Nrf2 Controls Constitutive and Inducible Expression of ARE-driven Genes through a Dynamic Pathway Involving Nucleocytoplastic Shuttling by Keap1*

Received for publication, March 21, 2005, and in revised form, June 29, 2005. Published, JBC Papers in Press, July 6, 2005, DOI 10.1074/jbc.M503074200

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Nrf2 regulates the expression of genes encoding antioxidant proteins involved in cellular redox homeostasis. Previous studies have suggested that activation of Nrf2 is mediated by mechanisms promoting its dissociation from Keap1, a cytosolic repressor that acts to sequester the transcription factor in the cytoplasm. As a short-lived protein, Nrf2 is also activated by mechanisms leading to its stabilization in cells under stress, and recent evidence indicates that Keap1 has an active role in the control of its stability. In this report, using immunocytochemistry, cell fractionation, and chromatin immunoprecipitation analyses, we found that Nrf2 is primarily a nuclear protein and that it is expressed and recruited to the chromatin constitutively to drive basal gene expression. Furthermore, we found evidence indicating that Keap1 may repress Nrf2 activity by transiently shuttling into the nucleus to promote its ubiquitylation. The data suggested that the steady-state level of Nrf2 is maintained by a dynamic pathway that balances its constitutive expression with a Keap1-regulated degradation process downstream of its role as a transcriptional activator. We suggest that the stabilization of Nrf2 in cells under stress represents the central regulatory response mediated by mechanisms that interfere with its interaction with Keap1, leading to the induction of antioxidant enzymes important to maintain cellular redox homeostasis.

Nrf2 is the central transcription factor involved in regulating the expression of antioxidant and phase II drug-metabolizing enzymes important in the protection of cells against oxidative damage caused by electrophiles and reactive oxidants. Impaired expression of these enzymes in Nrf2-deficient mice has been linked to the sensitivity of these animals to the toxic effects of various drugs and inflammatory compounds (1–6).

Nrf2 activates transcription of its genes through binding specifically to the antioxidant-response element (ARE) found in the gene promoters. The ARE is a cis-acting enhancer that mediates the transcriptional activation of genes in response to a diverse range of chemical agents including phenolic antioxidants and electrophilic compounds (7). In addition, the ARE also mediates the constitutive (or basal) expression of many of these genes under physiological conditions (8, 9), an observation that correlates well with the ubiquitous expression of Nrf2 at steady-state levels in various tissues and cell lines (10).

The involvement of Nrf2 in controlling both the constitutive and the inducible expression of its genes (11–13) suggests that its activity is controlled by a tightly regulated pathway. Nrf2 activity is repressed by the cytosolic protein Keap1, which has been proposed to act by sequestering and tethering the transcription factor in the cytoplasm. Activation of Nrf2 is thought to involve mechanisms that mediate its release from Keap1 and promote its translocation into the nucleus to drive gene transcription (14). This mechanism of Nrf2 activation has subsequently been reported in several other studies (15–17). In addition, Nrf2 is a labile protein and a substrate of the ubiquitin-dependent proteasome, and its stabilization in response to inducers of ARE-driven genes is believed to contribute to the activation process (18, 19).

More recently, new evidence has emerged showing that Keap1 can also repress Nrf2 activity by actively promoting its degradation (20–22). That Keap1 is directly involved in controlling Nrf2 stability is confirmed by several observations linking Keap1 activity to the Cullin 3-based E3 ubiquitin-protein isopeptide ligase complex (23–26). The fact that the Keap1 protein contains multiple cytochrome residues, many of which are potential sites of electrophilic attack by ARE inducers, has led to the suggestion that the Keap1-Nrf2 interaction constitutes a sensor of oxidative stress involved in triggering the ARE response to restore physiological redox status (14, 27–29). Indeed, the identification of cytochrome residues 151, 273, and 288 in Keap1 that are critical for its activity supports such a role (22, 30).

Thus, the widely held view that Keap1 forms a passive complex with Nrf2 in the cytoplasm appears to be contradicted by these recent findings. How Keap1 tethers Nrf2 in the cytoplasm without causing its degradation needs to be reconciled. Given its short half-life, a functional significance of tethering Nrf2 in the cytoplasm also needs to be addressed. In addition, such an upstream interaction between the two proteins would be prohibitive for Nrf2 to drive the basal expression of its genes. We have recently suggested that the repression of Nrf2 by Keap1 may constitute an actual downstream event that occurs only after Nrf2 has affected its transcriptional activity. Accordingly, upon synthesis, Nrf2 would translocate directly to the nucleus, activate gene transcription, and then be targeted for degradation by Keap1. Such a pathway would therefore redefine Nrf2 as predominantly a nuclear protein (31).

To explore the validity of this model, we have reexamined the localization and distribution of Nrf2 activity and investigated the cellular compartment in which its stability might be regulated. We found that Nrf2 is localized primarily in the nucleus and that its stability is regulated in this compartment. Furthermore, the normally cytosolic Keap1 appeared to repress Nrf2 activity by transiently shuttling into this com-

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‡ The abbreviations used are: ARE, antioxidant-response element; tBHQ, tert-butylihydroquinone; LMB, leptomycin B; γ-GCS, γ-glutamylcysteine synthetase heavy chain subunit; ICC, immunocytochemistry; IP, immunoprecipitation; IB, immunoblot; ChIP, chromatin immunoprecipitation; NE, nuclear extract; CE, cytosolic extract; CREB, cAMP-response element-binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Ub, ubiquitin; NES, nuclear export sequence; HA, hemagglutinin; CAT, chloramphenicol acetyltransferase; FAM, 6-carboxyfluorescein; TAMRA, 6-carboxy-tetramethylrhodamine.
partment to promote its ubiquitylation. We proposed that Nrf2 is part of a gene regulatory pathway mediated by mechanisms that control its stability downstream of its role as a transcriptional activator. This allowed Nrf2 to exert its dual function of controlling both the constitutive expression of ARE-driven genes under physiological conditions and their inducible expression in response to stress.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Antibodies, and Plasmid Constructs—**HepG2 and H4IIEC3 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics, all of which were obtained from Invitrogen. For overexpression studies, cells were transfected using Lipofectamine 2000 reagent according to the manufacturer’s instructions (Invitrogen). The following primary antibodies were used: αNrf2-C20 (Santa Cruz Biotechnology), αCREB (Santa Cruz Biotechnology), αUb (Santa Cruz Biotechnology), αGAPDH (Research Diagnostics), αHA (Roche Applied Science), αV5 (Invitrogen). In addition, a rabbit anti-Nrf2 antibody (designated αH9251) was raised against a peptide near the N terminus of the Nrf2 protein (19). The antibody was purified by two passages over an affinity matrix coupled with the immunizing peptide. Recognition of Nrf2 by this antibody was tested and confirmed by immunoblot experiments using as substrates a histidine-tagged Nrf2 protein purified from a bacterially expressed source (21) as well as in vitro translated Nrf2 produced using the TNT-coupled wheat germ extract system (Promega). The antibody recognizes Nrf2 from human, mouse, and rat. Horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology and Pierce. Alexa Fluor 488-conjugated antibodies were obtained from Invitrogen. Plasmid vectors expressing Nrf2 tagged with the HA epitope and Keap1 tagged with the V5 epitope at their C terminus were constructed by inserting PCR-amplified rat Nrf2 cDNA into the vector pMVH (Roche Applied Science) and rat Keap1 cDNA into the vector pCDNA3.1 V5-His (Invitrogen), respectively. Expression plasmid for the mutant Keap1ΔNES-V5 protein was obtained by site-directed mutagenesis in which leucine residues at positions 308 and 310 were changed to alanine using the QuikChange kit from Stratagene. The integrity of these constructs was confirmed by DNA sequencing analysis of the entire coding sequence.

**Immunocytochemistry—**HepG2 or H4IIEC3 cells were seeded on coverslips placed in 6-well dishes and allowed to recover for 20 h. Cells were either left untreated or treated with tert-butylhydroquinone (tBHQ, 50 μM, Sigma), MG-132 (10 μM, Calbiochem), and lactacystin (5 μM, Calbiochem) for 4 h. Treatment with leptomycin B (LMB, Sigma) was at 10 nM. Following washing with phosphate-buffered saline, cells were fixed in formaldehyde (3.7%, 10 min), permeabilized with Triton X-100 (1%, 5 min), and blocked with bovine serum albumin (0.5%, 1 h) in phosphate-buffered saline. Cells were incubated with αNrf2-NTR, αHA, or an αV5 antibodies as indicated for 1 h followed by incubation with Alexa Fluor 488-conjugated secondary antibodies for 30 min. For propidium iodide counterstaining of nuclei, cells were preincubated with RNase A for 15 min. The coverslips were mounted on slides using ProLong Gold antifade reagent (Invitrogen). Fluorescent images were obtained by laser scanning confocal microscopy (DM IRBE, Leica).

**Cell Fractionation—**Nuclear and cytosolic protein extracts were prepared as described (32) with minor modifications. Briefly, cells were seeded and cultured on 60-mm dishes to 90% confluency and treated with appropriate chemicals (see above) for 4 h or as indicated. Following washing, cells were harvested by scraping in ice-cold phosphate-buffered saline and collected by centrifugation at 500 x g for 5 min. Cells were lysed with Nonidet P-40 lysis buffer (50 mM Tris-HCl, 10 mM NaCl, 5 mM MgCl₂, 0.5% Nonidet P-40, pH 8.0) for 10 min on ice. Following centrifugation, the supernatant (or cytosolic extracts) was collected and subjected to centrifugation at 15,000 x g for 30 min to remove cellular debris. The nuclear pellet was washed three times with the same lysis buffer and resuspended by vortexing in high salt buffer (20 mM HEPES, 0.5 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, pH 7.9). The nuclear extracts were cleared of debris by centrifugation as before. Protein concentration was estimated using Coomassie Plus protein assay reagent (Pierce) and bovine serum albumin as standard. A small aliquot of these extracts was used for direct immunoblot analysis, and the remaining extracts were subjected to immunoprecipitation.

**Immunoprecipitation and Immunoblot Analysis—**Nrf2 or H4IIEC3 cells were cultured and treated as above. Following treatment, cells were washed twice with ice-cold phosphate-buffered saline buffer and whole cell lysates were obtained by direct lysis with immunoprecipitation (IP) buffer or with SDS sample buffer (for immunoblotting). For IP of subcellular fractions, nuclear (NE) and cytosolic (CE) extracts were diluted with IP buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 1 mM EDTA, pH 7.5), incubated with αNrf2-C20 for endogenous Nrf2 or with αHA-agarose for ectopically expressed Nrf2-HA for 16 h at 4°C. Immune complexes obtained with Nrf2-C20 were precipitated using protein A-Sepharose for 1 h. The precipitates were fractionated on 4–12% NuPAGE (Invitrogen) gels, transferred onto polyvinylidene difluoride membranes, and probed with an anti-ubiquitin (αUb) monoclonal antibody. Immune-reactive polypeptides were detected by chemiluminescence using SuperSignal luminol reagents (Pierce) and subsequent autoradiography. For detection of intact Nrf2 following cell fractionation, NE (5 μg) and CE (10 μg) were fractionated on 10% NuPAGE gels and probed with αNrf2-NTR. (Note: we have found that the mobility of Nrf2 on NuPAGE gels reflects its molecular weight more accurately than on gels using other buffer systems such as Tris-glycine.)

**Chromatin Immunoprecipitation Assays—**The chromatin immunoprecipitation (ChIP) assays were performed essentially as described (33–35). H4IIEC3 cells were seeded in 150-mm dishes (or in 245 x 245-mm dishes for multiple IPs) and incubated at 37°C for 16 h. Cells were treated with Me₂SO, tBHQ, or MG-132 (10 μM), and lactacystin (5 μM). ChIP was performed as described (33–35). H4IIEC3 cells were seeded in 150-mm dishes (or 245 x 245-mm dishes for multiple IPs) and incubated at 37°C for 16 h. Cells were treated with Me₂SO, tBHQ, or MG-132 (10 μM), and lactacystin (5 μM). ChIP was performed as described.
RESULTS

Nrf2 Is Localized in the Nucleus of HepG2 and H4IIEC3 Hepatoma Cells—The localization of Nrf2 to the cytoplasm previously has been inferred largely from several studies, including those from our laboratory, using an anti-Nrf2 antibody (αNrf2-C20, Santa Cruz Biotechnology) raised against a peptide derived from the C terminus of the protein (15–17). However, in addition to intact Nrf2, we have observed that the antibody cross-reacts with a number of other unidentified proteins on immunoblots, thus calling into question its common use in immunocytochemistry (ICC) and subcellular fractionation studies. We sought to reexamine the distribution of endogenous Nrf2 in two different cell lines, HepG2 and H4IIEC3, under stressed and non-stressed conditions. To this end, we performed ICC experiments using an antibody raised against a peptide near the N-terminal region (αNrf2-NTR) that reacts with high affinity and specificity for intact Nrf2 (19). Using confocal microscopy, we found that Nrf2 is localized in the nucleus of both HepG2 and H4IIEC3 cells under non-stressed conditions (Fig. 1A). This pattern of nuclear distribution is indistinguishable from that of cells exposed to the phenolic antioxidant tBHQ, an inducer of the Nrf2-dependent gene expression, or to the proteasome inhibitor MG-132 (Fig. 1A). These results demonstrated that Nrf2 is primarily a resident of the nucleus, and its translocation into this compartment following synthesis in the cytoplasm appears to be mediated by a constitutive process.

To confirm these findings, we employed cell fractionation procedures (32) to purify NE from CE and determine the distribution of Nrf2 by immunoblot (IB) analysis. In support of the ICC results, Nrf2 was detected principally in the NE fractions of both HepG2 (Fig. 1B) and H4IIEC3 (Fig. 1C) cells under non-stressed conditions. Consistent with the stabilizing effects induced by tBHQ (19), a significant increase in the Nrf2 protein level was observed in the NE from cells treated with this phenolic antioxidant. Results of the IB experiments using the anti-GAPDH antibody indicated that cross-contamination, if any, between the nuclear and cytosolic fractions during their preparations was not detectable. Significantly, Nrf2 was not detected in the CE fractions from unstimulated cells, in contrast to our previous observations showing the presence of this protein in cytosolic extracts using another antibody (16). Thus, our findings here not only demonstrated the nuclear localization of Nrf2 but suggested that its translocation into the nucleus following synthesis is inherently a constitutive and rapid process.

We also performed ChIP assays (33–35) to examine the interaction of endogenous Nrf2 with the ARE of the rat GST-A2 gene in H4IIEC3 cells. Using αNrf2-C20, we found that Nrf2 interacts specifically with its enhancer within the context of the chromatin in H4IIEC3 cells under non-stressed conditions. The recruitment of Nrf2 to this ARE is enhanced in cells stimulated with tBHQ (Fig. 2A), which correlates with the ability of this inducer to stabilize the transcription factor to increase transcription of ARE-driven genes (19). Control ChIP experiments were also performed in which IP of the lysates was carried out in the absence of antibodies or in the presence of αCREB. In either case, PCR amplification of the ARE sequence failed to yield a detectable product (Fig. 2B).

These data indicated that Nrf2 is constitutively recruited to the ARE, and its enhanced recruitment at this site in tBHQ-treated cells was a result of its stabilization.

On the basis of these results, we concluded that Nrf2 is principally a nuclear protein and that it translocates into the nucleus and interacts with the ARE constitutively to activate gene transcription. These data suggested that it is unlikely that Nrf2 forms a passive complex with Keap1 in the cytoplasm under non-stressed conditions.

Keap1 Promotes Nrf2 Ubiquitination in the Nucleus—Since Nrf2 is a short-lived protein (t1/2 ~ 15–30 min) and its stability is regulated by the ubiquitin-dependent proteasome (18–22), we explored whether ubiquitination of Nrf2 takes place in the nucleus or requires prior export of the protein to the cytoplasm. Using the cell fractionation procedures described above, we performed IP experiments to pull down Nrf2 from the NE and CE fractions prepared from HepG2 cells followed by IB analysis with an anti-ubiquitin monoclonal antibody. The results of these experiments showed that ubiquitylated forms of Nrf2 are found mainly in the NE and are undetectable from the CE fractions of HepG2 cells. These conjugated proteins accumulate only in cells treated with inhibitors of the proteasome (Fig. 3B), presumably because they would otherwise be rapidly degraded. Similarly, the unconjugated forms of Nrf2 were also detected primarily in the NE fractions from cells that were either left untreated or treated with tBHQ or proteasome inhibitors (Fig. 3A), consistent with the ICC results (Fig. 1A). The fact that
proteins in the NE fractions (Fig. 3). Co-expression of Keap1 led to further accumulation of the conjugated form of Nrf2-HA only in the NE fractions of the transfected cells. Interestingly, overexpressed Keap1-V5 was detected in both NE and CE fractions (Fig. 3D). Although the presence of Keap1-V5 in the CE was expected, its detection in the NE fractions was surprising and was suggestive of a close association between Keap1 and the outer face of the nuclear envelope. In contrast, intact Nrf2-HA was found primarily in the NE fractions, although its presence in the CE fractions was also observed (Fig. 3D). This was likely due to the fact that the Nrf2-HA protein was massively overproduced in the transfected cells, leading to leakage or spillover during the preparation of the subcellular fractions. Similar results have also been observed that were possibly due to the same technical problems (22).

**Transgenic Shuttling of Keap1 into the Nucleus**—The promotion of Nrf2 ubiquitylation in the nucleus by Keap1 implied that Keap1 must either enter this compartment or act through an intermediate across the nuclear envelope to carry out its function. Because repression of Nrf2 by Keap1 requires molecular interaction between the two proteins (14, 20–22, 46, 48), we focused on the possibility that Keap1 might be shuttling into the nucleus to promote Nrf2 ubiquitylation. During the preparation of this study, a new study emerged demonstrating the nucleocytoplasmic shuttling of Keap1 through the CRM1/exportin pathway in HeLa cells (37). As the trafficking of many proteins across the nuclear envelope is mediated by this pathway (38–41), we sought to reaffirm that Keap1 might engage in such shuttling activity in HepG2 cells. We transfected HepG2 cells with the plasmid expressing Keap1 tagged with the V5 epitope. Following transfection, we treated cells with LMB to inhibit the activity of the CRM1/exportin pathway and performed ICC using an anti-V5 antibody. The Keap1-V5 protein was found to accumulate substantially in the nucleus of cells exposed to LMB (Fig. 4A), indicating that Keap1 is capable of traversing the nuclear envelope to promote Nrf2 degradation. Since Keap1 does not appear to possess a nuclear localization signal, the precise mechanisms by which it enters the nucleus are not understood and remain to be investigated. The nuclear shuttling activity by Keap1, however, appeared to be of a transitory nature because its presence in the nuclear compartment could be detected only through the inhibition of the CRM1/exportin pathway. Treatment with tBHQ neither caused nuclear accumulation nor affected the LMB-induced accumulation of Keap1 in the nucleus (Fig. 4A), indicating that its nucleocytoplasmic shuttling activity is not specifically regulated by this ARE inducer.

Both ubiquitylated and unconjugated forms of Nrf2 are detected only in the NE fractions suggested that the ubiquitylation process takes place in the nucleus and does not require export of the transcription factor to the cytoplasm.

It has been demonstrated in a previous report that Keap1 acts by promoting Nrf2 ubiquitylation (22). Since degradation of Nrf2 has been shown to be mediated by both Keap1-dependent and Keap1-independent pathways (36), we next addressed the question of whether the ubiquitylation of Nrf2 in the nucleus could be promoted by Keap1. As a way to confirm the expression of this protein in HepG2 cells, we found by Taqman analysis that *Keap1* mRNA is expressed in these cells, although its levels are not affected by treatment with tBHQ (see Fig. 6C). Furthermore, since the regulation of Nrf2 stability by Keap1 involves a redox-sensitive mechanism, the fact that tBHQ induces Nrf2 stabilization in HepG2 cells (19) (Fig. 1B) indicates that Keap1 is expressed and functionally active in this cell line. To more directly assess the involvement of Keap1, we performed transient transfection experiments and examined the effects of Keap1 overexpression on Nrf2 ubiquitylation. A plasmid encoding Nrf2 fused with the HA epitope at its C terminus was constructed, and an anti-HA antibody was used to distinguish overexpressed Nrf2-HA from the endogenous protein. We found by ICC that Keap1 is expressed and functional in the nucleus of cells exposed to LMB (Fig. 3A), indicating that its nuclear localization signal, the precise mechanisms by which it enters the nucleus are not understood and remain to be investigated. The nuclear shuttling activity by Keap1, however, appeared to be of a transitory nature because its presence in the nuclear compartment could be detected only through the inhibition of the CRM1/exportin pathway. Treatment with tBHQ neither caused nuclear accumulation nor affected the LMB-induced accumulation of Keap1 in the nucleus (Fig. 4A), indicating that its nucleocytoplasmic shuttling activity is not specifically regulated by this ARE inducer.

Proteins that are exported from the nucleus through the CRM1/exportin pathway are recognized through a nuclear export sequence (NES). Analysis of the primary structure of Keap1 from human, rat, and mouse reveals a potential NES motif within its linker region that is highly conserved among species and among well-characterized NESs of other proteins (Fig. 3B). To support the LMB data, two key leucine residues (Leu-308 and Leu-310) within this motif were mutated, and the distribution of the mutant Keap1-V5 was examined by ICC and confocal microscopy. Indeed, mutation of these two leucine residues leads to an altered distribution of Keap1 and contributes significantly to its accumulation in the nucleus (Fig. 4C). These results confirm the LMB data and are consistent with and provide further support to the recent finding demonstrating the shuttling activity of Keap1 between the two cellular compartments through the CRM1/exportin pathway (37).

As inhibition of the exportin pathway by LMB causes Keap1 to be trapped in the nucleus (Fig. 4A), we sought to confirm that it would lead to a decrease in the protein level as well as transcriptional activity of endogenous Nrf2 in HepG2 cells. Using immunoblot analysis, we found that cells exposed to LMB had a lower level of the endogenous Nrf2 protein, despite being a cytosolic protein, is capable of promoting the ubiquitylation of Nrf2 in the nucleus. We have also performed IB experiments to examine expression levels of intact Nrf2-HA and Keap1-V5 in the transfected cells. Interestingly, overexpressed Keap1-V5 was detected in both NE and CE fractions (Fig. 3D). Although the presence of Keap1-V5 in the CE was expected, its detection in the NE fractions was surprising and was suggestive of a close association between Keap1 and the outer face of the nuclear envelope. In contrast, intact Nrf2-HA was found primarily in the NE fractions, although its presence in the CE fractions was also observed (Fig. 3D). This was likely due to the fact that the Nrf2-HA protein was massively overproduced in the transfected cells, leading to leakage or spillover during the preparation of the subcellular fractions. Similar results have also been observed that were possibly due to the same technical problems (22).
protein than untreated cells (Fig. 5A). In transfection experiments, expression of an ARE-CAT reporter gene was also found to be repressed by LMB (Fig. 5B). These repressive effects, however, were reversed in cells co-treated with either tBHQ or MG-132 (Fig. 5, A and B), indicating that the stability of the Nrf2 protein was adversely affected by the LMB treatment. These data confirmed our previous results and pro-
provided evidence linking the presence of Keap1 in the nucleus to its control of Nrf2 stability. We have also examined the effects of overexpression of the NES-mutated Keap1 (Keap1ΔNES-V5) protein on Nrf2 activity. In co-transfection experiments, overexpression of wild-type Keap1 caused a dose-dependent decrease in the Nrf2 protein levels as well as repression of Nrf2-driven activation of an ARE-CAT reporter gene. In contrast, overexpression of Keap1ΔNES-V5 had no apparent effects on either Nrf2 levels or reporter gene activity (Fig. 5, C and D). Since this absence of effects was observed across increasing levels of protein expression, it indicated that the intrinsic biological activity of Keap1 might have been adversely affected in the mutant protein. This was most likely due to a conformational change caused by the mutation of the two Leu residues within the critical linker region.

The Steady-state Level of Nrf2 Depends on Its Constitutive Expression—The steady-state level of Nrf2 in the cell is maintained through a balance between de novo protein synthesis and degradation (18, 19). To establish whether transcription of the Nrf2 gene might contribute to this process, we blocked transcription in HepG2 cells with actinomycin D and examined both Nrf2 protein and mRNA levels from these cells. Using IB analysis, we found that treatment of cells with actinomycin D caused a rapid decrease in the Nrf2 protein level within 4 h (Fig. 6A), a pattern of reduction that mirrors that of its corresponding mRNA as determined by Taqman analysis (Fig. 6B). Thus, Nrf2 transcription was required to maintain its cellular steady-state level. Although the rate of Nrf2 transcription is not apparently affected by ARE inducers (18, 19, 42, 43), its constitutive expression appears to be essential to enable it to control expression of ARE-driven genes at basal level. Subsequent targeting of Nrf2 for degradation by Keap1 and/or Keap1-independent pathways ensured that its excessive accumulation can be prevented.

DISCUSSION

The activation of Nrf2 depends on mechanisms that regulate its interaction with Keap1. Although Keap1 has been shown to repress Nrf2 by sequestering it from the nucleus (14), recent findings on the unstable nature of Nrf2 (18, 19) and a role for Keap1 in targeting this protein for degradation (20–22) have raised questions on the nature of the pathway that regulates Nrf2 activity.

In this report, we presented evidence demonstrating that Nrf2 is a nuclear protein and that its steady-state level as well as its stabilization in cells under stress is regulated by nuclear components of the ubiquitin-dependent 26 S proteasome. Our conclusion that Nrf2 is a nuclear protein is supported by consistent results using an antibody with specificity and high affinity for Nrf2 in the ICC and subcellular fractionation studies. Furthermore, the interaction of Nrf2 with the ARE of the GST-A2 gene under non-stressed conditions not only provides further evidence of its nuclear localization but is consistent with its involvement in the constitutive expression of this gene (11–13). It also indicated that the recruitment of Nrf2 to sites of transcription does not appear to be a regulated process, and its accumulation at these sites likely reflected its stabilization in cells under stress. These data suggested that Nrf2 stabilization is essential in the regulatory response, leading to the induction of antioxidant and drug-metabolizing enzymes by inducers of the ARE gene battery.

Although up-regulation of Nrf2-dependent genes helps to protect cells against oxidative damage and chemical stress, their down-regulation is equally critical to ensure that gene expression can be restored to constitutive levels to maintain redox homeostasis. The importance of
such gene down-regulation is evident, as demonstrated in a recent study on *keap1* (−/−) mice, showing that uncontrolled expression of Nrf2 and downstream genes at elevated levels leads to deleterious consequences (44). The finding that Keap1 actively targets Nrf2 for degradation (20–22) suggests that an important role for this repressor may be related to the process of gene down-regulation. Although Keap1 expression has been reported to be up-regulated in a previous report (45), we did not find evidence for this effect in HepG2 cells. Hence, Keap1 appeared to be a constitutively active protein and did not participate in a feedback-inhibition type of gene regulation.

The involvement of Keap1 in the down-regulation of gene expression implied that it acts downstream of the signaling pathway to promote Nrf2 degradation. This would be permissive for Nrf2 to fulfill its role as transcriptional activator prior to its degradation. Such a notion was consistent with the fact that as a nuclear protein it is unlikely that Nrf2 interacts with Keap1 in the cytoplasm under non-stressed conditions and upstream of its transactivational activity. Since Nrf2 is constitutively expressed in the cells, we envisaged that an important function of Keap1 might be to prevent Nrf2 from accumulating to an excessive level. This was supported by the finding that the Keap1-promoted ubiquitylation of Nrf2 occurs exclusively in the nucleus, a process that appears to be dependent on the nucleocytoplasmic shuttling by Keap1. Since Nrf2 is constitutively recruited to the ARE within the chromatin, this suggested that Keap1 is likely to target Nrf2 for degradation only after it has carried out its transactivational function. A question that arose from this scheme is how Nrf2 avoids being recognized by Keap1 following its synthesis in the cytoplasm. Although this remains to be investigated, one possible mechanism that would enable Nrf2 to bypass such recognition is the coupling of its nuclear translocation to the translation process, providing a direct route to its targeted destination. Given its short half-life in the cell, coupling translation with translocation would allow an efficient and rapid Nrf2 entry into the nucleus to effectively regulate its genes.

As protein stability is inherently determined by the rate of degradation, the stabilization of Nrf2 by ARE inducers may be brought about by a decrease in the rate of its turnover. Since Keap1 must shuttle into the nucleus to promote Nrf2 degradation, Nrf2 stabilization is likely to depend on mechanisms that interfere with the intranuclear interaction between the two proteins. These may involve post-translational modifications of either protein, including direct and/or indirect attacks by the electrophilic inducers to thiol-rich Keap1 via cysteine residues (28, 29) and/or phosphorylation of Nrf2 by protein kinases (46–49). Such modifications did not appear to affect the nucleocytoplasmic shuttling activity of Keap1. However, with Keap1 being constitutively active, its interaction with Nrf2 must be tightly regulated. The presence of an NES motif in Keap1 met the requirement for such restriction by ensuring that its nuclear shuttling activity is of a transitory nature. This is particularly important because otherwise, a prolonged Keap1 presence in the nucleus may lead to excessive and/or premature degradation of Nrf2, as demonstrated by the repressive effects of LMB on the endogenous Nrf2 activity. Interaction between Keap1 and Nrf2 in the nucleus may also be interfered with by prothymosin α, a nuclear protein found to putatively compete with Nrf2 for the same binding site on Keap1 (37). Additionally, Nrf2 stabilization may be governed by mechanisms regulating the association between Keap1 and the Cul3-dependent E3 ubiquitin-protein isopeptide ligase complex (23–26). Although Cul3 has been co-localized with Keap1 in the cytoplasm (24), its nuclear localization has also been observed (50), which would support its participation in promoting Nrf2 ubiquitylation in the nucleus. Finally, it is likely that Nrf2 stability is controlled by a complex regulatory network involving multiple mechanisms or pathways. For instance, although Nrf2 stabilization by tBHQ leads to a longer half-life, the stabilized transcription factor is still relatively unstable (19), suggesting the involvement of a Keap1-independent pathway in the regulation of Nrf2 stability. This is consistent with a recent report showing that Nrf2 is degraded by both Keap1-dependent and Keap1-independent pathways (36).

In summary, we propose that Nrf2 activity is regulated through a dynamic pathway of gene regulation (Fig. 7). Accordingly, Nrf2 is constitutively expressed in cells and translocates directly into the nucleus following its translation on ribosomes to activate gene transcription. By a mechanism yet to be determined, Nrf2 is then targeted for degradation by Keap1, a process that requires the transient shuttling of Keap1 into the nucleus. In cells under stress, the stabilization of Nrf2 in response to inducers is brought about by mechanisms that prevent Keap1 from targeting Nrf2 for degradation in the nucleus. The resulting decreased rate of Nrf2 degradation, combined with *de novo* protein synthesis, leads to the accumulation and direct recruitment of Nrf2 to the ARE, where it acts to increase transcription of its genes. The regulation of Nrf2 activity by such a pathway allows it to exert its dual function of controlling gene expression constitutively and inducibly.

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FIGURE 7. Schematic illustration of a proposed alternative model of the Nrf2-Keap1 pathway of gene regulation.
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J. Biol. Chem. 2005, 280:32485-32492.
doi: 10.1074/jbc.M503074200 originally published online July 6, 2005

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