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Modulation of oxidative phosphorylation of human kidney 293 cells by transfection with the internal rotenone-insensitive NADH–quinone oxidoreductase (*NDI1*) gene of *Saccharomyces cerevisiae*

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Abstract

In contrast to the mitochondrial proton-translocating NADH–quinone oxidoreductase (complex I), which consists of at least 43 different subunits, the internal rotenone-insensitive NADH–quinone oxidoreductase (Ndi1) of *Saccharomyces cerevisiae* is a single polypeptide enzyme. The *NDI1* gene was stably transfected into the human embryonal kidney 293 (HEK 293) cells. The transfected *NDI1* gene was then transcribed and translated in the HEK 293 cells to produce the functional enzyme. The immunochemical and immunofluorescence analyses indicated that the expressed Ndi1 polypeptide was located to the inner mitochondrial membranes. The expression of Ndi1 did not alter the content of existing complex I in the HEK 293 mitochondria, suggesting that the expressed Ndi1 enzyme does not displace the endogenous complex I. The NADH oxidase activity of the *NDI1*-transfected HEK 293 cells was not affected by rotenone but was inhibited by flavone. The ADP/O ratios coupled to NADH oxidation were lowered from 2.4 to 1.8 by *NDI1*-transfection while the ADP/O ratios coupled to succinate oxidation (1.6) were not changed. The *NDI1*-transfected HEK 293 cells were able to grow in media containing a complex I inhibitor such as rotenone and 1-methyl-4-phenylpyridinium ion. The potential usefulness of incorporating the Ndi1 protein into mitochondria of human cells is discussed.

Keywords: NADH dehydrogenase; Mammalian cell; Mitochondrial disease; Oxidative phosphorylation; Gene therapy

Abbreviations: Q, quinone; UQ10, ubiquinone-10; complex I, the mitochondrial proton-translocating NADH-Q oxidoreductase; NDH-1, the bacterial proton-translocating NADH-Q oxidoreductase; NDH-2, the bacterial NADH-Q oxidoreductase lacking the energy coupling site; EPR, electron paramagnetic resonance; Ndi1, internal rotenone-insensitive NADH-Q oxidoreductase of *S. cerevisiae* mitochondria; MPP^+, 1-methyl-4-phenylpyridinium ion; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; FP and IP, flavoprotein and iron–sulfur protein fractions of complex I; ROS, reactive oxygen species; SOD, superoxide dismutase; EGTA, ethylene glycol-bis-(β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetate; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; DMEM, Dulbecco's modified Eagle's medium; SDS, sodium dodecyl sulfate; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; HEK, human embryonic kidney; ORF, open reading frame; ECL, enhanced chemiluminescence

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1. Introduction

The NADH–quinone (NADH-Q) oxidoreductase of the respiratory chains can be divided into three groups [1–3]. They are the proton-translocating NADH-Q oxidoreductase (designated complex I in mitochondria and NDH-1 in bacteria) [1,3], the Na^+-translocating NADH-Q oxidoreductase (this type of enzyme complex has been reported in bacteria only) [4–8], and the NADH-Q oxidoreductase lacking an energy coupling site [1,3]. Mammalian mitochondria are believed to contain only complex I as the NADH dehydrogenase in the respiratory chain. It is generally accepted that mammalian complex I is composed of at least 43 unlike subunits and has the most intricate structure of the membrane-associated enzyme complexes [2,9–12]. Of these 43 subunits, seven are encoded by mitochondrial DNA and the others are encoded by nuclear DNA [13,14]. Complex I bears noncovalently bound FMN and at least eight iron–sulfur clusters as prosthetic groups [15,16]. At the present time, at least five EPR-detectable iron–sulfur clusters have been identified [9,10]. This enzyme complex is sensitive to rotenone, piericidin A, rolliniastatin, and capsaicin [17,18]. Complex I has been shown to be a one-electron reaction enzyme [19]. In contrast to mammalian mitochondria, mitochondria of *Saccharomyces cerevisiae* lack complex I but instead have two rotenone-insensitive NADH-Q oxidoreductases [20–22]. These two NADH-Q oxidoreductases in *S. cerevisiae* do not function as proton-translocating enzymes; one faces the intermembrane space (referred to as external rotenone-insensitive NADH-Q oxidoreductase). The other, like the mammalian complex I, faces the mitochondrial matrix (designated internal rotenone-insensitive NADH-Q oxidoreductase (Ndi1)) [20–22].

The Ndi1 enzyme of *S. cerevisiae* mitochondria is a single polypeptide enzyme with noncovalently bound FAD as a cofactor and no iron–sulfur clusters [20]. The *S. cerevisiae* Ndi1 is inhibited by flavone but not by rotenone [1,3,20]. Because neither FADH intermediates nor semi-quinone EPR signals have been detected in the Ndi1 enzyme [20], it has been suggested that, unlike complex I, the Ndi1 enzyme is a two-electron reaction enzyme. The *NDI1* gene encoding the enzyme has been cloned and sequenced [22]. The DNA sequence reveals the presence of an ORF of 1539 bp predicted to encode a precursor protein of 513 amino acid residues, which includes the NH_2 terminal 26-residue signal sequence for import into mitochondria. These 26 residues are cleaved off to form the mature protein [22] which is attached to the inner mitochondrial membrane on the matrix side.

As we have recently reported [23,24], *S. cerevisiae* Ndi1 is a versatile enzyme as demonstrated by the fact that the Ndi1 enzyme expressed in *Escherichia coli* acts as a member of the respiratory chain in the prokaryotic host cells. Furthermore, the Ndi1 enzyme expressed in complex I-deficient Chinese hamster cells restores the NADH oxidase activity to the parent cell level. These studies suggest that the *NDI1* gene may provide a potentially useful tool for gene therapy of mitochondrial diseases caused by complex I deficiency. As the next step to explore this potential, it was imperative to verify that the *NDI1* gene could be functionally expressed in mitochondria of human cells bearing their own inherent complex I. It would be of particular interest to investigate the following: (a) whether the expressed Ndi1 displaces complex I in the inner mitochondrial membranes; (b) whether the expression of the Ndi1 in human cells modulates the oxidative phosphorylation in host cell mitochondria (e.g., ADP/O ratios, rotenone sensitivity, etc.); and (c) whether the expressed Ndi1 enzyme protects cells against free radical complications resulting from complex I catalyzing a one-electron reaction. This latter point is of interest because it is known that free radicals are generated by mammalian mitochondrial respiratory chain activity at 1–2% of all electrons passing down the electron transport chain [25].

In this paper, we demonstrate that the *S. cerevisiae* *NDI1* gene can be transcribed and translated in human embryonal kidney 293 cells. The expressed Ndi1 was incorporated predominantly into mitochondria which was not unexpected given the presence of the leader sequence in the precursor Ndi1. In addition, the expressed enzyme modified the characteristics of oxidative phosphorylation in the host cells, although the content of complex I in HEK 293 mitochondria was unchanged by *NDI1* transfection. The NADH oxidase activity of Ndi1-overexpressed cells was insensitive to rotenone, but sensitive to flavone. The ADP/O ratios of oxidative phosphorylation sus-

tained by the NADH oxidase activity in the transfected HEK 293 cell mitochondria were decreased from 2.4 to 1.8 owing to the *NDI1* overexpression, whereas the Ndi1 overexpression did not affect the ADP/O ratios (1.6) associated with succinate oxidation. Finally, although the nontransfected HEK 293 cells could not survive in media containing rotenone or 1-methyl-4-phenylpyridinium ion (MPP^+), the *NDI1*-transfected cells were resistant to these reagents.

2. Materials and methods

2.1. Construction of NDI1-expression plasmid

A 5.5 kbp *Kpn*I/*Pst*I DNA fragment bearing the full-length *NDI1* was excised from the λ 7056 (approximately 17 kbp *S. cerevisiae* DNA inserted) and ligated into a *Kpn*I/*Pst*I-cut cloning vector pTZ19U. The resulting plasmid was designated pKP5.5. A 2.3 kbp *Sal*I/*Eco*RV fragment, again containing the full-length *NDI1*, was then isolated from pKP5.5 and ligated into a *Sal*I/*Sma*I-cut cloning vector pTZ18U. The resulting plasmid was designated pRVS2.3. Two oligonucleotide primers were employed. One was to generate a *Kpn*I recognition site 60 bp upstream from the initiation codon of the *NDI1* gene: 5′-TCAGGTAG*GGTACC*AGTT-3′ (the underlined bases represent variations from the *S. cerevisiae* DNA, and italicized bases indicate the *Kpn*I site). The other primer was to construct a *Bgl*II site 213 bp downstream from the termination codon of the *NDI1* gene: 5′-AGTGATCAA*CAGATC*TTG-3′ (the underlined bases were mutated from *S. cerevisiae* DNA, and italicized bases represent the *Bgl*II site). Using pRVS2.3 as the template, site-specific mutagenesis was carried out [23]. The resulting plasmid was designated pRVS(KpnI, BglII). The pRVS(KpnI, BglII) construct was cut with *Kpn*I and *Bgl*II, and the 1.9 kbp *Kpn*I/*Bgl*II fragment containing the full-length *NDI1* gene (1539 bp) was ligated into the *Kpn*I/*Bam*HI site in the mammalian expression vector pHook-2 (Invitrogen). The pHook-2 uses the Rous sarcoma virus promoter to express and display a single-chain antibody against a specific hapten on the surface of transfected cells. The pHook-2 also contains the human immediate early cytomegalovirus promoter in the upstream region of a multiple cloning site to express the gene of interest. This plasmid is useful for both transient and stable transfection. The resulting expression plasmid was designated pHook(NDI1). The construct was verified by DNA sequencing of both strands as described previously [24].

2.2. NDI1 gene transfection

The 293 cells of human embryonal kidney was grown in DMEM supplemented with 10% horse serum, 5 mM galactose, and 50 μg/ml gentamycin. Cells were maintained at 37°C in a 5% CO_2 atmosphere.

The HEK 293 cells (1×10^5) in 1 ml of DMEM containing 5 mM galactose and 10% horse serum were transfected with 8–10 μg of pHook(NDI1) by the calcium phosphate precipitation method [26]. The transfected cell lines were isolated by screening with 0.5 mg/ml of antibiotic G418 and 0.1 μM rotenone. Approximately 10 cell lines were isolated. The isolated cell lines exhibited various levels of *NDI1* expression. Five cell lines indicated high levels of *NDI1* expression. These selected cell lines have displayed the same properties. Of these, one cell line (designated H47) was used in this paper. One cell line (designated M49) showed medium level of *NDI1* expression. This cell line was also used in this paper. The *NDI1*-transfected HEK 293 cells were grown in DMEM+10% horse serum+0.5 mg/ml G418+0.1 μM rotenone in the presence of 5 mM galactose.

2.3. Isolation of mitochondria and mitochondrial membrane fraction

Mitochondria were isolated from freshly harvested cells essentially according to Trounce et al. [27]. The nontransfected and *NDI1*-transfected HEK 293 cells (approximately 1×10^9 cells) were washed twice with phosphate-buffered saline (PBS) and harvested by trypsinization. The pellets were suspended in 5 ml of a buffer containing 210 mM mannitol, 70 mM sucrose, 1 mM EGTA, 5 mM Hepes (pH 7.2), 0.2 mM PMSF, and 0.5% fatty acid free bovine serum albumin (isolation buffer). The cell suspensions were treated with 1–2 mg/ml of digitonin for 1 min on ice. The digitonin-treated suspension was then diluted

10-fold with isolation buffer and centrifuged at $3000 \times g$ for 5 min to remove excess detergent. The cell pellet was resuspended with the isolation buffer and homogenized using a tight fitting Dounce homogenizer (15–20 up/down strokes). The homogenate was centrifuged at $10\,000 \times g$ for 20 min at 4°C to separate cell membrane fragments from the remaining intact organelles. The pellet was suspended in 0.1 ml of the isolation buffer and used as the intact mitochondrial fraction. The intact mitochondria were briefly sonicated and centrifuged at $150\,000 \times g$ for 30 min at 4°C. The pellets were resuspended in 0.1 ml of the isolation buffer and used as the mitochondrial membrane fraction.

2.4. Digitonin-permeabilized cells

Digitonin, by binding to cholesterol in the eukaryotic plasma membrane, creates pores through which the soluble components of the cell can be released [28]. Because the intracellular membranes have a cholesterol content substantially lower than the plasma membrane, the mitochondria and the cytoskeleton are left intact [29]. Thus, permeabilization with digitonin allows the mitochondria ready access to the respiratory substrates and inhibitors (glutamate, succinate, etc.) to be tested. It should be noted, however, that in this assay system NADH added exogenously does not serve as a substrate for complex I because NADH provided exogenously is present on the cytoplasmic side and is unable to penetrate into the mitochondrial matrix. When malate plus glutamate are employed as respiratory substrates, the corresponding dehydrogenases generate NADH from NAD in the matrix compartment, which can then be oxidized by complex I. This assay procedure is reliable and is not disturbed by various diaphorases.

2.5. Other analytical procedures

Protein was estimated by the bicichoninic acid (BCA) method (Pierce). SDS–polyacrylamide gel electrophoresis was carried out by the modified method of Laemmli [30]. Immunoblotting [31–33], measurements of respiratory chain activities by digitonin-permeabilized cells [29] and intact mitochondria [27], and immunofluorescence [34] were done according to the references cited. Any variations from the procedures and other details are described in the figure legends.

2.6. Materials

Antisera specific to FP 51 kDa, FP 24 kDa, IP 49 kDa, and IP 30 kDa subunits of bovine heart complex I and to β-subunit of bovine F_1-ATPase were generous gifts from Prof. Youssef Hatefi (The Scripps Research Institute). Prototype antiserum specific to human mitochondria was a generous gift from Prof. Eng M. Tan (The Scripps Research Institute). This serum, from a patient with primary biliary cirrhosis, reacted with the pyruvate dehydrogenase complex E2 subunit (70 kDa) and the 55 kDa polypeptide in human mitochondria. Acrylamide, *N*,*N*′-methylenebis(acrylamide), SDS, SDS–PAGE calibration marker proteins, Coomassie Brilliant blue R-250, and the in vitro mutagenesis kit were from Bio-Rad; NADH, ADP, flavone, rotenone, and antimycin A were from Sigma; 1-methyl-4-phenylpyridium iodide (MPP^+) was from Research Biochemicals International (Natick, MA); enhanced chemiluminescence (ECL) kits were from Amersham; the pHook-2 vector was from Invitrogen.

3. Results

3.1. Expression of the yeast NDI1 in HEK 293 cells

We have previously reported that the yeast *NDI1* gene can be functionally overexpressed in *E. coli* membranes and that the expressed Ndi1 enzyme supplants the existing NDH-1 and NDH-2 functions in terms of electron transfer from NADH to Q [23]. In addition, we demonstrated that the Ndi1 enzyme could be functionally expressed in complex I-deficient Chinese hamster CCL16-B2 cells and that the expressed Ndi1 restored the NADH oxidation activity in the mitochondria [24]. In order to pursue the use of the *NDI1* gene as a potential therapy for complex I deficiency in human mitochondria, it is a prerequisite to investigate (a) whether the yeast *NDI1* gene can be stably transfected into human cells, (b) whether the yeast *NDI1* gene can be functionally expressed in human mitochondria, and (c) whether the expressed Ndi1 can control NADH oxidation in hu-

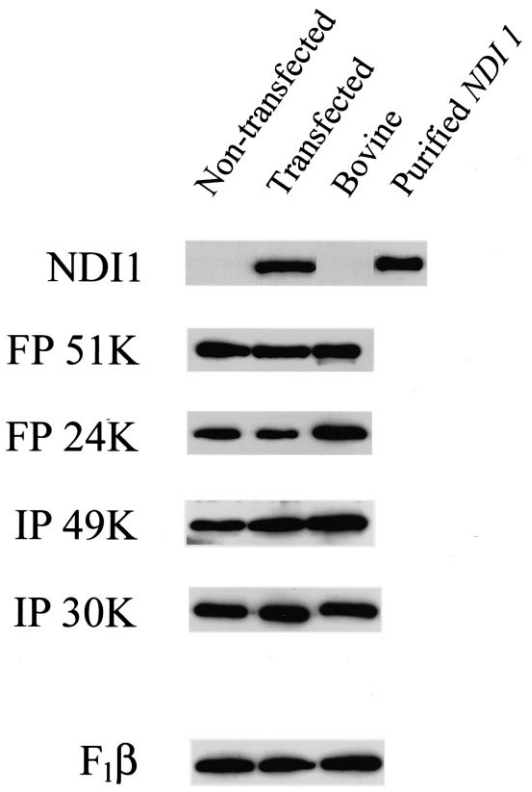


Fig. 1. Immunoblotting analyses of mitochondrial membranes isolated from nontransfected and *NDI1*-transfected HEK 293 cells, and bovine heart mitochondria by using antibodies specific to the Ndi1, complex I FP 51 kDa subunit, complex I FP 24 kDa subunit, complex I IP 49 kDa subunit, complex I IP 30 kDa subunit, and the β-subunit of bovine F_1-ATPase. The rightmost lane is the immunoblot of the Ndi1 protein containing the leader sequence as a standard. The amount of protein loaded in each lane was adjusted to give similar responses with respect to the β-subunit of F_1 ATPase. Immunoblotting was performed by use of the ECL system (Amersham).

man mitochondria. In order to pursue answers to these questions, we attempted to transfect a HEK 293 cell line with the yeast *NDI1* gene. The HEK 293 cell line was selected because it is known to be efficiently transfected by foreign DNA. Our efforts yielded several stable *NDI1*-transfected cell lines which were established from single colonies after selection with the antibiotic G418 and rotenone. Even after 4 months of culture the transfected cells retained their properties, providing evidence for stable transfection.

Immunofluorescence microscopic analyses using antibodies to human mitochondria and the yeast Ndi1 polypeptide were employed to identify the location of the expressed *NDI1* gene product in the cells. The data indicated that the expressed Ndi1 was predominantly localized in the mitochondria of the HEK cells. In order to confirm these results, the mitochondrial membranes were isolated from the *NDI1*-transfected and nontransfected HEK 293 cells and were subjected to SDS–PAGE followed by immunoblotting with the affinity-purified antibody to the yeast *NDI1* gene product (Fig. 1). The data indicate that the antibody specific to the yeast Ndi1 enzyme reacted with mitochondria of the transfected cells but did not react with mitochondria of nontransfected cells. These data suggest that the leader sequence of the yeast *NDI1* gene successfully guides

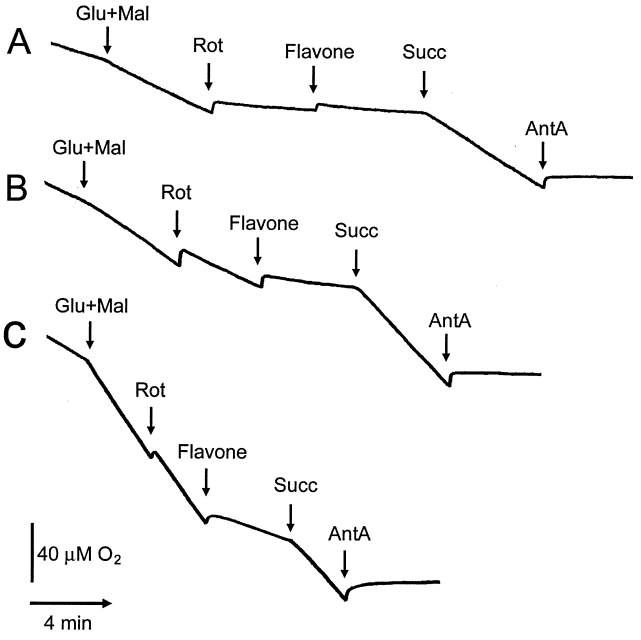
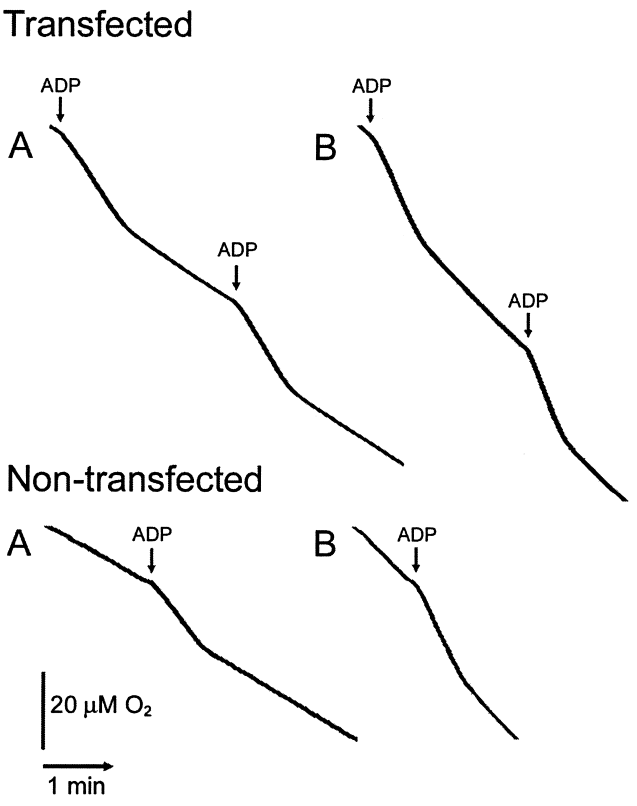


Fig. 2. Comparison of the NADH oxidase activity of digitonin-permeabilized nontransfected HEK 293 cells (A), the *NDI1*-transfected HEK 293 cells with medium level of expression (M49) (B), and the *NDI1*-transfected HEK 293 cells with high levels of expression (H47) (C) (at 4×10^7 cells/ml). The cells were harvested by trypsinization and resuspended in 1 ml of a medium containing 20 mM Hepes (pH 7.1), 250 mM sucrose, and 10 mM $MgCl_2$. The cells were treated with 50–150 μg of digitonin until more than 90% of the cells are stained by trypan blue. The digitonin-treated cells were washed with the same medium. Oxygen consumption was measured polarographically in 0.6 ml of the buffer containing 20 mM Hepes (pH 7.1), 250 mM sucrose, and 10 mM $MgCl_2$ by using a Clark electrode in a water-jacketed chamber maintained at 37°C. Where indicated, 5 mM glutamate (Glu), 5 mM malate (Mal), 5 μM rotenone (Rot), 0.5 mM flavone (Flavone), 5 mM succinate (Succ), and 5 μM antimycin A (AntA) were added.

the product to the mitochondria in both human cells and Chinese hamster cells [24]. Also shown in Fig. 1 is the result of immunoblotting with antibodies directed to the FP 51 kDa, FP 24 kDa, IP 49 kDa and the IP 30 kDa subunits of bovine heart complex I [9,10,31,35]. The data reveal that there is no apparent difference in mitochondria between nontransfected and the *NDI1*-transfected 293 cells in terms of the content of complex I subunits. This is not surprising because Ndi1-type enzyme and complex I are known to coexist in plant and certain fungal mitochondria as well as in bacteria [1,2].

3.2. *Effect of NDI1 expression on the electron transport activity*

As reported in our previous paper [24], the expressed yeast Ndi1 enzyme restores the NADH oxidase activity of complex I-deficient Chinese hamster CCL16-B2 cells. Furthermore, in the case of *E. coli*, the NADH oxidase activity derived from the expressed Ndi1 enzyme became significantly higher than that of the native NDH-1 and NDH-2 [23]. As described above, the expression of Ndi1 does not appear to affect the content of complex I in the mitochondria of HEK 293 cells. Therefore, it was of interest to investigate the contribution of complex I and the expressed Ndi1 to the overall electron transport activity of the transfected HEK 293 cells. The activity measurements were performed using digitonin-permeabilized nontransfected and *NDI1*-transfected HEK 293 cells and the results are shown in Fig. 2. The nontransfected cells exhibited the expected malate/glutamate-dependent respiratory activity, which was completely inhibited by rotenone. When succinate was added, the respiration was stimulated. This stimulation was completely inhibited by antimycin A and KCN. The respiration activity of the *NDI1*-transfected cells with medium levels of expression (M49) induced by malate/glutamate was partially inhibited by rotenone and then completely inhibited by addition of flavone. The respiratory activity of the *NDI1*-transfected cells with high levels of expression (H47) was increased significantly over that observed in the nontransfected cells by addition of malate and glutamate. This increase was not inhibited by rotenone, but was inhibited by flavone [20]. These results indicate that the NADH oxidase



Cells	ADP/O Ratios	
	Glu + Mal	Succinate
Transfected	1.75	1.56
Non-transfected	2.41	1.60

Fig. 3. Comparison of ADP/O ratios in nontransfected and *NDI1*-transfected (H47) HEK 293 cells using glutamate+malate and succinate as respiratory substrates. The *NDI1*-transfected mitochondria (2.3 mg/ml) and nontransfected mitochondria (1.7 mg/ml) were prepared as described in Section 2. In panel A, glutamate+malate were used as respiratory substrates; In panel B, succinate was used as the respiratory substrate. Glutamate, malate and succinate were added at 5 mM. Where indicated, 50 nmol ADP (pH 7.0) were added. The assay medium was composed of 225 mM mannitol, 75 mM sucrose, 10 mM KCl, 10 mM Tris–HCl (pH 7.2), 2.5 mM $MgCl_2$ and 10 mM KP_i (pH 7.2). The oxygraph measurements were carried out as described in Fig. 2 except that the reaction temperature was 30°C. The ADP/O ratios determined by the oxygraph analyses are summarized in the inset table (each ADP/O ratio is an average of five experiments).

activity observed in the *NDI1* expressed HEK 293 cells is ascribed almost entirely to the Ndi1 enzyme despite the fact that complex I is still present. NADH, which when added exogenously is unable

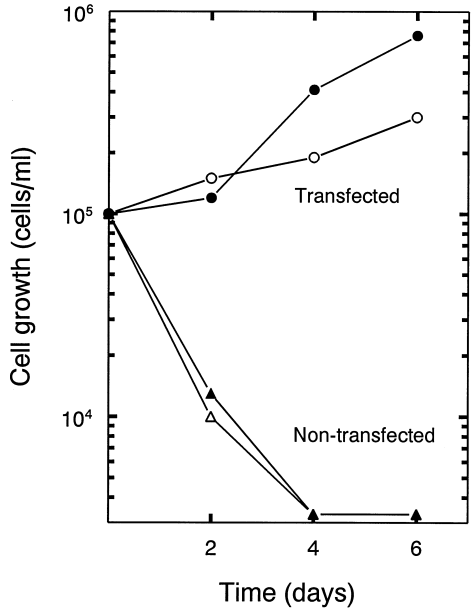


Fig. 4. Effects of rotenone on cell growth of nontransfected and *NDI1*-transfected (H47) HEK 293 cells. Both nontransfected and *NDI1*-transfected cells (10^5) were grown in 0.1 μM rotenone culture medium in the presence of 0.6 mM glucose (○, transfected; △, nontransfected) or 0.6 mM glucose+5 mM galactose (●, transfected; ▲, nontransfected). In the case of the *NDI1*-transfected HEK 293 cells, 0.5 mg/ml of the antibiotic G418 also was present. Cells were cultured at 37°C in a 5% CO_2 atmosphere. Cell viability was assessed by trypan blue exclusion, and cell numbers were determined by using a hemocytometer.

to cross the inner mitochondrial membrane, did not increase the respiration of the digitonin-permeabilized *NDI1*-transfected HEK 293 cells, suggesting that the NADH-binding site of the expressed Ndi1 faces the matrix compartment as was observed in the yeast mitochondria (data not shown) [21]. These results agree with our previous observations with the yeast *NDI1*-transfected Chinese hamster CCL16-B2 mutant cells [24]. To confirm that the yeast Ndi1 acts in concert with the other members of the respiratory chain in the HEK 293 cells, the ADP/O ratios of mitochondria isolated from nontransfected and *NDI1*-transfected cells were compared (see Fig. 3). Because the Ndi1 enzyme lacks an energy coupling site, if this enzyme is responsible for the NADH to UQ10 electron transfer, the observed ADP/O ratios with a NADH-related substrate are expected to be the same as that with succinate. The data (Fig. 3) demonstrate that this is, in fact, what was observed. The ADP/O ratios of nontransfected HEK 293 mitochondria, 2.41 and 1.60 by glutamate/malate and succinate, respectively, are consistent with previously reported results [27]. On the other hand, the ADP/O ratios of the *NDI1*-transfected HEK 293 (H47) mitochondria were 1.75 and 1.56, respectively, by glutamate/malate and succinate. These results support the hypothesis that the expressed Ndi1 is capable of NADH-Q oxidoreductase activity in the respiratory chain of HEK 293 mitochondria.

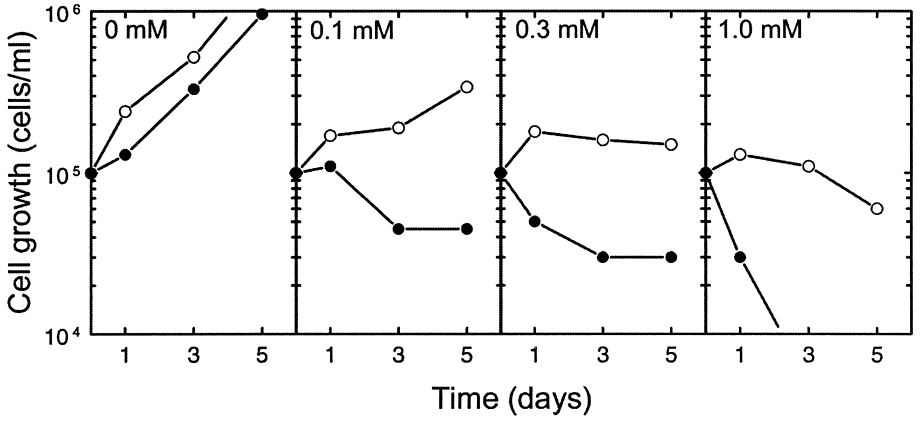


Fig. 5. Effect of MPP^+ on cell growth of nontransfected and *NDI1*-transfected (H47) HEK 293 cells. Nontransfected cells (10^5) (●) and *NDI1*-transfected cells (10^5) (○) were cultured in a medium containing 0.6 mM glucose, 5 mM galactose, and various concentrations of MPP^+ (0, 0.1, 0.3, and 1.0 mM). 0.5 mg/ml of G418 was added to the *NDI1*-transfected HEK 293 cell culture medium. Cell viability and cell numbers were determined as described in Fig. 4.

3.3. Effect of rotenone and MPP^+ on the cell growth

As anticipated, the *NDI1*-transfected 293 cells (H47) can grow in the media containing rotenone (Fig. 4). This is more evident when oxidative phosphorylation was enhanced by the addition of 5 mM galactose to the growth medium [24]. On the other hand, the nontransfected cells cannot survive in the presence of rotenone. The effect of MPP^+ on the viability of the *NDI1*-transfected and nontransfected HEK 293 cells was also examined. MPP^+ is known to inhibit complex I and to cause Parkinson's disease-like symptoms in humans and primates [36]. The data (Fig. 5) reveal that in the culture media containing 0.1 mM MPP^+, the *NDI1*-transfected cells were able to grow but the nontransfected cells could not survive. When the concentration of MPP^+ was increased to 0.3 mM, the *NDI1*-transfected cells grew initially and continued to survive for up to 5 days whereas 70% of the nontransfected cells were dead under the same conditions. At 1.0 mM MPP^+, the *NDI1*-transfected cells survived for up to 3 days before beginning to die. However, the nontransfected cells were 70% dead after 1 day of culture in 1.0 mM MPP^+, and were completely dead by 3 days in culture. These data clearly indicate that the *NDI1*-transfected HEK 293 cells are more insensitive to MPP^+ than the nontransfected HEK 293 cells. This is believed to be due to the fact that MPP^+ is not a potent inhibitor of the Ndi1 enzyme expressed in the HEK 293 cells. One possible explanation for the lack of effect of MPP^+ on the Ndi1 enzyme is that the Ndi1 enzyme and complex I catalyze a two-electron reaction and a one-electron reaction, respectively.

4. Discussion

In previous papers [23,24], we have shown that the *S. cerevisiae* Ndi1 enzyme can be functionally expressed and can exhibit NADH-Q oxidoreductase activity in the respiratory chains of *E. coli* membranes and complex I-deficient Chinese hamster CCL16-B2 mitochondria. Our ultimate goal is to use the yeast *NDI1* gene to restore the NADH oxidase activity in human mitochondria with complex I deficiency. In this paper, it is demonstrated that the yeast *NDI1* gene can be functionally expressed in the human embryonic kidney cell line, HEK 293. Despite the presence of endogenous complex I, the expressed Ndi1 accounts almost entirely for the electron transfer from NADH to UQ10 in the mitochondria of these cells. Recently, two respiration-deficient Chinese hamster cell mutants have been characterized. Both were shown to have a mutation in the MWFE subunit of complex I. One mutant contains a deletion which produces a truncated MWFE subunit. The other has a missense mutation which substitutes a lysine for an arginine at the 50th position. In both mutant cells, complex I activity was almost entirely absent. Although complementation of the truncated MWFE mutant with hamster NDUFA1 cDNA (encoding the MWFE subunit) restored the NADH oxidase activity to 100%, complementation of the missense mutant (R50K) restored the NADH oxidase activity to only 40% (H.C. Au, B.B. Seo, A. Matsuno-Yagi, T. Yagi, I.E. Scheffler, unpublished results). These results suggest that under certain conditions (e.g., point mutation etc.) the defective subunit can still bind and occupy its site within the enzyme complex, making it difficult to implant the substitute introduced. By contrast, complementation with the yeast *NDI1* gene does not suffer from this limitation. As described above, the *NDI1*-transfected 293 cells showed NADH oxidase activity which was actually higher than the nontransfected cells. This is clearly one of the advantages of using the *NDI1* gene as the rescue gene.

Another advantage of the Ndi1 protein is that it is believed to catalyze two-electron reactions [20] and may, thus, alleviate possible complications caused by reactive oxygen species (ROS). The fact that the *NDI1*-transfected cells are much more resistant to MPP^+ than nontransfected cells supports this hypothesis. Although the actual impact of MPP^+ in neurotoxicity remains to be demonstrated in living animals, it has been shown that intracellular MPP^+ can be taken up and concentrated within the mitochondria where it blocks complex I, which in turn decreases the production of ATP and increases the formation of free radicals such as superoxide [36]. The role of free radical production in neurotoxicity has recently been assessed by testing MPTP toxicity in transgenic mice with increased activity of Cu/Zn-superoxide dismutase (SOD), the key enzyme in the

detoxification of superoxide. These transgenic mice have 2.5–3 times greater brain SOD activity than do wild-type animals. MPTP administration was shown to cause substantial damage in the wild-type animals. In contrast, no significant damage was detected in the transgenic animals with increased SOD [36]. It may be expected that similar results will be observed in *NDI1*-transgenic mice. This hypothesis will be verified in the future.

The decrease in ADP/O ratio of the mitochondrial respiratory chain in the transfected cells represents yet another potential advantage of transfection with the *NDI1* gene. Given the major contribution of NADH oxidation to the overall ATP synthesis in mitochondria, the lowered ADP/O ratio should lead to decreased production of ATP in the cell. This would make the Ndi1 enzyme a likely therapeutic tool for obesity. One of the possible therapies currently being considered for obesity is to uncouple oxidative phosphorylation [37–39]. However, previous efforts to treat obesity by uncoupling oxidative phosphorylation using thyroid hormone, dinitrophenol or massive doses of sympathetic agonists have failed because mitochondrial uncoupling occurred at unwanted, as well as at desired, sites [37]. It is envisioned that, unlike the use of uncouplers, with the Ndi1 enzyme it should be easier to attenuate the level of ATP because it only affects the first coupling site of the respiratory chain leaving the other sites fully functional.

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