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Original article

Identification of novel mitochondrial localization signals in human Tafazzin, the cause of the inherited cardiomyopathic disorder Barth syndrome

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A B S T R A C T

Mutations in the gene *tafazzin* (TAZ) result in Barth syndrome (BTHS). Patients present with hypotonia, cyclic neutropenia, 3-methyglutaconic aciduria, and cardiomyopathy, which is the major cause of mortality. The recessive, X-linked TAZ gene encodes a mitochondrial membrane-associated phospholipid modifying enzyme, which adds unsaturated fatty acid species to monolysocardiolipin to generate mature cardiolipin in the mitochondrial membrane that is essential for mitochondrial morphology and function. To identify intrinsic mitochondrial localization sequences in the human TAZ protein, we made sequential TAZ peptide-eGFP fusion protein expression constructs and analyzed the localization of eGFP fluorescence by confocal microscopy. We assessed these fusion proteins for mitochondrial localization through cotransfection of H9c2 cells with plasmids encoding organellar markers linked to TdTomato. We have identified two peptides of TAZ that are independently responsible for mitochondrial localization. Using CRISPR-generated TAZ knock out cell lines, we found that these peptides are able to direct proteins to mitochondria in the absence of endogenous TAZ. These peptides are not located within the predicted enzymatic clefts of TAZ, implying that some BTHS disease causing mutations may affect mitochondrial localization without affecting transacylase activity. These novel peptides improve our understanding of TAZ intracellular trafficking, provide insight into the molecular basis of BTHS and provide molecular reagents for developing targeted mitochondrial therapies.

1. Introduction

Barth syndrome is an X-linked recessive disorder that presents clinically with 3-methylglutaconic aciduria, neutropenia, hypotonia and cardiomyopathy. Causative genetic mutations have been mapped to the *tafazzin* (TAZ) gene, which encodes a phospholipid transacylase that regulates the maturation of cardiolipin (CL) [1,2]. The TAZ gene lies on the short arm of chromosome X and is encoded by 11 exons. Four splice variants have been identified and validated: full length (TAZ FL), a variant lacking exon 5 (TAZ Δ5), one lacking exon 7 (TAZ Δ7) and one variant lacking both exon 5 and exon 7 (TAZ Δ5Δ7). Studies show that although all four variants localize to mitochondria, only the former two have transacylase activity, with TAZ Δ5 being predominant. TAZ FL is present only in primates, whereas TAZ Δ5 is highly conserved [3,4].

While the function of the non-enzymatically active isoforms of TAZ has yet to be determined, TAZ FL and TAZ Δ5 catalyze the final maturation step of the ubiquitous mitochondrial phospholipid, CL [5,6]. TAZ is localized to mitochondrial membranes in mammals, where it acts by exchanging acyl chains between CL and other phospholipids, the final result being a CL molecule with predominantly unsaturated fatty acyl chains [7]. It has been postulated that TAZ activity is promiscuous and the observed specificity of its action is conferred by the restraints of the membrane space where it is most active [8].

CL is a unique phospholipid that is exclusively synthesized and functions in mitochondrial membranes [7,9]. It consists of a diphosphatidylglycerol molecule that binds four acyl chains. TAZ remodels CL by exchanging its initially saturated acyl chains for unsaturated acyl chains, making the molecule more thermodynamically favorable for membranes with a high degree of curvature [8]. Tetralinoleoyl CL (L4CL) is the most abundant mature species in mammalian muscle. In BTHS patient tissues, mature CL species are decreased and the immature monolysocardiolipin (MLCL), a CL molecule with only three acyl chains, accumulates [10], CL interacts with protein complexes involved in the formation and maintenance of mitochondrial inner membrane cristae, as well as with respiratory supercomplexes in the inner mitochondrial membrane (IMM) [11,12]. Failure to properly remodel CL ultimately results in defects of mitochondrial morphology and function, both of which have been implicated in cardiovascular disease

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by numerous studies [13,14].

Critically, as CL is only synthesized in mitochondria, correct targeting of TAZ must occur in order for CL to undergo its final step of remodeling. Previous work in yeast has identified a 28 amino acid peptide in the C-terminus of the TAZ is necessary for mitochondrial targeting. However, the exact mitochondrial targeting sequence for human TAZ is yet unknown [15]. Since heart failure is known to be associated with various types of mitochondrial dysfunction [16], identification of mitochondrial targeting reagents will facilitate development of mitochondrially-targeted therapies for heart failure.

In this study, we investigated the internal sequences that are responsible for targeting TAZ to the mitochondria. Using various regions of TAZ fused with eGFP, we found two distinct fragments that were capable of localizing to mitochondria independently. TAZ (84–95) confers exclusive targeting to mitochondria of eGFP, while TAZ (185–220) results in partial targeting to mitochondria along with other subcellular compartments.

2. Materials and methods

2.1. DNA constructs

The tdTomato open reading frame (ORF) was isolated by PCR from pCAG-ChromsonR-tdTomato (a gift from Edward Boyden, Addgene plasmid #59169, [17]) and cloned into peGFP-N1 to replace the eGFP ORF. To construct expression plasmids encoding organelle-specific fluorescent markers that localize to lysosomes, peroxisomes, mitochondrial inner and outer membranes, the ORFs of LAMP2b (NP_054701.1), PXMP2 (NP_061133.1), TIMM23 (NP_006318.1), TOMM20 (NP_055580.1), and TAZ (isoform 1, NP_000107.1 and isoform 2, NP_851828.1), respectively, were isolated from 293 T cell cDNA by PCR with corresponding primers. The ORFs of organelle-specific markers were cloned in-frame at the N-terminus of tdTomato at *Bgl* II and *Sal* I sites, driven by the CMV early promoter. The ORF of wild type TAZ was cloned into peGFP-N1 (Clontech, Mountain View, CA) at *Bgl* II and *Sal* I sites to generate the TAZ full length phTAZv1-eGFP and the TAZ Δ5 isoform 2 phTAZ-eGFP.

For constructing the N-terminal deletion series of human TAZ-eGFP fusion proteins, we selected endogenous codons encoding methionines as initiation codons to create in-frame fusions with eGFP at *Bgl* II and *Sal* I sites. The C-terminal TAZ deletion series were generated by PCR using hTAZ-eGFP as template and a reverse primer complementary to TAZ to create TAZ C-terminal mutations; the forward primer encoded a linker peptide (Gly-Gly-Gly) between the TAZ fragment and eGFP. The PCR products were isolated, digested with *Sal* I and ligated for transformation into STBL3 bacteria. For generating the single point mutations of hTAZ-eGFP, recombinant DNAs were generated by PCR of plasmid phTAZ-eGFP with the mutated nucleotides in primers according to the BTHS human TAZ gene variants database (Barth Syndrome Foundation, available at http://www.barthsyndrome.org/english/view.asp?x=1(Accessed July 15, 2017), [4]).

To generate a plasmid for clustered, regularly interspaced, short palindromic repeat RNA-guided (CRISPR) targeting of TAZ in female rat cardiomyoblast H9c2 cells, two sets of primers for gRNA expression were used to target TAZ exon 3: 5′-TTTCAGGATCCCTACGAAAA-3′ and 5′-CTGAAGTTGATGCGTTGGTG-3′ and were both cloned into pSQT1313 for the gRNA multiplex expression.

Plasmid constructs were confirmed by sequencing (Eurofins, Louisville, KY).

2.2. Cell culture and cell transfection

Rat cardiomyoblast H9c2 cells were cultured in 10% FBS/DMEM on a cover slide. For fluorescence colocalization experiments, H9c2 cells were seeded at 25000 cells/well on a 12 mm diameter coverslide in a 24-well plate and allowed to attach overnight. Equal amounts of plasmid DNAs, encoding mutated- or full length hTAZ- eGFP and organelle-specific tdTomato, were used to transfect cells with Lipofectamine 3000 (ThermoFisher Scientific, Waltham, MA) according to the manufacturer's instructions. Cells were harvested and fixed with 4% paraformaldehyde/PBS and mounted with DAPI-Vectashield (Vector Laboratories, Burlingame, CA) or DAPI Fluoromount-G (Southern Biotech, Birmingham, AL).

2.3. Tafazzin CRISPR knockout cell lines

To generate female rat cardiomyoblast H9c2 Tafazzin (NM_001025748) knockout lines, we used a dimeric CRISPR RNA-guided *Fok*I nuclease approach for genome editing [18]. This strategy reduces the potential for off-target effects by fusing the dimerization-dependent FokI nuclease to a catalytically inactive Cas9 (dCas9) to induce double stranded DNA breaks. This approach requires two separate guide RNAs on opposing strands of DNA. The H9c2 cells were plated in a 24-well plate at a density of 25,000 cells/well and transfected with 250 ng of the plasmid pSQT-rTAZ-exon3 and 750 ng of pSQT1601-*Fok*I-dCAS9 to create indels, and co-transfected with 80 ng pIRES-hrGFP-Neo or with 120 ng pLKO-scramble for cell selection, using lipofectamine 3000 (ThermoFisher Scientific, Waltham, MA). Single colonies resistant to 500 μg/mL G418 for 7-days or 1 μg/mL puromycin for 2-days were screened by PCR with primers flanking rTAZ exon 3 in introns 2 and 3 (Table 1) which generated a 120 bp PCR product in wild type cells. To verify the TAZ alleles, the PCR products were resolved in 8% polyacrylamide (19:1 acrylamide:bisacrylaminde) in 0.5 × TBE buffer. The PCR products from each colony were cloned into pJET1.2 (ThermoFisher Scientific, Waltham, MA) and verified by sequencing (Eurofins, Louisville, KY). The candidate clones were further tested for the 10 most probable predicted off-targets (http://crispr.mit.edu) by PCR with 10 sets of primers (Table 1).

2.4. Confocal microscopy

The confocal images were taken with a 60 × oil lens under a Nikon A1R confocal mounted on a Nikon TiE inverted microscope. The images are shown with maximal intensity projection of z-planes.

2.5. Western blotting

H9c2 cells were cultured to confluency in 15 cm plates. Cells were then collected by scraping and centrifuged at 600*g* for 5 min and the pellet resuspended in mitochondria isolation buffer (250 mM sucrose, 10 mM Tris pH 7.4, 0.1 mM EDTA). Cells were homogenized in a glass dounce homogenizer until 90% of cells showed trypan blue staining (70 strokes). Homogenates were then spun at 600 g for 10 min to pellet nuclei and cell debris and then at 7600 g to pellet the crude mitochondrial fraction. The pellet was re-suspended in radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 1% Triton X-100, 0.25% SDS) with protease inhibitor cocktail (Sigma P8340) added. Protein concentration was measured by Bradford assay and 20 micrograms of protein from each sample were resolved by SDS-PAGE (10%). Proteins were transferred onto nitrocellulose membranes, which were then probed with antibodies against succinate dehydrogenase (SDHA, MitoSciences) and TAZ [19], generously shared by S. M. Claypool. The SDHA antibody was used at a dilution of 1:10,000 and the TAZ antibody was used at a dilution of 1:1000. The membrane was then blotted with secondary antibodies horse anti-mouse and goat anti-rabbit IgG HRP-conjugates (Cell Signaling Technologies, both at 1:5000 dilution). The membranes were incubated with enhanced chemiluminescence agents (Pierce) and analyzed by a ChemiDoc Image System (Bio−Rad).

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