Phosphorylation of Tyrosine 568 Controls Nuclear Export of Nrf2*

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Nuclear factor Nrf2, under normal conditions, is retained in the cytosol by INrf2. Antioxidants and oxidants antagonize this interaction, resulting in the release of Nrf2. Nrf2 translocates to the nucleus binds to ARE and activates a battery of chemopreventive genes. Once this is achieved, Nrf2 is exported out of the nucleus, binds with INrf2, and degrades. Nrf2 contains well defined signals that control nuclear import and export of Nrf2. The present studies demonstrate that phosphorylation of tyrosine 568 is required for Crm1-mediated nuclear export of Nrf2. Mutation of tyrosine 568 to phenylalanine interaction with Crm1 and abrogation of nuclear export of Nrf2. Further studies revealed that Fyk1-mediated nuclear export and degradation of Nrf2. Nuclear factor Nrf2, under normal conditions, is retained in the cytosol by INrf2. Antioxidants and oxidants antagonize this interaction, resulting in the release of Nrf2. Nrf2 translocates to the nucleus binds to ARE and activates a battery of chemopreventive genes. Once this is achieved, Nrf2 is exported out of the nucleus, binds with INrf2, and degrades. Nrf2 contains well defined signals that control nuclear import and export of Nrf2. The present studies demonstrate that phosphorylation of tyrosine 568 is required for Crm1-mediated nuclear export of Nrf2. Mutation of tyrosine 568 to phenylalanine interaction with Crm1 and abrogation of nuclear export of Nrf2. Further studies revealed that Fyk1-mediated nuclear export and degradation of Nrf2.

This article has been retracted by the publisher. An investigation at the University of Maryland, Baltimore determined in both the submitted but unpublished and in the published versions of Fig. 2, empty lanes were purported to depict absence of labeling in pcDNA. Examining the unedited version shows that the image was obtained from a region outside the gel, where no labeling can occur. The figure does not support that there is no labeling after transfection with the empty pcDNA vector and the claim that transfection with the active constructs had a specific effect. The investigation also determined that in Fig. 6, in the versions submitted to EMBO, JBC (first submission), and to Mol. Cell. Biol., images of transfected Hepa-1 cells were obtained from a single sample, captured from a single microscope field, and then presented as if they originated from different samples. Whereas in the originally submitted versions, the treatment is described as “LMB,” in the published version, the same data are purported to show effects of PP2.

A cytosolic inhibitor of Nrf2, INrf2 (inhibitor of Nrf2) or KEAP1 (Kelch-like ECH-associated protein1), was reported (8–9). INrf2 retains Nrf2 in the cytoplasm. The INrf2-Nrf2 complex serves as cellular sensor of oxidative and electrophilic stress generated from endogenous reactions and exogenous chemicals, xenobiotics, drugs, UV, and ionizing radiation (6–7).

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2 The abbreviations used are: Nrf2, NF-E2-related factor; ARE, antioxidant response element; NQO1, NAD(P)H:quinone oxidoreductase 1; f-BHQ, tert-butyl hydroquinone, LMB, leptomycin B; PKC, protein kinase C; siRNA, small interference RNA; CMV, cytomegalovirus; PBS, phosphate-buffered saline; LDH, lactate dehydrogenase; RIPA, radiomune precipitation assay; DMEM, Dulbecco’s modified Eagle’s medium; PP2, 4-amino-5-(N4-chlorophenyl)-7-(N1-propyl)pyrazolo[3,4-d]pyrimidine.
action of mutant Nrf2 with Crm1 and abrogation of nuclear export. Hydrogen peroxide treatment initially increased nuclear import of Nrf2, presumably to increase the ARE-mediated gene expression to prevent oxidative/electrophilic stress. This was followed by increase in phosphorylation and nuclear export of Nrf2. Further studies with siRNA revealed that Fyn kinase phosphorylated Nrf2Y568, which facilitated nuclear export of Nrf2 for binding to INrf2 and degradation.

MATERIALS AND METHODS

Construction of Plasmids—The construction of pGL2B-NQO1-ARE and pcDNA-Nrf2 has been previously described (18). The pcDNA-Nrf2 was used as a template to construct Nrf2 mutants. The forward 5′-GGAGGTGATGAGAAACCCATGCAGCCCGAA-3′ and reverse 5′-GTCTTCTCTTCTGCTGTCCTG-3′ primers were used to amplify Nrf2 coding region without stop codon. The PCR-amplified product was used as a template to construct Nrf2 mutants. The forward and reverse 5′-ACTGCTATGAGAATTCCG-3′ primers, forward (5′-CCAGTGAA-3′) and reverse (5′-GTTGATAGAATTCCG-3′) primers, forward 5′-GTTGATAGAATTCCG-3′, and reverse 5′-AGGCTTTCCATCCTCATACGTAACATGCT-3′ were used to amplify the fragment corresponding to Nrf2Y568A. The PCR fragment containing the desired mutation was gel-purified and transferred into maximum efficiency DH5α chemically competent cells. A similar strategy was used to generate the Nrf2Y568A mutant. The plasmids were confirmed by sequencing.

Modified pCMV vectors were used to clone the FLAG- and Crm1 proteins. A modified polylinker containing two FLAG epitopes placed adjacent to each other and stop codons in each frame was cloned into the pCMV vector to generate the FLAG-vector. Mouse INrf2 was amplified from the IMAGE clone obtained from ATCC using the primers, forward (5′-GTTGATAGAATTCCGACCTCATCCTCATACGTAACATGCT-3′) and reverse (5′-AGGCTTTCCATCCTCATACGTAACATGCT-3′). The PCR-amplified DNA contained 5′- and 3′-ends, respectively. The PCR-amplified DNA was ligated with the primers, forward (5′-GGAGGTGATGAGAAACCCATGCAGCCCGAA-3′) and reverse (5′-GGAGGTGATGAGAAACCCATGCAGCCCGAA-3′) using the IMAGE clone obtained from ATCC using the primer, forward (5′-GTTGATAGAATTCCG-3′) and reverse (5′-GTTGATAGAATTCCG-3′). The PCR-amplified DNA contained 5′- and 3′-ends, respectively. The amplified DNA was digested with XbaI and BamHI restriction sites at 5′- and 3′- ends, respectively. The amplified DNA was digested with XbaI and BamHI and subcloned into the FLAG vector digested with similar enzymes. The resultant plasmids were designated as pCMV-FLAG-mINrf2 and pCMV-FLAG-mCrm1.

Cell Culture, Co-transfection of Expression Plasmids, and Luciferase Reporter Assay—Human hepatoma (HepG2) cells were grown in monolayer cultures in 6-well plates in minimum essential medium-α supplemented with 10% fetal bovine serum. Transient transfections were done in cells grown to ∼50% confluence using the Effectene Transfection reagent (Qiagen). Cells were co-transfected with 0.2 μg of reporter construct (human NQO1-ARE-Luc) and ten times less quantities of firefly Renilla luciferase encoded by plasmid pRL-TK. Renilla luciferase was used as the internal control in each transfection. Wherever indicated, the cells were also co-transfected with 0.5 μg of pcDNA expression plasmids encoding wild-type Nrf2 or mutant Nrf2Y568A. To analyze the effect of tyrosine kinase inhibitors on NQO1-ARE activity, the transfected cells were pretreated for 8 h with the indicated kinase inhibitor (Genistein or AG18 or PP2) in the concentrations mentioned in the figures. All the inhibitors were purchased from Calbiochem and were of the highest purity available. Cells were then treated with Me2SO or induced with t-BHQ (50 μM) for 16 h in the media containing the indicated kinase inhibitors. After the treatment for specified time, the cells were washed with 1× PBS and lysed in 1× Passive lysis buffer from the Dual-Luciferase Reporter Assay System Kit (Promega, Madison, WI). The luciferase activity was measured using the procedures described previously (18). Pre-designed siRNA against mouse Fyn protein and control scrambled siRNA were purchased from Ambion and transfected in Hepa-1 cells using the Effectene transfection reagent following the manufacturer’s suggested protocol.

Subcellular Fractionation, Western Blotting, and NQO1 Activity—Hepa-1 cells were transfected with 2.0 μg of either pcDNA-Nrf2-V5 or pcDNA-Nrf2Y568A-V5 plasmids using the Effectene transfection reagent as described above. 24 h after transfection, the cells were treated with Me2SO, t-BHQ, or PP2 as indicated in the figures. To analyze the localization of endogenous Nrf2, HepG2 cells were seeded in 100-mm plates and treated with Src inhibitor PP2 with or without Me2SO or antioxidant t-BHQ, H2O2 with or without nuclear export inhibitor leptomycin B (LMB), or PP2 as indicated in the figures. At the end of treatment, cells were washed twice with ice-cold PBS, scraped in PBS using a rubber policeman, and centrifuged at 500 rpm for 5 min. Biochemical fractionation of the cells was done using the Nuclear Extract Kit (Active Motif, Carlsbad, CA) following the manufacturer’s protocol. The protein in the nuclear fraction was determined using the Protein Assay reagent (Bio-Rad, Hercules, CA). 100 μg of the cytosolic and 50 μg of the nuclear proteins were fractionated on a 10% SDS-PAGE, Western blotted, and probed with anti-lactate dehydrogenase (Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-V5-HRP antibody (Invitrogen), and β-actin antibody (Sigma). To confirm the purity of subcellular fractions, the Western blot blotted with cytoplasm-specific anti-lactate dehydrogenase (LDH) antibody (Chemicon International, Temecula, CA), nuclear specific anti-lamin B antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The levels of protein on a Western blot were quantitated by using QuantityOne image software (ChemiDoc XRS, Bio-Rad) and normalized against proper loading controls. The NQO1 activity was determined by previously described procedures (19).

In Vitro Binding—The in vitro transcription/translation of the plasmids encoding Nrf2-V5, Nrf2Y568A-V5, and INrf2 were performed using the TNT-coupled rabbit reticulocyte lysate system (Promega). Redivue [-35S]methionine (Amersham Biosciences) was substituted for methionine in the reactions to radiolabel the translated proteins. The plasmid encoding luciferase provided in the kit was used as a control for the transcription translation reaction. After the coupled transcription/translation, the proteins were checked for their correct size by SDS-PAGE, autoradiography, and Western analysis with V5-HRP antibody. V5 antibody was used to detect the V5-tagged wild-type Nrf2 and Nrf2Y568A proteins. All of the in vitro translated/proteins gave same size bands. Binding assay: 5 μl of each in vitro translated protein (Nrf2-V5 + INrf2 or Nrf2Y568A-V5 + INrf2) in protein binding buffer (1 mM Tris, pH 7.5, 2 mM NaCl, 10% glycerol, 10% Nonidet P-40, 1 mM sodium vanadate supplemented with protease inhibitors) were mixed and incubated at 37°C for 30 min. This was followed by addition of 2.5 μg of anti-V5 antibody and sufficient protein binding buffer to make the volume to 100 μl, and the mixture was incubated overnight at 4°C with shaking. After incubation, 40 μl of washed Protein A beads (Santa Cruz Biotechnology) were added, and the mixture was incubated for 1 h at 4°C with shaking. The slurry was centrifuged at 10,000 rpm for 30 s, and the supernatant was discarded. The beads were washed twice with the protein binding buffer. Finally, the beads were boiled in SDS sample dye and analyzed by SDS-PAGE as described above.
Degradation of Nrf2—Hepa-1 cells were grown in 100-mm tissue culture plates and were co-transfected with 2.0 μg of either pcDNA-Nrf2-V5 or pcDNA-Nrf2Y568A-V5 plasmids. 24 h after transfection, the cells were pretreated with either Me6SO or MG132 (20 μM) for 8 h. Cells were washed twice with media and treated with 30 μg/ml cycloheximide for different time points (0.5, 1, 2, or 3 h). One set of the cells was left untreated with MG132 alone. After treatment for the indicated time points, the cells were washed twice with ice-cold 1× PBS and lysed in RIPA buffer. 100 μg of lysate was resolved on a 10% SDS-PAGE, Western blotted, and probed with anti-V5, anti-LDH, anti-lamin B, and anti-β-actin antibodies.

Immunoprecipitation—Cells either transfected or treated for appropriate times were washed two times with ice-cold PBS and harvested. Cytosolic, nuclear, or whole cell fractions were prepared. Five hundred micrograms of extract was used for immunoprecipitation. Briefly, extract was incubated with either mouse IgG, anti-V5 antibody (Invitrogen) or anti-phospho-tyrosine (anti-pTyr) antibody (clone 4G10, Upstate Biotechnology, Waltham, MA). The immunoprecipitation reaction was performed in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, and 1 μM sodium vanadate supplemented with tyrosine phosphatase inhibitor mixture (Sigma) and protease inhibitors. The extract was incubated with 2.5 μg of antibody overnight at 4 °C with shaking. 40 μl of washed Protein A beads (Santa Cruz Biotechnology) was added, and the mixture was incubated for 1 h at 4 °C with shaking. The slurry was centrifuged at 10,000 rpm for 30 s, and the supernatant was discarded. The beads were washed twice with RIPA buffer. 25 μl of SDS-sample dye was added, the mixture was boiled, and immunoprecipitates were resolved on a 10% SDS-PAGE. Western blotting with anti-V5HRP, anti-pTyr, anti-Nrf2, or anti-FLAG antibodies. FLAG immunoprecipitation was done using the FLAG-agarose beads (Sigma).

Phosphorylation Analysis—Hepa-1 cells transfected with Nrf2-V5 or Nrf2Y568A-V5 were lysed in RIPA buffer supplemented with tyrosine phosphatase inhibitor mixture and protease inhibitor mixture. HepG2 cells treated with H2O2 and PP2 were also lysed in a similar manner. HepG2 cells co-transfected with Fyn siRNA were harvested in the same manner. HepG2 cells treated with H2O2 and PP2 were lysed in RIPA buffer to determine the phosphorylation status of endogenous Nrf2. 500 μg of total cell lysate was used to immunoprecipitate with mouse IgG, anti-V5, or anti-pTyr antibodies as described above. The input and immunoprecipitates were boiled in SDS-sample dye and resolved on 10% SDS-PAGE and immunoblotted with respective antibodies.

Pulse-Chase Assay—Hepa-1 cells were transfected with Nrf2-V5 or Nrf2Y568A-V5. The cells 24 h after transfection were incubated with methionine-deficient DMEM (Sigma) for 30 min. The cells were then labeled with methionine-deficient DMEM containing ~200 μCi of [S-35]methionine mixture (Express35S, PerkinElmer Life Sciences), for 1 h at 37 °C (Pulse). After rinsing with normal culture medium (DMEM supplemented with 10% fetal bovine serum), the cells were chased by normal culture medium supplemented with 100 μg/ml l-methionine for 0, 0.5, 1, 2, and 4 h. MG132 (20 μM) was added wherever indicated. Cells were rinsed once with PBS and lysed in RIPA on ice for 30 min. Insoluble cellular debris was cleared by centrifugation at 10,000 rpm for 5 min at 4 °C. After centrifugation, the supernatants were used for immunoprecipitation with anti-V5 antibody as described earlier. Immunoprecipitates were boiled in 1× SDS buffer and resolved on 10% SDS gel. The gel was treated with Amplify solution to enhance the 35S signal, dried, and autoradiographed. The band intensities were quantified using the QuantityOne image software, and the percent Nrf2 or Nrf2Y568A remaining was plotted against time.
but failed to immunoprecipitate tyrosine to alanine Nrf2Y568A-V5 or tyrosine to phenylalanine Nrf2Y568F-V5 mutants of Nrf2 (Fig. 1F). Genistein-induced nuclear accumulation of Nrf2 raised questions regarding enhanced nuclear import because of altered interaction of mutant Nrf2Y568A with INrf2 and/or loss of nuclear export of Nrf2.

Interaction of Nrf2 and Mutant Nrf2Y568 with INrf2—We performed in vitro and in vivo experiments to investigate Nrf2 and mutant Nrf2Y568 interaction with INrf2 (Fig. 2). Luciferase (Luc control), Nrf2-V5, Nrf2Y568A-V5, and Nrf2Y568F-V5, and INrf2 all were successfully co-precipitated with FLAG-INrf2 (Fig. 2, E), immunoprecipitation/Western analysis. Nrf2Y568 but not mutants Nrf2Y568A and Nrf2Y568F are phosphorylated. Hepa-1 cells were transfected with Nrf2-V5 or Nrf2Y568A-V5 or Nrf2Y568F-V5. The cells were harvested 24 h after transfection, lysed in RIPA buffer, and immunoprecipitated with anti-V5 or anti-phosphotyrosine antibodies. The input (one of five) and immunoprecipitates were analyzed by SDS-PAGE, Western blotting, and probing with anti-V5 and anti-phosphotyrosine antibodies (J, input).

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**FIGURE 1. Nrf2 tyrosine 568 is phosphorylated.** Hepa-1 cells were co-transfected with NQO1-ARE luciferase reporter and firefly luciferase as described under “Materials and Methods.” 36 h after transfection, the cells were pretreated with either Me2SO or t-BHQ (50 μM) for 16 h in the medium containing Genistein or AG18. Cells were harvested, lysed, and split into cytosolic and nuclear fractions. The results are presented as ± S.E. of three independent experiments, and each experiment was done in triplicate. C, subcellular localization of Nrf2 after treatment with either Me2SO or 50 μM Genistein for 2 h. Cells were fixed and Nrf2 localization was determined by immunofluorescence staining with an anti-Nrf2 antibody followed by fluorescence tagged secondary antibody. The cells were stained with Hoechst to visualize the nuclei in blue filter. Top panel, green fluorescence respective of Nrf2 localization. Middle panel, nucleus stained with Hoechst. Lower panel, phase contrast. The staining pattern of Nrf2 in untreated or Genistein treated cells was scored for 100 cells in three separate experiments. Graph shows the percentage of cells with the indicated Nrf2 staining patterns. D, schematic presentation of wild-type Nrf2 and mutant Nrf2Y568A and Nrf2Y568F. Various protein domains of Nrf2 are labeled, and position of tyrosine mutation is indicated. E and F, immunoprecipitation/Western analysis. Nrf2Y568 but not mutants Nrf2Y568A and Nrf2Y568F are phosphorylated. Hepa-1 cells were co-transfected with Nrf2-V5 or Nrf2Y568A-V5 or Nrf2Y568F-V5. The cells were harvested 24 h after transfection, lysed in RIPA buffer, and immuno-precipitated with anti-V5 or anti-phosphotyrosine antibodies. The input (one of five) and immunoprecipitates were analyzed by SDS-PAGE, Western blotting, and probing with anti-V5 and anti-phosphotyrosine antibodies (J, input).
panels). Therefore, the results from in vitro translated and overexpressed proteins in transfected cells clearly demonstrate that both mutant Nrf2Y568A and Nrf2 proteins interact with INrf2 in both cytosol and nuclear fractions. Therefore, Genistein-induced nuclear accumulation is not due to the loss of interaction of mutant Nrf2Y568A with INrf2. The results also demonstrated that INrf2 exists in the nucleus and binds to Nrf2 in similar fashion as in the cytosol.

Nrf2 and Nrf2Y568A Interaction with Crm1 and Nuclear Export of Nrf2—The studies were extended to determine the role of phosphorylation of Nrf2Y568A in nuclear export of Nrf2. Immunohistochemistry and Western assays were performed to investigate subcellular localization of Nrf2-V5 and mutant Nrf2Y568A-V5 in transfected Hepa-1 cells (Fig. 3, A and B). Both assays demonstrated distribution of Nrf2-V5 between cytosol and nucleus. Treatment with nuclear export inhibitor leptomycin B (LMB) led to inhibition of nuclear export and accumulation of Nrf2-V5 in the nucleus (Fig. 3, A and B). On the contrary, the mutant Nrf2Y568A localized predominately in the nucleus. LMB had no effect on nuclear localization of Nrf2Y568A-V5. A small amount of Nrf2Y568A-V5 observed in cytosolic fractions (Fig. 3B) is due to cytosolic retention of mutant protein by INrf2. These results combined with results in Fig. 1 led to the conclusion that tyrosine 568 phosphorylation is required for nuclear export of Nrf2. Next, we determined the mechanism of the role of tyrosine 568 phosphorylation in nuclear export of Nrf2. Crm1, also known as exportin 1, is known to bind to several proteins and export them out of the nucleus (20). Immunoprecipitation followed by immunoblotting was used to analyze the interaction of Nrf2-V5 and Nrf2Y568A-V5 with FLAG-Crm1 in transiently transfected Hepa-1 cells (Fig. 3C). Because it is known that antioxidant t-BHQ induces nuclear export of Nrf2 at 4 h of treatment (17), we used t-BHQ to enhance interaction between Nrf2 and Crm1. Immunopre-

FIGURE 2. Nrf2 and mutant Nrf2Y568A both interact with INrf2. A, in vitro transcription/translation of Nrf2-V5, Nrf2Y568A-V5, and INrf2. Nrf2-V5, Nrf2Y568A-V5, INrf2, and luciferase (control) plasmids were in vitro transcribed/translated using the TNT coupled reticulocyte lysate system. 5 μl of the translated proteins was loaded on the gel (lanes 1–4), resolved on a 10% SDS-PAGE, treated with Amplify solution to enhance the 35S signal, dried, and autoradiographed (upper panel) or Western blotted and probed with anti-V5 antibodies (lower panel). For in vitro binding assay, equal amounts of in vitro translated proteins were mixed in binding buffer in combinations as displayed (lanes 5–12), incubated at 37 °C for 30 min, and immunoprecipitated with either mouse IgG or anti-V5 antibody. The immunoprecipitates along with input controls were analyzed for 35S signal by autoradiography (upper panel) and by SDS-PAGE, Western blotting, and probing with anti-V5 antibodies (lower panel). B–D, co-immunoprecipitation assay. Hepa-1 cells were co-transfected with Nrf2-V5 or Nrf2Y568A-V5 along with FLAG-INrf2 in a 4:1 ratio. The cells were harvested 24 h after transfection and cytosol, and nuclear extracts were prepared by standard procedures. 50 μg of cytosol/nuclear extracts (input) were immunoblotted with anti-V5 and anti-FLAG antibodies, the same blot was also reprobed with anti-lamin B and anti-LDH antibodies to confirm the purity of fractionation. 500 μg of extracts was immunoprecipitated with IgG, anti-V5, or anti-FLAG antibody. The input (one of five) and immunoprecipitates were resolved on a 10% SDS-PAGE and Western blotted with anti-V5 and anti-FLAG antibodies, respectively. IP, immunoprecipitation; WB, Western blotting.
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FIGURE 3. Phosphorylation of Nrf2Y568 is essential for interaction with Crm1 and nuclear export of Nrf2. A and B, transfection/immunohistochemistry/Western analysis. Mutant Nrf2Y568A predominately localize in the nucleus due to loss of nuclear export. A, Hepa-1 cells were transfected with wild-type Nrf2-V5 or mutant Nrf2Y568A-V5, treated with or without 20 ng/ml LMB for 8 h, fixed and analyzed by immunohistochemistry using fluorescein isothiocyanate tagged anti-V5 antibody (green fluorescence in upper panel). The nuclei were stained with Hoechst (middle panel). The upper and middle panels are superimposed in lower panel. B, Hepa-1 cells transfected with Nrf2-V5 and Nrf2Y568A-V5 were harvested and cytosolic/nuclear extracts prepared and analyzed by SDS-PAGE/Western blotting and probing with anti-V5-HRP antibody. The blot was also probed with lamin B and LDH antibodies to demonstrate purity of nuclear and cytosolic fractions respectively. C, transfection/co-immunoprecipitation analysis. Hepa-1 cells were co-transfected with Nrf2-V5 or Nrf2Y568A-V5 with FLAG-Crm1, treated with MeSO or 50 μM antioxidant t-BHQ for 4 h, lysed in RIPA buffer and immunoprecipitated with IgG, anti-V5 or anti-FLAG antibodies. The immunoprecipitate was analyzed by SDS-PAGE/Western blotting and probing with anti-V5 and anti-FLAG antibodies.

FIGURE 4. Hydrogen peroxide-induced tyrosine phosphorylation and nuclear export of Nrf2. A, Western analysis. Hydrogen peroxide-induced nuclear export of Nrf2. Hep-G2 cells were treated with 400 mM H2O2 for 30 min, 2 h, or 4 h without leptomycin B (−LMB) or with 20 ng/ml nuclear export inhibitor leptomycin B (+LMB). Cells were harvested at the end of treatments, and nuclear fractions were prepared by standard procedures. Nuclear extracts were resolved on 10% SDS-PAGE and Western blotted with anti-Nrf2 and anti-lamin B antibodies. Nrf2 levels were normalized to lamin B levels by using QuantityOne image software and the percent Nrf2 in the nucleus in H2O2 with or without LMB-treated cells at different time points is shown. The densitometry results are presented as ± S.E. of three independent experiments and representative blot is shown. B and C, Western/phosphorylation analysis. Hydrogen peroxide-stimulated phosphorylation of Nrf2 was inhibited with Src kinases inhibitor PP2. HepG2 cells were either untreated or treated with 400 μM H2O2 for the indicated time intervals. In related experiments, the cells were pretreated with 1 μM PP2 for 8 h followed by treatment with 400 μM H2O2 plus 1 μM PP2 for 2 h. The cells were harvested, and nuclear extract was immunoblotted with Nrf2 and lamin B antibodies. Nrf2 levels were normalized to lamin B levels by using QuantityOne image software, and the percent Nrf2 in the nucleus in H2O2 with or without PP2-treated cells at different time points is shown. The densitometry results are presented as ± S.E. of three independent experiments and representative blot is shown. In a similar experiment the nuclear extracts were immunoprecipitated with phosphotyrosine antibodies followed by Western analysis with Nrf2 antibodies (C). IP, immunoprecipitation; WB, Western blotting. *, nonspecific band.
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A.

B.

Hydrogen Peroxide-mediated Induction of Tyrosine 568 Phosphorylation and Nuclear Export of Nrf2

- Hydrogen peroxide treatment of Hep-G2 cells led to nuclear import of Nrf2 within 30 min of treatment, presumably to increase expression of Nrf2 downstream genes to provide cellular protection against hydrogen peroxide-induced oxidative stress (Fig. 4A, -LMB). Once this is achieved, Nrf2 started exiting the nucleus 2 h after hydrogen peroxide treatment and was reduced to lower than control (untreated) levels by 4 h after treatment (Fig. 4A, -LMB). The nuclear export of Nrf2 was LMB-sensitive, as there was little or no clearance of Nrf2 from the nucleus when treated with H2O2 in the presence of LMB (Fig. 4A, right panel, +LMB). The nuclear levels of Nrf2 were quantitated by densitometry and displayed as a graph, which demonstrates a significant reduction in the amount of Nrf2 from the nucleus at 4 h of H2O2 treatment but not in the presence of LMB (Fig. 4A, right panel, last columns, p > 0.005). Interestingly, the Src family of tyrosine kinase inhibitor PP2 significantly blocked the hydrogen peroxide-induced nuclear export of Nrf2 beginning at 2 h (p < 0.005) (Fig. 4B, left panel and graph). This observation with PP2 was similar to that observed with nuclear export inhibitor LMB (Fig. 4B, left panel and graph).
4, compare A with B). In addition, PP2 also blocked the tyrosine phosphorylation of Nrf2 at 2 h of hydrogen peroxide treatment (Fig. 4C). These results suggested that hydrogen peroxide-induced nuclear export of Nrf2 is mediated via tyrosine phosphorylation of Nrf2 by Src kinase(s).

Nuclear Export and Degradation of Nrf2—The experiments in Fig. 2 indicated that both Nrf2 and Nrf2Y568A interacted with Irf2. Irf2 is known to function as cul3-based E3 ligase in Nrf2 degradation (21, 22). Experiments were performed to compare the rate of degradation of Nrf2 and mutant Nrf2Y568A so as to determine the role of phosphorylation of tyrosine 568 in Nrf2 degradation. Based on the assumptions that Nrf2 is degraded in the cytoplasm, we hypothesized that Nrf2Y568A, which is deficient in export, will be more stable than Nrf2. The results of the degradation experiment showed that the rate of degradation of mutant Nrf2Y568A was significantly slower than wild-type Nrf2 (Fig. 5A, –LMB compare top lanes of left and right panels). Blocking of the nuclear export of Nrf2 with LMB showed similar slower rate of degradation as mutant Nrf2Y568A (Fig. 5A, +LMB, left panel). In the same experiment, LMB had no effect on rate of degradation of mutant Nrf2Y568A with compromised nuclear export (Fig. 5A, right panel). The results are also displayed as graphs plotted for percent Nrf2 remaining in the cells against time (Fig. 5A). The results clearly demonstrate that nuclear export of Nrf2 is required for degradation of Nrf2. Therefore, both the mutation and LMB treatment reduced the rate of degradation of Nrf2 due to blocking of nuclear export. To further elucidate these observations, we performed pulse-chase analysis in Hepa-1 cells after transient transfection with Nrf2-V5 and Nrf2Y568A-V5 (Fig. 5B). The results from pulse-chase analysis were in agreement with the in vivo degradation analysis. Nrf2 degraded much faster compared with Nrf2Y568A (Fig. 5B). This suggested that Nrf2Y568A phosphorylation leads to nuclear export of Nrf2 that binds to Irf2 and degrade.

PP2-mediated Inhibition of Nuclear Export of Nrf2 and Activation of Nrf2 Downstream Genes—Western and immunohistochemistry assays revealed that treatment of transfected Hepa-1 cells with PP2 led to time-dependent nuclear accumulation of Nrf2-V5 (Fig. 6A, left panel...
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A. Fyn siRNA

B. Tyrosine kinase Fyn phosphorylated Nrf2Y568 leading to nuclear export and degradation of Nrf2. In related experiments, PP2 treatment increased ARE-mediated luciferase activity in Nrf2-V5-overexpressing cells but not in cells overexpressing mutant Nrf2Y568A-V5 (Fig. 6C). In further experiments, PP2 treatment induced nuclear accumulation of endogenous Nrf2 in Me2SO- and t-BHQ-treated cells (Fig. 6, D and graph). The PP2-induced nuclear accumulation of Nrf2 was followed by increased expression of Nrf2 downstream gene NQO1 (Western analysis in Fig. 6E) and NQO1 activity (Fig. 6F). PP2 is a known specific inhibitor of Src tyrosine kinase family of enzymes (23). PP2-mediated inhibition of Nrf2Y568 phosphorylation (Fig. 4) and nuclear export of Nrf2 (Fig. 6) indicated that one or more members of the Src family of tyrosine kinases might have a role in phosphorylating Nrf2Y568.

Fyn siRNA-mediated Inhibition of Nuclear Export and Degradation of Nrf2 and Activation of ARE-luciferase—Four members of the Src family, namely Fyn, Src, Lyn, and Yes, are ubiquitously expressed as Nrf2 (4, 23). Among the Src kinases, Fyn kinase is interesting, because it is known to be phosphorylated in response to UV followed by localization of phosphorylated Fyn in the nucleus (24). Therefore, we studied the role of Fyn in phosphorylation of Nrf2Y568. Hepa-1 cells were transfected with Fyn siRNA or control siRNA and analyzed for Fyn expression, Nrf2Y568 phosphorylation, nuclear localization, and stability of Nrf2 and ARE-mediated luciferase gene expression and induction in response to antioxidant t-BHQ (Fig. 7). Western analysis revealed that Fyn siRNA transfection caused dose-dependent inhibition of Fyn, which was unaffected by control siRNA (Fig. 7A). Fyn siRNA also catalyzed dose-dependent inhibition of phosphorylation of Nrf2-V5 (Fig. 7B, blot and the graph in the lower panel). In addition, Fyn siRNA led to dose-dependent stabilization (Fig. 7C) and nuclear accumulation of Nrf2 (Fig. 7D) but had no effect on stability and localization of mutant Nrf2Y568A (Fig. 7, C and D). Furthermore, Fyn siRNA but not control siRNA showed dose-dependent increase in ARE-mediated luciferase gene expression in mock and Nrf2-V5-transfected cells (Fig. 7E). The Fyn siRNA-mediated increase in ARE-mediated luciferase gene expression and induction was absent in cells overexpressing mutant Nrf2Y568A-V5 (Fig. 7E). The slight increase in luciferase activity observed was expected because of endogenous Nrf2. These results indicated that Fyn, a member of the Src family of tyrosine kinases, phosphorylated Nrf2Y568 and this phosphorylation is required for nuclear export and degradation of Nrf2.

DISCUSSION

The studies have shown that 1Nrf2-Nrf2 complex serves as an oxidative sensor generated from chemicals, xenobiotics, drugs, UV, and radiation (4). This leads to dissociation of Nrf2 from INrf2. Nrf2 moves to the nucleus and binds to ARE. This results in coordinated activation of a battery of greater than 100 chemopreventive genes essential for protection against oxidative stress, cellular transformation, neoplasia, and other adverse effects. Nrf2 contains well defined signals that control its...
nuclear import and export (17). PKC-mediated phosphorylation of Nrf2S40 is known to dissociate Nrf2 from INrf2 leading to its nuclear import and activation of ARE-mediated gene expression (15, 16). However, what regulates nuclear export of Nrf2 is unknown.

The present studies demonstrate that tyrosine 568 in Nrf2 is phosphorylated, and this phosphorylation is essential for Nrf2 binding with Crm1 and nuclear export. Mutation of tyrosine 568 to alanine or phenylalanine resulted in the loss of phosphorylation and interaction of Nrf2 with Crm1 and abrogation of nuclear export of Nrf2. The wild-type Nrf2 and mutant Nrf2Y568A both interacted with INrf2 and were released/imported in the nucleus in response to endogenous cellular stressors. The mutant Nrf2Y568A lacking the tyrosine phosphorylation accumulated in the nucleus due to the loss of nuclear export of mutant protein. This was clearly evident from the observations that accumulation of mutant protein inside nucleus was insensitive to nuclear export inhibitor leptomycin B (LMB) and was similar to nuclear accumulation of wild-type Nrf2 protein in response to leptomycin B. The studies also indicated that hydrogen peroxide initially led to nuclear accumulation of Nrf2, presumably to activate chemoprotective genes, and later induced phosphorylation of tyrosine 568 for enhanced nuclear export of Nrf2. The mechanism of phosphorylated Nrf2 interaction with Crm1 remains unknown. It is expected that phosphorylation of Nrf2Y568 leads to structural changes that expose the leucine-rich nuclear export signal region (amino acid 545–554) for interaction with Crm1. The results further indicated that exported Nrf2 binds to INrf2 and degrades. The mutant Nrf2Y568A failed to exit the nucleus and degraded at a more reduced rate than wild-type Nrf2. The regulation of nuclear export and degradation of Nrf2 is one mechanism of high significance that controls Nrf2 abundance inside the nucleus. This is especially important because cells face constant everyday challenge of oxidative stress that continuously leads to import of Nrf2 that after use has to be exported out and degrade. The accumulation of Nrf2 inside the nucleus for longer time is lethal to cell survival (12).

It is well established that INrf2 binds to Nrf2 and retains it in the cytosol until the signal is received to release Nrf2. INrf2 is also known to function as Cul3-based E3 ligase in proteasomal degradation of Nrf2 (21, 22). The results from present studies demonstrate that tyrosine 568 phosphorylation is not essential for binding of Nrf2 with INrf2, because both Nrf2 and mutant Nrf2Y568A interacted with INrf2. This observation leads to an interesting hypothesis that INrf2 could recognize and differentiate between de novo synthesized unphosphorylated Nrf2 and the nuclear exported tyrosine-phosphorylated Nrf2. This recognition might allow INrf2 to target the nuclear exported phosphorylated Nrf2 for degradation and hold de novo synthesized unphosphorylated Nrf2 until a signal is received for its release. The viability of this reasonable hypothesis remains to be determined by experiments. The only result that requires explaining to fit in this hypothesis is the observation of INrf2-mediated degradation of mutant Nrf2Y568A inside the nucleus. It is possible that INrf2 recognized the mutant Nrf2Y568A the same as phosphorylated Nrf2 and targeted it for degradation.

The present studies suggest that a member of the Src family of tyrosine kinases, Fyn, phosphorylates Nrf2Y568 and regulates nuclear export and degradation of Nrf2. Fyn siRNA inhibited Fyn kinase expres-

FIGURE 8. Model depicting the role of phosphorylation of tyrosine 568 in regulation of Nrf2.

3 A. K. Jain and A. K. Jaiswal, unpublished observation.
Phosphorylation Controls Nuclear Export of Nrf2

 phosphorylation of Nrf2, and led to stability and nuclear accumulation of Nrf2 due to abrogation of nuclear export of Nrf2. It is possible but unknown if other Src kinases, including Src, Lyn, and Yes are also capable of phosphorylating tyrosine 568 in Nrf2. Fyn-mediated phosphorylation of Nrf2 also raises questions regarding mechanism by which Fyn kinase receives signals from hydrogen peroxide and other chemicals leading to activation and nuclear export of Nrf2.

In conclusion, we demonstrated that Fyn kinase-mediated phosphorylation of tyrosine 568 regulates Nrf2 interaction with Crm1 and nuclear export. The phosphorylated Nrf2 is degraded in the cytosol by binding to INrf2. A model is described in Fig. 8. PKC-mediated phosphorylation of Nrf2S40 leads to the release and nuclear translocation of Nrf2. Nrf2 binds with ARE and activates gene expression. Once this is done, Nrf2Y568 is phosphorylated by Fyn and presumably by other Src kinases leading to nuclear export of Nrf2 that binds to INrf2 and degrades. INrf2 could also translocate inside the nucleus, bind to Nrf2, and degrade Nrf2 inside the nucleus, and/or INrf2-Nrf2 complex is transported out for degradation of Nrf2 in the cytosol. The mutant Nrf2Y568A accumulates in the nucleus due to lack of nuclear export. INrf2 binds and degrades mutant Nrf2Y568A inside the nucleus or might bring it out in the cytosol and degrade it.

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