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Abstract

Nucleoside diphosphate kinase (NDPK/Nm23), responsible for intracellular di- and triphosphonucleoside homeostasis, plays multiple roles in cellular energetics, signaling, proliferation, differentiation and tumor invasion. The only human NDPK with a mitochondrial targeting sequence is NDPK-D, the NME4 gene product, which is a peripheral protein of mitochondrial membranes. Subfractionation of rat liver and HEK 293 cell mitochondria revealed that NDPK-D is essentially bound to the inner membrane. Surface plasmon resonance analysis of the interaction using recombinant NDPK-D and model liposomes showed that NDPK-D interacts electrostatically with anionic phospholipids, with highest affinity observed for cardiolipin. Mutation of the central arginine (Arg-90) in a surface-exposed basic RRK motif unique to NDPK-D strongly reduced interaction with anionic phospholipids. Due to its symmetrical hexameric structure, NDPK-D was able to cross-link anionic phospholipid-containing liposomes, suggesting that NDPK-D could promote intermembrane contacts. Latency assays with isolated mitochondria and antibody binding to mitoplasts indicated a dual orientation for NDPK-D. In HeLa cells, stable expression of wild type but not of the R90D mutant led to membrane-bound enzyme in vivo. Respiration was significantly stimulated by the NDPK substrate TDP in mitochondria containing wild-type NDPK-D, but not in those expressing the R90D mutant, which is catalytically equally active. This indicates local ADP regeneration in the mitochondrial intermembrane space and a tight functional coupling of NDPK-D with oxidative phosphorylation that depends on its membrane-bound state.
THE NUCLEOSIDE DIPHOSPHATE KINASE D (NM23-H4) BINDS THE INNER MITOCHONDRIAL MEMBRANE WITH HIGH AFFINITY TO CARDIOLIPIN AND COUPLES NUCLEOTIDE TRANSFER WITH RESPIRATION

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Running head: NDPK-D (Nm23-H4) - membrane interaction

Key words: mitochondria, cardiolipin, oxidative phosphorylation, phospholipid

Nucleoside diphosphate kinase (NDPK/Nm23), responsible for intracellular di- and triphosphonucleoside homeostasis, plays a multifaceted role in cellular energetics, signaling, proliferation, differentiation and tumor invasion. The only human NDPK with a mitochondrial targeting sequence is NDPK-D, the NME4 gene product, which is a peripheral protein of mitochondrial membranes. In this study, subfractionation of rat liver and HEK 293 cell mitochondria revealed that NDPK-D is essentially bound to the inner membrane. Surface plasmon resonance analysis of the interaction using recombinant NDPK-D and different phospholipid liposomes showed that NDPK-D interacts electrostatically with anionic phospholipids, with highest affinity observed for cardiolipin. Mutation of the central arginine (R90) in a surface exposed basic RRK motif unique to NDPK-D strongly reduced phospholipid interaction with model liposomes. NDPK-D was also able to cross-link anionic phospholipid-containing liposomes due to its symmetrical hexameric structure exposing three R90 on two opposite faces suggesting that NDPK-D could promote intermembrane contacts. Latency assays with isolated mitochondria and antibody binding to mitoplasts indicated a dual orientation for NDPK-D at the inner membrane. In HeLa cells naturally almost devoid of NDPK-D, stable expression of wild type but not of R90D mutant led to membrane-bound enzyme in vivo. Respiration was significantly stimulated by the NDPK substrate TDP in mitochondria containing wild-type NDPK-D, but not in those expressing R90D mutant that is catalytically equally active. This indicates local ADP regeneration in the mitochondrial intermembrane space and a tight functional coupling of NDPK-D with oxidative phosphorylation that depends on its membrane-bound state.

Nucleoside diphosphate kinases (NDPK), encoded by NME genes (also called NM23), catalyze the exchange of γ-phosphate between di- and triphosphonucleosides and participate in the regulation of intracellular nucleotide homeostasis. They mainly utilize ATP formed by oxidative phosphorylation to synthesize the other triphosphonucleosides, in particular GTP (1). Given the poor substrate selectivity of NDPKs, it is assumed that specificity could arise from the presence of different isoforms at different subcellular localizations. Associated in networks with other nucleotide metabolizing enzymes such as adenylyl kinases, creatine kinases and glycolytic enzymes, NDPKs participate in high-energy phosphoryl transfer and signal communication in the cell (2). Up to now, nine genes encoding NDPK or NDPK-like proteins have been identified (3,4), but little is known about their respective role within the cell. The most studied, NDPK-A and -B, encoded by NME1 and NME2 genes respectively, play a key role in tumor progression and metastasis dissemination (5,6).

NDPK activity has been found associated with different cellular compartments, such as cytosol, nucleus, plasma membrane and mitochondria. Precise localization in the latter organelles has been a matter of debate. Depending on species and tissue examined, NDPK activity was reported in both the matrix and the intermembrane/cristae space (7),
including the so-called contact sites between inner and outer membrane (8-10). In mammalian liver (rat and rabbit), the NDPK activity was mainly associated with an extra-matrix compartment, probably the intermembrane/cristae space, while in heart, activity was more abundant in the matrix (11). For mitochondrial NDPK in matrix, many functions have been proposed, ranging from nucleus supply for mitochondrial nucleic acid and protein synthesis, to functional interaction with the Krebs cycle succinyl thiokinase (STK) and the catabolism of short chain fatty acids (12,13). Very recent data point to yet undescribed role of a matrix NDPK in synthesizing GTP important for iron homeostasis (14).

It is not known whether the various reported mitochondrial localizations of NDPK activity are due to different mitochondrial compartment-specific isoenzymes or a dual localization of the same NDPK isoenzyme. In yeast, a NDPK isoform without specific targeting signal is present in cytosol and mitochondrial intermembrane space (15). Other known mitochondrial NDPK proteins possess a canonical mitochondrial targeting sequence in amoeba (16), plants (17), green alga (18), pigeon (19), mouse (20) and human (21,22). However, these enzymes apparently differ in their intramitochondrial localization, which would be matrix in pigeon (19), but intermembrane/cristae space in plants (23,24) and amoeba (16). In mammals, membrane association has been reported without information on enzyme orientation (22).

The human mitochondrial NDPK (NDPK-D), encoded by the NME4 gene, targets mitochondria via an amino-terminal specific sequence, which is cleaved to reveal catalytic activity (22). It is ubiquitously expressed (21) and active as a hexamer, like the cytosolic NDPK-A, B and C isoforms (22,25). Information on its precise localization and function is scarce, except its peripheral association with mitochondrial membranes (22), a putative association with STK (26) and an overexpression noted in colorectal carcinomas (27). The aim of the present study was to characterize the structural basis of NDPK-D/mitochondrial membrane interactions as well as to define sub-mitochondrial localization of NDPK-D and its functional consequences.

**Experimental Procedures**

**Materials**- The human recombinant NDPK-D missing the first 33 amino acids and fused to a tag at its N terminus was obtained as described in (22). Purified human recombinant cytosolic NDPK-A and -B were a kind gift of Prof. I. Lascu. The antibodies were all raised against human proteins. The polyclonal antibodies against NDPK-D were obtained by immunizing rabbits as described in (22). For immunocytochemistry and immunoprecipitation experiments, we used antibodies, which were affinity purified using recombinant NDPK-D.

The polyclonal antibodies for VDAC1 (porin), ANT and ANT2 were from Calbiochem and Santa-Cruz. The polyclonal antibodies against Mn-SOD were from Abcam. The monoclonal antibody for OPA1 was from BD Biosciences. The affinity purified antibodies against NDPK-A and -B were prepared using the recombinant proteins as described (28). The mitochondria-selective dye MitoTracker TM Red CMXRos, AlexaFluor 488-conjugated goat anti-rabbit IgG and Biotin X DHPE were from Molecular Probes. The magnetic beads coated with sheep anti-rabbit IgG (Dynabeads® M-280) were from Invitrogen. Formalin solution (10%, neutral buffered), 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), P.P'. Di(adenosine-5')pentaphosphate (Ap5A), 5,5'- Dithiobis (2-nitrobenzoic acid) (DTNB) and succinyl-CoA were from Sigma. Lipids were from Avanti Polarlipids (Alabaster, USA). The protease inhibitor mixture and lactate dehydrogenase were from Roche Applied Science. Pyruvate kinase was obtained from Fluka.

**Plasmid construction and site directed mutagenesis**- The mutant NDPK-D protein with an aspartate residue instead of an arginine at position 90 was generated using the pET-28a(+) plasmid constructed to express the his-tagged NDPK-D33 protein (22) and the Transformer TM site-directed mutagenesis kit (Clontech). The mutagenic primer designed to produce the desired point mutation was 5'-CAGGACCTGCAGGACAAGCCTTCTAC-3' (the altered nucleotides are in boldface type). The mutated recombinant protein was purified by chromatography through a nickel-nitrilotriacetic acid agarose column under native conditions following the procedure provided by Qiagen (22). For inducible NDPK-D expression in eukaryotic cells, the cDNA encoding the full-length NDPK-D protein was inserted in the pcDNA4/TO vector (T-Rex™ system, Invitrogen). The insert was generated by PCR
amplification using the pET-21b plasmid constructed to express the full length NDPK-D protein (22) as template and the following primers (5'-CCAGCTTAAGCCACCATGGGCGGCCTCTTC-3' and 5'-CATTGGATCCTTTCAAGGTTGATGCT-3') to introduce AJ11 and BamHI sites for subcloning of the insert at the corresponding sites into the pCDNA4/TO vector. For eukaryotic expression of the R90D mutant, the Eco47III-BamHI fragment of the pCDNA4/TO construct expressing the wild type NDPK-D was replaced by the same fragment of the pET-28a(+) construct expressing the mutant.

**Cell Culture and transfection-** Cell lines were grown at 37 °C in a 5% CO₂ atmosphere in Gibco media (Invitrogen), supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. HEK 293 cells were grown in Dulbeccoo's modified Eagle's medium (D-MEM), containing 4.5 g/l glucose, Glutamax and 25 mM HEPES. T-Rex™-HeLa cells (Invitrogen) stably expressing the Tet repressor encoded by the plasmid pcDNA6/TR (Invitrogen) were grown in Minimum Essential Medium (MEM) containing Earle's salts, 2 mM glutamine and zeocin (500 µg/ml) to maintain the pCDNA6/TR vector. They were transfected by calcium phosphate/DNA precipitation with the pcDNA4/TO vector, empty or encoding the wild type NDPK-D. After 48 h transfection, cells were seeded into fresh medium with 80 µg/ml digitonin/mg protein. After washing three times with buffer A by 5 min centrifugation at 10,000xg, mitoplasts (0.2 mg proteins) were resuspended in two tubes containing 200 µl buffer A and 1 µg of either affinity purified anti-NDPK-D or anti-Mn-SOD antibodies for 1h at 4°C on a rotating wheel. After washing three times in buffer A to remove unbound antibodies, mitoplasts were suspended in 200 µl RIPA buffer (150 mM NaCl, 50mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 0.5 % deoxycholic acid, 0.1% SDS, 1 mM PMSF supplemented with protease inhibitor mixture), added with 80 µl of magnetic beads, previously rinsed with RIPA buffer and incubated for 1h at 4°C. Beads linked to the target antibody/antigen complexes (accessible in intact mitoplasts) were recovered rinsed three times with RIPA and suspended in Laemmli buffer. The supernatants, containing unbound antigens i.e. only accessible to antibodies after mitoplast lysis, were saved and further incubated for 1h at 4°C with beads previously linked to anti-NDPK-D and anti-Mn-SOD antibodies. Beads were recovered and suspended in Laemmli buffer. All samples were further analyzed by Western blotting.

**Western blot analysis-** Proteins from cell and mitochondrial extracts and from immunoprecipitates and purified recombinant proteins were electrophoretically separated on 10% or 12.5% polyacrylamide gels and transferred onto Immobilon P membrane (0.1 µm, Millipore) for 2 h at 22 V in 10 mM CAPS...
buffer, pH 11, 10 % methanol for NDPK-D and Mn-SOD, as described in Milon et al. (22) for NDPK-A and -B, or onto nitrocellulose membrane for 90 min at 50 V in 0.025 M Tris-base, 0.192 M glycine, 20% methanol and 0.02% SDS for the other proteins. The polyclonal anti-NDPK-D (1/7500), anti-Mn-SOD (1/2000), anti-cytochrome c (1 µg/ml), anti-ANT (1/2000), anti-NDPK-A and -B (1 µg/ml) and the monoclonal anti-cytochrome oxidase (0.5 µg/ml), anti-VDAC (1/1,000) and anti-OPA1 (1/2000) antibodies were reacted, in the secondary reaction, with peroxidase conjugated anti-rabbit (1/10,000) or anti-mouse antibodies (1/2,000) obtained from P.A.R.I.S., Compiègne, France. The blots were revealed using the Amersham ECL Plus™ Western blotting detection system from GE Healthcare.

Fluorescence microscopy and immunocytochemistry- For mitochondria staining and immuno-detection of NDPK-D in stably transfected T-Rex™-HeLa, cells cultivated on microscope glass slides were incubated with 25 nM MitoTraker for 30 min at 37°C, fixed in formalin solution for 2 h at room temperature, permeabilized in PBS containing 0.5 % Triton, incubated with anti-NDPK-D affinity purified antibody (1/500) for 1 h at 37°C and with the AlexaFluor 488-conjugated goat anti-rabbit antibodies for 2 h at room temperature. After mounting on glass slides with VECTASHIELD (Vector Laboratories, Burlingame, CA), fluorescence was observed with a Leica HC microscope.

Assay of NDP kinase and succinyl thiokinase activities- Both activities were measured spectrophotometrically in mitochondrial extracts. For NDPK we used a coupled pyruvate kinase-lactate dehydrogenase assay (32) with 0.2 mM ATP and 0.2 mM TDP as co-substrates and 100 µM Ap5A to inhibit endogenous adenylate kinase. STK was assayed according to (33) with 1 mM ADP (HEK293) or GDP (liver).

Generation of liposomes- For preparation of liposomes, we used pure PC or 84% PC and 16% of a second lipid: PE, CH, PI, PS, PG or CL. Aliquots of the required natural lipids in chloroform solution were combined in the desired ratio. In addition, 0.1% (w/w) Biotin-X-DHPE was incorporated for Biacore experiments. Large unilamellar vesicles (LUV) were prepared by hydration and a combination of freeze/thawing and extrusion as previously described (34). Briefly, dry lipids were hydrated (5-20 mg/ml) in standard buffer (10 mM TES pH 7.0, 50 mM K-acetate) and finally dispersed by vortexing to produce multilamellar vesicles. The lipid suspension was subjected to 6-10 freeze/thaw cycles and then extruded 19 times through polycarbonate membranes (Nuclepore) with 0.4 then 0.2 µm diameter pores using a mini-extruder. The resulting LUV have a diameter of approximately 160 nm as analyzed by electron microscopy and were used within 3 days.

Surface plasmon resonance spectroscopy:Binding of NDPK isoenzymes and NDPK-D-R90D mutant to a model lipid membrane was measured by surface plasmon resonance spectroscopy (SPR) with a Biacore 2000™ instrument (Biacore, Uppsala) according to (34), using standard buffer with 2 mM β-mercaptoethanol. Briefly, a carboxymethyl sensor chip CM5 (Biacore, Uppsala) was covered with 20 000 response units (RU) avidin to immobilize 500 RU biotinylated liposomes. NDPK association (on) and dissociation (off) kinetics were recorded at 25°C and a flow rate of 0.3 ml h⁻¹ with NDPK concentrations ranging from 5 to 320 nM. A final injection of 0.5% SDS was performed to recover the chip-avidin surface. Unchanged kinetics with increased flow rate revealed the absence of mass transport limitations. Kinetic data were corrected for background binding to the avidine-liposome-coated chip by subtracting the signal for pure PC liposomes. Simultaneous fitting of on- and off-kinetics by single- or double-exponential rate equations with BIA-evaluation software v.4.1. as described in (34) yielded unsatisfactory results due to the kinetic stability (very slow off-rate) of the measured interactions. The affinity constants (Kₐ) were therefore derived from the concentration-dependency of the extrapolated equilibrium response Rₑq (Scatchard plots).

Light scattering measurement- Light scattering was analyzed as described in (35). Vesicle cross-linking was induced by addition of 30 nM NDPK and followed for 1000 s. The data were normalized to the scattering signal just after addition of NDPK.

Respiration measurements- Mitochondrial oxygen consumption was measured at 25°C with a high performance OROBOROS oxygraph (Anton Park, Innsbruck, Austria) in a respiration buffer (250 mM sucrose, 10 mM Tris/Mops, pH 7.4) supplemented with 10 mM Pₐₐ, 2 mM MgCl₂, and 2 µM rotenone. Resting respiration (State 4) was initiated by 5 mM succinate, and subsequently stimulated by sequential addition
of increasing amounts of ADP (5-100 μM) in absence and in presence of 0.5 mM TDP. Stimulation of respiration by ADP was followed for 10 min and the rate of oxygen consumption was analyzed by fitting the initial slope of the curves after ADP addition. Cytochrome c was added at the end of the measurements to control the intactness of the outer membrane. Mean and standard deviation were calculated from 6-7 measurements of two independent mitochondrial isolations of different cell culture cycles.

RESULTS

NDPK-D is bound to the mitochondrial inner membrane. Since the localization of NDPK isoforms in mitochondria has been a matter of debate, we have examined this issue by analyzing Percoll-purified mitochondria from rat liver and HEK 293 cell cultures using immunoblotting and activity assays (Fig. 1). When contact formation between mitochondrial membranes was suppressed with glycerol-containing buffer (30), highly pure preparations of inner and outer membrane from rat liver mitochondria could be obtained. NDPK-D co-purified exclusively with inner membrane (Fig. 1A). Only traces of cytosolic NDPK-B, if any, were detectable in the mitochondrial membrane fractions. Similar results were obtained with HEK 293 mitochondria (Fig. 1B). Immunogold labeling of HEK 293 mitochondria confirmed association of NDPK-D with inner mitochondrial membranes (data not shown). A NDPK activity test in intact HEK mitochondria and submitochondrial fractions (Fig. 1C) showed NDPK activity associated essentially with the inner membrane and accessible to the intermembrane/cristae space in contrast to the matrix STK activity (36).

NDPK-D binding to anionic phospholipid containing liposomes. The peripheral association of NDPK-D with mitochondrial membranes (22) was characterized quantitatively in vitro with surface plasmon resonance, using liposomes containing various phospholipid amounts and recombinant human NDPK-D. Binding of NDPK-D to artificial liposomes was clearly dependent on the presence of 16% anionic phospholipids like PS, PG and CL as indicated by the equilibrium binding response (Fig. 2). Phospholipids with neutral net charge like PC and PE did not interact with the kinase. The 16% CL/84% PC ratio mimicks the mitochondrial membrane composition (37). Increasing the salt concentration of the running buffer gradually reduced NDPK-D/phospholipid interaction and the CL-binding drug doxorubicin efficiently displaced NDPK-D from CL-containing liposomes (not shown). These data revealed a purely electrostatic interaction of NDPK-D with CL, the predominant phospholipid of the mitochondrial inner membrane.

Identification of the NDPK-D residue involved in the CL binding. Examination of the electrostatic surface potential of the enzyme identified some largely basic surface patches (blue in Fig. 3A). A basic triad in each monomer (Fig. 3B) contains an arginine (R90) unique to NDPK-D (Fig. 3C & D). Three such motifs are exposed on top and bottom side of the hexameric structure (Fig. 3B) and thus represent putative candidates for interaction with acidic phospholipids. Note that the central basic cavity is normally buried by the C termini, which are missing in the structure because they were not resolved in the electron density map (22). The putative binding motif was examined by mutating R90 to aspartate (R90D), thus changing the positive charge into a negative one and mimicking the motifs found in cytosolic NDPK-A and -B (Fig. 3C). We obtained a fully catalytically active mutant (not shown) indicating a correctly folded, hexameric structure, since this is required for NDPK activity (38).

High affinity NDPK-D binding to CL-containing model membranes abolished by the R90D mutation. While the wild-type NDPK-D bound CL-containing liposomes with high affinity, the R90D mutant failed almost completely to bind CL (Fig. 4A & B) and presented a membrane interaction even lower than cytosolic NDPK-A and -B (Fig. 4C & D). Thus, switching a single basic residue into an acidic amino acid, resulting in 3 mutations per binding face in the hexamer, is sufficient to prevent membrane interaction. The NDPK-D interaction with CL was characterized by a very high equilibrium binding response (Fig. 4E), a high affinity of 29±3 nM, and very slow dissociation kinetics (Fig. 4A). Cytosolic NDPK isoforms showed 10-25 times lower affinity (NDPK-A: 708±93 nM, NDPK-B: 219±32 nM) and 4-6 times lower equilibrium response. A low affinity for anionic phospholipids could explain the faint interaction of cytosolic isoenzymes, in particular of NDPK-B, with mitochondrial membranes (Fig. 1A). Interaction of wild type NDPK-D with acidic phospholipids other than CL, although showing a similar equilibrium response, was characterized by lower affinity.
NDPK-D induces liposome cross-linking. Since NDPK-D only exists as a symmetrical hexameric structure, the complex exposes always two opposite, identical binding faces (Fig. 3A) which could simultaneously interact with two phospholipid-containing membranes. This suggests that the hexamer may cross-link inner and outer mitochondrial membranes to form the naturally occurring contact sites. A classical light scattering assay (Fig. 5) showed that with liposomes consisting of 84% PC/16% CL, wild type NDPK-D rapidly produced liposome aggregation, detectable as scattering decrease, while the R90D mutant was much less effective. The cross-linking kinetics showed an initial rapid phase (ca 100 s), followed by a long lasting slow phase. With the R90D mutant, the fast phase was entirely lacking, and only a slow rate cross-linking took place. NDPK-D was unable to cross-link liposomes containing only PC. The changes in the scattering signal were not due to liposome fusion, as we already showed in a parallel study (48).

**NDPK-D orientation.** Orientation of NDPK-D in mitochondria was analyzed by latency tests and by accessibility of NDPK-D in mitoplasts to immunoprecipitation. Latency tests determine the accessibility of enzyme activity across the outer-membrane that is permeable for metabolites in the intact organelle. We used mitochondria from liver, HEK 293 cells and from HeLa cells induced to overexpress NDPK-D. HeLa cells are missing immunodetectable amounts of NDPK-D. However, lines stably transfected with the NM23-H4 gene under the control of a tetracycline-inducible promoter can achieve high NDPK-D levels when induced by tetracycline (Fig. 6A). In the absence of tetracycline, trace amounts of immunodetectable NDPK-D were observed reflecting some leakage of the promoter. Staining with anti-NDPK-D antibodies of T-Rex™-HeLa cells induced to express NDPK-D, exhibited a typical mitochondrial pattern (Fig. 6B, left) which coincided with the mitochondrial marker MitoTracker (Fig. 6B, middle) as shown in merged images (Fig. 6B, right). A similar immunostaining was observed in T-Rex™-HeLa cells induced to overexpress the R90D NDPK-D mutant (not shown).

Mitochondrial preparations kept in isoosmotic buffer were 85-95% intact as determined by the measurable activity of matrix enzyme STK. The assay of NDPK activity showed a clear bipartite behavior (Fig. 6C). While most NDPK activity (about 70-80%) was accessible in intact mitochondria from liver and HEK 293 cells and thus localized in the intermembrane and cristae space, a consistent fraction of about 20-30% was hidden and could be only revealed by detergent. This indicates a partial localization of the enzyme in the matrix space. It might be argued that this activity corresponds to another isoform of NDPK not examined here. We used T-Rex™-HeLa cells, induced to overexpress NDPK-D, to address this issue. A very low NDPK activity was observed with and without detergent in the control T-Rex™-HeLa cells transfected with the empty vector. Much higher activity was observed in the cells induced to overexpress NDPK-D by tetracycline. Again, only about 50% NDPK-D activity was accessible in the intact organelle, while the remaining activity was only revealed by addition of detergent.

To further corroborate the orientation of NDPK-D at the inner membrane, mitoplasts were prepared by digitonin treatment of purified mitochondria (29) and incubated in isotonic buffer either with antibodies against NDPK-D or the matrix enzyme Mn-SOD as a control. After washing and lysis in RIPA buffer, antibody complexes were trapped with anti-rabbit IgG covalently bound to magnetic beads. The same immunoprecipitation was repeated after lysing the mitoplasts. As shown in Fig. 6D, more than 50% of NDPK-D were accessible in intact mitoplasts while less than 20% of Mn-SOD was immunoprecipitated, corresponding to residual lysis of mitochondria during treatment. Only after mitoplastic lysis, the remaining 45% of NDPK-D, which did not react initially with the antibodies, could be detected, along with over 80% of Mn-SOD. Altogether, these data suggest a dual orientation of NDPK-D, towards the intermembrane/cristae space and towards the matrix space.

**The NDPK-D membrane interaction in vivo is strongly reduced by the R90D mutation.** Binding to mitochondrial membranes was analyzed in T-Rex™-HeLa cells overexpressing the wild-type and R90D mutant NDPK-D (Fig. 7). While an important fraction of the wild type NDPK-D remained bound to the membranes after lysing the organelle, almost all the mutant was soluble. Na2CO3 treatment solubilized the wild-type protein showing again the electrostatic nature of the interaction.

Only membrane-bound NDPK-D fully couples NDPK-D with respiration. Since NDPK-
D appears to be predominantly exposed towards the mitochondrial intermembrane space, it should, in principle, be able to locally regenerate ADP from ATP through its NTP synthesis activity, thus stimulating oxidative phosphorylation. To examine whether wild-type and R90D mutant differ in this respect, we analyzed respiration of purified, energized mitochondria from HeLa cells stably transfected either with empty vector (control) or with NDPK-D vector coding for wild-type or R90D protein (Fig. 8). After induction by tetracycline, the latter cell lines showed comparable expression levels of wild-type and R90D protein in mitochondria (Fig. 8G). Respiration was then analyzed by titrating ADP in absence or presence of the NDPK substrate TDP to determine the apparent K_m for ADP. In absence of TDP, all mitochondria showed a similar K_m(ADP) of 20-25 µM. However, marked differences were found in presence of TDP. With mitochondria from HeLa control cells lacking NDPK-D, the ADP titration curve and the resulting K_m(ADP) remained unchanged (Fig. 8F). Expression of NDPK-D wild-type significantly shifted the ADP titration curve and decreased K_m(ADP) by about 50%, to below 10 µM (Fig. 8D). By contrast, expression of comparable levels of equally active R90D mutant reverted the ADP titration curve and K_m(ADP) almost to control values (Fig. 8E). These data reveal a functional coupling of NDPK-D with respiration and thus with the oxidative phosphorylation process that is largely dependent of the membrane-bound state of NDPK-D.

**DISCUSSION**

NDPK-D is the only mammalian isoform exhibiting a mitochondrial targeting sequence (22). Examination of highly pure mitochondrial subfractions showed unequivocally a NDPK-D localization at the inner mitochondrial membrane. This confirms our previous data identifying NDPK-D as a peripheral membrane protein detachable from the mitochondrial membranes by alkali treatment (22). Using mitochondria-mimetic liposomes, we now report that the association occurs through a strong ionic interaction between the R90 of NDPK-D and acidic phospholipids, with an especially high affinity for CL. R90 is central in a triad of basic residues located at a surface-exposed loop connecting alpha helices αA and α2 and which is unique to NDPK-D. This residue is absent in the sequence of cytosolic A and B NDPKs and of other human NME proteins sequences. Properties of the NDPK-D loop carrying the basic binding motif make it particularly suitable to act as an interaction domain. First, it belongs to the most divergent part of the NDPK sequences suspected to provide specificity for cellular functions. Second, as seen by the poor definition in the electron density map, this loop is relatively mobile, unless it is stabilized by substrate binding (39). It could thus provide the necessary flexibility for docking to CL, similarly to the CL-binding domain of MtCK (40). It should be noted that, in NDPK-A, B and C, the R90 position is occupied by acidic residues (aspartate and glutamate), also part of a charged motif which could interact with yet unknown intracellular targets.

CL has been described as membrane anchor for peripheral membrane proteins such as cytochrome c (41), mitochondrial creatine kinase (MtCK) (40) and truncated Bid (tBid) (42), and as a glue for supercomplex formation in the mitochondrial respiratory chain (43,44). However, specific sequence motifs responsible for the involved interactions have rarely been identified. Pairs of adjacent basic residues could interact with the two phosphate headgroups of CL, and such motifs can be found in cytochrome c (41) and the C-terminal phospholipid interaction domain of MtCK (40). In the case of NDPK-D, the x-ray structure reveals a distance of 5-10 Å between the ionized groups of the two adjacent arginines (R89 and R90), which would fit very well the distance between the two negatively charged phosphate groups of CL.

A basic residue equivalent to R90 can be found in NDPK-D orthologue sequences from mammals, fish and *Xenopus*, as well as, curiously, in mitochondrial NDPK from *Dictyostelium discoideum* but not in the mitochondrial NDPK of birds and of lower organisms such as *Drosophila*, *Caenorhabditis elegans* and bacteria. Mitochondrial NDPK from plants even has an acidic residue at the equivalent site like mammalian cytosolic NDPKs. This suggests that specific mitochondrial membrane association appeared late in evolution and could be important for vertebrate mitochondrial functions. Similarly, the MtCK ability to interact structurally or functionally with mitochondrial membranes or the ATP/ADP translocator (ANT) has been acquired at the dawn of vertebrate evolution (40,45).

Since NDPK-D forms symmetrical homohexamers, it presents three interacting motifs on
each of the two identical opposite ("top" and "bottom") faces. This structure enables NDPK-D to cross-link vesicles containing 16% CL. A similar cross-linking property has been described for octameric MtCK, which forms a symmetrical, cuboidal structure exposing four binding domains at each of its top and bottom faces (40). In fact, many properties of NDPK-D observed in our study are reminiscent to those reported for MtCK, although these two proteins are structurally and phylogenetically unrelated. Since MtCK is known to be located in the intermembrane space (46) and was shown to be enriched in contact sites (10), to promote contact formation and to stabilize mitochondrial membranes (47), similar functions are very likely to occur for the NDPK-D hexamer. Moreover, recent in vitro data show that both proteins, MtCK and NDPK-D, are able to use intermembrane contacts to transfer phospholipids between two model membranes, a process that is not due to membrane fusion (48). In addition, MtCK promotes formation of CL domains, a phenomenon proposed to occur at contact sites (49). The role of NDPK in phospholipid transfer and clustering in vivo is currently under study. In fact, CL plays a key role in apoptosis by translocating from the inner to the outer mitochondrial membrane and then even further to other cellular membranes (50). Proapoptotic tBid then binds to the outer membrane CL and induces release of pro-apoptotic factors such as cytochrome c, itself bound to CL of the inner membrane.

Another functional property of NDPK-D shared with MtCK is the ability to stimulate respiration through local synthesis of ADP in the intermembrane/cristae space and close so-called functional coupling to respiration via ANT. This process is well described for MtCK as creatine-stimulated respiration and was shown to involve formation of proteolipid complexes containing MtCK and ANT (51; reviewed in: 46, 52). Indeed, supply of TDP, a NDPK-specific substrate, to intact mitochondria containing NDPK-D significantly enhanced respiration and reduced its apparent $K_m$ for ADP, confirming that NDPK-D is closely coupled to ANT. In plants, a close interaction between both proteins had been suggested by co-immunoprecipitation (24). The R90D mutant, which only differs by lower affinity to anionic phospholipids, lacks a significant TDP-dependent effect on respiration in our experimental conditions. Therefore, the present study provides the first evidence that such close functional coupling depends on the membrane-bound state of the NDPK-D hexamer. Such a specific role for the membrane-bound enzyme has also been proposed in case of octameric MtCK (51), albeit not shown experimentally to date.

In the intermembrane space, NDPK is proposed to couple ATP export through ANT to the synthesis of the other nucleoside triphosphates (53). Mitochondria are compartmentalized organelles with a dynamic structure, which can change (fusion and fission) upon cellular bioenergetic state, cell cycle and apoptosis under the control of GTPases bound to the inner and outer mitochondrial membranes (54). Local regeneration of GTP by NDPK-D bound to the inner membrane could thus be involved in these processes. It should be noted that cristae remodeling is a vertebrate feature (55) and thus evolved together with the membrane association of NDPK-D (see above).

All NDPK-D orthologues possess a canonical mitochondrial targeting sequence, but their final mitochondrial destination depends on the tissue of origin or the species. Although our functional evidence (stimulation of respiration and membrane cross-linking) confirms a localization of NDPK-D at the outer leaflet of the inner membrane thus facing the intermembrane space, latency tests and antibody detection of NDPK-D in intact mitoplasts point to a dual orientation towards intermembrane/cristae space and towards the matrix space (see Fig. 9). This NDPK distribution was clearly distinct from the one of the matrix enzymes STK and Mn-SOD and was not only observed in liver, but also in HEK 293 cells in which no cytosolic NDPKs could be detected (22) and HeLa cells forced to overexpress NDPK-D. The mechanisms leading to this dual orientation are unknown. Given the presence of a canonical mitochondrial targeting sequence, it could be the result of incomplete import into the matrix space or of an incomplete re-export from the matrix into the intermembrane space (56).

Taken together, NDPK-D could be defined as a peripheral membrane protein of the inner mitochondrial membrane with dual orientation towards the matrix and the intermembrane space (see Fig. 9). The former location will allow all the classical functions such as e.g. nucleoside triphosphate supply for nucleic acid and protein synthesis. The latter location in conjunction with the membrane-binding and -cross-linking properties of NDPK-D leads to NDP-stimulated respiration and could
be involved in new functions such as contact site formation, lipid transfer and local NTP/NDP homeostasis. It is tempting to propose that this NDPK-D fraction participates in regulation of GTP-dependent mitochondrial membrane dynamics, energy flux control, and/or apoptosis.

REFERENCES

1. Lascu, I., and Gonin, P. (2000) *J. Bioenerg. Biomembr.* **32**(3), 237-246
2. Dzeja, P. P., and Terzic, A. (2003) *J. Exp. Biol.** **206**(Pt 12), 2039-2047
3. Lacombe, M. L., Milon, L., Munier, A., Mehus, J. G., and Lambeth, D. O. (2000) *J. Bioenerg. Biomembr.* **32**(3), 247-258
4. Sadek, C. M., Jimenez, A., Damdimopoulos, A. E., Kieselbach, T., Nord, M., Gustafsson, J. A., Spyrou, G., Davis, E. C., Okó, R., van der Hoorn, F. A., and Miranda-Vizuete, A. (2003) *J. Biol. Chem.* **278**(15), 13133-13142
5. Steeg, P. S., Bevilacqua, G., Kopper, L., Thorgeirsson, U. P., Talmadge, J. E., Liotta, L. A., and Sobel, M. E. (1988) *J. Natl. Cancer Inst.* **80**(3), 200-204
6. Boissan, M., Wendum, D., Arnaud-Dabernat, S., Munier, A., Debray, M., Lascu, I., Daniel, J. Y., and Lacombe, M. L. (2005) *J. Natl. Cancer Inst.* **97**(11), 836-845
7. Jacobus, W. E., and Evans, J. J. (1977) *J. Biol. Chem.* **252**(12), 4232-4241
8. Cherradi, N., Defaye, G., and Chambaz, E. M. (1994) *Endocrinology* **134**(3), 1358-1364
9. Brdizcka, D. (1991) *Biochim. Biophys. Acta* **1071**(3), 291-312
10. Brdizcka, D., Beutner, G., Ruck, A., Dolder, M., and Wallimann, T. (1998) *Biofactors* **8**(3-4), 235-242
11. Muhonen, W. W., and Lambeth, D. O. (1995) *Comp. Biochem. Physiol. Biochem. Mol. Biol.* **110**(1), 211-223
12. Krebs, H. A., and Wiggins, D. (1978) *Biochem. J.* **174**(1), 297-301
13. Lambeth, D. O. (2002) *IUBMB Life* **54**(3), 143-144
14. Gordon, D. M., Lyver, E. R., Lesuisse, E., Dancis, A., and Pain, D. (2006) *Biochem. J.* **400**(1), 163-168
15. Amutha, B., and Pain, D. (2003) *Biochem. J.* **370**(Pt 3), 805-815
16. Troll, H., Winckler, T., Lascu, I., Muller, N., Saurin, W., Veron, M., and Mutzel, R. (1993) *J. Biol. Chem.* **268**(34), 25469-25475
17. Escobar Galvis, M. L., Hakansson, G., Alexciev, K., and Knorpp, C. (1999) *Biochimie* **81**(12), 1089-1096
18. Anderca, M. I., Furuichi, T., Pinontoan, R., and Muto, S. (2002) *Plant Cell Physiol.* **43**(11), 1276-1284
19. Lambeth, D. O., Mehus, J. G., Ivey, M. A., and Milavetz, B. I. (1997) *J. Biol. Chem.* **272**(39), 24604-24611
20. Masse, K., Dabernat, S., Bourbon, P. M., Larou, M., Amrein, L., Barraud, P., Perel, Y., Camara, M., Landry, M., Lacombe, M. L., and Daniel, J. Y. (2002) *Gene* **296**(1-2), 87-97
21. Milon, L., Rousseau-Merck, M. F., Munier, A., Erent, M., Lascu, I., Capeau, J., and Lacombe, M. L. (1997) *Hum. Genet.* **99**(4), 550-557
22. Milon, L., Meyer, P., Chiadmi, M., Munier, A., Johansson, M., Karlsson, A., Lascu, I., Capeau, J., Janin, J., and Lacombe, M. L. (2000) *J. Biol. Chem.* **275**(19), 14264-14272
23. Sweetlove, L. J., Mowday, B., Hebestreit, H. F., Leaver, C. J., and Millar, A. H. (2001) *FEBS Lett.* **508**(2), 272-276
24. Knorpp, C., Johansson, M., and Baird, A. M. (2003) *FEBS Lett.* **555**(2), 363-366
25. Erent, M., Gonin, P., Cherfilis, J., Tissier, P., Raschella, G., Giartosio, A., Agou, F., Sarger, C., Lacombe, M. L., Konrad, M., and Lascu, I. (2001) *Eur. J. Biochem.* **268**(7), 1972-1981
26. Kowluru, A., Tannous, M., and Chen, H. Q. (2002) *Arch. Biochem. Biophys.* **398**(2), 160-169
27. Hayer, J., Engel, M., Seifert, M., Seitz, G., and Welter, C. (2001) *Anticancer Res.* **21**(4A), 2821-2825
28. Pinon, V. P., Millot, G., Munier, A., Vassy, J., Linares-Cruz, G., Capeau, J., Calvo, F., and Lacombe, M. L. (1999) *Exp. Cell Res.* **246**(2), 355-367
29. Hovius, R., Lambrechts, H., Nicolay, K., and de Kruijff, B. (1990) *Biochim. Biophys. Acta* **1021**(2), 217-226
FOOTNOTES

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The abbreviations used are: ANT, adenine nucleotide translocator; CH, cholesterol; CL, cardiolipin; LUV, large unilamellar vesicle; MIM, mitochondrial inner membrane; MOM, mitochondrial outer membrane; Mn-SOD, Mn-superoxide dismutase; NDPK-D, mitochondrial isoform of nucleotide diphosphate kinase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PMSF, phenylmethylsulfonyl fluoride; PS, phosphatidylserine; RIPA, radioimmuno precipitation assay; STK, succinyl thio kinase; TCA, tricarboxylic acid cycle; VDAC, voltage-dependent anion channel.
**FIGURE LEGENDS**

**Fig. 1.** NDPK-D is bound to the inner mitochondrial membrane. Highly pure mitochondria and mitochondrial subfractions from (A) rat liver and (B, C) HEK 293 cells were analyzed for NDPK isoforms by immunoblot (A, B) or NDPK activity (C). (A, B) Immunodetection of cytosolic NDPK-A and NDPK-B or mitochondrial NDPK-D, together with ANT (ANT2 or all ANT isoforms, MIM marker), VDAC (MOM marker) or CYT-C (cytochrome c, IS/MIM marker) in following fractions: D, recombinant human NDPK-D; LIV, liver homogenate; CYT, soluble cytosolic fraction; MIT, mitochondria; MOM, mitochondrial outer membrane; MIM, mitochondrial inner membrane; IS, soluble proteins of intermembrane space, MX soluble proteins of matrix space. Lanes for blots were loaded each with 20 µg protein in (A) and about 30 µg in (B). (C) Relative NDPK activity (normalized to the highest activity) measured in intact HEK 293 mitochondria (MIT) and in the resulting solubilized subfractions. Values are the mean of triplicate determination. NDPK (grey bars) activity is given together with the enzymatic activity of the matrix marker STK (open bars).

**Fig. 2.** NDPK-D binds cardiolipin and different anionic phospholipids. The relative association equilibrium response of 500 nM NDPK with liposomes has been measured by surface plasmon resonance spectroscopy (SPR). Liposomes consisted of pure PC, phosphatidylecholine, or 84% PC and 16% of a second lipid: PE, phosphatidylethanolamine, CH, cholesterol, PI, phosphatidylinositol, PS, phosphatidylserine, PG, phosphatidylglycerol, CL, cardiolipin. Note: all anionic phospholipids (dark grey bars) show a high degree of NDPK-D binding, while uncharged PC and PE (light grey bars) do not.

**Fig. 3.** NDPK-D exposes a basic RRK surface motif. (A) Human mitochondrial NDPK-D (Protein Data Bank, PDB, accession code: 1EHW) has a basic surface potential (basic, blue surface; acidic, red surface), most pronounced at surface-exposed loops containing a cationic RRK motif on “top” and “bottom” sides of the hexameric structure. (B) Enlarged ribbon presentation of the motif within the frame in (A). Within this motif, the central R90 is unique to NDPK-D in human species (C) and conserved in NDPK-D orthologues of vertebrate species (D). The accession numbers of the sequences were obtained from SwissProt (NDPK A: P15531; NDPK B: P22392; NDPK C: Q13232 and NDPK D: O00746) in (C) and from GenBank and SwissProt (M. musculus: NP_062705; R. norvegicus: NP_001102948; M. domestica: XP_001372617; X. tropicalis: CAJ82999; D. rerio: NP_957489; C. livia: P87355; P. sativum: Q9SP13; D. discoideum: P34093 and E. coli: P0A763) in (D).

**Fig. 4.** NDPK-D, but not the R90D mutant or cytosolic isoforms, shows high affinity binding to CL-containing model membranes. (A-D) SPR analysis of the interaction between human NDPKs and CL. On and off kinetics of (A) NDPK-D wild type, (B) NDPK-D R90D mutant, (C) NDPK-A and (D) NDPK-B at various NDPK concentrations. Traces show the binding to 84% PC/16% CL liposomes corrected for background binding to 100% PC liposomes. On top, the black bar indicates injection of NDPK, the grey bar injection of running buffer only. (E-F) Concentration dependence of the equilibrium response of NDPK isoforms that bind to liposomes consisting of (E) 84% PC/16% CL or (F) 84% PC/16% PG.

**Fig. 5.** NDPK-D mediates liposome cross-linking by simultaneous interaction of two opposite binding sites. Human NDPK wild type (black points and lines) and NDPK-D R90D mutant (grey points and lines) were injected into suspensions of liposome consisting of 84% PC/16% CL (solid lines) or 100% PC (dotted lines). The decrease in light scattering was followed for 15 min.

**Fig. 6.** NDPK-D expression and localization in mitochondria. (A) Immunoblot analysis for the presence of NDPK-D in mitochondrial extracts from HeLa cells stably transfected with empty vector (V) or with vector expressing NDPK-D in the absence (D-T) and in the presence (D+T) of tetracycline, as well as a recombinant NDPK-D (D rec, presenting a slightly higher Mr due to the hexahistidine tag). (B) immunofluorescence pictures of T-Rex™-HeLa cells stably transfected with NDPK-D expressing vector and induced to express the protein by addition of tetracycline. Cells were co-stained with anti-NDPK-D affinity purified antibodies (left) and the mitochondria marker MitoTracker (middle). NDPK-D was localized to mitochondria as shown by fluorescence overlapping in the merged images (right).
Examination was performed using a 63x magnification objective. (C) Latency assay of NDPK (grey bars) and STK activities (open bars) in intact mitochondria either intact or lysed with detergent (1 % Triton for liver and 0.5 % CHAPS for HEK and HeLa cells) of rat liver, as well as HEK 293, HeLa-V (empty vector) and HeLa-D cells (NDPK-D vector). (D) Accessibility of NDPK-D to immunoprecipitation. Mitoplasts were incubated either with antibodies against NDPK-D or against the matrix enzyme (Mn-SOD). After lysis of mitoplasts in RIPA buffer, antibody complexes were trapped by anti-rabbit IgG coupled to magnetic beads. The lysate was then subjected to a second immunoprecipitation to capture the remaining protein that was protected before, mainly in the mitoplast matrix space. The immunoprecipitated fractions corresponding to the intact (Int.) and lysed (Lys.) mitoplasts were analyzed by Western blotting using anti-NDPK-D (left) and anti-Mn-SOD (right) antibodies. The quantified signals are given in the bar graph. Note: NDPK-D in intact mitoplasts only partially reacts with anti-NDPK-D antibody; the remainder is only accessible after lysis. Intactness of mitoplasts is about 80% as indicated by the amount of non-accessible Mn-SOD.

Fig. 7. The NDPK-D membrane binding is strongly reduced by the R90D mutation. Mitochondria were purified from T-Rex™-HeLa cells stably transfected with vectors expressing the wild type (WT) and the mutant (R90D) NDPK-D and added with tetracycline. After hypotonic treatment in the absence or the presence of Na2CO3, sonication and high-speed centrifugation, the particulate (P) and soluble (S) fractions were analyzed by SDS-PAGE revealed with anti NDPK-D and anti OPA1 antibodies, the latter as a marker of the inner membrane fraction (57).

Fig. 8. NDPK-D wild-type but not R90D mutant reduces Km(ADP) of oxidative phosphorylation in the presence of the NDPK substrate TDP. (A-C) Typical oxygen consumption by mitochondria purified from HeLa cells stably transfected with vector coding for (A) NDPK-D wild-type, (B) NDPK-D R90D mutant and with empty vector (C) and induced by tetracycline. Measurements were done in 1 ml of iso-osmotic buffer using 0.25 mg mitochondrial protein and succinate as substrate (arrow “succ”) in absence (grey trace) or presence (black trace) of 0.5 mM TDP. ADP was sequentially added (arrows “ADP”), e.g. 1, 5, 10, 25, 50, and 100 µM in (A) or 5, 10, 25, 50, and 100 µM in (B,C). Intactness of the outer mitochondrial membrane was routinely checked by a final addition of cytochrome c (arrow “cyt c”). (D-F) Dependence of the respiratory control ratio RCR on ADP concentration (mean ± standard deviation, n=6-7). (G) Immunoblot of mitochondrial preparations obtained from the three different HeLa cell lines, probed with antibodies against NDPK-D and OPA1 (loading control). Note: Km(ADP) of respiration in presence of TDP decreases by about 50% in presence of NDPK-D wild-type, but not with NDPK-D R90D mutant. Also note the different time scale in (A).

Fig. 9. A putative model for NDPK-D function in mitochondria. NDPK-D is bound at the mitochondrial inner membrane mainly to CL, the major anionic phospholipid in this membrane. It thus may co-localize with some other proteins, e.g. ANT (49), in CL patches. Such a co-localization of ANT and NDPK-D would explain the coupling of intermembrane/cristae space NDPK-D to mitochondrial respiration. ATP exported by ANT would be immediately transphosphorylated to GTP or other NTPs. Since cytosolic NTP turnover is ensured by multiple cytosolic NDPKs, the mitochondrially generated NTPs may not leave the organelle via VDAC but rather supply a sink in the mitochondrial intermembrane/cristae space where they could be used by intramitochondrial GTPases to fulfill specific functions. For example, the dynamin-like GTPase OPA1, involved in mitochondrial membrane fusion, shares the same location as NDPK-D, namely the outer leaflet of the inner mitochondrial membrane (57). On the other hand, NDPK-D facing the matrix space could convert “spare” matrix GTP into “transport-competent” ATP for export via ANT. There again, this function would be facilitated by a common location (inner leaflet of the inner mitochondrial membrane) with a TCA enzyme synthesizing GTP, STK.
Figure 1

A

|     | D | LIV | CYT | MIT | MOM | MIM | IS-MX |
|-----|---|-----|-----|-----|-----|-----|-------|
| NDPK-A |    |     |     |     |     |     |       |
| NDPK-B |    |     |     |     |     |     |       |
| NDPK-D |    |     |     |     |     |     |       |
| ANT2  |    |     |     |     |     |     |       |
| VDAC1 |    |     |     |     |     |     |       |

B

|     | MIT | MOM | IS | MIM | MX |
|-----|-----|-----|----|-----|----|
| NDPK-D |    |     |    |     |    |
| ANT   |    |     |    |     |    |
| CYT-C |    |     |    |     |    |

C

|     | Relative activity [%] |
|-----|------------------------|
|     | NDPK                  |
|     | STK                   |

MIT | MOM | IS | MIM | MX |
Figure 2
Figure 3

C  Human group I
NDPK paralogues

NDPK A  .DLKDRPF..
NDPK B  .DLKDRPF..
NDPK C  .ELRERPF..
NDPK D  .DLRRKPF..

D  NDPK D orthologues

H. sapiens  .HYQDLRKKPFPYA..
M. musculus  .HYRLQKPPFYPA..
R. norv.  .HYRLQKPPFYPA..
M. domestica  .HYHDLKKPFPYN..
X. tropicalis  .HYHDLRRKPFYPA..
D. rerio  .HYVSLOKKPFYSS..
C. livia  .HYQQLRKKPFPYA..
P. sativum  .HYHDLKERPFNG..
D. discoideum  .HYEDLKKPFNG..
E. coli  .FYAEHDGKPFFDG..
Figure 6
Figure 7

\[
\begin{array}{c|c|c|c|c}
 & P & S & P & S \\
\hline
- \text{Na}_2\text{CO}_3 & & & & \\
\hline
+ \text{Na}_2\text{CO}_3 & & & & \\
\hline
\text{WT} & \text{R}_{90}\text{D} & & & \\
\hline
\text{NDPK D} & \text{NDPK D} & \text{OPA1} & & \\
\end{array}
\]
Fig. 8

A and B: Kinetic data for NDPK-D WT and NDPK-D R160D showing the effect of ADP and succinate (SUCC) on oxygen consumption (O2) in the presence of CYT C. The data points indicate the points of ADP addition.

C: Control experiments without NDPK-D.

D and E: Graphs showing the relative change rate (RCR) of oxygen consumption as a function of ADP concentration for NDPK-D WT and NDPK-D R160D.

F: Control experiments for RCR.

G: Western blot analysis showing NDPK D and OPA1 expression levels in NDPK-D WT, NDPK-D R160D, and control samples.
Figure 9

Diagram showing the mitochondrial inner membrane system with labels for various components such as cytosol, outer membrane, intermembrane space, inner membrane, matrix, porin (VDAC), NTP, ADP, cardiolipin, ATP, GDP, GTP, and adenylate translocator.