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Transcriptional Induction of the Alpha-1 Acid Glycoprotein (AGP) Gene by Synergistic Interaction of Two Alternative Activator Forms of AGP/Enhancer-Binding Protein (C/EBPβ) and NF-κB or Nopp140

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Alpha-1 acid glycoprotein/enhancer-binding protein (AGP/EBP) (C/EBPβ), a member of the C/EBP family, is one of the key transcription factors responsible for the induction of a wide array of genes, some of which are expressed during the acute-phase response. Both activator and repressor were shown to be encoded by the intronless *agp*/*ebp* or its rat and human homologs, which contain a common bZIP domain at their C-terminal regions. Expression of the AGP gene (*agp*) is regulated by AGP/EBP in liver during the acute-phase response. However, the molecular mechanism for this regulation is poorly understood. The experiments reported here demonstrate that two activator forms of AGP/EBP, one of which has an additional 21 amino acids at its N-terminal region, are expressed in liver as well as in a number of cell lines. We have also demonstrated that NF-κB and a phosphoprotein of 140 kDa, Nopp140, interact with different AGP/EBP activators synergistically, which results in induction of the *agp* gene in an AGP/EBP-binding-motif-dependent manner. Furthermore, extracellular stimuli that are known to be NF-κB inducers can selectively activate the *agp* gene by cooperating with one of the two activator forms of AGP/EBP. The physiological significance of differential regulation for the function of two activator forms of AGP/EBP through selective interaction with different transcription factors is discussed.

Alpha-1 acid glycoprotein/enhancer-binding protein (AGP/EBP) (10) is a mouse liver-enriched transcription factor that belongs to the C/EBP transcription factor family. NF-IL6 (1), LAP (12), IL-6DBP (28), and CRP2 (35) are factors closely related or homologous to AGP/EBP. Treatment of cells with either lipopolysaccharides (LPS) (10) or inflammatory cytokines (i.e., interleukin-1 [IL-1], IL-6, or tumor necrosis factor alpha) (2, 5) can dramatically induce expression of AGP/EBP in different mouse tissues. Autoregulated expression of the *agp*/*ebp* gene has been described previously (11).

It has been demonstrated that AGP/EBP is one of the key transcription factors responsible for the induction of acute-phase response proteins such as AGP, serum amyloid A, and angiotensinogen (7, 10, 15, 29). Furthermore, AGP/EBP or its homologs are also involved in the regulation of many early and immediate-early genes (2). Although substantial progress in the understanding of the function of AGP/EBP and its homologs has been made, the regulation remains to be elucidated. AGP/EBP is a member of the bZIP protein family. In vivo, AGP/EBP may form homodimers as well as heterodimers. It has been demonstrated that AGP/EBP can heterodimerize with C/EBP (8) or C/ATF (32) and can physically associate with NF-κB (30) through its bZIP region.

NF-κB has been found to be involved in the regulation of many genes, including those encoding cytokines and cytokine receptors, various viral genes (e.g., human immunodeficiency virus or cytomegalovirus [CMV] genes), and genes that are involved in the acute-phase and inflammatory responses (2). NF-κB or NF-κB-like factors were found to be involved in the regulation by IL-1 of some acute-phase protein genes (5–7, 29), such as angiotensinogen and serum amyloid A. NF-κB can be specifically activated by treatment of cells with cytokines, various growth factors, tumor promoters, protein synthesis inhibitors (e.g., cycloheximide), or free oxygen radicals (22, 31). NF-κB and C/EBP represent distinct families of transcription factors that target unique DNA enhancer elements. The heterodimeric NF-κB complex is composed of p50 and p65 polypeptides. All members of the NF-κB family, including the product of the proto-oncogene c-*rel*, are characterized by their highly homologous ~300-amino-acid N-terminal regions. This Rel homology domain mediates DNA binding, dimerization, and nuclear targeting of these proteins (3, 21). NF-κB and NF-IL6 have been shown to be involved in the synergistic regulation of inflammatory cytokines such as IL-6 and IL-8 (23) and human serum amyloid A (6, 15). These synergistic stimulations by NF-κB and NF-IL6 are mediated by both κB and NF-IL6 motifs. The bZIP regions of C/EBP family proteins can associate with the Rel homology domain of NF-κB (30). Therefore, complexes formed by NF-κB and members of the C/EBP family may be involved in the regulation of the acute-phase protein genes in three different manners: κB motif dependent, C/EBP motif dependent, and C/EBP motif independent. Recent experiments with AGP/EBP-interacting proteins resulted in the identification of a phosphoprotein of 140 kDa, Nopp140, that associates with AGP/EBP (25). Furthermore, Nopp140 was identified as a transcription factor. Thus, in addition to members of the C/EBP family, members of the NF-κB family, and Nopp140, other factors are likely to be identified as AGP/EBP-interacting proteins.

Recent findings indicate that activators and repressors can

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be encoded by the same gene (13, 16). There are many examples of the generation of a wide range of active proteins with diverse functions by alternative splicing from a single gene (16). Among these are a number of genes that encode transcription factors, but in many of these cases the functional significance of alternative splicing is unclear. In some cases, however, alternative splicing constitutes a switch that dramatically reverses function (16).

The intronless *lap* gene (the rat homolog of mouse *agp*/*ebp*) encodes both an activator (LAP) and a repressor (LIP). The *lap* gene is transcribed in many tissues, but the LAP protein is most abundant in the liver. A repressor form, LIP, is generated by an alternative translation starting site within the coding sequence, which leads to translation of a protein with a truncated N terminus (13). LAP and LIP can form heterodimers that bind DNA with an efficiency equivalent to that of the homodimers. However, the LIP-specific repression seems to depend on the heterodimer formation, and the production of LIP is a dynamic and developmentally regulated process (13). Three forms of NF-IL6, corresponding to polypeptides generated by in-frame translational initiation sites from different AUGs of the same NF-IL6 mRNA species, are also detected in human embryonal carcinoma NT2/D1 cells; the production of these forms is regulated by IL-6 and retinoic acid (33). The functional regulation of these proteins remains obscure. In this report, we demonstrate that two activators are encoded by the *agp*/*ebp* gene. There are two major differences in the functional activities of these two AGP/EBP activators: (i) basal activity and (ii) functional interaction with NF-κB and Nopp140. NF-κB can interact with AGP/EBP(f), while Nopp140 can interact with AGP/EBP(m), synergistically to activate the transcription of the *agp* gene. The synergistic interaction is dependent on the AGP/EBP-binding motif. Extracellular stimuli that are known NF-κB inducers activate the *agp* gene through AGP/EBP(f).

MATERIALS AND METHODS

Plasmids. The AGP-CAT and CMV-AGP/EBP plasmids were obtained as described elsewhere (10). Expression vectors for NF-κB were kindly provided by Joe DiDonato. Plasmids containing the mutated AGP promoter sequence were as described previously (19, 20). AGP/EBP-dZIP is a derivative of internal deletion of *Pvu*II fragment, which generates a 14-amino-acid deletion in the leucine zipper. For site-directed mutagenesis, full-length cDNA of AGP/EBP was subcloned into M13mp18. AGP/EBP(m) cDNA was derived from the deletion of 49 nucleotides of full-length cDNA. A single-stranded cDNA template was prepared from transformed *Escherichia coli* CJ236 and used for site-directed mutagenesis (9). The following primers were used for the mutagenesis: mutant 1 (m1), 5′-CGGGCCCCGCCACCATGGACCGCCTGCT-3′; mutant 2 (m2), 5′-TTTAGACCCTTGGAAGTGG-3′; and S239L, 5′-GTGCGCAAGCTTCGCG ACAAG-3′. Detailed procedures for cloning of rat Nopp140 (24) and construction of the CMV-Nopp140 expression vector will be published elsewhere (25).

Expression and purification of recombinant proteins. An *Xba*I fragment of NF-κB p50 was generated from CMV-p50 and subcloned into pGEM. An *Eco*RI fragment isolated from pGEM was partially digested with *Pst*I and cloned into pRSET (Invitrogen, San Diego, Calif.). Mouse AGP/EBP(f), AGP/EBP(m), AGP/EBP(s), and AGP/EBP-dZIP were cloned into pRSET. The His-tagged recombinant protein was expressed in *E. coli* BL21(DE3) and purified with an Ni column.

Preparation of nuclear extracts, antibodies, and Western blot (immunoblot) analysis. Liver nuclei and nuclear extract were isolated from p388D1 cells or mouse livers as described previously (13). Ten micrograms of nuclear extract was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), blotted onto a Hybond-C membrane (Amersham), and probed with a monoclonal antibody (1:2,000 dilution) to AGP/EBP. Polyclonal and monoclonal (A16) anti-AGP/EBP antibodies were as detailed elsewhere (10). Polyclonal antibodies against the N-terminal 21 amino acids (N21) were generated by immunization of a rabbit with Keyhole limpet hemocyanin-conjugated peptide NH_2-MHR LLAWDAACLPPPPAAFRP-COOH (GeneMed Biotech, South San Francisco, Calif.). Detection was with an enhanced chemiluminescence kit (Amersham).

Cell cultures, transfections, and CAT assay. BHK, HepG2, and C127 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. DNA transfections were performed by the calcium phosphate precipitation method. For each 6-cm-diameter petri dish, the calcium phosphate-DNA precipitate contained 2 μg of target plasmid with 50 ng of CMV-AGP/EBP plus CMV-p50 and p65 NF-κB (125 ng each) or 50 ng of CMV-AGP/EBP alone. pGEM plasmid DNA (Promega) was used to give a final amount of 3 μg of DNA for the transfection. For UV irradiation, cells were exposed to UVC (20 J/m^2) (14) at 16 h posttransfection. For IL-1β- or LPS-conditioned macrophage medium treatment, cells were changed to serum-free Dulbecco's modified Eagle's medium at 24 h posttransfection in the presence of 4 ng of IL-1β per ml or 30% (vol/vol) macrophage medium. Cells were harvested at 48 h posttransfection. Chloramphenicol acetyltransferase (CAT) activity was determined from autoradiograms of thin-layer chromatography plates with the Molecular Dynamics Image analysis system.

Coimmunoprecipitation. For coimmunoprecipitation analysis, 0.5 μg of each affinity column-purified AGP/EBP and p50 were mixed in buffer (12 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.6], 60 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.5 mM dithiothreitol, and 10% glycerol) containing 1 mg of bovine serum albumin per ml, and the mixture was incubated for 15 min at room temperature. After the reaction, the chemical cross-linker dithio-bis(succinimidyl-propionate) (Pierce) was added to a final concentration of 2 mM, and the mixture was incubated for 30 min at room temperature. The reaction was quenched by the addition of 100 mM ethanolamine, and the mixture was diluted with phosphate-buffered saline (PBS) buffer containing 1% Triton X-100. The reaction mixture was precleared by addition of normal rabbit serum and protein A-Sepharose and incubated for 1 h at 4°C. Anti-NF-κB p50 antibody (Santa Cruz Biotech) and protein A-Sepharose were then added to the supernatant and incubated for 2 h at 4°C. The protein A-Sepharose was washed four times with PBS–1% Triton X-100, incubated with SDS sample buffer, and subjected to SDS-PAGE. The separated polypeptides were blotted onto a Hybond-C membrane (Amersham). The blots were probed with antibodies, and detection was with an enhanced chemiluminescence kit (Amersham).

In vitro transcription-translation. The cDNA from AGP/EBP(f) was cloned into pGEM4 and linearized with *Bam*HI. In vitro transcription-translation experiments were performed according to the manufacturer's instructions (Promega). Three microliters of the final reaction mixture from a nuclease-treated reticulocyte lysate was subjected to SDS-PAGE and autoradiography.

RESULTS

Three polypeptides are encoded by the intronless *agp*/*ebp* gene. To resolve the issue of whether two or three polypeptides are encoded by the intronless *agp*/*ebp* gene, we performed in vitro transcription-translation experiments and Western blot analysis. Polypeptides with molecular masses of 38, 34, and 21 kDa were detected by the in vitro transcription-translation system (Fig. 1A, lane 2). These results agree with that of Western blot analysis with polyclonal antibodies to AGP/EBP (Fig. 1A, lane 1). These data suggest that three polypeptides can be encoded by the full-length cDNA of AGP/EBP. Two longer forms of AGP/EBP were detected in nuclear extracts of mouse liver (Fig. 1B, lane 9) and p388D1 cells (lane 10) with monoclonal (A16) antibodies. To demonstrate unequivocally that these two forms of AGP/EBP are derived from de novo synthesis rather than from posttranslational modifications, the blots were probed with N21 antibodies (Fig. 1B). The larger form (38 kDa) of AGP/EBP was detected in the nuclear extracts of liver (lane 5), p388D1 cells (lane 6), and AGP/EBP(f)-transfected HepG2 cells (lane 8) by N21. Nontransfected HepG2 cells (lanes 3 and 7) failed to express the 38-kDa polypeptide that is recognized by N21 antibodies. N21 antibodies do not recognize NF-IL6 (data not shown). In addition to these two forms of AGP/EBPs, the 21-kDa polypeptide detected by in vitro transcription-translation and rabbit polyclonal antibodies (Fig. 1A, lane 2) has previously been shown to be an alternative translation product of the *lap* gene (rat homolog of *agp*/*ebp*) and functions as a transcriptional repressor (13). These results suggest that three polypeptides could be expressed from the *agp*/*ebp* gene. A schematic representation of Ala/Pro, Ala, Pro, and Ser stretches of these polypeptides is shown in Fig. 1C. All the AGP/EBP forms contain a common bZIP domain.

Synergistic induction of the *agp* gene by NF-κB may be mediated by its physical interaction with AGP/EBP. Previous studies have shown that AGP/EBP is an activator for AGP-

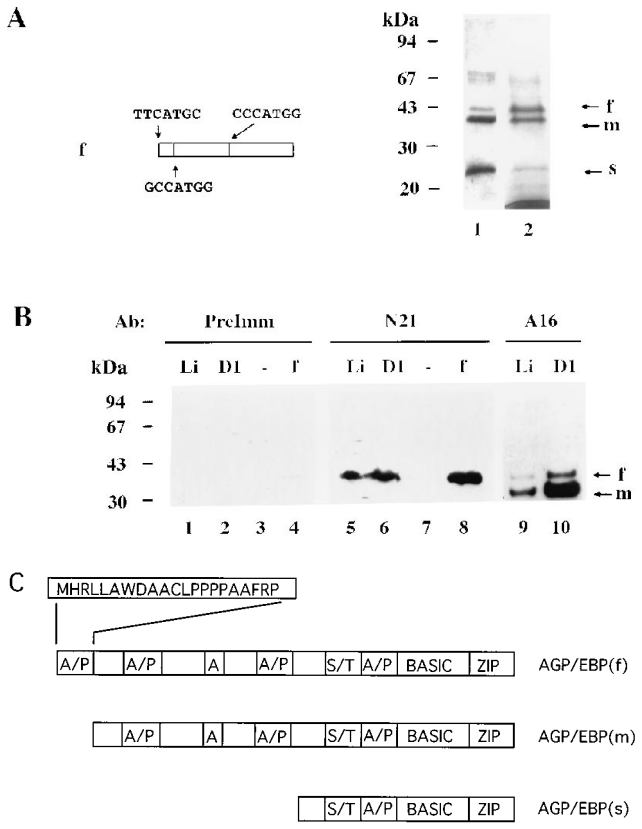

FIG. 1. Three polypeptides are encoded by the intronless *agp*/*ebp* gene. (A) The left panel shows a schematic representation of AGP/EBP cDNA open reading frame containing three in-frame potential translational initiation sites, which was used for construction of plasmids for in vitro transcription and translation and expression vectors for transfection. Lane 1, p388D1 nuclei (optical density at 260 nm = 2) were subjected to SDS-PAGE and blotted onto a Hybond-C membrane (Amersham). The blot was probed with polyclonal antibodies to AGP/EBP. Positions of molecular mass markers are indicated. Arrows indicate the 38-kDa (f), 34-kDa (m), and 21-kDa (s) forms, respectively. Lane 2, three polypeptides of 38, 34, and 21 kDa were detected by in vitro transcription and translation. (B) Detection of f and m forms of AGP/EBP by Western blot analysis with N21 antibodies (Ab) (lanes 5 to 8) or monoclonal antibodies (A16) (lanes 9 and 10). The N-terminal antibody (N21) was derived from immunization of a rabbit with a synthetic polypeptide conjugated to keyhole limpet hemocyanin (detailed in Materials and Methods). Preimmune serum (PreImm) (lanes 1 to 4) was derived from the same rabbit immunized with the polypeptide antigen. Li and D1, 40 μg of liver nuclear extract and 30 μg of p388D1 cell nuclear extract, respectively. Lanes 3 and 7 (−) and lanes 4 and 8 (f), nuclear extracts from the nontransfected and the AGP/EBP(f)-transfected HepG2 cells, respectively. (C) Schematic representation of three polypeptides encoded by the *agp*/*ebp* gene. The Ala/Pro-, Ala-, Pro/Ser-, and Ser-rich stretches are shown. An additional Ala/Pro stretch of the 21-amino-acid sequence located at the N terminus of AGP/EBP(f) is indicated.

CAT in a transfection assay. However, a suboptimal dose of the AGP/EBP expression vector has a minimal activation effect on AGP-CAT (20). To investigate the putative activation of the *agp* gene by NF-κB, we performed cotransfection experiments using expression vectors of NF-κB (p50 and p65) and the reporter plasmid AGP-CAT. NF-κB (p50 and p65) alone cannot stimulate CAT expression (Fig. 2A). However, when the expression vectors of NF-κB and a suboptimal dose of AGP/EBP(f) (50 ng) were cotransfected with AGP-CAT, synergistic activation of CAT expression was observed (Fig. 2A). Members of the C/EBP family were previously shown to have physical and functional interactions with NF-κB (p50 or p65) (18, 30). To determine the biochemical basis for the synergistic activation of the *agp* gene by AGP/EBP(f) and NF-κB (p50 and p65), we investigated their physical interactions. Mixtures of recombinant NF-κB and AGP/EBPs were chemically cross-linked by a reversible cross-linker, dithio-bis(succinimidyl-propionate), to stabilize the complexes during immunoprecipitation. All three forms of AGP/EBP were efficiently coimmunoprecipitated with p50 of NF-κB by using anti-p50 antibodies (Fig. 2B, lanes 1 to 4). In contrast, bZIP domain-truncated AGP/EBP could not be coimmunoprecipitated (Fig. 2B, lane 5). Similar results were obtained for interactions between NF-κB p65 and AGP/EBPs (data not shown). These results suggest that the synergistic interaction between AGP/EBP(f) and NF-κB (p50 and p65) depends on their physical interactions. Similar results were also obtained for interactions between FLAG-tagged recombinant NF-κB p50 protein and AGP/EBPs. FLAG-tagged recombinant NF-κB protein was incubated with in vitro-translated AGP/EBP(f) (Fig. 2C, lanes 3 and 5) and AGP/EBP-dbZIP (lanes 4 and 6). All three forms of AGP/EBP were efficiently precipitated with p50 by using an anti-FLAG-M2 affinity gel (Fig. 2C, lane 3). In contrast, bZIP domain-truncated AGP/EBP could not be precipitated by FLAG-tagged NF-κB p50 (Fig. 2C, lane 4). These results suggest that the synergistic interaction between AGP/EBP(f) and NF-κB (p50 and p65) depends on their physical interactions.

Synergistic activation of *agp* by NF-κB and AGP/EBP depends on the AGP/EBP-binding motif. Four separate AGP/EBP-binding motifs (A, C, D, and E) have been identified in the 180-bp 5′ upstream regulatory region of *agp* (20). Although the sequence of the D motif, 5′-GGGAAATGTC-3′, is similar to that of the canonical κB site (5′-GRGRNNYYYY-3′), oligonucleotides containing the D motif cannot be recognized by NF-κB in a mobility shift assay (unpublished results). The functional importance of these sites for AGP/EBP transactivation has been established by our previous work (20). To determine the functional roles of these motifs in the synergistic activation of the *agp* gene by NF-κB and AGP/EBP, we conducted transfection experiments using reporter plasmids containing mutations in the C, D, and E sites and deletion of the C, D, and E sites (Fig. 3A). Mutations in the C and E sites and deletion of the C, D, and E sites cause more-reduced synergistic activation of *agp* by NF-κB and AGP/EBP than a single mutation of the D site (Fig. 3A). Our previous data indicate that both C and E motifs are essential for the maximal induction of the *agp* gene by AGP/EBP (20). The present results showing reduced synergistic activation of *agp* by NF-κB (p50 and p65) and AGP/EBP(f) due to mutations of C and E sites agree nicely with our previous results (20).

Furthermore, a mutant of AGP/EBP, carrying an S-239→L (S239L) mutation (which was shown to lack the sequence-specific motif-binding activity [26]) lost its activity upon cooperating with NF-κB (p50 and p65) in synergistic activation of the *agp* gene (Fig. 3B). These results demonstrate that the binding of AGP/EBP to its cognate motif is essential for its synergistic interaction with NF-κB (p50 and p65).

The N-terminal 21-amino-acid portion of AGP/EBP(f) plays important roles in the synergistic activation of *agp* by AGP/EBP(f) and NF-κB. When optimal amounts of the AGP/EBP(f) and AGP/EBP(m) expression vectors are used, both can activate AGP-CAT in a cotransfection assay. However, the basal activity of AGP/EBP(m) is much higher than that of AGP/EBP(f) (Fig. 4A). This difference may be attributed to the intrinsic properties and protein levels of AGP/EBP(f) and AGP/EBP(m) (13). However, NF-κB can activate the *agp* gene by its synergistic interaction with AGP/EBP(f) or AGP/EBP(m). The synergistic activation of AGP-CAT by AGP/EBP(f) and NF-κB is fourfold higher than that of AGP/EBP(m) and NF-κB (Fig. 4A, right panel). To determine the protein levels

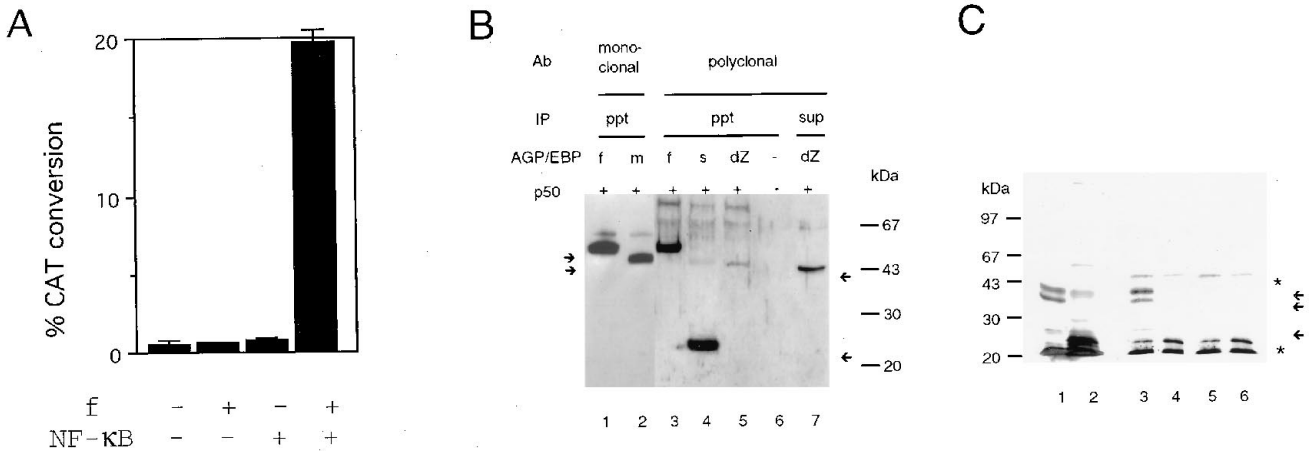

FIG. 2. Functional and physical interaction between AGP/EBP and NF-κB as demonstrated by cotransfection and coimmunoprecipitation (IP) experiments. (A) BHK cells were transfected with AGP-CAT (2 μg) in the presence of expression vectors for AGP/EBP(f) (50 ng), NF-κB p50 and p65 (125 ng each), or AGP/EBP(f) and NF-κB p50 and p65. (B) Purified, recombinant NF-κB p50 protein (0.5 μg) was incubated with recombinant f (0.5 μg) (lanes 1 and 3), m (lane 2), s (lane 4), and AGP/EBP-dZIP (dZ) (lane 5). The immunoprecipitates resulting from anti-p50 antibody (p50) (Santa Cruz Biotech) treatment were subjected to Western blot analysis. ppt, immunoprecipitates; sup, supernatant after separation of the immunoprecipitates; +, presence of anti-p50 antibody. The arrows indicate AGP/EBP proteins detected by Western blotting with anti-AGP/EBP monoclonal (lanes 1 and 2) and polyclonal (lanes 3 and 7) antibodies (Ab). (C) Lanes 1 and 2, in vitro-translated f and AGP/EBP-dbZIP, respectively. Purified, FLAG-tagged recombinant NF-κB p50 protein (0.5 μg) was incubated with in vitro-translated f (lanes 3 and 5) and AGP/EBP-dbZIP (lanes 4 and 6). The precipitates resulting from an anti-FLAG M2 affinity gel (IBI) (lanes 1 and 2) or protein A-beads without antibody (lanes 3 and 4) were subjected to SDS-PAGE and autoradiography. Arrows indicates the f, m, and s forms of AGP/EBP, while asterisks represent the nonspecific signal.

of AGP/EBPs in cells transfected with various expression vectors (Fig. 4A, left panel), Western blot analysis was used to detect proteins in the nuclear extracts of these transfected cells. Both anti-AGP/EBP monoclonal (A16) and N21 polyclonal antibodies were used as probes (Fig. 4B). The AGP/EBP(m) expression vectors do not express the f form as detected in Western blotting with N21 antibodies (Fig. 4B, right panel, lane 3). Although the protein levels in the transfected cells varied, no correlation between the activation by these vectors and their protein expression levels was observed (compare Fig. 4A, right panel, and Fig. 4B, left panel, lanes 2 and 5; right panel, lanes 2 and 3). As long as there is expression of AGP/EBP(f), the synergistic activation by these expression vectors and NF-κB (p50 and p65) is fourfold higher than for that which contains the m form alone (compare the results of Fig. 4A, right panel, and Fig. 4B, right panel). To prove that the sequences located in the N-terminal 21-amino-acid region are responsible for the observed greater synergistic activation of *agp* by AGP/EBP(f) and NF-κB than by AGP/EBP(m) and NF-κB, we created mutants that have either a perfect Kozak consensus sequence at the first translation initiation site (m1) or a silent second translation initiation site (m2) (Fig. 4A, left panel). Members of the first class of mutants preferentially produce AGP/EBP(f), whereas members of the second class, which possess TTG, do not produce AGP/EBP(m) at all (Fig. 4B, left panel, lanes 3 and 4). In cotransfection experiments, m1 behaves similarly to wild-type AGP/EBP(f) (Fig. 4A, right panel). However, synergistic activation of AGP-CAT and NF-κB in m2 is better than that of m1 and wild-type AGP/EBP(f) (Fig. 4A). Because of the lack of AGP/EBP(m), the basal activity of m2 is noticeably lower than that of m1 and wild-type AGP/EBP(f). These data indicate that the stretches located in the N-terminal 21-amino-acid region of AGP/EBP(f) play an important role in the synergistic activation of the *agp* gene.

Extracellular stimuli that activate NF-κB can preferentially cooperate with AGP/EBP(f) to activate the expression of the *agp* gene. A number of extracellular stimuli, such as proinflammatory cytokines (e.g., IL-1β), LPS-conditioned macrophage medium, tetradecanoyl phorbol acetate, and UV irradiation, can activate NF-κB (3, 4, 22). The results described above showed that NF-κB and AGP/EBP(f)/(m) can activate the *agp* gene synergistically. To further study whether the mechanism of this synergistic action is physiologically relevant, we performed cotransfection experiments to compare the responses of CMV-AGP/EBP(f) and CMV-AGP/EBP(m) to two reporters, AGP-CAT (*agp*, wild-type AGP promoter) and mutant AGP-CAT (mtE, the E motif-mutated AGP-CAT) in the presence or absence of various extracellular stimuli. In the presence of LPS-conditioned macrophage medium, only AGP-CAT was stimulated (Fig. 5A, left panel); mutant AGP-CAT showed no response to LPS-conditioned medium. The LPS-conditioned medium could enhance the synergistic reaction of cotransfected CMV-AGP/EBP(f) and wild-type AGP-CAT (Fig. 5A, left panel). Similarly, cotransfected CMV-AGP/EBP(f) responds well to UV irradiation for stimulation of AGP-CAT, while CMV-AGP/EBP(m) shows no response (Fig.

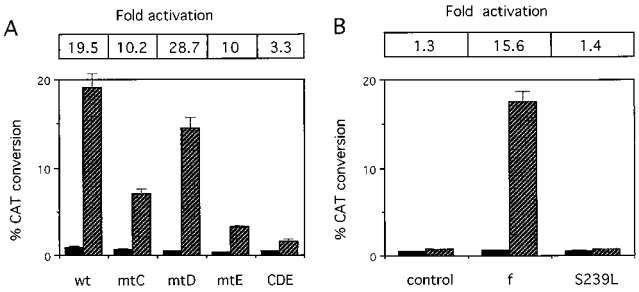

FIG. 3. Synergistic activation of the *agp* gene by AGP/EBP(f) and NF-κB is dependent on the AGP/EBP-binding motif. (A) Functional assays of the reporter plasmids derived from wild-type (wt) and mutant *agp* 5′ upstream regulatory sequences cotransfected with CMV-AGP/EBP(f) (50 ng) (solid bars) or CMV-AGP/EBP(f) (50 ng) plus NF-κB p50 and p65 (125 ng each) (hatched bars). Four mutants were created by site-directed (mtC, mtD, and mtE) or deletion (CDE) mutagenesis as detailed in Materials and Methods (19, 20). (B) A mutant of AGP/EBP(f) (S239L) that cannot bind to the AGP/EBP motif has lost its synergistic activation of the *agp* gene with NF-κB p50 and p65. Functional assays of transfections of AGP-CAT alone (control) or with CMV-AGP/EBP(f) or mutant CMV-AGP/EBP(f)-S239L with CMV-NF-κB p50 and p65 were performed.

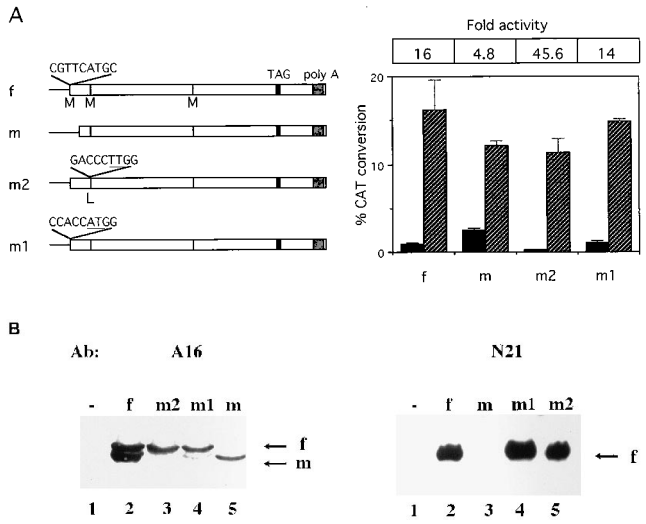

FIG. 4. Synergistic activation of AGP-CAT by NF-κB and various AGP/EBPs and AGP/EBP protein levels in transfected cells. (A) Functional synergism of AGP/EBPs containing mutations within or around the first two ATGs with NF-κB. Left panel, partial sequence of the expression vectors used for the transfection studies. f, wild-type CMV-AGP/EBP(f); m, CMV-AGP/EBP(m), which is lacking the N-terminal 21 amino acids; m2, the second ATG of CMV-AGP/EBP(f) was mutated to TTG; m1, the first translation initiation context of CMV-AGP/EBP(f) was mutated to a perfect Kozak consensus context. Right panel, activation of AGP-CAT by cotransfection of CMV-NF-κB p50 and p65 (125 ng each) with expression vectors (50 ng each) f, m, m1, and m2 (hatched bars) or with these expression vectors in the absence of CMV-NF-κB p50 and p65 (solid bars). (B) Western blot analysis of nuclear extracts prepared from the transfected cells. Ten micrograms of CMV-AGP/EBP(f), m, m1, or m2 was transfected into HepG2 cells. A nuclear extract (5 μg) was subjected to SDS-PAGE and blotted onto Hybond-C (Amersham). The probe was monoclonal antibodies (Ab) to AGP/EBP (A16) or N21 antibodies (1:1,000 dilution).

5A, right panel). Finally, IL-1β can also stimulate the activation of AGP-CAT in the presence of AGP/EBP(f) (Fig. 5A, right panel). None of these extracellular stimuli can enhance AGP/EBP(m)-mediated activation of AGP-CAT. These results show that extracellular stimuli can preferentially cooperate with AGP/EBP(f) for the induction of the *agp* gene. To further demonstrate that NF-κB plays an essential role in this synergistic interaction, we deleted the activation domain of p65, to give p65dAD (Fig. 5B, left panel). When AGP-CAT was cotransfected with CMV-AGP/EBP(f), CMV-p50, and CMV-p65dAD, the synergistic interaction between these expression vectors was abolished (Fig. 5B, right panel). These results indicate that the synergistic interaction of AGP/EBP(f) and NF-κB is essential for the activation of the *agp* gene.

A novel transcription factor, Nopp140, interacts with AGP/EBP(m) preferentially, which results in synergistic activation of the *agp* gene. We have identified Nopp140 as an AGP/EBP-interacting protein by immunoaffinity chromatography followed by liquid chromatography-mass spectrum (LC/MS/MS) analysis. Nopp140 has also been shown to be a transcription factor (25). Biochemical characterizations indicated that the bZIP domain of AGP/EBP is critical for its interaction with Nopp140 (25). To investigate the potential differences in functional interaction between Nopp140 and AGP/EBP(f) or AGP/EBP(m), we performed cotransfection experiments using expression vectors of CMV-Nopp140, CMV-AGP/EBP(f), and CMV-AGP/EBP(m) and the reporter plasmid AGP-CAT. Nopp140 cooperates with AGP/EBP(m), but not AGP/EBP(f), which results in synergistic activation of AGP-CAT (Fig. 6). Consistent with the results described above, the basal transcriptional activity of AGP/EBP(m) is higher than that of AGP/EBP(f) (Fig. 6).

DISCUSSION

The discovery that one gene can encode activators and repressors has important implications for our understanding of the regulation of positive and negative transcription factors in the cell. There are many examples of proteins with diverse function derived from a single gene by alternative splicing mechanisms (16). Alternative splicing of the proto-oncogene *erbA*α generates three different transcripts. Their encoded proteins are identical throughout the N-terminal 370 amino acids but diverge thereafter. These different isoforms exhibit a tissue-specific distribution and thyroid hormone response. Detailed analyses of alternative splicing of genes that encode activators and repressors have been described (16). The CREM mRNA isoforms generated by alternative splicing encode both activators (CREMτ) and repressors (CREMα, -β, and -γ). These isoforms illustrate the regulation of alternative splicing on the developmental function of a transcription factor in a tissue-specific manner (16). Descombes and Schibler (13) have demonstrated that the *lap* gene (a rat homolog of mouse *agp*/*ebp* and the human NF-IL6 gene) encodes different proteins (LAP and LIP) by using alternative translation start sites.

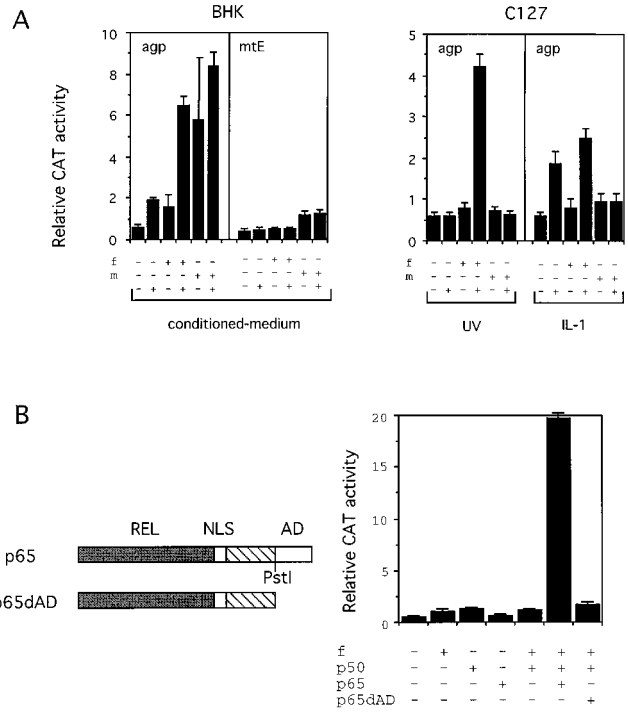

FIG. 5. Extracellular stimuli that induce NF-κB can also stimulate AGP/EBP(f). (A) AGP-CAT can be activated by treatment of CMV-AGP/EBP(f)-transfected cells with LPS-conditioned macrophage medium, UV irradiation, or IL-1β. Left panel, BHK cells were transfected with CMV-AGP/EBP(f) or CMV-AGP/EBP(m) with (+) or without (−) LPS-conditioned macrophage medium treatment. agp and mtE, wild-type AGP-CAT and E-site-mutated AGP-CAT, respectively. Right panel, C127 cells were used for transfection followed by UV irradiation or IL-1β (IL-1) treatment. (B) Transactivation domain of p65 is required for synergistic interaction between NF-κB p50 and p65 and AGP/EBP(f). Left panel, domain organizations of wild-type p65 and transactivation domain (AD) deletion mutant of p65 (p65dAD). Right panel, CAT activities of cells transfected with AGP-CAT and CMV-AGP/EBP(f) or with AGP-CAT and CMV-AGP/EBP(f) and CMV-NF-κB (containing wild-type or mutant p65). NLS, nuclear localization signal.

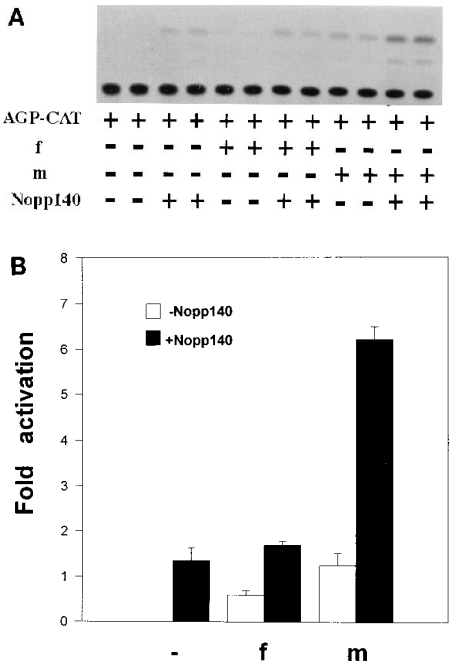

FIG. 6. Synergistic activation of the *agp* gene by AGP/EBP(m) and Nopp140. BHK cells were cotransfected with AGP-CAT (2 μg) and CMV-AGP/EBP(f) (0.1 μg) or AGP/EBP(m) (0.1 μg) in the absence or presence of CMV-Nopp140 (0.5 μg). Duplicate experiments were performed. (A) Autoradiogram; (B) Fold activation.

The original discovery revealed that LAP is an activator while LIP is a repressor. It was unclear whether two LAPs were indeed produced in vivo. Descombes and Schibler (13) suggested that the larger LAP may be derived from posttranslational modifications of LAP. However, several lines of evidence suggest that this is not the case. In the original study (13), when cells were transfected with LAP, only LAP was produced (i.e., no modified form of LAP could be produced). However, cDNA containing the wild-type LAP (i.e., both full-length and mid-length LAP can be produced) could encode two larger forms of LAP. In the present paper, we have demonstrated clearly that both forms [i.e., AGP/EBP(f) and AGP/EBP(m)] can be encoded by the *agp/ebp* gene. Furthermore, it has been suggested that three forms corresponding to our AGP/EBP(f), AGP/EBP(m), and AGP/EBP(s) were produced by the human homolog of LAP, NF-IL6 (33).

agp/ebp gene expression may be regulated at different levels in living cells, such as transcriptional, translational, and posttranslational modifications of AGP/EBP and combined interactions of AGP/EBP with other proteins. This study demonstrates that the hitherto-neglected form of AGP/EBP (i.e., the f form) can be expressed by the intronless *agp/ebp* gene. Two lines of evidence indicate the presence of AGP/EBP(f): (i) Western blotting of nuclear extracts with N21 antibodies and (ii) in vitro transcription and translation of AGP/EBP cDNA (full length). The preparation of rabbit antibodies against a peptide of the N-terminal 21 amino acids is crucial for the identification of AGP/EBP(f) unequivocally. These same antibodies can be used to identify proteins that may preferentially interact with AGP/EBP(f) rather than AGP/EBP(m). This should enable us to study the functional regulation of AGP/EBP(f) and AGP/EBP(m) and their potential interaction with other proteins.

The basal activity of AGP/EBP(m) is much higher than that of AGP/EBP(f) (13) (Fig. 4A). This difference may be due to higher protein levels of AGP/EBP(m) than of AGP/EBP(f) in the cells. The levels of AGP/EBP(m) in most cells or in liver are indeed higher than those of AGP/EBP(f). However, whether this accounts for the higher basal activity of AGP/EBP(m) than of AGP/EBP(f) remains to be resolved. It is not yet known if the differences observed in basal activity and synergy are relevant to the regulation of an endogenous, inducible gene. It is also not yet known if the higher basal activity of the m form has an impact on the regulation of one or more endogenous genes or if the higher basal activity is irrelevant. However, a newly discovered AGP/EBP(m)-interacting transcription factor, Nopp140, is relatively abundant compared with the inducible factor NF-κB. This may account for the higher basal activity of AGP/EBP(m) in transfection assays. These results suggest that intrinsic properties of these activators may be responsible for the differences in their functional activities. It is not yet known if the f form preferentially targets genes that contain an adjacent NF-κB site, despite the fact that the f form seems to synergize with NF-κB somewhat more strongly than the m form. The actual picture is much more complex than the present oversimplified interpretations. The only difference between AGP/EBP(f) and AGP/EBP(m) is that the former has an additional 21 amino acids at its N-terminal region. Our data clearly indicate that this difference in sequence is responsible for their functional diversity. An alternative explanation is that this may be due to more-effective repression by dimer formation of AGP/EBP(f) and AGP/EBP(s) than of AGP/EBP(m) and AGP/EBP(s). NF-κB has been shown to interact with members of the C/EBP family physically and functionally (30). Although both AGP/EBP(f) and AGP/EBP(m) interact with NF-κB, which results in the synergistic activation of the *agp* gene, the degree of synergism for AGP/EBP(f) is much higher than that for AGP/EBP(m). This may be due to the additional alanine/proline-rich stretch located at the N-terminal region of AGP/EBP(f). This additional stretch of sequence may account for the overall conformational difference between AGP/EBP(f) and AGP/EBP(m). Recently obtained data indicate that C/EBPβ assumes a tightly folded conformation in the DNA binding and activation domains, which are masked by interactions with the regulatory domain (34). The protein must undergo activation to function efficiently in certain types of cells (34). Thus, the synergistic interactions of AGP/EBP and NF-κB are likely due to the induced conformational changes of AGP/EBP. It is unlikely that NF-κB has higher affinity with AGP/EBP(s), which may result in the removal of repressor.

This study provides evidence for the importance of the sequences of the 21-amino-acid stretch located at the N terminus of AGP/EBP(f). This region is alanine and proline rich. For a number of other proteins or transcription factors, Ala/Pro-rich domains have been implicated in their normal functions (17, 36). Several lines of evidence show that the Ala/Pro stretch of the N-terminal 21 amino acids of AGP/EBP(f) is crucial for the synergistic interaction between NF-κB and AGP/EBP(f). (i) The functional synergism of NF-κB and m2 is much greater than that of NF-κB and wild-type AGP/EBP(f) (Fig. 4A). This result can be attributed to the production of AGP/EBP(f) [with no AGP/EBP(m)] by m2 vector, while wild-type AGP/EBP(f) vector can encode both AGP/EBP(f) and AGP/EBP(m) (Fig. 4B). (ii) The functional synergism of AGP/EBP(m) and NF-κB is reduced. This is due to the inability of mutant m to produce AGP/EBP(f). The basal activity of AGP/EBP(m) and its functional synergism with NF-κB in activating the *agp* gene cannot be altered by changing its expression levels (data not shown). In contrast, the basal activity of AGP/EBP(f) is low when the

full-length AGP/EBP expression vector is used for transfection studies, and it can be even lower when the second ATG is mutated to TTG (Fig. 4A) [only AGP/EBP(f) was produced]. Thus, it is most likely that the 21-amino-acid stretch of AGP/EBP(f) may be a transactivation domain responsible for the synergistic interaction of AGP/EBP(f) with NF-κB. This also explains the much lower degree of synergistic interaction between AGP/EBP(m) and NF-κB than between AGP/EBP(f) and NF-κB, which is most likely due to the common Pro/Ala-rich domains in AGP/EBP(f) and AGP/EBP(m). These interpretations are further strengthened by results obtained from transfection studies with the mutants m1 and m2. This is most likely due to the expression of AGP/EBP(f) but lack of AGP/EBP(m). Functional synergism is not affected when m2 and m1 are cotransfected with NF-κB (Fig. 4). Both m2 and m1 can result in the production of AGP/EBP(f); therefore, no detrimental effect on their synergistic interaction with NF-κB is observed. However, we cannot rule out the possibility that the synergistic effect of NF-κB and AGP/EBP(f) is due to the sequestration of AGP/EBP(s) by NF-κB. The data of Descombes and Schibler (13) showed that m1 and m2 did not produce LIP. It is unlikely that the sequestration occurs. AGP/EBP is a liver-enriched transcription factor; the basal activity of AGP/EBP(m) may play important roles in maintaining the normal liver homeostasis, while AGP/EBP(f) may have crucial functions during the acute-phase response. Our conclusion about the differential activation of AGP/EBP(f) and AGP/EBP(m) by NF-κB is further supported by the preferential activation of AGP/EBP(f) by NF-κB inducers. These results have shown that the induction of the acute-phase response gene (i.e., *agp*) by physical and functional interactions of NF-κB and AGP/EBP(f) is physiologically important. Furthermore, NF-κB plays a key role in induction of acute-phase response genes whether they contain a κB motif or not. These results also demonstrate that combinatorial interactions between members of different families (27, 30, 31) are much more complex than previously realized.

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The first two authors contributed equally to this work.

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