ERK-mediated phosphorylation of BIS regulates nuclear translocation of HSF1 under oxidative stress

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B-cell lymphoma (BCL)-2-interacting cell death suppressor (BIS) has diverse cellular functions depending on its binding partners. However, little is known about the effects of biochemical modification of BIS on its various activities under oxidative stress conditions. In this study, we showed that H2O2 reduced BIS mobility on SDS–polyacrylamide gels in a time-dependent manner via the activation of extracellular signaling-regulated kinase (ERK). The combined results of mass spectroscopy and computational prediction identified Thr285 and Ser289 in BIS as candidate residues for phosphorylation by ERK under oxidative stress conditions. Deletion of these sites resulted in a partial reduction in the H2O2-induced mobility shift relative to that of the wild-type BIS protein; overexpression of the deletion mutant sensitized A172 cells to H2O2-induced cell death without increasing the level of intracellular reactive oxygen species. Expression of the BIS deletion mutant decreased the level of heat shock protein (HSP) 70 mRNA following H2O2 treatment, which was accompanied by impaired nuclear translocation of heat shock transcription factor (HSF) 1. Co-immunoprecipitation assays revealed that the binding of wild-type BIS to HSF1 was decreased by oxidative stress, while the binding of the BIS deletion mutant to HSF1 was not affected. These results indicate that ERK-dependent phosphorylation of BIS has a role in the regulation of nuclear translocation of HSF1 likely through modulation of its interaction affinity with HSF1, which affects HSP70 expression and sensitivity to oxidative stress.

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INTRODUCTION

B-cell lymphoma (BCL)-2-interacting cell death suppressor (BIS), also known as BCL-2-associated athanogene 3 or carboxyamido-triazole-stressed-1, is a BCL-2-interacting protein that enhances the anti-apoptotic activity of BCL-2.1–3 The pro-survival activity of BIS is shown by its increased expression in a variety of human cancers, including lymphocytic leukemia, melanoma, glioma and thyroid, pancreatic and ovarian cancers.4–9 BIS expression has also been shown to be induced by stress, such as heat shock, heavy metal treatment, oxidative stress and viral infections, suggesting a role for BIS in cellular homeostasis under conditions of cellular stress.10–12

The diverse functions of BIS are attributable to its ability to interact with various proteins via specific domains. For example, the BCL-2-associated athanogene domain is reported to interact with BCL-2 or heat shock protein (HSP) to exert anti-apoptotic and anti-stress effects,13 while the proline-rich domain is essential for interaction with phospholipase Cy and modulation of migration/invasion.14 Furthermore, two IPV motifs are required for the binding of BIS to HSPB8 to initiate autophagy.15

In addition to specific domains in the BIS protein, biochemical modification of the BIS protein has also been shown to influence the interactions between BIS and specific binding proteins and the subsequent cellular phenotypes. Tyr phosphorylation of BIS following epidermal growth factor stimulation is essential for phospholipase C binding,14 while phosphorylation of BIS at S136 or S172 is important for its interaction with 14-3-3.16 In addition, protein kinase (PK) Cδ-mediated phosphorylation of BIS at residue S187 has been implicated in epithelial–mesenchymal transition and the invasiveness of thyroid cancer cells.17 A recent study showed that following glucose stimulation, BIS was phosphorylated by focal adhesion kinase, which led to F-actin remodeling.18

In previous studies, we showed that BIS was markedly upregulated in reactive hippocampal astrocytes after transient forebrain ischemia19 and that downregulation of BIS sensitized C6 rat glioma cells to oxygen–glucose deprivation-induced...
reactive oxygen species (ROS) accumulation and cell death.\(^\text{20}\) Furthermore, BIS-haploinsufficient mice showed increased ROS and oxidative stress, resulting in aggravation of diabetic nephropathy.\(^\text{21}\) These results suggest that BIS protects against ROS, although the underlying mechanism is unclear.

The present study addressed this question by investigating the expression and phosphorylation of BIS under oxidative stress conditions in various cell lines. We found that BIS was phosphorylated by extracellular signal-regulated kinase (ERK) following \(\text{H}_2\text{O}_2\) treatment and that this post-translational modification is the mechanism by which BIS modulates the sensitivity of cells to ROS via regulation of heat shock factor (HSF) 1-dependent transactivation of HSP70.

**MATERIALS AND METHODS**

**Cell culture and treatment**

A172 (human glioma cells), A549 (human lung adenocarcinoma cells), and HK2 (human kidney tubule epithelial cells) were purchased from the American Type Culture Collection. A172 cells were cultured in Dulbecco’s Modified Eagle Medium, and A549 and HK2 cells were cultured in RPMI supplemented with 10% fetal bovine serum (Thermo Scientific, Waltham, MA, USA) and 1% penicillin–streptomycin (Thermo Scientific). Mouse embryonic fibroblasts were prepared from wild-type C57B6 mouse embryos\(^\text{22}\) and grown in Dulbecco’s Modified Eagle Medium with 20% fetal bovine serum. Cells were maintained at 37°C in a humidified incubator containing 5% \(\text{CO}_2\). After overnight culture, the cells were rinsed with phosphate-buffered saline (Sigma-Aldrich, St. Louis, MO, USA) and then exposed to \(\text{H}_2\text{O}_2\) at the indicated concentration in glucose/serum-free Dulbecco’s Modified Eagle Medium for the indicated times. In some experiments, cells lysates were treated with alkaline phosphatase, or the cells were pretreated with kinase inhibitors 1 h prior to \(\text{H}_2\text{O}_2\) treatment. U0126 (an extracellular signal-regulated kinase (ERK) inhibitor), SB203580 (a p38 inhibitor), and SP600125 (a c-Jun N-terminal kinase (JNK) inhibitor) were purchased from Calbiochem (San Diego, CA, USA). To determine the effects of a ROS scavenger, 1 \(\mu\text{M}\) of N-acetyl-L-cysteine (Sigma-Aldrich) was added to the cells before \(\text{H}_2\text{O}_2\) exposure.

**Western blot analysis**

Total cell lysates were prepared as described previously.\(^\text{23}\) Protein concentration was determined using a BCA protein assay (Pierce, Rockford, IL, USA). For subcellular fractionation, a nuclear and cytoplasmic extraction kit, NE-PER (Thermo Scientific), was used according to the manufacturer’s instructions. An equal amount of protein for each sample was separated by SDS–PAGE. Gels were stained with Instant Blue (Coomassie-based gel staining dye that is compatible with mass spectrometry analysis. Expendeon), and the visualized BIS band was excised. In-gel digestion was performed with modified porcine trypsin (Promega), and the peptides were analyzed by liquid chromatography–tandem mass spectrometry. The resulting peaks were searched against the SwissProt database (2014.07 release, 20210 sequences for human) using a Mascot Daemon search engine (version 2.4.0, Matrix Science), and the peptides of interest were quantified by peak area integration using the extracted ion chromatograms. We also predicted the phosphorylation motifs of BIS using the Motif Scan program\(^\text{24}\) and Group-based Prediction System 2.0\(^\text{25}\).

**Phosphorylation site analysis**

The BIS protein was immunoprecipitated from A172 cell lysates and separated by SDS–PAGE. Gels were stained with Instant Blue (Coomassie-based gel staining dye that is compatible with mass spectrometry analysis. Expendeon), and the visualized BIS band was excised. In-gel digestion was performed with modified porcine trypsin (Promega), and the peptides were analyzed by liquid chromatography–tandem mass spectrometry. The resulting peaks were searched against the SwissProt database (2014.07 release, 20210 sequences for human) using a Mascot Daemon search engine (version 2.4.0, Matrix Science), and the peptides of interest were quantified by peak area integration using the extracted ion chromatograms. We also predicted the phosphorylation motifs of BIS using the Motif Scan program\(^\text{24}\) and Group-based Prediction System 2.0\(^\text{25}\).

**Construction of the BIS plasmid and cell transfection**

The deletion mutant (Δ281-292 BIS) was prepared by a two-step PCR protocol and cloned into the pcAGGS vector\(^\text{1}\) or pcMV-myc (Clontech, Mountain View, CA, USA). The transfections of the full-length (FL) BIS or Δ281-292 BIS constructs were carried out using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions.

**ROS determination and cell viability assay**

Quantification of the ROS was performed with 10\(\mu\text{M}\) of CM-H\(_2\)DCFDA (Life Technologies, Eugene, OR, USA) using a BD FACSCalibur (BD Bioscience, San Jose, CA, USA). Cell viability was evaluated with a water soluble tetrazolium salt assay using an EZ-Cell Viability Assay kit (Istbio, Seoul, Korea) according to the manufacturer’s instructions. Viability was expressed as a percentage of the control group.

**Quantitative real-time PCR analysis**

Total RNA was isolated using RNAiso Plus (TaKaRa, Shiga, Japan), and complementary DNA was synthesized by reverse transcription using a ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan). Quantitative real-time PCR was performed to validate the expression levels of the target mRNAs using SYBR Premix Ex Taq (TaKaRa) on an ABI 7300 machine (Applied Biosystems, Carlsbad, CA, USA). The specific primers for real-time PCR include the following: HSP70 (forward primer : 5′-CGGGGGTAACCGCACTCATAACTCTGAACCCATCT-3′, reverse primer : 5′-GTTCACTCATGACGGGATCT-3′), HSP27 (forward primer : 5′-TGACGGTCCAGACCAATGGAAGT-3′, reverse primer : 5′-ATGGTGATCTCGTTGGACTG-3′), c-fos (forward primer : 5′-CAAGGGAGACAGAACACTCT-3′, reverse primer : 5′-AGTTCAGATCAAGGGAAGCA-3′) and β-actin (forward primer : 5′-AGTACTCGTGTGGATGGC-3′, reverse primer : 5′-C CGCTCGAGCGGTCTTCTCTGTC-3′). The relative values for the target mRNAs were calculated after normalization to the Ct value of β-actin in the same sample using the ddCt method.

**Confocal microscopy and immunofluorescence staining**

A172 cells were first seeded onto glass coverslips in complete medium and allowed to adhere overnight at 37°C before proceeding with the treatments. Cells were fixed using 4% paraformaldehyde (Bioseang, Sungnam, Korea) and incubated in 0.5% Triton X-100 for permeabilization. Then, the cells were incubated with an anti-HSF1 monoclonal antibody (1:1000, Promega). Immunoreactivity was visualized with Texas Red-conjugated anti-rabbit immunoglobulin G (Santa Cruz Biotechnology) using a Zeiss LSM 700 laser fluorescence confocal...
microscope with Zen software (Carl Zeiss Microimaging, Jena, Germany).

Co-immunoprecipitation analysis
A172 cells transfected with Myc-tagged FL-BIS or Myc-tagged Δ281–292 BIS were treated with H₂O₂ for 3 h. An equal amount of each protein lysate was incubated with normal rabbit immunoglobulin G or antibodies against HSF1 (Cell Signaling Technology) for 4 h at 4 °C, followed by incubation with 20 μl of protein A magnetic beads (Millipore) for 16 h at 4 °C. The immune complexes were analyzed by western blot analyses with anti-c-Myc antibodies (Santa Cruz Biotechnology). Protein lysates were also subjected to western blot analyses with the indicated antibodies.

Statistics
All experiments were repeated at least three times, and the data are shown as the mean ± s.e. Student’s t-tests were employed to compare two different groups. A P-value of <0.05 was considered to be statistically significant.

RESULTS
BIS mobility is reduced by H₂O₂ treatment
H₂O₂ treatment of A172 cells reduced BIS protein migration, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE). This mobility shift was apparent as early as 30 min after treatment, with the maximum effect observed at 3 h. Then, the mobility returned to baseline at 6 h. At least three different protein bands were observed, indicating that H₂O₂ induced stepwise and multiple modifications of BIS in a time-dependent manner. The magnitude of the mobility shift was independent of H₂O₂ concentration (Figure 1a).

H₂O₂-induced retardation of BIS mobility was also observed in a diverse range of cell types, including A549, HK2 cells mouse embryonic fibroblasts (Figure 1b). In subsequent experiments, we used A172 cells treated with 100 μM of H₂O₂ for 3 h.

Pretreatment of the cells with the ROS scavenger N-acetyl-L-cysteine abrogated the effect of H₂O₂ on BIS electrophoretic mobility (Figure 1c). BIS expression was upregulated by heat shock, but there was no shift in mobility under these conditions (Figure 1d). These results suggest that H₂O₂-mediated ROS accumulation, and not other types of cellular stress, is responsible for the change in BIS protein mobility induced by H₂O₂.

H₂O₂ induces BIS phosphorylation via ERK activation
Because phosphorylation is the most common biochemical modification of proteins, we examined whether phosphorylation accounted for the heterogeneity of BIS observed in SDS–PAGE following H₂O₂ treatment. Incubation of A172 cell lysates with alkaline phosphatase completely reduced the H₂O₂-induced mobility shift of BIS (Figure 2a), suggesting that BIS phosphorylation in the presence of H₂O₂ leads to its altered mobility. To determine which kinase is responsible, we pretreated A172 cells with the inhibitors U0126 (ERK inhibitor), SB203580 (p38 inhibitor) or SP600125 (JNK inhibitor) prior to H₂O₂ exposure. U0126 completely abolished H₂O₂-induced BIS phosphorylation; however, alteration in BIS mobility was detected in A172 cells pretreated with SB203580 or SP600125 (Figure 2b), although these agents abrogated the phosphorylation of their target proteins (Figure 2c). These

Figure 1  H₂O₂-induced mobility shift of BIS visualized by electrophoresis. The BIS protein was detected by western blotting, with β-actin serving as a loading control. (a) A172, (b) HK2, A549 and MEF cells were treated with the indicated concentrations of H₂O₂ for the indicated times. (c) A172 cells were treated with 1 mM N-acetyl-L-cysteine (NAC) for 1 h or were left untreated and were then exposed to 100 μM H₂O₂ for 3 h. (d) A172 cells were exposed to heat shock (43 °C for 30 min) and then allowed to recover by incubation at 37 °C for the indicated times. BIS, B-cell lymphoma (BCL)-2-interacting cell death suppressor; MEF, mouse embryonic fibroblasts.
results indicate that BIS phosphorylation in the presence of H$_2$O$_2$ is ERK dependent.

**BIS is phosphorylated at Thr285 and Ser289 under oxidative stress conditions**

We sought to identify the BIS residues that are phosphorylated under oxidative stress conditions using immunoprecipitation-coupled liquid chromatography/tandem mass spectrometry. Immunoprecipitation and western blot analysis were carried out using an anti-BIS antibody (Figure 3a); we confirmed that the band at ~70 kDa corresponded to BIS by mass spectrometry (data not shown). Several residues of the BIS protein were phosphorylated following H$_2$O$_2$ treatment; based on this information, along with computational predictions, Thr285 and Ser289 were identified as potential ERK target sites. We also carried out a quantitative analysis based on the ion chromatogram. As expected, the unphosphorylated peptide 283-SSTPLHSPSP-292 was more abundant in the control sample, while the phosphopeptide 283-SSpTPLHSPSP-292 was only detected in samples from the H$_2$O$_2$-treated cells (Figure 3b).

We then constructed a vector expressing recombinant human BIS with deletions in the residues Ala281 to Pro292, which encompassed the putative phosphorylation sites (Δ281–292 BIS; Figure 3c). A western blot analysis of Δ281–292 BIS-transfected A172 cell lysates showed two separate BIS bands with high and low molecular weights corresponding to endogenous BIS and Δ281–292 BIS, respectively. Following H$_2$O$_2$ treatment, a mobility shift was detected in both bands; however, the shift was reduced for Δ281–292 BIS, as shown by the increased distance between the two bands (Figure 3d). These results indicate that the H$_2$O$_2$-induced mobility shift of Δ281–292 BIS was partly abolished by the deletion of amino acids 281–292, suggesting that Thr285 and/or Ser289 phosphorylation is responsible for the shift.

We also evaluated whether phosphorylated BIS participates in the physiological redox signaling pathway involving ERK because previous studies showed that BIS could alter the activation of ERK, which is sensitive to the redox state of the cells.$^{26}$ We found that Δ281–292 BIS overexpression in A172 cells did not affect the level of phospho-ERK (p-ERK) and phospho-ETS domain-containing protein (ELK)-1 (p-ELK) proteins and the transactivation of c-fos, which are downstream targets of ERK, following treatment with H$_2$O$_2$ (Figures 3d and e).

Deletion of BIS residues 281–292 sensitizes A172 cells to H$_2$O$_2$-induced cell death

To determine the physiological significance of BIS phosphorylation under oxidative stress conditions, we evaluated the effects of Δ281–292 BIS on cell viability after H$_2$O$_2$ exposure. A172 cells transfected with the various constructs were exposed to 100 μM H$_2$O$_2$ for 3 h, and viability was assessed with water soluble tetrazolium salt assays. Overexpression of FL-BIS or Δ281–292 BIS had no effect on the basal level of A172 cell viability, whereas viability was decreased to 71.1%, 83.9% and 60.3% in the cells transfected with the empty vector, FL-BIS, and Δ281–292 BIS, respectively, after H$_2$O$_2$ exposure (Figure 4a). These results show that the susceptibility to H$_2$O$_2$-induced cell death was decreased by FL-BIS overexpression and increased by Δ281–292 BIS overexpression ($P = 0.0459$ and 0.0217 vs H$_2$O$_2$-treated empty vector-transfected cells).

To determine whether the sensitization resulting from deletion of residues 281–292 is due to a loss of anti-oxidant capacity,$^{20}$ we measured ROS accumulation in FL-BIS- and Δ281–292 BIS-transfected A172 cells following H$_2$O$_2$ treatment.
There was no difference between the two proteins in ROS levels, which were increased by ~1.7-fold ($P < 0.001$) in cells overexpressing either protein following treatment with 100 μM H$_2$O$_2$ for 3 h (Figure 4b). Taken together, these results suggest that overexpression of Δ281–292 BIS increases sensitivity to ROS without affecting intracellular ROS production and activation of the ERK-ELK-c-fos pathway induced by H$_2$O$_2$.

Deletion of BIS residues 281–292 causes downregulation of HSP70 after H$_2$O$_2$ treatment

Several studies have shown that HSP70, a BIS binding partner, has a protective function as a chaperone protein under oxidative stress conditions. 27,28 Given the decrease in cell viability induced by H$_2$O$_2$ upon deletion of BIS residues 281–292, we hypothesized that HSP70 along with BIS protects against H$_2$O$_2$. HSP70 levels were unaffected by H$_2$O$_2$ treatment in cells overexpressing the empty vector or FL-BIS; however, in Δ281–292 BIS-transfected cells, HSP70 expression was reduced under oxidative stress conditions. A quantitative analysis revealed that the HSP70 protein level was decreased by 19.6% ($P = 0.0111$) in cells expressing the BIS deletion mutant following H$_2$O$_2$ treatment but remained constant in the other groups (Figure 4c). Similar trends were observed by quantitative reverse transcriptase–PCR analysis; HSP70 mRNA levels were reduced by 45.3% ($P = 0.0312$) in Δ281–292 BIS-transfected cells exposed to H$_2$O$_2$ but were unchanged in the other groups (Figure 4d). However, HSP27 mRNA levels were

Figure 3 Identification of BIS residues phosphorylated under oxidative stress. (a, b) Immunoprecipitation coupled with liquid chromatography/tandem mass spectrometry (LC-MS/MS) was used to identify BIS phosphorylation sites following H$_2$O$_2$ treatment. A172 cells were treated with 100 μM H$_2$O$_2$ for 3 h, and the BIS protein was immunoprecipitated from cell lysates with an anti-BIS antibody. (a) An aliquot of the immunoprecipitated proteins was analyzed by western blotting using an anti-BIS antibody. (b) The band corresponding to BIS in the immunoprecipitated complex was subjected to trypsin digestion and analyzed by LC-MS/MS; data were compared to computational predictions. The abundance of the unphosphorylated peptide 283-SSTPLHSPSP-292 and the phosphopeptide 283-SSpTPLHpSPSP-292 was quantified by peak area integration using the extracted ion chromatograms. (c) Schematic representation of FL-BIS and mutant BIS (with deletion of residues Ala281 to Pro292; Δ281–292 BIS). (d) A172 cells were transfected with the pCAGGS vector (Vector) or Δ281–292 BIS and treated 48 h later with 100 μM H$_2$O$_2$ for 3 h. Cell lysates were subjected to western blotting to assess the levels of BIS, p-ERK, and p-ELK proteins, with β-actin used as a loading control. (e) Relative levels of c-fos mRNA were evaluated by quantitative real-time PCR. Values represent the mean ± s.e. of triplicate experiments. *$P<0.05$, **$P<0.01$. BIS, B-cell lymphoma (BCL)-2-interacting cell death suppressor; ERK, extracellular signaling-regulated kinase; ELK, ETS domain-containing protein; FL, full length.
Figure 4 Effect of Δ281–292 BIS overexpression on cell viability and HSP70 expression following H₂O₂ treatment. A172 cells were transfected with pCAGGS (Vector), FL-BIS, or Δ281–292 BIS and then treated 48 h later with 100 μM H₂O₂ for 3 h. (a) Cell viability was determined with the water soluble tetrazolium salt assay; viability of cells transfected with the empty vector was designated as 100%. Values represent the mean ± s.e. of triplicate experiments. *P<0.05. (b) Intracellular ROS levels were assessed by CM-H₂DCFDA staining and flow cytometry. Data are presented as the fold change in the mean value from three independent experiments with the s.e. ***P<0.001 vs untreated cells (c) Expression levels of BIS, HSP70, HSF1, and p-ERK were determined by western blotting (left panels). The results from a densitometric analysis of HSP70 protein levels from three independent experiments are shown in the right panels. The basal level of HSP70 protein in A172 cells transfected with the vector was arbitrarily set to 1. Relative levels of (d) HSP70 mRNA and (e) HSP27 mRNA were evaluated by quantitative real-time PCR. Values represent the mean ± s.e. of triplicate experiments. *P<0.05, **P<0.01. BIS, B-cell lymphoma (BCL)-2-interacting cell death suppressor; ERK, extracellular signaling-regulated kinase; HSF1, heat shock transcription factor 1; HSP70, heat shock protein 70; ROS, reactive oxygen species.
not significantly different among the three groups (Figure 4e). Taken together, these data demonstrate that blocking ERK-dependent BIS phosphorylation specifically suppresses HSP70 transcription under oxidative stress conditions.

**Deletion of BIS residues 281–292 blocks nuclear translocation of HSF1**

To determine whether BIS regulates HSP70 transactivation following H$_2$O$_2$ exposure, we assessed whether Δ281–292 BIS overexpression affects the activation of HSF1, a regulator of HSP70 transcription, in a process involving hyperphosphorylation, shuttling from the cytoplasm to the nucleus, and binding to target gene promoters. We found that Δ281–292 BIS overexpression in A172 cells had no effect on the HSF1 protein level or phosphorylation status (Figure 4c). However, the level of HSF1 in the nuclear fraction was reduced in these cells compared to those expressing the empty vector or FL-BIS, in which H$_2$O$_2$ treatment induced nuclear translocation.
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An immunocytochemical analysis revealed that Δ281–292 BIS-transfected cells showed impaired nuclear translocation of HSF1 after H₂O₂ treatment (Figure 5b). Then, we determined whether H₂O₂-induced BIS phosphorylation affected the interactions between BIS and HSF1 proteins. As shown in Figure 5c, without H₂O₂ treatment, both the FL-BIS and Δ281–292 BIS proteins were detected as a complex with HSF1. However, following H₂O₂ treatment, the interaction strength between wild-type BIS and HSF1 was notably reduced, whereas the binding affinity between Δ281–292 BIS and HSF1 was not changed. These findings clearly indicate that the phosphorylation status of BIS is critical in determining the efficiency of its interaction with HSF1, which affects the subcellular localization of HSF1.

DISCUSSION

BIS has been implicated in various cellular processes; however, little is known about the biological consequences of BIS protein modification. The present study demonstrated that H₂O₂ treatment induced ERK-dependent BIS phosphorylation. Deletion of 12 amino acids (Δ281–292) in the protein, including the potential phosphorylation sites Thr285 and Ser289, increased the sensitivity of A172 cells to H₂O₂-induced cell death. This is consistent with the effect of BIS knockdown on glioma or renal cell survival under oxidative stress conditions. However, biochemical modifications of amino acid side chains frequently led to conformational changes and subsequent aggregation of proteins, which facilitated proteasomal degradation of oxidized proteins. Thus, under oxidative stress, the verification of chaperones and co-chaperones was critical to relieve cells from conformational stress or from the excess burdens of misfolded proteins against oxidative stress-induced cell death. Our results showed that cells overexpressing Δ281–292 BIS showed a decrease in HSP70 levels, which was accompanied by the inhibition of HSF1 nuclear translocation. We, therefore, speculated that the suppression of HSP70 expression resulting from the inhibition of HSF1 nuclear translocation might contribute, at least in part, to increasing the sensitivity to oxidative stress exhibited by cells expressing Δ281–292 BIS.

HSF1 activates BIS gene transcription by binding heat shock elements in the promoter region in response to various stimuli, including heat shock and proteasome inhibition. However, a proteomic analysis based on quantitative immunoprecipitation combined with gene knockdown demonstrated a direct interaction between the HSF1 and BIS proteins. Our results using co-immunoprecipitation assays clearly showed that exogenously expressed wild-type BIS as well as the Δ281–292 BIS proteins could be detected in a complex with HSF1. Interestingly, while the interaction between wild-type BIS and HSF1 was weaker after H₂O₂ treatment, the association between Δ281–292 BIS and HSF1 was not apparently changed by oxidative stress (Figure 5c). These findings suggest that BIS Thr285 and/or Ser289 phosphorylation following H₂O₂ treatment may decrease the binding affinity between BIS and HSF1 and/or another interaction partner, releasing HSF1 for nuclear translocation. The loss of these phosphorylation sites on BIS may promote the persistent sequestration of HSF1, preventing the nuclear translocation and subsequent activation of target genes. Thus, phosphorylation of BIS may modulate the interaction affinity with HSF1 by which BIS modulates HSF1 activity under oxidative stress. A recent study also showed that BIS may have a key role in the nucleocytoplasmic shuttling of HSF1 under heat stress. However, based on the observation that heat shock did not induce BIS phosphorylation in our study, an alternative mechanism could regulate the localization of HSF1 based on cellular stress.

Although we observed inhibition of HSF1 nuclear translocation in parallel with a reduction in HSP70 levels in Δ281–292 BIS-expressing cells, HSP70 was not induced by H₂O₂.
treatment in cells transfected with the empty vector or the wild-type BIS-expressing vector, whereas HSF1 was activated, as shown by its phosphorylation and nuclear translocation (Figure 4c). HSF1 activation is critical for the induction of HSPs in response to a variety of cellular stressors, including heat shock, oxidative stress, infection and inflammation, although several investigators have reported that HSF1 activation by some oxidants is not always accompanied by HSP70 upregulation. An earlier study showed that oxidizing agents exert dual effects on the regulation of HSF1  in vitro— that is, they promote its nuclear translocation but also decrease its DNA-binding activity. Furthermore, H_2O_2 treatment resulted in rapid, global, but transient repression of transcription by some oxidants is not always accompanied by HSP70 induction.54,55 Although target genes, which is supported by previous studies showing of HSF1 as a transcriptional regulator is not equivalent against with HSF1. However, the details of how BIS post-translational phosphorylation via ERK, which has a role in the regulation of may be different with classical heat shock stress responses. In addition, HSP27 induction was not affected by the phosphor-

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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