Hypoxia induces cyclophilin B through the activation of transcription factor 6 in gastric adenocarcinoma cells

KWON JEONG[1], KIYOON KIM[1], HUNSUNG KIM[1], YOOJUNG OH[1], SEONG-JIN KIM[2], YUNHEE JO[2] and WONCHAE CHOE[1]

[1]Department of Biochemistry and Molecular Biology (BK21 project), Medical Research Center for Bioreaction to Reactive Oxygen Species and Biomedical Science Institute; [2]Neurodegeneration Control Research Center, School of Medicine, Kyung Hee University, Seoul 130-701, Republic of Korea

Received April 15, 2014; Accepted February 2, 2015

DOI: 10.3892/ol.2015.3102

Abstract. Hypoxia is an important form of physiological stress that induces cell death, due to the resulting endoplasmic reticulum (ER) stress, particularly in solid tumors. Although previous studies have indicated that cyclophilin B (CypB) plays a role in ER stress, there is currently no direct information supporting the mechanism of CypB involvement under hypoxic conditions. However, it has previously been demonstrated that ER stress positively regulates the expression of CypB. In the present study, it was demonstrated that CypB is transcriptionally regulated by hypoxia-mediated activation of transcription factor 6 (ATF6), an ER stress transcription factor. Subsequently, the effects of ATF6 on CypB promoter activity were investigated and an ATF6-responsive region in the promoter was identified. Hypoxia and ATF6 expression each increased CypB promoter activity. Collectively, these results demonstrate that ATF6 positively regulates the expression of CypB by binding to an ATF6-responsive region in the promoter, which may play an important role in the attenuation of apoptosis in the adaption to hypoxia. These results suggest that CypB may be a key molecule in the adaptation of cells to hypoxic conditions.

Introduction

An immediate reaction to hypoxia is a reduction in the rate of global protein synthesis, which is thought to reduce energy demands when oxygen and adenosine triphosphate levels are low (1). This response triggers stressors that alter the environment of the endoplasmic reticulum (ER) by impairing nascent ER protein glycosylation, disulfide bond formation or calcium level interference with protein folding, resulting in the ER stress response (2-4). To prevent the deleterious effects of ER stress, cells have evolved numerous protective strategies, including activating signal transduction pathways that trigger complex transcriptional and translational responses, which is known as the unfolded protein response (UPR) (5). The UPR is mediated by three distinct ER-transmembrane signaling proteins that act as major proximal sensors of the ER stress response, consisting of inositol-requiring transmembrane kinase and endonuclease 1α, activation of transcription factor 6 (ATF6) and protein kinase-like ER kinase (6-8). Full-length p90-ATF6 is located in the ER membrane, and in the absence of ER stressors, the protein is maintained in an inactive form by binding to glucose-regulated protein (GRP)78 (9). When activated, cleaved ATF6, or p50-ATF6, then translocates into the nucleus, where it activates the transcription of ER stress response-associated genes, including GRP78 (10,11).

Cyclophilin B (CypB) is a 21-kDa protein belonging to the cyclophilin family of peptidyl-prolyl *cis*-trans isomerases (12). CypB has been identified in the ER and nucleus and is also present in notable levels in the blood and breast milk (13). It has previously been revealed that CypB plays important roles in the response to ER stress-mediated cell death (14). Although it has been reported that the expression of CypB is regulated under physiological and pathophysiological states, the mechanism of the regulation is largely unknown.

The aim of the present study was to explore the possible functional mechanisms underlying the induction of CypB under hypoxic conditions. In addition, the transcription factors involved in the increase of CypB expression under hypoxia have yet to be reported in the literature. In the present study, it was demonstrated that hypoxia transcriptionally upregulates CypB through ATF6. Therefore, understanding the molecular basis of the regulation of CypB under hypoxic conditions is of clinical importance for the removal of gastric adenocarcinoma cells. These results indicate that CypB may be a key molecule in the adaptation of cells to hypoxia.

Correspondence to: Professor Wonchae Choe, Department of Biochemistry and Molecular Biology (BK21 project), Medical Research Center for Bioreaction to Reactive Oxygen Species and Biomedical Science Institute, School of Medicine, Kyung Hee University, 1 Hoegi-dong, Dongdaemoon-gu, Seoul 130-701, Republic of Korea
E-mail: wchoe@khu.ac.kr

Key words: cyclophilin B, activation of transcription factor 6, endoplasmic reticulum stress

Meterials and methods

Reagents and antibodies. Fetal bovine serum (FBS) and RPMI-1640 medium were purchased from Lonza (Walkersville, MD, USA). X-tremeGENE DNA transfection reagents were purchased from Roche Applied Science (Mannheim, Germany). The cyclophilin B antibody was purchased from Abcam (Cambridge, UK). Actin, human influenza hemagglutinin (HA)-tag and an enhanced chemiluminescence western blotting kit were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Cyclophilin B, calnexin, ATF6 and ATF4 were purchased from Abcam. All other chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Plasmid constructions. cDNA encoding the C-terminal 670 amino acids of ATF6 was subcloned into the pcDNA vector (Invitrogen, Carlsbad, CA, USA), between the *Bam*HI and *Xho*I sites for protein expression, which tagged the protein with an HA-tag at the C-terminal end. The human CypB promoter reporter constructs (-974, -800, -350, -250 and -100) were kindly provided by Dr Sung Soo Kim (Kyung Hee University, Seoul, Korea). All constructs were verified by sequencing.

Cell culture and in vitro hypoxia model. The human gastric carcinoma AGS cells (American Type Culture Collection, Manassas, VA, USA) were maintained in RPMI-1640 medium supplemented with 10% FBS, in humidified air containing 5% CO_2 at 37˚C. The cells were incubated under either normoxic (20% O_2) or hypoxic conditions (0.1% O_2). Cultured AGS cells were subjected to hypoxic conditions at 37˚C for the time periods indicated, while controls were maintained in normoxic conditions at 37˚C for the same time periods.

Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative PCR analysis. Total cellular RNA was extracted from cells using TRIzol reagent (Invitrogen). The cDNA was synthesized from 1 μg of total RNA using M-MLV reverse transcriptase (Fermentas, Hanover, MD, USA). The specific primers used for RT-PCR are as follows: CypB forward, 5'-AATTCCATCGTGTAATCAAGGACTT-3' and reverse, 5'-TCTTGACTGTCGTGATGAAGAACT-3'; and β-actin forward, 5'-GTACTTGCGCTCAGGAGGAG-3' and reverse, 5'-TCGTGCGTGACATTAAGGGG-3'. The PCR products were analyzed by agarose gel electrophoresis and visualized using ethidium bromide. The signals were quantitated using ImageJ software (version 1.45; National Institutes of Health, Bethesda, MD, USA). Quantitative PCR was performed using an ABI prism 7300 Sequence Detection System (Applied Biosystems Life Technologies, Foster City, CA, USA) with SYBRGreen PCR Master Mix (Applied Biosystems). The PCR reaction was carried out for 40 thermal cycles of denaturation at 94˚C for 30 sec, annealing at 55˚C for 30 sec and extension at 72˚C for 30 sec. Expression of the target gene was analyzed by an absolute quantification method and normalized using β-actin levels.

Luciferase assay. The CypB promoter sequence was analyzed using Genomatix MatInspector (Genomatix Software GmbH, Munich, Germany). One ER stress element (ERSE) candidate was located at 222 bp, ~209 bp from the CypB open reading frame. The AGS cells were transfected with 1.0 μg of the pGL3 basic-derived plasmids together with the pCMV-β-galactosidase internal control plasmid (Promega, Madison, WI, USA). The activity of luciferase and β-galactosidase was measured using 50 μl of each cell lysate and a fluorescence microplate reader (Victor 1420 Multilabel Counter; Wallac, Turku, Finland). The luciferase activity was normalized on the basis of the β-galactosidase values. The transfection experiments were performed in triplicate and repeated at least three times.

Western blot analysis. The cells were washed twice with cold PBS on ice and harvested by scraping with a rubber cell scraper. The cells were pelleted by centrifugation at 4˚C and resuspended directly into a lysis buffer, which consisted of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.5% Igepal CA-630, 2 mM EDTA, 10 mM NaF, 2 mM Na_3VO_4 and 0.01% protease inhibitor cocktail. The cell lysates were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. Subsequent to blocking in 5% skim milk and Tris-buffered saline with 0.1% Tween-20, signals were detected and analyzed by a Kodak X-OMAT 2000 image analyzer. Densitometric analysis was performed using ImageJ software.

Statistical analysis. The results were expressed as the mean ± standard deviation, which was obtained from at least three independent experiments. Statistical analyses were conducted using a Student's *t*-test. By convention, $P<0.05$ was considered to indicate a statistically significant difference.

Results

Hypoxia results in the transcriptional upregulation of CypB expression. Since overexpression of CypB has been demonstrated in numerous types of cancer cells, the present study investigated whether the elevated CypB level is caused by transcriptional induction. RT-PCR was performed using AGS cells that were subjected to hypoxic conditions for 0, 6 and 18 h (Fig. 1A). Upregulated CypB mRNA expression was observed during a 6 h exposure to hypoxia, and up to a five-fold increase in CypB mRNA expression was identified in the cells exposed to an 18 h period of hypoxia, compared with the negative control. In addition, the treatment of AGS cells with 5 μg/ml actinomycin D, an mRNA synthesis inhibitor, abolished the hypoxia-mediated induction of CypB mRNA expression, indicating that upregulated CypB mRNA is mainly induced, not by mRNA stability, but by the *de novo* synthesis of mRNA under hypoxia. The quantitative PCR also demonstrated upregulated CypB mRNA levels under hypoxia, consistent with the results shown in Fig. 1A and B. To further understand the molecular mechanisms by which CypB mRNA is induced by hypoxia, the CypB promoter activity was monitored using the wild-type CypB-974 promoter in AGS cells (Fig. 1C). As expected, the luciferase activity of the wild-type CypB-974 promoter also demonstrated a significant increase under hypoxic conditions compared with normoxic conditions. These results indicate that the CypB gene is transcriptionally induced under hypoxic conditions in AGS cells.

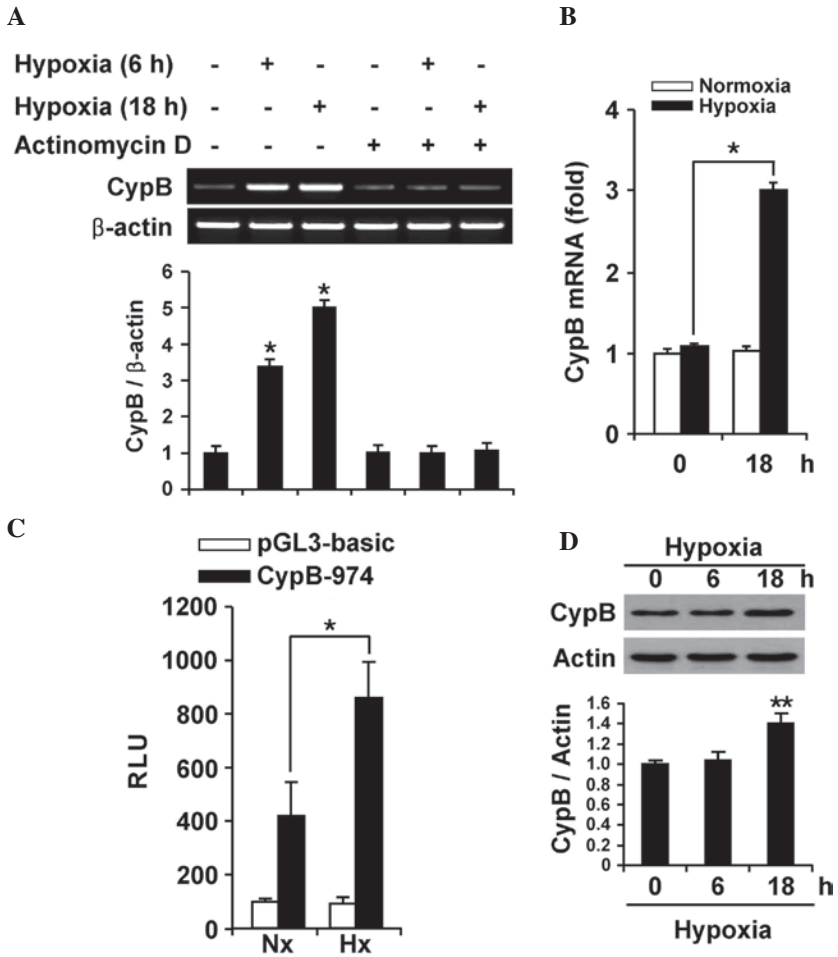

Figure 1. Transcriptional upregulation of CypB under hypoxic conditions. (A) The AGS cells were exposed to hypoxia for the indicated times in the absence or presence of actinomycine D (5 μg/ml). The expression of CypB mRNA was analyzed by RT-PCR, with β-actin as a loading control. Quantitative analysis of RT-PCR was performed using the ImageJ program (lower panel). The data are reported as the mean ± SD from three independent experiments. (B) Quantitative PCR analysis of CypB mRNA expression in AGS cells cultured under hypoxic conditions for 0 and 18 h. The data are reported as the mean ± SD from three independent experiments. *P<0.05 vs. 0 h under hypoxic conditions. (C) The AGS cells were transiently transfected with wild-type CypB-974 promoter constructs, and further incubated for 24 h under hypoxic conditions. The luciferase activity is expressed relative to that of normoxic cells. The data are reported as the mean ± SD from three independent experiments. *P<0.05 vs. CypB-974 cells under normoxic conditions. (D) The AGS cells were exposed to hypoxia for the indicated times and the expression of the CypB protein was analyzed by western blot analysis. CypB, cyclophilin B; RT-PCR, reverse transcription-polymerase chain reaction; SD, standard deviation; Nx, normoxia; Hx, hypoxia; RLU, relative light units.

The CypB protein level was subsequently monitored in AGS cells under hypoxic conditions, and it was found that CypB protein expression was significantly increased (Fig. 1D). A time-course experiment demonstrated that CypB protein expression under hypoxic conditions reached a maximum level after 6 h and was sustained until the cells had been exposed to hypoxia for 18 h. Densitometric analysis revealed that the CypB protein expression was higher in AGS cells exposed to hypoxic conditions when compared with the control, suggesting a crucial role of CypB in hypoxia. These results indicate that CypB expression is upregulated by hypoxia in AGS cells.

Hypoxia-mediated induction of CypB requires an ATF6 transcription factor. The promoters of ER chaperones contain ERSEs (10,15) and previous studies have revealed that the human CypB promoter contains ER stress-responsive elements that are induced by ATF6-dependent pathways (16). To identify the region of the CypB promoter that is responsible for hypoxia-induced transcriptional upregulation, the luciferase activity was measured using incrementally truncated reporter plasmids, comprising pGL3-CypB-974, -800, -350, -250 and -100, that were transfected into AGS cells (Fig. 2A). The luciferase activity in each assay was normalized to that of a co-transfected pGL3-basic plasmid, pCMV-β-galactosidase luciferase. The pCMV-β-galactosidase luciferase plasmid did not respond to hypoxia, but the pGL3-CypB-250, -350, -800 and -974 reporter plasmids demonstrated significantly increased luciferase activity under hypoxic conditions. The empty pGL3-basic vector and pGL3-CypB-100 plasmid did not demonstrate hypoxia-induced luciferase activity. These results suggest that the *cis*-element responsible for the response to hypoxic conditions is within the region 250 bp upstream of the transcription start site. Within this region, the ERSE element was located using a promoter analysis program. Therefore, it was hypothesized that the ERSE element is associated with hypoxia-induced transcription activity. In the following promoter experiments, pGL3-CypB-250, a plasmid containing the ERSE element of the CypB promoter, was used to test this hypothesis in detail.

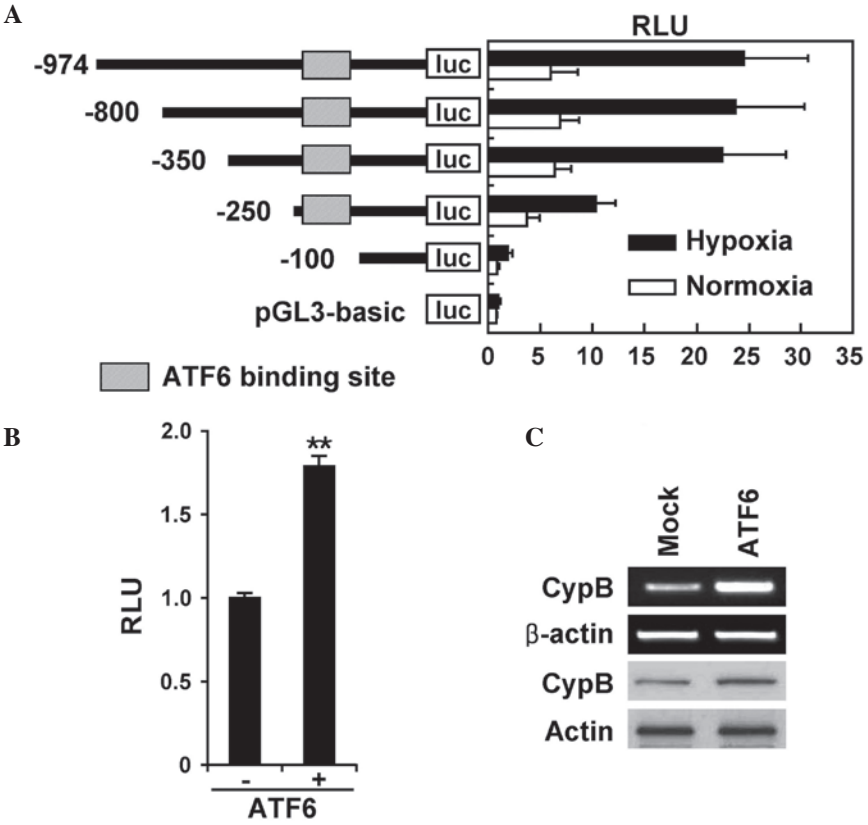

Figure 2. ATF6 mediates hypoxia-induced transcriptional upregulation of CypB. (A) Schematic representation of deletion constructs for the human CypB promoter, revealing the location of ATF6. The AGS cells were transfected with various deletion constructs of pGL3-CypB, comprising pGL3-CypB-974, -800, -350, -250 and -100, together with 0.4 mg pCMV-β-galactosidase vector. The cells were incubated under normoxic or hypoxic conditions for 24 h, and assayed for luciferase activity. (B) The AGS cells were transfected with the pGL3-CypB-250 plasmid and the active form-ATF6 (p50ATF6) expression plasmid. Subsequent to transfection, the cells were incubated under normoxic or hypoxic conditions for 24 h and assayed for luciferase activity. The data are reported as the mean ± standard deviation from three independent experiments. (C) The AGS cells were transfected with p50ATF6 in a dose-dependent manner. The expression of the CypB protein was analyzed by western blot analysis. ATF6, activation of transcription factor 6; CypB, cyclophilin B; luc, luciferase; RLU, relative light units.

ATF6 is a UPR transducer that binds to the CCACG region of ERSE elements in the promoter region of UPR-responsive genes (15). ATF6 is constitutively synthesized as a 90-kDa protein, termed p90ATF6, that is converted to a 50-kDa protein, termed p50ATF6, particularly in ER-stressed cells prior to the induction of GRP78. p50ATF6 translocates into the nucleus, where it specifically activates the transcription of ER stress response-associated genes, including GRP78 (17). To determine whether CypB is regulated by p50ATF6 at the transcriptional level under hypoxic conditions, the AGS cells were transiently co-transfected with ATF6 transcription factor expression plasmids and pGL3-CypB-250 plasmids. The luciferase activity of these transfected cells was subsequently analyzed (Fig. 2B). It was found that p50ATF6 expression plasmids increased the luciferase activity in the transfected cells compared with cells transfected with the pcDNA3 empty vector. RT-PCR and western blot analysis revealed that CypB protein expression is increased by the expression of p50ATF6 in AGS cells (Fig. 2C). These results indicate that the hypoxia-mediated activation of CypB transcription requires ATF6.

Discussion

In the present study, the ER stress-mediated ATF6 pathway of CypB expression was demonstrated to be one of the mechanisms induced by hypoxia in AGS cells. It has previously been reported that CypB activation was found to protect AGS cells against hypoxia through the attenuation of ER stress-mediated cell death. However, the mechanism for the induction of the ER stress pathways following exposure to hypoxia have not been clearly identified.

In the present study, ATF6, an ER stress-associated transcription factor, was found to mediate the upregulation of CypB under hypoxic conditions (Fig. 1). ERSE, ERSE-II and ATF6-binding *cis*-acting element have been reported as consensus sequences for the binding of p50ATF6 (15,18,19). The ER stress response element CCAAT-N9-gtaaCGTGG (ERSE-III), which is located within the CypB promoter region and is similar to the conventional ERSE-I motif of CCAAT-N9-CCACG (19), was consistently used for ATF6 binding under hypoxic conditions, as demonstrated by the luciferase assay. Hypoxia was found to significantly upregulate CypB mRNA expression in a time-dependent and ATF6-dependent manner. The stimulation of ERSE-mediated transcription activity by ATF6 requires the integrity of the tripartite structure of the ERSE, a high-affinity CCAAT binding site for NF-Y/CBF and a functional NF-Y complex, which is consistent with the present results.

It has been established that ER stress favors survival and induces apoptotic signaling in various types of cells. Therefore, the decision between survival and apoptosis may depend on the

balance between survival signaling and apoptotic signaling. In future studies, the involvement of ER stress-specific apoptotic signaling in hypoxia in AGS cells may be examined.

In summary, ATF6 upregulates CypB gene expression through an increase in the promoter activity of CypB in AGS cells, which may demonstrate anti-apoptotic properties under hypoxic conditions. Future studies investigating the mechanism through which CypB prevents ER stress may aid the present understanding of the nature of hypoxia-associated diseases, including solid tumors.

Acknowledgements

This study was supported by a grant from the Kyung Hee University in 2012 (grant no., KHU-20121733), and a grant from the Basic Science Research Program through the National Research Foundation of Korea (NRF), which is funded by the Ministry of Education (grant no., NRF-2013R1A1A2060694).

References

1. Hochachka PW, Buck LT, Doll CJ and Land SC: Unifying theory of hypoxia tolerance: Molecular/metabolic defense and rescue mechanisms for surviving oxygen lack. Proc Natl Acad Sci USA 93: 9493-9498, 1996.
2. Lee AS: The glucose-regulated proteins: Stress induction and clinical applications. Trends Biochem Sci 26: 504-510, 2001.
3. Wu J and Kaufman RJ: From acute ER stress to physiological roles of the Unfolded Protein Response. Cell Death Differ 13: 374-384, 2006.
4. Xu C, Bailly-Maitre B and Reed JC: Endoplasmic reticulum stress: Cell life and death decisions. J Clin Invest 115: 2656-2664, 2005.
5. Kaufman RJ: Stress signaling from the lumen of the endoplasmic reticulum: Coordination of gene transcriptional and translational controls. Genes Dev 13: 1211-1233, 1999.
6. Harding HP, Calfon M, Urano F, Novoa I and Ron D: Transcriptional and translational control in the Mammalian unfolded protein response. Annu Rev Cell Dev Biol 18: 575-599, 2002.
7. Breckenridge DG, Germain M, Mathai JP, Nguyen M and Shore GC: Regulation of apoptosis by endoplasmic reticulum pathways. Oncogene 22: 8608-8618, 2003.
8. Schröder M and Kaufman RJ: The mammalian unfolded protein response. Annu Rev Biochem 74: 739-789, 2005.
9. Shen J, Chen X, Hendershot L and Prywes R: ER stress regulation of ATF6 localization by dissociation of BiP/GRP78 binding and unmasking of Golgi localization signals. Dev Cell 3: 99-111, 2002.
10. Li M, Baumeister P, Roy B, Phan T, Foti D, Luo S and Lee AS: ATF6 as a transcription activator of the endoplasmic reticulum stress element: Thapsigargin stress-induced changes and synergistic interactions with NF-Y and YY1. Mol Cell Biol 20: 5096-5106, 2000.
11. Yoshida H, Okada T, Haze K, Yanagi H, Yura T, Negishi M and Mori K: ATF6 activated by proteolysis binds in the presence of NF-Y (CBF) directly to the cis-acting element responsible for the mammalian unfolded protein response. Mol Cell Biol 20: 6755-6767, 2000.
12. Price ER, Zydowsky LD, Jin MJ, Baker CH, McKeon FD and Walsh CT: Human cyclophilin B: A second cyclophilin gene encodes a peptidyl-prolyl isomerase with a signal sequence. Proc Natl Acad Sci USA 88: 1903-1907, 1991.
13. Price ER, Jin M, Lim D, Pati S, Walsh CT and McKeon FD: Cyclophilin B trafficking through the secretory pathway is altered by binding of cyclosporin A. Proc Natl Acad Sci USA 91: 3931-3935, 1994.
14. Oh Y, Kim EY, Kim Y, et al: Neuroprotective effects of overexpressed cyclophilin B against Aβ-induced neurotoxicity in PC12 cells. Free Radic Biol Med 51: 905-920, 2011.
15. Yoshida H, Haze K, Yanagi H, Yura T and Mori K: Identification of the cis-acting endoplasmic reticulum stress response element responsible for transcriptional induction of mammalian glucose-regulated proteins. Involvement of basic leucine zipper transcription factors. J Biol Chem 273: 33741-33749, 1998.
16. Kim J, Choi TG, Ding Y, et al: Overexpressed cyclophilin B suppresses apoptosis associated with ROS and Ca2+ homeostasis after ER stress. J Cell Sci 121: 3636-3648, 2008.
17. Ye J, Rawson RB, Komuro R, Chen X, Davé UP, Prywes R, Brown MS and Goldstein JL: ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBPs. Mol Cell 6: 1355-1364, 2000.
18. Kokame K, Kato H and Miyata T: Identification of ERSE-II, a new cis-acting element responsible for the ATF6-dependent mammalian unfolded protein response. J Biol Chem 276: 9199-9205, 2001.
19. Wang Y, Shen J, Arenzana N, Tirasophon W, Kaufman RJ and Prywes R: Activation of ATF6 and an ATF6 DNA binding site by the endoplasmic reticulum stress response. J Biol Chem 275: 27013-27020, 2000.