Pro-inflammatory Cytokines Tumor Necrosis Factor α and Interleukin-6 and Survival Factor Epidermal Growth Factor Positively Regulate the Murine GSTA4 Enzyme in Hepatocytes

Received for publication, December 24, 2001, and in revised form, February 19, 2002
Published, JBC Papers in Press, March 7, 2002, DOI 10.1074/jbc.M112351200

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We hypothesized that glutathione transferases could be induced and may participate to cellular defenses against the oxidative stress occurring during liver regeneration. Here, we evidenced that murine GSTA1 (mGSTA1), A4, Pi, and Mu are up-regulated during mouse liver regeneration, exhibiting a biphasic pattern of induction correlating early G1 phase and G1/S transition of the cell cycle. Using confocal microscopy immunolocalization and subcellular fractionation, mGSTA4 was demonstrated in both mitochondria and cytosol and found preferentially increased in cytosol during liver regeneration. In addition, mGSTA4 was induced in vivo and in cultured hepatocytes by tumor necrosis factor α (TNFα), interleukin-6 (IL-6), and epidermal growth factor (EGF), factors that play crucial roles in hepatocyte survival and proliferation during liver regeneration. However, the mitogenic effect of EGF was not responsible for the induction of mGSTA4. In transient transfections, IL-6 and EGF, but not TNFα, transactivated the human GSTA4 (hGSTA4) promoter cloned upstream of the luciferase reporter gene suggesting that IL-6 and EGF up-regulated hGSTA4 at a transcriptional level, whereas TNFα could rather act at a post-transcriptional level. The inhibition of phosphoinositide 3-kinase, p38 MAPK, and MEK/ERK signaling pathways, using specific inhibitors, prevented EGF-dependent induction of mGSTA4 and transactivation of hGSTA4 promoter. Altogether, these data favor the conclusion that, in regenerating hepatocytes, several GST isozymes are induced and that cytokines TNFα and IL-6 and survival factor EGF positively regulate mGSTA4 via survival signaling pathways.

Quiescent, differentiated hepatocytes are able to re-enter the cell cycle and proliferate to restore the liver mass following liver defects resulting from surgical removal or caused by chemicals and viruses. Entry into and progression through early G1 phase of the cell cycle, also called priming (1), are induced by the cytokines tumor necrosis factor α (TNFα) (2, 3), and interleukin-6 (IL-6) (4) and are required for the hepatocytes to fully respond to growth factors (5). TNFα binding to its type 1 receptor successively activates NFKB (5), IL-6 expression, and STAT3 (6, 7) in the early G1 phase, which constitute a key signaling pathway during hepatocyte proliferation (4, 8). The late G1 progression and commitment to DNA replication are controlled by growth factors (9–11) including hepatocyte growth factor, transforming growth factor-α, and epidermal growth factor (EGF) (1) through the activation of the MEK/ERK pathway (11).

Several lines of evidence indicate that partial hepatectomy (PH) is rapidly followed by an oxidative stress due to increased reactive oxygen species (ROS) and nitric oxide (NO) production leading to lipid peroxidation (12–14).

Regarding ROS, TNFα is now recognized as playing a crucial role in the production of these species during liver regeneration. In support of such a role, it is worth noting that the multimerization of TNFα type I receptor following binding of TNFα has indeed been shown to lead to recruitment of TRAF (TNF receptor-associated protein), a protein involved in the signaling pathway regulating ROS production in mitochondria (15), probably through the inhibition of complex III of the electron transport chain (16).

Both ROS and NO have been demonstrated to contribute significantly to induce hepatocyte proliferation. Indeed, in transgenic mice with targeted disruption of TNFα type I receptor (TNFRI) or of the type II nitric-oxide synthase (iNOS), the enzyme that catalyzes the formation of NO from arginine, hepatocyte proliferation after PH is strongly impaired (2, 12). In this context, it then appears that TNFα-related oxidative stress functions as a signaling pathway rather than eliciting deleterious effects. Such a function is mediated through the activation of redox-sensitive proteins, especially the transcription factor NFκB (17), which on one hand allows the occurrence of proliferation by transactivating cell cycle genes such as c-fos and c-jun controlling the G1/G0 transition, and on the other hand, attenuates deleterious responses resulting from oxidative stress (e.g. activation of caspases by trans-activating the anti-apoptotic gene Becl2, that prevents activation of caspases (18)).
NFκB also induces the mitochondrial uncoupling protein UCP-2 (13) and iNOS (19), both of which contribute to reduce oxidative stress (1). UCP2, an inner mitochondrial channel for protons, plays a major role in limiting production of ROS (13) by dissipating the electrochemical gradient. NO, whose production is enhanced by induction of iNOS, although it participates in lipid peroxidation (14), also exhibits a cytoprotective effect by preventing apoptosis through S-nitrosylation of caspases, which strongly inhibits caspase activities (20). This last mechanism is most likely involved in liver regeneration because in iNOS knock-out mice, PH is followed by a strongly increased caspase-3 activity and hepatocyte death (12). Along with the TNAα-dependent activation of antioxidant defenses, it is worth noting that cytokine IL-6 and growth factors have also been shown to favor hepatocyte survival during liver regeneration through stimulation of anti-apoptotic gene products (21, 22).

In normal hepatocytes, an excess of ROS is also neutralized through the action of thiols, especially glutathione (GSH), of which the content is increased during liver regeneration (21). In addition, glutathione S-transferases (GST) of the alpha class detoxify organic hydroperoxides and protect cells against oxidative stress (22). For instance, transfection of hGSTA2 in K562 cells results in cell protection toward H2O2-induced lipid peroxidation (23). The GSTA3 enzyme, the α subunit that exhibits the highest activity against 4-hydroxynonenal (4-HNE) (24, 25), also efficiently protects against oxidative damage mediated by this cytotoxic product of lipid peroxidation generated by ROS overproduction (26). In addition, we have recently demonstrated that ROS overproduction induced by hepatic iron overload is correlated with an increase in mGSTA4 expression (27).

These observations suggest that GST enzymes could participate in defenses against oxidative stress during liver regeneration. However, to date, little is known about regulation of GST expression and activity during liver regeneration. Lee and Boyer (28) have reported a decrease in mRNA levels of several GSTs at 12 h post-PH, whereas Mori et al. (29) have shown a higher expression of GSTP1 after 2 and 3 days post-PH. To our knowledge, no study has attempted to correlate the expression of GSTs with the oxidative stress occurring during the first hours following PH.

The aim of this paper was, first, to study the expression and activities of several GST subunits, including the mGSTA4 isoform, during the liver regeneration after a two-thirds hepatocotomy in mouse. Then, we investigated whether oxidative stress, the pro-inflammatory cytokines TNFα and IL-6, and growth/survival factor EGF, which are involved in proliferation and survival of hepatocytes during liver regeneration, may regulate the expression of mGSTA4 both in vitro and in hepatocytes in primary culture.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—The rabbit polyclonal antibody against a mGSTA4 peptide was characterized in our laboratory (30). The other antibodies were purchased as follows: rabbit polyclonal anti-human GSTA1, GSTM1, and GSTP1 antisera from Biotrin, Dublin, Ireland; rabbit polyclonal anti-cytochrome c from Santa Cruz Biotechnology, Santa Cruz, CA; monoclonal anti-cytochrome c oxidase from Molecular Probes, Eugene, OR; monoclonal anti- phospho-p44/42 MAPK (Thr202/Tyr204) and anti-p44/42 MAPK from Cell Signaling Technology, Beverly, MA; and anti-albumin from ICN Pharmaceuticals, Orsay, France; horseradish peroxidase-linked rabbit antisera from Bio-Rad, Ivory sur Seine, France; and mouse and goat anti-rabbit rhodamine-conjugated IgG from Santa Cruz Biotechnology.

**Animal Experiments**—Balb/c male mice (8 weeks old, Janvier laboratories, Le Genest, France) were subjected to 70% partial hepatectomy under ether anesthesia and were killed at various times post-PH. As controls, animals underwent a sham operation consisting of a laparotomy without tissue resection. 1-N^1-(1-Iminoethyl)lysine dihydrochloride (NIL; Calbiochem) was injected intraperitoneally at a dosage of 40 µg/g of body weight in normal and hepatotomized animals 1 h before the surgery. Animals were killed 2 h after NIL injection. TNFα, IL-6 (Promocell, Heidelberg, Germany), and EGF (Promega, St. Quentin Fallavier, France) were given i.p. in saline (0.9% NaCl) containing 5% bovine serum albumin at 40, 80, and 125 µg/g of body weight, respectively. Control animals received the corresponding sterile saline vehicle. Animals were killed 2 h after TNFα and IL-6 injection and 6 or 24 h after EGF injection.

**Isolation and Culture of Hepatocytes**—Mouse hepatocytes were isolated in two-step procedure using 0.25% collagenase I (Roche Diagnostics, Meylan, France) as previously described (31). Isolated hepatocytes were resuspended in Williams’ medium E (Invitrogen) containing penicillin (100 IU/ml), 1 mg/ml serum bovine albumin, 5 µg/ml bovine insulin, and 10% fetal calf serum (Invitrogen), plated at a density of 6.10^4/cm^2, and maintained at 37°C in a humidified 5% CO2/95% air. EGF was added at 50 ng/ml and TNFα and IL-6 at 20 ng/ml 24 h after cell seeding. Specific inhibitors Ly 294002, SB 203580 (Calbiochem), and U0126 (Promega) of PI3K, p38 MAPK, and MEK, respectively, were added 1 h prior to EGF stimulation at final concentrations of 15, 12, and 50 µM, respectively.

**RNA Extraction and Northern Blot Analysis**—Total RNA from cells or liver biopsies was isolated using an SV RNA extraction kit (Qiagen, Courtabeuf, France). Ten µg of total RNA from each sample were used for Northern blot analysis. Blots were hybridized with the corresponding 32P-labeled cDNA probe (la-3PIdCTP, 3000 Ci/mmol, Amersham Biosciences) at 65°C overnight. Blots were washed at moderate stringency and exposed to radiograph films. The 18S ribosomal probe was used as control.

**Protein Extraction and Western Blot Analysis**—Cultured liver biopsies were homogenized in lysis buffer (HEPEs, pH 7.5, 50 mm, NaCl 150 mm, EDTA 1 mm, EGTA 2.5 mm, 0.1% Tween 20, 10% glycerol, β-glycerophosphate 10 mm, sodium fluoride 1 mm, sodium orthovanadate 0.1 mm, phenylmethylsulfonyl fluoride 0.1 mm, leupeptin 10 µg/ml, and aprotonin 10 µg/ml). CDK1 was purified from liver extracts using p9CKS412 beads and recovered with sample buffer as described previously (9). Protein concentrations were determined using the Bradford method. Proteins were fractionated by SDS-PAGE (12.5%), transferred to a polyvinylidene difluoride membrane and incubated further for 2 h in PBS containing 3% bovine serum albumin and then overnight with primary antibody. Membranes were washed twice before incubation with secondary antibody for 1 h. Proteins of interest were visualized using the chemiluminescence reagent ECL (Interchim). Detection of Intraacellular MDA and 4-HNE Levels—MDA and 4-HNE levels were determined using lipid peroxidation assay kits (Calbiochem). Liver biopsies were washed in ice-cold 0.9% NaCl and sonicated in 20 mm Tris-HCl, pH 7.4, to −10% (w/v). Liver extracts were centrifuged at 3000 x g for 10 min at 4°C, and the supernatant was collected prior to determination of total protein concentration and the MDA and 4-HNE colorimetric assays.

**Isolated Mitochondria**—Mitochondria from liver mitochondria were isolated from freshly harvested livers by differential centrifugation in ice-cold H medium containing 210 mM mannitol, 70 mM sucrose, and 2 mM HEPES buffer (pH 7.4) (32). The purified pellets were suspended in 200 µl of buffer containing 150 mM KCl, 0.5 mM malonate, 0.1 mM oxoglutarate, and 10 mM HEPES.

**GST Activity Assays**—GST activities toward 1-chloro-2,4-dinitrobenzene (CDNB) and ethacrynic acid (EA) were determined by spectrometric analysis (33, 34) of total proteins. GST activity toward 4-HNE was quantified using a high pressure liquid chromatography method as described previously (27).

**Immunohistochemistry**—Mice were anesthetized and perfused through the portal vein with 4% paraformaldehyde in 0.1 M sodium cacodylate for 15 min at a flow rate of 10 ml/min. Tissue fragments were washed in 0.1 M PBS for 4 h and in 10% glycerol-PBS overnight and were frozen in liquid nitrogen-cooled isopentane. Frozen tissue sections were mounted on glass slides coated with 10% gelatin in PBS and incubated in PBS containing 3% bovine serum albumin for 30 min. They were then covered with a solution of anti-rabbit GSTA4 (1/100) or anti-rabbit cytochrome c (1/100) antiserum for 1 h at room temperature. Sections were washed with PBS, incubated for 30 min with rabbit IgG conjugated to rhodamine (1/200), washed, and mounted. Serial z-axis optical analysis of sections was done at 1-µm intervals using a laser scanning confocal microscope (Confocal Leica TCS NT).
Cells were sonicated in PBS, and \(^{3}H\)thymidine incorporation was measured after DNA precipitation in 15% trichloroacetic acid.

**Reporter Gene Constructs and Transient Transfection in Primary Cultured Mouse Hepatocytes**—Two DNA fragments corresponding to the 5'-flanking region of the \(hGSTA4\) gene (1571 and 165 bp upstream of exon 1) characterized previously (35) were cloned into the pGL3 basic vector (Promega) upstream of the firefly luciferase reporter gene. The pRL-CMV vector encoding Renilla luciferase and pGL3-GSTA4-(1–1571) or pGL3-GSTA4-(1–165) were co-transfected in 24-h cultured hepatocytes using cationic lipids according to Gilot et al. (36). Dual luciferase assays (firefly and Renilla) were done using a Promega kit. pGL3 basic (a promoterless pGL3-luciferase construct) and pGL3 promoter (pGL3-luciferase construct with the SV40 promoter) vectors were transfected providing negative and positive firefly luciferase controls.

**Statistical Analysis**—Values were expressed as the mean ± S.D. Student’s t test was used for the estimation of statistical significance. A p value less than 0.05 was considered statistically significant.

**RESULTS**

**Induction of Several GST Isoforms during Liver Regeneration**—The expression of the different GST isoforms was analyzed during liver regeneration over a 96-h period after a two-thirds hepatectomy of Balb/c mice. To ensure that liver regeneration occurred as previously described, the relative mRNA levels of the two cell cycle genes, cyclin D1 and CDK1, were analyzed. A clear induction of cyclin D1 transcripts was observed between 30 and 96 h, whereas the level of CDK1 mRNA was increased between 48 and 96 h post-PH with a maximal expression at 72 h (Fig. 1A) as previously reported (37).

Levels of mGSTA4, A1, and Pi mRNAs were augmented during liver regeneration exhibiting a biphasic induction of expression (Fig. 1B). An initial increase was observed within 1 h post-PH, and mRNA levels remained high for 12 to 24 h. The second peak of induction took place at 48 and 96 h post-PH with a maximal expression at 72 h. In sham-operated mice, mGSTA4, A1, and Pi mRNA levels showed a transient and moderate increase between 1 and 8 h post-PH but not thereafter. mGSTMu mRNAs were detected at very low levels, and no significant change was observed during the first 96 h of regeneration.

mGSTA4, A1, Pi, and Mu proteins were further investigated by Western blot (Fig. 2A). Expression of mGSTA4, A1, and Mu proteins exhibited a biphasic pattern as observed for mRNAs. The first induction occurred between 0.5 and 4 h after PH; mGSTA4 was very transiently induced at 0.5 h, whereas up-regulation of mGSTA4, Mu, and Pi remained elevated between 0.5 and 4 h. The second induction of the four GST proteins took place between 24 and 72 h depending upon the GST isoforms. In sham-operated mice, a slight increase of mGSTA4, A1, Mu, and Pi proteins was also detected at 1 and 12 h post-PH as observed with the corresponding mRNAs, which returned to control values thereafter except for mGSTPi, which remained elevated until 48 h.

To confirm the induction of several GSTs, during the first hours post-PH, we measured GST activities using three different substrates: CDNB, a common substrate for mGSTA4, A1, Mu, and Pi; and 4-HNE and EA, specific substrates for GSTA4 and GSTPi, respectively (22). GST activities measured with CDNB, 4-HNE, and EA were all significantly increased between 0.5 and 2 h after PH compared with sham-operated animals. The induction of GST expression observed between 24 and 72 h was also correlated with a strong induction of GST activities using CDNB as a substrate (Fig. 2B).

**mGSTA4 Is Located in the Cytosol and Mitochondria of Hepatocytes**—The subcellular localization of mGSTA4 was analyzed in normal mouse liver using the indirect immunofluorescence technique and confocal microscopy (Fig. 3A). A punctuated staining typical of a mitochondrial distribution was observed for cytochrome c whereas mGSTA4 exhibited both a
punctuated and an intense homogeneous cytosolic staining.

To confirm these results, mGSTA4 protein expression was analyzed by Western blot in the cytosol and mitochondria of normal or regenerating livers after cell fractionation. The reliability of the cell fractionation was demonstrated in cell extracts from normal and 24-h post-PH regenerating livers by performing Western blots of the mitochondrial cytochrome c and cytochrome oxidase proteins, which were detectable mainly in mitochondrial fractions while albumin was observed only in cytosols (Fig. 3B).

By Western blot the mGSTA4 protein was detectable in both mitochondrial and cytosolic fractions from normal liver, regenerating livers 1 and 24 h post-PH, and sham-operated livers 24 h post-laparotomy (Fig. 3C). The signal obtained in cytosols was much higher than in mitochondria and was increased at 1 and 24 h post-PH compared with normal and sham-operated animals. No change in mGSTA4 level was found in mitochondrial extracts during liver regeneration even after a longer exposure time of the blots (data not shown).

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hepatocytes (1), could be involved in this induction. TNFα and IL-6 injections to normal mice induced expression of mGSTA4 but not mGSTA1, used as control, compared with mice injected with the vehicle only (Fig. 4C).

In normal livers at 6 and 24 h after EGF administration, expression of mGSTA4 was significantly induced compared with the levels of mGSTA4 in livers of mice injected with the vehicle and noninjected animals (Fig. 4D). To determine whether EGF also induced proliferation of liver cells, expression of CDK1 protein, a cell cycle marker of S, G2, and M phases (9, 10), was studied and compared with its expression in regenerating livers at 40 and 72 h post-PH. In livers of control and EGF-treated mice, CDK1 was not detected, whereas its expression was strongly induced in regenerating livers. These results indicated that TNFα, IL-6, and EGF up-regulated mGSTA4 expression in normal liver.

**TNFα, IL-6, and EGF Induce mGSTA4 Expression in Primary Cultures of Mouse Hepatocytes**—Primary cultures of mouse hepatocytes were used to confirm the up-regulation of mGSTA4 by TNFα, IL-6, and EGF and analyze the level of regulation. The addition of TNFα and IL-6 to the culture medium at 24 h led to induction of the mGSTA4 protein, whereas the levels of mGSTA1 and albumin, used as controls, were unaffected (Fig. 5A). Stimulation by EGF at 4 or 24 h after plating transiently induced mGSTA4 at 24 and 48 h, respectively, whereas the level of mGSTA1 was not increased after EGF stimulation (Fig. 5B). To verify if stimulation by these factors resulted in an increase in mGSTA4 mRNA levels, a Northern blot analysis using mGSTA4 cDNA was performed on EGF-, TNFα-, and IL-6-treated hepatocytes. Induction of mGSTA4 mRNAs by EGF, TNFα, and IL-6 was confirmed (Fig. 5C).

To determine whether the induction of mGSTA4 by these soluble factors occurred at the transcriptional level, transfection experiments were performed using the human GSTA4 promoter, which we had previously isolated and characterized (35). A significant increase in luciferase activities was observed after stimulation with EGF and IL-6, but not with TNFα, when cells were transfected with the pGL3-GSTA4-(1–1571) plasmid carrying 1571 base pairs of the hGSTA4 promoter upstream of the luciferase reporter gene (Fig. 5D), indicating that induction of hGSTA4 by EGF and IL-6 was most likely due to a transcriptional activation of the GSTA4 gene.

**Proliferating and/or Survival Pathways Are Involved in the Induction of mGSTA4 by EGF**—To confirm that proliferating and/or survival pathways were involved in the induction of mGSTA4 expression by EGF, we used inhibitors of different signaling pathways, namely Ly 294002, SB 203580, and U0126 which inhibit PI3K, p38 MAPK, and MEK, respectively, and analyzed mGSTA4 expression by Western blot.

Treatments of EGF-stimulated hepatocytes by Ly 294002, SB 203580, and U0126 led to a strong decrease in mGSTA4 protein expression, whereas GSTA1 was not modified (Fig. 6A). Because interference between the PI3K and MEK/ERK pathways has been evidenced in many cell types, the effects of these inhibitors on the phosphorylation of AKT (P-AKT) and ERK1/2 (P-ERK), respective substrates of PI3K and MEK, were investigated by Western blot (Fig. 6A). Ly 294002 was found to strongly diminish P-AKT and P-ERK levels, whereas U0126 almost completely abolished P-ERK and slightly decreased P-AKT. SB 203580 affected neither P-AKT nor P-ERK. In addition, these three molecules strongly inhibited DNA replication in EGF-stimulated hepatocytes (Fig. 6B).

The ability of these inhibitors to down-regulate hGSTA4 transcriptional activity by transient transfection of pGL3-GSTA4-(1–1571) plasmid was investigated. Four hours after transfection, hepatocytes were treated with SB 203580, Ly 294002, and U0126 in the absence or presence of EGF for 24 h. As expected, luciferase activity was enhanced in the presence of EGF. SB 203580 slightly decreased luciferase activities in both nonstimulated and EGF-stimulated cells, whereas treatments...
by Ly 294002 and U0126 strongly down-regulated reporter gene activity in both nonstimulated and EGF-stimulated cultures (Fig. 6C).

**DISCUSSION**

Liver regeneration following partial hepatectomy is associated with an overproduction of ROS, which probably play a critical role in the induction of hepatocyte proliferation (1). Several mechanisms of defense are activated to neutralize this ROS excess (12, 13). Among the protective defense systems in hepatocytes, GSTs, particularly GSTA4, are recognized to play an important role in the elimination of lipoperoxidation products in various physiopathological situations (39, 40).

Only a few studies have dealt with the regulation of GSTs during liver cell growth without detailed kinetics of GST expression during the first 3 days post-PH (28, 29). Our results have clearly demonstrated for the first time that several GSTs belonging to distinct classes, in particular mGSTA4, exhibit a biphasic pattern of induction concomitant with the two critical steps of liver regeneration, i.e. entry into the cell cycle (the so-called priming) and the commitment to DNA synthesis, which are controlled by the pro-inflammatory cytokines TNFα and IL-6 and by growth factors, respectively (1).

Oxidative stress, demonstrated by H₂O₂ production in mitochondria (13) and increased levels of MDA and 4-HNE products in cytoplasm detected in this study, is observed in early G₁ phase during liver regeneration. We postulated that this increase in lipid peroxidation products was associated with an increase in mitochondrial and/or cytosolic mGSTA4 content. Several recent studies have dealt with immunolocalization of GSTA4 in hepatocytes and led to contradictory results. This enzyme has been reported to be located in both mitochondria and cytosol (41), only in mitochondria (42), or predominantly at or near the plasma membrane (43). Our observations based on both immunofluorescence confocal microscopy and subcellular fractionation support a distribution of mGSTA4 in both mitochondria and cytosol and a preferential increase of mGSTA4 content in the cytosol of regenerating hepatocytes. The mitochondrial and cytosolic localization of mGSTA4 is compatible with the formation of ROS in mitochondria and lipid peroxidation-derived aldehydes in membranes that diffuse within the cell and attack targets far from the site of their original production.

The induction of mGSTA4 during the first hour post-PH could result from oxidative stress, because 4-HNE is known to
be substrate and inducer of this GST isoform (25, 38). However, our results show that, in both normal and regenerating liver, NIL, a specific inhibitor of iNOS, diminished the levels of 4-HNE and MDA, whereas mGSTA4 protein content was unchanged or augmented in regenerating and normal liver, respectively. These findings favor the conclusion that the expression of mGSTA4 is not strictly correlated to the levels of lipid peroxidation and more precisely to the contents of 4-HNE and/or MDA. Nevertheless, it cannot be totally excluded that free radicals rather than lipoperoxidation products may contribute to the up-regulation of mGSTA4 during liver regeneration.

In the regenerating liver, induction through NFκB activation (17) of cellular defenses involved in the neutralization of the excess of ROS and lipid peroxidation products, such as the mitochondrial UCP-2 protein (13), iNOS (14), and the anti-apoptotic proteins BclXL and Akt (44, 45), are dependent upon stimulation by the pro-inflammatory cytokines TNFα, TNFα, or IL-6. Treatments were performed for 6 h starting 24 h after plating. Data are representative of three independent experiments. Here, we show that injection of TNFα, IL-6, and EGF to normal mice resulted in an induction of mGSTA4, whereas mGSTA1 remained unchanged. The biphasic induction of mGSTA4 during the first hours after PH could result from the overproduction of the pro-inflammatory cytokines TNFα and IL-6 involved in the cell cycle priming and activation of survival pathways by growth factors such as EGF. This hypothesis is strongly reinforced by our in vitro data, demonstrating that mGSTA4 expression was also increased after stimulation by TNFα, IL-6, and EGF. The second peak of induction of mGSTA4, occurring between 24 and 48 h post-PH, could be due to the increase in growth factors in plasma that promotes the G1/S transition (11). However, our data indicated that mGSTA4 induction by EGF is not necessarily correlated to progression in late G1 or S phases. Indeed, in vivo injection of EGF induced mGSTA4, whereas hepatocytes did not proliferate, as previously reported (1) and confirmed in this study by the absence of CDK1 expression, a cell cycle marker of S, G2, and M phases (9).

Previous studies have demonstrated modulation of GST expression by cytokines and growth factors in the liver. An increase in hGSTA1 and A2 by IL-4 in cultured human hepatocytes (46) and a marked decrease in rGSTA2 and M1 mRNA levels by IL-1β in rat hepatocytes (47) have been evidenced. Moreover, rGSTP1 expression is strongly induced by EGF in cultured rat hepatocytes (48). However, this is the first time, to our knowledge, that induction of several GSTs has been evidenced during liver regeneration and that a correlation has been established between this induction and the soluble factors essential for hepatocyte survival and proliferation.

Our results also suggest that IL-6 and EGF positively regulate hGSTA4 expression at the transcriptional level, whereas TNFα could act rather at a post-transcriptional level as previ-
upstream of the transcription start site of the hGSTA4 gene. 0.5 kb upstream of the promoter region used for our transfection assays (data not shown). Therefore, we cannot rule out that TNFα could activate hGSTA4 transcription via the putative NFκB binding site located upstream of the 1.5-kb promoter sequence.

The p42/p44 ERK1/2 (11), p38 MAPK (49), and PI3K (44) signaling pathways activated during liver regeneration are essential for both the proliferation and survival of hepatocytes during the hepatic regenerative process. Recently, several reports have indicated that MAPK pathways are involved in the regulation of GST enzymes. Indeed, Kang et al. (50) have demonstrated that the activation of p38 MAPK and PI3K during oxidative stress leads to the induction of hGSTA2. In addition, Yin et al. (51) have demonstrated that GSTαi has protective effects against H2O2-mediated cell death via activation of p38 MAPK, ERK, and NFκB and repression of the c-jun N-terminal kinase signaling pathways. Here, we have shown that the use of the specific inhibitors Ly 294002, SB 203580, or U0126, respectively, prevented the induction of hGSTA4 expression and the transcriptional activation of the hGSTA4 promoter-luciferase construct by EGF.

 Altogether, these data demonstrate the induction of several GST isoforms, including GSTM1, A4, Mu, and Pi, during liver regeneration. They also strongly suggest that the pro-inflammatory cytokines TNFα and IL-6 and the growth/survival factor EGF, which control hepatocyte survival and proliferation during liver regeneration, might be involved in the up-regulation of hGSTA4 via PI3K and/or MAPK pathways. Thus, mGSTA4 could be a target gene induced by survival factors and could contribute to cellular defenses against oxidative stress in hepatocyte.

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![Fig. 6. Inhibition of mGSTA4 expression by inhibitors of PI3K (Ly 294002), P-p38 MAPK (SB 203580), and MEK (U0126) in EGFr-stimulated hepatocytes.](image-url)
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Pro-inflammatory Cytokines Tumor Necrosis Factor α and Interleukin-6 and Survival Factor Epidermal Growth Factor Positively Regulate the Murine GSTA4 Enzyme in Hepatocytes

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J. Biol. Chem. 2002, 277:17892-17900.
doi: 10.1074/jbc.M112351200 originally published online March 7, 2002

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