Nuclear Import and Export Signals in Control of Nrf2*

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Nrf2 binds to the antioxidant response element and regulates expression and antioxidant induction of a battery of chemopreventive genes. In this study, we have identified nuclear import and export signals of Nrf2 and show that the nuclear import and export of Nrf2 is regulated by antioxidants. We demonstrate that Nrf2 contains a bipartite nuclear localization signal (NLS) and a leucine-rich nuclear export signal, which regulate Nrf2 shuttling in and out of the nucleus. Immunofluorescence and immunoblot analysis revealed that Nrf2 accumulates in the nucleus within 15 min of antioxidant treatment and is exported out of nucleus by 8 h after treatment. Nrf2 mutant lacking the NLS failed to enter the nucleus and displayed diminished expression and induction of the downstream NAD(P)H:quinone oxidoreductase 1 gene. The Nrf2 NLS sequence, when fused to green fluorescent protein, resulted in accumulation of green fluorescence protein in the nucleus. The Nrf2 NES sequence, when fused to a heterologous protein, demonstrated that NES-mediated nuclear export of Nrf2 when fused to a heterologous protein. Further studies and could function as an independent export signal. The Nrf2 NES was sensitive to leptomycin B which caused Nrf2 to accumulate predominantly in the nucleus. The Nrf2 NES was sensitive to leptomycin B, demonstrating that this signal sequence was sufficient to direct nuclear accumulation of Nrf2 in the normal cell. The Nrf2 NES-mediated nuclear export of Nrf2 demonstrated that this signal sequence was sufficient to direct nuclear accumulation of Nrf2 in the normal cell. The Nrf2 NES mediated nuclear export of Nrf2 demonstrated that this signal sequence was sufficient to direct nuclear accumulation of Nrf2 in the normal cell. The Nrf2 NES-mediated nuclear export of Nrf2 demonstrated that this signal sequence was sufficient to direct nuclear accumulation of Nrf2 in the normal cell.

INrf2, leading to the loss of the association of Nrf2 with nuclear import and export signals of Nrf2 is particularly important in favor of activation of antioxidant enzymes. When this signal is achieved, Nrf2 exits the nucleus for binding to INrf2 and degradation.

The family of NF-E2-related factors includes three members, Nrf1, Nrf2, and Nrf3 (1–3). Nrf2 is most potent among the three protein factors in regulation of basal and induced expression of antioxidant enzyme genes (1). The studies have provided clear evidence that Nrf2/ARE-mediated coordinated expression and induction is a mechanism of critical importance in cellular protection against oxidative stress and neoplasia (1–3). Mice lacking the Nrf2 gene exhibited a marked decrease in the expression and induction of antioxidant enzyme genes, including NAD(P)H:quinone oxidoreductase 1 (NQO1) and increased sensitivity to chemically induced neoplasia (4, 5). INrf2 (inhibitor of Nrf2) or KEAP1 (Kelch-like ECH-associated protein 1) retains Nrf2 in the cytoplasm (1, 6, 7). INrf2 leads to the proteosomal degradation of Nrf2 in the normal cell cytoplasm (8–13). The exposure to antioxidants leads to dissolution of Nrf2/ARE-mediated expression and induction in response to a variety of stimuli including antioxidants (1–3). Nrf2 is most potent among the three protein factors in regulation of basal and induced expression of antioxidant enzyme genes (1). INrf2 (inhibitor of Nrf2) or KEAP1 (Kelch-like ECH-associated protein 1) retains Nrf2 in the cytoplasm (1, 6, 7). INrf2 leads to the proteosomal degradation of Nrf2 in the normal cell cytoplasm (8–13). The exposure to antioxidants leads to dissolution of Nrf2/ARE-mediated expression and induction in response to a variety of stimuli including antioxidants (1–3). Nrf2 is most potent among the three protein factors in regulation of basal and induced expression of antioxidant enzyme genes (1).

This article has been retracted by the publisher. An investigation at the University of Maryland, Baltimore determined that contrast and brightness were enhanced to selectively conceal LDH and Lamin B bands in Fig. 4. This drastically reduced the apparent presence of Lamin B in the cytosol fraction and of LDH in the nuclear fraction, thereby artificially increasing the difference in the proteins compared to that in the other fraction examined.

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Nrf2 in the nucleus have not been fully characterized. The nuclear localization signal that regulates nuclear translocation of Nrf2 is uncharacterized. In addition, the nuclear export of Nrf2 and the nuclear export signal in Nrf2 remain unknown. Furthermore, the antioxidant regulation of import and export signals of Nrf2 also remains unknown.

The purpose of the current study was to examine the nuclear import and export of Nrf2. Toward this end, a bipartite nuclear localization signal (NLS) was identified in the C terminus of Nrf2, which is required for Nrf2 nuclear import and which promoted nuclear import when fused to a heterologous green fluorescence protein (GFP). Deletion of the NLS led to the loss of nuclear localization of Nrf2 and diminished transcriptional activity of Nrf2 on downstream genes, including NQO1 in transfected cells, as compared with wild type Nrf2. A nuclear export signal (NES) was also identified in the C terminus of Nrf2, the deletion of which caused accumulation of Nrf2 in the nucleus. This NLS could function as an independent export signal when fused to a heterologous protein and was sensitive to leptomycin B. Antioxidant treatment led to nuclear accumulation of endogenous and transfected wild type Nrf2 but not an NLS mutant within 15 min. Nrf2 but not an NES mutant exited the nucleus by 8 h after antioxidant treatment. This exiting of Nrf2 was sensitive to leptomycin B. Further studies showed that NLS-mediated nuclear export of Nrf2 is required for degradation of Nrf2 in the cytosol. These results led to the conclusion that both nuclear import and export of Nrf2 play a significant role in basal expression and antioxidant induction of a battery of chemopreventive proteins, crucial for cellular growth and survival.

EXPERIMENTAL PROCEDURES

Plasmid DNAs—The construction of pcDNA-Nrf2 was previously described (28). As a template to construct several deletion constructs (Fig. 1). The forward 5'-GCAGGACTCAATGCGATCTTTTGC-3' and reverse 5'-GGTTTCTGTATGAGGAGGACATGAGCA-3' primers were used to amplify the Nrf2 coding region. The amplified product was TA-cloned into the pcDNA-Nrf2-V5 vector (Invitrogen). pcDNA-Nrf2-NLS-V5 was constructed by cloning the fragments just before and after the NLS region of Nrf2 primers forward (5'-GCAGGACATGCGATCTTTTGC-3') and reverse (5'-TATACTGCAGATCAATCGTGTAGACG-3') into pcDNA-Nrf2-V5 vector. The resulting amplified fragments were ligated and TA-cloned into the pcDNA3.1/V5-His vector following the protocol as suggested by the manufacturer. pcDNA-Nfr2-N2NES-V5 was constructed by PCR amplifying the fragments just before and after the NES region using the primers forward (5'-GCAGGACATGCGATCTTTTGC-3') and reverse (5'-GTTTTCTGTATGAGGAGGACATGAGCA-3') primers. The amplified fragments were ligated and TA-cloned into the pcDNA3.1/V5-His vector as described above. For constructing the ΔNeh2 mutants of pcDNA-Nrf2-V5 and pcDNA-Nfr2-NLS-V5, these plasmids were used as template for PCR amplification to amplify respective fragments using forward (5'-ACATGCGACATACATGCAGACGAGC-3') and reverse (5'-TATACTGCAGATCAATCGTGTAGACG-3') primers followed by cloning into pcDNA 3.1/V5-His TOPO vector. All of the plasmids were confirmed by sequencing and expressed a V5 epitope tag at their C terminus.

GFP, an NLS Fusion Protein—A DNA fragment corresponding to amino acids 494–511 of the nuclear localization signal (NLS) of Nrf2 was made by PCR. The primers used for PCR were forward (5'-TCAGGAGAATAAGTTAATGCGG-3') and reverse (5'-CCCTGCTTTCTCTCAGTCTG-3'), respectively. The resulting PCR product was cloned into pcDNA3.1 TOPO vector (Invitrogen). The plasmid was then digested with Kpn1 and Xba1, and the digested fragment was subcloned into EGFP-C1 vector (Clontech). The construct was named GFP-NLS, having GFP at the N terminus of the NLS.

Two Yellow Fluorescent Protein (2YFP), an NES Fusion Protein—The 2YFP vector was a gift from Dr. Yanping Zhang (M.D. Anderson Cancer Center, Houston, TX). A DNA fragment corresponding to amino acids 545–554 of the nuclear export signal (NES) of Nrf2 was made using the oligonucleotides, which were then annealed. The resulting fragment had Nhel and AgeI overhangs and was cloned into the corresponding sites in pc2YFP vector. The construct was confirmed by sequencing and named 2YFP-NES.

Cell Culture, Co-transfection of Expression Plasmids, and Luciferase Reporter Assays—Human hepatoma (HepG2) cells were grown in monolayer cultures in 6-well plates in minimum essential medium-alpha supplemented with 10% fetal bovine serum, both from Invitrogen. Transient transfections were done in cells grown to ~50% confluence using the Effectene Transfection reagent (Qiagen, Valencia, CA) following the manufacturer's protocol. Cells were co-transfected with 0.2 μg of reporter construct (human NQO1-ARE-Luc) mixed with pcDNA and different deletion constructs of Nrf2 (pcDNA-Nrf2-V5, pcDNA-ΔNeh2Nrf2-V5, pcDNA-Nrf2-ΔNLS-V5, and pcDNA-ΔNeh2N2NES-Nrf2-V5) in the quantities mentioned in the figures. The plasmid pRL-TK encoding Renilla luciferase was used as the internal control in each transfection. 36 h after transfection, the cells were induced with 50 μM t-butyldihydroquinone (t-BHQ) dissolved in Me2SO for 16 h. One set of transfected cells were treated with Me2SO for the same period of time and used as vehicle controls. To study the effect of leptomycin B on ARE activity for different time points, 36 h after transfection with reporter plasmids, cells were pretreated with 8 h with 20 ng/ml leptomycin B. After 8 h of pretreatment, cells were then treated with vehicle control Me2SO or 50 μM t-BHQ with or without 20 ng/ml leptomycin B for the indicated time periods. After treatment, the cells were washed with 1× phosphate-buffered saline (PBS) and fixed in 1× phosphate-buffered saline (PBS) on a System Kit (Promega, Madison, WI). Immunofluorescence—Mouse hepatoma (Hepa-1) cells were grown in Lab-Tek II chamber slides in Dulbecco's modified essential medium supplemented with 10% fetal bovine serum, both from Invitrogen. Cells were transfected with the desired plasmid using the procedures described above. For studying the localization of endogenous Nrf2, the cells were treated with 50 μM t-BHQ for 15 min and for 1, 2, 4, 6, and 8 h. The transfected cells were treated with t-BHQ in the presence or absence of 20 ng/ml leptomycin B (Sigma) for 8 h. Cells were then fixed in formalin (Polysciences, Inc., Warrington, PA) and permeabilized with cold aceton (Fisher). The antibody used for immunostaining the V5-tagged protein was anti-V5-FITC (Invitrogen), and for visualizing the endogenous protein, the cells were probed with Nrf2 antibody, (Santa Cruz Biotechnology). The fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibody (Chemicon International, Temecula, CA) was used as secondary antibody by procedures previously described (29). To visualize the nuclei, the cells were stained with Hoechst stain (Bio-Rad). The fluorescent images were captured using appropriate filters in a Nikon eclipse TE 2000-U fluorescent microscope fitted with a Photometrics CoolSnap CF camera, and images were enhanced using Adobe Photo-Deluxe software. In some experiments, the localization of Nrf2 and mutant Nrf2 was also determined by SDS-PAGE, Western blotting, and probing with Nrf2 antibody as described (12).

Subcellular Fractionation and Western Analysis—Hepa-1 cells were grown in 100-mm tissue culture plates, and after reaching 50% confluence, these cells were transfected with 2.0 μg of pcDNA-Nrf2-V5, pcDNA-Nfr2-NLS-V5, or pcDNA-Nfr2-ΔNLS-V5 plasmids using the Effectene transfection reagent as described above. Twenty-four hours
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Fig. 1. Schematic presentation of Nrf2 deletion mutants. Six discrete domains of the mouse Nrf2 protein are designated as Neh2 (amino acids 17–89), transactivation domain (amino acids 90–447), Cap ‘n’ Collar (amino acids 448–483), DNA-binding domain (amino acids 484–516), leucine zipper (amino acids 517–552), and Neh3 domain (amino acids 553–600). The top panel also shows wild type Nrf2 with the putative NLS (amino acids 494–511) and NES (amino acids 545–554). pcDNA-Nrf2ΔNeh2-V5 lacks the Neh2 domain (amino acids 1–90); pcDNA-Nrf2ΔNLS-V5 lacks the NLS; pcDNA-Nrf2ΔNeh2ΔNLS-V5 lacks both the Neh2 domain and the NLS; pcDNA-Nrf2ΔNES-V5 lacks the NES. GFP-NLS is the Nrf2 NLS sequence fused to the EGFP plasmid. p2YFP-NES is the Nrf2 NES sequence fused to the p2YFP plasmid.

After transfection, the cells were treated with Me2So, t-BHQ, or leptomycin B as indicated in the figures. At the end of treatment, cells were washed twice with ice-cold phosphate-buffered saline, scraped in phosphate-buffered saline 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. Briefly, the cell pellet was resuspended in 1× hypotonic buffer (cytoplasmic buffer) supplemented with complete protease inhibitor mixture (Roche Applied Science), incubated for 15 min, vortexed in the presence of detergents, and centrifuged.

A supernatant (cytoplasmic fraction) was collected into a precooled microcentrifuge tube; remaining is the nuclear pellet. The nuclear pellet was washed twice with the cytoplasmic buffer in the lysis buffer supplemented with 1× protease inhibitors. The suspension was incubated on ice for 30 min. The suspension was vortexed at 14,000 × g at 4 °C. The supernatant was collected. The protein concentration was determined using a BCA assay reagent (Bio-Rad). 100 µg of the nuclear extract was used on a 10% SDS-PAGE, Western blotted, and probed with antibody against horseradish peroxidase (Invitrogen) and β-actin (Sigma). To confirm the purity of subcellular fractionation, protein extracts were immunoblotted with cytoplasmic and nuclear markers. To detect Nrf2 in different subcellular compartments, we added and incubated primary antibodies complexes were washed twice with 1× SDS sample buffer, boiled with 1× SDS sample dye, and resolved by immunoblotting with antibody to Nrf2, anti-GFP, and anti-FLAG-horseradish peroxidase antibodies.

RESULTS

We generated several V5-tagged deletion mutants of Nrf2 to identify and characterize nuclear import and export signals of Nrf2 and show that the nuclear import and export of Nrf2 is regulated by antioxidants. We further demonstrate that NLS-mediated nuclear import is required for activation of ARE-mediated gene expression. We also demonstrate that NES-mediated nuclear export is required for rapid degradation of Nrf2 in the cytosol. The structures of wild type Nrf2 and Nrf2 deletion mutants used in the present study are shown in Fig. 1. Prosite search of Nrf2 amino acid sequence tentatively identified a single copy each of the NLS and NES region at the C terminus of the protein (Fig. 1). The NLS was found located between amino acids 494 and 511, and the NES was between amino acids 545 and 554.

Hep-G2 cells were co-transfected with reporter plasmid NQO1 gene ARE-Luc and different concentrations of expression plasmids encoding Nrf2 or Nrf2-V5 and Nrf2ΔNES-V5 as shown in Fig. 2 to determine the role of NLS on Nrf2 regulation of ARE-mediated gene expression. Western analysis analyzed
Fig. 2. Effect of deletion of NLS on Nrf2 regulation of ARE-mediated gene expression and induction in response to t-BHQ. A–C, Western blotting showing the overexpression of transfected plasmids. 100-μg lysates of HepG2 cells transfected with different concentrations of pcDNA-Nrf2 (A), pcDNA-Nrf2V5 (B), or pcDNA-Nrf2ΔNLS-V5 (C) were resolved on 10% SDS-PAGE and immunoblotted with anti-Nrf2, anti-V5, horseradish peroxidase, or β-actin antibodies. *, nonspecific band with anti-Nrf2 antibody.

D–F, pcDNA-Nrf2; pcDNA-Nrf2V5; pcDNA-Nrf2ΔNLS-V5; cells transfected with pcDNA-Nrf2-V5; F, cells transfected with pcDNA-Nrf2V5; pcDNA-Nrf2ΔNLS-V5.

The overexpression of Nrf2, Nrf2V5, and Nrf2ΔNLS-V5 in transfected cells (Fig. 2, A–C). The transfection of HepG2 cells with plasmids encoding wild type Nrf2, Nrf2V5, or Nrf2ΔNLS-V5 overexpressing Nrf2 and Nrf2-V5 both up-regulated ARE-mediated luciferase expression and induction in response to t-BHQ in Fig. 2, D–E). These results demonstrated that increased levels of Nrf2 and Nrf2ΔNLS-V5 in transfected cells failed to increase basal and/or antioxidant-induced expression of ARE-mediated gene expression with an increase in overexpression of Nrf2ΔNLS-V5 in transfected cells (Fig. 2, D–E). However, the transfection with 0.5 μg of Nrf2ΔNLS-V5 showed insignificant but persistent increase in the basal and antioxidant-induced ARE activity (Fig. 2F, last panel). This increase in ARE activity is presumably a result of the binding of Nrf2ΔNLS-V5 with INrf2 and titration of endogenous Nrf2. Nrf2ΔNLS-V5 has the INrf2-binding Neh2 domain and expected to bind to INrf2. As a result, some of the endogenous Nrf2 is titrated out and translocates to the nucleus, leading to ARE activation. To explore this possibility, we generated Nrf2 and Nrf2ΔNLS-V5 mutants lacking the Neh2 domain that binds with INrf2. These were designated as Nrf2ΔNeh2-V5 and Nrf2ΔNeh2ΔNLS-V5 (Fig. 1). These plasmids were transfected in Hep-G2 cells to determine the role of Neh2 and NLS domains in the Nrf2 regulation of ARE-mediated luciferase and endogenous NQO1 gene expression and induction in response to t-BHQ. Western analysis with V5 antibody revealed overexpression of Nrf2 and Nrf2 deletions in transfected cells (Fig. 3A). Overexpression of wild type Nrf2 showed a significant increase in basal and induced expression of luciferase and endogenous NQO1 genes compared with wild type Nrf2 (compare lanes 1 and 2 in Fig. 3, D–E). Deletion of the Neh2 INrf2-binding domain from Nrf2 also led to an Nrf2ΔNeh2V5-induced increase in basal and t-BHQ-induced expression of ARE-luciferase and endogenous NQO1 genes (compare lanes 1 and 3 in Fig. 3, F–D). However, the deletion of the NLS domain from Nrf2 led to significant loss in basal and induced expression as compared with wild type Nrf2 (compare lanes 2 and 4 in Fig. 3, F–D). Interestingly, the deletion of both Neh2 and NLS domains from Nrf2 resulted in the complete loss of capacity of Nrf2 to activate ARE-mediated luciferase and endogenous NQO1 gene expression and induction in response to t-BHQ (compare lanes 1 and 5 in Fig. 3, F–D). The minor increase in basal and induced expression of luciferase and the NQO1 gene in the case of Nrf2ΔNLS-V5 in Figs. 2F and 3, F–D (lane 4), was not observed with Nrf2ΔNeh2ΔNLS-V5 (Fig. 3, F–D, lane 5). This is because the Nrf2ΔNeh2ΔNLS-V5 mutant failed to bind to INrf2 and release endogenous Nrf2 by titration.

The Nrf2 NLS (amino acids 494–511) was aligned with previously characterized NLS from nucleoplasmin, p73, and p53 (Fig. 4A). Results revealed that Nrf2 NLS was highly homologous to NLS from nucleoplasmin, p73, and p53. Nrf2 NLS showed conservation of basic amino acid clusters and was bipartite as observed with nucleoplasmin, p73, and p53 NLS. Hepa-1 cells were transfected with Nrf2V5 or Nrf2ΔNLS-V5 to analyze the subcellular localization of Nrf2 with or without the NLS. The transfected Hepa-1 cells were biochemically fractionated into nuclear and cytoplasmic fractions. The two fractions were then immunoblotted with anti-V5 antibody. Western analysis revealed that in MeSO-treated cells, wild type Nrf2-V5 was present both in cytoplasm and nucleus (Fig. 4B), whereas the Nrf2ΔNLS-V5 was only present in the cytoplasm (Fig. 4B). The Nrf2ΔNLS-V5 was absent in nuclear fraction. The treatment of transfected cells with t-BHQ for 15 min showed Nrf2 localization predominantly in the nucleus (Fig. 4B).
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Effect of deletion of Nrf2-binding domain Neh2 and NLS on ARE-mediated luciferase and endogenous NQO1 gene expression and induction. A–D, lane 1, pcDNA vector; lane 2, pcDNA-Nrf2-V5; lane 3, pcDNA-Nrf2ΔNeh2-V5; lane 4, pcDNA-Nrf2ΔNLS-V5. A, Western blotting showing the overexpression of transfected plasmids. HepG2 cells were transfected with pcDNA empty vector, wild type pcDNA-Nrf2-V5, or Nrf2 deletion mutants. SDS-PAGE and immunoblotted with anti-V5-horseradish peroxidase antibodies. B, ARE-luciferase assay. HepG2 cells were seeded in 6-well plates, and 24 h after incubation, the cells were co-transfected with NQO1 ARE luciferase reporter plasmid and Renilla luciferase plasmid along with the pcDNA-Nrf2-V5, pcDNA-Nrf2ΔNeh2-V5, or pcDNA-Nrf2ΔNLS-V5 as described. C, NQO1 activity. D, Western blot analysis of NQO1 protein. Cytosolic extracts from transfected HepG2 cells treated with Me2SO or t-BHQ for 8 h showed localization of Nrf2 both in cytoplasm and nucleus (Fig. 5, LMB). However, the treatment of cells with leptomycin B (LMB) blocked nuclear export of endogenous Nrf2 in Hepa-1 cells (Fig. 5C, +LMB). The treatment of Hepa-1 cells with t-BHQ for 8 h showed localization of Nrf2 both in cytoplasm and nucleus (Fig. 5D, −LMB). These results clearly demonstrated that leptomycin B blocked nuclear export of endogenous Nrf2 in Me2SO-treated as well as t-BHQ-treated Hepa-1 cells. The results also suggested that Nrf2 is actively exported out from the nucleus in a leptomycin B-sensitive manner.

We used nuclear export inhibitor leptomycin B to determine the role of NES in nuclear export and localization of endogenous Nrf2. Hepa-1 cells were treated with Me2SO and t-BHQ in the absence or presence of leptomycin B and probed with anti-Nrf2 followed by FITC-tagged secondary antibody to determine localization of endogenous Nrf2 protein in the absence and presence of leptomycin B. Endogenous Nrf2 was found localized both in cytoplasm and nucleus in the absence of leptomycin B (Fig. 5C, −LMB). However, the treatment of cells with leptomycin B led to nuclear accumulation of Nrf2 in Me2SO-treated cells (Fig. 5C, +LMB). The treatment of Hepa-1 cells with t-BHQ for 8 h showed localization of Nrf2 both in cytoplasm and nucleus (Fig. 5D, −LMB). These results clearly demonstrated that leptomycin B blocked nuclear export of endogenous Nrf2 in Me2SO-treated as well as t-BHQ-treated Hepa-1 cells. The results also suggested that Nrf2 is actively exported out from the nucleus in a leptomycin B-sensitive manner.

We generated a mutant Nrf2ΔNES-V5 deficient in NES by internal deletion of NES and used this mutant to further confirm the role of NES in nuclear export of Nrf2 in transfected cells. The Hepa-1 cells were transfected with pcDNA-Nrf2-V5 or pcDNA-Nrf2ΔNES-V5, treated with either Me2SO or t-BHQ for 8 h, and probed with FITC-tagged V5 antibodies. In related
transfected with pcDNA-Nrf2-V5 or pcDNA-Nrf2-NLS-V5. 24 h after transfection, cells were treated with either Me2SO or t-BHQ (50 μM) for 15 min. Cells were then harvested, and cytosol and nuclear extracts were prepared and immunoblotted against anti-V5 antibody as described under "Experimental Procedures." The immunoblots were stripped and reprobed with anti-lamin B and anti-lactate dehydrogenase antibodies to confirm equal loading. C, immunohistochemistry. Hepa-1 cells in chamber slides were transfected with plasmids encoding either pcDNA-Nrf2-V5 or pcDNA-Nrf2-NLS-V5 as described under "Experimental Procedures." 36 h after transfection, the cells were fixed and visualized for green fluorescence for GFP, showing Nrf2 localization; lower panel, stained nucleus with the Hoechst stain. D, immunoblot analysis. Hepa-1 cells were transfected with plasmids encoding GFP alone or the plasmid encoding the GFP fused to Nrf2 NLS as described under "Experimental Procedures." The transfected cells were fixed and visualized for green fluorescence for GFP. Left panel, green fluorescence of the GFP plasmid alone; middle panel, stained nucleus with the Hoechst stain; right panel, merged images from green and blue filters. Each experiment was repeated at least three times, and representative results are shown.

experiments, the transfected cells were subcellularly fractionated, and nuclear and cytosolic fractions were prepared by standard procedures. The nuclear and cytosolic proteins were separated on SDS-PAGE, Western blotted, and probed with V5 antibodies. The purity of nuclear and cytosolic fractions was tested by probing Western blots with anti-lamin B and anti-lactate dehydrogenase antibodies, respectively. The results of immunofluorescence are shown in Fig. 6, A and B, and results of Western analysis are shown in Fig. 6, C and D. The results showed immunofluorescence both in the cytosol and the nucleus of Me2SO-treated Hepa-1 cells expressing wild type Nrf2-V5 (Fig. 6A, Me2SO-LMB). This showed that Nrf2-V5 localized both in cytosolic and nuclear fractions. The results with Me2SO-treated Hepa-1 cells expressing mutant Nrf2ΔNES-V5 protein showed localization of mutant Nrf2 protein deficient in NES predominantly in the nucleus (Fig. 6B, Me2SO-LMB). The treatment of transfected cells with t-BHQ for 8 h showed similar localization of wild type Nrf2-V5 and mutant Nrf2ΔNES-V5 proteins as observed with Me2SO-treated cells. The results with Nrf2-V5 were similar as those observed with endogenous Nrf2 protein in Fig. 5A and indicated that t-BHQ-induced nuclear import of Nrf2-V5 was followed by nuclear export of Nrf2-V5 (Fig. 5B, t-BHQ-LMB). Interestingly, the Hepa-1 cells transfected with pcDNA-Nrf2ΔNES-V5 deficient in NES showed a prominent nuclear fluorescence in cells treated with t-BHQ for 8 h, indicating the absence of nuclear export of Nrf2ΔNES (Fig. 6B, t-BHQ-LMB). The pretreatment of transfected cells with leptomycin B blocked the nuclear export of Nrf2 in Me2SO- and t-BHQ-treated cells as evident from the absence of immunofluorescence in cytosol (Fig. 6A, Me2SO + LMB and t-BHQ + LMB). However, no effect of leptomycin B on nuclear localization of Nrf2ΔNES mutant was observed in Me2SO- and t-BHQ-treated cells (Fig. 6B, Me2SO + LMB and t-BHQ + LMB). Western analysis supported immunofluorescence studies (Fig. 6, C and D).

We performed in vitro and in vivo experiments and evaluated Nrf2 and Nrf2ΔNES mutant interaction with INrf2 to confirm that the predominant nuclear localization of Nrf2ΔNES is because of the deficiency of export signal and not because of the loss of interaction with INrf2 (Fig. 7). The plasmids encoding Nrf2-V5, Nrf2ΔNES-V5, and INrf2 were in vitro transcribed and translated. The translated proteins ran at required size on a 10% gel (Fig. 7A, lanes 1–3). The in vitro translated proteins were mixed together in equal amounts and then immunoprecipitated using the anti-V5 antibody and autoradiographed for 35S signal. The results demonstrated that Nrf2ΔNES interacted with INrf2 similar as wild type Nrf2 (Fig. 7A, lanes 5 and 7). Nrf2ΔNES interaction with INrf2 was also determined in in vivo experiments as shown in Fig. 7B. Hepa-1 cells were co-

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**FIG. 4. Requirement of nuclear localization signal for antioxidant-induced Nrf2 nuclear import.** A, alignment of Nrf2 NLS with other protein NLSs. The amino acid sequence of the bipartite NLS of nucleoplasmin, p73, and p53 proteins. Homologous amino acids are shown in boldface type and those mutated in Nrf2-NLS by site-directed mutagenesis are shown in italic. B, Nucleoplasmic, p73, and p53 proteins, and Nrf2 were transcribed with t-BHQ (50 μM) for 15 min. Cells were then harvested, and cytosol and nuclear extracts were prepared and immunoblotted against anti-V5 antibody as described under "Experimental Procedures." The immunoblots were stripped and reprobed with anti-lamin B and anti-lactate dehydrogenase antibodies to confirm equal loading. C, immunohistochemistry. Hepa-1 cells in chamber slides were transfected with plasmids encoding either pcDNA-Nrf2-V5 or pcDNA-Nrf2-NLS-V5 as described under "Experimental Procedures." 36 h after transfection, the cells were fixed and visualized for green fluorescence for GFP, showing Nrf2 localization; lower panel, stained nucleus with the Hoechst stain. D, immunoblot analysis. Hepa-1 cells were transfected with plasmids encoding GFP alone or the plasmid encoding the GFP fused to Nrf2 NLS as described under "Experimental Procedures." The transfected cells were fixed and visualized for green fluorescence for GFP. Left panel, green fluorescence of the GFP plasmid alone; middle panel, stained nucleus with the Hoechst stain; right panel, merged images from green and blue filters. Each experiment was repeated at least three times, and representative results are shown.

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**FIG. 5. Effect of t-BHQ on localization of Nrf2.** A, green fluorescence showing Nrf2 localization; lower panel, stained nucleus with the Hoechst stain. B, immunofluorescence studies were performed on Hepa-1 cells expressing wild type Nrf2-V5 (Fig. 5A, Me2SO-LMB). This showed that Nrf2-V5 localized both in cytosolic and nuclear fractions. The results with Me2SO-treated Hepa-1 cells expressing mutant Nrf2ΔNES-V5 protein showed localization of mutant Nrf2 protein deficient in NES predominantly in the nucleus (Fig. 5B, Me2SO-LMB). The treatment of transfected cells with t-BHQ for 8 h showed similar localization of wild type Nrf2-V5 and mutant Nrf2ΔNES-V5 proteins as observed with Me2SO-treated cells. The results with Nrf2-V5 were similar as those observed with endogenous Nrf2 protein in Fig. 5A and indicated that t-BHQ-induced nuclear import of Nrf2-V5 was followed by nuclear export of Nrf2-V5. Interestingly, the Hepa-1 cells transfected with pcDNA-Nrf2ΔNES-V5 deficient in NES showed a prominent nuclear fluorescence in cells treated with t-BHQ for 8 h, indicating the absence of nuclear export of Nrf2ΔNES. This showed that Nrf2-V5 localized both in cytosol and the nucleus of Me2SO-treated Hepa-1 cells expressing wild type Nrf2-V5. The results with Me2SO-treated Hepa-1 cells expressing mutant Nrf2ΔNES-V5 protein showed localization of mutant Nrf2 protein deficient in NES predominantly in the nucleus. The pretreatment of transfected cells with leptomycin B blocked the nuclear export of Nrf2 in Me2SO- and t-BHQ-treated cells as evident from the absence of immunofluorescence in cytosol. However, no effect of leptomycin B on nuclear localization of Nrf2ΔNES mutant was observed in Me2SO- and t-BHQ-treated cells. Western analysis supported immunofluorescence studies. We performed in vitro and in vivo experiments and evaluated Nrf2 and Nrf2ΔNES mutant interaction with INrf2 to confirm that the predominant nuclear localization of Nrf2ΔNES is because of the deficiency of export signal and not because of the loss of interaction with INrf2. The plasmids encoding Nrf2-V5, Nrf2ΔNES-V5, and INrf2 were in vitro transcribed and translated. The translated proteins ran at required size on a 10% gel. The in vitro translated proteins were mixed together in equal amounts and then immunoprecipitated using the anti-V5 antibody and autoradiographed for 35S signal. The results demonstrated that Nrf2ΔNES interacted with INrf2 similar as wild type Nrf2. Nrf2ΔNES interaction with INrf2 was also determined in in vivo experiments as shown in Fig. 7B. Hepa-1 cells were co-
transfected with V5-tagged wild type Nrf2 along with FLAG-tagged INrf2. INrf2 was immunoprecipitated with IgG or anti-V5 antibody. These results clearly demonstrate that INrf2 co-immunoprecipitates with both Nrf2 and Nrf2ΔNES (Fig. 7B, left and right panel). Therefore, both in vitro and in vivo assays showed that Nrf2ΔNES bound to INrf2 the same as wild type Nrf2 and that predominant localization of Nrf2ΔNES in the nucleus was not due to loss of interaction with INrf2 but was due to the loss of NES from Nrf2ΔNES mutant protein.

The NES domain of Nrf2 was synthesized and cloned in frame with two copies of YFP to generate plasmid p2YFP-NES. Immunofluorescence analysis of Hepa-1 cells transfected with p2YFP-NES vector alone showed localization of YFP in the cytosol and nucleus, mostly in the nucleus, with no effect of leptomycin B treatment on localization of p2YFP (Fig. 8A). Interestingly, p2YFP-NES in a similar assay was found localized predominantly in cytosol (Fig. 8B, left panel). Nuclear localization of YFP-NES was not detected. Interestingly, the pretreatment of cells with leptomycin B blocked the nuclear export of YFP-NES, and YFP-NES was only detected in the nucleus (Fig. 8B, right panel).

We also analyzed the effect of leptomycin B on expression and t-BHQ induction of the NQO1 ARE-mediated luciferase gene to demonstrate the role of NES in Nrf2 regulation of basal expression and antioxidant induction of downstream genes. The transfection of the NQO1 gene ARE-Luc in HepG2 cells expressed ARE-mediated luciferase activity that was induced in response to t-BHQ (Fig. 9, compare MeSO with t-BHQ). The t-BHQ-induced expression was highest at 8 h after treatment and plateaued thereafter at 16, 24, and 36 h after t-BHQ treatment. In other words, the induction was the same between 8 and 36 h of t-BHQ treatment. This finding is in agreement with our Nrf2 localization data, where Nrf2 attains a normal localization pattern 8 h after t-BHQ induction. Interestingly, the treatment of cells with leptomycin B significantly increased basal expression and t-BHQ induction of ARE-mediated gene expression, especially at 24 and 36 h after MeSO and t-BHQ treatment (Fig. 9, compare MeSO + LMB with t-BHQ + LMB).

We followed the stability of Nrf2 and Nrf2ΔNES proteins in transfected cells to determine the functional mechanism of the role of nuclear export of Nrf2 in degradation of Nrf2. The degradation pattern of Nrf2ΔNES was compared with wild type Nrf2 in whole cell lysates and cytosolic and nuclear extracts. Hepa-1 cells were transfected with Nrf2V5 or Nrf2ΔNES-V5. The cells were pretreated with MG132 for 8 h to initially inhibit the proteasomal degradation of Nrf2, leading to accumulation of Nrf2 for degradation studies. The cells were then treated with cycloheximide for different time points to block new protein synthesis and let the accumulated Nrf2 degrade in the cell via proteasomal degradation. This design is frequently used to study the stability/degradation of proteins (11). Whole cell lysate, cytosolic, and nuclear extracts prepared from these cells were immunoblotted with anti-V5 antibody. Whole cell lysate, wild type Nrf2 stabilized after treatment with MG132 and disappeared within 2 h of cycloheximide treatment (Fig 10A, top left panel). On the contrary, Nrf2ΔNES degradation was significantly slower as compared with wild type Nrf2. This was evident from the observation that mutant Nrf2ΔNES was visible in significant amounts even 3 h after cycloheximide treatment (Fig. 10A, top right panel). It is also noteworthy that
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Fig. 6. C-terminal NES in Nrf2 is required for Nrf2 export and is sensitive to leptomycin B. A and B, immunohistochemistry of transfected cells. Hepa-1 cells were transfected with plasmids encoding either wild type Nrf2-V5 or V5-tagged mutant Nrf2ΔNES as described under “Experimental Procedures.” 36 h after transfection, cells were harvested, fractionated, and examination. Nrf2 localization was determined by immunofluorescence with anti-V5-FITC antibody as described under “Experimental Procedures.” The cells were also stained with Hoechst stain to visualize the nuclei in a blue filter. A, cells transfected with pcDNA-Nrf2-V5 plasmid; B, cells transfected with pcDNA-Nrf2ΔNES-V5 plasmid; middle panel, stained nucleus with Hoechst stain; right panels, merged images respective of Nrf2 and mutant Nrf2 detected with anti-V5 antibodies. Western blots were also probed with anti-lamin B and anti-lactate dehydrogenase antibodies to test the purity of nuclear and cytosolic fractions, respectively. Western blots were probed with V5 antibodies. Western blots were also probed with V5 antibodies. Western blots were also probed with V5 antibodies. Western blots were also probed with V5 antibodies.

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In addition, the amount of Nrf2-V5 in MG132-treated cytosol, both wild type Nrf2-V5 and mutant Nrf2ΔNES-V5, were degraded rapidly (Fig. 10A, cytosolic extract panels). However, the amount of Nrf2-V5 in the MG132 lane was higher than Nrf2ΔNES-V5 (compare lanes 2 in the cytosolic extract panels in Fig. 10A). In the nucleus, the wild type Nrf2 degradation was as compared with Nrf2ΔNES-V5 in MG132-treated whole cell extract (compare lane 2 in whole cell lysate panels in Fig. 10A). The higher amount of Nrf2-V5 in MG132-treated cytosolic extract despite a lower amount in whole cell extract was due to stabilization of newly synthesized and nuclear exported Nrf2-V5. On the other hand, the lower content of Nrf2ΔNES-V5 in MG132-treated whole cell lysate extract (compare lane 2 in whole cell lysate panels in Fig. 10A). The higher amount of Nrf2-V5 in MG132-treated cytosolic extract despite a lower amount in whole cell extract was due to stabilization of newly synthesized and nuclear exported Nrf2-V5. On the other hand, the lower content of Nrf2ΔNES-V5 in MG132-treated whole cell lysate extract (compare lane 2 in whole cell lysate panels in Fig. 10A). The higher amount of Nrf2-V5 in MG132-treated cytosolic extract despite a lower amount in whole cell extract was due to stabilization of newly synthesized and nuclear exported Nrf2-V5. On the other hand, the lower content of Nrf2ΔNES-V5 in MG132-treated whole cell lysate extract (compare lane 2 in whole cell lysate panels in Fig. 10A). The higher amount of Nrf2-V5 in MG132-treated cytosolic extract despite a lower amount in whole cell extract was due to stabilization of newly synthesized and nuclear exported Nrf2-V5. On the other hand, the lower content of Nrf2ΔNES-V5 in MG132-treated whole cell lysate extract (compare lane 2 in whole cell lysate panels in Fig. 10A). The higher amount of Nrf2-V5 in MG132-treated cytosolic extract despite a lower amount in whole cell extract was due to stabilization of newly synthesized and nuclear exported Nrf2-V5. On the other hand, the lower content of Nrf2ΔNES-V5 in MG132-treated whole cell lysate extract (compare lane 2 in whole cell lysate panels in Fig. 10A). The higher amount of Nrf2-V5 in MG132-treated cytosolic extract despite a lower amount in whole cell extract was due to stabilization of newly synthesized and nuclear exported Nrf2-V5. On the other hand, the lower content of Nrf2ΔNES-V5 in MG132-treated whole cell lysate extract (compare lane 2 in whole cell lysate panels in Fig. 10A). The higher amount of Nrf2-V5 in MG132-treated cytosolic extract despite a lower amount in whole cell extract was due to stabilization of newly synthesized and nuclear exported Nrf2-V5. On the other hand, the lower content of Nrf2ΔNES-V5 in MG132-treated whole cell lysate extract (compare lane 2 in whole cell lysate panels in Fig. 10A).

Discussion

Nrf2-mediated expression and coordinated induction of a battery of defensive genes including detoxifying enzymes is a mechanism of critical importance in protection against chemically induced oxidative stress and neoplasia (1). Therefore, the signals/mechanisms that regulate nuclear availability of Nrf2 are extremely important for the regulation of expression and induction of defensive genes. In the current study, we identified and characterized a bipartite NLS, which is required for Nrf2 nuclear localization and can promote the nuclear localization of a normally cytoplasmic Nrf2 protein. In addition, we demonstrated, for the first time, that Nrf2 undergoes active nuclear export and that this export is leptomycin B-sensitive and mediated by an NES located in the Nrf2 C terminus. Nrf2 is normally retained in the cytoplasm by its inhibitor IκBα (6, 7). Antioxidants antagonize this interaction leading to the release of Nrf2 from IκBα. Nrf2 translocates in the nucleus. This leads to increased ARE-mediated gene expression. A search of the Nrf2 amino acid sequence identified an NLS domain in the C terminus of the Nrf2 protein between amino acids 494 and 511 (amino acid sequence RRRGKQKVAANQCRKRR). Nrf2 NLS sequence aligned perfectly with well characterized NLS sequences from nucleoplasmins, p73, and p53. The NLS was required for nuclear translocation of Nrf2. This was clearly evident from several observations. Nrf2 mutant lacking the NLS failed to enter the nucleus and displayed...
brane and immunoblotted with anti-V5 antibody. In a similar experiment, the samples were transferred to the nitrocellulose membrane and immunoblotted with anti-V5 antibody to enhance the 35S signal, dried, and autoradiographed. In a similar experiment, the samples were transferred to the nitrocellulose membrane and immunoblotted with anti-V5 antibody.

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FIG. 7. In vitro and in vivo interaction of Nrf2 and Nrf2ΔNES with INrf2. A, in vitro interaction of Nrf2 and Nrf2ΔNES with INrf2. pDNA-Nrf2-V5, pDNA-Nrf2ΔNES-V5, and pDNA-INrf2 plasmids were in vitro transcribed/translated using the TNT coupled reticulocyte lysate system by procedures described under “Experimental Procedures.” 5 μl of the translated proteins were loaded in the input lane. Equal amounts of proteins were mixed with the binding buffer as indicated and incubated at 37 °C for 30 min, and the mixture was immunoprecipitated with either mouse IgG or anti-V5 antibody by shaking at 4 °C overnight. The input and immunoprecipitates were resolved on a 10% SDS-PAGE, treated with Amplify solution to enhance the 35S signal, dried, and autoradiographed. In a similar experiment, the samples were transferred to the nitrocellulose membrane and immunoblotted with anti-V5 antibody.

B, in vivo interaction of Nrf2 and Nrf2ΔNES with INrf2. Hepa-1 cells were seeded in 100-mm plates and co-transfected with plasmids encoding wild type Nrf2-V5 or Nrf2ΔNES-V5 along with FLAG-INrf2. 36 h after transfection, cells were either left untreated or nuclear export was inhibited by leptomycin B for 8 h before fixing and examination. Cells were visualized in a green filter for YFP fluorescence and a blue filter for Hoescht stained nuclei. A, cells transfected with p2YFP empty vector; B, cells transfected with p2YFP-NES plasmid. Top panel, green fluorescence of the YFP; middle panel, stained nucleus with the Hoechst stain; lower panel, merged images from green and blue filters. Each experiment was repeated three times, and representative results are shown here.

FIG. 8. Nrf2 C terminus NES as an autonomous export signal. A and B, immunohistochemistry. Hepa-1 cells were transfected with plasmids encoding tandem copies of YFP (2YFP) alone or the plasmid encoding the 2YFP fused to Nrf2 NES as described under “Experimental Procedures.” 36 h after transfection, cells were either left untreated (−LMB) or treated (+LMB) with leptomycin B for 8 h prior to fixing and examination. Cells were visualized in a green filter for YFP fluorescence and a blue filter for Hoechst stained nuclei. A, cells transfected with p2YFP empty vector; B, cells transfected with p2YFP-NES plasmid. Top panel, green fluorescence of the YFP; middle panel, stained nucleus with the Hoechst stain; lower panel, merged images from green and blue filters. Each experiment was repeated three times, and representative results are shown here.

FIG. 9. Effect of leptomycin B on Nrf2-mediated ARE activity. HepG-2 cells were seeded in 6-well plates 24 h after incubation; the cells were co-transfected with NQO-1 ARE luciferase reporter plasmid and Renilla luciferase plasmid. 36 h after transfection, cells were pretreated with 20 ng/ml leptomycin B for 8 h. Cells were then treated with Me2SO or t-BHQ (50 μM) with or without leptomycin B for 8, 16, 24, and 36 h. Cells untreated or only pretreated with leptomycin B were harvested and lysed as 0-h readings. Cells were then harvested and lysed to analyze the luciferase activity. The results are presented ± S.E. of three independent experiments, and each experiment was done in triplicate.

diminished expression and induction of downstream genes including NQO1. The small amount of expression and induction observed in Fig. 2 was the same as in vector (pDNA)-transfected control and was due to endogenous Nrf2. In similar experiments, the wild type Nrf2 significantly increased basal and induced expression of the downstream NQO1 gene. The Nrf2 NES sequence fused to GFP resulted in the nuclear accumulation of GFP, indicating that this signal sequence was sufficient to direct nuclear localization of Nrf2. It appears that Nrf2 contains a single NLS, since no other NLS was found by sequence analysis, and deletion of NLS led to complete loss of nuclear localization of Nrf2 in the nucleus.

Immunofluorescence studies on endogenous Nrf2 proteins in Hepa-1 cells treated with antioxidants demonstrated nuclear localization of Nrf2 within 15 min of antioxidant treatment. Immunofluorescence analysis also demonstrated nuclear export of Nrf2 to the cytoplasm that might have started as early as 1 h after antioxidant treatment and was clearly visible at 8 h after antioxidant treatment. One can argue that newly synthesized Nrf2 might have contributed to the cytoplasmic appearance of Nrf2 in Hepa-1 cells 8 h after antioxidant treatment. This is possible; however, our data indicate that the contribution of new synthesis has to be minimal, because pretreatment of cells with leptomycin B failed to demonstrate the cytosolic presence of Nrf2. Therefore, a majority of the cytosolic presence of Nrf2 in Hepa-1 cells 8 h after treatment with antioxidant was due to nuclear export of Nrf2. The immunofluorescence of Nrf2-V5-transfected Hepa-1 cells with V5 antibody also supported the above conclusions on nuclear export of Nrf2. Amino acid sequence analysis of Nrf2 identified a leucine-rich NES consensus sequence at the C terminus of the Nrf2 protein between amino acids 545 and 554 (amino acid sequence LKRRLTLYL). The NES consensus sequence was determined from previously reported nuclear export signals from several proteins, including IkB-α, TFIIA, hDM2, p53, p73, and PKI-α proteins (30). The studies with mutant Nrf2 lacking NES confirmed that Nrf2 NES is functional. Nrf2 was more prominent in the nucleus when its C-terminal NES was deleted by mutation or when nuclear export was inhibited by leptomycin B treatment. The predominant localization of NES-deficient mutant Nrf2ΔNES in the nucleus was not due to lack of its interaction with INrf2, because Nrf2ΔNES interacted with INrf2 the same as wild type Nrf2. Our studies also demonstrated that the Nrf2 C-terminal NES can function as an au-
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were washed and treated with 30 μg/ml cycloheximide (CHX) for different time points. For the experiments with inhibitors, cells were transfected with either pcDNA-Nrf2-V5 or pcDNA-Nrf2ΔNES-V5. 24 h after transfection, the cells were pretreated with either MG132 (20 μM) or leptomycin B (20 ng/ml) for 8 h. MG132-treated cells were then harvested, and whole cell lysate or cytosolic and nuclear fractions were prepared as described under "Experimental Procedures." 100 μg of cell lysate or cytosolic extract and 50 μg of nuclear extract were resolved on 10% SDS-PAGE and immunoblotted with anti-V5, anti-lactate dehydrogenase, anti-lamin B, or anti-β-actin antibodies. B, in a similar experiment as described for A, the cells were pretreated with leptomycin B (20 ng/ml) along with MG132 (20 μM) for 8 h followed by CHX + LMB for the indicated times. Cells were harvested, and whole cell lysate was probed with anti-V5 and anti-actin antibodies.

FIG. 10. Functional mechanism of Nrf2 NES. A, Hepa-1 cells were seeded in 100-mm plates and transfected with either pcDNA-Nrf2-V5 or pcDNA-Nrf2ΔNES-V5. 24 h after transfection, the cells were pretreated with either MG132 (20 μM) or leptomycin B alone. Wild-type Nrf2 is expressed at low levels in most normal cells and, at least in some cell types, is localized in the cytoplasm. In response to oxidative stress and various other stresses, Nrf2 levels increase, and the Nrf2 protein deficient in NES was more prominent in the nucleus. The stress-induced, nuclear accumulation of Nrf2 is likely to result from both diminished nuclear export and continued nuclear import. These results suggested that nuclear and cytosolic distribution of Nrf2 is a balance of nuclear import and export of Nrf2. The studies also raise interesting questions regarding mechanisms that regulate nuclear import and export of Nrf2. It is believed that oxidative stress-mediated sulfhydryl modifications of INrf2 and/or phosphorylation of Nrf2 regulates release and nuclear transport of Nrf2 (1). It is expected that nuclear export of Nrf2 is also regulated by unknown modifications of Nrf2 and remains to be determined. Unknown modifications might include phosphorylation of Nrf2 as observed with other proteins including p53 and p73 (31). Interestingly, a recent report showed nuclear import and export of Keap1 (INrf2) (32). The impact of nuclear shuttling of INrf2 on localization and fate of Nrf2 remains unknown and is an exciting area of investigation.

In conclusion, our results indicate that subcellular localization of Nrf2 is controlled by both nuclear import and export signals and suggest that the overall distribution of Nrf2 is likely to result from the balance between these two processes. Proper control of nuclear import and export is likely to be an important regulatory determinant of Nrf2, since Nrf2 availability in the nucleus has significant impact on cell survival and growth.

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