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Endoplasmic reticulum stress and N-glycosylation modulate expression of WFS1 protein

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Abstract

Mutations of the *WFS1* gene are responsible for two hereditary diseases, Wolfram syndrome and low frequency sensorineural hearing loss. The WFS1 protein is a glycoprotein located in the endoplasmic reticulum (ER) membrane but its function is poorly understood. Herein we show WFS1 mRNA and protein levels in pancreatic islets to be increased with ER-stress inducers, thapsigargin and dithiothreitol. Another ER-stress inducer, the N-glycosylation inhibitor tunicamycin, also raised WFS1 mRNA but not protein levels. Site-directed mutagenesis showed both Asn-663 and Asn-748 to be N-glycosylated in mouse WFS1 protein. The glycosylation-defective WFS1 protein, in which Asn-663 and Asn-748 had been substituted with aspartate, exhibited an increased protein turnover rate. Consistent with this, the WFS1 protein was more rapidly degraded in the presence of tunicamycin. These data indicate that ER-stress and N-glycosylation play important roles in WFS1 expression and stability, and also suggest regulatory roles for this protein in ER-stress induced cell death.

Keywords: Wolfram syndrome; Low frequency sensorineural hearing loss; WFSI; ER-stress; N-Glycosylation

The *WFS1* gene, encoding a transmembrane protein of the endoplasmic reticulum (ER) [1], is mutated in two hereditary diseases, autosomal recessive Wolfram syndrome (OMIN:222300) [2,3] and autosomal dominant low frequency sensorineural hearing loss (LFSNHL) (OMIM:600965) [4,5]. The former is also known as DIDMOAD, summarizing the most frequent symptoms; diabetes insipidus, diabetes mellitus, optic atrophy, and deafness. More than 100 mutations of the *WFS1* gene have been identified to date in patients with these diseases [6]. WFS1 protein, also called wolframin, consists of 890 amino acids [2,3] and its homologues are found in several organisms; *Drosophila melanogaster* (CG4917), *Anopheles gambiae* (EBIP3764), *Ciona intestinalis* (Cin.16116), *Fugu rubripes* (SINFRUP82345), and *Xenopus laevis* (Xl.3995). However, these proteins share no homology with known proteins, making it difficult to speculate as to their functions.

We recently established a murine model with a disrupted *wfs1* gene [7]. Mutant mice exhibited impaired glucose homeostasis due to defective insulin secretion in vivo. Studies using isolated islets revealed that mutant islet cells were prone to apoptosis induced by insults which impair ER functions and trigger the so-called unfolded protein response (UPR) [8,9]. Therefore, it was suggested that WFS1 protein plays a role in modulation of apoptotic processes that arise from impairment of ER function [7]. In addition, isolated islets from WFS1-deficient mice exhibited defective insulin

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secretion which was accompanied by decreased calcium responses to glucose. Conversely, wolframin-overexpressing islets showed increased insulin secretion, indicating that wolframin also participates in regulation of stimulus-secretion coupling in insulin exocytosis [7]. It has recently been reported that WFS1 protein/wolframin expression in *Xenopus* oocytes conferred cation channel activity and increased cytosolic calcium levels [10]. This observation is intriguing since intracellular calcium regulation plays important roles in modulating both apoptotic and exocytotic processes. Despite these advancements, however, little is known about the mechanisms by which WFS1 protein actually alters these processes.

To understand the role that WFS1 protein/wolframin plays in the regulation of apoptotic and exocytotic events as well as in other as yet unknown cellular processes, information on the structure and function of this protein must be obtained. The amino acid sequence suggests that WFS1 protein is a multi-membrane spanning protein with hydrophilic amino (N)- and carboxy (C)-terminal regions [2,3]. In addition, biochemical and immunocytochemical analyses showed WFS1 protein to be an ER membrane glycoprotein [1].

In the present studies, we first examined WFS1 protein expression after treatment with agents that trigger UPR. We found WFS1 mRNA and protein levels to be increased by thapsigargin or dithiothreitol (DTT). Treatment with tunicamycin, an inhibitor of N-glycosylation, also raised WFS1 mRNA levels, suggesting that UPR increases WFS1 mRNA levels. However, the WFS1 protein level is not increased by tunicamycin. Subsequent analyses demonstrated protein stability to be reduced in the glycosylation-defective WFS1 protein. These results contribute to further understanding of the functions of this enigmatic protein.

Materials and methods

Reagents and antibodies. Tunicamycin, thapsigargin, DTT, and anti-actin antibody were purchased from Sigma–Aldrich Japan (Tokyo, Japan). Anti-HA and anti-CHOP antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-WFS1 N-terminus antibody was described previously [11].

Pancreatic islet isolation and treatment with ER-stress inducers. Pancreatic islets were isolated from male C57BL/6 mice by retrograde injection of collagenase (Sigma–Aldrich Japan, Tokyo, Japan) into the pancreatic duct. Approximately 100 (for Western blot analyses) or 200 (for RNA extraction) islets were treated with 2 μg/ml thapsigargin, 5 mM DTT, or 5 μM tunicamycin for 36 h in RPMI1640 medium. Total RNA was extracted using Isogen reagent (NipponGene, Toyama, Japan). Quantitative real-time PCR analysis for WFS1 mRNA levels was performed using primers, 5′-CTGGAAACTCAACCCCAAGA-3′ and 5′-TTGGATTCACTGCTGACGAG-3′.

Plasmids. pHA-mWFS1 encodes a fusion protein consisting of an initiator methionine, the HA epitope tag (YPYDVPDYA), and amino acids 2–890 of mouse WFS1 protein. To generate this plasmid, a fragment encoding a *Sal*I restriction site and amino acids 2–484 was amplified by PCR. Using the PCR method, pmWFS1-HA encoding mouse WFS1 protein with an HA tag between residues 830 and 831 was also generated. pHA-mWFS1(N633D) and pHA-mWFS1 (N748D), which encode HA-tagged WFS1 protein with a mutation of asparagine 633 to aspartate and asparagine 748 to aspartate, respectively, were generated using PCR-based mutagenesis on pHA-mWFS1. pHA-mWFS1(N633D/N748D) encoding a mutant protein with mutation of both asparagine residues was generated using pHA-mWFS1(N633D).

Cell culture and transient transfection. MIN6 [12] and COS7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum, 50 U/ml penicillin, and 50 μg/ml streptomycin sulfate. Transfection of plasmids was carried out using FuGENE6 (Roche, Indianapolis, IN) diluted in OPTI media (Invitrogen, Carlsbad, CA). Cells were harvested for Western blot or proceeded to immunostaining analysis 36 h after transfection. Immunostaining was performed using anti-HA antibody and FITC-conjugated anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA).

Trypsin treatment. COS7 cells transfected with either pHA-mWFS1 or pmWFS1-HA were homogenized in a buffer containing 270 mM sucrose, 2 mM EDTA, and 50 mM Hepes (pH 7.5). Cellular membranes were recovered by centrifuging the homogenate at 17,400*g* for 15 min. Membranes (100 μg) were then incubated with trypsin at various concentrations at 4 °C. After a 30 min incubation, homogenates were boiled and subjected to SDS/PAGE and Western blot analysis.

Endoglycosydase cleavage. COS7 cells transfected with either wild-type WFS1 cDNA or mutant constructs were dissolved in denaturing buffer (0.5% SDS, 1% β-mercaptoethanol), boiled for 10 min at 100 °C, then incubated at 37 °C for 1 h with endoglycosidase H (500 U), and subjected to electrophoresis on NuPAGE 3–8% Tris–acetate gel (Invitrogen).

Metabolic labeling. MIN6 cells were labeled with [^{35}S]methionine and [^{35}S]cysteine (100 μCi/ml; EXPRE^{35}S^{35}S labeling mix, Perkin–Elmer-New England Nuclear, Boston, MA) in DMEM with either methionine or cysteine in the presence or absence of 5 μg/ml tunicamycin for 3 h. Cells were then chased for different periods in complete medium with or without tunicamycin. COS7 cells transfected with pHA-mWFS1 or pHA-mWFS1(N633D/N748D) were also labeled with [^{35}S]methionine and [^{35}S]cysteine for 3 h. Cells were then chased for different periods in complete medium. MIN6 and COS7 cells were lysed in a buffer containing 100 mM NaCl, 0.5 mM EDTA, 20 mM Tris (pH 7.5), and 0.5% NP-40. Lysates were incubated with 10 μl protein A/G–Sepharose (Amersham Biosciences, Piscataway, NJ) for 2 h and then briefly centrifuged. The resulting supernatant was incubated with anti-WFS1 N-terminus or anti-HA antibodies overnight and then incubated with protein A/G–Sepharose for 2 h. The beads were washed three times and bound WFS1 proteins were eluted in SDS-sample buffer and subjected to SDS/PAGE (10%).

Statistical analyses. Data are presented as means ± SE. Differences were assessed by Student's *t* test.

Results and discussion

Effect of ER-stress inducers on WFS1 expression in pancreatic islets

We recently reported that WFS1-deficient islets exhibited increased susceptibility to apoptosis due to impaired ER function [7]. Therefore, in this study, we first determined WFS1 expression in isolated mouse pancreatic islets treated with the ER-stress inducers, thapsigargin, DTT, and tunicamycin. Thapsigargin is an inhibitor of the sarco(endo)plasmic reticulum Ca^{2+} pump and

depletes ER Ca^{2+}, which affects the functions of Ca^{2+}-dependent ER chaperone proteins. DTT and tunicamycin affect protein folding by disrupting disulfide bonds and inhibiting N-glycosylation, respectively. These compounds therefore cause mis-folding of proteins (ER-stress) and induce UPR [13]. As shown in Fig. 1, WFS1 protein expression was increased in islets treated with 2 μM thapsigargin (Fig. 1A) or 5 mM DTT (Fig. 1B) for 36 h. Greater than threefold increases in WFS1 mRNA levels were also observed by quantitative RT-PCR analyses in islets treated with these agents (data not shown). Another ER-stress inducer, tunicamycin (5 μg/ml), did not raise WFS1 protein levels in isolated islets (Fig. 1C). However, quantitative RT-PCR analyses revealed WFS1 mRNA levels to be increased by 72% in islets treated with tunicamycin (Fig. 1D). These data suggest that WFS1 mRNA expression increases in response to the ER-stress.

WFS1 protein has been shown to be a glycoprotein [1] like the inositol trisphosphate receptor, another ER membrane resident protein essential for cellular calcium homeostasis and signaling [14]. The unaltered WFS1 protein levels despite increased mRNA levels in islets treated with tunicamycin raise the possibility that inhibition of N-glycosylation affects WFS1 protein stability. To address this possibility, pancreatic β-cell derived MIN6 cells were labeled for 3 h with [^{35}S]methionine/cysteine and chased with unlabeled methionine and cysteine for different intervals in the continuous absence or presence of tunicamycin. As shown in Fig. 1E, WFS1 protein in tunicamycin-treated cells was more rapidly degraded, suggesting that inhibition of N-glycosylation reduces WFS1 protein stability.

Membrane topology of WFS1 protein

To study the roles of N-glycosylation more specifically, we first sought to determine N-glycosylation site(s) of WFS1 protein/wolframin. Since N-glycosylation occurs in the ER, it was prerequisite to know the membrane topology of this protein. The initial hydropathy plot studies did not provide a definitive answer; WFS1 protein contains 9 or 10 transmembrane segments, with long hydrophilic stretches on both the N- and the C-termini [2,3].

To localize the N- and the C-termini of WFS1 protein with respect to the ER membrane, we transiently expressed, in COS7 cells, mouse WFS1 protein tagged with an HA-epitope in either the N- or the C-terminal stretch (designated HA-mWFS1 or mWFS1-HA, respectively,

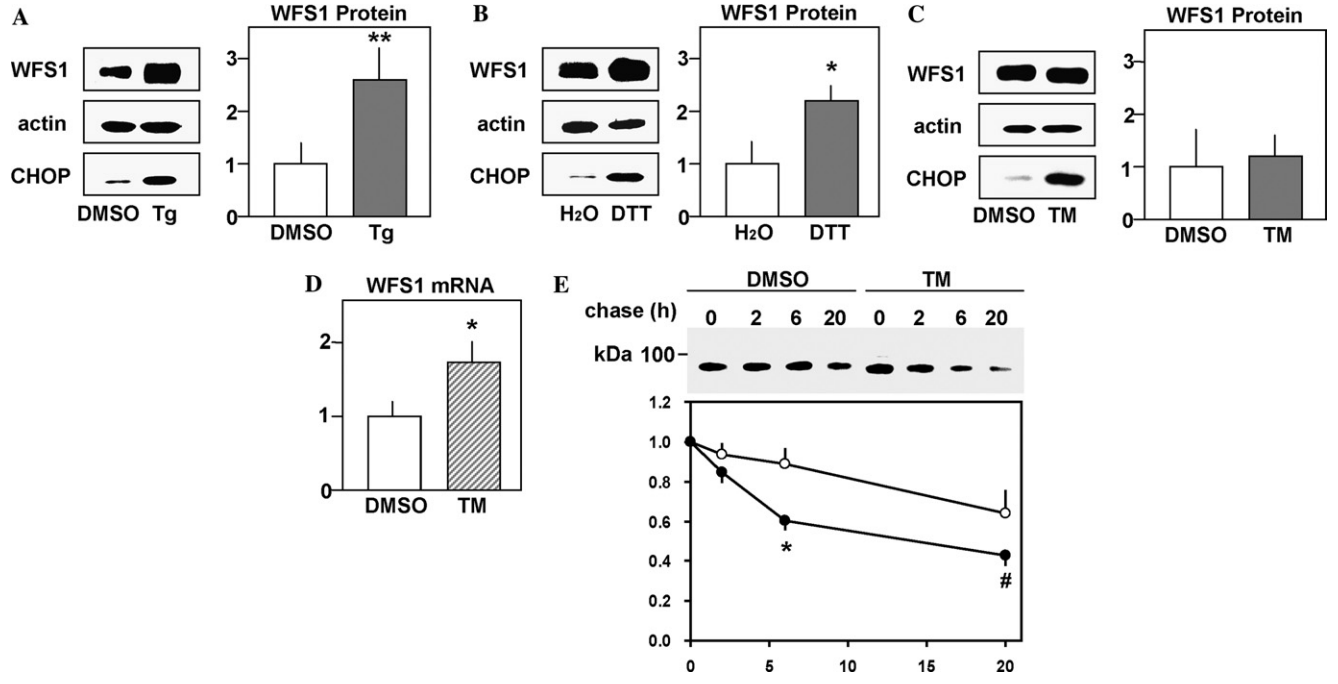

Fig. 1. WFS1 expression in mouse pancreatic islets in response to ER-stress inducers. (A–C) Isolated mouse islets were challenged with 2 μM thapsigargin (Tg) (A, $n = 4$), 5 mM DTT (B, $n = 3$) or 5 μg/ml tunicamycin (TM) (C, $n = 5$). After a 36-h incubation, the islets were subjected to SDS/PAGE and blotted using antibodies against the WFS1 N-terminus, actin, or CHOP. Representative blots are shown in the left panels. Increased CHOP expression indicated successful induction of ER-stress mediated apoptosis. WFS1 protein/actin levels are summarized in the right panels. Data are expressed as the expression relative to those of a control islet preparation. (D) Total RNA was extracted from isolated mouse islets treated with 5 μg/ml tunicamycin for 36 h. WFS1 and GAPDH mRNA levels were determined by quantitative real-time PCR. WFS1 mRNA levels were normalized to those of GAPDH. Data were obtained using three independent sets of islet preparations. (E) MIN6 cells were pulse-labeled for 3 h without or with 5 μg/ml tunicamycin and chased for up to 20 h in the continuous absence or presence of the drug. A representative result from three independent experiments is shown in the upper panel. Data from three experiments are summarized, after normalization to time zero of the chase in the lower panel. Open circles, DMSO-treated MIN6 cells. Closed circles, tunicamycin-treated cells. $^{\#}P = 0.0634$, $^{*}P < 0.05$, $^{**}P < 0.01$.

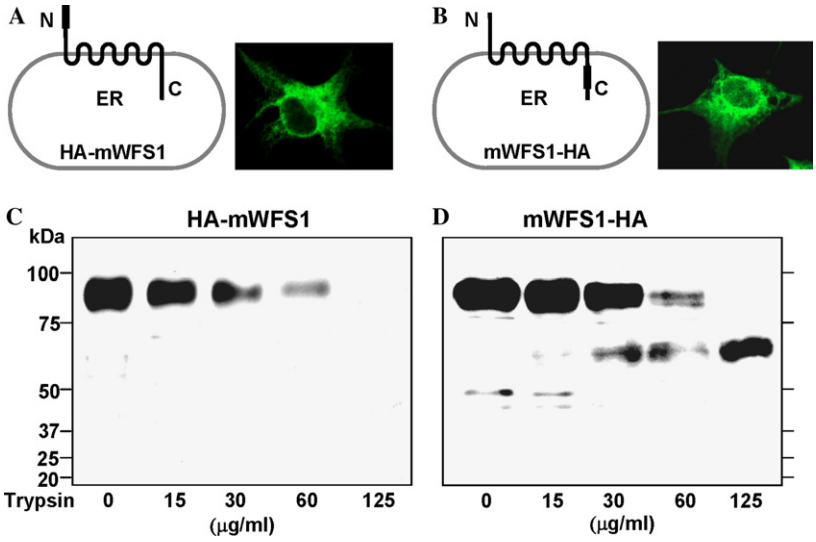

Fig. 2. Membrane orientations of the N- and the C-termini of WFS1 protein as determined by trypsin proteolysis. WFS1 protein tagged with the HA epitope at either the N- (A,C) or the C-terminus (B,D) was expressed in COS7 cells. (A,B) Schematic illustration and immunocytochemical demonstration of the HA-tagged WFS1 proteins used in (C) and (D). (C,D) Membranes from COS7 cells transfected with a plasmid encoding either HA-mWFS1 or mWFS1-HA were treated with the indicated amounts of trypsin. After incubation for 30 min at 4 °C, the reactions were stopped by boiling for 5 min, and subjected to SDS/PAGE and immunoblot analysis with anti-HA antibody.

Figs. 2A and B). HA-mWFS1 and mWFS1-HA proteins were successfully expressed and localized to the ER, as demonstrated by reticular staining in the cytoplasm (Figs. 2A and B). Membrane preparation of cells expressed with either HA-mWFS1 or mWFS1-HA proteins was then subjected to trypsin digestion followed by SDS/PAGE and immunoblotting with an antibody against the HA epitope. The HA-epitope tagged at the N-terminus was completely digested with increasing concentrations of trypsin (Fig. 2C). This was not due to loss of membrane vesicle integrity, since no changes in an ER-resident chaperone protein, GRP78, were detected using an antibody against GRP78 in the same membrane preparations (data not shown). In contrast, the C-terminal HA epitope was protected from trypsin (Fig. 2D). These results indicated that WFS1 protein has odd numbers of transmembrane segments with the orientation of the N-terminus toward the cytoplasm and that of the C-terminus toward the ER lumen. A similar conclusion was obtained by trypsin-digestion of the membrane, followed by detection with C-terminal or N-terminal antibodies [15].

Determination of N-glycosylation sites.

Mouse WFS1 protein has six asparagine residues with the consensus sequence for N-glycosylation (N-X-S/T, where X is any amino acid except for proline). According to a 9-transmembrane model with N-terminus cytosolic/C-terminus luminal orientation, asparagines 663 and 748 would be localized in the ER. Therefore, we mutated these two asparagine residues to aspartate in order to determine whether one or both are N-linked glycosylation site(s). Mutant WFS1 proteins in which asparagine 663 and/or asparagine 748 was mutated to aspartate [designated HA-mWFS1(N663D), HA-mWFS1(N748D), and HA-mWFS1(N663D/N748D)] were successfully expressed in the ER (Fig. 3A). When these WFS1 mutant proteins were subjected to SDS/PAGE, HA-mWFS1(N663D/N748D) migrated faster than the HA-wild-type mWFS1 protein, while both HA-mWFS1(N663D) and HA-mWFS1(N748D) mutants were between the two (Fig. 3B). These results suggested that both asparagine residues, 663 and 748, are glycosylation sites and place the C-terminal stretch of WFS1 protein within the intraluminal compartment. Furthermore, as shown in Fig. 3C, although endoglycosidase H (Endo H) treatment of the wild-type WFS1 protein resulted in faster migration of the protein, the mobility of the double mutant [WFS1 (N663D/N748D)] protein was not affected by treatment with Endo H. In addition, the double mutant protein exhibited the same mobility as the wild-type WFS1 protein treated with Endo H. These data demonstrated that no asparagine residue other than N663 and N748 is glycosylated.

Reduced protein stability of N-glycosylation-defective WFS1 protein

N-glycosylation reportedly plays an important role in the stability of proteins such as the T cell antigen receptor α-subunit [16], the α-subunit of the nicotinic acetylcholine receptor [17], and apolipoprotein B [18]. Therefore, to assess the role of N-glycosylation in WFS1 protein stability, we performed pulse-chase

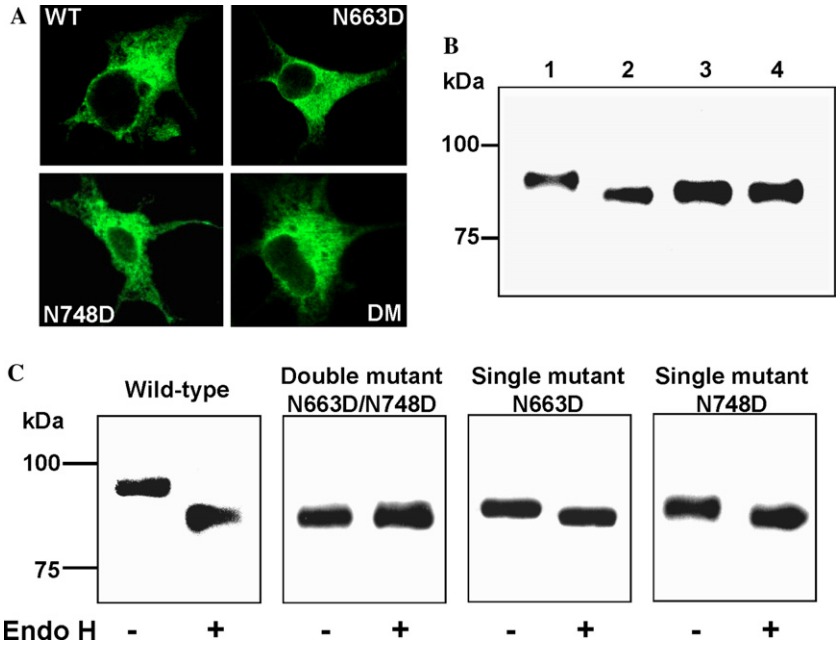

Fig. 3. Electrophoretic mobility and effect of endoglycosidase digestion on HA-tagged wild-type and mutant WFS1 proteins. (A) Immunofluorescence localization of HA-tagged WFS1 proteins: upper left, wild-type HA-mWFS1; upper right, HA-mWFS1(N663D); lower left, HA-mWFS1(N748D); lower right, HA-mWFS1(N663D/N748D). (B) COS7 cells transfected with 0.5 μg plasmids encoding either HA-tagged wild-type or mutant WFS1 proteins were lysed, subjected to SDS/PAGE, and probed with anti-HA antibody: lane 1, HA-mWFS1; lane 2, HA-mWFS1 (N663D/N748D); lane 3, HA-mWFS1(N663D); and lane 4, HA-mWFS1(N748D). (C) Lysates of COS7 cells transfected with either wild-type WFS1 cDNA or mutant constructs were incubated at 37 °C for 1 h with or without endoglycosidase H (500 U) and were subjected to electrophoresis on NuPAGE 3–8% Tris–acetate gel.

experiments. In these experiments, COS7 cells transiently transfected with either the HA-wild-type mWFS1 or mutant HA-mWFS1(N663D/N748D) cDNAs were labeled for 3 h with [^{35}S]methionine/cysteine and chased for different intervals. As shown in Fig. 4, the wild-type mWFS1 protein was relatively stable; $65 \pm 6\%$ ($n = 3$) of the protein remained 18 h after its synthesis. In contrast, only $44 \pm 5\%$ ($n = 3$) of mWFS1(N663D/N748D) remained after 18 h. These data showed protein stability to be reduced when both N-glycosylation sites were disrupted. One could argue an increased turnover rate of mWFS1(N663D/N748D) to be due to introducing aspartate residues rather than lack of glycosylation. To study the roles of N-glycosylation in various glycoproteins, asparagine residues in the consensus motif have been substituted with a variety of amino acids, such as aspartate ([19,20], this study), glutamine [14,21], alanine [19,22], threonine [23], and isoleucine [24]. None of these replacements were perfect, because introducing different residues might have its own effects. In this study, we also observed that WFS1 protein in MIN6 cells was more rapidly degraded when treated with a glycosylation inhibitor, tunicamycin (Fig. 1E). Thus, both molecular and biochemical approaches indicated that glycosylation-defective WFS1 protein has reduced stability. We therefore conclude that N-glycosylation affects WFS1 protein levels. A previous study using the ER resident glycoprotein ribophorin showed N-glycosylation to be necessary for calnexin binding, which prevents the glycoprotein from being rapidly degraded [25]. Such a mechanism could also be operative in WFS1 protein.

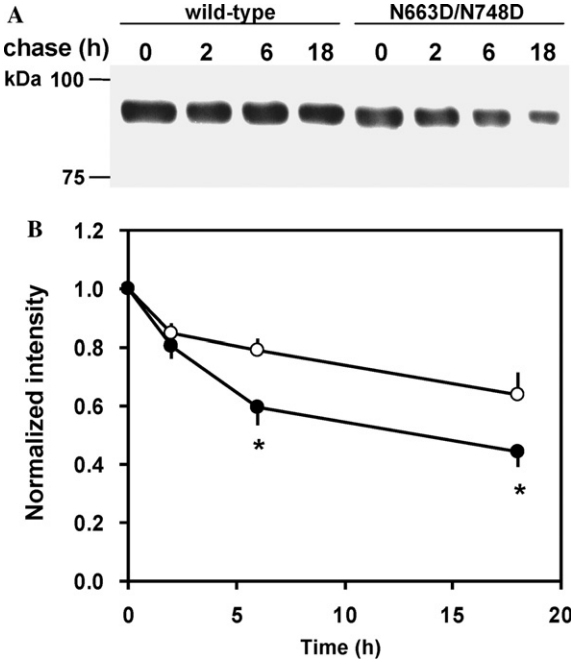

Fig. 4. Decreased stability of glycosylation-defective WFS1 protein. (A) HA-mWFS1 and HA-mWFS1(N663D/N748D) profiles of radiolabeled bands as a function of time of chase. A representative result from three experiments is shown. (B) Data from three experiments are summarized after normalization to time zero of chase. *$P < 0.05$.

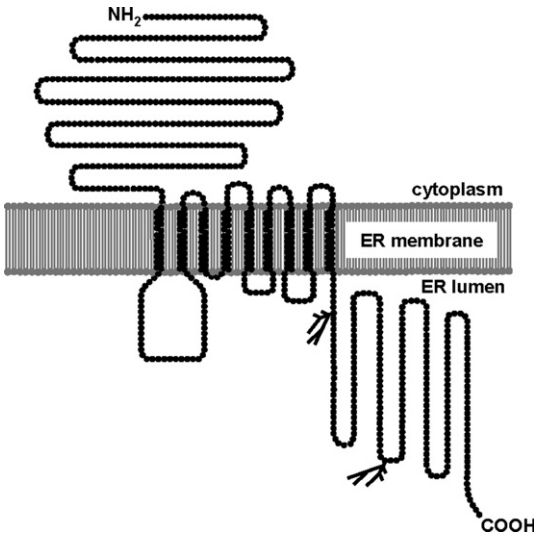

Fig. 5. Transmembrane topology model for mouse WFS1 protein with glycosylation sites. Membrane topology of mouse WFS1 protein as based on analysis using the SOSUI computer program [26] and data obtained in this study. Two N-glycosylation sites in the C-terminus stretch are depicted.

Herein, we defined the membrane topology of WFS1 by analyzing protease protection susceptibility: the orientation of the N-terminus in the cytoplasm and the C-terminus in the ER. This topology was supported by determining the N-glycosylation sites to be N663 and N748. A schematic diagram of WFS1 protein deduced from the current study and others is shown in Fig. 5. WFS1 protein/wolframin is a type II transmembrane protein and has long N-terminal and C-terminal stretches. These stretches could interact with other molecule(s), thereby mediating specific functions. Further study of these actions is clearly warranted. N663 and N748 residues in murine WFS1 protein correspond to N661 and N746 in the human orthologue. Although more than 100 mutations have been identified in WFS1 protein/wolframin and span the entire coding sequence, no mutations in these asparagine residues or adjacent amino acids were found in patients with Wolfram syndrome or LFSNHL. Given that loss of the functions of this protein is at least one of the pathogenic mechanisms of Wolfram syndrome [6,15], future survey of genomic sequences in patients with these diseases might identify mutations in these glycosylation sites.

The present data also provide evidence that increased WFS1 expression is the primary response of this protein to ER stress. There is no increase in WFS1 protein expression in response to tunicamycin, despite increased WFS1 mRNA levels. This is apparently attributable to the combined effects of increased mRNA levels and reduced protein stability. The increased WFS1 expression in response to ER-stress raises the possibility that WFS1 protein is a component of the UPR and plays a protective role against ER stress. This notion is supported by our recent findings that islets isolated from WFS1-deficient mice exhibited increased susceptibility to ER stress-induced apoptosis [7].

Acknowledgments

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