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Wolfram syndrome-associated mutations lead to instability and proteasomal degradation of wolframin

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Abstract **Wolfram syndrome is caused by mutations in *WFS1* encoding wolframin, a polytopic membrane protein of the endoplasmic reticulum. Here, we investigated the molecular pathomechanisms of four missense and two truncating mutations in *WFS1*. Expression in COS-7 cells as well as direct analysis of patient cells revealed that *WFS1* mutations lead to drastically reduced steady-state levels of wolframin. All mutations resulted in highly unstable proteins which were delivered to proteasomal degradation. No wolframin aggregates were found in patient cells suggesting that Wolfram syndrome is not a disease of protein aggregation. Rather, *WFS1* mutations cause loss-of-function by cellular depletion of wolframin.**

Keywords: Wolfram syndrome; *WFS1*; Wolframin; Mutation; Endoplasmic reticulum

1. Introduction

Wolfram syndrome is a rare autosomal-recessive disorder defined by the association of early-onset diabetes mellitus and optic atrophy. Additional neurological and endocrinological manifestations may be present [1]. The disease phenotype is caused by mutations in *WFS1* on 4p16 [2,3] encoding the 100 kDa protein wolframin. Secondary structure analysis predicted wolframin as a multispanning membrane protein with nine transmembrane segments [3]. Wolframin is a resident component of the endoplasmic reticulum (ER) [4] with an N_{cyt}/C_{lum} orientation in the ER membrane [5]. Recent studies suggested an involvement in the regulation of ER stress [6–8].

To date, a wide spectrum of different mutations in *WFS1* is known to cause Wolfram syndrome [9]. Most of them represent inactivating mutations implying that they cause a loss-of-function phenotype. Consistently, our previous study and that of others showed lack of wolframin in fibroblasts derived from patients with genotypes W371X/R629W, fs343X396/fs343X396 [5] or G695V/W648X [7]. However, there is no current concept about how wolframin is inactivated. We proposed that instability of *WFS1* transcripts is associated with truncating mutations W371X and F343fsX396, whereas mutation R629W causes instability of wolframin protein. Fonseca and co-authors [7] suggested that missense mutations G695V and P724L cause inactivation by formation of wolframin aggregates. Here, we investigated the effects of six different missense mutations and truncating mutations on the expression level, stability, degradation and the intracellular fate of wolframin. We show that all these *WFS1* mutations result in unstable proteins. Proteasomal degradation but not formation of aggregates contributes to cellular depletion of wolframin.

2. Materials and methods

2.1. Generation of WFS1 constructs

Wolframin mutants were generated by the megaprimer mutagenesis protocol. A mutated megaprimer was amplified using an outside wild-type (WT) primer (forward or reverse) and a middle mutagenic primer (forward or reverse). This megaprimer was used in a second round of PCR together with the appropriate outside primer. Mutants F883X and P885L were generated by a single PCR step. Human *WFS1* in pIRES2 [5] was used as template for PCR. Primers were: WT-F, 5′-atggactccaacactgctccg-3′; WT-R, 5′-tcaggccgccgacaggaatc-3′; P504L-R, 5′-catagagcaggcacaggacgctg-3′; R629W-F, 5′-ctgacgtggagctccatgg-3′; W700X-R, 5′-gaagcggccggtctacgtg-3′; P724L-F, 5′-catcaacatgctcctgttcttcatc-3′; F883X-R, 5′-tcagaaaaagaagtcgaagcc-3′; P885L-R, 5′-tcaggccgccgacaggaataggaagaa-3′. A high fidelity Taq polymerase (Roche) was used for all PCR amplifications. *WFS1* fragments were ligated into pIRES2 (Clontech) and sequenced using an automated ABI310 sequencer.

2.2. Cell culture and transfection

Fibroblasts derived from a healthy individual as well as from Wolfram syndrome patients carrying mutations W371X/R629W, fs343X396/fs343X396 [5] or W700X/W700X were used in this study. Fibroblasts and COS-7 cells were cultured at 37 °C in DMEM supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine. COS-7 cells were transfected with *WFS1* constructs in 12-well tissue culture plates using Metafectene (Biontex). Twenty-four hours post-transfection, cells were either used for metabolic labelling experiments or harvested by trypsinisation. When indicated, cells were grown in the presence of lactacystin (Calbiochem) or E-64 (Sigma).

2.3. Pulse-chase experiments

Pulse-chase experiments were performed as described previously [5]. In brief, construct-transfected COS-7 cells were pulse-labeled for 30 min with 100 μCi/ml ^{35}S-labeled methionine/cysteine (NEN) and then chased for indicated time periods. At each time point, cells were lysed in IP buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 10% glycerol) containing 1% SDS and protease inhibitors (mini complete, Roche) for 30 min at 0 °C. Cleared extracts were subjected to immunoprecipitation with anti-WoN antibody bound to Protein A-sepharose and analysed by SDS/PAGE (3–8% NuPage Tris–acetate gels, Invitrogen) and autoradiography.

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2.4. Immunofluorescence

Fibroblasts were grown until sub-confluency and then fixed in ice-cold methanol (100%) for 20 min. Blocking was done in 1 × PBS containing milk powder (5%) followed by incubation with anti-WoN (1:100) in blocking solution for 90 min at room temperature. Incubation with secondary antibody was performed for 60 min using anti-rabbit IgG-FITC (1:360, Sigma). Slides were mounted in fluorescent mounting medium (DAKO), sealed and then analysed in a confocal microscope with the appropriate filter.

2.5. Detergent extraction

Transfected COS-7 cells (~1×10^5 cells) were lysed in 100 µl 1 × PBS pH 7.4, 10% glycerin, 2 mM EDTA, 1% Nonidet P-40 (Sigma) and protease inhibitors (mini complete, Roche) for 30 min on ice. Insoluble material was recovered by centrifugation at 20 000 × *g* for 15 min and solubilised in 50 µl 10 mM Tris/HCl pH 7.4, 1% SDS for 10 min at room temperature. After addition of 200 µl detergent buffer, samples were sonicated for 20 s. TCA-precipitated proteins derived from soluble and insoluble fractions were then separated on 7% Tris–acetate gels (Invitrogen) and analysed by immunoblotting.

2.6. RT-PCR analysis

RNA from cultured fibroblasts was extracted using TRIZOL (Invitrogen) and treated with DNAse (Promega) according to the manufactures recommendations. First-strand cDNA was synthesised from total RNA using oligo (d)T primers and Superscript II (Invitrogen). RT-PCR amplification was performed with human *WFS1*-specific primers (F: 5′-cctgccactgcgtctgaag-3′; R: 5′-ccacatccaggttgggctc-3′) and *GAPDH*-specific primers (F: 5′-gtccactggcgtcttcacca-3′; R: 5′-ggtggcagtgatggcatggac-3′) and analysed after different cycle numbers. The *WFS1* primers used here amplify a region of the gene which spans an intron; the *GAPDH* primers correspond to published primers for pseudogene-free amplification of the *GAPDH* gene [10].

3. Results

3.1. WFS1 mutations

In this study, we investigated four missense and two truncating *WFS1* mutations. The missense mutations correspond to sequence portions encoding transmembrane helical (P504L), cytoplasmic loop (R629W) and lumenal C-terminal (P724L, P885L) domains of wolframin. Of these, P504L, R629W and P885L belong to the known rare homozygous missense mutations [2,11,12]. The W700X mutation represents a novel inactivating mutation present in homozygosity in a female patient. It leads to truncation of a major portion of the hydrophilic C-terminus. The F883X mutation causes deletion of the last eight amino acid residues and mimics a group of frameshift mutations affecting the very C-terminus of wolframin [2,11,13].

3.2. WFS1 mutations lead to cellular depletion of wolframin

To assess steady-state concentrations of wolframin mutants, we used transient expression of wild-type and mutated constructs in COS-7 cells. Cells expressing the wild-type wolframin showed very strong signals at about 100 kDa (Fig. 1A). In contrast, the steady-state concentrations of mutants were significantly reduced when compared to the wild-type protein. The direct analysis of endogenous mutated wolframin in patient cells revealed a more drastic reduction (Fig. 1B): signals corresponding to $\text{wolframin}_{\text{W700X}}$ were almost undetectable in respective patient cells and $\text{wolframin}_{\text{R629W}}$ was markedly reduced compared to control cells. We conclude that *WFS1* mutations, both truncating and missense mutations, cause cellular depletion of wolframin.

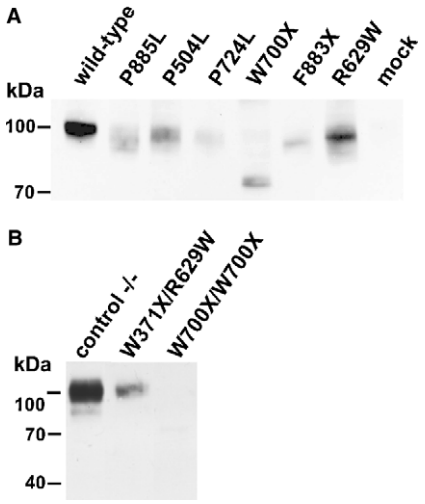

Fig. 1. Steady-state levels of mutated and truncated forms of wolframin. (A) Construct-transfected COS-7 cells were lysed in SDS buffer and analysed by immunoblotting with anti Wo-N. (B) Immunoblot analysis of control fibroblasts and of two patients (W371X/R629W, W700X/W700X). Aliquots (5×10^4 cells) were resolved by SDS/PAGE and blotted with anti-WoN and anti-β actin.

3.3. Truncating mutations of WFS1 are associated with stable transcripts

Nonsense and frameshift mutations generate premature stop codons which may cause mRNA degradation through nonsense-mediated mRNA decay (NMD) and thus, absence of the encoded protein [14]. We asked whether late truncating mutations W700X and F883X cause instability of *WFS1* transcripts. Since reliable RT-PCR analysis of construct-transfected cells is hampered by residual plasmid DNA, we directly determined levels of *WFS1* transcripts in W700X/W700X fibroblasts. For comparison, we re-analysed patient fibroblasts with F343fsX396 present in homozygosity [5]. RT-PCR analysis revealed stable *WFS1* transcripts in W700X/W700X cells (Fig. 2). Surprisingly, we also obtained stable *WFS1* transcripts in F343fsX396/F343fsX396 fibroblasts what was in contrast to our previous findings [5]. False-positive results due to amplification from residual genomic DNA could be excluded. We speculate that the interference of pseudogene sequences by the use of different *GAPDH* primers in the former study has

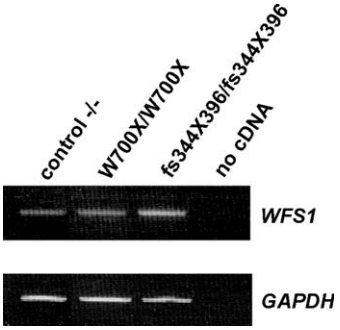

Fig. 2. Semi-quantitative RT-PCR analysis of *WFS1*. First-strand cDNAs derived from two patients (W700X/W700X, fs343X396/fs343X396) and a control were analysed by PCR using *WFS1*- or *GAPDH*-specific primers as a control. PCR products corresponding to 26 cycles are shown.