Regulation of CCAAT/Enhancer-binding Protein (C/EBP) Activator Proteins by Heterodimerization with C/EBPγ (Ig/EBP)*

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The CCAAT/enhancer-binding proteins (C/EBPs) are basic leucine zipper transcription factors that play important roles in regulating cell growth and differentiation. C/EBP proteins form leucine zipper-mediated homodimers but are also capable of heterodimerizing with other C/EBPs in vitro. Here we show that C/EBPβ occurs predominantly as a heterodimer that displays rapid mobility in gel shift assays. Biochemical fractionation and antibody supershift assays demonstrate that the C/EBPβ heterodimeric partner is C/EBPγ (Ig/EBP), a C/EBP protein that has been implicated as an inhibitor of other family members. Although most cell types express C/EBPβ/C/EBPγ heterodimers, macrophages contain a C/EBPβ partner that is serologically distinct from C/EBPγ. We found that C/EBPγ blocked the ability of C/EBPβ and C/EBPγ to activate a reporter gene in L cell fibroblasts but did not inhibit a chimeric C/EBPβ protein containing the GCN4 leucine zipper. Repression by C/EBPγ occurs at the level of transactivation and requires heterodimerization with the C/EBP partner. C/EBPγ was an ineffective repressor in HepG2 hepatoma cells despite forming C/EBP heterodimers, and C/EBPα was not effectively inhibited in either L or HepG2 cells. Our findings demonstrate that C/EBPγ modulates C/EBP activity in a cell- and isoform-specific manner.

Eukaryotic transcription factors commonly occur in families whose members share similar DNA binding specificities and other functional properties. Many transcription factors are dimeric and can form homodimers as well as heterodimers with other family members. The capacity to heterodimerize provides a means of enhancing regulatory diversity, as the various dimeric species within a protein family may exhibit distinct functional properties (1–4). Thus, it is important to elucidate the dimerization status of transcription factors in vivo to understand the full range of their biological activities and regulation. In the present study, we have examined the dimerization properties of CCAAT/enhancer-binding proteins (C/EBPs) in cells. C/EBPs are a family of basic leucine zipper (bZIP) DNA-binding proteins (5) consisting of five core members: C/EBPα, C/EBPβ, C/EBPδ, C/EBPγ, and C/EBPδ (Ig/EBP). C/EBP proteins bind to DNA as dimers and display highly related DNA binding and dimerization specificities (reviewed in Refs. 6–8). C/EBPs are involved in the regulation of many cellular processes. C/EBPα, C/EBPβ, and C/EBPδ regulate gene expression including stress and inflammatory signals, including regulation of acute phase response genes in hepatocytes and expression of proinflammatory cytokine genes in monocytes (9–13). Overexpression experiments and analysis of knockout mice demonstrate that C/EBP proteins also control cell growth and differentiation (6–8, 14). For example, forced expression of C/EBPα and/or C/EBPβ in precursor cells of the adipocyte, granulocyte, and keratinocyte lineages causes growth arrest and induces cellular differentiation (15–17). In other contexts, C/EBPγ has been reported to stimulate cell growth (18, 19). Thus, C/EBP proteins regulate a variety of cellular phenotypes in a wide range of cell types.

In addition to forming homodimers, C/EBP proteins are capable of heterodimerizing with the other family members in vitro (20–22). Heterodimerization could potentially alter several functional activities of C/EBP proteins, including DNA binding, transactivation potential, responsiveness to signaling pathways, and the ability to cooperate with other transcription factors. It has been assumed that heterodimers between C/EBP family members occur in vivo and possess regulatory activities that are distinct from the homodimeric forms. However, there is no evidence for such heterodimers in vivo. An association between C/EBPα and C/EBPβ was observed in transient overexpression experiments (20), and evidence has been reported for C/EBPα/C/EBPβ heterodimers in liver nuclear extracts (23) and mononuclear cells (24). In addition, C/EBPs appear to heterodimerize with proteins from other bZIP subfamilies, including Fos/Jun (25) and ATF/CREB (26–28).

Here we report that C/EBP proteins in cell and tissue extracts are found predominantly as heterodimers with C/EBPγ. C/EBPγ is a ubiquitously expressed member of the C/EBP family that was first identified by its affinity for cis-regulatory sites in the Ig heavy chain promoter and enhancer (29). C/EBPγ contains a C/EBP-like bZIP region but lacks an amino-terminal transactivation domain (30, 31) and can inhibit transcriptional activation by C/EBPα or C/EBPβ (31). We show that C/EBPγ can repress C/EBPβ- and C/EBPδ-mediated transactivation of a reporter gene in fibroblasts in a leucine zipper-dependent manner, indicating that the repression by C/EBPγ involves heterodimerization with its partner. Interestingly, C/EBPγ did not repress transactivation of C/EBPβ or C/EBPδ in HepG2 hepatoma cells, nor did it inhibit C/EBPα activity in either cell type. Thus, the ability of C/EBPγ to inhibit C/EBPβ...
activators is cell-specific and differs for the various C/EBP family members.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Culture**—L cell fibroblasts were cultured in Dulbecco's modified Eagle's medium (BioWhittaker, Inc.) supplemented with 10% fetal bovine serum (HyClone, Inc.) in the presence of kanamycin, streptomycin, and penicillin. HepG2 hepatoma cells (ATCC HB-8065) were maintained in minimum essential Eagle's medium (BioWhittaker, Inc.) supplemented with nonessential amino acids, sodium pyruvate, 10% fetal bovine serum (HyClone, Inc.) in the presence of kanamycin, streptomycin, and penicillin. Other cell lines used for analysis of nuclear extracts were C6-2B (rat glioma; Ref. 32), IC-21 (murine macrophage; Ref. 33; ATCC TIB 186), and EMT6 (murine mammary tumor). P388-Cβ and P388-C6-C1 cells are stably transfected P388 lymphoblasts (ATCC CCL 46) expressing C/EBPβ and C/EPFδ, respectively (34, 35). P388 cell lines were grown in RPMI 1640 (BioWhittaker) supplemented with 10% fetal clone 1 serum (HyClone, Inc.), glutamine, kanamycin, streptomycin, and penicillin.

**Antibodies**—C/EBPβ antisera specific for the COOH terminus (C-19) was obtained from Santa Cruz Biotechnology. Peptide antisera recognizing the NH2 terminus of C/EBPβ (20) and the NH2 terminus of C/EBPδ (36) have been described. A polyclonal antisera against bacterially expressed C/EBPγ (37) was kindly provided by K. Calame. Two peptide antisera were raised against murine C/EBPβ by immunizing rabbits with synthetic peptides corresponding to the amino-terminal (Ser-Lys-Leu-Ser-Gln-Pro-Ala-Thr-Thr-Pro-Gly-V al-A sn-Gly-Cys) and carboxy-terminal peptide (Cys-Ile-Ser-Thr-Glu-Thr-Thr-Ala-Thr-Asn-Asp)

**Plasmid Constructs**—The C/EBPβ coding sequence was amplified by PCR from a plasmid containing a C/EBPβ clone of murine origin (29). Two oligonucleotide primers were used, one overlapping the initiation codon with an introduced NcoI restriction site and the second spanning the termination codon with an introduced Hind III restriction site. The PCR product was digested with NcoI (partial) and Hind III (complete) and subcloned into pMEX expressing C/EBPβ. A bacterial expression construct, pJL6-C/EBPβ, was generated by inserting the digested PCR product into the expression vector pJL6 as described (38). The GAL4-C/EBPβ hybrid construct was described previously (38).

**Transient Transfections**—Transfections were carried out using 30–40% confluent monolayers in 10-cm dishes using FuGENE™ (Roche Molecular Biochemicals). For co-transfection experiments, a constant amount of C/EBP reporter plasmid (DE1–, alb- luc (2.5 μg) (38) and C/EBP expression constructs pMEX-C/EBPβ, pMEX-C/EBPδ-G2, pMEX-C/EBPδ, or pMEX-C/EBPβ (0.75 μg) were transfected with varying quantities of C/EBP expression construct pMEX-C/EBPγ (0.5–8.5 μg). pRSV-β-galactosidase (0.5 μg) was co-transfected to normalize for transfection efficiency. The total amount of DNA used for transfections (12.25 μg) was kept constant by adding an appropriate amount of the pMEX vector. After 48 h, the cells were lysed and analyzed for luciferase activity using the Enhanced Luciferase Assay Kit (PharMingen International) and for β-galactosidase activity using the luminescence β-galactosidase Genetic Reporter System II (CLONTECH Laboratories, Inc.).

GAL4-C/EBPβ transfection assays were conducted using 30–40% confluent monolayers in 60-mm wells using FuGENE™ (Roche Molecular Biochemicals). One μg of (G3)2 E1B-luc reporter plasmid (38), 5 ng of GAL4-C/EBPβ vector, and 25 ng of Ha-Ras (12V) vector were transfected with varying quantities of the pcDNA3.1 C/EBPγ expression vector (13.3 ng to 1.25 μg). The Renilla luciferase vector, pRL-TK (Promega), was co-transfected as an internal standard for transfection efficiency. Sixteen hours prior to harvesting the cells, the medium was removed and replaced with serum-free media. Cells were collected 48 h after transfection, lysed, and analyzed using the Dual-Luciferase® assay system (Promega).

**Nuclear Extracts**—Nuclear extracts from cell lines and transfected cells were prepared by a detergent lysis method. Transfected cells were washed once with buffer, scraped, and then divided. 20% of the cells from 10-cm dishes were used for luciferase assays by resuspension in detergent lysis solution (100 mM potassium phosphate (pH 7.8), 0.2% Triton X-100, 1 mM diethiolether (DTT); CLONTECH Laboratories, Inc.), incubation at room temperature for 5 min and centrifugation. The remaining 80% of the cells were used to make nuclear extracts by resuspension in lysis buffer (20 mM HEPES (pH 7.9), 1 mM EDTA, 10 mM NaCl, 1 mM DTT, 0.1% (w/v) Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, 5 μg/ml apro tinin, 5 μg/ml antipain) and incubation on ice for 10 min. Nuclei were centrifuged at 3,500 rpm for 10 min. Proteins were extracted from nuclei by incubation in high salt buffer (25 mM HEPES (pH 7.9), 0.2 mM EDTA, 0.42% NaCl, 0.2 mM DTT, 25% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, 5 μg/ml antipain) at 4°C for 20 min with vigorous shaking. Nuclear debris was pelleted by centrifugation at 14,000 rpm for 5 min, and the supernatant was collected and stored at −70°C. Nuclear extracts from mouse tissues were prepared by an Nonidet P-40 lysis procedure as described previously (39).

**Electrophoretic Mobility Shift Assay (EMSA)**—The following double-stranded oligonucleotides containing the wild-type consensus C/EBP site (bold) or a mutant C/EBP site (bold) were used as probes or competitor DNAs.

**Wild-type:** 5′-GATCCATATCCCTCCATGCGCCATTAGGCTAAAA

**Mutant:** 5′-GATCCATATCCCTCAGGGCCCTTAGGCTAAAA

The probe was end-labeled using [32P]dCTP and Klenow polymerase. DNA-protein complexes were formed as described (38). The following double-stranded oligonucleotides containing the wild-type consensus C/EBP site (bold) or a mutant C/EBP site (bold) were used as probes or competitor DNAs.

**Wild-type:** 5′-GATCCATATCCCTCCATGCGCCATTAGGCTAAAA

**Mutant:** 5′-GATCCATATCCCTCAGGGCCCTTAGGCTAAAA

**RESULTS**

C/EBPβ Occurs in Cells Predominantly as a Rapidly Migrating EMSA Complex—To examine the dimeric state of C/EBP proteins in cells, we performed EMSA on a series of nuclear extracts using a consensus C/EBP binding site oligonucleotide as the probe. We initially focused our analysis on C/EBPβ.
because this protein is widely expressed in cell lines and tissues. We examined nuclear extracts from cell lines representing glioma (C6-2B), mammary epithelia (EMT6), and macrophages (IC-21) (Fig. 1A). The cell line extracts contained multiple species that bound specifically to the C/EBP motif, as determined by the ability of the unlabeled wild-type probe, but not a mutated oligonucleotide, to compete for binding (Fig. 1B and data not shown). To identify complexes containing C/EBPβ, we performed supershift analysis using a C/EBPβ antibody. As shown in Fig. 1A, C/EBPβ binding activities were observed in all extracts, and the C/EBPβ EMSA complexes occurred in two forms. One complex exhibited the same mobility as a bacterially expressed C/EBPβ homodimer (lane 7), whereas a second species (indicated by asterisks) displayed considerably faster mobility in the gel. In all cases this faster migrating form was the predominant C/EBPβ species. We also analyzed extracts from P388-Cβ cells, which express C/EBPβ from a retroviral vector (34, 35). P388 is a lymphoblastic tumor cell that contains very low levels of endogenous C/EBP proteins except C/EBPγ (Ref. 41; see below). P388-Cβ nuclear extracts

**FIG. 1.** C/EBP proteins in cells occur primarily as rapidly migrating EMSA complexes. **A**, EMSA of nuclear extracts from cell lines. A consensus C/EBP binding site was used as the probe. Extracts (3–12 µg) were assayed in the presence or absence of the NH2-terminal C/EBPβ antibody. Positions of the rapidly migrating species are indicated by asterisks. Bacterially expressed C/EBPβ was analyzed in parallel (lane 7) to show the mobility of the C/EBPβ homodimer. **B**, specificity of C/EBP-DNA complexes. Increasing amounts of unlabeled C/EBP competitor probe (lanes 7–12) or a mutant oligonucleotide with a disrupted C/EBP site (lanes 1–6) were added to binding reactions containing nuclear extracts from L cells. **C**, EMSA of nuclear extracts from stably transfected P388 lymphoblasts. Extracts from P388 cells and a P388 transfectant expressing C/EBPβ (P388-Cβ) were analyzed as described in panel A.
Identification of C/EBP\(\gamma\) as the C/EBP\(\beta\) Heterodimeric Partner—The rapid mobility of C/EBP\(\beta\) heterodimers and their presence in all cell types examined indicate that the dimeric partner is a small, ubiquitously expressed protein. These features suggested that the partner might be C/EBP\(\gamma\) (Ig/EBP), a 16.4-kDa protein that can dimerize with other C/EBP proteins and is expressed at the mRNA level in many cell types (29, 31). A C/EBP\(\gamma\) polyclonal antiserum raised against recombinant C/EBP\(\gamma\) (31) did not supershift the C/EBP\(\beta\) heterodimer, although it shifted a more rapidly migrating complex that corresponds to a C/EBP\(\gamma\) homodimer (data not shown). However, biochemical purification of the rapidly migrating complex to near homogeneity suggested that the heterodimerizing factor may indeed be C/EBP\(\gamma\). Therefore, we generated additional antisera using synthetic peptides corresponding to the C/EBP\(\gamma\) amino and carboxyl termini and tested the antibodies in EMSA supershift experiments. Both C/EBP\(\gamma\) antisera supershifted a partially purified, rapidly migrating C/EBP\(\beta\) complex isolated from P388-C/EBP\(\beta\) cells, as well as a putative C/EBP\(\gamma\) homodimer also present in this preparation (Fig. 3A). Thus, these two peptide antibodies recognize C/EBP complexes that appear to contain C/EBP\(\gamma\).

To determine whether the rapidly migrating C/EBP\(\beta\) complexes in cell extracts are C/EBP\(\gamma\) heterodimers, we tested a panel of cell extracts in antibody supershift assays using the COOH-terminal C/EBP\(\gamma\) antibody. Fig. 3B (upper panel) shows that rapidly migrating C/EBP\(\beta\) complexes from glioma cells (lanes 1 and 2) and mammary epithelial cells (lanes 3 and 4), as well as from P388-C\(\beta\) cells (lanes 7 and 8), were supershifted by the C/EBP\(\gamma\) antibody. Similarly, a C/EBP\(\beta\) heterodimeric complex from P388-C\(\beta\)-C1 cells (lanes 9 and 10) reacted with the C/EBP\(\gamma\) antibody. Interestingly, the rapidly migrating C/EBP\(\beta\) complex from IC-21 macrophages was only weakly supershifted by the COOH-terminal C/EBP\(\gamma\) antibody (lanes 5 and 6) and by the NH\(_2\)-terminal C/EBP\(\gamma\) antibody (data not shown), despite the fact that this complex migrates identically with C/EBP\(\beta\)/C/EBP\(\gamma\) heterodimers from other cells. C/EBP\(\beta\) complexes from two other monocytic/macroage cell lines also did not react appreciably with C/EBP\(\gamma\) antibodies (data not shown). These findings indicate that a distinct, but functionally related, protein heterodimerizes with C/EBP\(\beta\) in monocytic cells.

Western blot experiments using the same panel of cell extracts (Fig. 3B, lower panel) confirmed that a protein of ~18 kDa, identical in size to ectopically expressed C/EBP\(\gamma\) (lane 9), was detected in all cells examined including macrophages.

Because our analysis of C/EBP dimerization thus far used transformed or immortalized cell lines, we next wished to determine whether C/EBP\(\beta\) heterodimerizes with C/EBP\(\gamma\) in normal tissues. We prepared nuclear extracts from mouse liver, brain, ovary, and spleen and analyzed them by EMSA and antibody supershift experiments. As shown in Fig. 3C, each extract contained a rapidly migrating C/EBP\(\beta\) complex (denoted by an asterisk) that could be supershifted by the C/EBP\(\beta\) and C/EBP\(\gamma\) antibodies. Nuclear extracts from L cells transfected with C/EBP\(\gamma\) (lane 14) or C/EBP\(\beta\) plus C/EBP\(\gamma\) (lane 15) were analyzed on the same gel to confirm the identities of the homo- and heterodimeric C/EBP\(\beta\) and C/EBP\(\gamma\) EMSA species. In summary, the experiments of Fig. 3 show that C/EBP\(\beta\) occurs mainly as a heterodimer with C/EBP\(\gamma\) in cell lines as well as normal tissues.

C/EBP\(\gamma\) Causes Cell-specific Repression of C/EBP-mediated Transcription—We next investigated whether heterodimerization with C/EBP\(\gamma\) affects the transcriptional activity of C/EBP\(\beta\). Initially, we examined the effect of C/EBP\(\gamma\)...
C/EBP Heterodimerization

Fig. 3. The rapidly migrating C/EBP heterodimers contain C/EBPγ. A, the C/EBPβ heterodimer reacts with two peptide antisera specific for C/EBPγ. A partially purified fraction containing the C/EBPβ heterodimer isolated from P388-C/EBPβ cells (data not shown) was analyzed by EMSA supershift using antisera raised against peptides corresponding to the amino or carboxyl termini of C/EBPγ (lanes 2 and 3, respectively). B, upper panel, nuclear extracts from the cell lines used in Fig. 1 were assayed by EMSA supershift using the COOH-terminal C/EBPγ antibody. P388-C-C1 is a P388 derivative expressing C/EBPβ. Positions of the C/EBP/C/EBPγ heterodimers and C/EBPγ homodimers are shown on the right. Lower panel, Western blot analysis of C/EBPγ in cell extracts. The nuclear extracts (~30 μg of protein) from the upper panel were analyzed by Western blotting using the COOH-terminal C/EBPγ antibody. Nuclear protein (1 μg) from untransfected (lane 6) or C/EBPγ-transfected (lane 7) L cells was analyzed in parallel to demonstrate that overexpressed C/EBPγ is identical to the endogenous protein. C, EMSA of C/EBP complexes in nuclear extracts from mouse tissues. Each extract was analyzed in the absence and presence of C/EBPβ and C/EBPγ antibodies. Bands corresponding to C/EBP/C/EBPγ heterodimers are indicated by asterisks. The last three lanes contain extracts from untransfected (lane 13), C/EBPγ-transfected (lane 14), and C/EBPβ- plus C/EBPγ-transfected (lane 15) L cells. The identities of the EMSA complexes are indicated on the right.

on C/EBPβ-mediated transactivation using a C/EBP-dependent promoter-reporter construct ([DE1]α-lb-Luc; Ref. 38) in HepG2 hepatoma cells (Fig. 4A, left panel). C/EBPβ alone increased reporter expression by ~15-fold. Co-transfecting increasing amounts of a C/EBPγ expression vector did not significantly diminish reporter gene expression, even at the highest dose of C/EBPγ (5.6 μg), which is an 11.3-fold excess of C/EBPγ over the C/EBPβ vector. This result was unexpected, because C/EBPγ was previously found to inhibit the transactivation function of C/EBPγ and C/EBPβ in B lymphoma cells, 3T3 fibroblasts, and promonocytic cells (31). Therefore, we performed a similar C/EBPγ titration experiment in L fibroblastic cells (Fig. 4A, right panel). In these cells C/EBPγ clearly inhibited C/EBPβ-mediated transactivation of the (DE1)α-lb-Luc reporter in a dose-dependent manner. Luciferase activity decreased linearly with the amount of C/EBPγ vector added and reached 22% of the control level at the maximal dose of C/EBPγ. Similar results were obtained using a reporter construct containing two copies of a consensus C/EBP binding site (data not shown). Thus, C/EBPγ is capable of inhibiting C/EBPβ activity in L cells but not in HepG2 hepatoma cells.

To assess the levels of homo- and heterodimeric C/EBP complexes in the transfected cells, we prepared nuclear extracts and subjected them to EMSA analysis (Fig. 4B). C/EBPβ homodimer and heterodimer levels were increased in the transfected cells. Heterodimers were observed in cells transfected with C/EBPβ alone, resulting from dimerization with endogenous C/EBPγ (lane 2). The amount of heterodimeric complex increased with the addition of C/EBPγ vector, as did the levels of C/EBPγ homodimer. The EMSA complexes were quantitated by phosphorimaging, and C/EBPγ homodimer levels were calculated either as the percentage of total C/EBP binding activity (heterodimer plus the two homodimeric species) or as the fraction of C/EBPγ binding activity (C/EBPβ homodimer plus heterodimer) (Fig. 4A). The proportion of C/EBPβ homodimer decreased with added C/EBPγ, and the dimerization curves were similar in HepG2 cells (no transcriptional repression by C/EBPγ) and L cells (repression). Western blotting showed that C/EBPγ did not alter C/EBPβ levels in the nuclear extracts (Fig. 4C). These experiments show that C/EBPβ/C/EBPγ heterodimers are formed in both cell types, suggesting that heterodimers are transcriptionally active in HepG2 cells but not in L cells.

To further examine whether inhibition of C/EBPβ by C/EBPγ requires heterodimerization, we used the zipper-swap mutant, C/EBPβ-G12Z, which is unable to dimerize with C/EBPγ. Fig. 5A shows that C/EBPγ did not repress C/EBPβ-G12Z activity in either HepG2 or L cells, whereas in HepG2 cells transactivation was actually enhanced at the highest dose of C/EBPγ. EMSA (Fig. 5B) verified that C/EBPβ-G12Z/C/EBPγ heterodimers were not formed in the transfected cells, although homodimers were observed. Thus, the ability of C/EBPγ to inhibit C/EBPβ activity requires heterodimerization between the two proteins and is not the result of competitive binding of transcriptionally inactive C/EBPγ homodimers to the promoter.

Because the DNA binding activity of C/EBPβ was not repressed by heterodimerization, it seemed likely that C/EBPγ inhibits transactivation. To investigate this possibility, we used a GAL4-C/EBPβ fusion protein whose ability to activate a US-dependent reporter gene (G5-E1b-luc) depends on the transactivation domain (TAD) of C/EBPβ (38). Normally the activity of full-length C/EBPβ fused to GAL4 is very low because of strong repression of the TAD by inhibitory sequences located in COOH-terminal regions of the molecule, including the bZIP domain (38, 44). However, GAL4-C/EBPβ can be
activated by coexpression of oncogenic Ha-Ras. Therefore, we transfected GAL4-C/EBPβ with a Ha-Ras(12V) vector and the G5E1b-luc reporter into L cells along with increasing amounts of the C/EBPγ vector. As shown in Fig. 6, C/EBPγ potently inhibited GAL4-C/EBPβ activity in a dose-responsive manner. This result demonstrates that heterodimerization with C/EBPγ suppresses the ability of the C/EBPβ TAD to stimulate transcription, even when C/EBPβ is tethered to DNA through a heterologous DNA-binding domain. C/EBPγ did not inhibit GAL4-C/EBPβ activity in HepG2 cells (data not shown), further supporting the observation that C/EBPγ repression is cell-specific.

We next asked whether C/EBPγ could repress transactivation by other C/EBP family members (Fig. 7). Neither C/EBPα nor C/EBPδ was inhibited by C/EBPγ in HepG2 cells, and, in fact, C/EBPδ activity was stimulated nearly 2-fold at the max-

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3 J. D. Shuman and P. F. Johnson, unpublished results.
imal C/EBP dose. This enhanced activity was associated with increased expression of C/EBPδ (data not shown), the mechanism of which is unknown. Similar to C/EBPβ, C/EBPδ transactivation was repressed by C/EBPγ in L cells. In contrast, C/EBPα activity was unaffected by C/EBPγ in L cells (Fig. 7) despite the fact that the two proteins formed heterodimers (data not shown). Collectively, our data indicate that C/EBPα heterodimers are active in L cells, whereas C/EBPβ/C/EBPγ and C/EBPδ/C/EBPγ dimers are repressed.

DNA-independent Association of C/EBPβ and C/EBPγ in Vitro and in Vivo—The observation that C/EBP proteins in cells occur predominantly as heterodimers with C/EBPγ suggested that they might preferentially dimerize with C/EBPγ. Using recombinant His-tagged C/EBPβ and C/EBPγ proteins, we performed coimmunoprecipitation assays to compare the ability of C/EBPβ to self-dimerize and to heterodimerize with C/EBPγ in vitro (Fig. 8A). Self-dimerization was examined by mixing full-length (p52) C/EBPβ with a truncated C/EBPβ protein (C/EBPβ-(192–276)) containing only the bZIP portion of the molecule. Mixtures of full-length C/EBPβ and either C/EBPγ or C/EBPβ-(192–276) were immunoprecipitated with an antibody directed against the amino terminus of C/EBPβ. The immunoprecipitates were analyzed by Western blotting using a reagent that detects the polyhistidine tag. In the absence of full-length C/EBPβ, neither of the other proteins was immunoprecipitated (lanes 1 and 2), as expected. When full-length C/EBPβ was added to the mixtures, C/EBPγ and C/EBPβ-(192–276) were detected in the precipitated fraction (lanes 3 and 4). Both proteins were immunoprecipitated with similar efficiency, suggesting that the C/EBPβ leucine zipper has comparable affinity for itself and for C/EBPγ. Thus, the predominance of heterodimers in vivo may be the result of a molar excess of C/EBPγ in cells, or, alternatively, dimerization might be regulated by a cellular mechanism such as phosphorylation.

We next examined the association between C/EBPβ and C/EBPγ in transfected cells using a co-immunoprecipitation assay. Epitope-tagged C/EBPβ, which contains the NH₂-terminal 13 amino acids of GCN4 fused to its amino terminus, was expressed in L or HepG2 cells, either alone or with C/EBPγ. Nuclear extracts were prepared and subjected to immunoprecipitation with an antibody recognizing the GCN4 tag (36),

![Fig. 5. Repression of C/EBPβ activity by C/EBPγ requires the C/EBPβ leucine zipper. A, co-transfection experiments were performed in HepG2 and L cells as described in Fig. 4, except that pMEZ-C/EBPβ-G₁₂ was used instead of wild-type C/EBPβ. Data are the average (± S.E.) of three experiments. B, EMSA of nuclear extracts from the transfected cells. Positions of C/EBPβ-G₁₂ homodimers and C/EBPγ homodimers are indicated.](image)
followed by Western blotting for C/EBP α. As shown in Fig. 8B (bottom panel), ectopic C/EBP γ co-immunoprecipitated with C/EBP β in L cells and in HepG2 cells (lanes 3 and 6). Thus, in both cell types C/EBP β and C/EBP γ are associated in the absence of DNA. These findings further support the conclusion that impaired heterodimerization does not explain the inability of C/EBP γ to repress transcription in HepG2 cells.

**DISCUSSION**

Our studies demonstrate that C/EBP activator proteins exist predominantly as heterodimers with C/EBP γ in vivo. By comparing EMSA complexes generated with recombinant C/EBP β with those from nuclear extracts, we observed that C/EBP β in cell lines and tissues occurs mainly as a rapidly migrating heterodimer. Antibodies specific for the NH2 and COOH termini of C/EBP γ supershifted the rapidly migrating C/EBP species, confirming that the heterodimers contain C/EBP γ. Our characterization of C/EBP heterodimers in this study has focused on C/EBP β, because this isoform is expressed in many...
cell lines. However, C/EBPβ and C/EBPα also occurred as rapidly migrating heterodimers in P388 transfectants that stably express these proteins (Fig. 3B and data not shown), as well as in transiently transfected L cells (data not shown). Thus, it seems likely that all of the C/EBP activators heterodimerize with C/EBPγ in vivo.

Heterodimerization with C/EBPγ did not detectably alter the DNA-binding specificity of its C/EBP partner, because homodimers and heterodimers bound efficiently to a consensus C/EBP element. The major effect of dimerization with C/EBPγ was to repress C/EBP transactivation function. In L cells, co-expression of C/EBPγ inhibited the ability of C/EBPβ and C/EBPβ to activate transcription from a C/EBP-dependent promoter. A C/EBPβ chimera containing the GCN4 leucine zipper that cannot heterodimerize with C/EBPγ was resistant to repression. C/EBPγ also suppressed transactivation by a GAL4-C/EBPβ fusion protein. Collectively, these results indicate that heterodimerization with C/EBPγ inhibits the transcriptional activity of C/EBPβ. At present it is unclear how heterodimerization with C/EBPγ suppresses transactivation. C/EBPγ lacks a TAD and by itself neither activates nor represses transcription of target genes (31). It is possible that, because C/EBP heterodimers contain only one activating subunit, they cannot efficiently stimulate transcription. Alternatively, heterodimerization with C/EBPγ might block access to a coactivator protein for which association with the C/EBP activator involves sequences in the leucine zipper and/or basic region. This possibility is currently under investigation.

The fact that C/EBPγ did not repress transactivation by any of the C/EBPβs in HepG2 hepatoma cells is noteworthy. Analysis of the C/EBPβ dimeric species expressed in transfected cells showed that heterodimers were produced and their levels increased in proportion to the amount of transfected C/EBPβ vector. A homodimeric C/EBPβ complex was observed in both L and HepG2 cells, and this complex was not appreciably diminished with increased C/EBPγ expression (Fig. 4B). It is possible that a pool of homodimers exists that is resistant to heterodimerization, perhaps because of a specific post-translational modification. However, because the occurrence of these persistent homodimers did not differ in the two cell types, their presence cannot account for the differential repression by C/EBPγ.

Because there was no difference in heterodimer formation in HepG2 and L cells, at least as assessed by EMSA and coimmunoprecipitation experiments, we postulate that C/EBP/C/EBPγ heterodimers are transcriptionally active in HepG2 cells but not in L cells. There are several potential explanations for this difference in activity. Heterodimers could be the target of activating kinases in HepG2 cells but not in L cells, whereas homodimers might be effective substrates in both cells. Such modifications could occur on either the C/EBP activator protein or the C/EBPγ subunit. It is also conceivable that protein:protein interactions mediated by the bZIP region are necessary for transcriptional activation and that heterodimeric bZIP domains are differentially active for this function in the two cell types. Irrespective of the mechanism, the ability of C/EBPγ to affect the transactivation potential of C/EBP activators in a cell-specific manner represents a novel means of controlling C/EBP activity.

A mouse strain carrying a null mutation at the C/EBPγ locus has been developed (45). Homozygous mutant animals show grossly normal embryonic development and are initially viable after birth. However, the majority of mutant mice die within 48 h of postnatal development. Although the cause of mortality was not determined, the lethal phenotype shows that C/EBPγ has an essential function in newborn animals and presumably also in adult mice. It remains to be determined whether the lethality of C/EBPγ-deficient mice results from the absence of C/EBP heterodimers, leading to formation of homodimers with altered regulatory activities. Considering the involvement of C/EBP proteins in many biological processes and the predominance of C/EBP/C/EBPγ heterodimers in cells, it is not surprising that deletion of C/EBPγ would have severe phenotypic consequences. An additional function for C/EBPγ in lymphoid cells was revealed by analysis of bone marrow chimeras generated from C/EBPγ null donor cells (45). Natural killer cells derived from mutant donors display reduced cytolytic activity and impaired production of interferon-γ in response to interleukin-12 or interleukin-18 stimulation. Nevertheless, the molecular basis for defective interferon-γ gene expression in C/EBPγ-deficient natural killer cells has not been elucidated.

In another study examining C/EBPγ function in vivo, Zafarana et al. (46) created transgenic mice overexpressing C/EBPγ in erythroid cells. Animals homozygous for the C/EBPγ transgene displayed increased fetal γ-globin gene expression compared with adult β-globin expression, indicating that C/EBPγ positively regulates γ-globin transcription. However, when C/EBPγ expression was increased further by making the transgenic allele homozygous, fetal erythropoiesis was eliminated and the embryos did not survive beyond embryonic day 14.5. These results demonstrate that C/EBPγ stoichiometry critically affects development of the erythroid lineage. We suggest that the developmental defects associated with high ectopic C/EBPγ expression may result from decreased levels of C/EBP homodimers in erythroid precursor cells.

In addition to regulating transcription, C/EBPγ proteins can induce cell growth arrest (47–49). In proliferating cell lines, C/EBP proteins occur primarily as heterodimers, raising the possibility that heterodimerization with C/EBPγ mitigates the growth arrest activity of these proteins. In experiments to create P388 cell lines expressing the zipper swap mutant, C/EBPβ-G12z, only minimal amounts of the mutant protein were detected in stable transfectants whereas the wild type protein could be expressed at much higher levels (41). This result is consistent with the idea that C/EBPβ must heterodimerize with C/EBPγ for its expression to be tolerated in proliferating cells. Furthermore, HepG2 hepatoma cells express significantly lower levels of C/EBPα and C/EBPβ than are found in normal, terminally differentiated hepatocytes (50). We speculate that C/EBPγ may be unable to suppress C/EBP-mediated growth arrest in hepatoma cells, similar to its inability to inhibit C/EBP-dependent transcription in these cells. Thus, conversion of hepatocytes to proliferating hepatoma cells might require strong down-regulation of C/EBPα and C/EBPβ expression. In future studies it will be informative to examine the ability of C/EBPγ to modulate C/EBP-mediated cell growth arrest in various cellular contexts.

The observation that C/EBP activity can be inhibited by heterodimerization with C/EBPγ suggests that C/EBP dimerization might be regulated to control gene transcription. In this regard, calcium-regulated phosphorylation of a serine residue in the leucine zipper of C/EBPβ has been linked to its increased transcriptional activity (51). Although the molecular mechanism underlying this activation event has not been elucidated, the authors raised the possibility that phosphorylation of the C/EBPβ zipper might control dimerization. Our studies indicate that C/EBPγ could be involved in this putative regulatory mechanism. Although our experiments thus far have focused on artificial promoters, future studies will address potential differences between C/EBP homodimers and heterodimers in activating authentic promoters, in addition to the possibility
that C/EBPβ heterodimerization is regulated by developmental cues or other physiological signals.

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