Biochemical and Biophysical Research Communications 463 (2015) 870–875

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Lack of GCN5 remarkably enhances the resistance against prolonged endoplasmic reticulum stress-induced apoptosis through up-regulation of Bcl-2 gene expression

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ARTICLE INFO

Article history:
Received 27 May 2015
Accepted 4 June 2015
Available online 15 June 2015

Keywords:
Apoptosis
Bcl-2
ER stress
GCN5
Gene targeting

ABSTRACT

The endoplasmic reticulum (ER), a complex membrane structure, has important roles in all eukaryotic cells. Catastrophe of its functions would lead to ER stress that causes various diseases such as cancer, neurodegenerative diseases, diabetes and so on. Prolonged ER stress could trigger apoptosis via activation of various signal transduction pathways. To investigate physiological roles of histone acetyltransferase GCN5 in regulation of ER stress, we analyzed responses of homozygous GCN5-deficient DT40 mutants, ΔGCN5, against ER stress. GCN5-deficiency in DT40 caused drastic resistance against apoptosis induced by pharmacological ER stress agents (thapsigargin and tunicamycin). Pharmaceutical analysis using specific Bcl-2 inhibitors showed that the drastic resistance against prolonged ER stress-induced apoptosis is, in part, due to up-regulation of Bcl-2 gene expression in ΔGCN5. These data revealed that GCN5 is involved in regulation of prolonged ER stress-induced apoptosis through controlling Bcl-2 gene expression.

1. Introduction

Endoplasmic reticulum (ER) is an important organelle for lipid synthesis, drug metabolism, calcium storage and protein synthesis (translation, folding and maturation) in eukaryotic cells [1–3]. Catastrophe of these functions would lead to ER stress that causes various diseases such as cancer [4], neurodegenerative diseases (e.g. Alzheimer's disease, amyotrophic lateral sclerosis and Parkinson's disease) [5,6] and diabetes [7,8]. Prolonged ER stress could trigger apoptotic cell death through various signal transduction pathways [5,9,10]. Upon chronic ER stress, caspase-12, the ER membrane associated protease belonging to the caspase family, is activated, followed by promoting apoptosis via initiating caspase-mediated limited proteolysis cascade [11]. On the other hand, protein kinase RNA-like ER kinase (PERK) promotes apoptosis through translation of activating transcription factor 4 (ATF4) [12]. Sequentially, ATF4 induces expression of C/EBP-homologous protein (CHOP) gene, resulting in apoptosis progression via suppressing gene expression of anti-apoptotic B-cell lymphoma 2 (Bcl-2) and activating that of pro-apoptotic B-cell lymphoma-associated X (Bax) [13]. The CHOP gene is also up-regulated by various unfolded protein response-related transcription factors such as activating transcription factor 6 (ATF6) and X-box binding protein-1 (Xbp-1) [10,14]. In addition, unmitigated ER stress also activates apoptosis

Abbreviations: AFT4, activating transcription factor 4; AFT6, activating transcription factor 6; Bax, B-cell lymphoma-associated X; Bcl-2, B-cell lymphoma 2; Bcl-xL, B-cell lymphoma extra-large; CHOP, C/EBP-homologous protein; ER, endoplasmic reticulum; GRP78, glucose-regulated protein 78; IRE1, inositol-requiring protein 1; PERK, protein kinase RNA-like ER kinase; SAGA, Spt-Ada-GCN5 acetyltransferase; Xbp-1, X-box binding protein-1.

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http://dx.doi.org/10.1016/j.bbrc.2015.06.027
0006-291X/

signaling pathway through interaction of inositol-requiring protein 1 (IRE1) with tumor necrosis factor receptor-associated factor 2 and apoptosis signal-regulating kinase [15]. In contrast, glucose-regulated protein 78 (GRP78), also called as binding immunoglobulin protein, protects cell against ER stress-induced apoptosis [16,17]. GRP78 plays an extremely important role in the cell survival against ER stress, while CHOP acts as the principal executor of various pro-apoptotic events upon severe ER stress. Studies on ER stress-induced apoptosis are rapidly developing. However, the mechanisms of ER stress-induced apoptosis remain poorly understood even now, from the viewpoint of epigenetic regulation such as histone acetylation catalyzed by histone acetyltransferases.

To investigate the physiological roles of GCN5, a prototypical histone acetyltransferase [18], we generated homozygous GCN5-deficient DT40 mutants, ΔGCN5, by gene targeting techniques [19]. The techniques used are excellent methods to study physiological roles of various genes including histone modification-related genes [20,21]. Our previous studies have revealed that GCN5-deficiency caused not only delayed growth rate, suppressed cell cycle progression at G1/S phase transition, and down- or up-regulated various G1/S phase transition-related genes [19], but also drastic resistance against B cell receptor-mediated apoptosis associated with negative selection of immature B cells [22]. In addition, GCN5 activated phosphatidylinositol 3-kinase/Akt survival pathway in cells exposed to oxidative stress via controlling gene expressions of tyrosine protein kinases Syk and Btk [23], promoted the superoxide-generating system in leukocytes via controlling the gp91-phox gene expression [24] and participated in tolerance against UV-irradiation via controlling gene expression of DNA polymerase η [25]. Recently, we showed that GCN5 is essential for interferon regulatory factor-4 gene expression followed by transcriptional activation of Blimp-1 during B cell development [26].

In this study, we investigated effects of GCN5-deficiency on prolonged ER stress-induced apoptosis. Our results revealed that GCN5 is involved in regulation of prolonged ER stress-induced apoptosis through controlling Bcl-2 gene expression.

2. Materials and methods

2.1. Materials

Thapsigargin (Nacalai Tesque, Kyoto, Japan), tunicamycin (Calbiochem, San Diego, CA, USA), Bcl-2 inhibitor ABT-199 and HA14-1 (Selleck Chemicals, Houston, TX, USA), Trizol reagent (Invitrogen, Carlsbad, CA, USA), Ex *Taq* DNA polymerase (Takara Bio, Shiga, Japan), proteinase K (Wako, Osaka, Japan), RNase A (Sigma–Aldrich, St. Louis, MO, USA) and cDNA synthesis kit ReverTra Ace-α (Toyobo, Osaka, Japan) were obtained.

2.2. Cell cultures and apoptosis induction

Generation of ΔGCN5 was described in our previous report [19]. DT40 cells and all subclones were cultured essentially as described [19]. ER stress-mediated apoptosis was induced as follows: cells (1.5×10^6) in 10 ml of culture medium were incubated with 1 μM thapsigargin or 5 μg/ml tunicamycin at 37 °C up to 24 h. Treatment with Bcl-2 inhibitor ABT-199 [27] was carried out as follows. Cells (1.5×10^6) in 10 ml of culture medium were treated with ABT-199 (2 or 5 μM) at 37 °C for 30 min and subsequently incubated with 1 μM thapsigargin at 37 °C for 24 h in the presence of ABT-199. Viable cells were counted by trypan blue dye exclusion method as described [28]. DNA fragmentation assay was carried out as described [28]. In brief, cells were lysed in 50 mM Tris-HCl (pH 8.0) containing 2 mM EDTA, 100 μg/ml proteinase K and 1% SDS. DNA was extracted, re-suspended in 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA and 20 μg/ml RNase A, and incubated at 37 °C for 2 h. DNA samples were applied to 1.5% agarose gel electrophoresis.

2.3. Semiquantitative RT-PCR

Total RNA was isolated from DT40 and its subclones using Trizol reagent. RT was performed with a first strand cDNA synthesis kit at 42 °C for 20 min, followed by heating at 99 °C for 5 min. Semiquantitative RT-PCR was performed as described [19,22–26] using forward and reverse primers for appropriate genes listed in Supplementary Table S1, which were synthesized according to the sequence data deposited in GenBank. Chicken GAPDH gene was used as internal control. PCR products were subjected to 1.5% agarose gel electrophoresis. Data obtained by semiquantitative RT-PCR before reaching the plateau were analyzed by Multi Gauge software (densitometrical analysis mode) using a luminescent image analyzer LAS-3000 (Fujifilm, Tokyo, Japan).

2.4. Detection of spliced and unspliced Xbp-1 mRNA

To detect both spliced and unspliced chicken Xbp-1 mRNA, RT-PCR was performed using forward (5′-ATGTGAAGGAATCCCAGGTG-3′) and reverse (5′- CATTTCTGGGTCCAGACTGT-3′) primers. Chicken GAPDH gene was used as control. PCR products were subjected to 1.5% agarose gel electrophoresis. Data obtained by RT-PCR before reaching the plateau were analyzed as described above.

2.5. Statistical analysis

Results (viability and PCR) are presented as averages of three separate experiments. Error bars indicate standard deviation. Statistical differences were calculated with Student's *t* test.

3. Results and discussion

3.1. Effects of GCN5-deficiency on progression of pharmacological ER stress agents-induced apoptosis of DT40 cells

To know the effects of GCN5-deficiency on pharmacological ER stress agents-induced apoptosis, we first examined influences of thapsigargin treatment on apoptosis of ΔGCN5 and wild type DT40 cells (Fig. 1A). Thapsigargin is an inhibitor of sarcoplasmic/ER Ca^{2+} ATPases which act as calcium ion transporters [11]. The viability of both ΔGCN5 and DT40 remained unchanged in the absence of thapsigargin, but the viability of DT40 (~12% at 24 h) was remarkably affected as compared to that of three independent ΔGCN5 clones (~92% at 24 h) in the presence of 1 μM thapsigargin (Fig. 1A, upper graph). The DNA fragmentation in ΔGCN5 was more suppressive compared with accelerated level of that in DT40 (Fig. 1A, lower electrophoretic profile). The suppressive DNA fragmentation pattern was similarly obtained from two other independent ΔGCN5 clones (data not shown). Next, we examined effects of tunicamycin treatment on apoptosis of ΔGCN5 and DT40 (Fig. 1B). Tunicamycin is an inhibitor of N-linked protein glycosylation [11]. The viability of ΔGCN5 and DT40 was not altered in the absence of tunicamycin, but in the presence of this agent (5 μg/ml) the viability of DT40 (~42% at 24 h) was remarkably lower than that of three independent ΔGCN5 clones (~90% at 24 h) (Fig. 1B, upper graph) in almost the same manner as upon thapsigargin treatment. The DNA fragmentation in ΔGCN5 was also more repressive compared with prominent level of that in DT40 (Fig. 1B, lower electrophoretic profile). The repressive DNA fragmentation pattern was also observed from two other independent ΔGCN5 clones (data not shown). Analyses using these two agents with different modes of

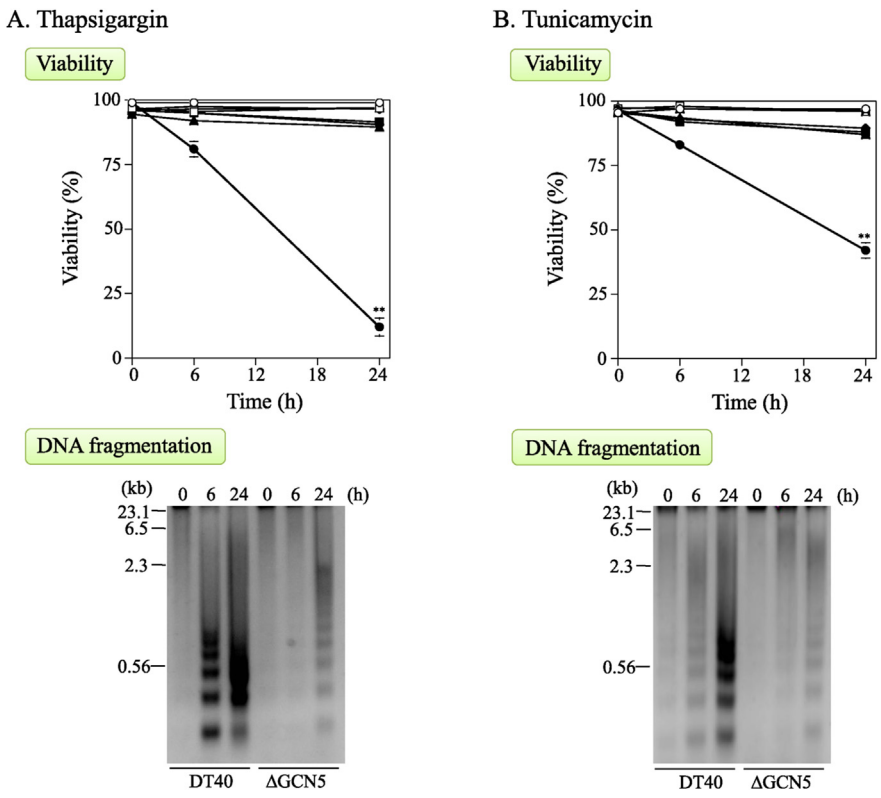

Fig. 1. Effects of pharmacological ER stress agents on apoptosis of ΔGCN5. Effects of thapsigargin (A) and tunicamycin (B) on viability and DNA fragmentation were analyzed. Upper graphs: Viable cells were counted by the trypan blue dye exclusion method. Viability of DT40 (circles) and three independent ΔGCN5 clones (squares, triangles and lozenges). Cells in DMEM medium containing 10% (v/v) fetal bovine serum were treated with (filled symbols) or without (open symbols) 1 μM thapsigargin or 5 μg/ml tunicamycin at 37 °C up to 24 h. Lower electrophoretic profiles: Effects of treatments with thapsigargin (A) and tunicamycin (B) on DNA fragmentation in ΔGCN5 and DT40. DNAs isolated from ΔGCN5 and DT40 were analyzed by 1.5% agarose gel electrophoresis. The sizes of λ-DNA digested with *Hin*dIII are indicated in kilobase pairs.

action suggested that GCN5 is indeed necessary for progression of prolonged ER stress-induced apoptosis of DT40.

3.2. Effects of GCN5-deficiency on transcriptions of ER stress-induced apoptosis signaling pathway-related genes

To know the effects of GCN5-deficiency on gene expressions of ER stress-induced apoptosis signaling pathway-related genes identified in chickens, we carried out semiquantitative RT-PCR on total RNAs prepared from three independent ΔGCN5 clones and DT40 (Fig. 2). GCN5-deficiency showed significant influences on transcriptions of some ER stress-induced apoptosis signaling pathway-related genes; Caspase-12 (to ~140%), CHOP (to ~205%), GRP78 (to ~195%), IRE1 (to ~140%) and Xbp-1 (to ~60%). However, these data are insufficient for understanding the mechanisms of the drastic resistance of ΔGCN5 against pharmacological ER stress agents-induced apoptosis.

3.3. Effects of thapsigargin treatment on transcriptions of various ER stress-induced apoptosis signaling pathway-related genes and splicing of Xbp-1 mRNA in ΔGCN5 and DT40 cells

To clarify the molecular mechanisms for drastic resistance against pharmacological ER stress agents-induced apoptosis of ΔGCN5, ΔGCN5 and DT40 cells were cultured in the presence of thapsigargin for 6 h, and RT-PCR was carried out on various ER stress-induced apoptosis signaling pathway-related genes (Fig. 3A). In both ΔGCN5 and DT40 cells, thapsigargin treatment elevated the amounts of mRNAs of all genes tested. These results show that thapsigargin-induced cellular signaling response, resulting in up-regulation of transcriptions of these genes, nearly equally progresses regardless of presence/absence of GCN5. However, interestingly, there are significant differences between ΔGCN5 and DT40 in the amounts of mRNAs of Caspase-12 and CHOP. It is notable that

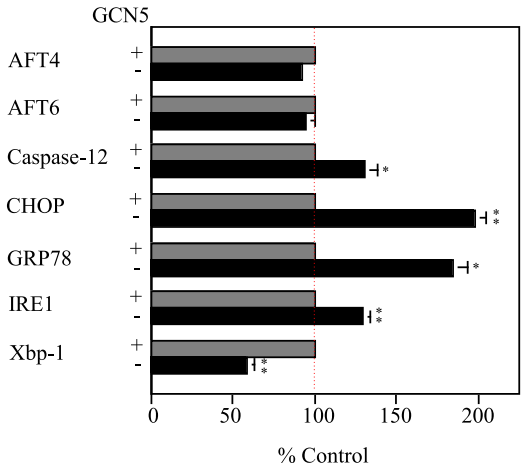

Fig. 2. Effects of GCN5-deficiency on transcription of ER stress-induced apoptosis signaling pathway-related genes. Total RNA was extracted from three independent ΔGCN5 (solid bars) and DT40 (gray bars), and mRNA levels of ER stress-induced apoptosis signaling pathway-related genes were determined by semiquantitative RT-PCR using appropriate primers (see Supplementary Table S1). Chicken GAPDH gene was used as internal control. The gel images obtained were analyzed by LAS-3000. Data calibrated with the internal control are indicated as percentages of control values (100%) obtained from DT40. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control values.

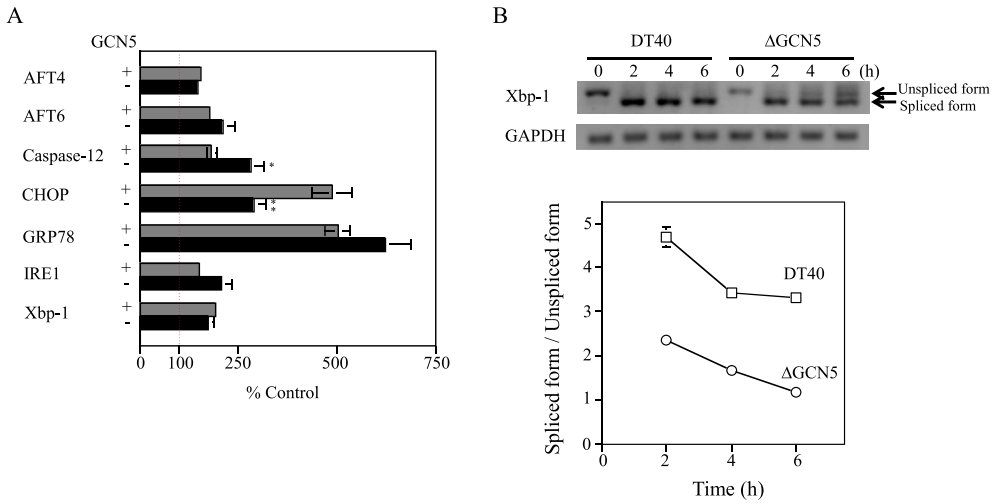

Fig. 3. Effects of GCN5-deficiency on transcription of various ER stress-induced apoptosis signaling pathway-related genes and splicing of Xbp-1 mRNA upon thapsigargin treatment. (A) Effects of GCN5-deficiency on transcriptions of various ER stress-induced apoptosis signaling pathway-related genes upon thapsigargin treatment. Total RNA was extracted from thapsigargin-treated ΔGCN5 (solid bars) and DT40 (gray bars) for 6 h, and mRNA levels of appropriate genes were determined by semiquantitative RT-PCR. Chicken GAPDH gene was used as internal control. Data calibrated with the control are indicated as percentages of control values (100%) obtained from untreated DT40. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the data of thapsigargin-treated DT40. (B) Effects of GCN5-deficiency on Xbp-1 mRNA splicing upon thapsigargin treatment. Upper electrophoretic profiles: Total RNAs were extracted from thapsigargin-treated ΔGCN5 and DT40 up to 6 h. To detect spliced and unspliced chicken Xbp-1 mRNAs, RT-PCR was performed, and PCR products were subjected to 1.5% agarose gel electrophoresis. Chicken GAPDH gene was used as control. Lower graph: The ratio of spliced to unspliced Xbp-1 mRNAs in ΔGCN5 (circles) and DT40 (squares).

thapsigargin treatment increased the CHOP mRNA level to 5-fold in DT40, in contrast to only 1.3-fold in ΔGCN5. The difference in the CHOP mRNA level between ΔGCN5 and DT40 may be reflected in their sensitivity to thapsigargin.

On the other hand, ER stress induces IRE1-catalyzed Xbp-1 mRNA splicing, which is known as a useful marker of ER stress [29]. To know influences of GCN5-deficiency on ER stress-induced Xbp-1 mRNA splicing, we carried out RT-PCR on total RNAs prepared from thapsigargin-treated ΔGCN5 and DT40 (Fig. 3B). It seems that Xbp-1 mRNA splicing was suppressed to about half in ΔGCN5 compared with DT40, although there are insignificant differences between ΔGCN5 and DT40 in the amounts of mRNA of IRE1 that splices Xbp-1 mRNA (see Fig. 3A). We have no data that can dissolve this discrepancy so far. However, GCN5-deficiency seems to participate in suppression of spliced Xbp-1-mediated ER stress signaling. Under ER stress conditions, spliced Xbp-1 is required for transcriptional activation of CHOP [14]. On stimulation with thapsigargin, the lower amounts of CHOP mRNA in ΔGCN5 (see Fig. 3A) may be due to the suppression of splicing of Xbp-1 mRNA. However, these data are also not able to fully explain the mechanisms of the drastic resistance of ΔGCN5 against pharmacological ER stress agents-induced apoptosis.

3.4. Involvement of Bcl-2 in drastic resistance against thapsigargin-induced apoptosis of ΔGCN5

Bcl-2 family can play important roles in prolonged ER stress-induced apoptosis [5,10,30]. In our previous reports [19,22], we showed that the mRNA level of Bcl-2, an anti-apoptotic gene, was drastically increased in ΔGCN5. In contrast, the mRNA level of B-cell lymphoma extra-large (Bcl-xL) was decreased and that of Bax was not altered in ΔGCN5. Taken together, there is a possibility that the remarkably increased Bcl-2 could bring about the drastic resistance against pharmacological ER stress-induced apoptosis of ΔGCN5. To examine this possibility, first, we studied Bcl-2 transcription level. The mRNA level of Bcl-2 was remarkably increased in ΔGCN5 (to ~2000% of the control value obtained from DT40). While the mRNA level of Bcl-2 in thapsigargin-treated ΔGCN5 at 6 h was markedly decreased but was still higher (to ~1000%) than the control value obtained from untreated DT40. In contrast, the mRNA level of Bcl-2 was not significantly affected by thapsigargin in DT40 (Fig. 4A), although the mRNA level of CHOP, which inhibits the expression of Bcl-2, was remarkably increased (to ~550% of the control value obtained from untreated DT40) in thapsigargin-treated DT40 cells (see Fig. 3A). There is no evidence that can address this problem so far. Since some available anti-Bcl-2 antibodies did not bind to chicken Bcl-2 protein, immunoblot assay could not be carried out. Next, we studied effects of specific Bcl-2 inhibitor ABT-199 on thapsigargin-induced apoptosis. ABT-199 (up to 5 μM) showed insignificant effects on cell viability of ΔGCN5 in the absence of thapsigargin (Supplementary Fig. S1). On the other hand, addition of ABT-199 significantly accelerated apoptotic cell death in a dose dependent manner in the presence of 1 μM thapsigargin (Fig. 4B and C). Similar results were obtained using an other Bcl-2-selective inhibitor HA14-1 [31] (Supplementary Figs. S1 and S2). These results suggest that remarkable up-regulation of Bcl-2 could contribute to the drastic resistance against thapsigargin-induced apoptosis of ΔGCN5.

3.5. Concluding remarks

As mentioned above, our data suggest that GCN5 is involved in regulation of prolonged ER stress-induced apoptosis via controlling Bcl-2 gene expression. However, there are some problems that remain to be solved. First, some ER stress-induced apoptosis-related factors, including PERK, have not been identified in chickens yet. The possibility that the unidentified factors may participate in the drastic resistance against prolonged ER stress remains to be solved. Second, the mechanism of up-regulation of Bcl-2 gene expression is unknown in ΔGCN5. GCN5 is generally considered to directly participate in up-regulation of its target genes as a transcriptional coactivator with intrinsic histone acetyltransferase activity [18,32,33]. Therefore, there is a high probability that some factors whose expressions are activated by GCN5 would strongly suppress Bcl-2 gene expression. Needless to say, identification of these putative factors should be done in the future.

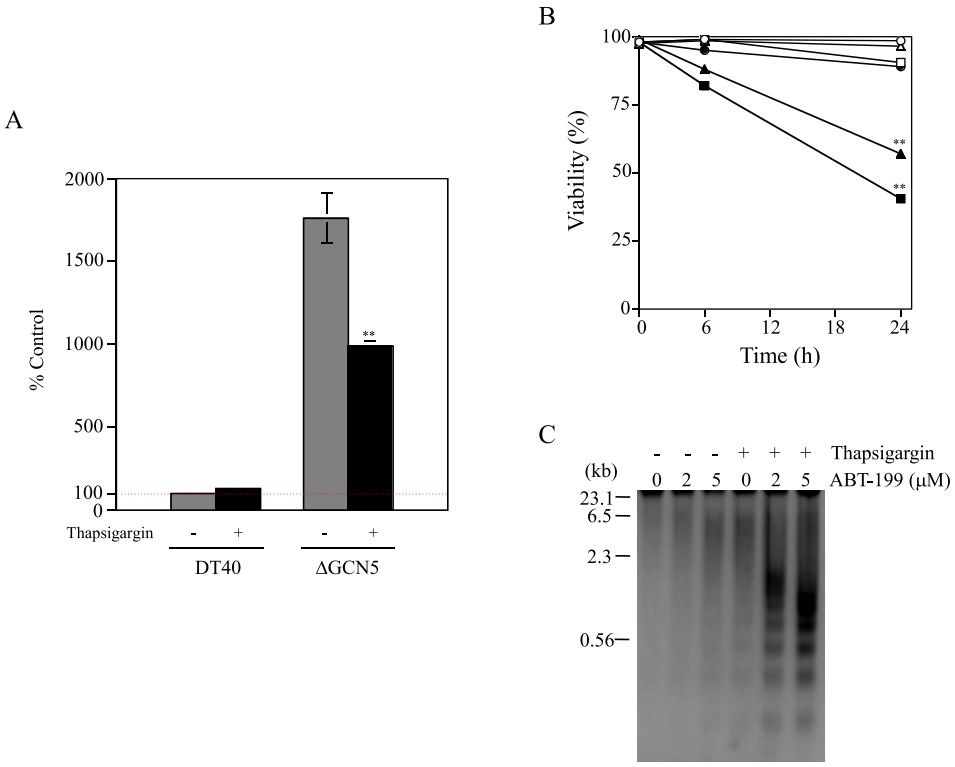

Fig. 4. Studies on involvement of Bcl-2 in drastic resistance against thapsigargin-induced apoptosis of ΔGCN5. (A) Effects of thapsigargin on Bcl-2 transcription. Total RNAs were extracted from ΔGCN5 and DT40 incubated in the presence (solid bars) or absence (gray bars) of thapsigargin for 6 h, and mRNA levels of Bcl-2 were determined by semiquantitative RT-PCR. Chicken GAPDH gene was used as internal control. Data calibrated with the control are indicated as percentages of control values (100%) obtained from untreated DT40. $^{**}P < 0.01$ compared with the data of thapsigargin-untreated ΔGCN5. (B) Effects of Bcl-2 inhibitor on the viability of ΔGCN5 in the presence of thapsigargin. ΔGCN5 cells were treated with (filled symbols) or without (open symbols) 1 μM thapsigargin in the absence (circles) or presence of 2 (triangles) or 5 μM (squares) Bcl-2 inhibitor ABT-199 up to 24 h. Viable cells were counted by the trypan blue dye exclusion method. $^{**}P < 0.01$ compared with the data of thapsigargin-treated ΔGCN5 in the absence of ABT-199. (C) Effects of Bcl-2 inhibitor on DNA fragmentation in ΔGCN5 in the presence of thapsigargin. ΔGCN5 cells were treated with 2 or 5 μM Bcl-2 inhibitor ABT-199 in the presence of 1 μM thapsigargin for 24 h. DNAs were isolated from the cells and analyzed by 1.5% agarose gel electrophoresis. The sizes of λ-DNA digested with *Hin*dIII are indicated in kilobase pairs.

Recently, involvement of GCN5 in ER stress is being investigated by degrees. It has been shown that Spt-Ada-GCN5 acetyltransferase (SAGA), a multisubunit coactivator complex containing GCN5, can be recruited to the promoters of ER stress target genes [34], and SAGA-associated factor 29 has a dual role in ER stress survival [35]. Very recently, it has also been revealed that GCN5 acetylates spliced Xbp-1 protein and enhances its nuclear retention and stability [36]. Taken together with these previous studies, our results will be significantly useful for elucidating epigenetic mechanisms of the ER stress-induced signaling pathways regulated by GCN5. Furthermore, GCN5 participates in regulation of various cell death pathways. GCN5-deficiency in DT40 cells shows acceleration of cell death caused by hydrogen peroxide [23] and UV-irradiation [25], while it suppresses cell death mediated by B cell receptor-signaling [22] and ER stress. Thus, GCN5 may be one of the most powerful supervisor in cell life and death decision mechanisms in vertebrates. Studies on GCN5 will become more important for elucidation of mechanisms of life and death regulation.

Acknowledgments

We thank C. Kawai for technical support (DNA sequencing) and H. Madhyastha and R. Madhyastha for editorial reading of the manuscript. This work was supported in part by Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 25430170, to H. K.). This work was also partly supported by the Grant for Joint Research Project of the Institute of Medical Science, the University of Tokyo.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.06.027.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.06.027.

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