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Characterization of the novel protein KIAA0564 (Von Willebrand Domain-containing Protein 8)

Moulun Luo [a], April E. Mengos [b], Wuqiong Ma [a], Jean Finlayson [a], Rocio Zapata Bustos [a], Yuan Xiao Zhu [c], Chang-Xin Shi [c], Tianna M. Stubblefield [d], Wayne T. Willis [a], Lawrence J. Mandarino, PhD [a, *]

[a] Division of Endocrinology, University of Arizona, Tucson, AZ, United States
[b] Division of Laboratory Medicine, Mayo Clinic Arizona, Scottsdale, AZ, United States
[c] Division of Hematology-Oncology, Mayo Clinic Arizona, Scottsdale, AZ, United States
[d] Mouse Metabolic Phenotyping Laboratory, Mayo Clinic Arizona, Scottsdale, AZ, United States

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ABSTRACT

The VWA8 gene was first identified by the Kazusa cDNA project and named KIAA0564. Based on the observation, by similarity, that the protein encoded by KIAA0564 contains a Von Willebrand Factor 8 domain, KIAA0564 was named Von Willebrand Domain-containing Protein 8 (VWA8). The function of VWA8 protein is almost unknown. The purpose of this study was to characterize the tissue distribution, cellular location, and function of VWA8. In mice VWA8 protein was mostly distributed in liver, kidney, heart, pancreas and skeletal muscle, and is present as a long isoform and a shorter splice variant (VWA8a and VWA8b). VWA8 protein and mRNA were elevated in mouse liver in response to high fat feeding. Sequence analysis suggests that VWA8 has a mitochondrial targeting sequence and domains responsible for ATPase activity. VWA8 protein was targeted exclusively to mitochondria in mouse AML12 liver cells, and this was prevented by deletion of the targeting sequence. Moreover, the VWA8 short isoform overexpressed in insect cells using a baculovirus construct had *in vitro* ATPase activity. Deletion of the Walker A motif or Walker B motif in VWA8 mostly blocked ATPase activity, suggesting Walker A motif or Walker B motif are essential to the ATPase activity of VWA8. Finally, homology modeling suggested that VWA8 may have a structure most confidently similar to dynein motor proteins.

1. Introduction

The Von Willebrand Factor A Domain Containing 8 (VWA8) gene originally was identified by the Kazusa cDNA project and given the designation KIAA0564. Sequence analysis showed that the protein encoded by the KIAA0564 gene likely contains a VWA domain in its C-terminus. VWA8 has two predicted isoforms, a long (VWA8a) and short (VWA8b) isoform. The human VWA8 gene is localized to Chromosome 13 while the mouse VWA8 gene is on Chromosome 14. The human and mouse VWA8 amino acid sequences are 86 and 89% identical for the long and short isoforms, respectively. Although VWA8 protein is essentially uncharacterized, the VWA8 gene appears as a possible signal in three genome wide association studies, for serum calcium concentrations [1], autism [2], and bipolar disorder with comorbid migraine [3]. The promoter of the VWA8 gene also is differentially methylated in acute myeloid leukemia [4]. One proteomics study found that VWA8 associates with TRβ2 (Thyroid hormone receptor β2), but not TRβ1 [5]. We previously found, in an unbiased proteomic screening of livers from high fat fed, obese, insulin resistant mice compared to lean, standard diet fed control C57BL/6J mice that VWA8 protein abundance was increased in livers from the obese mice [6]. As an initial step in determining whether VWA8 protein might be involved in dysregulation of metabolism in obesity, this study was undertaken to characterize the tissue distribution, cellular location, and potential function of VWA8.

* Corresponding author. Division of Endocrinology, Department of Medicine, University of Arizona College of Medicine, 1501 N Campbell Ave Tucson, AZ, 85724, United States.
E-mail address: mandarino@deptofmed.arizona.edu (L.J. Mandarino).

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2. Materials and methods

2.1. Animals, diets, and adenovirally driven expression of VWA8 in mouse liver

All procedures were approved by the Mayo Clinic Institutional Animal Care and Use Committee (Protocol No. A38109). Male C57BL/6J mice were purchased from Harlan Teklad (Houston, TX) and housed under controlled temperature (23 °C) and lighting (10 h light:14 h dark) conditions with free access to water and food. Details of diets and mice are as previously described [6]. To produce diet-induced obesity and fatty livers, 6 week C57BL/6J mice were fed an irradiated rodent diet (10% fat diet D12450B2018S; or 60% fat diet D12492, *at libitum*) for 10 weeks. VWA8 also was expressed ectopically in mouse liver *in vivo* for further functional analysis. For these studies, 10 week C57BL/6J mice were infected via retro orbital injection with empty vector (Ad-Vec) or an adenoviral vector construct encoding the short isoform of VWA8 (Ad-VWA8b). Three days after injection, the mice were sacrificed and livers were harvested and homogenized [6] for further analysis.

2.2. Real time PCR for VWA8 mRNA expression

Quantitative real time PCR analyses for VWA8a (long) and VWA8b (short) isoform mRNAs were performed as described [7]. Total mRNA was isolated from livers of low fat diet and high fat diet mice (RNeasy Mini Kit, cat. no. 74104, QIAGEN) and then converted into cDNA. For VWA8a, the primers were 5′-GGCTGACCAAGGGATTATCA-3′ (Forward) and 5′-TCTGGCAGAGGAAACTCCTT-3’ (Reverse). For VWA8b, the primers were 5′-GGCTGACCAAGGGATTATCA-3′ (Forward) and 5′-GTTCTACCAATAGTGCGTTTCCTTAC-3′ (Reverse).

2.3. VWA8 cloning and expression constructs

Total RNA was isolated from the liver of a 10 week old C57BL6/J mouse (RNeasy Mini Kit, cat. no. 74104, QIAGEN). The RT–PCR approach was utilized to convert mRNA into single-stranded cDNA (LongRange 2Step RT-PCR Kit, cat. no. 205922, QIAGEN). Total cDNA was used to amplify specific cDNA with specific primers by PCR and then the amplified cDNA was inserted into a vector. For cloning of mouse VWA8 long isoform cDNA, the sense primer was 5′ GATCGCCTCCTGCCCGGTCCAGGGACTG-3′ and antisense primer 5′-GCTGGACATGGCACAGGTCGGCTTGGTCG-3′. For cloning of mouse VWA8 short isoform cDNA, the sense primer was 5′-GATCGCCTCCTGCCCGGTCCAGGGACTG-3′ and antisense primer 5′-GGACAATGCTCAAGGTTCTACCAATAGTG-3′. The sequencing results revealed that the cloned mouse VWA8a cDNA exactly matched NM_027906.1 and VWA8b matched NM_173758.3.

Mouse VWA8a and VWA8b cDNA were subcloned into pCMV6-AC-HA plasmid (Origene #PS100004) to generate pCMV-mVWA8a-HA and pCMV-mVWA8b-HA. A deletion of the first 34 amino acids in N-terminus of long isoform from its wild type pCMV-mVWA8a-HA by PCR with primers 5′- GCTAGCGATCGCCATGGGCGGGGACCAGCAGCGG -3′ and 5′- GCTAGCGGCCGCGTGATACTGGACAGCATGGTGG-3′ generated a construct pCMV-mVWA8a (35-1905)-HA. A deletion of GGKGCGKT (Walker A motif) from the wild type pCMV-mVWA8b-HA by PCR with primers 5′-GTTCTTAGCGATGACAATTAAGCATATGTC-3′ and 5′- GACATATGCTTAATTGTCATCGCTAAGAAC-3′ generated a construct pCMV-mVWA8b-(ΔGKT)-HA. A deletion of LVLLDG (Walker B motif) from the wild type pCMV-mVWA8b-HA by PCR with primer 5′-GTTGACGCGGTGGATTTTGCCTTCCCGGGC-3′ and 5′-GCCCGGGAAGGCAAAATCCACCGCGTCAAC-3′ generated a construct pCMV-mVWA8b-(ΔLVL)-HA. All of the constructs and mutant were verified by DNA sequencing.

2.4. Generation of adenovirus and baculovirus vectors

Ad-mVWA8a with an HA tag, Ad-mVWA8b with an HA tag and an adenovirus empty vector Ad-Vec (as a control), were created by following a protocol described previously [8]. The baculovirus Bac-mVWAb with a His tag was produced using the Bac-to-Bac Baculovirus Expression System (Invitrogen #10359-016). Mouse VWA8b cDNA with a His tag was subcloned into pFastBac 1 vector and the construct was transformed into DH10Bac competent cells (containing Bacmid and Helper) to obtain recombinant Bac-mVWA8b. The recombinant Bacmid DNA Bac-mVWA8b was used to transfect Sf21 insect cells to express the protein. The expressed protein mouse VWA8b with a His tag was purified using Ni-NTA Agarose (Invitrogen #R901-15) and eluted.

2.5. Cell culture transfection and immunoprecipitation

AML12 cells were purchased from ATCC and cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Hela cells were purchased from ATCC and grown in EMEM (ATCC #30-2003), also supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Transfection and immunoprecipitation were performed as described previously [8].

2.6. Immunofluorescence staining

AML 12 cells were cultured on glass cover slips in 6-well dishes. Cells were live-stained with 200 nM MitoTracker™ Red (Invitrogen #M7512) for 30 min before the fixation with 3% paraformaldehyde in PBS for 20 min. Cells then were permeabilized with 0.5% triton X-100 in PBS for 5 min, quenched with 100 mM glycine in PBS for 20 min, and blocked with 1% BSA in PBS for 1 h. Cells were then exposed to primary antibodies for 2 h at room temperature. Following three washes with PBS, the cells were treated for 1 h with Alexa Fluor secondary antibodies (Invitrogen #A21202) diluted 1:1000. Samples were mounted on glass slides with Vectashield mounting medium and examined under an inverted confocal microscope.

2.7. ATPase activity assay and Western blotting

ATPase activity was measured using an ATPase Assay Kit (Fisher #60-101-20) according to the manufacturer's instructions. Expressed VWA8b with an HA tag from mouse liver or Hela cells was immunoprecipitated by anti-HA antibody and Protein A beads. Immunoprecipitates were added to SB Mix (containing ATP) to incubate at 37 °C for 15 min. After 15 min, beads were spun down and supernatant was transferred to a new tube. The reaction was stopped and colored by adding Gold Mix. Absorbance was measured at 600 nm. Proteins bound to beads were eluted by heating at 95 °C for 4 min in SDS sample loading buffer. Eluted proteins were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and detected by Western blotting with primary antibody followed by horseradish peroxidase-conjugated secondary antibodies. This ATPase assay also was used for baculovirus-expressed mVWA8b solution eluted from Ni-NTA.

2.8. Bioinformatics and homology modeling

VWA8 amino acid sequences were analyzed for the presence of a mitochondrial targeting sequence using MitoProt [9]. For homology modeling, VWA8a and VWA8b primary amino acid sequences were analyzed using the Phyre2 server [10].