Published in final edited form as:

Free Radic Biol Med. 2007 June 15; 42(12): 1797–1806.

MOLECULAR MECHANISM OF HUMAN NRF2 ACTIVATION AND DEGRADATION: ROLE OF SEQUENTIAL PHOSPHORYLATION BY PROTEIN KINASE CK2

Jingbo Pi^{*,†}, Yushi Bai[†], Jeffrey M. Reece[‡], Jason Williams[§], Dianxin Liu[‡], Michael L. Freeman[¶], William E. Fahl[¶], David Shugar[#], Jie Liu^{*}, Wei Qu^{*}, Sheila Collins[†], and Michael P. Waalkes

*Laboratory of Comparative Carcinogenesis, NCI at NIEHS, NIH, RTP, North Carolina 27709; †Endocrine Biology Program, The Hamner Institutes for Health Sciences, RTP, North Carolina 27709; ‡Laboratory of Signal Transduction, NIEHS, NIH, RTP, North Carolina 27709; §Laboratory of Structure Biology, NIEHS, NIH, RTP, North Carolina 27709; Department of Radiation Oncology, Vanderbilt-Ingram Cancer Center, Vanderbilt University, Nashville, Tennessee 37232; ¶McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin, 53706; #Institute of Biochemistry & Biophysics, Polish Academy of Sciences, 02-106 Warsaw, Poland.

Abstract

Nrf2 is a key transcription factor in the cellular response to oxidative stress. In this study we first identify two phosphorylated forms of endogenous human Nrf2 after chemically-induced oxidative stress and provide evidence that protein kinase CK2-mediated sequential phosphorylation plays potential role in Nrf2 activation and degradation. Human Nrf2 has a predicted molecular mass of 66 kDa. However, immunoblots showed that two bands at 98 and 118 kDa, which are identified as phosphorylated forms, are increased in response to Nrf2 inducers. In addition, human Nrf2 was found to be a substrate for CK2 which mediated two steps of phosphorylation, resulting in two forms of Nrf2 migrating with differing Mr at 98 kDa (Nrf2–98) and 118 kDa (Nrf2–118). Our results support a role in which calmodulin binding regulates CK2 activity, in that cold (25 °C) in Ca²⁺-free media (cold/Ca²⁺-free) decreased both cellular calcium levels and CK2-calmodulin binding and induced Nrf2–118 formation, the latter of which was prevented by CK2 specific inhibitors. Gel-shift assays showed that the Nrf2–118 generated under cold/Ca²⁺-free conditions does not bind to the antioxidant response element, indicating that Nrf2–98 has transcriptional activity. In contrast, Nrf2–118 is more susceptible to degradation. These results provide evidence for phosphorylation by CK2 as a critical controlling factor in Nrf2-mediated cellular antioxidant response.

Keywords

Nrf2; CK2; phosphorylation; oxidative stress; arsenic

Corresponding Author: Michael P. Waalkes, Ph.D, NCI at NIEHS, National Institutes of Health, MD F0-09, 111 Alexander Dr., Research Triangle Park, NC 27709, USA. Phone: (919) 541-2328; Fax: 919-541-3970. E-mail: waalkes@niehs.nih.gov.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

INTRODUCTION

Excessive and/or sustained increases in reactive oxygen species (ROS) production or electrophilic stress are implicated in the pathogenesis of many chronic diseases including cancer, diabetes, atherosclerosis, Alzheimer's, and Parkinson's, as well as in aging [1]. Induction of a family of oxidative stress related genes that protect against the damage by electrophiles and ROS is a key element in the maintenance of cellular redox homeostasis and in reducing oxidative damage [2,3]. These genes, encoding for various detoxification and antioxidant enzymes, are coordinately regulated through consensus *cis*-elements called antioxidant response elements (AREs) or electrophile-response elements (EpREs) in their 5'-flanking promoter regions [2]. Transcription factor NF-E2-related factor 2 (Nrf2), a member of the Cap'n' Collar family of bZIP proteins, is a central regulator in both constitutive and inducible ARE-related gene expression [2,3]. Nrf2 knockout mice show a deficiency in this coordinated genetic program and have a higher susceptibility to oxidative damage [4–6].

Nrf2 is ubiquitously expressed in a wide range of tissues and cell types. Previous studies [7], including our own recent results [8], suggest Nrf2 may be regulated at multiple levels by a coordinated process. Cytoplasm-nuclear translocation, up-regulated transcription, and/or decreased degradation have been proposed as mechanisms for Nrf2 activation [2,9]. In addition, extensive studies have demonstrated that protein phosphorylation is a potential mechanism for the activation of Nrf2-ARE mediated pathways [2,7]. To date, several cytosolic kinases, including protein kinase C (PKC) [10,11], phosphatidylinositol 3-kinase (PI3K) [12], mitogen-activated protein kinase (MAPK) [13], and ER-localized pancreatic endoplasmic reticulum kinase (PERK) [14,15] have been shown to modify Nrf2 and potentially involve in Nrf2-mediated signal transduction at AREs. Phosphorylation of Nrf2 at Ser-40 through a PKC-based mechanism has been reported to play a critical role in the dissociation of Nrf2 from Keap1, an negative regulator of Nrf2 [10,11]. The PI3K pathway regulates the rearrangement of actin microfilaments and depolymerization of actin facilitates the nuclear translocation of Nrf2 [12]. Furthermore, MAPK pathways activated by extracellular signal-regulated kinase kinase 1, transforming growth factor-beta-activated kinase, and apoptosis signal-regulating kinase are reported to be signaling pathways for Nrf2 activation [13]. Quite recently, it was shown that the accumulation of unfolded proteins in endoplasmic reticulum activates Nrf2 via the direct phosphorylation of Nrf2 by PERK [14,15]. Though Nrf2 can potentially be phosphorylated at multiple sites by different protein kinases, and such phosphorylation seems to be an important part of Nrf2-mediated ARE activation, the identity of the activating protein kinase(s) involved remains elusive. Furthermore, to date, endogenously phosphorylated forms of Nrf2 have not been isolated *in vivo* or *in vitro*.

Protein kinase CK2 is a highly conserved protein kinase with a growing list of more than 300 substrates, the majority of which are proteins implicated in nuclear functions such as signal transduction, gene expression, RNA synthesis, ubiquitination and cell survival [16,17]. Although CK2 is functionally pleiotrophic and appears to be central to many of the molecular switches that protect the cell against stress [18], a possible role for CK2 in Nrf2 regulation has not been defined. In the present study, we first identified two endogenous phosphorylated forms of Nrf2 in human cells and then provided evidence that protein kinase CK2-mediated Nrf2 phosphorylation plays a pivotal role in Nrf2 activation as well as degradation. These data provide compelling evidence for phosphorylation by CK2 as a critical controlling factor in the Nrf2-mediated cellular response to electrophilic or oxidative stress.

MATERIALS AND METHODS

Chemicals

Sodium *m*-arsenite (NaAsO₂), *tert*-butyl hydroxyquinone (tBHQ), isopropyl-1-thio- β -D-galactopyranoside, Ly294002, wortmannin, 5,6-dichlorobenzimidazole riboside (DRB) and calmodulin (CaM) were obtained from Sigma (St. Louis, MO). 4,5,6,7-tetrabromobenzotriazole (TBB) was synthesized as described previously [19]. Phosphatases including λ -PPase, PP1, LAR, and YOP, as well as recombinant human CK2, were purchased from New England BioLabs, Inc. (Beverly, MA). Genistein, staurosporine, SB203580, and calphostin C were obtained from Merck Biosciences AG (Laefelfingen, Switzerland). Antibodies for Nrf2 (H-300, C-20, T-19), calmodulin (CaM), Sam 68, β -actin, CK2 α , α' , and β subunits were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Plasmid Construction and Recombinant Nrf2 Expression

The cDNA encoding full length human Nrf2 (NM_006164) in the pCMV6-XL4 vector was purchased from Origene Technologies, Inc. (Rockville, MD). The *Escherichia coli* expression plasmid for glutathione S-transferase (GST) fusion protein containing full-length human Nrf2 were constructed by subcloning the human Nrf2 cDNA into a pGEX4T1 (Amersham Biosciences, Piscataway, NJ) fusion expression vector. pGEX4T1-Nrf2 was introduced into *Escherichia coli* BL21 (DE3) (Stratagene, La Jolla, CA) by transformation and the cells were cultured at 37 °C for 16 hrs. After the *E. Coli* cells were treated with 1 mM of isopropyl-1-thio- β -D-galactopyranoside for 2.5 hrs, the cells were harvested and then lysed by sonication. Recombinant GST-Nrf2 was purified by RediPack GST Purification Modules (Amersham Biosciences, Piscataway, NJ) and the GST part was cleaved by thrombin protease (Amersham Biosciences, Piscataway, NJ) on column according to the manufacturer's recommendation.

Cell Culture, Transfection and Point Mutation

The human keratinocyte (HaCaT) cell line is a spontaneously immortalized human epithelial cell line developed by Boukamp *et al.* [20]. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U penicillin/ml, and 100 μ g streptomycin/ml. Cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere. HepG2, H69, G-361, HUV-EC and COS-7 cell lines were obtained from ATCC (Manassas, VA) and were cultured according to the manufacturer's recommendation. Transfection and point mutation were conducted by Effectene Transfection Reagent (QIAGEN Inc., Valencia, CA) and QuickChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), respectively.

Preparation of Protein Extracts and Western Blotting

The whole cell extracts were obtained by using Cell Lysis Buffer (Cell Signaling Technology, Inc., Beverly, MA) with 0.5% of Protease Inhibitor Cocktail (Sigma) and 1% of Phosphatase Inhibitor Cocktail I (Sigma). Nuclear and cytosolic fractions were separated by TransFactor Extraction Kit (BD Biosciences Clontech, Palo Alto, CA). All the protein fractions were stored at -70 °C until use. Proteins were separated by Novex 4–12%, 4–20% or 6% Tris-Glycine Gel (Invitrogen, Carlsbad, CA) and transferred onto nitrocellulose membranes. The blots were probed with the primary antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Antibody incubations were performed in Blocker^{TM} BLOTTO in TBS (Pierce, Rockford, IL). Immunoreactive proteins were detected by chemiluminescence using ECL reagent (Amersham Pharmacia, Piscataway, NJ) and subsequent autoradiography. Quantitation of the results was performed by Bio-Rad Gel Doc 2000^{TM} Systems with Bio-Rad TDS Quantity One software.

Nrf2 Immunoprecipitation and Phosphorylation of Nrf2 by CK2 in vitro

Nuclear extracts (20 mg protein) from arsenic-treated HaCaT cells were precleared by incubation with 50 μ l of rabbit serum and 100 μ l of protein G-Sepharose beads (50% slurry) for 30 min at 4°C. After centrifugation at 3,000 $\times$ g for 1 min, the supernatants were incubated with the antibody against Nrf2 (H-300, 40 μ g) overnight at 4°C with rotation. Following incubation of the preparation with 200 μ l of protein G beads for 2 hrs at 4°C with rotation, the beads were washed four times with 1 ml of immunoprecipitation buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 10% Glycerol, 1% Igepal with protease and phosphatase inhibitors) on ice. The beads were then resuspended with 200 μ l of the immunoprecipitation buffer and 5 μ l was used in each assay as the substrate of human CK2.

Recombinant human Nrf2 (3 μ g) or immunoprecipitation products were incubated with recombinant human CK2 in CK2 buffer (pH 7.2) containing 20 mM MOPS, 25 mM β -glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 25 mM magnesium chloride, 100 μ M ATP, 0.2 mM substrate peptide, 0.4 μ M PKA inhibitor cocktail, and 100 μ Ci of [γ -³²P]ATP (PerkinElmer Life Sciences, Inc., Boston, MA) for 5 and 30 min at 37 °C. Proteins were then resolved by SDS-PAGE and visualized by autoradiography.

Monitor of cellular calcium mobilization

Intracellular Ca²⁺ was monitored with the fluorescent probe Fluo-4 acetoxymethylester (Fluo-4 AM, Molecular Probes, Eugene, OR). HaCaT cells were grown on glass bottom culture dishes (MatTek, Ashland, MA) for 24 hrs and then incubated for 20 min at 37 °C in serum-free medium containing fluo 4 AM (2 μ M) and Pluronic F-127 (0.2 mg/ml; Molecular Probes). Then cells were viewed immediately with a laser scanning confocal microscope (LSM 510 NLO) mounted on an Axiovert 100M microscope (Carl Zeiss, Inc., Thornwood, NY). The images were obtained simultaneously using the 488 nm line from the included Ar laser as the excitation source, and the Zeiss Plan-Apo 63x oil N.A.=1.4 as objective lens. For the fluorescence emission, a LP 505 nm long pass filter was used, along with a pinhole of 0.6 Airy units, corresponding to a z-resolution of 0.6 microns. All fluorescent images in the same figure were acquired with the same settings. The software used for acquisition was Zeiss LSM510 ver 3.2 for Windows 2000.

Co-immunoprecipitation of CaM and CK2 Catalytic Subunits and CK2 Kinase Assay

The nuclear extracts (3 mg protein) were immunoprecipitated with CaM antibody (5 μ g) as above. The immunoprecipitation products were detected by immunoblots with CK2 α and CK2 α' antibodies. *In vitro* CK2 kinase assay was conducted using recombinant human CK2. After CK2 (5 U/each tube) was incubated with CaM (150 U) and/or calcium (Ca²⁺, 1 mM) at 37°C for 30 min, the activities were determined with the Casein Kinase 2 Assay Kit (Upstate, Lake Placid, NY).

Gel shift assay

Single-stranded oligonucleotides (Sigma-Genosys, Woodlands, TX) were annealed by PCR to form consensus oligomers containing the γ -glutamyl cysteine synthetase (γ -GCS_h) promoter specific Nrf2 -binding site (ARE, underlined): (5'-GCACAAAGCGCTGAGTCACGGGGAGG-3'; 5'-GTCCCCGTGACTCAGCGCTTTGTGCG - 3'). Gel shift was performed by incubating nuclear extracts (5 μ g protein) for 20 min at RT with 8 fmol of ³²P-labeled double-stranded oligonucleotides in binding buffer (50 mM Tris, pH 7.5, 13 mM MgCl₂, 1mM EDTA, 5% glycerol, 1mM dithiothreitol). Antibody against Nrf2 was added for the supershift for 30 min at 4°C before the incubation with oligonucleotides. The DNA-protein complex was separated in 6% native polyacrylamide gels in 0.5% Tris-Glycine running buffer, dried under vacuum

and followed by autoradiography. Quantitation of the results was performed by Bio-Rad Gel Doc 2000™ Systems with Bio-Rad TDS Quantity One software.

Statistics

Data are expressed as mean $\pm$ SEM of $n = 3$ to 6 in all cases. A one-way ANOVA, followed by Dunnett's multiple comparison test, was used to compare the treated groups to the control. A P value of ≤ 0.05 was considered significant in all cases. All the images showed are representatives of 3 to 6 experiments.

RESULTS

Endogenous human Nrf2 is a phosphoprotein

Human Nrf2 contains 605 amino acids with a predicted molecular mass of 66 kDa. However, prior work has shown that transcription and translation of the full-length Nrf2 cDNA produces a major band at approximately 96 kDa, a discrepancy likely due to an abundance of acidic residues in Nrf2, leading to anomalous migration in SDS/PAGE [21]. Unexpectedly, our immunoblots (Fig. 1A) showed two clear bands with apparent M_r of 98 and 118 kDa increased in response to oxidant stress resulting from treatment with As or tBHQ, two well-known chemical inducers of Nrf2, in nuclear and cytosolic fractions from HaCaT cells, a human keratinocyte line. Although other bands were observed, such as NS85 in nuclear and NS85 and NS41 in cytosolic fractions, these did not correspond to Nrf2 and were unaltered by As or tBHQ exposure (Fig. 1A). They likely represent non-specific (NS) binding of the antibody used for western blot analysis. The two Nrf2 bands in HaCaT cells were also observed in a variety of other human epithelial or mesenchymal cell lines including HepG2, H69, G-361 and HUV-EC cells, particularly after As exposure (Fig. 1B). Furthermore, forced over-expression of Nrf2 by transfection into COS-7 and HaCaT cells produces the 98 and 118 kDa Nrf2 bands (Fig. 1C). Importantly, the two Nrf2 bands are detected by two distinct Nrf2 antibodies (H-300 and C-20, Santa Cruz).

To ascertain if the bands with higher apparent M_r might represent phosphorylated forms of Nrf2, we treated the nuclear fractions isolated from As-treated HaCaT cells, which contain high levels of Nrf2, with different phosphatases (Fig 2). The dual specific λ protein phosphatase (λ -PPase) removes phosphate from serine (Ser), threonine (Thr) and tyrosine (Tyr) residues and is inhibited by EDTA and vanadate. PP1 is a protein phosphatase with activity towards phosphoSer/Thr residues. LAR and YOP are protein phosphatases that remove phosphate specifically from Tyr residues. As shown in Fig. 2, λ -PPase treatment resulted in the loss of the two higher M_r Nrf2 bands and the appearance of a new, faster migrating band (apparent M_r approximately 92 kDa). Inclusion of the phosphatase inhibitors vanadate (as Na_3VO_4) and EDTA prevented this change. Similar results were observed after exposure to PP1 but not with LAR and YOP. Thus, the higher M_r bands are clearly due to phosphorylation occurring at Ser and/or Thr sites and both Nrf2 bands typically detected by western blots are phosphorylated.

Nrf2 is phosphorylated by CK2

Phosphoacceptor sites in proteins phosphorylated by CK2 are specified by multiple acidic residues, with the acidic residue at position +3 relative to the target Ser/Thr phosphoacceptor residue being of particular relevance [17]. Human Nrf2 is an acidic protein containing 17.1% acidic residues (Supplement 1). Our Prosite search revealed the presence of thirteen putative CK2 sites in human Nrf2, eleven of which are highly conserved among species (Supplement 1). This suggests Nrf2 is a potential substrate for CK2. To determine if CK2 is involved in the phosphorylation of Nrf2, CK2 kinase assays were performed using purified, recombinant, human Nrf2 and Nrf2 immunoprecipitation products from the nuclear extracts of acute arsenic-treated HaCaT cells as the substrates. As shown in Fig. 3A, two clear phosphorylated bands at

the same apparent Mr (98 and 118 kDa) as phosphorylated endogenous Nrf2 were formed using recombinant human Nrf2 as substrate in a CK2-dependent fashion. In addition, two similar [γ - ^{32} P]ATP incorporated bands were detected using Nrf2 immunoprecipitation products of human cells as the substrates (Fig. 3B). Furthermore, MALDI-MS and MALDI-MS/MS analyses identified two bands isolated from CK2-treated recombinant human Nrf2 (Fig. 3C) with apparent Mr approximately 98 and 118 kDa as human Nrf2 (accession # 20149576). These data indicate that human Nrf2 is a CK2 substrate and that CK2 mediates two steps of Nrf2 phosphorylation (Nrf2 $\rightarrow$ Nrf2-98 $\rightarrow$ Nrf2-118). Individual points mutations of each of the highly conserved putative CK2 phosphorylation sites (Supplement 1) did not affect the migration of the two Nrf2 bands (data not shown), suggesting that multiple sites may be phosphorylated and that loss of phosphorylation at any single site is not sufficient to alter the apparent Mr of the phosphorylated Nrf2 forms.

Furthermore, to examine whether constitutive CK2 activity is critical for Nrf2 activation in response to oxidative stress, we examined acute arsenic-induced nuclear Nrf2 accumulation in HaCaT cells pretreated with two specific CK2 inhibitors. One of these is DRB, which is widely employed as a CK2 inhibitor, although its selectivity has not been strictly defined. We have therefore also employed TBB, which is not only a more potent ATP-competitive CK2 inhibitor [19,22], but also, when tested against a panel of more than 30 Ser/Thr and Tyr protein kinases, exhibited very high selectivity for CK2. TBB or DRB pretreatment dramatically reduced arsenic-induced nuclear Nrf2 accumulation (Supplement 2A). In addition, inhibitors of PI3 kinase (Ly294002 or wortmannin) and MAP kinase p38 (SB203580), but not PKC (staurosporine or calphostin C), also marginally inhibited the arsenic-induced Nrf2 accumulation. These results suggest that multiple kinases may be involved in arsenic-induced Nrf2 activation.

CK2 activation causes endogenous Nrf2 phosphorylation

Although the mechanism by which CK2 activity is regulated is unknown, a search of the calmodulin (CaM) Target Database (<http://calcium.uhnres.utoronto.ca>) indicates a highly conserved CaM binding site in CK2 catalytic subunits α and α' (Supplement 3). In fact, our results show CaM inhibits purified CK2 kinase activity in a Ca^{2+} -dependent fashion (Fig. 4A), suggesting Ca^{2+} mobilization may affect CK2 activity. Further, HaCaT cells exposed to cold shock (25 °C) in Ca^{2+} -free conditions (termed cold/ Ca^{2+} -free conditions) quickly lose intracellular Ca^{2+} (Fig. 4B) while cold shock or Ca^{2+} -free alone only marginally decreases intracellular Ca^{2+} (not shown). When the HaCaT cells pretreated with As to induce Nrf2 were then exposed to cold/ Ca^{2+} -free conditions, the binding of CaM-CK2 α' was markedly decreased in the nuclear fraction (Fig. 4C), although the nuclear protein levels of CK2 subunits α , α' , β and CaM showed no change. In addition, As-exposure alone increased CaM-CK2 α' binding compared with control cells. These data strongly suggest that CaM is a Ca^{2+} -dependent negative regulator of CK2 activity.

Accordingly, cold/ Ca^{2+} -free conditions caused the complete loss of the Nrf2-98 band and a coordinate increase of the Nrf2-118 band (Fig. 5A). Cold shock or Ca^{2+} -free conditions alone only modestly increases the ratio of Nrf2-118 to Nrf2-98. Furthermore, the change of Nrf2 induced by cold/ Ca^{2+} -free conditions was totally prevented by the CK2 specific inhibitors TBB or DRB. However, inhibitors of other protein kinases such as PI3K (Ly294002 or wortmannin), MAP kinase p38 (SB203580), and PKC (staurosporine or calphostin C) did not impact disappearance of Nrf2-98 induced by cold/ Ca^{2+} -free conditions (Supplement 4). In addition, as shown in Fig. 5B, forced over-expression of Nrf2 by transfection into COS-7 cells which were pretreated with CK2 inhibitor DRB resulted in a significantly lowered ratio of the two bands (Nrf2-118/Nrf2-98).

CK2-mediated phosphorylation is potentially involved in Nrf2 activation and degradation

Pretreatment by As dramatically increases nuclear Nrf2 accumulation (both Nrf2–98 and Nrf2–118) in HaCaT cells while cold/ Ca^{2+} -free conditions induce a loss of Nrf2–98 and a concomitant increase in Nrf2–118 (Fig. 6A). A gel-shift assay using an oligonucleotide containing the human γ -GCS_h ARE showed Nrf2–98, but not Nrf2–118 generated by cold/ Ca^{2+} -free conditions, has ARE binding activity (Fig. 6B). It should be noted that we could not have a clear supershift band using commercial available Nrf2 antibodies, although the anti-Nrf2 (H-300) can reduce the DNA banding (data not shown).

Nrf2 has been reported as a target for proteasome-mediated degradation, and increased protein stability is a post-transcriptional mechanism that enhances Nrf2-mediated induction of the antioxidant response [7]. To examine the contribution of phosphorylation in endogenous Nrf2 clearance, Nrf2 was transiently induced in HaCaT cells by As, and the cells were then exposed to cold/ Ca^{2+} -free conditions. The cells were then placed under normal culture conditions and the turnover of Nrf2 was examined by immunoblot (Fig. 6C). The Nrf2 accumulated in HaCaT cells after As treatment was phosphorylated by cold/ Ca^{2+} -free conditions. The phosphorylation promotes Nrf2 clearance (Fig. 6D), which was totally blocked by the treatment with proteasome inhibitors MG115 and MG132 (data not shown), indicating that Nrf2 is rapidly degraded through the proteasome pathway. Together, these results indicate that cold/ Ca^{2+} -free conditions induced CK2 activation which results in ‘hyper-phosphorylation’ of Nrf2 thereby decreasing Nrf2 transcriptional activity and accelerating Nrf2 degradation.

DISCUSSION

Nuclear accumulation of Nrf2 is one mechanism, and apparently an essential mechanism, by which Nrf2 acts as a transcriptional activator [7]. Although multiple mechanisms appear to be involved in Nrf2 activation, including both transcriptional and post-transcriptional events, these are incompletely defined. The results of the present study provide evidence that endogenous human Nrf2 is constitutively phosphorylated, that the extent of this phosphorylation controls, at least in part, activity and degradation, and that CK2 is the major kinase for this phosphorylation. Furthermore, electrophilic- or ROS-induced nuclear Nrf2 accumulation is highly dependent on constitutive CK2 activity, suggesting CK2 is critical in regulating Nrf2-mediated cellular antioxidant response.

The results of the present study indicate that phosphorylation of several of the CK2 sites in human Nrf2 is a key controlling factor in Nrf2 activity or degradation. A considerable, largely unsuccessful, effort has been expended to find a physiological modulator of CK2. In this regard, cold/ Ca^{2+} -free conditions, although non-physiological, provide us with a highly effective method to regulate intracellular Ca^{2+} which has a dramatic impact on CK2 activity. In part through this ability to control CK2 activity, our results indicate CK2 is a major kinase involved in the phosphorylation of endogenous Nrf2 in human cells. Indeed, cold/ Ca^{2+} -free conditions resulted in a dramatic phosphorylation of Nrf2 in intact cells and this was totally inhibited by the specific CK2 inhibitors DRB or TBB. Human Nrf2 contains thirteen putative CK2 phosphorylation sites and the precise sites of phosphorylation cannot be defined from the present work, although it appears phosphorylation occurs at Ser and/or Thr residues. Regardless of the precise site, our results suggest phosphorylation of Nrf2 is constitutive and messenger-independent, as is known for most CK2-mediated phosphorylations [16].

CK2 is composed of two catalytic subunits ($\alpha\alpha$, $\alpha'\alpha'$ or $\alpha\alpha'$), and a dimer of two regulatory β subunits [16], but the molecular mechanisms by which CK2 activity and its subunits are regulated in mammalian cells are incompletely defined [23]. The present data indicate there is a Ca^{2+} -dependent interaction between CaM and CK2 α and α' units that clearly impacts CK2 activity. These results support a model in which Ca^{2+} mobilization may control local CK2

activity by affecting CaM binding to CK2. CK2 catalytic subunits α and α' contain a highly conserved CaM binding site, and CaM could inhibit CK2 activity in a Ca^{2+} -dependent fashion. In addition, cold/ Ca^{2+} -free conditions, which dramatically decrease intracellular Ca^{2+} levels, reduced CaM-CK2 α' binding and enhanced CK2-dependent Nrf2 phosphorylation. Although the cold/ Ca^{2+} -free conditions are non-physiological, protein phosphorylation and/or dephosphorylation in response to cold-induced stress or alternation of intracellular Ca^{2+} has been well studied [24,25]. In addition, an inhibitory effect of spermine, a CK2 stimulator [26], on mast cell secretion was found to be highly dependent on a condition with low temperature and Ca^{2+} -free [27].

CaM is a ubiquitously expressed, highly conserved acidic protein involved in number of intracellular Ca^{2+} signaling and phosphorylation pathways [28]. Although there are no reports indicating CaM modulates CK2 activity in mammalian cells, Ca^{2+} -inhibited catalytic subunit of CK2 has been identified in *Paramecium tetraurelia* [29]. In addition, convincing evidence has indicated that CaM is a CK2 substrate [30], thus, CaM-CK2 binding may competitively inhibit CK2 activity towards other substrates. Indeed, Apel et al. [31] indicated that CaM at physiologically significant concentrations inhibits the phosphorylation of neuromodulin by CK2. Thus, the results of our present work provide evidence for an additional-control system for the cellular antioxidant response in which CaM when combined with Ca^{2+} , influences CK2 activity which, in turn, dictates Nrf2 activation and degradation.

The level of CK2 appears to be tightly regulated in normal cells, and cells resist a change in their intrinsic level of CK2. However, a consistent elevation of CK2 has been observed in rapidly proliferating tissues and a wide variety of tumors [16,32]. In this regard, our previous studies [33] indicated that As-induced malignant transformation of human keratinocytes is associated with an increased expression and enzymatic activity of CK2 in nuclear fractions. Consistent with the roles of CK2 on Nrf2 phosphorylation, the As-transformed cells showed a higher ratio of the bands of Nrf2-118 and Nrf2-98 after the cells were acutely exposed to Nrf2 activators. In particular, the Nrf2-98 was significant lower in transformed cells than in control cells under the same conditions. Furthermore, expression of the genes downstream of Nrf2, including NAD(P)H:quinone oxidoreductase 1, heme oxygenase-1 and $\gamma\text{-GCS}_h$ was dramatically increased by acute arsenic treatment in control cells. In contrast, the increases in these antioxidant response genes were significantly lower in the As-transformed cells. In addition, consistent with the high activity of CK2 in leukemia cells [34], we find high ratios of Nrf2-118 to Nrf2-98 in cultured leukemia cell lines, NB4 and U937 (data not shown).

Recent results demonstrated that phosphorylation of Nrf2 at Ser-40 through a PKC-based mechanism plays a critical role in the dissociation of Nrf2 from Keap1 [10,11], a saturable cytoplasmic repressor of Nrf2 [2,3]. The present results clearly indicate that PKC is not responsible for constitutive Nrf2 phosphorylation nor nuclear Nrf2 accumulation in HaCaT cells. The PKC inhibitors staurosporine and calphostin C did not inhibit Nrf2 phosphorylation caused by cold/ Ca^{2+} -free conditions and also did not affect nuclear accumulation of Nrf2 induced by As. A Prosite search also revealed the presence of two Tyr phosphorylation sites in human Nrf2. However, Tyr phosphorylation is not necessary for constitutive Nrf2 phosphorylation since treatment by LAR and YOP (Tyr-specific phosphatases) had no effect on the two phosphorylated Nrf2 bands. Collectively, these results provide the first convincing evidence that CK2 is responsible for endogenous phosphorylation of Nrf2, which, in turn, is a key regulatory event in Nrf2-mediated processes.

Our results with CaM-CK2 α' co-immunoprecipitation indicate As treatment increased CaM-CK2 α' binding, suggesting As potentially inhibits nuclear CK2 activity and subsequently decreases CK2-mediated Nrf2 phosphorylation and degradation. Similarly, Torres and Pulido [35] reported that the C terminus of the tumor suppressor phosphatase and tensin homologue

deleted on chromosome ten (PTEN) is constitutively phosphorylated by CK2 at several residues in intact cells and the phosphorylation modulates PTEN stability to proteasome-mediated degradation. Previous studies have suggested that Keap1 plays essential roles in the Nrf2-AREs stress response system, not only as a sensor of oxidative and electrophilic stress but also as a regulator of Nrf2 degradation [36]. Keap1 is a stress sensor protein that functions directly as an adaptor molecule in the Cul3-based E3 ligase system in the rapid degradation of Nrf2 [37]. However, since Keap1 is expressed exclusively in cytoplasm, it cannot play a direct role in nuclear Nrf2 clearance. Alternative pathways may exist that mediate nuclear Nrf2 degradation, and it is possible that CK2-mediated phosphorylation helps nuclear Nrf2 translocate back to the cytoplasm for Keap1-mediated degradation. However, a connection between CK2-mediated Nrf2 phosphorylation and Keap1's function has not been established and will require further investigation. Regardless, our results support the function of CK2 as a central regulatory molecule for Nrf2 transcriptional activity in response to oxidative stress as well as its eventual degradation.

The ubiquitin-dependent proteolysis system controls the abundance of cellular proteins and serves a central regulatory function in many biological processes, including cell cycle progression, signal transduction, and transcription [38]. Extensive studies indicate that Nrf2 is a short-lived signaling molecule degraded rapidly by the ubiquitin-proteasome pathway [2, 36,37,39–41]. Nrf2 stabilization alone seems to be sufficient to trigger its nuclear accumulation [7]. The extent of Nrf2 seems to be important for its stability. Indeed, our current results provide evidence that CK2-mediated phosphorylation promotes Nrf2 degradation.

In summary, the present data indicate that CK2-mediate endogenous Nrf2 phosphorylation which, in turn, regulates Nrf2 activity. Thus, phosphorylation is a key event in the control of the Nrf2-mediated antioxidant response, providing for both its' activation and eventual degradation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Dr. G. Bird for his assistance with the calcium analysis, Dr. R. Petrovich for his help with Nrf2 purification, and Drs. K. Itoh and E. Leslie for their valuable discussions concerning this work. The authors are also grateful to Drs. L.K. Keefer and J. Shen for their critical reviews of this manuscript.

This research was supported in part by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does the mention of trade names, commercial products, or organizations imply endorsement by the United States Government.

References

1. Dhakshinamoorthy S, Jaiswal AK. c-Maf negatively regulates ARE-mediated detoxifying enzyme genes expression and anti-oxidant induction. *Oncogene* 2002;21:5301–12. [PubMed: 12149651]
2. Nguyen T, Sherratt PJ, Huang HC, Yang CS, Pickett CB. Increased protein stability as a mechanism that enhances Nrf2-mediated transcriptional activation of the antioxidant response element. Degradation of Nrf2 by the 26 S proteasome. *J Biol Chem* 2003;278:4536–41. [PubMed: 12446695]
3. Itoh K, Tong KI, Yamamoto M. Molecular mechanism activating Nrf2-Keap1 pathway in regulation of adaptive response to electrophiles. *Free Radic Biol Med* 2004;36:1208–13. [PubMed: 15110385]
4. Chan K, Kan YW. Nrf2 is essential for protection against acute pulmonary injury in mice. *Proc Natl Acad Sci U S A* 1999;96:12731–6. [PubMed: 10535991]

5. Ramos-Gomez M, Kwak MK, Dolan PM, Itoh K, Yamamoto M, Talalay P, Kensler TW. Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice. *Proc Natl Acad Sci U S A* 2001;98:3410–5. [PubMed: 11248092]
6. Chan K, Han XD, Kan YW. An important function of Nrf2 in combating oxidative stress: detoxification of acetaminophen. *Proc Natl Acad Sci U S A* 2001;98:4611–6. [PubMed: 11287661]
7. Nguyen T, Yang CS, Pickett CB. The pathways and molecular mechanisms regulating Nrf2 activation in response to chemical stress. *Free Radic Biol Med* 2004;37:433–41. [PubMed: 15256215]
8. Pi J, Qu W, Reece JM, Kumagai Y, Waalkes MP. Transcription factor Nrf2 activation by inorganic arsenic in cultured keratinocytes: involvement of hydrogen peroxide. *Exp Cell Res* 2003;290:234–45. [PubMed: 14567983]
9. Goldring CE, Kitteringham NR, Elsby R, Randle LE, Clement YN, Williams DP, McMahon M, Hayes JD, Itoh K, Yamamoto M, Park BK. Activation of hepatic Nrf2 in vivo by acetaminophen in CD-1 mice. *Hepatology* 2004;39:1267–76. [PubMed: 15122755]
10. Huang HC, Nguyen T, Pickett CB. Phosphorylation of Nrf2 at Ser-40 by protein kinase C regulates antioxidant response element-mediated transcription. *J Biol Chem* 2002;277:42769–74. [PubMed: 12198130]
11. Bloom DA, Jaiswal AK. Phosphorylation of Nrf2 at Ser40 by protein kinase C in response to antioxidants leads to the release of Nrf2 from INrf2, but is not required for Nrf2 stabilization/accumulation in the nucleus and transcriptional activation of antioxidant response element-mediated NAD(P)H:quinone oxidoreductase-1 gene expression. *J Biol Chem* 2003;278:44675–82. [PubMed: 12947090]
12. Kang KW, Choi SH, Kim SG. Peroxynitrite activates NF-E2-related factor 2/antioxidant response element through the pathway of phosphatidylinositol 3-kinase: The role of nitric oxide synthase in rat glutathione S-transferase A2 induction. *Nitric Oxide* 2002;7:244–53. [PubMed: 12446173]
13. Yu R, Chen C, Mo YY, Hebbar V, Owuor ED, Tan TH, Kong AN. Activation of mitogen-activated protein kinase pathways induces antioxidant response element-mediated gene expression via a Nrf2-dependent mechanism. *J Biol Chem* 2000;275:39907–13. [PubMed: 10986282]
14. Cullinan SB, Diehl JA. PERK-dependent activation of Nrf2 contributes to redox homeostasis and cell survival following endoplasmic reticulum stress. *J Biol Chem* 2004;279:20108–17. [PubMed: 14978030]
15. Cullinan SB, Zhang D, Hannink M, Arvisais E, Kaufman RJ, Diehl JA. Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival. *Mol Cell Biol* 2003;23:7198–209. [PubMed: 14517290]
16. Pinna LA. Protein kinase CK2: a challenge to canons. *J Cell Sci* 2002;115:3873–8. [PubMed: 12244125]
17. Meggio F, Pinna LA. One-thousand-and-one substrates of protein kinase CK2? *Faseb J* 2003;17:349–68. [PubMed: 12631575]
18. Ahmed K, Gerber DA, Cochet C. Joining the cell survival squad: an emerging role for protein kinase CK2. *Trends Cell Biol* 2002;12:226–30. [PubMed: 12062170]
19. Zien P, Bretner M, Zastapilo K, Szyska R, Shugar D. Selectivity of 4,5,6,7-tetrabromobenzimidazole as an ATP-competitive potent inhibitor of protein kinase CK2 from various sources. *Biochem Biophys Res Commun* 2003;306:129–33. [PubMed: 12788077]
20. Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J Cell Biol* 1988;106:761–71. [PubMed: 2450098]
21. Moi P, Chan K, Asunis I, Cao A, Kan YW. Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. *Proc Natl Acad Sci U S A* 1994;91:9926–30. [PubMed: 7937919]
22. Sarno S, Reddy H, Meggio F, Ruzzene M, Davies SP, Donella-Deana A, Shugar D, Pinna LA. Selectivity of 4,5,6,7-tetrabromobenzotriazole, an ATP site-directed inhibitor of protein kinase CK2 ('casein kinase-2'). *FEBS Lett* 2001;496:44–8. [PubMed: 11343704]
23. Olsten ME, Litchfield DW. Order or chaos? An evaluation of the regulation of protein kinase CK2. *Biochem Cell Biol* 2004;82:681–93. [PubMed: 15674436]

24. Soto T, Beltran FF, Paredes V, Madrid M, Millar JB, Vicente-Soler J, Cansado J, Gacto M. Cold induces stress-activated protein kinase-mediated response in the fission yeast *Schizosaccharomyces pombe*. *Eur J Biochem* 2002;269:5056–65. [PubMed: 12383265]
25. Donelan MJ, Morfini G, Julyan R, Sommers S, Hays L, Kajio H, Briaud I, Easom RA, Molkentin JD, Brady ST, Rhodes CJ. Ca^{2+} -dependent dephosphorylation of kinesin heavy chain on beta-granules in pancreatic beta-cells. Implications for regulated beta-granule transport and insulin exocytosis. *J Biol Chem* 2002;277:24232–42. [PubMed: 11978799]
26. Leroy D, Heriche JK, Filhol O, Chambaz EM, Cochet C. Binding of polyamines to an autonomous domain of the regulatory subunit of protein kinase CK2 induces a conformational change in the holoenzyme. A proposed role for the kinase stimulation. *J Biol Chem* 1997;272:20820–7. [PubMed: 9252407]
27. Vliagoftis H, Mak L, Boucher W, Theoharides TC. Dual effect of spermine on mast cell secretion exhibits different calcium and temperature requirements. *Int J Immunopharmacol* 1999;21:547–59. [PubMed: 10501624]
28. Benaïm G, Villalobo A. Phosphorylation of calmodulin. Functional implications. *Eur J Biochem* 2002;269:3619–31. [PubMed: 12153558]
29. Vetter D, Kissmehl R, Treptau T, Hauser K, Kellermann J, Plattner H. Molecular identification of a calcium-inhibited catalytic subunit of casein kinase type 2 from *Paramecium tetraurelia*. *Eukaryot Cell* 2003;2:1220–33. [PubMed: 14665457]
30. Arrigoni G, Marin O, Pagano MA, Settimo L, Paolin B, Meggio F, Pinna LA. Phosphorylation of calmodulin fragments by protein kinase CK2. Mechanistic aspects and structural consequences. *Biochemistry* 2004;43:12788–98. [PubMed: 15461451]
31. Apel ED, Litchfield DW, Clark RH, Krebs EG, Storm DR. Phosphorylation of neuromodulin (GAP-43) by casein kinase II. Identification of phosphorylation sites and regulation by calmodulin. *J Biol Chem* 1991;266:10544–51. [PubMed: 1828073]
32. Unger GM, Davis AT, Slaton JW, Ahmed K. Protein kinase CK2 as regulator of cell survival: implications for cancer therapy. *Curr Cancer Drug Targets* 2004;4:77–84. [PubMed: 14965269]
33. Pi JDBA, Qu W, Liu J, Bai Y, Fahl WF, Yamauchi H, Collins S, Waalkes MP. Role of protein kinase CK2 in perturbed Nrf2-mediated oxidative stress response in human keratinocytes during arsenic-induced malignant transformation. *The Toxicologist* 2006;90:#58.abstract
34. Phan-Dinh-Tuy F, Henry J, Boucheix C, Perrot JY, Rosenfeld C, Kahn A. Protein kinases in human leukemic cells. *Am J Hematol* 1985;19:209–18. [PubMed: 3860003]
35. Torres J, Pulido R. The tumor suppressor PTEN is phosphorylated by the protein kinase CK2 at its C terminus. Implications for PTEN stability to proteasome-mediated degradation. *J Biol Chem* 2001;276:993–8. [PubMed: 11035045]
36. Kang MI, Kobayashi A, Wakabayashi N, Kim SG, Yamamoto M. Scaffolding of Keap1 to the actin cytoskeleton controls the function of Nrf2 as key regulator of cytoprotective phase 2 genes. *Proc Natl Acad Sci U S A* 2004;101:2046–51. [PubMed: 14764898]
37. Zhang DD, Lo SC, Cross JV, Templeton DJ, Hannink M. Keap1 is a redox-regulated substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex. *Mol Cell Biol* 2004;24:10941–53. [PubMed: 15572695]
38. Hershko A, Ciechanover A. The ubiquitin system. *Annu Rev Biochem* 1998;67:425–79. [PubMed: 9759494]
39. Aono J, Yanagawa T, Itoh K, Li B, Yoshida H, Kumagai Y, Yamamoto M, Ishii T. Activation of Nrf2 and accumulation of ubiquitinated A170 by arsenic in osteoblasts. *Biochem Biophys Res Commun* 2003;305:271–7. [PubMed: 12745069]
40. McMahon M, Itoh K, Yamamoto M, Hayes JD. Keap1-dependent proteasomal degradation of transcription factor Nrf2 contributes to the negative regulation of antioxidant response element-driven gene expression. *J Biol Chem* 2003;278:21592–600. [PubMed: 12682069]
41. Stewart D, Killeen E, Naquin R, Alam S, Alam J. Degradation of transcription factor Nrf2 via the ubiquitin-proteasome pathway and stabilization by cadmium. *J Biol Chem* 2003;278:2396–402. [PubMed: 12441344]

Abbreviations

Nrf2	Nuclear factor E2-related factor 2
DMEM	Dulbecco's modified Eagle's Medium
PBS	phosphate buffered saline
tBHQ	<i>tert</i> -butyl hydroxyquinone
DRB	5,6-dichlorobenzimidazole riboside
TBB	4,5,6,7-tetrabromobenzotriazole
CaM	calmodulin
CK2	casein kinase 2
γ-GCS_h	γ -glutamyl cysteine synthetase
As	arsenite

Fig 1.

(A) Representative images of Nrf2 immunoblots with nuclear and cytosolic fractions derived from control and As- or tBHQ-exposed HaCaT cells. C, control; As, cells were treated with 10 μ M of arsenite for 6 hrs; tBHQ, cells were treated with 15 μ M of tert-butyl hydroxyquinone for 6 hr. Cellular fractions (nuclear protein 20 μ g; cytosolic protein 60 μ g) were separated on 4–12% Tris-Glycine gels and detected using anti-Nrf2 (H-300, Santa Cruz). The characteristic double bands at 98 and 118 kDa and a non-specific binding at 85 kDa (termed as NS85) were observed in nuclear fractions, while in the cytosol apparent non-specific binding also occurred at 41 kDa (NS41). Sam 68 and β -actin were used as loading controls. (B) Western blot analysis showing two Nrf2 bands (98 and 118 kDa) in different human cell lines. C, control; As, cells were treated with 10 μ M of arsenite for 6 hrs. β -actin was used as loading control. (C) Over-expression of Nrf2 by transfection showing two clear Nrf2 bands by immunoblots. The cell lysates of COS-7 and HaCaT cells were detected using Nrf2 antibodies H-300 and C-20 (Santa Cruz). Sam 68 and β -actin were used as loading controls. C, control; O, over-expression.

Fig 2.

Evidence that endogenous human Nrf2 is a constitutively phosphorylated protein. Nuclear protein (20 μ g protein) was incubated with λ -PPase, PP1, LAR, or YOP at 30 °C for 30 min and then were detected by immunoblotting with anti-Nrf2 (H-300). Sam 68 was used as loading control.

Fig 3.

Human Nrf2 is a substrate of CK2. Recombinant human Nrf2 (A) and Nrf2 immunoprecipitation products from the nuclear extracts of acute As-exposed HaCaT cells (B) were incubated with recombinant human CK2 (1000 U/ml) in CK2 buffer with [γ - ^{32} P]ATP for the indicated times, and resulting proteins were then resolved by SDS-PAGE and visualized by autoradiography. TBB (4,5,6,7-tetrabromobenzotriazole) is a specific CK2 inhibitor. (C) SDS-Page of recombinant Nrf2 after CK2 treatment *in vitro*. Recombinant human Nrf2 (1 mg protein) were incubated with recombinant human CK2 (1000 U/ml) in CK2 buffer with ATP for 30 mins. P-Nrf2, phosphorylated Nrf2.

Fig 4.

(A) Inhibitory effects of CaM (150 U/ml) and Ca²⁺ (1 mM) on the activity of purified CK2. *, $p < 0.05$ compared with control. (B) Cold (25°C)/Ca²⁺-free conditions caused a quick decrease of intracellular Ca²⁺ in HaCaT cells. Ca²⁺ dynamics in same cells were monitored by confocal microscope with Flou-4 AM: a, normal; b and c, cold/Ca²⁺-free conditions for 3 min and 6 min, respectively; d and e, replacement of the Ca²⁺-free media with media containing normal Ca²⁺ levels (1.8 mM) and the Ca²⁺ ionophore, ionomycin (10 μM) for 3 min and 6 min, respectively. (C) Effects of arsenic followed with cold/Ca²⁺-free conditions on CK2α'-CaM binding as well as CK2 subunits and CaM protein levels in nuclear fractions. C, control; As, cells pretreated with As (10 μM, 4 hrs) were cultured in normal culture condition for 1.5 hrs; As+X, cells pretreated with As (10 μM, 4 hrs) were exposed to cold/Ca²⁺-free conditions for 1.5 hrs. CaM-CK2α' binding were determined by immunoblotting with anti-CK2α' after the nuclear fractions were co-immunoprecipitated with CaM antibody. Lower panel, quantitative results of CaM-CK2α' binding analysis. *, $P < 0.05$ compared with control; #, $P < 0.05$ compared with As alone.

Fig 5.

(A) Cold/Ca²⁺-free conditions cause Nrf2 phosphorylation which is inhibited by CK2 specific inhibitors. HaCaT cells were pretreated with As (10 μ M, 4 hrs) and then treated with cold (25° C) and/or Ca²⁺-free conditions with or without CK2 inhibitors 5,6-dichlorobenzimidazole riboside (DRB) or 4,5,6,7-tetrabromobenzotriazole (TBB). CK2 inhibitors were added prior to manipulation of culture conditions. The nuclear fractions were used for immunoblots. (B) CK2 inhibitor decreases the ratio of the two bands of Nrf2 overexpressed in COS-7 cells. COS-7 cells, which have high transfection efficiency, were pretreated with DRB for 1 hr and followed by Nrf2 transfection. The culture media containing 30 or 90 μ M DRB were refreshed every 4 hrs. Whole cell lysates harvested at 16 hrs after transfection were used for immunoblots.

A

B

Fig 6.

Phosphorylation of Nrf2 by CK2 regulates Nrf2 activation and degradation. (A) Western blot measurement of nuclear Nrf2 accumulation. C, control; As, cells pretreated with As (10 μ M, 4 hrs) and then cultured under normal conditions for 1.5 hrs; As+X, cells pretreated with As (10 μ M, 4 hrs) and then treated with cold/Ca²⁺-free conditions for 1.5 hrs. (B) Gel shift with γ -GCS_h oligonucleotide containing ARE. C, control; As, cells pretreated with As (10 μ M, 4 hrs) and then cultured under normal conditions for 1.5 hrs; AX, cells pretreated with As (10 μ M, 4 hrs) were then treated at cold/Ca²⁺-free conditions for 1.5 hrs. Lower panel is the quantitative analysis of the gel shift results. *, $p < 0.05$ compared with C; #, $p < 0.05$ compared with As. (C) Phosphorylation of Nrf2 promotes its clearance. Cells were pretreated with As (10 μ M, 4 hrs), and then exposed to cold/Ca²⁺-free conditions or continued incubation under normal culture conditions for 1.5 hrs. Whole cell lysates extracted at 0, 2 and 6 hrs post cold/

Ca²⁺-free conditions were determined by immunoblots. The image is a representative of three blots. (D) Quantitative analysis of Western blot results.