 was previously termed N135; however, resequencing of this mutant indicated that its coding region contained residues 6–150.) Assays for chloramphenicol acetyltransferase activity (CAT), β-galactosidase activity, and protein determination were performed as previously described (22).

Co-immunoprecipitation Assay—Co-immunoprecipitation of C/EBPα and CA150 was performed using mouse liver nuclear extract prepared as described previously (28). 400 μg of nuclear extract was precleared with 50 μl of a 50% slurry of Protein G-agarose for 1 h at 4°C in a total volume of 400 μl of co-immunoprecipitation buffer (20 mM Tris, pH 7.2, 1 mM EDTA, 0.1% Triton X-100, 150 mM NaCl) plus 1 mg/ml bovine serum albumin. The pre-cleared extract was then incubated overnight at 4°C with the specific antibody that had been pre-attached to Protein G-agarose beads. The beads were pelleted and washed three times with 1 ml of co-immunoprecipitation buffer. The pellet was resuspended in SDS-PAGE loading buffer, boiled, and subjected to SDS-PAGE and Western blot analysis.

Western Blot Analysis—Western blot analysis was performed as previously described (29), using Western Lightning™ chemiluminescent reagent (PerkinElmer Life Sciences) to detect the antigen-antibody complexes. Anti-C/EBPα (14AA) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-CREB antibody (9192) was purchased from Cell Signaling, and anti-CA150 antibodies (B456) were purchased from Bethyl Laboratories (Montgomery, TX).

Preparation of Nuclear Extracts—Nuclear extracts were prepared from several mouse tissues as described previously (30). Briefly, tissues were homogenized in 5 volumes of buffer with a Dounce homogenizer, and the crude nuclear pellet was isolated by centrifugation at 12,000 × g for 5 min. The pellet was resuspended in buffer containing a final concentration of 400 mM NaCl and placed on ice for 15 min. This mixture was centrifuged at 12,000 × g for 20 min. The supernatant contained the soluble nuclear protein fraction.

Growth Arrest Assay—Growth-arrest assays were performed in COS7 and HEK293 cells as previously described (30). Briefly, cells were transfected with pAdTrack-C/EBPα, which expresses both green fluorescent protein and C/EBPα from distinct mRNAs. As indicated in the figure and corresponding legend, other experiments involved co-transfection with full-length CA150 or mutCA150, both expressed as hemagglutinin-tagged proteins using a vector described by King et al. (31). After 3 days, the number of green cells per colony (1, 2, or >2) as a percent of total green cells was assessed. Preliminary co-transfection experiments were performed to determine an optimal ratio of C/EBPα and CA150 under which the inhibitory effect of CA150 is maximal. The data shown were obtained by using a 1:4 ratio of C/EBPα to CA150 expression plasmids.

Chromatin Immunoprecipitation Assay—The chromatin immunoprecipitation assay was performed with mouse liver tissues using the ChIP-IT kit (Active Motif). Briefly, the chromatin solutions were sheared by enzymatic digestion according to the instruction manual. The size of DNA fragments produced averaged between 500 and 1000 bp in length. Antibodies against C/EBPα (14AA), CA150 (N19), and cdk2 (all purchased from Santa Cruz Biotechnology) were added to each aliquot of precleared chromatin and incubated overnight. Protein G-agarose beads were added, and the mixture was incubated for 1.5 h at 4°C. After reversing the cross-links, DNA was isolated and used for PCR reactions with primers specific for PEPCK and HNF6 promoter regions that contain the C/EBPα binding sites (−204/−3 and −175/+140, respectively). The sequences of the primers for these promoters are as follows. PEPC-K/C/EBP-F: 5′-GGCTCCTCCCAA-CATTCATTAAC-3′; PEPC-K/C/EBP-R: 5′-GTAGGCCGCGCCTCTTGCTTTA-3′; HNF6-C/EBP-F: 5′-GCTCGAGCTGGCGGGCGGCACAGGCGG-3′; HNF6-C/EBP-R: 5′-AGGAGTCCAGTCTCATCAGTGGCTG-3′. As a control for the appropriate shearing of DNA, primers were designed for a region −3.4 kb upstream of the C/EBP site in the

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PEPCK promoter (−3429/−3081) and ~1.5 kb upstream in the HNF6 promoter (−1579/−1210). The sequences of these primers are as shown below. PEPCK-Control-F: 5’-AACCAACTGACCCTAATCTCACAGA-3’, PEPCK-Control-R: 5’-GCTGCAGTCCAGCTAATGCAACAA-3’, HNF-Control-F: 5’-AGGCAGGCACTTGGGTTAAGAGAT-3’, HNF-Control-R: 5’-AGAGCCTTGTCTGCTTAGGTGCTT-3’. PCR mixtures were amplified for 1 cycle of 95°C for 5 min, annealing temperature for primers (62°C) for 5 min, and 72°C for 2 min. Then PCR mixtures were amplified for 34 cycles of 95°C for 1 min, annealing temperature for 2 min, and 72°C for 1.5 min. PCR products were separated by 1.5% agarose gel electrophoresis or by 4% PAGE.

RESULTS

To search for co-regulators of C/EBPα, we employed a yeast two-hybrid approach to screen a human liver cDNA library. The “bait” used to screen the library was a fusion protein consisting of the GAL4 DNA-binding domain fused to a mutated transactivation domain of C/EBPα. This transactivation domain contained amino acids 6–217 of C/EBPα, with three amino acids (Tyr-67, Phe-77, and Leu-78) mutated to alanines. The three point mutations have been shown previously to abrogate the constitutive activity of the transactivation domain (3), yet not significantly alter the integrity of the domain based on the observation that the protein kinase A-inducible activity of this mutant remains functional (5). The use of this mutant as the bait was necessary, because the wild-type C/EBPα transactivation domain displayed significant activity in yeast (data not shown) and thus gave a high background of reporter gene expression.

Several potential interactors were obtained by this screen and sequenced, and one of these was a previously identified gene product called CA150 (32). CA150 was first characterized as a nuclear protein from HeLa cells that was associated with RNA polymerase II and involved in Tat-activated transcription of the human immunodeficiency viral promoter. It contains glutamine- and alanine-rich repeats that are characteristic of transcriptional regulators. CA150 has been shown to repress RNA polymerase II transcription, albeit in a specific fashion, because it doesn’t repress transcription of all genes (24). CA150-mediated repression of elongation requires a TATA box, and overexpression of the TATA-binding protein alleviates the repression, although there appears to be no direct physical interaction between the TATA-binding protein and CA150 (24). CA150 has been shown to bind directly to the phosphorylated carboxyl-terminal domain of RNA polymerase II.

The strength of the genetic interaction observed in the yeast two-hybrid assay between the transactivation domain of C/EBPα and CA150 was quantified and compared with that of two, well described interactors using a liquid β-galactosidase assay. This assay quantifies the amount of lacZ reporter gene activity in yeast that results from the bait and prey proteins interacting on a GAL4-driven promoter. As expected, the empty bait vector alone, expressing only the GAL4 DNA-binding domain, resulted in only a small amount of β-galactosidase activity (Fig. 1). Transformation of yeast with the CA150 prey vector (expressing the GAL4 transactivation domain fused to CA150), alone or in combination with the empty bait vector, also resulted in little activation of the lacZ reporter gene. When yeast were transformed with the mutant C/EBPα bait vector, some stimulation of β-galactosidase activity was observed, but this was not increased further with co-transformation with the empty prey vector. However, when the C/EBPα bait and
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CA150 prey vectors were co-transformed, a significant induction of lacZ reporter gene activity was observed, suggesting that an interaction between the C/EBPa and CA150 occurred (Fig. 1). Comparison of this interaction with that of the positive control TD1 and VA3 vectors, which express a p53 bait protein and large T-antigen prey protein, respectively, indicated that the degree of genetic interaction between C/EBPa and CA150 is relatively strong.

One study examining the expression of CA150 suggested that the levels of mRNA were very low in liver (33), raising the question of whether it is expressed at significant levels in this tissue. To address this issue, nuclear extracts from several mouse tissues were prepared, and Western blots were performed to assess protein levels of CA150 and to compare them with the relative levels ofCREB, a ubiquitously expressed transcription factor. As shown in Fig. 2, CA150 protein levels in liver were easily detectable and were similar to those present in kidney, brain, and lung. Heart, skeletal muscle, and spleen showed low levels of CA150, however, an abundant protein of molecular mass ~200 kDa was detected in muscle extracts. No CA150 was detected in cytosolic fractions, confirming that it localizes to nuclei (data not shown, see Fig. 9A). CREB showed a similar pattern of expression to CA150 except that spleen expression was abundant. Thus, CA150 appears to be widely expressed albeit at varying levels.

We next performed co-immunoprecipitation assays to see if we could detect complexes between C/EBPa and CA150. Mouse liver nuclear extracts were incubated with antibodies for C/EBPa, CREB, or with no antibodies, and assessed for the presence of CA150 in the immunoprecipitate. The efficacy of C/EBPa depletion of the nuclear extract was first assessed. As shown in Fig. 3A, addition of Protein G-agarose beads alone (Pre-cleared input), or co-immunoprecipitation with no antibody or CREB antibody, did not deplete the extract of C/EBPa. However, immunoprecipitation with anti-C/EBPa effectively depleted the extract. The immunoprecipitates were then analyzed for the presence of CA150 (Fig. 3B). CA150 was detected only in the immunoprecipitates obtained using anti-C/EBPa.

C/EBPa shares some amino acid sequence similarity with C/EBPa, including sub-regions within the transactivation domain (3, 6). This suggested that CA150 might also interact with this isoform. This hypothesis was tested by quantitative yeast two-hybrid analysis (Fig. 4). Transformation of yeast with the CA150 prey vector alone produced no stimulation of β-galactosidase activity. As expected, the C/EBPa bait vector, expressing a fusion protein consisting of the GAL4 DNA-binding domain fused to the transactivation domain (amino acids 1–108) of C/EBPa, stimulated the β-galactosidase reporter gene, indicating that this transactivation domain is active in yeast. When the CA150 prey and C/EBPa bait vectors were co-transformed, a significant induction of reporter gene activity was observed, suggesting that the GAL4 transactivation domain on the CA150 prey protein was recruited to the reporter gene. The specificity of this interaction was demonstrated by the lack of activation of the reporter gene observed when a CREB bait vector and CA150 prey vector were co-transformed into yeast (Fig. 4).

We next examined what effect CA150 had on the transactivation potential of C/EBPs, using transient transfection of reporter genes in HepG2 cells. Initially, we assessed the activity of CA150 on −68GAL4X4, which consists of the GAL4 DNA binding domain fused to the transactivation domain (amino acids 6–217) of C/EBPa, linked to a minimal promoter. This, it is a promoter that is highly sensitive to C/EBP-dependent transactivation. Overexpression of C/EBPa or C/EBPβ in HepG2 cells induced the activity of this promoter by ~20-fold, respectively (Fig. 5). When increasing amounts of a CA150 expression vector were co-transfected with the expression vector for C/EBPa or C/EBPβ, a dose-dependent inhibition of the transactivation produced by both C/EBP isoforms was observed (Fig. 5).

Because CA150 was shown to interact with the transactivation domain of C/EBPa and C/EBPβ in the yeast two-hybrid assays, we next examined whether CA150 could inhibit the transactivation produced by GAL4-C/EBP chimeras. To assess this, we used a GAL4 reporter gene −68GAL4X4, which consists of four binding sites for GAL4 linked to a minimal promoter. Co-transfection of HepG2 cells with a CA150 expression vector had no significant effect on the activity of this reporter gene in the absence of expression of GAL4 fusion proteins (Fig. 6). Expression of CA150, which consists of the GAL4 DNA binding domain fused to the transactivation domain (amino acids 6–217) of C/EBPa, resulted in a significant induction of reporter gene activity. This transactivation was inhibited by co-expression of CA150. Similarly, the reporter gene was activated by expression of βGa, the GAL4 fusion protein containing the transactivation domain (amino acids 1–108) of C/EBPβ, and this transactivation was inhibited ~50% by CA150 (Fig. 6). Consistent with the lack of interaction of CA150 with CREB noted in the yeast two-hybrid analysis shown in Fig. 4, CA150 had no effect on the transactivation produced by co-expression of GAL-CREB (Fig. 6).
G-CREB) and the catalytic subunit of protein kinase A, the latter which phosphorylates and activates CREB. The transactivation produced by G-Sp1, a GAL4-Sp1 chimera, was also unaffected by CA150 (Fig. 6), which further supports our hypothesis that CA150 is a specific co-repressor for C/EBPs.

The domain of C/EBPα through which CA150 interacts was next examined using a quantitative yeast two-hybrid assay to assess the ability of C/EBPα mutants (Fig. 7A) to interact with CA150. Initially, a series of C-terminal deletion mutants were examined and compared with the intact transactivation domain of C/EBPα (amino acids 6–217). Similar
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FIGURE 6. CA150 inhibits the transactivation produced by GAL4-C/EBP chimeras. HepG2 cells were transfected with the CAT reporter gene –68GX4 (3.5 μg), along with an expression plasmids for CA150 (8 μg), G6 (50 ng), Gβ (50 ng), G-CREB (1 μg), and the catalytic subunit of protein kinase A (PKA; 1 μg), or G-Sp1 in the combinations indicated. RSV-βgal (1 μg) was included in all mixtures to monitor transfection efficiency. CAT values are reported relative to the amount of CAT activity obtained when the reporter gene alone (–) was transfected, which was set to a value of 1.0. Values shown are averages ± S.E. of three independent experiments.

to that presented in Fig. 1, the CA150 prey and 6–217 domain of C/EBPα (bait) showed a strong genetic interaction in the yeast two-hybrid assay (Fig. 7B). The deletion mutant 6–150 also displayed a strong interaction with the CA150 prey, however the deletion mutant 6–135 showed a complete loss of interaction with CA150, suggesting that amino acids 135–150 of C/EBPα contain an important interaction motif. Two additional C-terminal deletion mutants, 6–124 and 6–50, also showed no interaction with CA150 (Fig. 7B). Amino-terminal deletion mutants were also analyzed to further delineate the CA150 interaction domain. A mutant containing amino acids 51–217 (i.e. lacking the first 50 amino acids) showed significant ability to interact with CA150, although not as effective as the intact transactivation domain. Further deletion to residue 97 (97–217) resulted in a significant decrease in reporter gene activity, although some interaction activity persisted with the carboxyl-terminal deletion analysis, which suggested that residues 135–150 are important for the interaction. Together, these data suggest an important role for the 135–150 domain of C/EBPα in mediating the interaction with CA150, with perhaps some contribution from the 51–96 domain.

The co-activator p300 has been reported to enhance both C/EBPα- and C/EBPβ-dependent transactivation. Therefore, we examined whether CA150 could inhibit the co-activator effect of p300 in reporter gene transfection experiments. As expected, C/EBPβ produced a strong transactiva-

tion of the pDR4 reporter gene (Fig. 8), which has multiple C/EBP binding sites in the promoter. Co-expression of p300 with C/EBPβ further enhanced this transactivation. Co-expression of CA150 was shown to significantly inhibit the transactivation produced by C/EBPβ alone and by the combination of C/EBPβ and p300 (Fig. 8). These data suggest that CA150 has a dominant effect over the co-activator function of p300. It should be noted that we were unable to consistently observe any co-activator effect of p300 on C/EBPα-dependent transactivation, despite testing a variety of different C/EBPα/p300 plasmid ratios and different reporter genes in the transfection assay (data not shown).

Chromatin immunoprecipitation (ChIP) analysis of mouse liver was employed to examine whether C/EBPα and CA150 could be detected on promoters of genes that are known to be either activated or repressed by C/EBPα. Initially we established that CA150 was localized to the nucleus in mouse liver. The Western blot shown in Fig. 9A indicated that nuclear extracts, but not cytosolic extracts, contain CA150 protein. We then performed ChIP analysis of the PEPCk promoter, which is transcriptionally activated by C/EBPα (23). As shown in Fig. 9B, PEPCk promoter fragments containing the C/EBP binding site could be detected in immunoprecipitates from nuclear extracts (prepared from two individual mice (#1 and #2)). However, anti-CA150 did not immunoprecipitate this PEPCk promoter region. As controls for the immunoprecipitation, we also repeated the ChIP using anti-cdc2 or with no antibody, which as expected resulted in no PEPCk promoter signal. As an additional control, we performed the PCR with a control set of primers that amplified a region of the PEPCk promoter ~3.4 kb upstream of the C/EBP binding sites. No signal was obtained in any of the immunoprecipitated samples. These data confirmed previous ChIP analysis (34), which showed that C/EBPα binds to the PEPCk promoter in vivo, and further suggest that CA150 is not present on this promoter.

We reasoned that CA150, as a co-repressor of C/EBPα, might only be recruited to promoters that are repressed by this transcription factor. Therefore, we next examined the HNF6 gene promoter, because it has been shown to be repressed by C/EBPα (35). As shown in Fig. 9B, both C/EBPα and CA150 were shown to be present on this promoter. No signal was obtained using primers that amplified a region 1.5 kb upstream of the C/EBP binding site, nor when no antibody was used in the immunoprecipitation step. These results suggested that CA150 is recruited to promoters that are repressed by C/EBPα.

We next examined whether CA150 could repress the growth-arrest activity of C/EBPα, in addition to its ability to repress its transactivation function. As shown in Fig. 10 (A and B), COS7 cells transfected with an empty pAdTrack vector continue to proliferate: 5 days post-transfection, >70% of the colonies consisted of two or more cells. Co-transfection of this empty vector with an expression vector for CA150 did not significantly alter the proliferative state of the cells. When C/EBPα was overexpressed in these cells, >90% of the colonies consisted of a single cell 5 days after transfection, indicating that growth arrest had occurred (Fig. 10, A and B). Co-transfection with full-length CA150 resulted in the release of cells from growth arrest, as indicated by a significant decrease in the number of colonies containing a single cell and a corresponding increase in the number of colonies containing two or more cells. C/EBPα levels were unaffected by co-expression of CA150 (data not shown), indicating that CA150 inhibited the activity of C/EBPα and not its abundance.

To test the specificity of this effect of CA150 on the growth-arrest activity of C/EBPα, a CA150 deletion mutant was generated that lacked the amino-terminal 642 amino acids, i.e. it contained amino acids 643–1098. The rationale for generating this mutant was that, because the original CA150 clone pulled out of the yeast two-hybrid screen coded
FIGURE 7. Mutational analysis of C/EBPα identifies a CA150 interaction domain. A, a schematic of the GAL4-C/EBPα bait proteins used in the yeast two-hybrid analysis. B, quantitative yeast two-hybrid analysis was performed as described in the legend to Fig. 1 and under “Experimental Procedures,” using the C/EBPα bait and CA150 prey plasmids indicated. Values shown are averages ± S.E. of at least six clones picked from two separate transformations, with the β-galactosidase reporter gene activity for each clone assayed in duplicate. Values shown are averages ± S.E. of three independent experiments.
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FIGURE 8. CA150 inhibits the p300-mediated enhancement of C/EBPβ transactivation. HepG2 cells were transfected with the CAT reporter gene –68FX4 (3.5 µg), along with an expression plasmid for CA150 (8 µg), p300 (5 µg), and C/EBPβ (0.25 µg) in the combinations indicated. RSV-βgal (1 µg) was included in all mixtures to monitor transfection efficiency. CAT values are reported relative to the amount of CAT activity obtained when the reporter gene alone (−) was transfected, which was set to a value of 1.0. Values shown are averages ± S.E. of three independent experiments.

FIGURE 9. CA150 associates with a promoter that is repressed by C/EBPα. ChIP analysis was performed on mouse liver DNA. A, a Western blot was performed to identify the presence of CA150 in cytosolic (C) or nuclear (N) fractions (100 µg) of the mouse liver homogenates. The corresponding positions of the molecular weight markers are shown on the left. B, the results of ChIP analysis from two mouse livers (#1 and #2) are shown. The cross-linked DNA fragments were immunoprecipitated with either no antibody (No ab), or with anti-C/EBPα, -CA150, or -cdk2. The immunoprecipitated fragments were then subjected to PCR with primers directed against the C/EBP binding region of the PEPCK or HNF6 promoters, or with control primers directed at a region ~1.5 kb upstream of each corresponding C/EBP site. The PCR signal from each input set of DNA samples is shown in the first lane.

for amino acids 89–480, we hypothesized that the carboxyl-terminal region of CA150 would not possess the interaction domain. This mutant was first tested in the quantitative, two-hybrid β-galactosidase assay, and shown to have significantly reduced ability to interact with C/EBPα relative to wild-type CA150 (Fig. 10C). Furthermore, this mutant was unable to repress the transactivation of Gα when tested by reporter gene assay in HepG2 cells, whereas full-length CA150 with overexpression of C/EBPα resulted in >90% of the colonies consisting of single cells, the same as that observed with overexpression of C/EBPα alone.

The ability of CA150 to reverse C/EBPα-dependent growth arrest was also observed in HEK293 cells (Fig. 11, A and B). In these cells, ectopic expression of C/EBPα resulted in >90% of the colonies consisting of one cell. Co-expression of CA150 co-expression released cells from this growth arrest, such that almost 80% of the colonies consisted of two or more cells, whereas the mutant CA150 lacked this ability. Similar results were obtained using 3T3-L1 cells (data not shown). Thus, the ability of CA150 to inhibit C/EBPα-dependent growth arrest does not appear to be a cell-type specific.

It is possible that CA150 overrides C/EBPα-mediated growth arrest without any direct effect on C/EBPα itself but through a different mechanism. To examine this issue, we took advantage of the findings from one of our earlier studies that showed that the growth-arrest inhibitory domain of C/EBPα lies within a short proline-rich region extending from residue 175 to 217 and that this domain alone is sufficient to inhibit cell proliferation (12). Because our two-hybrid data in Fig. 7B suggested that CA150 interacts with C/EBPα through residues 135–150, it was predicted that CA150 should not be able to reverse the growth-arrest activity of this short domain if it exerts its effects directly on C/EBPα, but would be able to if it exerted its effects through an indirect mechanism. COS7 cells were transfected with a GAL4–175-217 fusion (Fig. 12A) expression plasmid, and the number of cells per colony was assessed after 5 days. Similar to our previous observations, this short region mediated growth arrest, with >90% of colonies consisting of only one cell (Fig. 12B). Interestingly, co-expression of CA150 was able to reverse the growth arrest produced by this short domain, suggesting that this short domain does not contain the CA150 interaction domain, consistent with our two-hybrid interaction data. Furthermore, it suggests that CA150 regulates cell proliferation by direct targeting of C/EBPα.

DISCUSSION

Eukaryotic transcription factors have been demonstrated to exert their effects by making contact with one or more components of the preinitiation complex, which through a variety of mechanisms lead to altered rates of transcription of their target gene. In many cases, transcription factors contact the preinitiation complex indirectly through so-called “bridging factors” or co-regulators. Certain co-regulators...
mediate the induction of transcription and thus are appropriately termed co-activators, whereas others lead to the inhibition of transcription and are thus classified as co-repressors (36). These co-regulators exert their effects by several mechanisms. They have been shown to possess transactivation or inhibitory domains, which directly contact components of the preinitiation complex. Additionally, these co-regulators can possess histone-modifying activities and/or recruit other proteins that possess these activities, which can lead to altered transcription through chromatin remodeling mechanisms (37).

The C/EBP family of transcription factors, themselves larger members of the basic region-leucine zipper family of proteins, have been extensively investigated. The α and β members have received special attention because of their role in initiating and regulating adipocyte differentiation, terminal differentiation of certain other cell types, and their involvement in metabolic gene regulation (38, 39). While their role in regulating these processes is reasonably well understood, precisely how they exert their transcriptional effects is not. What we do know is that their transactivation domains lie in the amino terminus, whereas the DNA binding and dimerization motifs lie in the carboxyl terminus (1). Moreover, studies using GAL4 fusion approaches suggest that, for the most part, these two domains function independently of one another (4, 8, 9, 40). In the case of C/EBPα, the transactivation domain has been shown to be capable of physically interacting with two components of the preinitiation complex, the TATA-binding protein and TFII B (3). Point mutations in C/EBPα residues within a domain encompassing amino acids 67–78 severely abrogate the strength of these interactions and reduce the transactivation potential of C/EBPα (3). These findings suggest that one mechanism whereby C/EBPα transactivates genes is through these contacts made with the preinitiation complex.

Several co-activators for C/EBPα have been identified. The first was p300/CBP, which was based on the observation that overexpression of p300 enhanced the transactivation potential of C/EBPα (15). A similar potentiation of C/EBPα transactivation of the leptin promoter by p300 was observed by Erickson et al. (14). In this study, analysis of C/EBPα mutants suggested that p300 functionally interacted with multiple regions of the transactivation domain (amino acids 1–240). The Retinoblastoma protein is also a potential co-regulator of C/EBPα. Chen et al.
(16) showed that the Retinoblastoma protein enhances the binding activity of C/EBPα in vitro and potentiates the transactivation potential of C/EBPα. Moreover, a physical interaction between the two proteins was demonstrated by co-immunoprecipitation. An additional biological piece of evidence comes from studies showing that 3T3-L1 preadipocytes that are deficient in Retinoblastoma protein are defective in their ability to differentiate into adipocytes, a process that also requires C/EBPα activity (41). In the case of C/EBPβ, p300 appears to be a co-activator, based on its ability to enhance C/EBPβ-dependent transactivation and physically interact with multiple regions in the amino terminus of C/EBPβ (15). TIF1β has also been demonstrated to be a co-activator of C/EBPβ (42). However, this co-activator appears to interact with the bZIP domain of C/EBPβ.

In the present study, we report the discovery and characterization of CA150 as a co-repressor for both C/EBPα and -β. CA150 was first identified as a nuclear protein from HeLa cells that was associated with RNA polymerase II and regulated Tat-activated transcription of the human immunodeficiency virus promoter (32). CA150 contains glutamine- and alanine-rich repeats that are characteristic of transcriptional regulators. CA150 has been identified as an elongation factor that represses RNA polymerase II transcription, although in a specific fashion, i.e. it does not repress transcription of all genes (24). CA150-mediated repression of elongation requires a TATA box, and overexpression of TATA-binding protein can alleviate the repression, although no direct physical interaction between TATA-binding protein and CA150 has been demonstrated (24). CA150 has been shown to bind directly to the phosphorylated carboxyl-terminal domain of RNA polymerase II, consistent with it being associated with the elongation form of RNA polymerase II (43).

A previous study examining the tissue expression pattern of human CA150 by Northern blot analysis suggested that CA150 was widely expressed, but lowest levels were observed in liver and lung (33). In the present study, we examined protein levels in nuclear extracts from several mouse tissues, and found that these two tissues along with brain and kidney contained some of the highest amounts of CA150. It is unclear whether these differences reflect species differences or post-transcriptional regulatory control of CA150 expression. Most importantly, though, our analysis did confirm CA150 expression in liver, which at least affords it the opportunity to be a co-regulator of liver-enriched C/EBPα.

Several different pieces of evidence are presented in this report to support the hypothesis that CA150 is a co-repressor for C/EBP proteins. First, the results from two-hybrid analysis provided a genetic approach that suggested that the two proteins interact with each other, which was confirmed by co-immunoprecipitation experiments. Transient transfection assays indicated that CA150 specifically repressed the transactivation produced by C/EBPs, because transactivation by protein kinase A-activated CREB and Sp1 were unaffected by CA150. Suné and Garcia-Blanco (24) also showed that CA150 does not repress transcription from all promoters but rather shows selectivity. Thus, while the cytomegalo-
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A virus, SV40, RSV, and herpes simplex virus thymidine kinase promoters were unaffected by CA150, the human immunodeficiency virus-1 promoter displayed sensitivity to repression by CA150. Third, the data in Fig. 10 indicated that mutant CA150, which was defective in its ability to interact with C/EBP in a two-hybrid assay and repress C/EBP-mediated transactivation, was also defective in its ability to release cells from C/EBP-dependent growth arrest. Thus, a significant body of evidence supports our hypothesis that CA150 interacts with C/EBP transcription factors, thereby repressing their activities.

The mutational analysis of C/EBPs that was performed to identify the CA150 interacting domain(s) suggested a critical role for residues 135–150, with a secondary role for the domain extending from 51 to 96. Examination of these regions indicated that both contain an LXXL motif. Moreover, two LXX(L/I) motifs are also contained within amino acids 1–108 of C/EBPB, which was shown in the present study to both interact with CA150 in a two-hybrid assay (Fig. 4) and to be sensitive to transcriptional repression by CA150 (Fig. 5). Thus, the LXXL motifs within C/EBPs may serve as the interface through which CA150 binds. It is of interest to note that a similar motif, LXXLL, was initially characterized in nuclear receptors, where it resides within the ligand-binding domain and mediates interactions with co-regulators (44). Thus, the LXXLL-related motif may serve as a general co-regulator docking site used by a variety of transcription factors.

A question of biological relevance that arises from the present study is why would a transcription factor like C/EBPα that transactivates genes require a co-repressor? While it is true that C/EBPα has generally been shown to function as a transcriptional activator, there have been at least two reports of transcriptional repression by C/EBPα. Rastegar et al. (35) reported that C/EBPα binds to the HNF6 promoter at two sites, and when C/EBPα was expressed ectopically, it was able to inhibit an HNF6 luciferase reporter gene in hepatoma cells. In the present study, we showed by ChIP analysis that both C/EBPα and CA150 were bound to the HNF6 promoter. However, when the PEPCK promoter, which is activated by C/EBPα (23), was examined, C/EBPα alone was detected. Thus, CA150 may only be recruited to promoters that are repressed by C/EBPα, thereby providing a potential mechanism to explain how this transcription factor can in some cases act as a repressor. Other potential gene targets for repression by the C/EBPα-CA150 complex exist, such as the rat placental glutathione S-transferase gene promoter, which is repressed by C/EBPα in normal liver (45). What remains to be determined is what recruits CA150 to promoters that are targets for repression by C/EBPα and not to those that are activated.

The other interesting finding reported herein is that CA150 repressed both biological activities of C/EBPα. C/EBPα is unique as a transcription factor in that it plays two distinct roles within the cell. Although its transcriptional activity was the first to be characterized, more recently it has been uncovered that it also plays a role in regulating the cell cycle. When overexpressed in cultured cells, it represses cell proliferation, which is consistent with it being highly expressed in terminally differentiated cells such as adipocytes and hepatocytes, and poorly expressed in proliferating cells. This growth-arrest activity of C/EBPα, which does not require its transcriptional activity (12), resides within residues 175–217, which is proline-rich. This short domain of C/EBPα blocks cell proliferation by interacting with cdk2 and cdk4 and forming inactive

FIGURE 12. CA150 is unable to repress the activity of the minimal growth-arrest domain of C/EBPα. A, a schematic of full-length, wild-type C/EBPα is shown, with the growth-arrest inhibitory domain (amino acids 175–217) shown in black and the bZIP domain highlighted by diagonal lines. Below this is a schematic of the GAL4-175-217 fusion protein. B, the bar graph shows a summary of three independent experiments in which the growth-arrest activity of the GAL4-175-217 fusion protein was examined in COS7 cells, in the absence and presence of CA150 as described in the legend to Fig. 10. Values shown are the means ± S.E.
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cell cycle control. The ability of CA150 to block C/EBPα-mediated growth arrest suggests yet another layer of regulation for cell cycle control.

Precisely how CA150 exerts its inhibitory effects on C/EBP is not known. However, the fact that it represses two very distinct activities of C/EBPα, which are conferred by different domains of this transcription factor, without altering the abundance of C/EBPα suggests that the mechanism whereby CA150 represses C/EBPα is through a general effect. Possibilities include inducing a conformational change in C/EBPα that affects overall protein structure and function and/or by simultaneously, due to its larger size, preventing co-activators, general transcription factors, and Cdk5 from interacting with C/EBPα. Future experiments will be aimed at further delineating the one or more mechanisms whereby CA150 is able to repress the dual activities of C/EBPα.

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