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

Michaella Velichkova* and Tama Hasson

Section of Cell and Developmental Biology, Division of Biological Sciences, University of California at San Diego, La Jolla, California 92093

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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 ubiq-

* Corresponding author. Mailing address: University of California at San Diego, Division of Biological Sciences, Section of Cell and Developmental Biology, 2129 Bonner Hall, MC 0368, 9500 Gilman Drive, La Jolla, CA 92093-0368. Phone: (858) 822-3033. Fax: (858) 822-3034. E-mail: velichko@biomail.ucsd.edu.

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uitination 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 phosphorylation at serine 40 target Nrf2 to the nucleus (3). Therefore, although it is clear that Keap1 maintains Nrf2's location in the cytoplasm where it targets the complex for degradation, it remains unclear how Nrf2 is activated and targeted to the nucleus.

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% CO_2 in Dulbecco's modified Eagle's medium (Gibco-Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS) and 1% Fungizone (Omega Scientific, Tarzana, CA). HepG2 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 hydroxyquinone (tBHQ; 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) or 3 nM leptomycin B (LMB; LC Laboratories, Woburn, MA) was added to the culture media.

Immunocytochemistry. Cells were plated on acid-washed coverslips. For experiments focusing on the location of Nrf2, cells were methanol fixed as described in reference 22. For experiments focusing on the location of cytoskeletal proteins, cells were fixed with formaldehyde as described previously (40). In both cases, antibody staining was undertaken as previously described (40). Secondary antibodies (fluorescein isothiocyanate [FITC]- or rhodamine-conjugated donkey anti-mouse and donkey-anti rabbit) were purchased from Jackson Immunoresearch Laboratories, West Grove, PA, and used at a concentration of 1:100. Hoechst (Sigma-Aldrich, St. Louis, MO) was used at a concentration of 10 μg/ml to label nuclear DNA. For immunostaining, rabbit anti-Keap1 antibody (40) was used at 10 μg/ml, rabbit anti-Nrf2 antibody (Santa Cruz, Paso Robles, CA) was used at 1:100, mouse anti-vinculin antibody (Sigma-Aldrich, St. Louis, MO) was used at 1:400, mouse anti-β-catenin antibody (BD Biosciences, San Diego, CA) was used at 1:50, rabbit anti-glutathione *S*-transferase (GST) alpha (Alpha Diagnostic, Inc., San Antonio, TX) was used at 1:50. Rhodamine-conjugated phalloidin (Molecular Probes, Eugene, Oregon) was used at a concentration of 80 nM. Images were acquired using a Leica DMR fluorescence microscope fitted with an ORCA digital camera as described previously (40).

Cell transfection. For nuclear export experiments, NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS and transfected with Keap1-green fluorescent protein (GFP) constructs using the Lipofectamine Plus (Invitrogen, Carlsbad, CA) reagent according to the manufacturer's instructions. After 16 h of expression, LMB was added to a final concentration of 3 nM for 3 h (28). Cells were fixed and examined by immunocytochemistry as described above. For quantification of Keap1-GFP nuclear redistribution, 200 cells were counted for each construct and cytoplasmic versus nuclear location scored.

Plasmids. cDNA encoding the entire open reading frame of human Keap1 as well as fragments of Keap1 were amplified using standard PCR methods from KIAA0132 (40). The 5′ and 3′ primers contained HindIII and EcoRI restriction sites, respectively, allowing directional insertion into digested pEGFP-N1 vector (Clontech, La Jolla, CA). The QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to generate the nuclear export signal (NES) mutants. All clones were confirmed by sequencing and by immunoblot analysis using rabbit anti-Keap1 antibody (40) and mouse anti-GFP antibody (Clontech, La Jolla, CA) (see Fig. S1 in the supplemental material).

Subcellular fractionation. NIH 3T3 cells grown in T-225 flasks were serum starved for 16 h and treated with or without 100 μM DEM for 1 h or 3 nM LMB overnight. Cells were washed three times with phosphate-buffered saline (PBS) on ice, scraped into PBS, and collected by centrifugation for 5 min at 500 × *g*. Cell pellets were incubated in 5 volumes hypotonic buffer (10 mM HEPES, pH 7.8, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA) for 30 min on ice and lysed by homogenization with a loose-fitting pestle of a Dounce homogenizer. Nuclei were collected by centrifugation at 1,000 × *g* for 5 min, and cell lysis was monitored by inspection under a microscope. The homogenization procedure was repeated three times until pure nuclear fractions were obtained. The final nuclear pellets were resuspended in nuclear extraction buffer (50 mM HEPES, pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol) and incubated on ice for 20 min. The nuclear extracts were clarified by centrifugation for 20 min at 14,000 × *g*.

Cytoplasmic and cytoskeletal fractions were prepared as follows. A 1/10 total volume of cytoskeletal isolation buffer (10% Triton X-100, 1 M KCl, 10 mM HEPES, 2 mM $MgCl_2$, 5 mM EDTA, pH 7.8) was added to the nuclear-free supernatants, followed by vortexing and incubation on ice for 20 min. The samples were centrifuged at 14,000 × *g* for 10 min and separated into an insoluble cytoskeletal pellet and a soluble cytoplasmic supernatant. The cytoskeletal pellets were then resuspended in hypotonic buffer, and the protein concentrations of all three fractions (nuclear, cytoskeletal, and cytoplasmic) were determined by bicinchoninic acid assay (Bio-Rad, Hercules, CA). Extracts were mixed with 10× sodium dodecyl sulfate (SDS)-gel sample buffer. An equal amount of protein was loaded in each lane on a 4 to 20% gradient SDS-polyacrylamide gel. The gels were transferred to nitrocellulose and analyzed by immunoblot analysis as described previously (40). For immunoblot analysis, anti-Keap1 antibody was used at a concentration of 1 μg/ml, anti-Nrf2 antibody was used at 1:1,000, mouse antiactin antibody (Sigma) was used at 1:2,500, antilamin antibody (a kind gift of the Gerace lab, TSRI, La Jolla, CA) was used at 1:1,000, rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (RDI, Flanders, NJ) was used at 0.05 μg/ml, and rabbit anti-GST alpha (Alpha Diagnostic Inc, San Antonio, TX) was used at 1:1,000.

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 actin 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-

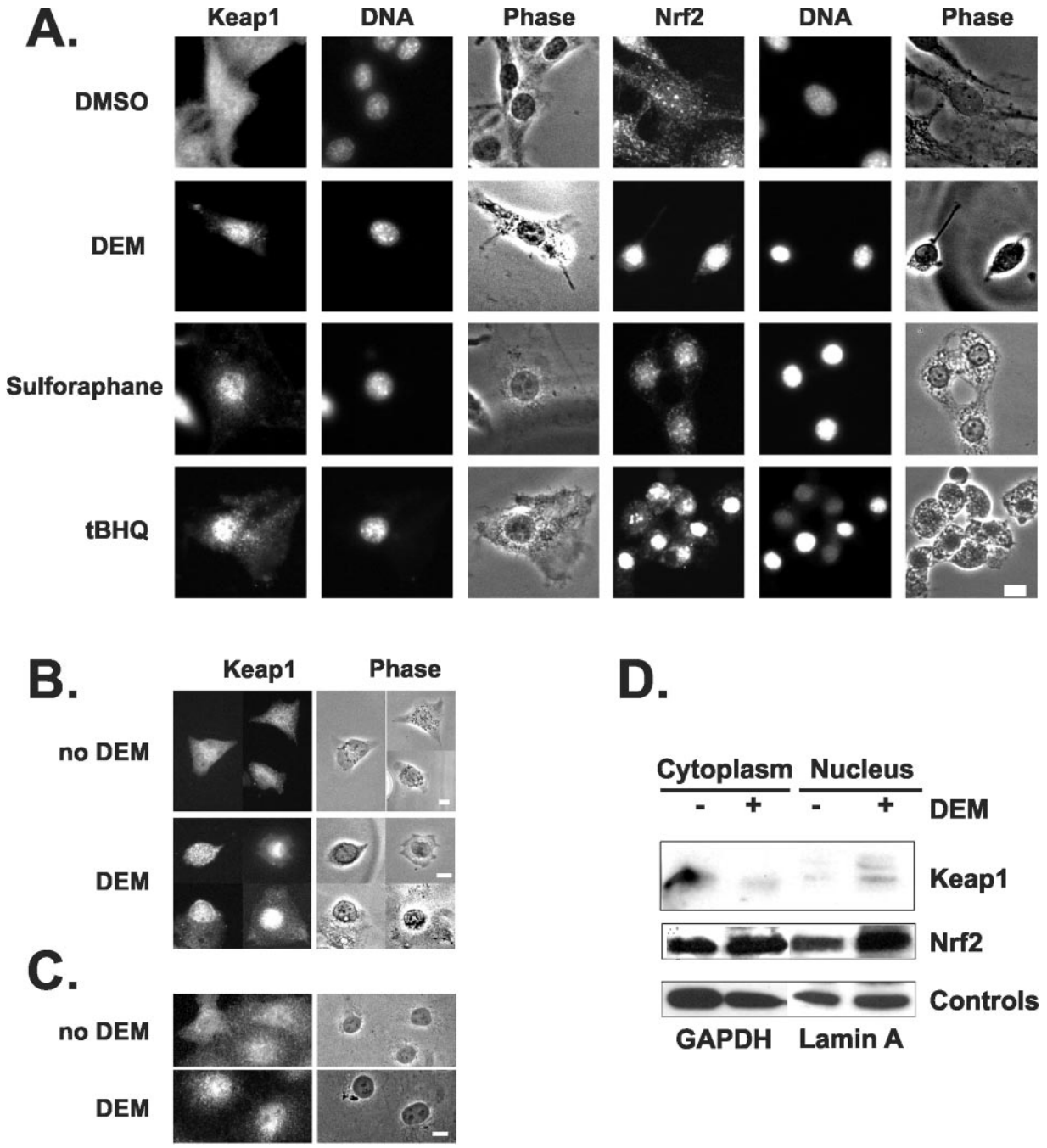

FIG. 1. Keap1 and Nrf2 both redistribute to the nucleus after oxidative stress. (A) Indirect immunofluorescence localization of Keap1 and Nrf2 in NIH 3T3 cells serum starved for 2 h followed by treatment for 24 h with vehicle (dimethyl sulfoxide [DMSO]) or activators of the oxidative stress pathway, 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 colocates 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 colocation 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 retain Keap1 in the cytoplasm under basal conditions. Analysis of the Keap1 protein sequence revealed a potential NES for the nuclear export factor CRM1/exportin encompassed by amino acids 272 to 322 (Fig. 4A). The exportin binding site is characterized by closely spaced hydrophobic amino acids, usually leucine or isoleucine, with the consensus sequence $Lx_{(1-3)}$

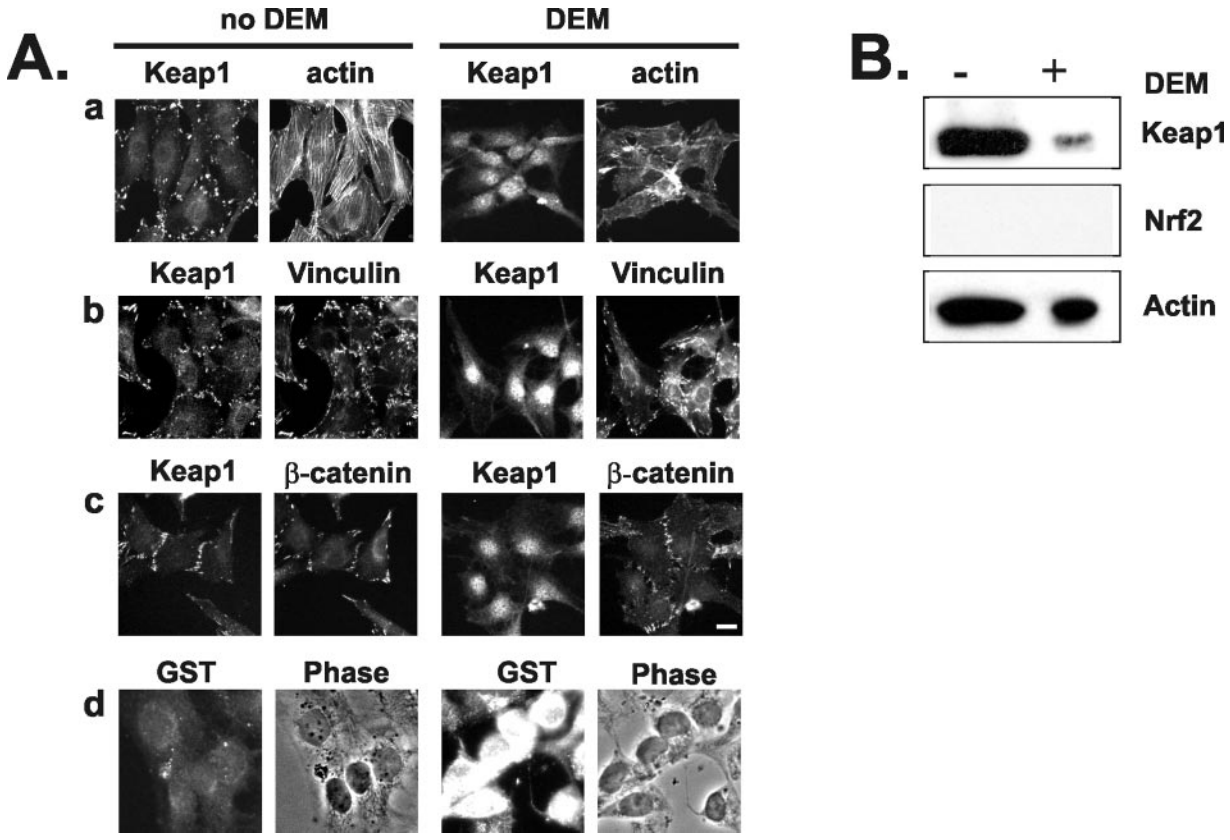

FIG. 2. Keap1 exits the actin cytoskeleton after oxidative stress, and this exit is not due to disruption of the adhesion structures. (A, a) Localization of Keap1 and F-actin in NIH 3T3 under conditions that promote assembly of the actin cytoskeleton. NIH 3T3 cells were serum starved for 16 h followed 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, a 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 is 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. GSTα, a protein regulated by Nrf2 at the transcriptional level, was visualized with a GSTα-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.

$Lx_{(2-4)}LxL$ (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 an 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.

To further confirm that this difference in localization was due to nuclear export, the transfected cells were treated with the inhibitor LMB. LMB binds directly to CRM1/exportin and inhibits its association with NES-containing proteins. As a result, LMB treatment leads to the nuclear accumulation of NES-bearing proteins that should normally be exported to the cytoplasm (28). Quantification of the location of GFP in cells expressing the full-length and truncated GFP-Keap1 fusion constructs after LMB treatment revealed that redistribution to the nucleus occurred for constructs containing the putative NES (Fig. 4C and D). For example, 95% of Keap1-GFP-expressing cells had a predominantly cytoplasmic localization

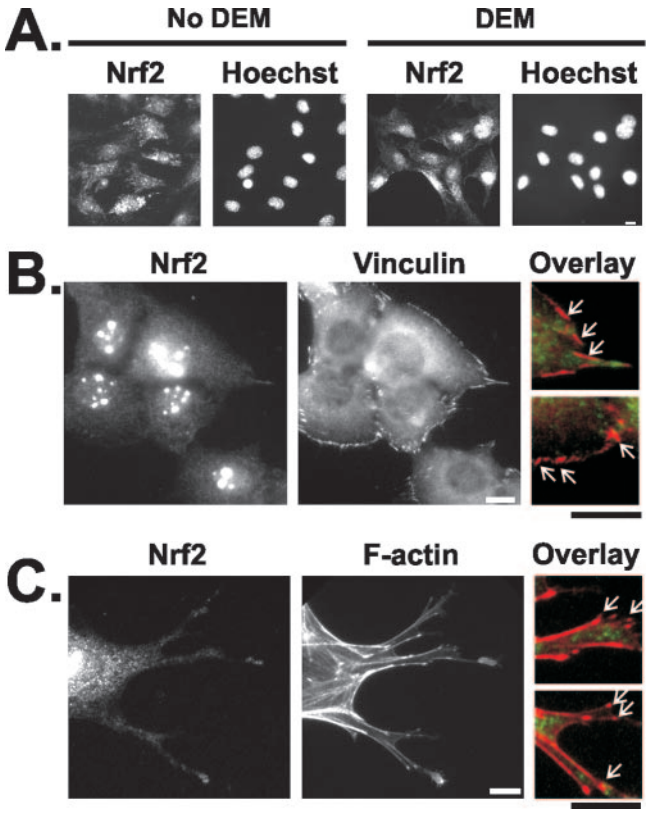

FIG. 3. Nrf2 is not associated with the cytoskeleton before or after oxidative stress. (A) Immunolocalization of Nrf2 in NIH 3T3 cells with assembled actin cytoskeleton. NIH 3T3 cells were cultured as described for Fig. 2 and treated with or without DEM. Cells were methanol fixed and immunostained for Nrf2 and DNA as described for Fig. 1. Even after serum treatment and actin assembly, Nrf2 maintained the ability to relocate to the nucleus after oxidative stress. Nrf2 was localized with a rabbit anti-Nrf2 antibody, followed by an anti-rabbit FITC-conjugated secondary antibody. DNA was visualized with Hoechst dye. (B) Immunolocalization of Nrf2 and vinculin under nonoxidative conditions. Cells were PFA fixed before staining for Nrf2 as described above (shown in green). Staining for vinculin is as described in the legend to Fig. 2B (shown in red). Arrows in the overlay point out the lack of overlap between the two proteins. (C) Staining for Nrf2 and F-actin under nonoxidative conditions. Cells were fixed with PFA. Nrf2 was visualized as described above (shown in green), and the F-actin was visualized with rhodamine-conjugated phalloidin (shown in red). Arrows in the overlay point out the lack of overlap between F-actin and Nrf2 in peripheral areas. Bars, 10 μm.

of the fusion protein; after LMB treatment, 78% of cells now exhibited a primarily nuclear localization of the GFP fusion protein (Fig. 4C and D). Constructs lacking the NES were not affected by LMB treatment, and comparison of the truncate boundaries suggests that a minimal CRM1-binding site lies between amino acids 272 and 315. Therefore, Keap1's predominant cytoplasmic location is maintained by nuclear export through CRM1/exportin.

To characterize and confirm the activity of Keap1's NES, we introduced point mutations in the putative NES in the IVR domain (amino acids 272 to 312). The hydrophobic amino acids L301, I304, L308, and L310 were changed to alanine residues. Cysteine residues in proximity to the hydrophobic stretch of leucines are an integral part of functional NES in 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 cysteine 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 (5), 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

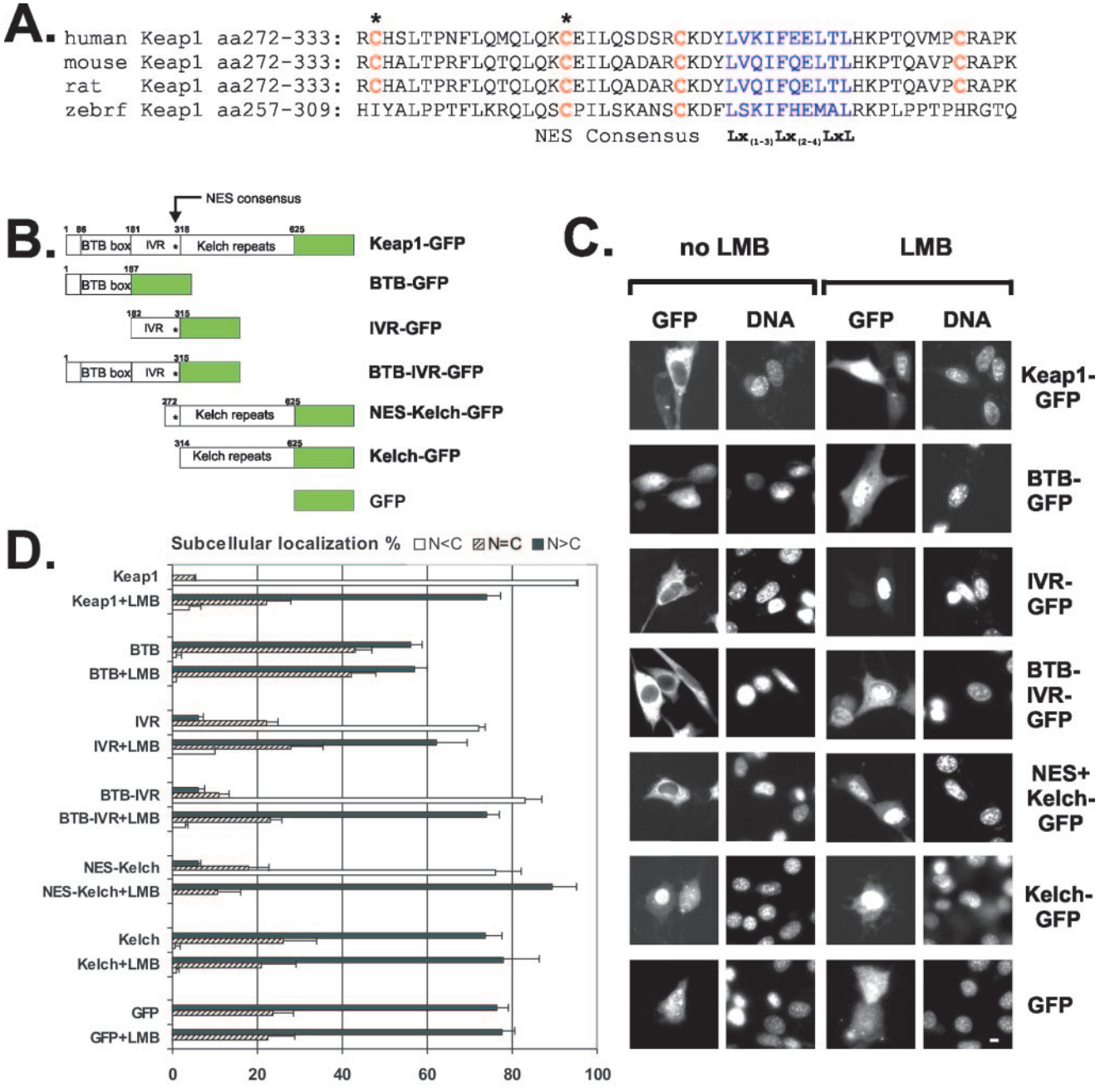

FIG. 4. Keap1 localization to the cytoplasm is due to Crm1/exportin-mediated nuclear export. (A) Alignment of human, mouse, rat, and zebra fish (zebrf) Keap1 protein sequences. The proposed NES is highlighted in blue. A consensus NES, taken from reference 11, is shown below for comparison. Conserved 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.

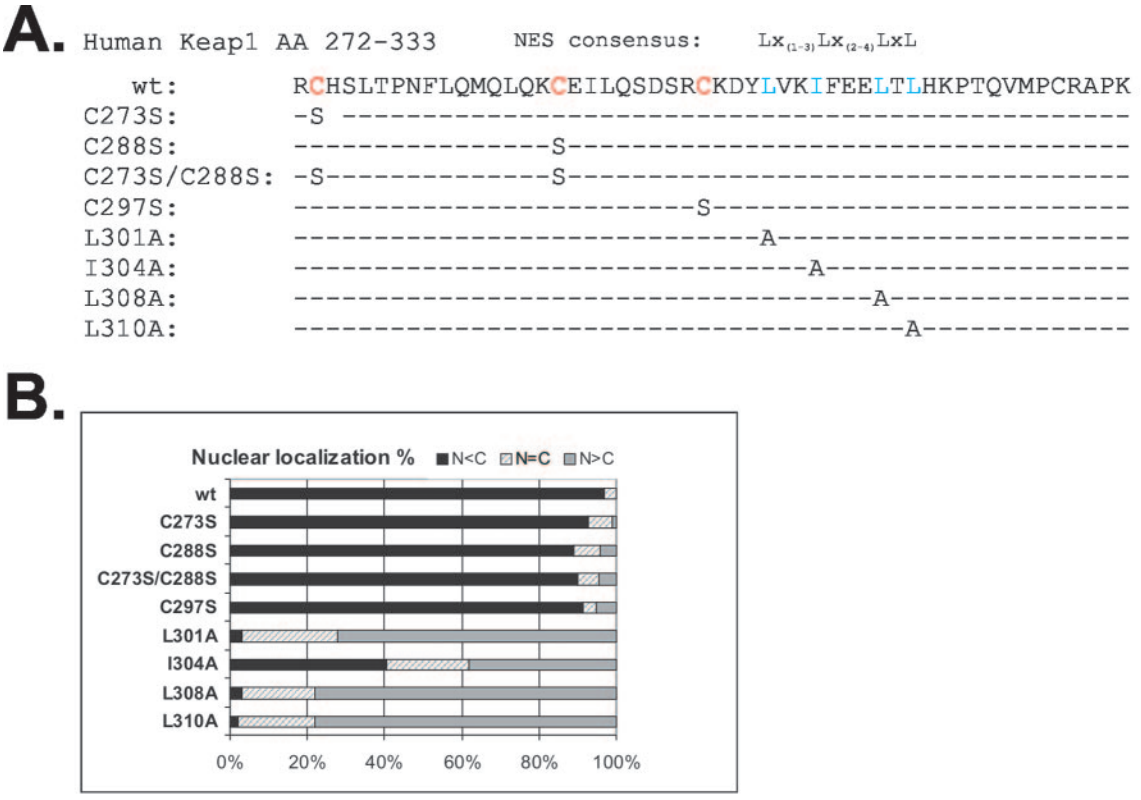

FIG. 5. Mutations in the putative NES of Keap1 result in Keap1 and Nrf2 translocation to the nucleus. (A) Sequence of the human Keap1 amino acid (AA) residues 272 to 333 and NLS consensus sequence as described in the legend to Fig. 4. Altered hydrophobic amino acids are shown in blue; altered cysteine 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.

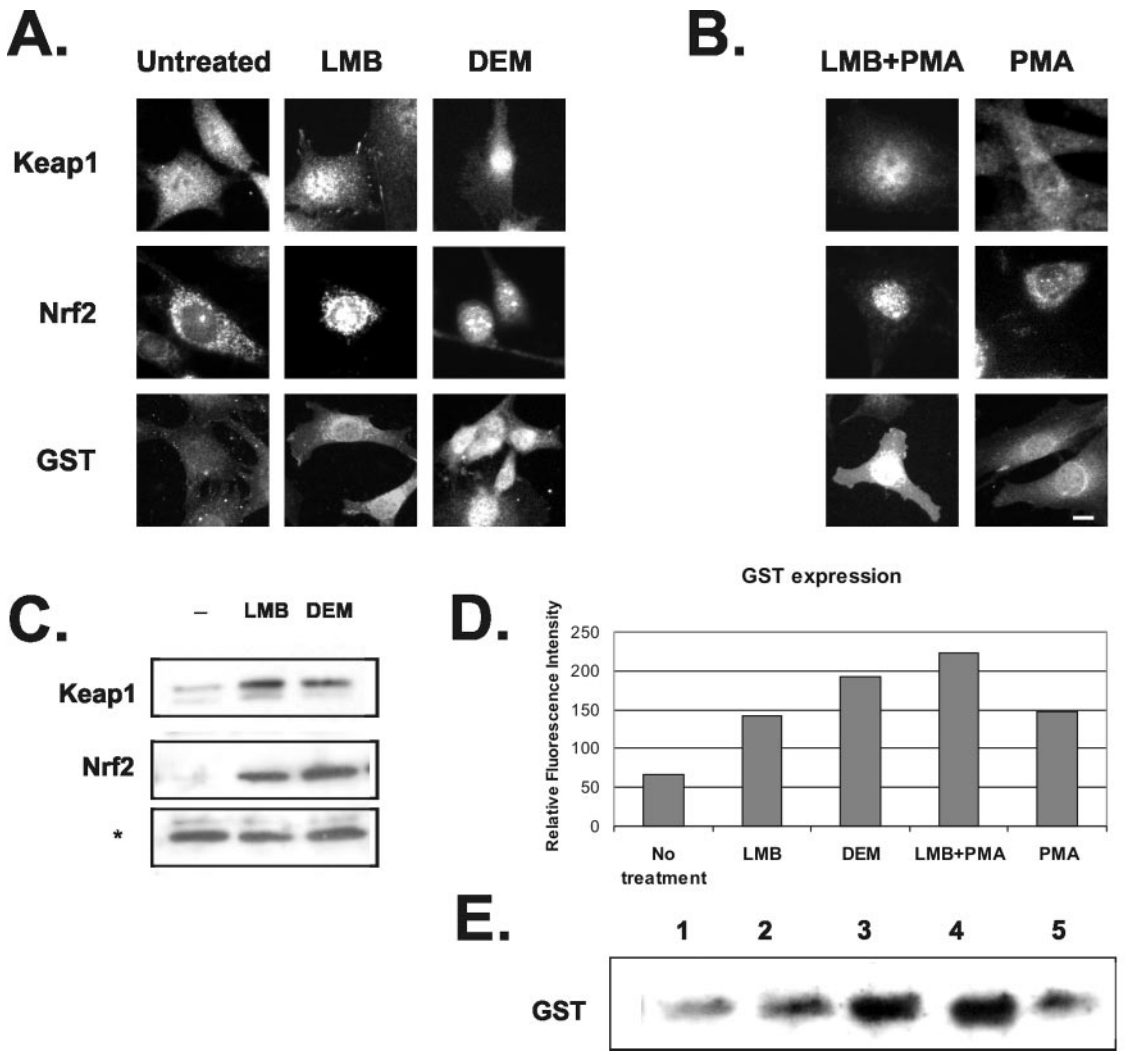

FIG. 6. LMB treatment redistributes Keap1 and Nrf2 to the nucleus, resulting in partial activation of the oxidative stress pathway. (A, B) NIH 3T3 cells were cultured as described for Fig. 2 and treated with or without 3 nM LMB for 16 h, 100 μM DEM for an hour, and 100 nM PMA for 30 min. Cells were PFA or methanol fixed and immunostained for endogenous Keap1, Nrf2, and GSTα as described for Fig. 1 and 2. Both Keap1 and Nrf2 redistributed to the nucleus after LMB treatment. Bar, 10 μm. (C) Western blot of nuclear extracts from HepG2 cells treated with 3 nM LMB and 100 μM DEM. Both Keap1 and Nrf2 concentrate in the nucleus after LMB treatment. *, loading control; −, no treatment. (D) GST expression levels were quantified by measuring fluorescence intensity per pixel in cells stained for GSTα using Photoshop software. (E) GST protein levels in cells treated with LMB, PMA, and DEM. NIH 3T3 cells were treated as described for panels A and B. Cells were washed with PBS and lysed in 5× sample buffer. Equal amounts of cell lysates were loaded in each lane, and the amount of GST was evaluated by immunoblotting with an anti-GSTα antibody. Lanes: 1, no treatment; 2, 3 nM LMB; 3, 100 μM DEM; 4, 3 nM LMB plus 100 nM PMA; 5, 100 nM PMA.

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).

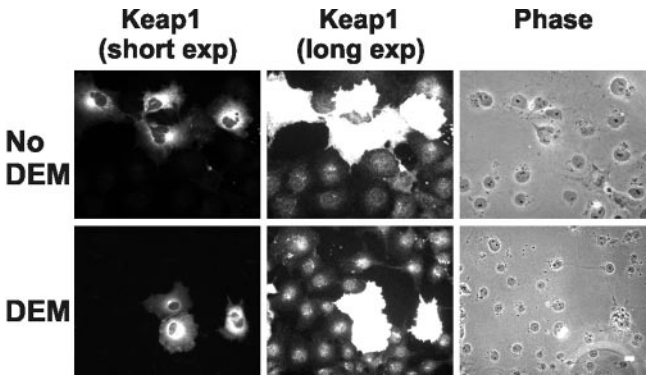

FIG. 7. Keap1-GFP is insensitive to DEM treatment. COS7 cells were transfected with full-length Keap1-GFP. Twenty-four hours after transfection, 100 μM DEM was added for 1 hour. Cells were fixed with PFA as described for Fig. 2 and stained with a rabbit anti-Keap1 antibody, followed by incubation with 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 Keap1were 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.

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 NES 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 inactivation of Keap1's NES have identical outcomes, nuclear accumulation of both Keap1 and Nrf2, we envision that Keap1 uses a similar mechanism to regulate Nrf2's location.

In the past, Nrf2 had been considered to be regulated by a unique, nuclear export-independent mechanism, by virtue of its anchoring to the cytoskeleton by Keap1 (22, 24). As we have shown that Keap1 does not anchor Nrf2 to the cytoskeleton, our study suggests that Nrf2 might in fact belong to the same category of conditional transport-regulated transcription factors as the antioxidant response activators mentioned above. Sequence analysis suggests that Nrf2 itself does not possess a classic NES. Instead, Keap1 serves this purpose, and we envision that oxidation inactivates Keap1's NES, allowing for nuclear accumulation of both Keap1 and Nrf2. A unique feature of the Nrf2/Keap1 regulatory system, however, is that the NLS and NES are situated on two separate proteins as opposed to within the transcription factor alone.

We have found that nuclear accumulation of Nrf2 by treatment with LMB to inactivate export partially activates expression of the GSTα antioxidant response gene. This activation was evident in the absence of oxidative stress. However, nuclear enrichment of Nrf2 alone was not sufficient to induce the levels of GST protein expression observed following oxidation. This supports the hypothesis that there are additional oxidation-sensitive factors required to achieve the full transcriptional potential of the ARE genes. One possible candidate is protein kinase C, which has been shown to phosphorylate Nrf2 (3, 15). Our observation that PMA treatment activates further GSTα expression in LMB-treated cells supports this hypothesis. Phosphorylation by PKC may stimulate the disruption of the Nrf2/Keap1 complex or may activate parallel pathways that allow Nrf2 to heterodimerize with small-Maf proteins and turn on ARE response genes.

Based on our study, we propose a new model for Keap1-mediated regulation of Nrf2 (Fig. 8). Keap1 and Nrf2 exist as a complex that can move between the nucleus and the cytoplasm. Nrf2 has an NLS, while Keap1 possesses an NES that is dependent on the Crm/exportin1 pathway. Under basal, nonoxidative conditions, the NES signal is dominant over the NLS and the Keap1/Nrf2 complex is located exclusively in the cytoplasm, where it is targeted for ubiquitin-mediated degradation. A small fraction of the Keap1/Nrf2 complex shuttles to the nucleus, however, accounting for the low basal level of expression of ARE genes and the low levels of Keap1 and Nrf2 evident in the nucleus.

After LMB treatment, the nuclear export pathway is blocked and, as a result, the Keap1/Nrf2 complex is targeted to the nucleus where ARE genes are partially activated. A similar redistribution is seen after oxidative stress, where the nuclear export pathway for Keap1 is specifically blocked due to oxidation, and as a result, both Nrf2 and Keap1 accumulate in the nucleus.

The precise mechanism of nuclear entry after LMB treatment or oxidative stress still remains to be discerned. It has been proposed that oxidative stress disrupts the Keap1/Nrf2 complex in the cytoplasm (9, 46). Therefore, each protein may enter the nucleus separately, Nrf2 by virtue of its NLS and Keap1 through a yet unidentified mechanism. Alternatively, as a similar redistribution is seen after LMB treatment under nonoxidative conditions, the complex may remain intact and

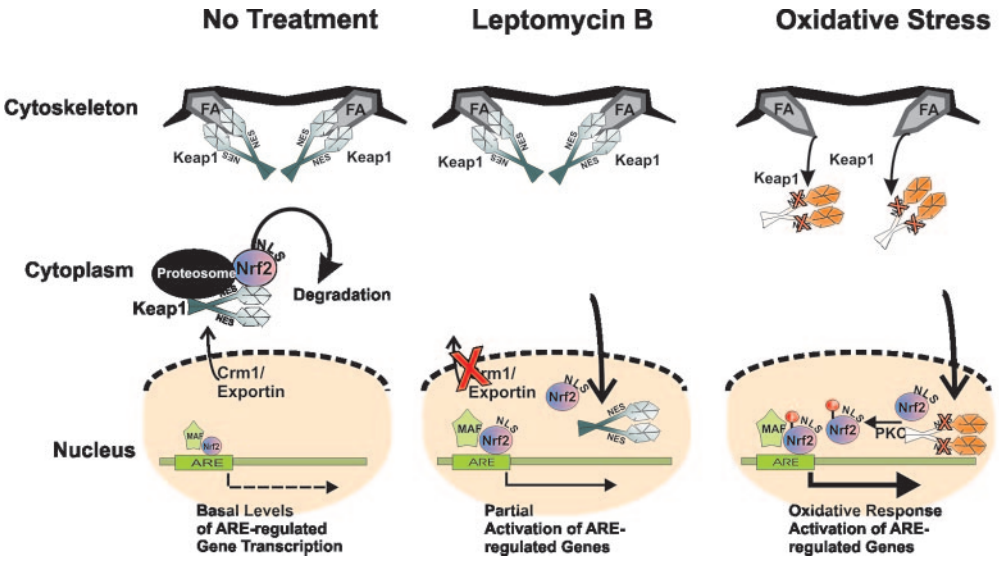

FIG. 8. Model for Keap1 regulation of Nrf2. Under nonoxidative conditions, Keap1 is evident in focal adhesions (FA). In addition, Keap1 and Nrf2 form a complex in the cytoplasm. This cytoplasmic location is actively maintained by Crm1/exportin, which recognizes an NES present in Keap1. In the cytoplasm, Keap1/Nrf2 is associated with the proteosome, where it is targeted for ubiquitin-mediated degradation. A small amount of the complex shuttled to the nucleus via Nrf2's NLS ensures basal levels of ARE-regulated gene transcription. Inhibition of Crm1/exportin by treatment with LMB results in nuclear translocation of both Keap1 and Nrf2. This represents influx of the cytoplasmic pool of Keap1, as the cytoskeletal pool remains in focal adhesions. Nuclear accumulation of Keap1 and Nrf2 results in the partial activation of the ARE genes. Oxidative stress modifies cysteine residues found within Keap1. This results in the release of Keap1 from the cytoskeleton and from the degradation machinery. All pools of Keap1 and Nrf2 concentrate in the nucleus. A second oxidation sensitive signal, possibly activation of PKC, which phosphorylates Nrf2 (red P), leads to full activation of ARE-regulated genes.

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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