WFS1 Is a Novel Component of the Unfolded Protein Response and Maintains Homeostasis of the Endoplasmic Reticulum in Pancreatic β-Cells*

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In Wolfram syndrome, a rare form of juvenile diabetes, pancreatic β-cell death is not accompanied by an autoimmune response. Although it has been reported that mutations in the WFS1 gene are responsible for the development of this syndrome, the precise molecular mechanisms underlying β-cell death caused by the WFS1 mutations remain unknown. Here we report that WFS1 is a novel component of the unfolded protein response and has an important function in maintaining homeostasis of the endoplasmic reticulum (ER) in pancreatic β-cells. WFS1 encodes a transmembrane glycoprotein in the ER. WFS1 mRNA and protein are induced by ER stress. The expression of WFS1 is regulated by inositol requiring 1 and PKR-like ER kinase, central regulators of the unfolded protein response. WFS1 is normally up-regulated during insulin secretion, whereas inactivation of WFS1 in β-cells causes ER stress and β-cell dysfunction. These results indicate that the pathogenesis of Wolfram syndrome involves chronic ER stress in pancreatic β-cells caused by the loss of function of WFS1.

In 1938, Wolfram and Wagener (1) analyzed four siblings with the combination of juvenile diabetes and optic atrophy, thus providing the first report of Wolfram syndrome. Because a significant portion of patients with Wolfram syndrome develop diabetes insipidus and auditory nerve deafness, this syndrome is also referred to as the diabetes insipidus, diabetes mellitus, optic atrophy, and deafness syndrome (2, 3). Its pathogenesis is still unknown.

Although patients with Wolfram syndrome are not generally obese and do not have insulinitis, the β-cells in their pancreatic islets are selectively destroyed (4). Families that exhibit Wolfram syndrome share mutations in a gene encoding the WFS1 protein, a transmembrane protein in the endoplasmic reticulum (ER) (5, 6). WFS1 serves as an ER calcium channel (7), suggesting that this molecule may have a function in ER homeostasis. Therefore, inactivation of WFS1 may cause imbalances in ER homeostasis.

The ER is an important cellular compartment for the folding of newly synthesized secretory proteins such as proinsulin. Imbalance in ER homeostasis elicits stress in this organelle. ER stress is defined as an imbalance between the actual folding capacity of the ER and the demand placed on this organelle. The unfolded protein response (UPR), an adaptive response that counteracts ER stress, has three components as follows: gene expression, translational attenuation, and ER-associated protein degradation system (8–10). Accumulating evidence suggests that a high level of ER stress or defective ER stress signaling (i.e. the UPR) causes β-cell death during the development of diabetes (9, 11–13).

Inositol requiring 1 (IRE1), a sensor for unfolded and misfolded proteins in the ER, is a central regulator of the UPR. IRE1α, which is expressed ubiquitously, has a high level of expression in the pancreas and placenta (14, 15); IRE1β is expressed only in epithelial cells of the gastrointestinal tract (16, 17). The presence of unfolded proteins in the ER causes dimerization, trans-autophosphorylation, and consequent activation of IRE1. Activated IRE1 splices XBP-1 (X-box binding protein-1) mRNA, leading to synthesis of the active transcription factor XBP-1 and up-regulation of UPR genes (18, 19). In contrast, prolonged ER stress activates the cell-death pathway through IRE1. Under chronic ER stress, IRE1 recruits tumor necrosis factor receptor-associated factor 2 (20), which activates apoptosis-signaling kinase 1 (ASK1) (21, 22). Activated ASK1 leads to the activation of c-Jun N-terminal protein kinase and, in the presence of extreme ER stress, induces apoptosis (23). It has been suggested that this pathway is important for insulin resistance in patients with type 2 diabetes (24). Obesity causes ER stress in the liver and leads to hyperactivation of c-Jun N-terminal protein kinase signaling. This causes serine phosphorylation of insulin receptor substrate-1 and inhibits insulin action in liver cells. In addition, tumor necrosis factor receptor-associated factor 2 recruitment by IRE1 causes clustering and activation of caspase-12 at the ER membrane (25). Activated caspase-12 induces apoptosis under pathological ER stress conditions (26).

Two more upstream components in the UPR, PKR-like ER kinase (PERK) and activating transcription factor 6 (ATF6) (27–29), are also sensors of unfolded or misfolded proteins and are activated by the accumulation of such proteins in the ER. PERK is highly expressed in pancreatic islets (29, 30). Activated PERK phosphorylates the α subunit of eukaryotic translation initiation factor 2 (eIF2α), which leads to the attenuation of general protein translation. This reduces the ER workload and protects cells from apoptosis resulting from ER stress (31). In Wolcott-Rallison syndrome, a rare form of juvenile diabetes, mutations

* The abbreviations used are: ER, endoplasmic reticulum; UPR, unfolded protein response; IRE1, inositol requiring 1; PERK, PKR-like ER kinase; eIF2α, eukaryotic translation initiation factor 2; siRNA, small interfering RNA; ATF6, activating transcription factor 6.

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in the EIF2AK3 gene encoding PERK have been reported (32). PERK knock-out mice also develop diabetes because of the high level of ER stress in the pancreas (33, 34), strongly suggesting that H9252-cell death in patients with Wolcott-Rallison syndrome is caused by ER stress. ATF6 is a bZIP-containing transcription factor in the ER. Under ER stress, ATF6 is cleaved and released from the ER. The bZIP domain of ATF6 then translocates into the nucleus and up-regulates the UPR-specific downstream genes. The physiological role of ATF6 in pancreatic H9252-cells is not yet known.

Increasing evidence suggests that a high level of ER stress and defective ER stress signaling are important in the pathogenesis of diabetes. It is highly likely that downstream components of ER stress signaling maintain ER homeostasis in pancreatic β-cells. Therefore, defective ER stress signaling could cause a high level of ER stress in pancreatic β-cells and lead to β-cell dysfunction and diabetes. Pancreatic β-cells are specialized in proinsulin folding and insulin secretion. It is possible that β-cells have a unique downstream component of ER stress signaling. In this study we investigated whether Wolfram syndrome gene 1 (WFS1) is a component of ER stress signaling and has a function in maintaining ER homeostasis in β-cells.

EXPERIMENTAL PROCEDURES

Plasmids, Cell Culture, and Transfection—INS-1 832/13 cells were a gift from Dr. Christopher Newgard (Duke University Medical Center). INS-1 832/13 cells were maintained in RPMI with 10% fetal bovine serum and transfected with siRNA for WFS1 using the Cell Line Nucleofector™ T kit and the Nucleofector device (Amaxa Biosystems, Gaithersburg, MD). siRNAs were designed and synthesized at Qiagen (Valencia, CA) as follows: for rat WFS1–1, AAGGCATGAAGGTCTAACATT; for rat WFS1–2, AAGGCCATGCTGCCTCAAT. COS7 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and then transfected with WFS1 expression vectors using FuGENE (Roche Applied Science). Full-length human WFS1 cDNA, as well as P724L and G695V mutant WFS1 cDNA, were tagged with a FLAG epitope and subcloned to a pcDNA3 plasmid under the control of the cytomegalovirus promoter. The P724L and G695V mutations were introduced using the GeneTailor site-directed mutagenesis system (Invitrogen). Mouse embryonic fibroblasts were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. Human fibroblasts of a patient with Wolfram syndrome and a control individual were obtained, respectively, from Coriell Institute (Camden, NJ) and from Dr. Alan Permutt (Washington University School of Medicine). Human fibroblasts were maintained in Eagle's modified essential medium with 10% fetal bovine serum.

Immunostaining—COS7 cells and frozen sections of mouse pancreata were fixed in 2% paraformaldehyde for 30 min at room temperature and then permeabilized with 0.1% Triton X-100 for 2 min. The fixed cells were washed with phosphate-buffered saline, blocked with 10% bovine serum albumin for 30 min, and incubated in primary antibody overnight at 4 °C. The cells were washed three times in phosphate-buffered saline/Tween 0.1% and incubated with secondary antibody for 1 h at room temperature. Images were obtained with a Leica TCS SP2 AOBS confocal microscope with LCS software. FLAG M2 antibody was
purchased from Sigma. Anti-WFS1 antibody was generated as described previously (35).

Immunoblotting—Fibroblasts and INS-1 832/13 cells were lysed with M-PER (Pierce) containing protease inhibitors. COS7 cells were lysed for 15 min in ice-cold buffer (20 mM Hepes, pH 7.5, 1% Triton X-100, 150 mM NaCl, 10% glycerol, 1 mM EDTA) containing protease inhibitors. Insoluble material was recovered by centrifugation at 13,000 × g for 15 min and solubilized in 10 mM Tris-HCl and 1% SDS for 10 min at room temperature. After the addition of 4 volumes of lysis buffer, samples were sonicated for 10 s. Lysates were separated and normalized for total protein (20 μg per lane) using 4–20% linear gradient SDS-PAGE (Bio-Rad) and then electroblotted. FLAG M2 antibody was purchased from Sigma. Anti-WFS1 antibody was generated as described previously (35).

Isolating Islets from Mouse Pancreas—Mice were anesthetized, and their pancreatic islets were then isolated by pancreatic duct injection of 5 ml (0.85 mg/ml) of collagenase solution followed by digestion at 37 °C for 25 min with mild shaking. Digestion was stopped by adding ice-cold RPMI with 1% horse serum. Islets were washed several times with RPMI, separated from acinar cells on a Histopaque gradient, and handpicked using a dissecting microscope.

Real Time PCR—Total RNA was isolated from the cells by RNeasy (Qiagen, Valencia, CA). 1 μg of total RNA from cells was reverse-transcribed with oligo(dT) primer. For the thermal cycle reaction, the ABI prism 7000 sequencer detection system (Applied Biosystems, Foster City, CA) was used at 50 °C for 2 min, 95 °C for 10 min, then 40 cycles at 95 °C of 15 s each, and at 60 °C for 1 min. By using mouse actin for mouse embryonic fibroblasts, human glyceraldehyde-3-phosphate dehydrogenase for human fibroblasts, mouse actin for mouse islets, and rat actin for INS-1 832/13 cells as a control, the PCR was performed in triplicate for each sample and then repeated twice for all experiments. Cyber Green (Bio-Rad) and the following sets of primers were used for real time PCR: for mouse actin, GCAAGTGCTTCTAGGCGGAC and AAGAAAGGGTGTAACGCAGC; for mouse WFS1, CCATCAAGCATTCCGCTGC and GGGTAGGCCTCGCCATACA; for rat actin, GCAAATGCTTCTAGGCGGAC and AAGAAAGGGTGTAACGCAGC; and for rat WFS1, CATCACCAAGGACATCGTCCT and AGCACGTCCTTGAACTCGCT.

RESULTS

WFS1 Is a Component of the IRE1 Signaling Pathway—The pathogenesis of Wolfram syndrome has been attributed to mutations in the WFS1 gene (35, 36). The WFS1 gene encodes a 100-kDa glycoprotein containing 9–10 transmembrane domains that localizes to the ER (5, 6). Membrane proteins in the ER are often involved in the UPR (8, 37). Measuring the expression levels of WFS1 by real time PCR, we found that WFS1 mRNA is induced by ER stress and is under the control of IRE1α and PERK. In wild-type mouse fibroblasts, induction of WFS1 mRNA was increased 3–5-fold by two ER stress inducers, tumicamycin and thapsigargin (Fig. 2).
In Ire1α−/− and Perk−/− cells, WFS1 induction was attenuated (Fig. 1A). By measuring WFS1 protein expression levels by immunoblot using anti-WFS1 antibody, we found that WFS1 protein expression was decreased in Ire1α−/− and Perk−/− cells as compared with wild-type cells (Fig. 1B). Although there was a marked induction of WFS1 mRNA in wild-type cells by both inducers of ER stress (Fig. 1A), induction of WFS1 at the protein level in tunicamycin-treated cells in which N-glycosylation was inhibited was modest (Fig. 1B), suggesting that the WFS1 protein is unstable when N-glycosylation is inhibited. Also, there was no significant difference in WFS1 mRNA content between wild-type cells and both knock-out cells, although at the protein level, both Ire1α−/− and Perk−/− cells exhibited a profound decrease in WFS1 protein expression. This suggests further that WFS1 protein becomes unstable by chronic high levels of ER stress because there exists a higher base-line ER stress level in Ire1α−/− and Perk−/− cells, which are deficient in ER stress signaling. These results indicate that WFS1 is a component of the UPR and that its mRNA expression is regulated by the IRE1 and PERK signaling pathways.

Mutant WFS1 Does Not Accumulate on the ER Membrane—It has been reported that WFS1 gene mutations lead to loss of function of WFS1 protein. Nonsense or frameshift mutations of the WFS1 gene lead to a complete absence of WFS1 protein because of instability of the mutant protein (36). To extend this observation, we examined the cellular localization of mutant WFS1 protein. Most of the WFS1 gene mutations in patients with Wolfram syndrome occur in exon 8, which encodes the transmembrane of the protein and in C-terminal luminal domains (5, 6). We cloned the full-length human WFS1 gene by using human EST clones and then introduced into it the P724L and G695V mutations, which occur in Wolfram syndrome, by means of PCR-based mutagenesis. Like most WFS1 mutations in Wolfram syndrome patients, the P724L and G695V mutations occurred in exon 8.

We then determined the cellular localization of wild-type and mutant WFS1 by immunostaining cells transfected with an expression vector for wild-type, P724L, or G695V WFS1 tagged at its C terminus with a FLAG epitope. Immunostaining of cells expressing wild-type WFS1 showed a diffuse reticular pattern that co-localized with the ER marker ribophorin I (Fig. 2, A–C). However, immunostaining with anti-FLAG antibody of cells expressing mutant WFS1 showed a punctate staining pattern in the ER, suggesting that WFS1 tends to aggregate there (Fig. 2, D–F). Part of WFS1 P724L showed a diffuse reticular pattern and was co-localized with ribophorin I, suggesting that this part of WFS1 P724L is localized to the ER membrane (Fig. 2, D–F). These staining patterns suggest that, in contrast to wild-type WFS1, most of the newly synthesized WFS1 P724L protein aggregates and thus is not expressed on the ER membrane. We obtained similar results by expressing WFS1 G695V (data not shown).

When we assessed the aggregation of WFS1 P724L by SDS-PAGE immunoblot analysis of detergent-soluble and detergent-insoluble lysates from COS7 cells transiently expressing these proteins, we found that the formation of insoluble and high molecular weight complexes was much more prominent in cells expressing WFS1 P724L than in cells expressing wild-type WFS1 (Fig. 2G, lower panel).

We also measured WFS1 protein expression levels in fibroblasts from a patient with Wolfram syndrome and a control individual. We found that WFS1 protein could not be detected in the patient sample (Fig. 2H), suggesting that mutant WFS1 protein in patients with Wolfram syndrome does not accumulate in cells or can no longer be detected by the antibody due to a conformational change. Our results indicate that most of the newly synthesized mutant WFS1 protein tends to aggregate and does not fold into a proper three-dimensional structure. Therefore, it is likely that Wolfram syndrome is caused by a loss of function of WFS1.

**FIGURE 3. WFS1 maintains ER homeostasis in pancreatic β-cells.** A, distribution of WFS1 in mouse pancreas analyzed by immunohistochemistry using anti-WFS1, anti-insulin, and anti-glucagon antibodies. Merged image shows the co-localization of WFS1 and insulin (upper panel) or WFS1 and glucagon (lower panel). Scale bars, 50 μm. B, WFS1 mRNA is up-regulated by insulin secretagogues. Mouse islets were pretreated with 2.5 mM glucose for 1 h and stimulated with 16.7 mM glucose and 30 mM KCl for 1 h. Expression levels of WFS1 and actin were then measured by real time PCR (n = 3; values are mean ± S.E.). C, WFS1 protein is up-regulated by high glucose. Mouse islets were pretreated with 2.5 mM glucose for 2 h and stimulated with 16.7 mM glucose. The expression levels of WFS1 and actin were measured by immunoblot (IB).

WFS1 Is Important in Sustaining ER Homeostasis in Pancreatic β-Cells—In immunohistochemistry experiments on mouse pancreata using anti-WFS1, anti-insulin, and anti-glucagon antibodies, we detected WFS1 mainly in the islets, where it co-localized with insulin (Fig. 3A). However, WFS1 did not co-localize with glucagon, indicating that WFS1 is especially important in the function of β-cells.

It has been shown that WFS1 has an important function in stimulus-secretion coupling in insulin secretion (38). To determine WFS1 gene expression levels during insulin secretion, we pretreated mouse islets for 1 h with 2.5 mM glucose and then stimulated these cells for insulin secretion with 16.7 mM glucose and 30 mM KCl. WFS1 gene expression increased after treatment with both 16.7 mM glucose and 30 mM KCl but not after treatment with 2.5 mM glucose (Fig. 3B), suggesting that WFS1 up-regulation is important for insulin secretion. By measuring WFS1...
protein expression levels by immunoblot using anti-WFS1 antibody, we confirmed this WFS1 induction by 16.7 mM glucose in mouse islets (Fig. 3C).

ER homeostasis is important for insulin secretion because proinsulin, the insulin precursor, must be folded into its proper three-dimensional structure in the ER in order to become mature insulin. As a direct means of examining the relationship between the loss of function of WFS1 and ER homeostasis, we knocked down WFS1 expression in a \( \text{H9252} \)-cell line, INS-1 832/13, using siRNA for WFS1. The suppression of WFS1 caused an increase in the expression of BiP (Fig. 4A), Ero1α (Fig. 4B), spliced XBP-1 (Fig. 4C), total XBP-1 (Fig. 4D), Chop (Fig. 4E), and WFS1 (Fig. 4F), markers for ER stress in INS-1 832/13 cells. This suppression also increased the expression of another ER stress marker, Chop (Fig. 4E). However, the induction of Chop mRNA was modest as compared with its usual up-regulation under ER stress. Because Chop is a downstream component of Perk signaling (39), this suggests that eIF2α phosphorylation by Perk in response to WFS1 suppression is modest. Indeed, we could not detect eIF2α phosphorylation by suppressing WFS1 expression in INS-1 832/13 cells (data not shown). These results indicate that WFS1 has an important function in mitigating high levels of ER stress and in maintaining ER homeostasis in pancreatic \( \beta \)-cells. Therefore, suppression of WFS1 in \( \beta \)-cells could cause chronic ER stress in these cells.

To analyze the WFS1 expression level under pathological conditions, we measured WFS1 mRNA induction in islets from the ob/ob diabetes mouse model. We isolated islets from diabetic ob/ob mice and control C57Bl6 mice and measured WFS1 mRNA induction by treating the cells with 16.7 mM glucose. We found that induction of WFS1 mRNA was significantly more attenuated in ob/ob mice than in control mice (Fig. 5). This suggests that \( \beta \)-cells in ob/ob mice are in a state of chronic ER stress and that WFS1 induction is saturated.
to pancreatic $\beta$-cells, causing them to malfunction in patients with Wolfram syndrome. Indeed, we found that transient expression of pathogenic WFS1 mutants caused ER stress in a pancreatic $\beta$-cell line, MIN6 (data not shown). However, stable expression of pathogenic WFS1 in MIN6 cells did not cause ER stress and did not change the viability of these cells. These observations suggest that the expression of mutant WFS1 causes ER stress in pancreatic $\beta$-cells under specific conditions. It is also possible that ER stress response caused by transient and incomplete suppression of WFS1 with siRNA might differ from that caused by chronic and complete WFS1 deficiency. We plan to undertake additional studies to explore these possibilities.

The high levels of ER stress and pancreatic $\beta$-cell death in patients with Wolfram syndrome may be related to the $\beta$-cell dysfunction in patients with type 2 diabetes. The pathogenesis of type 2 diabetes is a result of peripheral resistance to the action of insulin, which may lead to a prolonged increase in insulin biosynthesis. Because the folding capacity of the ER is then overwhelmed, this peripheral resistance to insulin may activate the ER stress signaling pathways. For this reason, chronic ER stress in $\beta$-cells may lead to $\beta$-cell death in patients with type 2 diabetes who are genetically susceptible to ER stress. Further investigations of ER stress in the pathogenesis of diabetes are needed to obtain an understanding of the relationship between ER stress and type 2 diabetes.

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