Glutamate cysteine ligase (GCL) catalyzes the rate-limiting reaction in the de novo synthesis of glutathione (GSH), an important antioxidant that contributes to the maintenance of cellular redox status. GCL is a holoenzyme composed of two subunits, the heavier subunit (73 kDa) provides the catalytic function of the enzyme (GCL-C, catalytic) and the lighter subunit (28 kDa) is responsible for modulating the kinetic properties of the catalytic subunit (GCL-M, modulatory).

Glutamate cysteine ligase modulatory (GCLM), also known as γGCS, γ-glutamylcysteine synthetase, light subunit, is transcriptionally regulated through an Electrophile Response Element (EpRE) in response to various oxidative stresses (Cai et al., 1997; Moinova and Mulcahy, 1998; Wild et al., 1999). EpREs or EpRE-like sequences are also present in the promoters of numerous phase-II genes such as NQO1 (NADPH-Quinone Oxido Reductase 1) and glutathione S-transferases (Favreau and Pickett, 1991; Rushmore et al., 1990, 1991; Rushmore and Pickett, 1990; Wasserman and Fahl, 1997) as well as other antioxidant genes including both GCL genes and heme-oxygenase 1 (Alam et al., 1999; Kim et al., 2001). The presence of EpREs in the promoters of multiple detoxification genes results in a coordinated induction of these protective enzymes in response to pro-oxidants or electrophiles such as pyrrolidine dithiocarbamate (PDTC) (Prestera et al., 1993).

Treatment of HepG2 cells with PDTC results in time- and dose-dependent increases in GCLM expression in a mechanism mediated through the EpRE (Mulcahy et al., unpublished data; Wild and Mulcahy, 1999). Therefore, investigations into the mechanism of PDTC induction of GCLM will yield information that can be applied toward a better understanding of other EpRE-regulated genes and enzymes.

The core consensus sequence of the EpRE was originally established by Rushmore et al. (1991) as an 11-bp sequence: 5′-G/ATGAG/CnnnGCG/A-3′. This core sequence was later expanded by Wasserman and Fahl to include additional flanking sequences, resulting in the 20-bp element: 5′-G/ATGAG/CnnnGCG/A-3′ (Wasserman and Fahl, 1997). The EpRE sequence bears a high degree of similarity to the Maf response element (MARE-5′-TGCTGAGTCAGCA-3′), suggesting that similar transcription factors may bind both EpREs and MAREs (Igarashi et al., 1994). Subsequent studies have confirmed that heterodimers consisting of Cap-N-Collar family members (Nrf1, Nrf2, NFE2-p45) and other b-zip transcription factors (Fos, Jun, Mafs) can bind at both MAREs and EpREs (Alam et al., 1999; Dhakshinamoorthy and Jaiswal, 2000, 2002). The EpRE sequence is transactivating an EpRE-containing GCLM/luciferase reporter transgene. Collectively these functional assays suggest that Nrf2 is involved in both basal and induced expression of multiple EpRE-containing genes.

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Erk Activation Is Required for Nrf2 Nuclear Localization during Pyrrolidine Dithiocarbamate Induction of Glutamate Cysteine Ligase Modulatory Gene Expression in HepG2 Cells

Pyrrolidine dithiocarbamate (PDTC) induction of the human glutamate cysteine ligase modulatory (GCLM) gene is dependent on activation of the mitogen-activated protein kinases (MAPKs) extracellular regulated kinase (Erk) and p38, and is not affected by protein kinase C (PKC) or PI3K inhibitors. Nrf2 binding to the electrophile response element (EpRE) located within the GCLM promoter is decreased after MAPK inhibition, suggesting that Nrf2 could be a downstream target of activated MAPK. To evaluate this hypothesis, a series of Nrf2 proteins harboring mutations in conserved consensus MAPK phosphorylation sites were developed and used in multiple functional assays. All mutated Nrf2 proteins tested interacted with the cytoplasmic repressor Keap1 in a manner indistinguishable from wild-type Nrf2. Furthermore, the mutant and wild-type Nrf2 proteins were similarly capable of transactivating an EpRE-containing GCLM/luciferase reporter transgene. Collectively these functional assays suggest that Nrf2 is not likely to be a direct downstream target of activated MAPK in vivo. However, treatment of HepG2 cells with MAPK inhibitors PD98059 and/or SB202190 prior to exposure to PDTC, reduced Nrf2 translocation to the nucleus, suggesting that MAPK-directed phosphorylation is a requirement for nuclear localization during PDTC induction of GCLM gene expression.

Key Words: EpRE; phosphorylation; Erk, p38.
encoding detoxification enzymes, as well as the genes for human GCLC (glutamate cysteine ligase catalytic) and GCLM (Chan and Kan, 1999; Chan and Kwong, 2000; Chan et al., 2001; Enomoto et al., 2001; Hayes et al., 2000; Ishii et al., 2000; Itoh et al., 1997; Kwak et al., 2001; McMahon et al., 2001; Ramos-Gomez et al., 2001). Nrf2 is normally sequestered in the cytoplasm of cells bound to Keap1, a cytoskeletal binding protein related to the Drosophila actin-binding Kelch protein, which under basal conditions anchors Nrf2 in the cytoplasm (Itoh et al., 1999). Itoh et al. demonstrated that after an oxidative/electrophilic stress, Nrf2 translocates from the cytoplasm to the nucleus and transactivates EpRE-containing genes. The mechanism by which the Nrf2/Keap1 complex senses the oxidative/electrophilic stress and triggers Nrf2 release and subsequent nuclear translocation is still unclear. It has been proposed that Keap1, which is rich in reactive cysteines, may directly sense the oxidative insult via thiol modification and undergo a conformational change that releases Nrf2 (Itoh et al., 1999).

An alternate hypothesis is that Nrf2 release from Keap1 is mediated by specific kinases that are activated after an oxidative stress. Both protein kinase C (PKC) and PI3K have been demonstrated to regulate the nuclear translocation of Nrf2 (Huang et al., 2000, 2002; Lee et al., 2001). Mitogen-activated protein kinases (MAPK) have also been demonstrated to be involved in Nrf2 binding to an EpRE sequence (Zipper and Mulcahy, 2000). A cross-species comparison of Nrf2 amino acid sequence identified several conserved consensus MAPK phosphorylation sites in Nrf2. We hypothesized that these conserved phosphorylation sites might function in Nrf2 activation in one of several ways: (a) phosphorylation of Nrf2 by MAPK at conserved sites could be required for release of Nrf2 from Keap1, (b) MAPK phosphorylation could be involved in Nrf2 nuclear translocation, or (c) direct phosphorylation of Nrf2 by MAPK could be required for Nrf2/DNA binding and subsequent transactivation. We generated Nrf2 mutants lacking the conserved MAPK phosphorylation sites and examined the effect of these Nrf2 mutants on Nrf2/Keap1 release and Nrf2 binding/transactivation of the GCLM reporter transgene. The results demonstrate that the PKC and PI3K pathways do not mediate GCLM upregulation in response to PDTC in HepG2 cells. Furthermore, while nuclear translocation of Nrf2 is decreased following MAPK inhibition, suggesting MAPK-dependent phosphorylation events may be required for Nrf2 nuclear translocation, it is unlikely that Nrf2 itself is a direct target for MAPK phosphorylation as mutation of the conserved MAPK consensus phosphorylation sites in Nrf2 failed to alter Nrf2 transactivation of GCLM gene expression or Nrf2 interaction with the cytoskeletal-binding protein, Keap1.

MATERIALS AND METHODS

Plasmids and reagents. The GFP-Nrf2 fusion protein was generated by blunt ending BspH1-digested pCIneoNrf2, followed by digestion with Smal.

The resulting Nrf2 fragment was ligated into pEGFPC1 (Clontech) at the Smal site in the multiple cloning region of the vector. The generation of the pG3L-1.9GCLM-luciferase transgene containing 1927 bp of upstream promoter sequence cloned into the pG3L3-basice luciferase vector (Promega), has been described previously (Moinova and Mulcahy, 1998). The pTFI-hNQO1 luciferase enhancer construct was generated by ligating an oligonucleotide containing the antioxidant response element (ARE) from hNQO1 (5'-CAGTGACAGTCTGATCAGACTGAACTCTG-3') into pTFI (ATCC) as described previously (Wild et al., 1998). The AP-1-luciferase enhancer was generated by ligating a double stranded AP-1 site probe (5'-GGAGAATCTGAAGCAATCAGTACGACTG-3') into the Smal site of pTFI. Four AP-1 inserts were designed by sequence analysis of the ligated vector resulting in a 4XAP-1 enhancer. The Nrf2 cDNA clone was obtained from Dr. Etsuro Ito (Hirosaki University), and subsequently recloned into pCIneo to obtain pCIneoNrf2 as described elsewhere (Wild and Mulcahy, 1999). The pEF-mKeap1 expression plasmid was kindly provided by Dr. Masayuki Yamamoto (University of Tsukuba, Japan). The Kinase inhibitors RO-32-0432, RO-31-8425, LY294002, SB202190, and PD98059 were obtained from Calbiochem. Staurosporine and wortmannin were purchased from Sigma. The Nrf2 antibody was from Santa Cruz Biotechnology.

Cell culture and transient transfection. HepG2 cells were maintained in Dulbecco’s modified Eagle medium, supplemented with 10% (v/v) fetal bovine serum and 50 µg/ml gentamycin. HepG2 cells were obtained from ATCC and are derived from a human hepatocellular carcinoma. Cells of liver origin are highly metabolic and express high levels of many detoxification enzymes including GCLM (Gipp et al., 1995), thereby facilitating the study of mechanisms of transcriptional regulation. Cells were transfected using a standard calcium phosphate and glycerol shock procedure as described previously (Moinova and Mulcahy, 1998; Wild and Mulcahy, 1999). Thirty-six h after transfection, the medium was replaced with fresh medium containing PDTC and/or specific inhibitors, as indicated in figure legends. In experiments with kinase inhibitors, cells were pretreated with the inhibitor for 1 h prior to a 6-h exposure to 100 µM PDTC (dissolved in DMSO at 1000× stock) or an 18-h exposure to tert-buty1 hydroquinone (tBHQ) (dissolved in DMSO at 1000× stock). After treatment of cells with the indicated agents and/or inhibitors, cells were lysed in 1X lysis buffer (Promega), and extracts were assayed for luciferase activity, as described by the manufacturer. Luciferase activity was monitored using a Monolight 2010 luminometer. Transfection experiments were normalized to β-gal expression by cotransfection of pRSV-β-gal (A. Jaiswal, Baylor College of Medicine). Experiments were performed in triplicate and results were expressed as luciferase/β-gal. The mean of at least 2–3 independent experiments (± standard deviation, or standard error, respectively) are presented. Student’s t-test was performed and p values < 0.05 were considered significant.

Immunofluorescence, GFP imaging, and Western blotting. For immunocytochemistry experiments, HepG2 cells were grown on sterile glass cover slips in 6-well plates and pretreated with 25µM PD98059 and/or SB202190, or with vehicle (DMSO) for 1 h prior to a 6-h exposure to PDTC. The coverslips were washed with PBS before fixation in 1.4% formaldehyde for 30–60 min at 4°C. Cells were washed with 0.2% Triton X-100 in PBS three times for 5 min at room temperature, in order to permeabilize the cells. The coverslips were then incubated with 2% goat serum/PBS for 1–3 h at 4°C, and incubated overnight at 4°C with Nrf2 antibody diluted 1:200 in 2% goat serum/PBS. The coverslips were washed with PBS prior to incubation with a 1:200 dilution of anti-rabbit-FITC antibody (ZyMax). Coverslips were washed, mounted on glass slides using fluorescent mounting medium (Dako), and visualized by fluorescence microscopy. For experiments using the GFP-Nrf2 construct, HepG2 cells were transiently transfected as described above. Thirty-six–forty-eight h after transfection, cells were stained with 5 µg/ml Hoechst #33342 for 10 min at 37°C, washed with PBS, and visualized with a fluorescence microscope to determine subcellular localization of GFP-Nrf2.

Western blots were performed using nuclear extracts prepared from HepG2 cells pretreated with SB202190 and/or PD98059 before exposure to PDTC, as described previously (Zipper and Mulcahy, 2000). 25 µg of nuclear protein
was separated on 7% acrylamide gels and transferred to nitrocellulose membranes using a Trans-blot Semi-Dry Transfer Cell (Biorad). Membranes were blocked in 5% nonfat dry milk/TBS/Tween 0.1% for 1–3 h at room temperature before incubation with 1:200 dilution of Nrf2 antibody (Santa Cruz) overnight at 4°C. Membranes were washed in TBS/Tween 0.1% and incubated with anti-rabbit-HRP (Pierce) before development with SuperSignal chemiluminescent substrate (Pierce). Nonspecific bands recognized by the Nrf2 antibody served as loading controls for each of the nuclear extracts. The Nrf2 standard was produced by incubating purified GST-Nrf2 protein with thrombin protease (Calbiochem) at 22°C for 20 h. An aliquot of the cleaved GST-Nrf2 products was run in a lane alongside the nuclear extracts to identify the Nrf2-specific band.

Site-directed mutagenesis. Site-directed mutagenesis was performed with commercially available kits (Stratagene and Promega) using the following primers as the sense strand (specific bases mutated are indicated in lowercase):

- S198A: 5'CCATGTTCCAgTCAGAGAGC-3', S226A: 5'GTAGTGAACgTGcTCACATTTG-3', S340A: 5'CCAGGTTGCGaACgCACAGAACAC-3', S561A: 5'GGAAACCTTAGCTCCTAGTGG-3', S392A: 5'GGTACAACCCTTGcCACCATCTCAGG-3', S417A: 5'CCAGAGAAAGAATTGCCTGTAgcTCCTGGTCAT-3'.

Primers were synthesized at the University of Wisconsin Biotechnology Center. All mutations were confirmed by sequence analysis.

RESULTS

Much controversy exists over which signal transduction cascades are involved during the induction of EpRE/ARE mediated genes. Recent work by Huang et al. have demonstrated that tBHQ induction of NQO1 luciferase activity requires PKC activation (Huang et al., 2000). To determine if PKC pathways were involved in PDTC induction of GCLM, HepG2 cells were transiently transfected with a GCLM reporter transgene. Transfected cells were treated with three different PKC inhibitors, as shown in Figure 1. Staurosporine is a nonspecific PKC inhibitor, which, in addition to blocking PKC, also inhibits protein kinase A, protein kinase G, and CaM kinase. Both RO-31-8425 and RO-32-0432 are more selective PKC inhibitors, which inhibit multiple PKC isozymes with a slightly higher specificity for PKCα. The general PKC inhibitor, staurosporine, was effective in blocking the threefold induction of the GCLM luciferase reporter (p < 0.05). However, the two specific PKC inhibitors (RO-31-8425 and RO-32-0432) had no effect on GCLM gene induction following PDTC exposure (Fig. 1).

To determine if the doses of the specific PKC inhibitors used were indeed able to block PKC activity, a 4XAP-1 luciferase reporter gene was constructed and transiently transfected into HepG2 cells. The transfected cells were pretreated with the PKC inhibitors before exposure to TPA, a potent PKC activator. All inhibitors significantly decreased (RO-32-0432) or eliminated (staurosporine and RO-31-8425) TPA induction of AP-1 luciferase activity (data not shown). Collectively these data suggest that PDTC induction of GCLM in HepG2 cells is not dependent on PKC activation.

Lee et al. (2001) have demonstrated that in IMR-32 neuroblastoma cells the PI3 kinase pathway is the major signaling cascade involved in EpRE gene induction. In IMR-32 cells, PDTC and tBHQ induction of GCLM are reduced ~50% by LY294002, suggesting PI3K mediates partial induction in this cell line (Fig. 2A, p < 0.05). In contrast, in HepG2 cells, the PI3K pathway inhibitors were ineffective at blocking PDTC (Figs. 2B and 2C) or tBHQ (Fig. 2C) induction of GCLM. PDTC treatment of HepG2 cells induced GCLM reporter gene activity ~twofold. Pretreatment with the PI3K inhibitor wortmannin, prior to PDTC exposure, had no effect on the magnitude of PDTC induction (Fig. 2B). Similarly, pretreatment of HepG2 cells with either 25 or 50 μM LY294002 had no effect on PDTC or tBHQ induction of the GCLM luciferase transgene. PDTC increased expression of the luciferase constructs ~twofold, and tBHQ induced ~fivefold, even in the presence of the inhibitor (Fig. 2C).

Previous studies from our laboratory have demonstrated that MAPK inhibitors PD98059 and SB202190 decreased or eliminated PDTC induction of GCLM, and also reduced Nrf2 binding to the GCLM EpRE sequence (Zipper and Mulcahy, 2000). Collectively, these data suggested that Erk and p38, but not PKC or PI3K phosphorylation events are involved in Nrf2 transactivation of the EpRE sequence in the promoter of the human HMG gene in HepG2 cells. Since many common substrates of MAP kinases are themselves transcription factors, we hypothesized that phosphorylation of Nrf2 by extracellular regulated kinase (Erk) or p38 might be required for one or more steps in Nrf2 activation.

A comparison of the primary amino acid sequence for chicken, rat, mouse, and human Nrf2 identified three potential phosphorylation sites (P-X-S/T-P or minimally S/T-P) conserved in all four species and three additional conserved sites in the mouse, rat, and human proteins (Fig. 3). A series of Nrf2 mutant proteins were developed by mutating the serine in each conserved phosphorylation site to an alanine. Functional properties of the mutant Nrf2 proteins were then compared with

![GCLM-Luciferase](image)
those of wild-type Nrf2 to determine if disruption of one or more MAPK phosphorylation sites modulated Nrf2 function in vivo.

We first investigated whether the conserved MAPK phosphorylation sites in Nrf2 were necessary for transactivation of a GCLM promoter/luciferase transgene containing a Nrf2-responsive EpRE. When HepG2 cells were co-transfected with wild-type Nrf2 cDNA and the GCLM reporter transgene, luciferase activity increased ~17-fold (Fig. 4) by comparison to the level of expression detected in cells transfected with the empty vector. GFP-Nrf2 was concentrated in the nucleus (Fig. 5B). In contrast, when Keap1 was cotransfected along with GFP-Nrf2, nuclear fluorescence was greatly diminished and prominent cytoplasmic fluorescence was observed, confirming the ability of recombinant Keap1 to sequester Nrf2 in the cytoplasm.

Keap1 inhibition of Nrf2 transactivation was used as another functional assay to determine whether mutation of potential phosphorylation sites on Nrf2 altered Nrf2/Keap1 interaction. Transfection of HepG2 cells with either the wild type or the M6 Nrf2 mutant (in which all six potential phosphorylation sites are mutated) increased luciferase expression approximately tenfold by comparison with cells transfected with the empty vector (Fig. 5C). Transfection with the cDNA for the Nrf2 S561A mutant produced a ~sevenfold increase in reporter expression. Cotransfection with Keap1 suppressed the transactivating potential of the wild-type and mutant Nrf2 proteins to a similar extent; to levels comparable to those observed in cells not transfected with the Nrf2 expression vector.

In HepG2 cells overexpressing Nrf2, PDTC exposure failed to further increase GCLM reporter transgene expression. In contrast, GCLM reporter expression was elevated ~2.6-fold following PDTC exposure in cells expressing both Keap1 and wild-type Nrf2, signifying that PDTC treatment had resulted in release of Nrf2 from Keap1. The level of GCLM expression obtained from PDTC-mediated Nrf2 release from Keap1 was more, when the S561A or S417A mutants were combined with any other mutation, wild-type transactivating potency was observed.

Since Nrf2 is normally sequestered in the cytoplasm bound to Keap1, Nrf2 must be released prior to nuclear translocation and subsequent transactivation. It is possible that phosphorylation of Nrf2 might alter the Nrf2/Keap1 interaction rather than influence Nrf2-transactivation potential, directly. While the Nrf2 transfection experiments addressed the role of potential MAPK phosphorylation sites on the transactivation potential of free Nrf2 protein (Fig. 4), they did not address a possible role for phosphorylation events in the dynamics of the Nrf2/Keap1 interaction. To evaluate this possibility, we first confirmed that expression of recombinant Keap1 could repress EpRE activation by exogenous Nrf2. We co-transfected HepG2 cells with either of two different EpRE-mediated luciferase reporter vectors (pGL3-1.9-GCLM or pT81-hARE-NQO1), 1.0 μg of an Nrf2 expression vector, and increasing amounts of Keap1 cDNA. As shown in Figure 5A, co-transfection of Keap1 decreased Nrf2 transactivation of both luciferase transgenes. To determine whether the observed decrease in Nrf2 transactivation by Keap1 was indeed due to cytoplasmic sequestration of Nrf2, we examined the subcellular localization of a GFP-tagged Nrf2. In HepG2 cells transfected with the cDNA for GFP alone, fluorescence was uniformly distributed throughout the cell (Fig. 5B). When the cDNA encoding the GFP-Nrf2 fusion protein was introduced into HepG2 cells in the absence of exogenous Keap1, the GFP-Nrf2 was concentrated in the nucleus (Fig. 5B). In contrast, when Keap1 was cotransfected along with GFP-Nrf2, nuclear fluorescence was greatly diminished and prominent cytoplasmic fluorescence was observed, confirming the ability of recombinant Keap1 to sequester Nrf2 in the cytoplasm.
not as great as that observed in cells overexpressing Nrf2 alone. These results are consistent with previous reports which also demonstrate that in the presence of overexpressed Keap1, exposure to oxidants/electrophiles only partially restores Nrf2 transactivation potential (Itoh et al., 1999; Sekhar et al., 2002).

Reporter gene expression following PDTC exposure was equivalent in cells expressing wild-type Nrf2, the S561A, or M6 Nrf2 mutants, indicating that these sites have no direct influence on the dynamics of the Nrf2 binding to Keap1 or on Nrf2 release following PDTC exposure.

Since mutation of potential MAPK phosphorylation sites present in Nrf2 failed to alter Nrf2-mediated transactivation of reporter genes or the dynamics of Keap1/Nrf2 interactions, we examined the effect of the MAPK inhibitors on Nrf2 nuclear translocation. Increased nuclear levels of Nrf2 were detected by Western blotting of nuclear extracts prepared from PDTC-treated cells (Figs. 6A and 6B). When PDTC-treated HepG2 cells were preincubated with 25 μM of the Erk pathway inhibitor, PD98059 (a dose previously shown to reduce induction by PDTC, Zipper and Mulcahy, 2000), levels of nuclear Nrf2 were reduced dramatically (Fig. 6A). Preincubation with the p38 inhibitor, SB202190, only slightly decreased Nrf2 levels evident in the nucleus following exposure to PDTC (Fig. 6B). Simultaneous incubation with both inhibitors prior to PDTC exposure reduced the level of nuclear Nrf2 to that of untreated controls (Fig. 6B).

FIG. 3. Cross-species comparison of Nrf2 amino-acid sequence indicating the presence of conserved MAPK phosphorylation sites. Nrf2 amino acid sequences were obtained from NCBI and aligned using DNA Star MegAlign program. Conserved MAPK phosphorylation sites (P-X/S/T-P, or S/T-P) are boxed, and DNA binding domain is also indicated.

FIG. 4. Nrf2 proteins with MAPK phosphorylation site mutations are able to transactivate a GCLM luciferase reporter transgene. One μg of Nrf2 wt or mutant constructs were transiently transfected into HepG2 cells, and transactivation of a GCLM luciferase reporter transgene was measured. Nrf2 mutations are listed as the amino acid number corresponding to the conserved serine in the MAPK phosphorylation site that was mutated to alanine. Luciferase activity was calculated as RLU/β-gal, and results were plotted as fold increase relative to cells transfected with empty vector (neo). Experiments were performed at least 3 times and are reported as mean ± SE. Asterisks denote p < 0.05 compared to wild-type (wt)-Nrf2 transactivation of the GCLM luciferase transgene.
To assess the possible role of MAPKs in Nrf2 nuclear translocation, we exposed HepG2 cells to PDTC and MAPK inhibitors and examined the sub-cellular location of endogenous Nrf2 by immunofluorescence using a Nrf2 antibody. In untreated cells, fluorescence was evident in both the nucleus and cytoplasm (Fig. 7A). Following exposure to 100 μM PDTC and MAPK inhibitors, fluorescence was primarily localized to the cytoplasm (Fig. 7B). These results suggest that MAPK activation is required for Nrf2 nuclear localization.
oxidative or electrophilic insults. Although a comprehensive
bind this sequence to induce gene transcription in response to
EpREs, within promoters of detoxification enzymes, allows coordinat
investigation. The presence of EpREs or sequences similar to
genes is a complex process that is only beginning to yield to
exposure to PDTC for an additional 1 h. Western blots were performed on 25
both inhibitors (B). Cells were pretreated for 1 h with inhibitors prior to
PDTC
extracts were harvested from control HepG2 cells or cells treated with
PDTC, nuclear fluorescence persisted but cytoplasmic staining
was markedly reduced (compare Figs. 7A vs. 7B). However, even though cytoplasmic fluorescence decreased, it was not possible to detect a significant increase in nuclear fluorescence following PDTC exposure. Pretreatment of HepG2 cells with 25 μM PD98059 prior to PDTC exposure blocked the decrease in cytoplasmic fluorescence evident following treatment with PDTC alone (Fig. 7D vs. 7B). SB202190 was less effective at inhibiting the PDTC-induced decrease in Nrf2 cytoplasmic localization (data not shown). Cytoplasmic Nrf2 fluorescence in cells preincubated with both inhibitors prior to PDTC exposure, was indistinguishable from control cells or cells pre-
treated with PD98059 alone (data not shown).

DISCUSSION

The regulation of phase II enzymes and other detoxification genes is a complex process that is only beginning to yield to investigation. The presence of EpREs or sequences similar to EpREs, within promoters of detoxification enzymes, allows coordinated induction of these genes as part of an adaptive response to oxidative/electrophilic insults such as tBHQ or PDTC exposure (Prestera et al., 1993). Soon after the identification of the core EpRE consensus sequence, research focused on identification of the trans-factors that recognize and bind this sequence to induce gene transcription in response to oxidative or electrophilic insults. Although a comprehensive list of trans-factors has not been confirmed, an overwhelming body of evidence indicates that the Cap-N-Collar (CNC) family member Nrf2 binds at EpREs to upregulate transcription of EpRE containing genes, following exposure to many inducing agents (Alam et al., 1999; Dhakshinamoorthy and Jaiswal, 2000; Jeyapaul and Jaiswal, 2000; Kim et al., 2001; Moinova and Mulcahy, 1998; Myhrstad et al., 2001; Venugopal and Jaiswal, 1996, 1998; Wild et al., 1999). Although Nrf1 is also capable of transactivating EpRE-containing genes, Nrf2 is a much more potent transcriptional activator (Jeyapaul and Jaiswal, 2000; Mulcahy et al., unpublished data; Myhrstad et al., 2001). Perhaps the most compelling evidence that Nrf2 functions in this capacity in vivo is the recent work demonstrating that Nrf2 knockout mice express decreased levels of several key detoxification enzymes and fail to mount a robust adaptive response after oxidative stress (Chan and Kan, 1999; Chan and Kwong, 2000; Chan et al., 2001; Enomoto et al., 2001; Hayes et al., 2000; Itoh et al., 1997; Kwak et al., 2001; McMahon et al., 2001; Ramos-Gomez et al., 2001).

In 1999, Itoh et al., reported that Nrf2 was regulated by nuclear exclusion (Itoh et al., 1999). In unstimulated cells, Nrf2 is localized in the cytoplasm bound to the cytoskeletal binding protein Keap1, which itself contains double-glycine-repeats (also known as Kelch repeats) capable of binding the actin cytoskeleton (Adams et al., 2000; Lecuyer et al., 2000, Zipper and Mulcahy, 2002). In response to an oxidative challenge, Nrf2 is released from Keap1 repression and accumulates in the nucleus. Itoh et al. (1999) have hypothesized that electrophiles or reactive oxygen species (ROS) may react with one or more of the multiple cysteines on Keap1, resulting in a conformational change that liberates Nrf2. More recent
experiments have resulted in the identification of several cysteines that are modified by oxidative insult and are implicated in Nrf2 release from Keap1 (Dinko-Kostova et al., 2002).

Alternatively, Nrf2 or Keap1 may serve as a substrate for one or more of the many kinase pathways that are activated following exposure to oxidants/electrophiles. Many different candidate pathways have been suggested to be involved in EpRE/ARE gene induction by pro-oxidants such as tBHQ and PDTC. The results presented here demonstrate that in HepG2 cells, PDTC induction of a GCLM reporter transgene can be inhibited by the general PKC inhibitor, staurosporine. However, more specific PKC inhibitors failed to affect PDTC induction. Since staurosporine also inhibits other protein kinases, its effect on GCLM gene expression cannot be attributed solely to PKC. Both RO-32-0432 and RO-31-8425, however, are specific inhibitors of multiple PKC isoforms. The failure of the more specific PKC inhibitors to alter GCLM gene expression suggests PKC is not mediating PDTC induction of GCLM. These results conflict with the findings of Huang et al. (2000) who observed that the specific PKC inhibitor RO-32-0432 blocked NQO1 gene induction by ~60% in HepG2 cells. One potential explanation for this difference could be related to differences in the reporter transgene employed in the respective studies. We utilized the GCLM luciferase reporter, which contains a 1.9 kb of promoter sequence, whereas Huang et al. used a 31-bp EpRE enhancer derived from the NQO1 promoter. Furthermore, Huang et al. (2000) exposed cells to tBHQ, whereas we induced expression with PDTC. It is conceivable that these two classes of inducing agents activate different kinase cascades as a consequence of generation of distinct biochemical signals. In support of this explanation, we have observed that PDTC induction of the GCLM reporter can be prevented by preincubating cells with the antioxidant N-acetyl cysteine (NAC) prior to PDTC exposure. However, NAC pretreatment is ineffective at inhibiting tBHQ induction of the same luciferase reporter construct (Mulcahy et al., unpublished data). The activation of different kinase cascades by different inducers (PDTC and tBHQ) could explain why PKC inhibitors are effective against tBHQ induction but not PDTC induction of GCLM.

We also tested the ability of PI3K inhibitors to effect GCLM gene induction HepG2 cells and IMR-32 cells. PI3K inhibitors were unable to prevent induction of the GCLM luciferase transgene by either tBHQ or PDTC in HepG2 hepatoblastoma cells. However, in IMR-32 neuroblastoma cells, the PI3K inhibitor LY294002 decreased GCLM induction by ~50%. These observations are in agreement with Lee et al. (2001), who observed that the PI3K pathway mediates NQO1 induction through the EpRE sequence in IMR-32 cells. We previously demonstrated that inhibition of Erk and/or p38 kinases reduced or eliminated PDTC induction of GCLM transcription and also decreased Nrf2 binding to the GCLM EpRE (Zipper and Mulcahy, 2000). Collectively, these data suggest that the activation of multiple kinase cascades can culminate in activating EpRE-containing genes, although the specific cascade responsible can vary in a cell line-specific manner. In the case of PDTC induction of GCLM expression in HepG2 cells, activation of the Erk and p38, but not PKC or PI3K pathways mediates signal transduction, culminating in upregulation of the GCLM gene.

The decreased Nrf2-EpRE binding detected after MAPK inhibition suggested the possibility that Nrf2 might be a direct downstream target of MAPKs, as has been demonstrated for other transcription factors such as c-jun. In vitro studies, purified Nrf2 protein was phosphorylated by immunoprecipitated Erk 1 and 2 kinases (data not shown). Although Nrf2 has not been previously identified as a MAPK substrate, our in vitro phosphorylation studies, coupled with the presence of multiple conserved MAPK phosphorylation sites within Nrf2, lend support to the hypothesis that Nrf2 could be a substrate of activated MAPKs. MAPK-dependent phosphorylation of Nrf2 could contribute to EpRE-mediated GCLM gene activation by at least 3 distinct mechanisms following oxidative/electrophilic stress: (1) phosphorylation of one or more MAPK sites on Nrf2 might mediate Nrf2 release from Keap1; (2) Nrf2 phosphorylation may be required for nuclear translocation; or (3) phosphorylation could be a prerequisite for DNA-binding and/or transactivation. We reasoned that if Nrf2 phosphorylation was required to affect the release of Nrf2 from Keap1, individual Nrf2 proteins harboring mutations in one or more of the potential MAPK phosphorylation sites might differ in their ability to mediate activation of EpRE reporter transgenes. However, no differences among the various Nrf2 mutants and wild-type Nrf2 were detected with respect to Keap1 binding or reporter gene induction following PDTC exposure, effectively discounting a role for these conserved MAPK sites in Nrf2 binding and release from Keap1.

Once released from Keap1, in response to an as yet unidentified signal, Nrf2 has been shown to translocate from the cytoplasm to the nucleus. It has not yet been determined whether post-translational modification of Nrf2 is required for this step of the signal transduction pathway. Nrf2 was detected in the cytoplasm and nucleus of untreated HepG2 cells by immunofluorescence. The presence of endogenous Nrf2 in the nucleus differs from previous reports, which failed to detect nuclear Nrf2 in untreated cells (Huang et al., 2000), but is consistent with Western blots (Fig. 6) and our previous super-shift analyses, which detected Nrf2 in nuclear extracts prepared from control cells (Zipper and Mulcahy, 2000). Following PDTC treatment, there was a significant decrease in Nrf2 fluorescence evident in the cytoplasm, but a corresponding increase in nuclear fluorescence was difficult to appreciate by microscopy. However, Western blots did reveal a significant increase in nuclear Nrf2 protein following PDTC exposure. Both the decrease in cytoplasmic Nrf2 fluorescence (observed with immunofluorescence) and the nuclear Nrf2 accumulation (measured with Western blots) were inhibited by PD98059, and, to a lesser extent, by inhibition of p38 with SB202190. Simulta-
neous treatment with both inhibitors abrogated Nrf2 translocation, consistent with our previous report that combination of the two inhibitors effectively blocked GLC gene induction and binding of Nrf2 to the GCLM EpRE (Zipper and Mulcahy, 2000).

Collectively these data suggest that the Erk and p38 pathways mediate signal transduction, culminating in upregulation of the GCLM subunit gene (and presumably other EpRE-regulated genes) following PDTC exposure, at least in part by regulating the nuclear translocation of Nrf2. While Erk or p38 inhibition prevents accumulation of Nrf2 in the nucleus following PDTC exposure, this effect is not likely to be related to Nrf2 phosphorylation per se, since other experiments described in this report indicate that the mutated Nrf2 proteins were as effective at transactivating reporter transgenes as was wild-type Nrf2.

The exact mechanism(s) by which Erk regulates Nrf2 nuclear localization and transcriptional activation has yet to be elucidated. We recently discovered that Keap1 dimerization is required to sequester Nrf2, and that disruption of the Keap1 dimer results in Nrf2 release, nuclear translocation and subsequent activation of Nrf2 target genes (Zipper and Mulcahy, 2002). Disruption of the Keap1 complex was not dependent on Erk activation, suggesting MAPKs act downstream of Nrf2 phosphorylation per se, since other experiments described in this report indicate that MAPKs act downstream of Nrf2 and Nrf2 are not direct targets of MAPK phosphorylation, nor is MAPK phosphorylation the trigger for Nrf2 release from Keap1. However, MAPK activation is involved in Nrf2 nuclear translocation.

We have developed a model (Fig. 8) that illustrates some potential mechanisms by which phosphorylation could regulate Nrf2 nuclear entry. Once Nrf2 is released from Keap1 (a mechanism that is independent of Erk activation, Zipper and Mulcahy, 2002), Erk-mediated phosphorylation of a Nrf2 chaperone or some other type of accessory protein might be required for Nrf2 nuclear translocation (Fig. 8, pathway B). Precedence for such a mechanism of MAPK-regulated nuclear translocation exists in the case of nuclear factor of activated T cells (NFAT). Calcium signals induce NFAT nuclear translocation in a process regulated by calcineurin, which is cotransported with NFAT to form a transcriptionally active complex. Recent work has demonstrated that c-Jun N-terminal kinase (Jnk) regulates NFAT nuclear translocation through phosphorylation of calcineurin, the NFAT chaperone protein (Chow et al., 2000; Gomez del Arco et al., 2000).

Alternatively, phosphorylation of one of the previously identified Nrf2 binding partners (e.g., small Maf, JunD) (Jeyapaul and Jaiswal, 2000; Wild et al., 1999), or an as-yet-unidentified binding partner (labeled Nrf2-B.P. in Fig. 8) could be required for Nrf2 heterodimerization and nuclear translocation (Fig. 8, pathway A). Phosphorylation-induced transcription factor dimerization is a common theme in biology and has been demonstrated for many other transcription factors, including Erk2 (Khokhlatchev et al., 1998), the Smads, and the Stats. Phosphorylation of these proteins results in dimerization and subsequent nuclear translocation. It is conceivable then, that MAPK-dependent phosphorylation of one or more other critical protein partners might regulate Nrf2 nuclear localization, thereby accounting for the disruptive effect of MAPK inhibition on Nrf2 transactivation potential.

REFERENCES


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