Biochemical and Biophysical Research Communications 422 (2012) 527–533

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

Endoplasmic reticulum chaperone GRP78 suppresses the aggregation of proteins containing expanded polyglutamine tract

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ARTICLE INFO

Article history:
Received 11 May 2012
Available online 22 May 2012

Keywords:
Polyglutamine diseases
GRP78
Protein aggregation
ER stress

ABSTRACT

Polyglutamine (polyQ) diseases are inherited neurodegenerative diseases characterized by the aggregation of proteins containing expanded polyQ tract. It has been shown that expanded polyQ tract-containing proteins impair the functions of other cellular proteins. However, quantitative changes of cellular proteins in cells expressing expanded polyQ tract-containing proteins have not been performed. Here, we performed proteomic analysis of cells expressing expanded polyQ tract-containing proteins, and showed that GRP78, the endoplasmic reticulum (ER) chaperone, was significantly decreased in the cells expressing enhanced green fluorescent protein with a pathological-length polyQ tract (EGFP–polyQ97), but not with a non-pathological-length polyQ tract (EGFP–polyQ24). In addition, we revealed that down-regulation of GRP78 expression resulted in increase of the aggregation of EGFP–polyQ97. Conversely, the aggregation of EGFP–polyQ97 was suppressed by the overexpression of GRP78 in the cells. Furthermore, it seemed that the decreased GRP78 expression in the cells expressing EGFP–polyQ97 was due to the enhanced protein degradation of GRP78 through the ubiquitin–proteasome pathway. These findings indicated that GRP78, which has an inhibitory effect on the aggregation of proteins containing expanded polyQ tract, may be an effective target for the treatment of polyQ diseases.

1. Introduction

Polyglutamine (polyQ) diseases are a group of inherited neurodegenerative disorders sharing the common feature of glutamine tract expansion within unrelated proteins as their mutational basis and are responsible for at least nine diseases, including Huntington's disease, spinal and bulbar muscular atrophy, dentatorubral pallidoluysian atrophy, and spinocerebellar ataxia types 1, 2, 3, 6, 7, and 17 [1,2]. All of these disorders show late onset of neurological symptoms with progressive neuron dysfunction and eventual neuronal loss, although the susceptible regions in the nervous system differ among them. A common characteristic feature of polyQ diseases is the formation of insoluble aggregates of proteins in the vulnerable neurons [1,2]. The aggregates including expanded polyQ proteins are found in both the nucleus and the cytoplasm, and the aggregates represent a pathological event correlated with disease progression [3–7]. Additionally, these aggregates accumulate the components of transcription factors, the ubiquitin–proteasome system, and molecular chaperones, and numerous cellular pathways are impaired by the expression of proteins containing an expanded polyQ tract [8–11]. It remains unclear, however, which of these cellular defects are initial and specific triggers of the expanded polyQ tract-containing proteins.

Endoplasmic reticulum (ER) is a large membrane-enclosed intracellular organelle in which secretory and membrane-bound proteins are synthesized and folded into their final three-dimensional structures [12,13]. Perturbation of ER function due to glucose deprivation, aberrant Ca^{2+} regulation, or accumulation of misfolded proteins leads to the unfolded protein response (UPR) to cope with this imbalance of ER homeostasis. The UPR results in activation of three linked signaling pathways emanating from three ER stress sensors: inositol-requiring protein 1α (IRE1α), PKR-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6α (ATF6α) [14,15]. The combined actions of these signaling cascades serve to reduce ER stress through induction of chaperone and attenuation of protein translation [15]. Recently, alterations in the function of ER have been shown to be involved in cell stress and in different types of human disorders including polyQ diseases [16–18]. However, the molecular event linking the aggregation of polyQ tract-containing proteins to ER stress response is unknown.

Here, we performed proteomic analysis of cellular proteins prepared from HeLa cells expressing enhanced green fluorescent protein with a pathological-length polyQ tract (EGFP–polyQ97) or a non-pathological-length polyQ tract (EGFP–polyQ24), and found that GRP78, the ER chaperone, was significantly decreased in the

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0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.05.078

cells expressing enhanced green fluorescent protein with EGFP–polyQ97, but not EGFP–polyQ24. In addition, we revealed that the expression levels of GRP78 were inversely related to the aggregation of EGFP–polyQ97. These findings suggest that GRP78 has an inhibitory effect on the aggregation of proteins containing expanded polyQ tract.

2. Materials and methods

2.1. Cell culture

HeLa-tetQ24 and HeLa-tetQ97 cells, which overexpress either EGFP–polyQ24 or EGFP–polyQ97 by the removal of DOX, have been described previously [19]. These cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 μg/ml hygromycin, 100 μg/ml geneticin, and 1 μg/ml doxycycline (DOX) at 37 °C with 95% air and 5% CO_2.

The induction of EGFP–polyQ24 and EGFP–polyQ97 expression was performed as follows. HeLa-tetQ24 and HeLa-tetQ97 cells were trypsinized, washed twice with 10 ml of fresh medium, and then grown in fresh medium without DOX for 24 h. The following day, the cells were washed once with phosphate-buffered saline (PBS) and incubated in fresh medium without DOX at 37 °C for the indicated period.

2.2. Plasmids and transfection

To construct expression plasmids for GRP78 (pcDNAGRP78), GRP78 cDNA was amplified from the total RNA of human HEK293 cells by a reverse transcriptase-polymerase chain reaction (RT-PCR) using the RNA PCR kit (AMV) ver. 3.0 according to the manufacturer's instructions (TaKaRa, Shiga, Japan). The following oligonucleotides were used as primers of RT-PCR: 5′-GCGGATCC-ACTGGCTGGCAAGATGAAGC-3′ and 5′-CGCTCGAGTGCAGATCTGT-GTCTACAACT-3′, with the restriction enzyme sites underlined. Then, the PCR products were digested with *BamHI* and *XhoI*, and subcloned between *BamHI* and *XhoI* sites of the mammalian expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA, USA).

For plasmid transfection, HeLa-tetQ24 and HeLa-tetQ97 cells were grown in DMEM medium without DOX for 48 h, then trypsinized and divided into 24-well plates (7×10^4 cells/well). The following day, the cells were transiently transfected with 2 μg of pcDNAGRP78 using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instructions. For the knockdown of GRP78 expression, HeLa-tetQ24 and HeLa-tetQ97 cells were grown in 24-well plates (7×10^4 cells/well) without DOX for 24 h, and then transfected with 20 pmol of the GRP78 siRNA (Invitrogen) or RISC-free control siRNA (Dharmacon, Chicago, IL, USA) using Lipofectamine 2000.

2.3. Detection of the cells with protein aggregates

Cells grown on collagenized coverslips (2×10^4 cells/cm^2 in 24-well plates) were fixed with 4% paraformaldehyde for 30 min at room temperature. After washing with PBS, the cells were stained with 10 μM Hoechst 33342 for 10 min in the dark and observed using a fluorescence microscope (Nikon, Tokyo, Japan). The number of cells with visible aggregates and the number of transfected cells without aggregates were counted independently in randomly chosen microscopic fields in different areas of each coverslip. Approximately 600–1000 transfected cells were analyzed in each experiment.

2.4. Proteomic analysis

Preparation of protein extracts and proteomic analysis were performed as described previously [20]. Briefly, each protein extract (700 μg of protein) was loaded onto dry immobilized pH gradient strips (17 cm, pH 3–10 nonlinear, Bio-Rad Laboratories, Hercules, CA, USA) under conditions of passive rehydration. Isoelectric focusing was performed at a maximum of 10,000 V for a total of 80,000 V-h. Two-dimensional electrophoresis (2-DE) was performed in 12% SDS-polyacrylamide gels, and the gels were stained with a SYPRO Ruby solution (Bio-Rad Laboratories). To check for reproducibility, the experiment was repeated at least five times using independently purified samples. Differentially expressed spots were excised and digested with Trypsin, and the resultant peptides were extracted and analyzed using a Voyager-DE™ MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA). Proteins were identified with peptide mass fingerprinting using an MS-Fit search engine.

2.5. Western blotting

Cells (5×10^5 cells/35 mm dish) were lyzed with 0.1% SDS and boiled for 5 min. Aliquots (20 μg of protein) of cell extracts in SDS-sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.00125% bromophenol blue) were subjected to SDS–polyacrylamide gel electrophoresis, then transferred onto nitrocellulose membranes by electrotransfer. Immunodetection was performed with a chemiluminescent detection method (ECL plus, GE Healthcare Bioscience, Piscataway, NJ, USA). Antibodies used in this study were as follows: anti-GRP78 (BD Biosciences Pharmingen, San Diego, CA, USA), anti-GFP (G6539, Sigma Chemical, St. Louis, MI, USA), anti-α-tubulin (T9026, Sigma Chemical) and anti-multi-ubiquitin (D058-3, Medical & Biological Laboratories, Nagoya, Japan).

2.6. Indirect immunofluorescence

Cells grown on collagenized coverslips (2×10^4 cells/cm^2 in 24-well plates) were fixed with 4% paraformaldehyde for 30 min at room temperature and permeabilized with 0.1% Triton X-100 in PBS. After blocking with 5% bovine serum albumin in PBS for 1 h, anti-GRP78 (H-129, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:100 dilution was added to the coverslip and incubated in a moist chamber for 1 h at 37 °C. After washing with PBS, rhodamine red-conjugated anti-rabbit IgG antibody at a 1:200 dilution (Invitrogen) was added to the coverslip and incubated further at 37 °C for 1 h. After washing with PBS, the cells were observed using a fluorescence microscope.

2.7. RNA isolation and gene expression

Total RNA was isolated using GenElute mammalian total RNA miniprep kit (Sigma Chemical). The RNA was reverse-transcribed to single-strand cDNA using RNA PCR kit (TaKaRa) and the resultant cDNA was used for RT-PCR analysis. Data were normalized to GAPDH and are presented as the mean ± SD from four independent experiments.

2.8. ^{35}S-Methionine labeling and immunoprecipitation

HeLa-tetQ24 and HeLa-tetQ97 cells were grown in DMEM medium with or without DOX for 72 or 120 h, then trypsinized and transferred to 60 mm dishes (7×10^5 cells/dish) at 24 h before the experiment. The following day, the cells were washed once with PBS and incubated in fresh medium without methionine and cysteine at 37 °C for 1 h, and then incubated in medium

containing 100 mCi/ml ^{35}S-Methionine (EasyTag EXPRE^{35}S^{35}S Protein Labeling Mix, Perkin Elmer, Waltham, MA, USA) for 6 h at 37 °C. Then, the cells were washed twice with PBS and incubated in fresh medium. At the indicated time points, the cells were washed twice with PBS and lysed in ice-cold lysis buffer. The lysates were then centrifuged at 20,000*g* for 10 min at 4 °C, and the aliquot of supernatant (500 μg of protein) was used for immunoprecipitation using anti-GRP78 (Santa Cruz Biotechnology) or control anti-rabbit IgG antibody. The immunoprecipitated proteins were resolved by SDS–PAGE, and the dry gel was exposed to X-ray film (Fuji Film, Kanagawa, Japan) at −80 °C for 24–72 h.

3. Results

3.1. Expression of GRP78 decreases in the expanded polyQ protein-expressing HeLa cells

We established the stable HeLa cell lines HeLa-tetQ24 and HeLa-tetQ97 cells, which overexpress either EGFP–polyQ24 or EGFP–polyQ97 under the control of a tetracycline-regulated promoter [19]. Fig. 1A shows the expression levels of EGFP–polyQ24 and EGFP–polyQ97 in these cells grown with or without DOX. At 3 or 5 days after DOX removal from the medium, the expression of EGFP–polyQ24 and EGFP–polyQ97 was induced in HeLa-tetQ24 and HeLa-tetQ97 cells, respectively. In addition, when these cells was observed by fluorescence microscopy, EGFP–polyQ97 but not EGFP–polyQ24 aggregated in the cytoplasm and/or the nucleus of the cells, and proportions of cells with aggregates increased depending on the incubation time after DOX removal (Fig. 1B, C).

To identify proteins that are differentially expressed in the cells with aggregation of the proteins containing an expanded polyQ tract, we next analyzed the proteome profile in HeLa-tetQ24 and HeLa-tetQ97 cells with or without EGFP–polyQ24/97 expression using gel-based proteomics technology. A representative pair of resulting 2-DE gels is shown in Fig. 2A. About 500 spots in each image were matched and quantified using PDQuest analyzing software, and at least three protein spots were expressed at a different level in the cells expressing EGFP–polyQ97, compared with EGFP–polyQ24. The proteins were analyzed by peptide mass fingerprinting after in-gel digestion with trypsin, and were identified as GRP78 (Fig. 2B), prohibition and interleukin-1 family member 8 (data not shown). Among these proteins, we focused on GRP78 because GRP78 is involved in protein folding in ER lumen and prevents the aggregation of unfolded proteins [12,13]. The decreased levels of GRP78 were further confirmed by Western blot analysis in the cells expressing EGFP–polyQ97 (Fig. 2C).

3.2. GRP78 suppresses expanded polyQ tract-induced protein aggregation in HeLa cells

To study the role of GRP78 in the aggregation of proteins containing a pathological-length polyQ tract, we next examined the effects

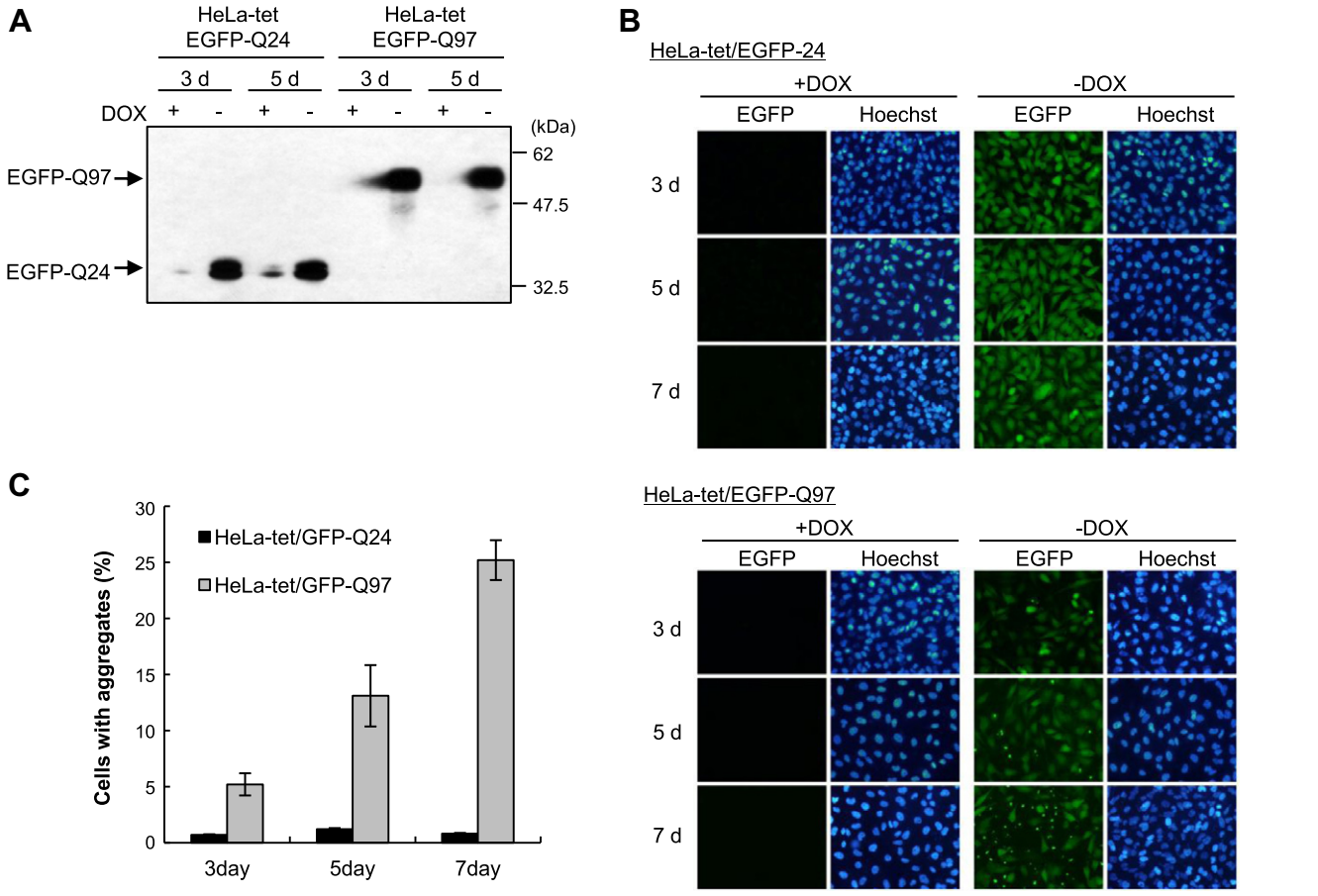

Fig. 1. Establishment and characterization of HeLa cells that overexpress either EGFP–polyQ24 or EGFP–polyQ97 using the tet-off system. HeLa-tetQ24 and HeLa-tetQ97 cells were grown in medium with or without DOX for the indicated period. (A) Cells were harvested, protein extracts (20 μg each) were separated by SDS–PAGE, and the expression levels of EGFP–polyQ24 and EGFP–polyQ97 were determined by western blotting using anti-GFP antibody. (B) The cells were fixed, stained with Hoechst 33342, and observed using a fluorescence microscope. EGFP (green) shows the distribution of EGFP–polyQ24 or EGFP–polyQ97. (C) The proportions of cells containing protein aggregates in GFP-positive cells at various times after the removal of DOX are represented as the mean ± S.D. of four independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

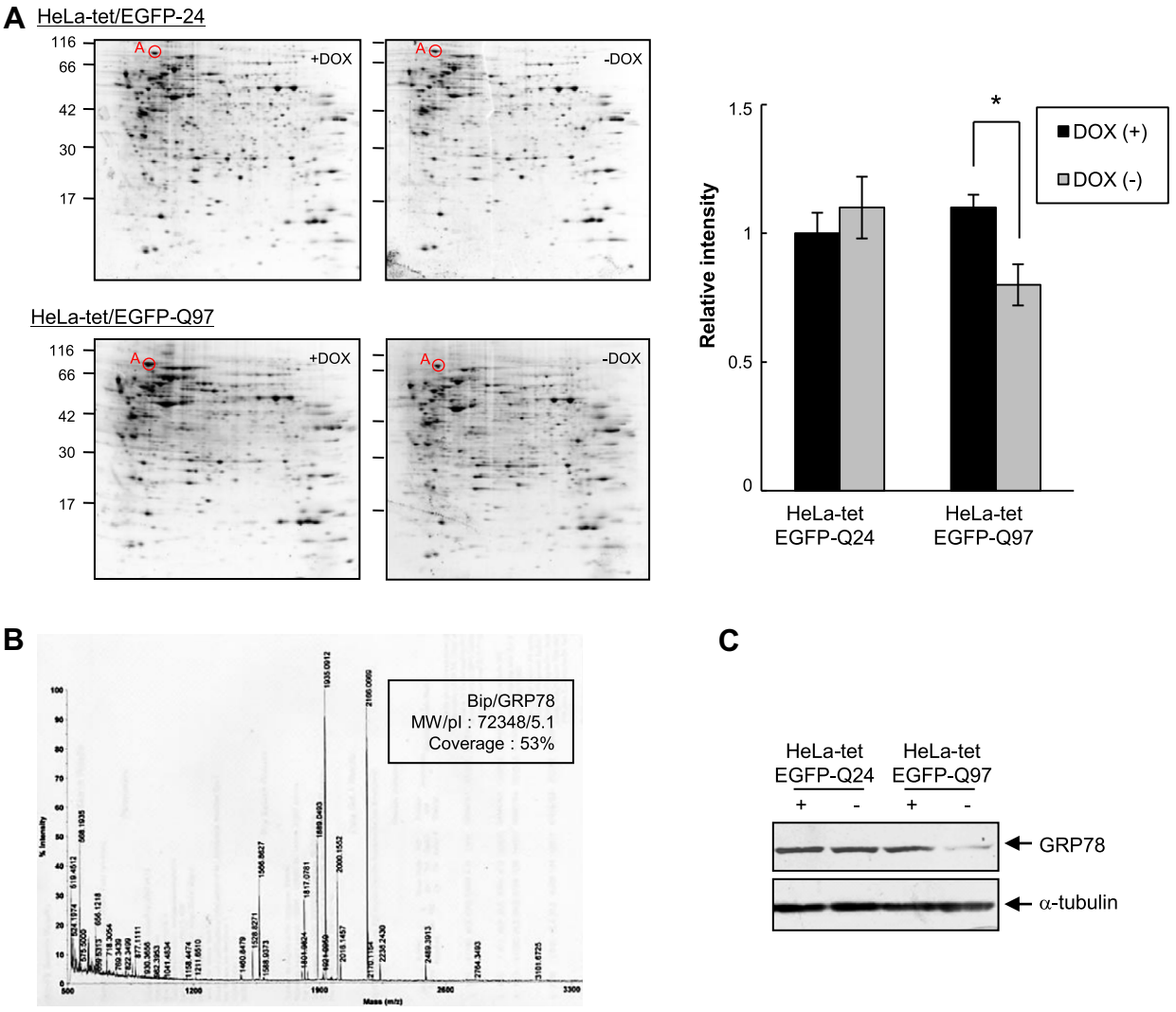

Fig. 2. Expression of GRP78 was decreased by the expression of EGFP–polyQ97 in HeLa cells. (A) Protein extracts (700 μg of proteins) from HeLa-tetQ24 and HeLa-tetQ97 cells grown in the medium with or without DOX for 5 days were separated by 2-DE and stained with SYPRO Ruby solution. A representative spot (red circle) indicates the protein whose expression was remarkably reduced when expression of EGFP–polyQ97, but not EGFP–polyQ24, was induced in HeLa cells. Relative intensity of spot A was quantified with PDQuest analyzing software, and is represented as the mean ± S.D. of four independent experiments. The significance of differences was assessed using unpaired Student's *t*-test. An asterisk indicates significance at $p < 0.05$. (B) Spot A was excised from the stained 2-DE gels, digested with trypsin in-gel. The resultant peptides were extracted and analyzed using a MALDI-TOF mass spectrometer. Mass values obtained from peaks were used to search databases with the software MS-fit. (C) HeLa-tetQ24 and HeLa-tetQ97 cells were grown in the medium with or without DOX for 7 days. Protein extracts (20 μg each) were separated by SDS–PAGE and the levels of GRP78 and α-tubulin were analyzed by Western blotting using the respective antibodies. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of GRP78 knockdown on the aggregate formation of EGFP–polyQ97. Transfection of GRP78 siRNA significantly reduced endogenous GRP78 expression, and the aggregation of EGFP–polyQ97 was increased in HeLa-tetQ97 cells (Fig. 3A and B). Conversely, when GRP78 was overexpressed in HeLa-tetQ97 cells, the aggregation of EGFP–polyQ97 was suppressed (Fig. 3C and D). Thus, the expression levels of GRP78 seemed to be inversely correlated to the aggregation of proteins containing an expanded polyQ tract.

3.3. Decreased GRP78 expression by the expression of EGFP–polyQ97 is due to increased GRP78 protein degradation through the ubiquitin–proteasome pathway

To elucidate how the expression level of GRP78 was decreased in the cells expressing EGFP–polyQ97, we next examined the expression level of GRP78 mRNA in HeLa-tetQ97 cells grown with or without DOX. As shown in Fig. 4A, the levels of GRP78 mRNA were not changed by the induction of EGFP–polyQ97 at 3 or 5 days after DOX removal. In addition, when the synthesis of GRP78 was measured by the labeling of cells with ^{35}S-methionine and immunoprecipitation using anti-GRP78 antibody, the level of GRP78 synthesis was not affected by the expression of EGFP–polyQ97 (Fig. 4B). Therefore, we analyzed the degradation rate of GRP78 protein by a pulse-chase experiment using ^{35}S-methionine in HeLa-tetQ97 cells. As shown in Fig. 4C, the degradation of GRP78 protein was dramatically enhanced by the expression of EGFP–polyQ97. In addition, when the cells were treated with MG132, an inhibitor of the ubiquitin–proteasome pathway, decreased GRP78 protein by the expression of EGFP–polyQ97 was suppressed (Fig. 4D). Thus, the decreased GRP78 expression in the cells with proteins containing an expanded polyQ tract seemed to be due to the increased GRP78 protein degradation through the ubiquitin–proteasome pathway.

4. Discussion

Aggregation of proteins containing expanded polyQ tract is a common characteristic feature of polyQ diseases, resulting in

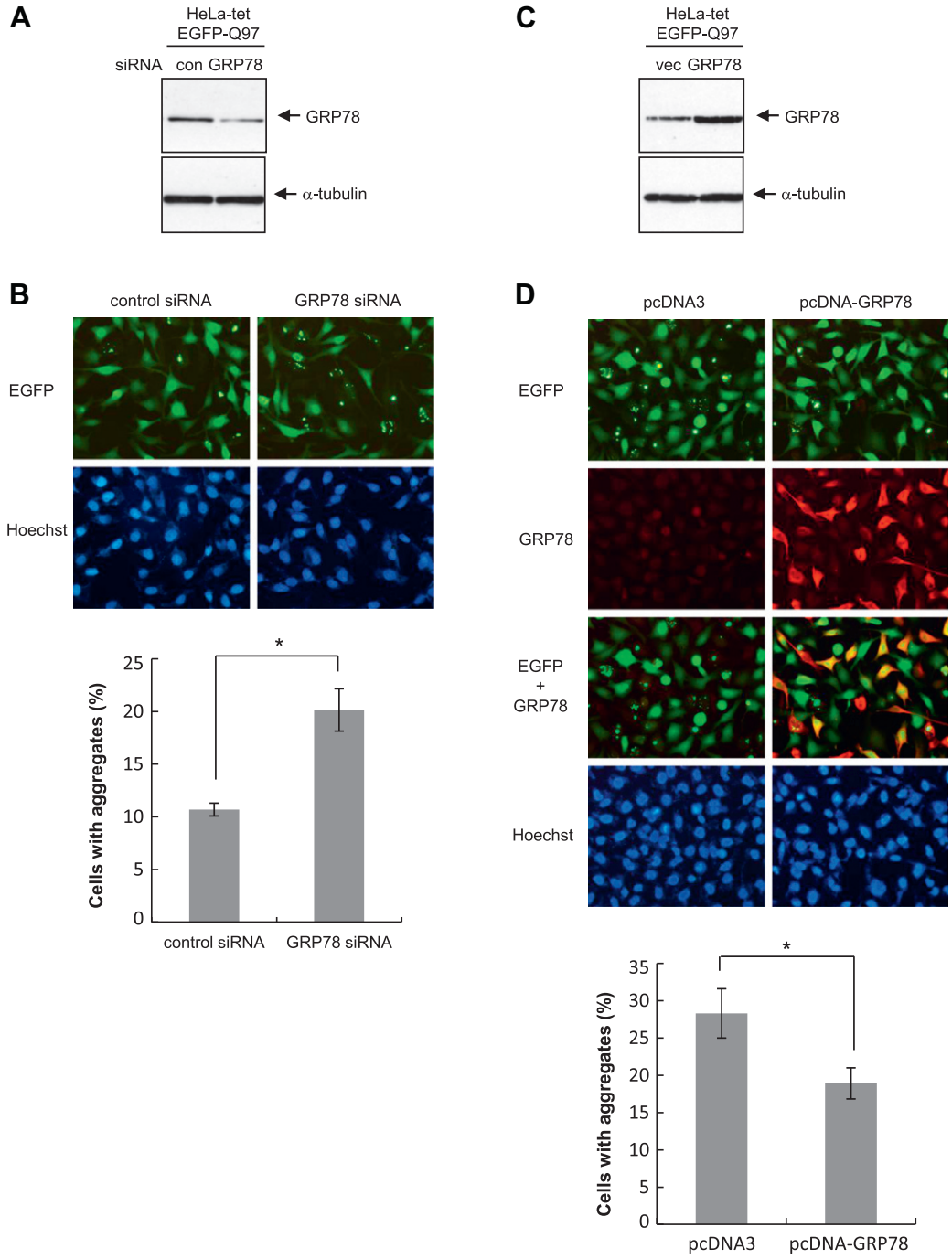

Fig. 3. Expression levels of GRP78 are inversely related to the aggregation of EGFP–polyQ97. (A) HeLa-tetQ97 cells were grown in medium without DOX for 24 h. The cells were transfected with GRP78 siRNA or RISC-free control siRNA. At 72 h after transfection, the cells were harvested and GRP78 and α-tubulin were detected by western blotting using respective antibodies. (B) At 72 h after siRNA transfection, the cells were fixed with paraformaldehyde, stained with Hoechst 33342, and observed using a fluorescence microscope. The proportions of cells containing protein aggregates in EGFP-positive cells are represented as the mean ± S.D. of four independent experiments. The significance of differences was assessed using unpaired Student's *t*-test. An asterisk indicates significance at $p < 0.01$. (C) HeLa-tetQ97 cells were grown in medium without DOX for 48 h. The cells were transfected with pcDNAGRP78 plasmid or pcDNA vector. At 48 h after transfection, the cells were harvested and GRP78 and α-tubulin were detected by western blotting using respective antibodies. (D) At 48 h after plasmid transfection, the cells were fixed with paraformaldehyde, stained with Hoechst 33342, and observed using a fluorescence microscope. The expression of exogenous GRP78 (red) was observed by indirect immunofluorescence using an anti- GRP78 antibody. The proportions of cells containing protein aggregates in GFP-positive cells are represented as the mean ± S.D. of four independent experiments. The significance of differences was assessed using unpaired Student's *t*-test. An asterisk indicates significance at $p < 0.01$.

impairment of the functions of some cellular proteins. In this study, we identified that an ER chaperone, GRP78, was significantly decreased in the cells expressing the proteins with a pathological-length polyQ tract, but not those with a non-pathological-length polyQ tract, by proteomic analysis. In addition, GRP78 suppresses the aggregation with proteins containing expanded polyQ tract. Furthermore, the decreased GRP78 expression in the cells with proteins of an expanded polyQ tract seemed to be due to the increased GRP78 protein degradation through the ubiquitin–proteasome pathway. GRP78 is a member of the HSP70 family abundant

in the lumen of ER, and plays important roles as a molecular chaperone to remove malfolded proteins in ER lumen [12,13]. Overexpression of GRP78 has been shown to increase ER stress resistance and to have beneficial effects in several cell types [21–23]. Therefore, the decreased expression of GRP78 by the expression of expanded polyQ tract may impair 'quality control' in the ER, resulting in the dysfunction of some cellular proteins in polyQ diseases.

Previous studies showed that the expression of proteins with expanded polyQ repeats in neuronal cells induces ER stress with elevation of GRP78 expression, cleavage of caspase-12, and activation of the c-Jun-N-terminal kinase (JNK) pathway for ER stress [24–26]. Kouroku et al. showed that the polyQ72 with perinuclear aggregates, cytoplasmic inclusions and nuclear inclusions was observed in about 50% of EGFP-positive cells by transiently transfection with pEGFP-72CAG plasmid in the neuronal C2C5 cells, and

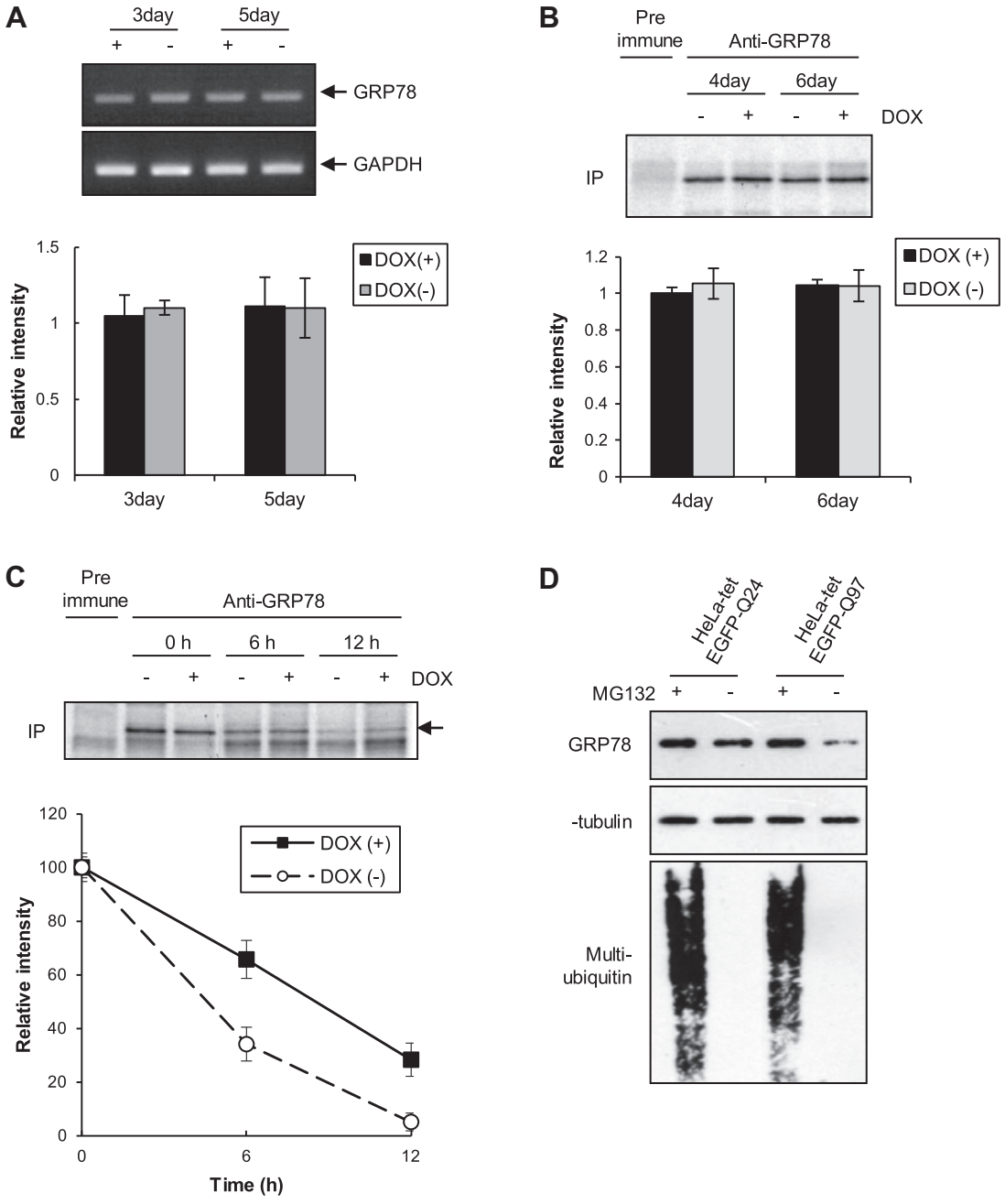

Fig. 4. GRP78 expression was decreased by the expression of EGFP–polyQ97 through increased GRP78 protein degradation through the ubiquitin–proteasome pathway. (A) Total RNA (0.5 μg each) was reverse-transcribed to single-stranded cDNA and analyzed by PCR using primers specific for *grp78* mRNA and *GAPDH* mRNA. Expression values of *grp78* mRNA were normalized to that of *GAPDH* mRNA. The values are the mean ± SD from four independent experiments. Statistical significance was determined using unpaired Student's *t*-test. (B) After labeling with ^{35}S-methionine for 6 h, the cells were lysed and GRP78 was immunoprecipitated using anti-GRP78 antibody. The immunoprecipitates were resolved with SDS–PAGE and the synthesized GRP78 was detected by autoradiography. The values are the mean ± SD from four independent experiments. (C) After labeling with ^{35}S-methionine for 6 h, the cells were washed with PBS and then chased in the absence of ^{35}S-methionine for different time periods. Then, the cells were lysed and GRP78 was immunoprecipitated using anti-GRP78 antibody. The values are the mean ± SD from four independent experiments. The significance of differences was assessed using unpaired Student's *t*-test. (D) HeLa-tetQ24 and HeLa-tetQ97 cells were grown in the medium with or without DOX for 7 days. Then, the cells were treated with 1 mM MG132 for 6 h. Protein extracts (20 μg each) were separated by SDS-PAGE and the levels of GRP78, α-tubulin, and multi-ubiquitinated proteins were analyzed by Western blotting using the respective antibodies.

these aggregates were induced the polyQ aggregate-mediated cell death through the activation of caspase-12 [24]. Transient expression of *N*-terminal huntingtin fragment proteins containing expanded polyQ tract also induces protein aggregation and cell death in the neuronal PC6.3 cells [26]. In these models, upregulation of GRP78 and activation of JNK, which stimulate ER stress, were induced in the cells expressing the proteins with an expanded polyQ tract. However, Thomas et al. showed that the *N*-terminal androgen receptor fragment with an expanding polyQ tract induces an ER stress-responsive promoter activity in a polyQ length-dependent manner, but failed to enhance the levels of GRP78 expression [27]. Thus, the level of GRP78 expression was controversial, although the aggregation with proteins containing expanded polyQ tract induced the UPR. Here, we showed the level of GRP78 expression was reduced by the long term expression of expanded polyQ tract using the cells, which overexpress EGFP–polyQ97 by the removal of DOX. Previously, we and other showed that the level of GRP78 expression was reduced in the diabetic animal under the prolonged ER stress conditions [28,29]. Therefore, the level of GRP78 expression may be also decreased by the prolonged ER stress induced the aggregation with proteins containing expanded polyQ tract, involving in the pathogenesis of neurodegenerative disorders with polyQ expansion.

In summary, we show here that the levels of GRP78 expression are inversely related to the aggregation of proteins containing an expanded polyQ tract. We have recently found that the protein aggregation of EGFP–polyQ97 was suppressed by the treatment with naringenin, a compound that up-regulated GRP78 without ER stress (Yamagishi et al. unpublished data). Thus, GRP78 is an effective target for the treatment of polyQ diseases, and small molecules that induce GRP78 expression may help to develop an effective approach for the treatment of polyQ diseases including Huntington's disease and spinal and bulbar muscular atrophy.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research (C) (No. 1750903 to T.H., No. 23590094 to N.Y.) and Program for the Strategic Research Foundation at Private Universities (2008-2012) from the Ministry of Education, Culture, Sports, Science and technology of Japan.

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