AMERICAN SOCIETY FOR MICROBIOLOGY | Molecular and Cellular Biology

Phosphatidylinositol 3,4,5-Trisphosphate Phosphatase SKIP Links Endoplasmic Reticulum Stress in Skeletal Muscle to Insulin Resistance

Takeshi Ijuin,[a] Tetsuya Hosooka,[b] Tadaomi Takenawa[c]

Division of Biochemistry, Kobe University Graduate School of Medicine, Kobe, Japan[a]; Division of Diabetes and Endocrinology, Department of Internal Medicine, Kobe University Graduate School of Medicine, Kobe, Japan[b]; Division of Membrane Biology, Biosignal Research Center, Kobe University, Kobe, Japan[c]

Insulin resistance is critical in the pathogenesis of type 2 diabetes. Endoplasmic reticulum (ER) stress in liver and adipose tissues plays an important role in the development of insulin resistance. Although skeletal muscle is a primary site for insulin-dependent glucose disposal, it is unclear if ER stress in those tissues contributes to insulin resistance. In this study, we show that skeletal muscle kidney-enriched inositol polyphosphate phosphatase (SKIP), a PIP_3 (phosphatidylinositol-3,4,5-trisphosphate) phosphatase, links ER stress to insulin resistance in skeletal muscle. SKIP expression was increased due to ER stress and was higher in the skeletal muscle isolated from high-fat-diet-fed mice and *db/db* mice than in that from wild-type mice. Mechanistically, ER stress promotes activating transcription factor 6 (ATF6) and X-box binding protein 1 (XBP1)-dependent expression of SKIP. These findings underscore the specific and prominent role of SKIP in the development of insulin resistance in skeletal muscle.

In type 2 diabetes mellitus, insulin resistance is characterized by an impairment of glucose uptake in skeletal muscle (1–3). Skeletal muscle insulin resistance is considered to be an initial metabolic defect in the development of this disease (4, 5). A number of studies have suggested a relationship between human skeletal muscle insulin resistance and the pathogenesis of type 2 diabetes (6–8). A marked decrease in the insulin-induced Akt phosphorylation in the normal glucose tolerance in offspring of type 2 diabetes parents is observed in comparison to those of healthy subjects (8). This implies that the molecular basis of muscle insulin resistance is already established at the early stages of type 2 diabetes. Despite extensive study, the molecular basis of the initial defects in muscle insulin resistance is not well established. Therefore, identification of the key molecules that contribute to the development of muscle insulin resistance should provide important insights into the treatment of the disease.

Previous studies have shed light on the links between obesity, endoplasmic reticulum (ER) stress, insulin action, and type 2 diabetes, and the molecular mechanism of these links in the liver and adipose tissue has been identified (9–11). Excessive fat storage stimulates ER stress, which leads to the deregulation of insulin signaling and plays a significant role in obesity-related pathogenesis (10). Release of nonesterified fatty acids from adipose tissue triggers ER stress, which induces inflammation through the protein kinase R-like ER kinase (PERK)-eukaryotic translation initiation factor 2α (eIF2α) pathway (12). The resultant expression of cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) induces increased activation of inositol-requiring enzyme 1 (IRE1) and c-Jun N-terminal kinase (JNK), which results in serine phosphorylation of insulin receptor substrate 1 (IRS-1) to develop insulin resistance in adipose tissue (10, 13). Although muscle insulin resistance is considered an early stage in this pathogenesis (6–8), the molecular basis of initial defects is not well established. Previous studies suggested that the unfolded protein response (UPR) was very weak in skeletal muscle and ER stress was not involved in the regulation of insulin resistance (14–16). However, increased UPR and expression of ER stress markers have recently been demonstrated in skeletal muscle isolated from exercising mice and mice fed on a high-fat diet (HFD) (14, 17). The UPR is mediated by three ER transmembrane proteins, PERK, IRE1, and activating transcriptional factor 6 (ATF6). Upon ER stress, RNase activity of IRE1 cleaves *XBP1* mRNA to form a shorter spliced form (*XBP1-s*) that is translated into the transcription factor XBP1s. XBP1s, alone or together with ATF6α, increases the transcription of chaperones, such as GRP78 (18). ATF6 is constitutively expressed as an ER transmembrane full-length 90-kDa protein (p90ATF6), which is cleaved to a 50-kDa protein (p50ATF6) in response to ER stress and acts as a transcriptional factor that leads to the expression of XBP1 and CCAAT/enhancer binding protein homologous protein (Chop) (18, 19).

In skeletal muscle, insulin stimulation leads to the activation of phosphatidylinositol (PI) 3-kinase and generates phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which activates Akt and glucose uptake (20, 21). Akt translocates from cytosol to the plasma membrane through its binding with PIP_3, where it is phosphorylated at Ser-473 and Thr-308 (20, 22), which are both required for full activation of Akt. Among the three Akt family kinases, only Akt2 is implicated in the regulation of insulin-dependent glucose homeostasis. PIP_3 phosphatases hydrolyze PIP_3 and attenuate the PI 3-kinase signaling pathway. Skeletal muscle and kidney-enriched inositol polyphosphate 5-phosphatase (SKIP) is one of the PIP_3 phosphatases that negatively regulate insulin-dependent glucose uptake in skeletal muscle (23–26). SKIP heterozygous knockout mice exhibit improved systemic insulin sensitivity and are resistant to diet-induced insulin resistance (25). A euglycemic clamp study showed an increase in the rates of glucose infusion

Received 5 October 2015 **Accepted** 6 October 2015

Accepted manuscript posted online 19 October 2015

Citation Ijuin T, Hosooka T, Takenawa T. 2016. Phosphatidylinositol 3,4,5-trisphosphate phosphatase SKIP links endoplasmic reticulum stress in skeletal muscle to insulin resistance. Mol Cell Biol 36:108–118. doi:10.1128/MCB.00921-15.

Address correspondence to Takeshi Ijuin, tijuin@med.kobe-u.ac.jp.

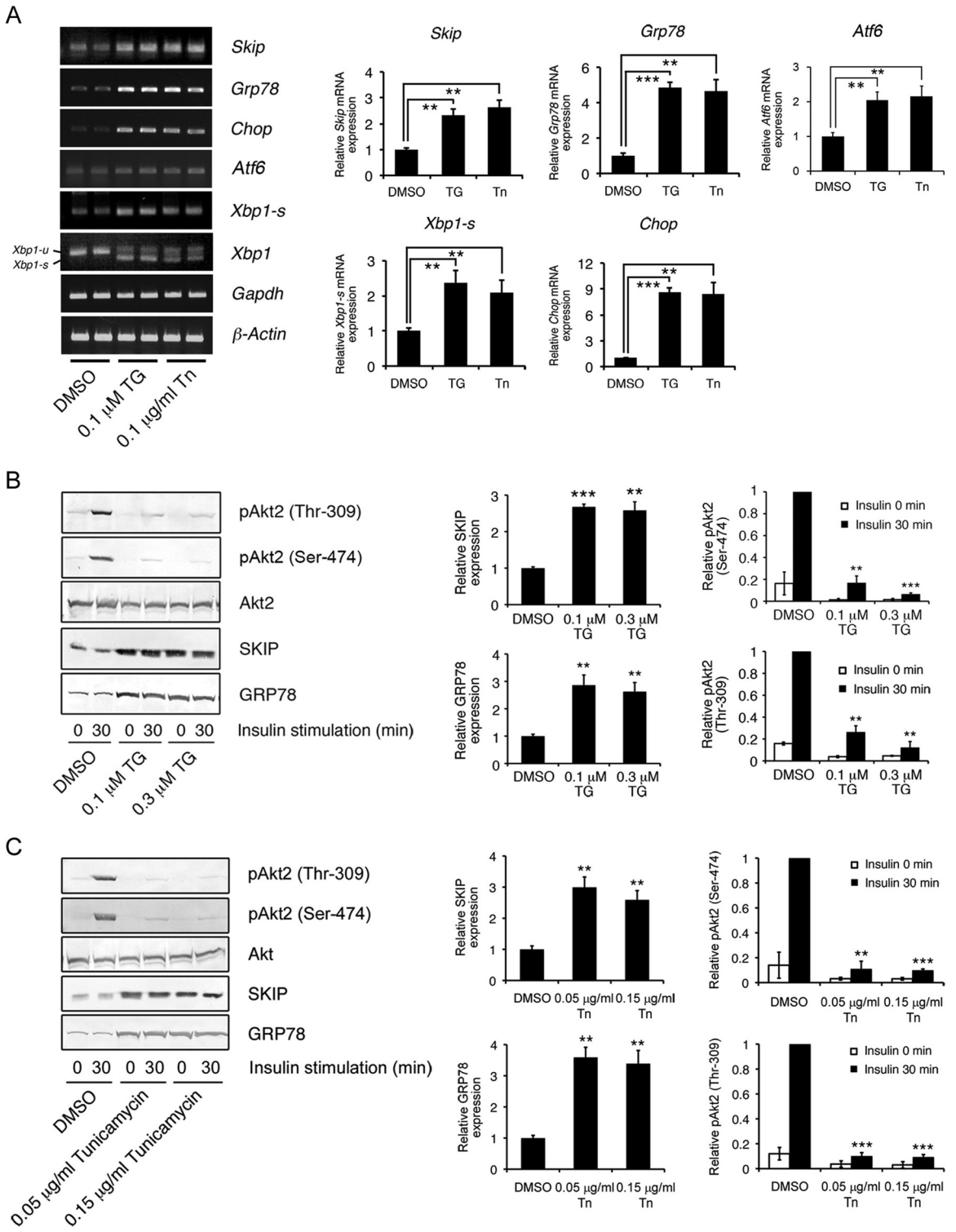

FIG 1 ER stress increased SKIP expression. (A) RT-PCR analysis of C2C12 myoblast cells treated with thapsigargin or tunicamycin. *Skip*, *Grp78*, *Chop*, *Atf6*, *Xbp1*, and *Xbp1-s* mRNA levels were measured by semiquantitative RT-PCR. Samples were loaded in duplicate. Relative amounts of these mRNAs were quantified by densitometry and normalized to the amount of *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase gene) mRNA. **, $P < 0.01$; ***, $P < 0.001$. (B) Expression of SKIP, GRP78, and phospho-Akt2 in C2C12 cells treated with thapsigargin (0.1 or 0.3 μM) for 24 h and insulin (100 nM) for 30 min; protein levels were measured by densitometry. Results are presented as the means ± SEM from three independent experiments. **, $P < 0.01$; ***, $P < 0.001$. (C) C2C12 myoblast cells were treated with tunicamycin (0.05 or 0.15 μg/ml) for 24 h and then stimulated with insulin (100 nM) for 30 min. Lysates were used for Western blot analysis. Expression of SKIP, GRP78, phospho-Akt2 (Ser-474), and phospho-Akt2 (Thr-309) were measured by densitometry. Results are presented as the means ± SEM from three independent experiments. **, $P < 0.01$; ***, $P < 0.001$. (D) RT-PCR analysis of C2C12 cells treated with palmitate (2 mM) for 18 h. *Skip*, *Grp78*, *Chop*, *Atf6*, *Xbp1*, and *Xbp1-s* mRNA levels were measured by semiquantitative RT-PCR. Relative amounts of these mRNAs were quantified by densitometry and normalized to the amount of *Gapdh* mRNA. **, $P < 0.01$. (E) C2C12 myoblast cells were treated with palmitate (2 mM) for 18 h prior to stimulation with insulin (100 nM) for 30 min. Lysates were used for Western blot analysis. Expression of SKIP, GRP78, phospho-Akt2 (Ser-474), and phospho-Akt2 (Thr-309) were measured by densitometry. Results are presented as the means ± SEM from three independent experiments. *, $P < 0.05$.

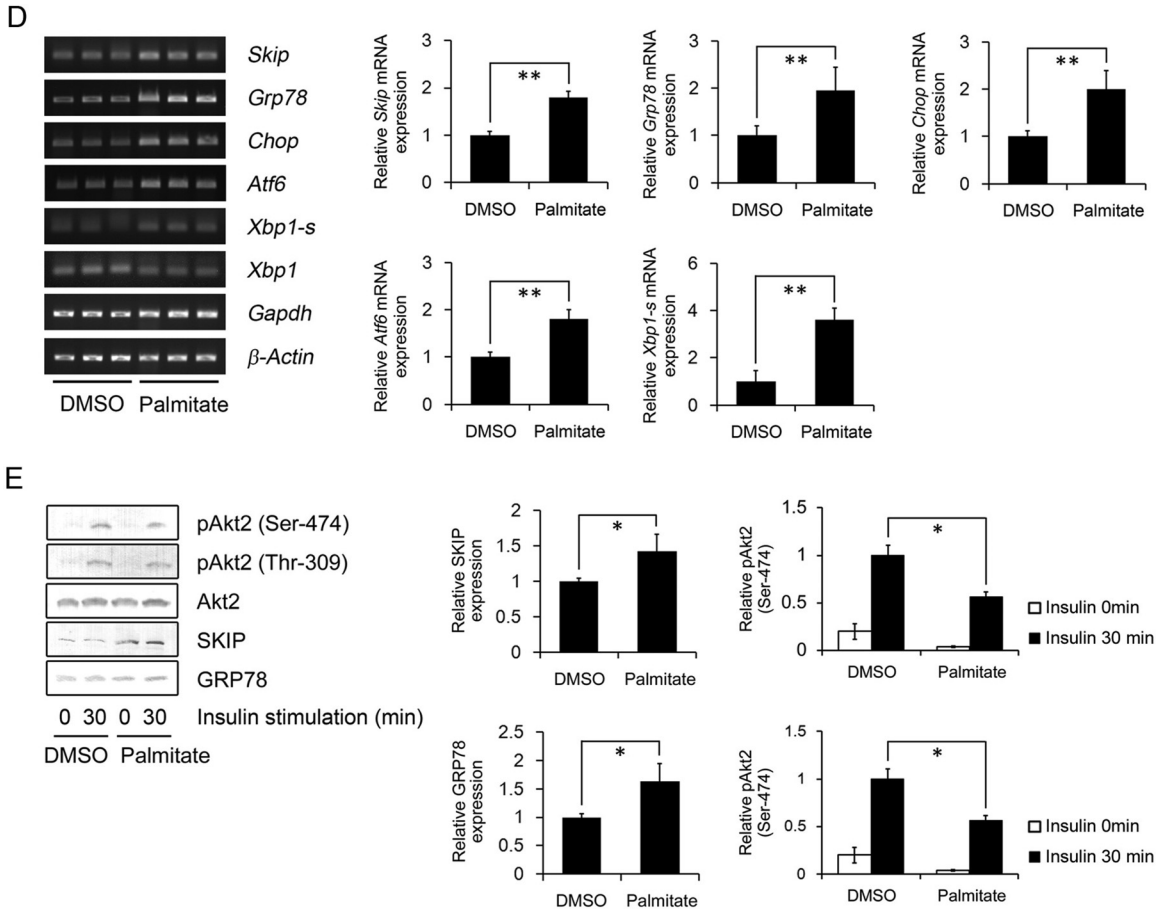

FIG 1 continued

and insulin-stimulated glucose disposal in these mice without any abnormalities in hepatic glucose production (25). Isolated soleus muscle from these mice shows enhancement of insulin-dependent Akt phosphorylation and glucose uptake (25). Mechanistically, SKIP formed a complex with GRP78 under resting conditions (27). SKIP relocates from the ER to the plasma membrane upon insulin stimulation, where it binds to the activated form of p21-activated kinase 1 (PAK1) and forms a complex with Akt2 (3). The location of SKIP proximal to these PIP_3 effectors determines the efficiency and specificity of the termination of insulin signaling (26). Therefore, it appears likely that SKIP is the specific regulator for diet-induced insulin resistance in skeletal muscle (26). Thus, the expression status of SKIP is likely to contribute to the development of insulin resistance in this tissue. Here we show new evidence of how diet-induced insulin resistance is developed in skeletal muscle. ER stress increased the expression of SKIP in skeletal muscle, which leads to an inactivation of insulin-induced PI 3-kinase signaling.

MATERIALS AND METHODS

Materials. Antibodies raised against the C-terminal region of SKIP were purchased from LifeSpan Biosciences, Inc. (Seattle, WA). Antibodies specific for Akt, Akt2, phospho-Akt (Ser-473), and phospho-Akt (Thr-308) were purchased from Cell Signaling Technology (Beverly, MA). Anti-insulin receptor β, GRP78, and IRS-1 antibodies were purchased from BD Biosciences (Franklin Lakes, NJ). Anti-XBP1 and ATF6 antibodies for immunoblotting were purchased from Novus Biologicals (Littleton, CO). Palmitate was purchased from Wako Chemicals Inc. (Osaka, Japan). Thapsigargin and tunicamycin were purchased from Nacalai Tesque Inc. (Kyoto, Japan).

Plasmids. A 3×FLAG-tagged SKIP expression vector was generated by cloning human SKIP into p3xFLAG-CMV8 vector (Sigma-Aldrich, St. Louis, MO). Expression vectors for PGC-1α, ATF6, XBP1, and GRP78 were generated by cloning mouse cDNAs into pcDNA3.1(+) vector (Life Technologies, Carlsbad, CA).

Transfection and RNA interference. Small interfering RNA (siRNA) duplexes were purchased from Life Technologies (Carlsbad, CA). The following oligonucleotides were used in this study: control, 5′-GAGCAACTGCGTGTCGAATCTCTTA-3′; mouse SKIP #1, 5′-CATAAGCCTTCTTTCTGATGCTGCT-3′; mouse SKIP #2, 5′-GAGTCAACGTCTGCCTGAAGCTTTA-3′; rat SKIP #1, 5′-CCATGGAGCAGTTTCTTCATGGATA-3′; rat SKIP #2, 5′-TCGAGGACTTTGGGCTGCTCTTTGT-3′; mouse ATF6 #1, 5′-GGAGTCGACGTTGTTTGCTGAACTT-3′; mouse ATF6 #2, 5′-AGAACATGGACTGACTCCAAAGAAA-3′; mouse XBP1 #1, 5′-TCTTCCCATGGACTCTGACACTGTT-3′; mouse XBP1 #2, CAGAGTCTGCTAGTCTGGAGGAACT-3′. For transfections, 20 nmol of control, SKIP, GRP78, ATF6, or XBP1 siRNA duplexes was transfected into mouse C2C12 or rat L6 myoblast cells. Cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) for 24 h and then serum starved for another 24 h. Cells were stimulated with insulin (100 nM) at the indicated times.

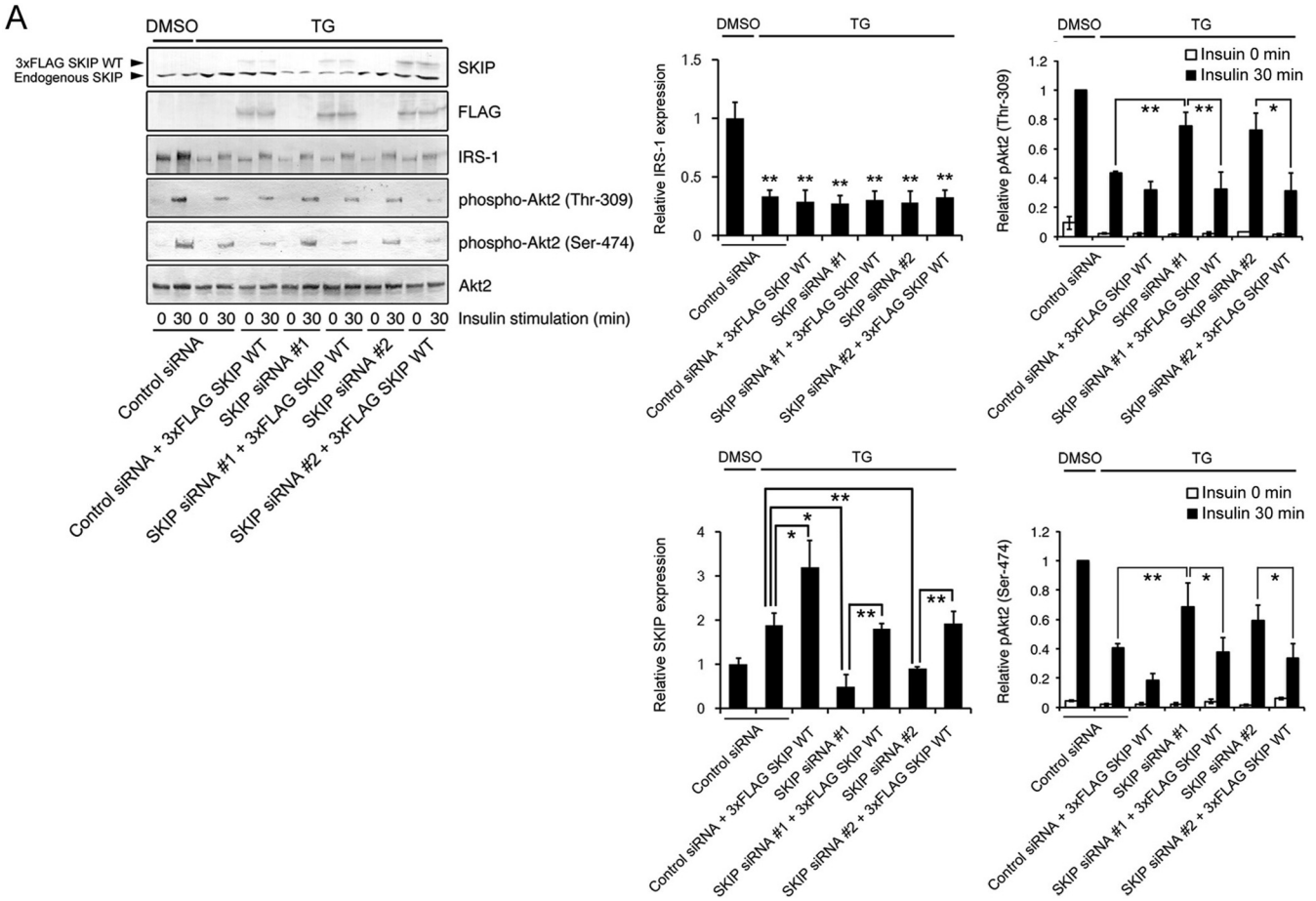

FIG 2 Silencing of SKIP restored ER stress-induced inhibition of insulin-dependent glucose uptake. (A) Effect of SKIP silencing and expression of 3×FLAG-SKIP on insulin-induced phosphorylation of Akt2 at Ser-474 and Thr-309 in C2C12 cells by treatment with thapsigargin (0.1 μM, 12 h). Expression of IRS-1, Akt2, phospho-Akt2 (Ser-474), phospho-Akt2 (Thr-309), and SKIP was measured by densitometry. Values shown have been normalized to total Akt2. *, $P < 0.05$; **, $P < 0.01$. (B) Effects of SKIP silencing on insulin-induced phosphorylation of Akt2 at Ser-474 and Thr-309 in C2C12 cells by treatment with thapsigargin (0.1 μM, 24 h). Expression of IRS-1, Akt2, phospho-Akt2 (Ser-474), phospho-Akt2 (Thr-309), and SKIP was measured by densitometry. Values shown have been normalized to total Akt2. **, $P < 0.01$; ***, $P < 0.001$. (C) Glucose uptake analysis in thapsigargin-treated L6 cells silencing SKIP and/or expression of 3×FLAG-SKIP. Results are presented as the means ± SEM. *, $P < 0.05$.

Immunoblotting. Cells were washed with phosphate-buffered saline (PBS) and lysed with a cell lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 5 mM NaF, 1 mM Na_3VO_4, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1% Triton X-100. Cell lysates were solubilized in SDS sample buffer, boiled for 5 min, resolved by SDS-PAGE, and transferred to a nitrocellulose membrane. Western blotting was carried out using the primary antibodies described above. Alkaline phosphatase-conjugated anti-mouse and anti-rabbit secondary antibodies (Promega, Madison, WI) were utilized. Densitometry was used to quantify protein levels.

Measurement of Akt2 phosphorylation by immunoprecipitation. Akt2 phosphorylation at Thr-309 and Ser-474 was measured as described previously (3). Cell lysates were immunoprecipitated with Akt2 antibody for 1 h at 4°C, and immunoprecipitates were washed with wash buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 50 mM NaF, 1 mM Na_3VO_4, and 1 mM PMSF 5 times before they were analyzed by Western blotting using antibodies for phospho-Akt (Thr-308) or phospho-Akt (Ser-473). Densitometry was used to quantify protein levels.

Glucose incorporation analysis. The glucose cellular uptake measurement kit (Cosmo Bio, Japan) was used to measure glucose incorporation into L6 myoblast cells according to the manufacturer's protocol. Cells were stimulated with insulin (10 nM) for 60 min.

Treatment of cells with palmitate, thapsigargin, or tunicamycin. C2C12 cells grown up to 80% confluence were treated with palmitate (2 mM) for 18 h, thapsigargin (0.1 or 0.3 μM), or tunicamycin (0.05 μg/ml or 0.15 μg/ml) for 12 or 24 h and then stimulated with insulin for 60 min.

ChIP assay. Chromatin immunoprecipitation (ChIP) assays were performed as described previously (15) using the XBP1 M-186 antibody (Santa Cruz Biotechnology, Dallas, TX), ATF-6 70B1413 antibody (Abcam, Cambridge, MA), and the SimpleChIP Plus Enzymatic Chromatin IP kit (Cell Signaling Technology, Beverly, MA) according to the manufacturer's protocol.

Statistical analysis. Prior to analysis, we visualized all data to ensure that they were normally distributed. Differences between treatments were investigated using Student's *t* tests. All values are listed as the means ± standard errors of the means (SEM).

Animals. Mouse experiments were performed according to the guidelines of the animal ethics committee of Kobe University Graduate School of Medicine. Male C57BL/6J mice, *db*/+ mice, and *db*/*db* mice (10 to 12 weeks old) were purchased from CLEA Japan (Tokyo, Japan). The mice were kept on a 12-h day/night cycle, were housed in cages, and had free access to water and normal chow or a high-fat diet (60% of calories from fat; CLEA Japan). At 8 weeks of age, mice were switched from the normal diet to the high-fat diet. The 8-week-old *db*/+ and *db*/*db* mice and 32-

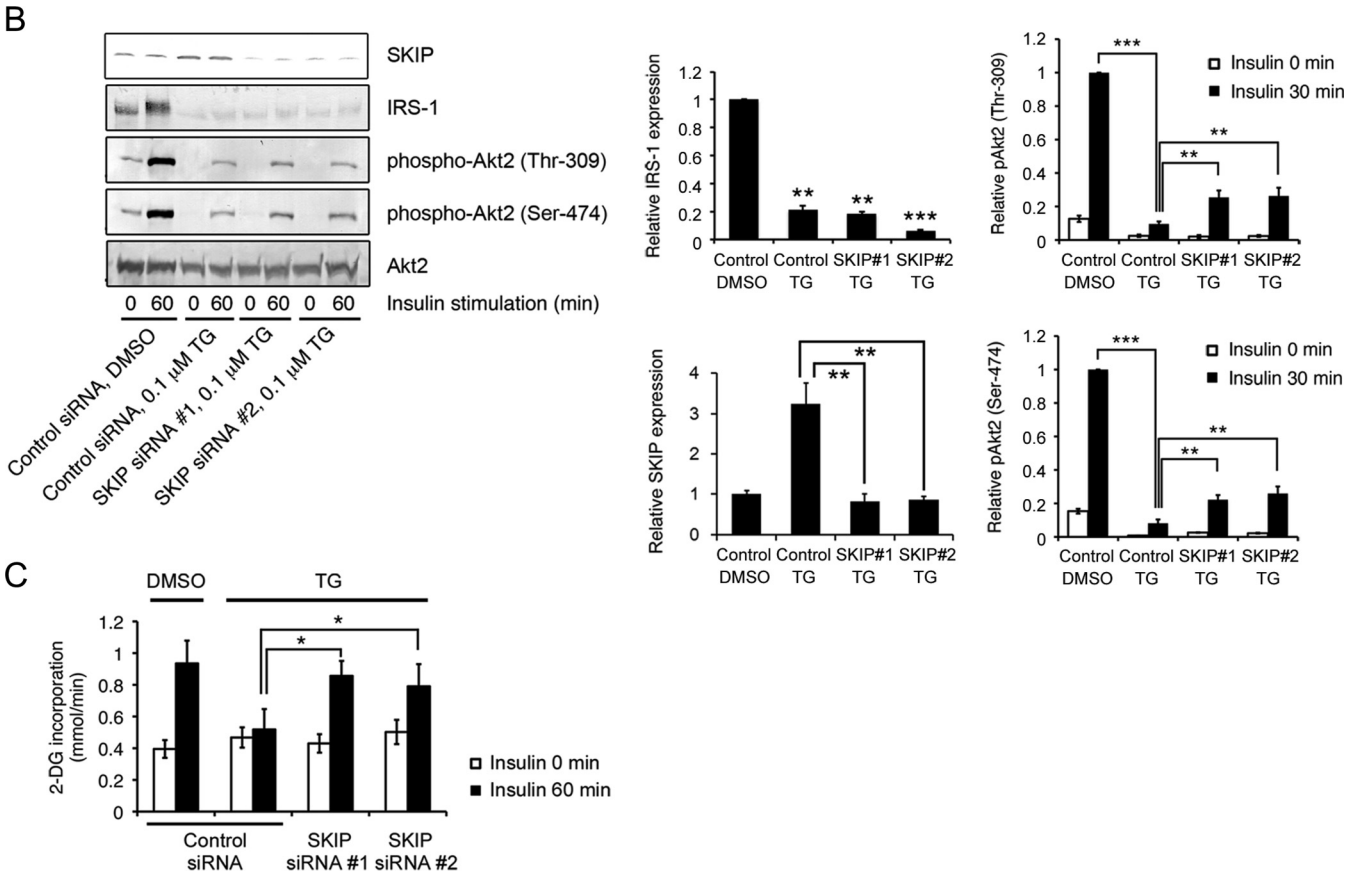

FIG 2 continued

week-old C57BL/6J mice were used for the isolation of gastrocnemius skeletal muscle.

RESULTS AND DISCUSSION

To investigate the underlying mechanisms by which SKIP regulates insulin resistance in skeletal muscle, we sought to identify whether SKIP expression is regulated by ER stress and the UPR. Treatment for 24 h with thapsigargin or tunicamycin, agents commonly used to induce acute ER stress, induced increased expression of *SKIP* mRNA and the UPR markers *GRP78*, *Chop*, and *Xbp1-s* (Fig. 1A), which was accompanied by a marked decrease in insulin-dependent Akt2 phosphorylation at Thr-309 and Ser-474, both of which are PIP_3 dependent (Fig. 1B and C). A high concentration of nonesterified fatty acids in plasma triggers ER stress and insulin resistance in peripheral tissues (28, 29), and treatment of C2C12 cells with the saturated fatty acid palmitate induces inactivation of insulin signaling (29). We therefore evaluated the effect of palmitate on SKIP expression in C2C12 cells. Treatment with 2 mM palmitate for 18 h increased *Skip* mRNA and SKIP protein expression by approximately 50% in comparison to control dimethyl sulfoxide (DMSO)-treated cells (Fig. 1D and E). In these cells, expression levels of *Atf6* and *Grp78* mRNA were increased (Fig. 1D) and insulin-induced Akt2 phosphorylation was decreased (Fig. 1E), showing an induction of the UPR. These results suggest that ER stress triggers expression of SKIP. Next, to examine whether these increases in SKIP expression contribute to the suppression of insulin signaling, C2C12 cells were transfected with SKIP siRNAs. In these cells, SKIP expression levels were reduced to a level approximately 20 to 50% that of control cells (Fig. 2A). Insulin resistance is characterized by an inactivation of IRS-1 (10). Following thapsigargin treatment for 12 h, IRS-1 expression and Akt2 phosphorylation were decreased to a level 40% of that of control DMSO-treated cells; silencing of SKIP under these conditions markedly increased the insulin-dependent phosphorylation of Akt2, to a level approximately 60 to 80% of that of control DMSO-treated cells (Fig. 2A). In contrast, expression of SKIP under these conditions decreased Akt2 phosphorylation to a level of 20 to 40% of that of control DMSO-treated cells (Fig. 2A). Furthermore, in cells treated with thapsigargin for 24 h, only 10% of IRS-1 was expressed compared to control cells, while knockdown of SKIP increased Akt2 phosphorylation 2-fold, to the level in control siRNA-transfected cells (Fig. 2B). Expression of IRS-1 was not altered by the knockdown of SKIP (Fig. 2A and B). In addition, in L6 myocytes, thapsigargin treatment for 12 h markedly decreased insulin-mediated glucose uptake, which was restored by the silencing of SKIP (Fig. 2C). These results suggest that ER stress-dependent inhibition of insulin action is mediated not only by inactivation of IRS-1 but also by increased expression of SKIP.

Certain of the transcription factors are activated upon activation of the UPR, including ATF6 and XBP1 (30, 31). To identify if SKIP expression is under the control of the transcriptional factors ATF6, XBP1, and PGC1α, expression vectors bearing these genes were transfected into C2C12 cells and the level of *Skip* mRNA was

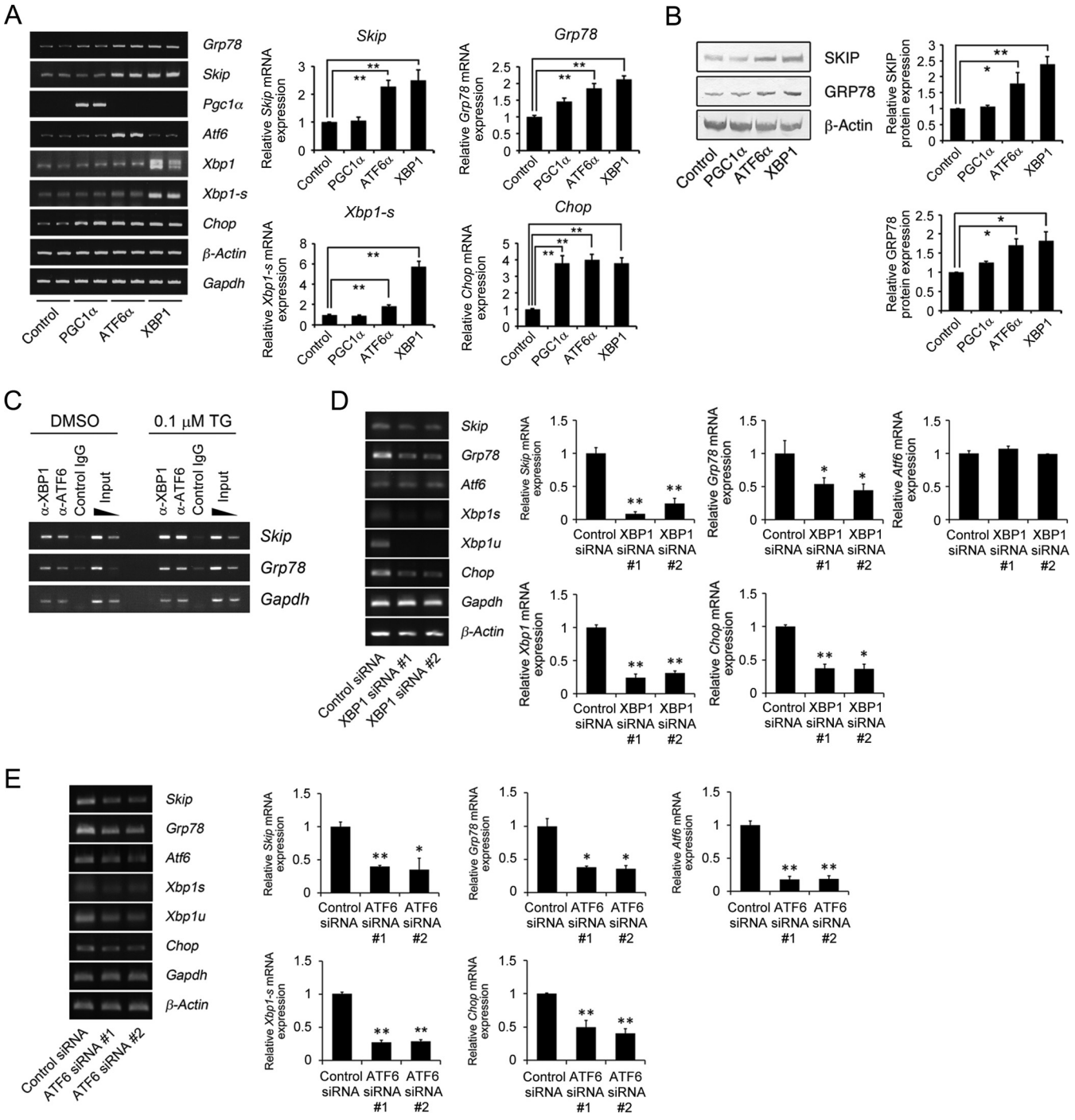

FIG 3 SKIP expression is under the control of the XBP1 transcription factor. (A and B) Induction of SKIP by ATF6 and XBP1 expression in C2C12 cells. Results of semiquantitative RT-PCR (A) and Western blot analysis (B) of C2C12 cells transfected with plasmids expressing PGC-1α, ATF6α, and XBP-1. Samples were loaded in duplicate. Relative amounts were quantified by densitometry, which were normalized to vector-transfected cells. *, $P < 0.05$; **, $P < 0.01$. Results are presented as the means ± SEM from four independent experiments. (C) ChIP assay for ATF6α, XBP1, and PGC1α in C2C12 myotube cells treated with DMSO or thapsigargin. For input, PCRs were performed with 0.5% or 0.25% of total starting material. (D and E) Effects of knockdown of XBP1 (D) and ATF6 (E) on *SKIP* mRNA expression in C2C12 cells. *Skip*, *Grp78*, *Xbp1*, and *Atf6* mRNA levels were measured by semiquantitative RT-PCR. Relative amounts of these mRNAs were quantified by densitometry, which were normalized to the amount of *Gapdh* mRNA. *, $P < 0.05$; **, $P < 0.01$. (F and G) Effects of silencing of XBP1 (F) and ATF6 (G) on SKIP expression and insulin-induced phosphorylation of Akt2 at Ser-474 and Thr-309 upon thapsigargin (0.1 μM, 12 h) treatment in C2C12 cells. Cells were stimulated with insulin (100 nM) for 30 min. Expression levels of phospho-Akt2 (Ser-474), phospho-Akt2 (Thr-309), and SKIP were measured by densitometry. Values shown have been normalized to total Akt2. *, $P < 0.05$; **, $P < 0.01$. (H and I) Effects of silencing XBP1 (H) and ATF6 (I) on SKIP expression and insulin-induced phosphorylation of Akt2 at Ser-474 and Thr-309 in C2C12 cells in the absence of thapsigargin treatment. The results of immunoblotting are shown.

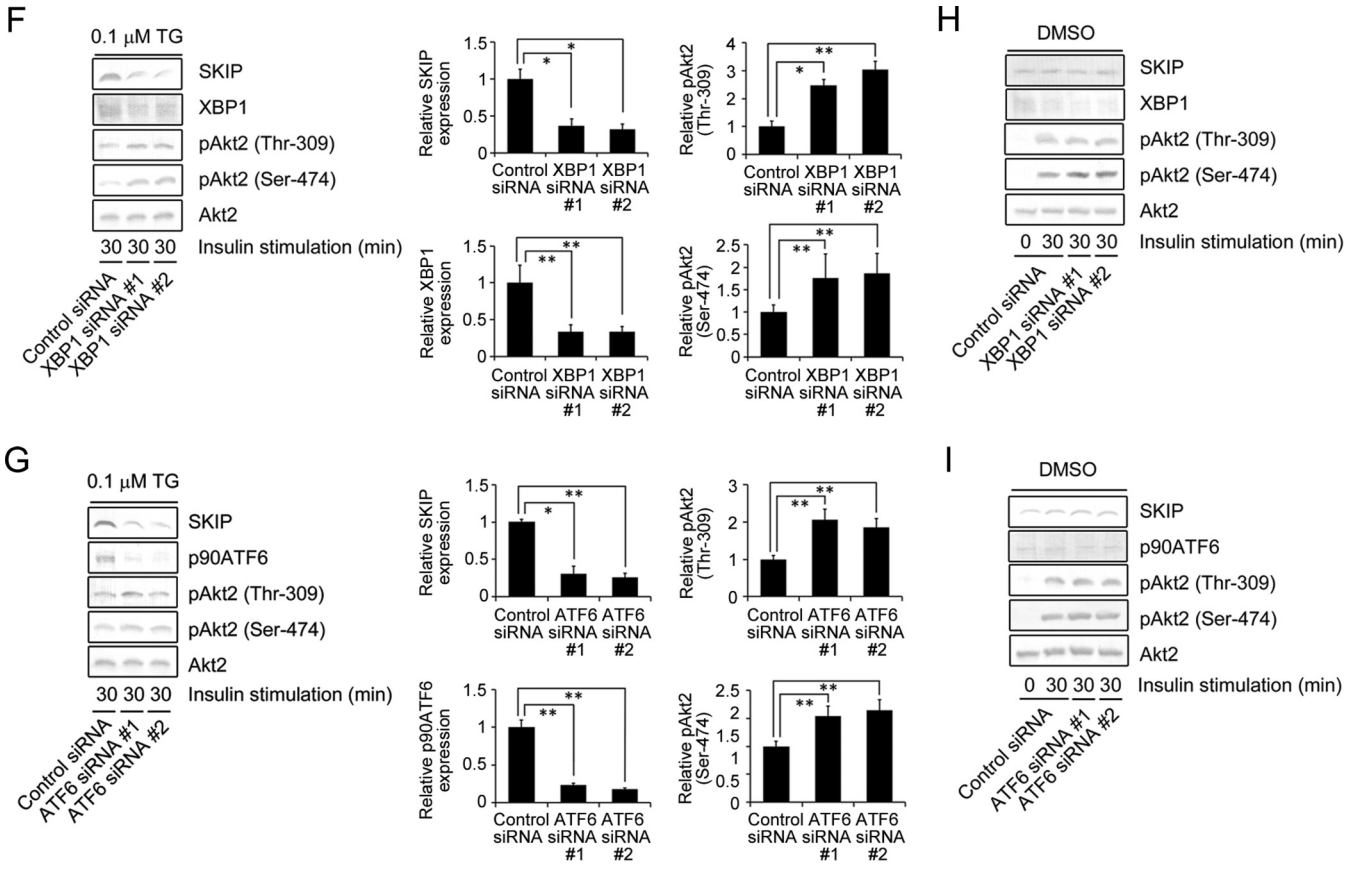

FIG 3 continued

determined by semiquantitative reverse transcription (RT)-PCR. ATF6 is a membrane-bound transcription factor that activates genes in the UPR (32), and overexpression of this factor in C2C12 cells resulted in increased levels of *Skip*, *Grp78*, and *Chop* mRNA (Fig. 3A). Overexpression of the unspliced form of XBP1, whose mRNA expression is induced upon ER stress (18), induced *Skip* and *Grp78* mRNA expression (Fig. 3A). SKIP protein expression was also increased by the expression of ATF6α and XBP1 (Fig. 3B). In contrast, expression of the transcriptional coactivator PGC-1α, which protects against metabolic disease, did not change *Skip* mRNA levels. ChIP and semiquantitative PCR showed that *SKIP* is a target gene of XBP-1 and ATF6α (Fig. 3C) and treatment with thapsigargin led to increases in promoter occupancy. Next, the effects of XBP1 and ATF6 knockdown on *SKIP* expression were examined. Knockdown of XBP1 and ATF6 in C2C12 cells decreased expressions of *Grp78* and *Chop* mRNA, implying the decreases in UPR in these cells (Fig. 3D and E). *Skip* mRNA levels were decreased by approximately 75% following knockdown of XBP1 and ATF6 in comparison to control siRNA-transfected C2C12 cells treated with 0.1 μM thapsigargin for 24 h (Fig. 3D and E), which were accompanied by a marked decrease in SKIP protein expression and by increases in insulin-dependent Akt2 phosphorylation at Thr-309 and Ser-474 in comparison to control siRNA-treated cells (Fig. 3F and G). The effects of XBP1 and ATF6 knockdown on *SKIP* expression and on insulin signaling in the absence of thapsigargin treatment were also examined. However, these cells did not show changes in SKIP protein expression and in Akt2 phosphorylation upon insulin stimulation (Fig. 3H and I). These results suggest that ER stress promotes *SKIP* expression at both the translational and transcriptional levels, which is dependent on ATF6 and XBP1 transcriptional factors. Previous studies have reported an activation of the UPR in skeletal muscle (14). A 70% HFD for 6 weeks led to an induction of the UPR in soleus and gastrocnemius skeletal muscle in mice (14); however, this effect is very weak in skeletal muscle. In order to confirm an induction of UPR and induction of SKIP expression in skeletal muscle in mouse models of obesity, isolated gastrocnemius muscle was analyzed by semiquantitative RT-PCR and immunoblotting. In the gastrocnemius skeletal muscle in dietary (HFD) models of mouse obesity, *Grp78* and *Skip* mRNA expression was increased to a level more than 2-fold that of control mice fed a normal diet (Fig. 4A). Furthermore, in mouse genetic models of obesity (*db/db* mice), *Grp78* and *Skip* mRNA levels in gastrocnemius skeletal muscle were increased to a level approximately twice that of control (*db/+*) mice (Fig. 4B). These increases might be owing to an increase in UPR in skeletal muscle, which was confirmed by increased expression of *ATF6* and *Xbp1-s* mRNA (Fig. 4A and B). Expression of SKIP protein, as well as of GRP78, XBP1, and p90ATF6/p50ATF6, was also greater in these dietary and genetic models of mouse obesity than that in the control mice, respectively (Fig. 4C and D). Although the spliced form of XBP1 protein was not detected due to a weak UPR in skeletal muscle, these results suggested increased UPR in skeletal muscle of dietary and genetic models of obesity. Since a 50% reduction of SKIP expres-

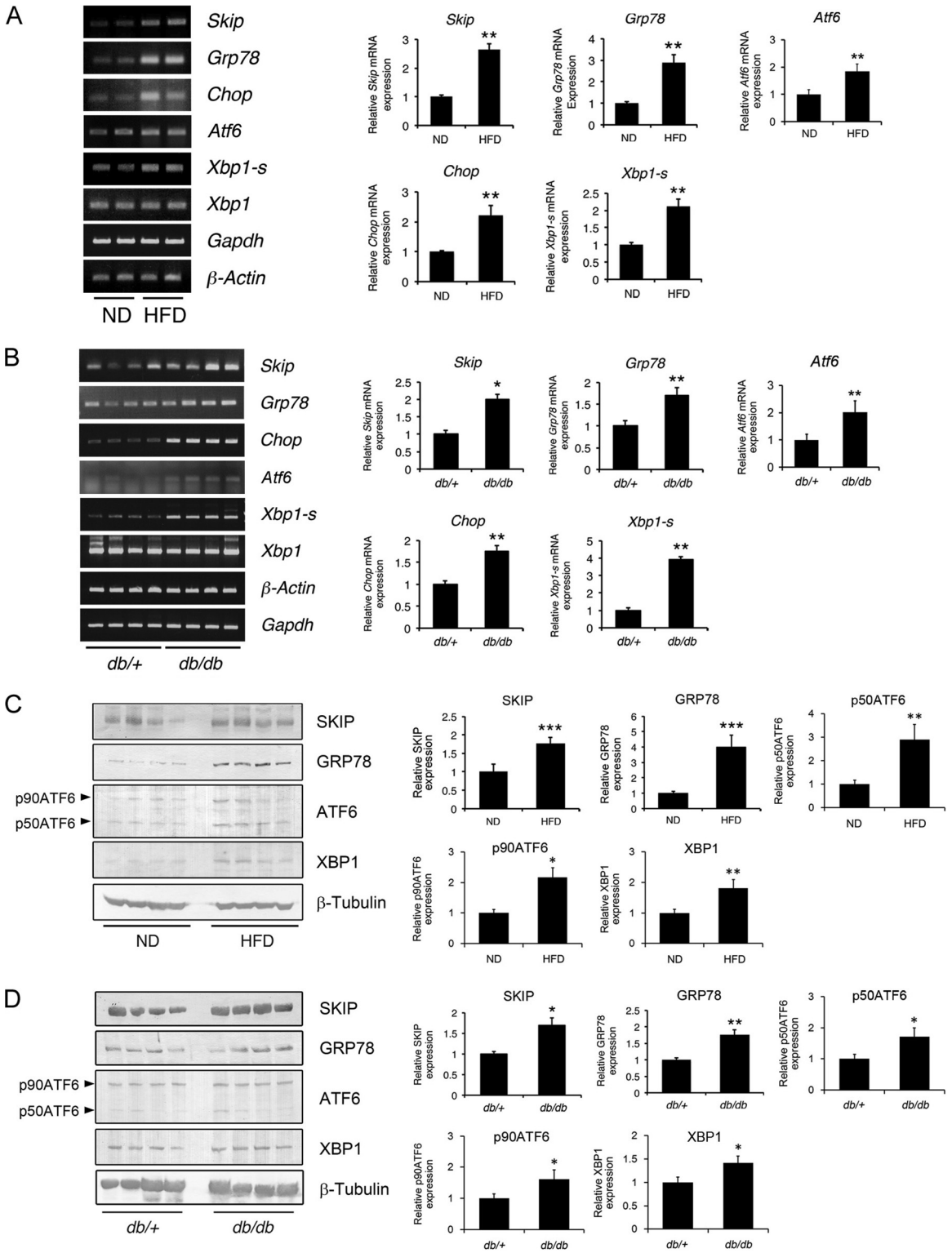

FIG 4 Increased SKIP expression in skeletal muscle in diet-induced and genetic models of obesity in mice. (A and B) Semiquantitative RT-PCR analysis of *Skip*, *Grp78*, *Chop*, *Atf6*, *Xbp1-s*, and *Xbp1* mRNA in the gastrocnemius skeletal muscle of mice with diet-induced obesity (A) and *db/db* mice (B). *, $P < 0.05$; **, $P < 0.01$ (*t* test). Results are presented as the means ± SEM from four independent experiments. (C and D) SKIP and GRP78 expression levels were examined in lysates isolated from gastrocnemius muscle of male C57BL/6 mice kept on a normal chow diet (ND) or a high-fat diet (HFD) for 28 weeks (C) and male diabetic model (*db/db*) and control (*db/m*+) mice that were kept for 7 to 8 weeks (D). The results of immunoblot analysis of SKIP, GRP78, ATF6, and XBP1 of the gastrocnemius skeletal muscle isolated from these mice were shown. The positions of p90ATF6 and p50ATF6 are indicated on the left. β-Tubulin was examined as a loading control. Results are presented as the means ± SEM from four independent experiments. *, $P < 0.05$; **, $P < 0.01$ (*t* test).

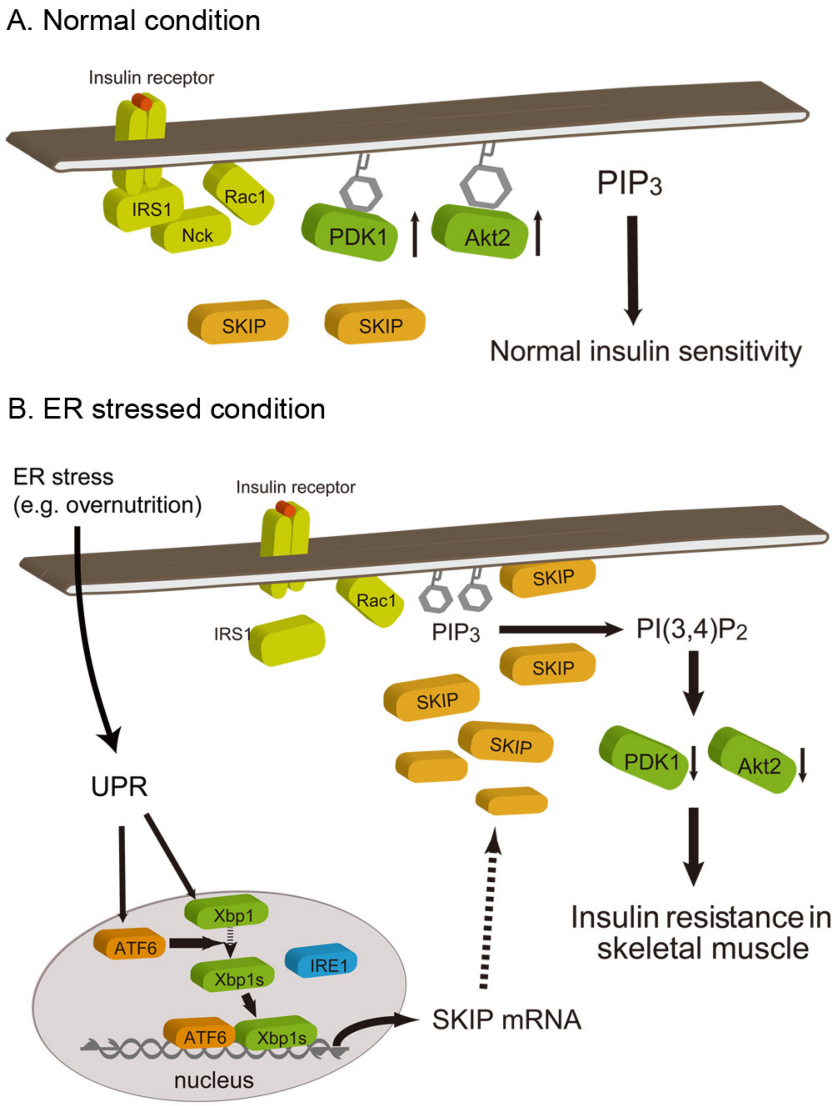

FIG 5 Working model for induction of insulin resistance under ER-stressed conditions. (A) Under resting conditions, SKIP localizes to the ER through binding with GRP78, and insulin stimulation induces translocation of the SKIP-GRP78 complex from the ER to the plasma membrane. (B) ER stress triggers the unfolded protein response, and splicing of *Xbp1* mRNA induces the expression of SKIP and GRP78. Increased amounts of SKIP induce insulin resistance in skeletal muscle cells.

sion in heterozygous knockout mice resulted in a significant increase in insulin signaling (25), a 2-fold increase in SKIP expression might be enough for the alteration of insulin signaling and the development of insulin resistance. Taken together, an increased expression of SKIP by ER stress contributes to an early onset of the development of insulin resistance, and therefore SKIP should be considered an effector of the UPR.

In conclusion, our results provide the first evidence underlying the molecular basis for the regulation of insulin resistance by SKIP, which in turn explains the mechanisms by which SKIP mediates the early onset of skeletal muscle insulin resistance through an activation of the UPR. Under normal conditions, expression of SKIP is comparably low (Fig. 5A). Under ER stress conditions, such as diet-induced obesity, ATF6 and the IRE1-XBP1 system are activated, and as a result, expression of SKIP is increased, leading to suppression of the insulin signaling pathway (Fig. 5B). Among the main canonical ER stress transducers, ATF6 and the IRE1-XBP1 system are likely to be essential for the expression of the genes involved in lipid biosynthesis and protein folding, including GRP78. Our previous results have shown that an accumulation of SKIP at the plasma membrane contributes to the suppression of insulin signaling and glucose uptake; membrane-localized SKIP interacts with an active Pak1, leading to the hydrolysis of PIP_3 bound to Akt2 and PDK1 (3). Accumulation of SKIP in proximity to these PIP_3 effectors might mediate an effective inhibition of insulin signaling under ER-stressed conditions. Elucidation of the process by which SKIP is transferred to the plasma membrane will lead to a better understanding of the regulation of insulin action in skeletal muscle. Our current results suggest that induction of ER stress in skeletal muscle triggers an increase in SKIP, which is a key link between ER stress and insulin resistance in

skeletal muscle, thereby making SKIP a promising target for efforts aimed at improving insulin sensitivity and treating diabetes mellitus.

ACKNOWLEDGMENTS

We are grateful to Yoshikazu Tamori (Kobe University Graduate School of Medicine) for helpful discussion and assistance.

T.I. and T.H. performed experiments and analyzed the data. T.I. and T.T. designed the study and wrote the paper. All authors discussed the results and commented on the manuscript.

We declare that we have no competing interests.

FUNDING INFORMATION

Japan Society for the Promotion of Science provided funding to Takeshi Ijuin under grant number 25460365. Japan Diabetes Foundation provided funding to Takeshi Ijuin. Novo Nordisk Pharma provided funding to Takeshi Ijuin. Hyogo Science and Technology Association provided funding to Takeshi Ijuin. Ministry of Education, Culture, Sports, Science, and Technology (MEXT) provided funding to Tadaomi Takenawa under grant number 23227005.

This work was supported in part by grants to T.I. from the Japan Society for the Promotion of Science (JSPS; Kakenhi grant number 25460365), the Japan Diabetes Foundation, Novo Nordisk Pharma Ltd., and the Hyogo Science and Technology Association and by a grant-in-aid for scientific research (S) to T.T. by the Ministry of Education, Culture, Sports, Science and Technology, Japan (Kakenhi grant number Scientific Research 23227005). The funders had no role in study design, data collection and analysis, decision to publish, or presentation of the manuscript.

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