

Tubulin binding blocks mitochondrial voltage-dependent anion channel and regulates respiration

Tatiana K. Rostovtseva^{a,1}, Kely L. Sheldon^a, Elnaz Hassanzadeh^a, Claire Monge^b, Valdur Saks^b, Sergey M. Bezrukov^a, and Dan L. Sackett^c

^aLaboratory of Physical and Structural Biology and ^cLaboratory of Integrative and Medical Biophysics, Program in Physical Biology, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20892; and ^bLaboratory of Fundamental and Applied Bioenergetics, Joseph Fourier University, 2280 Rue de la Piscine, BP53X, 38041, Grenoble Cedex 9, France

Edited by Lynn Margulis, University of Massachusetts, Amherst, MA, and approved October 8, 2008 (received for review July 2, 2008)

Regulation of mitochondrial outer membrane (MOM) permeability has dual importance: in normal metabolite and energy exchange between mitochondria and cytoplasm and thus in control of respiration, and in apoptosis by release of apoptogenic factors into the cytosol. However, the mechanism of this regulation, dependent on the voltage-dependent anion channel (VDAC), the major channel of MOM, remains controversial. A long-standing puzzle is that in permeabilized cells, adenine nucleotide translocase (ANT) is less accessible to cytosolic ADP than in isolated mitochondria. We solve this puzzle by finding a missing player in the regulation of MOM permeability: the cytoskeletal protein tubulin. We show that nanomolar concentrations of dimeric tubulin induce voltage-sensitive reversible closure of VDAC reconstituted into planar phospholipid membranes. Tubulin strikingly increases VDAC voltage sensitivity and at physiological salt conditions could induce VDAC closure at <10 mV transmembrane potentials. Experiments with isolated mitochondria confirm these findings. Tubulin added to isolated mitochondria decreases ADP availability to ANT, partially restoring the low MOM permeability (high apparent K_m for ADP) found in permeabilized cells. Our findings suggest a previously unknown mechanism of regulation of mitochondrial energetics, governed by VDAC and tubulin at the mitochondria-cytosol interface. This tubulin-VDAC interaction requires tubulin anionic C-terminal tail (CTT) peptides. The significance of this interaction may be reflected in the evolutionary conservation of length and anionic charge in CTT throughout eukaryotes, despite wide changes in the exact sequence. Additionally, tubulins that have lost significant length or anionic character are only found in cells that do not have mitochondria.

evolution | microtubules | oxidative phosphorylation | VDAC | tubulin C-terminal

Oxidative phosphorylation requires transport of metabolites, including cytosolic ADP, ATP, and inorganic phosphate, across both mitochondrial membranes for F_1F_0 -ATPase to generate ATP in the matrix. Voltage-dependent anion channel (VDAC, also called mitochondrial porin) is the most abundant protein in mitochondrial outer membrane (MOM) and is known to be primarily responsible for ATP/ADP flux across the outer membrane (1, 2). Until recently, VDAC was generally viewed as a part of the pathway for release of cytochrome *c* and other apoptogenic factors from the mitochondrial intermembrane space into the cytosol at the early stage of apoptosis. The recent genetic studies undermined this view (3) but still left open a lot of questions concerning the role of VDAC in MOM permeabilization in apoptosis (4–6). A conserved property of VDACs *in vitro* is the ability to adopt a unique fully open state and multiple states with significantly smaller conductance (7). It was demonstrated that the latter, so called “closed states” are impermeable to ATP but still permeable to small ions (8), including Ca^{2+} (9).

In isolated mitochondria, respiration is characterized by an apparent K_m for exogenous ADP that is ≈ 10 -fold lower than in

permeabilized cells with oxidative metabolism (10, 11). The discrepancy is most likely caused by a lower permeability of the MOM (and hence VDAC) for ADP in the permeabilized cells and may be the result of the interaction of mitochondria with some cytoskeletal protein(s). Such a conjecture is supported by the action of mild trypsin treatment of permeabilized cells, which causes decrease in apparent K_m for ADP accompanied by mitochondrial rearrangement, implicating the existence of a protein factor(s) associated with the cytoskeleton (10). The decreased value of apparent K_m for ADP after mild proteolytic treatment was similar to K_m in isolated mitochondria and purified preparations of adenine nucleotide translocase (ANT) (10), thus showing that the kinetic values of ANT are similar in all preparations. Therefore, high values of apparent K_m for ADP in permeabilized cells are related to decreased permeability of MOM, possibly because of interaction with cytosolic proteins.

Tubulin, the subunit of microtubules, is a plausible candidate because this cytoskeletal protein is known to interact with mitochondria (12, 13). In addition to mitochondrial membranes, tubulin has been shown to interact with proteins associated with other membranes (14, 15). Tubulin is a heterodimer, composed of similar 50-kDa globular α and β subunits. Each subunit possess a negatively charged, extended C-terminal tail (CTT) that represents $\approx 3\%$ of the subunit mass and $\approx 40\%$ of the subunit charge (16, 17). Tubulin shows saturable, high-affinity binding to intact mitochondria (12) and hence to MOM, although the region of tubulin responsible for binding has not yet been identified. Immunoprecipitation experiments demonstrated a specific association of VDAC with tubulin (13), which indicates that VDAC could be a receptor for tubulin binding to MOM. Exploring this possibility, we studied the functional interaction between VDAC and tubulin to show that nanomolar concentrations of dimeric* tubulin induce highly voltage-sensitive reversible closure of VDAC reconstituted into planar phospholipid membranes.

Author contributions: T.K.R., V.S., S.M.B., and D.L.S. designed research; T.K.R., K.L.S., E.H., and C.M. performed research; T.K.R., C.M., V.S., and D.L.S. analyzed data; and T.K.R., V.S., S.M.B., and D.L.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

¹To whom correspondence should be addressed at: Laboratory of Physical and Structural Biology, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, 9000 Rockville Pike, Building 9, Room 1E-106, Bethesda, MD 20892. E-mail: rostovtt@mail.nih.gov.

*Tubulin in all experiments was dimeric, not polymerized, because there was no GTP or Mg^{2+} added, and the total tubulin concentration was at least 100-fold below the critical concentration for polymerization in the presence of GTP and Mg^{2+} .

This article contains supporting information online at www.pnas.org/cgi/content/full/0806303105/DCSupplemental.

© 2008 by The National Academy of Sciences of the USA

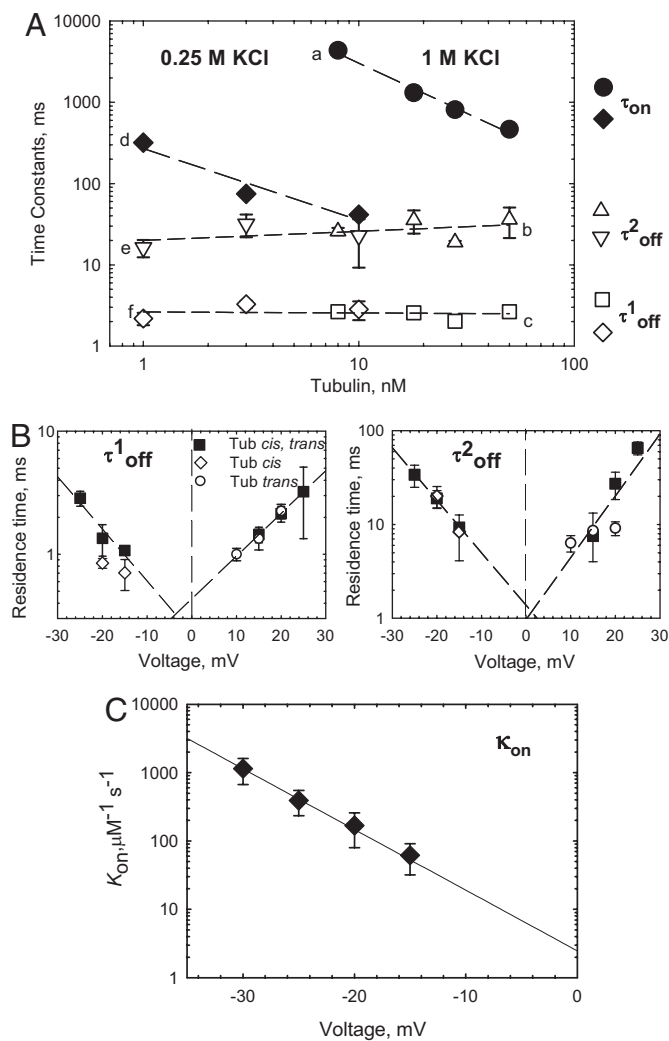


Fig. 2. The binding parameters for tubulin-induced VDAC closure depend on tubulin concentration, electrolyte concentration, and applied voltage. (A) (a and d) VDAC open time between successive blockages, τ_{on} , linearly decreases with tubulin concentration and depends on salt concentration. The medium consisted of 1 M KCl (a, filled circles) and 0.25 M KCl (d, filled diamonds). Both components of tubulin residence (closed) time, $\tau_{off}^{(1)}$ and $\tau_{off}^{(2)}$, are independent of tubulin or salt concentration (b, c, e, and f, open symbols) and are equal to 2.8 ± 0.5 ms and 23.7 ± 6.3 ms, respectively. The applied voltage was -20 mV. (B) Voltage dependence of VDAC residence times, $\tau_{off}^{(1)}$ and $\tau_{off}^{(2)}$, in the presence of 50 nM tubulin in *cis* (open diamonds), *trans* (open circles), or both sides (filled squares) of the membrane. Residence time in extrapolation to 0 voltage does not depend on 1-side (*cis* or *trans*) or 2-side tubulin addition. (C) Voltage dependence of the on-rate, κ_{on} , with 50 nM tubulin added to the *cis* side. The line is an exponential fit to $\kappa_{on} = \kappa_0 \exp(nVF/RT)$ with $n = 4.97$. Each time value presents the characteristic time of 9 different log probability fitting procedures \pm SE. The medium consisted of 1 M KCl (B and C) and buffered with 5 mM Hepes at pH 7.4. VDAC was isolated from *N. crassa* mitochondria. Bilayer membranes were formed from DPhPC.

from both sides of the channel and that anionic CTT are mainly responsible for VDAC blockage (see Fig. 4).

Role of CTT in Tubulin–VDAC Interaction. To test the CTT-dependent mechanism, we studied the effect of tubulin with truncated C termini, tubulin-S, on VDAC conductance. Comparison of VDAC current traces in the presence of tubulin-S (Fig. 3A) and intact tubulin (*viz.* “tubulin”) (Fig. 3C), obtained under the same experimental conditions, shows a striking difference. Tubulin-S did not induce reversible blockage typical for tubulin, but instead generated

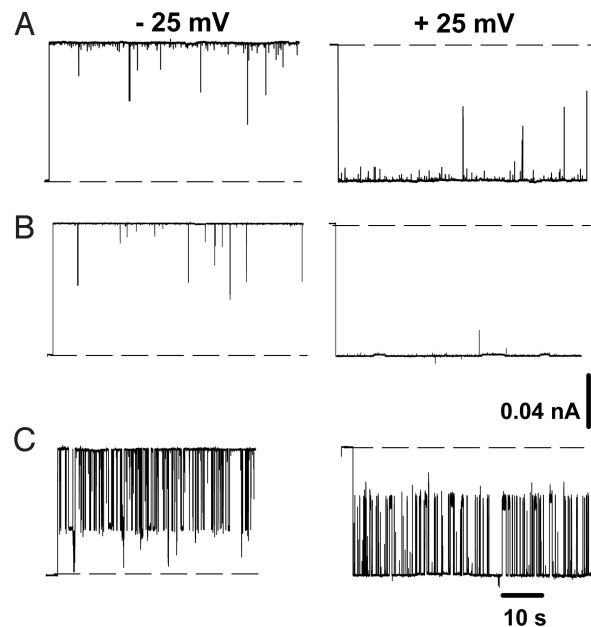


Fig. 3. Tubulin interaction with VDAC requires the presence of C-terminal tails of tubulin. Tubulin (50 nM) with truncated CTT, tubulin-S (A), or a mixture of 10 μM 2 synthetic peptides of mammalian α - and β -brain tubulin CTT (B) does not induce channel blockage characteristic for intact tubulin (10 nM), with fast reversible blockage to 1 closed state (C). Representative current traces through single VDAC were obtained at ± 25 mV of applied voltage in 1 M KCl solutions buffered with 5 mM Hepes at pH 7.4. Tubulin, tubulin-S, and CTT peptides were added to both sides of the membrane. Other experimental conditions were as in Fig. 2 B and C.

shorter current interruptions with $\tau_{closed} < 1$ ms and rather broad distribution of conductances of the closed states [supporting information (SI) Fig. S1a]. An excess of current noise in the presence of tubulin-S indicates that the tubulin globular body interacts with VDAC even without CTT. However, these interactions most likely are of different nature than those with intact tubulin. Accordingly, experiments on multichannel membranes confirmed that tubulin-S does not affect VDAC voltage-gating parameters such as gating charge and the characteristic voltage at which half-channels are open and half-closed (Fig. S1b and Table S1). Results similar to tubulin-S (*i.e.*, the absence of reversible channel blockage) were obtained with actin (Fig. S2), also an acidic protein but lacking CTT. This agrees with a previous report of actin interaction with VDAC from *Neurospora crassa* (19).

It was shown that a number of different synthetic and natural polyanions nonspecifically increase VDAC voltage gating (7). This raises a question about specificity of VDAC closure by tubulin, considering that CTT are highly negatively charged. To address this, we studied the effect of CTT synthetic peptides on VDAC properties. Two synthetic peptides with the sequences of mammalian α and β brain tubulin CTT did not induce detectable channel closure up to micromolar concentrations. In the experiment presented in Fig. 3B, a mixture of 2 synthetic peptides (10 μM each) corresponding in sequence to mammalian α and β brain tubulin CTT was added to both sides of the membrane, but the current traces through single channels were indistinguishable from control traces. This suggests that CTT peptides translocate through the channel too fast, in a < 0.1 ms time scale, and do not block it in a time-resolved manner. Experiments on multichannel membranes confirmed that VDAC voltage-gating parameters were also not affected by CTT peptides (Fig. S3). Thus, the results with tubulin-S, actin, and CTT synthetic peptides support our model of tubulin CTT interaction with VDAC leading to the voltage-dependent channel closure.

When tubulin CTT penetrates into the VDAC lumen (Fig. 4), it

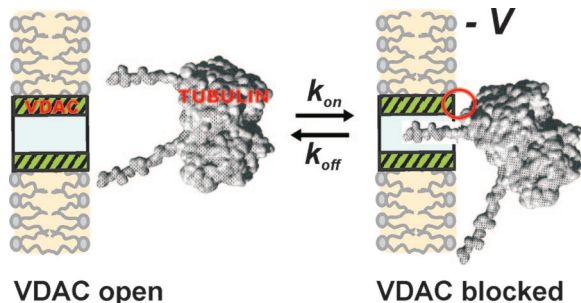


Fig. 4. Model of tubulin–VDAC interaction. One tubulin CTT partially blocks channel conductance by entering VDAC pore. This process is voltage-dependent and could be described by the 1st-order reaction of one-to-one binding of tubulin to VDAC. Some additional interaction between tubulin globular body and VDAC may be involved. The model of a tubulin dimer was redrawn from ref. 17.

negative charge changes channel ion selectivity. The residual conductance of the channel during tubulin block was cation-selective with the permeability ratio of $P_K/P_{Cl} = 2.4 \pm 0.7$ and reversal potential of (-14 ± 4) mV in 1.0 M *cis*/0.2 M *trans* gradient of KCl. This should be compared with the anion selectivity of VDAC open state, which is characterized by $P_K/P_{Cl} = 0.5 \pm 0.02$ and reversal potential of (12 ± 1) mV. The selectivity inversion further supports the proposed model where VDAC closure arises from interaction of negatively charged CTT with net positively charged channel interior (Fig. 4). A similar effect on VDAC selectivity was described when negatively charged phosphorothioate oligonucleotides blocked VDAC (20).

The effect of tubulin blockage of VDAC was confirmed with VDAC isolated from mitochondria from rat liver (see traces in Fig. 1A) and heart and from *N. crassa* (see traces in Fig. 3C), and with tubulin isolated from bovine and rat brain, which gave indistinguishable results. Tubulin isolated from *Leishmania tarentolae* and *Leishmania amazonensis* exhibited VDAC blockage qualitatively similar to mammalian tubulins, but quantitatively there were some differences, possibly because of the extensive posttranslational modifications (PTMs) in CTT of *Leishmania* tubulin.

Tubulin Decreases Respiration Rate of Isolated Mitochondria. Our results demonstrate that tubulin can induce reversible blockage of VDAC and hence should be able to regulate ATP/ADP flux across MOM. Can tubulin do so in the intact mitochondria? To answer this question, we conducted experiments on isolated mitochondria. Oxygen consumption of isolated brain and heart mitochondria in response to successive additions of ADP was measured by using oxygraph. The value of apparent K_m for exogenous ADP represents the availability of ADP for ANT to activate oxidative phosphorylation. The classic value of the K_m for ADP of isolated mitochondria is ≈ 10 – $20 \mu\text{M}$ (21), and the linearization of its kinetic of respiration is shown in Fig. 5A. Addition of $1 \mu\text{M}$ tubulin resulted in the appearance of a second component of mitochondria respiration kinetics with K_m of $(169 \pm 52) \mu\text{M}$, >20 times higher than the first component ($7 \pm 2 \mu\text{M}$; Fig. 5B and Table 1). The high K_m component accounts for $\approx 30\%$ of the total ADP flux in mitochondria. Similar results were obtained with mitochondria from heart (Table 1). Thus, there is tubulin binding to mitochondria as seen from the second component with high K_m . Furthermore, the absence of variation of the maximal rate of respiration, V_{max} (Table 1) between the control and the second component of the respiration kinetics in presence of tubulin, highlights the integrity of the respiratory chain (22). A possible explanation for the persistence of the first component (population of VDAC unaffected by tubulin) could be different affinity of tubulin to VDAC isomers 1, 2, and 3. There could also be other factors that compete with tubulin for VDAC binding, like hexokinases, which have been reported to bind

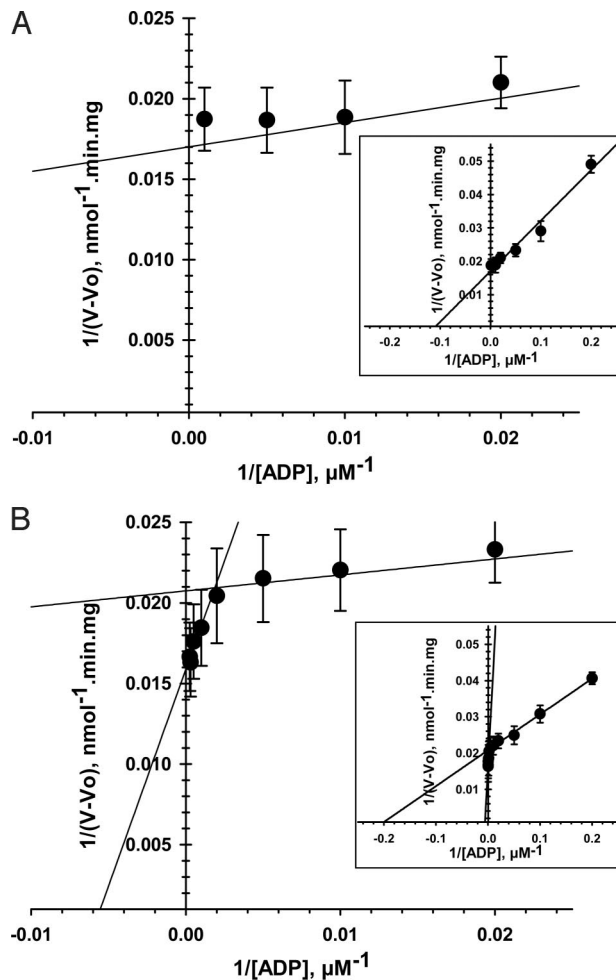


Fig. 5. Tubulin dramatically increases apparent K_m for ADP in regulation of respiration of isolated brain mitochondria. Shown are double-reciprocal representations of the respiration kinetics of brain mitochondria activated by ADP in control (A) and in the presence of $1 \mu\text{M}$ tubulin (B). The 2 straight lines represent 2 different respiration kinetics in the presence of tubulin (B). (Insets) Enlargements of A and B. Each data point is a mean of 6–9 independent experiments \pm SE.

to MOM directly through VDAC or close by (23), possibly obstructing tubulin access to VDAC. However, the precise nature of the 2 components is not clear at this time and needs further careful studies.

The interaction of other cytosolic proteins with VDAC may be important for regulation of tubulin–VDAC binding. Tubulin is an abundant and stable protein, present in many cells at concentrations higher than those shown here to block VDAC. We suggest that tubulin–VDAC interaction may be modulated through competition with other proteins that bind to VDAC, preventing tubulin binding. Another possibility is that PTM of VDAC or tubulin may alter this interaction. This possibility is particularly intriguing because PTMs of tubulin mostly occur on the CTT (24), the locus of interaction with VDAC. Under physiological conditions, tubulin concentration does not change dramatically. However, it is worth mentioning here that in cells exposed to microtubule-targeting drugs such as colchicine or paclitaxel (Taxol), the balance between tubulin dimers and polymerized tubulin can change. This might affect cytosolic tubulin dimer concentration and alter tubulin–VDAC interaction.

Evolutionary Considerations. These results raise interesting evolutionary questions. Tubulin is a very old protein, found in all eukaryotes (25). This raises the question of whether the interaction

Table 1. Kinetic parameters of respiration in brain and heart isolated mitochondria with 1 μ M tubulin and without it

Brain mitochondria (n = 6–9)	Apparent K_m (ADP), μ M	V_{max} , nmol O_2 /min/mg
Without tubulin	9 \pm 1	55 \pm 5
With tubulin		
First component	7 \pm 2	49 \pm 15
Second component	169 \pm 52	61 \pm 15
Heart mitochondria (n = 4–6)		
Without tubulin	11 \pm 2	216 \pm 11
With tubulin		
First component	9 \pm 5	136 \pm 9
Second component	330 \pm 47	241 \pm 10

Each value is a mean of n experiments \pm SE.

we show here plays a role throughout the eukaryotic world. In support of this, we have shown that similar results are obtained with VDACS from rat or *Neurospora*, and with tubulin from rat or *Leishmania*. We can also approach this question by comparing the sequences of VDAC and tubulin from many species.

VDAC sequences (nearly 250) are known from plants, animals, fungi, stramenopiles, alveolates, rhodophyta, and charophyta, and functional data exist for VDAC from a number of organisms (26). Functional and structural properties of VDAC are preserved, although a great deal of sequence variation is found.

Tubulin sequences are known from throughout eukaryotes and reveal remarkable sequence conservation (27). A notable exception is the region of tubulin in the CTT, which shows significant sequence variation, both between isoforms in a given species and between species.

Although CTT sequences vary significantly, the functional properties that we found to be involved in VDAC–tubulin interaction are remarkably conserved throughout eukaryotic α - and β -tubulins, with a few significant exceptions to be discussed below. This is shown in alignments of β -CTT in Fig. 6A and in more extensive alignments of α - and β -CTT in Fig. S4. Our analysis shows that CTT length and charge are highly conserved in cells that contain mitochondria. Summary statistics for \approx 100 α - and β -CTT sequences from our alignments and from ref. 27 are presented in Fig. 6B.

Tubulins with highly altered length or charge and that are the sole α or β gene in the organism are found only in organisms that do not have mitochondria (although not all organisms that lack mitochondria have altered CTT; see Fig. S4). Examples of altered tubulin CTT are from obligate intracellular eukaryotes that lack functional mitochondria and contain tubulin CTT much shortened in length and reduced in charge, including total loss of charge (Fig. 6 and Fig. S4). In addition, genes for α - and β -tubulins are found in bacteria (*Prostheco bacter* species) (28), likely the result of horizontal gene transfer from an unknown eukaryote (29). Although their function in the bacterial cell is unclear, these tubulins have essentially the same 3D structure as mammalian tubulin and exhibit GTP-dependent polymerization (29). However, the CTT differ radically from mammalian tubulin, perhaps in part because of the absence of mitochondria and hence loss of selection to maintain a CTT that can interact with VDAC. Both length and charge of the CTT, features that are highly conserved in eukaryotes, seem free to vary widely in *Prostheco bacter*, ranging from total loss to significant extension in length and acquisition of positive charges (Fig. 6A).

We suggest that the conservation of CTT length and charge in tubulins from organisms with functional mitochondria is caused in part to selective pressure to maintain tubulin CTT–VDAC interaction and thereby cytoplasmic regulation of mitochondrial energetics [although clearly other factors are also important, such as the evolutionarily conserved PTM that occur here (30)]. Conversely, the divergence in length and charge seen in some eukaryotes that lack functional mitochondria and in bacteria is because of the absence of this selective pressure.

Conclusions

We find that nanomolar concentrations of tubulin vastly increase VDAC sensitivity to voltage and could induce VDAC closure at low transmembrane potentials. Tubulin interaction with VDAC requires the presence of anionic CTT on the intact protein. This suggests a completely new role for dimeric tubulin and its charged CTT. Established roles for tubulin CTT have been dominated by interactions with microtubule-severing proteins (31), motor proteins (32), and plus-end-binding proteins (33), as well as being the

A Sequence alignments of beta-tubulin CTT

Eukaryotes with mitochondria	
<i>Homo sapiens</i>	ATADEQGFEEGEDEA
<i>Gallus gallus</i>	ATANDGEEAFEDDEEINE
<i>Danio rerio</i>	ATADDEADFGEEGE
<i>Xenopus laevis</i>	ATADEQGFEEDEEA
<i>Drosophila melanogaster</i>	ATADEDAEFEEEQEAFVDEN
<i>Homarus americanus</i>	ATADDEAEFEEEGFVNGEYA
<i>Zea mays</i>	ATAEHYDEEEQDGEERHD
<i>Schizosaccharomyces pombe</i>	AGIDRGDELYEIEEEKPEPLY
<i>Brugia malayi</i>	ATADEEQDLQEGESYIQQEE
<i>Tetrahymena thermophila</i>	ATAEEEGEFEEEGGN
<i>Leishmania tarentolae</i>	ATVEEEGEYDEEEPT
Eukaryotes lacking mitochondria	
<i>Guillardia theta</i>	AKMNDIAFELQQLY
<i>Enterocytozoon bieneusi</i>	SQVGGY
<i>Encephalitozoon cuniculi</i>	ATIADADFLVN
Bacteria	
<i>Prostheco bacter de joneii</i> (BTubB)	---
<i>Prostheco bacter de joneii</i> (BTubA)	SQAALAVQISAGTQMIAAAA-GVSDAAGSMSLALVDRRA

B Summary of alpha and beta CTT properties

	Alpha (n = 86)	Beta (n = 132)
Charge ¹	-7.2 \pm 1.4	-10.3 \pm 1.5
Length (residues) ¹	9.9 \pm 1.6	19.2 \pm 2.8

1. Values are mean \pm s.d.

Fig. 6. Analysis of tubulin CTT sequences. (A) Sequence alignments of β -tubulin CTT. Full sequences were aligned, but only the CTT, defined as the residues from C-terminal to the last residue in the crystal structure, are shown. Acidic residues are shown in red, basic residues in blue. More extensive alignments of both α - and β -CTT are in Fig. S4. (B) Summary of length and charge values for α - and β -CTT, taken from our alignments and from the data in ref. 27. Each value is a mean \pm SD; n , number of species.

site of PTM (34). We propose a model for tubulin–VDAC interaction in which the tubulin CTT penetrates into the channel lumen, potentially reaching through the channel because of the length of the CTT, interacting with a positively charged domain of VDAC. Because of this interaction, VDAC has a high affinity for CTT, which partially blocks channel conductance (Fig. 4). Identification of which CTT, α , β , or both, blocks VDAC is a subject of future research.

The tubulin-induced increase of K_m for ADP in isolated mitochondria is evidence of a significant drop of the availability of ADP to ANT and partial restoration of the K_m found in permeabilized cells. Thus, a mechanism for increasing K_m by decreasing ATP/ADP permeability through MOM is closure of VDAC by tubulin. By this type of control, tubulin may selectively regulate metabolic fluxes between mitochondria and cytoplasm (35, 36).

Conservation of tubulin CTT length and charge and VDAC folding pattern throughout mitochondria-containing eukaryotes suggest that this interaction is widespread and ancient. Our experimental results with tubulins and VDAC from distantly related organisms demonstrate this. The loss of tubulin CTT properties only in organisms that lack mitochondria reinforces this view. Our results not only identify a previously unknown mechanism of regulation of MOM permeability and mitochondria respiration, but also identify a functional role for the cytoskeletal protein, dimeric tubulin.

Materials and Methods

Protein Purification. VDAC from rat liver or *N. crassa* mitochondrial outer membranes was isolated and purified as described in ref. 37 and were a generous gift of M. Colombini (University of Maryland, College Park, MD). Bovine brain tubulin and rabbit muscle actin were obtained from Cytoskeleton. Rat brain tubulin was purified as described in ref. 38 (see *SI Materials and Methods*). Tubulin-S was produced by subtilisin cleavage of rat brain tubulin as described in ref. 39. Peptides corresponding to the CTT of α - and β -tubulins were obtained from Sigma–Genosys. The sequence of the synthetic peptide corresponding to the CTT

is that of the $\beta\alpha$ -1 isotype, EVGVHSEVEGEGEEGEEY, whereas the β -sequence is that of the β I isotype, DATAEEEDFGEEAEEEA.

Electrophysiological Recordings. Planar lipid membranes were formed from monolayers made from 1% (wt/vol) of lipids in hexane on 70- to 80- μ m-diameter orifices in the 15- μ m-thick Teflon partition that separated 2 chambers as described in ref. 40. The lipid-forming solutions contained diphytanoyl phosphatidylcholine (DPhPC) or asolectin and cholesterol (10:1 wt/wt). DPhPC and asolectin (soybean phospholipids) were purchased from Avanti Polar Lipids, and cholesterol was purchased from Sigma. VDAC insertion was achieved by adding 0.1–1.5 μ L of a 1% Triton X-100 solution of purified VDAC to the 1.2-mL aqueous phase in the *cis* compartment while stirring. Potential is defined as positive when it is greater at the side of VDAC addition (*cis*). After channels were inserted and their parameters were monitored, tubulin was added to one or both sides of the membrane under constant stirring for 2 min. Conductance measurements were performed as described in ref. 40 (see *SI Materials and Methods*). Amplitude, lifetime, and fluctuation analysis was performed by using Clampfit 9.2 (Axon Instruments).

Mitochondria Respiration Measurements. Brain and cardiac mitochondria were isolated as described (11, 41) (see *SI Materials and Methods*). All measurements of respiration rates were performed by using the high-resolution respirometry (Oroboros Oxygraph) with substrates of 10 mM succinate for brain mitochondria and 5 mM glutamate and 2 mM malate for heart mitochondria. Oxygen solubility in the Mitomed solution was taken to be 225 μ M at 25 °C. Kinetics of activation of respiration in mitochondria was studied by increasing successively the final concentrations of ADP (5–10–20–50–100–200–500–1,000–2,000–3,000 μ M) in the absence or in presence of 1 μ M tubulin, with 0.2% of BSA and 1 U/ml apyrase for ADP regeneration. Mitochondria were incubated with 1 μ M tubulin 30 min at room temperature (25 °C) before introduction in the oxygraphy chamber. Mitochondrial apparent K_m for ADP and proportion of each component of respiration kinetics were calculated as described in ref. 42.

ACKNOWLEDGMENTS. This work was supported by the Intramural Research Program of the Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health (T.K.R., K.S., E.H., S.M.B., and D.L.S.) and by Agence National de la Recherche Project BLAN07–2.188128, Institut National de la Santé et de la Recherche Médicale, France (to C.M. and V.S.). V.S. was also supported by Estonian Science Foundation Grants 6142 and 7117.

- Colombini M (2004) VDAC: The channel at the interface between mitochondria and the cytosol. *Mol Cell Biochem* 256–257:107–115.
- Lemasters JJ, Holmuhamedov E (2006) Voltage-dependent anion channel (VDAC) as mitochondrial governor: Thinking outside the box. *Biochim Biophys Acta* 1762:181–190.
- Baines CP, Kaiser RA, Sheiko T, Craigen WJ, Molkenin JD (2007) Voltage-dependent anion channels are dispensable for mitochondrial-dependent cell death. *Nat Cell Biol* 9:550–555.
- Rostovtseva TK, Tan W, Colombini M (2005) On the role of VDAC in apoptosis: Fact and fiction. *J Bienerg Biomembr* 37:129–142.
- Galluzzi L, Kroemer G (2007) Mitochondrial apoptosis without VDAC. *Nat Cell Biol* 9:487–489.
- Yagoda N, et al. (2007) RAS–RAF–MEK-dependent oxidative cell death involving voltage-dependent anion channels. *Nature* 447:864–886.
- Colombini M, Blachly-Dyson E, Forte M (1996) VDAC, a channel in the outer mitochondrial membrane. *Ion Channels*, ed Narahashi T (Plenum, New York), pp 169–202.
- Rostovtseva T, Colombini M (1996) ATP flux is controlled by a voltage-gated channel from the mitochondrial outer membrane. *J Biol Chem* 271:28006–28008.
- Tan W, Colombini M (2007) VDAC closure increases calcium ion flux. *Biochim Biophys Acta* 1768:2510–2515.
- Appaix F, et al. (2003) Possible role of cytoskeleton in intracellular arrangement and regulation of mitochondria. *Exp Physiol* 88:175–190.
- Saks VA, et al. (1995) Control of cellular respiration in vivo by mitochondrial outer membrane and by creatine kinase. A new speculative hypothesis: Possible involvement of mitochondrial–cytoskeleton interactions. *J Mol Cell Cardiol* 27:625–645.
- Bernier-Valentin F, Rousset B (1982) Interaction of tubulin with rat liver mitochondria. *J Biol Chem* 257:7092–7099.
- Carré M, et al. (2002) Tubulin is an inherent component of mitochondrial membranes that interacts with the voltage-dependent anion channel. *J Biol Chem* 277:33664–33669.
- Rasenick MM, Donati RJ, Popova JS, Yu JZ (2004) Tubulin as a regulator of G-protein signaling. *Methods Enzymol* 390:389–403.
- Santander VS, et al. (2006) Tubulin must be acetylated in order to form a complex with membrane Na^+ , K^+ -ATPase and to inhibit its enzyme activity. *Mol Cell Biochem* 291:167–174.
- Sackett DL, Bhattacharyya B, Wolff J (1985) Tubulin subunit carboxyl termini determine polymerization efficiency. *J Biol Chem* 260:43–45.
- Priel A, Tuszynski JA, Woolf NJ (2005) Transitions in microtubule C-termini conformations as a possible dendritic signaling phenomenon. *Eur Biophys J* 35:40–52.
- Lemeshko VV (2006) Theoretical evaluation of a possible nature of the outer membrane potential of mitochondria. *Eur Biophys J* 36:57–66.
- Xu X, Forbes JG, Colombini M (2001) Actin modulates the gating of *Neurospora crassa* VDAC. *J Membr Biol* 180:73–81.
- Tan W, Loke YH, Stein CA, Miller P, Colombini M (2007) Phosphorothioate oligonucleotides block the VDAC. *Biophys J* 93:1184–1191.
- Vignais P (1976) Molecular and physiological aspects of adenine nucleotide transport in mitochondria. *Biochim Biophys Acta* 456:1–38.
- Nicholls D, Ferguson SJ (2002) *Bioenergetics* (Academic, London).
- Robey RB, Hay N (2006) Mitochondrial hexokinases, novel mediators of the antiapoptotic effects of growth factors and Akt. *Oncogene* 25:4683–4696.
- Hammond JW, Cai D, Verhey KJ (2008) Tubulin modifications and their cellular functions. *Curr Opin Cell Biol* 20:71–76.
- Margulis L, Chapman M, Guerrero R, Hall J (2006) The last eukaryotic common ancestor (LECA): Acquisition of cytoskeletal motility from aerotolerant spirochetes in the Proterozoic Eon. *Proc Natl Acad Sci USA* 103:13080–13085.
- Young MJ, Bay DC, Hausner G, Court DA (2007) The evolutionary history of mitochondrial porins. *BMC Evol Biol* 7:31.
- Tuszynski JA, et al. (2006) The evolution of the structure of tubulin and its potential consequences for the role and function of microtubules in cells and embryos. *Int J Dev Biol* 50:341–358.
- Jenkins C, et al. (2002) Genes for the cytoskeletal protein tubulin in the bacterial genus *Prostheobacter*. *Proc Natl Acad Sci USA* 99:17049–17054.
- Schlieper D, Oliva MA, Andreu JM, Löwe J (2005) Structure of bacterial tubulin BtubA/B: Evidence for horizontal gene transfer. *Proc Natl Acad Sci USA* 102:9170–9175.
- Westermann S, Weber K (2003) Post-translational modifications regulate microtubule function. *Nat Rev Mol Cell Biol* 4:938–947.
- Roll-Mecak A, Vale RD (2008) Structural basis of microtubule severing by the hereditary spastic paraplegia protein spastin. *Nature* 451:363–367.
- Skinioitis G, et al. (2004) Modulation of kinesin binding by the C termini of tubulin. *EMBO J* 23:989–999.
- Mishima M, et al. (2007) Structural basis for tubulin recognition by cytoplasmic linker protein 170 and its autoinhibition. *Proc Natl Acad Sci USA* 104:10346–10351.
- Verhey KJ, Gaertig J (2007) The tubulin code. *Cell Cycle* 6:2152–2160.
- Saks V, et al. (2006) Cardiac system bioenergetics: Metabolic basis of the Frank–Starling law. *J Physiol* 571:253–273.
- Saks V, Vendelin M, Aliev MK, Kekelidze T, Engelbrecht J (2007) Mechanisms and modeling of energy transfer between and among intracellular compartments. *Brain Energetics: Integration of Molecular and Cellular Processes*, eds Gibson GF, Dielen G (Springer, Berlin), pp 815–860.
- Blachly-Dyson E, Peng SZ, Colombini M, Forte M (1990) Alteration of the selectivity of the VDAC ion channel by site-directed mutagenesis: Implication for the structure of a membrane ion channel. *Science* 247:1233–1236.
- Wolff J, Sackett DL, Knippling L (1996) Cation-selective promotion of tubulin polymerization by alkali metal chlorides. *Protein Sci* 5:2020–2028.
- Knippling L, Hwang J, Wolff J (1999) Preparation and properties of pure tubulin S. *Cell Motil Cytoskeleton* 43:63–71.
- Rostovtseva TK, Kazemi N, Weinrich M, Bezrukov SM (2006) Voltage gating of VDAC is regulated by nonlamellar lipids. *J Biol Chem* 281:37496–37506.
- Booth RF, Clark JB (1978) A rapid method for the preparation of relatively pure metabolically competent synaptosomes from rat brain. *Biochem J* 176:365–370.
- Saks V, et al. (1998) Permeabilized cell and skinned fiber techniques in studies of mitochondrial function in vivo. *Mol Cell Biochem* 184:81–100.