Nrf2 Possesses a Redox-insensitive Nuclear Export Signal Overlapping with the Leucine Zipper Motif

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Basic leucine zipper (bZIP) protein Nrf2 is a key transcription factor mediating the antioxidant response. Under homeostatic conditions Nrf2 is anchored to cysteine-rich Keap1 and sequestered in the cytoplasm. When challenged with oxidative stress, Keap1 functions as a redox-sensitive switch and releases Nrf2. Subsequently, Nrf2 translocates into the cell nucleus and binds to a cis-acting enhancer called the antioxidant response element located in the promoters of a battery of cytoprotective genes and initiates their transcription. In this study we identify a canonical nuclear export signal (NES) (537LKKQLSTLYL250) located in the leucine zipper (ZIP) domain of the Nrf2 protein. The enhanced green fluorescent protein-tagged ZIP domain of Nrf2 (amino acids 509–599) exhibited a CRM1-dependent cytosolic distribution that could be abrogated by site-directed mutations or treatment with the nuclear export inhibitor, leptomycin B. Ectopic expression of the Nrf2-NES could also exclude the GAL4 DNA binding domain into the cytoplasm. This NES overlapped with the ZIP motif in Nrf2, suggesting that the formation of heterodimers between Nrf2 and other bZIP proteins may simultaneously mask the NES and attenuate Nrf2 nuclear export. The Nrf2-NES appeared to be redox-insensitive. Neither oxidants (sulforaphane and diethyl maleate) nor reducing compounds (N-acetyl-L-cysteine and reducing glutathione) could disrupt the cytosolic distribution of Nrf2-NES. Because Nrf2 activation is generally redox-sensitive, the redox insensitivity of this Nrf2-NES indicates the importance of Keap1 retention as a rate-limiting step in Nrf2 activation. The characterization of the Nrf2 NES may help decipher the mechanisms governing nuclear localization and subsequent transcriptional activation of Nrf2-mediated cytoprotective genes.

Mammalian liver cells possess elaborate and highly efficient cytoprotective machinery. When challenged with oxidative or electrophilic stress, these cells respond with rapid and coordinated expression of antioxidant gene products, including heme oxygenase 1 (HO-1)1 (1) and γ-glutamylcysteine synthetase and NAD(P)H:quinone oxidoreductase 1 (2–4) as well as phase II-detoxifying enzymes such as glutathione S-transferase (GST) (2, 3), quinone reductase (4), and UDP-glucuronosyltransferase (3).

Pivotal to this antioxidant response is a transcriptional factor Nrf2 (NF-E2-related factor 2) (5). Nrf2 knock-out mice display attenuation of constitutive and inducible expression of GST and HO-1 (6, 7). The Nrf2 null mice also show an increased susceptibility to injury when exposed to various chemical toxins (8, 9). Nrf2 is a member of the basic leucine zipper (bZIP) transcription factor subfamily featuring a Cap “n” collar (CNC) motif (5). Under homeostatic conditions, Nrf2 is mainly sequestered in the cytoplasm by tethering to a cytoskeleton-binding protein (10) called Kelch-like erythroid CNC homologue (ECH)-associated protein 1 (Keap1) (11, 12). When challenged with oxidative stress, Nrf2 is quickly released from Keap1 retention and translocates to the nucleus (13). Nrf2 is required to form heterodimers with small MaP proteins (14, 15) to bind specifically to a cis-acting enhancer called the antioxidant-responsive element (ARE) (16) or the electrophile-responsive element (EpRE) (17). ARE has been identified in the 5′-flanking regions of many cytoprotective genes including HO-1 (18, 19), γ-glutamylcysteine synthetase (20), NAD(P)H:quinone oxidoreductase 1 (21, 22), and GST (11, 23).

Recently, extensive studies have begun to elucidate the mechanisms of transcriptional regulation of the antioxidant response. It appears that Keap1 retention in the cytosol and subsequent release of Nrf2 is a critical step in the initial antioxidant response (24–28). The Keap1 binding domain of Nrf2 is the Nrf2-ECH homology domain 2 (Neh2) located in the N-terminal end (11). A four-amino acid motif (ETGE) in the Neh2 domain is critical in mediating Nrf2-Keap1 binding, and the deletion of this motif abolishes Keap1 retention of Nrf2 in the cytoplasm (21). Keap1 is a cysteine-rich protein. One or more of its 27 cysteine residues may be subjected to thio-oxidation by a variety of thiol-reactive compounds (13, 29). In vitro, the Keap1-Neh2 complex is only formed under strong reducing condition, and the electrophoretic mobility of the Keap1-Neh2 complex can be altered by the thiol-reactive phyto-oxidant sulforaphane (30). These in vitro observations are supported by in vivo studies showing that the antioxidant response elicited by sulforaphane can be effectively inhibited by reducing compounds (30).

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§ The abbreviations used are: HO-1, heme oxygenase 1; ARE, antioxidant response element; bZIP, basic leucine zipper; CNC, cap-and-collardomain; DBD, DNA binding domain; DEM, diethyl maleate; GFP, green fluorescent protein; EGFP, enhanced GFP; GSH, reduced glutathione; GST, glutathione S-transferase; Keap1, Kelch-like ECH-associated protein 1; NAC, N-acetyl-l-cysteine; Neh2, Nrf2-ECH homology domain 2; Nrf2, NF-E2-related factor 2; NLS, nuclear location signals; NES, nuclear export signals; CRM1, chromosome region maintenance; LMB, leptomycin B; DAPI, 4′,6-diamidino-2′-phenylindole; IP, SUL, sulforaphane.
Identification of a NES in the C Terminus of Nrf2

TABLE I

| Constructs | Primers | Sequence |
|------------|---------|----------|
| EGFP-Nrf2zip | + | 5'-A AACT CGA CGT GAA AAT TAGA GAA CTA C-3' |
| Nrf2zip L537A | + | 5'-T GAC AAA ACG CTC CTA GCC AAA AAA AAA CTC ACC ACC A-3' |
| Nrf2zip L541A | - | 5'-AAC TCG GTA ATT GGT GCT GGC TTT TTT TAG CAG TGA TGT T-3' |
| Nrf2zip L544A | + | 5'-CTG AAA AAA CAA CGT ACC ACC GCA TAT CCA GAA GGT TGC TAT GGC ATG-3' |
| Nrf2zip L546A | + | 5'-CAT GCT GAA AAC TTC GAT ACG ATG GAT GGT GCT TAT TTT TTT CAG-3' |
| Nrf2zip 4P mutant | + | 5'-G TAG CAT GCT GAA AAT GCC GCAC TGT GCC CAA GAA GGT CAG TAT T-3' |
| EGFP-Gal4DBD | + | 5'-AAC ACT CGA GCT AAT CTA CTG GCT GAC-3' |
| EGFP-NesGal4DBD | - | 5'-AA AAA GCC TTC AAT CGA TAC ACT CAA CAG TTG-3' |

Sens primers are designated as +, and antisense primers are designated as -.

Some authors, therefore, hypothesize that Keap1 functions as a redox-sensitive switch to control the initiation step of the antioxidant response (29, 30).

Although solid progress has been made in the understanding of the Keap1-Nrf2 cytosolic interaction, the mechanism governing nuclear translocation of Nrf2 is largely unknown. Great progress has been made in elucidating the mechanisms underlying nucleocytoplasmic transport. Active nucleocytoplasmic transport through nuclear pores is mediated by a variety of nuclear importin and exportin proteins (32). In a Ran GTPase-dependent fashion, these nuclear importin or exportin proteins recognize the nuclear location signals (NLSs) or nuclear export signals (NESs) on cargo proteins and facilitate their transport. In the case of nuclear export, CRM1 (chromosome region maintenance) has been characterized as a nuclear exportin (37, 38). CRM1 can effectively export human immunodeficiency virus Rev protein in a Ran-regulated manner (38).

The first leucine-rich NESs were identified in human immunodeficiency virus Rev protein (35) and in an inhibitor of cAMP-dependent protein kinase (34). Using a randomization and selection approach to compare a variety of functional NES motifs, a consensus leucine-rich NES motif has been proposed (36). CRM1-mediated nuclear export can be effectively inhibited by the cytotoxic leptomycin B (LMB) (39). LMB can bind covalently to a cysteine residue in CRM1 (39) and interfere with the binding of both Ran and the cargo proteins (37, 40). Several functional NES motifs have been identified in some bZIP proteins. One NLS and one redox-sensitive NES have been identified in the CNC/bZIP proteins Bach 1/2 (41) that have been identified in the bZIP protein Tcf11 (45).

Plasmid Constructions—The full-length wild type cDNA of human Nrf2 was kindly provided by Drs. Yue W. Kan and Jefferson Chan (University of California at San Francisco). The ZIP domain of Nrf2 (amino acids 503–589) was PCR-amplified (Table I) and subcloned into pEGFP-C1 vector (Clontech, Palo Alto, CA) by Xhol/BamHI digestion. The resulting plasmid was digested using green fluorescent protein (GFP)-Nrf2zip. The GAL4 DNA binding domain (DBD) was amplified using PCR-amplified pGAL4DBD (Table I) and subcloned into pEGFP-NesGal4DBD via PstI/BamHI digestion to form a EGFP-Nrf2-NES-GAL4DBD fusion protein. PCR-amplified GAL4DBD (Table I) was also subcloned into pEGFP-C1 as an Xhol/BamHI fragment to form an EGFP-GAL4DBD fusion protein.

Site-directed Mutagenesis—Alanine substituted mutations were performed using the QuikChange XL site-directed mutagenesis kit purchased from Stratagene (La Jolla, CA) according to the manufacturer's instruction with few modifications. Briefly, both sense and antisense mutagenic oligonucleotide primers (Table I) were designed to mutate leucine to alanine. The primers were synthesized and PAGE/purified by Integrated DNA Technologies, Inc. (Coralville, IA). Mutagenesis reactions were performed in 50-µl reaction solutions containing 100 ng of template DNA, 125 ng of sense and antisense mutagenic primers, 1× reaction buffer with dNTP supplements, 3 µl of QuikSolution, 2.5 µl of Pfu Turbo DNA polymerase, and double distilled water. Mutagenesis reactions were performed as follows: denaturation at 95 °C for 1 min, 18 cycles of 95 °C for 50 s, 60 °C for 50 s, and 68 °C for 7 min and concluded by a 7-min extension at 68 °C. The parental methylated dsDNA plasmids in the reaction mix were digested with DpnI at 37 °C for 3 h. Afterward, the reaction products were transformed into XL10-Gold cells (Stratagene). The transformed plasmids were extracted and sequenced.

Transient Transfection and Reporter Gene Activity Assays—HeLa or HEK293 cells were plated in six-well plates at a density of about 4.0 × 10^3 cells/well. Twenty four hours after plating, cells were transfected using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Briefly, 2 µg of pH6-Nrf2 were added into 250 µl of Opti-MEM together with 0.5 µg of ARE-Luc reporter and 0.5 µg of pRSV-β-galactosidase plasmid, which was included for the normalization.
Identification of a NES in the C Terminus of Nrf2 and the Design of Fusion Proteins. +, basic region featuring tandem of positively charged amino acids; GAL4DBD, the DNA binding domain of yeast GAL4 protein; LLLLL, leucine zipper domain; TAD, transactivation domain.

Gradient SDS-polyacrylamide gel (Bio-Rad) electrophoresis and transferred to polyvinylidene difluoride membrane using a semi-dry transfer system (Fisher). The membrane was blocked with 5% nonfat milk in Tris-buffered saline with Tween 20 containing 20 mg/ml Tris-HCl, pH 7.6, 8 mg/ml NaCl, and 0.2% Tween 20 at room temperature for 1 h. The membrane was then probed with the primary antibody (1:500), anti-Nrf2 (C-20) (1:500), goat anti-HO-1 (1:500), or anti-β-actin (1:5000) in 3% nonfat milk, Tris-buffered saline with Tween 20 at 4 °C overnight. After washing 3 times with Tris-buffered saline with Tween 20, the membrane was blotted with peroxidase-conjugated anti-rabbit or anti-goat secondary antibody (1:5000 dilution) at room temperature for 1 h. The protein was detected using the ECL mixture from Bio-Rad.

**Epifluorescence Microscopy**—The expression and subcellular distribution of EGFP-tagged Nrf2zip and Nrf2zip mutants were examined using a Nikon Eclipse E600 epifluorescence microscope and a Nikon C-SHG1 UV light source purchased from Micron-Optics (Cedar Knolls, NJ). HepG2 cells were cultured on ethanol-sterilized glass coverslips (Cronos, Rockland, ME) and imaged in a Leica TCS-SP confocal microscope (Leica, Wetzlar, Germany) or a Bio-Rad MRC 1024 system (Bio-Rad). Images were superimposed using Las software (version 2). Images were digitized using a Nikon DXM1200 camera and Nikon ACT-1 software (version 2). Images were superimposed using SPOT 3.5.2 software (Diagnostic Instrument Inc.). For time-lapse imaging, HepG2 cells transfected with EGFP-Nrf2zip were maintained at 37 °C and 5% CO2 in 35-mm glass-bottomed dishes (MatTek Corp., Ashland, MA) and subjected to the treatments of LBM or redox compounds. The fluorescent signals were examined using a Zeiss Axiovert 200M inverted epifluorescent microscope. The fluorescent images were captured using an AxioVision MR monochrome camera and assigned pseudo-color using AxioVision 4.1 software.

**RESULTS**

**Structure of Nrf2 and Design of Fusion Proteins**—The Nrf2 protein can be divided into two parts (Fig. 1). The C terminus contains the CNC, basic region, and ZIP domains. The N terminus contains the acidic transactivation domain and a Keap1 binding Neh2 domain (11, 21). There are three putative NLSs (33) in human Nrf2. In the N terminus a region from amino acids 26–37 that is enriched in basic amino acids may function as a NLS. In the C terminus there is a canonical bipartite NLS located in the basic region (amino acids 486–502) featuring 2 tandem of 3–4 basic amino acids separated by 10 amino acids (33). Our preliminary data found that this putative bipartite NLS indeed functioned strongly as an NLS. In addition, there is another putative NLS (35) located at the end of the C terminus. In searching for a NES we found embedded in the leucine zipper domain a leucine-rich motif (36) that conforms to the canonical NES motif of XXYXXXXXVX (34–36), where X stands for long chain hydrophobic amino acid residues, and Y can be any amino acid. Interestingly, two key residues of this

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2 W. Li, M. R. Jain, and A. N. T. Kong, unpublished data.

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Fig. 1. Schematic drawing of the molecular structure of Nrf2 and the design of fusion proteins. mRNA-1 construct was obtained from IMAGE cloning consortium. PCR primers were designed (sense, 5′-CGGT GGA TCC ATG CCA GCA ATT-3′; anti-sense, 5′-CGG TGG TAC CTT AAT CAC-3′) using a Sirius luminometer (Berthold Detection System). The ARE-luciferase activity was normalized by luciferase activity.
transfected with 1 

subjected to fluorescent microscopic examination. A-C, cytosolic distribution pattern of EGFP-Nrf2zip. A, the green fluorescence of Nrf2zip was confined in the cytoplasm. B, DAPI staining to show the positions of cell nuclei (arrowheads). C, superimposed image to confirm the nuclear position of the cell in A. D-F, site-directed mutation abolished the nuclear export of Nrf2zip. Mutating Leu-544 (D), Leu-537 and Leu-546 (E), and all four key leucine residues (F) converted the distribution of EGFP-Nrf2zip into a whole cell distribution pattern. Scale bar, 10 μm.

Identification of a NES in the C Terminus of Nrf2

Fig. 2. Nrf2 possesses a functional NES motif. HepG2 cells were transfected with 1 μg of EGFP-Nrf2zip or its mutant derivatives and subjected to fluorescent microscopic examination. A-C, cytosolic distribution pattern of EGFP-Nrf2zip. A, the green fluorescence of Nrf2zip was confined in the cytoplasm. B, DAPI staining to show the positions of cell nuclei (arrowheads). C, superimposed image to confirm the nuclear position of the cell in A. D-F, site-directed mutation abolished the nuclear export of Nrf2zip. Mutating Leu-544 (D), Leu-537 and Leu-546 (E), and all four key leucine residues (F) converted the distribution of EGFP-Nrf2zip into a whole cell distribution pattern. Scale bar, 10 μm.

Putative NES, leucines 537 and 544 (asterisks), are important amino acids in the zipper motif.

To examine whether the putative NES plays a functional role in Nrf2 translocation, we designed and constructed a probe protein containing this NES. Because the position of this NES is very close to the bipartite NLS (amino acids 486–502), we dissected them from each other by constructing a fusion protein called EGFP-Nrf2zip. EGFP-Nrf2zip consists of an EGFP tag and a segment of the C terminus of human Nrf2 (amino acids 503–589), which begins right after the bipartite NLS in the basic region and preserves the entire ZIP domain (Fig. 1).

Nrf2 Possesses a Functional NES Located in the ZIP Domain—In the majority of HepG2 cells, EGFP-Nrf2zip proteins exhibited a predominantly cytosolic distribution pattern (Fig. 2, A–C) in which green fluorescence was confined to the cytoplasm, with cell nuclei devoid of fluorescence (Fig. 2A). The positions of cell nuclei were confirmed by DAPI staining (Fig. 2B, arrows) superimposed with the green fluorescent image (Fig. 2C). In contrast, when EGFP alone was expressed, green fluorescence was observed throughout the cell (data not shown). We found that EGFP-Nrf2zip proteins were confined in cytoplasm in more than 85% of the transfected HepG2 cells (Table II). In 13.9% of the cells an evenly distribution pattern was observed (Table II). There were barely any cells showing nuclear distribution. Similar results were also found when EGFP-Nrf2zip was expressed in HeLa and HEK293 cells, although the cytosolic distribution of EGFP-Nrf2zip was more pronounced in HeLa and HEK293 cells (data not shown).

To further examine the possibility that the observed cytosolic distribution was mediated by the NES, we performed site-directed mutagenesis of the NES. We found that a single-point mutation, leucine 544 to alanine, could convert the cytosolic distribution into a whole cell distribution pattern (Fig. 2D; Table II). Similar results were also found with L537A, L541A, and L546A mutants (data not shown). The percentages of cells with a whole cell distribution pattern were similar among these four single point mutants (Nrf2zip-1p) (data not shown) and in strong contrast to the results with EGFP-Nrf2zip. Two point mutations of L537A and L546A of Nrf2 also exhibited an evenly distributed pattern (Fig. 2E; Table II). Furthermore, we constructed a mutant ablating all four of these leucines and observed the same whole-cell distribution pattern (Fig. 2F; Table II). Thus, it appears that single point mutations in the Nrf2-NES are sufficient to disable the nuclear-exporting function. No further increase in disruption was observed with increasing multiple-point mutants relative to the mutant carrying a single substitution.

One might have predicted that disruption of the NES by mutation would lead to a nuclear condensation pattern rather than a whole cell distribution pattern because the construct has a putative NLS (residues 580KSKK583) to promote nuclear localization. Import in the absence of an NES for export might lead to accumulation in the nucleus. There are two possible explanations. One is that 580KSKK583 is either not a functional NLS or is a weak NLS. However, the 580KSKK583 motif, where the lysine residue (K) can be substituted with another basic amino acid arginine (R), is conserved in all Nrf2 molecules cloned from different species (see Fig. 7), suggesting functional importance. Alternatively, Nrf2zip may possess additional unidentified NESs or other unknown inhibitory elements to tightly control the nuclear localization of Nrf2zip. Further experiments are needed to solve this puzzle.

The Nuclear Exporting Activity of Nrf2-NES Is CRM1-dependent—Next, we examined whether the function of Nrf2-NES is mediated by the binding to the nuclear Exporting protein, CRM1 (40). Our in vitro GST pull-down assay showed clearly that GST-Nrf2zip bound to CRM1 (Fig. 3A). In contrast neither GST nor the GST-Nrf2zip-4p mutant bound to CRM1 (Fig. 3A). Our in vitro CRM1 pull-down results also showed that only Nrf2zip bound to CRM1 (data not shown). In agreement with our GST assay result, our immunoprecipitation assay also showed that when 100 ng of CRM1 protein were incubated with 500 μg of the lysate of HepG2 cells transfected with EGFP-Nrf2zip or EGFP-Nrf2zip-4p in the presence of mouse anti-GFP antibody and protein A conjugated-Sepharose beads, only the co-precipitation of CRM1 and EGFP-Nrf2zip was detected (Fig. 3B). In contrast, no binding was detected between CRM1 and the Nrf2zip-4p mutant (Fig. 3B). To further examine the CRM1-dependent nuclear export activity of Nrf2-NES, we treated HepG2 cells expressing EGFP-Nrf2zip with LMB, an inhibitor of CRM1 (39). Time-lapse imaging showed that when treated with 10 nM LMB, the fluorescence of EGFP-Nrf2zip migrated into the nucleus within 10–20 min (Fig. 3C). A similar time course of disruption of nuclear export of transcription factor Bach 2 by 10 ng/ml LMB was reported previously (41).

Percentages of HepG2 cells that displayed nuclear (N), whole cell (N + C) and cytoplasmic (C) distribution of EGFP-tagged proteins are presented. The single, 2-point, and 4-point mutants of Nrf2zip are abbreviated as Nrf2zip-1p, -2p, and -4p, respectively. LMB stands for 10 nM LMB treatment for 2 h. For each construct ∼300 cells were randomly chosen, counted, and pooled as a result of three independent experiments.

| Constructs          | Subcellular distribution |
|---------------------|--------------------------|
|                     | N  | N + C | C    |
| Nrf2zip only        | 0.0| 13.9  | 86.1 |
| Nrf2zip + LMB       | 20.0| 80.0  | 0.0  |
| Nrf2zip-1p          | 2.4| 97.6  | 0.0  |
| Nrf2zip-2p          | 3.5| 96.5  | 0.0  |
| Nrf2zip-4p          | 3.2| 96.8  | 0.0  |
| Gal4DBD             | 70.3| 24.2  | 5.5  |
| NES-Gal4DBD         | 5.9| 30.8  | 63.3 |
| NES-Gal4DBD + LMB   | 9.3| 89.1  | 1.6  |
| Nrf2(L544A)-Gal4DBD| 3.9| 84.8  | 11.3 |
| Nrf2                | 1.6| 81.1  | 17.4 |
| Nrf2 + LMB          | 84.1| 15.9  | 0.0  |
Identification of a NES in the C Terminus of Nrf2

The nuclear exporting activity mediated by Nrf2-NES is CRM1-dependent. A, GST pull-down assay showed that GST-Nrf2zip bound to CRM1. In contrast, neither GST nor the GST-Nrf2zip 4-point (4p) mutant bound to CRM1. 2 μg of GST or GST-Nrf2zip/4p proteins were incubated with 100 ng of CRM1 proteins with glutathione-Sepharose-4B beads at 4 °C for 2.5 h. After an extensive wash, the eluents were subjected to immunoblotting (WB) analysis. B, Nrf2zip could co-precipitate with CRM1 protein. In contrast, Nrf2zip 4-point mutant failed to co-precipitate with CRM1. Protein A-conjugated-Sepharose beads were incubated overnight at 4 °C with 500 μg of cell lysates overexpressing EGFP-Nrf2zip or EGFP-Nrf2zip 4p mutant together with 100 ng of purified CRM1 in the presence of mouse anti-GFP antibody. The beads were then extensively washed. The bound proteins were eluted and subjected to immunoblotting (IB) analysis. C, time-lapse imaging shows that treatment with 10 nm LMB for 0, 10, and 20 min disrupted the cytosolic distribution of EGFP-Nrf2zip in HepG2 cells. Scale bar, 10 μm.

These data suggest that the observed cytosolic distribution pattern of EGFP-Nrf2zip was very likely maintained by the active expulsion mediated by the NES in a CRM1-dependent manner.

Ectopic Expression of Nrf2-NES Can Expel GAL4DBD into the Cytoplasm—To further examine whether the Nrf2-NES alone is sufficient to exert nuclear exporting function, we expressed the Nrf2-NES (amino acids 503–567) ectopically in a well known nuclear protein, the DNA binding domain of yeast protein GAL4 (Gal4DBD) (48). We constructed an EGFP-tagged fusion protein by fusing the Nrf2-NES to the N terminus of GAL4DBD (48). We used time-lapse imaging to detect the nuclear distribution of GAL4DBD out of the nucleus in HepG2 cells. A, EGFP-GAL4DBD proteins accumulated in the cell nucleus. B, EGFP-Nrf2-NES-GAL4DBD showed a cytosolic distribution pattern. C, site-directed mutation of the leucine residue equivalent to Leu-544 in Nrf2zip abolished the nuclear export activities. D, treatment with 10 nm LMB also converted the cytosolic distribution into a whole cell distribution pattern (arrow), with only a few percentage of cells (arrowhead) showing nuclear accumulation of EGFP-Nrf2-NES-GAL4DBD. Scale bar, 10 μm.

These ectopic expression data clearly showed that Nrf2-NES could expel a heterologous nuclear protein into the cytoplasm. Considering the fact that GAL4DBD possesses an innate NLS (48), our data suggest that the Nrf2-NES appears to be a stronger driving force than the NLS of GAL4DBD. Failure to restore the nuclear condensation pattern of GAL4DBD when the Leu-544 residue of Nrf2-NES is mutated is puzzling. There may be some unidentified NES or other inhibitory element(s) that deter the complete nuclear import of Nrf2-NES (L544A)-GAL4DBD. Further studies are needed to address this issue.

The Nrf2-NES Appears to Be Redox-insensitive—Because Nrf2 plays a pivotal role in the antioxidant response, we examined whether the nuclear export activity mediated by Nrf2-NES would respond to redox signals. Previous studies have shown that phyto-oxidant sulforaphane (SUL), a natural isothiocyanate, and DEM could induce ARE-luciferase activity mediated by Nrf2 (31, 49). The maximal induction elicited by 12.5 μM SUL could be effectively inhibited by the pretreatments with 5 mM reducing compounds NAC or conjugating agent GSH (31). Our time-lapse imaging data showed that when HepG2 cells expressing EGFP-Nrf2zip were treated with 12.5 μM SUL for 1 h, the cytosolic distribution pattern of EGFP-Nrf2zip was unchanged (Fig. 5A–D). Similar results were also found in treatments with 100 μM DEM (data not shown). A cell percentage assay also showed that virtually no difference could be detected between EGFP-Nrf2zip-expressing cells with or without the treatments of SUL and DEM for 2 h (Table III). In addition, treatment of SUL with reducing compounds of 5 mM GSH for 1 h also failed to disrupt the cytosolic distribution pattern of EGFP-Nrf2zip in HepG2 cells (Fig. 5, E–H; Table III). Similarly, treatment with 5 mM NAC for 1 h did not alter the cytosolic distribution of Nrf2zip (data not shown). In contrast, treatment with 10 nm LMB for 20 min did disrupt the cytosolic distribution of EGFP-Nrf2zip (Fig. 3B, Table II). These data suggest that the Nrf2-NES is redox-insensitive. Further experiments such as challenging EGFP-Nrf2zip-expressing cells with redox agents with different chemical structures will reveal whether this Nrf2-NES is truly redox-insensitive.
Identification of a NES in the C Terminus of Nrf2—In the present study we identified for the first time a canonical leucine-rich NES in Nrf2. This Nrf2-NES mediated pronounced CRM-1-dependent nuclear export activities, which were abolished by LMB treatments and site-directed mutations. When expressed ectopically, this Nrf2-NES could convert the nuclear distribution pattern of Gal4DBD into a cytosolic distribution pattern. These data show that the Nrf2-NES may play a functional role in determining the subcellular localization of Nrf2. It is also quite unusual that the position of the NES overlaps with the leucine zipper motif of Nrf2. Furthermore, this Nrf2-NES appears to be redox-insensitive. Taken together these discoveries help expand our understanding of the mechanisms underlying the transcriptional activation of Nrf2.

Up until now, the mechanistic studies of Nrf2 activation were mainly focused on Keap1 (for review, see Ref. 50). Virtually nothing is known about Nrf2 nuclear translocation after it is released from Keap1. In our current study the discovery of a functional NES in Nrf2 shows that Nrf2 translocation is not a

**DISCUSSION**
Among these four stability (53). In the Nrf2-NES motif, the interaction forms a hydrophobic core essential for dimer formation and a residue in the opposite monomer, respectively (52). To be hydrophobic residues. In the process of dimerization, the heptad of ZIP domain, respectively. The position a and d need to be hydrophobic amino acids to exert nuclear export function.

The NES motif we identified in Nrf2 conforms to the canonical leucine-rich NES (34–36). Canonical leucine-rich NES feature a 10-amino acid motif formulated as **Sequence alignment of the C-terminal ends of Nrf1, -2, and -3 including the ZIP domain.** The NES motif observed in human Nrf2 is also conserved in mouse, rat, chick, and frog. The positions of key residues of NES are highlighted in black boxes. The Nrf2-NES is not conserved in Nrf1 and Nrf3, with one key leucine residue changed in Nrf1 and Nrf3. Furthermore, there is no NES-like motif in the ZIP domain of Nrf1 and Nrf3. The leucine residues for leucine zipper are designated with asterisks. A conserved putative NLS motif found in Nrf2 in different species is also highlighted in a transparent box.

 Moose Nrf1: LDTILNLREDELDQRQARLREKREVFRLSRLQGQSWGKQGSRFRGRLRDECHRGYPSSHQALYVAQSGDSVLLIRPT
 Human Nrf1: LDTILNLREDELDQRQARLREKREVFRLSRLQGQSWGKQGSRFRGRLRDECHRGYPSSHQALYVAQSGDSVLLIRPT
 Rat Nrf1: LDTILNLREDELDQRQARLREKREVFRLSRLQGQSWGKQGSRFRGRLRDECHRGYPSSHQALYVAQSGDSVLLIRPT
 Human Nrf2: LENVLEQDLDHKLDEKREKLKEKENDSLHLHKLEKEDGFPMVEFLMLRDECKGYSYSELYLQTTDRDNSVFVKPSSKQDPDKKN
 Mouse Nrf2: LENVLEQDLDHKLDEKREKLKEKENDSLHLHKLEKEDGFPMVEFLMLRDECKGYSYSELYLQTTDRDNSVFVKPSSKQDPDKKN
 Rat Nrf2: LENVLEQDLDHKLDEKREKLKEKENDSLHLHKLEKEDGFPMVEFLMLRDECKGYSYSELYLQTTDRDNSVFVKPSSKQDPDKKN
 Chick ECH1: LENVLEQDLDHKLDEKREKLKEKENDSLHLHKLEKEDGFPMVEFLMLRDECKGYSYSELYLQTTDRDNSVFVKPSSKQDPDKKN
 Frog Nrf2: LENVLEQDLDHKLDEKREKLKEKENDSLHLHKLEKEDGFPMVEFLMLRDECKGYSYSELYLQTTDRDNSVFVKPSSKQDPDKKN
 Zebra fish Nrf2: LENIVGLEYEYDLKKEEEKLKMERKSLNKLKQGSLYCGVEFGLMRDECKAFSPNEFSLQHTADTFVFLVERKL

FIG. 7. Sequence alignment of the C-terminal ends of Nrf1, -2, and -3 including the ZIP domain. The NES as identified in human Nrf2 is also conserved in mouse, rat, chick, and frog. The positions of key residues of NES are highlighted in black boxes. The Nrf2-NES is not conserved in Nrf1 and Nrf3, with one key leucine residue changed in Nrf1 and Nrf3. Furthermore, there is no NES-like motif in the ZIP domain of Nrf1 and Nrf3. The leucine residues for leucine zipper are designated with asterisks. A conserved putative NLS motif found in Nrf2 in different species is also highlighted in a transparent box.

passive or an automatic process. In fact, even though Nrf2 is released from Keap1 in the cytoplasm, our results suggest that there may be active nuclear export of Nrf2 to retain it in the cytoplasm. This NES may need to be masked to achieve nuclear localization. In other words, Keap1 may not be the only arresting force to sequester Nrf2 in the cytoplasm.

The NES motif identified in Nrf2 conforms to the canonical leucine-rich NES (34–36). Canonical leucine-rich NES feature a 10-amino acid motif formulated as $\Phi^4XX\Phi^3X\Phi^4$, where 4 positions of $\Phi$ are required to be hydrophobic amino acids residues such as leucine, isoleucine, valine, methionine, and phenylalanine, whereas $X$ can be any amino acid (34–36). Among these four $\Phi$ residues, $\Phi^3$ and $\Phi^4$ are critical and must be hydrophobic amino acids to exert nuclear export function (36). In the present study our functional assays provided several lines of evidence that mutation of each of these four $\Phi$ residues alone or in combination was sufficient to disable the NES function in Nrf2-NES.

One salient feature of this Nrf2-NES is its overlapping position with the leucine zipper domain (Fig. 7). Under physiological conditions, for the formation of homo- or heterodimer, the leucine zipper domain of each bZIP monomer assumes a conformation of parallel coiled coil (51) that consisted of 4–6 heptads formulated as (abcdefg)4–6. The position a and d need to be hydrophobic residues. In the process of dimerization, the a and d residues in one monomer interact with the complementary d and a residue in the opposite monomer, respectively (52). The interaction forms a hydrophobic core essential for dimer stability (53).

In the Nrf2-NES motif, $\Phi^3$ (Leu-541) is located at the d position in the fifth and sixth heptads formulated as (abcdefg)4–6. The position a and d need to be hydrophobic amino acids to exert nuclear export activity. A careful search in the ZIP domain of Nrf1 and Nrf3 failed to identify any leucine-rich NES motif (Fig. 7). Therefore, this NES motif does not appear to be conserved in Nrf1 and Nrf3 (Fig. 7). This NES motif is also employed by other Nrf proteins, i.e., Nrf1 and Nrf3. When the amino acid sequences of the C-terminal ends of Nrf1, Nrf2, and Nrf3 from various species are aligned, it is very interesting to note that the NES motif observed in human Nrf2 is highly conserved in all known Nrf2 sequences with the exception of zebrafish (Fig. 7). The high cross-species conservation of the NES motif underlines the functional importance of this NES motif in Nrf2 activation. In contrast, the Nrf2-NES motif does not appear to be conserved in Nrf1 and Nrf3 (Fig. 7).

A careful search in the ZIP domain of Nrf1 and Nrf3 failed to identify any leucine-rich NES motif (Fig. 7). Therefore, this leucine-rich NES appears only conserved for Nrf2 molecules. Nrf1 and Nrf3 have some similar amino acids to the Nrf2 motif possessing hydrophobic residues in position $\Phi^1$, $\Phi^2$, and $\Phi^3$. However, in position $\Phi^4$, Nrf1 and Nrf3 have polar residues (glutamine in Nrf1 and glutamine or histidine in Nrf3) (Fig. 7). $\Phi^4$ is critical for NES function (36), which is consistent with our results showing that alanine substitution of Leu-546 was sufficient to abolish the nuclear export activity of human Nrf2-NES. Thus, it seems likely that the polar residues at position 4 of Nrf2-NES may be sufficient to abrogate the NES activity in Nrf1 and Nrf3. This observation of differences among Nrf molecules may challenge the conventional notion of functional redundancy among the Nrf molecules. In gene knock-out experiments, ablation of the Nrf2 molecule did not result in any severe phenotypic changes (54), and it was attributed to redundancy conferred by other Nrf molecules. However, the apparently different NES signals among Nrf molecules as described above suggests that functional redundancy may be more complicated than originally conceived, and further investigation is needed.

Although the NES is an important element in determining the subcellular localization of Nrf2, complete ablation of this NES by a four-point mutation failed to result in the accumulation of Nrf2-NES in the nucleus. A similar result was observed in ectopically expressed Nrf2-NES. Point mutations of Nrf2-NES could not restore the nuclear distribution pattern of GAL4DBD even though GAL4DBD has an NLS (48). In addition, LMB treatment also did not accumulate Nrf2-NES in the

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nucleus. This incomplete nuclear import behavior suggested the possibility that other unidentified NESs or inhibitory elements, which are Irrespective to LMB treatments, may exist in Nrf2zip that deter further nuclear import. One possibility may be constitutive phosphorylation of Nrf2zip. There is a consensus mitogen-activated protein kinase site (S(T/Y)PS/S) in EGFP-Nrf2zip as well as in EGFP-Nrf2-NES-GAL4DBD constructs. In our laboratory we found that a GST-tagged C-terminal segment of Nrf2 was constitutively phosphorylated. Because all of the NLS motifs described to date are tandem of positively charged amino acids, it was proposed that negatively charged phosphorylated groups can deter nuclear import. This hypothesis was proven for the transcription factor NFAT1. NFAT1 is constitutively phosphorylated under basal conditions, and dephosphorylation can indeed facilitate its translocation to the nucleus (55). Further studies will be needed to determine whether phosphorylation of Nrf2 has a role in nuclear translocation. For example, treatment with specific mitogen-activated protein kinase inhibitors or co-expression of phosphatases with EGFP-Nrf2zip NES mutants might result in the accumulation of Nrf2zip in the nucleus, supporting this model. Alternatively, substitution of the phosphate acceptor serine 561 with a neutral amino acid may help to solve this puzzle.

In our present study the subcellular distribution pattern of EGFP-Nrf2zip appeared not to be affected by treatment with oxidants (SUL and DEM) or reducing compounds (NAC and GSH). However, more experiments using other structurally different redox compounds would be needed to conclude that this Nrf2-NES is truly redox-insensitive. Furthermore, other putative NLS and NES in Nrf2 may also need to be examined for redox sensitivity. However, our current results indicating that the Nrf2-NES located in the ZIP domain is redox-insensitive are in striking contrast to the previous finding that the conditional nuclear export of CNC/bZIP protein Bach 2 was mediated by a redox-sensitive NES (41). Igarashi and co-workers (41) illustrated that the redox sensitivity of the NES in Bach 2 was attributed to two key cysteine residues. The fact that there is no cysteine residue in our newly identified Nrf2-NES or the whole segment of Nrf2zip may explain the difference between Nrf2-NES and the Bach 2-NES. If the NES in the ZIP domain as well as other putative NES motifs in Nrf2 are truly redox-insensitive, then Nrf2 may exemplify a different redox-responsive activation mechanism.

Nrf2 signaling, when triggered by oxidants, is a multistep activation process. It is well established that the initial step, the release of Nrf2 from Keap1 retention, could be a redox-sensitive step, at least in the in vitro setting (29, 30). If the subsequent steps are redox-insensitive, it could underlie the vital importance of the initial step as the rate-limiting step for Nrf2 activation. This may also explain in part why the ablation of the Keap1 gene, which leads to loss of control of Nrf2 activation, is lethal in the mice (56). So far our knowledge on the redox sensitivity of Nrf2 is still quite limited. Recently, a redox-sensitive cysteine was identified in the basic region right in the middle of the bipartite NLS and DNA binding domain (57). Mutation of this cysteine residue did not alter the Keap1 retention and nuclear translocation of Nrf2. However, the mutation attenuated the DNA binding capability of Nrf2 (57). The dissection and analysis of redox sensitivity of individual functional elements of Nrf2 in the future may help to draw a more complete picture of Nrf2 activation triggered by oxidative stress. The identification of a functional NES in Nrf2 will not only help us to understand the activation of Nrf2, it may also help us decipher the mechanism of deactivation of Nrf2. Although all the efforts up to now have focused on the activation of Nrf2, the effective termination of Nrf2 signaling in the nucleus may also be equally important. Hyperactivation of Nrf2 and a long-lasting antioxidant response may be equally harmful as to the failure to respond to oxidant attacks. Indeed, Nrf2 has a very rapid turnover rate. The measured half-life of Nrf2 is as short as 15 min (58, 59). Accumulating evidence shows that after fulfilling its transactivation function, Nrf2 is destined for proteasomal degradation in the cytoplasm (58, 59), although some weak degradation activity may also exist within the nucleus (60). Therefore, Nrf2 signaling can be turned on and off rapidly to match rapid changes of the redox status of the cells. Our present study has identified Nrf2-NES as a potential candidate for the deactivation signal of Nrf2. In the process of nuclear localization, the Nrf2-NES could be masked, probably by heterodimer formation with MafG/K via the ZIP domain. After Nrf2 fulfills its transactivation function in the nucleus, the mechanism in exposing the Nrf2-NES, leading to its nuclear export, remains to be elucidated. It will certainly be an interesting topic for future study.

In summary, the present study identified a functional NES in Nrf2 localized in the leucine zipper dimerization domain. It binds to CRM1 and is sensitive to LMB treatments. It could also exclude nuclear protein Gal4DBD into cytoplasm. Furthermore, we also found that this Nrf2-NES appeared to be redox-insensitive. Because the nucleocytoplasm translocation of transcription factors is the consequence of a dynamic equilibrium of multivalent NLS and NES, the characterization of the NES in Nrf2 is the first step to delineate the complex mechanisms underlying the nuclear import and export of Nrf2, which has a critical impact on the transcription regulation of ARE-mediated cellular cytoprotective genes.

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Nrf2 Possesses a Redox-insensitive Nuclear Export Signal Overlapping with the Leucine Zipper Motif
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