CCAAT/Enhancer-binding Proteins $\alpha$ and $\beta$ Interact with the Silencer Element in the Promoter of Glutathione S-Transferase P Gene during Hepatocarcinogenesis

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We have previously identified a silencer in the glutathione S-transferase P (GST-P) gene which is strongly and specifically expressed during chemical hepatocarcinogenesis. At least three trans-acting factors bind to multiple cis-elements in the silencer. One of them, Silencer Factor-B (SF-B), is identical with CCAAT enhancer-binding protein $\beta$ (C/EBP$\beta$) and binds to GST-P Silencer 1 (GPS1). Many C/EBP$\beta$ binding sites are recognized by the three C/EBP isoforms. Western blot analysis of C/EBP isoforms during chemical hepatocarcinogenesis revealed a decrease of C/EBP$\alpha$ expression. However, there was no change in C/EBP$\beta$ level. In the nuclear extracts from normal liver, C/EBP$\alpha$ was the dominant form that bound to GPS1, whereas both C/EBP$\alpha$ and C/EBP$\beta$ bound to GPS1 in the nuclear extracts from carcinogenic liver. Furthermore, transfection assays showed that C/EBP$\alpha$ not only repressed the GST-P promoter activity but also attenuated the transcriptional stimulation by C/EBP$\beta$. These observations strongly suggest that the ratio of C/EBP$\alpha$ to C/EBP$\beta$ is one of the important factors for the GST-P silencer activity, and the decrease of this ratio during hepatocarcinogenesis reduces the silencer activity and, consequently, increases the GST-P expression.

The rat glutathione S-transferase P (GST-P) gene, belonged to $\pi$-class GST, is strongly and specifically expressed during chemical hepatocarcinogenesis, and is considered to be an excellent tumor marker. The level of human GST-$\pi$ has been reported to be significantly increased in a number of tumors including colon, stomach, esophagus, and lung. However, GST-$\pi$ levels are not increased in hepatocellular carcinomas. The GST-$\pi$ is expressed constitutively in hepatocytes.

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$^\ddagger$The abbreviations used are: GST-P, glutathione S-transferase P; GPS1, GST-P Silencer 1; GPS4, GST-P Silencer 4; SF-A, Silencer Factor A; SF-B, Silencer Factor B; SF-C, Silencer Factor C; C/EBP, CCAAT enhancer-binding protein; LAP, liver-enriched transcriptional activator protein; LIP, liver-enriched transcriptional inhibitory protein; bZIP, basic region and leucine zipper region; MSV, Moloney sarcoma virus; CMV, cytomegalovirus; GR, glucocorticoid receptor; IL, interleukin.

Therefore, induction of GST-P gene during hepatocarcinogenesis is a rat-specific event (1).

The rat GST-P gene consists of two enhancers and a silencer (2). One of the enhancers, termed GST-P enhancer I, is a strong positive regulatory element and has two phorbol 12-O-tetradecanoic acid/phorbol 13-acetate responsive element (TRE)-like sequences. The GST-P gene expression is mediated mainly by this GST-P enhancer I (3, 4). The GST-P promoter also contains TRE and GC box and shows high activities in several cell lines (2, 3, 5). Nevertheless, mRNA of the GST-P gene, as well as the protein, is undetectable in normal liver (6, 7). These observations prompted us to investigate the silencer that had been identified just upstream of the TRE and the GC box of the promoter (2, 8).

At least three transcription factors bind to multiple cis-elements located in this silencer. One of these factors, Silencer Factor-B (SF-B) that binds to GST-P Silencer 1 (GPS1), has been cloned by a Southwestern protocol (8, 9). The nucleotide sequence of SF-B is identical with that of liver-enriched transcriptional activator protein (LAP) (10) and IL6-DBP (11). SF-B/LAP/IL6-DBP belongs to the CCAAT enhancer-binding protein family whose members have highly similar carboxyl-terminal DNA-binding domains and leucine zipper dimerization domains. Homologs of SF-B/LAP/IL6-DBP have also been cloned as NF-IL6 (12), AGP/EBP (13), C/EBP$\beta$ (14), and CRP2 (15). To avoid confusion, these proteins will be collectively called C/EBP$\beta$. Other C/EBP members include the following: C/EBP$\alpha$ (16); C/EBP$\delta$, also called CRP3 (15), NF-IL6 (17), and CELF (18); C/EBP$\gamma$, also known as IgEBP-1 (19), GPE-BP (20), and CRP1 (15), and CHOP-10 (21).

C/EBP$\alpha$ can trans-activate several genes encoding adipose-specific proteins, including a fatty acid-binding protein various termed 422 or ap2, stearoyl-CoA desaturase, and the insulin-responsive glucose transporter (22, 23). C/EBP$\beta$ and C/EBP$\delta$ promote the transcription of the acute phase proteins such as $\alpha_2$-acid glycoprotein, serum amyloid A, and kininogen (24–26). The basic region and the leucine zipper region (bZIP) of C/EBP isoforms show high similarity, and some cis-elements are recognized by each of the C/EBP isoforms (11, 14, 15, 17). Therefore, the expression profile of C/EBP$\beta$ isoforms is important to understanding the gene expression which is controlled by them. For example, the accumulation of C/EBP$\beta$ and C/EBP$\delta$ reached a maximal level during the first 2 days of adipocyte differentiation and declined sharply before the onset of C/EBP$\alpha$ accumulation. The expression profiles of the adipocyte-specific mRNA are similar in pattern (14).

In this paper, the pattern of expression of C/EBP isoforms and the profile of GPS1 binding protein during chemical hepatocarcinogenesis were examined by Western blot analysis and
gel mobility shift assay. We also describe functional analyses which demonstrate that C/EBPβ not only represses the GST-P promoter but also attenuates the transcriptional stimulation by C/EBPα (LAP).

MATeRIALS AND METHODS

Animals and Treatments—Carcinogenic experiments were according to the Solt-Farber protocol (27). Experiments were initiated by injecting 200 mg of diethylnitrosamine per kg into 6-week-old Wistar male rats. After feeding a basal diet for 2 weeks, the diet was changed to basal diet containing 0.02% 2-acetylaminofluorene. Partial hepatectomy was performed at the beginning of the 3rd week, and the rats were sacrificed at the 7th and 8th weeks after diethylnitrosamine treatment. Control rats were injected with 0.9% NaCl solution and fed a basal diet.

Preparation of Cytosol Fraction and Nuclear Extracts from Rat Liver and HepG2 Cells and Western Blot Analysis—Rat livers were excised, and 1 g was homogenized in 2 ml of cold 0.25 % Tris-HCl (pH 7.5) and centrifuged at 10,000 × g for 10 min at 4 °C. The supernatants were used as the cytosol fractions. Nuclear extracts from rat liver were prepared according to the method of Lichtsteiner et al. (28). Finally, the precipitates from the nuclear extracts were dialyzed against 25 mm Hapes (pH 7.6), 0.1 mm EDTA, 40 mm KCl, 10% glycerol, and 1 mm dithiothreitol. HepG2 nuclear extracts were prepared by the following procedure. Cells were washed with three times with phosphate-buffered saline, harvested, and centrifuged. The cell pellet (∼1 volume) was suspended in 8 volumes of lysis buffer containing 20 mm Hapes (pH 7.9), 1 mm EDTA, 0.5 mm spermidine, 1 mm dithiothreitol, 10% glycerol, 1 mm phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 0.3 μg/ml antipain, lyzed by the addition of 3/100 volume of Nonidet-P-40, and incubated for 5 min on ice. The lysate was centrifuged for 10 min at 12,000 rpm. The precipitates were suspended in 1 volume of 20% KCl, incubated for 30 min on ice, and centrifuged for 30 min at 98,000 rpm. The supernatant was applied to Sephadex G-50 spin column equilibrated lysis buffer plus 0.05 % KCl, centrifuged, and collected. Ten micrograms of protein and 0.2 μg of HepG2 nuclear extracts, as determined by the protein assay dye reagent kit (Bio-Rad), were electrophoresed by SDS-polyacrylamide gel electrophoresis containing 12% acrylamide. Proteins were electrophoretically transferred to nitrocellulose filter (Schleicher & Schuell). After transfer, GST-P and CMV-LAP, C/EBPα expression vector, was kindly provided by Dr. S. L. McKnight, and CMV-LAP, C/EBPα-36 (LAP) expression vector, by Dr. U. Schibler. In each case, pBluescript (Stratagene) plasmid was used to bring the final amount of plasmid to 6 μg. Luciferase activities were measured by PicaGene (Toyoin Mfg. Co., Ltd). All the transfection experiments were performed at least three times by using two or three different preparations of DNA, and the regulation ratio was derived from the mean values of the results. In some experiments, the transfection efficiency was checked by cotransfection with pRSV γ-1, an eukaryotic expression vector which contained the Escherichia coli β-galactosidase (lacZ) structural gene controlled by Rous sarcoma virus long terminal repeat. β-Galactosidase activity was assayed as described (31). It was confirmed that the variation of transfection efficiency was less than 20%.

RESULTS

The Expression Profiles of C/EBP Isoforms during Hepatocarcinogenesis—SF-B (C/EBPβ), a member of the C/EBP family that binds specifically to GPS1, has been cloned from a normal rat liver cDNA library by a Southwestern method (9). It is known that C/EBPβ binding sites are recognized by other members of the C/EBP family and the pattern of expression of C/EBP isoforms changes during cell differentiation (14). To examine the expression of C/EBP isoforms during hepatocarcinogenesis, carcinogenic rats were prepared according to the Solt-Farber procedure (Ref. 27 and Fig. 1). At the end of 7 or 8 weeks, rats were killed, the livers which had a large number of foal and nodules were excised, and then the cytosol fractions and nuclear extracts were prepared. As a control, rats were injected with saline and were fed basal diet. We first checked the reproducibility of the carcinogenic experiments. Western blotting analysis of the cytosol fractions with GST-P antibody indicated that GST-P protein was induced at the 7th and 8th weeks after diethylnitrosamine was injected, but no GST-P protein was detected in the control rats as described previously (Ref. 32 and Fig. 2A).

Then we performed Western blot analysis by using the nuclear extracts. For determination of expression profiles of C/EBP isoforms, antibodies specific to each of the C/EBP isoforms were used (Fig. 2, B and C). It is reported that C/EBPα and C/EBPβ share the common feature that, besides the full-
transcriptional regulation of silencer by C/EBP family

FIG. 2. Immunoblot analysis of cytosol GST-P and nuclear C/EBP isoforms during hepatocarcinogenesis. 10 μg of protein from cytosol fraction (A) and nuclear extracts (B and C) was electrophoresed on a denaturing polyacrylamide gel, transferred to nitrocellulose by Western blotting, and probed with antibodies specific to GST-P (A), C/EBPα (B), and C/EBPβ (C). Lane 1, control; lanes 2 and 3, 7th & 8th weeks after diethylnitrosamine treatment; lane 4, 0.2 μg of HepG2 nuclear extracts transfected with CMV-LAP (C/EBPβ-36) and CMV-LIP (C/EBPβ-20). The arrows indicate the positions of migration of GST-P, C/EBPα-42, and C/EBPβ-36 (LAP), respectively, and the arrowheads indicate that of C/EBPα-30 and C/EBPβ-20 (LIP), respectively. Cross-reacting materials (CRM) observed in samples probed with antibody specific to C/EBPβ are indicated. Molecular masses are shown in kDa.

FIG. 3. GPS1 formed complexes with C/EBPα and C/EBPβ in the normal rat liver nuclear extracts. Gel shift analyses of normal rat liver nuclear extracts were performed with GPS1 as a probe and with or without antibodies specific for three isoforms of C/EBP. GPS1 or GPS4 was used as a competitor. Lane 1, probe only; lane 2, control (without competitor); lanes 3 and 4, specific competitor GPS1 was used at 50- and 250-fold molar excess, respectively; lane 5, nonspecific competitor GPS4 was used at 250-fold molar excess; lanes 6–9, nuclear extracts plus preimmune serum, anti-C/EBPα, anti-C/EBPβ, and anti-C/EBPδ, respectively; lanes 10–13, probe plus preimmune serum, anti-C/EBPα, anti-C/EBPδ, and anti-C/EBPδ, respectively, without nuclear extracts, respectively. The relative shift ratios (control = 100) of complex I and complex II were also prepared, analyzed by SDS-polyacrylamide gel electrophoresis, and immunoblotted with a C/EBPβ peptide antibody. This antibody reacted with C/EBPβ-36 and C/EBPβ-20. One 45-kDa immunoreactive band of rat liver nuclear extracts and two bands of HepG2 nuclear extracts are presumed to be cross-reacting materials. Immunoreactive bands of HepG2 nuclear extracts may be derived from human C/EBPβ (NF-IL6). No C/EBPα was detected in the normal and carcinogenic liver nuclear extracts (data not shown).

Identification of C/EBPα as the Major GPS1 Binding Protein in Normal Rat Liver—Members of the C/EBP family can form homodimers and heterodimers that exhibit similar binding specificities (11, 14, 15, 17). To analyze trans-acting factors binding to GPS1 of the GST-P silencer, we prepared nuclear extracts from normal rat liver for gel mobility shift assay (Fig. 3). When the normal liver nuclear extracts were incubated with the labeled GPS1, two different mobility shift complexes (complex I and complex II) were detected. It is likely that complex II consists of more than two bands. Competition analyses using the specific competitor, GPS1, and the nonspecific competitor, GPS4, indicate that these two complexes contain binding factors specific to GPS1 (Fig. 3, lanes 2–5).

To analyze further which members of the C/EBP protein family were involved in these complexes, antibodies against each of these proteins were used in gel mobility shift analysis. When extracts were incubated with antibody specific to C/EBPα, both complex I and complex II almost disappeared. C/EBPβ antibody also decreased the activity of the complex I and complex II. Preimmune serum and anti-C/EBPδ antisera did not affect the mobility of the complexes. Owing to the high sequence conservation within the dimerization region of the different C/EBP family members, the several proteins encoded by c/ebpα and c/ebpδ genes could form many homodimers and heterodimers. Although we could not clarify which isoform of C/EBP family composed two complexes, we revealed that these complexes contain both C/EBPα and C/EBPβ.

Profile of GPS1 Binding Proteins during Hepatocarcinogen-
To analyze the factors which bind to GPS1 in nuclear extracts prepared from the carcinogenic rat liver, we carried out a gel mobility shift assay (Fig. 4). While incubation of radiolabeled GPS1 with normal rat liver nuclear extracts generated two retarded complexes (Fig. 3), complex I completely disappeared from hepatocarcinogenic rat livers (Fig. 4, lanes 2 and 7). The isoforms of the C/EBP family that recognize GPS1 were examined by the gel mobility shift analysis using the specific antibody. Complex II derived from hepatocarcinogenic rat liver nuclear extract with GPS1 also involved C/EBPα and C/EBPβ, but not C/EBPγ (Fig. 4, lanes 4–6 and 9–11).

Transcriptional Regulation of C/EBPα and C/EBPβ-36 (LAP) on GPS1—

C/EBPα generally acts as a transcriptional activator. But C/EBPα repressed the transcription through GPS1 in the GST-P silencer. To make sure that C/EBPα activates the transcription through its binding site, C/EBPα and C/EBPβ-36 (LAP) expression vectors were cotransfected with the reporter plasmids containing the multimerized GPS1 with GST-P promoter (Fig. 5A). Both C/EBPα and C/EBPβ-36 (LAP) activated transcription on multimerized GPS1. Thus, C/EBPα repressed the transcription through GPS1.
Transcriptional Regulation of Silencer by C/EBP Family

GS1 in the GST-P silencer, but acted as a transcriptional activator on multimerized GS1.

DISCUSSION

Gene expression in the liver appears to be controlled by combinatorial mechanisms derived from the liver-enriched transcription factors, including C/EBPα and C/EBPβ. The GST-P gene is strongly and specifically expressed during hepatocarcinogenesis, but neither mRNA of the GST-P gene nor its protein is detectable in the normal liver (6, 7). We have previously shown that SF-B (C/EBPβ), which binds to GS1, one of the multiple negative elements of the GST-P silencer (8). Members of the C/EBP family can form homodimers and heterodimers that exhibit similar binding specificities (11, 14, 15, 17). Expression of the C/EBP proteins is important for cell differentiation and growth (14). To elucidate the function of GS1, we analyzed the expression profiles of the C/EBP proteins during hepatocarcinogenesis and the transcriptional regulation of GS1 by members of the C/EBP family.

To analyze trans-acting factors binding to GS1, which is found within the GST-P silencer, we prepared nuclear extracts from normal and carcinogenic rat livers for Western blot and gel mobility shift assays. The expression of the C/EBPα-42 was drastically reduced and C/EBPα-30 slightly decreased during hepatocarcinogenesis. C/EBPβ-36 (LAP) was detected in the normal and carcinogenic rat liver, and C/EBPβ-39 (LAP) and C/EBPβ-20 (LIP) were undetectable. Together with the results of gel shift assays, it is suggested that C/EBPα-42 and C/EBPα-30 mainly occupy the GS1 site in the normal rat liver and act as negative regulators. The binding activity of C/EBPα to GS1 reduced significantly in the liver during hepatocarcinogenesis, while the activity of C/EBPβ did not change. Although little is known about the repression activity of C/EBPα, repressor and attenuator domains have been identified within the trans-activation domains of C/EBPα (36, 37). Furthermore, in the normal liver, C/EBPα occupies the acute phase-responsive element in the α1-acid glycoprotein gene which is only expressed at a very low level (24).

We performed the transfection analysis in HepG2, a human hepatoma cell line. Expression level of C/EBPβ is higher than that of C/EBPα in HepG2 cells, which was generally used to observe the effect of the transcriptional regulation of C/EBPα and C/EBPβ on its binding site (35, 38). The observed levels of repression and activation are weak, since endogenous factors respond to GS1. GS1 in the GST-P silencer showed repression activity with C/EBPα and activation activity with C/EBPβ-36 (LAP). These data suggest that GS1 acts as a silencer or an enhancer depending on its binding factors. Furthermore, C/EBPα attenuated the transcriptional activity by C/EBPβ-36 (LAP). Therefore, C/EBPα acts as a negative regulator rather than a transcriptional activator on GS1 in the GST-P silencer, although we do not know the repression mechanisms of C/EBPα. These observations strongly suggest that the ratio of C/EBPα to C/EBPβ is an important factor for silencer activity in the GST-P gene promoter, and the decrease of this ratio during hepatocarcinogenesis reduces the silencer activity and, consequently, increases the GST-P expression.

Overexpression of C/EBPα and C/EBPβ-36 (LAP) in HepG2 cells increased the reporter activity driven by GS1 multimers. C/EBPβ-36 (LAP) mediates the transcriptional activation of the GS1 located both in the silencer and multimerized GS1. However, the function of C/EBPβ is dependent on the elements which bind to cis-element around GS1, since overexpression of C/EBPα decreased reporter activity driven by GS1 in the GST-P silencer and mediated the transcriptional activation of the multimerized GS1. It is important that C/EBP family members interact, directly or indirectly, with other trans-acting factors. For example, human C/EBPβ (NF-IL6) and glucocorticoid receptor (GR) synergistically activate transcription of the rat α1-acid glycoprotein gene via direct protein-protein interactions which is probably generated from the bZIP domain of C/EBPβ (NF-IL6) and near the zinc finger region of GR (39). Analyses of the gene expression of human serum amyloid A, interleukin 6, and interleukin 8 have demonstrated that C/EBPα and NF-κB transcriptionally synergize with each other (25, 40). LeClair et al. (41) reported that the NF-κB p50 subunit physically interacts with the bZIP domain of C/EBPβ (NF-IL6). The bZIP domain, which is highly conserved among C/EBP family members, is important for the protein-protein interaction.

In addition to SF-B binding to the GS1 site in the GST-P silencer, SF-A and SF-C also bind to the other cis-elements in this region and function as a negative regulator. SF-A binds to GS4 with a strong affinity and several other cis-elements in the silencer region with a weak affinity. SF-C binds to the GS2 site, which partially overlaps GS1 and GS3, binding sites of SF-B and SF-A, respectively (8). We previously reported that SF-A, SF-B, and SF-C all might be important for maximum silencer activity (8). Characterization of the protein-protein interactions, including these factors, is required for further elucidation of the mechanism of repression in normal liver and activation during hepatocarcinogenesis.

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