A mass spectrometric approach to investigate cardiolipin metabolism in Barth syndrome
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CHAPTER 8

Only One Splice Variant of the Human TAZ Gene Encodes a Functional Protein with a Role in Cardiolipin Metabolism.

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ABSTRACT Barth syndrome (BTHS) is an X-linked recessive disorder caused by mutations in the TAZ gene and is characterized by cardiomyopathy, short stature, neutropenia, and 3-methylglutaconic aciduria. Recently it was found that BTHS patients exhibit a profound cardiolipin deficiency although the biosynthetic capacity to synthesize this lipid from its precursor phosphatidylglycerol is entirely normal. Like BTHS patients, a Saccharomyces cerevisiae strain, in which the yeast orthologue of the human TAZ gene has been disrupted, exhibits an abnormal cardiolipin profile as determined by tandem mass spectrometry.

Additionally, this yeast strain grows poorly on non-fermentable carbon sources. We have used both properties of this yeast disruptant as a read-out system to test the physiological functionality of each of 12 different splice variants that have been reported for the human TAZ gene. Our results demonstrate that only the splice variant lacking exon 5 was able to complement the retarded growth of the yeast disruptant on selective plates and restore the cardiolipin profile to the wild type pattern. We conclude that this splice variant most likely represents the only physiologically important mRNA, at least with regard to cardiolipin metabolism.

INTRODUCTION Barth syndrome (BTHS1; MIM 302060 [OMIM]) is an X-linked recessive disorder, which clinically is characterized by cardiomyopathy, skeletal myopathy, growth retardation, and neutropenia. Additional laboratory findings include intermittent lactic acidemia, low blood cholesterol, and increased urinary excretion of 3-methylglutaconic acid, 3-methylglutaric acid, and 2-ethylhydracrylic acid (1). Moreover, mitochondria of BTHS patients have an abnormal ultrastructure, and several different respiratory chain defects in muscle and fibroblasts have been reported (1). The disease is often fatal in childhood because of cardiac failure or sepsis. The clinical expression of the disease, however, is quite variable in severity and may show profound intrafamilial variability (1–3).
The TAZ gene (previously known as G4.5), which is mutated in this disorder, is located on Xq28 (4) and is postulated to contain 11 exons based on the alignment of cDNA sequences with genomic data (5). At the transcriptional level, six different mRNAs have been identified containing different combinations of exons 5–7 because of differential splicing. Two variants containing either all three exons or lacking exon 5 are consistently more abundant in most tissues examined (5). Alternative splicing also has been reported to produce two different 5'-ends of the transcripts leading to two possible translation initiation sites (1st ATG at position 1–3 and 2nd ATG at position 277–279 of the full-length open reading frame). As a consequence, there are at least 12 possible mRNAs, which are present in different amounts in different tissues (5). Whether these splice variants all give rise to functional proteins awaited the identification of the physiological function of the encoded tafazzins, which has not been established so far.

In 1997, Neuwald (6) hypothesized that tafazzins share homology with a family of acyltransferases that are involved in phospholipid metabolism. In line with this hypothesis, our group found abnormal levels of cardiolipin and phosphatidylglycerol in BTHS cells, whereas all other phospholipid classes are normal (7). Cardiolipin is an acidic polyglycerophospholipid, which is almost exclusively found in the inner mitochondrial membrane and is synthesized from phosphatidylglycerol (8).

The structure of cardiolipin is shown in Figure 1. After synthesis of cardiolipin, its fatty acid side chains are remodeled by a deacylation-reacylation cycle to obtain different cardiolipin species, which contain mainly mono-unsaturated and di-unsaturated chains of 16–18 carbons in length (8). Cardiolipin is required for optimal functioning of the respiratory chain complexes and several other mitochondrial inner membrane proteins, including the ATP/ADP transporter and the carnitine acylcarnitine translocase (9–12). Investigations in cultured skin fibroblasts of BTHS patients showed that the rate of biosynthesis of cardiolipin from phosphatidylglycerol is normal but that the cardiolipin pool size is considerably reduced as compared with control cells. Furthermore, the incorporation of linoleic acid, which is the characteristic acyl side chain found in mammalian cardiolipin, into both phosphatidylglycerol and cardiolipin was significantly reduced (7).

These results suggest that the TAZ gene encodes one or more acyl-transferases involved in the remodeling of cardiolipin or its precursors. Direct evidence that the tafazzins are involved in cardiolipin metabolism was unavailable until now.
EXPERIMENTAL PROCEDURES

DNA MANIPULATIONS

The 12 TAZ splice variants were amplified by PCR from primary human fibroblast cDNA using the following primers: two KpnI-tagged forward primers 5'-GGT ACC ATG CCT CTG CAC GTG AAG T-3' and 5'-GGT ACC ATG GAC GAC CCT CAT CTC TG-3' for the first (position 1-3) and the second (position 277-279) translation initiation site, respectively, and a Sall-tagged reverse primer 5'-GTC GAC CTA TCT CCC AGG CTG GAG GTG-3'. As described previously (5), five PCR products were observed on agarose gel, which subsequently were subcloned in the pGEM-T vector (Promega).

Inserts were sequenced to exclude sequence errors introduced by Taq polymerase, and the different splice variants were cloned downstream of the yeast PGK promoter into the KpnI and Sall sites of the yeast expression vector pYPGK18. This vector is derived from the pHVXII vector (13) after replacing the original multiple cloning site by that of pUC18.

Yeast tafazzin (YTAZ) was amplified from W303 genomic DNA using a KpnI-tagged forward primer 5'-GGT ACC ATG TCT TTT AGG GAT GTC CTA G-3' and a Sall-tagged reverse primer 5'-GTC GAC TCA ATC ATC TCT ACC TCT TGG-3' and cloned into the KpnI and Sall sites of pYPGK18. The insert was sequenced to exclude sequence errors.

GENERATION OF THE YPR140W DISRUPTANT

To construct the Δtaz deletion mutant, the entire YPR140w open reading frame was replaced by the kanMX4 marker gene (14). The PCR-derived construct for disruption consisted of the kanMX4 gene flanked by short regions of homology corresponding to the YPR140w 3' and 5' non-coding regions. pKan was used as template with the YPR140w primers (5'-ATG TCT TTT AGG GAT GTC CTA GAA AGA GGA GAT GAA TTT TTA GAA GCC TAG CGT ACG CTG CAG GTC GAC-3' and 5'-TCA ATC ATC TCT ACC CTT TGG TTT ACC CTC TGG AGG CAG AAA CTT TTG ATC GAT GAA TTC GAG CTC G-3').

The resulting PCR fragments were introduced into Saccharomyces cerevisiae wild type W303 strain (15). G418-resistant clones were selected by growth on YPD plates containing G418 (200 mg/liter) (14).

EXPRESSION OF TAZ SPLICE VARIANTS AND COMPLEMENTATION ANALYSIS

The different pYPGK18 constructs were transformed to Δtaz using the lithium acetate procedure (16). Transformed yeast cells were grown on minimal glucose medium (0.67% yeast nitrogen base, 2% glucose) at 30 °C and harvested in mid-exponential phase. For the complementation analysis, transformants were streaked on rich ethanol plates (2% peptone, 1% yeast extract, 2% agar, and 2% ethanol) and incubated at 37 °C for 1 week.

For cardiolipin and immunoblot analysis, spheroplasts were prepared using zymolyase
Onl vy  On e  Splic e  Varian t o f  th e  Huma n  TAZ  Gen e  Encode s  a  Functional Protein with a Role in Cardiolipin Metabolism

Figure 2: Cardiolipin profiles of yeast strains and selected transformants.

A, wild type and taz. Cardiolipins are present in the region from m/z 620 to m/z 740 as indicated. The cardiolipin profile of taz is abnormal; the tetra-unsaturated cardiolipins species are deficient. Note m/z 699.5, corresponding to (C16:1)(C18:1)3-CL, which is present in wild type but virtually absent in taz (indicated by an arrow). Monolysocardiolipins (MLCLs) accumulate in taz in the region from m/z 550 to m/z 600 but are almost absent in wild type.

B, taz expressing YTAZ, HTAZ-1stATG-full, HTAZ-1stATG-ex5, and HTAZ-1stATG-ex7. Expression of YTAZ and HTAZ-1stATG-ex5 fully restores the cardiolipin profile. Again, note m/z 699.5 (indicated by an arrow).

Expression of HTAZ-1stATG-full partially restores the cardiolipin profile; however, the monolysocardiolipins are still present at elevated levels. HTAZ-1stATG-ex7, which displays a taz cardiolipin profile, is representative of all other tested splice variants.
according to Franzusoff et al. (17) and lysed in a 10 mM sodium phosphate buffer, pH 7.4, containing 140 mM NaCl and protease inhibitor mixture (Roche Applied Science). After sonication, protein determination was performed by the method of Bradford (18).

Table 1: Composition of the different splice variants and used abbreviations

<table>
<thead>
<tr>
<th>Splice variant</th>
<th>Abbreviation</th>
<th>First initiation site</th>
<th>Second initiation site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full length</td>
<td>HTAZ-1stATG-full</td>
<td></td>
<td>HTAZ-2ndATG-full</td>
</tr>
<tr>
<td>Lacking exon 5</td>
<td>HTAZ-1stATG-ex5</td>
<td></td>
<td>HTAZ-2ndATG-ex5</td>
</tr>
<tr>
<td>Lacking exon 7</td>
<td>HTAZ-1stATG-ex7</td>
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<td>HTAZ-2ndATG-ex7</td>
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<td>HTAZ-1stATG-ex5,7</td>
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<td></td>
<td>HTAZ-2ndATG-ex5,6,7</td>
</tr>
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CARDIOLIPIN ANALYSIS BY HIGH PRESSURE LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

Lipids were extracted using a modification of the method of Folch et al. (19). Briefly, 0.4 nmol of CL(C14:0)₄ (Avanti Polar Lipids, Alabaster, AL) was added to the equivalent of 1 mg of protein in a total volume of 1 ml of distilled water, and this aqueous phase was extracted twice with 3 ml of chloroform/methanol (2:1 v/v) followed by centrifugation at 1000 x g. The combined organic phases were evaporated under a nitrogen stream, and the residue was taken up in 100 μl of chloroform/methanol/water (50:45:5 v/v/v) containing 2.5 ml/liter NH₄OH. Five microliters of this lipid extract was injected into a straight-phase high pressure liquid chromatography system, and cardiolipin analysis was performed by on-line electrospray tandem mass spectrometry as described previously (20). By using bovine heart monolysocardiolipin (consisting mostly of trilinoleylmonolysocardiolipin, Avanti Polar Lipids, Alabaster, AL), we determined that with this method monolysocardiolipins elute about 0.2 min later than the cardiolipins. For the acquisition of the cardiolipin spectra of both cardiolipins and monolysocardiolipins, a continuous negative scan was made from 400 to 1000 m/z in a time window of 2.5 min, during which both types of compounds elute from the column.

In a separate analysis, using the same extract, single ion monitoring was used to quantify selected monolysocardiolipins and cardiolipins. The area of each (monolyso)cardiolipin
peak \( A_{(ML)CL} \) and that of the added internal standard \( A_{IS} \) was quantified using MassLynx 3.3 (Micromass, Manchester, UK). The amount of (monolysocardiolipins was calculated by the formula: \( A_{(ML)CL}/A_{IS} \times 0.4 \) nmol (the amount of internal standard added). These data were used to calculate the relative cardiolipin and monolysocardiolipin levels (Figure 4) in comparison to wild type levels.

**Figure 3:**
A, immunoblot analysis of cell homogenates of the different transformants (each corresponding to 10 \( \mu \)g of protein) using -human TAZ antibody.
B, complementation analysis of the various transformants. Transformants were streaked from selective plates to rich ethanol plates and incubated at 37\(^\circ\)C for 1 week. Wild type (WT) and taz transformed with HTAZ-1stATG-full, YTAZ, and pYPGK18 are present on each plate to facilitate the comparison with other transformants. Clearly, only YTAZ and HTAZ-1stATG-ex5 complement the growth phenotype.

**TAFAZZIN ANTIBODY GENERATION**

The portion of the TAZ cDNA corresponding to exons 8–11 was amplified by PCR from human fibroblast cDNA using Taq polymerase and the following primers: a BamHI-tagged forward primer 5'-GGA TCC ATC GGG CGC CTG ATT GCT GAG-3' and an HindIII-tagged reverse primer 5'-AAG CTT CTA TCT CCC AGG CTG GAG GTG-3'. The PCR product was cloned downstream of the isopropyl-1-thio- -D-galactopyranoside-inducible PTAC promoter into the BamHI and HindIII sites of the bacterial expression vector pMAL-C2X, to express the last 98 amino acids of tafazzin fused to maltose-binding protein. This protein sequence is present in all of the protein products of the putative splice variants of the TAZ gene. The insert was sequenced to exclude sequence errors introduced by PCR, after which the
construct was transformed to the Escherichia coli strain BL21. Protein expression, purification, and antibody generation was performed as described previously (21). For affinity purification of the crude antiserum, the same tafazzin fragment was cloned downstream of the isopropyl-1-thio-D-galactopyranoside-inducible PTAC promoter into the BamHI and HindIII sites of the bacterial expression vector pGEX-4T in order to express it as a fusion to glutathione S-transferase.

This protein was expressed, purified, coupled to CNBr-activated Sepharose (Amersham Biosciences), and used for affinity purification of the-TAZ antibodies as described previously (22). This resulting antibody solution was diluted 1:100 for immunoblotting, which was performed according to Vaz et al. (21).

Figure 4:
Comparison of the cardiolipin and monolysocardiolipin levels in selected transformants. Values are shown as ratios to wild type (WT) levels (e.g. wild type levels are set to 1).

A. tetraunsaturated cardiolipins: m/z 685.5 = (16:1)₄-CL, m/z 699.5 = (16:1)₃(18:1)-CL, m/z 713.5 = (16:1)₂(18:1)₂-CL, and m/z 727.5 = (18:1)₄-CL.

B. monolysocardiolipins. The two most abundant ions were selected: m/z 568.5 representing (16:0)(16:1)(18:1)-MLCL and m/z 582.5, which corresponds to (16:0)(18:1)₂-MLCL.

**RESULTS AND DISCUSSION**

To investigate the functionality of the different human splice variants of the TAZ gene, we have generated a strain of S. cerevisiae in which the yeast orthologue of the TAZ gene, YPR140w, has been disrupted. This yeast deletion strain is unable to grow on non-fermentable carbon sources, such as ethanol, at 37°C. As in BTHS patients (7, 20), taz displays an abnormal cardiolipin profile. Figure 2A shows the cardiolipin profile of wild type W303 strain and that of the disruption mutant taz. In each cardiolipin cluster, the peak with the lowest m/z represents tetra-unsaturated cardiolipin. For example, the ion with m/z 727.5 represents a cardiolipin containing four oleic acid
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(C18:1) acyl side chains. To interpret the cardiolipin spectra shown in Figure 2, one should keep in mind that cardiolipins are measured as doubly charged ions, which implies that the mass over charge ratio (m/z) corresponds to half of the actual cardiolipin mass and that the distance between the molecular ion peak and its first isotope peak is 0.5 Da. Therefore, the next cardiolipin within the 727.5 cluster has an m/z of 728.5. This cardiolipin has one unsaturation less and represents tri-unsaturated cardiolipin with three oleic acid (C18:1) acyl side chain and one stearic acid (C18:0) acyl side chain.

The next cluster, which contains an ion with m/z 713.5, consists of cardiolipins with one C16 and three C18 fatty acyl side chains, where m/z 713.5 represents cardiolipin containing one palmitoleic acid (C16:1) and three oleic acid (C18:1) acyl side chains. Figure 2A shows that, as observed for BTHS, tetra-unsaturated cardiolipin species are either completely absent (m/z 727.5) or present in decreased levels (m/z 685.5, 699.5, and 713.5) indicating that this yeast disruptant is a good model for BTHS. In addition, there is accumulation of monolysocardiolipins (m/z 567.5/568.5 and 581.5/582.5), which thus far has not been observed in BTHS.

Because, with regard to the cardiolipin profile, the yeast phenotype is similar to that observed in BTHS patients, we used this yeast system to investigate the functionality of the different TAZ mRNA splice variants by complementation analysis. Table I gives a description of the various splice variants that were tested and the abbreviations used for them. Yeast tafazzin (YTAZ) was used as a positive control for the complementation analysis. All constructs were transformed to taz, including pYPGK18 without insert, which was used as a negative control. Immunoblot analysis using an affinity-purified polyclonal antibody that we raised against the invariant C-terminal part of human tafazzin confirmed that the splice variants were correctly expressed (Figure 3A).

**Figure 5:**
ClustalW alignment of part of human tafazzin containing exon 5 and tafazzin orthologues of Mus musculus (mouse, MmTAZ), Rattus norvegicus (rat, RnTAZ), Xenopus laevis (African clawed frog, XITAZ), Caenorhabditis elegans (nematode, CeTAZ), Anopheles gambiae (mosquito, AgTAZ), Drosophila melanogaster (fruit fly, DmTAZ), Arabidopsis thaliana (thale cress, AtTAZ), and S. cerevisiae (baker's yeast, YTAZ).
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First, it was determined whether the transformants had regained the ability to grow on ethanol plates at 37 °C. Figure 3B shows that only the expression of YTAZ and HTAZ-1stATG-ex5 was able to complement the growth phenotype, whereas transformants expressing the other splice variants remained retarded in growth similar to taz. Second, cardiolipin analysis was performed in cell homogenates, and this showed that all transformants had the same cardiolipin profile as Ataz, except for those expressing YTAZ and HTAZ-1stATG-ex5 and HTAZ-1stATG-full, which show either a complete or partial normalization of the cardiolipin profile (Figure 2). By virtue of the added internal standard, the amount of the most abundant monolysocardiolipins (m/z 568.5 and 582.5) and that of the tetra-unsaturated cardiolipins containing C16 and C18 acyl side chains (m/z 685.5, 699.5, 713.5, and 727.5) was determined and related to the levels found in the wild type strain (Figure 4). The quantitative results presented in this figure confirm that the growth-phenotype correction also corresponds to a complete restoration of the cardiolipin profile for YTAZ and HTAZ-1stATG-ex5.

Interestingly, in contrast to these two latter strains, the transformant expressing HTAZ-1stATG-full displays a partial restoration of the cardiolipin profile, i.e. cardiolipin species containing a majority of C18 fatty acids (cardiolipin clusters m/z 727.5 and m/z 713.5) are still deficient, whereas cardiolipin species containing mostly C16 fatty acids (m/z 699.5 and m/z 685.5) display a pattern similar to that of wild type (Figure 4A). This partial restoration of the cardiolipin profile is also reflected by the monolysocardiolipin levels, which in the transformants expressing YTAZ or HTAZ-1stATG-ex5 are comparable with wild type but in the transformant expressing HTAZ-1stATG-full are still elevated (Figure 4B). Taken together, our results suggest that only HTAZ-1stATG-ex5 and HTAZ-1stATG-full are functional splice variants, which are directly involved in cardiolipin metabolism. There are several reasons, however, to question whether HTAZ-1stATG-full really represents a physiologically relevant mRNA. Figure 5 shows a ClustalW alignment of amino acids residues 94–172 of HTAZ-1stATG-full with tafazzin orthologues of very distinct organisms, including mammals, flies, yeast, and plant. This sequence region includes amino acids residues 123–153 encoded by
Onl y O n e S p l i c e V a r i a n t o f t h e H u m a n T A Z G e n e E n c o d e s 
 a F u n c t i o n a l P r o t e i n w i t h a R o l e i n C a r d i o l i p i n M e t a b o l i s m

exon 5. Note that none of the orthologous sequences have any sequence homology to this 
region, whereas in the sequences flanking the amino acids encoded by exon 5 a high 
homology with all orthologues is observed (Figure 5). This is also true at the genomic DNA 
level, as concluded from translated blast analysis (tBlastn) using the full-length human 
tafazzin as query in the mouse and rat genome data base, which identifies all exons, except 
exon 5 (not shown).

This lack of homology with both related and distant organisms in conjunction with 
the inability of HTAZ-1stATG-full to restore the growth phenotype on selective conditions 
and the partial restoration of the cardiolipin profile strongly suggests that exon 5, the existence 
of which was postulated on the basis of its presence in some human mRNA splice variants, 
is a result of aberrant splicing and in fact does not lead to a functionally relevant protein. 
This is also supported by the observation that of the 42 different mutations that currently 
have been identified in the TAZ gene, no mutation is located in exon 5 (Figure 6) (2, 3, 5, 
23-28). The results from the complementation analysis also show that none of the tafazzins 
produced from the second translation initiation site were able to complement the phenotype, 
not even HTAZ-2ndATG-ex5. This is in agreement with the observation that patients with 
mutations in exons 1 and 2, i.e. before the second translation initiation site which is sup-
posed to be used for the production of the shorter tafazzins, also suffer from BTHS and do 
not display a milder phenotype when compared with patients with mutations in exons 3–10 
(3, 5, 24, 25).

In conclusion, by using a heterologous functional complementation assay, we have 
demonstrated that of all the different TAZ mRNAs splice variants reported previously, only 
the variant lacking the postulated exon 5 sequence results in a physiologically functional 
enzyme involved in cardiolipin metabolism. These results have important implications for 
the interpretation of mutational data of patients. Moreover, they pave the way for specific 
studies aimed at determining the exact physiological role of the encoded tafazzin in cardio-
lipin biosynthesis.

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