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Mitochondrial Respiratory Chain Supercomplexes Are Destabilized in Barth Syndrome Patients

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Mutations in the human *TAZ* gene are associated with Barth Syndrome, an often fatal X-linked disorder that presents with cardiomyopathy and neutropenia. The *TAZ* gene encodes Tafazzin, a putative phospholipid acyltranferase that is involved in the remodeling of cardiolipin, a phospholipid unique to the inner mitochondrial membrane. It has been shown that the disruption of the Tafazzin gene in yeast (*Taz1*) affects the assembly and stability of respiratory chain Complex IV and its supercomplex forms. However, the implications of these results for Barth Syndrome are restricted due to the additional presence of Complex I in humans that forms a supercomplex with Complexes III and IV. Here, we investigated the effects of Tafazzin, and hence cardiolipin deficiency in lymphoblasts from patients with Barth Syndrome, using blue-native polyacrylamide gel electrophoresis. Digitonin extraction revealed a more labile Complex I/III_2/IV supercomplex in mitochondria from Barth Syndrome cells, with Complex IV dissociating more readily from the supercomplex. The interaction between Complexes I and III was also less stable, with decreased levels of the Complex I/III_2 supercomplex. Reduction of Complex I holoenzyme levels was observed also in the Barth Syndrome patients, with a corresponding decrease in steady-state subunit levels. We propose that the loss of mature cardiolipin species in Barth Syndrome results in unstable respiratory chain supercomplexes, thereby affecting Complex I biogenesis, respiratory activities and subsequent pathology.

Keywords: mitochondria; supercomplex; membrane protein; cardiolipin; blue native PAGE

Introduction

Barth Syndrome (BTHS; MIM 302060) is an X-linked recessive disease that presents with cardioskeletal myopathy, neutropenia and 3-methylglutaconic aciduria.[1] Dilated cardiomyopathy commonly occurs within the first year, with mortality high in infants or in early childhood.[2] The disease was mapped to the *TAZ* gene at position Xq28, with mutations in the gene identified soon after.[3,4] The *TAZ* gene product, Tafazzin, is a putative phospholipid acyltranferase that is conserved in many species from yeast to *Caenorhabditis elegans*.[5] Patients with Barth Syndrome exhibit defects in cardiolipin, an anionic phospholipid consisting of two phosphatidyl groups linked together by glycerol, which is found almost exclusively in the inner mitochondrial membrane. The incorporation of the characteristic acyl side-chain linoleic acid is affected, resulting in reduced levels of mature tetralinoleoyl-cardiolipin and the accumulation of a monolyso species.[6,7] Reduction of mature cardiolipin in Barth Syndrome patients has been correlated with a variety of reported defects, including mitochondrial morphological abnormalities,[8] respiratory chain dysfunction, including reduced Complex III (ubiquinol-ferricytochrome *c* oxidoreductase, EC 1.10.2.2) and Complex IV (cytochrome *c* oxidoreductase, EC 1.9.3.1) activities,[2,9] increased mitochondrial proliferation[10] and decreased mitochondrial membrane potential.[10]

Abbreviations used: BN-PAGE, blue native polyacrylamide gel electrophoresis; DDM, *n*-dodecyl-β-D-maltoside; TX-100, Triton X-100; TIM, translocase of the inner membrane; TOM, translocase of the outer membrane.

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It has been reported that cardiolipin is important for promoting and/or stabilizing higher-ordered forms of respiratory chain complexes called supercomplexes (or respirasomes).[11–14] In mammals, the supercomplexes contain Complexes I (NADH-ubiquinone oxidoreductase, EC 1.6.5.3), III, and IV in different combinations. These supercomplexes have been proposed to provide catalytic enhancement by reducing diffusion distances of substrates and/or substrate channeling.[15] Their other major function is to increase the stability and aid in the assembly of the respiratory chain complexes I, III and IV.[16] In some patients with mitochondrial respiratory chain dysfunction, the lack of fully assembled Complex III can result in an unstable Complex I, highlighting the importance for these complexes to exist in a supercomplex structure for stability.[16,17]

Studies of *Saccharomyces cerevisiae* have revealed decreased stability of Complex III/IV supercomplexes in cardiolipin-deficient *crd1*Δ mutants.[13] More recently, the stability and assembly of Complex IV was found to be reduced in yeast cells lacking Taz1, the orthologue of human Tafazzin.[18] While instructive, yeast models of mitochondrial respiratory disease have limitations due to the lack of a true Complex I and the fact that human Complex I forms supercomplexes with Complex III and Complex IV. Here, we examine the steady-state levels of the respiratory chain complexes and the supercomplexes in cells from Barth Syndrome patients. The cardiolipin defects present in Barth Syndrome patients result in destabilization of the supercomplex by weakening the interactions between Complexes I, III and IV, and by reducing the levels of Complex I. We propose that supercomplex instability is an important factor in the mitochondrial defects and subsequent disease observed in Barth Syndrome.

Results

It has been reported that cardiolipin is required for stabilization, but not formation, of respiratory supercomplexes in yeast cells.[13] More recently, the investigation of Complex IV in yeast *taz1*Δ mutants revealed a decreased stability of the III_2/IV_2 supercomplex.[18] We investigated whether a similar defect could be seen in cells from Barth Syndrome patients. We used primary lymphoblasts from three patients (P1–P3) with known *TAZ* gene mutations expected to result in no production of Tafazzin protein. P1 and P2 are siblings with the same premature stop codon mutation (c.655C>T), while P3 has a splice site mutation (c.527-1G>A). Control lymphoblasts were also used. Analysis of mitochondria from patient lymphoblasts using HPLC/mass spectrometry revealed that the cardiolipin levels were decreased by sevenfold and the amount of monolysocardiolipin was 115 times higher compared to control lymphoblasts.[36] For blue native polyacrylamide gel electrophoresis (BN-PAGE) analysis, cells were solubilized using the mild detergent digitonin, which maintains respiratory supercomplexes intact. Following electrophoresis, the gel was transferred and immunodecorated with antibodies specific for Complex I and Complex II (as loading control). As can be seen, the ~1.7 MDa $CI/CIII_2/CIV$ supercomplex was observed in all cell lines but at reduced levels (approximately 75–84%) in the three Barth patients (Figure 1(a), lanes 2–4) compared to controls (Figure 1(a), lanes 1 and 5). This coincided with the appearance of the smaller ~1.5 MDa $CI/CIII_2$ supercomplex,[19] which was approximately twice as abundant in the Barth Syndrome patients (Figure 1(b)).

The results presented in Figure 1 suggest that the cardiolipin defects present in these Barth Syndrome

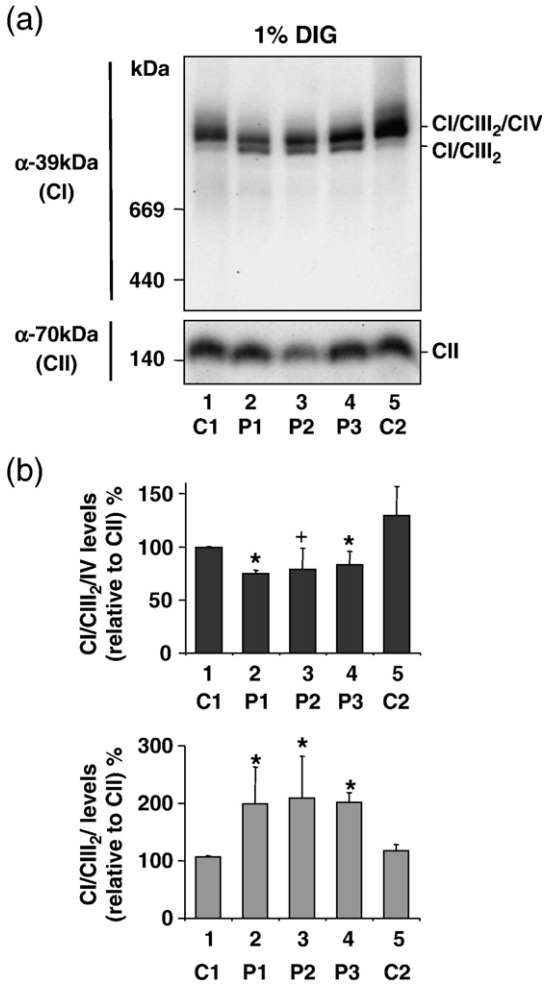

Figure 1. $CI/CIII_2/CIV$ supercomplex levels are reduced in Barth patient lymphoblasts. (a) Control (C1 and C2, lanes 1 and 5) or Barth Syndrome patient lymphoblasts (P1-P3, lanes 2–4) (120 μg total protein) were solubilized in 1% (w/v) digitonin (DIG), separated by BN-PAGE, transferred to PVDF membrane and immunodecorated using anti-Complex I (α-39 kDa) and Complex II (α-70 kDa) subunit antibodies. $CI/CIII_2/CIV$ = Complex I, III, IV supercomplex, $CI/CIII_2$ = Complex I, III supercomplex, CII = monomeric Complex II. (b) Quantification of the $CI/CIII_2/CIV$ supercomplex and the $CI/CIII_2$ supercomplex relative to Complex II. Data are mean(±SD), n = three separate blots, $^*p<0.05$, $+p=0.07$.

patients cause the destabilization of the supercomplex due to dissociation of Complex IV. To test this hypothesis, Complex IV and its supercomplexes were analyzed using BN-PAGE and Western blot analysis using anti-subunit I Complex IV antibodies. The levels of Complex II were also assessed as loading control (Figure 2(a)). In control cells, the majority of Complex IV is found in its monomeric form, with the remainder spread between the CI/$CIII_2$/CIV and $CIII_2$/CIV supercomplexes as well as an unknown species (labeled *) at approximately 500 kDa (Figure 2(a), lanes 1 and 5). In contrast, all supercomplex forms of Complex IV are virtually absent from the cell extracts of Barth Syndrome patients (Figure 2(a), lanes 2–4). Monomeric Complex IV is present at similar levels in both the control and patient extracts, indicating that it remains stable when not part of a supercomplex. Of note, Complex IV appeared to migrate slightly faster in the Barth Syndrome patients compared to the controls. To confirm that the faster migration was not an artefact of the gel electrophoresis, control and Barth Syndrome patient extracts were applied to alternating lanes on the blue-native gel (Figure 2(b)). The migration of Complex IV in the Barth Syndrome patient mitochondria is clearly faster than Complex IV in the control samples (Figure 2(b), compare patient lanes 1, 3, 5 with control lanes 2 and 4). The increased mobility correlates with the absence or reduction of cardiolipin bound to Complex IV in patients with Barth Syndrome.

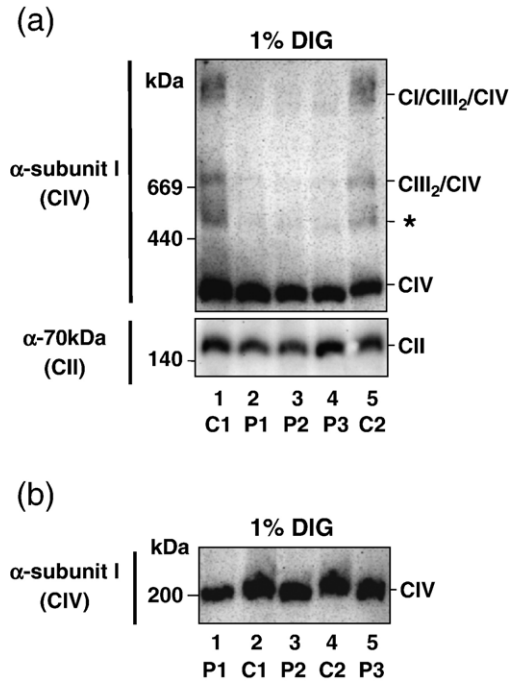

Figure 2. Complex IV is stripped from the supercomplex in Barth Syndrome patients. Control (C1 and C2) or Barth Syndrome patient lymphoblast (P1–P3) cells ((a), 120 μg total protein) or isolated mitochondria ((b), 50 μg total protein) were solubilized in 1% (w/v) digitonin (DIG), separated by BN-PAGE, transferred to PVDF membrane and immunodecorated using anti-Complex IV (α-subunit I) and Complex II (α-70 kDa) subunit antibodies. CI/$CIII_2$/CIV = Complex I, III, IV supercomplex, $CIII_2$/CIV = Complex III/IV dimer, CIV = monomeric Complex IV, CII = monomeric Complex II. The asterisk (*) indicates an unidentified supercomplex containing Complex IV.

We next investigated whether other respiratory complexes besides Complex IV are affected in Barth Syndrome lymphoblasts. Cells were solubilized in the non-ionic detergent *n*-dodecyl-β-D-maltoside (DDM) and separated by BN-PAGE followed by Western blot analysis (Figure 3(a) and (b)). The different properties of DDM in comparison to digitonin result in the liberation of Complex IV from its supercomplex forms and the resultant appearance of the smaller CI/$CIII_2$ supercomplex, as well as monomeric CI (Figure 3(a), left panel) and the $CIII_2$ homodimer (Figure 3(b), left panel). In Barth Syndrome lymphoblasts, the CI/$CIII_2$ supercomplex is markedly reduced in comparison to controls, suggesting a decreased stability of this complex. Indeed, in Barth patients the levels of the CI/$CIII_2$ supercomplex (standardized to CII) ranged from 30–45% of controls (Figure 3(a), right panel). Also evident in the Barth Syndrome patients is a decrease in monomeric CI (Figure 3(a), left panel, lanes 2–4). Conversely, the $CIII_2$ homodimer appears to be more abundant than in controls after DDM solubilization (Figure 3(b), left panel lanes 2–4). In fact, the $CIII_2$ homodimer is 2.5–2.8 times more abundant in patients than controls (Figure 3(b), right panel). In contrast to the decreased levels of III_2/IV supercomplex observed with the Complex IV antibody (Figure 2), the $CIII_2$/IV supercomplex does not appear to be affected after DDM solubilization and immunodecoration for Complex III. This may be a result of differing detergents employed that affect complex solubility and/or the different antibodies used to detect the complexes.

Factors contributing to the detection of native complexes on BN-PAGE are the ratio of solubilizing detergent to total protein and the total membrane content within the sample.[20] To examine the stability of the supercomplexes and the effects of reduced cardiolipin in Barth Syndrome patient cells in more detail, a titration of different detergent to protein ratios was performed using DDM. In control cells, Complex I is found mainly in its I/III_2 supercomplex form until a detergent to protein ratio of 2.25 g/g was used (Figure 3(c), lane 4). In contrast, monomeric Complex I is seen mainly in Barth Syndrome cells (from patient 2) across the detergent range. The I/III_2 supercomplex is barely detectable at 1.25 g/g (Figure 3(c), lane 6) and totally absent at 1.75 g/g and 2.25 g/g (Figure 3(c), lanes 7 and 8). The CI/$CIII_2$ supercomplex is less stable in Barth Syndrome extracts at a wide range of DDM detergent to protein ratios, with the liberation of more monomeric Complex I, resulting in a higher Complex I to CI/$CIII_2$ supercomplex ratio than in controls.

It was observed that the CI/$CIII_2$ supercomplex is destabilized in Barth Syndrome patients, and that levels of monomeric Complex I appear to be reduced (Figure 3(a), lanes 2–4). To investigate this

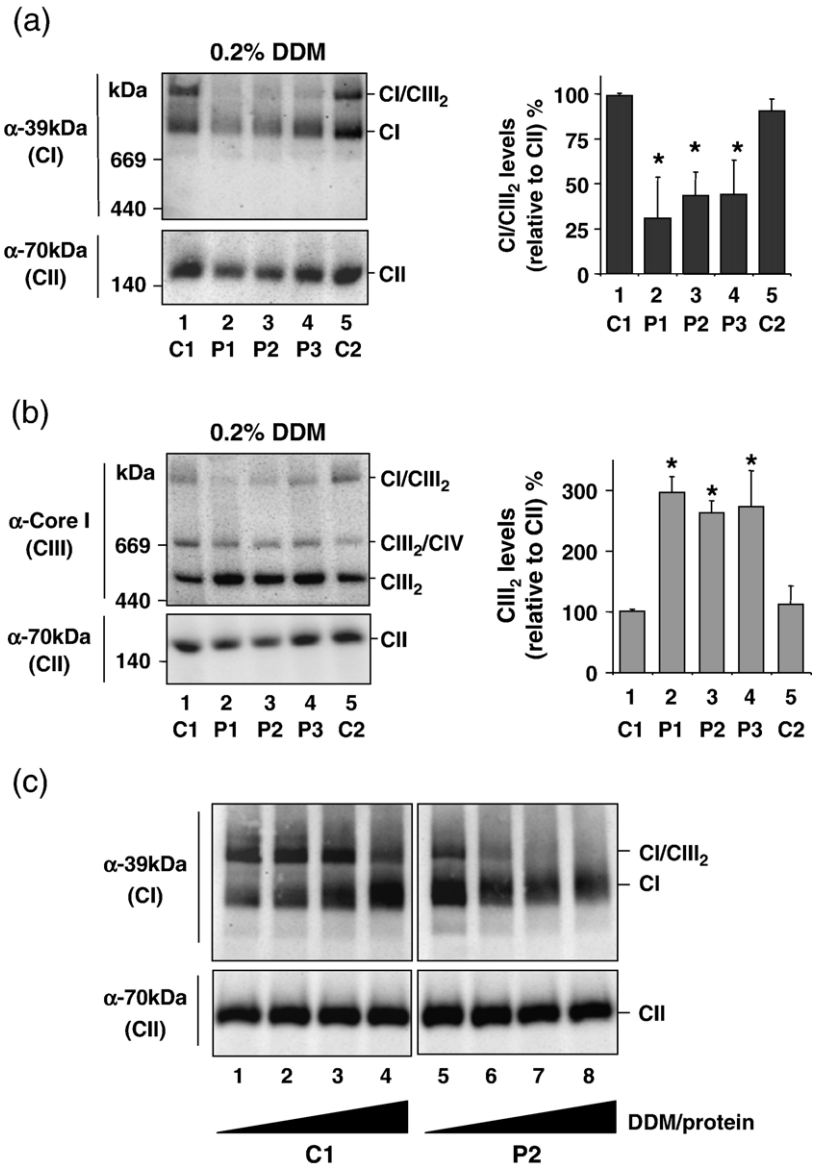

Figure 3. $CI/CIII_2$ supercomplex stability is reduced in Barth patient lymphoblasts. Control or Barth Syndrome patient lymphoblasts (120 μg total protein) were solubilized in 0.2% (w/v) dodecyl-β-D-maltoside (DDM), separated by BN-PAGE, transferred to PVDF membrane and immunodecorated for (a) Complex I (anti-39 kDa subunit) or (b) Complex III (anti-Core I subunit). Complex II was used as a control (anti-70 kDa subunit). $CI/CIII_2$ = Complex I, III supercomplex, CI = monomeric Complex I CII = monomeric Complex II, $CIII_2/CIV$ = Complex III/IV dimer, $CIII_2$ = Complex III homodimer, CII = monomeric Complex II. Quantification of $CI/CIII_2$ supercomplex ((a) right panel) and Complex III homodimer ($CIII_2$) ((b) right panel) is relative to Complex II. Data are mean(±SD), (a) n = four separate blots, (b) n = three separate blots. *$p < 0.05$. (c) Control (C1, lanes 1–4) or Barth Syndrome patient (P2, lanes 5–8) cells (100 μg of total protein/lane) were solubilized in DDM at a detergent to protein ratio of 0.75 g/g (lanes 1 and 5), 1.25 g/g (lanes 2 and 6), 1.75 g/g (lanes 3 and 7) and 2.25 g/g (lanes 4 and 8), separated by BN-PAGE, transferred to PVDF membrane and immunodecorated using anti-Complex I (α-39 kDa) or Complex II (α-70 kDa) subunit antibodies.

further, control and patient lymphoblasts were solubilized in the non-ionic detergent Triton X-100 (TX-100), the strongest of the detergents used here. This detergent totally solubilizes the supercomplexes, thereby liberating Complex I in its monomeric form such that more direct comparisons of its levels can be made. Triton X-100 solubilization revealed that the Barth Syndrome patients have decreased levels of Complex I compared to controls (Figure 4(a)). Quantification relative to Complex II revealed a reduction in Complex I with levels 63–75% to that of controls. A possible explanation for the reduced levels of Complex I is that the cardiolipin defects present may reduce detergent solubility. However analysis of the TX-100 insoluble pellet showed no difference between control and Barth Syndrome patient samples with virtually all Complex I solubilized in all samples (data not shown).

To establish if the reduction of Complex I was associated with reduced steady-state levels of individual subunits, SDS-PAGE and Western blot analysis were performed. The levels of the 30 kDa and 17 kDa Complex I subunits were reduced in the Barth Syndrome patients by around 35–50% when compared to the control (Figure 4(b)). This reduction is similar to the reduced levels of mature Complex I holoenzyme observed in Figure 4(a). In contrast, the levels of the Complex III core I subunit were similar in both control and Barth Syndrome patient samples (Figure 4(b)).

Cardiolipin defects in yeast have been shown to affect other complexes in the inner mitochondrial membrane apart from those in the respiratory chain, in particular the ADP/ATP carrier.[18] To determine whether the cardiolipin defects in Barth Syndrome have a global destabilizing effect on all complexes in the inner mitochondrial membrane, a non-respiratory chain complex, the TIM23 complex (translocase of the inner membrane), was examined (Figure 4(c)). The levels of the TIM23 complex, standardized to the TOM (translocase of the outer membrane) complex, were not significantly different in the Barth Syndrome patients (P1, P2, and P3) compared

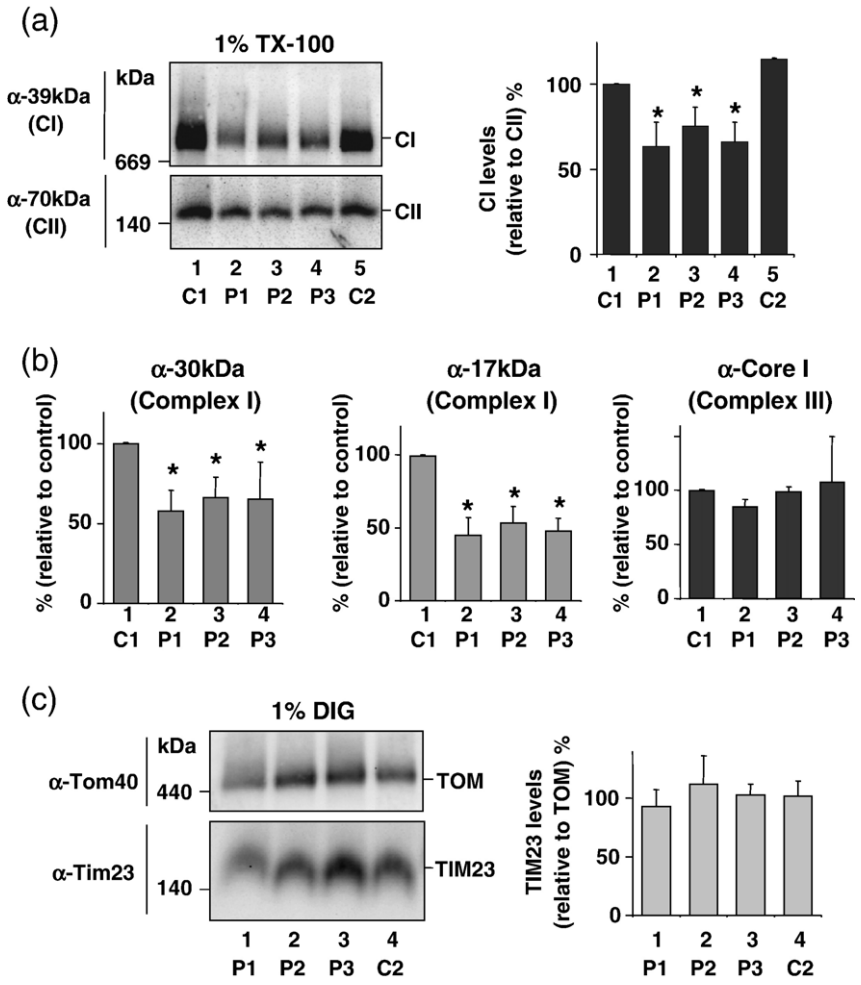

Figure 4. Complex I levels are reduced in lymphoblasts from Barth Syndrome patients. (a) Control (C1 and C2, lanes 1 and 5) or Barth Syndrome patient lymphoblasts (P1–P3, lanes 2–4) (120 μg total protein) were solubilized in 1% (w/v) Triton X-100 (TX-100), separated by BN-PAGE, transferred to PVDF membrane and immunodecorated using anti-Complex I (α-39 kDa) and Complex II (α-70 kDa) subunit antibodies. CI = monomeric Complex I, CII = monomeric Complex II. Quantification of monomeric Complex I levels relative to Complex II are shown (right panel). Data are mean(±SD), n = three separate blots, $^*p < 0.05$. (b) Proteins from control (C1) or Barth Syndrome patient (P1–P3) lymphoblast mitochondria (40 μg) were separated by SDS-PAGE and immunodecorated using anti-Complex I (α-30 kDa and α-17 kDa), Complex II (α-70 kDa) or Complex III (α-Core I) subunit antibodies before quantification. Subunit levels were standardized to the 70 kDa Complex II subunit. Data are mean(±SD), n = three separate blots, $^*p < 0.05$. (c) Control (C2) or Barth Syndrome patient lymphoblasts (P1-P3) (50 μg mitochondria) were solubilized in 1% (w/v) digitonin (DIG), separated by BN-PAGE, transferred to PVDF membrane and immunodecorated using anti-TOM Complex (α-Tom40) or anti-TIM23 Complex (α-Tim23) subunit antibodies (left panels). Quantification of TIM23 Complex levels was performed relative to the TOM Complex (right panels). Data are mean(±SD), n = three separate blots.

to the control. This suggests that the decreased levels of Complex I observed in Barth Syndrome patient lymphoblasts is not due to a general destabilization of all complexes in the inner membrane.

Discussion

Cardiolipin is a mitochondrial-specific phospholipid located in the inner membrane, where it aids in the proper functioning of protein complexes involved in energy generation and in the maintenance of mitochondrial shape.[2,8–10] A number of studies have shown that cardiolipin is an important factor in the function of respiratory complexes of the mitochondrial inner membrane,[21–24] while in yeast it has been found to be important in the formation and/or stability of the respiratory supercomplexes.[11–13,18] In particular, cardiolipin has been found to be required for the association between Complexes III and IV, and this is highlighted by the presence of cardiolipin in the crystal structures of both Complex III[25] and Complex IV.[26]

Defects in the putative phospholipid acyltransferase Tafazzin result in cardiolipin remodeling defects, with increased levels of the monolysocardiolipin precursor found in both yeast *taz1*Δ mutants[27] and in human Barth Syndrome.[28] While oxidation of cardiolipin has been suggested to free a pool of cytochrome *c* from the inner membrane to accelerate apoptosis,[29] the increase in monolysocardiolipin species in Barth Syndrome patient mitochondria has been shown not to increase susceptibility of cells to apoptosis.[28] Thus, it appears that the reduction in the levels of mature tetralinoleoyl-cardiolipin is the more important factor in disease pathogenesis. Although cardiolipin deficiency has been shown to disrupt Complex III/IV supercomplexes in yeast *taz1*Δ mutants,[18] the stability of the respiratory complexes and the different supercomplex forms have not been investigated before in Barth Syndrome patient cells.

We show here that cardiolipin defects in lymphoblasts from Barth Syndrome patients cause instability of the CI/$CIII_2$/CIV supercomplex, resulting in the liberation of Complex IV monomer.

Furthermore, we observed that monomeric Complex IV in Barth Syndrome patients migrates faster than normal Complex IV on BN-PAGE after solubilization by TX-100. Three to four cardiolipin molecules are thought to bind to bovine heart Complex IV, with two high-affinity binding sites near subunits VIIa, VIIc (and possibly VIII) and a low-affinity binding site near subunit VIa.[26] Depletion of cardiolipin from bovine heart Complex IV results in reduced enzymatic activity and decreased stability, along with the dissociation of subunits VIa (9.4 kDa) and VIb (10 kDa) from the complex.[24] The loss of cardiolipin, in conjunction with the possible loss of subunits VIa and VIb, would explain the faster migration of Complex IV on BN-PAGE observed here in Barth Syndrome patients. In yeast, cardiolipin is critical for the stability of Complex III/IV supercomplexes,[12] binding tightly at the putative interface comprised of the transmembrane helices of cytochromes *b* and c_1.[13] Similarly, we find human supercomplexes require cardiolipin, with the deficiency in Barth Syndrome patients resulting in a destabilized CI/$CIII_2$/CIV supercomplex.

Ten tightly bound cardiolipin molecules per native Complex I have been identified; however, their exact structural or functional role is undetermined.[30] Unlike for Complex IV, we did not observe a faster migration of Complex I in Barth Syndrome patient cells. Nevertheless, our data here suggest that cardiolipin is important for interactions between Complex I and the Complex III homodimer, with levels of the CI/$CIII_2$ supercomplex and monomeric Complex I reduced in Barth Syndrome patients. Reduction of Complex I and its associated supercomplexes has been observed in mitochondrial disease patients that harbor Complex III assembly defects, and hence reduced levels of the CI/$CIII_2$ supercomplex.[16,17] Our results suggest that the association between Complex III and Complex I within a supercomplex is important for Complex I biogenesis.

Cardiolipin has been shown to be important for Complex I (and Complex III) enzymatic function, with depletion resulting in reduced activity.[31] We could not detect any substantial difference in Complex I enzyme activity in lymphoblasts from Barth Syndrome patients here, which is not surprising, given the modest decrease of Complex I observed in Figure 4 (25–37%). However, the activities of Complexes I and IV were found to be reduced in skeletal muscle from patient P1 (data not shown), suggesting that respiratory complexes show variable tissue sensitivities to cardiolipin abnormalities. This may depend on the ratio of respiratory complexes to cardiolipin content in mitochondrial membranes, or in their rates of subunit synthesis or turnover. Alternatively, the tissue variation in enzyme activities may reflect variation in the levels and composition of cardiolipin species. Indeed, cardiolipin content in Barth Syndrome patients was found to be decreased to a greater extent in skeletal muscle than in lymphoblasts (fourfold compared with twofold).[32]

The lack of cardiolipin in Barth Syndrome patients may contribute to defects in respiratory chain function in a number of ways. Firstly, enzymatic function of Complexes I, III and IV is affected directly. Secondly, Complex IV stability is reduced, which in turn destabilizes the CI/$CIII_2$/CIV supercomplex, and thirdly, the destabilization of the supercomplex results in lower levels of Complex I, further reducing enzymatic activity. These factors may all contribute to the mitochondrial respiratory deficiency and subsequent clinical pathology associated with Barth Syndrome.

Materials and Methods

Cell lines and culture conditions

Lymphoblast cell lines were derived from Barth Syndrome patients and controls. Patients P1 and P2 are siblings, with a premature stop codon mutation in the *TAZ* gene (c.655C>T, R123X). Patient P1 presented at four months of age with failure to thrive (FTT), muscle weakness and dilated cardiomyopathy. Patient P2 had fetal hydrops, bilateral pleural effusions and an enlarged heart, and did not respond to resuscitation after delivery. Patient P3 has a *TAZ* splicing defect (c.527-1G>A) and presented at four months of age with short stature, neutropenia and dilated cardiomyopathy.

Cells were grown at 37 °C and 5% (v/v) CO_2 in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 5–10% (v/v) fetal bovine serum (FBS, Invitrogen) and 50 μg/ml of uridine.

Mitochondrial isolation

Cell pellets were resuspended in isolation solution (20 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (Hepes) (pH 7.6), 220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 0.5 mM PMSF, 2 mg/ml of bovine serum albumin (BSA)) and incubated on ice for 15 min. Cells were lysed using 30 passes of a drill-fitted pestle in a 1 ml glass homogeniser at 4 °C. Lysates were centrifuged at 750*g* to remove cell debris/nuclei, with the mitochondria subsequently pelleted from the supernatant at 10,000*g* before resuspension in the appropriate buffer for BN-PAGE.

SDS-PAGE

Tris-Tricine SDS-PAGE was performed as described.[33] Cell pellets (120 μg total protein) were prepared for electrophoresis by suspension in 50 mM Tris–HCl (pH 6.8), 2% (w/v) (SDS), 100 mM DTT, 10% (v/v) glycerol before separation on a 10–16% (w/v) polyacrylamide Tris-Tricine gradient gel at 100 V and 25 mA for 14 h.

Blue native polyacrylamide gel electrophoresis (BN-PAGE)

BN-PAGE was performed essentially as described,[34] but with minor modifications. Cell pellets (120 μg of protein) or mitochondria (50 μg of protein) were solubilized for 30 min on ice in 50 μl of 20 mM Bis-Tris (pH 7.4), 50 mM NaCl, 10% (v/v) glycerol containing either 1% (w/v)

digitonin (Merck), 1% (w/v) TX-100 (Sigma) or 0.2% (w/v) DDM (Sigma). For whole cell solubilization, detergent to protein ratios were typically 4 g/g for digitonin, 0.8 g/g for DDM and 4 g/g for TX-100. Insoluble material was removed by centrifugation at 18,000*g* for 5 min at 4 °C, with the soluble component combined with BN-PAGE loading dye (final concentrations: 0.5% (w/v) Coomassie brilliant blue G-250, 50 mM ε-amino n-caproic acid (Sigma), 10 mM Bis-Tris, pH 7.0) and separated on a 4%–13% polyacrylamide BN-PAGE gel made - in 70 mM ε-amino n-caproic acid, 50 mM Bis-Tris (pH 7.0). For separation, the cathode buffer (15 mM Bis-Tris (pH 7.0), 50 mM Tricine) containing 0.02% (w/v) Coomassie brilliant blue G-250 was used until the dye front had reached approximately one-third of the way through the gel before exchange with colorless cathode buffer. The anode buffer was 50 mM Bis-Tris (pH 7.0). Native complexes were separated at 100 V and 5 mA for 13.5 h at 4 °C.

Western transfer and immunodetection

Following electrophoresis, gels were soaked in 48 mM Tris–HCl, 39 mM glycine, 0.0037% (w/v) SDS, 20% (v/v) methanol for 20 min before semi-dry transfer to polyvinylidene fluoride (PVDF) membrane (Immobilon, Millipore) at 20 V and 400 mA for 1.5 h. Membranes were stained with 0.1% (w/v) Coomassie brilliant blue R-250, 50% (v/v) methanol, 7% (v/v) acetic acid before destaining first with 50% (v/v) methanol, 7% (v/v) acetic acid and then with 90% (v/v) methanol, 10% (v/v) acetic acid. For immunodetection, PVDF membranes were re-wetted briefly with 100% methanol then blocked with 10% (w/v) skim milk powder in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) containing 0.05% (v/v) Tween-20 for 2 h. Membranes were washed before incubating with primary mouse monoclonal antibodies overnight at 4 °C. Mouse monoclonal antibodies to mitochondrial respiratory chain subunits (Invitrogen) included the 30 kDa (Complex I), 70 kDa (Complex II), Core I (Complex III) and subunit I (Complex IV). Rabbit polyclonal antibodies to the human Complex I 39 kDa (NDUFA9) and 17 kDa (NDUFB6) subunits were raised against recombinant, bacterially expressed protein as described.[35] Other antibodies used include the α-Tim23 mouse monoclonal (BD Biosciences Pharmingen) and α-Tom40.[35] Secondary probing with an anti-mouse or anti-rabbit HRP conjugated antibody (Sigma) was performed for 2 h followed by detection using ECL reagents (Amersham) and a Chemi genius Bioimaging system (Syngene).

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