The export of certain nuclear proteins is involved in the regulation of various nuclear functions, including transcription. In some cases, the export of target proteins is induced upon environmental or cellular cues, resulting in conditional gene expression. The small Maf proteins appear to be critical regulators of heme oxygenase (HO)-1, an antioxidant defense enzyme that degrades heme into iron, carbon monoxide, and biliverdin. Although ho-1 is repressed by Bach1/small Maf heterodimers, it is activated by Nrf2/small Maf heterodimers, indicating that Bach1 and Nrf2 compete with each other. We anticipated that the nuclear concentration of Bach1 might be regulated to ensure that the entire system effectively responds to various stimuli. We carried out detailed domain analysis of Bach1 in an effort to understand how various inducers of HO-1 inactive Bach1. We show here that cadmium, a strong inducer of HO-1, activates the nuclear export of Bach1. This cadmium-induced export of Bach1 was mediated in trans by its C-terminal region that is conserved between Bach1 and Bach2. The nuclear export of Bach2 was also induced by cadmium, indicating that the cadmium responsibility is shared between Bach1 and Bach2. The nuclear export of Bach1 was dependent on Crm1/Exportin-1 as well as the extracellular signal-regulated kinase-1/2 (ERK1/2) activity. These results indicate that the nuclear export of Bach1 constitutes an important regulatory mechanism to relieve the Bach1-mediated repression of genes such as ho-1.

Cells possess an enormous repertoire of gene responses to cellular and environmental stimuli. To a large extent, gene responses consist of programmed interactions between transcription factors and their target cis-DNA sites. Oxidative stress responses are critical to cell survival, because cells cannot avoid reactive oxygen species as long as they use molecular oxygen. Heme oxygenase-1 (HO-1)1 provides an interesting system for understanding inducible gene expression upon oxidative stress (1, 2). HO-1 is one of the rate-limiting enzymes involved in heme degradation, generating ferrous iron, carbon monoxide, and biliverdin, which is rapidly reduced to bilirubin. Carbon monoxide, biliverdin, and bilirubin have antioxidant and anti-inflammatory activities in vitro (1, 2). Thus, HO-1 is an antioxidant defense enzyme that converts heme into antioxidants. Although its role in lower eukaryotes is not clear, HO-1 is essential for higher eukaryotes to cope with various aspects of cellular stress and to regulate cellular iron metabolism (3). Transcription of ho-1 is robustly induced in mammalian cells by oxidative stress, various pro-inflammatory stimulants such as cytokines, heavy metals, heat shock, UV light, and lipopolysaccharide (4–8). As in the classic view of metabolic control, the expression of ho-1 is also induced by the heme substrate (6, 9, 10). However, the precise mechanism by which ho-1 is induced in response to diverse stimuli remains unclear.

Recent studies have revealed that the induction of ho-1 expression involves the interplay of various basic-leucine zipper (bZip) transcription factors on critical enhancer elements, including the StRE (stress responsive element (11)) or MARE (Maf recognition element (12)). StRE and MARE are similar to each other and embed the 12-O-tetradecanoylphorbol-13-acetate-responsive element (TRE), a binding motif for AP-1 transcription factors (12). In general, MARE can be bound in vitro by various heterodimeric bZip factors, including NF-E2, NF-E2-related factors (Nrf1, Nrf2, and Nrf3), Maf and AP-1 families (13–21). Recent studies have shown that the regulation of ho-1 through MARE/StRE involves the small Maf proteins (MafF, -G, and -K), Nrf, and Bach1. The small Maf proteins form heterodimers with Nrf proteins to activate MARE-dependent gene expression (22–24). Bach1 forms heterodimers with the small Maf proteins that bind to MARE just like the Nrf/small Maf heterodimers (25). However, Bach1 heterodimers function as transcription repressors (25). Gene targeting experiments in mice revealed that, under normal conditions, Bach1 binds to the enhancers of ho-1 to repress its expression (26). Genetic experiments further indicated that Bach1 repressor activity is dominant over activators of HO-1, such as Nrf2, effectively keeping HO-1 expression at low levels under normal conditions (26). Thus, there is a competitive interplay between the Bach1-containing repressor dimers and Nrf-containing activator dimers. For the system to be effective in regulating inducible gene expression, the activities or nul-
clear concentrations of Bach1 and/or Nrf proteins must be under dynamic control. We recently reported that heme regulates the DNA binding activity of Bach1 through a direct interaction in vitro (26, 27). The Bach1-heme interaction is mediated by evolutionarily conserved multiple heme regulatory motifs, including the cysteine-proline (CP) dipeptide sequence in Bach1. Increased levels of heme abrogate this repressor function of Bach1 by binding to Bach1 and thus inhibiting its DNA-binding activity. In the absence of Bach1 activity, other factors such as Nrf2 appear to bind to MAREs and activate HO-1 expression (28). Thus, the regulation of ho-1 involves a direct sensing of heme levels by Bach1, analogous to the sensitivity of the lac repressor to lactose, generating a simple feedback loop whereby the substrate affects the repressor.

Notwithstanding the recent progress, it remains unclear how ho-1 is induced by the vast array of stimuli. Aside from heme, conditions resulting in induction may increase the activities of MARE activators such as Nrf2. Indeed, cadmium stabilizes Nrf2 by inhibiting its degradation through the ubiquitin-proteasome pathway (28). In addition to the regulation of Nrf2 activity, other inducers may also inhibit Bach1 activity. In this respect, a comparison of Bach1 with its related factor, Bach2, may yield some clues. We previously showed that the subcellular localization of Bach2 is regulated by nuclear export (29, 30). In cultured cells under normal conditions, Bach2 is localized in the cytoplasm through its C-terminal conserved cytoplasmic localization signal (CLS). CLS directs leptomycin B-sensitive and Crm1/Exportin1-dependent nuclear export. Oxidative stressors abort CLS activity and induce the accumulation of Bach2 in the nucleus. The CLS-like sequence is highly conserved among mouse as well as human Bach2 and Bach1 (29). To address the possibility that Bach1 is regulated by conditional nuclear export, we carried out a detailed domain analysis of Bach1 in an effort to identify regions that might specify its subcellular localization. Our results indicated that at least two inducers of ho-1, cadmium and heme, inactivate the repressor Bach1 in distinct ways to achieve conditional gene expression and suggest that the interpretation of various stimuli by Bach1 is critical in ho-1 regulation.

EXPERIMENTAL PROCEDURES

Reagents—Tissue culture media were from Sigma, and fetal bovine serum was obtained from JRH Biosciences. Restriction endonuclease and other DNA-modifying enzymes were purchased from either New England Biolabs or Takara. Oligonucleotides were synthesized by Invitrogen. CdCl2 was purchased from Wako. Kinase inhibitors and reagents for luciferase assays were purchased from Promega. All other chemicals were reagent grade.

Plasmids—Plasmid pEGFP-C2 was obtained from Clontech. The Bach1, MaF and FLAG expression plasmids pcMV-Bach1, pEGF-MaF, and pcDNA3.1B-FLAG, respectively) used were as previously described (20, 26, 31). Plasmids constructed in this study are shown in Fig. 1. The mouse Bach1 cDNA sequence (20) was inserted into the KpnI site of pcDNA3.1B-FLAG or pEGFP-C2, resulting in pcDNA3.1B-FLAG-Bach1 or pEGFP-Bach1. The pcDNA3.1B-FLAG-Bach1 plasmid was digested with EcoRI and then self-ligated to yield pcDNA3.1B-FLAG-Bach1ΔT18. The pcDNA3.1B-FLAG-Bach1ΔT18 plasmid was digested with SalI site and inserted into the XhoI site of pcDNA3.1B-FLAG, resulting in pcDNA3.1B-FLAG-Bach1ΔT18 plasmid. The Bach1 cDNA sequences lacking the coding domain for the C-terminal amino acids was amplified by PCR, digested with NotI, and inserted into the EcoRI/KpnI site of pcDNA3.1B-FLAG. The resulting plasmids were pcDNA3.1B-FLAG-Bach1ΔC1, pcDNA3.1B-FLAG-Bach1ΔC2, pcDNA3.1B-FLAG-Bach1ΔC3, and pcDNA3.1B-FLAG-Bach1ΔC4. The pcDNA3.1B-FLAG-Bach1ΔC1 plasmid was digested with EcoRI and then self-ligated to yield pcDNA3.1B-FLAG-Bach1ΔC1/T2BAC1. The primers used were as follows: ΔC1, 5′-TTAAAGGTACCTGAGAT-CACAGCTCTGCAGCG-3′; and ΔC4, 5′-TTAAAGGTACCTTTAGGTGGGCTAGGGCAG-3′; and ΔC3, 5′-TTAAAGGTACCTTTAGGTGGGCTAGGGCAG-3′; and ΔC3, 5′-TTAAAGGTACCTTTAGGTGGGCTAGGGCAG-3′; and ΔC4, 5′-TTAAAGGTACCTTTAGGTGGGCTAGGGCAG-3′; and ΔC4, 5′-TTAAAGGTACCTTTAGGTGGGCTAGGGCAG-3′; and ΔC4, 5′-TTAAAGGTACCTTTAGGTGGGCTAGGGCAG-3′; and ΔC4, 5′-TTAAAGGTACCTTTAGGTGGGCTAGGGCAG-3′; and ΔC4, 5′-TTAAAGGTACCTTTAGGTGGGCTAGGGCAG-3′; and ΔC4, 5′-TTAAAGGTACCTTTAGGTGGGCTAGGGCAG-3′; and ΔC4, 5′-TTAAAGGTACCTTTAGGTGGGCTAGGGCAG-3′.

Cell Culture—293T cells and GM02063 cells were cultured in Dulbecco’s modified Eagle’s medium (Nissui) with 10% fetal bovine serum (JRH Biosciences), 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen). The cells were treated with chemical reagents at the indicated concentrations.

Immunochemistry—293T cells were transfected with various Bach1 expression plasmids and cultured for 24 h. The cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature. After fixing, the cells were washed in PBS and permeabilized in 0.5% Triton X-100 in PBS for 5 min. Cells were washed and treated for 30 min at 37 °C with the anti-FLAG antibody (Sigma) at a dilution of 1:500–1,000. Cells were then washed three times in PBS and then treated with fluorescein-conjugated anti-mouse IgG antibodies (Sigma) diluted in 1% bovine serum albumin in PBS at a concentration of 1:200 at 37 °C for 30 min. After washing, cells were counterstained with 10 μM Hoechst 33342 and mounted in Vectashield (Vector). The cells were then examined by fluorescence microscopy.

GFP Observation—293T cells were transfected with the GFP fusion expression plasmids and cultured for 24 h. The subcellular localization of GFP fusion proteins was examined under fluorescence microscopy after fixation in 4% paraformaldehyde. To examine the effects of reagents, transfected cells were treated with 20 ng/ml leptomycin B or 10 μM CdCl2 for the indicated periods at the end of incubation.

Image Acquisition—The images were taken with a Leica epifluorescence microscope equipped with a charge-coupled device camera controlled by Qfluoro software (Leica). Adobe Photoshop was used for the presentation of the images.

Reporter Assays—The MARE reporter pRBGP2 possesses three copies of the MARE derived from the chicken β-globin enhancer as previously described (15). 293T cells were seeded in 12-well dishes 16 h before transfection. The cells were transfected with the reporter and effector plasmids by lipofection. To examine the effects of various reagents, transfected cells were cultured for an additional 16 h and treated with vehicle or inducing agents for 4 h. To examine the effects of kinase inhibitors, cells were cultured in serum-free medium. Kinase inhibitors were added 12 h prior to the addition of CdCl2, and maintained during the remainder of the incubation period. The preparation of cell extracts and measurement of luciferase activity were carried out as previously described (29). Three independent experiments, carried out in duplicate, were performed, and the results were averaged and diagrammed with the standard errors.

RESULTS

Bach1 Export from Nuclei Is Dependent on Crm1/Exportin—When overexpressed in various mammalian cells, Bach1 accumulates within the cytoplasmic region with clear nuclear exclusion. To test whether the cytoplasmic-dominant accumulation involves nuclear export, we expressed FLAG-tagged Bach1 in human embryonic kidney 293 cells and treated the cells with leptomycin B (LMB), an inhibitor of Crm1. As shown in Fig. 1A, LMB induced efficient nuclear accumulation of Bach1, establishing that Bach1 is regulated by nuclear export. Based on this observation, we then examined how and when the subcellular localization of Bach1 is regulated.

Multiple Domains of Bach1 Regulate Subcellular Localization—the nuclear export of Bach2 is mediated by the CLS (29). The CLS is a non-canonical type of nuclear export signal (NES) whose activity is inhibited by oxidative stressors such as diethyl maleic acid. Because the corresponding region of Bach1 is well conserved, we hypothesized that a deletion of the putative CLS from Bach1 would result in nuclear accumulation. To define regions involved in the regulation of Bach1 subcellular localization, we constructed various truncated versions of Bach1 tagged with FLAG at the N terminus (Fig. 1B). Bach1ΔC1 lacked the C-terminal 14 amino acids and thus the entire putative CLS from Bach1 would result in nuclear accumulation. To define regions involved in the regulation of Bach1 subcellular localization, we constructed various truncated versions of Bach1 tagged with FLAG at the N terminus (Fig. 1B). Bach1ΔC1 lacked the C-terminal 14 amino acids and thus the entire putative CLS from Bach1 would result in nuclear accumulation. To define regions involved in the regulation of Bach1 subcellular localization, we constructed various truncated versions of Bach1 tagged with FLAG at the N terminus (Fig. 1B). Bach1ΔC1 lacked the C-terminal 14 amino acids and thus the entire putative CLS from Bach1 would result in nuclear accumulation. To define regions involved in the regulation of Bach1 subcellular localization, we constructed various truncated versions of Bach1 tagged with FLAG at the N terminus (Fig. 1B). Bach1ΔC1 lacked the C-terminal 14 amino acids and thus the entire putative CLS from Bach1 would result in nuclear accumulation. To define regions involved in the regulation of Bach1 subcellular localization, we constructed various truncated versions of Bach1 tagged with FLAG at the N terminus (Fig. 1B). Bach1ΔC1 lacked the C-terminal 14 amino acids and thus the entire putative CLS from Bach1 would result in nuclear accumulation. To define regions involved in the regulation of Bach1 subcellular localization, we constructed various truncated versions of Bach1 tagged with FLAG at the N terminus (Fig. 1B). Bach1ΔC1 lacked the C-terminal 14 amino acids and thus the entire putative CLS from Bach1 would result in nuclear accumulation. To define regions involved in the regulation of Bach1 subcellular localization, we constructed various truncated versions of Bach1 tagged with FLAG at the N terminus (Fig. 1B). Bac...
Fig. 1. Bach1 and its derivatives. A, 293T cells were transfected with FLAG-tagged Bach1-expression plasmid, cultured for 24 h, and treated with [lower panels] or without [upper panels] LMB for 2 h. Images are DNA [right] and Bach1 [left]. B, schematic representation of mouse Bach1 and truncated derivatives tagged with FLAG epitope. A fragment spanning residues 636–685 was fused with EGFP. CP motifs (27) are indicated with circled numbers. C, indicated proteins were expressed in 293T cells and analyzed by immunoblotting using anti-FLAG antibody. Bach1 proteins are indicated with dots above or below the bands. Arrowheads indicate cross-reactive bands that were also present in the non-transfected cells (the left-side lane). Note that deletions of CLS caused slower mobility (∆C1 and ∆BTBAC1).

sequentially removing the C-terminal region, we found that Bach1AC2 was excluded from nuclei, whereas Bach1ΔC3 showed considerable accumulation in nuclei (Fig. 2, A and B). Thus, the region between residues 636 and 685 is critical for cytoplasmic accumulation. Further deletion (∆C4) completely abolished cytoplasmic staining, suggesting that the leucine zipper inhibits nuclear accumulation in this assay.

Bach1 and Bach2 possess the BTB/POZ domain in their N-terminal regions. The BTB/POZ domain is found in various nuclear and cytoplasmic proteins and is known to mediate protein-protein interactions (for a review, see Ref. 32). This domain mediates transcriptional repression through its ability to recruit the corepressors SMRT and N-CoR, as demonstrated for BCL-6 and PLZF (33–35). The Bach1 BTB domain mediates oligomer formation, generating a multivalent DNA-binding complex (25, 32). Surprisingly, removing the BTB domain of Bach1 (Bach1ΔBTB) resulted in nuclear accumulation (Fig. 2). These results indicated that the cytoplasmic localization of Bach1 is regulated by multiple domains. Even in the absence of the putative CLS, other regions such as amino acids 636–685 and the BTB domain inhibit nuclear accumulation. This is in clear contrast to Bach2, where the deletion of CLS is sufficient to cause nuclear accumulation of Bach2 in many cell types (Ref. 29 and data not shown).

One of the interesting observations described above is that the LMB treatment resulted in the nuclear accumulation of Bach1, whereas the deletion of CLS was not sufficient to cause nuclear accumulation. This fact suggests that Bach1 also possesses an additional NES. To examine whether the region spanning amino acids 636–685 contains a NES, we fused the region to EGFP (see Fig. 1) and examined its subcellular localization in the absence or presence of LMB. Like EGFP, the fusion protein was distributed in both nuclear and cytoplasmic compartments (Fig. 3). LMB did not increase the nuclear signal or diminish the cytoplasmic signal. These results indicate that the region examined does not function as a NES. One possibility is that this region facilitates interaction with a cytoplasmic protein. Although the fusion protein tested in Fig. 3 did not show a clear nuclear exclusion, the small size of the protein may allow diffusion into nuclei, obscuring cytoplasmic retention. Alternatively, the region 636–685 may collaborate with other regions on Bach1 to strengthen cytoplasmic interaction. This is consistent with above observations that ∆C4 showed more efficient nuclear accumulation than ∆C3 (Fig. 2). We recently found a cytoskeletal protein that binds Bach1 depending on the presence of this region. Together, these observations suggest that the region spanning amino acids 636–685 is not a NES and rather plays a role in the cytoplasmic anchoring of Bach1.

Cadmium Activates the Nuclear Export of Bach1—Our previous observations suggested that ho-1 is repressed by Bach1 under normal conditions. In a simple model, Bach1 may accumulate within nuclei under normal conditions. This model predicts that the subcellular localization of Bach1 may be further regulated in trans through interaction with co-functioning proteins. As previously reported (36), the overexpression of MafK together with Bach1 resulted in significant nuclear accumulation of Bach1 (Fig. 4A, upper panel, and B). Heterodimer formation may stabilize nuclear accumulation through DNA binding, because Bach1 displays poor DNA binding by itself. In any case, a substantial amount of Bach1 may be localized in the nucleus when MafK or other small Maf proteins are available.

Having established that the localization of Bach1 is determined by multiple layers of regulation, we wished to examine the subcellular localization of Bach1 and MafK upon cadmium treatment, a condition that induces HO-1. As shown in Fig. 4 (A and B), when transfected cells were treated with 10 μM cadmium for 4 h, Bach1 accumulated in the cytoplasmic region and excluded from the nuclear region. Although MafK showed a cytoplasmic staining upon cadmium treatment, it did not show a clear nuclear exclusion like Bach1 and a significant nuclear staining of MafK remained. This observation suggested that Bach1 and MafK affected each other’s localization. The translocation of Bach1 was inhibited by simultaneous treatment with LMB, suggesting the involvement of Crm1-dependent nuclear export in this process (Fig. 4B). Bach2 responded to MafK expression and cadmium in a similar way as Bach1 (Fig. 4C), indicating that both Bach1 and Bach2 share similar responses toward cadmium.

Cadmium may inhibit the interaction between Bach1 and MafK, allowing for the nuclear export of liberated Bach1. To test this possibility, we examined the effect of cadmium upon the Bach1ΔBTB that had accumulated in the nuclear region.

\*C. Yamazaki and K. Igarashi, unpublished data.
independent of MafK under normal culture conditions. We found that, although Bach1/H9004BTB showed nuclear-dominant accumulation when expressed alone, it displayed cytoplasmic-dominant accumulation upon cadmium treatment (Fig. 5A, compare upper two panels). Quantification of the distribution under the two conditions clearly indicated that Bach1/H9004BTB did not accumulate within the nuclear compartment in the presence of cadmium (Fig. 5B). This observation indicates that the observed cadmium effect on Bach1 does not involve changes in the Bach1-MafK interaction and that the BTB domain is not necessary for the cadmium effect. To examine whether CLS is involved in the cadmium-induced nuclear export of Bach1, we constructed Bach1/H9004BTB/H9004C1 that lacks both the BTB and CLS domains (see Fig. 1). Bach1/H9004BTB/H9004C1 appeared to accumulate in nuclei more efficiently than Bach1/H9004BTB under normal culture conditions and showed virtually no cytoplasmic-dominant accumulation (Fig. 5B). When the cells were treated with cadmium, Bach1/H9004BTB/H9004C1 remained within the nuclei (Fig. 5A, lower two panels, and B), clearly indicating that CLS is critical for the cadmium-induced nuclear export of Bach1. These results indicate that cadmium induces the nuclear export of Bach1, but not of MafK, and that the translocation requires CLS. This selective export of the inhibitory subunit of MARE-binding complexes may allow for rapid subunit exchange from Bach1-containing small Maf complexes to Nrf2- or other activator-containing complexes.

To fully address the biological significance of the Bach1 response to cadmium, we examined effects of various concentrations of cadmium on the wild-type Bach1 and Bach1/H9004C1 in MafK-overexpressing cells. As shown in Fig. 6 (left column), as low as 1 µM cadmium induced cytoplasmic accumulation of Bach1 in GM02063 human fibroblastic cells (37). Three micromolar cadmium was sufficient for clear nuclear exclusion of Bach1 in most of the transfected cells. Considering that maximum induction of HO-1 in chick embryo liver cells is achieved at 2 µM cadmium (38), the dose-responsibility suggests that the effect of cadmium upon subcellular localization of Bach1 is biologically significant.

In a similar experimental setting, we observed that cadmium induced cytoplasmic accumulation of Bach1/H9004C1 (right column), but its responsibility was significantly reduced. The effect of 1 µM cadmium on Bach1/H9004C1 was less drastic as compared with that upon the wild-type Bach1. The results corroborated the critical role of CLS in cadmium-induced nuclear export especially at lower, and thus physiologically more relevant, concentrations of cadmium. In the presence of higher concentration of cadmium (i.e. 10 µM), we observed clear nuclear exclusion of Bach1/H9004C1 in most of the transfected cells. These results suggest the presence of another, CLS-independent mechanism that mediates nuclear export of Bach1 in response to higher concentrations of cadmium (see "Discussion").

**Fig. 2. Subcellular distribution of Bach1 and Bach1 derivatives.** A, 293T cells were transfected with the indicated expression plasmids. Images for DNA (right), Bach1 detected with anti-FLAG antibody (middle), and merged (left) are shown. B, subcellular localization of Bach1 and its derivatives were classified into three categories: C>N, cytoplasmic-dominant accumulation (gray bar); C=N, roughly equal distribution in cytoplasmic and nuclear compartments (white bar); and C<N, nuclear-dominant accumulation (black bar). Results of counting 200 cells are shown. Similar results were obtained in several independent experiments.

**Fig. 3. The region spanning amino acid residues 636–685 is not an NES.** 293T cells were transfected with the expression plasmid for a fusion of EGFP and residues 636–685, and treated with (lower) or without (upper) LMB for 2 h. Images are for DNA (right) and EGFP (right).
Cadmium Inactivates Bach1 via Mechanisms Other Than Nuclear Export—The above results strongly suggested that translocation of Bach1 to the cytoplasmic region is important for the regulation of \( \text{ho-1} \) expression in response to cadmium. We further tested this model by examining the effects of Bach1 and Bach1/H9004C1 on a \( \text{ho-1} \) reporter expression. The reporter plasmid \( p\text{HO15luc} \) contains the upstream 15-kb DNA of \( \text{ho-1} \) and is repressed by Bach1 (26). We co-transfected the reporter and FLAG-tagged Bach1 or FLAG-tagged Bach1/H9004C1 expression plasmids. In the absence of cadmium, both Bach1 and Bach1/H9004C1 inhibited the reporter gene expression (Fig. 7). One micromolar cadmium strongly induced the reporter \( p\text{HO15luc} \). Ten micromolar cadmium showed less but significant activation of the reporter gene expression. In the presence of these concentrations of cadmium, both Bach1 and Bach1/H9004C1 failed to inhibit the reporter gene expression. These results indicate that, although cadmium inactivates the repressor activity of Bach1, the CLS-directed nuclear export is not the sole mechanism for the cadmium effect.

Involvement of MAPK Pathway in the Cadmium-induced Nuclear Export of Bach1—Cadmium rapidly activates p38 and ERK1/2 (39). p38 was previously shown to be essential for the stimulation of Nrf2 activity upon cadmium treatment (39). Considering its critical repressive role in \( \text{ho-1} \) regulation, we hypothesized that the subcellular localization of Bach1 may be regulated by p38 or additionally by ERK1/2. We examined whether p38 and ERK1/2 activities were required for the cadmium-induced nuclear export of Bach1 by treating cells expressing Bach1ΔBTB or Bach1ΔBTBΔC1 with U0126, an inhibitor of MEK1/2 (activators of ERK1/2), or with SB203580, an inhibitor of p38. Upon treating cells with U0126, Bach1ΔBTB remained within the nuclei even in the presence of cadmium (Fig. 8). In contrast to this, the cadmium-induced nuclear export of Bach1 was not inhibited in the presence of SB203580 (data not shown).

We next examined the effect of U0126 on the regulatory activity of Bach1 in reporter assays (Fig. 9A). The reporter
pRBGP2 contains three copies of MAREs and is induced by oxidative stressors. Although Bach1 repressed the reporter gene activity, cadmium counteracted this inhibitory effect. However, by treating cells with U0126, the effect of cadmium upon Bach1-mediated repression was lost and the reporter gene expression was maintained at lower levels. Unexpectedly, SB203580 showed effects similar to U0126. Although SB203580 did not affect the nuclear export of Bach1, it may affect other activities of Bach1 such as DNA binding. Alternatively, SB203580 may inhibit the reporter gene activation through its effect upon the activators such as Nrf2 (39). To confirm that nuclear export of Bach1 is important for the cadmium effect upon the reporter gene expression, we examined the effect of LMB. As shown in Fig. 9B, LMB also inhibited cadmium-induced reporter gene expression in the presence of Bach1. Taken together, these results established that cadmium induces the nuclear export of Bach1 and that this translocation is important for MARE-dependent gene activation.

**DISCUSSION**

We show here that the cytoplasmic accumulation of Bach1 is regulated through its multiple regions (Fig. 10). These include the region encompassing residues 636–685, the BTB/POZ domain, and CLS. The fact that at least three domains regulate the cytoplasmic accumulation of Bach1 points to the importance of cytoplasmic compartmentalization as a means of regulating the nuclear concentration of Bach1. Because CLS-dependent nuclear export is induced by cadmium, the nuclear concentration of Bach1 can change in response to cadmium.

An intriguing feature of ho-1 gene regulation is its response to diverse signals. This responsiveness is dependent on the multiple MAREs/StRE sequences present in the upstream enhancer regions (40). We previously showed that Bach1 is the critical repressor of the ho-1 enhancers under normal conditions and is required to maintain a low level of ho-1 expression (26). Loss of bach1 is sufficient to uncouple ho-1 from the stress-responsive control, resulting in robust expression even without stress (26). Heme, one of the inducers of ho-1, inhibits the DNA-binding activity of Bach1 via direct binding (26, 27). This regulatory mechanism clearly exemplifies that one way to achieve ho-1 induction is to inactivate the Bach1 repressor. Our results described here further extend the regulatory mecha-
nisms of Bach1 and show that Bach1, the “brake” of ho-1 expression, is relieved not only by heme but also by cadmium, another inducer of ho-1. Thus, at least two inducers with no structural similarities converge upon Bach1 to induce ho-1 expression with distinct mechanisms. If we consider the ho-1 enhancers and their binding protein complexes as a regulatory node, the node possesses a simple structure with complex calculating capacities for integrating various inputs as detailed below.

The activating arm of small Maf complexes, Nrf2, is regulated by several mechanisms. Keap1 traps Nrf2 within the cytoplasmic region under normal conditions. Upon oxidative stress, the inhibitory interaction is suppressed, resulting in the nuclear accumulation of Nrf2 (41). Several studies indicate that Nrf2 is also regulated by degradation (28, 42, 43). Under normal conditions, Nrf2 is degraded rapidly. Oxidative stress and heme somehow stabilize Nrf2, increasing its transactivating function. Thus, there are multiple reactions that conditionally activate Nrf2.

A striking feature of Bach1 is its response to two inducers of ho-1, cadmium and heme, via distinct mechanisms. Although heme inhibits the DNA-binding activity of the Bach1-small Maf heterodimer as a ligand of Bach1 (26, 27), we show here that cadmium induces the nuclear exclusion of Bach1 by activating its nuclear export. The cadmium-induced nuclear export of Bach1 was dependent on CLS and the nuclear exporter Crm1. Its nuclear export. The cadmium-induced nuclear export of cadmium induces the nuclear exclusion of Bach1 by activating ally activate Nrf2.

The regulatory program is wired as a competitive interplay of the bZip transcription factor network. The activating signals, resulting in changes in the competitive interplay of Bach1 and Nrf2 (and possibly other activators). Furthermore, in contrast to Nrf2, whose activation by cadmium in MCF-7 epithelial cells depends on the p38 kinase (39), the cadmium-induced nuclear export of Bach1 was dependent on ERK1/2 activity. The results of reporter assays confirmed that the activation of both p38 and ERK1/2 is important for the enhancement of MARE-dependent gene expression by cadmium. Although p38 is required for the activation of Nrf2 (39), ERK1/2 is required to inactivate Bach1. This interpretation is consistent with the results described in Fig. 9A: SB203580 may have exerted an inhibitory effect upon the reporter gene activation by suppressing Nrf2 or related activators. It remains also feasible that SB203580 precludes cadmium-responsive inhibition of Bach1 such as negative regulation of DNA binding as discussed above. At present, we do not know the exact target that is modified by ERK1/2 or p38. Although further studies are obviously required to resolve this issue, the results described in this study indicate Bach1 is an interesting downstream effector of MAPK cascades in stress responses. Because MARE embeds TRE (12), a binding site for Jun/Fos heterodimers, Bach1 may also play a role in AP-1-dependent stress responses.

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