An Assembled Complex IV Maintains the Stability and Activity of Complex I in Mammalian Mitochondria*

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In the mammalian mitochondrial electron transfer system, the majority of electrons enter at complex I, go through complexes III and IV, and are finally delivered to oxygen. Previously we generated several mouse cell lines with suppressed expression of the nuclearly encoded subunit 4 of complex IV. This led to a loss of assembly of complex IV and its defective function. Interestingly, we found that the level of assembled complex I and its activity were also significantly reduced, whereas levels and activity of complex III were normal or up-regulated. The structural and functional dependence of complex I on complex IV was verified using a human cell line carrying a nonsense mutation in the mitochondrially encoded complex IV subunit 1 gene. Our work documents that, although there is no direct electron transfer between them, an assembled complex IV helps to maintain complex I in mammalian cells.

The mitochondrial electron transfer chain consists of four multiprotein complexes, NADH-ubiquinone oxidoreductase (complex I), succinate-ubiquinone oxidoreductase (complex II), ubiquinone-cytochrome c oxidoreductase (complex III), and cytochrome c oxidase (complex IV). Substantial information has been obtained on the molecular structures of each individual respiratory complex, but the organization of overall respiratory chain is still not clear (1). One favored model is that the respiratory complexes are embedded in the lipid bilayer of inner mitochondrial membrane and are connected electronically by diffusing ubiquinone and cytochrome c (2). However, with the recent development of blue native (BN)4-PAGE, a method for isolating intact membrane-bound protein complexes and thus particularly useful for investigating the integrity of mitochondrial respiratory complexes (3, 4), supercomplexes that contain multiple respiratory complexes have been reported in bacteria (5), in yeast (6), and in mammalian (6) and plant mitochondria (7), suggesting an alternative model of direct channeling between complexes.

Mammalian complex I is the largest and least understood component of the mitochondrial oxidative phosphorylation system (8, 9). It is composed of 45 subunits encoded by both nuclear and mitochondrial genomes (10). Among mitochondrial defects that have been associated with various human diseases and normal aging process, complex I deficiency is the most frequent phenotype (8, 9, 11, 12). This phenomenon has been attributed to the large genetic capacity that controls the biogenesis of complex I. However, it has been shown recently that complex III deficiency could also lead to a defective complex I activity (13). We report here that an assembled complex IV is required to maintain the stability of complex I in a mouse cell line with suppressed expression of subunit 4 of complex IV and a human cybrid carrying a nonsense mutation in mtDNA-encoded subunit 1 of complex IV.

EXPERIMENTAL PROCEDURES

Cell Culture—The cell line A9 is a derivative of the L mouse fibroblast cell line (14). It was grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. C1, C2, and C3 cells were maintained in the above medium supplemented with 50 μg/ml of uridine and 4 μg/ml of puromycin. Cybrids containing 0 and 100% of G6930A mutant mtDNA were generated by fusion of platelets from a patient with the G6930A mtDNA mutation to human osteosarcoma 143B cells lacking mtDNA ("p cells") (15, 16) and were cultured in Dulbecco’s modified Eagle’s medium with 5% fetal bovine serum supplemented with 50 μg/ml of uridine.

Preparation of Mitochondria Fractions—Mitochondria were isolated according to procedures previously described (17). Cells were incubated for 3 min in 10 mM Tris-HCl, pH 6.7, 10 mM KCl, and 1.5 × 10−7 M MgCl2 and processed in a Potter-Elvejem homogenizer. The homogenate was then brought to 0.25 M sucrose and centrifuged for 5 min at 1200 × g to remove large debris and nuclei. The mitochondria were collected by centrifugation for 10 min at 8000 × g.

Western Blot—The following antibodies were utilized in Western blot analysis: MS103 for complex I subunit GRIM-19, MS111 for complex I subunit NDUFA9, MS204 for complex II subunit Fp, MS304 for complex III subunit core 2, MS404 for

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Defective Complex IV Destabilizes Complex I

complex IV subunit COX I, MS407 for complex IV subunit COX IV, and MS 507 for complex V. F1F0 ATP synthase alpha subunit. Antibody MSA03 against porin (voltage-dependent anion channel) was utilized to control the loading. All the antibodies were from Mitosciences Inc. The Western blot was carried out according to the protocols provided by Mitosciences.

Mitochondrial Protein Synthesis—Pulse-labeling experiments were performed according to protocol described previously (17). Samples of 2 × 10^6 cells were incubated with [35S]methionine for 30 min at 37 °C in the presence of the cytoplasmic translational inhibitor emetine at 100 μg/ml. In pulse-chase labeling experiments, labeling was carried out as described above, except that emetine was replaced with cycloheximide and incubation time with [35S]methionine was extended to 2 h; thereafter, the cells were subjected to a 19-h chase in complete unlabeled medium in the absence of cycloheximide. The labeled cells were lysed, and 50 μg of protein were electrophoresed through an SDS-polyacrylamide gel (15–20% exponential gradient).

Blue Native Gel Electrophoresis—Mitochondria protein concentration was measured by the Bradford method. BN-PAGE (18, 19) was used for separation of respiratory complexes with 4–13 or 4.5–13% gradient. 40 μg of mitochondrial proteins were loaded on a minigel of 1.5 × 70 × 82 mm (Bio-Rad). The gels were run for 30 min at 40 V and then at 80 V until the dye reached the end of the gel. The protein complexes were detected by in-gel staining: for complex I activity, 0.1 mg/ml NADH and 2.5 mg/ml nitrotetrazolium blue in 2 mM Tris-HCl reached the end of the gel. The protein complexes were electrophoresed through a 5–13% BN-PAGE.

Enzymatic Assays—The enzymatic activities were assayed following the protocol by Birch-Machin and Turnbull (21). The rotenone-sensitive complex I activity was measured with 0.1 μM rotenone by following the decrease in absorbance due to the oxidation of NADH at 340 nm (ε = 6.22 × 10^3 M⁻¹ cm⁻¹) in assay buffer by using a DU-640 spectrophotometer (Beckman Instruments). Similarly, complex III activity was measured by reduction of cytochrome c(III) at 550 nm (ε = 21 mM⁻¹ cm⁻¹) using d-ubiquinol-2 as an electron donor, and the KCN-sensitive complex IV activity was measured with 2 mM KCN by oxidation of cytochrome c(II) at 550 nm (ε = 19.1 M⁻¹ cm⁻¹).

Results

In a previous study, we successfully suppressed expression of the gene encoding subunit 4 of cytochrome c oxidase (COX IV) by introducing a COX IV-targeted RNA interference species from the plasmid pSIREN-DNR-COXIV in mouse A9 cells (22). Three independent cell lines expressing pSIREN-DNR-COXIV (designated C1, C2, and C3) were established. The absence of COX IV led to a failure in assembly of that protein complex and a defective respiratory function. To investigate the potential interactions between different respiratory complexes, we decided to take advantage of the availability of these cell lines to study the effects of a defective complex IV on the stability and function of other complexes.

We first carried out Western blot analysis to examine the steady state levels of several respiratory complex subunits in A9 and C1, C2, and C3 cells with mitochondrial porin (voltage-dependent anion channel) as loading control. As shown previously, in C1, C2, and C3 cells, the levels of COX I, subunit 1 of complex IV, were dramatically decreased due to defective assembly of the protein complex (22). No significant changes were found in levels of subunits of complexes II, III, and V, as shown by immunoblot with antibodies against the flavoprotein subunit (SDH1) of complex II, subunit core 2 of complex III, and subunit α of complex V (Fig. 1A). However, two subunits of complex I exhibited considerable decreases in C1, C2, and C3 cells, a 52–88% decrease for subunit GRM-19, and a 59–80% decrease for subunit NDUF9 as determined by densitometry.

To verify whether the reduction in protein level is due to a decrease in protein synthesis or an decrease in protein stability, we carried out both mitochondrial protein pulse and pulse-chase experiments (17). Newly synthesized mitochondrial translation products were labeled with [35S]methionine for 30 min in the presence of emetine to inhibit cytoplasmic protein synthesis. The mouse mtDNA-encoded proteins have been identified previously by comparing the electrophoretic patterns with human products and analysis of mouse mtDNA mutants (23–25). As shown in Fig. 1B, there was no obvious difference in overall mitochondrial protein synthesis (including all detectable subunits of complex I) between the C1–C3 cells and the control A9 cell. This result indicated that there was no difference in protein synthesis between A9 and C1–C3 cells. However, in pulse-chase experiments where cells were labeled for 2 h with [35S]methionine in the presence of cycloheximide, a reversible inhibitor of cytoplasmic protein synthesis, followed...
by a 19-h chase in complete unlabeled medium in the absence of the inhibitor, as expected levels of three complex IV subunits (COX I, COX II, and COX III) were markedly decreased; due to the defective assembly of this protein complex, the identifiable mtDNA-encoded subunits of complex I (ND2 and ND4) were also significantly reduced in C1–C3 cells. At the same time, in the cells with defective complex IV assembly, the recognizable mtDNA-encoded subunits of complex III (cytochrome b) and complex V (A6) were present in somewhat increased amounts as compared with the control line, probably reflecting a compensatory effect. Taken together, these results pointed to a considerably lower stability of mtDNA-encoded complex I subunits in cells with defective complex IV assembly, resulting possibly from unstable complex I assembly.

The stability of complex I was further examined using BNG electrophoresis (3). Mitochondrial samples electrophoresed using the BN-PAGE system were subjected to immunoblot analysis for respiratory complexes I, III, and IV (Fig. 2A) and to in-gel activity assays for complex I and complex IV (Fig. 2B). As shown in Fig. 2A, complex IV levels in C1–C3 cells as indicated by b” were significantly decreased as previously reported (22). Consistent with the result from pulse-chase experiments, complex III levels (as a”) were dramatically reduced. b, c, and d were likely the subcomplexes of complex I, recently reported by Vogel et al. (26). Interestingly, in C1–C3 cells, subcomplexes 4 and 5, which had the molecular mass of ~300- and 500-kDa as identified by Vogel et al. (26), were accumulated. a’ and a” were likely the supercomplex composed of complexes III and IV as it was detected by the antibodies against both complexes. Very low levels of assembled complexes I and IV were also verified by the in-gel staining method as shown in Fig. 2B. These results indicated that the very low levels of complex I subunits observed in the pulse-chase experiments were due to the failure of these subunits to be incorporated into the fully assembled complex I.

The low complex I level could be the result of the failure of assembly of this complex or the increase of its turnover. The accumulation of subcomplexes suggested that the defective assembly could be more important. To further investigate, we carried out a BNG analysis on pulse-chased mitochondrial proteins. A9, C1, C2, and C3 cells were labeled with [35S]methionine for 2 h. Cells were then chased for 2, 5, and 18 h in the complete unlabeled medium to allow the incorporation of the labeled subunits into the complexes. The mitochondrial proteins were isolated from the labeled cells and then subjected to BNG analysis. As shown in Fig. 3, no respiratory complex assembly was observed after 2 h of labeling but no chase. There was a report using Chinese hamster NDUFA1 mutant cells as a conditional complex I assembly system that suggested that complex I assembly might take considerable time (>18 h) (27). However, we found that after 2 h of chase, complexes I and IV already started to emerge in A9 cells, but not in C1, C2, and C3 cells. After 18 h of chase, significant amounts of all five respira-
Defective Complex IV Destabilizes Complex I

The failure to detect significant amounts of complex I level at short chase times (2 or 5 h (data not shown)) or at a longer time (18 h) also suggested a defective assembly of complex I.

To check whether the reduction in complex I assembly was due to the lack of ATP supply resulting from defective respiration or to the necessity for successful electron transfer for complex I assembly, we treated A9 cells with various inhibitors of mitochondrial respiration, including 0.1 mM of the complex I inhibitor rotenone, which completely obstructs electron transfer through complex I as indicated by results of measurement of malate/glutamate-driven respiration; 20 nM antimycin, which blocks complex III-dependent respiration; 5 mM sodium azide or 2 mM potassium cyanide, which inhibit electron flow from complex IV to oxygen; or 15 mg/ml of the complex V inhibitor oligomycin, which reduces ATP levels even lower than those observed in C3 cells (data not shown). As shown in Fig. 4A, although there were very low levels of subcomplex 4 (as b) that appeared in A9 cells treated with various inhibitors, the overall assembly of complexes I (as a), III (as b'), IV (as b''), and supercomplex with complexes III and IV (as a' and a'') was not affected by treatment with any of these inhibitors. To confirm the effectiveness of inhibitors on the ATP/ADP ratio and on membrane potential, a long term (5 h) incubation experiment was also carried out. Again, no decreases in assembled complex I or accumulation of its subcomplexes were observed in cells treated with inhibitors (Fig. 4B). These results indicate that respiratory defects will not lead to destabilization of complex I.

The effect of disrupted complex IV on the stability of complex I could also be mediated by some common mechanisms. For example, the effect might be caused by competition for chaperones in matrix, where complex IV destabilization competes a putative chaperone in binding incoming complex IV subunits, and thus incoming complex I subunits are improperly chaperoned and fail to assemble correctly, or it could be because of up-regulation on proteases that are needed to clean up incorporated complex IV subunits, which is also used for complex I subunits. If this is correct, one might expect that destabilization of complex I could also lead to the defects in complex IV assembly. To test these possibilities, we took advantage of the availability of a cell line that exhibited defective complex I assembly, a previously isolated 4A cell carrying a frameshift mutation in mtDNA-encoded complex I subunit, ND6 gene (23). A BNG analysis was performed in 4A cells and its wild type control A9 cells. As shown in Fig. 5, although complex I assembly was totally disrupted, no abnormal complex IV assembly was detected in 4A cells. So far, all these results suggest that the steady state level of complex I was significantly reduced in C1, C2, and C3 cells due to a defective assembly of complex IV.

To confirm predictions from protein analyses, we also studied the activities of respiratory complexes. A specific measurement of the activities of the different complexes was carried out by using biochemical enzymatic assays. Mitochondrial membranes were partially purified from isolated mitochondria (9). Complex I (NADH ubiquinone oxidoreductase) activity was determined by following the oxidation of NADH with ubiquinone as the electron acceptor (21). Complex III (ubiquinone-cytochrome c oxidoreductase) activity was measured as reduc-

FIGURE 4. Respiratory complex analysis with various inhibitors. Cells were treated with antimycin (20 mM) (An), azide (5 mM) (Az), KCN (2 mM) (Kc), oligomycin (15 μg/ml) (O), and rotenone (0.1 mM) (R) for 5 min (A) and 5 h (B) at 37 °C, and the mitochondrial proteins were subjected to BNG analysis with immunoblot analysis with antibodies specific for the GRIM-19 subunit of complex I, the core 2 protein of complex III, and subunit 1 of complex IV.

FIGURE 5. Complexes I and IV assembly in A9 and 4A cells. Complexes I and IV assembly was analyzed on BNG by Western blot with antibodies specific for the GRIM-19 subunit of complex I and subunit 1 of complex IV.
mitochondrial proteins). In isolated mitochondria (42 nmol/min/mg mitochondrial protein) confirmed by an 80% reduction in complex I enzymatic activity supercomplex was completely abolished in mutant cybrids. The nature of this supercomplex was confirmed by Western blot analysis of mitochondrial proteins separated by second dimension, denaturing gel electrophoresis (not shown). This was observed the presence of a supercomplex III containing 0 and 100% of the G6930A mutation. As shown in Fig. 6, the KCN-sensitive cytochrome c oxidase activities in COX IV-crossed cells were decreased to 6–21% of control activity. Consistent with other results described above, whereas complex III activity was up-regulated in the C1–C3 cells, possibly as a compensatory effect, the rotenone-sensitive NADH-Q oxidoreductase activity is significantly compromised, decreasing by 64–80%.

To examine the generality of the effect of a defect in complex IV on the stability and activity of complex I and to determine whether this result is also significant in humans, we took advantage of the existence of human transmitochondrial cell lines (cybrids) from a patient with a multisystem mitochondrial disorder with a G6930A nonsense mutation in the COX I gene (15). The G to A transition at mtDNA nucleotide 6930 changes a glycine to a stop codon, resulting in a loss of the C-terminal 170 amino acids (33%) of the protein. It has been shown that the G6930A mutation causes a disruption in the assembly and defective activity of complex VI (15, 16).

A BNG analysis was carried out comparing a pair of cybrids containing 0 and 100% of the G6930A mutation (16). As shown in Fig. 7, complex IV assembly was disrupted as described previously (16). The steady state level of assembled complex I in cybrids with 100% G6930A is significantly reduced as compared with wild type cybrids. In addition, in wild type cells, we observed the presence of a supercomplex III+IV, which reacted with both antibodies against COX I and core 2 of complex III. The nature of this supercomplex was confirmed by Western blot analysis of mitochondrial proteins separated by second dimension, denaturing gel electrophoresis (not shown). This supercomplex was completely abolished in mutant cybrids. Defective complex I assembly in COX I mutant cybrids was confirmed by an 80% reduction in complex I enzymatic activity in isolated mitochondria (42 nmol/min/mg mitochondrial proteins) as compared with control cybrids (200 nmol/min/mg mitochondrial proteins).

**DISCUSSION**

The interdependence among respiratory complexes was first noticed when complex III was shown to be required to maintain complex I in mammalian mitochondria (13). This phenomenon might be not that surprising considering that direct electron transfer between these two complexes might be facilitated by physical interactions. However, complex IV has not been shown to directly interact with complex I. Contrary to our current observations, it has been reported that in muscle from a SURF-1 patient defective complex IV did not affect the levels of complexes I and III (28). The discrepancy is probably due to some threshold in the “assembling level” of complex IV. Some residual complex IV and significant subcomplex assembly was observed in SURF-1 patients (29). Although the steady state levels of holocomplex in SURF-1-deficient samples were just under 10%, which was even less than we saw with our samples, there were significant amounts of complex IV subcomplexes (29). It is possible that some subcomplexes of complex IV could also stabilize the complex I assembly. The other difference between the SURF-1-deficient samples and our cells was the cell type. It might be true as previously suggested (30) that the dependence of complex I assembly on complex IV is more pronounced in rapidly dividing cells. The generality that a severe defect in complex IV assembly could disrupt complex I assembly was further supported by observations in two systems, one in mouse cell lines with RNA interference suppression of nuclear-encoded COX IV and one in human cybrids carrying a nonsense mutation in a mtDNA-encoded COX I gene.

Because both complex III assembly and activity were not disrupted, current results seem not to be mediated by an unstable complex III. It therefore seemed possible that active electron transfer among the respiratory complexes helps to stabilize complex I assembly or, because complex I is the largest respiratory complex, its assembly might require more energy than is available in complex IV-defective cells. We excluded these two possibilities by conducting experiments with various respira-
Defective Complex IV Destabilizes Complex I

tory inhibitors. We also ruled out the explanation that the effect was due to some common mechanisms that regulate the stabilities of both complexes I and IV.

More likely, the accumulation of subcomplexes 4 and 5 in C1–C3 cells seems to suggest the difficulty of incorporating the complex I subunits into the membrane (26). Although more experiments are required, our results seem to indicate that complex I may not be stable unless it is assembled into a supercomplex also containing complexes III and IV. Because complex I is the major entry point for electron transfer (31), it is likely that this supercomplex is the central functional unit for mitochondrial respiration. It is interesting to note that in Paracoccus denitrifi-
cans, a bacterium utilizing an electron chain that consists of a full complement of mitochondrial respiratory complexes, complex I is stabilized by assembly into a supercomplex containing both complex III and complex IV (5).

Mitochondrial diseases are estimated to occur with a prevalence of 10–15 cases/100,000 persons (32). Mitochondrial dysfunction has further been suggested to play a role in more common diseases such as type II diabetes (33), cancer (34), and even in the normal aging process (35). Among mitochondrial defects, complex I deficiency is emerging as the most frequent phenotypes (8, 11, 12). This phenomenon has been attributed to the substantial genetic capacity that controls the biogenesis of complex I, because complex I is the largest (45 subunits) component of the respiratory chain. Another implication from this study is that complex III and complex IV deficiencies can also lead to a complex I defect and, in this sense, complex I deficiency might be truly characteristic of mitochondrial dysfunction.

While preparing this report, we found that, consistent what we describe here, Diaz et al. (30) reported that in COX 10 knock-out mouse fibroblast where complex IV is unable to assemble and there is no detectable complex IV activity the levels of respiratory complex I were significantly reduced. They further showed that transduction of COX 10, a protoheme: heme O farnesyl transferase that is essential for the incorporation of heme a in COX I, restored the complex I function.

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