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Dimerization of pro-oncogenic protein Anterior Gradient 2 is required for the interaction with BiP/GRP78

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ABSTRACT

Anterior Gradient 2 (AGR2), an ER stress-inducible protein, has been reported to be localized in endoplasmic reticulum (ER) and its level is elevated in numerous metastatic cancers. Recently, it has been demonstrated that AGR2 is involved in the control of ER homeostasis. However, the molecular mechanism how AGR2 regulates ER stress response remains unclear. Herein we show that AGR2 homo-dimerizes through an intermolecular disulfide bond. Moreover, dimerization of AGR2 attenuates ER stress-induced cell death through the association with BiP/GRP78. Thus, these results suggest that dimerization of AGR2 is crucial in mediating the ER stress signaling pathway.

1. Introduction

The unfolded protein response (UPR) signaling pathway is a cellular stress response initiated by BiP/GRP78, a central regulator of ER processes including protein biogenesis, signal transduction, and calcium homeostasis [1–5]. In the resting state, BiP/GRP78 is associated with ER stress transducers such as inositol-requiring kinase 1α (IRE1α), protein kinase RNA-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6). Upon ER stress, the UPR signaling pathway is triggered by dissociation of BiP/GRP78 from IRE1α and PERK, which are then activated by oligomerization and phosphorylation in the ER outer membrane [6]. The activated UPR signaling pathway attenuates the translation of newly synthesized protein to prevent further translational loading and to restore normal ER function. Furthermore, activation of UPR signaling contributes to tumor cell survival [7]. BiP/GRP78 knockdown suppresses tumor growth in nude mice [8]. XBP1 and PERK are also required for tumor growth. XBP1 knockdown does not yield transplantable tumors in nude mice [9]. Similarly, PERK knockdown reduces the rate of tumor growth *in vivo* [10,11].

Human Anterior Gradient 2 (AGR2) was identified as a homologue of *Xenopus laevis* XAG-2, which serves an essential role during neural development [12,13]. In humans, AGR2 is elevated in most metastatic adenocarcinomas (e.g., breast, pancreas, prostate, gastric, esophagus, liver, lung, and colon) [14–21]. AGR2 is induced by various stresses such as hypoxia, serum depletion, and ER stress [22,23]. The malignant tumor environment, which produces hypoxic stress, triggers ER stress; this stress response in solid tumors is linked to the unfolded protein response (UPR) signaling pathway [7,10,24–28]. Over-expression of AGR2 promotes tumor growth and a metastatic phenotype *in vivo* and *in vitro* [29]. AGR2 silencing also affects not only the UPR signaling pathway but also ER stress induced autophagy [30]. Although numerous reports claim that AGR2 is involved in the UPR signaling pathway and ER stress-induced cell death, the molecular mechanism linking AGR2 to the UPR signaling pathway has not been described yet.

In the present study, we demonstrated that AGR2 forms a homo-dimer through an intermolecular disulfide bond in human colon cancer cell, Hct8. We also showed that dimerization of AGR2 attenuates ER stress induced cell death and its dimerization is required for the interaction with BiP/GRP78. Thus, these results may imply that dimerization of AGR2 is critical step to modulate UPR signaling pathway.

2. Materials and methods

2.1. Cell culture and treatment

Hct8 (human colon adenocarcinoma cell line) and HeLa cells were cultured in Dulbecco's Modified Eagle Media (DMEM) containing 10% fetal bovine serum (FBS) at 37 °C under a 5% CO_2

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atmosphere. Cells ($0.5–1 \times 10^6$ cells/well) were plated and then incubated for various periods with 2 μg/mL tunicamycin (Sigma), 0.25 μM thapsigargin (Sigma) to induce ER stresses. All experiments were repeated at least 3 times to ensure reproducibility.

2.2. Expression plasmids and transfection

Full-length cDNA clones encoding human AGR2 and BiP/GRP78 were purchased from Invitrogen and subcloned into a mammalian expression vector. The AGR2 and BiP/GRP78 coding sequences were inserted into pCMV-Tag 1 (Agilent Technologies) with no tag and pcDNA3.1 zeo (+) with an N-terminal FLAG (or HA) using the *BamH*I/*Xho*I sites. A FLAG tag was inserted into the full AGR2 sequence, based on a previously reported FLAG-AGR2 construct [31]. pCMV-Tag 1 and pcDNA3.1 zeo (+) AGR2 [C81S] mutant constructs were generated by PCR-based site-directed mutagenesis. All constructs were verified by DNA sequencing from both directions. Transfection of the plasmids (1 μg of DNA unless otherwise indicated) into cells was conducted using Lipofectamine 2000 reagent (Invitrogen).

2.3. Western blotting

Approximately 48 h after transfection, cells were harvested, washed twice with ice-cold PBS, and lysed in NP-40 lysis buffer (20 mM Tris–HCl, pH 7.5; 137 mM NaCl; 1 mM EDTA; 1% NP-40; 10% glycerol) supplemented with protease inhibitor and phosphatase inhibitor (Roche). The cell lysate was clarified by centrifugation (13,000 rpm) for 20 min at 4 °C. The proteins (10–30 μg) in the supernatant were separated by SDS–PAGE, transferred to PVDF membrane (Millipore), probed with the appropriate antibodies, and then visualized by using an ECL kit (Pierce). Tubulin was used as a loading control in all Western blots. Primary antibodies used were as follows: AGR2 (Santa Cruz and Abcam), tubulin, FLAG (Sigma), p-PERK (Santa Cruz), p-IRE1α (Abcam), BiP/GRP78, HA, PERK, IRE1α, PARP, or caspase-3 (Cell Signaling).

2.4. Immunoprecipitation

Immunoprecipitation of FLAG-tagged AGR2 and BiP/GRP78 was performed using anti-FLAG M2 affinity gels (Sigma). To isolate FLAG-tagged proteins, 500–600 μg of cell lysate was mixed with 20 μL of affinity gel at 4 °C for 6 h with mild shaking. The antigen–antibody complexes were collected by centrifugation at 3000 rpm for 1 min, washed 3–5 times with NP-40 lysis buffer, and then boiled with 1× SDS sampling buffer for 5 min at 95 °C to elute the proteins from the affinity gels. The eluted proteins were analyzed by Western blotting.

2.5. Cell viability assay

Cell viability under ER stress conditions was determined using the Cell Counting Kit-8 (CCK8, DOJINDO Laboratories). Briefly, cells (5×10^3 cells/well) cultured in a 96-well plate were treated with tunicamycin or thapsigargin for 60 h. After treatment, 10 μL of CCK8 assay solution was added and the cultures were incubated for 1 h. Absorbance at 450 nm was measured using an ELISA reader. Percent viability was calculated using empty vector as 100%.

2.6. Chemical crosslinking

Immediately before use, disuccinimidyl suberate (DSS) or bis[sulfosuccinimidyl] suberate (BS^3)was dissolved in DMSO or water. Cells were harvested and washed 3 times with ice-cold PBS. The pelleted cells were resuspended in 1 mL PBS, and then chemical cross-linkers were added to each cell suspension. The cross-linked cells were incubated for 30–60 min with mild shaking at room temperature, and then quenched by quenching buffer (1 M tris, pH 7.5) to a final concentration of 15 mM for 15 min at room temperature to stop the chemical reaction. The chemically cross-linked cells were subjected to Western blot analysis.

2.7. Statistical analysis

All quantitative data were analyzed using an independent Student's *t*-test and considered significant at $p < 0.05$.

3. Results

3.1. AGR2 forms a homo-dimer through an intermolecular disulfide bond

Our group had previously identified that AGR2 is associated with the high metastatic potential in human gastric cancer cell [32]. During in pursuit of the function of AGR2, we observed that bacterially expressed AGR2 protein migrates approximately 34 kDa, corresponding to its dimeric form in the gel filtration chromatography (data not shown). AGR2 contains a protein disulfide isomerase (PDI)-like domain that consists of a CXXS domain, called the Tx-like domain (Fig. 1A). Although it has been reported that AGR2 forms heterodimers with MUC2 through the disulfide bond in mucus-producing cells using Cys81 residues [31], this results led us to examine this idea that AGR2 may form a dimer in cells. To test this idea, chemical crosslinking agents (DSS and BS3 intra- and extracellular cross-linkers, respectively) were applied to Hct8 cells and cell extracts were analyzed by Western blotting. Consequently, 17 and 34 kDa bands were observed, corresponding to monomeric and dimeric AGR2, respectively (Fig. 1B). To investigate whether this dimerization is derived from the intermolecular disulfide bond, we prepared an AGR2 mutant in which cysteine 81 was substituted with serine (CS). FLAG tagged wild-type AGR2 and CS mutant AGR2 were overexpressed in Hct8 cells. Then cell extracts were subjected to non-reducing SDS–PAGE and were analyzed by Western blotting using anti FLAG antibodies. The dimer form of CS-mutant AGR2 was not seen in non-reducing SDS–PAGE whereas that of wild-type AGR2 was observed (Fig. 1C), indicating that AGR2 forms a homo-dimer through an intermolecular disulfide bond.

3.2. Dimerization of AGR2 attenuates ER stress-induced cell death

AGR2 has been identified as a tumorigenic and metastatic marker [15,17,20,33–35]. In tumor cells, elevated AGR2 level was correlated with cell proliferation [29,36,37], although the underlying mechanism remains unclear. As the silencing of AGR2 impaired the UPR pathway [30], we speculated whether overexpression of AGR2 may also affect the UPR signaling pathway. Thus, we examined the levels of phosphorylated PERK and phosphorylated IRE1α when AGR2 was over-expressed. In the absence of ER stresses, overexpression of FLAG-tagged AGR2 induced phosphorylation of both PERK and IRE1α in a dose-dependent manner (Fig. 2A). To examine the role of AGR2 dimerization, wild-type AGR2 or the CS mutant was over-expressed. When wild-type AGR2 was expressed, phosphorylated PERK and phosphorylated IRE1 increased. However, over-expression of the CS mutant did not induce either PERK or IRE1 phosphorylation (Fig. 2B), indicating that dimerization of AGR2 is important for the activation of the UPR signaling pathway. We then examined whether over-expression of ARG2 influences cell viability upon ER stresses such as tunicamysin and thapsigargin. Over-expression of wild-type AGR2 rendered cells resistant to ER stresses (Fig. 2C), which is contrast to the effects

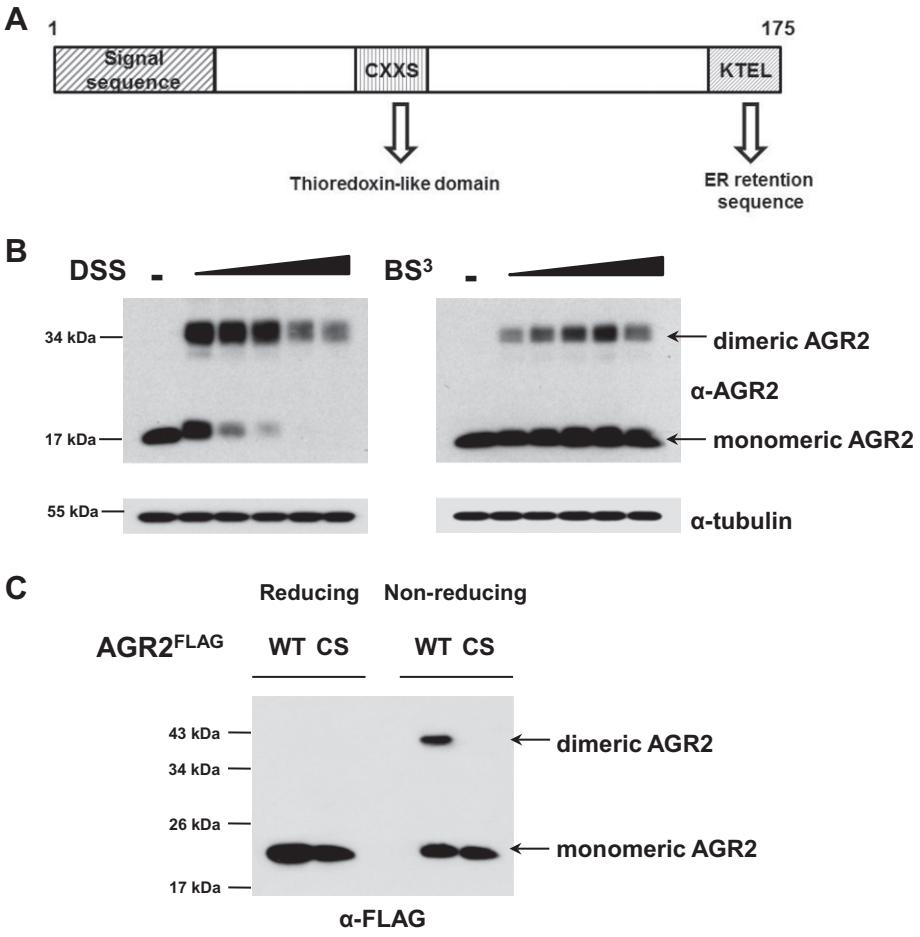

Fig. 1. AGR2 forms a homo-dimer through an intermolecular disulfide bond. (A) Diagram of AGR2 protein showing its domains and motifs. (B) Chemical crosslinkers (DSS, intracellular crosslinker; BS^3, extracellular crosslinker) were added to each cell suspension at final concentrations of 0, 0.1, 0.25, 0.5, 1.0, and 1.5 mM. Monomeric and dimeric AGR2 were detected by Western blotting with AGR2 antibody. (C) FLAG-tagged wild-type and CS-mutant AGR2 were transfected into Hct8 cells. The cell extracts were reduced by 2-mercaptoethanol or left non-reduced. FLAG-tagged proteins were detected by Western blotting with FLAG antibody.

of siAGR2 (30). However, over-expression of CS mutant did not exhibit any change in cell viability (Fig. 2C). We also checked ER stress induced caspase 3 activation. In response to prolonged ER stress, caspase 3 was activated via its cleavage as shown in Fig. 2D and cells undergo cell death. Wild-type AGR2 over-expression attenuates the activation of caspase-3 under ER stress, whereas the CS mutant overexpression did not affect the cleavage of caspase 3, compared to mock transfection (Fig. 2D). Thus, this result implies that AGR2 dimerization attenuates ER stress-induced cell death.

3.3. Dimerization of AGR2 is required for the interaction with BiP/GRP78

The previous results led us to examine the possibility that AGR2 may interact with UPR signaling pathway regulators such as BiP/GRP78, IRE1α, and PERK. Thus, exogenous FLAG-tagged AGR2 was expressed in Hct8 cells and was immunoprecipitated using FLAG M2 beads. The immunoprecipitated complexes were analyzed by Western blotting using anti-BiP/GRP78, IRE1α, or PERK antibodies. BiP/GRP78, an initiator in the UPR pathway and a central molecular chaperone in all ER processes, is co-immunoprecipitated with AGR2 (Fig. 3A). However, PERK and IRE1αwere not co-immunoprecipitated with AGR2 (data not shown). We wondered whether the physical interaction between ARG2 and BiP/GRP78 is affected under ER-stressed conditions. Endogenous AGR2 was immunoprecipitated with anti-AGR2 antibody and the immunoprecipitated complex was analyzed using anti BiP/GRP78 antibody. As shown in Fig. 3B, the interaction seemed to be enhanced by treatment with tunicamycin. This may be due to the increased levels of BiP/GRP78 and AGR2 in response to tunicamycin (Fig. 3B). In order to elucidate how the dimerization of AGR2 affects the interaction with BiP/GRP78, wild-type AGR2 or CS mutant AGR2 and HA tagged BiP/GRP78 were expressed and co-immunoprecipitation experiment was carried out. Consistent with previous data, wild-type AGR2 binds toBiP/GRP78. However, the interaction between CS-mutant AGR2 and BiP/GRP78 was weakened (Fig. 3C), although the expression levels of wild-type and CS appeared same. Taken together with previous and present results, AGR2 dimerization is required for binding to BiP/GRP78 and the interaction between AGR2 and BiP/GRP78 triggers the activation of the UPR signaling pathway, attenuating ER stress-induced cell death.

4. Discussion

We previously showed that AGR2 is up-regulated in the OCUM-2MLN metastatic gastric cell line and is associated with invasiveness in gastric cancer [32]. A recent report indicated that the sole cysteine residue of AGR2 forms mixed disulfide bonds with MUC2, and AGR2 itself is required for the expression of MUC2 protein in secretory cells of the normal mouse intestine, suggesting that AGR2 is essential for the production of mucin [31]. However,

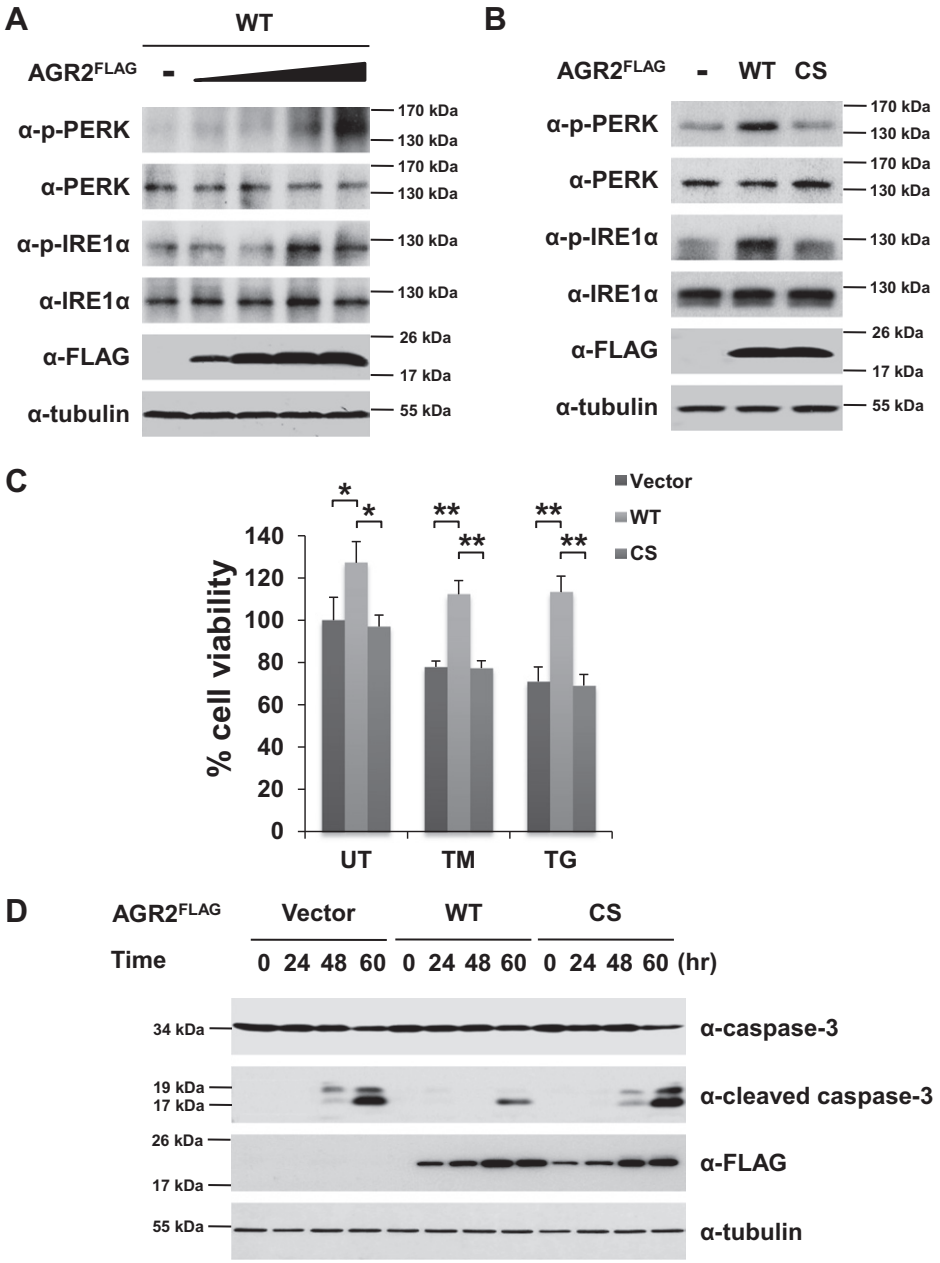

Fig. 2. Dimerization of AGR2 attenuates ER stress-induced cell death. (A) FLAG-tagged AGR2 (0, 0.25, 0.5 1.0, and 2.0 μg) was transfected into HeLa cells. Cell extracts were prepared and analyzed by Western blotting using anti FLAG, p-PERK, PERK, p-IRE1, IRE1, and tubulin antibodies. (B) FLAG-tagged AGR2 (wild-type and mutant) and empty vector were transfected into HeLa cells. Cell extracts were analyzed as describe in (A). (C) FLAG-tagged AGR2 (wild-type and mutant) and empty vector were transfected into HeLa cells. Cells was treated with 2 μg/mL tunicamycin and 0.25 μM thapsigargin for 60 h or left untreated. Cell viability was determined by CCK-8. Statistically significant differences between scrambled and AGR2-silenced cells; $^{*}p < 0.05$ and $^{**}p < 0.005$, respectively. (D) FLAG-tagged AGR2 (wild-type and mutant) and empty vector were transfected into HeLa cells. Cells were treated with 2 μg/mL tunicamycin for the indicated durations or left untreated. Caspase-3 activation was detected by Western blotting.

we observed that AGR2 make a homo-dimer using its sole cysteine residue in human colon cancer cells, Hct8. It might be due to the fact that MUC2 would not be expressed in Hct8 cells. In the structural point of view, based on the crystal structure of AGR3 which contain 71% of sequence homology with AGR2, sole cysteine residue of AGR2 would be exposed to the surface presumably. Although AGR3 does not make a dimer, thioredoxin-like protein, ERp19 which has similar structure with AGR3 exist as a dimer using its cysteine residues on the Tx-domain [38].

It has been reported that BiP/GRP78 is associated with IRE1α and PERK in the resting state, and BiP/GRP78 dissociates from IRE1α and PERK upon ER stress conditions. This dissociation triggers the phosphorylation of IRE1α and PERK, which transmit downstream signals [28,39]. Thus, we investigated the possibility that AGR2 influences the interaction between BiP/GRP78 and IRE1α or PERK. However, we have not observed a stable interaction between BiP/GRP78 and IRE1α or PERK in the resting condition (data not shown). Presumably, this is due to the fact that the UPR pathway is constitutively activated in a number of tumor cell lines, such that BiP/GRP78 is dissociated from either IRE1α or PERK.

Elevated levels of AGR have been correlated with metastasis and tumorigenesis including cancer cell growth, proliferation, and anti-apoptosis. Silencing of AGR2 reduces cell proliferation

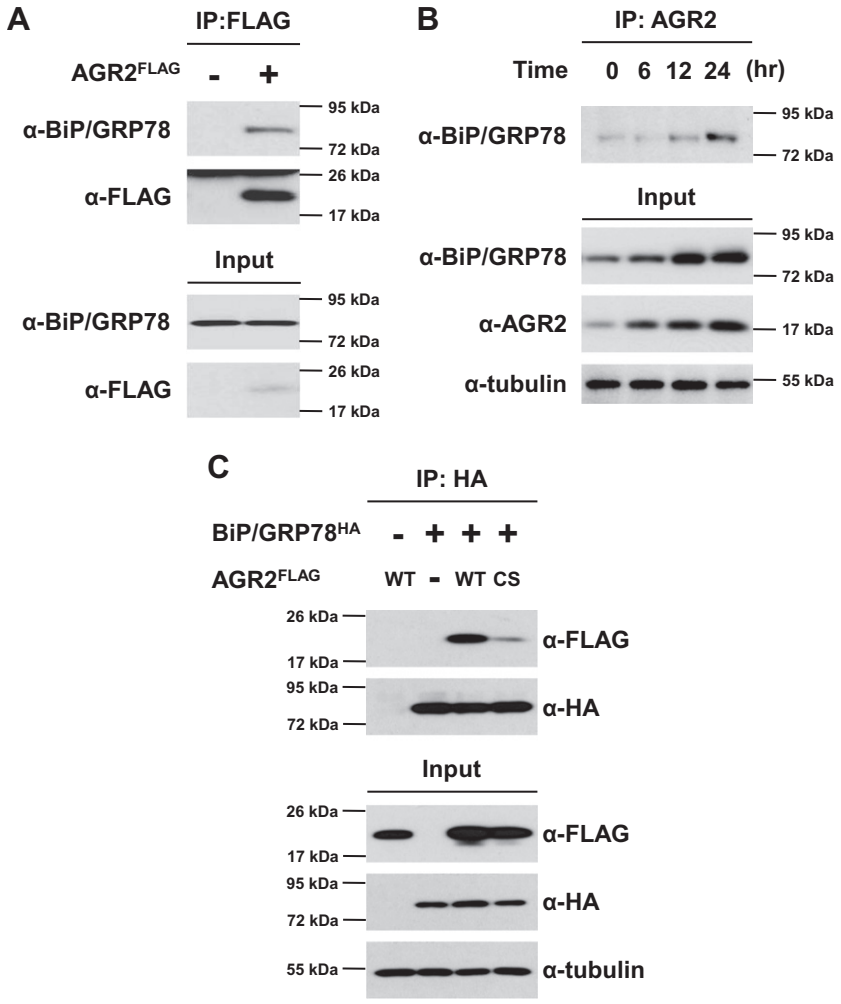

Fig. 3. Dimerization of AGR2 is required for the interaction with BIP/GRP78. (A) FLAG-tagged AGR2 or empty vector (−) was transfected into Hct8 cells. FLAG-tagged AGR2 was immunoprecipitated by anti-FLAG agarose. The precipitated immune complexes were blotted with anti-BiP/GRP78 antibody. (B) Hct8 cells were treated with 2 μg/mL tunicamycin for the indicated durations or left untreated. Endogenous AGR2 was immunoprecipitated and the precipitated immune complexes were blotted with anti-BiP/GRP78 antibody. (C) FLAG-tagged AGR2 (wild-type or CS mutant) and HA-tagged BiP/GRP78 were co-transfected into HeLa cells. HA-BiP/GRP78 complexes were immunoprecipitated with anti-HA agarose and the precipitated immune complexes were blotted with anti-FLAG antibody.

and induces apoptotic cell death in estrogen receptor-positive breast cancer cell lines T47D and ZR-75-1 [40]. In these reports, AGR2 stimulates cell growth and survival by modulating expression of cyclin D1, estrogen receptor-α, and survivin. In a study of AGR−/− mice, the absence of AGR2 led to disruption of enterocyte homeostasis by reduced proliferation and induced apoptosis in the small intestine and colon [23]. We show that over-expression of AGR2 attenuates ER stress-induced cell death (Fig. 3C and D). It should be noted that these results are consistent with previous reports that have correlated AGR2 levels with cell proliferation and metastasis in tumor cells.

In conclusion, we have demonstrated that dimerization of AGR2 plays crucial roles in mediating the UPR signaling pathway through its interaction with BiP/GRP78. These results may provide a cue explaining how AGR2 functions in cells.

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