Phosphorylation of Nrf2 at Ser40 by Protein Kinase C
Regulates Antioxidant Response Element-mediated Transcription

H.-C. Huang, Truyen Nguyen, and Cecil B. Pickett*
Schering-Plough Research Institute, Kenilworth, NJ 07033

(running title: Nrf2 Phosphorylation by PKC)

*To whom correspondence should be addressed:
Schering-Plough Research Institute
2015 Galloping Hill Road, Kenilworth, NJ 07033
Tel: 908-740-7300; Fax: 908-740-7514
E-mail: cecil.pickett@spcorp.com
SUMMARY

Nrf2, a basic leucine zipper transcription factor, is an essential activator of the coordinated transcription of genes encoding antioxidant enzymes and phase II detoxifying enzymes through the regulatory sequence termed antioxidant response element (ARE). Recently we reported evidence for the involvement of protein kinase C (PKC) in phosphorylating Nrf2 and triggering its nuclear translocation in response to oxidative stress. We show here that phosphorylation of purified rat Nrf2 by the catalytic subunit of PKC was blocked by a synthetic peptide mimicking one of the potential PKC sites. Accordingly, Nrf2 bearing a Ser-to-Ala mutation at amino acid 40 (S40A) could not be phosphorylated by PKC. The S40A mutation did not affect *in vitro* binding of Nrf2/MafK to the ARE. However, it partially impaired Nrf2 activation of ARE-driven transcription in a reporter gene assay when Keap1 was overexpressed. *In vitro* transcribed/translated Keap1 could be coimmunoprecipitated with Nrf2. Phosphorylation of wild-type Nrf2 by PKC promoted its dissociation from Keap1, whereas the Nrf2(S40A) mutant remained associated. These findings together with our prior studies suggest that the PKC-catalyzed phosphorylation of Nrf2 at Ser40 is a critical signaling event leading to ARE-mediated cellular antioxidant response.
INTRODUCTION

The antioxidant response element (ARE)\(^1\) is a regulatory sequence involved in the coordinated transcriptional activation of genes coding for a number of antioxidant enzymes and phase II detoxifying enzymes (1-6). Reactive oxygen species and electrophiles are potent activators of genes containing an ARE, mediated by the basic leucine zipper (bZIP) transcription factor Nrf2 (NF-E2-related factor 2) (7-9). Accumulated evidence from studies of nrf2-null mice has established that Nrf2 is an essential ARE-binding factor involved in both constitutive and inducible gene expression via the ARE (9-11). An important regulatory step leading to ARE activation is the oxidative stress-induced nuclear translocation of Nrf2, which normally appears to be sequestered in the cytoplasm by the cytoskeleton-binding Keap1 protein (12-14). However, the precise mechanism by which ARE-activating signals reach Nrf2 and cause dissociation of the putative inhibitory Nrf2-Keap1 complex remains unclear.

Several protein kinase pathways have been implicated in transducing oxidative stress signals to gene expression mediated through the ARE. A number of reports have addressed a possible role for extracellular signal-regulated kinase (ERK1/2) in ARE activation. The findings have however remained controversial: ERK1/2 has been found to regulate the ARE positively in certain hepatoma cells (15-17) but negatively in others (18). Similarly, p38 MAP (mitogen-activated protein) kinase has also been shown to affect ARE activity, either positively (17, 19, 20) or negatively (16, 21). More recently, phosphatidylinositol 3-kinase (PI3-kinase) and its downstream target Akt/PKB have been linked to activation of the ARE in hepatoma (18, 19) and neuroblastoma (22) cell lines. However, none of the known cellular components involved in ARE regulation have been shown to be targets of any of these kinases.

Recently, we reported several findings that indicate an important role for protein kinase C (PKC) in the ARE-mediated gene expression (23). 1) Phorbol 12-myristate 13-acetate (PMA), a
potent PKC-activating phorbol ester, stimulates ARE-driven transcription, which is blocked by selective PKC inhibitors. 2) Nuclear translocation of Nrf2 is induced by PMA but arrested by PKC inhibitors. 3) Both Nrf2 nuclear translocation and activation of the ARE by tert-butylhydroquinone (tBHQ) treatment are suppressed by PKC inhibitors. 4) Nrf2 is phosphorylated \textit{in vitro} by purified PKC or immunoprecipitated PKC from tBHQ-induced cells. 5) Nrf2 phosphorylation in HepG2 cells is enhanced by PMA and tBHQ, but abolished by PKC inhibitors. Together these results suggest that one critical step in the signaling cascade towards ARE activation may be the phosphorylation of Nrf2 by PKC, which promotes the nuclear translocation of this transcription factor in response to oxidative stress. The present study is a continuation of our investigation into the involvement of PKC in regulating the ARE. We sought to identify the site of phosphorylation in Nrf2 by PKC, and to characterize the mechanistic significance of Nrf2 phosphorylation.
EXPERIMENTAL PROCEDURES

Purification of Nrf2. A high-level expression plasmid of rat Nrf2 gene linked at its N-terminus to a His₆ tag was obtained by cloning the rat Nrf2 cDNA (GenBank Accession Number AF037350) into the pQE-30 vector (QIAGEN). S40A mutant (AGT->GCT) was obtained by the PCR mutagenesis method and cloned into the same expression plasmid. Nrf2 wild-type or S40A mutant expression was induced by the addition of 0.5 mM isopropylthio-β-D-galactoside (IPTG) for 6 hr at 30°C in *Escherichia coli* M15 cells. Cell pellets were suspended and sonicated in a buffer containing 100 mM NaH₂PO₄/10 mM Tris HCl (pH 8.0), 300 mM NaCl, 0.1 mM EDTA, 5 mM β-mercaptoethanol, 10 mM imidazole, 0.5% Tween-20, and 15% glycerol. After centrifugation at 14,000xg for 20 min, soluble lysates were loaded onto Ni-NTA column. A 40 mM imidazole wash was followed by elution at 250 mM imidazole in the same buffer. Eluted fractions were pooled and dialyzed against 20 mM HEPES (pH 7.5), 200 mM NaCl, 0.1 mM EDTA, 1 mM DTT, and 20% glycerol and stored at –80°C.

Kinase Assays. Purified His₆-tagged rat Nrf2 wild-type and S40A mutant proteins were used as substrates in *in vitro* kinase assays with catalytic subunits of rat brain PKC (Calbiochem). 50 µl reactions were carried out at 30°C in a buffer containing 25 mM HEPES (pH 7.5), 10 mM MgCl₂, 200 µM ATP, and 2 µCi [γ-³³P]ATP, and stopped at the indicated times by the addition of sample buffer for SDS-PAGE analysis. The level of [³³P]ATP incorporation into Nrf2 was determined by autoradiography or by a PhosphorImager (Fujifilm FLA-2000). [γ-³³P]ATP was omitted from kinase reactions whose products were subsequently used in EMSA or in immunoprecipitation studies as described below.

Electrophoretic Mobility Shift Assays (EMSA). EMSA were performed essentially as described previously (24). A double-stranded oligonucleotide containing the rat *QR* gene ARE
was used as probe after end-labeling with $[\gamma-^{32}P]ATP$ by T4 polynucleotide kinase. The sequence of the DNA probe was 5'-GATTTCAGTCTAGAGTCACAGTGACTTGGC-AAAATCTGAGCCG-3' (ARE core sequence highlighted in bold). Purified rat Nrf2 wild-type or S40A mutant protein was preincubated with rat MafK (unless otherwise indicated) for 20 min at 25°C before the addition of DNA probe for another 20 min at 30°C. MafK proteins were produced in vitro by the TNT coupled transcription/translation wheat germ extract system (Promega) (24). DNA-protein interactions were detected by electrophoresis on non-denaturing 6% polyacrylamide gels in Tris borate-EDTA (TBE) buffer, followed by autoradiography. For competition experiments, a 200-fold molar excess of either unlabeled probe or a random 43-base oligonucleotide was included in the preincubation mixture at 25°C prior to the addition of the labeled probe. For supershift assay, an anti-Nrf2 antibody (sc-722X; Santa Cruz Biotechnology) was added after the binding reaction for 4 hr at 4°C before electrophoresis. To determine the effect of PKC phosphorylation of Nrf2 on ARE binding, Nrf2 wild-type or S40A mutant protein was first incubated in kinase assay buffer for 1 hr at 30°C in the presence or absence of PKC, before an aliquot was taken for incubation with MafK and the labeled probe for EMSA.

**Cell Culture, Transfection, and Reporter Assays.** HepG2 cells were obtained from the American Type Culture Collection, and maintained as previously described (1). Transient transfection was performed as before on cells in 6-well plates at approximately 70% confluency using LipofectAMINE Plus Reagent (Life Technologies). 1 μg of an expression plasmid containing rat QR ARE linked to chloramphenicol acetyltransferase (CAT) reporter gene was co-transfected with 0.12 μg of pcDNA3 plasmid (Invitrogen) bearing wild-type or S40A mutant Nrf2 and, where indicated, with 60 ng of pcDNA3 plasmid containing rat Keap1 (GenBank Accession Number AF304364). Total amount of transfected DNA was kept constant
at 2 µg by the addition of pcDNA3 vector to the DNA mixture. Cells were incubated for 18 hr after overnight recovery from transfection and harvested in M-PER Mammalian Protein Extraction Reagent (Pierce). Cell lysates were assayed for CAT activity as described (23), and the results were quantitated by a PhosphorImager.

**In vitro Association Assays.**  
In vitro transcription/translation of the pcDNA3 plasmids bearing Nrf2 and Keap1 was carried out using the TNT coupled wheat germ extract system (Promega) in the presence of [35S]methionine according to the manufacturer’s instructions. The products were then incubated together for 15 min at 30°C before immunoprecipitation by an anti-Nrf2 antibody in an IP buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS for 4 hr at 4°C, followed by the addition of Protein A-Trisacryl beads (Pierce). The mixture was rotated at 4°C for 1 hr, and washed extensively in IP buffer containing 0.3 M NaCl. Precipitates were resolved by SDS-PAGE, and detected by autoradiography. Quantitation of band intensity was performed on a PhosphorImager. To determine the effect of PKC phosphorylation on Nrf2-Keap1 interaction, equal amount of labeled Nrf2 wild-type or S40A mutant was incubated with PKC and Keap1 in kinase assay buffer for 30 min at 30°C before immunoprecipitation.
RESULTS

A Peptide Inhibitor of Nrf2 Phosphorylation by PKC

In prior studies we showed that Nrf2 is phosphorylated in HepG2 cells (23). Nrf2 phosphorylation was activated by the phorbol ester PMA but blocked by the PKC inhibitor staurosporine, suggesting that Nrf2 may be a PKC target. Accordingly, in vitro PKC assays revealed that purified rat Nrf2 was phosphorylated by the catalytic subunit of rat brain PKC, or by PKC immunoprecipitated from HepG2 lysates. To identify the specific site(s) of phosphorylation in Nrf2 by PKC, a serine/threonine kinase, synthetic peptides mimicking potential Nrf2 PKC sites were used as competitors for in vitro phosphorylation of Nrf2 by PKC. Rat Nrf2 is a 597 amino acid residue protein containing seven potential PKC phosphorylation sites according to the canonical pattern [S/T]-X-[R/K], where X is any residue. Six 8-residue peptides (36-43, 375-382, 414-421, 435-442, 585-592, 590-597) were synthesized corresponding to potential PKC target sites at S40, S378, T417, T418, S439, S589, and T594 respectively (Fig. 1A). All were readily soluble in 20% DMSO except peptide 36-43, whose solubility was greatly improved when it was extended at both ends by one residue to a 10-mer (EVFDFSQRQK). In vitro kinase assays were performed as described previously (23), using commercially obtained catalytic subunits of rat brain PKC and purified rat Nrf2 as substrate. Although Nrf2 phosphorylation by PKC appeared unaffected or even enhanced by several of the peptides, it was reduced by more than 90% in the presence of 5 mM peptide 35-44 (Fig. 1B). PKC activity against a standard substrate was not suppressed by this peptide (not shown), indicating that its effect was not on the enzyme itself. These findings suggest that Ser40 of Nrf2 is an authentic site of phosphorylation by PKC.

A Nrf2 Mutant Defective for PKC Phosphorylation

To confirm these peptidomimetics data a site-directed mutagenesis approach was utilized. Rat Nrf2 gene bearing a AGT->GCT (Ser-to-Ala) mutation at amino acid position 40 (nrf2-S40A) was cloned into a high-level
expression plasmid containing a His$_6$-tag N-terminal to the insert. *Escherichia coli*-expressed Nrf2-S40A protein was purified to near homogeneity by metal chelate affinity chromatography (Fig. 2). The prominent band ~90 kD was confirmed to be Nrf2 by Western blot using an antibody against Nrf2 (not shown). Nrf2-S40A was then used in parallel with wild-type Nrf2 as substrates in *in vitro* PKC assays. As shown in Fig. 3, the single amino acid change from Ser to Ala at position 40 completely abolished PKC phosphorylation of Nrf2. The lack of residual phosphorylation in this mutant indicates that Ser40 is the only PKC site, consistent with the peptide competition data.

It should be noted that Ser40 is also one of four potential PKC sites in human Nrf2 (GenBank accession number Q16236), which is highly homologous to the rat Nrf2. It is likely that PKC phosphorylates human Nrf2 at the same site, and that the phosphorylation of Nrf2 observed in human hepatoma HepG2 cells (23) is at least in part catalyzed by PKC on Ser40.

**Binding of Nrf2/MafK Complex to the ARE Is Independent of PKC**  
We and others have previously shown that *in vitro* transcribed/translated Nrf2 binds with high affinity to the ARE as part of a heterodimeric complex with small Maf proteins (9, 24, 25). To examine whether interaction of Nrf2 with the ARE is affected by the mutation abolishing PKC phosphorylation, *in vitro* EMSA with *E. coli*-expressed His$_6$-Nrf2-S40A and *in vitro* translated MafK were performed, using ARE sequence derived from the rat QR gene as probe. As expected, Nrf2 (wild-type or S40A) or MafK alone could not bind to the ARE. In the presence of MafK, however, both Nrf2-wt and Nrf2-S40A formed shifted complexes with the ARE probe. These complexes were supershifted upon incubation with an antibody against Nrf2 (Fig. 4A). The intensity and mobility of the wild-type and S40A mutant complexes are virtually indistinguishable. Furthermore, formation of both types of complexes was completely blocked by excess unlabeled QR ARE but not by random oligonucleotides (Fig. 4B). Therefore the S40A
mutation did not alter the specific high-affinity interaction between Nrf2/MafK and the ARE. Indeed, PKC-phosphorylated Nrf2 bound to the ARE in a similar manner as non-phosphorylated wild-type Nrf2 or the S40A mutant defective for PKC phosphorylation (Fig. 4C). Thus the formation of ARE-binding transcriptional complex containing Nrf2 and small Maf proteins does not appear to be regulated by the phosphorylation of Nrf2 by PKC.

**Keap1 Is Involved in the Impaired ARE Activation by Nrf2-S40A**

To determine whether the Nrf2-S40A mutant has an effect on Nrf2 transactivation of the ARE, we conducted transient transfection experiments in HepG2 cells. Introduction of Nrf2-overexpressing plasmid into a number of hepatoma cell lines has previously been shown to result in dose-dependent activation of the ARE-mediated transcription (16, 24). A rat QR ARE-linked CAT reporter gene was co-transfected with a high-copy plasmid vector bearing wild-type Nrf2. An approximately 12-fold activation of ARE-driven CAT activity was observed using 0.12 µg of Nrf2 plasmid DNA. Co-transfection with an equal amount of Nrf2-S40A resulted in comparable level of activation (Fig. 5).

Since Keap1 has been shown to repress Nrf2 activity by sequestering it in the cytoplasm (12), we asked if any functional defect of the Nrf2-S40A mutant might involve Keap1. As expected, co-transfection of Keap1 with wild-type Nrf2 decreased Nrf2-dependent ARE activation. Interestingly, overexpression of Keap1 resulted in a partial impairment of ARE activation by the Nrf2-S40A mutant that was not seen with endogenous levels of Keap1 in HepG2 cells. ARE activation by Nrf2-S40A was reduced to a level less than 50% of that achieved with wild-type Nrf2, from an approximately 6-fold activation to 2.5-fold (Fig. 5). In these transfected cells, Nrf2 wild-type and S40A proteins were expressed to comparable levels as
verified by Western blot using an anti-Nrf2 antibody (not shown). These findings indicate a role for Keap1 in the apparent transactivation defect exhibited by the Nrf2-S40A mutant.

**Phosphorylation of Nrf2 by PKC Promotes its Dissociation from Keap1**

Studies from Itoh et al. (12) have shown that Nrf2 interacts with Keap1 through a region of about 100 amino acid residues at its N-terminus (named Neh2 domain). Since PKC phosphorylates Nrf2 at Ser40, we explored whether the Keap1-dependent deficiency of the S40A mutant in ARE activation is attributable to its altered interaction with Keap1. We first tested if *in vitro* transcribed/translated Nrf2 and Keap1 proteins could associate with each other. Nrf2 and Keap1 were labeled in the presence of $[^{35}\text{S}]$methionine during separate translation reactions. The products were then incubated together, followed by immunoprecipitation with an anti-Nrf2 antibody, and the precipitated products subjected to SDS-PAGE and autoradiography. Keap1 could be quantitatively co-precipitated with Nrf2 by the anti-Nrf2 antibody (Fig. 6A).

We then investigated the interaction between Keap1 and the Nrf2-S40A mutant. Similar amounts of Keap1 co-precipitated with both wild-type and S40A Nrf2 (Fig. 6B). However, when immunoprecipitation was carried out after incubation of these components in the presence of PKC, the amount of Keap1 associated with wild-type Nrf2 was reduced by about 50%. By contrast, the S40A mutant interacted with Keap1 to a similar extent with or without PKC (Fig. 6B). Furthermore, the dissociation of Keap1 from wild-type Nrf2 was abolished when PKC was preincubated in the presence of 10 nM staurosporine, a potent inhibitor of PKC (not shown). We therefore conclude that phosphorylation of Nrf2 by PKC at Ser40 plays a critical role in facilitating the release of Nrf2 from Keap1. Consequently, the impaired ability of the Nrf2-S40A mutant to activate ARE-mediated transcription is most likely due to a defect in the dissociation of Nrf2 from its cytoplasmic inhibitor Keap1.
**DISCUSSION**

We recently reported that phosphorylation of Nrf2 by PKC induces nuclear translocation of this transcription factor and activation of the ARE in response to oxidative stress (23). In the present study, we further characterized the molecular mechanisms of Nrf2 phosphorylation and its functional significance in ARE activation. Our data demonstrate that PKC phosphorylates Nrf2 at Ser40 and facilitates its release from the cytoplasmic anchor Keap1. Together with our earlier findings, these results suggest a mechanistic model of ARE-mediated cellular antioxidant response involving the PKC-catalyzed phosphorylation of Nrf2 at Ser40 as a specific trigger for the nuclear translocation of this transcription factor.

Previous studies from Itoh et al. (12) using cell-based overexpression systems demonstrated that Nrf2 is normally retained in the cytoplasm by its association with the cytoskeleton-binding protein Keap1. Electrophilic agents liberate Nrf2 from the Nrf2-Keap1 cytosolic complex, allowing it to traverse into the nucleus to activate ARE-driven gene expression. Deletion mapping experiments further indicated that Keap1 binds through the Neh2 domain of Nrf2, comprising approximately 100 N-terminal amino acids (12). Our *in vitro* results here provide a mechanistic explanation for the importance of this domain in Nrf2-Keap1 interaction. A critical residue within this region appears to be Ser40, whose phosphorylation by PKC upon oxidative stress promotes the dissociation of Nrf2 from Keap1. It remains to be tested in intact cells whether Ser40 phosphorylation is sufficient, or whether other molecular events and components must also be involved, for Nrf2 to disengage from its cytoplasmic inhibitor and translocate into the nucleus. It is also not known whether Keap1 itself is a PKC target. Rat Keap1 possesses 5 potential PKC sites, four within the KELCH and C-terminal regions which have been identified as important for binding to Nrf2 (13). However, since we did not observe a reduction in the amount of Keap1 co-precipitated with the Nrf2-S40A mutant upon incubation
with PKC, apparently no PKC-induced alterations occurred on Keap1 that would weaken the Keap1-Nrf2 interaction \textit{in vitro}.

The present findings suggest that the functional significance of PKC phosphorylation of Nrf2 is likely to involve specifically the nuclear-cytoplasmic shuttling step. The defect exhibited by the Nrf2-S40A mutant in ARE-driven reporter gene assay appears to correlate with the amount of the available cytoplasmic inhibitor Keap1. Thus the repression of ARE-mediated gene expression by overexpression of Keap1 became more pronounced in the presence of Nrf2-S40A, presumably due to the dissociation defect of this mutant. However, the formation of ARE-binding complex with Nrf2/MafK in the gel shift assay was not affected by the state of PKC-catalyzed phosphorylation of Nrf2, or when the S40A mutant was used. Therefore PKC phosphorylation does not appear to play a role in either the association of Nrf2/MafK heterodimer with the ARE sequence, or dimerization of Nrf2 with MafK. PKC has also been implicated in the aryl hydrocarbon receptor (AhR)-mediated transcriptional regulation (26, 27). While PKC action in that pathway appears to involve a nuclear event, it does not impact the transcription factor’s DNA-binding activity (26). It will be interesting to determine if the Nrf2 proteins present in the nucleus are predominantly of the phosphorylated form, even though phosphorylation by PKC does not seem to be required for high-affinity binding of Nrf2 to the ARE. The simplest model based upon our results would suggest that phospho-Nrf2, after release from Keap1, is directly transported into the nucleus for binding to the ARE sequence. However, it is possible that yet unidentified protein phosphatases act before the binding of Nrf2 to its target sequence, and promote the export of this transcription factor back into the cytoplasm as part of a regulatory cycle of phosphorylation-dependent nuclear-cytoplasmic shuttling of Nrf2. In studies of other systems, oxidants have been shown to activate kinases and inactivate phosphatases \textit{in concert} (28).
Our prior studies suggesting the involvement of PKC in ARE activation by Nrf2 were performed with pharmacological inhibitors of PKC such as staurosporine. In identifying a site-specific mutant (S40A) that abrogates PKC-catalyzed phosphorylation of Nrf2 and impairs its dissociation from Keap1, we have confirmed an essential role for PKC phosphorylation of Nrf2 in the signaling pathway leading to ARE activation. The present results support a direct role for PKC, rather than its downstream kinases, in the regulation of Nrf2 activity. An important question is whether PKC itself may be a sensor for oxidative stress. An early report showed that catechol and hydroquinone, which are potent inducers of the ARE, also activate PKC (29). PKC possesses structural features that suggest it as an excellent target for direct redox-sensitive modifications (30). Indeed there have been several reports indicating oxidation of reactive cysteines in both its N-terminal regulatory and C-terminal catalytic domains that serves to regulate PKC activity (30). Although it is at present unclear what isoform(s) of PKC phosphorylates Nrf2, a likely candidate may be PKCδ, which has been implicated in a number of cellular processes involving oxidative stress (31, 32).

Reports from several laboratories have indicated the involvement of other kinase pathways in ARE-mediated transcription (17-22). Transfection studies using wild-type and dominant-negative MAPK and Nrf2 have suggested a positive role for MAPK in ARE regulation in HepG2 cells (15, 16). However, inhibition of ERK in H4IIE cells resulted in increased expression of the GSTA2 gene, suggesting a negative role for ERK in ARE-mediated activity (18). The exact role for the p38 kinase in ARE regulation is also confusing from existing literature, as several laboratories have reported a positive effect (17, 19, 20), while another showed that a p38 inhibitor caused the activation of ARE-dependent reporter gene (21). Recently, PI3 kinase and its downstream Akt kinase have been shown by inhibitor studies to be
positive regulators of ARE activity in H4IIE hepatoma (18) and IMR-32 neuroblastoma cells (22). Further elucidation of the precise roles of these kinases will be facilitated by the identification of specific cellular targets known to be involved in ARE regulation. It should be noted that in IMR-32 cells, ARE-mediated transcription has been reported to be PKC-independent (33). However, the same experimental system was also shown to be oxidative-stress independent, in contrast to the many studies from hepatocytes, where oxidative stress-induced ARE-mediated gene expression has been firmly established.

While the EMSA results indicate that Nrf2/MafK interaction with ARE is independent of PKC phosphorylation, it remains possible that binding of Nrf2 with a nuclear factor other than small Maf proteins to the ARE may be subjected to regulation by PKC. Our earlier studies have suggested that such alternative factors may exist, as nuclear extract from HepG2 and H4IIEC3 cells formed complexes with the ARE that are distinct from those formed by in vitro translated Nrf2/MafK proteins (24). There have been several reports of alternative Nrf2 partners binding to the ARE (34-37). CBP (cAMP Responsive Element Binding Protein) has recently been shown to bind Nrf2 via two transactivation domains on Nrf2 in a cooperative manner to activate ARE transcription synergistically (36). ATF4 has also been demonstrated to interact with Nrf2 in regulating ARE-driven heme oxygenase-1 gene expression (37).

It should be noted that while the nuclear translocation of Nrf2 has been shown to be a major mechanism for ARE activation in all cell types examined, Nrf2 also has a documented role in constitutive ARE-mediated gene expression. We have shown that Nrf2 is present in the nucleus without tBHQ stimulation, and its essential role in mediating the basal activity of the ARE has been reported in a cell-free system (24) and in nrf2-null mice (11). Future investigations should reveal molecular details that comprise the coordinated transcriptional
activation of antioxidant enzymes, of which phosphorylation of Nrf2 at Ser40 by PKC is but one of many critical regulatory steps.
REFERENCES

1. Rushmore, T. H. and Pickett, C. B. (1990) J. Biol. Chem. 265, 14648-14653

2. Friling, R. S., Bensimon, A. Tichauer, Y., and Daniel, V. (1990) Proc. Natl. Acad. Sci. USA 87, 6258-6262

3. Favreau, L. V. and Pickett, C. B. (1991) J. Biol. Chem. 266, 4556-4561

4. Li, Y. and Jaiswal, A. K. (1992) J. Biol. Chem. 267, 15097-15104

5. Wild, A. C., Moinova, H. R., and Mulcahy, R. T. (1999) J. Biol. Chem. 274, 33627-33636

6. Alam, J., Stewart, D., Touchard, C., Boinapally, S., Choi, A. M. K., and Cook, J. L. (1999) J. Biol. Chem. 274, 26071-26078

7. Moi, P, Chan, K., Asunis, I., Cao, A., and Kan, Y. W. (1994) Proc. Natl. Acad. Sci. USA 91, 9926-9930

8. Igarashi, K., Kataoka, K., Itoh, K., Hayashi, N., Nishizawa, M., and Yamamoto, M. (1994) Nature (London) 367, 568-572

9. Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., Oyake, T., Hayashi, N., Satoh, K., Hatayama, I., Yamamoto, M., and Nabeshima, Y. (1997) Biochem. Biophys. Res. Commun. 236, 313-322

10. Kwak, M. K., Itoh, K., Yamamoto, M., Sutter, T. R., and Kensler, T. W. (2001) Mol. Med. 7, 135-145

11. McMahon, M., Itoh, K., Yamamoto, M., Chanas, S. A., Henderson, C. J., McLellan, L. I., Wolf, C. R., Cavin, C., and Hayes, J. D. (2001) Cancer Res. 61, 3299-3307

12. Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J. D., and Yamamoto, M. (1999) Genes Dev. 13, 76-86

13. Dhakshinamoorthy, S. and Jaiswal, A. K. (2001) Oncogene 20, 3906-3917
14. Sekhar, K. R., Spitz, D. R., Harris, S., Nguyen, T. T., Meredith, M. J., Holt, J. T., Guis, D., Marnett, L. J., Summar, M. L., and Freeman, M. L. (2002) *Free Radic. Biol. Med.* **32**, 650-662
15. Yu, R., Lei, W., Mandlekar, S., Weber, M. J., Der, C. J., Wu, J., and Kong, A.-N. T. (1999) *J. Biol. Chem.* **274**, 27545-27552
16. Yu, R., Chen, C., Mo, Y. Y., Hebbar, V., Owuor, E. D., Tan, T.-H., and Kong, A.-N. T. (2000) *J. Biol. Chem.* **275**, 39907-39913
17. Zipper, L. M. and Mulcahy, R. T. (2000) *Biochem. Biophys. Res. Commun.* **278**, 484-492
18. Kang, K. W., Cho. M. K., Lee, C. H., and Kim, S. G. (2001) *Mol. Pharmacol.* **59**, 1147-1156
19. Kang, K. W., Ryu, J. H., and Kim, S. G. (2000) *Mol. Pharmacol.* **58**, 1017-1025
20. Alam, J., Wicks, C., Stewart, D., Gong, P., Touchard, C., Otterbein, S., Choi, A. M., Burow, M. E., and Tou, J. (2000) *J. Biol. Chem.* **275**, 27694-27702
21. Yu, R., Mandlekar, S., Lei, W., Fahl, W. E., Tan, T.-H., and Kong, A.-N. T. (2000) *J. Biol. Chem.* **275**, 2322-2327
22. Lee, J.-M., Hanson, J. M., Chu, W. A., and Johnson, J. A. (2001) *J. Biol. Chem.* **276**, 20011-20016
23. Huang, H.-C., Nguyen, T., and Pickett, C. B. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 12475-12480
24. Nguyen, T., Huang, H.-C., and Pickett, C. B. (2000) *J. Biol. Chem.* **275**, 15466-15473
25. Dhakshinamoorthy, S. and Jaiswal, A. K. (2000) *J. Biol. Chem.* **275**, 40134-40141
26. Chen, Y.-H. and Tukey, R. H. (1996) *J. Biol. Chem.* **271**, 26261-26266
27. Long, W. P., Chen, X., and Perdew, G. H. (1999) *J. Biol. Chem.* **274**, 12391-12400
28. Meng, T. C., Fukada, T., and Tonks, N. K. (2002) *Mol. Cell* **9**, 387-399
29. Gopalakrishna, R., Chen, Z.-H., and Gundimeda, U. (1994) *Proc. Natl. Acad. Sci. USA* 91, 12233-12237

30. Gopalakrishna, R. and Jaken, S. (2000) *Free Rad. Biol. & Med.* 28, 1349-1361

31. Jain, N., Zhang, T., Kee, W. H., Li, W., and Cao, X. (1999) *J. Biol. Chem.* 274, 24392-24400

32. Sun, X., Wu, F., Datta, R., Kharbanda, S., and Kufe, D. (2000) *J. Biol. Chem.* 275, 7470-7473

33. Lee, J.-M., Moehlenkamp, J. D., Hanson, J. M., and Johnson, J. A. (2001) *Biochem. Biophys. Res. Commun.* 280, 286-292

34. Montano, M. M., Wittmann, B. M., and Bianco, N. R. (2000) *J. Biol. Chem.* 275, 34306-34313

35. Zhu, M. and Fahl, W. E. (2001) *Biochem. Biophys. Res. Commun.* 289, 212-219

36. Katoh, Y., Itoh, K., Yoshida, E., Miyagishi, M., Fukamizu, A., and Yamamoto, M. (2001) *Genes Cells* 6, 857-868

37. He, C. H., Gong, P., Hu, B., Stewart, D., Choi, M. E., Choi, A. M., and Alam, J. (2001) *J. Biol. Chem.* 276, 20858-20865
FOOTNOTES

1. The abbreviations used are: ARE, antioxidant response element; Nrf2, NF-E2-related factor 2; PKC, protein kinase C; MAP kinase, mitogen-activated protein kinase; ERK1/2, extracellular signal-regulated kinase; PI3 kinase, phosphatidylinositol 3-kinase; PMA, phorbol 12-myristate 13-acetate; tBHQ, tert-butylhydroquinone; QR, NAD(P)H:quinone oxidoreductase; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay.
ACKNOWLEDGMENTS

We thank Dr. Philip Sherratt for useful advice and discussions.
FIGURE LEGENDS

Fig. 1. Peptide Inhibition of Nrf2 Phosphorylation by PKC.  A. Amino acid sequence of rat Nrf2. Seven potential PKC phosphorylation sites (S/T-X-R/K) are shown in bold. Synthetic peptides used to mimic these sites are underlined.  B. In vitro kinase assays were performed using 2 μM purified Nrf2, 5 nM catalytic subunit of rat brain PKC, 2 μCi [γ-33P]ATP, in the presence of 5 mM of the indicated peptides, for 20 min at 30°C. 33P-labeled Nrf2 was resolved by SDS-PAGE and subjected to autoradiography.

Fig. 2. Expression and Purification of His6-tagged Nrf2-S40A Protein. Nrf2-S40A (AGT→GCT) mutant was cloned into pQE30 expression vector (Qiagen). Nrf2-S40A fusion protein containing a His6 tag at the N-terminus was overexpressed in E. coli upon induction for 6 hr at 30°C in the presence of 0.5 mM IPTG, and purified by Ni-NTA affinity chromatography. Coomassie brilliant blue G-250 stain of samples resolved by SDS-PAGE is shown: total lysate before (lane 1) and after (lane 2) IPTG induction; soluble lysate loaded on Ni-NTA column (lane 3); Ni-NTA column flow-through (lane 4); 40 mM imidazole wash (lane 5); 250 mM imidazole elution (lane 6). Protein band indicated by arrow was confirmed to be Nrf2 by Western blot with an anti-Nrf2 antibody (not shown).

Fig. 3. PKC phosphorylates Nrf2 at Ser40. PKC activity was assayed as in Fig. 1B using 2 μM purified wild-type Nrf2 or Nrf2-S40A mutant protein as substrate for the indicated times at 30°C. Western blot using an anti-Nrf2 antibody was performed for an aliquot of the same samples as those shown in the autoradiogram above.

Fig. 4. Binding of Nrf2/MafK to the ARE Is PKC-independent. Electrophoretic mobility shift assays (EMSA) were performed as described under Experimental Procedures using 32P-labeled rat QR ARE sequence as probe. His6-Nrf2 wild-type and S40A proteins were purified
from *E. coli* as described in Fig. 2. *In vitro* transcribed/translated MafK proteins were produced from TNT coupled wheat germ extract system (Promega). **A.** Supershift analysis was carried out by adding an anti-Nrf2 antibody to the incubation mixture for 4 hr at 4°C prior to electrophoresis. **B.** Competition experiments were performed in the presence of 200-fold molar excess of unlabeled oligonucleotides containing the rat *QR* ARE or a random sequence. **C.** EMSA was preceded by incubating Nrf2 wild-type and S40A proteins for 1 hr at 30°C in a PKC kinase assay buffer with or without PKC.

**Fig. 5. Defective ARE Activation by Nrf2-S40A Involves Keap1.** Rat *QR* ARE-CAT reporter construct (1 µg) was co-transfected with pcDNA3 expression plasmid (0.12 µg) bearing wild-type or S40A Nrf2 into HepG2 cells on 6-well tissue culture plates. Where indicated, 60 ng of Keap1-pcDNA3 plasmid was co-transfected. CAT assays were performed as previously described, and the results quantitated by a PhosphorImager. Activity is shown as fold activation over the level obtained with transfection of an empty pcDNA3 vector in the absence of Keap1 plasmid. The data represent means of three independent experiments.

**Fig. 6. PKC-dependent Interaction between Nrf2 and Keap1.** **A.** Nrf2 (wild-type) and Keap1 were *in vitro* transcribed and translated in the presence of [35S]methionine and incubated together where indicated for 15 min at 30°C before immunoprecipitation by an antibody against Nrf2. Amount of Keap1 used in incubation with Nrf2 was doubled in the last lane. Precipitated products were washed, resolved by SDS-PAGE, and autoradiographed. **B.** Similar immunoprecipitation experiments as in **A** were performed using wild-type or S40A Nrf2 and Keap1. A 30 min incubation of Nrf2 and Keap1 at 30°C in a PKC assay buffer in the presence or absence of PKC preceded immunoprecipitation. Band intensity was quantified by a PhosphorImager. The amounts of Nrf2 precipitated were normalized, and the amount of Keap1
that co-precipitated under each condition was expressed relative to that co-precipitated with Nrf2-WT without PKC (set as 100). The results shown are typical of at least three separate experiments.
A

1  MMDLELPFG  LQSQQDMDSL  DILWRQDIDL  GVSREVFDFS  QRQKDYELEK
51  QKKLEKERQE  QLQKEQKAF  FAQLQLDEET  GEFLPIQPAQ  HIQTDTSGSV
101  SYSQVAHIPK  QDALYFEDCM  QLAAETFPPV  DDHESLALDI  PSHVESSVFT
151  TPDQAQSLSDS  SLETAMTLSI  SQQDMEQVW  QELFSIPELQ  CLNTEKQQA
201  ETTTVPSPEA  TLTEMDNSNYH  FYSSIPSLEK  EVDSCSPHFL  HGFEDSFSSI
251  LSTDDASQLN  SLDSNPTLNT  DFGDEFYSAF  LAEPSGGGS  PSSAASQSL
301  SELLGPIEIG  CDLSLCKAFN  QKHTEGTVEF  NDSDSGISLN  TSPSRASPEH
351  SVESSYGDPS  PPFSFSDEME  ELDSAPGSVK  QNGPAQTPH  SSGDTVQPLS
401  PAQGHSAAVH  ESQCENTKK  EVPNPSGHQK  VPTKDKHSS  RLEAHLTRDE
451  LRAKALHIPF  PVEKINLPV  DDFNEMMSKE  QFNEAQLALI  RDIRRRGKNK
501  VAAQNRKRK  LENIVELEQG  LGLKNDERK  LLREKENDR  NLHLLKRLS
551  TLYLEVFSML  RDEDGKPYSP  SEYSSLQQTRD  GNVFLVPKSK  KPDTKKN

B

| Peptide | none | 35-44 | 375-382 | 414-421 | 435-442 | 585-592 | 590-597 |
|---------|------|-------|---------|---------|---------|---------|---------|
| Phosphorylated Nrf2 | | | | | | | |
Fig. 2

MW (kD)  1  2  3  4  5  6

[Image of a gel electrophoresis pattern with MW markers and His$_6$-Nrf2S40A indicated]
Fig. 3

Time (min) | WT | S40A
---|---|---
0 | 5 | 15 | 30 | 60 | 0 | 5 | 15 | 30 | 60

\[^{33}P\]Nrf2

His\textsubscript{6}-Nrf2

\[ \text{Nrf2 Phosphorylation by PKC} \]

\[ \text{time (min)} \]
| A | B | C |
|---|---|---|
| **His$_{6}$-Nrf2** | WT | S40A |
| MafK | - | - | + | + | + | + | - |
| Anti-Nrf2 | - | - | - | - | + | + | - |

**Fig. 4**
Fig. 5

Bar graph showing Relative CAT Activity for different conditions: pNrf2 only and pNrf2 + pKeap1.

- **vector**: Low activity for both conditions.
- **Nrf2-wt**: Higher activity compared to vector, with a peak in pNrf2 + pKeap1.
- **Nrf2-S40A**: Lower activity compared to vector and Nrf2-wt, with a slight increase in pNrf2 + pKeap1.

The y-axis represents Relative CAT Activity, ranging from 0 to 15.
Fig. 6

**A**

| IP: ctrl | Anti-Nrf2 |
|----------|-----------|
| $^{35}$S-Nrf2 | + + - + + + |
| $^{35}$S-Keap1 | + - + + ++ |

Amount of Keap1 co-precipitated with Nrf2

**B**

| Nrf2 | WT S40A | WT S40A | PKC | - | - | + | + |
|-------|---------|---------|-----|---|---|---|---|

Relative Intensity (Arbitrary Units)