Antioxidant-induced Phosphorylation of Tyrosine 486 Leads to Rapid Nuclear Export of Bach1 That Allows Nrf2 to Bind to the Antioxidant Response Element and Activate Defensive Gene Expression*

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Antioxidants cause stabilization and nuclear translocation of NF-E2-related factor 2 (Nrf2), where it binds to the antioxidant response element (ARE) and induces up-regulation of defensive genes that protect cells against oxidative and electrophilic stress. Bach1, the negative regulator of Nrf2, competes with Nrf2 for binding to the ARE in the human NQO1 promoter. In this study, we demonstrate that Bach1 exits the nucleus within 1–2 h upon antioxidant treatment. Genistein, an inhibitor of tyrosine kinases, blocked nuclear export of Bach1. Site-directed mutagenesis and immunoprecipitation assays identified tyrosine 486 that was phosphorylated in response to the antioxidant and was essential for nuclear export of Bach1. Chromatin immunoprecipitation assays revealed a competitive interplay between Bach1 and Nrf2 at 1–2 h for binding to the human NQO1 ARE. Luciferase and real time PCR assays showed a significant decrease in antioxidant induction of reporter activity and mRNA levels in cells transfected with mutant Bach1 compared with wild type. This decrease was due to the absence of nuclear export of the mutant protein. Bach1 levels inside the nucleus returned to normal at 4 h after antioxidant treatment in the absence but not in the presence of protein synthesis inhibitor cycloheximide. In addition, antioxidant treatment increased the transcription of Bach1 as shown by pulse chase and real time PCR experiments. Taken together, these results indicate that increased synthesis of Bach1 restored its nuclear levels to normal at 4 h. In conclusion, antioxidant-induced tyrosine 486 phosphorylation leads to nuclear exit of Bach1, thus allowing Nrf2 access to the ARE.

Bach1 (BTB and CNC homology 1, basic leucine zipper transcription factor 1) is a transcription repressor that is conserved and ubiquitously expressed in tissues (1–4). In the absence of cellular stress, Bach1 heterodimers with small Maf proteins that bind to the antioxidant response element (ARE)2 and repress defensive gene expression (1, 2, 5). Bach1 may function as a sensor of oxidative stress that may mediate gene induction upon its inactivation (6–8). Bach1 allows defensive gene induction, such as heme oxygenase 1, upon its release from the ARE and subsequent replacement by NF-E2-related factor 2 (Nrf2). The cellular stress causes Nrf2 to be released from its cytosolic inhibitor, INrf2, and translocation into the nucleus leading to the activation of ARE-mediated gene expression (9, 10). Nrf2 cannot bind to the ARE as a monomer, but requires dimerization with one of the small Maf or Jun proteins to bring about transactivation (11, 12). Nrf2 binds to the ARE and regulates expression and coordinated induction of a myriad of genes encoding chemopreventive proteins, including detoxifying enzymes NAD(P)H:quinone oxidoreductases (NQO1 and NQO2), glutathione S-transferase Ya subunit, γ-glutamylcysteine synthetase, and heme oxygenase 1 (13). There appears to be a competitive interplay between the Bach1-containing repressor dimers and Nrf2-containing activator dimers (2, 14).

Nuclear export is an important event in the regulation and induction of many different proteins (15). To protect the cell from oxidative stress and other cellular insults, cells may react by exporting specific proteins from the nucleus. Bach1, a transcriptional repressor of the heme oxygenase gene, localizes in the nucleus under normal conditions. The heavy metal cadmium and heme induced chromosomal region maintenance 1 (Crm1)-dependent nuclear export of Bach1 (16, 17). The nuclear export of Bach1 is vital to cellular survival when exposed to heavy metals induced-stress.

Bach1 also contains two different nuclear export signals (NESs), each mediating specific responses to cadmium and heme (16, 17). However, the antioxidant-induced nuclear export of Bach1 is unknown. In addition, the molecular mechanism that controls nuclear export of Bach1 in response to heavy metals or antioxidant remains unclear.

In this report, we demonstrate that tert-butyldihydroquinone (t-BHQ), an antioxidant, induced rapid export of Bach1 from the nucleus to allow free access of the ARE to incoming Nrf2, which led to activation of antioxidant gene expression. We also demonstrate that antioxidant-induced phosphorylation of tyrosine 486 is essential for the nuclear export of Bach1. Further

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2 The abbreviations used are: ARE, antioxidant response element; ChIP, chromatin immunoprecipitation; DMSO, dimethyl sulfoxide; LDH, lactate dehydrogenase; LMB, leptomycin B; NES, nuclear export signal; NQO1, NAD(P)H:quinone oxidoreductase 1; Nrf2, NF-E2-related factor 2; RIPA, radiimmune precipitation assay; t-BHQ, tert-butyldihydroquinone; WT, wild type; CLS, cytoplasm localization signal; Crm1, chromosomal region maintenance 1.
experiments showed that once the Nrf2 task was completed, newly synthesized Bach1 entered the nucleus, leading to the suppression of ARE-mediated gene expression down to normal levels.

MATERIALS AND METHODS

Cell Cultures—Human hepatoblastoma (HepG2) and mouse hepatoma (Hepa-1) cells were obtained from the American Type Culture Collection. HepG2 cells were grown in minimum essential α medium and Hepa-1 in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin (40 units/ml), and streptomycin (40 μg/ml). The cells were grown in a monolayer in an incubator at 37 °C in 95% air and 5% CO2.

Plasmid Construction—The mouse Bach1 was amplified from a mouse liver marathon ready cDNA library using Bach1-specific primers (forward, 5′-ACCATGTCTGAGTGAAGTGCAGTGCCG; and reverse, 5′-CTGGTCAGTACGTCACTTTGTCAGAC). The cDNA was subcloned into pcDNA 3.1/V5-His Topo vector by TA cloning (Invitrogen). The cDNA encodes the V5-tagged Bach1 wild type (WT) protein designated Bach1-WT. Two tyrosine residues (Tyr431 and Tyr486) present in Bach1-WT were mutated to alanine by using a site-directed mutagenesis kit (Invitrogen). Mutant Y431A was generated by PCR using mutant forward primer 5′-GACGGCCCAAGAAAGGGCGCATCGCAAAGGCGAG-3′ and reverse primer 5′-GACGCTGCTGAGCAGTGCT-3′. Mutant Y486A was generated by PCR using mutant forward primer 5′-GAA-CTGGGTGAACATAGGCACTCAGAGATCCT-3′ and reverse primer 5′-ATGGGATCCCAATTTCCAAGTTGCTTGA-3′. These plasmids will be designated Bach1-Y431A-V5 and Bach1-Y486A-V5. Two tyrosine residues (Tyr431 and Tyr486) present in Bach1-WT were mutated to alanine by using a site-directed mutagenesis kit (Invitrogen). Mutant Y431A was generated by PCR using mutant forward primer 5′-GACGGCCCAAGAAAGGGCGCATCGCAAAGGCGAG-3′ and reverse primer 5′-GACGCTGCTGAGCAGTGCT-3′. Mutant Y486A was generated by PCR using mutant forward primer 5′-GAA-CTGGGTGAACATAGGCACTCAGAGATCCT-3′ and reverse primer 5′-ATGGGATCCCAATTTCCAAGTTGCTTGA-3′. These plasmids will be designated Bach1-Y431A-V5 and Bach1-Y486A-V5. The cytoplasmic localization sequence was TGA. These plasmids will be designated Bach1-Y431A-V5 and Bach1-Y486A-V5.

In Vitro Transcription/Translation—In vitro transcription/translation of the plasmids encoding tyrosine mutations was performed using the TnT-coupled rabbit reticulocyte lysate system (Promega). 0.2 μg of plasmid DNA was incubated with 25 μl of TnT-coupled rabbit reticulocyte lysate supplied with 40 μM 1-Methionine at 30 °C for 90 min. The plasmid encoding luciferase provided in the kit was used as a control for the transcription/translation reaction. After the coupled transcription/translation, the proteins were checked for their correct size on 10% SDS-PAGE followed by immuno blotting. All of the in vitro transcribed/translated proteins gave the expected size bands.

Subcellular Fractionation and Western Blotting—Hepa-1 and HepG2 cells, seeded in 100-mm plates and treated/transformed as displayed in the figures, were washed twice with ice-cold phosphate-buffered saline, trypsinized, and centrifuged at 1500 rpm for 5 min. For making whole cell lysates, the cells were lysed in RIPA buffer (50 mM Tris, pH 8.0. 150 mM NaCl, 0.2 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholic acid, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate supplemented with protease inhibitor mixture (Roche Applied Science)). Cytoplasmic and nuclear biochemical fractionation of the cells was done using the Nuclear Extract kit (Active Motif, Carlsbad, CA) following the manufacturer’s protocol. The protein concentration was determined using protein assay reagent (Bio-Rad). 50–60 μg of protein were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk and incubated with anti-Bach1 C-20 (1:500) purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-V5 horseradish peroxidase (1:5000) purchased from Invitrogen, anti-phosphotyrosine (1:1000), and anti-actin (1:5000) purchased from Sigma. The membranes were washed three times with TBST (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20), and immunoreactive bands were visualized using the ECL system (Amersham Biosciences). The intensity of the protein bands was quantitated by using QuantityOne 4.6.3 Image software (Chemi Doc XRS; Bio-Rad) and normalized against proper loading controls. To confirm the purity of nuclear-cytoplasmic fractionation, the membranes were reprobed with cytoplasm-specific anti-lactate dehydrogenase (LDH) (Chemicon) and nucleus-specific anti-lamin B antibodies (Santa Cruz Biotechnology). In related experiments, the cells were treated with 100 μM t-BHQ (Sigma), 20 ng/ml leptomycin B (LMB), cycloheximide, 100 μM MG132, 50 μM genistein (Calbiochem), 10 μg/ml cadmium chloride (Sigma), or DMSO as a vehicle for different time intervals.

Immunoprecipitation—For immunoprecipitation, 1 mg of whole cell lysates was equilibrated in RIPA buffer, precleared by protein AG-agarose (Santa Cruz Biotechnology), and then extracts were incubated with respective antibodies (1 μg) at 4 °C overnight. Immune complexes were collected by addition of protein AG-agarose for 2 h and were washed three times with RIPA buffer containing 0.25% Nonidet P-40. Proteins were resolved by 10% reducing SDS-PAGE and transferred to nitrocellulose membrane. The membranes were blocked with 3% bovine serum albumin and incubated with their respective primary and secondary antibodies. Immunoreactive bands were visualized using the ECL system.

Transient Transfection and Luciferase Assay—HepG2 cells were seeded in 100-mm plates at a density of 1 × 106 cells/plate 24 h prior to transfection. The cells were transfected with 1 μg of the indicated plasmids using Effectene transfection reagent (Qiagen) according to the manufacturer’s protocol. After 24 h of transfection, cells were treated, harvested, and cell-specific protein regulation was examined by Western blotting. For the luciferase reporter assay, Hepa-1 cells were seeded in 12-well plates at a density of 8 × 104. The cells were then co-transfected with 0.1 μg of human NQO1 pGL2-hARE and varying concentrations of either Bach1-WT or Bach1-Y486A plasmids and 10× less quantity of firefly Renilla luciferase encoded by plasmid pRL-TK. Renilla luciferase was used as an internal control in each transfection. After 24 h of transfection, the cells were washed with 1× phosphate-buffered saline and lysed in 1× passive lysis buffer from the Dual Luciferase® reporter assay system kit (Promega, Madison, WI). The luciferase activity was measured using a Packard LumiCount Plate reader according to the manufacturer’s instructions.
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**RESULTS**

**Antioxidant Treatment Induces Nuclear Export of Bach1**—HepG2 cells were treated with either DMSO (vehicle control) or the antioxidant t-BHQ at different time points. The subcellular localization of endogenous Bach1 was followed by immunoblotting (Fig. 1A; see also quantitative densitometry graphs below the figures). Antioxidant treatment of HepG2 cells led to thionine for 4 h and then treated with t-BHQ at different time points. The cells were rinsed once with phosphate-buffered saline and lysed in RIPA on ice for 30 min. Insoluble cellular debris was cleared by centrifugation at 10,000 rpm for 5 min at 4 °C. After centrifugation, the supernatants were used for immunoprecipitation with anti-V5 antibody as described earlier. Immunoprecipitates were boiled in 1× SDS buffer and resolved on 10% SDS gel. The gel was treated with Amplify Fluorographic Reagent (Amersham Biosciences) to enhance the 35S signal, dried, and autoradiographed.

**Chromatin Immunoprecipitation (ChIP) Assay**—A ChIP assay was performed using a kit from Active Motif as per protocol. Briefly, 70% confluent HepG2 cells were treated with DMSO or 100 μM t-BHQ for 2 or 4 h and then fixed in 1% formaldehyde for 15 min. Cells were lysed and nuclei pelleted by centrifugation. Nuclei were resuspended and sheared using a sonicator (Misonix, Farmingdale, NY) with five pulses of 20 s at 25% of maximum output. Sheared chromatin was immunoprecipitated with 2 μg of anti-Nrf2, anti-Bach1, or control IgG antibody. The cross-links reversed overnight at 65 °C and deproteinized with 20 μg/ml proteinase K. PCR was performed with a primer pair spanning the human NQO1 gene ARE. The primers as follows: forward, 5'-CAGTGCGATGCCACCCAGGGA-3', and reverse, 5'-GCATGCCCTTTTAAGCCTGGCA-3'. The PCR condition used for ChIP assay was 37 cycles of a denaturing step at 94 °C for 30 s, an annealing step at 65 °C for 30 s, and an extension step at 72 °C for 30 s. PCR products (227 bp with NQO1 ARE primers) were separated on 2% agarose gel containing ethidium bromide and imaged using a ChemiDoc XRS.

**Statistical Analysis**—The data from luciferase assays, real time PCR, and immunoblotting band intensities were analyzed using a two-tailed Student’s t test. Data are expressed as mean ± S.D. of three independent experiments. Significance values are represented as *, p < 0.05; **, p < 0.005; and ***, p < 0.0001 and are shown in the figures.

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**Real Time PCR**—Hepa-1 cells were seeded in 100-mm plates. 24 h later, cells were treated with either t-BHQ or 2 μg/ml actinomycin D (Sigma) and harvested. RNA was extracted using RNeasy mini kit (Qiagen). RNA was converted to cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer’s instructions. cDNA was used with Taqman Master Mix (Applied Biosystems) and Bach1 Primer and Probe amplicon Mm00476079_m1 or GusB amplicon Mm00446953_m1 as a control (Applied Biosystems). Total mix was run on 7500 Real Time System (Applied Biosystems) using relative quantitation according to the manufacturer’s instructions.

**Pulse Chase Assay**—Hepa-1 cells were plated in 6-well plates and allowed to adhere. Cells were then transfected with 500 ng of Bach1-V5/well for 24 h. Cells were incubated with methionine-deficient Dulbecco’s modified Eagle’s medium (Sigma) for 30 min. The cells were then labeled with methionine-deficient Dulbecco’s modified Eagle’s medium containing ~200 μCi of [S35]methionine mixture (Expre35S35S; PerkinElmer Life Sciences), for 1 h at 37 °C (pulse). After rinsing with normal culture medium (Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum), the cells were chased by normal culture medium supplemented with 100 μg/ml t-methionine for 4 h and then treated with t-BHQ at different time points. The cells were rinsed once with phosphate-buffered saline and lysed in RIPA on ice for 30 min. Insoluble cellular debris was cleared by centrifugation at 10,000 rpm for 5 min at 4 °C. After centrifugation, the supernatants were used for immunoprecipitation with anti-V5 antibody as described earlier. Immunoprecipitates were boiled in 1× SDS buffer and resolved on 10% SDS gel. The gel was treated with Amplify Fluorographic Reagent (Amersham Biosciences) to enhance the 35S signal, dried, and autoradiographed.

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Nuclear Export Inhibitors and Tyrosine Kinase Inhibitors Block Bach1 Nuclear Export—To investigate the mechanism of nuclear export of Bach1, HepG2 cells treated with the antioxidant along with LMB, a specific inhibitor of proteins containing NESs, and genistein, a tyrosine kinase inhibitor, were used. After treatment of t-BHQ and LMB, subcellular localization of endogenous Bach1 was followed by immunoblotting. LMB stymied nuclear export of endogenous Bach1 and caused no significant changes in protein levels within the nucleus (Fig. 2A). It has also been shown that LMB blocks the function of Crm1, a receptor for the NES (18). Treatment with DMSO or t-BHQ and genistein also blocked the nuclear export of endogenous Bach1 (Fig. 2B). The results demonstrate that nuclear export is likely due to tyrosine phosphorylation and a possible interaction with the Crm1 receptor.

**Tyrosine Phosphorylation Mutation Causes Nuclear Accumulation of Bach1—**Procite and NetPhos analysis of the Bach1 amino acid sequence identified two different tyrosine phosphorylation sites shown in Fig. 3A (upper panel). It was hypothesized that Tyr$^{486}$ is critical for the nuclear export of Bach1 based on the fact that only Tyr$^{486}$ is conserved among all three species presented (Fig. 3A, right panel). Tyrosine mutations followed by subcellular localization experiments were performed to investigate which tyrosine residue may be implicated in the nuclear export. Bach1Y431A-V5 displayed an antioxidant-mediated nuclear export (Fig. 3B) comparable with Bach1-V5 (compare Figs. 1B and 3B). Cells transfected with the mutant Bach1Y486A-V5 and treated with t-BHQ did not show nuclear export of endogenous Bach1 within 2 h, presumably to allow Nrf2 to bind to the ARE. There was no accumulation of endogenous Bach1 in the cytosol. Overexpression of Bach1-V5 in HepG2 cells treated with either DMSO or t-BHQ also exhibited nuclear export of Bach1 (Fig. 1B). The overexpression seemed to change the kinetics of the protein export, speeding up from 2 h to 1 h (compare Fig. 1, A and B). Cells treated with the proteasome inhibitor MG132 along with the antioxidant showed nuclear export of endogenous Bach1 protein at 2 h. With the treatment of MG132, endogenous Bach1 seemed to accumulate in the cytoplasm (Fig. 1C), suggesting that Bach1 exiting the nucleus is being degraded within the cytosol (compare Fig. 1, A and C). Overexpression of Bach1 WT-V5 in cells treated with MG132 and either DMSO or t-BHQ also showed nuclear export of Bach1-V5 at 1 h (Fig. 1D). These results demonstrate that t-BHQ can cause nuclear export of Bach1 and subsequent degradation in the cytosol.

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FIGURE 4. Bach1 CLS deletion and Crm1 interaction. A, cells were transfected with 1 μg of Bach1 CLS and then treated with either 100 μM DMSO or 100 μM t-BHQ for the indicated time points. Cells were harvested, and nuclear and cytosolic extracts were prepared. Lysates were immunoblotted. Densitometry measurements of bands were quantitated and are shown in a graph on the right. Error bars indicate ± S.E. of triplicate samples. B, HepG2 cells were co-transfected with 1 μg of Bach1 WT-V5 or Bach1Y486A-V5 and 1 μg of Crm1-FLAG for 24 h and then treated with either DMSO or 100 μM t-BHQ for the indicated time points. Cells were harvested and lysed in RIPA buffer. 1 mg of lysate was immunoprecipitated (IP) with anti-V5 antibody and Western blotted (WB) with anti-FLAG and anti-V5 (top two panels). 1 mg of lysate was immunoprecipitated with anti-FLAG antibody and Western blotted with anti-V5 and anti-FLAG antibodies (bottom two panels).

FIGURE 5. Subcellular localization after cadmium treatment. Cells were transfected with either Bach1 WT-V5 or Bach1Y486A-V5 and treated with either DMSO or 10 μg of cadmium chloride (CdCl2) for the indicated time points. Cells were harvested, and nuclear and cytosolic extracts were prepared. Lysates were immunoblotted. Densitometry measurements of bands were quantitated and are shown in graphs below the blots. Anti-V5, anti-LDH, and anti-lamin B were probed. Error bars indicate ± S.E. of triplicate samples.

Cadmium Treatment Causes Nuclear Accumulation of Bach1Y486A—Previous reports have shown that cadmium induces the nuclear export of Bach1 (16). Thus far, we have shown that antioxidant treatment causes the nuclear export of Bach1 similarly to cadmium. To investigate whether Bach1 can be exported out of the cell in response to cadmium, we transfected Bach1-V5 and Bach1Y486A and then treated the cells with cadmium. As anticipated, cadmium induced Bach1-V5 nuclear export starting at 1 h (Fig. 5A). However, Bach1Y486A-V5 was unable to translocate out of the nucleus (Fig. 5B), suggesting that Tyr486 is a possible target mechanism in the nuclear export of Bach1.

Immunoprecipitation of Bach1-V5 Shows Tyrosine Phosphorylation—Immunoprecipitation followed by immunoblotting was used to investigate tyrosine phosphorylation of endogenous Bach1, Bach1-V5, and Bach1Y486A. Cells were treated with t-BHQ, and immunoprecipitation was performed with anti-Bach1 antibodies. Western blots were probed with anti-phosphotyrosine antibodies and then reprobed with anti-Bach1 antibodies. Anti-Bach1 antibodies immunoprecipitated phosphotyrosine at 1 and 2 h (Fig. 6A, top panel). The reverse export (Fig. 3C). Instead, Bach1Y486A-V5 showed nuclear accumulation, opposite of what Bach1-V5 and Bach1Y431A-V5 demonstrated. These observations suggest that tyrosine phosphorylation of Bach1Y486 may be required for nuclear export of Bach1.

Bach1 Nuclear Export Is Dependent upon CLS and Crm1—Previous studies have identified a CLS domain within the Bach1 gene that is responsible for nuclear export (16). Deletion of the CLS and treatment with cadmium have previously shown nuclear retention (16). Experiments to investigate whether t-BHQ affects Bach1 lacking a functional CLS were performed. Cells were transfected with Bach1ΔCLS and treated with antioxidants for the indicated time points. As expected, t-BHQ did not induce nuclear export of Bach1ΔCLS (Fig. 4A). These results indicate that translocation requires the CLS. To address fully the mechanism of nuclear export, we examined whether Bach1-V5 and Bach1Y486A-V5 could interact with Crm1. Cells co-transfected with Bach1-V5 constructs and Crm1-FLAG were treated with the antioxidant for the indicated time points and immunoprecipitated followed by immunoblotting. Anti-V5 antibodies immunoprecipitated FLAG-tagged Crm1 at 1 and 2 h (Fig. 4B, left panel). In the reverse experiment, anti-FLAG antibodies immunoprecipitated Bach1-V5 at 1 and 2 h. In a similar experimental setting, cells were co-transfected with Bach1Y486A and Crm1-FLAG. Anti-V5 antibodies were unable to immunoprecipitate Crm1-FLAG. Together, these results show that Bach1-V5, and not Bach1Y486A, was able to interact with Crm1, suggesting that Bach1 nuclear export is dependent upon Crm1 interaction and tyrosine phosphorylation.
immunoprecipitation confirmed an interaction between endogenous Bach1 and phosphotyrosine (Fig. 6A, bottom panel). Immunoprecipitation with anti-V5 antibodies behaved similarly to endogenous Bach1. Anti-V5 antibodies immunoprecipitated phosphotyrosine at 1 and 2 h (Fig. 6B, top panel). Bach1Y486A-V5 was immunoprecipitated with anti-V5 antibodies but failed to immunoprecipitate phosphotyrosine (Fig. 6B, top panel). In the reverse experiments, the anti-phosphotyrosine antibodies successfully immunoprecipitated Bach1-V5 (Fig. 6B, bottom panel) but failed to precipitate Bach1Y486A-V5 (Fig. 6C, bottom panel). These observations suggest that endogenous Bach1 and Bach1-V5 are tyrosine-phosphorylated but that Bach1Y486A is not capable of tyrosine phosphorylation. This confirms that Bach1Y486E is required for nuclear export of Bach1.

**Bach1Y486A-V5 Represses ARE-mediated NQO1 Activity—**To investigate the hypothesis that Bach1 nuclear export allows Nrf2 to bind to the ARE, we performed a ChIP assay in HepG2 cells. These results demonstrated that Bach1, and not Nrf2, was able to bind to the ARE at basal levels (Fig. 7A). However, once cells were treated with t-BHQ for 2 h, Bach1 binding to the ARE decreased due to its nuclear export. At the same time, Nrf2 binding increased, allowing for defensive gene induction. The Nrf2 binding rapidly decreased at 4 h because of Bach1 reentry into the nucleus.

Luciferase assays were used in the determination of whether Bach1-V5 activity differs from Bach1Y486A-V5 activity on t-BHQ-induced expression of ARE-mediated luciferase expression. Cells were co-transfected with either Bach1-V5 or Bach1Y486A-V5 and human NQO1 pGL2-hARE-Luc and treated with either DMSO or t-BHQ for 1 h. Luciferase activity showed a steady decrease when overexpression of Bach1-V5 was increased compared with control (Fig. 7B). However, when Bach1Y486A-V5 was overexpressed, luciferase was null regardless of the concentration of Bach1-V5 that was transfected. Western analysis with NQO1 antibody revealed results similar to the luciferase assay (Fig. 7C). A dose-dependent decrease in NQO1 expression when cells were overexpressed with Bach1-V5 was observed. When cells were overexpressed with Bach1Y486A-V5, NQO1 expression decreased. These results suggest that because Bach1-V5 is still able to export out of the nucleus at 1 h, NQO1 activity will remain high compared with the control. Bach1Y486A-V5 cannot export from the nucleus, so NQO1 activity will have no dose-dependent responses and minimal activity regardless of how much Bach1Y486A-V5 was transfected. Cells transfected with increasing concentrations of Bach1-V5 showed a significant nuclear increase in protein levels. Transfection of Bach1Y486A-V5 showed a nuclear accumulation regardless of concentration of Bach1Y486A-V5, suggesting that the mutant cannot export out of the nucleus. The presence of nuclear Bach1-V5 and Bach1Y486A protein levels corresponds to NQO1 luciferase activity and NQO1 protein expression (compare Fig. 7, B and D). Real Time PCR analysis of NQO1 mRNA expression was also performed. Cells transfected with either Bach1-V5 or Bach1Y486A-V5 were treated with antioxidants at varying time points and analyzed (Fig. 7E). NQO1 mRNA increased almost 6-fold at 1 and almost 4-fold at 2 h when transfected with Bach1-V5. This increase in activity is due to Bach1 nuclear export and subsequent Nrf2 nuclear import. NQO1 mRNA returned to basal levels at 4 and 8 h because of reentry of Bach1 into the nucleus and subsequent down-regulation of ARE-mediated gene expression. When cells were transfected with Bach1Y486A, NQO1 mRNA levels remained low compared with control. The decreased NQO1 levels are because of Bach1Y486A inability to export out of the nucleus, which inhibits Nrf2 from binding to the ARE. Taken together, these data demonstrate the dominant negative effects of the Bach1Y486A mutant.

**Cycloheximide Represses Bach1 Protein Expression—**To investigate the degradation of Bach1, cells were treated with cycloheximide, a protein synthesis inhibitor, in the absence of antioxidant. Endogenous nuclear Bach1 levels decreased significantly by 1 h, and by 4 h levels were down to nearly 10% compared with control (Fig. 8A). HepG2 cells were then pretreated with cycloheximide and treated with t-BHQ at different time points. Endogenous nuclear Bach1 protein starts to export out of the nucleus at 1 h and is nearly complete at 2 h (Fig. 8B). However, at 4 h, nuclear Bach1 protein is not present compared with t-BHQ treatment alone (compare Figs. 4A and 8B). These results suggest that Bach1 protein at 4 h is from new synthesis (Fig. 1A) because new synthesis is being blocked by cycloheximide. Endogenous Bach1 levels were also more stable in the absence of antioxidant but diminished more rapidly in the pres-
ence of antioxidant. The half-life of Bach1 was decreased in the presence of t-BHQ (Fig. 8C). In a similar experiment, HepG2 cells were plated in 12-well plates at a density of 8 * 10^4 well prior to transfection. Cells were then co-transfected with 100 ng of human NQO1 pGLO2-hARE-Luc and the indicated concentrations of Bach1-V5 or Bach1Y486A-V5/well. 10 ng of pRL-TK plasmid encoding Renilla luciferase plasmid DNA was used as an internal control of transfection efficiency. 24 h later, cells were treated with either DMSO or 100 μM t-BHQ for 1 h. Cells were then lysed, and relative luciferase activity was measured and plotted. All experiments were repeated three times, and the mean ± S.E. (error bars) is presented. C, cell lystate from B was quantified and Western blotted. Membrane was probed with anti-actin and anti-NQO1. D, Hepa-1 cells were transfected with either Bach1-V5 or Bach1Y486A-V5. 24 h after transfection, cells were treated with 100 μM DMSO or t-BHQ. Cells were harvested, and nuclear and cytosolic extracts were prepared. Lysates were immunoblotted with anti-Bach1 and anti-lamin B. E, Hepa-1 cells were transfected with either Bach1 WT or Bach1Y486A and treated with 100 μM t-BHQ. RNA was extracted and converted to cDNA. 50 ng of cDNA was mixed with 1 * Taqman Master Mix and NQO1 primers and probes and GusB primers and probes as control. Relative quantitation of mRNA was measured and plotted. Experiments were repeated three times, and the mean ± S.E. (error bars) is presented. Comparison between DMSO and t-BHQ within each transfection concentration shows a significant difference. **, p < 0.005; ***, p < 0.0001 by a two-tailed Student’s t test.

**Antioxidant Induction of Bach1 Protein Expression and Bach1 mRNA**—To investigate the physiological significance of the antioxidant-induced export of Bach1, we looked at the protein and mRNA expression levels. HepG2 cells were treated with a constant concentration of t-BHQ at varying time points, and total protein expression was followed. Endogenous Bach1 is inducible with antioxidant treatment reaching maximum protein levels at 16 h (Fig. 9A). Hepa-1 and HepG2 cells were treated with varying concentrations of t-BHQ, and Bach1 protein expression was highest with the highest concentration of antioxidant present (Fig. 9B). Because t-BHQ is inducing Bach1 protein levels, we studied the effects of t-BHQ on the newly synthesized Bach1 using pulse chase experiments. HepG2 cells were metabolically labeled with [35S]methionine for 1 h and chased with unlabeled methionine for 4 h. Cytosolic and nuclear fractions were also separated. Labeled Bach1-V5 showed no increase between DMSO treatment and 1 h of antioxidant treatment (Fig. 9C). However, labeled nuclear and cytosolic Bach1-V5 at 2 and 4 h showed an increase in expression.

**FIGURE 7. Bach1-V5 and Bach1Y486A-V5 effects on ARE-mediated activity.** A, ChIP assay. HepG2 cells were treated with 100 μM t-BHQ for 2 and 4 h, fixed with formaldehyde, cross-linked, and sheared the chromatin. The chromatin was immunoprecipitated (IP) with anti-Bach1, anti-Nrf2 antibody, and control IgG. Bach1 and Nrf2 binding to NQO1 promoter was analyzed by PCR with specific primers for the human NQO1 ARE region. The ARE region of the NQO1 promoter was also amplified from 5 μl of purified soluble chromatin before immunoprecipitation to show input DNA. B, Hepa-1 cells were plated in 12-well plates at a density of 8 * 10^4 well prior to transfection. Cells were then co-transfected with 100 ng of human NQO1 pGLO2-hARE-Luc and the indicated concentrations of Bach1-V5 or Bach1Y486A-V5/well. 10 ng of pRL-TK plasmid encoding Renilla luciferase plasmid DNA was used as an internal control of transfection efficiency. 24 h later, cells were treated with either DMSO or 100 μM t-BHQ for 1 h. Cells were then lysed, and relative luciferase activity was measured and plotted. All experiments were repeated three times, and the mean ± S.E. (error bars) is presented. C, cell lystate from B was quantified and Western blotted. Membrane was probed with anti-actin and anti-NQO1. D, Hepa-1 cells were transfected with either Bach1-V5 or Bach1Y486A-V5. 24 h after transfection, cells were treated with 100 μM DMSO or t-BHQ. Cells were harvested, and nuclear and cytosolic extracts were prepared. Lysates were immunoblotted with anti-Bach1 and anti-lamin B. E, Hepa-1 cells were transfected with either Bach1 WT or Bach1Y486A and treated with 100 μM t-BHQ. RNA was extracted and converted to cDNA. 50 ng of cDNA was mixed with 1 * Taqman Master Mix and NQO1 primers and probes and GusB primers and probes as control. Relative quantitation of mRNA was measured and plotted. Experiments were repeated three times, and the mean ± S.E. (error bars) is presented. Comparison between DMSO and t-BHQ within each transfection concentration shows a significant difference. **, p < 0.005; ***, p < 0.0001 by a two-tailed Student’s t test.
Tyrosine Phosphorylation Control of Nuclear Export of Bach1

Nuclear levels at 2 h are less than nuclear levels at 4 h, and cytosolic levels at 4 h are less than at 2 h, suggesting that at 4 h, most of the newly synthesized Bach1 is in the nucleus. Cycloheximide was used at 2 h as a control. To confirm the antioxidant induction of Bach1, mRNA was also measured using real time PCR. Treatment of cells with t-BHQ for the indicated time points showed almost a 2-fold induction of Bach1 mRNA at 4 h (Fig. 9D). Bach1 mRNA levels remained significantly higher over 8 and 16 h over DMSO control. In a similar experiment, preincubation with the transcription inhibitor actinomycin D blocked the t-BHQ-mediated induced expression of Bach1 mRNA (Fig. 9E). Taken together, these results indicate that after the nuclear export of Bach1 has taken place, t-BHQ induces Bach1 transcription and subsequent increases in protein levels. Levels of protein, labeled protein, and mRNA are increasing, presumably to replenish the Bach1 that was exported out of the nucleus and lost because of degradation.

**DISCUSSION**

Nrf2-mediated expression and coordinated induction of defensive genes, including detoxifying enzymes, are a mechanism of critical importance in protection against chemical- and radiation-induced oxidative stress and neoplasia (13). Therefore, the signals and mechanisms that regulate nuclear availability of Nrf2 are extremely important for the regulation of expression and induction of defensive genes (13, 19). Bach1 belongs to the Cap'n collar (CNC)-related basic region leucine zipper (bZip) factor family, which includes Nrf2 (19–22). Bach1 is a negative regulator of Nrf2-mediated NQO1 expression (2). ChIP assays have shown a direct competition between Bach1 and Nrf2 for binding to ARE and leading to repression or activation of gene expression, respectively (2).

Present studies demonstrated that t-BHQ stimulated rapid export of Bach1 from the nucleus, which allowed Nrf2 to bind to the ARE and activate NQO1 gene expression. The antioxidant-induced nuclear export of Bach1 appears to be an integral part of the ARE/Nrf2-mediated activation of defensive genes. If Bach1 export is disturbed resulting in higher levels of Bach1 in the nucleus, Nrf2 will be unable to bind to the ARE appropriately, which will compromise induction of defensive genes (2). Bach1 that is exported out of the nucleus is degraded in the cytosol, which was evident from the absence of cytosolic Bach1 after nuclear export has taken place and cytosolic Bach1 accumulation in cells pretreated with protease inhibitor MG132. The studies also demonstrated a role of antioxidant-induced phosphorylation of tyrosine 486 in the control of the nuclear export of Bach1. Mutation of tyrosine 486 led to nuclear accumulation of Bach1 because nuclear export of Bach1 is an early event that takes place before Nrf2 is imported inside the nucleus. Entry of Fyn into the nucleus and phosphorylation of Nrf2 tyrosine 568 are late events (24). Therefore, the tyrosine kinase that is implicated in the phosphorylation of tyrosine 486 remains unknown. Additionally, it has been shown previously that the cadmium-induced nuclear export of Bach1 was dependent upon extracellular signal-regulated protein kinase (ERK1/2) activity in the mitogen-activated protein kinase pathway (16). It may be possible that ERK1/2, acting as an upstream kinase, may be responsible for phosphorylation and activation of the unknown tyrosine kinase involved in the antioxidant-induced Bach1 nuclear export. We believe that the tyrosine kinase that phosphorylates Bach1 has to be stress-responsive and is rapidly activated in response to antioxidants.

Furthermore, treatment with LMB blocked the nuclear export of Bach1. LMB acts as an inhibitor of Crm1, a NES-dependent export protein. Therefore, it appears that the antioxidant treatment causes Bach1 nuclear export that is dependent on Crm1. Blocking the nuclear export by mutation of tyrosine 486 does not allow Crm1 to interact with Bach1, allowing accumulation within the nucleus. Previous findings also showed the nuclear exit of Bach1, via CLS and NES, in response to cadmium...
and heme treatment, respectively (16, 17). Treatment with cadmium also causes nuclear retention of Y486A because tyrosine phosphorylation is unable to take place. Also, the Bach1 mutant lacking the CLS did not undergo nuclear export when treated with t-BHQ. These findings indicate that antioxidant-induced nuclear export appears to function through the CLS and subsequently Crm1. In addition, it is unknown at this time whether antioxidants can induce nuclear export via the heme-dependent NES. Because the CLS is essential for cadmium-induced nuclear export of Bach1 and not essential for the relocalization of Bach1 in response to heme (17), it is probable that antioxidants would act analogously to cadmium and not be responsible for nuclear export via the heme-dependent NES. Thus, cadmium- and antioxidant-induced nuclear export are probably through shared mechanisms.

Interestingly, upon Bach1 nuclear export, subsequent accumulation in the cytosol was absent. When cells were treated with MG132, Bach1 accumulation could be seen in the cytosol alluding to the conclusion that antioxidants may induce proteasome-dependent degradation upon nuclear export. Previous reports have shown that hemin is also responsible for proteasome-dependent degradation of Bach1 in the cytoplasmic region following nuclear export (25), suggesting that both heme and antioxidants have similar mechanisms that cause proteasome-dependent degradation. We also found that the half-life of Bach1 was decreased in the presence of antioxidant, which corresponds to previous reports that heme also decreases Bach1 half-life (25). Nuclear Bach1 levels were restored back to normal within 4 h of antioxidant treatment, which required increases in transcription and synthesis. Bach1 transcription, like other Nrf2 downstream genes, is up-regulated in response to antioxidant. Therefore, Bach1 is expected to be a member of the ARE/Nrf2-regulated battery of genes (13).

In summary, antioxidant-induced nuclear export and import of Bach1 is a significant component of the oxidative/electrophilic stress-induced Nrf2 signaling cascade. Antioxidant activates an unknown tyrosine kinase that phosphorylates

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**FIGURE 9. Effect of t-BHQ on Bach1 protein and gene expression.** A, Hepa-1 cells were treated with 100 μM DMSO or t-BHQ for the indicated time points. Cells were harvested and lysed in RIPA buffer. Lysates were immunoblotted with anti-Bach1 and anti-actin antibodies. B, Hepa-1 and HepG2 cells were treated with the indicated concentrations of t-BHQ or DMSO for 16 h. Cells were harvested and lysed in RIPA buffer, and lysates were immunoblotted with anti-Bach1 and anti-actin antibodies. C, pulse chase assay is shown. Hepa-1 cells transfected with 0.5 μg of Bach1-V5 were metabolically labeled with [S35]methionine (pulse). 1 h later the medium was replaced with complete medium containing sufficient cold methionine, and the cells were harvested at 4 h (chase). 100 μM t-BHQ and/or 30 μg/ml cycloheximide was added to the medium at different time points. 500 μg of nuclear (N) or cytosolic (C) cell lysate was immunoprecipitated (IP) with 2 μg of anti-V5 antibody. The immunoprecipitates were resolved on 10% SDS-PAGE and autoradiographed for 35S signal. D and E, Hepa-1 cells were treated with DMSO or 100 μM t-BHQ and/or 2 μg/ml of actinomycin D (ActD) for varying time points. Cells were collected, and RNA was extracted. 50 ng of cDNA was mixed with 1 × Taqman Master Mix and Bach1 primers and probes, and GusB primers and probes as control. Relative quantitation of mRNA was measured and plotted. Experiments were repeated three times, and the mean ± S.E. (error bars) is presented. Comparison between DMSO and t-BHQ, or DMSO + ActD and t-BHQ + ActD treated time points shows a different significance. **, p < 0.005 by two-tailed Student’s t test.
Bach1Y486. This results in a rapid export of Bach1 from the nucleus and degradation in the cytosol. In the meantime, the antioxidant stabilizes Nrf2, which causes translocation into the nucleus where it binds to the ARE and activates coordinated expression of a myriad of defensive genes. The antioxidant also leads to an increase in synthesis of Bach1 that is imported into the nucleus to achieve the normal level of Bach1 and repression of NQO1 gene expression.

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