Regulation of Adipogenesis by a Transcriptional Repressor That Modulates MAPK Activation*

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Mitogen-activated protein kinase (MAPK) is required for cell growth and cell differentiation. In adipogenesis, MAPK activation opposes the differentiation process. The regulatory mechanisms or the cellular factors that regulate the switch between growth and differentiation in the adipogenic lineage have been largely unelucidated. We show here that AEBP1, a transcriptional repressor that is down-regulated during adipogenesis, complexes and protects MAPK from its specific phosphatase in mammalian cells. We further show evidence that the modulation of MAPK activation by AEBP1 is a biologically relevant process in adipogenesis. Our results suggest that modulation of MAPK activation by the protective effect of AEBP1 may constitute a critical part in the determination between cell growth and differentiation in the adipogenic lineage. The proposed mode of action by which a transcription factor regulates MAPK activation is novel.

Adipogenesis is a complex process regulated by a variety of hormones, growth factors, and cytokines that act via specific receptors to transduce external growth and differentiation signals through a cascade of intracellular events. The initiation of differentiation of preadipocytic cell lines, such as 3T3-L1, into mature adipocytes is induced by continuous exposure to pharmacological doses of insulin or physiological doses of IGF-1. In addition, cAMP and glucocorticoids are generally considered necessary for the induction of differentiation. However, the mechanistic actions of insulin and other ligands on differentiation are not clearly defined (1). The determination that the activation of the IGF-1 receptor is a potent inducer of the differentiation process implies that a tyrosine-kinase-mediated signaling pathway is involved in adipocyte differentiation. Furthermore, constitutive expression of Ras or Raf-1 (two components of receptor tyrosine kinase pathways) can induce preadipocyte differentiation in the absence of insulin or IGF-1 (2, 3). However, Raf-1 was able to induce only partial differentiation, indicating that Raf-independent pathways downstream of Ras may be involved in adipocyte differentiation. Moreover, the signals generated by Raf-1 did not activate MAPK or a 90-kDa S6 kinase, suggesting a functional dissociation between Raf-1 and MAPK/S6 kinase activation in Ras signaling pathways leading to 3T3-L1 differentiation (4). Moreover, it was demonstrated that MAPK activation is not required for, but rather antagonizes, the differentiation process (5). In sharp contrast, studies by Sale et al. (6) showed that MAPK is a key element in adipogenesis, with MAPK activation being required for signaling initiated by insulin and serum stimulation, for activation of DNA synthesis, and for the induction of differentiation in 3T3-L1 cells.

MAPK is activated by phosphorylation at specific threonine and tyrosine residues on the MAPK molecule by the upstream kinase, MAPK kinase (MAPKK, or MEK (7–9)), and if this activation is sustained, MAPK translocates to the nucleus. However, if this activation is transient, MAPK is prevented from translocating (10). MAPK phosphorylation (activation) is a reversible process, in which protein phosphatases play a crucial role in controlling cellular activities. Because MEK is known to be transiently activated by phosphorylation, the regulation of prolonged or transient MAPK activation may be through phosphatases (10, 11). An emerging class of dual-specificity phosphatases that directly and specifically regulate MAPK family members has been characterized. Among them is the dual-specificity phosphatase PYST1 (also called MKP3 or rVH6) that is selective for inactivation of the extracellular-signal-regulated kinase (ERK) family MAPKs (ERK1 and ERK2) (12–14).

We have previously identified a novel transcriptional repressor (termed AEBP1) with carboxypeptidase (CP) activity (15) that regulates an adipocyte-specific gene (aP2); furthermore, we showed that its CP activity is vital for the transcriptional repression function (16). In this paper, we use coimmunoprecipitation experiments to demonstrate that AEBP1 complexes with ERK in vivo and in vitro. We also use ectopically expressed AEBP1, along with the MAPK-specific phosphatase PYST1/MKP3/rVH6, to demonstrate that AEBP1 attenuates MAPK inactivation, resulting in increased MAPK activity. Finally, we show evidence that the modulation of MAPK activation by AEBP1, which is independent of its transcriptional function, is a biologically relevant process in adipogenesis. This modulation of MAPK activation by the protective effect of AEBP1 illustrates a potential new mode of regulation in adipogenesis. The notion that a transcription factor can also function as a regulator of MAPK activation is unexpected and unprecedented.

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EXPERIMENTAL PROCEDURES

Cell Culture, Transfection, and Plasmids—COS-7, 3T3-L1, and NIH-3T3 cells were cultured in 90-mm dishes and transacted at 80% confluence by the polybrene procedure as described previously (17). Detailed descriptions of plasmid constructions and the generation of the overexpressing and the antisense cell lines are available from the author on request. The HA-tagged expression plasmids pJ3H-AEBP1, pJ3H-AEBP1(−), pJ3H-AEBP1LDLD, pJ3H-AEBP1ΔHic, and pJ3H-AEBP1ΔSty were derived from pJ3H (18). The retrovirus plasmids pAEBP1Neo, pAEBP1HicNeo, pAEBP1StyNeo, and the antisense plasmid pASNeo were derived from pWZLNeo (19). The retrovirus plasmids pAEBP1DLLD/Puro and pAEBP1DLLD(−)/Puro were derived from pBabe-puro (20).

Immunoprecipitation and Kinase Assay—Cell extracts were prepared in cold radioimmuno precipitation buffer containing 1 mM phenethylmethylsulfonyl fluoride, 10 μg aprotonin/ml, 10 μg leupeptin/ml, 5 mM EDTA, and 5 mM EGTA. Cell extracts were incubated with protein A-agarose (Santa Cruz Biotechnology) for 1 h, after which the beads were discarded and the supernatants were incubated with specific antibodies (2 μg/ml anti-HA, anti-ERK1, anti-MEK1, or anti-IGFR, or 2.5 μg/ml of either affinity-purified anti-AEBP1 antibodies or IgG from preimmune serum) for 1 h and then overnight with protein A-agarose. Samples were collected and washed four times with radioimmuno precipitation buffer. The precipitated samples were resolved by SDS-PAGE and analyzed by Amersham Pharmacia Biotech’s ECL blotting system.

Phosphatase Protection Assay—For the in vivo assay, COS-7 cells transfected with plasmids expressing AEBP1, PYST1, and HA-ERK1 were starved for 2 h in Dulbecco’s modified Eagle’s medium (DMEM) containing 0.1% calf serum, then stimulated for 15 min with 10 ng/ml of EGF (Sigma). Cell lysates were prepared from both quiescent (lane 1) and mitogen (lane 2) activated 3T3-L1 cells. Activation (phosphorylation) of MAPK, which can be determined by the slower migration of the phosphorylated form in SDS-PAGE (Fig. 1A, bottom) is approximately similar to the amount of MAPK asso-
ciated with ERKs (lane 3).

Adipocyte Differentiation and Lipid Staining—Stable cell lines were maintained in DMEM containing 10% calf serum and 200 ng of G418/ml (Life Technologies, Inc.). At confluence, cells were treated with DMEM containing 10% cosmetic calf serum (HyClone Laboratories), 0.5 mM dexamethasone, 5 μg insulin/ml, 0.5 mM 1-methyl-3-isobutylxanthine for 2 days then maintained in DMEM containing 10% cosmetic calf serum and 5 μg insulin/ml for 6 days. Cells were washed three times with phosphate-buffered saline, fixed by incubating in 4% paraformaldehyde for 10 min, and stained with a saturated oil Red O in 60% triethyl phosphate solution (BDH).

RESULTS

AEBP1 Is Phosphorylated by, and Associated with, MAPK—AEBP1, besides being a novel carboxypeptidase with transcrip
tional repression function, has a serine, threonine, proline (STP)-rich region with a few possible MAPK proline-directed phosphor
ylation sites in the C terminus. Indeed, the recombinant AEBP1 protein can be phosphorylated by immunoprecipitated MAPK, and the phosphorylation site(s) may be located at the C terminus, because no phosphorylation was detected with a truncated mutant form of AEBP1 (AEBP1ΔSty (16)), which lacks the C-terminal domain (data not shown). However, it is possible that conformational changes caused by the truncation in this mutant may mask potential phosphorylation sites in other regions of AEBP1. Nevertheless, these results have suggested that one kinase responsible for AEBP1 phosphorylation is MAPK.

Immunoprecipitation and immunoblot experiments with antibodies toward AEBP1 and phosphorylated tyrosine have indicated that AEBP1 was not phosphorylated by a tyrosine kinase. Interestingly, two phosphoproteins with the approximate molecular weights of the MAPK’s ERK1 and ERK2 were detected when AEBP1 immunoprecipitates were immunoblotted with anti-phosphotyrosine antibody (data not shown). These observations prompted us to examine the possibility that MAPK can be coprecipitated with AEBP1. To verify this intriguing possibility, we utilized cell extracts from different stages of adipocyte differentiation for coimmunoprecipitation, because AEBP1 expression is abolished in adipocytes (16). Fig. 1A (top) shows the immunoprecipitation analysis of AEBP1 abundance during 7 days after exposure to differentiation conditions. Expression of AEBP1 was decreased on day 1 and persisted until 4 days after exposure to differentiation medium, then decreased on day 5 and was subsequently abolished at the terminal stage of adipocyte differentiation on days 6 and 7. Because AEBP1 is undetectable on days 6 and 7, we would not expect to observe any coprecipitation of MAPK on these days, and MAPK was not detected in the AEBP1 immunoprecipitates from days 6 and 7 (Fig. 1A, middle). Immunoblotting of the cell lysates showed no significant change in the content of MAPK during adipocyte differentiation (Fig. 1A, bottom).

To further assess the interaction between AEBP1 and MAPK we carried out reciprocal coimmunoprecipitation experiments. Cell extracts were immunoprecipitated with either anti-AEBP1 or anti-ERK1 antibodies, and then the post-precipitated cell extracts were further immunoprecipitated with the opposite antibodies. The precipitated samples were then analyzed by immunoblotting with anti-AEBP1 and anti-ERK1 antibodies, respectively. Fig. 1B shows that the amount of AEBP1 that remains unbound (top, lane 2) is approximately similar to the amount of AEBP1 associated with ERKs (top, lane 1). Conversely, the amount of MAPK that remains unbound (bottom, lane 4) is approximately similar to the amount of MAPK associated with AEBP1 (bottom, lane 3). These results indicate that about 50% of AEBP1 and MAPK molecules interact with each other in mammalian cells.

In light of the above studies, it would be interesting to determine whether AEBP1 interacts with the phosphorylated, dephosphorylated, or both forms of MAPK to determine whether the AEBP1-MAPK interaction was further involved in regulation of MAPK. To determine the phosphorylation state of the MAPK molecules that complex with AEBP1, we used cell extracts from quiescent and from serum-stimulated 3T3-L1 cells. Activation (phosphorylation) of MAPK, which can be detected by the slower migration of the phosphorylated form in SDS-polyacrylamide gels, was induced by serum stimulation (Fig. 1C, top). AEBP1 was then isolated from cell extracts by immunoprecipitation with anti-AEBP1 antibody and MAPK was detected by immunoblot analysis. Fig. 1C (bottom) shows that ERKs are detected in the AEBP1 immunoprecipitates prepared from both quiescent (lane 3) and the serum-stimu-
AEBP1 associates with MAPK in mammalian cells. A, confluent 3T3-L1 cells (day 0) were subjected to the differentiation protocol, and total cell extracts were prepared at the times indicated during differentiation. The extracts were immunoprecipitated with anti-AEBP1 antibody and then immunoblotted with either anti-AEBP1 (top) or anti-ERK1 antibodies (middle). Bottom, Western blot analysis of the cell extracts with anti-ERK1 antibody (Santa Cruz Biotechnology) that also cross-reacts with ERK2. B, total protein (1 mg) from 3T3-L1 cell extracts was subjected to immunoprecipitation with either anti-AEBP1 or anti-ERK1 antibodies (1st) and the post-immunoprecipitation extracts were further immunoprecipitated with the opposite antibodies (2nd). Both sets of precipitated samples were analyzed by immunoblotting with anti-AEBP1 (top) and anti-ERK1 (bottom) antibodies, respectively. As a control, the same amount of total protein was immunoprecipitated with either anti-IGFR antibody (lanes 5) or pre-immune IgG (lanes 6). C, top, 3T3-L1 cells were starved in DMEM containing 0.1% calf serum for 24 h, then treated with DMEM containing 15% fetal bovine serum for various lengths of time. Five micrograms of total cell lysate protein was resolved by SDS-PAGE. Both unphosphorylated and phosphorylated forms of ERK1 and -2 were detected by immunoblotting with anti-ERK1 antibody (200 ng/ml). Bottom, cell extract from quiescent (lanes 1, 3) or serum-stimulated (lanes 2, 4) cells was immunoprecipitated with anti-ERK1 antibody (200 ng/ml). Both, cell extract from quiescent (lanes 1, 3) or serum-stimulated (lanes 2, 4) cells was immunoprecipitated with anti-ERK1 antibody (200 ng/ml). Reciprocal immunoprecipitation with anti-ERK1 antisera and immunoblotting with anti-HA antisera (top) demonstrated that HA-AEBP1 was detected from pJ3H-AEBP1-transfected cells (lane 2) but not from cells transfected with the control plasmid (lane 5). Similar coimmunoprecipitation experiments with an upstream component of the MAPK signaling module showed that AEBP1 does not interact with MEK1 (data not shown). However, we cannot rule out the possibility that AEBP1 interacts with other upstream components of the MAPK pathway.

To locate a domain(s) of AEBP1 responsible for MAPK interaction, we generated constructs encoding mutant derivatives of the HA-tagged AEBP1 protein (Fig. 2B); these constructs were then transfected individually and analyzed for the ability of the encoded AEBP1 polypeptides to interact with MAPK by coimmunoprecipitation experiments. Fig. 2C (bottom) shows that ERK1 was coprecipitated from cells transfected with the wild-type (lane 1) and most mutant derivatives (lanes 3–5) but not from cells transfected with either the mutant derivative pJ3H-AEBP1ΔLDL (lane 2), which expresses an HA-tagged AEBP1 that lacks the N-terminal 233 amino acids (Fig. 2B) or the empty vector pJ3H (lane 6). Interaction with MAPK was abolished neither by deletion in the carboxypeptidase domain (pJ3H-AEBP1ΔHic, lane 4) nor by truncation at the C terminus (pJ3H-AEBP1ΔSty, lane 5). Significantly, ERK1 was coprecipitated from cells transfected with the mutant derivative pJ3H-AEBP1ΔLDL (lane 3), which expresses only the N-terminal 204 amino acids (residues 32–235) of AEBP1 (Fig. 2B). Therefore, these results indicate that the domain of AEBP1 responsible for MAPK interaction is located at the N terminus, which contains sequences that were previously termed discordant-like domain (DLD (16)), and suggest that phosphorylation is not required for AEBP1 to interact with MAPK.

We developed an in vitro coimmunoprecipitation system for assessing the interaction to determine whether AEBP1 interacts with MAPK directly or via intermediates. The wild-type and mutant derivatives of the His-tagged recombinant AEBP1 proteins (16) were mixed individually with the recombinant ERK2 protein, then the mixture was immunoprecipitated with anti-ERK or anti-AEBP1 antisera. The precipitates were then analyzed by immunoblotting with anti-AEBP1 or anti-ERK antisera. As shown in Fig. 2D, the wild-type (lane 3) and all the mutant derivatives of His-AEBP1 (lanes 5–7), except the mutant ΔLDL with amino-terminal deletion (lane 4), were detected in the immunoblot. Specifically, a mutant derivative, which consists of only the N-terminal 204 amino acids (DLD), was able to coprecipitate with the recombinant ERK2 protein (lane 5). The apparent inability of the N-terminal deletion mutant to interact with MAPK is due neither to its inability to be recognized by the anti-AEBP1 antisera nor to its prevention of immunoprecipitation by anti-ERK antisera (data not shown). Taken together these results suggest that MAPK directly interacts with AEBP1 at the N terminus. Examination of the sequences in the N-terminal domain of AEBP1 did not reveal any MAPK docking sites described previously (21–23). However, a putative MAPK-docking site, which is characterized by a cluster of positively charged amino acids (24), is present in the N-terminal domain of AEBP1.

Modulation of MAPK Activation by AEBP1—Because AEBP1 associates specifically with MAPK, AEBP1 may participate in the MAPK signaling pathway by modulating MAPK activation. We asked whether MAPK activity could be modulated by altering the abundance of AEBP1 in 3T3-L1 cells. The pooled stable 3T3-L1 cell lines were generated with a retrovirus directing expression of either wild-type (AEBP1/Neo-P) or the mutant derivatives (AEBP1ΔHic/Neo-P and AEBP1ΔSty/Neo-P) of AEBP1. Fig. 3A (second row) shows the amount of phosphorylated ERK in the stable cells. The AEBP1/Neo-P cells contained the highest level among the stable cell lines, whereas
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**Fig. 2.** AEBP1 directly interacts with MAPK through its N-terminal domain. A, equal amounts of COS-7 cell extracts, transfected with 10 μg of p3H-AEBP1 (lanes 1–3) or the control plasmid p3H-AEBP1(−) (lanes 4–6), were used in immunoprecipitation studies with anti-HA antibody (lanes 1, 4), anti-ERK1 antibody (lanes 2, 5), or anti-IGFR antibody (lanes 3, 6). The immunoprecipitated samples were immunoblotted with either anti-HA antibody (top) or anti-ERK1 antibody (bottom). B, schematic representations of only AEBP1 coding regions of HA-AEBP1 fusion-protein derivatives. Small black boxes represent sequences created by frameshift mutations. The numbers indicate amino acid residues of AEBP1. The presence (+) and absence (−) of interaction with MAPK is shown. C, COS-7 cells were individually transfected with 10 μg of p3H-AEBP1 (lane 1) and its mutant derivatives as indicated (lanes 2–5). Lane 6 contains material from cells transfected with the empty vector pJ3H. Cell extracts from transfected cells were analyzed by immunoblotting with anti-HA antibody (top). The asterisk indicates HA-AEBP1 and its mutant derivatives. Cell extracts were also immunoprecipitated with anti-HA antibody and then analyzed by immunoblotting with anti-ERK1 antibody (bottom). D, 300 ng of the His-tagged recombinant AEBP1 (lanes 1–3) and its mutant derivative (lanes 4–7) proteins was individually mixed with (lanes 3–7) or without (lanes 1 and 2) the recombinant ERK2 protein (300 ng). The mixtures were immunoprecipitated either with preimmune IgG (lane 1) or p44/42 MAPK antibody (lanes 2–7). The precipitates were immunoblotted with anti-AEBP1 antibody. The asterisk indicates His-AEBP1 and its mutant derivatives.

The above results suggest that AEBP1 can modulate MAPK activation. However, because the conclusions are based on results obtained with AEBP1-transfected cell lines, it cannot be concluded whether AEBP1 operates similarly at physiological concentrations. It would thus be important to examine whether suppression of endogenous AEBP1 by antisense RNA expression can lead to decreased MAPK activity. Cells were transfected with an expression vector (pAS/Neo) that constitutively expresses an antisense RNA corresponding to the 5′-700-bp region of the AEBP1 transcript. We established two stable cell lines (AS/Neo-7 and -11), which showed decreased levels of AEBP1 in comparison to the level in the control cell line (Neo-12) established with the empty vector. Of the two clones, AS/Neo-11 exhibited a lower level of AEBP1 expression (Fig. 3B, top row). As predicted, AS/Neo-11 exhibited lowest MAPK activity (24% of the control level) (Fig. 3B, second row), but MEK activity (detected by phospho-MEK1 antibody) was similar among these cell lines (Fig. 3B, fourth row). These results suggest that AEBP1 specifically modulates MAPK activation, and the modulation may be physiologically relevant.

AEBP1 Specifically Modulates MAPK Activation by Protecting It from Phosphatase—A possible mechanism of MAPK modulation by AEBP1 is through protection of MAPK from its phosphatase. Because AEBP1 was observed at similar levels in the nuclear and post-nuclear soluble fractions (25), we predict that AEBP1 may protect MAPK from the cytosolic MAPK-specific phosphatase PYST1/MKP3/rVH6. To test this hypothesis we used ectopically expressed AEBP1, along with ERK1 and PYST1, to demonstrate that AEBP1 attenuates ERK1 inactivation. To establish a system that allows assessment of MAPK regulation by AEBP1, we expressed PYST1 and measured mitogen-stimulated ERK1 activity in transfected COS-7 cells. Fig. 4A (top) shows that PYST1 expressed in COS-7 cells blocked heterologously expressed ERK1 (pECE/HA-ERK1) activation following stimulation with epidermal growth factor (EGF), and this inhibition increased when cells were transfected with increasing amounts of PYST1 plasmid (pSG5/
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Fig. 3. Modulation of MAPK activation by AEBP1. A, total protein (20 μg) from the stable cells was analyzed by immunoblotting with AEBP1 (top), Phospho-p44/42 MAPK monoclonal (upper middle), and p44/42 MAPK (lower middle) antibodies. Cell extracts were also analyzed by immunoblotting with anti-PPARγ antibody (Santa Cruz Biotechnology) at 7 days after the confluent cells were subjected to the differentiation protocol (bottom). B, AEBP1 protein level in each anti-sense stable cell line was determined by Western blot analysis (top). ERKs were immunoprecipitated with anti-ERK1 antibody and the MAPK activity analysis was carried out by the immunocomplex assay using MBP (second row) as described under “Experimental Procedures.” The radiolabeled MBP bands were quantitated by Molecular Imager (Bio-Rad) and the percent activity was estimated for each sample. Phosphorylated MEK1 (fourth row) and total MEK1 (fifth row) were detected by immunoblotting of the cell extracts with Phospho-MEK1/2 and MEK1 antibodies (New England BioLabs), respectively.

PYST1-Myc (12)). Next, we tested whether expressing AEBP1 restored the EGF-stimulated ERK1 activation under conditions where the PYST1-mediated inhibition would be maximal. Fig. 4B (top) shows that EGF-stimulated ERK1 activation was recovered in a dose-dependent manner when COS-7 cells were transfected with the plasmid expressing AEBP1 (lanes 4–6) but not with the control plasmid (lanes 1–3). The PYST1 inhibition is unlikely mediated by AEBP1 binding to the phosphatase, because coimmunoprecipitation experiments did not reveal such interaction (data not shown). To rule out the possibility that the transcriptional repression activity of AEBP1 is involved in the modulation of MAPK activation, we repeated the protection experiments with mutant derivatives of AEBP1 defective in transcriptional repression activity but still able to interact with MAPK. We predicted that the mutants ΔHic and ΔSty are defective in transcriptional function, because ΔHic lacks the C-terminal DNA-binding domain whereas ΔHic lacks the CP activity that is required for transcriptional function (16). Indeed, both mutants were defective in transcriptional repression activity (Fig. 4C). Importantly, the EGF-stimulated ERK1 activation (detected by phospho-ERK1 antibody), under conditions where inhibition by PYST1 is maximal, was recovered in a dose-dependent manner when COS-7 cells were transfected with the plasmid expressing the mutant derivative ΔHic (Fig. 4D, top, lanes 4–6). These results are in agreement with the MAPK modulation activity retained by the mutant derivative ΔHic (Fig. 3A). In agreement with the results in Fig. 4A, the protection by ΔHic was not as strong as that conferred by wild-type AEBP1 (Fig. 4D, top, lanes 7–9), perhaps due to the large deletion in the ΔHic protein (Fig. 2B). We did not observe any protection effect with the ΔSty mutant (Fig. 4D, top, lanes 1–3), which suggests that the protection domain is located at the C terminus that is missing in the ΔSty protein (Fig. 2B). These results are consistent with the data showing lack of MAPK modulation activity in cells overexpressing the ΔSty mutant (Fig. 3A). The EGF-stimulated MEK1 activation (detected by Phospho-MEK1/2 antibody) was not affected in any of the transfected cells (Fig. 4D, second row). Also, the decreased protection activity of ΔHic and ΔSty are not due to defects in their expression, because the expression level of each mutant was similar to the wild-type level (Fig. 4D, third row). These results indicate that AEBP1 can protect MAPK from a MAPK-specific phosphatase and suggest that the protection is most likely mediated by protective interaction not by transcriptional function.

To further substantiate the conclusion that AEBP1 modulates MAPK activation by a protective interaction, we utilized another mutant derivative (DLD) that consists of only the interaction domain (Fig. 2) as a dominant-negative mutant. We would predict that this mutant competes away MAPK from the wild-type and attenuates the protective activity exhibited by the wild-type in a dominant-negative fashion. Indeed, DLD expressed in COS-7 cells attenuated the protective activity of AEBP1 in a dose-dependent manner (Fig. 4E, top). Taken together, these results strongly suggest that AEBP1 modulates MAPK activation through protective interaction.

To test whether AEBP1 alone is sufficient for blocking dephosphorylation of activated MAPK by a MAPK phosphatase, an in vitro phosphatase assay was designed. Recombinant phospho-ERK2 protein was treated with the recombinant rVH6, a MAPK phosphatase, after incubation with either wild-type or mutant derivatives of the recombinant AEBP1 protein. The treated MAPK was then analyzed by immunoblotting with the phospho-MAPK antibody, and we found that the recombinant AEBP1 protein alone is not sufficient for the protection (data not shown). These results suggest that one or more accessory proteins are required, along with AEBP1, for stabilizing the phosphorylation status of MAPK and making it immune to dephosphorylation.

The MAPK-protective Activity of AEBP1 Modulates the Adipogenic Process—MAPK maintained in its active phosphorylated form by protection from phosphatases may cause overstimulation of MAPK pathways. Several recent reports showed that activation of the MAPK pathway phosphorylates a nuclear hormone receptor, PPARγ, and inhibits adipogenesis (26–29). Thus, we tested the hypothesis that the modulation of MAPK activity by AEBP1 is a biologically relevant process in adipogenesis.

The stable cell lines that overexpressed either wild-type or mutant derivatives of AEBP1 (Fig. 3A) were induced to differentiate, and these cultures were monitored for the differentiated phenotype. Examination of these cultures indicated striking differences in the ability of the cells to accumulate lipid, a feature of mature adipocytes. It was clear that the stable cell line AEBP1/Neo-P, generated by a virus harboring AEBP1 and showing MAPK protection activity (Fig. 4D), was unable to convert to the adipocyte phenotype (Fig. 5A). Importantly, AEBP1ΔSty/Neo-P cells, which did not show any MAPK modulation (Fig. 3A) and protection (Fig. 4D) activities, were able to differentiate as efficiently as the control cell line Neo-P, in which more than 90% of the cells accumulated lipid. In contrast, the stable cell line AEBP1ΔHic/Neo-P, generated by the virus harboring the mutant derivative ΔHic and showing MAPK protection activity (Fig. 4D), was not able to differentiate efficiently. As predicted from the differentiated phenotype, expression of the differentiation-specific gene PPARγ in the AEBP1ΔHic/Neo-P cells was much lower than the level in the control cells following the differentiation protocol (Fig. 3A, bottom). The amount of PPARγ in the AEBP1ΔSty/Neo-P cells was similar to the level in the control cells, but the level in the AEBP1/Neo-P cells was much lower. Because AEBP1ΔHic is defective in transcription function (Fig. 4C) the reduced level of...
PPARγ in the AEBP1ΔHic/Neo-P and AEBP1/Neo-P cells is most unlikely due to transcriptional repression of PPARγ expression by overexpression of AEBP1. These results suggest that the MAPK-protection activity is responsible for the anti-adipogenic effect.

Because expression of the dominant-negative mutant DLD caused an attenuation of the MAPK protection activity of AEBP1 (Fig. 4E), we tested whether the anti-adipogenic effect in the AEBP1/Neo-P cell line can be attenuated by ectopic expression of the dominant-negative mutant DLD. The stable cell line AEBP1/DLD(+), which was derived from the AEBP1/Neo-P cell line and infected with the virus expressing DLD (AEBP1DLD/Puro), was able to differentiate, albeit less efficiently than the Neo-P cells (Fig. 5B). The control stable cell line AEBP1/DLD(−), which was generated with the control virus (AEBP1DLD(−)/Puro), was unable to differentiate as in the case of the parental cell line AEBP1/Neo-P. As predicted, the amount of the differentiation-specific gene PPARγ was increased in the AEBP1/DLD(+) cells following the differentiation protocol (Fig. 5C, bottom). The amount of phosphorylated ERK in AEBP1/DLD(+) but not in AEBP1/DLD(−) cells was significantly decreased from the level in the parental AEBP1/Neo-P cells (Fig. 5C, second row). However, the MEK activity was not affected by ectopic expression of DLD (data not shown). These results strongly suggest that adipogenesis is regulated by the MAPK-protection activity of AEBP1.

**DISCUSSION**

MAPK localizes to the cytoplasm by binding to the cytoplasmic anchor MAPKK under quiescent conditions. After stimulation, MAPK becomes phosphorylated by MAPKK and dissociates from MAPKK. The dissociated MAPK then enters the nucleus by either passive diffusion or active transport mechanisms (30). It has been postulated that the nuclear accumulation of MAPK may require a putative nuclear anchor(s) (31). AEBP1 may be a mammalian nuclear anchor that is responsible for the nuclear accumulation of MAPK. The translocation is also dependent on the protection of ERK from MKP3, a cytosolic phosphatase. Protein-protein interaction between AEBP1 and ERK may protect ERK from MKP3 in the cytoplasm, thus allowing ERK to be translocated to the nucleus. It has been demonstrated that MKP3 is activated by direct binding to MKP3, a cytosolic phosphatase. Protein-protein interaction between AEBP1 and ERK may protect ERK from MKP3 in the cytoplasm, thus allowing ERK to be translocated to the nucleus. Thus, the protection by AEBP1 may be mediated by a simple competitive interaction between MKP3 for ERK and through rapid nuclear translocation.

The persistent activation of MAPK as a result of the protec-
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The apparent conflicting roles of MAPK, in which MAPK activation in preadipocytes appears to be required for differentiation (6, 45), but MAPK is also capable of repressing PPARγ function (26, 28) and opposes cell differentiation in 3T3-L1 cells (5), may be resolved by examining the differences in stimulation of MAPK, because MAPK will translocate to the nucleus upon prolonged activation, or by examining the activation of MAPK by different receptor types that utilize similar signaling pathways but cause drastic differences in cellular outcomes (37). Adipogenic agents such as insulin may cause transient MAPK activation, whereas mitogenic agents such as growth factors may cause prolonged MAPK activation. In support of this proposal, overexpression of the IRS-1, the major substrate of the insulin receptor tyrosine kinase (46), causes the activation of MAPK and induces cell proliferation in fibroblasts (47). Insulin normally causes these cells to differentiate, but prolonged activation of the IRS-1 component of the insulin signaling pathway induces cell proliferation, again indicating an activation-dependent function of MAPK in adipogenesis.

Our studies provide new information that the modulation of MAPK activation could constitute a critical part of the molecular mechanism of adipogenesis. The finding that the MAPK signal-transduction pathway is stimulated in response to factors that initiate cell differentiation, and the finding that MAPK physically interacts with AEBP1, strongly implies that this interaction is important in adipogenesis. AEBP1 may function to block inappropriate signals by balancing the level of activated MAPK in conjunction with regulation by specific phosphatases, thus maintaining the preadipocyte phenotype. When the cells are stimulated to differentiate, the protective effect of AEBP1 on MAPK activation may be attenuated, thus allowing the signal to begin the differentiation process. Our

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2 S.-W. Kim, and H.-S. Ro, unpublished data.
results demonstrate a novel function of AEBP1 as an important mediator, through its protective effect on MAPK, in the adipogenic signal-transduction pathway.

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