Delayed Mechanism for Induction of γ-Glutamylcysteine Synthetase Heavy Subunit mRNA Stability by Oxidative Stress Involving p38 Mitogen-activated Protein Kinase Signaling*

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Expression of the γ-glutamylcysteine synthetase heavy subunit (γ-GCSH), which encodes the rate-limiting enzymes for glutathione biosynthesis, is regulated by many cytotoxic agents. Moreover, γ-GCSH mRNA expression is elevated in colorectal cancer, but how γ-GCSH expression is regulated is not completely understood. By using actinomycin D, which inhibits new RNA synthesis, we showed that treatment of human colorectal cancer cells with the prooxidant sulindac increased the half-life of γ-GCSH mRNA. By using a tetracycline-regulated γ-GCSH mRNA assay system, we systematically dissected the cis-acting sequence and trans-acting factors that regulate the stability of γ-GCSH by cytotoxic prooxidants. We demonstrated that a HuR recognition sequence, AUUUA, in the 3′-untranslated region is responsible for the decay of γ-GCSH mRNA. Oxidative stress enhanced cytoplasmic content of HuR. Overexpression of HuR by transfection stabilized γ-GCSH mRNA, whereas overexpression of a dominant-negative HuR mutant suppressed the induced stability. Furthermore, prooxidant-induced γ-GCSH mRNA stabilization and HuR binding were blocked by p38 mitogen-activated protein kinase inhibitors. We provide the first evidence that reduction-oxidation regulation of γ-GCSH expression, itself a reduction-oxidation sensor and regulator, is mediated at least in part by the p38 mitogen-activated protein kinase signaling through the HuR RNA-binding protein.

Reactive oxygen species play important roles in the regulation of cell growth, differentiation, apoptosis, aging, and other physiological functions. Under normal physiological conditions, there is a balance between oxidants and antioxidants that constitutes reduction-oxidation (redox) homeostasis. Elevated production of reactive oxygen species exerts oxidative stress, leading to a host of pathologic consequences. One of the ways a cell regulates redox homeostasis is through the glutathione system that exists in both the reduced form (GSH) and the oxidized form (GSSG) (1). Under oxidative stress, GSH is oxidized by GSH peroxidase to GSSG that is eliminated by the MRPs (multidrug resistance associated protein) efflux pump or is catalytically reduced back to GSH by the NAPDH-dependent GSH reductase. GSH also regulates the activities and biosynthesis of other redox-regulating enzymes, such as superoxide dismutases and DT-diphorases (NAPDH quinone oxidoreductases 1 and 2). Because of the intracellular abundance of glutathione (1–10 mM) (2, 3), the GSH/GSSG system is the main redox regulator of cells.

The biosynthesis of GSH is regulated mainly by the rate-limiting enzyme γ-glutamylcysteine synthetase (γ-GCS). The mammalian γ-GCS is a heterodimer consisting of one 73-kDa heavy (or catalytic) subunit (γ-GCSH) (4, 5) and one 28-kDa light (or regulatory) subunit (6, 7). Hereditary γ-GCS deficiency is associated with anemia, jaundice, and neurological abnormalities (8), and total γ-GCSH deficiency is embryonic lethal in knock-out mice (9).

We demonstrated previously that expression of γ-GCSH could be induced by many cytotoxic agents, including antioxidant agents (10, 11), heavy metals (11), carcinogens (12), and prooxidants (13–16). All of these treatments, to various extents, induce intracellular reactive oxygen species imbalance. Moreover, γ-GCSH mRNA is frequently overexpressed in human colon cancers (17), which are associated with redox imbalance (18), suggesting that the GSH/γ-GCS system is a molecular sensor of intracellular redox homeostasis. Although elevated expression of γ-GCSH catalyzes enhanced expression of GSH, we observed that increased GSH expression provides feedback for the down-regulation of steady-state γ-GCSH mRNA expression (16). In addition, high GSH expression has been reported previously to suppress γ-GCSH enzymatic activities (19). This feedback mechanism underscores the importance of γ-GCSH as a redox regulator.

Studies of γ-GCSH gene regulation have focused on transcriptional levels. Transcriptional up-regulation of γ-GCSH is mediated by oxidative stress-response elements (ORE) located within −3802 bp (20, 21), although other investigators have also reported the involvement of an AP-1 (activator protein-1)-binding site (22–24) at the 5′ side of the γ-GCSH gene. The oxidative stress-response element contains the consensus sequence 5′-TGAGTCA, which is a target of the leucine zipper.

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‡ The abbreviations used are: redox, reduction-oxidation; γ-GCS, γ-glutamylcysteine synthetase; γ-GCSH, γ-glutamylcysteine synthetase heavy subunit; UTR, untranslated region; MAPK, mitogen-activated protein kinase; ASK1, apoptosis signal-regulating kinase 1; MAPKAPK2, MAPK-activated protein kinase 2; MKK3, MAPK kinase 3; PDTC, pyrrolidine dithiocarbamate; tBHQ, tert-butyldihydroquinone; 2-AAF, 2-acetylaminofluorene; nt, nucleotide; hnRNP, heterogeneous nuclear ribonucleoprotein; ORE, oxidative stress-response elements; WT, wild type.
Induction of γ-GCS mRNA Stability by Oxidative Stress

transcription factor Nrf2 (22). Nrf2 is normally bound to keap1, which contains a cysteine-rich domain and is anchored to the cytoplasmic actin cytoskeleton (25, 26). Oxidative stress disrupts keap1-Nrf2 interactions by modifying the two critical cysteine residues of keap1 (26), resulting in the release of Nrf2, which subsequently translocates into the nucleus. Nrf2 then associates with the small Maf proteins (MafK or MafG) (27–29), and the Nrf2-Maf complex transactivates γ-GCS expression (30–32). This mechanism has been shown to regulate many other so-called phase II detoxifying enzymes (NADPH quinine oxidoreductase 1, γ-GCS light chain, glutathione S-transferase α1, and heme oxygenase-1) (33, 34).

We reported previously that steady-state expression of γ-GCS mRNA increases by severalfold in human glioma cells treated with the alkylating antitumor agent 1-(4-amino-2-methyl-5-pyrimidinyl) methyl-3-(2-chloroethyl)-3-nitrosourea (10). However, nuclear run-on assays revealed less than a 0.5-fold increase in the transcriptional rate. These results suggest that post-transcriptional regulation is also involved in the increased steady-state expression, perhaps depending upon prooxidants and cell sources. The mechanism involved in this post-transcriptional regulation has yet to be elucidated. The objective of the current study was to gain insight into the post-transcriptional mechanism of redox-induced γ-GCS up-regulation in a cultured cell system. We found that oxidative stress-induced γ-GCS expression is mediated by the mRNA-stabilizing protein HuR, which interacts with an AU-rich sequence in the 3′-untranslated region (UTR) of γ-GCS mRNA through the p38 mitogen-activated protein kinase (MAPK) signal transduction pathway.

MATERIALS AND METHODS

Plasmid DNA and Construction of Deletion Mutants—The ~3802/GCSh5′-luc recombinant DNA (20), which contains nucleotides (nt) ~3802 to ~465 of the γ-GCS sequence in the luciferase reporter vector pGL3 basic (Promega, Madison, WI), was obtained from R. T. Mulcahy (University of Wisconsin Medical School, Madison, WI). A series of progressively deleted flanking sequences was created by PCR by using ~3802/GCSh5′-luc DNA as a template and appropriate primer sets. The PCR products were ligated into the pGL3 basic vector, generating ~814/GCSh5′-luc, ~202/GCSh5′-luc, ~149/GCSh5′-luc, and ~22/GCSh5′-luc recombinants.

Wild-type and antisense HuR (35) were obtained from M. Gorospe (National Institute on Aging, Bethesda, MD). Plasmids encoding the constitutively active and dominant-negative forms of apoptosis signal-regulator (ASPx3, and Ala-207/H9004 and HA-ASK1-KM (36), respectively) and HA-ASK1 (36) were transformed into Elevin-Blue supercompetent cells (Stratagene). The plasmid DNAs were sequenced by the manufacturer's instructions. Briefly, pTRE-γ-GCS(2983) DNA was used as the template in PCRs with primers containing ~2780/AGGGA and ~2595/AGGGA to replace ~2780/AUUA (site II) and ~2595/AUUA (site I), respectively. The methylated, wild-type DNA (template) was digested by DpnI, and the nonmethylated, mutated DNA (PCR product) was transformed into ElevLin-Blue supercompetent cells (Stratagene). All the plasmid DNAs were sequenced by the manufacturer's instructions.

Cell Culture and Treatments with Prooxidants—HT-29 and HCT-15 human colorectal cancer cells were purchased from the American Type Culture Collection (Manassas, VA). The cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37 °C in a 5% CO2 atmosphere. Cells at exponential growth conditions were treated with prooxidants at the following concentrations: 100 μM 2-acetylaminofluorene (2-AAF; Sigma), 50 μM menadione (Sigma), 100 μM pyridoline dithiocarbamate (PDTc; Sigma), 100 μM tert-butylhydroquinone (tBHQ; Sigma), and 800 μM sulindac (Sigma). In experiments where inhibitors were required, the cells were treated with 25 μM N-acetylcycteine (NAC) 2 h before the addition of sulindac, whereas 20 μM SB203580 (Calbiochem) and 20 μM PD98059 (Calbiochem) were added simultaneously with sulindac. Total RNA was extracted from the cells, and levels of γ-GCS mRNA were determined by the RNase protection assay.

Transient Transfection—Transient transfection was performed by using lipofectamine (Invitrogen) according to the manufacturer's instructions. Briefly, HEK293T cells (1 × 106 cells) were seeded into 60-mm Petri dishes; 24 h later, recombinant pTRE-γ-GCS plasmid DNA and EC1214A plasmid, which expresses tTA regulation factor, were introduced into cells by using 8 μg of Lipofectamine (the amount of DNA was kept constant at 4.5 μg within experiments by adding empty vector as required). After 5 h of incubation, the medium was removed, and Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum was added. To shut off mRNA synthesis, tetracycline (2 μg/ml, Sigma) was added 24 h after transfection, and cells were harvested 0, 2, 4, 6, 9, and 12 h later in guanidine thiocyanate lysis buffer (STAT-60; Tel-Test, Friendswood, TX). RNA was extracted through phenol/chloroform extraction and isopropanol alcohol precipitation.

Luciferase Assay—HEK293T cells (2 × 105 cells) were seeded in 24-well plates, and 24 h later, 0.5-fold increase in the transcriptional rate. These results suggested that post-transcriptional regulation is also involved in the increased oxidative stress-induced γ-GCS expression is mediated by the mRNA-stabilizing protein HuR, which interacts with an AU-rich sequence in the 3′-untranslated region (UTR) of γ-GCS mRNA through the p38 mitogen-activated protein kinase (MAPK) signal transduction pathway.

To determine site-directed mutations in the AU-rich sites, we used the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Briefly, pTRE-γ-GCS(2983) DNA was used as the template in PCRs with primers containing ~2780/AGGGA and ~2595/AGGGA to replace ~2780/AUUA (site II) and ~2595/AUUA (site I), respectively. The methylated, wild-type DNA (template) was digested by DpnI, and the nonmethylated, mutated DNA (PCR product) was transformed into ElevLin-Blue supercompetent cells (Stratagene). All the plasmid DNAs were sequenced by the manufacturer's instructions.
and γ-GCSH mRNA bands were adjusted independently within the linear range of the signals.

Western Blot Analysis—HEK293T cells (2 × 10⁶ cells) were seeded onto a 100-mm Petri dish. Twelve h later, cells were treated with sulindac (800 μM), PDTC (100 μM), or TBHQ (100 μM) alone or with SB203580 (20 μM) or PD98059 (20 μM) for 12 h. Cells were washed three times with phosphate-buffered saline and harvested. Cell extracts were resuspended in 200 μl of phosphate-buffered saline containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors (Complete; Roche Applied Science) and centrifuged at 12,000 rpm for 10 min at 4 °C. Protein concentrations were determined using a protein assay kit (Bio-Rad). Aliquots (40 μl) of protein were resolved by SDS-PAGE and transferred to poly(vinylidene difluoride) membranes (Millipore, Bedford, MA) by using standard procedures. The membranes were then subjected to Western blotting, and the blots were developed with the enhanced chemiluminescence system (Amersham Biosciences).

To investigate the effect of prooxidant on intracellular distribution of HuR between the cytoplasm and nucleus, we treated the cells with 800 μM sulindac and 100 μM TBHQ in the presence or absence of the inhibitors SB203580 or PD98059 for 12 h. Cytoplasmic and nuclear fractions were prepared from the treated cells using the NE-PER extraction kit (Pierce) and were processed by a standard Western blot using mouse monoclonal antibodies against β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a loading control. Phosphorylated and nonphosphorylated phospho-p38 MAPK (Thr-180/Tyr-182, 1:1,000 dilution), phospho-MKK3/6 (Ser-189/207, 1:1,000) and the enhanced chemiluminescence system (Amersham Biosciences). The reaction mixture was centrifuged for 2 min at 14,000 rpm at 4 °C. Levels of γ-GCSH mRNA were determined by the RNase protection assay using 18 S RNA as loading control. B, promoter sequence contributes minimally to the transcriptional up-regulation of γ-GCSH expression. Left, HT-29 cells were cotransfected with a series of γ-GCSH-luciferase reporter recombinant plasmids containing various lengths of γ-GCSH promoter sequences and control pRL-SV40 vector for 24 h. Right, luciferase activity was normalized to control Renilla luciferase activity and was presented as a fold induction. Open bars represent control group, and hatched bars represent treatment (100 μM) group. Each value represents mean ± S.D. of triplicate experiments.

To deplete HuR from the cell extracts, 10 μg of extract was incubated with 200 ng of anti-HuR antibody followed by an addition of 5 μl of protein A/G plus-agarose (Santa Cruz Biotechnology) for 2 h at 4 °C. The reaction mixture was centrifuged for 2 min at 14,000 rpm at 4 °C. The supernatants were subjected to Western blotting using anti-HuR antibody as described above.

To investigate whether the induced up-regulation of γ-GCSH by prooxidants is related to the transcriptional control, we analyzed the promoter activity of γ-GCSH using a −3802/−GCSh5′-luc reporter construct in response to the prooxidant TBHQ. −3802/−GCSh5′-luc plasmid contains ORE (20, 21) and AP-1 (23–24). Fig. 1B shows that treatment of 100 μM TBHQ failed to increase an appreciable amount of luciferase activities. Further analyses using recombinants with progressive deletions showed that treatment with TBHQ enhanced luciferase activity by no more than 0.5-fold. Similar results were obtained with sulindac (data not shown). Given the severalfold induction of steady-state γ-GCSH mRNA by TBHQ in cultured cells (Fig.

**RESULTS**

Post-transcriptional Regulation of γ-GCSH mRNA Expression Is Induced by Prooxidants—Fig. 1A shows the time course induction of γ-GCSH expression by various prooxidants, including sulindac, 2-AAF, TBHQ, and PDTC. Increased steady-state levels of γ-GCSH mRNA were observed 4–6 h and continued throughout the 10–24-h treatment time. Levels of induction ranged from 3- to 8.5-fold, depending upon the prooxidants. These results are consistent with our previous reports (14–16) that induction of γ-GCSH expression by prooxidants was not an early event.

**DISCUSSION**

To investigate whether the induced up-regulation of γ-GCSH by prooxidants is related to the transcriptional control, we analyzed the promoter activity of γ-GCSH using a −3802/−GCSh5′-luc reporter construct in response to the prooxidant TBHQ, −3802/−GCSh5′-luc plasmid contains ORE (20, 21) and AP-1 (23–24). Fig. 1B shows that treatment of 100 μM TBHQ failed to increase an appreciable amount of luciferase activities. Further analyses using recombinants with progressive deletions showed that treatment with TBHQ enhanced luciferase activity by no more than 0.5-fold. Similar results were obtained with sulindac (data not shown). Given the severalfold induction of steady-state γ-GCSH mRNA by TBHQ in cultured cells (Fig.

**Fig. 1.** Induction of γ-GCSH mRNA expression and analysis of γ-GCSH promoter sequence. A, up-regulation of γ-GCSH mRNA by cytotoxic agents is a late event. HT-29 cells were treated with sulindac (100 μM), 2-AAF (100 μM), TBHQ (100 μM), and PDTC (200 μM), for different time intervals as indicated. Levels of γ-GCSH mRNA were determined by the RNase protection assay using 18 S RNA as loading control. B, promoter sequence contributes minimally to the transcriptional up-regulation of γ-GCSH expression. Left, HT-29 cells were cotransfected with a series of γ-GCSH-luciferase reporter recombinant plasmids containing various lengths of γ-GCSH promoter sequences and control pRL-SV40 vector for 24 h. Right, luciferase activity was normalized to control Renilla luciferase activity and was presented as a fold induction. Open bars represent control group, and hatched bars represent treatment (100 μM) group. Each value represents mean ± S.D. of triplicate experiments.
1A), we conclude that transcriptional regulation by the proximal region of γ-GCS shows minimal transactivation. To investigate whether a post-transcriptional regulation mechanism is involved in γ-GCS expression, we used actinomycin D at the concentration (5 μg/ml) that inhibits RNA polymerase II activities (mRNA synthesis), but not RNA polymerase I activities (ribosomal RNA synthesis), to shut down polymerase II activities (mRNA synthesis), but not RNA polymerase I activities (ribosomal RNA synthesis), and then measured the stability of γ-GCS mRNA in the absence or presence of the prooxidant sulindac. The half-life of γ-GCS mRNA in HT-29 and HCT-15 colorectal cancer cells treated by actinomycin D alone was 6.3 ± 1.6 and 6.8 ± 1.0 h, respectively (Fig. 2, top). In the presence of sulindac, however, the respective values increased almost 2-fold to 12.1 ± 1.4 and 13.1 ± 1.3 h, respectively. These results suggested that post-transcriptional regulation is involved in the up-regulation of γ-GCS expression induced by sulindac.

To investigate the effects of protein synthesis inhibitors on γ-GCS mRNA expression, we treated HT-29 and HCT-15 cells with 10 μM cycloheximide with or without sulindac. In both cell lines, cycloheximide alone reduced γ-GCS mRNA levels over time, with half-life values of 6.5 ± 1.2 and 8.4 ± 0.7 h, respectively (Fig. 2, bottom). Simultaneous treatment with cycloheximide and sulindac initially decreased γ-GCS mRNA expression, but at 6 h and thereafter, the levels increased such that the half-life was >20 h. These results suggested a delayed mechanism of enhanced γ-GCS steady-state mRNA expression by sulindac.

To rule out the possibility of nonspecific inhibitor effects and to investigate post-transcriptional involvement in the regulation of γ-GCS mRNA expression, we constructed a tetracycline-regulated γ-GCS recombinant plasmid (pTRE-γ-GCS(2983)) that produces a transcript of 2543 nt (83 nt in a 5′-UTR, 1914 nt in a coding region, and 588 nt in a 3′-UTR) (Fig. 3A). In the presence of tetracycline, binding of tTA (encoded by the cotransfected EC1214A plasmid) to the promoter is blocked, and transcription of exogenously transcribed γ-GCS is inhibited (38, 39). We transfected pTRE-γ-GCS(2983) and EC1214 plasmid DNA into HEK293T cells. After 24 h, tetracycline alone or with sulindac was added into the culture medium. Cellular RNA was prepared at different time intervals thereafter. γ-GCS transcripts from the transfected DNA were determined by RNase protection assay using a probe that detected transcript from the transfected plasmid DNA but not from the endogenous counterpart. The half-life of the transcript was 7.8 ± 0.7 and 8.4 ± 1.0 h, respectively (Fig. 3, B and C). However, when sulindac was added 12 h before the addition of tetracycline, the half-life increased to >20 h (Fig. 3, B and C). We excluded the possibility that the concentration of tetracycline used here affected endogenous γ-GCS mRNA stability (data not shown). These results confirmed that sulindac enhances γ-GCS mRNA stability through a delayed mechanism.

3′-UTR Sequence of γ-GCS mRNA Is Important for Prooxidant-induced Delayed Stabilization—Sequences that control mRNA degradation can be located at the 5′-UTR, coding region, and 3′-UTR. We investigated whether the 3′-UTR of γ-GCS mRNA contains an unstable sequence that is a target of prooxidants. To this end, we constructed a 3′-UTR deletion mutant,
pTRE-\(\gamma\text{-GCS}\text{h}(2431)\), that lacked nt 2431–2983 (Fig. 4). This recombinant plasmid was transfected into HEK293T cells, and the stability of the transcript was similarly measured. The \(t_{1/2}\) was >20 h with or without sulindac (Fig. 4C). These results strongly suggested that the 3’-UTR from nt 2431 to 2983 contains unstable sequence(s).

To determine more precisely the unstable sequence, we constructed three additional recombinants, pTRE-\(\gamma\text{-GCS}\text{h}(2781)\), pTRE-\(\gamma\text{-GCS}\text{h}(2677)\), and pTRE-\(\gamma\text{-GCS}\text{h}(2517)\), whose 3’-UTR sequences ended at nt 2781, 2677, and 2517, respectively (Fig. 4A). These recombinant plasmids were transfected into HEK293T cells, and the \(t_{1/2}\) of the corresponding mRNA was determined. Deletion of nt 2781–2983 from the 3’-UTR stabilized \(\gamma\text{-GCS}\text{h}\) mRNA expression in the transfection assay, as the \(t_{1/2}\) increased from 7.8 \pm 0.8 to >20 h (Fig. 4B). These results suggest that nt 2781–2983 of \(\gamma\text{-GCS}\text{h}\) mRNA contains unstable sequence(s). Furthermore, when sulindac was included in the transfection assay, the \(t_{1/2}\) of the transcript increased to >20 h (Fig. 4B, top). This result indicated that the sequence responsible for sulindac-induced mRNA stabilization is located in the same region.

\textit{HuR Is Responsible for Sulindac-induced mRNA Stabilization}—Several trans-acting factors have been identified that control mRNA stabilization and destabilization. The most notable of these is the HuR factor, which recognizes AU-rich sequences (30, 40–42). By examining the sequence from nt 2781 to 2983 in the 3’-UTR of \(\gamma\text{-GCS}\text{h}\), we found a putative HuR-binding AU-rich sequence, \(\ast\text{2785AUUUA} \) (site II). To investigate whether this sequence is involved in \(\gamma\text{-GCS}\text{h}\) mRNA destabilization and is also the target for the sulindac-induced stabilization, we used site-directed mutagenesis in pTRE-\(\gamma\text{-GCS}\text{h}(2983)\) to replace this sequence with \(\ast\text{2785AGGGA}\). For comparison, we also replaced a sequence outside that region, site I (\(\ast\text{2595AUUUA} \) with \(\ast\text{2595AGGGGA} \), or replaced both wild-type sites with mutant sequences (Fig. 5A). We then transfected the respective plasmids under tetracycline-regulated conditions and analyzed the stabilities of the transcripts. Mutation of site I did not enhance stabilization of the \(\gamma\text{-GCS}\text{h}\) transcript, whereas mutations of site II or both sites I and II did (Fig. 5, B and C, \textit{untreated}), suggesting that the site II sequence is the mRNA-degrading element. More importantly, site II was also the target of sulindac-induced \(\gamma\text{-GCS}\text{h}\) mRNA stability, because only WT site II was responsive to sulindac induction (Fig. 5, B and C, \textit{sulindac}). Site II contains the HuR-responsive sequence because in the cotransfection experiment with HuR expression vector, only the wild-type and site I sequence conferred \(\gamma\text{-GCS}\text{h}\) mRNA stability (Fig. 5, B and C, \textit{HuR}). Taken together, these results strongly suggested that...
sulindac-induced γ-GCSH mRNA stability is mediated by the HuR-interacting 2785 AUUUA (site II) sequence.

Other Prooxidants Induce γ-GCSH mRNA Stability—To investigate whether prooxidants other than sulindac can induce stabilization of γ-GCSH mRNA, we used the tetracycline-regulated system to determine the t1⁄2 of pTRE-γ-GCSH transcripts in transfected HEK293T cells treated with 2-AAF, menadione, or t-BHQ, following the procedure as described in Fig. 3. All four prooxidants, like sulindac, induced stabilization of γ-GCSH transcripts in delayed mechanism (Fig. 6). Because these agents can induce redox imbalance, our results are consistent with the idea of redox regulation of γ-GCSH mRNA stability.

p38 MAPK Pathway Is Involved in Induction of γ-GCSH mRNA Stability—To investigate the signal transduction mechanism by which redox conditions influence γ-GCSH mRNA stability, we used an activator and several inhibitors of various signal transduction pathways (Fig. 7A). We envisioned that inhibitors of the involved pathways would suppress the induction of γ-GCSH mRNA stability. To this end, HT-29 colorectal cancer cells were treated with a prooxidant (sulindac, PDTC, or tBHQ) and/or the activator or inhibitors. Expression levels of γ-GCSH mRNA were determined by the RNAse protection assay and quantified by densitometry, using the signal from 18 S RNA as a control for sample loading. The expression of endogenous γ-GCSH mRNA was increased by treatment with prooxidants alone (Fig. 7B). This increase was not suppressed by the addition of genistein, H7, or staurosporine, which inhibit protein kinases A and C, and protein kinase A, C, and G, and calmodulin kinase pathways, respectively. The induction of γ-GCSH expression was moderately suppressed by sodium azide, an activator of AMP-activated protein kinase, and by PD98059, an inhibitor of MAPK/ERK kinase signals. SB203580, a specific inhibitor of the p38 MAPK pathway, was the most potent inhibitor overall. None of the inhibitors by themselves, at the concentrations used, enhanced the expression of γ-GCSH. These results suggest that p38 MAPK is an important signaling pathway involved in the induction of γ-GCSH expression by prooxidants, although the results also suggested other signaling mechanisms may be involved to a lesser degree.

To investigate whether the p38 MAPK pathway regulates γ-GCSH mRNA stability, we again used the tetracycline-regulated system in the transfection assay. Fig. 8 shows that sulindac-induced mRNA stability was inhibited by cotreatment with SB203580 but not with PD98059. N-Acetylcysteine, an inhibitor of oxidative stress, was also a potent inhibitor of sulindac-induced γ-GCSH mRNA stability. These results, together with those described above, strongly suggest that prooxidant-induced γ-GCSH mRNA stability is regulated by p38 MAPK sig-
Fig. 6. Enhanced stability of γ-GCS\(_\text{h}\) mRNA by various prooxidants. pTRE-γ-GCS\(_\text{h}\) (2.25 μg) was cotransfected with EC1214A (2.25 μg) into HEK293T cells. Twelve h later, cells were treated with 100 μM 2-AAF, 50 μM menadione, 500 μM sulindac, 100 μM tBHQ for an additional 12 h and followed by 1 μg/ml tetracycline. Total RNA was prepared, and the stability of γ-GCS\(_\text{h}\) mRNA expression was measured by RNase protection assay. Values shown represent mean ± S.D. A, graphical representation of the data in B. Each experiment was performed at least twice. γ-GCS\(_\text{h}/18\) S ratios were plotted as percentage of the maximum value at the time of tetracycline addition.

A. 

| Reagent     | Function and target | Dose  |
|-------------|---------------------|-------|
| Genistein   | Inhibitor of protein tyrosine kinase | 20 μM |
| H7          | Inhibitor of protein kinase A and C | 50 nM |
| Staurosporine | Inhibitor of protein kinase A, C, and G and calmodulin kinase | 40 nM |
| Sodium azide | Activator of AMP-activated protein kinase | 2 mM |
| SB203580    | Inhibitor of p38 MAPK | 20 μM |
| PD98059     | Inhibitor of MAPK/ERK kinase | 20 μM |

B. 

| Treatment | 
|-----------|
| Sulindac  | 
| PDTC      | 
| tBHQ      |
| γ-GCS\(_\text{h}\)  |

Fig. 7. Signal transduction pathways involved in prooxidant-induced stabilization of γ-GCS\(_\text{h}\) expression. A, function, target, and dose of activator and inhibitors used. B, HT-29 cells were treated with 800 μM sulindac, 100 μM PDTC, or 100 μM tBHQ for 12 h with or without indicated inhibitors. Total RNA was prepared, and the stability of γ-GCS\(_\text{h}\) mRNA expression was measured by RNase protection assay. †, prooxidant treatment; −, no prooxidant treatment.

Prooxidants Increase Cytoplasmic HuR and Binding to γ-GCS\(_\text{h}\) mRNA—To strengthen the involvement of HuR in the prooxidant-induced, p38 MAPK-mediated stabilization of γ-GCS\(_\text{h}\) mRNA, we treated HEK293T cells with sulindac or tBHQ in the presence of SB203580 or PD98059. Cytoplasmic and nuclear fractions were prepared from the treated cells and subjected to Western blotting using nuclear (lamin B) and cytoplasmic (α-tubulin) markers. The amounts of cytoplasmic and nuclear HuR were determined by Western blotting. No detectable cytoplasmic contamination of the nuclear fraction was evident (Fig. 9). Treatments with sulindac or tBHQ increased cytoplasmic content of HuR in the absence of any inhibitors. This increase was suppressed by SB203580 but not by PD98059. These results demonstrated that the prooxidant-induced increase in cytoplasmic HuR is specifically mediated by p38 MAPK signaling.

To demonstrate that the increase in HuR was accompanied by an increase in its binding to the γ-GCS\(_\text{h}\) 3’-UTR sequence, we performed gel mobility shift assays by incubating various cytoplasmic extracts with \(^32\)P-labeled probes containing site I, site II, and their mutated sequences. Fig. 10A shows that sulindac-treated extract could only support mobility shift of site II WT probe but not with probes containing site I and their mutated sequences. This mobility shift was not seen in the extracts from SB203580-treated cells. Extracts from PD98059-treated cells did not diminish the mobility shift. These results demonstrated that the induced binding activity to site II sequence involved p38 MAPK signaling. Binding of site II probe could be competed efficiently by its cognate \(^{2785}\)AUUUA sequence but not by the mutant \(^{2785}\)AGGGA sequence within the same concentration range, demonstrating the sequence specificity of protein binding (Fig. 10B). Moreover, depleting the cell extract by using an anti-HuR antibody diminished the binding (Fig. 10C), demonstrating the involvement of HuR in the binding to site II sequence.

The in vivo interaction between γ-GCS\(_\text{h}\) mRNA and HuR protein was further confirmed by using UV cross-linking. Treatment of sulindac increased the amount of γ-GCS\(_\text{h}\) mRNA bound to HuR (Fig. 11, compare lanes 3 and lane 1), and cotreatment with SB203580 (lane 7), but not PD98059 (lane 5), reduced the amount of bound γ-GCS\(_\text{h}\) mRNA to the control level (lane 1). Only marginal amounts of γ-GCS\(_\text{h}\) mRNA were detected when UV was not used (Fig. 11, lanes 2, 4, 6, and 8). Taken together, these results demonstrated that the prooxidant-induced increase in the cytoplasmic distribution of HuR is mediated by p38 MAPK signaling and that HuR interacts specifically with the site II sequence of γ-GCS\(_\text{h}\) mRNA.

p38 Signal Transduction Pathway in Prooxidant-induced γ-GCS\(_\text{h}\) mRNA Stability—The p38 MAPK signaling pathway is composed of three sequentially activated kinase families as follows: MAPK, MAPK kinase (MKK), and MKK kinase families. MAPK phosphorylates substrates upon activation through phosphorylation by MKKs, which are themselves activated by MKK kinases (43). To demonstrate further the involvement of the p38 pathway in prooxidant-induced γ-GCS\(_\text{h}\) mRNA stability, we investigated whether sulindac, PDTC, and tBHQ can activate p38 and MKK activities. Treating HT-29 cells with these prooxidants indeed activated p38, MKK3, and MAPK activating protein kinase 2 (MAPKAPK2), as evidenced by Western blotting (Fig. 12). Activation of p38 MAPK was suppressed by SB203580 but not by PD98059, consistent with the results shown above that SB203580 but not PD98059 suppressed sulindac-induced γ-GCS\(_\text{h}\) mRNA stability. Activation of MAPKAPK2 by these prooxidants was suppressed by SB203580 but not by PD98059, consistent with the results that
Induction of γ-GCS mRNA Stability by Oxidative Stress

FIG. 8. Inhibition of sulindac-induced γ-GCS mRNA stabilization. pTRE-γ-GCS(2983) (2.25 μg) was co-transfected with EC1214A (2.25 μg) into HEK293T cells, which were then treated with 1 μg/ml tetracycline alone (control) or with 800 μM sulindac (alone or with 25 mM N-acetylcysteine, 20 μM SB203580, or 20 μM PD98059) 12 h before 1 μg/ml tetracycline. Total RNA was prepared, and the stability of γ-GCS mRNA expression was measured by RNase protection assay. Values shown represent mean ± S.D. of three independent experiments. A, graphical representation of the data shown in B.

MAPKAPK2 is a downstream effector of p38 signaling (43). SB203580 did not suppress MKK3/6 activation because this kinase is upstream of p38 MAPK.

The results presented thus far are consistent with the following signal transduction mechanisms for the induction of γ-GCS mRNA stability: prooxidants → MKK kinase (ASK1) → MKK (MKK3/6) → p38 MAPK → MAPKAPK2 → HuR → γ-GCS mRNA stabilization. To provide support for this proposition, we cotransfected constitutively active and dominant-negative recombinants encoding various kinases with the tetracycline-regulated γ-GCS mRNA stability assay system. As shown in Fig. 13, cotransfection with plasmid DNA encoding constitutively active ASK1, MKK3, p38 MAPK, and MAPKAPK2 stabilized γ-GCS mRNA (Fig. 13A), whereas cotransfection with plasmid DNA encoding dominant-negative forms of these kinases suppressed the induced stabilization of γ-GCS mRNA (Fig. 13B). Similarly, cotransfection of HuR expression plasmid induced γ-GCS mRNA stability, whereas cotransfection of antisense HuR mutant suppressed the induction (Fig. 13B, bottom). Thus, we conclude that induction of γ-GCS mRNA stability by prooxidants is controlled by the p38 MAPK pathway through HuR, which interacts with an AU-rich sequence located at the 3′-UTR of the mRNA.

DISCUSSION

Post-transcriptional Regulation of γ-GCS mRNA Expression by Prooxidants—Oxidative stress is known to regulate the expression of a number of genes (reviewed in Ref. 44). Recent studies demonstrated that changes in redox conditions can transcriptionally activate many cellular genes without effects on mRNA stability, including those encoding angiotensin II receptor (45), mitochondrial transporter (UCP2) (46), insulin-like growth factor binding protein-1 (47), and Rac1 GTPase (48). Moreover, redox-regulated gene expression can be controlled at the post-transcriptional levels by modulating mRNA stability. Oxidative stress induced by glucose deprivation in cultured cells increases vascular endothelial growth factor mRNA stability (49) but reduces insulin growth factor-1 mRNA stability (50). Increased stability of extracellular superoxide dismutase mRNA, but not manganese superoxide dismutase mRNA, is associated with changes of cellular redox conditions by the treatment of 17β-estradiol (51). These observations, collectively, suggest multiple effects on mRNA stability by oxidative stress, perhaps depending upon the context of genes, cell types, and redox-modulating agents. However, mechanisms that regulate mRNA stability within these contexts are not well understood.

γ-GCS mRNA is an important system for studying redox-regulated mRNA stability. It encodes the rate-limiting enzyme for the de novo biosynthesis of GSH. The increased GSH levels preserve cytoprotective functions under oxidative stress (52). Thus, γ-GCS, like glutathione S-transferase α2 and NADPH quinone oxidoreductase 1, can be considered as a phase II enzyme in the detoxification system of drug metabolism (33, 34). Previous studies on the regulation of genes encoding phase II enzymes have been mostly focused on transcriptional mechanisms (53, 54). Post-transcriptional regulation of the phase II genes has not been well studied.

The present demonstration that increased γ-GCS mRNA stability under oxidative stress conditions underscores the importance of the post-transcriptional mechanism in the regulation of this phase II gene expression. Actinomycin D inhibitor experiments demonstrated that sulindac treatment resulted in an ~2-fold increase in the $t_{1/2}$ values of γ-GCS mRNA stability. Results from transient transfection assays using the tetracycline-regulated system are consistent with these results. More importantly, we have demonstrated that this post-transcriptional regulation was activated by many oxidative stress-inducing agents, including sulindac, PDTC, tBHQ, 2-AAF, and menadione; and some of these agents are not known for transcriptional activation of the phase II enzymes-encoded genes. Thus, the post-transcriptional regulation mechanism described here is not spurious and represents an important mechanism of γ-GCS gene regulation under stress conditions.

One of the important findings presented in this work is the identification of HuR as a target of prooxidant-induced γ-GCS mRNA stability. We first identified an AU-rich sequence...
The instability of γ-GCSH mRNA is responsible for the instability of γ-GCSH mRNA. Many AU-rich binding proteins have been identified, but HuR functions as an mRNA-stabilizing factor (35, 40–42). Indeed, overexpression of HuR by transfection stabilizes γ-GCSH mRNA using anti-HuR antibody, and the other was used for Western blotting of HuR as reference of sample loading. γ-GCSH mRNA levels were measured by RNase protection assay.

(2785AUUUA) located in the 3’-UTR of γ-GCSH mRNA that is responsible for the instability of γ-GCSH mRNA. Many AU-rich binding proteins have been identified, but HuR functions as an mRNA-stabilizing factor (35, 40–42). Indeed, overexpression of HuR by transfection stabilizes γ-GCSH mRNA degradation. HuR is a ubiquitously expressed, predominantly nucleus-located member of the elav (embryonic-lethal abnormal visual in Drosophila melanogaster) family of RNA-binding proteins (56). It has been shown that HuR translocates to the cytoplasm and stabilizes ARE-containing mRNAs (35, 57). Consistent with this mechanism, we demonstrated that upon treatment of prooxidants, the cytoplasmic contents of HuR are increased. This is associated with the enhanced binding of HuR to γ-GCSH mRNA as demonstrated by the in vivo cross-linking procedure. Another AU-rich RNA binding factor hnRNP D has also been reported to up-regulate mRNA stability in a cell type-specific manner (55). Whether hnRNP D plays a role in prooxidant-induced γ-GCSH mRNA stabilization remains to be investigated.

Redox Regulation of p38 MAPK Kinase Signaling in the Post-transcriptional Regulation of γ-GCSH mRNA Stability—Another important finding from the present study is the identification that the p38 MAPK pathway is involved in the oxidative stress-induced γ-GCSH mRNA stability. This was first demonstrated by using a panel of inhibitors to various signal transduction pathways, including SB203580 at concentration (20 μM) that has been demonstrated to be highly selective to the p38 MAPK2 enhanced γ-GCSH mRNA stabilization, whereas dominant-negative recombinant suppressed prooxidant-induced stabilization. Previous studies have shown that overexpression of MAPKAPK2 increases cytoplasmic HuR accumulation, which is associated with dramatic changes in the formation of HuR and ARE complexes (42). How MAPKAPK2 regulates cytoplasmic HuR accumulation remains to be elucidated, al-
was delayed in the time course analysis of scripts can only be observed when the prooxidants are added side effects of actinomycin D. This discrepancy might be attributable to unknown expression in cells treated with cycloheximide and sulindac.

It has been demonstrated that under normal physiologic conditions, the reduced form of thioredoxin is complexed with ASK1 (also known as MKK kinase 5) that inhibits ASK1 activity (63). Upon oxidative stress, thioredoxin is oxidized and dissociated from ASK1, which subsequently activates downstream signaling, by sequential phosphorylation of MKK3/6 and p38 MAPK (64). This mechanism also explains the association between cellular oxidative stress and the MAPK pathway activation as shown in Fig. 14.

This signal transduction pathway emphasizes the sequential events for redox-induced mRNA stabilization. The involvement of many steps of activation in the process may explain the delayed mechanism of induction as described here. The increased steady-state γ-GCSH mRNA expression induced by various prooxidants is a late event; stabilization of γ-GCSH transcripts can only be observed when the prooxidants are added before the switch to the tetracycline-off system; and induction was delayed in the time course analysis of γ-GCSH mRNA expression in cells treated with cycloheximide and sulindac. However, this delayed effect was not seen when actinomycin D was used. This discrepancy might be attributable to unknown side effects of actinomycin D.

We demonstrated previously that expression of γ-GCSH is itself regulated by redox conditions (16). The pathway described in Fig. 14 can also explain the redox-induced feedback inhibition of γ-GCSH biosynthesis. Stabilization of γ-GCSH mRNA may increase the biosynthesis of GSH, resulting in a reduced intracellular redox status. This in turn may suppress the oxidation of thioredoxin and its downstream p38 MAPK kinase pathway, thereby suppressing the induction of γ-GCSH mRNA stability.

**Post-transcriptional Regulation of γ-GCSH Expression in Cancer**—We observed previously that γ-GCSH mRNA expression is correlated with colorectal tumor progression, being low in adenomas and high in carcinomas (15, 17). The post-transcriptional regulation mechanism is likely to involve the increased γ-GCSH mRNA levels in colon cancer from the following considerations. First, it has been suggested that increased oxidative stress is associated with colorectal carcinogenesis (18). Second, by using immunohistochemical staining, it has been shown that the cytoplasmic abundance of HuR increases with malignancy, particularly in colorectal carcinomas (65). Moreover, the development of colon cancer in experimental animals correlates with HuR content. Colon cancer cells overexpressing HuR produced significantly larger tumors than those arising from control cells, whereas those with reduced HuR levels through small interference RNA- or antisense HuR-based approaches developed significantly more slowly (65). Third, constitutively elevated p38 MAPK expression is frequently associated with cancer progression (57). These findings, together with those described in this study, suggest that the pathway elicited in Fig. 14 may be the underlying mechanism for the enhanced expression of γ-GCSH mRNA in colon carcinomas. We have observed previously that expression of MRP1, an ATP-dependent efflux pump for eliminating diverse anticancer drugs, is tightly associated with that of γ-GCSH (13–16), including colon cancers (15, 17). It is plausible that the post-transcriptional regulation mechanism described here may be involved in the regulation of this important drug resistance transporter.

Taken together, our results have important implications in the redox regulation of colorectal carcinogenesis and the evolution of drug resistance during disease development. Further investigations are required to establish the clinical relevance of these observations. These studies may eventually lead to the development of intervention strategies for the management of colon cancer.
Induction of γ-GCS mRNA Stability by Oxidative Stress

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Delayed Mechanism for Induction of γ-Glutamylcysteine Synthetase Heavy Subunit
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Signaling
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