Keap1 Regulates the Oxidation-Sensitive Shuttling of Nrf2 into and out of the Nucleus via a Crm1-Dependent Nuclear Export Mechanism†

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Keap1 is a negative regulator of Nrf2, a transcription factor essential for antioxidant response element (ARE)-mediated gene expression. We find that Keap1 sequesters Nrf2 in the cytoplasm, not by docking it to the actin cytoskeleton but instead through an active Crm1/exportin-dependent nuclear export mechanism. Deletion and mutagenesis studies identified a nuclear export signal (NES) in the intervening region of Keap1 comprised of hydrophobic leucine and isoleucine residues in agreement with a traditional NES consensus sequence. Mutation of the hydrophobic amino acids resulted in nuclear accumulation of both Keap1 and Nrf2, as did treatment with the drug leptomycin B, which inactivates Crm1/exportin. ARE genes were partially activated under these conditions, suggesting that additional oxidation-sensitive elements are required for full activation of the antioxidant response. Based on these data, we propose a new model for regulation of Nrf2 by Keap1. Under normal conditions, Keap1 and Nrf2 are complexed in the cytoplasm where they are targeted for degradation. Oxidative stress inactivates Keap1’s NES, allowing entry of both Keap1 and Nrf2 into the nucleus and transcriptional transactivation of ARE genes.

Oxidative stress leads to DNA damage and neoplasia due to the increased production of reactive oxygen and thiol species that interact readily with intracellular molecules (12, 25). To counteract oxidative insult, cells induce phase I and phase II enzymes, a series of gene products that reduce the reactive electrophiles and detoxify carcinogens (27, 34). Of these, the phase II enzymes are transcriptionally regulated in a coordinated fashion. This regulation is mediated via a cis-acting enhancer sequence called the antioxidant response element (ARE) (34, 36). The transcription factor Nrf2 is required for the ARE-driven response to oxidative stress (reviewed in references 21 and 34). Nrf2 activation is induced by a diverse series of electrophilic compounds (18, 34).

Nrf2 is a member of the Cap’n’Collar subfamily of the bZIP transcription factors (19, 20). Nrf2 forms a heterodimer with a member of the small Maf proteins, also members of the bZIP family, to bind to the ARE DNA sequence and activate transcription (16, 19, 20, 31, 33). Gene targeting experiments have shown that Nrf2 mediates both the basal and the inducible activity of the ARE genes and dramatically influences their susceptibility to carcinogens. Nrf2-deficient mice have low, uninducible levels of phase II enzymes (17) and, as a result, are much more sensitive to acute toxic agents (10), hyperoxia (6), or carcinogens (35). Therefore, activation of Nrf2 activity is essential for a successful oxidative stress response.

Nrf2 regulation occurs posttranslationally, as Nrf2 is constitutively produced and its transcription does not change in response to antioxidants or xenobiotics (8, 17). Under basal conditions, it is sequestered in the cytoplasm where it is targeted for ubiquitin-mediated proteolysis (23, 32, 37, 39, 46). After oxidative stress, Nrf2 is stabilized and translocates to the nucleus where it activates ARE-responsive genes (4, 8, 17, 22, 23, 46). Both of these forms of regulation are mediated through an N-terminal domain of Nrf2, the Neh2 domain. Removal of the Neh2 domain produces a constitutively active, nucleus-localized product no longer regulated by oxidative stress (8, 22).

Keap1 is the regulatory factor that binds to the Neh2 domain and mediates Nrf2 activation following oxidation (8, 22). Under basal conditions, Keap1 binds to Nrf2 and retains it in the cytoplasm, where Nrf2 is targeted for ubiquitin-mediated degradation (23, 26, 32, 37, 39, 46). Confirming this role, Keap1 knockout mice exhibit constitutive activation of the Nrf2-dependent antioxidant genes due to nuclear accumulation of Nrf2 (42). As Keap1 is a cytoskeletal protein, present in a variety of adhesion structures in tissues and cultured cell lines (40, 41), it has been commonly proposed that sequestration of Nrf2 in the cytoplasm by Keap1 is due to its tethering to the actin cytoskeleton (22, 24).

Keap1 is comprised of three major domains, an N-terminal Broad complex, Tramtrack, and Bric-a-brac (BTB) domain, a central intervening region (IVR), and a series of C-terminal kelch repeats. The kelch repeats of Keap1 directly bind the Neh2 domain of Nrf2 (8, 22), whereas the IVR and BTB domains are required for the oxidation-sensitive regulation of Nrf2 via Keap1 (46, 47) due to a series of reactive cysteines present throughout these regions (9, 46). Three specific cysteines, one in the BTB region (C151) and two in the IVR (C273, C288) have been shown to be required for Nrf2 regulation. C273 and C288 are required for Keap1-mediated ubiqui-
utilization of Nrf2, whereas C151 is required to release Nrf2 from this pathway (46). However, the mechanism that links the oxidation state of Keap1 to changes in Nrf2 activity has not been identified.

Early studies proposed that Keap1 oxidation released Nrf2, allowing it to enter the nucleus and bind to ARE-containing genes (8, 22, 23, 47). In agreement, in vitro oxidation of Keap1 causes a change in the affinity of Keap1 for the Neh2 domain, releasing Nrf2 (9). Recent in vivo studies have reported that oxidation does release the Nrf2/Keap1 complex (46). However, an alternative model proposed that phosphorylation on serine 40 of Nrf2 by protein kinase C (PKC), and not oxidation per se, reduces the affinity of Keap1 for Nrf2 (3, 15). Phosphorylation alone does not activate Nrf2-mediated transcription, nor does oxidation per se, cause a change in the affinity of Keap1 for the Neh2 domain, releasing Nrf2 (9). Recent in vivo studies have reported that oxidation does release the Nrf2/Keap1 complex (46). However, the mechanism that links the oxidation state of Keap1 to changes in Nrf2 activity has not been identified.

In sum, the present model for Nrf2 regulation by Keap1 suggests that Keap1 sequesters Nrf2 in the cytoskeleton, where it is targeted for ubiquitin-mediated degradation. Upon oxidation, it is suggested that the Keap1/Nrf2 complex is dissolved, perhaps in concert with PKC-mediated phosphorylation, thus releasing Nrf2 to enter the nucleus where it can turn on ARE-containing genes. In the present study, we test this model. We find that Keap1 does not sequester Nrf2 in the cytoskeleton. Instead, Keap1 maintains Nrf2’s cytoplasmic location through oxidation-sensitive Crm1/exportin-dependent nuclear export.

MATERIALS AND METHODS

Cell culture. NIH 3T3 fibroblasts were maintained in 5% CO2 in Dulbecco’s modified Eagle’s medium (Gibco-Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. HeLa cells were maintained in modified Eagle’s medium, Earle’s salts, 1 mM sodium pyruvate, and 10% FBS, all from Gibco-Invitrogen (Carlsbad, CA).

In some experiments, cells were serum starved for 2 h, after which different oxidative agents were added to the culture medium for 24 h. Agents were added at the following concentrations, all of which, according to published reports, induce oxidative stress: 100 μM diethylmaleate (DEM; Sigma-Aldrich, St. Louis, MO) (22), 25 μM tert-butyl hydroperoxide (tBHP; Sigma-Aldrich, St. Louis, MO) (46), 4 μM sulforaphane (LKT Laboratories, St. Paul, MN) (46). To induce the oxidative stress response under conditions of cytoskeletal assembly, cells were serum starved and serum supplemented as described previously (41), after which DEM was added to the culture medium for 2 h at 100 μM. In some experiments, 100 nM phorbol 12-myristate 13-acetate (PMA; Calbiochem, La Jolla, CA) was added to the culture media.

Immunocytochemistry. Cells were plated on coverslips and incubated overnight in Dulbecco’s modified Eagle’s medium. The next day, cells were rinsed with PBS and incubated with primary antibodies. After incubation with primary antibodies, cells were rinsed 3 times with PBS and then incubated with secondary antibodies (fluorescein isothiocyanate [FITC]- or rhodamine-conjugated donkey anti-mouse and donkey-anti rabbit) were purchased from Jackson Immunoresearch, Inc., San Antonio, TX. Images were acquired using a Leica DMR fluorescence microscope fitted with an ORCA digital camera as described previously (40).

RESULTS

Both Keap1 and Nrf2 redistribute to the nucleus after oxidative stress. To test the existing model for Keap1 regulation of Nrf2, we used the NIH 3T3 fibroblast cell line. This cell line has been used to analyze the effects of oxidative stress on exogenously expressed Nrf2 and Keap1 (46) and also to characterize the targeting of endogenous Keap1 to the cytoskeleton (41). In mock-treated NIH 3T3 cells, endogenous Nrf2 was found in the cytoplasm, with a smaller population also present in the nucleus, as judged by immunofluorescence of methanol-fixed cells (Fig. 1A) and subcellular fractionation into the cytoplasmic and nuclear fractions (Fig. 1D). After treatment with known activators of the oxidative stress pathway, DEM, tBHQ, or sulforaphane, Nrf2 redistributed to the nucleus (Fig. 1A and D).

In NIH 3T3 cells, the endogenous Keap1 was found primar-
FIG. 1. Keap1 and Nrf2 both redistribute to the nucleus after oxidative stress. (A) Indirect immunofluorescence localization of Keap1 and Nrf2 in NIH 3T3 cells serum starved for 2 h followed by treatment for 24 h with vehicle (dimethyl sulfoxide [DMSO]) or activators of the oxidative stress pathway, DEM, tBHQ, and sulforaphane. Cells were fixed with methanol before incubation with antibodies specific to Keap1 or Nrf2 followed by incubation with an FITC-conjugated secondary antibody. For each panel, an image of the cell nuclei stained with the DNA-specific dye Hoechst (DNA) and a phase-contrast image of the cells (Phase) are also presented. (B) Localization of Keap1 in untreated HepG2 cells and cells treated for 24 h with DEM. Cells were fixed and stained as described for panel A. The corresponding immunofluorescence and phase-contrast images are shown. Bars, 10 μm. (C) Localization of Keap1 in untreated COS7 cells and cells treated with DEM for 2 h. (D) Fractionation of NIH 3T3 cells into nuclear and cytoplasmic fractions. Serum-starved NIH 3T3 cells were left untreated (−) or treated for 2 h with 100 μM DEM (+). Cells were isolated and fractionated into nuclear and cytoplasmic extracts (see Materials and Methods). Equal amounts of protein extracts were loaded in each lane, resolved by SDS-polyacrylamide gel electrophoresis, and probed for Keap1 and Nrf2 by immunoblot analysis. As loading controls, cytoplasmic extracts were probed for GAPDH and nuclear fractions were probed for Lamin A.
ily in the cytoplasm, with a small fraction evident in the nucleus, as judged by both immunofluorescence of methanol-fixed cells (Fig. 1A) and subcellular fractionation studies (Fig. 1D) using a Keap1-specific antibody. The specificity of this affinity-purified anti-Keap1 antibody was confirmed in both immunofluorescence and immunoblot studies by comparison with preimmune serum derived from the same rabbit (see Fig. S1 in the supplemental material); the Keap1-specific antibody recognized both endogenous Keap1 and exogenously expressed Keap1-GFP fusion proteins.

Current models state that Keap1 remains in the cytoplasm after oxidative stress. However, after oxidative stress induced by a variety of agents, Keap1 redistributed and accumulated in the nucleus (Fig. 1A and D). This redistribution was not unique to NIH 3T3 fibroblasts. In the human hepatoblastoma cell line HepG2 (Fig. 1B), as well as in Cos7 fibroblasts (Fig. 1C), endogenous Keap1 also accumulated in the nucleus after DEM treatment. Therefore, a conserved feature of Keap1 is the ability to respond to oxidative stress by redistributing to the nucleus.

**Keap1 exits the cytoskeleton after oxidative stress.** As Keap1 is primarily a cytoskeletal protein in tissues and cultured cells (40, 41), it has been proposed that sequestration of Nrf2 by Keap1 is achieved through attachment to the cytoskeleton (24). However, the methanol fixation method commonly used to visualize Nrf2 by immunofluorescence (e.g., the methods used for Fig. 1) removes actin filaments (data not shown), precluding visualization of either Keap1 or Nrf2 association with the cytoskeleton. Therefore, to study the potential association of Keap1 with Nrf2 in the cytoskeleton, we looked at Keap1 and Nrf2 using cell culture conditions and formaldehyde (PFA) fixation methods that maintain the actin cytoskeleton in NIH 3T3 cells (41).

Serum starvation followed by serum supplementation stimulates actin polymerization and results in integration of Keap1 into the cytoskeletal adhesion structures at the cell periphery (41) (Fig. 2A). Keap1 overlaps with vinculin and F-actin in the peripheral focal adhesions, and colocalizes with β-catenin in adherens junctions, but is not associated with the actin stress fibers (Fig. 2A, panels a, b, c; see Fig. S2 in the supplemental material). Keap1’s association with the cytoskeleton was confirmed in fractionation studies; the cytoplasmic fraction, presented in Fig. 1D, was treated with Triton X-100 and the insoluble cytoskeletal fraction isolated. As shown on Fig. 2B, untreated cells had a high concentration of Keap1 in the insoluble cytoskeletal fraction.

Treatment with DEM resulted in Keap1’s dissociation from the cytoskeleton, as evident from immunofluorescence experiments (Fig. 2A; see Fig. S2 in the supplemental material) and subcellular fractionation (Fig. 2B). F-actin was not dramatically altered after DEM treatment, although the cells appeared slightly more contracted (Fig. 2Aa; see Fig. S2A in the supplemental material). The focal adhesion component vinculin remained at sites of attachment, suggesting that focal adhesions were not disrupted by DEM treatment (Fig. 2Ab; see Fig. S2B in the supplemental material). Cell-cell contacts were altered in shape after DEM treatment, however β-catenin was retained at the residual sites of cell-cell contact (Fig. 2Ac; see Fig. S2C in the supplemental material). Therefore, Keap1 departs all actin-based adhesion structures upon exposure to DEM, but this is not due to global cytoskeletal disassembly. Serum treatment did not alter the ability of NIH 3T3 cells to respond to oxidative agents. As shown in Fig. 2Ad, the expression levels of GSTα (a Nrf2-inducible gene) (5) are markedly elevated in DEM-treated cells under conditions promoting cytoskeletal assembly.

In addition to its association with the cytoskeleton, a population of Keap1 was evident in the cytoplasm and also within the nucleus by both indirect immunofluorescence (Fig. 2; see Fig. S2 in the supplemental material) and by subcellular fractionation (Fig. 1D). Therefore, there are at least three populations of Keap1 within the cell, one within the nucleus, one associated with the cytoskeleton, and one within the cytoplasmic pool.

**Keap1 does not sequester Nrf2 in the cytoskeleton.** Using PFA fixation conditions that maintained the actin cytoskeleton, we evaluated the location of Nrf2 using a Nrf2-specific antibody. Unfortunately, this technique produced a nonspecific staining in the nucleus not seen in methanol-fixed cells (Fig. 3B). This staining was seen in all cell types tested, including cells that do not express abundant Nrf2 as judged by immunoblotting (data not shown), and was evident in both serum-starved and serum-treated cells (data not shown). The staining that appeared specific to the Nrf2 antibody was a diffuse cytoplasmic and nuclear staining, similar to that seen after methanol fixation (Fig. 3A). Focusing on this population, we utilized PFA fixation to analyze Nrf2 colocalization with cytoskeletal structures (Fig. 3B and C).

If the Keap1/Nrf2 complex is tethered to the cytoskeleton under basal conditions, we would expect Nrf2 to colocalize with Keap1 in the peripheral focal adhesions and adherens junctions in PFA-fixed cells. Available antibodies to Nrf2 and Keap1 are both rabbit derived, precluding direct double-staining experiments. Therefore, we evaluated whether Nrf2 was present in focal adhesions that contained vinculin and F-actin, components that overlap in location with Keap1 in these adhesion structures (Fig. 2). Double staining for Nrf2 and vinculin revealed no colocalization between these two proteins at peripheral focal adhesions or adherens junctions (Fig. 3B). There was also no colocalization between Nrf2 and F-actin in adhesion sites, although there was some apparent enrichment for Nrf2 at the tips of cytoplasmic extensions (Fig. 3C). This lack of cytoskeletal association was confirmed in biochemical fractionation experiments; Nrf2 was not evident in cytoskeletal fractions isolated from serum-treated NIH 3T3 cells (Fig. 2B). These results suggest that Keap1 and Nrf2 are not complexed in the cytoskeleton under basal conditions as previously proposed.

**Keap1 location is regulated by CRM1/exportin mediated nuclear export.** We have shown that sequestration of Nrf2 in the cytoplasm by Keap1 is not due to physical tethering of the complex to the actin cytoskeleton. To explain the cytoplasmic distribution of the Keap1/Nrf2 complex, we theorized that Keap1 might have an NES that acted in a dominant fashion to exclude Nrf2 from the nucleus, as judged by both indirect immunofluorescence of methanol-fixed cells (Fig. 1A and D). This redistribution was not unique to NIH 3T3 fibroblasts. In the human hepatoblastoma cell line HepG2 (Fig. 1B), as well as in Cos7 fibroblasts (Fig. 1C), endogenous Keap1 also accumulated in the nucleus after DEM treatment. Therefore, there are at least three populations of Keap1 within the cell, one within the nucleus, one associated with the cytoskeleton, and one within the cytoplasmic pool.

Current models state that Keap1 remains in the cytoplasm after oxidative stress. However, after oxidative stress induced by a variety of agents, Keap1 redistributed and accumulated in the nucleus (Fig. 1A and D). This redistribution was not unique to NIH 3T3 fibroblasts. In the human hepatoblastoma cell line HepG2 (Fig. 1B), as well as in Cos7 fibroblasts (Fig. 1C), endogenous Keap1 also accumulated in the nucleus after DEM treatment. Therefore, a conserved feature of Keap1 is the ability to respond to oxidative stress by redistributing to the nucleus.
Lx_{1-4}LxL (reviewed in reference 11). This potential NES is conserved in the intervening region of Keap1 across all species (Fig. 4A). Of note, this sequence is flanked by a series of conserved cysteine residues, two of which have been shown to be required for Keap1-mediated degradation of Nrf2 in the cytoplasm (Fig. 4A) (46).

To test the activity of this potential NES, a series of GFP-Keap1 fusion constructs were created and their location in the nucleus or cytoplasm visualized by fluorescence microscopy of live cells (Fig. 4B). GFP alone exhibits a nuclear enrichment in NIH 3T3 cells, suggesting the presence of a cryptic nuclear localization signal (Fig. 4C and D), so we predicted that any constructs lacking the NES would be found in the nucleus. Quantification of the location of the Keap1-GFP deletion constructs revealed that all the constructs that contained the putative NES (Fig. 4B) were evident in the cytoplasm (Fig. 4C and D), whereas constructs lacking the NES exhibited a predominantly nuclear location.

FIG. 2. Keap1 exits the actin cytoskeleton after oxidative stress, and this exit is not due to disruption of the adhesion structures. (A, a) Localization of Keap1 and F-actin in NIH 3T3 under conditions that promote assembly of the actin cytoskeleton. NIH 3T3 cells were serum starved for 16 h followed by reapplication of serum for 2 h to induce actin assembly. Cells were left untreated or treated with 100 μM DEM for 2 h and then fixed with PFA. Keap1 was visualized by immunofluorescence using a Keap1-specific antibody and an FITC-conjugated secondary antibody. Actin was visualized with rhodamine-conjugated phalloidin. (b) Colocalization of Keap1 and vinculin, the marker for focal adhesions. Cells were cultured and fixed as described for panel a and double stained for Keap1 and vinculin. Keap1 staining was as described for panel a. Vinculin was visualized with a mouse anti-vinculin antibody followed by a rhodamine-conjugated secondary antibody. Vinculin does not exit focal adhesions after DEM treatment, suggesting maintenance of focal adhesion integrity after DEM treatment. (c) Double staining for Keap1 and β-catenin, a marker for cell-cell contacts. Keap1 staining was as described for panel a. β-Catenin was visualized with a mouse anti-β-catenin antibody followed by a rhodamine-conjugated secondary antibody. β-Catenin remains associated with the residual adherens junction. (d) The oxidative stress pathway is active under conditions promoting actin cytoskeleton assembly. GSTs, a protein regulated by Nrf2 at the transcriptional level, was visualized with a GSTs-specific antibody and an FITC-conjugated secondary antibody. A phase-contrast image is also presented (Phase). The level of expression of GST increased dramatically after DEM treatment. Bars, 10 μm. (B) Biochemical evaluation of Keap1 and Nrf2 association with the cytoskeleton in the presence or absence of oxidative agents. NIH 3T3 cells were cultured as described for panel A in the presence (+) or absence (−) of DEM. The cytoskeleton was isolated as a detergent-insoluble fraction of the cytoplasmic extract. Fractions were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting as described in the legend to Fig. 1C. Keap1 is evident in the cytoskeletal fraction but leaves this fraction after DEM treatment. In contrast, Nrf2 is not present in the cytoskeleton under any conditions. β-Actin was used as a loading control.
some systems (28, 38), so three cysteine residues, C273, C288, and C297, were changed to serine (Fig. 5A). Wild-type and mutant Keap1-GFP constructs were introduced in NIH 3T3 cells, and the nuclear versus cytoplasmic localization of the expressed GFP protein was scored (Fig. 5B). As expected, wild type Keap1-GFP was located predominantly in the cytoplasm (Fig. 5B). In contrast, the constructs with mutated hydrophobic residues showed increased nuclear localization, implicating amino acids L301, I304, L308, and L310 as critical for Keap1’s nuclear export (Fig. 5B and C). None of the constructs with mutated hydrophobic residues displayed enriched nuclear localization. In addition, these constructs retained their sensitivity to LMB (Fig. 5D), suggesting that these cysteines are not included in the nuclear export signal.

**Nuclear targeting of Keap1 in the absence of oxidative stress redistributes Nrf2 to the nucleus and partially activates oxidative stress genes.** If Keap1 is required to sequester Nrf2 in the cytoplasm, then we predicted that cells transfected with Keap1-GFP constructs with a mutated NES should exhibit a redistribution of Nrf2 to the nucleus. As shown in Fig. 5C, that is indeed the case. Mutations of the hydrophobic NES residues resulted in Nrf2 redistribution to the nucleus. In contrast, mutations of cysteine residues in Keap1 did not increase Nrf2 localization in the nucleus.

To test whether the redistribution of both Keap1 and Nrf2 to the nucleus after NES mutation is not due to ectopic overexpression of Keap1-GFP, we treated NIH 3T3 cells with LMB and evaluated the location of the endogenous Nrf2 and Keap1 proteins (Fig. 6A). After overnight LMB treatment, both endogenous Keap1 and Nrf2 redistributed to the nucleus. Similar results are achieved after a shorter 3-h LMB treatment (see Fig. S3 in the supplemental material). The nuclear redistribution of Keap1 and Nrf2 after LMB treatment was confirmed by subcellular fractionation (Fig. 6C). Both Keap1 and Nrf2 displayed nuclear concentration after LMB treatment in a pattern similar to that seen after DEM oxidation. The Keap1 pool present in focal adhesion and adherens junctions was not affected by LMB treatment, however, suggesting that this pool of Keap1 was anchored to the actin cytoskeleton and was not actively cycling.

To test whether the presence of Keap1 and Nrf2 in the nucleus in the absence of oxidative stress is sufficient to turn on the expression of the ARE genes, we compared the expression levels of GSTα, a gene under the regulation of Nrf2 (3), in untreated cells and cells treated with LMB and DEM (Fig. 6A). The intensity of GST fluorescence in LMB-treated cells is higher than in control, untreated cells but lower than in DEM-treated cells, suggesting that nuclear trapping of Keap1/Nrf2 is not sufficient to activate the oxidative stress response to the levels induced by strong oxidants such as DEM (Fig. 6D).

**Recent studies have suggested that activation of PKC and phosphorylation of Nrf2 is required for activation of ARE genes (3, 15).** Therefore, we tested the effects of PMA, a PKC activator, on Keap1 and Nrf2 localization. PKC activation by PMA did not result in Keap1 and Nrf2 concentration in the nucleus (Fig. 6B). PMA treatment alone did slightly elevate the expression of GSTα, similar to that seen after LMB treatment. Of significance, the combined effect of LMB and PMA induced both redistribution of Nrf2 and Keap1 to the nucleus and high levels of GSTα expression comparable to that seen...
FIG. 4. Keap1 localization to the cytoplasm is due to Crm1/exportin-mediated nuclear export. (A) Alignment of human, mouse, rat, and zebrafish (zebrf) Keap1 protein sequences. The proposed NES is highlighted in blue. A consensus NES, taken from reference 11, is shown below for comparison. Conserved cysteine residues found in this region are shown in red. Cysteine residues required for Keap1’s regulation of Nrf2 are marked by asterisks (46). aa, amino acid. (B) GFP fusion constructs used to analyze the NES of Keap1. The amino acid boundaries of each construct above each diagram and the positions of the BTB, IVR, and Kelch repeat domains are shown. The position of the NES is shown with an asterisk. (C) Localization of Keap1-GFP fusion constructs in untreated NIH 3T3 cells and cells treated with 3 nM LMB for 3 h. Images were taken of live cells. Fluorescence images are arranged with the GFP fluorescence in the left panel (GFP) and the Hoechst stain of DNA in the right panel (DNA). Keap1-GFP and NES-Kelch-GFP are predominantly cytoplasmic but redistribute to the nucleus after LMB treatment. Other constructs are unaffected by LMB treatment and exhibit both nuclear and cytoplasmic locations. Bar, 10 μm. (D) A histogram quantifying the localization of the GFP constructs to the nucleus. Two hundred cells were counted for each construct. N < C, cells with GFP fusion proteins predominantly located in the cytoplasm; N = C, cells with GFP equally distributed between the cytoplasm and the nucleus; N > C, cells with GFP fusion protein predominantly concentrated in the nucleus.
FIG. 5. Mutations in the putative NES of Keap1 result in Keap1 and Nrf2 translocation to the nucleus. (A) Sequence of the human Keap1 amino acid (AA) residues 272 to 333 and NLS consensus sequence as described in the legend to Fig. 4. Altered hydrophobic amino acids are shown in blue; altered cysteine residues are shown in red. The amino acid substitutions to serine and alanine are indicated below the sequence. wt, wild type. (B) Quantification of the nuclear localization of Keap1-GFP with altered NES. NIH 3T3 cells were transfected with wild-type and mutant Keap1-GFP constructs as described for Fig. 4. N>C, cells with GFP fusion proteins predominantly located in the cytoplasm; N=C, cells with GFP equally distributed between the cytoplasm and the nucleus; N>C, cells with GFP fusion protein predominantly concentrated in the nucleus. One hundred cells were counted for each construct. (C) NIH 3T3 cells transfected with mutant Keap1-GFP constructs were fixed with methanol and stained for Nrf2 with a rabbit anti-Nrf2 antibody. (D) NIH 3T3 cells transfected with Keap1-GFP or GFP-tagged Keap1 cysteine mutants were treated with 3 nM LMB for 3 h. Cells were incubated with Hoechst, and images were taken of live cells. Bar, 10 μm.
after oxidation (Fig. 6B and D). Similar results were obtained when GSTα protein levels were directly assayed in immunoblot analysis (Fig. 6E). Therefore, redistribution of Keap1 and Nrf2 to the nucleus is not sufficient for ARE gene activation, and PKC may contribute to achieving full gene activation.

To explore the relationship between Keap1’s NES and oxidative stress, we treated cells overexpressing Keap1-GFP with DEM, expecting nuclear accumulation similar to that observed for the endogenous protein. However, overexpressed Keap1-GFP apparently loses its sensitivity to DEM, while the endogenous protein is still able to translocate to the nucleus after oxidation (Fig. 7). This result suggests that a limiting additional factor is required for Keap1’s redistribution following oxidation. The identity of this factor remains to be determined.

**DISCUSSION**

Keap1 represses the activity of Nrf2 under nonoxidizing conditions by sequestering Nrf2 in the cytoplasm. Current models postulate that Keap1 anchors Nrf2 to the actin cytoskeleton where it targets Nrf2 for ubiquitin-mediated degradation (21). Here we explain the mechanism of this sequestration, showing that Keap1 does not sequester Nrf2 in the cytoskeleton and instead maintains a cytoplasmic location through an active nuclear export pathway.

We have demonstrated that the IVR domain of Keap1 contains an NES between amino acids 272 and 312 with a conserved leucine-rich sequence (amino acids 301 to 310) similar to that seen in other proteins exported by Crm1/Exportin (11).
Previous studies suggested that the IVR of Keap1 was necessary for the degradation of Nrf2 in the cytoplasm (46). Expression of a construct that contained the BTB box and kelch repeats of Keap1, with deleted IVR, led to constitutive nuclear accumulation of Nrf2 and activation of oxidation response genes (46). Here we explain these results by showing that deletion or mutation of the IVR region results in nuclear accumulation of both Keap1 and Nrf2. A similar outcome is seen after inactivation of the Crm1/exportin pathway by LMB, suggesting that nuclear export is the primary mechanism for cytoplasmic sequestration of Nrf2. After inactivation of the NES, Keap1 is no longer exported constitutively to the cytoplasm, and therefore, it is no longer able to expose Nrf2 to the protein degradation machinery. As a result, Nrf2 accumulates stably within the nucleus. Therefore, the IVR of Keap1 is essential for Nrf2 degradation and oxidation-sensitive nuclear entry because it is required to maintain the location of these two proteins within the cytoplasm.

A characterized mechanism of activation of the antioxidant response is conditional nuclear transport of transcription factors. In yeast, the basic leucine zipper transcription factors yAP-1 and Pap1, two players in the oxidative stress response, are both translocated from the cytoplasm to the nucleus in response to oxidative stress (28–30). Both transcription factors contain a nuclear localization signal (NLS) and a dominant NES. Under normal conditions, yAP-1 and Pap1 interact with Crm1/exportin through their NES and are exported from the nucleus. After oxidative insult, the NES-dependent nuclear export is inhibited and the transcription factors are accumulated in the nucleus, where they activate antioxidant response genes. A similar mechanism of regulation through oxidation-dependent nuclear export has been described for the mammalian transcription factor Bach2; Bach2 possesses both an NLS and an NES and accumulates in the nucleus after oxidation inhibits the NES's function (14). Since oxidation and inactiva-

![Figure 7](http://mcb.asm.org/)

**FIG. 7.** Keap1-GFP is insensitive to DEM treatment. COS7 cells were transfected with full-length Keap1-GFP. Twenty-four hours after transfection, 100 μM DEM was added for 1 hour. Cells were fixed with PFA as described for Fig. 2 and stained with a rabbit anti-Keap1 antibody, followed by incubation with a rhodamine-conjugated anti-rabbit secondary antibody. Transfected cells overexpressing GFP-Keap1 were visualized by a short exposure (exp) (0.8 second), while nontransfected cells expressing endogenous Keap1 were visualized by a long exposure (3.5 seconds). Keap1-GFP is cytoplasmic with or without DEM treatment, while endogenous Keap1 translocates to the nucleus after DEM exposure. Phase-contrast images (Phase) are also shown.
nuclear entry of the Keap1/Nrf2 complex may be solely due to the NLS of Nrf2.

Even though the enrichment of both Keap1 and Nrf2 in the nucleus is evident in both immunofluorescence experiments and in biochemical fraction studies, we were unable to immunoprecipitate the two proteins as a complex from the nuclear extracts of cells treated with LMB or oxidative agents (data not shown). Therefore, it is most likely that the complex falls apart upon entry into the nucleus. This leaves Nrf2 free to dimerize with Maf proteins, forming the transcriptional activation complex required for partial activation of ARE gene expression. Additional oxidation-sensitive factors are required to fully activate expression of ARE genes.

Keap1’s three-dimensional structure is essential for its function. It can dimerize through its BTB domain, and this dimerization is apparently important for Keap1’s Nrf2 repression function. Keap1 also has multiple cysteine residues capable of forming intra- and intermolecular disulfide bonds. We hypothesize that under reducing conditions, the NES of Keap1 is exposed, ensuring that Keap1 is maintained in the cytoplasm. We further envision that Keap1 undergoes a conformational change that masks the NES upon oxidation of its reactive cysteines. In most cases where nuclear export is oxidation sensitive, there are cysteine residues in immediate proximity to the NES. In the case of the yeast factor Yap-1, however, the oxidation-sensitive disulfide bond that induces the conformational change that regulates NES function is formed between two parts of the molecule that are separated by 300 amino acids of intervening sequence (45). Therefore, it is possible that the cysteine residues regulating Keap1’s NES are not in an immediate vicinity to the NES. It has been shown that 5 cysteine residues can be directly modified by oxidative agents (9, 42). In addition, functional significance has been shown for at least 3 cysteine residues (C151, C273, and C288). We have only tested three possible cysteine candidates for involvement in Keap1’s nuclear export (C273S, C288S, and C297S). Other cysteine residues, or other amino acids affected by oxidation, can participate in the masking/unmasking of the NES in Keap1.

In recent years, several cytoskeletal proteins that are components of cell-ECM or cell-cell adhesion complexes have been shown to shuttle to the nucleus and potentially transmit signals from the exterior of the cell to the nucleus. Cell adhesion molecules that are involved in nucleocytoplasmic trafficking include the cell-cell contact components β-catenin, p120-catenin, ajuba, and ZO-1 as well as the focal adhesion components Hic-5, paxillin, and zyxin (1, 43). These adhesion molecules can interact with a variety of transcription factors and nuclear proteins, and it has been widely speculated that the nuclear accumulation of adhesion components activates transcription for specific signaling pathways.

A large number of the shuttling adhesion molecules either possess an NES within their sequence (Hic-5, paxillin, zyxin) (43) or interact with other NES-containing proteins (β-catenin) (7, 44). Since Keap1 is incorporated into focal adhesions and shuttles to the nucleus via an NES-dependent mechanism, it appears that it could potentially function in a similar fashion, by sensing signals through adhesion complexes and transmitting them to the nucleus where it would initiate a transcriptional response. However, we have shown that LMB treatment does not affect Keap1 that is associated with the focal adhe-
sions. Our data strongly suggest that the cytoplasmic pool of Keap1, not the cytoskeletal pool, shuttles to the nucleus. β-catenin, like Keap1, has three pools: cytoskeletal, cytoplasmic, and nuclear. In the case of β-catenin, the protein interacts with specific binding partners in each subcellular compartment (reviewed in reference 13). At the plasma membrane, β-catenin interacts with cadherin molecules and links them to the actin cytoskeleton. In the cytoplasm, β-catenin is complexed with axin and APC glycogen synthase kinase, which target it for ubiquitination and rapid degradation. In addition, APC and axin have both been shown to shuttle between the cytoplasm and the nucleus through an active Crm1/exportin-dependent nuclear export mechanism (2, 7, 13, 44), thus potentially serving as a molecular chaperones for β-catenin and regulating its nuclear-cytoplasmic distribution. Within the nucleus, β-catenin binds the transcription factor LEF-1 and serves as a transcriptional transactivator. It is evident that nucleocytoplasmic transport of β-catenin is essential for its diverse functions (reviewed in reference 13).

We envision that, similar to β-catenin, Keap1 might be associated with specific partners in each subcellular pool, each capable of response to different stimuli from the external environment. In the cytoplasm, there is abundant evidence for association of Keap1 with Nrf2 and potentially also with components of the degradative machinery (45). The component that associates with Keap1 in an oxidation-sensitive fashion in focal adhesions remains to be identified, as do nuclear components that bind Keap1.

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