MAPK Modulates the DNA Binding of Adipocyte Enhancer-Binding Protein 1[†]

Peter J. Lyons, Aleixo M. Muise,[‡] and Hyo-Sung Ro*

Department of Biochemistry and Molecular Biology, Dalhousie University, Halifax, Nova Scotia B3H 4H7, Canada

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ABSTRACT: Adipocyte enhancer-binding protein 1 (AEBP1) is a down-regulator of adipogenesis through its transcriptional repression activity, as well as through its interaction with mitogen-activated protein kinase (MAPK), which protects MAPK from its specific phosphatases. This study increases our understanding of the mechanisms of DNA binding by AEBP1, the first step in its function as a transcriptional repressor. We show that DNA binding by AEBP1 requires both the N- and C-terminal domains of AEBP1, and MAPK interaction with AEBP1 (through its N terminus) results in enhanced DNA binding. A threonine at position 623 within the C-terminal domain of AEBP1 plays an important role in DNA binding by AEBP1, because the mutation results in decreased DNA binding by AEBP1, which leads to a decrease in the transcriptional repression ability of AEBP1. We also show that *in vitro* phosphorylation of AEBP1 by MAPK is greatly reduced upon mutation of T623. These results suggest that MAPK regulates the transcriptional activity of AEBP1 by a novel dual mechanism, in which MAPK interaction enhances and subsequent phosphorylation decreases the DNA-binding ability of AEBP1.

The differentiation of fibroblast-like preadipocytes into fully developed, fat-filled adipocytes is regulated by a number of hormones, growth factors, and cytokines that transmit external signals through receptors to trigger a cascade of intracellular events. These intracellular events include the regulation of genes encoding master regulators of adipogenesis such as peroxisome proliferators-activated receptor γ (PPARγ)[1] and C/EBPα, as well as other transcription factors such as C/EBPβ and C/EBPδ, and ADD1/SREBP1c (*1*−*3*). Adipocyte enhancer-binding protein 1 (AEBP1) also plays an important role in the regulation of adipogenesis. AEBP1 was originally characterized as a transcription factor that binds the adipocyte enhancer-1 (AE-1) site of the aP2 promoter to repress transcription of this gene (*4*), which encodes the adipocyte fatty acid binding protein, an important marker of adipocyte differentiation (*5*, *6*). AEBP1 was found to be downregulated upon adipocyte differentiation, while constitutive overexpression of AEBP1 resulted in an inhibition of adipogenesis (*7*), suggesting that it is an important player in the regulation of fat cell growth and differentiation. The most striking feature of this protein is its carboxypeptidase (CP) domain, which was found to be necessary for transcriptional repression (*4*). It appears that the CP activity of AEBP1, also found to be stimulated by DNA binding (*8*), is the mediator of its active repression upon sequence-specific DNA binding.

As well as being a transcription factor regulating the expression of a critical gene involved in adipogenesis, AEBP1 is also involved in the signaling processes leading up to the regulation of gene expression. AEBP1 has been found to interact with the extracellular-regulated kinase (ERK) MAPK (*7*). MAPKs are serine/threonine kinases, which are activated upon phosphorylation by upstream dual-specificity kinases (MEK, in the case of ERK) at the end of a cascade of phosphorylation events. They then enter the nucleus, where they phosphorylate a large number of target proteins, often transcription factors, leading to changes in gene expression. The involvement of MAPK in adipogenesis has been somewhat unclear in past years. While some reports indicate that ERK activation leads to inhibition of adipogenesis (*9*, *10*), others report that ERK activation causes a stimulation of adipogenesis (*11*). These seemingly contradictory results indicate that the duration of phosphorylation has a major impact on the outcome. While transient phosphorylation may lead to stimulation of adipogenesis, prolonged activation of ERK may lead to inhibition of adipogenesis and greater proliferation. The interaction of AEBP1 with ERK is critically important in the regulation of adipogenesis, because it results in sustaining the phosphorylation of ERK. This protective effect results in inhibition of adipogenesis caused by high levels of MAPK phosphorylation over a longer duration and complements the function of AEBP1 as a transcriptional repressor of the aP2 gene in adipogenesis (*7*).

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* To whom correspondence should be addressed. Telephone: (902) 494-2367. Fax: (902) 494-1355. E-mail: hsro@dal.ca.

[‡] Presently at the Division of Gastroenterology and Nutrition, Department of Pediatrics, Hospital for Sick Children, University of Toronto, Toronto, Canada.

[1] Abbreviations: ADD1, adipocyte differentiation and determination factor; AE-1, adipocyte enhancer-1; AEBP1, adipocyte enhancer-binding protein 1; CAT, chloramphenicol acetyltransferase; C/EBP, CCAAT/enhancer-binding protein; CP, carboxypeptidase; EMSA, electrophoretic mobility shift assay; ERK, extracellular-regulated kinase; MAPK, mitogen-activated protein kinase; PPARγ, peroxisome proliferators-activated receptor γ; SREBP, sterol regulatory element-binding protein.

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In addition to the protection of MAPK activity by AEBP1, it was also found that AEBP1 is an *in vitro* substrate for MAPK (*7*). Thus, it seemed that AEBP1 could be added to the long list of ERK substrates, which includes other kinases and phosphatases, enzymes, and many transcription factors (*12*). The present study suggests that AEBP1 is phosphorylated by MAPK *in vitro* at a conserved phosphorylation site, threonine 623. Mutation of this residue results in a decrease in the ability of AEBP1 to bind DNA, leading to a decrease in its ability to act as a transcriptional repressor. Additionally, we find that interaction of AEBP1 with MAPK increases its ability to bind DNA. Thus, the transcription activity of AEBP1 may be regulated by MAPK through a novel dual mechanism.

MATERIALS AND METHODS

Sequence Analysis and Mutagenesis. Sequences were analyzed for phosphorylation site motifs using the scansite search algorithm, available on the World Wide Web (*13*). Mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene) and verified by sequencing.

Recombinant Protein Purification. Recombinant protein was purified as described previously (*8*) with Talon metal-affinity resin (Clontech), with some modifications. The extraction/wash buffer used was 50 mM sodium phosphate (pH 7.0), 6 M guanidine-HCl, and 300 mM NaCl. Second-batch wash and subsequent washing on the column was done with the extraction/wash buffer at pH 6.7. Elution was performed with imidizole elution buffer (45 mM sodium phosphate at pH 7.0, 5.4 M guanidine-HCl, 270 mM NaCl, and 300 mM imidizole), followed by stepwise dialysis as described previously.

Electrophoretic Mobility Shift Assay (EMSA). AE-1 probe was radiolabeled with [α-^{32}P]ATP by Klenow fill-in. Recombinant proteins were incubated with a probe for 25 min at room temperature in binding buffer [10 mM Tris at pH 7.5, 10 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 2.5% glycerol]. Samples were resolved on 5% 0.25× TBE mini-gels, which were then dried and exposed to film. EMSA using nuclear extracts was performed as above but with 2 μg of nuclear extracts along with 0.1 μg of poly dI/dC.

Kinase Assay. Recombinant AEBP1 (1 μg) was incubated with 50 units p42 MAPK (NEB) and 10 μCi [γ-^{32}P]ATP in the supplied reaction buffer [50 mM Tris-HCl at pH 7.5, 10 mM $MgCl_2$, 1 mM ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 2 mM DTT, and 0.01% Brij 35] for 30 min at 30 °C. The reaction mixture was then analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) on an 8.5% gel followed by transfer to nitrocellulose membrane (Amersham) and autoradiography.

Cell Culture, Transfection, and Preparation of Cell Extracts. NIH/3T3 cells were grown in Dulbecco's modified Eagle medium supplemented with 10% calf serum and penicillin/streptomycin. Transfections were done in 60 mm dishes with Polyfect (Qiagen) according to the protocol of the manufacturer. pJ3H-AEBP1, pJ3H-AEBP1(−), and mutants were derived from pJ3H (*14*). Cell extracts were prepared in cold radioimmune precipitation buffer (RIPA buffer: 50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 50 mM $Na_4P_2O_7$, 0.25% sodium deoxycholate, 0.1% Nonidet P-40, 1 mM Na_3VO_4, and 1 mM NaF) containing 1 mM phenylmethylsulfonyl fluoride, 5 mM ethylenediaminetetraacetic acid, 5 mM EGTA, and protease inhibitor cocktail (Roche). Nuclear extracts for EMSA and subcellular fractionation studies was made as described previously (*15*).

Reporter Assays. Transfection of NIH/3T3 cells was performed with 3 μg of pJ3H-AEBP1 or other pJ3H derivative expression plasmid, 200 ng of paP2(3AE-1/-120)-CAT reporter plasmid (*16*), along with 200 ng of pHermes-lacZ, which expresses the lacZ gene under the control of the CMV promoter. Cells were harvested 48 h after transfection. β-Galactosidase activity was assayed to normalize for transfection efficiency, and CAT activity was assayed as described previously (*4*).

RESULTS

Both N and C Termini of AEBP1 Are Necessary for DNA Binding. AEBP1 consists of three distinct domains. The central carboxypeptidase-like domain is flanked by an N-terminal discoidin-like domain (DLD) and a structurally uncharacterized domain in the C terminus (Figure 1A). Previous EMSA studies on AEBP1 have shown that a C-terminal deletion mutant of AEBP1, AEBP1ΔSty, which lacks the C-terminal 205 amino acids, is unable to bind DNA (*4*, Muise and Ro, unpublished results). This suggests that the DNA-binding domain of AEBP1 is located in the C-terminal domain, unless this C-terminal deletion causes a conformational change in other domains of the protein necessary for DNA binding. The C terminus of AEBP1 contains a basic region, rich in arginine, which is predicted to have α-helical secondary structure (parts A and B of Figure 1) similar to that found in the DNA-binding domain of the basic leucine zipper proteins such as CAAT/enhancer-binding protein (C/EBP, Figure 1C). To determine if other regions of AEBP1 might be involved in DNA binding, EMSAs were performed using recombinant N-terminally truncated AEBP1 (AEBP1ΔDLD) and ^{32}P-labeled AE-1 probe. No DNA binding was detected by this truncation mutant as well (lanes 4−6 of Figure 2A). These results suggest that both the C- and N-terminal domains of AEBP1 are involved in DNA binding or at least are necessary to retain the proper conformation needed for DNA binding.

DNA Binding and Transcriptional Activities of AEBP1 Are Inhibited by MAPK Phosphorylation Site Mutation. Not only does MAPK interact with AEBP1, leading to protection of MAPK from its specific phosphatases and stronger DNA binding for AEBP1 (see below), but MAPK also phosphorylates AEBP1 *in vitro*. This phosphorylation does not occur when the C terminus of AEBP1 is truncated (*7*), indicating that either the phosphorylation site is in the C terminus or that this truncation causes a conformational change in AEBP1 not allowing phosphorylation to occur. Sequence analysis of mouse AEBP1 revealed several potential MAPK phosphorylation sites based on the consensus motif PXS/TP (see Figure 1). When compared with all known mammalian AEBP1 protein sequences, only one putative phosphorylation site in the C terminus of AEBP1 (T623) is conserved. In fact, on the basis of the consensus motif for MAPK phosphorylation, threonine 623 is the only putative phos-

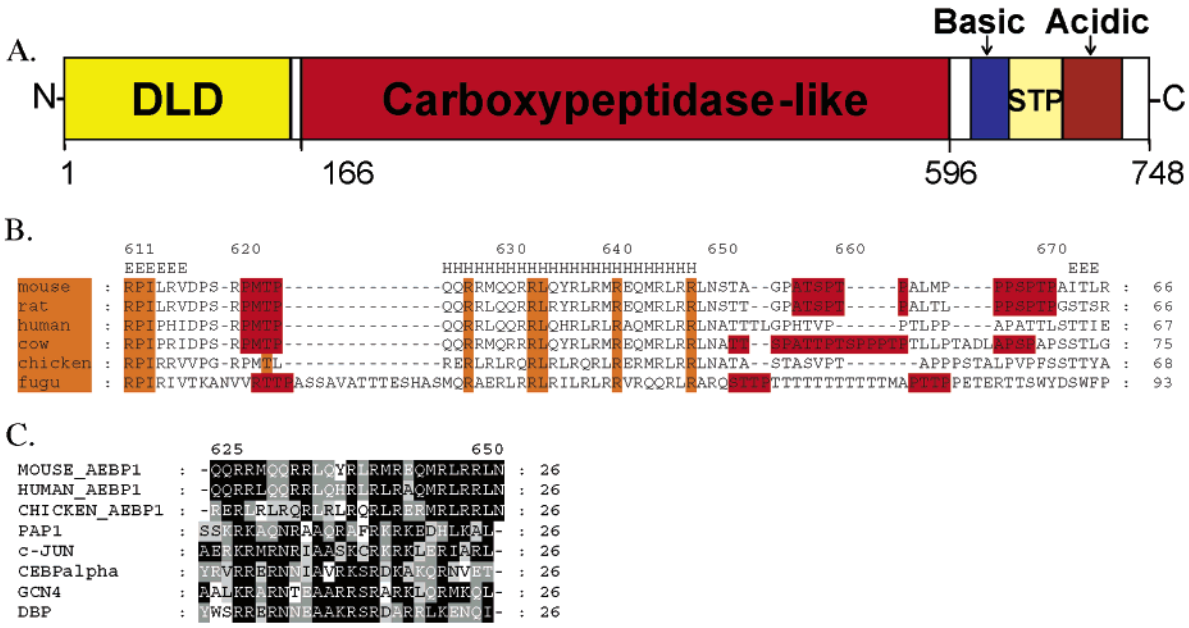

FIGURE 1: AEBP1 multiple alignments. (A) Domain structure of AEBP1 consists of an N-terminal DLD, a carboxypeptidase-like domain, and a C-terminal domain made up of basic, serine-threonine-proline-rich, and acidic regions. (B) Multiple alignment of a portion of the C terminus of AEBP1. The top row contains a secondary structure prediction obtained from PhDsec (*23*), in which E = β-strand and H = α-helical secondary structure. Conserved residues are colored orange, while potential MAPK phosphorylation sites are colored red. (C) Alignment of the DNA-binding basic regions of representative proteins of the bZIP family with that of AEBP1. This figure is adapted from ref *17*. The grayscale shading from black to white indicates progressively lower amino acid identity/similarity.

phorylation site present in the entire human AEBP1 sequence. Because phosphorylation by MAPK is known to regulate the activity of many proteins, we wanted to investigate the possibility that the activity of AEBP1 is modulated through phosphorylation by MAPK. We performed site-directed mutation on this threonine to mutate it to both alanine (T623A) and aspartate (T623D) to mimic constitutive dephosphorylation and phosphorylation, respectively. MAPK assays using equivalent amounts of recombinant AEBP1 (normalized by quantitation of a stained gel) showed that phosphorylation of recombinant AEBP1 T623A by active recombinant MAPK was about half that of wild-type (WT) AEBP1 (compare lanes 1 and 2 in Figure 2B). As well, kinase assays performed with AEBP1ΔDLD exhibit even less phosphorylation (approximately 6-fold less than WT AEBP1, lane 3 of Figure 2B), most likely because of the fact that AEBP1ΔDLD does not interact with MAPK (*7*). Altogether, MAPK assays with recombinant AEBP1 suggest that T623 of AEBP1 is an *in vitro* MAPK phosphorylation site, although there must be at least one other phosphorylation site in mouse AEBP1. This other site(s), however, is not conserved in human AEBP1.

The T623 AEBP1 phosphorylation site is just N-terminal to the basic helix found in the C terminus of AEBP1 (see Figure 1) and thought to be important for DNA binding, suggesting that phosphorylation of this site may lead to changes in the ability of AEBP1 to bind DNA. EMSAs of purified WT and mutant AEBP1 with labeled AE-1 probe show that both T623A and T623D mutants display greatly reduced DNA-binding ability (Figure 2A). This suggests that threonine 623 is important for strong DNA binding by AEBP1 and that mutation of this threonine to alanine or aspartate eliminates the hydroxyl group of the threonine most likely necessary for hydrogen bonding with the DNA. Alternatively, this residue may be important in allosteric interactions with other regions of AEBP1 involved in DNA binding, such as the N-terminal domain mentioned above.

To see the effect of this mutation on DNA binding in the context of the cell nucleus, EMSA was performed with nuclear extracts isolated from NIH/3T3 cells, which had been transfected with a plasmid encoding an HA-tagged WT, T623A, or T623D AEBP1 or the same plasmid with the AEBP1 cDNA inserted in the opposite orientation as a negative control (Figure 2C). A shifted AEBP1/DNA complex was formed, which was found to be a specific interaction upon incubation with a specific (S, unlabeled AE-1 DNA) and nonspecific (NS) DNA competitor. The shifted complex in the negative control lanes is a complex of endogenous AEBP1 and AE-1 probe. As expected, this complex increases in intensity upon incubation with AEBP1-transfected nuclear extracts. However, it decreases even below endogenous levels upon incubation with T623A and T623D AEBP1-transfected nuclear extracts. This may be due to a requirement for dimerization for AEBP1 to bind DNA. Previous *in vitro* EMSA experiments have shown the ability of AEBP1 to multimerize upon an increase in salt concentrations (Muise and Ro, unpublished results). It appears that mutant AEBP1, which is unable to bind DNA, titrates endogenous AEBP1 away from the DNA probe through a dimerization interaction. Experiments to verify dimerization of AEBP1 through immunoprecipitation have been unsuccessful. This may be due to the need for DNA for AEBP1 to dimerize and/or the difficulty in optimizing the conditions for dimerization, DNA interaction, and immunoprecipitation in one reaction.

Next, we examined the transcriptional activity of AEBP1 and its mutant derivatives showing diminished DNA-binding activity. Transient transfection of NIH/3T3 cells with AEBP1 WT or mutant forms along with a reporter was followed by a chloramphenicol acetyl transferase (CAT) assay to test for transcriptional repression activity of AEBP1. The reporter

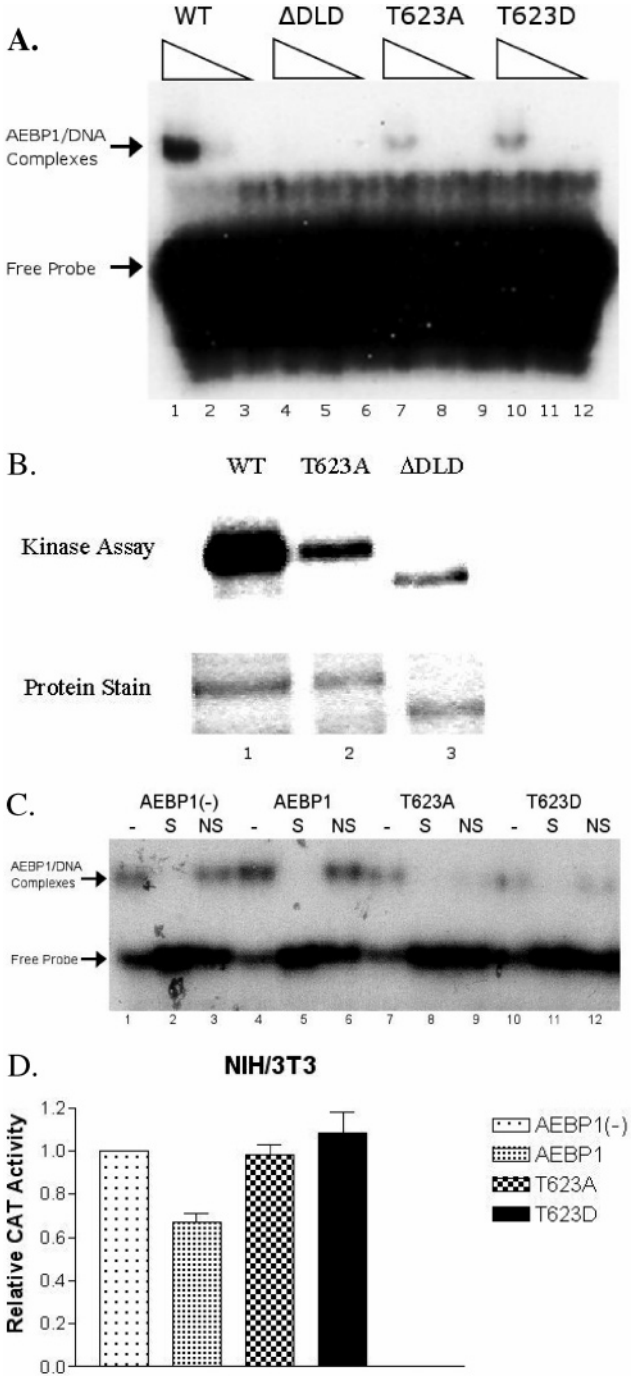

FIGURE 2: Mutation of AEBP1 threonine 623 results in a decreased *in vitro* phosphorylation by MAPK as well as a decreased DNA-binding and transcriptional repression ability. (A) EMSA was performed by incubating 0.15, 0.3, and 0.6 μg of recombinant AEBP1 (WT, ΔDLD, T623A, and T623D) protein with radiolabeled AE-1 probe and separating complexes on a nondenaturing gel followed by autoradiography. (B) Kinase assays were performed with 1 μg of WT, T623A, or ΔDLD AEBP1 and recombinant active ERK MAPK. Equivalent amounts of AEBP1 were run on SDS−PAGE and stained to verify the quantity of AEBP1. (C) NIH/3T3 nuclear extracts were incubated with radiolabeled AE-1 probe, as well as 50-fold excess specific competitor (lanes 2, 5, 8, and 11) or nonspecific competitor (lanes 3, 6, 9, and 12), resolved on a nondenaturing gel and visualized by autoradiography. (D) NIH/3T3 cells were separately transfected with the pJ3H-AEBP1 expression vector, the control plasmid pJ3H-AEBP1(−) containing the AEBP1 cDNA in the opposite orientation, or the T623 mutant derivatives, along with the reporter plasmid paP2(3AE-1/-120)CAT. Transcriptional activity was measured as relative levels of CAT activity. Data shown are the average of at least three different transfections ($n = 9$).

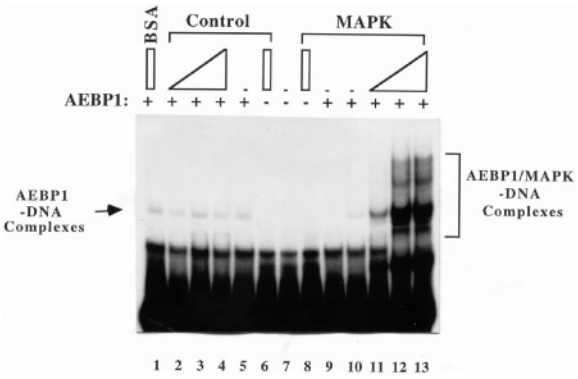

FIGURE 3: MAPK interaction with AEBP1 causes increased DNA-binding ability by AEBP1. Recombinant AEBP1 was incubated with radiolabeled AE-1 DNA. Lane 1 contains 2 μg of AEBP1 and 2 μg of bovine serum albumin (BSA). Lanes 2−4 contain 2 μg of AEBP1 and 0.5, 1.0, and 2.0 μg of control Rab-11, respectively. Lane 5 contains 2 μg of AEBP1, and lane 6 contains 2 μg of Rab-11 alone. Lane 7 contains AE-1 DNA alone. Lane 8 contains 2 μg of MAPK alone. Lane 9 contains 2 μg of AEBP1 plus unlabeled AE-1 DNA (competitive inhibitor). Lane 10 contains 2 μg of AEBP1 alone. Lanes 11−13 contain 2 μg of AEBP1 plus 0.5, 1.0, and 2.0 μg of MAPK, respectively. Complexes were resolved on a nondenaturing gel and detected by autoradiography.

used contained three copies of the AE-1-binding site, previously characterized as a site to which AEBP1 specifically binds within the aP2 promoter (*4*), upstream of the promoter region (−120/+21) of the aP2 gene (*16*). As expected, the decreased DNA-binding ability of the T623A and T623D AEBP1 mutants resulted in the elimination of transcriptional repression ability in NIH/3T3 cells (Figure 2D). Analysis of the localization of AEBP1 showed that the mutation of T623 does not affect its nuclear localization nor its expression level (data not shown). When the above results are taken together, they suggest that the transcriptional activity of AEBP1 may be regulated by phosphorylation on threonine 623.

DNA Binding by AEBP1 Is Stimulated by Interaction with MAPK. Previous work on AEBP1 has shown that AEBP1 interacts with MAPK through its discoidin-like domain in the N terminus of AEBP1, resulting in protection of the phosphorylation of MAPK from its MAPK-specific phosphatase (*7*). The C terminus of AEBP1 is also required for the protection activity (*7*). Because both of these domains are necessary for the protection of MAPK phosphorylation, as well as DNA binding, we wanted to determine whether the interaction of MAPK with AEBP1 affected its ability to bind DNA. Gel-shift assays, in which recombinant AEBP1 was preincubated with recombinant MAPK, show that interaction with MAPK actually stimulates the ability of AEBP1 to bind DNA. Lanes 11−13 of Figure 3 show that the DNA binding of 2 μg of AEBP1 (MW of 83-kDa) is maximally stimulated with 1 μg of MAPK (MW of 44-kDa), with an approximate 1:1 molar ratio. Doubling the amount of MAPK to 2 μg had no further effect on DNA binding by AEBP1. These results suggest that the interaction with MAPK, irrespective of phosphorylation, increases the ability of AEBP1 to bind DNA.

DISCUSSION

There are many conserved domains used by proteins to interact with a specific sequence of DNA. These are often

made up of, at least in part, a basic amphipathic helix, which inserts itself into the major groove of the DNA with positively charged side chains making contacts with the negatively charged DNA backbone. This is the case with C/EBP and other basic leucine zipper DNA-binding proteins (*17*), as well as for homeodomain-containing proteins such as Msx1/2 and others (*18*, *19*). Interestingly, there are similarities between both of these groups of proteins and AEBP1. AEBP1 contains a region in its C terminus that is predicted to form a basic helix and that has sequence similarity with many basic leucine zipper DNA-binding proteins. As well as this similarity in the putative binding domain, both proteins interact with the same stretch of DNA, the AE-1 site of the aP2 promoter (*4*, *20*). This suggests that they may have somewhat similar mechanisms of DNA binding. However, although there is some evidence for AEBP1 dimerization, the leucine zipper necessary for C/EBP dimerization is not present in AEBP1, suggesting a different mechanism for AEBP1 dimerization. It seems that the N-terminal DLD domain of AEBP1 may also be involved in DNA binding or in retaining a conformation necessary for DNA binding. The fact that MAPK interaction with the DLD domain causes an increase in DNA binding by AEBP1 suggests that this interaction may stabilize a more favorable DNA-binding conformation involving the N-terminal domain in cooperation with the C-terminal basic helix.

Just N-terminal to the basic helix in AEBP1 is a putative MAPK phosphorylation site, $_{621}$PMTP$_{624}$. We show in this report that threonine 623 of AEBP1 is important for DNA binding, because mutation to either an alanine or aspartate greatly reduces its ability to bind DNA in a gel-shift assay. Interestingly a very similar mutation in Msx2 (T147A) abolishes DNA binding by this protein (*21*). Msx2 is a homeodomain-containing protein. The homeodomain is a DNA-binding domain found in many proteins. It is made up of 60 amino acids that form three central helices, which interact with the major groove of DNA (helix 3 making the major contribution to this interaction), and an N-terminal extension, which lies across the minor groove (*22*). This N-terminal extension contains the crucial T147 as a part of the sequence KPRTP, which in the crystal structure for Msx1 makes a hydrogen bond to the DNA phosphate backbone (*22*). We believe a similar mechanism for DNA binding may be employed by AEBP1, with the basic helix in the major groove as in C/EBP and the RPMTP motif binding to the minor groove.

There is no indication that this threonine is phosphorylated in Msx1/2. We suggest that threonine 623 is a major *in vitro* phosphorylation site in AEBP1. This phosphorylation site is conserved between mouse, rat, human, and cow sequences. Recent database searches have found sequences for chicken and fugu AEBP1, which retain the threonine but do not conserve the MAPK consensus phosphorylation sequence. This suggests that phosphorylation at this residue may be important only in a mammalian system.

Altogether, the work presented here suggests that AEBP1 is phosphorylated *in vitro* at threonine 623 and that this site is important for DNA binding both by recombinant AEBP1 protein and transfected cell extracts. We also find that the N terminus of AEBP1 along with the C-terminal domain, is important for DNA binding and that interaction of MAPK with this N-terminal domain may optimize the conformation so that stronger DNA binding can occur. This would result in increased repression by AEBP1 at the aP2 promoter. Thus, AEBP1/MAPK interaction may stimulate cell proliferation both by enhancing MAPK phosphorylation as well as stimulating the DNA binding of AEBP1 at the aP2 promoter and thus inhibiting the expression of this gene, which is important for differentiation of preadipocytes. However, upon activation of MAPK and subsequent phosphorylation of AEBP1 by MAPK at threonine 623, AEBP1 would no longer be able to strongly bind DNA and would fall off. This explains a possible novel mechanism, whereby MAPK regulates both the initial binding of AEBP1 to DNA as well as the termination of this DNA binding, with the first through interaction with AEBP1 and the latter through phosphorylation of AEBP1.

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