Transcription factor Nrf2/MafK regulates rat placental glutathione S-transferase gene during hepatocarcinogenesis

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The rat GST-P (placental glutathione S-transferase), a phase II detoxifying enzyme, is not expressed in normal liver cells, but is highly and specifically induced during early hepatocarcinogenesis as well as in hepatocellular carcinoma cells. Results of previous studies indicated that *GST-P* gene activation was mainly controlled by an enhancer element, GPE1 (GST-P enhancer 1), but the specific activation mechanism of the *GST-P* gene was not fully understood [Morimura, Suzuki, Hoshi, Yuki, Nomura, Kitagawa, Nagatsu, Imagawa and Muramatsu (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 2065–2068; Suzuki, Imagawa, Hirabayashi, Yuki, Hisatake, Nomura, Kitagawa and Muramatsu (1995) Cancer Res. **55**, 2651–2655]. In the present study, we investigate the transcription factor Nrf2/MafK heterodimer (where Nrf2 stands for NF-E2 p45-related factor 2) as an activator of the *GST-P* gene through the action of GPE1 during hepatocarcinogenesis. Electrophoretic mobility-shift assay and footprinting analysis with wild-type GPE1 and GPE1 point mutants showed that the Nrf2/MafK heterodimer specifically bound GPE1. Reporter transfection assays indicated that Nrf2 strongly stimulated *GST-P* gene expression in mouse F9 embryonal carcinoma cells and H4IIE

rat hepatoma cells. Northern-blot analysis indicated that GST-P and Nrf2 mRNA increased in parallel with development of precancerous lesions and hepatocellular carcinoma. Keap1 (Kelch-like ECH-associated protein 1), an inhibitory factor of Nrf2, decreased the activation of GPE1 by Nrf2 and this suppression was restored after treatment with electrophilic compounds. *GST-P* mRNA expression in H4IIE cells was induced by electrophilic compounds, as was the expression of mRNAs of other phase II detoxifying enzymes. Chromatin immunoprecipitation analyses showed that antibodies both against Nrf2 and against MafK precipitated GPE1 from the chromatin of the pre-neoplastic hepatocytes and rat hepatoma cells (H4IIE and dRLh84), but not from normal hepatocytes. These results indicate that the Nrf2/MafK heterodimer regulates *GST-P* gene expression during early hepatocarcinogenesis and in hepatoma cells.

Key words: placental glutathione S-transferase gene, hepatoma, MafK, Nrf2 (NF-E2 p45-related factor 2), transcriptional control, tumour marker.

INTRODUCTION

GST (glutathione S-transferase) is a phase II detoxifying enzyme and catalyses the nucleophilic attack of GSH on electrophilic compounds [1,2]. Five subclasses of mammalian GST are recognized, namely Alpha, Mu, Pi, Sigma and Theta [3]. In contrast with isoenzymes of other classes, the rat placental Pi-class GST (GST-P) is not detected in normal liver and is not induced by common electrophilic drugs. However, GST-P is markedly increased during hepatocarcinogenesis [4–7] and has been used as a reliable tumour marker. This induction is mainly at the transcriptional level [8]. The molecular mechanism of GST-P expression may be closely related to the fundamental process of early hepatocarcinogenesis in rats.

We reported previously the transcriptional activation mechanism of the *GST-P* gene during hepatocarcinogenesis [9–12]. The *GST-P* gene has a strong enhancer element, GPE1 (GST-P enhancer 1), which is 2.5 kb upstream of the cap site [9,11,12]. This enhancer is a major control element for the expression of GST-P, specifically in pre-neoplastic lesions and in rat hepatoma. Muramatsu and co-workers [13,14] established a GPE1 transgenic rat line and showed that GPE1 is essential and nearly sufficient for the expression of GST-P during hepatocarcinogenesis. GPE1 is a specific enhancer of the rat *GST-P* gene and is not present

in the Pi-class *GST* gene of mouse or human, or in genes of the other GST isoenzymes [15–17], and expression patterns of human and mouse Pi-class GST differ markedly from those of the rat [18–21]. GPE1 consists of two TRE (phorbol-12-*O*-tetradecanoate-13-acetate responsive element; -TGAGTCA-) like elements with palindromic orientation (5'-TCAGTCAGTCACTATGATTCAGCAA-3'; TRE-like sequences are underlined) [11,12]. The GPE1 sequence is also similar to the ARE (antioxidant responsive element) sequence -GTGACTTGGA- [22] and the MARE (Maf recognition element) sequence -TGCTGACTCAGCT- [23]. These similarities suggest that transcription factors, such as Jun and Fos, Nrf2 (NF-E2 p45-related factor 2), Maf and their relatives, may bind to GPE1. We focused on Nrf2 as the candidate transcription factor responsible for GPE1 activation, since expression of other potential transcription factors did not correlate with the expression of GST-P.

Nrf2 is a member of the CNC (cap' n' collar) family of regulatory proteins. It dimerizes with small Maf proteins (i.e. MafK, MafG and MafF), members of the Maf family of transcription factors, through their bZip domain [24,25]. Small Maf proteins lack the transactivation domain present in the large Maf proteins (i.e. c-Maf, MafB, NRL and L-Maf/MafA). The Nrf2/small Maf heterodimer binds to ARE and MARE, and regulates downstream genes, including those of phase II detoxifying

Abbreviations used: ARE, antioxidant responsive element; ChIP, chromatin immunoprecipitation; DEM, dimethylmalate; EMSA, electrophoretic mobility-shift assay; GST, glutathione S-transferase; GST-P, placental (Pi-class) GST of the rat; GPE1, GST-P enhancer 1; Keap1, Kelch-like ECH-associated protein 1; MARE, Maf recognition element; β -NF, β -naphthoflavone; NQO1, NAD(P)H:quinone oxidoreductase 1; Nrf2, NF-E2 p45-related factor 2; TRE, phorbol-12-*O*-tetradecanoate-13-acetate responsive element.

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enzymes [26,27]. Experiments with *Nrf2* knockout mice showed that the basal expression of phase II detoxifying enzymes, such as GST and NQO1 [NAD(P)H:quinone oxidoreductase 1], was not significantly different from that of wild-type mice; however, induction of phase II detoxifying enzymes by electrophilic drugs was almost completely impaired in mutant mice [28]. Recently, Itoh and colleagues [29,30] identified Keap1 (Kleeh-like ECH-associated protein 1) as a key mediator in the induction of phase II detoxifying enzymes by electrophilic drugs. Keap1 suppresses Nrf2 activity by specific binding in the cytoplasm. An electrophilic agent liberates Nrf2 from Keap1 suppression, which allows Nrf2 to translocate to the nucleus and activate target genes.

In the present study, we examined the role of Nrf2/MafK in the expression of GST-P in rats. Interaction of Nrf2/MafK with GPE1 and activation of GPE1 by Nrf2 were analysed by EMSA (electrophoretic mobility-shift assay), footprinting analysis and reporter transfection analyses. Expression of GST-P and Nrf2 mRNA during the development of hepatocellular carcinoma was determined by Northern-blot analysis. Direct interaction of Nrf2 and MafK with GPE1 *in vivo* was determined by ChIP (chromatin immunoprecipitation) analysis. The combined results indicated that Nrf2/MafK binds to GPE1 and regulates GST-P in pre-neoplastic and neoplastic hepatocytes in the rat.

EXPERIMENTAL

Construction of plasmids

To construct the luciferase reporter genes, we used a promoterless luciferase plasmid vector (pGVB2; Nippon Gene, Toyama, Japan) that had the minimal promoter sequence of the rat GST-P gene (−50/+37 bp), containing a GC box and a TATA box [9]. The following sequences were inserted into the plasmid vector: ARE (at −473/−390 bp relative to the cap site of the rat NQO1 gene), GPE1 (5′-GTCAGTCACTATGATTGAGCAAC-3′), two mutated forms of GPE1, mGPE1 (5′-GTCAGTCACT-ACGATTGAGCAAC-3′) and mmGPE1 (5′-GTCAGTCTGCTA-TGATTGAGCAAC-3′; the mutated positions are underlined) and TRE (5′-ATGAGTCAGACAG-3′). The promoter region of the *GST-P* gene, containing ARE/TRE (at −143/+59 bp), was also cloned into pGVB2 and designated −143/Luc. The expression plasmids of Nrf2, MafK and Jun were described previously [21]. To isolate rat Keap1 cDNA by reverse transcriptase-PCR, total RNA from rat liver was used as a template and the primer sequences used were 5′-GGAATTCACCATGAGCCCC-GAACCAAG-3′ and 5′-GGGATCCTCAGCAGGTACAGTTT-TGTT-3′. The amplified Keap1 cDNA was inserted into an expression vector, pAβ2, which contained human β-actin promoter and enhancer [31].

Cell culture and transient transfections

Mouse F9 embryonal carcinoma and H4IIE rat hepatoma cell lines were maintained in Dulbecco's modified minimal essential medium (Nissui, Tokyo, Japan) with 7% (v/v) foetal bovine serum. For the reporter transfection assay, a total of 4 µg of DNA, including 1 µg of reporter plasmid and 0.5 µg of β-galactosidase expression plasmid (pSVβgal; Promega, Madison WI, U.S.A.), with or without 1 µg of effector expression plasmid (Nrf2, Keap1 or Jun) and pUC18 DNA, was co-transfected by the method of Chen and Okayama [32]. Cells were harvested 45 h post-transfection and then assayed for luciferase activity using a luciferase assay kit (Nippon Gene) and for β-galactosidase activity [33]. Luciferase activities were normalized to β-

galactosidase activities and all experiments were repeated at least twice and nearly identical results were obtained.

EMSA and DNase I footprinting analysis

The cDNA fragments encoding the DNA-binding domain of three transcription factors (amino acids 318–598 for Nrf2, 1–156 for MafK and 82–323 for MafB) were fused to the *Escherichia coli* maltose-binding protein gene of a pMAL-c2 vector (New England Biolabs, Beverly, MA, U.S.A.). The fusion proteins were expressed in *E. coli* and purified according to the vector manufacturer's instructions. The probes for EMSA and footprinting analyses were prepared by filling in the overhanging 5′-end of the appropriate restriction enzyme fragment using Klenow DNA polymerase I (Takara, Kyoto, Japan) and [α -³²P]dCTP. Double-stranded GPE1, mGPE1, mmGPE1 and TRE sequences were labelled with [γ -³²P]ATP by T4 polynucleotide kinase.

EMSA was performed as described previously [34]. Approx. 50 ng of the recombinant protein (Nrf2, MafK or MafB) was preincubated with 0.5 µg of poly(dI/dC) (Amersham Biosciences) for 10 min in 10 µl of binding buffer [20 mM Hepes, pH 7.9/20 mM KCl/1 mM EDTA/5 mM dithiothreitol/4 mM MgCl₂/15% (v/v) glycerol/100 µg/ml BSA]. ³²P-labelled DNA probe (1 × 10⁴ c.p.m.) was added and the mixture was incubated for another 10 min. Protein–DNA complexes were analysed by electrophoresis on 4% (w/v) polyacrylamide gel in a buffer consisting of 25 mM Tris/borate and 0.5 mM EDTA (pH 8.2). For the DNase I footprinting analysis, the probe (2 × 10⁵ c.p.m.) was incubated with or without Nrf2 and MafK fusion proteins (500 ng each) in 50 µl of binding buffer. Aliquots (50 µl) of DNase buffer (5 mM MgCl₂/5 mM CaCl₂/200 mM KCl/4 µg/ml sheared salmon sperm DNA) and 20 ng of DNase I (Takara) were added to the binding mixture and incubated for 3 min at room temperature (25 °C). The reaction was terminated by the addition of 100 µl of stop solution (20 mM EDTA/1% SDS/0.2 M NaCl). After proteinase K treatment (100 µg/ml, 15 min), DNA was extracted and analysed on a 6% (w/v) polyacrylamide gel containing 8 M urea. The guanine and adenine bases of the same probes were modified, digested by the Maxam–Gilbert method [41] and loaded as a marker ladder.

Animals and chemical hepatocarcinogenesis

Sprague–Dawley, LEA (a parent strain of LEC) and LEC rats were obtained from the Institute for Animal Experimentation, Hokkaido University Graduate School of Medicine. Rats with hyperplastic nodules, induced by the method of Solt and Farber [35,36], were kindly provided by Dr K. Satoh (Hirosaki University, Hirosaki, Japan). All animal experiments were conducted according to the Guidelines for Animal Experiments of Hokkaido University.

RNA analyses

Total cellular RNAs were isolated from rat tissues and cultured cells using an RNA extraction kit (ISOGEN; Nippon Gene). Northern-blot analysis was performed as described previously [37]. ³²P-labelled probes were prepared using a random-primed DNA-labelling kit (Takara) with [α -³²P]dCTP. RNase protection analysis was performed as described previously [9]. DNA fragments of a GST-P genomic clone (−55/+57 bp), NQO1 cDNA (204/755 bp), GST-Ya cDNA (97/300 bp) and Nrf2 cDNA (244/435 bp) were inserted into pBluescript II (Stratagene, San

Diego, CA, U.S.A.) vector, and antisense RNA was synthesized with [α - 32 P]UTP by T7 or T3 RNA polymerase *in vitro*.

ChIP analysis

An anti-Nrf2 antiserum was prepared by immunization of rabbits with the Nrf2 fragment (amino acids 84–146 fused to GST protein) expressed in *E. coli*. ChIP analysis was performed using the acetyl-histone H4 ChIP assay kit (Upstate Biotechnology, Lake Placid, NY, U.S.A.) according to the manufacturer's instructions. Briefly, cells were fixed in 1% formaldehyde at 37 °C for 10 min. After washing twice with ice-cold PBS containing protease inhibitors (1 mM PMSF, 1 μ g/ml aprotinin and 1 μ g/ml pepstatin), the cells were harvested, sonicated by an ultrasonic generator (Tomy Seiko, Tokyo, Japan) in SDS lysis buffer (1% SDS/10 mM EDTA/50 mM Tris/HCl, pH 8.1) and centrifuged. The supernatant chromatin fraction was diluted 10-fold with IP buffer (0.01% SDS/1.1% Triton X-100/1.2 mM EDTA/16.7 mM Tris/HCl, pH 8.1/167 mM NaCl) and precleared with 30 μ l of Protein A–Sepharose [50% (v/v) Protein A slurry, containing 20 μ g/ml sheared salmon sperm DNA and 1 mg/ml BSA; Amersham Biosciences]. The precleared chromatin fraction was incubated with either anti-Nrf2, anti-MafK (kindly provided by Dr K. Igarashi, Hiroshima University, Hiroshima, Japan), anti-acetyl-histone H3 or H4 antibody (06-599 and 06-866 respectively; Upstate Biotechnology) or preimmune serum for 16 h at 4 °C with gentle rotation. Immunocomplexes were mixed with 50 μ l of Protein A–Sepharose slurry and washed sequentially with wash buffer 1 (0.1% SDS/1% Triton X-100/2 mM EDTA/20 mM Tris/HCl, pH 8.1) containing 150 mM NaCl, next with wash buffer 1 containing 500 mM NaCl, then with wash buffer 2 (0.25 M LiCl/1% Nonidet P40/1% sodium deoxycholate/1 mM EDTA/10 mM Tris/HCl, pH 8.1) and, finally, twice with TE (10 mM Tris/HCl, pH 8.1/1 mM EDTA). Complexes were eluted from Protein A–Sepharose in 1% SDS and 0.1 M NaHCO₃, and cross-linking was reversed by heating to 65 °C for 4 h. DNAs were purified by proteinase K digestion and phenol/chloroform extraction. The specific promoter or enhancer regions were amplified by PCR using the following primer sequences: *NQO1* ARE (5'-AGACCCAAGCGTGACACCC-3' and 5'-GTCCTTGGTCAGATGTGGGA-3'), *GST-P* GPE1 (5'-T-GATTCTGCCATCTTCTGC-3' and 5'-CCAGCTTCTCTGG-ACAAACC-3') and *GST-P* promoter ARE/TRE (5'-CAGAC-TCCGGTCCAGCTGCT-3' and 5'-CGCGAACTTACTAGCTG-CTG-3'). The amplified DNAs were electrophoresed on 3% (w/v) agarose gel.

RESULTS

Nrf2/MafK binds and activates GPE1

GPE1 is a response enhancer element for the specific expression of the *GST-P* gene. First, we analysed the specific binding of Nrf2/MafK to GPE1. We performed EMSA using a GPE1 probe and recombinant proteins expressed in *E. coli* (Figure 1A). The ARE sequence of the rat *NQO1* gene promoter was used as a control probe. Nrf2 alone did not bind to either *NQO1* ARE or *GST-P* GPE1, but the Nrf2/MafK heterodimer bound strongly to both of these elements. The homodimers of MafB and MafK bound to both probes. Excess amounts of non-radioactive probe obscured the retarded complex bands (results not shown).

To confirm the binding of Nrf2/MafK to GPE1, DNase I footprinting analysis was performed (Figure 1B). The Nrf2/MafK heterodimer clearly protected the GPE1 core sequence (the two TRE-like sequences) from DNase I digestion. These results indicate that Nrf2/MafK strongly and specifically bound to GPE1.

Figure 1 The Nrf2/MafK heterodimer binds to GPE1

(A) EMSA of Nrf2, MafK and MafB with *GST-P* GPE1 and *NQO1* ARE. Approx. 50 ng of the indicated recombinant fusion protein was mixed with the indicated probes (2×10^4 c.p.m.) as described in the Experimental section. Some weak multimer bands are seen above the main bands. (B) DNase I footprinting analysis of Nrf2/MafK with GPE1 probe. Nrf2 and MafK fusion proteins (1 μ g each) were mixed and incubated with approx. 2×10^5 c.p.m. of probe and treated with DNase I as described in the Experimental section. Symbols (+) and (-) indicate the probe with or without Nrf2/MafK proteins respectively. Guanine and adenine residues were modified and digested by the Maxam–Gilbert method (M). Arrows indicate two palindromic TRE-like sequences of GPE1.

Figure 2 Activation of GPE1 by Nrf2 in F9 cells

The indicated luciferase constructs for *NQO1* ARE, GPE1 or -143/Luc (containing proximal ARE/TRE) were transfected into F9 cells with (+) or without (-) a co-transfected Nrf2 expression vector.

Transcriptional activation of GPE1 by Nrf2 was determined by reporter transfection analysis using mouse F9 embryonal carcinoma cells (Figure 2). Since GPE1 consists of two TRE-like sequences, a reporter gene containing GPE1 is highly expressed

Figure 3 Binding and activation of Nrf2/MafK with mutated GPE1

(A) EMSA was performed with Nrf2/MafK and GPE1, mGPE1, mmGPE1 or TRE probes as in Figure 1. The nucleotide sequences of probes are listed below the Figure and mutated nucleotides are underlined. (B) Nrf2 or c-Jun activation of the luciferase gene containing either GPE1 or mutated elements was analysed in F9 cells as described in the legend to Figure 2. The vector used was pGVB2, a promoterless luciferase plasmid.

in the hepatoma cells without co-transfection of an effector plasmid. In F9 cells, which are considered to lack AP1 activity, basal expression of GPE1 reporter gene is low, and effects of an exogenous factor can be clearly demonstrated. The co-transfection of Nrf2 expression plasmid resulted in strong stimulation of GPE1-mediated gene expression, and activation of the GPE1-luciferase gene was higher than that of the *NQO1* ARE-luciferase gene. Although Nrf2 usually functioned as a heterodimer with a small Maf protein (e.g. MafK), co-transfection of MafK-expression vector resulted in repression of GPE1- and ARE-mediated luciferase gene expression in a dose-dependent manner (results not shown). As reported previously, we assumed that small Maf proteins are ubiquitously expressed and that presence of excess amounts of small Maf protein leads to the formation of homodimers that do not contain a transactivation domain but inhibit the transcription of Nrf2-dependent genes by competing for the binding site [25]. Therefore we omitted the MafK expression vector in subsequent experiments.

The *GST-P* gene has one more ARE/TRE-like element located 61 bp upstream of the cap site, similar to the mouse Pi-class *GST* gene (*GST-P1*), a mouse homologue of the rat *GST-P* gene. Mouse *GST-P1* gene is stimulated by Nrf2 through the ARE/TRE element located 59 bp from the cap site in HepG2 cells [21]. In contrast, a rat *GST-P* reporter gene containing a proximal ARE/TRE sequence (–143/Luc) was barely stimulated by Nrf2 in F9 cells (Figure 2). As described later, ChIP analysis showed that Nrf2 and MafK did not bind to this region, in contrast with their binding to the GPE1 region (Figure 6A). These results indicate that the proximal ARE/TRE sequence in the rat does not function as an Nrf2-binding sequence.

To analyse further the DNA-binding specificity of Nrf2/MafK and the enhancer activity of GPE1, we used point-mutated GPE1 sequences (Figure 3). Previous studies have indicated that 5'-end nucleotides of the two inverted TRE-like sequences, 5'-TCAGTCACTATGATTCA-3' (TRE-like sequences are in upper-case and 5'-ends of TRE-like sequences are underlined), are important for the strong enhancer activity of GPE1 [11,12]. We prepared two point mutants, mGPE1 (5'-TCAGTCACTACGATTCA-3') and mmGPE1 (5'-TCAGTCGCTATGATTCA-3'; mutated nucleotides are underlined) for further analysis. EMSA showed

that the binding of Nrf2/MafK to the mGPE1 probe was fully abolished, but Nrf2/MafK binding to mmGPE1 was similar to that of wild-type GPE1 (Figure 3A). Both mGPE1 and mmGPE1 bound MafB homodimers in a similar manner to wild-type GPE1. TRE interacted with MafB, but not with Nrf2/MafK. To determine the activity of Nrf2 on the mutated GPE1s, reporter transfection analysis was performed using F9 cells (Figure 3B). As expected from EMSA, Nrf2 did not activate the mGPE1 reporter gene. Nrf2-dependent activation of mmGPE1 was also markedly lower than expected, despite the strong binding of Nrf2 to mmGPE1 shown by EMSA. EMSA and reporter transfection analysis with mutated GPE1s revealed that the Nrf2/MafK heterodimer binds to the 3'-side of the TRE-like sequences (5'-TCAGTCACTATGATTCA-3') of the GPE1 core element. The 5'-side of the TRE-like sequences (5'-TCAGTCACTATGATTCA-3') is also important for full enhancer activity of the GPE1. The finding that the 5'-end of both TRE-like sequences is important for binding and activation by Nrf2 is consistent with previous findings [12]. Furthermore, these results suggest that Nrf2 is a factor responsible for the enhancer activity of GPE1.

Nrf2 expression increases during hepatocarcinogenesis

Our results suggested that Nrf2 bound and activated GPE1. We next examined whether GST-P induction correlated with Nrf2 expression during hepatocarcinogenesis. We performed Northern-blot analysis on RNAs from chemically induced pre-neoplastic liver lesions and from livers of LEC rats of various ages. The LEC rat is a mutant strain characterized by development of spontaneous heredity hepatitis, followed by liver cirrhosis and hepatocellular carcinomas [38]. As shown in Figure 4(A), Nrf2 expression increased in the liver of LEC rats from the age of 8 months (stage of cirrhosis) to 2 years (stage of development of hepatocellular carcinomas). GST-P expression in the LEC rat correlated well with Nrf2 expression. Keap1, an inhibitor of Nrf2, was expressed in normal liver cells and did not change during the course of hepatocarcinogenesis (Figure 4B). GST-P mRNA was not detected in the normal liver cells from Sprague–Dawley and LEA rats or from 5-week-old (severe hepatitis stage) LEC rats. In livers bearing hyperplastic nodules, GST-P mRNA expression was extremely strong. Although the Nrf2 mRNA level was increased in cells from hyperplastic nodules when compared with those from normal liver, the extremely high level of GST-P expression was difficult to explain by increased Nrf2 expression alone. This result suggests that some other factors may also contribute to the extremely high expression of GST-P in pre-neoplastic liver lesions.

Keap1 suppresses the activation of GPE1 by Nrf2

To examine the effects of Nrf2 on the rat hepatoma cell line expressing GST-P, H4IIE, a transient transfection assay was performed (Figure 5A). The GPE1/luciferase gene was highly expressed in H4IIE cells, and co-transfection with Nrf2 induced a 4-fold activation similar to that of the *NQO1* ARE construct. A similar analysis using a different rat hepatoma cell line, dRLh84, produced similar results (results not shown).

Keap1, a cytoplasmic effector of Nrf2, inhibits the activation by Nrf2 [29]. Keap1 binds to Nrf2 in the cytoplasm and suppresses Nrf2 translocation to the nucleus. Electrophilic agents liberate Nrf2 from Keap1 and induce genes of phase II drug-metabolizing enzymes. We investigated whether this mechanism worked on the activation of GPE1 by using reporter transfection analysis in H4IIE cells. The reporter gene for GPE1 was strongly

Figure 4 Expressions of Nrf2, Keap1 and GST-P mRNA during hepatocarcinogenesis

Northern-blot analysis was performed using RNA from the liver of various rats and Nrf2 and GST-P (A) or Keap1 (B) mRNA probes. Total RNA was extracted from normal rat livers (NL), livers with hyperplastic nodules (HN), LEA rat livers (LEA) and livers of LEC rats at the indicated ages. RNA (20 µg) was loaded on to a 1.2% agarose gel containing formaldehyde, blotted and hybridized by the indicated probes. The same filter was rehybridized using the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe as a control. W, weeks; M, months; Y, years.

Figure 5 Effects of Nrf2, Keap1 and electrophilic agents on the GST-P expression in rat hepatoma cell line

(A) Reporter genes containing either GPE1 or NQO1 ARE were co-transfected with Nrf2 and Keap1 expression plasmid into H4IIE cells. After transfection, cells were treated with the indicated amounts of DEM for 24 h. (B) RNase protection analysis of H4IIE cells treated with drugs. RNAs were extracted from normal rat liver (Normal Liver), untreated H4IIE cells (H4IIE) and H4IIE cells treated for 24 h with either the solvent alone (ethanol; EtOH), 50 µM β-NF, 400 µM *t*-butylated hydroxyanisole (BHA) or the indicated micromolar concentrations of DEM. RNA (20 µg) was analysed with the indicated antisense RNA probes. All RNA samples, except Keap1, were detected by autoradiography. Keap1 mRNA was visualized by a Fuji BAS-2000 image analyser.

suppressed in cells co-transfected with the Keap1 expression vector, and activity was recovered by the addition of DEM (dimethylmalate) in a dose-dependent manner. Analysis of the

Figure 6 Nrf2 and MafK bind to GPE1 in hyperplastic nodules and hepatoma cells *in vivo*

ChIP assays were performed with anti-Nrf2 (A) and anti-MafK (B) antibodies in normal rat liver (Normal Liver), liver bearing hyperplastic nodules (HN), H4IIE cells and dRLh84 cells as described in the Experimental section. The DNAs were extracted from total sonicated nuclei (Input), Protein A bound without antibody (Protein A), Protein A bound with the indicated antibodies (Nrf2 or MafK) or Protein A bound with preimmune serum (Preimmune). Specific enhancer or promoter regions were amplified by PCR (33 cycles) with specific primers of the NQO1 ARE (NQO1ARE), the GST-P GPE1 (GPE1) and the proximal ARE/TRE region of GST-P gene (ARE/TRE).

luciferase gene that contained the NQO1 ARE gave results similar to those of GST-P GPE1.

To confirm the activation of the GST-P gene in response to drugs *in vivo*, we exposed H4IIE rat hepatoma cells to various drugs and determined the levels of GST-P mRNA by RNase protection analysis (Figure 5B). As expected from transfection analysis, GST-P mRNA expression was induced by β-NF (β-naphthoflavone), *t*-butylated hydroxyanisole and DEM treatment. Genes for the phase II detoxifying enzymes NQO1 and GST-Ya were also induced by these drugs. In contrast with genes for phase II detoxifying enzymes, genes for Nrf2 and Keap1 were not significantly induced by drugs, except by β-NF, as reported previously [39]. These results indicate that the Nrf2–Keap1 mechanism worked in regulating activation of GPE1.

Nrf2 and MafK bind to GPE1 in pre-neoplastic liver lesions and hepatoma cells *in vivo*

To examine the binding of Nrf2 and MafK to GPE1 of the GST-P gene *in vivo*, ChIP analyses were performed using anti-Nrf2 and anti-MafK antibodies as described in the Experimental section (Figure 6). We analysed the chromatin from cells of normal liver, of livers bearing pre-neoplastic nodules induced by the Solt–Farber system [35,36] and of rat hepatoma cell lines (H4IIE and dRLh84). The anti-Nrf2 antibody precipitated the NQO1 ARE from the chromatin of all samples. The anti-Nrf2 antibody precipitated the GST-P GPE1 from the chromatin of cells from liver with hyperplastic nodules and of hepatoma cells, but did not precipitate the GST-P GPE1 from the chromatin of normal liver cells. Detection of GPE1 by ChIP correlated well with GST-P expression. These results clearly indicate that Nrf2 was bound to the GPE1 in the cells expressing GST-P. The proximal

Figure 7 Acetylation of histones H3 and H4 in cells expressing GST-P

ChIP analyses were performed as described in the legend to Figure 6 using anti-acetyl-histone H3 and H4 antibodies in normal rat liver and H4IIE cells.

ARE/TRE-like sequence, located at -61 of the *GST-P* gene, was not precipitated by the anti-Nrf2 antibody. Consistent with the results of reporter transfection analysis (Figure 2), Nrf2/MafK did not bind and did not activate the proximal ARE/TRE-like element.

ChIP analysis was also performed using the anti-MafK antibody (Figure 6B). The results were completely consistent with those seen for anti-Nrf2 antibody. Anti-MafK antibody precipitated *NQO1* ARE from the chromatin fraction of all samples. GPE1 was precipitated by anti-MafK antibody from the cells of hyperplastic nodules and from hepatoma cells, but not from the normal liver cells. Anti-MafK antibody, similar to anti-Nrf2 antibody, did not precipitate the proximal ARE/TRE-like element of the *GST-P* gene. These results suggest that of the small Maf proteins (MafK, MafG and MafF), MafK is the predominant partner molecule for Nrf2 in liver and hepatoma cells. We performed ChIP analysis using anti-cJun antibody, but did not precipitate either GPE1 or the proximal ARE/TRE-like element from any sample (results not shown). These results strongly suggest that Nrf2/MafK is the activator responsible for GST-P expression during hepatocarcinogenesis.

Acetylation of histones H3 and H4 of the GST-P chromatin

Acetylation of histones H4 and H3 on the chromatin of GPE1 and promoter regions of the *GST-P* gene were investigated by ChIP analysis (Figure 7). Histones H3 and H4 were not acetylated in either GPE1 or promoter regions of the *GST-P* gene in normal liver. In the H4IIE hepatoma cell line, histones H3 and H4 were acetylated in both GPE1 and in the promoter regions, and this acetylation correlated with the activation of the *GST-P* gene.

DISCUSSION

Nrf2/MafK binds and activates the GST-P strong enhancer GPE1

GPE1 is the main enhancer element essential for the activation of the *GST-P* gene during hepatocarcinogenesis. In the present study, we identified Nrf2/MafK as an activator of GPE1. *In vitro* binding assays and reporter transfection analyses indicated that Nrf2/MafK specifically and strongly interacts with and activates GPE1 (Figures 1 and 2). EMSA using mutated GPE1 showed that the Nrf2/MafK heterodimer binds to the 3'-TRE-like sequence, but not to the 5'-TRE-like sequence, of the two inverted TRE-like sequences of GPE1 (Figure 3A). However, reporter transfection analyses showed that both the 3'- and 5'-TRE-like sequences were essential for strong activation of GPE1 (Figure 3B). From these results, one can speculate that another factor, which may interact with the 5'-TRE-like sequence and/or with the Nrf2/MafK, may participate in the GPE1 activation. Although increased expression of Nrf2 during hepatocarcinogenesis correlated well

with increased expression of GST-P in LEC rats, it is difficult to explain the extremely high expression of GST-P in hyperplastic nodules based on the increased quantity of Nrf2 alone (Figure 4). This result also suggests that additional mechanisms may be involved in the strong activation of the *GST-P* gene. By EMSA, we and others detected a factor that specifically bound to the 5'-TRE-like sequence of GPE1 in nuclear extracts from F9 and hepatoma cells (H. Ikeda, S. Nishi and M. Sakai, unpublished work) [40]. Involvement of this factor in the specific activation of the *GST-P* gene remains to be elucidated.

ChIP analyses clearly demonstrated that both Nrf2 and MafK bind GPE1 in cells expressing GST-P (hyperplastic nodules of liver and hepatoma cell lines). Histones H3 and H4 are acetylated both in GPE1 and in the proximal promoter regions in cells expressing GST-P. This result indicates that both GPE1 and the proximal promoter regions in these cells have an open chromatin structure. However, Nrf2 and MafK did not bind and activate to the proximal ARE/TRE region, as shown by ChIP (Figure 6A) and reporter transfection analyses (Figure 2). We examined previously the mouse Pi-class *GST* gene (*GST-P1*), a mouse homologue of the rat *GST-P* gene. In contrast with the rat *GST-P* gene, the mouse *GST-P1* gene was expressed in normal liver cells, predominantly in the liver of male mice, and GST-P1 is regulated by androgen. Nrf2/MafK binds to the ARE/TRE sequence located on the GST-P1 promoter (at -59 nt) and activates this gene [21]. Although mouse GST-P1 is also induced in pre-neoplastic foci of the mouse liver, the increase in expression is much less than that seen in the rat [20]. The main reason for this discrepancy is probably the presence of a strong enhancer element, GPE1, in the rat *GST-P* gene. The Pi-class *GST* genes of both the mouse and the rat are activated by Nrf2, but responsive elements for activation are the proximal ARE/TRE for the mouse and the distal GPE1 for the rat. The Pi-class *GST* gene has an ARE/TRE sequence having an almost identical sequence and location for both species. The ARE/TRE sequence is -TGACTCAG- for the rat and -TGAGTCAG- for the mouse, and it is located at -61 for the rat and at -59 for the mouse [9,21]. However, this element functions as an Nrf2-binding site in the mouse, but not in the rat. In the rat, the GPE1 element or surrounding promoter sequences may somehow interfere with the binding of Nrf2 to the proximal ARE/TRE.

The Keap1-Nrf2 pathway works on GPE1

Itoh et al. [29] identified Keap1 as a mediator protein for the induction of phase II detoxifying enzymes by electrophilic compounds. *Keap1* knockout mice have constitutive Nrf2 activation [30]. Transfection analysis showed that Keap1 suppressed GPE1-mediated transcription and the activity was recovered by treatment with DEM, an electrophilic compound (Figure 5A). Furthermore, endogenous GST-P mRNA expression was also stimulated in hepatoma cells by treatment with electrophilic drugs (Figure 5B). These results indicate that the Keap1-Nrf2 pathway regulates GST-P expression in hepatoma cells. Although a significant amount of Nrf2 is expressed in normal liver cells, GST-P expression is completely repressed by an unknown mechanism. The *GST-P* gene in normal liver cells is barely induced by treatment with electrophilic drugs [4]. However, our results indicated that these drugs induce the *GST-P* gene in hepatoma cells, although the extent of induction was not high as that in hyperplastic nodules. This discrepancy is probably because the completely repressed *GST-P* gene cannot be activated by the Nrf2-mediated induction mechanism in normal liver cells; however, in hepatoma cells, GST-P expression has been de-repressed. Therefore GST-P

expression can be further activated by the same pathway as that used to activate phase II detoxifying enzymes.

We thank Dr K. Igarashi for providing anti-MafK antibody and Dr K. Satoh for providing rats treated with the Solt–Farber system. This work was supported by a grant from Saitama Medical School Research Center for Genomic Medicine and by the Clark Memorial Foundation of Hokkaido University.

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Received 18 December 2003/10 February 2004; accepted 11 February 2004

Published as BJ Immediate Publication 11 February 2004, DOI 10.1042/BJ20031948