Expression of antioxidant and phase 2 xenobiotic metabolizing enzyme genes is regulated through cis-acting sequences known as antioxidant response elements. Transcriptional activation through the antioxidant response elements involves members of the CNC (Cap ‘n’ Collar) family of basic leucine zipper proteins including Nrf1 and Nrf2. Nrf2 activity is regulated by Keap1-mediated compartmentalization in the cell. Given the structural similarities between Nrf1 and Nrf2, we sought to investigate whether Nrf1 activity is regulated similarly to Nrf2. Nrf1 also resides normally in the cytoplasm of cells. Cytoplasmic localization however, is independent of Keap1. Co-localization analysis using green fluorescent protein-tagged Nrf1 and subcellular fractionation of endogenous Nrf1 and fusion proteins indicate that Nrf1 is primarily a membrane-bound protein localized in the endoplasmic reticulum. Membrane targeting is mediated by the N terminus of the Nrf1 protein that contains a predicted transmembrane domain, and deletion of this domain resulted in a predominantly nuclear localization of Nrf1 that significantly increased the activation of reporter gene expression. Treatment with tunicamycin, an endoplasmic reticulum stress inducer, caused an accumulation of a smaller form of Nrf1 that correlated with detection of Nrf1 in the nucleus by biochemical fractionation and immunofluorescent analysis. These results suggest that Nrf1 is normally targeted to the endoplasmic reticulum membrane and that endoplasmic reticulum stress may play a role in modulating Nrf1 function as a transcriptional activator.

Oxidative stress occurs as a result of imbalance between antioxidant defense and the production of reactive oxygen species (1). Sustained oxidative stress causes cellular damage and dysfunction, and it has been linked to various diseases. Antioxidant and phase 2 metabolizing enzymes protect cells from oxidants and electrophiles (2). Transcriptional regulation of these protective genes is regulated in part through DNA sequences known as antioxidant response elements (ARE)² (3). Members of small Maf and “Cap ‘n’ Collar” type of basic leucine zipper (CNC-bZIP) proteins mediate transcriptional regulation through the ARE (4). The CNC-bZIP family comprises a number of structurally related proteins that bind DNA as heterodimers. This family includes p45NFE2, Nrf1, Nrf2, Nrf3, Bach1, and Bach2 (5–9). Loss of function analysis in Nrf2 knock-out mice has demonstrated its importance in protection against oxidative stress-induced pathology in various organs (10–15). These and other studies have established that Nrf2 is an important regulator of ARE function. In mouse, Nrf1 is indispensable for viability during embryonic development (16). Analysis of Nrf1-deficient fibroblasts suggests that it is also involved in the oxidative stress response (17). Nrf1 has been shown to activate expression of both the catalytic and regulatory subunits of glutamyl-cysteine ligase genes (Gclc and Gclm) through AREs in the promoters of these genes (17, 18). The simultaneous loss of Nrf1 and Nrf2 results in early embryonic lethality (19). Expression of ARE-bearing genes was severely impaired in fibroblasts deficient in Nrf1 and Nrf2, resulting in marked sensitivity to oxidative stress. Together, these studies indicate that Nrf1 have overlapping functions with Nrf2 during development and in mediating ARE function in the oxidative stress response.

Controlling intracellular localization of transcription factors is an important mechanism for regulating gene expression. Nrf2 function appears to be regulated in part by Keap1-mediated compartmentalization and degradation of Nrf2 in the cell (20). Previous studies suggest that Nrf2 is maintained as a latent cytoplasmic transcription factor through binding Keap1 via the Neh2 domain of Nrf2 (21). Upon exposure to oxidative stress inducing stimulus, Nrf2 escapes Keap1-mediated repression and translocates to the nucleus where it activates transcription of target genes. Other studies have also shown that Nrf2 is a short-lived protein, and association with Keap1 promotes the degradation of Nrf2 through ubiquitin–proteosome-mediated pathway (22–26). In this model, Keap1 plays an active role in controlling the stability of Nrf2. More recent studies suggest that Nrf2 may also be regulated through nuclear export mechanism mediated by Keap1 (27, 28).

In contrast to Nrf2, little is known about how Nrf1 function is regulated. Beyond the CNC and bZIP domains, Nrf1 shares other structural similarities with Nrf2. In particular, Nrf1 also contains a Neh2-like domain near its N terminus, but the significance of this homology is not known. In addition, it has not

Nrf1 is Targeted to the Endoplasmic Reticulum Membrane by an N-terminal Transmembrane Domain

INHIBITION OF NUCLEAR TRANSLLOCATION AND TRANSCREATION FUNCTION

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been determined whether Nrf1 activity is also regulated through a mechanism involving differential localization in the cell. In this report, we present data to show that Nrf1 is found largely in the cytoplasm of cells. Although Nrf1 contains a functional Neh2 domain that interacts with Keap1, our results indicate that cytoplasmic localization is mediated through the N terminus region of Nrf1 that contains a putative transmembrane domain spanning amino acids 7–27 of the protein. The N terminus region of Nrf1 containing this hydrophobic transmembrane domain is sufficient to confer localization of a heterologous nuclear protein to the endoplasmic reticulum. Deletion of the Nrf1 transmembrane domain results in nuclear accumulation and strong activation of ARE-containing reporter gene. Tunicamycin-induced ER stress leads to generation of a smaller form of Nrf1 that correlated with nuclear accumulation of Nrf1. We propose that Nrf1 is normally localized to the endoplasmic reticulum and that ER stress may play an important role in regulating Nrf1 function through cleavage of the transmembrane domain.

EXPERIMENTAL PROCEDURES

Reagents—Tissue culture media, fetal calf serum, and Lipofectamine 2000 were purchased from Invitrogen. PCR primers and oligonucleotides were obtained from Operon Biotechnologies (Huntsville, AL). The Myc tag (9B11) mouse monoclonal antibody and horseradish peroxidase-linked anti-rabbit IgG and anti-mouse IgG antibodies were from Cell Signaling (Beverly, MA). Anti-FLAG (M2) was purchased from Sigma-Aldrich. Anti-Nrf2 (C200) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An antibody against Nrf1 was raised in rabbits using an Nrf1-glutathione transferase fusion protein (Proteintech Group, Chicago, IL). The antibody was affinity-purified using the immunizing protein, and specificity of the antibody was confirmed by immunoblot experiments using lysates from cells transfected with Myc-tagged Nrf1 (data not shown) and lysates from Nrf1 wild type and null fibroblasts. Chemiluminescent detection system for immunoblot (ECL) was purchased from Amersham Biosciences. Protease inhibitor mix was purchased from Pierce. Protein G-Sepharose beads were from Sigma-Aldrich. Restriction enzymes, Taq DNA polymerases, and other modification enzymes were purchased from New England Biosciences. Protease inhibitor mix was purchased from Pierce. Protein G-Sepharose beads were from Sigma-Aldrich. Restriction enzymes, Taq DNA polymerases, and other modification enzymes were purchased from New England Biosciences.

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Nrf1 Transcription Factor Is an ER Membrane Protein

TCT and TGGCGGCCGCTCTCACTTTCTCCGGTCCTT

TCTCTG and GGCTCACTTTACCGGTTCCTTTGGCTT

GACTACTTAACGGAAGGAC; Nrf1-(Δ30), CGGTCGACTACCTGACCTCAGCT; and Nrf1-(Δ86)-GACAGGGTCTGACGTGCAACC. The pCMVNrf1ΔNeh2-Myc was generated by digesting pCMVNrf1-Myc with BglII followed by religation to generate an in-frame deletion of the Neh2 domain in Nrf1 cDNA. M. Freeman at Vanderbilt University (Nashville, TN) kindly provided the FLAG-tagged Keap1 expression plasmid.

The pCMVNrf1n86/Npc/NLS/Myc was constructed by PCR amplification of Nrf1 (amino acids 1–86) by with TCAACCATGTTTTCCTGAGAAGAA and CACCTGGAAGTCCAGGCTCAGGCCG in which an NcoI and SalI site were incorporated onto the 5′ and 3′ primers, respectively, for cloning between the NcoI and SalI sites of pCMV/Npc/NLS/Myc. The CMV-Npc/NLS/Myc vector containing Xenopus laevis nucleoplasmin core linked to the SV-40 large T antigen NLS (amino acid sequence PKKKRKVEDP) that was tagged with Myc was generously provided by T. S. Yen at Veterans Affairs Medical Center (San Francisco, CA). The underlined ATG in the 5′ primer represents the initiator methionine, and the 3′ primer was designed so that the Nrf1 peptide is in-frame with the Npc/NLS/Myc cassette.

The construct containing enhanced green fluorescence protein (EGF)-tagged Nrf1 was constructed using pEGFP-N1 vector (Clontech, Palo Alto, CA), which allows in-frame fusion to the N terminus of EGFP. PCR amplification of Nrf1 was done using primers CGAATTCCTGACCATGGTT-TCTCTG and GGCTCACTTTACCGGTTCCTTTGGCTT corresponding to the N- and C-terminal regions of Nrf1 tagged with EcoRI and AgeI sites, respectively. The PCR product was digested with EcoRI and AgeI and subcloned into EcoRI and AgeI sites 3′ of the EGFP-coding region of pEGFP-N1.

Chimeras consisting of Nrf1 fused to the Gal4 DNA-binding and VP16 activation domain were generated by PCR amplification of the Gal4 of the Gal4-VP16 insert from a Gal4-VP16 plasmid using GTGCCCGTGCAAGATGAGCTACTG and CGCGCGCGCCGCCAGCTCTACTGTC in which a SalI and NotI site were incorporated onto the 5′ and 3′ primers, respectively. The amplified Gal4-VP16 insert was digested and cloned into the SalI and NotI sites in pCMV/Myc/Cyto to generate pCMVGal4VP16. The plasmid pCMVNrf1n86Gal4VP16 containing a Gal4 fusion to the C-terminal region of Nrf1 (amino acid residues 1–86) was generated by PCR amplification of the Nrf1 cDNA using the primers TCAACCATGTTT- TCTCTCATGCCAGAA and CTGGTTCAAGTGACGTGCAACC, and the pCMVNrf1n27Gal4VP16 plasmid containing
**Nrf1 Transcription Factor Is an ER Membrane Protein**

The putative transmembrane domain of Nrf1 (amino acid residues 1–86) was generated using TCAACCATGGTTTCTCTGAAGAAA and GTAAGTGTGACGTCAACCGGTAC. NcoI and Sall site were incorporated into the 5′ and 3′ primers, respectively, for cloning into the NcoI and Sall sites of pCMVGal4VP16.

**Immunoprecipitation**—Cells were lysed in cold radioimmune precipitation assay buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 5 μg/ml aprotinin, 5 μg/ml leupeptin) and cleared by centrifugation for 15 min at 4 °C. Protein concentrations were determined using Bio-Rad protein assay reagent. An equal volume of 2× SDS sample buffer (100 mM Tris, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromphenol blue, 10% 2-mercaptoethanol) was added to cell lysates and boiled for 5 min. The samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes. After blocking with 5% skim milk in TBS-T (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, and 0.05% Tween 20), the membranes were probed with the indicated antibodies. The antibody-antigen complexes were detected using the ECL system.

**Immunoblotting**—Cells were lysed in cold radioimmune precipitation assay buffer 48 h after transfection. The lysates were cleared by centrifugation for 15 min at 4 °C followed by overnight incubation with anti-FLAG or anti-Myc antibodies or control (IgG) antibodies. The next day, protein G-Sepharose beads were added followed by a 2-h incubation in the cold. The beads were collected by brief centrifugation and then washed extensively with radioimmune precipitation assay buffer. The proteins were eluted in 1× SDS sample buffer and heating at 95 °C for 5 min. The samples were separated by SDS-PAGE and transferred to nitrocellulose membrane, followed by immunoblotting with indicated primary antibodies and horseradish peroxidase-conjugated secondary antibodies. Detection of peroxidase signal was performed using the enhanced chemiluminescence method.

**Immunofluorescent Staining and Microscopy**—The cells were grown on coverslips, and the plasmids were transfected into cells using the Lipofectamine 2000. Two days after transfection, the coverslips containing the cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min, followed by three washes with PBS. The cells on slides were permeabilized with 0.1% Triton/PBS for 5 min and washed with PBS/Triton and blocked with 10% goat serum at room temperature. The slides were then incubated with 2-μg/ml anti-Myc monoclonal antibody labeled with AlexaFluor 488 using Zenon Mouse IgG-labeling kits according to the suppliers’ protocols (Molecular Probes, Eugene, OR). The slides were incubated for 1 h at room temperature, followed by several washes with PBS, mounted, and visualized with a Nikon epifluorescent microscope. For localization studies, Myc-tagged Nrf1 was also cotransfected with pcDNA-ER-DsRed (a generous gift from K. Moriishi, Osaka University, Osaka, Japan) that expresses calreticulin fused with DsRed protein. Confocal images were obtained using a Bio-Rad MRC 1024 UV Confocal Microscopy system. The images are single con-
Nrf1 Transcription Factor Is an ER Membrane Protein

RESULTS

Nrf1 Contains a Neh2 Domain—The similarity between Nrf1 and Nrf2 is highest within the CNC-bZIP domain that contains the Cap ‘n’ Collar homology region, as well as the basic DNA-binding and leucine zipper regions. In addition, Nrf1 also contains a region that has significant homology to the Neh2 domain of Nrf2 that has been shown to interact with Keap1 (Fig. 1). This region encompassing amino acids 171–244 of Nrf1 shows 72% homology of the Neh2 domain of Nrf2. In addition, sequence alignment revealed complete conservation in the hydrophobic section and the ETGE motif within the Neh2 domain. These observations suggest that Nrf1 may also interact with Keap1.

Nrf1 Interacts with Keap1—To determine whether Nrf1 could interact with Keap1, coimmunoprecipitation assays were performed. Human kidney 293 cells were transfected with Myc-tagged Nrf1 and FLAG-tagged Keap1 expression vectors, and cell lysates were subjected to immunoprecipitation with anti-FLAG or control IgG antibody, and Nrf1 was detected by anti-Myc immunoblotting.Using this assay, Nrf1 was found to coimmunoprecipitate with Keap1 but not with control rabbit IgG (Fig. 2 and data not shown). To confirm this result, we generated a mutant Nrf1 molecule (Nrf1ΔNeh2; Fig. 3a) in which the region encompassing the putative Neh2 domain (Δ173–286) was deleted and examined its binding with Keap1. As shown in Fig. 2, Nrf1ΔNeh2 did not exhibit binding to Keap1 by coimmunoprecipitation analysis. These data are consistent with the idea that Nrf1 also interacts with Keap1.

The N Terminus Determines Cytoplasmic Localization of Nrf1—We performed confocal analysis to further examine the localization of Nrf1 in the cell. Because the available antibodies were not of suitable sensitivity to detect endogenous Nrf1 by immunostaining, we examined NIH-3T3 cells transfected with a Myc-tagged full-length Nrf1-expressing construct to assess its subcellular localization (Fig. 3a). Confocal microscopy revealed that the Myc-tagged full-length Nrf1 is primarily located in the cytoplasm (Fig. 3b). Treatment with leptomycin B, a drug that inhibits CRM1-mediated nuclear export, failed to cause retention of full-length Nrf1 in the nucleus (data not shown). Thus, cytoplasmic localization of Nrf1 does not appear to be regulated by nuclear export. Next, we examined the localization of the mutant Nrf1 molecule in which the region containing the putative Neh2 domain was deleted (Fig. 3a). Expression of Nrf1ΔNeh2 was also found in the cytoplasm, indicating that cytoplasmic retention is not mediated via this domain (Fig. 3b). Therefore, a series of Myc-tagged deletion mutants of Nrf1 were generated to identify domains in Nrf1 that mediate its localization in the cytoplasm (Fig. 3a). The Nrf1Δ86 construct, which contains a deletion of 86 amino acids from the N terminus of Nrf1 but still retaining the Neh2 domain, was localized to the nucleus of cells (Fig. 3, b and c). Smaller deletions were then generated to better delineate the determinants of Nrf1 localization. Truncation of 30 amino acids from the N terminus (Nrf1Δ30) resulted in nuclear localization of Nrf1 (Fig. 3, b and c). However, a truncation of the first 6 amino acids (Nrf1Δ6) did not alter the cytoplasmic localization of Nrf1 (Fig. 3, b and c). Thus, the N-terminal region of Nrf1 encompassing the first 30 amino acids is necessary for cytoplasmic localization of the protein. Similar observations were made in COS-7 and 293 cells, thus excluding potential cell type-specific effects (data not shown).

N-terminal Region of Nrf1 Is Sufficient to Confer Cytoplasmic Localization to a Heterologous Protein—To determine whether the N terminus of Nrf1 is sufficient to target a heterologous protein to the cytoplasm of cells, we transfected cells with an expression construct containing the N-terminal amino acid residues 1–86 of Nrf1 fused to the nucleoplasmin protein of X. laevis (Npc) that also contains the SV40 NLS and Myc epitope...
**Figure 3. The N terminus of Nrf1 is required for cytoplasmic localization.**

*a*, schematic diagram of Nrf1 constructs used in immunofluorescence experiments. The full-length 741-amino acid Nrf1 is shown at the top, and the mutants, Nrf1ΔNeh2, Nrf1Δ6, Nrf1Δ30, and Nrf1Δ86, are shown below. The Myc epitope tag is fused to the C terminus of each protein and is not drawn to scale. 

*b*, Nrf1 constructs were transiently expressed in NIH-3T3 cells. Localization of the ectopically expressed proteins was determined by indirect immunofluorescence detection of the Myc epitope tag with mouse anti-Myc antibodies and fluorescein isothiocyanate-labeled anti-mouse antibody. The right panels show the same field showing DAPI fluorescence of nuclei. 

*c*, percentage of cells showing nuclear or cytoplasmic staining for each case was determined by counting at least 100 transfected cells/sample. WT, wild type.
tag (Fig. 4). Indirect immunofluorescence revealed that the N86-Npc/NLS chimeric protein was localized to the cytoplasm of transfected cells (Fig. 4). In contrast, expression of NPC/NLS was localized to the nucleus as expected. To rule out the possibility that cytoplasmic localization of N86-Npc/NLS was due to export from the nucleus through CRM1-mediated pathways, we examined its distribution in cells treated with the CRM1 inhibitor leptomycin B (29). Localization, however, was not affected after treatment with leptomycin B (data not shown). These results demonstrate that the N terminus of Nrf1 is sufficient to confer cytoplasmic localization to a heterologous protein.

**Nrf1 Is an Integral ER Membrane Protein**

—Sequence analysis of Nrf1 (www.cbs.dtu.dk/services/TMHMM-2.0/) revealed a potential transmembrane-spanning domain located between amino acid residues 7 and 24 of Nrf1 (Fig. 5a). Secondary structure analysis predicts an amphipathic α-helical structure that has been observed in membrane-associated proteins (30). When the amino acids were projected on to a helical wheel, hydrophobic residues (Leu, Phe, Ile, and Gly) are found on one face of the helix, and the other face consists of both hydrophilic and hydrophobic residues (Fig. 5b). This observation prompted us to examine the cellular localization of Nrf1. We expressed Nrf1 as a fusion protein with EGFP in NIH-3T3 cells and examined localization by confocal microscopy. As seen in Fig. 6a, fusion with Nrf1 caused GFP, which is normally distributed uniformly in the cell, to accumulate in the cytoplasm in a fine reticular pattern that was densest in the perinuclear region typical of endoplasmic reticulum-associated proteins. To further examine where Nrf1 is located in the cell, we performed colocalization studies with markers for mitochondria and endoplasmic reticulum. No overlap between Nrf1-EGFP fluorescence and the mitochondria was observed using MitoTracker Red as a mitochondrion-specific probe (data not shown). However, the distribution of Nrf1-EGFP was similar to the distribution of red fluorescent protein-tagged calreticulin, which is an endoplasmic reticulum protein, suggesting that Nrf1 is localized to the ER membrane (Fig. 6a). Similar results were observed in 293 and COS-7 cells (data not shown), indicating that this observation is not cell type-specific.

To ensure that the above results were not artifacts of overexpression, we sought to determine the localization of endogenous Nrf1 in cells, as well as determine whether Nrf1 is a peripheral or integral membrane protein. To do this, we prepared lysates of mouse embryonic fibroblasts using an aqueous two-phase partitioning technique that separates peripheral and membrane-bound proteins and analyzed the fractions for Nrf1 by Western blot. Fig. 6b shows that Nrf1 was detected predominantly in the membrane fraction of fibroblasts and migrated with an apparent mass of ~120 kDa that is consistent with previous results. Membrane fractions were also prepared from Nrf1 mutant fibroblasts to demonstrate the specificity of the antibody. No Nrf1 reactive band was detected in the membrane.
fractions from Nrf1 mutant fibroblasts. Although a small amount of the Nrf1 was observed in the soluble fraction of wild type fibroblast, this is likely due to contamination because immunoblotting detected the presence of calnexin, which is an established ER membrane protein (Fig. 6b). Importantly, the pattern differs from that of Nrf2, which is found in the soluble fraction consistent with its cytoplasmic localization in cells under basal conditions (Fig. 6b). To obtain additional biochemical evidence of ER localization, the location of endogenous Nrf1 in mouse embryonic fibroblasts was also examined by differential centrifugation to fractionate subcellular compartments for immunoblot analysis (Fig. 6c). Nrf1 was predomi-
nanty detected in the high speed pellet (P100) fraction, which is enriched in endoplasmic reticulum membrane. It is unlikely that the presence of Nrf1 in this fraction is due to contamination by Nrf1 from the nucleus, because no lamin B, a nuclear marker, was detected. Because calnexin was readily detected in the nuclear fraction, low levels of Nrf1 in this fraction may be caused by ER membrane contamination. Finally, no Nrf1 protein was detected in the cytosolic fraction.

To determine whether the N terminus of Nrf1 is sufficient to target a heterologous protein to the ER membrane, 293 cells transiently expressing N86/Npc/NLS/Myc was subjected to differential centrifugation to fractionate subcellular compartments for Western blotting. Immunoblots showed that the fusion protein was largely recovered in the P100 fraction containing endoplasmic reticulum membrane (Fig. 6d). No fusion protein was detected in the cytosolic (S100) fraction (Fig. 6d). Low levels of the fusion protein were also observed in the nuclei, S10 and mitochondrial (P10) pellet. However, these are likely due to contamination with membrane material as indicated by presence of calnexin. Cells expressing Myc-tagged Nrf1 protein containing a deletion of the membrane-spanning domain (Nrf1Δ86) were also examined as a control. Nrf1Δ86 was detected in the nuclear fraction, but not the ER-containing fraction. Based on these results, we conclude that the N terminus of Nrf1 is sufficient to target protein to the ER. Together, these results indicate that Nrf1 is located primarily in the ER as an integral membrane protein.

**N-terminal Deletion of Nrf1 Augments Activation of ARE-mediated Transcription**—The effect on gene activation was examined next to determine the functional significance of constitutive nuclear accumulation of Nrf1. N-terminally tagged Nrf1 constructs were used for these studies because we found that the Myc epitope placed at the C terminus of Nrf1 appeared to interfere with its transactivation function (data not shown). Luciferase reporter gene containing the antioxidant response element from the NQO1 gene was transfected along with either wild type Nrf1, Nrf1Δ39, or Nrf1Δ288 into COS-7 (Fig. 7a) or Hepa1c1c7 cells (Fig. 7b). Cells transfected with plasmid expressing the wild type Nrf1 showed minimal to no increase in reporter gene expression. In contrast, Nrf1Δ39 expression markedly increased reporter gene expression in both cell lines. No activation was detected with Nrf1Δ288, which is consistent with previous studies (31). The apparent differences in reporter gene activation were not due to differences in expression of the different constructs because comparable levels of proteins were expressed (Fig. 7c). Thus, deletion of the N-terminal region increases activation of reporter expression by Nrf1, which is consistent with the idea that membrane retention mediated by N-terminal domain inhibits the nuclear localization and therefore the transacting function of Nrf1.

**N-terminal Region of Nrf1 Attenuates Transcriptional Activation by Gal4VP16**—To confirm the inhibitory function of the N-terminal domain, we examined the effects of N terminus of Nrf1 fused to a heterologous transcription factor. We generated expression constructs containing Myc-tagged Gal4VP16 fused with 1–27 amino acids (pCMVNrf1n27GV) or 1–86 amino acids (pCMVNrf1n86GV) of Nrf1. Transactivation of reporter plasmid by Gal4VP16 was markedly inhibited by fusion with Nrf1. The addition of 1–27 amino acids of Nrf1 completely abrogated activation, and the addition of residues 1–86 attenuated reporter gene activation by 80% (Fig. 8a). Indirect immunofluorescence detection of the Myc tag revealed that Gal4VP16 was essentially nuclear as expected (Fig. 8b), whereas localization of Nrf1n27Gal4VP16

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**FIGURE 7. Nrf1 mediated transcription is augmented by N-terminal deletion of Nrf1.** Transcriptional activation of hNQO1-ARE by full-length Nrf1 compared with deletion mutants in COS-7 (a) and Hepa1c1c7 (b) cells. Luciferase activity was normalized to Renilla luciferase from pRL-TK. In each set of experiments, triplicate transfections were performed, and the relative Luciferase activities are presented. The bars depict the means ± S.E. (n = 3) of the luciferase activity relative to activity seen with vector control. c, expression of Myc-tagged Nrf1 and deletion mutant proteins in transfected COS-7 cells analyzed by Western blotting with anti-Myc antibody.
Processing of Nrf1 Is Induced by Tunicamycin—The observation that Nrf1 is an ER membrane protein suggests a possible relation between Nrf1 and ER stress. To explore this possibility, the effect of ER stress on Nrf1 localization in the cell was examined. To induce ER stress, cells transfected with Myc-tagged Nrf1 expression vectors were treated with tunicamycin prior to analysis. Whole cell extracts were prepared and analyzed by Western blotting with anti-Myc antibody. Similar to endogenous Nrf1, Myc-tagged Nrf1 also migrated at 120 kDa, and a doublet was observed that might represent different phosphorylation states of Nrf1 (Fig. 9a). In addition to the 120-kDa doublet bands, a faint band migrating at 110-kDa was seen on occasion in untreated cells. In tunicamycin-treated cells, increased amounts of the 110-kDa product were observed compared with untreated cells (Fig. 9a). To determine whether this smaller product might represent Nrf1 that is localized in nuclei of cells, we analyzed membrane and nuclear fractions of cells expressing Myc-tagged Nrf1 by Western blotting. The p120 doublet form of Nrf1 was found in the membrane fraction of untreated cells (Fig. 9b, second lane), and none were detected in the nuclear fractions (Fig. 9c). In contrast, the smaller 110-kDa form of Nrf1 was detected exclusively in the nuclear fraction with substantial amounts detected in lysates generated from cells treated with tunicamycin (Fig. 9c). This p110 form was noticeably absent in membrane fractions of both untreated and tunicamycin-treated cells (Fig. 9b), suggesting that ER stress induced by tunicamycin results in production of a nuclear form of Nrf1. To confirm this, we examined Nrf1 localization by confocal microscopy. In untreated cells, Myc-tagged Nrf1 expression showed a reticular pattern surrounding the nucleus that colocalized with the ER marker calretinin (Fig. 9d). In contrast, tunicamycin treatment caused Myc-tagged Nrf1 accumulation in the nucleus (Fig. 9d, lower panels). Together, these findings suggest that Nrf1 is released from the ER membrane to translocate to the nucleus upon ER stress.

DISCUSSION

Nrf1 is a member of the CNC basic leucine zipper (CNC-bZIP) family of transcription factors (7). CNC-bZIP factors are characterized by a highly conserved 43 amino acid homology domain immediately N-terminal to the basic DNA-binding domain that is also found in the Cap’n’Collar protein in Drosophila and the Skn protein in Caenorhabditis elegans. Currently, there are four closely related CNC-bZIP factors in mouse that include p45NFE2, Nrf1, Nrf2, and Nrf3. In addition to the CNC and bZIP domains, Nrf1 and Nrf2 share other regions of homology in their sequences (32). In particular, Nrf1 also contains a Neh2-like domain that has been shown to be important in controlling subcellular localization of Nrf2 mediated by interaction with the Keap1 protein. However, the functional significance of this homology in Nrf1 is not known.

In this study, we have demonstrated that Nrf1 is predominantly localized in the cytoplasm. Our experiments indicate that the subcellular localization of Nrf1 is regulated by a distinct mechanism than Nrf2 that involves Keap1, which functions to retain Nrf2 in the cytoplasm, as well as targeting it for proteasomal degradation. Although communoprecipitation studies showed that Nrf1 and Keap1 interact directly with each other through the Neh2-like domain of Nrf1, the Neh2-like domain is dispensable for cytoplasmic distribution of Nrf1 in the cell. This suggests that Keap1 does not play a role in tethering Nrf1 in the cytoplasm. It is possible that Keap1 interaction may be important in regulating Nrf1 stability or other aspects of Nrf1 function not examined here. Further studies are required to determine the significance of this interaction. We have instead shown that the N terminus of Nrf1 is essential for cytoplasmic localization.
distribution. Our analysis indicated that deletion of amino acid residues 1–30 of Nrf1 is sufficient to cause redistribution of Nrf1 to the nucleus and that the N-terminal domain of Nrf1 can confer nuclear exclusion of a heterologous, nuclear-targeted protein. It has been previously reported that the cellular distribution of TCF11, a longer isoform of human Nrf1 containing 772 amino acids, is regulated by Crm1-mediated nuclear export mechanism (33). However, cellular distribution of Nrf1 here is not likely to be directed by this mechanism, because nuclear export of TCF11 was dependent on nuclear export signal sequences located in an alternatively spliced exon encoding 30 amino acids that is absent in the Nrf1 isoform (742 amino acids) used in our studies. Consistent with this, blocking CRM1 pathway by leptomycin B failed to induce nuclear accumulation of Nrf1 in our analysis. Whether cytoplasmic distribution of TCF11 also involves the N-terminal domain is not known.

Exogenously expressed Nrf1 was localized to the endoplasmic reticulum by confocal microscopy. Transmembrane helices prediction based on the hidden Markov model transmembrane probability plot revealed a hydrophobic region at the N terminus of Nrf1 that is predicted to contain a single transmembrane region spanning amino acid residues 7–24. Modeling these amino acid residues on a helical wheel revealed an amphipathic structure that may play a role in targeting and anchoring Nrf1 in the ER membrane. In support of this prediction, biochemical fractionation showed that endogenous Nrf1 was found in fractions enriched in ER membrane, indicating that localization in the ER is not an artifact caused by overexpression of exogenously introduced genes. Fractionation studies also demonstrated that adding the N terminus of Nrf1 to NPC-NLS, which normally is targeted to the nucleus, is sufficient to direct the chimeric protein to the ER membrane, indicating that the N-terminal domain of Nrf1 contains a bona fide ER membrane targeting sequence. Moreover, Nrf1 RNA is highly enriched in membrane-bound polysomes (34). This observation is consistent with membrane-associated proteins being translated by polysomes, whereas cytosolic proteins are generated by free ribosomes in cells (35). Although Nrf1 and Nrf2 share a number of structural similarities, including the various Neh domains, it is interesting to note that the N-terminal region containing the transmembrane domain is missing in Nrf2. This N-terminal domain, however, is highly conserved among Nrf1 orthologs, suggesting that these sequences might play an important role in regulating Nrf1 function. In this regard, it is noteworthy that the activation function of full-length Nrf1 is blunted in comparison with Nrf1 that contains a deletion of the N-terminal domain, whereas its addition markedly attenuated transactivation function of the heterologous
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Gal4VP16 protein. Thus, the N-terminal domain negatively regulates Nrf1 activation.

Although Nrf1 is normally detected as a p120-kDa protein, a smaller form of the protein that migrated at 110 kDa was detected in tunicamycin-treated cells. The p110 product appears unstable that can be prolonged by inhibiting protein degradation. Accumulation of this smaller form of Nrf1 coincided with a decrease of the p120 form, and this p110-kDa form of Nrf1 was recovered as a soluble nuclear protein indicating its nuclear localization in cells. Consistent with this, increased nuclear accumulation of Nrf1 was also detected by immunofluorescent staining in cells that were treated with tunicamycin. Although we cannot exclude the possibility that this p110 product represents Nrf1 arising from alteration in phosphorylation or glycosylation status for example, one attractive possibility is that the faster migrating protein represents the proteolytic cleavage product of the membrane-bound form of Nrf1. Future studies are required to determine the molecular basis for the change in Nrf1 in cells exposed to ER stress stimuli.

An important mechanism in gene expression control is regulating intracellular distribution of transcription factors (36). Given that our findings suggest that Nrf1 is anchored in the ER membrane through its N-terminal transmembrane domain, we propose that Nrf1 is normally sequestered from the nucleus and that activation of function requires proteolytic cleavage to release it from the membrane. Examples of membrane tethered transcriptional regulators that illustrate this mode of regulation include ATF6, CREB-H, OASIS, and the SREBP family of transcription factors (37–42). SREBP proteins undergo processing under low sterol conditions that leads to their translocation into the nucleus where they activate gene expression. ATF6, CREB-H, and OASIS normally reside in the membrane of the endoplasmic reticulum and are activated through proteolytic cleavage in response to ER stress. Although Nrf1 mutant cells have been shown to be impaired in activating ARE-containing genes that function in protecting cells against oxidative stress, the p110 form of Nrf1 or its translocation into the nucleus was not induced by compounds that causes oxidative stress in cells (data not shown). Whether oxidative stress activates the Nrf1 pathway is currently unclear. It is possible that activation occurs in cell types or physiological conditions that are not tested here. The association of Nrf1 with the ER membrane and activation by tunicamycin suggests that Nrf1 is regulated differently compared with Nrf2 that may give rise to functional diversity between Nrf1 and Nrf2. In addition, these findings also suggest that Nrf1 may play a role in modulating response to ER stress. One possibility is that Nrf1 may regulate expression of genes such as chaperones and foldases that are vital for cells to maintain homeostasis under ER stress, thus acting as a downstream component of the ER stress-signaling pathway. Alternatively, activation of Nrf1 by ER stress may represent an antibody response. Activation of Nrf1 and ARE-driven genes encoding antioxidants allows the cell to respond to the impending oxidative stress that is induced by ER stress (43). Further experiments are required to sort out these possibilities.

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