Oxidative Stress Abolishes Leptomycin B-sensitive Nuclear Export of Transcription Repressor Bach2 That Counteracts Activation of Maf Recognition Element*

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The mammalian transcription activator Nrf2 plays critical roles in executing oxidative stress response by binding to the regulatory DNA sequence Maf recognition element. Bach2 is an Nrf2-related transcription repressor and a tissue-specific partner of the Maf oncoprotein family. We show here how Bach2 is regulated by an oxidative stress-sensitive conditional nuclear export. In cultured cells, Bach2 was localized in cytoplasm through its C-terminal evolutionarily conserved cytoplasmic localization signal (CLS). The CLS directed leptomycin B-sensitive nuclear export of reporter proteins, suggesting its dependence on the nuclear exporter Crm1/exportin 1. However, the CLS sequence does not bear a resemblance to the leucine-rich class of nuclear export signal, and mutagenesis analysis indicated that a stretch of nonhydrophobic amino acids is essential for its activity. Oxidative stressors aborted the CLS activity and induced nuclear accumulation of Bach2. Whereas oxidative stress is known to activate MARE-dependent transcription, overexpression of Bach2 in cultured cells silenced the inducibility of MARE. The results suggest that Bach2 mediates nucleocytoplasmic communication to couple oxidative stress and transcription repression in mammalian cells.

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† The abbreviations used are: bZip, basic region-leucine zipper; ARE, antioxidant response element; AP-1, activating protein-1; CLS, cytoplasmic localization signal; CNC, Cap’n’ collar; DEM, diethylmaleate; LMB, leptomycin B; MARE, Maf recognition element; NES, nuclear export signal(s); NLS, nuclear localization signal; Nrf2, NF-E2-related factor 2; redox, reduction-oxidation; PCR, polymerase chain reaction; GFP, green fluorescent protein; DBD, DNA binding domain.

Oxidative insults elicit various responses within cells including defense and cell death (1–4). To understand the mechanism by which cells transmit signals to nuclei in response to oxidative changes, it is important to identify specific regulatory targets of oxidative stress. In yeast, basic region-leucine zipper (bZip) transcription factors yAP-1 and Pap1, which are related to the vertebrate activating protein-1 (AP-1), play key roles in the oxidative stress response in Saccharomyces cerevisiae and Schizosaccharomyces pombe, respectively (5–10). However, the mechanism by which mammalian cells transmit signals to the nuclei in response to oxidative changes is largely unknown, because there are only a few specific regulatory targets of oxidative stress thus far identified. Among them are three well characterized transcription factors: NF-κB, AP-1, and the glucocorticoid receptor, whose activities are regulated by reduction-oxidation (redox) changes (11–13). Another interesting example is the vertebrate bZip transcription factor Nrf2 (14).

Analyses of Nrf2, yAP-1, and Pap1 have revealed the importance of translocation of the regulators between cytoplasm and the nucleus in response to oxidative stress. Nrf2 is essential for the antioxidant-responsive element (ARE)-mediated induction of phase II detoxifying and oxidative stress enzyme genes in mice (15). ARE is also known as an electrophile-responsive element (16). The activity of Nrf2 is repressed by binding to the cytoplasmic inhibitory factor, Keap1 (17). Oxidative stress liberates Nrf2 from Keap1, allowing Nrf2 to transverse to the nuclei and to activate ARE/MARE-dependent gene expression. Thus, the Keap1-Nrf2 complex is believed to constitute a cytoplasmic sensor system for oxidative stress (17). In contrast, both yAP-1 and Pap1 activities are regulated by a conditional nuclear export. In addition to a nuclear localization signal (NLS), they contain a nuclear export signal (NES) at their C termini (18–20), which resembles the leucine rich-class of NES (18, 20). yAP-1 and Pap1 interact with the nuclear export factor Crm1/exportin 1 through the NES and are exported from the nuclei under normal growth conditions. Upon oxidative stress, the NES-dependent nuclear export of yAP-1 and Pap1 is specifically inhibited, enabling them to accumulate in the nuclei and to execute an oxidative stress response (9, 18–21). The presence of cysteine residues within the NES have been suggested to confer the oxidative stress sensitivity (18, 20). Such a conditional nuclear export regulated by oxidative stress may be widespread in eukaryotes (18). However, there is no known evidence among higher eukaryotes.

The mammalian transcription factors Bach1 and Bach2 (22) belong to the Cap’n’Collar (CNC)-related bZip family that also includes Nrf2 (14, 23, 24). They form heterodimers with the Maf-related bZip proteins and thus bind to a Maf recognition element (MARE; Ref. 25), which resembles the ARE (16, 25–27), and a 12-O-tetradecanoylphorbol-13-acetate-responsive element, a binding motif for AP-1 transcription factors (25). The
similar specificity of the CNC-related factors in Maf interaction and DNA binding suggests that, besides Nrf2, some of them may be involved in the oxidative stress response as well.

Expression of Bach2 is specific to neural tissues and B lymphoid cells (22, 28). In these cells, Bach2 generates the major binding activity to MARE by dimerizing with a small Maf protein. To understand biological roles for Bach2, it is essential to place Bach2 within biological processes with known biochemical function. We report here that subcellular localization of Bach2 is regulated by a conditional nuclear export that is sensitive to oxidative stress. Mutagenesis analysis revealed that this regulation is operative through an evolutionarily conserved motif on Bach2. In contrast to Nrf2, Bach2 counteracted oxidative stress inducibility of MARE. The results suggest that Bach2 provides a simple mechanism that couples oxidative stress and transcription repression through MARE in mammalian cells.

EXPERIMENTAL PROCEDURES

Plasmids—Bach2 expression plasmid (pCMVBach2F) was described previously (22). The Bach2 cDNA sequence lacking coding domain for the C-terminal 21 amino acid residues was isolated as a EcoRI fragment from Bach2/F cDNA and was inserted into the Smal site of pCMV/KM, resulting in pCMVBach2A1. A stop codon was inserted into the EcoRI site of pCMV/KM cDNA so that the most C-terminal 21 amino acid residues were deleted using PCR. The mutagenic PCR primer was 5′-ctagctgaagctgagttc-3′ (the stop codon is underlined). The resulting PCR product was inserted into pGEM-T (Promega) and sequenced to confirm the mutation. The resulting mutated cDNA was isolated by XbaI/EcoRI digestion and inserted into the Smal site of pCMV/KM. Green fluorescent protein (GFP) fusion plasmids were constructed as follows. EcoRI 3.1-kilobase pair DNA was isolated from pBSBach2F and was inserted into the EcoRI site of pEGFPC1 (CLONTECH), resulting in pEGFPBach2 that encoded a fusion protein of EGFP and Bach2 (from amino acid residue 148 to 839). A 2.8-kilobase pair EcoRI DNA from the pCMVBach2/F vector was also inserted into the EcoRI site of pEGFPC2, resulting in pEFGBach2/C2 that encoded a fusion protein of EGFP and a portion of Bach2 (residues 148–818). Fusion proteins with the GALA DNA binding domain (DBD) and GFP were constructed as follows. First, a 0.8-kilobase pair HpaI DNA fragment was isolated from plasmid pBSBach2/F and inserted in the BamHI site of pGBT9 (CLONTECH) after blunting the DNA ends, resulting in a fusion of GAL4 DBD and the C-terminal 97 amino acid residues of Bach2 (pGBTB2C). Similarly, a 0.7-kilobase pair HpaI–EcoRI DNA fragment was isolated from plasmid pBSBach2/C2 and inserted in the BamHI site of pGAL3, resulting in a fusion of GAL4 DBD and the C-terminal 76 amino acid residues of Bach2 (pGALB2C). The GAL4 DBD and its fusion DNA were isolated by PCR, digested with KpnI, and ligated into the EcoRI site of pEFGBach2/C2. Primers for the PCR were 5′-ttttttaattataagctgagttc-3′ (the initiation methionine for GAL4 is underlined) and 5′-ttttttagccgaggtattttgttc-3′. The resulting PCR products were pEGFPG4, pEGFPG4B2C, and pEGFPG4B2C/AC2 and encoded GFP fused to GAL4 DBD, GAL4 DBD/Bach2 (residues 743–839), and GAL4 DBD/Bach2/C2 (residues 743–818), respectively.

To construct a fusion of GFP and Bach2 cytoplasmic localization signal (CLS), the C-terminal coding domain (amino acids 769–839) was amplified by PCR. The 5′ primer was 5′-tttttttattttattttctgcagagccaggagggc-3′, and the 3′ primer was 5′-tttttttattttattttactgcagagttc-3′. The PCR product was digested with PstI and BamHI and ligated with PstBamHI-digested pEGFPC1, resulting in pEGFPCLS.

Single amino acid substitution mutations were generated on pBSBach2/F by a site-directed mutagenesis system (CLONTECH) using mutagenic oligonucleotide primers. The primers were as follows: D815A, 5′-cagttctgatccgtctgctgagctgagtccaggtgagtccagggccc-3′; S820A, 5′-gtggatctgatccgtctgctgagctgagtccagggccc-3′; M823A, 5′-gaattcctgatccgtctgctgagctgagtccagggccc-3′; K826A, 5′-caaggaatcctgatccgtctgctgagctgagtccagggccc-3′; C820A, 5′-caaggaatcctgatccgtctgctgagctgagtccagggccc-3′; K826A, 5′-caaggaatcctgatccgtctgctgagctgagtccagggccc-3′; S820A, 5′-caaggaatcctgatccgtctgctgagctgagtccagggccc-3′; X827A, 5′-gaattcctgatccgtctgctgagctgagtccagggccc-3′; D820A, 5′-caaggaatcctgatccgtctgctgagctgagtccagggccc-3′; S820A, 5′-caaggaatcctgatccgtctgctgagctgagtccagggccc-3′; C820A, 5′-caaggaatcctgatccgtctgctgagctgagtccagggccc-3′; K826A, 5′-caaggaatcctgatccgtctgctgagctgagtccagggccc-3′; S820A, 5′-caaggaatcctgatccgtctgctgagctgagtccagggccc-3′; D820A, 5′-caaggaatcctgatccgtctgctgagctgagtccagggccc-3′; S820A, 5′-caaggaatcctgatccgtctgctgagctgagtccagggccc-3′.

Plasmids encoding amino acids 148–839 with the mutations were introduced into the EcoRI site of pEGFPC1, resulting in GFP fusion expression plasmids.

pXHC1 for cytomegalovirus promoter-directed expression of wild-type human CRM1 was constructed in pEGFP-N1 (CLONTECH) by replacing the GFP open reading frame with the CRM1 open reading frame. pXHC1 for LMB-resistant human CRM1 containing an amino acid replacement at Cys-528 with Ser, the human equivalent of the LMB-resistant fusion yeast Crn1-K1 (29), was constructed based on pXHC1 using PCR-mediated mutagenesis with primers 5′-gttcatctctgttatttttggatccctaggcataatctgactttgtcag-3′ and 5′-gtcttttttttggatccctaggcataatctgactttgtcag-3′ (mutations are underlined).

Immunocytochemistry—QT-6 cells were transfected with the Bach2 expression plasmids and cultured for 24–36 h. The cells were fixed with methanol and blocked in 2% goat serum in phosphate-buffered saline. The fixed cells were reacted for 1 h at room temperature with the anti-Bach2 antiserum (22) and then with the fluorescein isothiocyanate-conjugated anti-rabbit IgG (Zymed Laboratories Inc.). After washing, cells were stained with Hoechst 33342 and mounted in fluorescent mounting medium (DAKO). The cells were then examined by fluorescence microscopy.

GFP Observations—MNS-8 cells (30) were maintained in a medium containing a 1:1 mixture of Dulbecco’s modified Eagle’s medium and F-12 nutrients supplemented with 10% fetal bovine serum and 5% horse serum. MNS-8 cells or QT-6 cells were transfected with the GFP fusion expression plasmids and cultured for 36 h. Subcellular localization of GFP fusion proteins was examined using a fluorescence microscopy without fixation or with fixation in 4% paraformaldehyde. To prepare transfected cells, cells were treated with 10 ng/ml of leptomycin B or 1–2 μM of diethyl maleate (Sigma) for the indicated periods at the end of incubation.

Reporter Assays—QT-6 cells were seeded in 24-well dishes 24 h before transfection. The cells were transfected with the reporter and effector plasmids by the calcium phosphate precipitation method as described previously (23). After incubating cells with the DNA precipitate for 8 h, cells were washed in PBS and fed with fresh medium with or without 166 μM of diethyl maleate (Wako). Preparation of cell lysates was performed with the Luciferase Assay System (Promega) following the supplier’s protocol. Luciferase activities were measured with a Biotum Luminoimeter (Berthold). Firefly luciferase activity was normalized for transfection efficiency as determined by a control sea panay luciferase activity (28). The independent experiments, each carried out in duplicate, were performed, and the results were averaged and diagrammed with the S.E. values.

RESULTS

A Novel Domain Directs Bach2 to Cytoplasm—During analysis of subcellular localization of Bach2, we noticed that Bach2 localized in the cytoplasmic region and was excluded from the nuclear compartment within transfected cells (see Fig. 1B). The accumulation of Bach2 within cytoplasm suggests that the nuclear localization of this transcription factor might be negatively regulated. To test this hypothesis, we first examined subcellular localization of Bach2 and its derivatives using antibody staining after transfecting expression plasmids into quail fibroblast QT-6 cells. The wild-type Bach2 protein was localized in the cytoplasmic region and often excluded from the nuclei (Fig. 1, A and B). Thus, Bach2 appeared to contain a domain that inhibits nuclear accumulation. There are three conserved regions between Bach1 and Bach2, i.e. the BTB domain, the bZIP domain, and the most C-terminal region (Fig. 1A). Because no function has been assigned to the C-terminal region, we examined its possible involvement in the cytoplasmic localization of Bach2. Bach2ΔC1 lacked the C-terminal 97 amino acid residues, whereas Bach2ΔC2 lacked the conserved C-terminal 21 amino acid residues (Fig. 1A). Surprisingly, the C-terminal deletions led to accumulation of Bach2 in the nucleus in almost every transfected cell (Fig. 1, C and D). Virtually no signal was detected in the cytoplasmic region of transfected cells. Quantitative analyses of subcellular localization of Bach2 and its derivatives were shown in Fig. 1E and clearly indicate an important role for the C-terminal region in determining subcellular localization.

Bach2 expression is specific to neural tissues and B lymphoid cells (22, 28). To assess the function of the C-terminal region of Bach2 in living neural cells, we fused GFP and a portion of Bach2 with and without the C-terminal 21 amino acid residues
FIG. 1. The C-terminal region of Bach2 regulates its subcellular localization. A, schematic alignment of mouse Bach1 and Bach2 indicating three conserved regions: the BTB/POZ domain, CNC-related bZip domain, and the most C-terminal region. The C-terminal 97 or 21 amino acid residues were deleted in Bach2ΔC1 and ΔC2, respectively.

B–D, transfected QT-6 cells were examined for subcellular localization of Bach2 and its derivatives using antibody staining (left), or location of nuclei using Hoechst 33342 staining (right). E, wild-type Bach2; C, Bach2ΔC1; D, Bach2ΔC2. Results of quantitative analysis of subcellular localization of Bach2 and its derivatives. More than 200 transfected cells were observed for each expression plasmid and classified into three different categories: C > N, cytoplasmic dominant staining; C = N, roughly equal distribution between cytoplasmic and nuclear compartments; C < N, nuclear dominant staining.

The C-terminal region of Bach2 may inhibit nuclear localization of Bach2 by masking its NLS. In this case, the C-terminal region would function only in a specific protein context (i.e., on Bach2). To examine whether the C-terminal region is functional in a different protein context, we utilized a fusion protein of GFP and the DNA binding domain of yeast GAL4 (GG4) and its derivatives (Fig. 2A). When expressed in QT-6 cells, GG4 accumulated within nuclei, indicating that the GAL4 nuclear localization signal is functional in QT-6 cells (Fig. 2, C and D).

This nuclear accumulation was inhibited significantly by further fusing the C-terminal 97 amino acid residues of Bach2 that were missing in Bach2ΔC1 (GG4B2C; Fig. 2, C and D, and see Fig. 1A). A deletion of the C-terminal 21 amino acid residues (similar to that in Bach2ΔC2) eliminated the nuclear exclusion of the fusion reporter protein (GG4B2CΔC2; Fig. 2, C and D). Thus, the C-terminal region can impart cytoplasmic localization to a heterologous nuclear protein.

Based on these observations, we conclude that the subcellular localization of Bach2 is regulated by two components; one is the NLS within the basic region, and the other is the C-terminal region that autonomously directs the cytoplasmic localization of Bach2. We refer to the C-terminal region as CLS. A homology search of databases with the CLS sequence revealed no other protein with this motif except Bach1 and Bach2.

Leptomycin B-sensitive Nuclear Export of Bach2—The steady state nuclear accumulation of Bach2 could be regulated by either a conditional nuclear import or export. To distinguish these two possibilities, we examined the effect of leptomycin B (LMB). LMB binds to the nuclear export carrier protein Crm1/
export 1 and inhibits NES-dependent protein export (29, 31–34). QT-6 cells were transfected with the GFP-Bach2 fusion protein expression plasmid and then treated with LMB. As shown in Fig. 3, LMB induced rapid and efficient accumulation of GFP-Bach2 fusion protein within the nuclei. Similar results were obtained when the nonfusion, wild-type Bach2 was expressed and detected with an antibody (data not shown). The results indicate that Bach2 is normally exported from the nuclei through a LMB-sensitive export pathway. Since CLS of Bach2 was essential to impart cytoplasmic localization onto the heterologous reporter protein (Fig. 2), we conclude that CLS is responsible for the nuclear export of Bach2 (see below).

Oxidative Stress-regulated Nuclear Export of Bach2—Because Bach2 functions as a transcription regulator (22), the nuclear exclusion of Bach2 appeared conditional. Considering the fact that Nrf2 and MARE are involved in oxidative stress response (15, 17), we examined the effect of oxidative stressor diethylmaleate (DEM) on subcellular localization of the GFP-Bach2 reporter protein. As shown in Fig. 3C, DEM induced efficient nuclear accumulation of GFP-Bach2 in QT-6 cells. This nuclear accumulation was very rapid and was evident within 30 min after treatment with DEM. Within 1 h, almost every transfected cell accumulated GFP-Bach2 fusion protein within the nucleus and gave virtually no signal in the cytoplasmic region. Other reagents like dibutyryl cAMP, phorbol 12-myristate 13-acetate, and ionomycin did not show any effect on the cytoplasmic localization of GFP-Bach2 (data not shown). The results indicate that nuclear accumulation of Bach2 is induced by oxidative stress.

DEM may abolish cytoplasmic localization of Bach2 by either stimulating the nuclear import or inhibiting the nuclear export. To address this issue, we developed an assay system to monitor nuclear export activity of CLS independent of NLS activity. In this system, we used GFP as a reporter protein that localizes in both cytoplasmic and nuclear compartments by 20 min after transfection (time 0), GFP-CLS showed clear nuclear exclusion, whereas it was obtained when the nonfusion, wild-type Bach2 was expressed and detected with an antibody (data not shown). The results indicate that nuclear accumulation of Bach2 is induced by oxidative stress.

Nonfusion GFP was distributed in both nuclei as well as cytoplasm (data not shown), whereas the GFP-CLS reporter protein showed efficient nuclear exclusion (Fig. 4, B–D, time points 0). Time-lapse observation of transfected cells revealed that GFP-CLS redistributed in nuclei upon treatment with LMB. Nuclear accumulation was evident by 10 min after LMB treatment and completed by 20 min. These observations verified that the GFP-CLS fusion protein allows monitoring of nuclear export activity of CLS in living cells. Using this system, we examined the effect of DEM on the nuclear export activity of CLS. Time-lapse observations of the living transfected cells indicated that DEM induced rapid nuclear accumulation of GFP-CLS (Fig. 4C). Treatment of transfected cells with hydrogen peroxide also induced rapid nuclear accumulation of GFP-CLS (Fig. 4D). GFP-CLS did not show nuclear accumulation upon mock treatment (Fig. 4E). Thus, we conclude that the nuclear export mediated by Bach2 CLS is inhibited under oxidative stress conditions. Because a typical leucine-rich class of NES is not sensitive to oxidative stress in mammalian cells (18), the results suggest that Bach2 CLS directs mechanistically distinct nuclear exports.

CLS Contains Essential Cysteine and Nonhydrophobic Amino Acid Residues—One of the hallmarks of oxidative stress-sensitive NES of yAP-1 and Pap1 is that, in addition to the clusters of essential hydrophobic amino acids that are reminiscent of the typical NES, they contain juxtaposing essential Cys residues (18–20). A close inspection of the sequence of Bach2 CLS reveals that it contains two Cys residues that are well conserved among Bach1 and Bach2 from humans and mice (Fig. 5A and data not shown). Having approximately defined the CLS, we then examined the contributions of individual amino acid residues. The conserved residues were individually replaced with alanine in the context of the GFP-Bach2 fusion protein (Fig. 5A; see Fig. 2A for GFP-Bach2). Both of the C820A and C827A substitutions completely abolished cytoplasmic localization and resulted in a very efficient nuclear accumulation (Fig. 5, B and C; compare with Fig. 3A). Strikingly, most of the other conserved residues were also found to be essential for the cytoplasmic localization and nuclear exclusion (Fig. 5B). Thus, the CLS activity is based on an active nuclear export mecha-
Silencing of Oxidative Stress Response by Bach2

We show here that subcellular localization of Bach2 is regulated by LMB- and oxidative stress-sensitive nuclear export. This regulation is operative through the C-terminal conserved sequence of Bach2 (i.e. CLS). On the basis of the findings described here, we suggest that Bach2 functions as a biological switch that processes and transduces oxidative stress into nuclei (Fig. 8). In this model, we suggest that, although Bach2 contains both an NLS and CLS that mediates nuclear export, the CLS is more active than the NLS under normal conditions, resulting in net cytoplasmic localization of Bach2. Oxidative stress inhibits CLS activity to shift the balance between nuclear import and export activities of Bach2. Resulting rapid

DISCUSSION

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FIG. 5. Cys residues are essential for CLS activity. A, comparison of mouse Bach2 CLS with the corresponding region of mouse Bach1. B, effects of single amino acid substitution mutations on the CLS activity. Comparison of C-terminal sequences of mouse Bach1 and Bach2 is shown above. Identical residues are indicated with asterisks. The ΔC2 deletion junction is shown with an arrow for reference. Below are the individual mutations and their effects on the CLS activity as follows: C, mainly cytoplasmic with clear nuclear exclusion; N, exclusively nuclear. C. QT-6 cells were transfected with the GFP-Bach2C827A expression plasmid, and GFP was observed after fixation (left). Nuclei was stained with Hoechst 33342 (right).

FIG. 6. Crm1 directs nuclear export of Bach2. QT-6 cells were co-transfected with expression plasmids of GFP fusion reporter proteins in combination with expression plasmids for either wild-type hCrm1 or LMB-insensitive mutant hCrm1-K1. Cells were treated with 20 ng/ml of LMB for 8 h and then observed using inverted fluorescent microscopy. A, hCrm1 and GST-SV40 T NLS-GFP-Rev NES (G-NLS-F-NES; Ref. 33); B, hCrm1-K1 and G-NLS-F-NES; C, hCrm1 and GFP-Bach2; D, hCrm1-K1 and GFP-Bach2.

nism operating on the C-terminal conserved sequence containing a stretch of essential nonhydrophobic residues. This is in clear contrast to the typical LMB-sensitive NES that contains a cluster of essential hydrophobic residues without apparent contribution of hydrophilic residues (see “Discussion”).

Crm1 Directs CLS-mediated Nuclear Export—The above results indicated that CLS possessed sequence characters distinct from known NES, while its function was LMB-sensitive. To examine directly whether Crm1 can direct nuclear export of Bach2, we utilized a modified version of Crm1. A Cys residue at position 529 of S. pombe Crm1 is involved in LMB binding, and its substitution renders Crm1 insensitive to LMB (29). Activities of such an LMB-resistant Crm1 can be examined in the presence of LMB, which inhibits endogenous Crm1. A corresponding substitution was introduced into human Crm1 expression plasmid. The modified version of Crm1, hCrm1-K1, possessing Ser instead of Cys at the 529th position, can direct LMB-insensitive nuclear export of NES-bearing proteins when expressed in mammalian cells.2 Upon co-transfection of the expression plasmids, hCrm1-K1 directed nuclear export of a fusion protein of GST, NLS of large T antigen, GFP, and NES of Rev (G-NLS-F-NES; Ref. 33) even in the presence LMB, while wild-type hCrm1 failed to do so (Fig. 6, A and B). Thus, hCrm1-K1 was insensitive to LMB as expected. Using this system, we examined effects of hCrm1-K1 on the subcellular localization of GFP-Bach2. Although GFP-Bach2 accumulated within nuclei in the presence of LMB when expressed alone (see Fig. 3B) or with wild-type hCrm1 (Fig. 6C), GFP-Bach2 localized to the cytoplasmic region when co-expressed with hCrm1-K1 (Fig. 6D). These results clearly established that Crm1 mediated the nuclear export of Bach2.

Bach2 Antagonizes Oxidative Stress-induced Gene Expression—The functional contribution of Bach2 during oxidative stress response was examined in transfection reporter assays in QT-6 cells. As envisaged from the similarity between MARE and ARE (17, 25, 27), MARE was found to function as an oxidative stress response element (17). Treatment of the transfected cells with DEM induced the MARE-dependent reporter gene expression (Fig. 7A). QT-6 cells appear to contain an endogenous factor(s) that is induced upon oxidative stress and activates transcription by binding to MARE. This factor might be Nrf2 (17). Bach2 was found to efficiently silence the DEM inducibility in a dose-dependent manner (Fig. 7B). As a control, we examined effects of oxidative stress and Bach2 on a reporter plasmid containing mutated MARE (Fig. 7B). The control reporter showed a poor induction (only 3-fold) upon DEM treatment. Bach2 did not show any repressive effect on the reporter gene activity and caused slight activation. This binding site-independent activation by Bach2 was also observed when we tested the response of another reporter gene containing binding sites for nuclear hormone receptors (Fig. 7C). Because both Bach2 and nuclear hormone receptors are known to bind to co-repressors like the silencing mediator of retinoic acid and thyroid hormone receptor (28, 35), the binding site-independent activation may reflect squelching of co-repressors. Taken together, these results suggest that Bach2 functions as a transcription repressor through MARE under oxidative stress conditions. As reported previously (22), Bach2 repressed the basal activity of the MARE reporter gene in the absence of DEM (Fig. 7A). This repression may reflect the presence of a small fraction of Bach2 within nuclei.

FIG. 6. Crm1 directs nuclear export of Bach2. QT-6 cells were co-transfected with expression plasmids of GFP fusion reporter proteins in combination with expression plasmids for either wild-type hCrm1 or LMB-insensitive mutant hCrm1-K1. Cells were treated with 20 ng/ml of LMB for 8 h and then observed using inverted fluorescent microscopy. A, hCrm1 and GST-SV40 T NLS-GFP-Rev NES (G-NLS-F-NES; Ref. 33); B, hCrm1-K1 and G-NLS-F-NES; C, hCrm1 and GFP-Bach2; D, hCrm1-K1 and GFP-Bach2.

2 N. Kudo and M. Yoshida, unpublished results.
nuclear accumulation may allow Bach2 to regulate gene expression in response to oxidative stress.

Although the above model is similar to those proposed for yAP-1 and Pap1 (9, 18–21), detailed mechanisms appear distinct. Both yAP-1 and Pap1 possess sequences that are similar to a typical leucine-rich NES (18–20). In contrast, the Bach2 CLS lacks a cluster of essential hydrophobic amino acid residues such as LXXLXXXL motif, which is the consensus sequence for the LMB-sensitive, leucine-rich class of NES (36, 37). Rather, it contains several essential nonhydrophobic amino acid residues including Cys. Nonconsensus NES sequences that are inhibited by LMB have been described, suggesting that there is significant flexibility in the NES sequences bound by Crm1/exportin 1. Like the consensus NES, however, these atypical NES also contain clusters of hydrophobic amino acids (38). In contrast, our results indicated that nonhydrophobic residues are important for LMB-sensitive nuclear export of Bach2. Furthermore, unlike the leucine-rich class of NES, the entire conserved CLS sequence was virtually essential for nuclear export activity. Whereas Bach2 CLS is quite different from known NES in sequence characters, the results described in Fig. 6 clearly indicate that LMB-insensitive Crm1 can rescue nuclear export of Bach2 even in the presence of LMB. Together with the fact that that Crm1 is the only LMB-binding protein in HeLa cells (29), our results indicate that nuclear export of Bach2 is mediated by Crm1. Although CLS itself could be an NES, at present we cannot exclude the possibility that it mediates interactions with other NES-carrying proteins. Further analysis is necessary to clarify this issue and will lead to identification of a novel regulatory mechanism of nuclear export.

Functional significance of the CLS is reinforced by the fact that this region is highly conserved among Bach1 and Bach2 from humans and mice (22). This conservation suggests that Bach1 activity is also regulated by a similar mechanism. Indeed, we recently found that Bach1 is mainly cytoplasmic in transfected cells and that DEM induces its nuclear accumulation. A comparison of transcriptional activity of Bach2 with those of yeast AP-1 factors and mammalian Nrf2 suggests a unique role for Bach2. During oxidative stress, yeast AP-1 factors activate transcription of oxidative stress-inducible genes (5–9). In mice, Nrf2 functions as an activator of oxidative stress-inducible genes (15, 17). In contrast, our results suggest that Bach2 functions primarily as an inhibitory factor to repress a set of genes during oxidative stress. Because Nrf2 and Bach2 can compete for both Maf family partner proteins and target DNA sequences (14, 22, 23), combinatorial usage of such factors may generate diversity in oxidative stress response in various cell lineages. This is an interesting possibility because it is well known that different cells show distinct responses toward oxidative stress and electrophiles. For example, it has been reported that ARE-mediated gene regulation in neuroblastoma cells involves brain-specific transcription factors and/or signal transduction cascades (39). Because expression of Bach2 is specific to B cells and neural cells (22, 28), Bach2 may set thresholds for cellular reactions upon oxidative stress in these lineages, predisposing cells toward a certain outcome by inhibiting deployment of defense reactions. Future analyses of Bach2 may open opportunities to shed light on novel aspects of relationships among oxidative stress, gene expression, and development.

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