Bach1-dependent and -independent Regulation of Heme Oxygenase-1 in Keratinocytes*

Shukio Okada†, Akihiko Muto†, Eisaku Ogawa†, Ayako Nakanome§, Yasutake Katoh†, Shuntaro Ikawa*, Setsuya Aiba†, Kazuhiro Igarashi†, and Ryuhei Okuyama†\‡

From the Departments of †Dermatology and §Biochemistry, Tohoku University Graduate School of Medicine, Sendai 980-8574, the †Ikawa Group, Center for Interdisciplinary Research, Tohoku University, Sendai 980-8578, and the †Department of Dermatology, Shinshu University School of Medicine, Matsumoto 390-8621, Japan

Bach1 is a member of the basic leucine zipper transcription factor family, and the Bach1/small Maf heterodimer specifically represses transcriptional activity directed by the Maf recognition element (MARE). Because Bach1 is a repressor of the oxidative stress response, we examined the function(s) of Bach1 in keratinocytes subjected to oxidative stress. Oxidative stress induced by H2O2 led to an increase in MARE activity and expression of heme oxygenase-1 (HO-1), an inducible antioxidant defense enzyme. Bach1 depletion by small interfering RNAs or by deletion of Bach1 enhanced HO-1 expression in the absence of H2O2, indicating that Bach1 is a critical repressor of HO-1 in keratinocytes. Although Bach1-deficient or -reduced keratinocytes expressed higher levels of HO-1 than control cells in response to H2O2, Bach1 down-regulation did not attenuate the production of reactive oxygen species by H2O2. In contrast, Bach1 overexpression abolished HO-1 induction by H2O2, which led to increased reactive oxygen species accumulation. HO-1 was induced during keratinocyte differentiation, but MARE activity did not change during differentiation. Furthermore, Bach1 overexpression did not inhibit differentiation-associated induction of HO-1 expression, suggesting that HO-1 induction in differentiation is independent of Bach1. Thus, in response to oxidative stress, Bach1 regulates the oxidation state through the negative control of HO-1 expression prior to terminal keratinocyte differentiation. However, Bach1-mediated repression is negated during keratinocyte differentiation.

Redox regulation is critical to cell survival, because cells cannot avoid endogenous reactive oxygen species (ROS)2 that are generated as by-products of respiration (1). The skin is constantly exposed to harmful ROS that are generated by pro-oxidant agents in the environment, as well as by endogenous cellular oxidants. Thus, keratinocytes strongly and constitutively express ROS-detoxifying enzymes, including superoxide dismutase, catalase, glutathione peroxidase, and reductase, and contain substantial levels of the antioxidants tocopherol and ubiquinol (2, 3). Additionally, these cells possess an inducible defense system that includes heme oxygenase-1 (HO-1) (3–5). An imbalance between ROS and antioxidants can lead to the generation of high oxidant levels, which play a pivotal role in skin aging and disease (6).

The transcription factor Bach1 is a repressor of the oxidative stress response in higher eukaryotes (7, 8) and, together with its parologue Bach2, constitutes a subfamily of the basic leucine zipper family of proteins. Bach1 forms heterodimers with the basic leucine zipper subfamily of small Maf proteins (i.e. MafF, MafG, and MafK), which bind the Maf recognition element (MARE) in the promoter regions of genes such as HO-1, NADP(H) quinone (oxidoreductase), and β-globin (7, 9, 10), and thereby repress transcription in the absence of oxidative stress. In the presence of oxidative stress, Bach1 is inactivated (11, 12), which allows transcriptional activation of these genes by Nrf2 and other activators that also form heterodimers with small Maf proteins (7, 13). Thus, redox regulation is partly mediated by reciprocal DNA-MARE binding by the Bach1 repressor and other transcriptional activators.

Heme oxygenases HO-1 and HO-2 are the rate-limiting enzymes in heme degradation (14). HO-1 is an inducible enzyme, and HO-2 is constitutively expressed. Heme is a critical prosthetic group for many organisms and is synthesized in virtually all cells. However, free heme is a potent pro-oxidant that catalyzes the formation of ROS by the Fenton reaction (14). HO-1 converts excess heme into ferrous iron, carbon monoxide, and biliverdin, which is reduced to bilirubin by biliverdin reductase. Carbon monoxide and biliverdin, as well as bilirubin, have anti-oxidant and anti-inflammatory properties in vivo (15, 16), suggesting a role for HO-1 in the conversion of heme into antioxidants for defense against ROS. The transcription of Hmox-1, which encodes HO-1, is induced by oxidative stress (14). As the inducible enhancers of Hmox-1 carry multiple MAREs, HO-1 transcription is regulated by the balance between Bach1 and transcriptional activators.

The epidermis constitutes the primary defense mechanism of the body against oxidative damage. HO-1 is induced during keratinocyte differentiation and is highly expressed in the upper layers of the epidermis (17). However, the mechanism of Bach1-mediated HO-1 regulation in keratinocytes...
remains unclear. Here, we investigated the role of Bach1 in HO-1 regulation and ROS production.

EXPERIMENTAL PROCEDURES

Plasmids and Antibodies—The Bach1 expression plasmid (plasmid pCMV/Bach1) has been described previously (8). The pRBGP3 and pRBGP2 plasmids contain no copies or three copies, respectively, of the MARE region (5′-TCG ACC CGA AAG GAG CTG ACT CAT GCT AGC CC-3′) upstream of the thymidine kinase promoter in pGL2-TK (18). The reporter plasmids for the regulatory regions of HO-1 (pHO15luc) and Blimp1 possess MARE regions (10, 19, 20). The pHO15luc reporter plasmid contains the 15 kb of DNA upstream of the mouse HO-1 gene and was previously shown to recapitulate inducible expression of the HO-1 gene in response to heme, cadmium, and H2O2 (19). Polyclonal antisera against Bach1 (A1–6) was produced as described previously (21). The following antibodies were purchased and used as recommended by their suppliers: rabbit anti-HO-1 (Abcam, Tokyo, Japan); goat anti-lamin B (Santa Cruz Biotechnology, Tokyo, Japan); and mouse monoclonal anti-α-tubulin (Sigma). Horseradish peroxidase-conjugated goat anti-mouse IgG, goat anti-rabbit IgG (Amersham Biosciences), and rabbit anti-goat IgG (Santa Cruz Biotechnology) were used as secondary antibodies.

Mice, Cell Culture, Transient Transfection, and Adenoviral Infection—Primary keratinocytes were prepared from newborn ICR mouse epidermis, as described previously (22). In brief, the epidermis was separated from the dermis with 0.25% trypsin (Invitrogen) overnight at 4°C, plated in dishes precoated with type I collagen (Nitta Gelatin, Osaka, Japan), and cultured in minimum essential medium supplemented with 4% Chelex-treated fetal calf serum, epidermal growth factor (10 ng/ml; Invitrogen), and 0.05 mM CaCl2. Under these conditions, keratinocytes were maintained in an immature state, and differentiation was induced by addition of CaCl2 to a final concentration of 2 mM, as described elsewhere (23).

Primary keratinocytes were also prepared from the newborn mouse epidermis of Bach1−/− mice and Bach1+/+ mice using the procedure described above. The generation of Bach1−/− mice has been described previously (10). The study was carried out in accordance with international ethical guidelines for laboratory animals and was approved by the Institutional Animal Care and Use Committee. The following reagents were purchased from the indicated commercial sources: H2O2 (Wako, Osaka, Japan), doxycycline and the γ-secretase inhibitor N-[N-(3,5-difluorophenacyl)-l-alanyl]-S-phenylglycine t-butyl ester (Sigma), H2DCFDA (Invitrogen), the JNK inhibitor II, protein kinase C (PKC) α/β inhibitor Gö6976, the PKCβ/θ inhibitor rotterlin (Merck), the ERK inhibitor PD98059, and p38 MAPK inhibitor SB203580 (Cell Signaling Technology, Tokyo, Japan). The ATP assay kit was performed according to the supplier’s instructions (Bioassay Systems, Hayward, CA).

Transfections were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. To assess promoter activity, transfected keratinocytes were subjected to a Dual-Luciferase reporter assay (Promega, Tokyo, Japan) 72 h after transfection. Relative luciferase activities were normalized to Renilla luciferase activity. The small interfering RNAs (siRNAs) specific to Bach1 (B-1, B-2, and B-3) were purchased from Invitrogen, together with a stealth siRNA as a control. The HO-1-specific siRNA sequences were as follows: HO-1 siRNA1, 5′-AUC ACC AGC UUA AAG CCU UCU CUG G-3′; HO-1 siRNA2, 5′-CCA GAG AGC GCU UUA AGC UGG UGA U-3′. Recombinant adenoviruses expressing either Bach1 (Ad-Bach1) or lacZ (Ad-LacZ) were generated as described previously and used at a multiplicity of infection (m.o.i.) of 25 (22, 24). Ad-LacZ was used as a control. Bach1 expression was induced by a regulatory virus (Ad-T0; Adeno-X Tet-On; Clontech) with the addition of doxycycline (100 ng/ml).

Immunoblotting—Total cell proteins were extracted from cultured keratinocytes as described previously (22). Nuclear proteins were purified according to the manufacturer’s protocol (nuclear extract kit; Active Motif, Tokyo, Japan). The extracted proteins were resolved by SDS-PAGE and transferred to an Immobilon-P membrane (Millipore, Tokyo, Japan). The membranes were blocked with TBST (50 mM Tris, pH 7.5, 0.5% Tween 20) containing 5% nonfat dried milk, incubated with primary antibodies, rinsed with TBST, and incubated with horseradish peroxidase-conjugated secondary antibodies. After additional rinses, the blots were exposed to LumiGLO reagent (Cell Signaling Technology) and subsequently to x-ray film. Protein concentrations were normalized using a BCA protein assay (Pierce), and equal loading was assessed by α-tubulin immunoblot.
Bach1-dependent and -independent Regulation of HO-1

Real Time PCR—Total RNA was prepared from primary keratinocytes using the mRNAeasy mini kit (Qiagen, Tokyo) according to the manufacturer’s instructions. The cDNAs were synthesized using SuperScript II reverse transcriptase (Invitrogen) and 3 μg of total RNA as a template. Real time PCR analyses were performed with a Quantitect SYBR Green PCR kit (Qiagen) and an iCycler (Bio-Rad) using the following primers:

**Mcm5** promoter primers were as described previously (25). Bach1, 5′-GCC CGT ATG CTT GTG TGA TT-3′ and 5′-GCG GAC TTA TCT TAA ATG GCT-3′; and Nrf2, 5′-CAA GAC TTG GCC CAC TTA AAA GAC-3′ and 5′-AGT AAG GCT TTC CAT CCT CAC-3′.

Chromatin Immunoprecipitation (ChIP)—ChIP analyses were performed as described previously (25). In brief, keratinocytes were cross-linked with formaldehyde (1% final concentration) for 15 min at room temperature. Nuclear fractions were sonicated to break the DNA into fragments with an average length of ~200–500 bp and extracts subjected to immunoprecipitation with anti-Bach1 (A1–6) or normal rabbit serum. After releasing cross-linked DNA from the immunoprecipitated materials with 10 mM dithiothreitol, samples were incubated for 6 h at 65 °C to remove formaldehyde from the DNA. After purification with a QIAquick PCR purification kit (Qiagen), the recovered DNAs were subjected to PCR analyses. PCR analyses were performed using Takara LA Taq (Takara, Ohtsu, Japan) and an iCycler with primers capable of amplifying DNA fragments in the vicinity of the MARE motifs in the regulatory regions of HO-1, Blimp1, and Mcm5. The promoter of Mcm5, a gene adjacent to HO-1, was used as a control.

**H₂DCFDA Staining**—To quantify ROS, cultured keratinocytes were incubated with 100 or 500 μM H₂O₂ for 12 h and then stained with fluorescein isothiocyanate-conjugated H₂DCFDA. Analyses were performed using a FACS Calibur instrument with CellQuest software (BD Biosciences).

**Statistical Analyses**—Data were analyzed using an unpaired Student’s t test. Differences were considered statistically significant for p values less than 0.05.

**RESULTS**

**Inhibitory Effect of Bach1 on MARE Activity in Keratinocytes**—First, we examined the effect of Bach1 on transactivation in keratinocytes. The activity of Bach1 in keratinocytes was accessed by transfecting them with the MARE reporter pRBG2 and a Bach1 expression plasmid. Exogenous Bach1 suppressed MARE activity in a dose-dependent manner (Fig. 1A), suggesting that Bach1 acts to repress gene function. Bach1 did not affect the basal activity of the promoter (pRBG3 lacking MARE). Furthermore, we analyzed the effects of Bach1 on transactivation of HO-1 and Blimp1. Blimp1 is important for keratinocyte differentiation (26) and is repressed by Bach2, a parologue of Bach1, in B lymphoid cells (27). The HO-1 reporter pHO15Luc contains a region of 15 kb of DNA upstream of the murine HO-1 gene. The induction of HO-1 in response to heme releasing cross-linked DNA from the immunoprecipitated materials with 10 mM dithiothreitol, samples were incubated for 6 h at 65 °C to remove formaldehyde from the DNA. After purification with a QIAquick PCR purification kit (Qiagen), the recovered DNAs were subjected to PCR analyses. PCR analyses were performed using Takara LA Taq (Takara, Ohtsu, Japan) and an iCycler with primers capable of amplifying DNA fragments in the vicinity of the MARE motifs in the regulatory regions of HO-1, Blimp1, and Mcm5. The promoter of Mcm5, a gene adjacent to HO-1, was used as a control. The following primers were used for PCR amplification of reporters: HO-1 E1, 5′-TGA AGT TAA AGC CGT TCC GG-3′ and 5′-AGC GGC TTA TCT GCT TAG T-3′; HO-1 E2, 5′-GGG CTA GCA TGG GAA AG-3′ and 5′-AGA CTC CGC CCT AAG GGT TC-3′; Blimp1 promoter MARE, 5′-CTG ACA CTT ACA TGG TAT CTG TGC CC-3′ and 5′-AAAG TTC AGC GTC CTC GAG AGG-3′; and Blimp1 intron 5 MARE, 5′-GTT AAT CTT GTT CTC CGG TTG C-3′ and 5′-TCT TAA ATG GCT GTA GGC GGA C-3′. The Mcm5 promoter primers were as described previously (25).

**H₂DCFDA Staining**—To quantify ROS, cultured keratinocytes were incubated with 100 or 500 μM H₂O₂ for 12 h and then stained with fluorescein isothiocyanate-conjugated H₂DCFDA. Analyses were performed using a FACS Calibur instrument with CellQuest software (BD Biosciences).

**Statistical Analyses**—Data were analyzed using an unpaired Student’s t test. Differences were considered statistically significant for p values less than 0.05.

**RESULTS**

**Inhibitory Effect of Bach1 on MARE Activity in Keratinocytes**—First, we examined the effect of Bach1 on transactivation in keratinocytes. The activity of Bach1 in keratinocytes was accessed by transfecting them with the MARE reporter pRBG2 and a Bach1 expression plasmid. Exogenous Bach1 suppressed MARE activity in a dose-dependent manner (Fig. 1A), suggesting that Bach1 acts to repress gene function. Bach1 did not affect the basal activity of the promoter (pRBG3 lacking MARE). Furthermore, we analyzed the effects of Bach1 on transactivation of HO-1 and Blimp1. Blimp1 is important for keratinocyte differentiation (26) and is repressed by Bach2, a parologue of Bach1, in B lymphoid cells (27). The HO-1 reporter pHO15Luc contains a region of 15 kb of DNA upstream of the murine HO-1 gene. The induction of HO-1 in response to heme releasing cross-linked DNA from the immunoprecipitated materials with 10 mM dithiothreitol, samples were incubated for 6 h at 65 °C to remove formaldehyde from the DNA. After purification with a QIAquick PCR purification kit (Qiagen), the recovered DNAs were subjected to PCR analyses. PCR analyses were performed using Takara LA Taq (Takara, Ohtsu, Japan) and an iCycler with primers capable of amplifying DNA fragments in the vicinity of the MARE motifs in the regulatory regions of HO-1, Blimp1, and Mcm5. The promoter of Mcm5, a gene adjacent to HO-1, was used as a control.
MARE region (25). Bach1 repressed expression of both reporter genes (Fig. 1, B and C). Subsequently, we tested for binding of Bach1 to the HO-1 enhancer regions (E1 and E2) and Blimp1 regulatory regions (promoter MARE region and intron 5 MARE region (28)) by ChIP analysis (Fig. 1D). Bach1 bound to the E1 and E2 HO-1 enhancer regions. In contrast, no association of Bach1 with the Blimp1 regulatory regions was detected. However, we could not exclude the possibility of an association between Bach1 and the Blimp1 regulatory region. The association of MaFK with the Blimp1 regulatory region was much weaker than its association with the HO-1 enhancer, as detected by ChIP assay (data not shown). Therefore, the affinity of Bach1 for the Blimp1 regulatory region may be lower than that for the HO-1 enhancer.

Bach1 Regulation of HO-1 Expression in Response to Oxidative Stress—Because we detected a direct association between Bach1 and the HO-1 enhancer region, we next focused on oxidative stress-induced expression of HO-1. H2O2 increased both the amount of HO-1 protein (Fig. 2A) and the activity of the HO-1 enhancer region (Fig. 2B). MARE activation also increased in response to H2O2 (Fig. 2C). The increased MARE activity was consistent with HO-1 induction, because the HO-1 enhancer contains multiple MAREs. Bach1 activity is regulated at various levels, including ubiquitin-dependent degradation, nuclear localization, and changes in DNA affinity (7, 29). Next, we examined the amount of Bach1 in total and nuclear cellular fractions. Bach1 levels remained constant in total cellular fraction despite H2O2 addition and were slightly increased in nuclear cellular fraction (Fig. 2D), suggesting that neither degradation nor nuclear export contributed to the increase in MARE activation. Because Bach1 is regulated by the redox status of its cysteine residues, this regulation is likely in response to H2O2 (11).

In addition, we compared the levels of Bach1, Nrf2, HO-1, and HO-2 expression in primary keratinocytes and in primary dermal fibroblasts. Bach1 is expressed ubiquitously (8) and in keratinocytes (30, 31). Immunoblot analysis revealed that Bach1 levels were similar in primary keratinocytes and primary dermal fibroblasts (Fig. 2E). Nrf2 was also expressed at similar levels in both cell types. However, keratinocytes exhibited lower levels of HO-1 compared with dermal fibroblasts. Furthermore, lower HO-1 levels were present after treatment with H2O2 in keratinocytes (Fig. 2F). A similar level of HO-2 expression in these cells suggests that the differential expression of HO-1 is not a result of alterations in HO-2 expression. Although the levels of Bach1 and Nrf2 in keratinocytes were similar to those in dermal fibroblasts, Bach1 appeared to be the defining feature for the regulation of HO-1 expression in keratinocytes.

Bach1 Down-regulation Enhances HO-1 Expression but Does Not Reduce or Attenuate ROS Production—To investigate the function of Bach1 in the regulation of HO-1 in keratinocytes, we examined the levels of ROS in Bach1−/− keratinocytes. Consistent with earlier results showing that Bach1

FIGURE 3. Bach1 down-regulation does not decrease ROS levels despite the increase in HO-1 levels. A, HO-1 mRNA levels of Bach1−/− (solid bar) versus Bach1+/+ (white bar) keratinocytes. HO-1 mRNA levels were determined by real time reverse transcription-PCR with primers specific for the HO-1 sequence. Values were normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA levels and are expressed as arbitrary units. B, HO-1 protein expression in Bach1−/− versus Bach1+/+ keratinocytes. Keratinocytes were cultivated in the indicated concentrations of H2O2 and HO-1 expression was assessed by immunoblotting, with α-tubulin as a loading control. C, H2DCFDA expression in Bach1−/− (solid bar) versus Bach1+/+ (white bar) keratinocytes. Keratinocytes were incubated with H2O2 (0 or 500 μM) for 12 h. After H2DCFDA addition, trypsinized keratinocytes were analyzed using a cell sorter (left panel). The ratio of ROS-positive cells to total cells is also shown (right panel). Error bars represent the standard deviations. *p < 0.05. D, immunoblots of keratinocytes transfected with Bach1-specific siRNAs. HO-1 levels increased with Bach1 down-regulation by Bach1 sequence-specific (B-1, B-2, and B-3), but not control (C), siRNAs. The loading control was α-tubulin. E, H2O2 induces HO-1 expression in an additive manner in the siRNA-transfected keratinocytes. Primary keratinocytes transfected with specific siRNAs were incubated with the indicated concentrations of H2O2. HO-1 protein levels were determined by immunoblotting with anti-HO-1, and α-tubulin was used as a loading control. F, H2DCFDA expression in keratinocytes transfected with Bach1 sequence-specific (solid bar; B-1 and B-2) versus control siRNAs (white bar; C). Experiments were conducted as in C. Percentages indicate the ROS-positive fraction of the population. Standard deviations are indicated by error bars. *p < 0.05. Similar results were obtained in at least two independent experiments.
negatively regulates expression of the HO-1 reporter gene (Fig. 1B), Bach1−/− keratinocytes expressed higher levels of HO-1 mRNA and protein than wild-type cells under normal conditions (Fig. 3, A and B). Furthermore, H2O2 treatment resulted in much higher levels of HO-1 in Bach1−/− keratinocytes (Fig. 3B) than HO-1 levels in wild-type keratinocytes. These results indicate that Bach1 was responsible not only for the repression of basal HO-1 levels but also for the attenuation of HO-1 expression induced by oxidative stress conditions. Because increased HO-1 levels are predicted to protect against ROS, we examined whether Bach1 deletion and subsequent HO-1 increase was sufficient to protect keratinocytes against oxidative stress. ROS levels increase within the cell upon treatment with H2O2. However, despite the higher level of HO-1 expression, ROS accumulation was not attenuated by the loss of Bach1 (Fig. 3C). Rather, ROS levels were higher in Bach1−/− keratinocytes than control cells, regardless of H2O2 treatment.

To check effects of Bach1 down-regulation on ROS levels further, we next compared HO-1 expression before and after Bach1 knockdown by RNA interference (RNAi). Bach1 was knocked down by transient transfection with siRNA constructs that targeted three different regions of the Bach1 transcript. Similar to the Bach1−/− keratinocytes, reduced Bach1 levels resulted in increased HO-1 expression (Fig. 3D). H2O2 treatment resulted in higher levels of HO-1 in Bach1-depleted keratinocytes (Fig. 3E), but ROS accumulation was not decreased by Bach1 depletion (Fig. 3F). Similar to Bach1 gene deletion, RNAi inhibition of Bach1 expression increased ROS levels in the presence or absence of H2O2 treatment. Taken together, these results suggest that Bach1 down-regulation enhances HO-1 expression but is not sufficient to suppress ROS production.

To analyze the downstream effects of Bach1 down-regulated keratinocytes, we examined cell viability. Regardless of oxidative stress, the Bach1 down-regulation resulted in lowered keratinocyte viability (Fig. 4A). Furthermore, we quantified ATP levels because several heme proteins function in the mitochondria for energy production. The reduced Bach1 levels caused a decrease of ATP levels in both normal and oxidative stress conditions (Fig. 4B). These results suggest that HO-1 overexpression may lead to a disruption of energy production rather than protection against oxidative stress.

We further examined the effects of combined knockdown of Bach1 and HO-1 in keratinocytes by using HO-1-specific siRNA constructs. RNAi-mediated suppression of HO-1 mRNA decreased HO-1 levels even in the Bach1-depleted keratinocytes, regardless of oxidative stress (Fig. 4, C and D). The HO-1 knockdown attenuated the decreased cell viability by the Bach1 knockdown in both normal and oxidative stress conditions (Fig. 4E). In addition, the reduced HO-1 levels also abolished the decreased ATP levels in the Bach1-depleted keratinocytes (Fig. 4F). Thus, Bach1 appears to suppress HO-1 induction to maintain cellular homeostasis, particularly in energy production.

**Bach1 Up-regulation Enhances ROS Production by Blocking HO-1-induced Expression**—We next used an adenoviral transduction system to examine the effects of Bach1 overexpression. Using the Tet-On system, Bach1 expression was efficiently induced in keratinocytes (Fig. 5A). In the absence of oxidative stress, Bach1 overexpression did not alter the basal level of HO-1 expression in keratinocytes (Fig. 5B), showing that endogenous HO-1 expression is not dependent on the MARE elements in the E1 and E2 enhancer regions. In contrast, HO-1 induction in response to H2O2 was almost completely inhibited by overexpression of Bach1 in keratinocytes (Fig. 5C). Consequently, ROS levels were much higher in Bach1-overexpressing
cells than in control cells (Fig. 5D). Taken together, these results suggest that Bach1-mediated repression of HO-1 is specific to stress-induced HO-1 expression and was not effective in regulation of basal HO-1 levels under normal culture conditions. Interestingly, Bach1 up-regulation abolished cellular responses against oxidative stress, which resulted in ROS accumulation.

**Bach1-HO-1 Pathway during Keratinocyte Differentiation**—We next examined the role of Bach1 in the regulation of HO-1 during keratinocyte differentiation. Upon transfer of keratinocytes to high calcium medium, thereby forcing differentiation and the expression of keratins 1 and 10, loricrin, and filaggrin, the expression of HO-1 gradually and steadily increased at both the mRNA and protein levels (Fig. 6, A and B). In contrast, MARE reporter activity and HO-1 reporter activity did not increase during this differentiation period (Fig. 6, C and D). In addition, the level of the Bach1 transcript remained constant over this process (Fig. 6E). Similar to Bach1 levels, Nrf2 mRNA levels remained constant during differentiation. The protein levels of Bach1 and Nrf2 were increased in the nuclear fraction (Fig. 6F). Thus, HO-1 expression was induced by keratinocyte differentiation, but this induction was independent of both increased MARE activity and derepression of Bach1-mediated inhibition of HO-1 expression.

To further confirm that the differentiation-induced HO-1 expression was independent of Bach1, we overexpressed Bach1 in keratinocytes and examined HO-1 expression. The overexpression of Bach1 did not inhibit the calcium-induced expression of HO-1 (Fig. 6G). Furthermore, Bach1 did not affect basal HO-1 expression (i.e. in low calcium medium), supporting the finding that Bach1 is not relevant to HO-1 regulation under normal conditions. Thus, HO-1 induction is independent of Bach regulation during keratinocyte differentiation.

We next examined the impact of several signaling pathways on HO-1 induction during differentiation by the use of specific inhibitors. HO-1 induction was enhanced by inhibition of JNK, p38 MAPK, PKC/β, or γ-secretase that is necessary for Notch activation (Fig. 7). These signal pathways appeared to suppress HO-1 induction during differentiation. On the other hand, neither ERK nor PKC/β inhibitors affected HO-1 induction. Thus, HO-1 expression seemed to be repressed by several pathways that are known to regulate keratinocyte differentiation. The putative signaling pathway for accelerating HO-1 induction during calcium-induced differentiation remains to be identified.

**DISCUSSION**

Keratinocytes are constantly exposed to environmental pro-oxidants, which lead to harmful effects in the absence of appropriate protective cellular responses. Bach1 acts as a redox-sensitive repressor of genes that are under the control of the MARE element, but the role Bach1 plays in the environmental stress response in keratinocytes has not yet been reported. In this
study, we used keratinocytes with depleted Bach1 levels to demonstrate that Bach1 inhibits the oxidative stress-inducible expression of HO-1, a cytoprotective enzyme.

Although deletion of the Bach1 gene or RNAi resulted in higher expression of HO-1, Bach1 overexpression did not affect basal HO-1 expression. These apparently conflicting results are reconciled by the fact that even under normal culture conditions, cells are exposed to oxidative stress due to the presence of ~20% oxygen in the atmosphere (32). We hypothesize that the levels of Bach1 were sufficient to inhibit HO-1 induction in response to oxidative stress.

Because Bach1 and its competitors such as Nrf2 share small Maf proteins as their obligate heterodimer partners, a reduction in Bach1 levels would shift the equilibrium toward the formation of Nrf2/small Maf heterodimers, resulting in constitutively high levels of HO-1 expression. Hence, HO-1 may be readily activated by Nrf2 or other MARE-binding activators in Bach1-depleted keratinocytes. The higher levels of HO-1 generated by Bach1 down-regulation would protect cells against excessive oxidative damage. However, despite enhanced HO-1 expression, ROS levels in cultured keratinocytes were not decreased in Bach1-deficient keratinocytes or following RNAi depletion of Bach1. Rather, RNAi-mediated Bach1 down-regulation was sufficient to increase ROS levels. Therefore, there may be a limit to the cytoprotective function of HO-1 in keratinocytes. Because the cytoprotective function of HO-1 is manifested by reaction products such as bilirubin and carbon monoxide (14), the heme substrate could be the limiting factor in HO-1 function. Cells usually contain very little free heme (33), because the free heme molecule is cytotoxic due to its ability to catalyze the peroxidation of membrane lipids and formation of ROS. Higher HO-1 may promote degradation of not only free heme but also heme contained in heme protein complexes, leading to dysfunction of heme proteins. The increase of HO-1 levels by RNAi depletion of Bach1 resulted in a decrease of cell viability and a decrease in ATP levels in the presence or absence of high calcium. The loading control was \( \alpha \)-tubulin. Similar results were obtained in at least two independent experiments.
The epidermis is organized into several distinct overlying layers. As keratinocytes begin to differentiate, they cease cell growth and migrate to the upper layers (the spinous, granular, and cornified layers). HO-1 is induced during the terminal differentiation and is expressed in the upper part of the epidermis (17). These layers of the skin are directly exposed to the environment and require defense mechanisms against environmental insults, including oxidative stress and ultraviolet light. However, HO-1 induction during differentiation in vitro was independent of Bach1 inactivation and Nrf2 activation. Upon differentiation, HO-1 induction was not inhibited by Bach1 overexpression, suggesting that the differentiation-induced regulation of HO-1 is mediated by a MARE-independent mechanism under these conditions. Keap1-null mice, in which Nrf2 is constitutively activated, have a noticeably thicker cornified layer and express differentiation-specific proteins more abundantly than their wild-type littermates (45). Thus, Nrf2 overactivation alters some aspects of the differentiation process. However, it is unlikely that Nrf2 directly regulates keratinocyte differentiation under normal conditions, because the reporter activity of MARE was stable during normal differentiation. Instead, based on the observation that Nrf2 was activated in response to various stresses, the altered differentiation may be the result of an enhanced response to external stress, which would lead to increased keratinization as a defense. However, the mechanism responsible for the increased HO-1 expression in keratinocyte differentiation remains to be determined. Keratinocytes undergo complex changes during differentiation, which require the precise coordination of various molecular events (46). We did not identify a signaling pathway that accelerated HO-1 expression in differentiating keratinocytes. Unexpectedly, we found that inhibition of the JNK, p38 MAPK, PKCα/β, and Notch signal pathways affected HO-1 expression in keratinocytes. Considering the fact that the activation of these pathways is tightly connected to keratinocyte differentiation (47–50), the strict regulation of HO-1 may be critical for keratinocyte differentiation. Because the expression of epidermal proteins is precisely regulated in a temporal and spatial manner, HO-1 expression must be synchronized to that of other proteins during differentiation. However, HO-1 induction by Bach1 inactivation may be too rapid for differentiation and may be difficult to synchronize with the expression of other proteins.

Because Bach1 together with its competitor Nrf2 regulates cellular responses to oxidative stress, a fluctuation in the balance of these components may affect the pathological mechanisms underlying certain skin diseases. Interestingly, increased HO-1 expression is observed in psoriasis, a common skin disease marked by hyperproliferation and delayed keratinocyte differentiation (6). HO-1 expression seems to compensate for the abnormal features in psoriatic skin lesions, because HO-1 up-regulation attenuates keratinocyte hyperproliferation in an animal model of psoriasisform dermatitis (51). As high cell turnover occurs in psoriatic lesions, Bach1 may be inactivated by the increased concentrations of heme derived from degraded keratinocytes. Bach1 binds to heme through its heme regulatory motif, which results in the loss of its repressor activity (52) and the induction of HO-1. In addition, Bach1 has recently been reported to inhibit oxidative stress-induced cellular senescence by impeding the function of p53 (32). Bach1 function may affect.
not only the pathogenesis of skin diseases but also the process of skin aging.

REFERENCES

1. Finkel, T. (2003) Curr. Opin. Cell Biol. 15, 247–254
2. Briganti, S., and Picardo, M. (2003) J. Eur. Acad. Dermatol. Venereol. 17, 663–669
3. Hanselmann, C., Mauch, C., and Werner, S. (2001) Biochem. J. 353, 459–466
4. Vile, G. F., Basu-Modak, S., Waltner, C., and Tyrrell, R. M. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 2607–2610
5. Wagener, F. A., van Beurden, H. E., van den Hoff, J. W., Adema, G. J., and Figdor, C. G. (2003) Blood 102, 521–528
6. Wojas-Pelc, A., and Marcinkiewicz, J. (2007) Int. J. Exp. Pathol. 88, 95–102
7. Ishikawa, K., and Sun, J. (2006) Antioxid. Redox. Signal. 8, 107–118
8. Suzuki, H., Tashiro, S., Sun, J., Doi, H., Satomi, S., and Igarashi, K. (2003) J. Biol. Chem. 278, 6083–6095
9. Hintze, K. J., Katoh, Y., Igarashi, K., and Theil, E. C. (2007) J. Biol. Chem. 282, 34365–34371