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 readout 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.

Barth syndrome (BTHS, MIM 302060) 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 Fig. 1. After synthesis of cardiolipin, its fatty acid side chains are remodeled by a decacylation-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 acyltransferases 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'-GTT ACC ATG CCT

Received for publication, June 5, 2003, and in revised form, August 19, 2003
Published, JBC Papers in Press, August 20, 2003, DOI 10.1074/jbc.M305956200

Frédéric M. Vaz‡§, Rickelt H. Houtkooper‡, Fredoen Valianpour‡, Peter G. Barth¶, and Ronald J. A. Wanders‡

From the ‡Departments of Clinical Chemistry and Pediatrics, ¶Emma Children’s Hospital, Academic Medical Center, University of Amsterdam, P. O. Box 22700, 1100 DE Amsterdam, The Netherlands

© 2003 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in U.S.A.
Functionality of TAZ Splice Variants

The different pYPGK18 constructs were transformed to Δta2 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 GCT CTA GAA AGA GGA GAT GAA TTT TTA GAA GCC TAC ATC CCT TTT ACC CTC TGG AGG CAG AAA CTT TGG ATC GAT GAA GGA TGG CAG GCT G-3′) and Δta2 primer reverse 5′-GTC GAG AAG T-3′ for the first (position 1–3) and the second (position 277–279) translation initiation site, respectively, and a SalI-tagged reverse primer 5′-GTC GAC CTA TCT CCC AGG CTT 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 Kan1 and Sal1 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′-GTC ACC ATG AGC GAC CCT CAT CTC TG-3′ and a SalI-tagged reverse primer 5′-GTC GAC CTA TCT CCC AGG CTT 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 Kan1 and Sal1 sites of the yeast expression vector pYPGK18. The insertion was sequenced to exclude sequence errors.

Generation of the YPR140w Disruptant—To construct the Δta2 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 GCT CTA GAA AGA GGA GAT GAA TTT TTA GAA GCC TAC ATC CCT TTT ACC CTC TGG AGG CAG AAA CTT TGG ATC GAT GAA GGA TGG CAG GCT G-3′) and Δta2 primer reverse 5′-GTC GAG AAG T-3′ for the first (position 1–3) and the second (position 277–279) translation initiation site, respectively, and a SalI-tagged reverse primer 5′-GTC GAC CTA TCT CCC AGG CTT 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 Kan1 and Sal1 sites of the yeast expression vector pYPGK18. The insertion was sequenced to exclude sequence errors.

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), Δta2 displays an abnormal cardiolipin profile. Fig. 2A shows the cardiolipin profile of wild type W303 strain and that of the disruption mutant Δta2. In each cardiolipin cluster, the peak with the lowest mz represents tetra-unsaturated cardiolipin. For example, the ion with mz 727.5 represents a cardiolipin containing four oleic acid (C18:1) acyl side chains. To interpret the cardiolipin spectra shown in Fig. 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 mz 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 mz 713.5, consists of cardiolipins with one C16 and three C18 fatty acyl side chains, where mz 713.5 represents cardiolipin containing one palmitoleic acid (C16:1) and three oleic acid (C18:1) acyl side chains. Fig. 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/
FIG. 2. Cardiolipin profiles of yeast strains and selected transformants. A, wild type and Δtaż. Cardiolipins are present in the region from \(m/z\) 620 to \(m/z\) 740 as indicated. The cardiolipin profile of Δtaż is abnormal; the tetra-unsaturated cardiolipins species are deficient. Note \(m/z\) 699.5, corresponding to CL(C16:1)2(C18:1), which is present in wild type but virtually absent in Δtaż (indicated by an arrow). Monolysocardiolipins (MLCLs) accumulate in Δtaż in the region from \(m/z\) 550 to \(m/z\) 600 but are almost absent in wild type. B, Δtaż 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 Δtaż cardiolipin profile, is representative of all other tested splice variants.
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 Δtaz, 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 (Fig. 3A).

First, it was determined whether the transformants had regained the ability to grow on ethanol plates at 37 °C. Fig. 3B shows that only the expression of YTAZ and HTAZ-1stATG-ex5 complemented the growth phenotype.

| Splice variant | Abbreviation First initiation site | Abbreviation Second initiation site |
|---------------|-----------------------------------|-------------------------------------|
| Full length | HTAZ-1stATG-full | HTAZ-2ndATG-full |
| Lacking exon 5 | HTAZ-1stATG-ex5 | HTAZ-2ndATG-ex5 |
| Lacking exon 7 | HTAZ-1stATG-ex7 | HTAZ-2ndATG-ex7 |
| Lacking exons 5 and 7 | HTAZ-1stATG-ex5,7 | HTAZ-2ndATG-ex5,7 |
| Lacking exons 6 and 7 | HTAZ-1stATG-ex6,7 | HTAZ-2ndATG-ex6,7 |
| Lacking exons 5–7 | HTAZ-1stATG-ex5,6,7 | HTAZ-2ndATG-ex5,6,7 |

**Fig. 3.** A, immunoblot analysis of cell homogenates of the different transformants (each corresponding to 10 μg of protein) using α-human TAZ antibody. B, complementation analysis of the various transformants. Transformants were streaked from selective plates to rich ethanol plates and incubated at 37 °C for 1 week. Wild type (WT) and Δtaz 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.
whereas transformants expressing the other splice variants remained retarded in growth similar to Δtaz. Second, cardiolipin analysis was performed in cell homogenates, and this showed that all transformants had the same cardiolipin profile as Δtaz, 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 (Fig. 2). By virtue of the added internal standard, the amount of the most abundant monolysocardiolipins (m/z 585.5 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 (Fig. 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 (Fig. 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 (Fig. 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. Fig. 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 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 (Fig. 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 (Fig. 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 supposed 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

**Protein sequence encoded by exon 5**

| HTAZ  | 94 | RRPAAARLQKNSLGGTMSLGGSTTVDSAEFPQAENWEGKVVYDTGRMNPAGKXERSK | 172 |
|--------|----|--------------------------------------------------------------|----|
| MtTAZ  | 94 | RRPAAARLQKNSLGGTMSLGGSTTVDSAEFPQAENWEGKVVYDTGRMNPAGKXERSK | 142 |
| RnTAZ  | 94 | RRPAAARLQKNSLGGTMSLGGSTTVDSAEFPQAENWEGKVVYDTGRMNPAGKXERSK | 142 |
| XIrTAZ | 94 | RRPAAARLQKNSLGGTMSLGGSTTVDSAEFPQAENWEGKVVYDTGRMNPAGKXERSK | 142 |
| CeTAZ  | 94 | RRPAAARLQKNSLGGTMSLGGSTTVDSAEFPQAENWEGKVVYDTGRMNPAGKXERSK | 142 |
| AgTAZ  | 159 | RRPAAARLQKNSLGGTMSLGGSTTVDSAEFPQAENWEGKVVYDTGRMNPAGKXERSK | 208 |
| DmTAZ  | 96 | RRPAAARLQKNSLGGTMSLGGSTTVDSAEFPQAENWEGKVVYDTGRMNPAGKXERSK | 144 |
| AtTAZ  | 92 | RRPAAARLQKNSLGGTMSLGGSTTVDSAEFPQAENWEGKVVYDTGRMNPAGKXERSK | 140 |
| YTAZ  | 102 | RRPAAARLQKNSLGGTMSLGGSTTVDSAEFPQAENWEGKVVYDTGRMNPAGKXERSK | 151 |

**Fig. 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, *XlTAZ*), *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*).
exact physiological role of the encoded tafazzin in cardiolipin biosynthesis.

Acknowledgments—We thank Dr. G. Hakkaart and Dr. L. Nijtmans for the generation of the yeast deletion strain and Dr. H. R. Waterham and S. Ferdinandusse for critical comments on the manuscript.

REFERENCES
1. Barth, P. G., Wanders, R. J., Vreken, P., Janssen, E. A., Lam, J., and Baas, F. (1999) J. Inherited Metab. Dis. 22, 555–567
2. Orstavik, K. H., Orstavik, R. E., Naumova, A. K., D’Adamo, P., Gedeon, A., Bolhuis, P. A., Barth, P. G., and Toniolo, D. (1998) Am. J. Hum. Genet. 63, 1457–1462
3. Johnston, J., Kelley, R. I., Feigenbaum, A., Cox, G. F., Iyer, G. S., Funanage, V. L., and Proujansky, R. (1997) Am. J. Hum. Genet. 61, 1053–1058
4. Bolhuis, P. A., Henseleit, G. W., Hulsebos, T. J., Baas, F., and Barth, P. G. (1991) Am. J. Hum. Genet. 48, 481–485
5. Bione, S., D’Adamo, P., Maestrini, E., Gedeon, A. K., Bolhuis, P. A., and Toniolo, D. (1996) Nat. Genet. 12, 385–389
6. Neuwald, A. F. (1997) Curr. Biol. 7, R465–R466
7. Vreken, P., Valianpour, F., Nijtmans, L. G., Grivell, L. A., Plecko, B., Wanders, R. J., and Barth, P. G. (2000) Biochem. Biophys. Res. Commun. 279, 378–382
8. Hatch, G. M. (1998) Int. J. Mol. Med. 1, 33–41
9. Koshkin, V., and Greenberg, M. L. (2000) Biochem. J. 347, 687–691
10. Kadenbach, B., Mende, P., Kolbe, H. V., Stigan, I., and Palmieri, F. (1982) FEBS Lett. 139, 109–112
11. Robinson, N. C. (1993) J. Bioenerg. Biomembr. 25, 153–163
12. Noel, H., and Pande, R. V. (1986) Eur. J. Biochem. 155, 99–102
13. Swiegers, J. H., Vaz, F. M., Pretorius, I. S., Wanders, R. J., and Bauer, F. F. (2002) FEMS Microbiol. Lett. 210, 19–23
14. Wach, A. (1996) Yeast 12, 259–265
15. Mashkevich, G., Repetto, B., Gelernt, D. M., Jin, C., and Tzagoloff, A. (1997) J. Biol. Chem. 272, 14356–14364
16. Becker, D. M., and Guzante, L. (1991) in Guide to Yeast Genetics and Molecular Biology (Guthrie, C., and Fink, G. R., eds) pp. 182–187, Academic Press, Inc., San Diego
17. Franuzoff, A., Rothblatt, J., and Schekman, R. (1991) in Guide to Yeast Genetics and Molecular Biology (Guthrie, C., and Fink, G. R., eds) pp. 662–674, Academic Press, Inc., San Diego
18. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
19. Felch, J., Lees, M., and Stanley, G. M. (1957) J. Biol. Chem. 236, 497–509
20. Valianpour, F., Wanders, R. J., Barth, P. G., Overmars, H., and van Gennip, A. H. (2002) Clin. Chem. 48, 1390–1397
21. Vaz, F. M., Orman, R., Westings, K., Back, J. W., and Wanders, R. J. (2001) J. Biol. Chem. 276, 33512–33517
22. Hogenboom, S., Romeijn, G. J., Houten, S. M., Baas, M., Wanders, R. J., and Waterham, H. R. (2002) J. Lipid Res. 43, 90–98
23. Chen, R., Tsuji, T., Ichida, F., Bowles, K. R., Yu, X., Watanabe, S., Hirono, K., Tsubata, S., Hamamichi, Y., Ohta, J., Imai, Y., Bowles, N. E., Miyawaki, T., and Tsubin, J. A. (2002) Mol. Genet. Metab. 77, 319–325
24. Cantlay, A. M., Shokrallah, K., Allen, J. T., Lunt, P. W., Newbury-Ecob, R. A., and Steward, C. G. (1999) J. Pediatr. 135, 311–315
25. D’Adamo, P., Fassone, L., Gedeon, A., Janssen, E. A., Bione, S., Bolhuis, P. A., Barth, P. G., Wilson, M., Hasen, E., Orstavik, K. H., Patton, M. A., Green, A. J., Zamparchi, E., Donati, M. A., and Toniolo, D. (1997) Am. J. Hum. Genet. 61, 862–867
26. Ichida, F., Tsubata, S., Bowles, K. R., Haneda, N., Uese, K., Miyawaki, T., Dreyer, W. J., Messina, J., Li, H., Bowles, N. E., and Tsubin, J. A. (2001) Circulation 103, 1256–1263
27. Vesel, S., Stopar-Oberza, M., Trebusak-Podkrajsek, K., Jazbec, J., Podnar, T., and Battelino, T. (2003) Eur. J. Hum. Genet. 11, 97–101
28. Sakamoto, O., Ohura, T., Katsushima, Y., Fujisawa, I., Ogawa, E., Miyabayashi, S., and Inuma, K. (2001) Hum. Genet. 109, 559–563