CCAAT/Enhancer-binding Protein Family Members Recruit the Coactivator CREB-binding Protein and Trigger Its Phosphorylation*

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CCAAT/enhancer-binding protein (C/EBP) family members are transcription factors involved in important physiological processes, such as cellular proliferation and differentiation, regulation of energy homeostasis, inflammation, and hematopoiesis. Transcriptional activation by C/EBPα and C/EBPβ involves the coactivators CREB-binding protein (CBP) and p300, which promote transcription by acetylating histones and recruiting general transcription factors. In this study, we show that C/EBPδ is also using CBP as a coactivator. Based on sequence homology with C/EBPα and -β, we identify in C/EBPδ two conserved amino acid segments that are necessary for the physical interaction with CBP. Using reporter gene assays, we demonstrate that mutation of these residues prevents CBP recruitment and diminishes the transactivating potential of C/EBPδ. In addition, our results indicate that C/EBP family members not only recruit CBP but specifically induce its phosphorylation. We provide evidence that CBP phosphorylation depends on its interaction with C/EBPδ and define point mutations within one of the two conserved amino acid segments of C/EBPδ that abolish CBP phosphorylation as well as transcriptional activation, suggesting that this new mechanism could be important for C/EBP-mediated transcription.

The CCAAT/enhancer-binding protein (C/EBP) family is composed of pleiotropic transcription factors involved in tissue-specific metabolic gene transcription, in signal transduction activated by several cytokines, and in cell differentiation (for a review, see Refs. 1–7). Six members of the family have been described so far: C/EBPα, C/EBPβ, C/EBPδ, C/EBPγ, C/EBPε, and C/EBPζ (8). C/EBP isoforms bind to their cognate DNA element through a bipartite domain called bZIP. This domain consists of a basic region, contacting DNA, and a homo- or heterodimer-forming region called the leucine zipper (9). Because of the high conservation in the bZIP domain, C/EBP family members are able to form homo- or heterodimers, and all, except C/EBPζ, bind to the same cis-regulatory elements. C/EBPα, C/EBPβ, and C/EBPδ are involved in terminal differentiation of a variety of cells including adipocytes (10), hepatocytes (11, 12), gut epithelial cells (13), macrophages (14), myelomonocytes (15), and neurons (16, 17). In the nervous system, the role of C/EBP family members is not characterized as well as, for instance, in adipocytes or hepatocytes. However, a recent study suggests that they are essential for cortical progenitor cells to become postmitotic neurons (16). Interestingly, certain C/EBP isoforms appear to be involved in learning and memory processes (18–20), glial or neuronal cell functions (21–23), and neurotrophic factor expression (24).

Knock-out mice were generated for different C/EBP isoforms (reviewed in Refs. 7, 8, and 10). These C/EBP-deficient mice display various phenotypes extending from perinatal lethality (for C/EBPα) to subtle abnormalities. These different phenotypes suggest that C/EBP family members are not functionally redundant, which, to a certain extent, was confirmed by studies in cell cultures (reviewed in Ref. 7). Because the DNA binding domain of the C/EBP isoforms is highly similar, these functional differences must be mostly due to specific properties of their transactivation domain.

Relatively little is known about the way C/EBP family members activate transcription. C/EBPα interacts with TBP and TFIIIB, two major components of the general transcription machinery (25). Moreover, C/EBPα and C/EBPβ were shown to recruit the chromatin remodeling complex SWI/SNF (26, 27). Modification of chromatin is an important step in the activation of gene transcription. Unlike ATPase/helicase-type remodeling complexes such as SWI/SNF, other large complexes contain proteins with histone acetyltransferase (HAT) enzymatic activity (28). These coactivators acetylate the histone tails of nucleosomes, thus favoring chromatin remodeling and activation of transcription. CREB-binding protein (CBP) and p300 belong to this class of coactivators. CBP was first identified through its ability to bind to the transcription factor CREB (29). Since then, several other transcription factors were shown to require CBP for efficient transactivation (for a review, see Refs. 30–33). CBP functions as an adaptor between the tissue- and sequence-specific transcription factors and the general transcriptional machinery. Therefore, CBP is believed to activate gene transcription by recruiting basal transcription factors (TFIIB, TBP, and RNA polymerase II holoenzyme), by modifying chromatin structure via histone acetylation, and finally, by recruiting other histone acetyltransferases, such as SRC-1,
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EXPERIMENTAL PROCEDURES

Plasmids—pGL3-5×C/EBP was constructed by inserting five copies of a consensus C/EBP-binding site flanked by BamHI sites (5′-AGATCTCAGATGGCAATCTAGGATC-3′) into the BamHI site of pGL3-Promoter vector (Promega). pcDNA3-HA-CBPα, pcDNA3-HA-CBPβ, and pcDNA3-HA-CBPγ were constructed by inserting an oligonucleotide encoding the HA epitope at the N terminus of the C/EBP coding regions and ligating the fusion cDNAs into pcDNA3. pcDNA3-CBP-2×FLAG was constructed by inserting two FLAG epitopes at the C terminus of the C/EBP coding region. To create pGEX-C/EBPβ22-227, pGEX-C/EBPβ22-196, pGEX-C/EBPβ22-168, and pGEX-C/EBPβ168-196, PCR-generated fragments encoding amino acids 22–227, 22–196, or 22–168 of mouse C/EBPβ or amino acids 1–196 of mouse C/EBPα were inserted into the NotI/EcoRI sites of pGEX-4T3 (Amerham Biosciences). To construct pcDNA3-CBP1680-2241, 2×FLAG, pcDNA3-CBP1680-2241, 2×FLAG, pcDNA3-CBP1680-2241, 2×FLAG, or pcDNA3-CBP1680-2241, 2×FLAG, PCR-generated fragments encoding amino acids 1680–2241, or 1893–2441 of CBP were inserted into a modified pcDNA vector containing sequences encoding a nuclear localization sequence at the N terminus and two FLAG epitopes at the C terminus. MSV–LIP was described previously (21), pRC/RSV-FLAG-C/EBPβ41 was described by Loriaux et al. (41), and pRc/RSV-ATF-1 was described by Rehfs et al. (42). The Gal4-LUC (luciferase) reporter was a kind gift of G. Waeber (Department of Internal Medicine B, University Hospital, Lausanne, Switzerland) and was described by Bonny et al. (43). pcDNA3-Gal4 encodes the Gal4 DNA binding domain (amino acids 1–147). pcDNA3-Gal4-β1was generated by inserting a BamHI/HindIII fragment from pGEX-C/EBPβ168-196 into pcDNA3-Gal4. Deletions or alanine mutants of pGEX-C/EBPβ168-196 and pcDNA3-HA-CBPβ were generated by site-directed mutagenesis (Stratagene). The corresponding pcDNA3-Gal4-CBPβ168-112 mutants were constructed from the pGEX-C/EBPβ168-112 mutants. All of the constructs were verified by sequencing.

Cell Culture and Transfection Assays—HEK 293T cells were cultured on gelatin-coated plates in high glucose GLUTAMAX® Dulbecco’s modified Eagle’s medium’s (Invitrogen) containing 10% heat-inactivated newborn calf serum (Invitrogen), 100 units/ml penicillin G, and 100 μg/ml streptomycin sulfate (Invitrogen). Calcium phosphate transfection assays were performed according to an improved protocol described by Jordan et al. (44). Briefly, 293T cells were seeded at 8 × 10⁴ cells/ml 24 h prior to transfection and were transiently transfected with 8 μg of total DNA. The cell culture medium was changed 8 h later, and 24 h after transfection, cellular lysates were prepared for luciferase assays or immunoblot analysis.

Luciferase Assays—Transfected cells were washed with PBS, lysed in 250 μl of 1× cell culture lysis buffer (Promega), and centrifuged in a microcentrifuge for 2 min at 4 °C. To test the samples for luciferase activity, 20 μl of the 250× diluted supernatant was assayed in a Turner-Designs TD-20/20 luminometer using 100 μl of luciferase assay reagent (Promega). Luciferase activity was normalized to total cellular protein (Bio-Rad protein assay). All experiments were performed in triplicate.

Preparation of Cellular Extracts—PC12 cell nuclear extract was prepared according to Dignam et al. (45). To perform experiments with recombinant proteins expressed in 293T cells, whole cell extracts were prepared by lysing the cells in 50 mM Hepes, pH 7.6, 250 mM NaCl, 0.2 mM EDTA, 0.5% Nonidet P-40, 10 μM NaF, and 10 μM Na3VO4 for 30 min at 4 °C. Then the extracts were cleared by centrifugation at 16,000 × g for 10 min. To prevent proteolysis, a protease inhibitor mixture for mammalian tissue (Sigma) and calpain inhibitor were included in the lysis buffer.

Immunoblot Analysis—After separation on SDS-polyacrylamide gels (SDS-PAGE), the proteins were transferred to polyvinylidene difluoride membranes with a semidry blotting system (Bio-Rad) for 45 min at 20 V as in Cardinaux et al. (46). Blots were blocked overnight at 4 °C in TBST containing 10 mM Tris-HCl, 150 mM NaCl, pH 7.4, and 0.05% Tween 20, supplemented with 10% skim milk powder and 1% bovine serum albumin. Blots were subsequently incubated with a primary antibody in TBST plus 1% skim milk powder for 2 h at room temperature. Full-length CBP was detected with an anti-CBPα polyclonal antibody as in Cardinaux et al. (47). FLAG-tagged CBP fragments were detected with anti-FLAG M2 monoclonal antibody (Sigma), and HA-tagged CBP isoforms were detected with anti-HA rat monoclonal antibody (Roche Applied Science). Finally, polyvinylidene difluoride membranes were incubated with horseradish peroxidase-conjugated secondary antibodies and developed using chemiluminescence detection kits (ECL (Amerham Biosciences) or SuperSignal® West Femto (Pierce)).

RESULTS

CBP Coactivates C/EBPα, C/EBPβ, and C/EBPβ-mediated Transcription—p300 and CBP are transcriptional coactivators for C/EBPα and C/EBPβ (34–40). To investigate whether C/EBPβ is activating transcription through the same mechanisms, we performed a series of reporter gene assays using a synthetic C/EBP-responsive luciferase reporter gene (pGL3–5×C/EBP), which contained five copies of a consensus C/EBP-binding site in front of the SV40 promoter and the luciferase gene. 293T cells were transiently transfected with this reporter and the same amount of expression vectors encoding HA-tagged C/EBPα, C/EBPβ, or C/EBPβ (Fig. 1). C/EBPα and C/EBPβ increased luciferase gene expression by 2-fold, whereas C/EBPβ-mediated transcription was 12-fold higher than basal luciferase activity. Regardless of these differences in transcriptional activity, overexpression of CBP increased C/EBP-mediated transcription by 2–3-fold for each C/EBP fami.
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C/EBPα, C/EBPβ, and C/EBPδ Modify the Electrophoretic Mobility of CBP—While we were studying C/EBP-CBP interactions, we noticed that the migration of CBP in a SDS-polyacrylamide gel was affected when it was coexpressed with C/EBPα, C/EBPβ, or C/EBPδ (Fig. 3). To better characterize this change in mobility, we next tried to find a CBP fragment displaying a similar shift in the presence of C/EBPδ. We first tested a fragment encoding amino acids 1860–1892 of CBP, because we had shown in Fig. 2 that it was interacting with C/EBPδ. However, no mobility shift was observed with this CBP fragment (Fig. 4). Then we tested several CBP constructs, and, interestingly, we found that the electrophoretic mobility of CBP1860–2441, containing the E1A binding domain and the C terminus of CBP, was modified when it was coexpressed with C/EBPα, C/EBPβ, or C/EBPδ (Fig. 4B). CBP1893–2441 was not shifted in the presence of C/EBPδ, demonstrating that amino acids 1860–1892 were required to induce a mobility shift of CBP1860–2441. On the whole, these data suggest that by interacting with the E1A binding domain of CBP, C/EBP family members are able to recruit CBP.

C/EBPα, C/EBPβ, and C/EBPδ Interact with the E1A Binding Domain of CBP—The amino terminus of C/EBPβ interacts with the E1A binding domain of p300 (35). However, interactions of C/EBPβ or C/EBPδ with CBP have not yet been shown. To bridge this gap, we performed a series of GST pull-down experiments with various purified proteins containing the transactivation domain of C/EBPβ or C/EBPδ fused to GST (Fig. 2). First, we showed that the amino acids 1–196 of C/EBPδ were interacting with CBP from a PC12 cell nuclear extract (Fig. 2A), suggesting that C/EBPδ might recruit CBP in vivo. It was shown that C/EBPβ recruits p300 through its E1A binding domain (35). To test whether C/EBPβ or C/EBPδ interact with the same region of CBP, we then expressed in HEK 293T cells a FLAG-tagged protein containing CBP amino acids 1860–1892. A whole cell extract was prepared and incubated with GST-C/EBPδ1–196 or various deletions of the C/EBPδ transactivation domain fused to GST (Fig. 2B). A similar interaction with CBP1680–1892 was observed for C/EBPβ22–227, C/EBPβ22–193, and C/EBPδ1–196, whereas further deleting C/EBPδ to amino acid 103 reduced significantly its interaction with the E1A binding domain of CBP. Together, these experiments strongly suggest that C/EBPδ as well as C/EBPδ recruit the coactivator CBP through its E1A binding domain.

To confirm that C/EBPβ and C/EBPδ are able to interact with the E1A binding domain of CBP directly, without the help of accessory nuclear factors, CBP1680–1892 was translated in vitro and labeled with [35S]methionine (Fig. 2C). GST-C/EBPβ22–227 and GST-C/EBPδ1–196 both pulled down the CBP fragment, thus demonstrating a direct interaction between C/EBPβ or C/EBPδ and the E1A binding domain of CBP.

CBP Potentiates C/EBP-Mediated Transcription. HEK 293T cells were transfected as described under “Experimental Procedures” with 2 μg of pGL3-5×C/EBP and 0.25 μg of pcDNA3-HA-C/EBPα, β, or δ, or in the presence (+) of 4 μg of pcDNA3-CBP-2×FLAG. pcDNA3 was used to set the total amount of DNA to 8 μg for each condition. Results are displayed as the mean ± S.E. (n = 3) relative luciferase activity. Values are normalized for protein levels. Note that these results were obtained in the same experiment but that a different scale was used for C/EBPδ for presentation purposes.

Fig. 1. CBP potentiates C/EBP-mediated transcription. HEK 293T cells were transfected as described under “Experimental Procedures” with 2 μg of pGL3-5×C/EBP and 0.25 μg of pcDNA3-HA-C/EBPα, β, or δ, or in the presence (+) or in the presence (+) of 4 μg of pcDNA3-CBP-2×FLAG. pcDNA3 was used to set the total amount of DNA to 8 μg for each condition. Results are displayed as the mean ± S.E. (n = 3) relative luciferase activity. Values are normalized for protein levels. Note that these results were obtained in the same experiment but that a different scale was used for C/EBPδ for presentation purposes.

Fig. 2. CBP interacts with C/EBPβ and C/EBPδ through its E1A binding domain. A, binding of full-length CBP (CBPFL) from PC12 nuclear extract to GST-C/EBPδ1–196. The specificity of the interaction was confirmed with a negative control consisting of GST alone. B, binding of the E1A binding domain of CBP (CBP1680–1892) expressed in HEK 293T cells to various GST-C/EBPβ constructs or to GST-C/EBPδ1–196. FLAG-tagged CBP1893–2441 was detected by immunoblotting using monoclonal anti-FLAG antibody (top). Coomassie Brilliant Blue-stained GST proteins are shown in the bottom panel. The positions of molecular mass markers (in kDa) are shown on the left. C, GST pull-down experiment with in vitro translated CBP1680–1892 and the indicated GST proteins.

Fig. 3. C/EBP isoforms alter the electrophoretic mobility of CBP. HEK 293T cells were cotransfected with 1.5 μg of pcDNA3-CBP-2×FLAG and 7.5 μg of pcDNA3-HA-C/EBPα, β, or δ (or pcDNA3 (−)) using the calcium phosphate method. Cellular extracts were prepared 24 h after transfection, and proteins were analyzed by SDS-PAGE followed by immunoblotting using monoclonal anti-FLAG (top) or anti-HA (bottom) antibodies. The position of full-length CBP (CBPFL) in the absence of any C/EBP isoform is indicated by a black arrow, whereas the slow migrating form of CBP is marked by an empty arrow.
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HEK 293T cells were cotransfected with 1.5 μg of the indicated FLAG-tagged C/EBP fragments and 7.5 μg of the HA-tagged CBP isoforms (or pcDNA3 (−)) as in Fig. 3. Cellular extracts were prepared 24 h after transfection, and proteins were analyzed by SDS-PAGE followed by immunoblotting using monoclonal anti-FLAG antibody. The position of CBP1680–2441, in the absence of any C/EBP isoform is indicated by a black arrow, whereas the slow migrating forms of CBP1680–2441 are marked by an empty arrow.

The Mobility Shift of CBP1680–2441 Is Due to Phosphorylation—The most common covalent modification of proteins that induce a mobility shift in SDS-polyacrylamide gel electrophoresis is phosphorylation. To determine whether the slow migrating forms of CBP1680–2441 were actually generated by phosphorylation, we tested whether the mobility shift observed in the presence of the C/EBP family members was affected by phosphatase treatment. As shown in Fig. 5, shrimp alkaline phosphatase totally abolished the slow migrating forms of CBP1680–2441, observed when C/EBPα, C/EBPβ, or C/EBPδ was coexpressed with this CBP fragment. This experiment thus demonstrated that the C/EBP-induced mobility shift is due to CBP phosphorylation.

To exclude a nonspecific effect of protein overexpression, we then transfected a series of constructs expressing different proteins together with CBP1680–2441 (Fig. 6). First, we showed that the truncated form of C/EBPβ, called LIP (48), which does not contain a transactivation domain, did not trigger a mobility shift in CBP1680–2441. Moreover, CREB and ATF-1 that interact with the CREB-binding domain of CBP did not affect the mobility of the CBP fragment. These data thus confirmed that CBP phosphorylation was specifically triggered by the coexpression of transcriptionally active C/EBP family members.

The transcriptional activation of a protein kinase by the C/EBP isoforms could explain the appearance of the slow migrating forms of CBP. To test this possibility, we coexpressed CBP1680–2441 together with a fusion protein consisting of the Gal4 DNA binding domain and amino acids 1–152 of C/EBPδ (Fig. 6, last lane). The bZIP DNA binding domain of CBP is lacking in this deletion mutant; therefore, it cannot activate any cellular genes. The mobility shift of CBP was still induced by Gal4-C/EBPδ1–152 thus excluding a C/EBP-mediated transcriptional activation of a kinase. This last piece of data suggests that CBP phosphorylation is triggered by its interaction with the transactivation domain of C/EBPδ even if C/EBPδ is not bound to DNA.

Two Conserved Amino Acid Segments of C/EBPδ Are Critical for C/EBP Recruitment—Nerlov and Ziff (25) defined two regions highly conserved among C/EBPα, C/EBPβ, and C/EBPδ that they called box A and box B. Interestingly, deletions or mutations within these regions strongly decrease C/EBPα and C/EBPβ transcriptional activity (25, 36, 40, 49, 50). Therefore, we first asked whether these conserved regions, known to be necessary for C/EBPα- and C/EBPβ-mediated gene transcription, were equally important for C/EBPδ. As shown in Fig. 7A, in C/EBPδ, box A extends from amino acid 54 to 67, whereas box B consists of the amino acids 81–86. We performed a series of GST pull-down experiments and showed that deletion of box A or box B almost completely abolished the interaction of C/EBPδ with full-length CBP from PC12 cell nuclear extract and strongly diminished the interaction with CBP1680–152 (Fig. 7B). Furthermore, deletion of box A or box B markedly decreased the transcriptional activity of the corresponding Gal4-C/EBPδ1–152 mutants (Fig. 7C).

We then introduced some point mutations into C/EBPδ based on previously described mutations in C/EBPα (25). In this C/EBPδ isoform, Tyr67 of box A and Phe77 and Leu78 of box B were shown to be important for the interaction with TBP and TFIIIB as well as for the activation of transcription. Introducing corresponding alanine point mutations into C/EBPδ by changing Tyr67 (mut 1), Leu77, and Phe78 (mut 2), or all three amino acids (mut 3) strongly diminished binding of full-length CBP as well as the interaction with CBP1680–152 (Fig. 7B). Mutation of Leu61 and Phe62 in box B (mut 2) was clearly deleterious for the transcriptional activity of Gal4-C/EBPδ1–152 in contrast to the effect of Tyr67 mutation, which is more difficult to understand (Fig. 7C). Tyr67 mutation on its own did not impair Gal4-C/EBPδ1–152 activity, whereas the incorporation of this mutation into mut 2 further reduced its transcriptional activity (mut 3). Therefore, these data highlighted the importance of Leu61 and Phe62 for C/EBPδ-mediated transcription, showing a role...
C/EBP\(\beta\) Box B Is Required for CBP Phosphorylation—Having defined deletion and alanine mutations that affect CBP recruitment and transcriptional activity, we next tested the effect of these mutations on the ability of C/EBP\(\beta\) to induce CBP phosphorylation. Surprisingly, deletion of box A (Δ54–67), which strongly reduced the interaction with CBP in the GST pull-down experiments, did not abolish the mobility shift of CBP1680–2441 (Fig. 8A). In contrast, deletion of box B (Δ81–86) or both box A and box B resulted in a strong inhibition of CBP phosphorylation. These results therefore suggest that CBP phosphorylation mostly rely on the integrity of box B. This was confirmed by testing the effect of Gal4-C/EBP\(\beta\)1–152 alanine point mutations that were coexpressed with CBP1680–2441 in HEK 293T cells. As shown in Fig. 8B, replacement of Leu\(^{61}\) and Phe\(^{62}\) with alanine strongly reduced the mobility shift of the CBP fragment, whereas Tyr\(^{64}\) mutation had no effect. Taken together, our data thus suggest that C/EBP\(\beta\) is triggering CBP phosphorylation mostly through box B.

**FIG. 8.** Mutant forms of C/EBP\(\beta\) with reduced transactivation potential and CBP binding activity fail to induce CBP phosphorylation. A, HEK 293T cells were cotransfected with 1.5 μg of FLAG-tagged CBP1680–2441 and 7.5 μg of pcDNA3 (–), HA-tagged wild type C/EBP\(\beta\) (wt), or the indicated HA-tagged C/EBP\(\beta\) mutants. Transfection conditions and CBP detection were as in Fig. 4. Δ54–67, deletion of box A; Δ81–86, deletion of box B; Δ54–67 + Δ81–86, deletion of both box A and box B (see Fig. 7). B, HEK 293T cells were cotransfected with 1.5 μg of FLAG-tagged CBP1680–2441 and 7.5 μg of yeast Gal4 DNA binding domain alone (Gal), Gal4/C/EBP\(\beta\)1–152 (wt), or the indicated Gal4/C/EBP\(\beta\)1–152 mutants described in Fig. 7A. Transfection conditions and CBP detection were as in Fig. 4. The position of CBP1680–2441 in the absence of any C/EBP isoform is indicated by a black arrow, whereas the slow migrating forms of CBP1680–2441 are marked by an empty arrow.

**DISCUSSION**

Numerous studies have highlighted the presence of C/EBP binding sites in the promoter of genes involved in a variety of cellular processes (reviewed in Ref. 7). However, many questions remain about how C/EBP family members activate transcription. In this paper, we provide evidence that C/EBP-mediated transcription involves the recruitment of the coactivator CBP. We show that the interaction of C/EBP\(\beta\) with the E1A binding domain of CBP relies on two amino acid segments called box A and box B that are conserved between the activating members of the C/EBP family. Interestingly, C/EBP\(\alpha\), C/EBP\(\beta\), and C/EBP\(\delta\) induce slow migrating forms of full-length CBP, as well as of a C-terminal fragment of CBP, when they are coexpressed in HEK 293T cells. This mobility shift is due to CBP phosphorylation, because slow migrating forms disappear after phosphatase treatment. Finally, we show that deletion of box B or mutation of Leu\(^{61}\) and Phe\(^{62}\), which alters CBP recruitment and transcriptional activity, almost completely abolishes CBP mobility shift as well, suggesting a link between CBP phosphorylation and C/EBP-mediated gene acti-
vation. The effects of mutations that affect C/EBPβ box A are more difficult to interpret. Deletion of box A (Δ54–67) strongly reduces transcriptional activity and interaction with CBP but does not alter CBP phosphorylation. Moreover, mutation of Tyr464 within box A markedly decreases CBP binding but neither affects transcriptional activity nor CBP phosphorylation. As mentioned earlier, this apparent lack of data correlation might be due to the properties of the assays used to monitor CBP binding, C/EBP-mediated transcriptional activity, and CBP phosphorylation. A mutation that reduces the interaction with CBP in vitro might not significantly alter CBP phosphorylation in vivo, because the nuclear environment could favor weak and transient interactions that would not be visible in the GST pull-down assays. Alternatively, CBP could be phosphorylated very rapidly even if the C/EBP-CBP complex is not stable. Altogether, C/EBPβ mutations affect similarly CBP phosphorylation and transcriptional activity, except for the box A deletion mutant that strongly reduces transcriptional activity but not CBP phosphorylation. Other factors might interact with box A and participate in C/EBP-mediated transcriptional activation. Deletion of box A would thus impair recruitment of these factors, whereas Tyr464 mutation would not. These factors could be, for instance, TBP or TFIIIB, because they were shown to interact with this region in C/EBPα (25). However, the involvement of other coactivators is also possible.

We show that CBP phosphorylation relies both on the E1A binding domain, with which C/EBP isoforms interact, and the C-terminal glutamine-rich domain. This suggests that the interaction of C/EBP with CBP somehow recruits a protein kinase that phosphorylates one or several sites between amino acids 1893 and 2441. Recruitment of this yet uncharacterized kinase could occur in different ways. For instance, this kinase could be steadily associated with the C/EBP isoforms and could phosphorylate CBP when the C/EBP-CBP complex is built up. Alternatively, C/EBP binding may regulate the availability of a phosphorylation site by inducing a conformational change in CBP, or instead, the complex formed by C/EBP and CBP could be recognized by the kinase. Solving this issue will require identification of the protein kinase phosphorylating CBP in the presence of C/EBP. We tried to diminish CBP phosphorylation using many protein kinase inhibitors but were unable to determine which protein kinase is involved in this process.

A rather different interpretation of our data would be that C/EBP activates the transcription of a kinase gene whose product would then phosphorylate CBP. However, the following pieces of evidence argue against this possibility. First, our data suggest that CBP phosphorylation site(s) should be located between amino acids 1893 and 2441; nevertheless, CBP phosphorylation occurs only if the domain with which C/EBP interacts is present. If the role of C/EBP were solely to activate the transcription of a CBP kinase, then this domain would probably not be required. Second, deletion of box A (Δ54–67) drastically reduced CBP phosphorylation activity. Accordingly, this C/EBPβ mutant should not activate the kinase gene as efficiently as wild-type C/EBPβ, and thus CBP phosphorylation should be greatly reduced. However, CBP phosphorylation still occurred with this C/EBPβ mutant, suggesting that the transcriptional activation of a kinase is not involved. Finally, the best evidence so far comes from the data obtained with the Gal4-C/EBPβ1–152 construct. The bZIP DNA binding domain of C/EBPβ is lacking in this deletion mutant, and hence, it cannot bind to DNA or heterodimerize with endogenous C/EBP family members. Therefore, it is very unlikely that it could activate the gene of a CBP kinase. Nevertheless, CBP phosphorylation was induced by Gal4-C/EBPβ1–152, suggesting that CBP phosphorylation is triggered by its interaction with the transcription domain of C/EBPβ rather than by the transcriptional activation of a protein kinase.

During the preparation of this manuscript, Schwartz et al. (51) showed that C/EBP family members also trigger the phosphorylation of p300 C-terminal part. Conserved posttranslational modifications of CBP and p300 suggest that they are important for gene regulation mediated by C/EBP and potentially other transcription factors as well. Identifying which serines or threonines are phosphorylated in CBP1680–2441 is an important issue to determine the functional role of this phosphorylation. In this CBP fragment, two putative phosphorylation sites were previously identified: first, a protein kinase A phosphorylation site on serine 1772 (52) that might increase Pit-1 function in the presence of cAMP (53), although this issue remains controversial (54); second, a protein kinase B phosphorylation site (threonine 1872 in mouse CBP) that modulates the interaction of C/EBPβ with p300/CBP and mediates the effect of insulin on gene expression (38). We mutated Ser1772 or Thr1872 to alanine, but this had no effect on CBP phosphorylation induced by C/EBPβ (data not shown).

What could be the role of CBP phosphorylation in C/EBP-mediated gene transcription? It has been long known that CBP and p300 are phosphoproteins (reviewed in Refs. 31 and 33). For instance, cell cycle-dependent phosphorylation of p300 was first observed by Yaciuk and Moran (55). Likewise, retinoic acid and E1A induce phosphorylation of p300 during the differentiation of F9 cells (56). However, the huge size of p300 and CBP hindered the identification of phosphorylation sites within these coactivators. Consequently, only a few studies could correlate p300/CBP phosphorylation with a functional effect. For example, Ser301 of CBP was identified as a major target of CaMKIV phosphorylation in hippocampal neurons (57). Mutation of Ser301 impaired N-methyl-D-aspartate- and CaMKIV-stimulated transcription, demonstrating that CaMKIV signaling contributes to CREB/CBP-dependent transcription by phosphorylating CBP at Ser301. In addition, Ser66 of p300 was shown to be phosphorylated in vivo, most probably by protein kinase Co (58). Another report identified this site as a target of AMP-activated protein kinase (59). In both cases, Ser66 phosphorylation is inhibitory, since it either diminishes the p300 intrinsic HAT activity (60) or blocks the interaction of p300 with nuclear receptors (59). Several other studies reported regulation of p300 or CBP by protein kinases such as CaMKIV (61–63), MAPK (64–68), or cyclin E-Cdk2 (69–71), but none of them identified the amino acids targeted by these kinases. Interestingly, however, many of these studies showed that CBP or p300 were more important targets of protein kinases than the transcription factors themselves. For instance, MAPK appears to stimulate Elk-1-mediated gene expression by phosphorylating the C-terminal part of CBP rather than Elk-1 itself (64). Similarly, the activation of c-Jun by calcium does not require c-Jun phosphorylation but rather activation of CBP by calcium/calmodulin-dependent kinases (63). Pit-1-mediated regulation of the growth hormone and the prolactin genes involves the cAMP-protein kinase A pathway. However, mutating all the protein kinase A consensus sites in Pit-1 has no effect on the transcription of the growth hormone gene, suggesting that protein kinase A-mediated phosphorylation of CBP is responsible for cAMP activation (72). The prolactin gene is also inducible by cAMP without involving Pit-1 phosphorylation (68). In this case, cAMP was suggested to induce the MAPK family, which in turn phosphorylates CBP/p300 to stimulate transcription.

Stimulation of HAT activity was shown to be a way of increasing the coactivator function of CBP in a phosphorylation-dependent manner. A remarkable increase in histone acetyla-
tion was detected when the C-terminal region of CBP was phosphorylated in vitro by the p45 MAPK/extracellular signal-regulated kinase 1 (67). Similarly, the cyclin E-Cdk2 (to be consistent with C/EBP-CBP complex, which may target the same sites as MAPK, phosphorylates CBP in a cell cycle-dependent manner, thus enhancing its HAT activity (71). With this in mind, we measured the HAT activity of CBP coexpressed with C/EBPα, C/EBPβ, or C/EBPγ but found no significant difference with the activity of CBP expressed alone (data not shown).

In conclusion, one can speculate that C/EBP-mediated phosphorylation of CBP might have many diverse effects. For instance, it could induce a conformational change in CBP that might favor its interaction with components of the basal transcription machinery (e.g. TBP or TFIIIB). On the other hand, phosphorylation of CBP could trigger the recruitment of other chromatin remodeling complexes. Sorting out this issue will require identification of which protein kinase as well as which phosphorylation sites are involved in this process. However, regardless of what will result from these studies, one can already anticipate that CBP phosphorylation is an important modulatory mechanism in C/EBP-mediated gene transcription.

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CCAAT/Enhancer-binding Protein Family Members Recruit the Coactivator CREB-binding Protein and Trigger Its Phosphorylation
Krisztián A. Kovács, Myriam Steinmann, Pierre J. Magistretti, Olivier Halfon and Jean-René Cardinaux

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Additions and Corrections

**Vol. 278 (2003) 22136–22143**

Novel localization of the DNA-PK complex in lipid rafts. A putative role in the signal transduction pathway of the ionizing radiation response.

*Hector Lucero, Darren Gae, and Guillermo E. Taccioli*

Page 22136: The following grant statement should be included: “Dr. Lucero is supported by National Institutes of Health Grant GM31318.”

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Age-related changes in the biomolecular mechanisms of calvarial osteoblast biology affect fibroblast growth factor-2 signaling and osteogenesis.

*Catherine M. Cowan, Natalina Quarto, Stephen M. Warren, Ali Salim, and Michael T. Longaker*

The word “calvarial” was misspelled. The corrected title is shown above.

**Vol. 278 (2003) 36959–36965**

CCAAT/enhancer-binding protein family members recruit the coactivator CREB-binding protein and trigger its phosphorylation.

*Krisztián A. Kovács, Myriam Steinmann, Pierre J. Magistretti, Olivier Halfon, and Jean-René Cardinaux*

Page 36965, line 3: The sentence should read, “Similarly, the cyclin E-Cdk2 complex, which may target the same sites as MAPK, phosphorylates CBP in a cell cycle-dependent manner, thus enhancing its HAT activity (71).”

**Vol. 278 (2003) 40425–40428**

YXXM motifs in the PDGF-β receptor serve dual roles as phosphoinositide 3-kinase binding motifs and tyrosine-based endocytic sorting signals.

*Haiyan Wu, David A. Windmiller, Ling Wang, and Jonathan M. Backer*

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