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Human mitochondrial complex I assembles through the combination of evolutionary conserved modules: a framework to interpret complex I deficiencies

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With 46 subunits, human mitochondrial complex I is the largest enzyme of the oxidative phosphorylation system. We have studied the assembly of complex I in cultured human cells. This will provide essential information about the nature of complex I deficiencies and will enhance our understanding of mitochondrial disease mechanisms. We have found that 143B206 rho zero cells, not containing mitochondrial DNA, are still able to form complex I subcomplexes. To further address the nature of these subcomplexes, we depleted 143B osteosarcoma cells of complex I by inhibiting mitochondrial protein translation with doxycycline. After removing this drug, complex I formation resumes and assembly intermediates were observed by two-dimensional blue native electrophoresis. Analysis of the observed subcomplexes indicates that assembly of human complex I is a semi-sequential process in which different preassembled subcomplexes are joined to form a fully assembled complex. The membrane part of the complex is formed in distinct steps. The B17 subunit is part of a subcomplex to which ND1, ND6 and PSST are subsequently added. This is bound to a hydrophilic subcomplex containing the 30 and 49 kDa subunits, to which a subcomplex including the 39 kDa subunit is incorporated, and later on the 18 and 24 kDa subunits. At a later stage more subunits, including the 15 kDa, are added and holo-complex I is formed. Our results suggest that human complex I assembly resembles that of *Neurospora crassa*, in which a membrane arm is formed and assembled to a pre-formed peripheral arm, and support ideas about modular evolution.

INTRODUCTION

Complex I (NADH–ubiquinone oxidoreductase, EC 1.6.5.3) is the most frequently affected complex of the oxidative phosphorylation (OXPHOS) system leading to mitochondrial disease (1,2). The enzyme couples the transfer of two electrons from NADH to ubiquinone to the translocation of four protons across the mitochondrial inner membrane. The thus generated proton gradient is used by complex V to produce ATP. Mammalian complex I consists of 46 polypeptide subunits, seven encoded by the mitochondrial DNA and the remainder by the nuclear genome, a non-covalently bound flavomononucleotide (FMN) group and eight iron sulphur clusters (3). Although several mutations have been found in both nuclear and mitochondrial subunits (2,4–7) (Fig. 1), many complex I deficiencies remain to be explained (1). In striking contrast to complex IV, where the majority of deficiencies can be explained by mutations in genes encoding for specific assembly proteins, so far this has not been described for complex I. This discrepancy is probably caused by the absence of complex I in the yeast *Saccharomyces cerevisiae*, a model organism which enabled the identification of a dozen of complex IV assembly genes (8). Given the intricacy of complex I, it is very well possible that defective assembly proteins account for a number of complex I enzyme deficiencies. In a previous study, we have shown a

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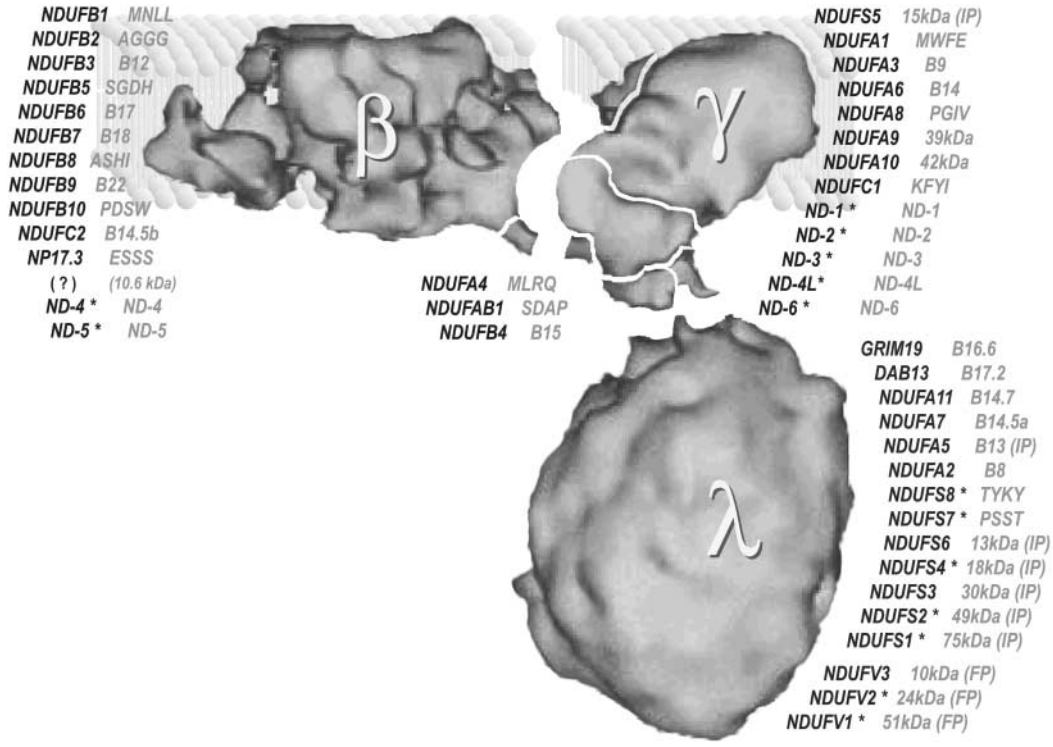

Figure 1. Schematic representation of human complex I subunits and their putative topology within the complex. Adapted from Carroll *et al.* (ref. 15). Bovine homologues are written in gray and human subunits in which mutations are found are marked with asteriks. The unknown (10.6 kDa) subunit has been identified in bovine, but its sequence has not been elucidated yet. [From Nijtmans *et al.* (2) © Springer, reprinted with permission of the publisher.]

decrease in the levels of intact complex I in six patients harbouring mutations in nuclear-encoded complex I subunits, indicating that complex I assembly and/or stability is compromised (9). Different patterns of low molecular weight subcomplexes are present in these patients; a finding that has also been demonstrated in other patient studies (7,10,11). Insight in complex I assembly in human cells will aid interpretation of these patient data and lead to a better understanding of the molecular mechanisms underlying these disorders.

Complex I, which awaits a crystal structure, is an L-shaped molecule with one arm embedded in the mitochondrial inner membrane and one arm protruding into the matrix, the peripheral arm (12). Although our knowledge of the biosynthesis of complex I is still limited, much information about the subunit topology has been obtained. Hatefi and coworkers were able to fractionate the bovine enzyme into three functional parts: the FMN containing part, the iron–sulphur cluster containing part and a membrane part (13). Over the years the group of Walker refined this fractionation into α, β, γ and λ parts (14,15) and more importantly they were able to identify all subunit components of these fractions by mass spectrometry (Fig. 1) (16). By using an immunocapture method in combination with mass spectrometry, Murray *et al.* (17) were able to identify the human homologues of 42 polypeptides of the 46 beef heart complex I subunits, suggesting an identical composition of the human and bovine complex I. To avoid confusion we use the bovine subunit-nomenclature in this paper. The conversion to the human nomenclature is given in Figure 1. Although the location of subunits within the complex only reveals physical associations of subunits, which does not necessarily reflect the physiological assembly, it provides vital information to construct an assembly pathway. Another aspect which can be taken into account when studying the assembly of complex I is the theory that the basic bacterial form of complex I has evolved from several functional modules: an NADH–dehydrogenase, a hydrogenase and a transporter module (18). It is temping to assume that at least part of the evolutionary origin of complex I is conserved in the mammalian assembly pathway (19).

Most of what is known about the assembly of complex I comes from studies carried out in the fungus *Neurospora crassa*, which contains 35 subunits (20). It has been shown that the peripheral arm can still be formed in the absence of mitochondrially encoded subunits (21). This independent formation of the membrane and protruding arm of the complex was also demonstrated in disruption mutants of this organism (22). In addition, the membrane arm appeared to be formed out of two subcomplexes, designated as the small and large intermediates (23). Interestingly, two non-subunit proteins, named CIA30 and CIA84, were bound to the large membrane arm assembly intermediate. Disruption of either of the proteins led to a specific block of complex I assembly and CIA30 and CIA84 are therefore regarded as complex I assembly proteins (24). A human homologue has only been found for CIA30, and despite sequence analysis of this gene in complex I-deficient patients, no pathogenic mutations have been described so far (25). To date, it is unclear whether complex I assembly in mammalian cells is comparable to the *N. crassa* model.

Consistent with this model is the finding that in metabolic labelling studies in combination with immunoprecipitations, some nuclear-encoded subunits can preassemble before mitochondrially encoded subunits are added to the complex (26). Nevertheless, in this study it is not clear which nuclear subunits form this scaffold for the mitochondrially encoded subunits. Immunopreciptations using a 49 kDa antibody demonstrated that no other mitochondrially encoded subunits were co-precipitated in human 143B and mouse A9 cybrid cells containing mutations in the ND4 and ND6 subunits (27,28). However, in cybrids which had mutations in ND5 all mitochondrially encoded subunits were co-precipitated, except ND5 (29). This suggests that ND4 and ND6 are essential for assembly, whereas ND5 is not. Compatible with these findings are observations in mutants of the green alga *Chlamydomonas reinhardtii.* Lack of ND1 and ND6 led to the absence of fully assembled complex I, but still a 160–210 kDa subcomplex could be formed, which contained homologues of the 49 and 75 kDa subunits and showed NADH–dehydrogenase activity. Besides the formation of this 160–210 kDa subcomplex, deletion of the ND4 and ND4/ND5 subunits also resulted in the formation of a 650 kDa subcomplex with NADH–dehydrogenase activity (30). An assembly pathway for human complex I has been recently described by performing two-dimensional blue native/sodium dodecyl sulphate polyacrylamide gel electrophoresis (2D BN/SDS–PAGE) of mitochondria from muscle biopsies of complex I-deficient patients. Several complex I subcomplexes were found depending on the antibodies used. Moreover, similar patterns of subcomplexes were found in different patients (10). This led to the suggestion that these subcomplexes are intermediates of assembly. By combining the patterns of the panel of antibodies an assembly pathway was deduced. In this model, subcomplexes of the peripheral arm and subcomplexes containing parts of both arms are found, suggesting that the peripheral and membrane arms are not assembled in separate ways. This is in sharp contrast to the *N. crassa* model, in which the peripheral arm is formed independently from the membrane arm.

In our study we have investigated whether partially assembled complex I subcomplexes could be formed in cells lacking mitochondrial DNA [143B206 rho zero (ρ^0) cells], as is the case in *N. crassa.* By using 2D BN/SDS–PAGE in combination with immunodetection several partially assembled subcomplexes were detected, illustrating that parts of the complex that do not contain any of the mitochondrially encoded complex I subunits can be formed. To address the dynamics of assembly, we created a conditional complex I assembly system by partially depleting 143B osteosarcoma cells of complex I and other OXPHOS complexes by treating them with doxycycline, an inhibitor of mitochondrial translation. After removal of this drug, complex I assembly resumed and the appearance of assembly intermediates was investigated. This approach has been successfully used to study the assembly of complexes V and IV (31,32). Besides the reappearing of complex I, we observed the appearance of partially assembled complex I intermediates, indicating that these subcomplexes were newly formed. On the basis of the alignment and analysis of these subcomplexes we propose a modular complex I assembly model, which largely resembles the assembly as described for *N. crassa* in that the membrane and peripheral parts can be preassembled independently.

RESULTS

Cells without mitochondrial DNA form partially assembled subcomplexes

To investigate whether subcomplexes of complex I can be formed in cells that do not express mitochondrial subunits, we analyzed 143B206 ρ^0 cells by 2D BN/SDS–PAGE electrophoresis in combination with western blotting (Fig. 2). Signals were obtained with antibodies against the 30, 39, 24 kDa and B17 subunits, but not with antibodies against the 15, 18 kDa and PSST subunits (data not shown), suggesting that the incorporation of these three proteins into the complex requires the presence of mitochondrially encoded subunits. Antibodies against the mitochondrially encoded ND1 and ND6 subunits were tested as negative controls with which no signals were obtained (data not shown). In control cells most of the signal appeared at the place where complex I runs on the first dimension (indicated in Fig. 2 as CI). As expected no fully assembled complex I could be detected in the ρ^0 cells. However, in ρ^0 cells the 30, 39, 24 kDa and the B17 subunits are present at higher molecular weight spots than the expected molecular weight of the monomeric subunit. This result shows that partially assembled subcomplexes can be formed in the absence of the mitochondrial complex I subunits. The 30 kDa subunit is part of at least five distinct subcomplexes. The first runs at the front and is probably the monomeric subunit. Next there are two subcomplexes at a molecular weight of ~80 and ~150 kDa and two subcomplexes at a molecular weight of ~250 and ~600 kDa. All these subcomplexes are also visible in the control cells. The 39 kDa subunit is detected as a long smear, which might reflect the hydrophobic nature of this subunit. A high molecular weight subcomplex can be distinguished also at 600 kDa. A subcomplex of a similar size is found in the control cells. The hydrophobic B17 subunit results in a smeary pattern as well; nevertheless six different subcomplexes can be distinguished. The first intense spot is likely to represent the unassembled subunit. The next subcomplexes run at 50, 200, 400, 650 and 800 kDa, respectively. These B17-containing subcomplexes observed in the ρ^0 cells are unexpected and there are several reasons to think that they are not assembly intermediates. First, the B17 subcomplexes in the ρ^0 cells are not observed in other cell systems we tested (see further sections). Second, a subcomplex of a molecular weight of 800 kDa (largest B17 spot) is difficult to explain given the fact that many subunits are lacking. For some reason the B17 subunit seems less prone to degradation than other subunits. It is therefore possible that these subcomplexes in the ρ^0 cells reflect aggregates of this hydrophobic subunit or aggregates of subcomplexes that contain this subunit. The 24 kDa subunit appears in two spots, the first one is connected by an arch to the second spot. These spots probably represent the monomeric molecule and the excess of blue dye, which runs at the front, causes the peculiar shape. An additional spot containing the 24 kDa subunit just before the front is also detected.

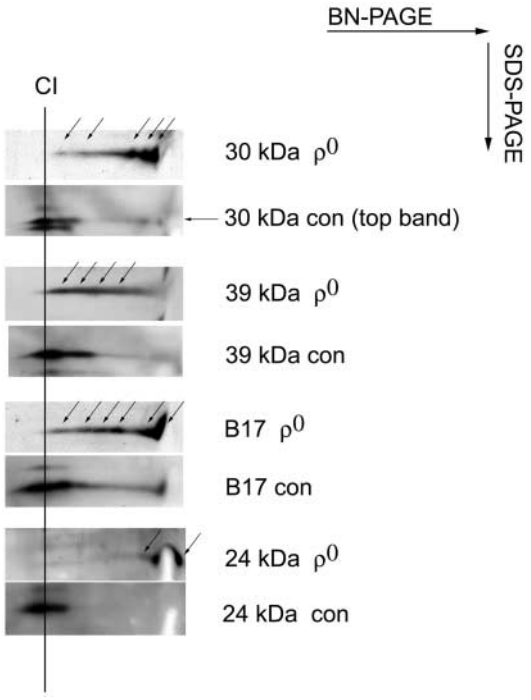

Figure 2. Complex I subcomplexes in ρ^0 cells. Crude mitochondrial fractions of control 143B osteosarcoma cells (con) and 143B206ρ^0 cells (ρ^0) were separated by 2D BN/SDS–PAGE electrophoresis (top-right arrows indicate the first and second dimension). The gels were blotted onto nitrocellulose and analyzed with antibodies against the complex I subunits 30, 39 kDa, B17 and 24 kDa. Subcomplexes are indicated with arrows. Fully assembled complex I is indicated as CI. In the panel for the 30 kDa subunit, a residual band of a previous antibody incubation of ND1 is still visible just below the 30 kDa spots. One should therefore focus on the top band (indicated in the figure).

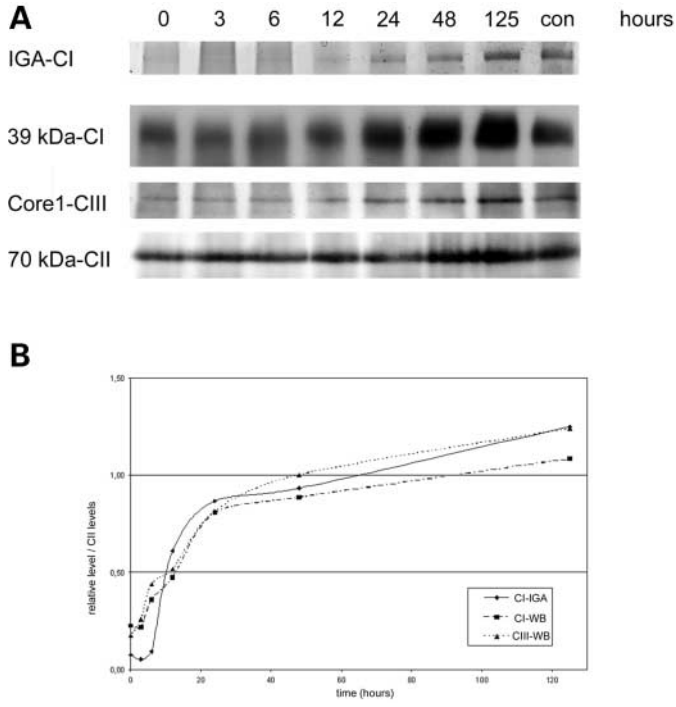

Figure 3. Appearance of complex I in 143B cells after doxycycline treatment. (**A**) Osteosarcoma 143B cells were treated for 6 days with doxycycline (an inhibitor of mitochondrial translation), the medium was replaced by doxycycline-free medium and cells were grown for the indicated time (in h). An aliquot of 40 μg of crude mitochondrial pellets were analyzed by BN electrophoresis in combination with complex I in-gel activity (IGA-CI, top panel). Duplicate gels were blotted and incubated with antibodies against the complex I subunit 39 kDa (39 kDa-CI, second panel), against the complex III core1 protein (Core1-CIII, third panel) and against the complex II 70 kDa subunit (70 kDa-CII, bottom panel). Control untreated 143B cells are indicated as con. (**B**). The signals for the in-gel activity assay and western blots were expressed as percentage of the untreated cells, normalized with the complex II 70 kDa subunit and plotted.

Reversibly blocking complex I assembly

Detection of assembly intermediates is difficult because these are transient products, which are likely to have short half-lives. Moreover, breakdown can occur simultaneously, complicating the identification of true assembly intermediates. To circumvent these problems we decided to deplete 143B osteosarcoma cells of complex I and other OXPHOS complexes containing mitochondrially encoded subunits, by reversibly blocking mitochondrial protein translation with the drug doxycycline. After 6 days of doxycycline treatment, we observed an ∼80% reduction of fully assembled complex I compared with untreated cells (Fig. 3A, 0 h lane). In agreement with previous reports (33), this indicates that the half-life of complex I is relatively long, especially taking into account that because of cell division the complex I pool is diluted. However, it needs to be mentioned that after 2–3 days of doxycycline treatment the growth of the cells slows down significantly and changes in metabolism likely influence the stability of complex I. Because further treatment with doxycycline affected cell viability in our culture conditions, we decided to treat the cells for 6 days. After the doxycycline treatment, the cells were washed, incubated with fresh medium and collected 3, 6, 12, 24, 48 and 125 h after for BN PAGE analysis (Fig. 3A). In-gel complex I activity and western blotting using an antibody against the 39 kDa subunit demonstrate the restoration of fully assembled complex I after 48–125 h (Fig. 3A and B). This rate of complex I restoration does not necessarily reflect the time required for the biosynthesis of complex I, since the assembly can make use of pools of subunits and partially assembled subcomplexes. The observed time-course for complex I assembly is comparable with the findings of Yadava *et al.* (33) in their conditional complex I assembly system in Chinese hamster fibroblasts. The fact that no complex I could be detected by the in-gel activity assay after 3 and 6 h can be explained by the lower sensitivity of this assay compared with immunodetection.

At 125 h after doxycycline removal, there is more fully assembled complex I compared with the control cells. Apparently, there is a compensatory mechanism in the cells as a response to the inhibition of mitochondrial protein synthesis. This might involve a general increase of mitochondrial mass because complex II (Fig. 3A), which only contains nuclear-encoded subunits, also shows such an increase at 125 h compared with the control cells. In this experiment we also tested complex III, since it has been recently demonstrated

that complex I stability is severely hampered when there is no fully assembled complex III present (34). As shown in Figure 3A and B, complex III is restored in a similar time-course as complex I. It is therefore unlikely that in our system the absence of complex III negatively affects complex I stability.

Reappearing of subunits

To follow the reappearance of subunits after the reversible block of assembly, samples taken at different time-points after removal of doxycycline were run on a SDS–PAGE, blotted on nitrocellulose and incubated with a panel of nine complex I antibodies (Fig. 4). Cells deprived of mtDNA (ρ^0 cells) were run as a negative control and untreated cells were included in the panel as a positive control. We observe remarkable differences in the steady-state levels of the different subunits. As expected, ρ^0 cells lack the mitochondrially encoded ND6 and ND1 subunits. Consistent with previous findings, also the 18, 15 kDa and PSST subunits were not detectable in ρ^0 cells and after 6 days of doxycycline treatment (lane 0) their abundance was very low (18 kDa subunit) or not detectable (15 kDa and PSST). These subunits start to reappear at 12–24 h and increase gradually to control levels at 125 h after release of inhibition. This result indicates that these subunits might not be stabilized in a preassembled subcomplex and they would enter the assembly process relatively late. In contrast, the 39, 30, 24 kDa and B17 subunits were still visible in ρ^0 cells and after 6 days of doxycycline treatment, suggesting that these subunits are more stable possibly because of the formation of subcomplexes. The steady-state levels of these subunits are comparable with the control levels at 6–12 h after removal of the doxycyline, which suggests that these subunits enter the complex I assembly pathway at a relatively early stage.

The mitochondrially encoded subunits ND1 and ND6 were still present at detectable levels after 6 days of doxycycline treatment, which is consistent with the small amounts (~20%) of assembled complex I observed (Fig. 3A, 0 h lane). Results show that these subunits gradually increase from 6–12 h until they reach the maximum at 125 h after doxycycline treatment. Since these subunits were directly targeted by the drug, which inhibits mitochondrial translation, no pools of preassembled subcomplexes could have been formed after the 6 days of doxycycline treatment. All subunits are more abundant after 125 h when compared with the untreated cells, probably as a compensatory mechanism as discussed in the previous section.

Dynamics of complex I subcomplexes

Immediately after removing the drug doxycycline, the synthesis of mitochondrially encoded subunits resumes and the assembly of complex I can start again. In this way complex I assembly is synchronized and intermediates are more likely to be detectable. To find out when subcomplexes start to appear and whether there is a change in the pattern during the assembly process, we monitored the subcomplexes by 2D BN/SDS–PAGE at all time-points. To allow a good overview about what is happening with time we grouped the

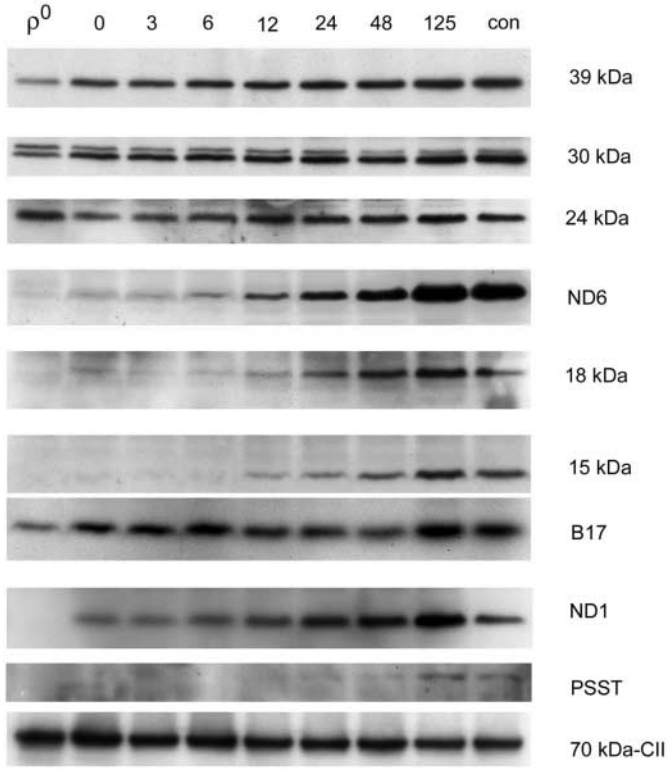

Figure 4. Kinetics of reappearance of complex I subunits. An aliquot of 40 μg of total cell lysate were loaded on a 10% SDS–PAGE, blotted on nitrocellulose filter and incubated with a panel of complex I antibodies (indicated on the right). An antibody against the complex II 70 kDa subunit was included as a loading control. The time (in h) after removing the doxycyline is indicated on the top. Cells without mitochondrial DNA (ρ^0) and control cells (con) were included as negative and positive controls, respectively.

subunits per time-point (Fig. 5). The molecular weights of the subcomplexes and possible co-localization of subunits within the same subcomplex will be discussed in more detail in the next section.

For all subunits investigated we observe residual holocomplex I (indicated with arrows) after 6 days of doxycyline treatment (time 0 h), which increases in amount with time. In addition, we observe subcomplexes for all investigated subunits. These subcomplexes increase in amount with time and subsequently the lower molecular weight subcomplexes decrease again, suggesting that these subcomplexes are true assembly intermediates and not breakdown products. Consistent with the results from the ρ^0 cells (Fig. 2) and the SDS gel (Fig. 4) we do not find subcomplexes smaller than 600 kDa for the 15 and 18 kDa subunits. This result suggests that these subunits enter the assembly pathway relatively late, possibly as monomeric subunit and not in a preassembled form. The subunits B17 and 30 kDa are present in low molecular weight subcomplexes, suggesting that these subunits enter the assembly route at an earlier stage. The B17 subunit is also detected as a smear with distinct thickenings suggesting subcomplexes. It is remarkable that shortly after the release of doxycycline there is much free unassembled subunit present at the front (right side) of the gel, suggesting that

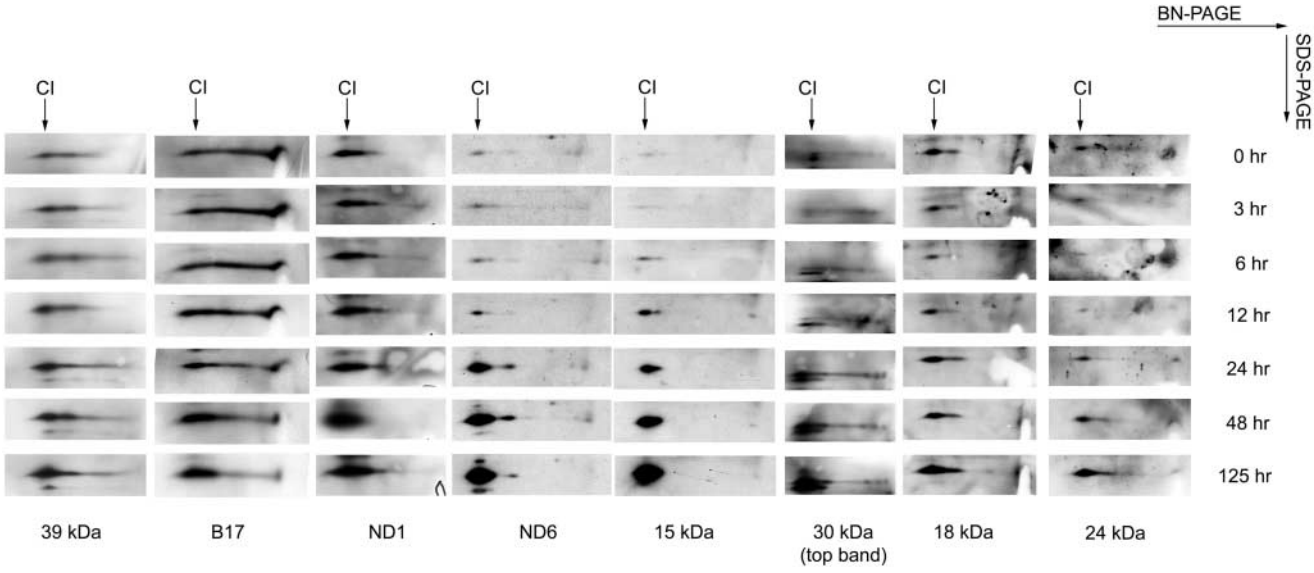

Figure 5. Dynamics of complex I subcomplexes. Cells were pretreated for 6 days with doxycycline and grown in the absence of the drug for the indicated times (specified on the right). An aliquot of 40 μg of crude mitochondrial pellets were analyzed by 2D BN/SDS–PAGE (arrows indicate the first and second dimension). Relevant parts of the blots were grouped per subunit (indicated on the bottom). The mobility of complex I in the first dimension is indicated with arrows (top). Complex I is indicated as CI. In the 30 kDa subunit panel an additional ND1 band is seen (Fig. 2).

there is a pool waiting for partners to participate in the assembly process. This is nicely illustrated by the fact that by increasing time larger subcomplexes appear and smaller subcomplexes disappear, indicating that higher molecular weight subcomplexes are formed. The 30 kDa subunit is detected in three characteristic low molecular weight subcomplexes comparable with the ρ^0 cells. The 39 kDa subunit is detected as a similar smear at the low molecular weight range, as seen in the ρ^0 cells. Although the 24 kDa subunit is visible after 6 days of doxycycline treatment in the SDS blot (Fig. 4), in the 2D BN/SDS blots no low molecular weight subcomplexes can be observed besides the small amounts of the monomeric subunit.

Composition of subcomplexes

In order to determine which subunits comigrate, we aligned the blots of several independent experiments and named A–G subcomplexes in which we observe more than one subunit (Fig. 6). The co-localization of subunits in a certain subcomplex is crucial for the interpretation of a possible assembly pathway (see Discussion). For this reason we also show other representative examples of a similar analysis in a 2D BN/SDS blot of a crude mitochondrial preparation of human embryonic kidney cells, HEK 293 (Fig. 6B) and control 143B cybrid cells (Fig. 6D). We observe the subcomplexes A–G with the same subunit composition in the different cell lines; however, the relative distribution of these subcomplexes differs, depending on the cell line and growth condition.

The 30 kDa subunit is part of three small subcomplexes, one running at the front, one running at ∼80 kDa (subcomplex H) and one at ∼150 kDa (subcomplex G) (Fig. 6A, B and D), as seen also in the ρ^0 cells (Fig. 2). The next co-localization is observed with subcomplexes F, D and B and fully assembled complex I (A). For the 49 kDa subunit we observe an identical pattern (Fig. 6D). As already discussed the 39 kDa subunit appears as a smear, possibly reflecting the hydrophobic nature of this subunit. The first distinguishable subcomplex appears at 250 kDa (Fig. 6B, subcomplex F). The next 39 kDa containing-subcomplex is subcomplex D, which has a molecular weight of 600 kDa (Fig. 6A–C). Another broad spot spans mobility from 950 to 1000 kDa (subcomplexes A and B). Although it seems to be one spot, we believe it actually consists of two spots that run closely together, which can be seen for some subunits shortly after removing doxycycline (ND6 and 15 kDa subunit panels, 6 h time-point, in Fig. 5). By adapting the gradient of the first dimension BN PAGE from 5–15 to 5–13%, a better separation of (sub)complexes A and B was obtained (Fig. 6C). Complex A represents fully assembled complex I. The 18 and 24 kDa subunits seem to comigrate with subcomplexes D, B and A. (Fig. 6A and C). The 15 kDa subunit is only present in subcomplexes A and B (Fig. 6A). Besides a spot at the front of the gel, which likely represents the monomeric subunit, subunit B17 shows a spot at ∼400 kDa (Fig. 6A and B, subcomplex E). Other subcomplexes in which this subunit is present are found at estimated molecular weights of 700 kDa (subcomplex C), 950 kDa (subcomplex B) and 1000 kDa (holo-complex I, A). ND1 comigrates with subcomplexes C, B and A, the same as ND6 and the PSST subunit (Fig. 6D). The subcomplexes and their subunit compositions are summarized in Table 1.

DISCUSSION

To better understand complex I assembly defects as seen in patients with a complex I deficiency (9,10), we aimed to

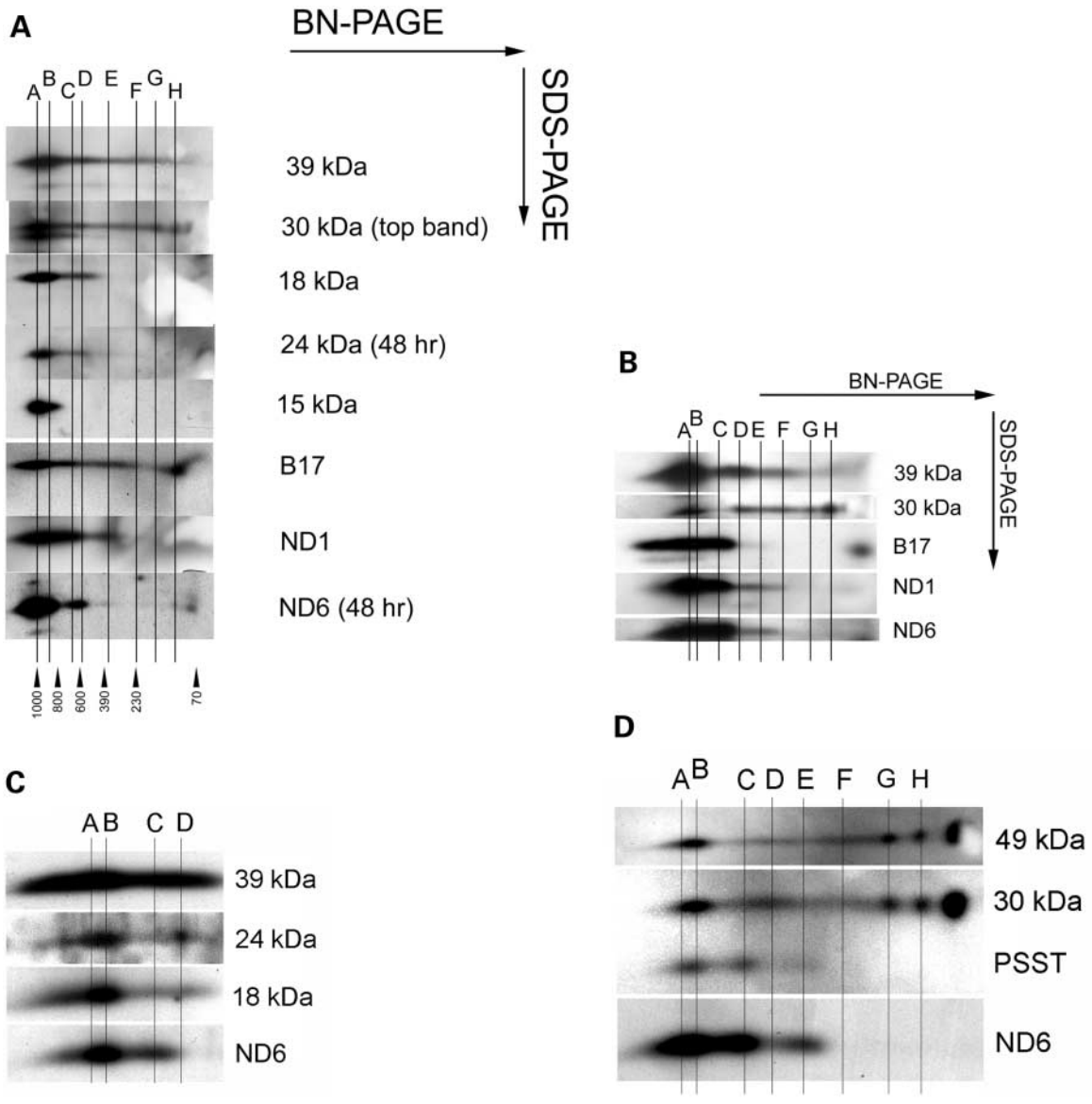

Figure 6. Co-localization of subunits in subcomplexes. (**A**) Crude mitochondrial fractions of 143B cells were analyzed by 2D BN PAGE at 24 or 48 h (indicated) after doxycycline removal. (**B**) The same procedure was followed with the untreated HEK 293 cells. (**C**) Untreated 143B cells analyzed on 2D BN/SDS–PAGE. A 5–13% acrylamide gradient was used in the first dimension in order to separate the higher molecular weight (sub)complexes A–D. (**D**) Control cybrids analyzed as described in (A) and (B). Arrows indicate the first and second dimension. The antibodies used are indicated on the right. Complex I (A) and subcomplexes B–H are indicated. Bottom arrows in (A) indicate the molecular weights of selected markers (complex I, 1000 kDa, complex III dimer + complex IV = 800 kDa, complex III = 600 kDa, F1-ATPase = 390 kDa, complex IV = 230 kDa and HSP70 = 70 kDa).

identify important steps in the assembly pathway of human mitochondrial complex I. We have used 2D BN/SDS–PAGE to investigate the appearance of complex I subcomplexes in cells devoid of mitochondrial DNA and in cells depleted of complex I and other OXPHOS complexes by the treatment of doxycycline, an inhibitor of mitochondrial translation. This approach has been successful in the identification of the assembly pathway of other respiratory chain complexes (31,32). These findings helped the interpretation of assembly intermediates in patient cells with defects in OXPHOS complexes, such as SURF1, ATP6, COX10, SCO1 and $tRNA^{leu}$ mutations (35–38).

Subcomplexes containing at least the 30 kDa subunit are observed in the ρ^0 cells, suggesting that the peripheral arm can partially be formed in the absence of a membrane arm. The subcomplexes observed in the ρ^0 cells (Fig. 2) and the doxycycline-treated cells (Fig. 6) are not necessarily the same because the ρ^0 cells are different from the doxycycline-treated cells. The ρ^0 cells are adapted to the fact that they do not contain mitochondrial DNA and cannot assemble any mitochondrial gene product containing complex, whereas the doxycycline-treated cells are only transiently partially depleted from mitochondrially encoded gene products. Also the ρ^0 cells cannot make mitochondrial mRNA anymore, whereas

Table 1. The presence of complex I subunits per observed subcomplex

Subunit	Subcomplex (estimated molecular mass)							
	H (80 kDa)	G (150 kDa)	F (250 kDa)	E (400 kDa)	D (600 kDa)	C (700 kDa)	B (950 kDa)	A (1 MDa)
15 kDa							x	x
18 kDa					x		x	x
24 kDa					x		x	x
30 kDa	x	x	x		x		x	x
49 kDa	x	x	x		x		x	x
39 kDa			x		x		x	x
B17				x		x	x	x
ND1				x		x	x	x
ND6				x		x	x	x
PSST				x		x	x	x

doxycyline-treated cells can. For this reason we base our model solely on the experiments with the doxycycline-treated cells.

We have observed that the assembly of complex I starts with different low molecular weight subcomplexes that differ in their subunit composition. This confirms that complex I assembly is a semi-sequential process in which subunits preassemble in different subcomplexes that are joined later in the assembly pathway. We can distinguish at least two distinct parts of complex I which are preassembled independently (Table 1). The first one contains the peripheral arm 30 kDa subunit, which immediately associates with the 49 kDa subunit. Next the 39 kDa, and later 18 and 24 kDa subunits are assembled. The second one contains the membrane arm subunit B17, to which subsequently the ND1, ND6 and PSST subunits associate. This peripheral and membrane parts are joined and additional subunits are inserted, including the 15 kDa subunit, to form a 950 kDa subcomplex which migrates closely to complex I. This 950 kDa subcomplex is completed to fully assembled complex I, possibly by the addition of subunits or conformational changes. In a recent paper Acín-Pérez *et al.* (34) observed also two closely migrating complex I bands on a BN PAGE after pulse-labelling mitochondrial translation products, which resemble (sub)complexes A and B. After longer chase times the upper complex I band increases in intensity compared with the lower complex I band, suggesting that the lower complex I band is converted into the higher complex I band. Our findings are consistent with the *N. crassa* model (21), for which it was proposed that complex I is assembled by combining different evolutionary modules (39). On the basis of our experiments and other cross-linking (40), fractionation (15), 2D BN/SDS (9,10) and evolutionary data (18), we propose an assembly pathway of complex I in human cells which is consistent with a modular assembly (Fig. 7). This model entails the formation of a NADH–dehydrogenase module, a hydrogenase module and a transporter module.

Assembly of the evolutionary conserved hydrogenase module of complex I starts with the 30 and 49 kDa subunits, which are located in the peripheral arm of complex I, and are two of the 14 mammalian core subunit homologues present in the basic complex from *Escherichia coli* (41). Three distinct low molecular weight subcomplexes were observed. Because the 30 kDa subunit has been shown to form a fusion protein with the 49 kDa subunit in *E. coli* (42) it is expected that these subunits are bound to each other early in assembly. The first two 30 kDa and 49 kDa spots might therefore represent the monomeric subunit and the subsequent association with each other to form subcomplex H. This is supported by the direct interaction of the 30 kDa and 49 kDa subunit in chemical cross-link studies in bovine (40). Consistent with this idea is the finding that in a complex I-deficient patient with a mutation in the 49 kDa subunit, the first 30 kDa spot accumulates, which suggests that the formation of the second spot is blocked (9). The third 30–49 kDa spot (subcomplex G) represents the subsequent association of another subunit to this 30–49 kDa subcomplex. Next, we observe association of the 39 kDa subunit and likely other subunits to this 30–49 containing subcomplex to form subcomplex F (Figs 6 and 7). The 39 kDa subunit fractionates with the membrane γ-part of complex I (Fig. 1) (14) and might therefore serve as a membrane anchor for the connection of the peripheral arm with the membrane arm.

Other subunits, including the 24 kDa, are bound to subcomplex F to form subcomplex D. Because the 24 kDa subunit constitutes the NADH–dehydrogenase module together with the 51 kDa subunit and the N-terminal segment of the 75 kDa subunit (43), it would be conceivable that these subunits preassemble in another intermediate complex. Although we do not observe a 24 kDa containing NADH–dehydrogenase subcomplex in our 2D BN/SDS–PAGE (Fig. 6), a 24 kDa containing low molecular weight product occurs in the ρ^0 cells (Fig. 2). This could imply that such a subcomplex exists, but it is rapidly assembled into subcomplex D. The 75 kDa subunit is likely to play a crucial role in connecting the NADH–dehydrogenase module to the hydrogenase module, since cross-linking studies show association with the 30 kDa subunit of the hydrogenase module, and with the 51 kDa subunit of the NADH–dehydrogenase module (40). The finding of a 160–210 kDa subcomplex in complex I-deficient mutants of *C. reinhardtii*, which contains the homologues of the 75 and 49 kDa subunits and displays NADH–dehydrogenase activity, again supports a previous assembly of the NADH–dehydrogenase/hydrogenase module (30).

To this NADH–dehydrogenase/hydrogenase-containing peripheral arm (subcomplex D) other subunits associate, including the 18 kDa subunit. This is in accordance with the observed cross-links between the 18 and 49 kDa subunits, and between the 18 and 30 kDa subunits (40). Patient cells lacking subunit 18 kDa are still able to assemble an 800 kDa

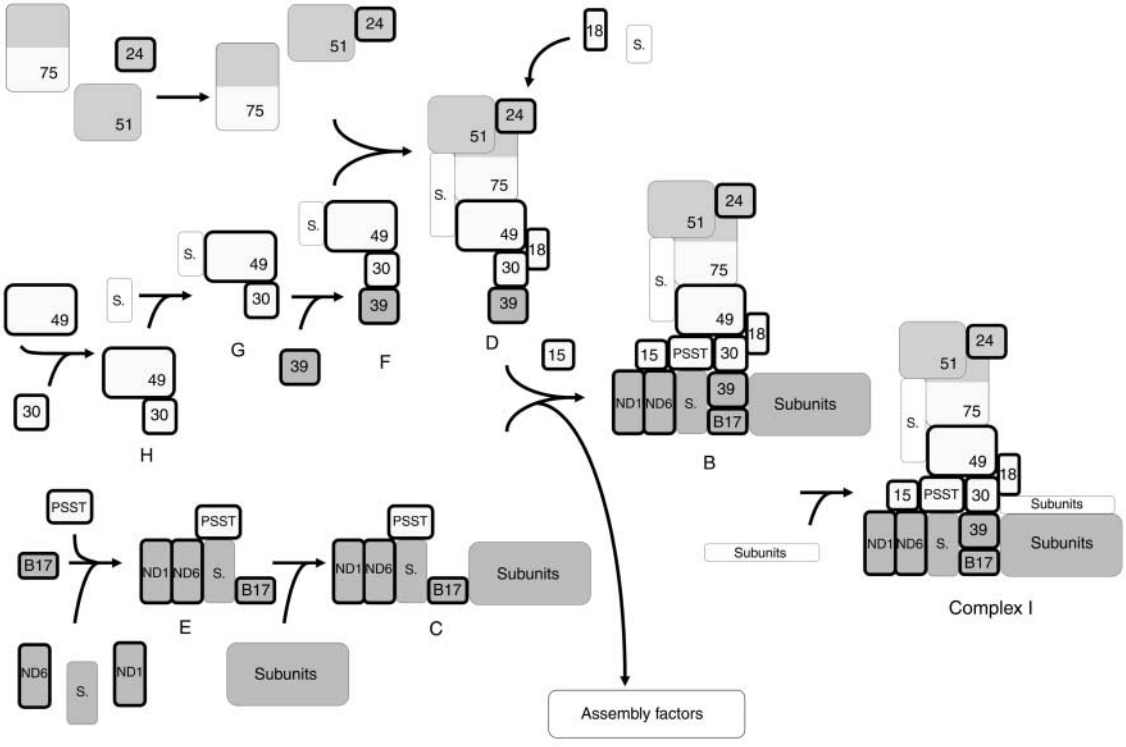

Figure 7. Proposed modular model of human complex I assembly. Subunits investigated in our study are boxed with a thick line. Subunits proposed on the basis of literature data (see Discussion) are boxed with a thin line. 'S.' and 'Subunits' indicate unidentified subunits. Observed complex I (A) and subcomplexes B–H are specified. Their molecular mass based on their electrophoretic mobility on a BN PAGE is indicated between brackets (see Discussion).

complex (9,11). This indicates that the 18 kDa subunit is acquired relatively late in the assembly, and that assembly can proceed without the 18 kDa subunit until an 800 kDa subcomplex is formed.

The membrane arm transporter module includes the B17 subunit, which is present in the β-part of the hydrophobic membrane arm (Fig. 1). B17 is accumulated as a monomeric subunit and in some possible low molecular weight subcomplexes. This subunit becomes part of the 400 kDa subcomplex E, to which other membrane arm subunits including ND1 and ND6 bind. Consistent with this finding is that there is a remarkable shift of B17 subcomplexes upon release of the mitochondrial translation block, indicating that mitochondrially encoded subunits are essential for this subunit to progress in the assembly. To the subcomplex E, also the N2 iron–sulphur cluster containing PSST subunit associates. There are several reports which are compatible with this membrane arm association of the PSST subunit. First, in mouse cells which lack ND6, the PSST subunit is absent (33). Second, chloramphenicol-treated *N. crassa* cells (21) and disruption mutants (44), in which only the peripheral arm was formed, also lack the N2 iron–sulphur cluster and thus the PSST subunit. Third, a functional coupling of PSST with ND1 also suggests a close association of these subunits (45). However, a PSST disruption mutant in *N. crassa* proved to be unable to assemble the peripheral arm (46). This suggests that PSST, which is located in the boundary between the peripheral and membrane arms, assembles to the membrane arm but is required for the stabilization of the peripheral arm. Interestingly, in methanogenic bacterium *Methanosarcina barkeri* (47) and photosynthetic bacterium *Rhodospirillum rubrum* (48), hydrogenases are organized in operons in which the gene for the PSST homologue is located next to the gene for the ND1 homologue, again illustrating a structural evolutionary conservation. Other genes of these operons are the homologues of ND5, TYKY, the 49 and 30 kDa subunits (only in *R. rubrum*).

Subsequently, other subunits associate to the membrane arm intermediate E and form subcomplex C (Figs 6 and 7). The peripheral arm (subcomplex D) and the membrane arm (subcomplex C) come together and form subcomplex B at a molecular weight of around 950 kDa. Paradoxically, two subcomplexes of 600 and 700 kDa add up to form a 950 kDa subcomplex; however, there are plausible explanations for this. First, the electrophoretic mobility of a protein or protein complex in a PAGE gel depends on charge and globular size of the molecule. When two protein complexes assemble into one complex, the resulting charge and globular size does not necessarily result in an electrophoretic mobility that corresponds to the sum of the two complexes. Second, it is described in *N. crassa* that complex I assembly proteins transiently associate with assembly intermediates (24), thereby contributing to the molecular weight of intermediate complexes but not to other subcomplexes which occur later in assembly. We also find in human cells co-localization of an assembly protein with intermediate subcomplexes but not with holo-

complex I (unpublished data). In a last step additional subunits, including the 15 kDa, are assembled to subcomplex B and finally fully assembled complex I is formed (A).

Recently, it has been demonstrated that the cyanobacteria *Synechocystis sp.* PCC 6803 contains a NDH-1 complex which resembles mitochondrial complex I (49). Although the electron import module or NADH–dehydrogenase part is not acquired in this enzyme, it contains a hydrogenase part and a transporter part. The assembly of the cyanobacterial NDH-1 was studied using 2D BN/SDS–PAGE. A hydrophilic subcomplex was observed containing the cyanobacterial homologues of the 30, 49 kDa, B13 and PSST subunits. A hydrophobic subcomplex was found which contains the homologues of ND1, ND2 and ND6. The finding of the PSST subunit in the hydrophilic part contradicts our findings, however, this can be explained by the chemical fractionation used. The authors propose that the separate modules are assembled independently. Our data support this conclusion and suggests that the modular assembly of complex I is preserved throughout evolution.

Recently, Antonicka *et al.* (10) proposed a model for complex I assembly on the basis of subcomplexes observed by 2D BN/SDS–PAGE in muscle samples of complex I-deficient patients. This model is in conflict with our proposed model at several points. First, the Antonicka model describes a 24 kDa containing subcomplex, which also contains the 18 and 20 kDa (PSST) subunits. The 20 kDa (PSST) subunit together with the 30, 49 kDa and ND1 subunits constitutes the core of the hydrogenase module. It is therefore very unlikely that the 20 kDa subunit is assembled in a subcomplex which does not contain any of these subunits and which is topologically located in another part of the complex (as discussed earlier). The 18 kDa subunit cross-links with the 49 and 30 kDa subunits (40). For this reason, it is more likely that these three subunits assemble together to form a subcomplex. Second, they propose the occurrence of a 310 kDa subcomplex containing the membrane arm subunit ND1 and the peripheral arm subunits 30–39–49 kDa, suggesting no separate formation of the peripheral and membrane arms. In contrast, we observed a co-localisation of the 30 and 39 kDa subunits with the ND1 subunit only late in the assembly, in the 950 kDa subcomplex B, which is compatible with the well-described *N. crassa* model. A possible explanation for these differences is that in the patient muscle samples investigated in the previous work, some of the proposed assembly intermediates are breakdown products resulting from an instable complex I. Increased instability of assembled complex I is known to occur for instance in cybrid cells containing an ND5 mutation (29).

In this study we present an alternative model for the assembly of complex I in the cultured human cells. The use of doxycycline-treated cultured cells allows following the dynamics of the assembly process, therefore avoiding the interference of breakdown products. This might give a more representative picture of the physiological assembly pathway of complex I. We confirm that complex I assembly is a semi-sequential process in which preassembled subcomplexes are joined to form holo-complex I. A preassembled peripheral arm is formed, which associates with a preassembled membrane arm to form an intermediate complex, to which other subunits are attached to form holo-complex I. Still much work needs to be done to elucidate more details of the assembly of all of the 46 subunits into complex I. New approaches, such as the construction of a viable complex I deletion strain of the complex I-containing yeast *Yarrowia lipolytica* (50), the generation of a mammalian conditional assembly system (33), the application of protein mass spectrometry and the analysis of newly identified Complex I-deficient patients, will aid this difficult task. Nevertheless, our proposed model for complex I assembly presents a starting point to further elucidate this intricate process and provides a framework to understand assembly defects in patients with a complex I deficiency. Moreover, information of the assembly status of complex I could provide a good prescreening method in diagnostics.

MATERIALS AND METHODS

Cell cultures

143B206 ρ^0 cells were cultured in DMEM (Life Technologies) supplemented with 5% fetal calf serum (FCS), antibiotics, 1 mM uridine and 100 μg/ml bromodeoxyuridine. HEK 293 human embryonic kidney cells and 143B osteosarcoma cells were cultured in DMEM supplemented with 10% FCS, antibiotics and 1 mM Uridine (51). 143B control cybrids (7) were cultured in DMEM (Life Technologies) supplemented with 10% FCS, antibiotics, glutamine and 1 mM sodium pyruvate. To block mitochondrial translation doxycycline was added at a concentration of 15 μg/ml (52). The cells were grown in exponential conditions and harvested at the indicated time-points.

BN electrophoresis and in-gel activity assays

BN 5–15 or 5–13% gradient gels were loaded with 20–40 μg of digitonin-isolated mitochondria as described before (52). After electrophoresis, the gels were further processed for in-gel activity assays, western blotting or second dimension 10% SDS–PAGE as described earlier (52). Proteins were transferred to a PROTAN® nitrocellulose membrane (Schleicher & Schuell).

SDS–PAGE analysis

Whole cell homogenates were prepared by resuspending 5×10^6 cells in 125 μl PBS containing 2% (w/v) *n*-dodecyl β-D-maltoside. Following a 15 min incubation on ice, homogenates were centrifuged (30 min, 12 000 g, 4°C). Next, the supernatant was mixed with an equal volume of Tricine sample buffer (Biorad laboratories, Hercules, USA) containing 2% (v/v) 2-mercaptoethanol. The mixture was kept at room temperature for 60 min. Protein (40 μg protein/lane) was separated on 10% polyacrylamide gel. Gels were blotted to PROTAN® nitrocellulose membrane (Schleicher & Schuell).

Antibodies

Western blotting was performed using primary antibodies raised against the following subunits of the human mitochondrial OXPHOS complexes: NDUFS7 (PSST), NDUFA3 (30 kDa), NDUFA9 (39 kDa), NDUFB6 (B17), NDUFS5 (15 kDa), UQCRC1, MTCO2, SDHA, (Molecular Probes), ND1

(a gift from Dr A. Lombes, France), ND6 and NDUFS4 (18 kDa) (a gift from Professor R. Capaldi, USA), NDUFV2 (24 kDa) (donated by Professor J. Walker, UK), NDUFS2 (49 kDa) (provided by Professor B. Robinson, Canada) and Hsp70 antibody (Affinity Bioreagents, Golden, USA). Peroxidase-conjugated anti-mouse IgGs or peroxidase-conjugated anti-rabit IgGs were used as secondary antibody (Molecular Probes). The signal was detected with ECL® plus (Amersham Biosciences) and the quantification of the blots was performed using ImagePro-Plus 4.1 image analysis software (Media Cybernetics, Silver Spring, MD, USA).

Protein assay

The protein concentration for BN PAGE and SDS–PAGE was determined in the *n*-dodecyl β-D-maltoside solubilized supernatants before adding Coomassie blue containing sample buffer, using a MicroBCA protein assay kit (Pierce).

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REFERENCES

1. Smeitink, J., van den Heuvel, L. and DiMauro, S. (2001) The genetics and pathology of oxidative phosphorylation. *Nat. Rev. Genet.*, **2**, 342–352.
2. Nijtmans, L.G.J., Ugalde, C., van den Heuvel, L.P. and Smeitink, J.A.M. (2004) Function and dysfunction of the oxidative phosphorylation system. In Bauer, M. Koehler, C. (eds), *Topics in Current Genetics. Mitochondrial Function and Biogenesis.* Springer, Heiderberg, Germany, Vol. 8, pp. 149–176.
3. Hirst, J., Carroll, J., Fearnley, I.M., Shannon, R.J. and Walker, J.E. (2003) The nuclear-encoded subunits of complex I from bovine heart mitochondria. *Biochim. Biophys. Acta*, **1604**, 135–150.
4. Triepels, R.H., van den Heuvel, L.P., Trijbels, J.M. and Smeitink, J.A. (2001) Respiratory chain complex I deficiency. *Am. J. Med. Genet.*, **106**, 37–45.
5. DiMauro, S. and Schon, E.A. (2003) Mitochondrial respiratory-chain diseases. *N. Engl. J. Med.*, **348**, 2656–2668.
6. Benit, P., Slama, A., Cartault, F., Giurgea, I., Chretien, D., Lebon, S., Marsac, C., Munnich, A., Rotig, A. and Rustin, P. (2004) Mutant NDUFS3 subunit of mitochondrial complex I causes Leigh syndrome. *J. Med. Genet.*, **41**, 14–17.
7. Ugalde, C., Triepels, R.H., Coenen, M.J.H., van den Heuvel, L.P., Smeets, R., Uusimaa, J., Briones, P., Campistol, J., Majamaa, K., Smeitink, J.A.M. and Nijtmans, L.G.J. (2003) Impaired complex I assembly in a Leigh syndrome patient with a novel missense mutation in the *ND6* gene. *Ann. Neurol.*, **54**, 665–669.
8. Barrientos, A., Barros, M.H., Valnot, I., Rotig, A., Rustin, P. and Tzagoloff, A. (2002) Cytochrome oxidase in health and disease. *Gene*, **286**, 53–63.
9. Ugalde, C., Janssen, R.J.R.J., Smeitink, J.A.M. and Nijtmans, L.G.J. (2004) Differences in assembly or stability of complex I and other mitochondrial OXPHOS complexes in inherited complex I deficiency. *Hum. Mol. Genet.*, **13**, 659–667.
10. Antonicka, H., Ogilvie, I., Taivassalo, T., Anitori, R.P., Haller, R.G., Vissing, J., Kennaway, N.G. and Shoubridge, E.A. (2003) Identification and characterization of a common set of complex I assembly intermediates in mitochondria from patients with complex I deficiency. *J. Biol. Chem.*, **278**, 43081–43088.
11. Scacco, S., Petruzzella, V., Budde, S., Vergari, R., Tamborra, R., Panelli, D., van den Heuvel, L.P., Smeitink, J.A. and Papa, S. (2003) Pathological mutations of the human *NDUFS4* gene of the 18-kDa (AQDQ) subunit of complex I affect the expression of the protein and the assembly and function of the complex. *J. Biol. Chem.*, **278**, 44161–44167.
12. Grigorieff, N. (1999) Structure of the respiratory NADH:ubiquinone oxidoreductase (complex I). *Curr. Opin. Struct. Biol.*, **9**, 476–483.
13. Galante, Y.M. and Hatefi, Y. (1978) Resolution of complex I and isolation of NADH–dehydrogenase and an iron–sulfur protein. *Methods Enzymol.*, **53**, 15–21.
14. Sazanov, L.A., Peak-Chew, S.Y., Fearnley, I.M. and Walker, J.E. (2000) Resolution of the membrane domain of bovine complex I into subcomplexes: implications for the structural organization of the enzyme. *Biochemistry*, **39**, 7229–7235.
15. Carroll, J., Shannon, R.J., Fearnley, I.M., Walker, J.E. and Hirst, J. (2002) Definition of the nuclear encoded protein composition of bovine heart mitochondrial complex I. Identification of two new subunits. *J. Biol. Chem.*, **277**, 50311–50317.
16. Carroll, J., Fearnley, I.M., Shannon, R.J., Hirst, J. and Walker, J.E. (2003) Analysis of the subunit composition of complex I from bovine heart mitochondria. *Mol. Cell. Proteomics*, **2**, 117–126.
17. Murray, J., Zhang, B., Taylor, S.W., Oglesbee, D., Fahy, E., Marusich, M.F., Ghosh, S.S. and Capaldi, R.A. (2003) The subunit composition of the human NADH–dehydrogenase obtained by rapid one-step immunopurification. *J. Biol. Chem.*, **278**, 13619–13622.
18. Friedrich, T. and Weiss, H. (1997) Modular evolution of the respiratory NADH: ubiquinone oxidoreductase and the origin of its modules. *J. Theor. Biol.*, **187**, 529–540.
19. Vogel, R., Nijtmans, L., Ugalde, C., van den Heuvel, L. and Smeitink, J. (2004) Complex I assembly: a puzzling problem. *Curr. Opin. Neurol.*, **17**, 179–186.
20. Videira, A. and Duarte, M. (2001) On complex I and other NADH: ubiquinone reductases of *Neurospora crassa* mitochondria. *J. Bioenerg. Biomembr.*, **33**, 197–203.
21. Tuschen, G., Sackmann, U., Nehls, U., Haiker, H., Buse, G. and Weiss, H. (1990) Assembly of NADH–ubiquinone reductase (complex I) in *Neurospora* mitochondria. Independent pathways of nuclear-encoded and mitochondrially encoded subunits. *J. Mol. Biol.*, **213**, 845–857.
22. Duarte, M., Sousa, R. and Videira, A. (1995) Inactivation of genes encoding subunits of the peripheral and membrane arms of *Neurospora* mitochondrial complex I and effects on enzyme assembly. *Genetics*, **139**, 1211–1221.
23. Schulte, U., Fecke, W., Krull, C., Nehls, U., Schmiede, A., Schneider, R., Ohnishi, T. and Weiss, H. (1994) *In-Vivo* dissection of the mitochondrial respiratory NADH–ubiquinone oxidoreductase (complex I). *Biochim. Biophys. Acta*, **1187**, 121–124.
24. Kuffner, R., Rohr, A., Schmiede, A., Krull, C. and Schulte, U. (1998) Involvement of two novel chaperones in the assembly of mitochondrial NADH:ubiquinone oxidoreductase (complex I). *J. Mol. Biol.*, **283**, 409–417.
25. Janssen, R., Smeitink, J., Smeets, R. and van den Heuvel, L. (2002) CIA30 complex I assembly factor: a candidate for human complex I deficiency? *Hum. Genet.*, **110**, 264–270.
26. Hall, R.E. and Hare, J.F. (1990) Respiratory chain-linked NADH–dehydrogenase. Mechanisms of assembly. *J. Biol. Chem.*, **265**, 16484–16490.
27. Hofhaus, G. and Attardi, G. (1993) Lack of assembly of mitochondrial DNA-encoded subunits of respiratory NADH–dehydrogenase and loss of enzyme activity in a human cell mutant lacking the mitochondrial *ND4* gene product. *EMBO J.*, **12**, 3043–3048.
28. Bai, Y.D. and Attardi, G. (1998) The mtDNA-encoded ND6 subunit of mitochondrial NADH–dehydrogenase is essential for the assembly of the membrane arm and the respiratory function of the enzyme. *EMBO J.*, **17**, 4848–4858.
29. Hofhaus, G. and Attardi, G. (1995) Efficient selection and characterization of mutants of a human cell-line which are defective in mitochondrial

DNA-encoded subunits of respiratory NADH–dehydrogenase. *Mol. Cell. Biol.*, **15**, 964–974.
30. Cardol, P., Matagne, R.F. and Remacle, C. (2002) Impact of mutations affecting ND mitochondria-encoded subunits on the activity and assembly of complex I in *Chlamydomonas*. Implication for the structural organization of the enzyme. *J. Mol. Biol.*, **319**, 1211–1221.
31. Nijtmans, L.G.J., Klement, P., Houstek, J. and van den Bogert, C. (1995) Assembly of mitochondrial ATP synthase in cultured human cells: implications for mitochondrial diseases. *Biochim. Biophys. Acta*, **1272**, 190–198.
32. Nijtmans, L.G.J., Taanman, J.W., Muijsers, A.O., Speijer, D. and van den Bogert, C. (1998) Assembly of cytochrome-c oxidase in cultured human cells. *Eur. J. Biochem.*, **254**, 389–394.
33. Yadava, N., Houchens, T., Potluri, P. and Scheffler, I.E. (2004) Development and characterization of a conditional mitochondrial complex I assembly system. *J. Biol. Chem.*, **279**, 12406–12413.
34. Acin-Perez, R., Bayona-Bafaluy, M.P., Fernandez-Silva, P., Moreno-Loshuertos, R., Perez-Martos, A., Bruno, C., Moraes, C.T. and Enriquez, J.A. (2004) Respiratory complex III is required to maintain complex I in mammalian mitochondria. *Mol. Cell*, **13**, 805–815.
35. Nijtmans, L.G., Henderson, N.S., Attardi, G. and Holt, I.J. (2001) Impaired ATP synthase assembly associated with a mutation in the human ATP synthase subunit 6 gene. *J. Biol. Chem.*, **276**, 6755–6762.
36. El Meziane, A., Lehtinen, S.K., Hance, N., Nijtmans, L.G.J., Dunbar, D., Holt, I.J. and Jacobs, H.T. (1998) A tRNA suppressor mutation in human mitochondria. *Nat. Genet.*, **18**, 350–353.
37. Tiranti, V., Galimberti, C., Nijtmans, L., Bovolenta, S., Perini, M.P. and Zeviani, M. (1999) Characterization of *SURF-1* expression and Surf-1p function in normal and disease conditions. *Hum. Mol. Genet.*, **8**, 2533–2540.
38. Williams, S.L., Valnot, I., Rustin, P. and Taanman, J.W. (2004) Cytochrome c oxidase subassemblies in fibroblast cultures from patients carrying mutations in *COX10*, *SCO1*, or *SURF1*. *J. Biol. Chem.*, **279**, 7462–7469.
39. Videira, A. (1998) Complex I from the fungus *Neurospora crassa*. *Biochim. Biophys. Acta*, **1364**, 89–100.
40. Yamaguchi, M. and Hatefi, Y. (1993) Mitochondrial NADH–ubiquinone oxidoreductase (complex-I). Proximity of the subunits of the flavoprotein and the iron sulfur protein subcomplexes. *Biochemistry*, **32**, 1935–1939.
41. Yagi, T., Yano, T., Di Bernardo, S. and Matsuno-Yagi, A. (1998) Procaryotic complex I (NDH-1), an overview. *Biochim. Biophys. Acta*, **1364**, 125–133.
42. Finel, M. (1998) Organization and evolution of structural elements within complex I. *Biochim. Biophys. Acta*, **1364**, 112–121.
43. Pilkington, S.J., Skehel, J.M., Gennis, R.B. and Walker, J.E. (1991) Relationship between mitochondrial NADH ubiquinone reductase and a bacterial NAD-reducing hydrogenase. *Biochemistry*, **30**, 2166–2175.
44. Nehls, U., Friedrich, T., Schmiede, A., Ohnishi, T. and Weiss, H. (1992) Characterization of assembly intermediates of NADH:ubiquinone oxidoreductase (complex I) accumulated in *Neurospora* mitochondria by gene disruption. *J. Mol. Biol.*, **227**, 1032–1042.
45. Schuler, F. and Casida, J.E. (2001) Functional coupling of PSST and ND1 subunits in NADH:ubiquinone oxidoreductase established by photoaffinity labeling. *Biochim. Biophys. Acta*, **1506**, 79–87.
46. Duarte, M., Populo, H., Videira, A., Friedrich, T. and Schulte, U. (2002) Disruption of iron–sulphur cluster N2 from NADH:ubiquinone oxidoreductase by site-directed mutagenesis. *Biochem. J.*, **364**, 833–839.
47. Kunkel, A., Vorholt, J.A., Thauer, R.K. and Hedderich, R. (1998) An *Escherichia coli* hydrogenase-3-type hydrogenase in methanogenic archaea. *Eur. J. Biochem.*, **252**, 467–476.
48. Fox, J.D., He, Y., Shelver, D., Roberts, G.P. and Ludden P.W. (1996) Characterization of the region encoding the CO-induced hydrogenase of *Rhodospirillum rubrum*. *J. Bacteriol.*, **178**, 6200–6208.
49. Prommeenate, P., Lennon, A.M., Markert, C., Hippler, M. and Nixon, P.J. (2004) Subunit composition of NDH-1 complexes of *Synechocystis sp.* PCC 6803: identification of two new ndh gene products with nuclear-encoded homologues in the chloroplast Ndh complex. *J. Biol. Chem.*, **279**, 28165–28173.
50. Kerscher, S.J., Eschemann, A., Okun, P.M. and Brandt, U. (2001) External alternative NADH:ubiquinone oxidoreductase redirected to the internal face of the mitochondrial inner membrane rescues complex I deficiency in *Yarrowia lipolytica*. *J. Cell Sci.*, **114**, 3915–3921.
51. King, M.P. and Attardi, G. (1989) Human-cells lacking mtDNA. Repopulation with exogenous mitochondria by complementation. *Science*, **246**, 500–503.
52. Nijtmans, L.G.J., Henderson, N.S. and Holt, I.J. (2002) Blue native electrophoresis to study mitochondrial and other protein complexes. *Methods*, **26**, 327–334.