The Nrf3 Transcription Factor Is a Membrane-bound Glycoprotein Targeted to the Endoplasmic Reticulum through Its N-terminal Homology Box 1 Sequence

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Transcription factor Nrf3 (NF-E2 p45-related factor 3) is targeted to the endoplasmic reticulum (ER). Mouse Nrf3 is subject to proteolysis, Asn glycosylation, and deglycosylation reactions. It is synthesized as a ~96-kDa protein that is subsequently converted into isoforms of ~90, 80, and 70 kDa. In the ER, the ~90-kDa glycoprotein is predominant and gives rise to ~80- and ~70-kDa isoforms. The ~90- and ~80-kDa polypeptides were observed in the nuclear envelope, whereas the ~70-kDa isoform was detected primarily in the nucleoplasm. Our experiments showed the N-terminal homology box 1 (NHB1, residues 12–31) is part of a tripartite signal peptide sequence, comprising n, h, and c regions. The n region (residues 12–23) was demonstrated to target Nrf3 to the ER and is necessary for its Asn glycosylation. The n region (residues 1–11) controlled the abundance of the ~90-kDa glycoprotein. The c region (residues 24–39) was found to contain a signal peptidase cleavage site that is responsible for production of the ~90-kDa mature Nrf3 glycoprotein from a ~96-kDa precursor. We have found that Nrf3 is activated by the ER stressors tunicamycin and brefeldin A, and that NHB1 is required for this response. Amino acids between the c region and NHB2 (residues 76–100) controlled the proteolytic processing of mouse Nrf3 into cleavage products of ~80-kDa (glycated) and ~70-kDa (non-glycated); by contrast, human Nrf3 lacked a signal peptidase cleavage site between its c region and NHB2. Lastly, data are presented suggesting that the NHB2 sequence in mouse Nrf3 may regulate the topology of the transcription factor within the ER membrane.

Nuclear factor-erythroid 2 p45-related factor 3 (Nrf3) is a member of the cap “n” collar (CNC) subfamily of basic-region-leucine zipper (bZIP) transcription factors (1, 2), which also includes Nrf1, Nrf2, and the NF-E2 p45 subunit. Like other members of the CNC bZIP family, Nrf3 can bind to the antioxidant response element (ARE, with a core consensus sequence 5’-TGA(C/G)nnnGC-3’) (1–5) and is therefore presumably involved in the regulation of antioxidant and detoxification genes, such as those for the glutamate cysteine ligase catalytic and modifier subunits, glutathione S-transferase (GST) isoenzymes and NAD(P)H:quinone oxidoreductase 1 (NQO1).

The physiological functions of Nrf3 remain elusive. It is expressed in the liver and a wide variety of other tissues, but is particularly abundant in the placenta (1, 6, 7). As Nrf3−/− mice have no obvious phenotype (8), it is possible that its loss can be compensated by Nrf1 and/or Nrf2. Interestingly, it has been found that the expression of Gclc, Gclm, and Ngo1 genes is not completely abolished in double Nrf1−/−:Nrf2−/− knockout mice (9), suggesting that the residual transcriptional activation may be mediated by Nrf3. This hypothesis is supported by the observation that Nrf3 mRNA levels were elevated between 4- and 5-fold in skin from Nrf2−/− mice, when compared with that in wild-type mice (10).

We and others have used bioinformatics to gain an insight into the in vivo activity of Nrf3. By definition, it contains a CNC and bZIP domain (collectively called Neh1) as well as an adjoining C-terminal Neh3L (Neh3-like) domain. We have noted previously that the N-terminal 126 residues of Nrf3 share certain similarities with the N-terminal domain (NTD) of Nrf1 (11, 12); this feature is absent from both NF-E2 p45 and Nrf2. Blank and his colleagues have reported that, uniquely among CNC bZIP factors, the human Nrf3 protein contains a PEST sequence between residues 158 and 172 (sharing homology with residues 137–152 of mouse Nrf3), which may contribute to its rapid turnover (13). These workers have also shown that the central

Discosoma sp. red fluorescent protein; ER, endoplasmic reticulum; GFP, green fluorescent protein; GST, glutathione S-transferase; hN126, N-terminal 126 residues of human Nrf3; LDS, lithium dodecyl sulfate; N66, N-terminal 66 residues; Neh3L, Neh3-like; Neh5L, Neh5-like; NHB1, N-terminal homology box 1; NQO1, NAD(P)H:quinone oxidoreductase 1; Nrf, nuclear factor-erythroid 2 p45 subunit-related factor; Nrf2, nuclear factor-erythroid 2 p45-related factor 2; ADAR2, acidic domain 2-like; ARE, antioxidant response element; BFA, brefeldin A; bZIP, basic-region leucine zipper; CNN, cap “n” collar; DsRed, leucine zipper (bZIP) transcription factors (1, 2), which also includes Nrf1, Nrf2, and the NF-E2 p45 subunit. Like other members of the CNC bZIP family, Nrf3 can bind to the antioxidant response element (ARE, with a core consensus sequence 5’-TGA(C/G)nnnGC-3’) (1–5) and is therefore presumably involved in the regulation of antioxidant and detoxification genes, such as those for the glutamate cysteine ligase catalytic and modifier subunits, glutathione S-transferase (GST) isoenzymes and NAD(P)H:quinone oxidoreductase 1 (NQO1).

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part of human Nrf3, between residues 298 and 399, contributes to transactivation. This region is herein included in the part of the protein designated TAD (Fig. 1 and supplemental Fig. S1), and sequence comparisons have revealed that it shares homology with three domains in Nrf1, namely, Neh5L, NST, and AD2. Lastly, Nrf3 contains a sequence that resembles the Neh6 domain in Nrf2 between its TAD and CNC domains.

Nrf3 can be divided into seven domains (i.e. NTD, PEST, TAD, Neh6L, CNC, bZIP, and Neh3L). As mentioned above, Nrf1 also contains NTD and TAD (i.e. Neh5L, NST, and AD2) regions that resemble those in Nrf3. In the case of Nrf1, its NTD is responsible for targeting and anchoring the factor to the endoplasmic reticulum (ER) through its NHB1 sequence and whether it is activated by ER stress. Importantly, the NHB1 in Nrf1 is part of a tripartite signal peptide, comprising an n region, h region, and c region. The n and c regions surrounding NHB1 are not highly conserved between Nrf1 and Nrf3. Although the signal peptide in Nrf1 is not cleaved by a signal peptidase (SPase), it is not known if the putative signal peptide in Nrf3 also fails to be cleaved from the bZIP protein. To identify the mechanism by which Nrf3 is modified, we have examined whether: (i) NHB1 and its adjacent residues conform to a signal peptide sequence that targets Nrf3 to the ER; (ii) this signal sequence contains a potential cleavage site for SPase; (iii) Nrf3 is integrated into the ER membrane and the nuclear envelope; and (iv) Nrf3 transactivation activity is increased by ER stress.

EXPERIMENTAL PROCEDURES

Chemicals, Enzymes, and Other Reagents—These were all of the highest quality and were readily available commercially. The ER extraction kit and all chemicals were purchased from Sigma-Aldrich. Endoglycosidase H (Endo H), peptide-N-glycosidase F (PNGase F), and proteinase K (PK) were obtained from New England Biolabs. Rabbit polyclonal antibodies against calreticulin (CRT), calnexin, and GFP were bought from Calbiochem (San Diego, CA) and Abcom PLC (Cambridge, UK), respectively. Mouse monoclonal antibody against the V5 epitope was from Invitrogen, whereas those against Sec61α, Lamin A/C, and retinoblastoma 1 gene protein were supplied by Upstate (Dundee, Scotland, UK). Goat polyclonal antibodies against Nrf3 (N14, V15, and L16) and small Maf (sMaf) proteins were bought from Santa Cruz Biotechnology (Santa Cruz, CA). The siRNA oligonucleotides against human Nrf3 were purchased from Ambion, Inc. (Austin, TX).

Expression Plasmids—The cDNA encoding mouse Nrf3, previously cloned into the pEF-BOS vector (1), was amplified in 50 µl of reaction buffer containing 1 unit of KOD Hot Start DNA polymerase (Merck Chemicals Ltd., Nottingham, UK), 0.5 mM MgSO4, 2% (v/v) DMSO, and 12.5 pmol of each of the forward and reverse primers. The first-strand cDNA encoding human Nrf3 was synthesized in the AccuScript™ High Fidelity RT-PCR system that contained 1 µg of total RNA extracted from human choriocarcinoma JAR cells. The double-stranded cDNA sequence, generated by the above PCR reaction, was cloned into pcDNA3.1/V5His B (Invitrogen) following KpnI/XhoI digestion. From this expression plasmid, various Nrf3 mutants were created by site-directed mutagenesis performed in the KOD Hot Start DNA PCRs containing the appropriate pairs of sense and antisense primers (14).

Nucleotide sequences encoding the NTD and N-terminal 66 amino acids (N66) of mouse Nrf3, as well as the N-terminal 126 residues of human Nrf3 (called hN126), along with several mutants, were generated by PCR using suitable primers. These products were ligated to the 5’-end of the cDNA for either Nrf2 or GFP in which their translation initiation sites had been mutated, to ensure that neither free Nrf2 nor free GFP were produced. Therefore, the cDNA for the fused protein was inserted into pcDNA3.1/V5His B (Invitrogen) following KpnI/XhoI digestion. From this expression plasmid, various Nrf3 mutants were created by site-directed mutagenesis performed in the KOD Hot Start DNA PCRs containing the appropriate pairs of sense and antisense primers (14).

Cell Culture, Transfection, and Luciferase Reporter Assays—Monkey kidney COS-1 cells were grown for 24 h in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. The JAR cells were cultured in RPMI 1640 containing 10% fetal bovine serum. After the cells reached 70% confluence, they were transfected with expression constructs for Nrf3 or its mutants, along with either P4tqng01-ARE-Luc or P3vq0GSTA2-6×ARE-Luc reporter plasmids, using Lipofectamine 2000 (Invitrogen) (12, 15). The pRL-TK plasmid encoding Renilla, as well as pcDNA4/HisMax/lacZ encoding β-galactosidase (β-gal), was used to control for transfection efficiency. In some experiments, transfected cells were treated for 24 h with chemicals before reporter gene activity was measured as described
previously (12). The significance of differences in the transactivation activity of wild-type Nrf3 and mutant forms of Nrf3 was determined using the Student’s t test.

siRNA Knockdown, Immunocytochemistry, and Confocal Microscopy—COS-1 and JAR cell lines were transfected with an expression construct for Nrf3, or siRNAs targeted against Nrf3. Approximately 24 h following transfection, the cells were examined by immunocytochemistry and confocal microscopy (11, 12).

Subcellular Fractionations—The intact ER, microsome-containing membrane, nuclear, and cytosolic fractions were prepared as described previously (11, 16, 17). The salt-extracted nuclear fraction was prepared according to an established method (18, 19). Briefly, purified nuclei were incubated with an extraction buffer (10 mM Hepes-KOH, pH 7.4, 0.42 M NaCl, 2.5% (v/v) glycerol, 1.5 mM MgCl₂, 0.5 mM sodium EDTA, 0.5 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1% complete protease inhibitor mixture). After being gently rotated for 60 min at 4 °C, the mixture was centrifuged at 14,000 × g for 5 min at 4 °C. The resulting supernatant was saved and is referred to as “salt-extracted nuclei.”

The nuclear envelope membrane fraction was obtained by the high-ionic-strength extraction method (20). In brief, purified nuclei were suspended at a concentration of 5 mg DNA/ml in the SMT buffer (250 mM sucrose, 50 mM MgCl₂, 50 mM Tris-HCl, pH 7.4, supplemented with 1 mM PMSF, and 1% complete protease inhibitor mixture), before they were gently homogenized by passing the mixture through a 25-gauge needle with 10 strokes. The nuclear homogenate (1 ml) was incubated with both DNase I and RNase (at a concentration of 250 μg of protein/ml) for 1 h at 4 °C before being sedimented by centrifugation at 1000 × g for 10 min at 4 °C. The resulting pellet was resuspended in an equal volume of MT buffer (0.2 mM MgCl₂, 10 mM Tris-HCl, pH 7.4, containing 1 mM PMSF and 1% complete protease inhibitor mixture), and was subsequently mixed with 4 volumes of high-NaCl buffer (2 M NaCl, 0.2 mM MgCl₂, 10 mM Tris-HCl, pH 7.4, supplemented with 1% (v/v) 2-mercaptoethanol, 1 mM PMSF, and 1% complete protease inhibitor mixture). After stirring for 30 min, the nuclear envelope membranes were pelleted by centrifugation at 16000 × g and 4 °C for 30 min.

Membrane Proteinase Protection Reactions—Briefly, either intact ER-rich or microsome-containing fractions were purified and resuspended in 100 μl of 1× isotonic buffer (10 mM Hepes, pH 7.8, containing 250 mM sucrose, 1 mM EDTA, 1 mM KCl, and 25 mM CaCl₂). An aliquot (30 μg of protein) of the membrane-containing preparation was incubated for 30 min on ice with protease K at a final concentration of 50 μg protein/ml. The reactions were terminated by heating at 90 °C for 10 min following the addition of 1 mM PMSF.

Deglycosylation Reactions, Co-immunoprecipitation, and Western Blotting—These were performed as reported elsewhere (11, 21). During Western blotting, some antibody-blotted nitrocellulose membranes were washed for 30 min with stripping buffer (7 M guanidine hydrochloride, 50 mM glycine, 0.05 M EDTA, 0.1 M KCl, and 20 mM 2-mercaptoethanol at pH 10.8) before being re-probed with an additional primary antibody (22). The intensity of some Western blots was calculated using the Java-based image-processing program (ImageJ) developed at the National Institutes of Health.

RESULTS

Nrf3 Transcriptionally Activates ARE-driven Gene Expression—Electrophoretic mobility shift assays have demonstrated that Nrf3 can bind ARE sequences as a heterodimer with Smad proteins (1, 2, 23). There are however conflicting reports about whether Nrf3 activates or suppresses ARE-driven gene expression. We found that Nrf3 activated a Pₓ-nqo1-ARE-Luc reporter gene about 1.7-fold (Fig. 2). By comparison, a parallel experiment using similar amounts of expression vector revealed that Nrf1 transactivated the same reporter gene ~4-fold, whereas Nrf2 increased the reporter activity 18-fold. Based on the mass of DNA transfected, Nrf3 demonstrated less transactivation activity than either Nrf1 or Nrf2. To determine whether differences in protein levels might account for the low activity of Nrf3, Western blotting was undertaken. It has been shown previously that post-synthetic modification of Nrf3 yields three electrophoretic bands, called A, B, and C, which in LDS-NuPAGE we estimate to have molecular masses of ~90, ~80, and ~70 kDa (Fig. 2B). Although Nrf3 exhibited less activity than either Nrf1 or Nrf2, both its ~90-kDa A and ~80-kDa B forms (which seemed to be present in similar amounts) appeared to be more abundant than either Nrf1 or Nrf2. The ~70-kDa Nrf3 C polypeptide was not always clearly discernible, probably because it is less abundant than the larger isoforms.
The NHB1 Sequence Targets Nrf3 to the ER—The low activity of Nrf3, like Nrf1, may be due to the fact that it contains an ER-targeting sequence in its N-terminal domain. Bioinformatic examination of human and mouse Nrf3 revealed that NHB1 and its adjacent residues conform to a classic tripartite ER-targeting signal peptide sequence. In the murine protein the n, h, and c regions are located between residues 1–11, 12–23, and 24–39, respectively (Fig. 3A). Residues between amino acids 7 and 27, which encompasses the h region, are predicted to fold into a hydrophobic α-helix that spans the membrane (Fig. 3B).

Whether these sequences contribute to the ER targeting of Nrf3 was examined by confocal microscopy. As shown in Fig. 3C, ectopic wild-type Nrf3 gave predominantly ER staining in 65% of COS-1 cells examined. A significant increase in ER staining was seen in 90% of cells that had been transfected with an expression construct for either the Nrf3^A2–11 or Nrf3^A24–39 mutants, in which the n region and the c region were deleted, respectively. By contrast, a marked increase in nuclear staining was observed upon transfection with either the Nrf3^A12–23 or Nrf3^A12–30 mutants, in which the h region and the essential NHB1 sequences were removed. Extracellular staining was not observed in the case of Nrf3^A2–39 as it was located exclusively in the nucleus (Fig. 3C).

We next examined whether the different subcellular distributions of wild-type murine Nrf3 and its mutants influence transactivation of the Ptenqo1-ARE-Luc reporter gene. Fig. 3D shows that the activity of Nrf3^A2–11 and Nrf3^A24–39 was significantly lower than the wild-type protein. Conversely, the Nrf3^A12–23, Nrf3^A12–30, and Nrf3^A2–39 mutants exhibited a modestly increased transcriptional activity when compared with the wild-type protein. To examine whether differences in transactivation of ARE-driven transcription affected by these various Nrf3 mutants might be due to their relative abundance or post-translational modification, Western blotting experiments were performed. By comparison with wild-type protein, ectopic Nrf3^A2–11 was expressed in COS-1 cells primarily as a ~90-kDa A polypeptide with a relative reduction in the amount of ~80-kDa B polypeptide (Fig. 3E). Conversely, the ~90-kDa protein was not detected upon transfection with an expression construct for the Nrf3^A12–23, Nrf3^A12–30, or Nrf3^A2–39 mutants. Production of the ~90-kDa protein was not altered in the case of the Nrf3^A24–39 mutant, whereas the amount of the ~80-kDa form was significantly reduced. These data suggest that the h region within NHB1 contributes to the negative regulation of Nrf3 by its NTD, presumably through association with the ER.

Endogenous Nrf3 Isoforms Are Located in the ER and the Nucleus—To confirm that endogenous Nrf3 is also localized in the ER, we performed immunocytochemistry using antibodies against an N-terminal region (N14) or an internal region (L16) of the Nrf3 protein. Confocal microscopy showed that human Nrf3 is located primarily in the extranuclear compartment of JAR cells, yielding a staining pattern similar to that obtained for...
calreticulin (Fig. 4A); this is consistent with the hypothesis that endogenous Nrf3 is associated with the ER. To support the immunocytochemistry data, knockdown experiments were performed. ER staining for Nrf3 was markedly diminished following transfection of JAR cells with two separate siRNA targeted against mRNA for the bZIP protein (Fig. 4A, right two lines). Western blotting using two different Nrf3 antibodies revealed that three immunoreactive polypeptides of ~50, 80, and 85 kDa were recovered in the ER and nuclear fractions (Fig. 4B). The level of all three polypeptides was significantly reduced following knockdown of Nrf3 mRNA using specific siRNA species.

Generation of the ~90-kDa Nrf3 Glycoprotein Requires the h Region—Differences in the molecular mass of Nrf3 isoforms may arise from ER-directed post-translational modification, such as Asn glycosylation. To test this possibility, we subjected wild-type mouse Nrf3 and its mutant proteins to enzymatic deglycosylation. As shown in Fig. 5A, while ectopic wild-type Nrf3 in COS-1 cells migrated during NuPAGE as two protein bands with molecular masses of ~80 kDa and ~90 kDa, digestion of the cell lysates with PNGase F resulted in a complete loss of the larger of the two proteins. This loss of the ~90-kDa band was accompanied by a modest increase in the amount of the ~80-kDa protein. Two similarly migrating proteins were resolved by NuPAGE from lysates prepared from COS-1 cells expressing the Nrf3\(^{2–11}\) or Nrf3\(^{2–24–39}\) mutants, although in both cases the ~90-kDa protein was predominant (Fig. 5A). Again, the largest ~90-kDa band from Nrf3\(^{2–11}\) or Nrf3\(^{2–24–39}\) disappeared following digestion with PNGase F and was replaced with substantially increased amounts of the ~80-kDa protein, along with a modest increase in the ~70-kDa band. Two similarly migrating proteins were resolved by NuPAGE from lysates prepared from COS-1 cells expressing the Nrf3\(^{3–24–39}\) or Nrf3\(^{3–24–75}\) mutants, although in both cases the ~90-kDa protein was predominant (Fig. 5A). A similar experiment using COS-1 cell lysates expressing Nrf3\(^{3–24–39}\) showed that either of the ~70- and ~80-kDa bands changed following digestion with PNGase F. Furthermore, when forms of Nrf3 lacking the entire n, h, and c regions were examined, the mutant Nrf3\(^{2–39}\) or Nrf3\(^{2–75}\) proteins each migrated as a single band of between ~70 and ~80 kDa. Digestion with PNGase F caused a slight increase in the electrophoretic mobility of the single Nrf3\(^{2–39}\) band, whereas the mobility of the single Nrf3\(^{2–75}\) band did not change.

Western blotting of subcellular fractions from COS-1 cells expressing ectopic wild-type mouse Nrf3 showed that the ~90-kDa protein was almost exclusively recovered in the intact ER-rich fraction, although a small amount of the ~80-kDa isofrom was also detected in this subcellular fraction (Fig. 5B). In vitro digestion with Endo H of Nrf3 in the ER resulted in its apparent molecular mass being reduced from ~90 to ~80 kDa (Fig. 5B). In addition, both the ~70- and ~80-kDa polypeptides were also detected in the microsome-rich membrane fraction, but no change in their molecular masses was observed following digestion with Endo H (Fig. 5B).

Taken together, the results in Fig. 5 suggest that the ~90-kDa Nrf3 A isoform represents a glycosylated protein, and its generation in the ER is dependent on the h region of the N-terminal signal sequence. The ~80-kDa Nrf3 B isoform may represent a glycosylated cleaved protein or a deglycosylated non-cleaved protein. The ~70-kDa Nrf3 C isoform may be a non-glycosylated, cleaved polypeptide. Examination of human Nrf3 protein showed that upon its expression in COS-1 cells two major isoforms of ~95 and ~80 kDa were observed, the former of which is a glycoprotein (Fig. 5C).

Nrf3 Can Heterodimerize with sMaf Proteins—To examine whether Nrf3 isoforms form heterodimers with sMaf proteins, we performed co-immunoprecipitation by incubating COS-1 lysates that had been co-transfected with expression constructs for both sMaf and Nrf3 with specific antibodies against these two bZIP proteins. Western blotting using antibodies against a V5 epitope revealed that a major immunoreactive Nrf3 isoform of ~80 kDa was immunoprecipitated by a sMaf antibody (Fig. 5D). Conversely, in a separate experiment, sMafK-V5 protein was precipitated by Nrf3 antibodies. Further, endogenous sMaf proteins from RL34 and JAR cell lysates were precipitated by Nrf3 antibodies (lower two panels); it was noted that the ~90-kDa Nrf3 protein was not observed in these pulldown experiments, possibly because it may be deglycosylated to become the ~80-kDa isoform by a glycosidase present in the immunoprecipitation buffer. Antibodies against the N-terminal region of Nrf3 (N14) precipitated neither the ~70-kDa protein nor an additional ~45-kDa Nrf3 isoform, but they were pulled down by antibodies against sMaf (Fig. 5D, upper two panels). These findings indicate that while cleavage of Nrf3 results in loss of

**FIGURE 3.** **Targeting of mouse Nrf3 to the ER by its N-terminal signal peptide.** A, diagrammatic representation of sequences deleted from the N terminus of mouse Nrf3. Residues placed on a dark background represent the NHB1 sequence. Within the putative signal peptide sequence, its N-terminal extension region, hydrophobic core region, and C-terminal polar region are abbreviated as n, h, and c regions. B, residues 7–27 were wheeled into a hydrophilic α-helix structure. Aromatic and hydrophobic amino acids are placed on a dark background, a basic arginine residue is on a blue background, a polar glutamine residue is on a light red background, two nucleophilic residues serine and threonine are on a green background, and four small glycine residues are shown on a gray background. C, COS-1 cells were transfected with 1.3 μg of an expression construct for V5-tagged wild-type Nrf3, or 1.3 μg of any of five different expression constructs for mutant forms of Nrf3 lacking portions of the N-terminal signal peptide, or 1.3 μg of an empty pCDNA3.1/V5His B vector. These expression vectors were each co-transfected with 0.7 μg of an ER/DSRed expression construct. Approximately 24 h after transfection, the subcellular location of the proteins was examined by immunocytochemistry followed by confocal imaging. Fluorescein isothiocyanate-labeled second antibody was used to locate V5-tagged proteins. Nuclear DNA was stained by 4′,6-diamidino-2-phenylindole (DAPI). The merge signal represents the results obtained when the three images were superimposed. The corresponding quantitative data shown here were calculated by determining the percentage of cells in which the extranuclear stain (i.e. cytoplasmic plus ER) was greater than or equal to the nuclear stain, as opposed to the percentage of cells in which the extranuclear stain was less than the nuclear stain. Bar = 20 μm. D, COS-1 cells were transfected with 1.2 μg of each of the expression constructs for wild-type or mutant Nrf3, or an empty pCDNA3.1/V5His B vector, together with 0.6 μg of P\(_3\)-neo1-ARE-Luc and 0.2 μg of a β-gal reporter plasmid. Approximately 36 h after transfection, the cells were harvested, and luciferase reporter assays were performed as described in the text. Luciferase activity was normalized for transfection efficiency, and data are presented as a fold change (mean ± S.D.) from three independent experiments, each performed in triplicate. Significant differences were determined using the Student’s t test and are shown as p values. E, total cell lysates were resolved using a 7% LDS-NuPAGE Tris acetate system. The V5-tagged proteins were visualized by Western blotting. The positions of the A, B, and C Nrf3 bands are indicated by arrows. The relative abundance of the three bands in both wild-type Nrf3 and the Nrf3\(^{3–24–39}\) mutant is shown.
portions of its NTD, the processed protein is still capable of forming a heterodimer with sMaf.

The NHB1 Sequence of Nrf3 Directs an NTD/Nrf2 Fusion Protein to the ER Where It Is Asn-glycosylated—

To confirm the functional significance of the NHB1-associated signal sequence within Nrf3, we created N66/Nrf2 and NTD/Nrf2 fusion proteins through attachment of residues 1–66 or 1–126 of the mouse bZIP factor to the N terminus of mouse Nrf2. Because the part of human Nrf3 that is equivalent to the c region of mouse Nrf3 contains significant amino acid differences, we also fused the NTD of the human bZIP factor to the N terminus of mouse Nrf2, giving hN126/Nrf2, as an additional control. Following transfection into COS-1 cells, ectopic wild-type Nrf2 was localized primarily in the nucleus (Fig. 6A). By contrast, both N66/Nrf2 and NTD/Nrf2 gave a predominantly ER stain in ~85% of COS-1 cells, whereas the remaining cells expressing these fusion proteins gave primarily a nuclear stain. Similar experiments using the hN126/Nrf2 chimeric fusion protein, showed it to be localized almost exclusively in the ER of all cells examined.

Deletion of the major portion of NHB1 from NTD/Nrf2, giving NTD/H9004 12–30/Nrf2, resulted in the ectopic mouse fusion protein becoming located exclusively in the nucleus of almost all cells. Similarly, a construct in which the NHB2 of mouse Nrf3 was deleted, NTD/H9004 76–100/Nrf2, was also localized preferentially in the nucleus of ~55% cells, although in this case the mutant fusion protein was observed in the ER of the remaining cells. These results suggest that the NHB1 of Nrf3 is required to target an NTD/Nrf2 fusion protein to the ER and that this process may also be modulated by NHB2.

To test whether the targeting of Nrf2 to different subcellular locations through Nrf3 sequences influences its activity, we performed luciferase reporter assays. Fig. 6B shows that transient transfection of COS-1 cells with wild-type mouse Nrf2 activated ARE-driven gene expression ~30-fold, whereas NTD/Nrf2 and N66/Nrf2 activated ARE-driven transcription by only ~18- and ~25-fold, respectively (left panel). By contrast, transfection with either NTD/H126–30/Nrf2 or NTD/H176–100/Nrf2 activated transcription to a similar extent as did wild-type Nrf2 (right panel). These results suggest that the targeting of CNC bZIP factors to the ER via NHB1 and NHB2 results in a partial inhibition of their activity.

Western blotting showed that cell lysates expressing NTD/Nrf2 produced a major ~95-kDa band and a relatively weaker band of ~85-kDa, but upon digestion with PNGase F their elec-
Flanking Residues 67–78 Control Proteolytic Processing of Mouse Nrf3—In the experiments described above, we found that N66/Nrf2 and NTD/Nrf2, both of which were tagged C-terminally with a peptide containing the V5 epitope, were each represented by two polypeptides of molecular masses that were equal to, or greater than, wild-type Nrf2. Because the two electrophoretic bands persisted after PNGase F digestion, it appeared likely that proteolytic cleavage of residues within the NTD of mouse Nrf3 accounted for the appearance of the two protein species because Nrf2 migrated during electrophoresis as a single band. We postulated that proteolysis might occur at a site that lies close to the C terminus of the N66 peptide.

Alanine and glycine are frequently found at the −1 position of an SPase cleavage site, whereas the −3 position is often occupied by alanine, serine, or valine (24). We therefore considered whether the consensus AAX site at Ala39 or Ala55 might be recognized by an SPase (Fig. 7A). To test this hypothesis, we mutated the potential 37AAA39 consensus site into VVV or NNN (to create Nrf3VVV or Nrf3NNN, respectively). We also mutated the other 53ASA55 site into VLV (to yield Nrf3VLV). As shown in Fig. 7B, wild-type Nrf3 migrated during NuPAGE in Tris acetate buffer primarily as the A and B isoforms. However, cell lysates expressing the hN126/Nrf2 chimera containing the V5 epitope, were each represented by two polypeptides of molecular masses that were equal to, or greater than, wild-type Nrf2. Because the two electrophoretic bands persisted after PNGase F digestion, it appeared likely that proteolytic cleavage of residues within the NTD of mouse Nrf3 accounted for the appearance of the two protein species because Nrf2 migrated during electrophoresis as a single band. We postulated that proteolysis might occur at a site that lies close to the C terminus of the N66 peptide.

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NuPAGE in a Tris-Bis buffer system (Fig. 7B, right panel). Further comparisons of the Nrf3VVV and Nrf3VVV/VLV mutants revealed that generation of the ~80-kDa type B isoform from the latter protein was diminished, but not completely abolished. By contrast, the Nrf3NNN mutant protein still produced substantial amounts of the ~80-kDa B protein (Fig. 7B, right panel), suggesting that it contains an additional SPase-recognized cleavage site. Further examination showed that the Nrf3VVV mutant did not influence production of the ~80-kDa B protein (Fig. 7B, right panel), suggesting that the 53ASA55 site is probably not recognized by an SPase.

To abolish its cleavage more completely, we deleted both the 37AAA39 SPase consensus site and residues 67–78, which contains two potential Site-1 protease recognition sites, to create Nrf337–39/67–78. As expected, the cleaved ~80-kDa Nrf3 isoform was not observed upon transfection of COS-1 cells with a construct for the double deletion mutant (Fig. 7C). Formation of this cleavage product was also blocked by a combined mutant Nrf3VVV/VLV/PxxV (in which PxxV indicates a mutant of RXXL70). It was however observed following transfection with constructs for either Nrf337–39/67–78 or Nrf337–70, indicating that there are two Site-1 protease cleavage sites between residues 67 and 78.

As shown in Fig. 7C (left panel), a mouse Nrf3 precursor protein of estimated molecular mass of ~96-kDa (called A') was generated upon ectopic expression of the Nrf3Δ37–39 mutant. This A' form could be converted by PNGase F digestion into a ~90-kDa form (Fig. 7C). The ~96- and ~90-kDa proteins were detected at relatively low levels following expression of the Nrf3Δ37–39 + Δ67–78 mutant, but the larger ~96-kDa protein was not observed from the Nrf3Δ37–39 + Δ67–70 mutant. These results demonstrate that the SPase-recognized 37AAA39 cleavage site is responsible for proteolytic processing of the ~96-kDa mouse Nrf3 precursor into a mature glycoprotein of ~90

**FIGURE 6.** The NHB1 within NTD redirects an Nrf2 fusion protein to the ER where it is Asn-glycosylated. A, the upper panel shows alignment of the N-terminal amino acids (residues 1–126) in mouse Nrf3 (mNrf3) and human Nrf3 (hNrf3), which were linked to the N terminus of mouse Nrf2. Identical residues are placed on a dark background, whereas residues possessing similar physicochemical properties are placed on a gray background. The positions of NHB1 and NHB2 are indicated. The lower panel shows the confocal images obtained from immunocytochemistry of COS-1 cells that were transfected with an expression construct for wild-type Nrf2, N66/Nrf2, NTD/Nrf2, mutants of NTD/Nrf2, hN126/Nrf2, or hNrf3. Each of these constructs was co-transfected with an ER/DsRed expression construct. Approximately 24 h after transfection, the subcellular location of proteins was examined by immunocytochemistry followed by confocal imaging. B, COS-1 cells were cotransfected with each of the indicated expression constructs along with the PsGvGST2-6X-ARE-Luc and pRL-TK reporter plasmids. Luciferase activity was determined 36 h later. Statistical significance was examined using the Student’s t test and is indicated as a p value. C, total cell lysates expressing the indicated protein were either incubated (+), or were not incubated (−), for 1 h with 500 units of PNGase F, before being resolved using 4–12% LDS/NuPAGE with Tris-Bis buffer and immunoblotted with antibodies against the V5 epitope.
Nrf3 Is a Membrane-bound Glycoprotein

**FIGURE 7.** The signal c region and its flanking residues between the NHB1 and NHB2 control proteolytic processing of mouse Nrf3. A, a sequence alignment of residues 1–101 from mouse Nrf3 with residues 1–115 of human Nrf3 and residues 1–107 of mouse Nrf3 is presented. The classic tripartite signal peptide comprises n, h, and c regions, and the sequences encompassed by these regions are indicated. The c region in mouse Nrf3 is overlined, whereas the c region in human Nrf3 is underlined. Within the c region, putative SPase-recognition sites are overlined and indicated by vertical solid-headed arrows, one of which consists of tri-alanine between position 37 and 39 that is absent from the human Nrf3. Additional cleavage sites for Site-1 protease were predicted in close proximity to the NHB2 sequence. These putative cleavage sites were deleted or mutated. B, COS-1 cells were cotransfected with each of expression constructs for Nrf3 and the indicated mutants, along with a V5 antibody. Western blotting of cell lysates expressing the murine Nrf3, mouse Nrf3, and its transactivation activity, we created a construct encoding mouse Nrf3A76–100. Confocal microscopy showed that, although this mutant protein localized to the ER, it gave a stronger nuclear stain than the wild-type bZIP factor (Fig. 9B). Nrf3A76–100, in which both the NHB1 signal sequence and the C-terminal region between the NHB1 and NHB2 were omitted, was localized predominantly in the nucleus, presumably because it lacks the ER signal peptide.

Western blotting of cell lysates expressing the murine Nrf3A76–100 mutant revealed that it, like wild-type Nrf3, also yielded three ~70-kDa, ~80-kDa, and ~88-kDa isoforms (Fig. 9C). Comparison with wild-type Nrf3 indicated that the level of the ~80-kDa Nrf3a B isofom was significantly reduced by deletion of the NHB2 sequence (Fig. 8C). The NTDVVV/Nrf2 fusion protein was targeted to the ER where it was glycosylated (Figs. 8, A and B). Targeting of the fusion protein to the ER was increased in cells expressing either the NTDVVV/Nrf2 or NTDVVV/VLV/Nrf2 mutants but no change was observed in the case of the NTDVVV/Nrf2 mutant (Fig. 8A). Deglycosylation reactions revealed that NTDVVV/Nrf2, like NTD/VNF2, was Asn-glycosylated, whereas neither NTDVVV/Nrf2 nor NTDVVV/VLV/Nrf2 were glycated (Fig. 8B). To help explain these findings, we proposed that NTD/VNF2 and NTDVVV/VNF2 adopt different conformations within the ER membrane (Fig. 8C). The NTDVVV/Nrf2 fusion protein was less active than NTD/VNF2 at transactivating Psiv40GSTA2–6xARE-Luc (Fig. 8D), suggesting that NTDVVV/Nrf2 may be improperly folded within the ER membrane.

**Nrf3 Activity Is Modulated by Its NHB2 Sequence**—As described above, attachment of the NTD of mouse Nrf3 to the N terminus of Nrf2 diminished its activity, whereas negative regulation by the NTD was completely abolished by deletion of NHB2 in the NTD76–100/Nrf2 mutant (Fig. 6B). These results suggest that Nrf3 may also be controlled by NHB2. Residues within and around NHB2 are predicted to adopt basic hydrophobic α-helix and β-sheet secondary structures, which could either lie in the plane of the membrane or span the membrane (Fig. 9A). To test whether the amphipathic NHB2 sequence controls subcellular distribution of Nrf3 and its transactivation activity, we created a construct encoding mouse Nrf3A76–100. Confocal microscopy showed that, although this mutant protein localized to the ER, it gave a stronger nuclear stain than the wild-type bZIP factor (Fig. 9B). Nrf3A76–100, in which both the NHB1 signal sequence and the C-terminal region between the NHB1 and NHB2 were omitted, was localized predominantly in the nucleus, presumably because it lacks the ER signal peptide.
ER membrane and allow cleavage to occur. In addition to these three forms of Nrf3\(^{76–100}\), two faster migrating bands were resolved that were estimated to have molecular masses of \(-54\) and \(-50\) kDa, called Nrf3 bands D and E, respectively (Fig. 9C). The D and E bands were identical with those of Nrf3\(^{1–172}\), indicating that they may have arisen through either translation from two internal start codons at Met\(^{173}\) and Met\(^{211}\) or proteolytic cleavage occurring within a central region.

We examined the effect that deleting NHB2 had on the activity of mouse Nrf3. Transactivation of the \(P_{Tr,\text{hqp}1}\)-ARE-Luc reporter gene was significantly reduced from \(-1.7\)-fold by wild-type Nrf3 to \(-1.2\)-fold activation by the Nrf3\(^{76–100}\) mutant (Fig. 9D). This loss of activity may be due to a failure to synthesize the \(-80\)-kDa Nrf3 isoform and a relative increase in the amount of the \(-88\)-kDa glycoprotein. Both the Nrf3\(^{2–75}\) and Nrf3\(^{2–100}\) mutants increased ARE-driven gene activity to \(-2.5\)- and \(-3.0\)-fold, respectively (Fig. 9D); this was accompanied by the appearance of ectopic polypeptides of between \(-70\) and \(-80\) kDa (Fig. 9C). Taken together, these results suggest that the \(-70\)- and \(-80\)-kDa Nrf3 polypeptides are both active, but the former is unstable.

Deletion of the NHB2 Sequence Results in the Retention of Nrf3 within the ER Lumen—To address the question of why mouse Nrf3\(^{76–100}\) is less active than wild-type Nrf3 (Fig. 9D), whereas the NTD\(^{76–100}\)/Nrf2 fusion protein has the same activity as wild-type Nrf2 (Fig. 6B, right panel), we examined whether Nrf3\(^{376–100}\) and NTD\(^{376–100}/Nrf2\) differ in their subcellular location and/or membrane topology. Fig. 10A shows that a small fraction of the \(-88\)-kDa Nrf3 in the context of the former protein, the wild-type c region is predicted to fold as a \(\beta\)-sheet, whereas in the latter protein the mutant c region could fold as a hydrophobic \(\alpha\)-helix. The scissors indicate a potential cleavage site. D, COS-1 cells were cotransfected with each of the indicated expression constructs along with the \(P_{\text{SvAGST}2\times6\times\text{ARE-Luc}}\) and pRL-TK plasmids. Approximately 36 h later, luciferase activity was measured and normalized for transfection efficiency. The data are presented as a \(-fold\) change (mean \pm S.D.) from three independent experiments, each performed in triplicate. Significant differences were determined using the Student’s \(t\) test and are shown as \(p\) values.
Nrf3 Is a Membrane-bound Glycoprotein

By contrast with the Nrf3^{12–30} mutant, an NTD^{12–30} mutant of Nrf2 fusion protein of ~92 kDa was predominantly recovered in both the nuclear and ER fractions (Fig. 10A, right panel). Although proteinase reactions revealed that the ~92-kDa protein was located in the lumen of the ER (Fig. 10C), NTD^{12–30} mutant of Nrf2 also yielded an additional cleaved polypeptide of ~85 kDa, with a similar abundance to wild-type Nrf2 (Fig. 10A, bottom panel). Subcellular fractionation showed that the ~85-kDa fusion protein was also localized in the nucleus (Fig. 10A, right panel).

Nrf3 Is a Membrane-associated Protein That Locates to the Nuclear Envelope—To explain why Nrf3 is less active than Nrf1, we examined whether wild-type Nrf3 is a luminal ER protein or a membrane-bound protein in the ER by performing protease protection reactions. Fig. 10D shows that following digestion of the ER fraction with PK, most of the ~90-kDa Nrf3 A isoform disappeared, to be replaced by a relatively weaker multiple-polypeptide ladder. This digested protein ladder comprised two major bands of ~70 and ~85 kDa and four additional bands of between ~12 and ~32 kDa. However, following digestion of the microsome-rich membrane fraction that primarily contained the ~80-kDa Nrf3 form, no polypeptide bands were detected indicating that it was not protected from proteolysis (Fig. 10D). In the same experiment, the ER-membrane protein Sec61α was completely digested by PK, whereas no change in the abundance of the luminal protein calreticulin was detected (Fig. 10D, lower panels). These results suggest that Nrf3 is translocated into the ER lumen and then inserted into the membrane through its hydrophobic and/or amphipathic regions. Once this bZIP protein is integrated into the membrane, we envisage it would either be released from the ER or it would be transported into the nuclear envelope. Consistent with the second of these proposals, both the ~80- and ~90-kDa Nrf3 proteins were recovered primarily in the nuclear envelope membrane fraction (Fig. 10E). By contrast, the salt-extracted soluble nuclear fraction was found to contain relatively small amounts of the ~70- and ~80-kDa Nrf3 forms, and none of the ~90-kDa protein.

The NHB1 Sequence Is Required to Enable Nrf3 Activity to Be Increased by ER Stressors—The biological significance of Nrf3 being targeted to the ER is unclear. We therefore tested whether it responds to ER stress. Fig. 11A shows that wild-type mouse Nrf3 was significantly activated by the ER stressors tunicamycin (TU) and brefeldin A (BFA), but not by thapsigargin (TG). Further, the increase in transactivation activity of Nrf3 produced by TU and BFA was prevented by ALLN (N-acetyl-L-leucyl-L-leucyl-L-norleucinal) (Fig. 11B), an inhibitor of proteolysis catalyzed by proteasomes (25). By contrast, ALLN markedly increased transactivation activity of the Nrf3^{12–30} mutant lacking NBH1, but this factor was not stimulated by TU, TG, and BFA (Fig. 11A). Moreover, both Nrf3 and Nrf3^{12–30} were significantly activated by co-
Western blotting showed that the ~90-kDa Nrf3 protein was not synthesized following treatment with TU or BFA, but in both cases a relatively weaker band of ~85-kDa was detected (Fig. 11, C and D, and see supplemental Fig. S3). This suggests that the ~90-kDa Nrf3 protein is produced by Asn glycosylation in both the ER and Golgi apparatus. In addition, TU treatment also increased expression of the ~80-kDa Nrf3 isoform. By contrast, neither the ~80-kDa nor the ~90-kDa Nrf3 proteins were markedly affected by treatment with TG or tBHQ (Fig. 11, C and D). Further, treatment with ALLN increased the abundance of both the ~70-kDa and the ~80-kDa polypeptides in COS-1 cells expressing Nrf3\textsuperscript{A12–30} protein (Fig. 11E). Taken together, these findings indicate that both the ~70-kDa and ~80-kDa Nrf3 polypeptides are active.

**Nrf3 Is a Membrane-bound Glycoprotein**

In the present report, we have demonstrated that Nrf3 is targeted to the ER through an NHB1 signal sequence, and that this motif is required to enable the activity of Nrf3 to be increased by the ER stressors TU and BFA.

**DISCUSSION**

In the present report, we have demonstrated that Nrf3 is targeted to the ER through an NHB1 signal sequence, and that this motif is required to enable the activity of Nrf3 to be increased by the ER stressors TU and BFA.
Nrf3 Is a Transcriptional Activator That Is Negatively Regulated by Its NTD

Our data show that mouse Nrf3 can transactivate an ARE-driven reporter gene in COS-1 cells, although it is less active than either Nrf1 or Nrf2. This is consistent with previous results obtained with a reporter construct based around ARE-containing sequences from the chicken β-globin gene (1). We have also presented evidence that the activity of Nrf3 is modestly increased by tBHQ and sulforaphane. Although Nrf3 is significantly less active than Nrf2, we found it does not blunt induction of ARE-driven gene expression mediated by the latter factor. These results appear to contradict the report of Sankaranarayanan and Jaiswal (23) that mouse Nrf3 negatively regulates expression of a reporter gene driven by an ARE from human NQO1. It should however be noted that the human NQO1-ARE contains an embedded AP-1 sequence (5’TGACTACGCT3’, the AP-1 motif is underlined), whereas our PtγhNqo1-ARE-Luc does not. Thus, the apparent discrepancy is likely to be due to the absence of an AP-1 motif within the ARE reporter construct we employed. We propose that, in the case of an ARE containing an AP-1 binding site, Nrf3 may outcompete Nrf2 through it being recruited to the AP-1 sequence as a heterodimer with c-Fos or Fra1, thereby causing negative regulation of the reporter gene. This does not, however, occur in the case of an ARE that lacks an AP-1 binding site.

Others have shown previously that human Nrf3 contains a transcription activation domain between residues 171 and 399 but that its activity is inhibited by the first 88 N-terminal residues (2). We have similarly found that attachment of residues 1–126 from human Nrf3 to the N terminus of Nrf2 reduced the activity of the fusion protein to only ~15% of that of wild-type Nrf2, indicating that NTD serves as a negative regulation domain. By contrast with the NTD of human Nrf3, the NTD of mouse Nrf3 was less effective at repressing Nrf2 when an equivalent fusion protein was created. The greater ability of the NTD from human Nrf3 to negatively regulate Nrf2, when compared with its murine counterpart, could be due to the absence of a proteolytic cleavage site in the hN126 sequence of the former bZIP protein.

Nrf3 Is a Membrane-bound Glycoprotein

Our present data have shown that Nrf3 is a membrane-bound glycoprotein in COS-1 cells, although it is less active than either Nrf1 or Nrf2. This is consistent with previous results obtained with a reporter construct based around ARE-containing sequences from the chicken β-globin gene (1). We have also presented evidence that the activity of Nrf3 is modestly increased by tBHQ and sulforaphane. Although Nrf3 is significantly less active than Nrf2, we found it does not blunt induction of ARE-driven gene expression mediated by the latter factor. These results appear to contradict the report of Sankaranarayanan and Jaiswal (23) that mouse Nrf3 in the hN126 sequence of the former bZIP protein.

Nrf3 Is Targeted to the ER through an NHB1 Signal Peptide—Our present data have shown that Nrf3, like Nrf1, is targeted to the ER through its NHB1-associated signal peptide (aa 1–39). Bioinformatics revealed that the signal peptide sequence in mouse Nrf3 comprises n, h, and c regions. The n region (aa 1–11) in Nrf3 may regulate its topology within the membrane. According to the positive-inside rule (26), the n region that adjoins the membrane-spanning α-helix should reside on the cytoplasmic side of the ER. Deletion of this region in Nrf3Δ12–30 caused an increase in the abundance of the ~90-kDa Nrf3 glycoprotein in the ER. This
suggests that the n region may serve as a signal for membrane trafficking of Nrf3 from the ER to the nucleus.

A putative transmembrane α-helix is formed by the h region (aa 12–23) and a few of its flanking residues. The h region probably transverses the core hydrophobic lipid tails within the membrane bilayer. We found that the Nrf3312–23 mutant, which lacks the h region, was neither localized in the ER nor did it generate the glycated ~90-kDa form. This indicates that the h region is required to target Nrf3 to the ER, and that this is necessary to enable its Asn glycosylation. However, unlike Nrf1, deletion of the h region in mouse Nrf3 did not substantially increase its ability to transactivate ARE-driven gene expression. This could be due to the fact that there exists an additional negative regulation domain (i.e. the PEST sequence) within Nrf3 that is absent from Nrf1.

The c region of a signal peptide sequence normally comprises 3–9 small polar amino acids (27). In the case of Nrf1, the c region of the signal peptide is not cleaved and thus represents an anchor sequence (11). By contrast, the signal peptide c region of mouse Nrf3 is cleaved at several potential SPase recognition sites (e.g. AXA). We have provided evidence that the NTD region of mouse Nrf3 is subject to proteolysis, but that no cleavage occurs in the NTD of human Nrf3. The37AAA39 site in mouse Nrf3 probably represents the cleavage site because it is absent from human Nrf3. Point mutation of the putative37AAA39 cleavage site in mouse Nrf3 resulted in a significant decrease in the amount of the ~80-kDa form produced but a concomitant increase in the level of the ~90-kDa isoform was not observed. However, deletion of the37AAA39 site resulted in the synthesis of a novel ~96-kDa precursor glycoprotein from the Nrf337–39 mutant. Further deletion of residues 67–78, located in the N-terminal border of NHB2, completely abolished production of the ~80-kDa Nrf3 protein. These findings indicate that in mouse Nrf3 the region between the NHB1 and NHB2 controls its cleavage by both an SPase and probably a Site-1 protease.

In addition, both Nrf332–39 and Nrf332–75, lacking the signal peptide and/or its flanking residues, gave rise to a single polypeptide of ~80 kDa, and did not yield the cleaved ~70-kDa polypeptide. These results suggest that in mouse Nrf3 residues 40–75 between the c region and NHB2 control the proteolytic cleavage to produce the ~70-kDa isoform, although this processing may occur within a region beyond the NTD. Finally, Fig.
study we have found that deletion of NHB2 in mouse Nrf3 (i.e. Nrf3<sup>Δ76−100</sup>) decreased its activity significantly. This was accompanied by a relative increase in the abundance of a ~88-kDa glycoprotein that was retained in the ER lumen, along with a decrease in the relative amount of the cleaved ~80-kDa isoform. These results suggest that the NHB2 sequence may be involved in the post-translational processing and sorting of Nrf3 protein within the ER, but further work is required to determine the molecular basis for this observation.

**Nrf3 Is Activated by Deglycosylation**—The principal ER-associated form of mouse Nrf3 is the ~90-kDa glycoprotein. We found that Asn glycosylation of mouse Nrf3 to yield the ~90-kDa isoform was largely prevented by TU, which selectively blocks Asn-linked glycosylation by inhibiting the core oligosaccharide synthesis (28), and BFA, which inhibits GDP/GTP-exchange factors to block membrane trafficking from the ER to Golgi apparatus (29). Further, we found that TU and BFA increased significantly the transactivation activity of Nrf3. However, glycosylation of Nrf3 and its activity were unaffected by TG, which inhibits the Ca<sup>2+</sup> transport ATPase to deplete the ER of calcium (30). The fact that TG did not increase Nrf3 activity suggests that the bZIP factor is not activated by ER stress per se, but rather its activity is influenced by glycosylation. These findings indicate that glycosylation of Nrf3 occurs in the ER and Golgi apparatus, and this modification negatively regulates its activity.

In addition, we discovered that the increase in transactivation activity of Nrf3 affected by TU or BFA was inhibited by ALLN. This inhibitor of proteasome enzyme activity (25) has been shown to block cleavage activity of the Site-1 protease (31). These findings are consistent with the hypothesis that mouse Nrf3 is processed from an inactive to an active form by a Site-1 protease.

**Topology of Nrf3 within the Membrane**—Membrane proteinase K protection assays have shown that mouse Nrf3 is inserted into membranes through several hydrophobic and/or

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**FIGURE 13.** A proposed model to describe the processing of mouse Nrf3. Mouse Nrf3 is synthesized as a precursor form that is targeted to the ER through the NHB1-associated signal peptide. If the signal c region is not cleaved by an SPase when its recognition site is deleted, the precursor protein can be translocated into the ER lumen where it is Asn-glycosylated to become the ~96-kDa form. This precursor form is not readily detected in cells expressing wild-type mouse Nrf3. Instead, the cells express three major isoforms: a mature glycoprotein of ~90 kDa with a low activity, along with two smaller active glycosylated and cleaved as well as non-glycosylated and cleaved forms of ~80 kDa and ~70 kDa, respectively. Thus during translocation the signal c region of Nrf3 is first cleaved, and its remaining portion is Asn-glycosylated in the ER lumen to become the ~90-kDa isoform. The mature form is integrated into the membrane through several amphipathic regions (e.g. TM1, NHB2, TMi, and TMc) and sorted into the nuclear envelope. It is proposed that the ~90-kDa glycoprotein is processed to become a major active ~80-kDa isoform either through deglycosylation during retrotranslocation from the lumen to the nucleoplasm, or by a proteolytic cleavage occurring between residues 67 and 78 that contains two putative Site-1 protease (S1P) recognition sites. The c region and its flanking residues in the proximity of NHB2 also control production of the unstable ~70-kDa isoform, but this proteolytic processing is likely to occur within a domain other than the NTD. In addition, following cleavage of the signal peptide c region, a fraction of Nrf3 protein might also be released from the ER and processed in the extra-ER compartment to become an active form of ~70 or ~80 kDa.

13 represents a proposed model to describe the processing of mouse Nrf3 within the ER.

The NHB2 Sequence in Nrf3 Is Required for Its Activity and Protein Sorting within the ER—Hitherto, the functional significance of the NHB2 peptide was not known. During the present

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amphipathic regions. The first transmembrane region (called TM1) is likely folded by residues 7–25 and is orientated in an N_cyt/C_lum fashion (Fig. 8C). However, if the TM1-adjoining signal peptide is cleaved by an SPase, the remaining portion of Nrf3 might either be released from the membrane to the cytoplasm or may translocate into the lumen of the ER.

Our observation that membrane proteinase protection yielded two bands of ~70-kDa and ~85-kDa suggests that an additional region within the NTD of Nrf3 may be exposed to the cytoplasmic side of the ER. The region in question may comprise residues within and around NHB2. This suggestion is supported by our finding that, upon deletion of the NHB2 sequence, a ~88-kDa protein is recovered from the proteinase protection assay.

During the proteinase protection experiments we observed a C-terminally V5-tagged Nrf3 peptide of ~12 kDa. The recovery of this peptide suggests that the bZIP protein contains a C-terminal transmembrane (called TMc) region with an N_cyt/C_lum orientation (supplemental Fig. S4). The TMc region may be formed by residues 361–379 insert into the membrane lipids, they may fold into a transmembrane amphipathic α-helix (called TMi), which could either lie on the plane of the membrane or span the lipid bilayer (Fig. 13).

Concluding Comments—In this study we have shown that both mouse and human Nrf3 are targeted to the ER through the NHB1 sequence within their respective N-terminal domains. Mouse Nrf3 is subject to N-terminal proteolytic cleavage, whereas the human protein is not. Both proteins are glycosylated in the ER and are also located in the nuclear envelope. Interestingly, although Nrf1 is also targeted to the ER and located in the nuclear envelope (32), our data suggest that Nrf3 is integrated into membranes in a more complete fashion. Proteinase protection assays have shown that the topology of Nrf3 within the membrane is modulated by NHB2 within the NTD.

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