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Comprehensive Invited Review

Ultrastructure of the Mitochondrion and Its Bearing on Function and Bioenergetics

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ABSTRACT

The recently ascertained network and dynamic organization of the mitochondrion, as well as the demonstration of energy proteins and metabolites subcompartmentalization, have led to a reconsideration of the relationships between organellar form and function. In particular, the impact of mitochondrial morphological changes on bioenergetics is inseparable. Several observations indicate that mitochondrial energy production may be controlled by structural rearrangements of the organelle both interiorly and globally, including the remodeling of cristae morphology and elongation or fragmentation of the tubular network organization, respectively. These changes are mediated by fusion or fission reactions in response to physiological signals that remain unidentified. They lead to important changes in the internal diffusion of energy metabolites, the sequestration and conduction of the electric membrane potential ($\Delta\Psi$), and possibly the delivery of newly synthesized ATP to various cellular areas. Moreover, the physiological or even pathological context also determines the morphology of the mitochondrion, suggesting a tight and mutual control between mitochondrial form and bioenergetics. In this review, we delve into the link between mitochondrial structure and energy metabolism. *Antioxid. Redox Signal.* 10, 1313–1342.

I. INTRODUCTION

A. The mitochondrial oxidative phosphorylation system

The rapid expansion of mitochondrial research within several domains of biology and medicine has brought together biochemists, neuroscientists, cell biologists, and clinicians. Prior to examining the link between mitochondrial form and function, we should briefly introduce the current state of knowledge in bioenergetics, followed by a descriptive analysis of common theories of mitochondrial structure.

In most human tissues, mitochondria provide the energy necessary for cell growth, and biological activities. It has been estimated that ~90% of mammalian oxygen consumption is mitochondrial, which primarily serves to synthesize ATP, although in variable levels according to the tissue considered and the organism's activity status. Mitochondria intervene in the ultimate phase of cellular catabolism, following the enzymatic reactions of intermediate metabolism that degrade carbohydrates, fats, and proteins into smaller molecules such as pyruvate, fatty acids, and amino acids, respectively (Fig. 1A). Mitochondria further transform these energetic elements into NADH and/or $FADH_2$, through β-oxidation and the Krebs cycle. Those reduced equivalents are then degraded by the mitochondrial respiratory chain in a global energy converting process called oxidative phosphorylation (OXPHOS) (141), where the electrons liberated by the oxidation of NADH and $FADH_2$ are passed along a series of carriers regrouped under the name of "respiratory chain" or "electron transport chain" (ETC), and ultimately transferred to molecular oxygen (Fig. 1B). ETC is located in mitochondrial inner membrane, with an enrichment in the cristae (65). ETC consists of four enzyme complexes (complexes I to IV), and two mobile electron carriers (coenzyme Q and cytochrome c). These complexes are composed of numerous subunits encoded by both nuclear genes and mitochondrial DNA at the exception of complex II (nuclear only). It was demonstrated that these complexes can assemble into supramolecular assemblies called "supercomplexes" or respirasomes (143, 144, 148).

In the presence of energy substrate (NADH or $FADH_2$), the transfer of electrons from complex I (or II) to complex IV mediates the extrusion of protons from the matrix to the intermembrane space, thus generating an electrochemical gradient of protons ($\Delta\tilde{\mu}H^+$) which is finally used by the F_1-F_o ATP synthase (*i.e.,* complex V) to produce adenosine triphosphate (ATP), the energetic currency of the cell. This gradient has two components: an electric potential ($\Delta\Psi$) and a chemical potential ($\Delta\mu_H{}^+$) that can also be expressed as a pH gradient (ΔpH). According to the chemiosmotic theory (114), $\Delta\tilde{\mu}H^+ = \Delta\Psi - Z\Delta pH$, with $Z = -2.303$ RT/F. Under physiological conditions, mitochondrial energy production can alternate between two energy steady-states: basically, at state 4, respiration is slow and ATP is not produced ($\Delta\Psi$ is high), whereas during state 3, respiration is faster and ATP is largely produced ($\Delta\Psi$ is lower). In particular conditions, such as mitochondrial inner membrane permeabilization or the use of a chemical uncoupler, $\Delta\Psi$ can be totally dispersed. As a consequence, respiration is accelerated and ATP production annihilated. The inhibition of respiratory chain complexes also generally decreases $\Delta\Psi$. Under physiological conditions, it is considered that mitochondria produce ATP in an intermediate state lying between state 3 and state 4. ATP is the only form of energy used by the cell, and when produced in the mitochondrion it is exported to the cytosol by the adenine nucleotide translocator (ANT) in exchange for cytosolic ADP. Generally, the transport of energy metabolites, nucleotides, and cofactors into and out of the mitochondrial matrix is performed by transporters located in the inner membrane (for review, see ref. 127). Mitochondria also contain "shuttle" systems that permit the transport of NADH (29).

The mitochondrial oxidative phosphorylation system is alternatively implicated in the generation of reactive oxygen species (ROS). These molecules can be considered as important products of mitochondrial energy metabolism that intervene in cell signaling (22). However, excessive amounts of ROS are deleterious for the cell, contributing to a variety of pathological processes. In most cells, the mitochondrial respiratory chain is recognized as the major site of ROS production in the form of superoxide, hydrogen peroxide, and the hydroxyl free radical (for review, see ref. 85). Complexes I and II generate superoxide within the mitochondrial matrix, Complex III generates superoxide at the Q_o site, resulting in the release of superoxide into either the intermembrane space or the matrix, and Complex IV has not been reported to generate ROS. Cy-

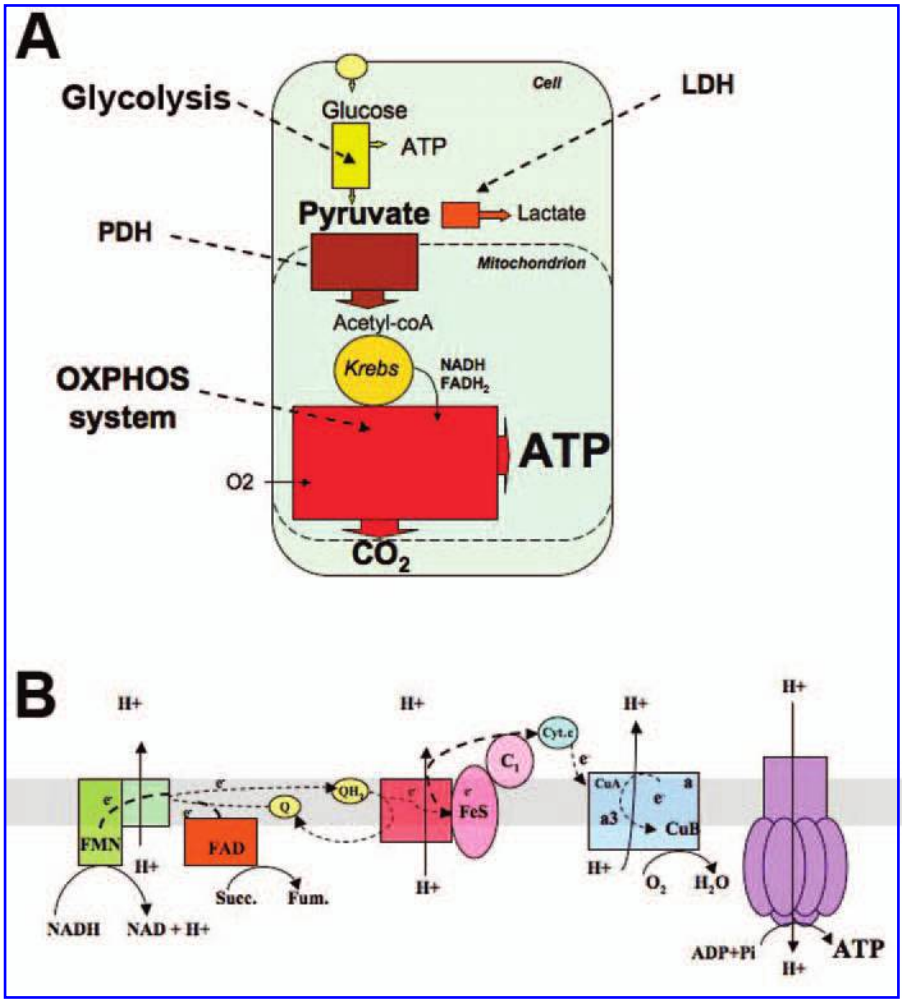

FIG. 1. Mitochondria as the power plant of the cell. Cellular energy metabolism and the mitochondrial oxidative phosphorylation system (**B**). In (**A**) the main chemical reactions of energy metabolism are presented in a mammalian cell (*light green rectangle*). The two main metabolic pathways [glycolysis (*yellow rectangle* in the cytosol) and oxidative phosphorylation (*red rectangle*) in mitochondria (*blue rectangle dashed line*)], are linked by the enzyme complex pyruvate dehydrogenase (*brown rectangle*, in mitochondria). Briefly, glucose is transported inside the cell and oxidized to pyruvate. Under aerobic conditions, the complete oxidation of pyruvate occurs through the TCA cycle to produce NADH and/or $FADH_2$. These reduced equivalents are oxidized further by the mitochondrial respiratory chain (**B**) to establish an electrochemical gradient of protons, that is finally used by the F 1F0-ATP synthase to produce ATP, the only form of energy used by the cell. For mammals, the respiratory chain consists of four enzyme complexes (complexes I–IV) and two intermediary substrates (coenzyme Q and cytochrome c). In this figure, complex I is *colored green*, complex II in *orange*, complex III in *pink*, complex IV in *blue*, and complex V (F1F0 ATP synthase) in *purple*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

tochrome *c* was also recently demonstrated to participate in the generation of hydrogen peroxide. The level of ROS generation depends closely on mitochondrial energy state and is favored by high membrane potential values ($\Delta\Psi$) (93). It is also largely increased in cases of respiratory chain inhibition, as observed in mitochondrial disease, or on experimental and animal models of OXPHOS deficiencies (111). However, in such cases, it can be demonstrated that threshold inhibition for complex I (30%), or complex III (70%), must be reached before to observe significant ROS production (151). Due to the proximity of the mitochondrial respiratory chain to mtDNA in the mitochondrial interior, ROS production can result in the set-up of a vicious cycle of oxidative damage, causing a progressive alteration of mtDNA and mitochondrial functions, that lead in turn to energy deprivation, redox imbalance, and cell dysfunction. Accordingly, the mitochondrial theory of aging predicts that ROS production contributes to the aging process, by generating damage that leads to respiratory chain dysfunction and hence further augmented ROS production (81). This vicious cycle is proposed to cause an exponential increase of mtDNA mutations over time, resulting in aging and associated degenerative diseases.

At the cellular level, the damaging effect of ROS overproduction occurs through oxidative degradation of cellular proteins, lipids, and DNA, resulting in a condition known as "oxidative stress." To counter this damaging effect of ROS, cells contain a multilayered system of antioxidant defenses, consisting of three types of enzymes: the superoxide dismutases (SODs), catalases (CATs), and peroxidases, of which glutathione peroxidase (GPX) is the most common. Two major types of SODs, manganese SOD (MnSOD) and copper–zinc SOD (CuZnSOD), are found in mammalian cells. MnSOD is localized in mitochondria, whereas CuZnSOD is found in the nucleus and cytosol. Similarly, GPX, which is selenium-de-

pendent, is found in the nucleus, mitochondria, and cytosol, while CAT is localized to the peroxisomes and cytosol. The radical species H_2O_2 is typically produced in peroxisomes as a byproduct of reactions catalyzed by oxidases. Catalase is also found in heart mitochondria. Excessive levels of ROS can also trigger cell death by opening the mitochondrial permeability transition pore (mPTP) that releases pro-apoptotic factors (94).

B. From mitochondria to the mitochondrial network

First of all, we need to define the terminology and the differences between mitochondria, mitochondrial network, and mitochondrial particles. Mitochondria were first identified 120 years ago in eukaryotic cells (14), described as a collection of free-floating individual vesicles existing in hundreds of copies, remaining freely in the cytosol (Fig. 2A). The reader can refer to the book written by Immo Scheffler (146) to follow the history of mitochondrial discovery and advances in the description of the organelle's interior. Such textbook descriptions have originated from pioneering observations of tissue sections by electron microscopy techniques that allowed the depiction of mitochondrial internal compartments (125, 126). These descriptions include the following: the outer membrane (OM), the inner membrane (IM) with its convolutions (cristae), the intermembranous space (IMS), and the matrix. More recently, the utilization of electron tomography permitted to refine the definition of the mitochondrial interior with the discovery of tiny junctions that separate the cristae from the inner boundary membrane (54, 55). In the past decade, the development of cell culture and fluorescence microscopy, along with mitochondrial specific fluorescent probes, have allowed the gathering of a more accurate, three-dimensional view of the mitochondrion in living human cells from various tissues (15, 68, 168). In these studies, the mitochondria look strikingly different, as an organelle that appeared more like a wide network of long tubules rather than a collection of small individual vesicles. In Fig. 2A, we see the arborescence of the mitochondrial network and the continuity of each tubule, as well an appreciation of the existence of subnetworks that reveal the complexity of this overall organization. In Fig. 2B, the link between the mitochondrial network and the more classic representation of mitochondrial infrastructure is illustrated. It has become more evident that what we used to call mitochondria correspond, in fact, to the sectional view of a single mitochondrial tubule.

Consideration of the three-dimensional reticular architecture of mitochondria created a revolution in mitochondrial research, and the term "mitochondrial network" progressively replaced the original name "mitochondria" in order to designate the organelle in a living cell. For Roderick A. Capaldi, this initiated the "changing face of mitochondrial research" (25), and even the next edition of the famous biochemistry textbook by Lubert Stryer describes the mitochondrion as a network (mt-network), thanks to an illustration provided by Michael Yaffe (168), a great contributor to the field of mitochondrial dynamics. Ironically, the terminology of "mitochondrial network" was already found in the original work of Lewis published in 1914 entitled "Mitochondria in tissue cultures" (103). The pioneering observations of whole cultured human cells by high voltage electron microscopy (HVEM) made by KR Porter and co-workers also revealed the networked architecture of the mitochondrion (167).

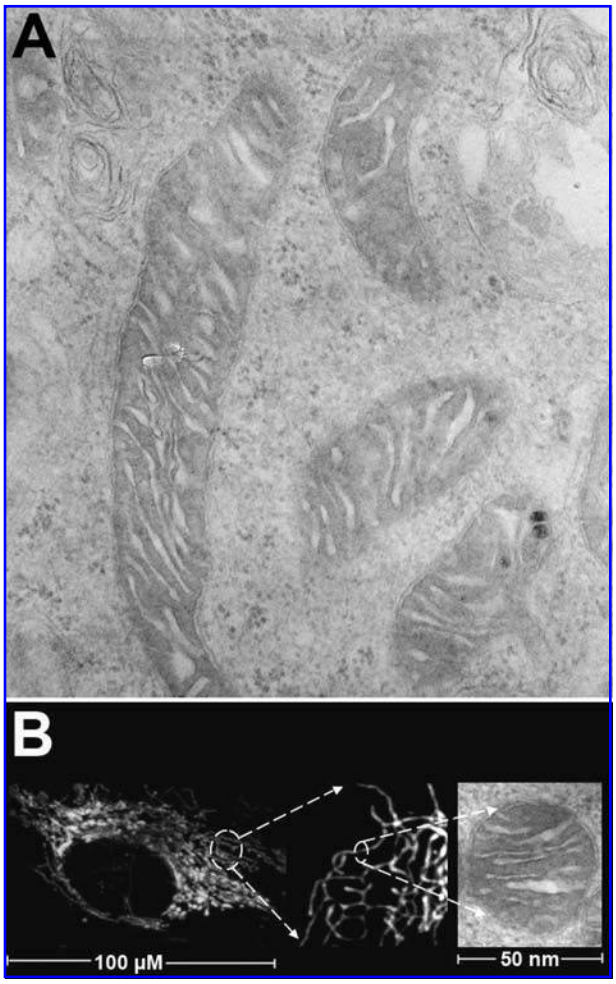

FIG. 2. From mitochondria to the mitochondrial network. (**A**) Electron micrograph of the sectional view of a human HeLa cell by Robert Gilkerson, showing four mitochondrial profiles according to the classic representation of Palade. (**B**) The mitochondrial network was imaged by fluorescence microscopy (bi-photonic), using a matrix-targeted GFP. The *middle panel* shows a magnification of the tubules and subnetworks. On the *right*, we figured the section of a tubule, showing the classic view of the mitochondrion as depicted in (**A**).

Subsequent studies on cultured human cells performed by the group of Vladimir Skulachev further stressed this notion with the term of "mitochondrial reticulum" (9, 134). Currently, various light microscopic methods and specific staining can be used to easily study mitochondrial network morphology and dynamic changes in living human cells. More specialized studies on mitochondrial dynamics include technological approaches such as FRET imaging, 4Pi- and STED- microscopy. An exhaustive review article by Stefan Jakobs (82) covers these methodological aspects and provides a complete discussion of the limitations in optical resolution that will have to be overcome in order to study mitochondrial dynamics via microscopy *in vivo*. Also, the combination of different microscopy techniques can be used to analyze mitochondrial organization, such as the association of three-dimensional light and electron microscopy that permits the study of concomitant changes in mi-

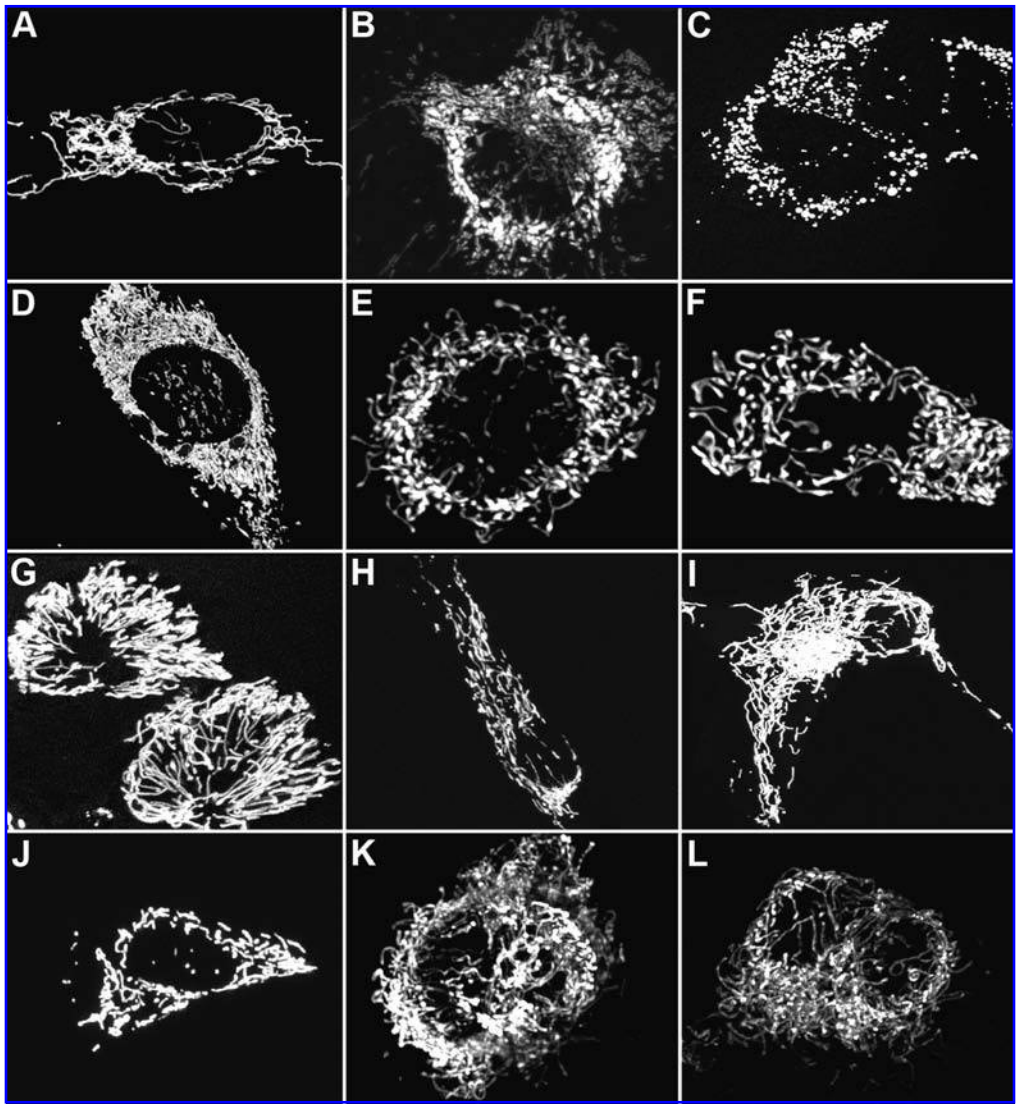

FIG. 3. Different shapes of the mitochondrial network can be observed in one cell population under pseudo-physiological conditions. HeLa cells were grown in glucose, with the exception of panel (**B**) where glucose-deprived DMEM was used. The numerous shapes include the classic filamentous mitochondrial network (**A**), the outgrowth (**B**), the late fragmented (**C**), the nuclear extended (**D**), the perinuclear (**E**), the intermediate (**F**), the radiating (**G**), the cytosol extended (**H**), the dense (**I**), the early fragmented (**J**), the early dividing (**K**), and the late dividing (**L**). All the shapes were obtained without the addition of any inhibitor of the OXPHOS system.

tochondrial interior and overall organization during apoptosis (155). Likewise, the combination of cryoelectrontomography with cryofluorescence microscopy has allowed the investigation of mitochondrial structure in cardiomyocytes at a resolution of 6–8 nm (69).

Beside these rapid advances in biological imaging, the understanding of organelle (dys)function also progressed within the last decade and has helped to elucidate the role of mitochondria in cell death, aging, oxidative stress, and neurological diseases. However, the field of bioenergetics has only just started to explore the structural aspects of mitochondrial network organization by focusing on the description of the respiratory chain in supramolecular assembly and the control of energy production by post-translational modifications. The challenge in bioenergetics is to now explain the impact of organelle morphological changes on the modalities of ATP production, or the role of fusion and fission of the mitochondrial tubules on the control of oxidative phosphorylation. For instance, real-time observations of the mitochondrial network *in situ* reveal that its morphology can alternate rapidly between a tubular reticulum or a collection of small circular fragments (68), but the functional consequences for energy production remain unknown. Likewise, the topological distribution of respiratory chain complexes within the mitochondrial interior has generated a large number of speculative models, but an all-encompassing model remains elusive. To better understand the physiological control and regulation of mitochondrial energy production, it is necessary to incorporate the development of complex studies that combine bioenergetics, protein, and lipid biochemistry, with mitochondrial imaging. In particular, dy-

namic and multiparameter approaches that permit the identification of concomitant changes in mitochondrial morphology, membrane potential, respiration, ROS production, and ATP synthesis, will help to unlock the link between organellar form and function.

Hence, a long time neglected and overdue, the morphological features of the mitochondrion are now at the forefront of understanding organelle physiology and physiopathology. In this review, we will present the current understanding of a field that seeks to demonstrate an intimate connection between mt-network structure and its complex bioenergetics.

II. DIVERSITY OF MITOCHONDRIAL NETWORK MORPHOLOGY

A. Variable morphology of the mitochondrial network

To understand the physiological importance of mitochondrial morphogenesis and dynamics for bioenergetics, one can try to observe first-hand the structural changes of the mitochondrial network under various situations in living human cells. This has been made possible by the utilization of fluorescent dyes or proteins such as green fluorescent protein (GFP) or red fluorescent protein (RFP), specifically targeted to the mitochondrial network matrix (82). However, even a simple observation of mitochondrial morphology in a cell culture dish already shows a large number of profiles (Fig. 3). For instance, we can observe different organizations that qualify as normal-tubular (Fig. 3A), outgrowth (Fig. 3B), fragmented (Fig. 3C), extended in the nucleus (Fig. 3D), perinuclear tight (Fig. 3E) intermediate (Fig. 3F and J), dividing (Fig. 3G, K, and L), or extended in the cytosol (Fig. 3H). Such diversity of mitochondrial morphology might be explained by differences in the physiological state. They include the cell cycle phase or the initiation of apoptosis, since these processes are not naturally synchronized in cell culture models. The study of Margineantu et al. (107) has shown that mt-network fragmentation could occur synchronously with the cell cycle in human fibroblasts. Similar conclusions were observed on yeast during meiosis and sporulation (67). This might reflect the need for mitochondrial dissociation before segregation to the daughter cells, and a possible coordination of organelle morphogenesis with the cell cycle. However, these observed changes in mt-network configuration between the G, S, and M phases might also correspond to variations in energy needs and utilization during the cell cycle. More generally, as fundamental cellular processes require the hydrolysis of ATP, changes in oxidative phosphorylation activity should be thought to affect mitochondrial morphogenesis in different physiological situations. For instance, it was shown that mitochondrial fragmentation is a crucial step in the apoptotic cascade (170), and that changes in mt-network configuration modulate calcium waves and subsequent intracellular signaling (157). The extent of energy production could also be impacted by, or even trigger these morphological changes, and is certainly related to both apoptosis and calcium signaling. Thus, the study of relationships between mitochondrial structure and bioenergetics is a prerequisite for a better understanding of biological processes as fundamental as cell growth, division, and apoptosis. It is also required for explaining the natural differences in mitochondrial network organization, observed for the same cell type in a single culture dish (Fig. 3A–L).

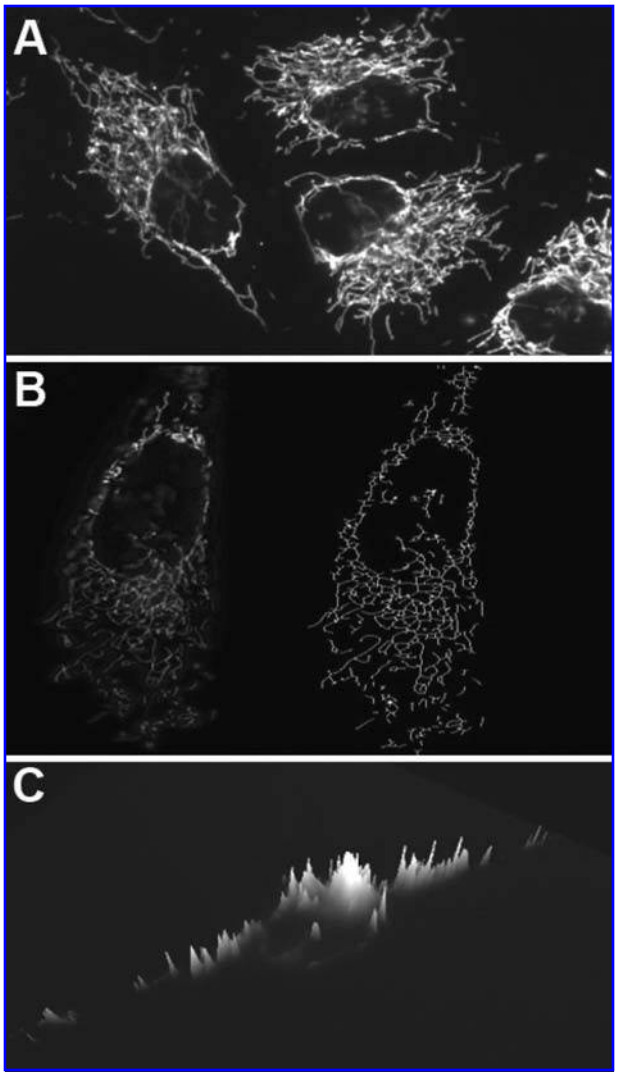

FIG. 4. Mitochondrial network morphofunctional analysis. (**A**) The difference in the shape of the mitochondrial network of four HeLa cells grown in glucose medium shows the complexity for a morphometric analysis. (**B**) Fluo'up software (Explora Nova, La Rochelle, France) was used to calculate the skeleton of the mitochondrial network and analyze the degree of branching, as well as the length of the tubules and the number of fragments. (**C**) A three-dimensional (confocal) image of the mitochondrial network, stained with TMRM ($\Delta\Psi$ sensitive fluorescent probe) was analyzed with Fluo'up to plot the fluorescence intensity along the tubule. It shows the heterogeneity of mitochondrial $\Delta\Psi$ along the tubule, with a region of higher intensity around the nucleus.

B. The mitochondrial network in tissues, species, and during differentiation

The observation of mitochondrial ultrastructure by transmission electron microscopy generally shows important differences according to the tissue considered. For instance, we compared

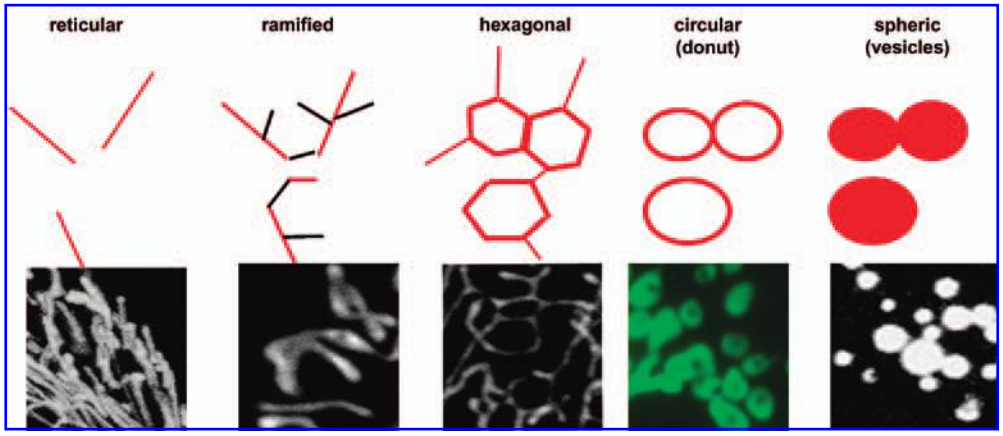

FIG. 5. Mitochondrial network ultrastructural motives. High magnification imaging of the mt-network of HeLa cells grown in different situations shows the variable shape of the tubules. These include the normal shape (reticular in glucose medium), the highly ramifying and branching in glucose-deprived medium, hexagonal regions, circular shapes after rapid rotenone treatment, and the vesicular organization *in* situations of severe OXPHOS impairment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

the mitochondrial profile in muscle, heart, liver, kidney, and brain taken from rats (13). It revealed a large diversity in mitochondrial shape and organization according to tissue, with an important variability of organellar section profile, intracellular localization, and heterogeneity. In muscle, the mitochondria appeared to be contained within membranous compartments all along the myofibers, and looked highly compacted. In heart, they were more numerous, and presented with a larger section. In liver, mitochondria were difficult to distinguish, as they presented with a less clear envelope and fewer cristae. They also looked more perinuclear, and closely intricated with the Golgi apparatus. In kidney, there was a large number of mitochondria essentially located in the interdigitations of the tubules, where the ion pumping activity is very active. In brain, mitochondrial sections were more dispersed throughout the cytosol, with an important variability in section profiles. The subsequent observation of mitochondrial internal organization also revealed differences between tissues concerning the number of cristae and matrix density (13). Again, this could be explained by a close dependency of mitochondrial form on energy state, as these differences were observed between organs that present with highly specialized functions and variable energy demands, as well as preferences in the type of energy substrates. The differences in mitochondrial morphology might also be linked to variations in organellar content between tissues, that depend on the cellular energy demand (13) and during development (137). In contrast with the above mentioned tissue-specific variability of mitochondrial ultrastructure, the overall networked and ramified architecture of this organelle could be conserved throughout species. For instance, in some eukaryotic microorganisms such as *Trypanosoma*, the mitochondrion is typically observed as a single, highly ramified organelle (129). Likewise, in *Toxoplasma*, the three-dimensional reconstruction of serial optical sections of CMXRos-stained tachyzoites observed by confocal laser scanning microscopy and of serial thin sections examined by transmission electron microscopy revealed that the protozoan presented only one ramified mitochondrion (110). Lastly, some studies indicate a possible role of mitochondrial dynamics in fundamental cell developmental processes, such as polarization and chemotaxism. In this line, Campello *et al*. showed that mitochondria specifically concentrate at the uropod during lymphocyte migration, by a process involving rearrangements of their shape (24). In this study, mitochondrial fission facilitated relocation of the organelles and promoted lymphocyte chemotaxis, whereas mitochondrial fusion inhibited both processes. These data might suggest that mitochondrial redistribution could be required to regulate the motor of migrating cells. In *Drosophila*, the polar granules are tightly associated with mitochondria in early embryos, suggesting that mitochondria could contribute to pole cell formation (3). It was even reported that mitochondrial large and small rRNAs (mtrRNAs) are transported from mitochondria to polar granules prior to pole cell formation. This transport of mtrRNAs from mitochondria to polar granules could be mediated by Tudor protein. To understand the importance of mitochondrial dynamics during embryogenesis, one can use *Ascidian* eggs which are one of the best materials for studying the phenomenon of cytoplasmic localization. In this manner, Fujiwara and Satoh examined the distribution pattern of mitochondria from oocytes to tailbud embryos of the ascidian, *HaloCynthia roretzi* (58). They showed that mitochondria contained in the ascidian egg are so unevenly partitioned that there occurs a remarkable quantitative difference among blastomeres. Indeed, following the ooplasmic segregation, mitochondria are mainly distributed to the myoplasmic region. More studies are required to unravel the role of mitochondrial dynamics in the regulation of developmental processes.

C. Morphometry of the mitochondrial network

The difficulty in characterizing mt-network organization has led to the utilization of a large number of qualitative terms such as "fragmented," "outgrowth," "thin," "pearls on a necklace . . . ," which lacks quantitative features (see Fig. 3A–L). To better describe the fine changes in mt-network architecture could help to understand the mechanisms involved in its morphogenesis. The group of Werner Koopman developed such a quantitative analysis of mitochondrial morphology by video-rate laser-scanning confocal microscopy (89, 91, 92). Briefly, the mitochondrial network is stained with fluorescent probes such as Rhodamine-123 or Mitotracker, and images are ac-

quired on a confocal inverted microscope, on living cells grown on coverslips and mounted in an incubation thermostated chamber. Then, to identify the mitochondrial structures (tubules of different length and fragments), an image-processed algorithm is applied. It consists of three main steps that include sequentially (a) the optimization of the contrast by reassigning the gray values of the pixels to cover the entire range available from 0 to 255, and (b) the application of a "top-hat" spatial filtering, which isolates bright features from a dark background, and (c) a threshold operation that removes distinct spherical objects of intermediate intensity introduced by the top-hat filter. In this manner, the fluorescent images are ready for an automated shape-analysis of mitochondrial structures. Two parameters descriptive of the mitochondrial network architecture can be obtained for each object: the form factor (F) and the aspect ratio (AR). The total number of mitochondrial particles, as well as the overall area per cell, can also be determined. In this manner, the authors showed that AR is more a measure of mitochondrial tubule length, whereas F is a measure of both length and the degree of branching. To give an example, they treated normal fibroblasts with rotenone at 100 nM, which is a potent respiratory chain complex I inhibitor, for 72 h. They observed important changes of the mt-network, with a dose-dependent increase in F, suggesting an increase in mitochondrial length and branching following rotenone treatment.

This work also highlights the difference between adaptive changes of the mt-network *versus* rapid changes than can occur directly after rotenone addition. Accordingly, we showed that cells treated with 4–6 ng/ml rotenone for only 4 h presented a dramatic change in mitochondrial network morphology characterized by vesicularization of the tubules and the apparition of numerous donut-like interdigitations (12). Higher doses of rotenone induced mitochondrial network fragmentation that appeared as a collection of stacked large rings (donuts). At last, the mitochondrial network was fully fragmented and constituted by numerous brightly fluorescent rings that turned into vesicles, and finally disrupted to let the matrix GFP diffuse in the cytosol. This sequence of mt-network destruction is also concentration dependent and differs from the adaptive changes observed in cells treated for long periods (139). To further characterize and quantify the changes in mt-network organization, we recently developed an automated analysis, by using software developed by Explora Nova in France. First, it requires an image of the mitochondrial network, as typically obtained by fluorescence microscopy (Fig. 4A). It allows us to calculate the "skeleton" of the mitochondrial network (Fig. 4B) by using a dedicated algorithm. These data are further processed to obtain information on the degree of branching and length of the tubules and fragments. Alternatively, the analysis of three-dimensional images (confocal stacks) permits visualization of the spatial changes in fluorescence intensity along a single tubule. In Fig. 4C, we show the results obtained on cells labeled with TMRM, which reveal a heterogeneous signal of that fluorescent $\Delta\Psi$-sensitive probe, throughout the mitochondrial network. It can be seen, for instance, that a more intense $\Delta\Psi$. is observed around the nucleus. Last, we show the various motifs that can be discovered at the level of mitochondrial network fine ultrastructure (Fig. 5). They can be visualized by zooming on the images acquired by two-photon microscopy, on human fibroblasts transfected with a matrix targeted GFP. The reticular, ramified, hexagonal, circular, or spherical shapes of the tubules are indicative of important reorganizations of the mitochondrion, with corresponding modifications of the interior (cristae and inner boundary membranes). To understand how these different profiles connect with the energy status of a particular cell will help to unravel the complex link between mitochondrial form and function. This is discussed below.

III. INTERNAL ORGANIZATION OF THE MITOCHONDRIAL NETWORK

A. Spatial organization of the oxidative phosphorylation system

To assess the spatial organization of the respiratory chain components inside mitochondrial tubules is crucial for the understanding of energy transduction processes. In Fig. 6, we illustrate the complexity of mitochondrial internal organization, as observed by electron microscopy (Fig. 6A), or electron tomography (Fig. 6C). In Fig. 6B, the different mitochondrial membranes and compartments are shown on a schematic representation. However, there is a great difficulty to obtain information on the protein composition of these different membranes, as no biochemical and imaging techniques allow the visualization of the organization of OXPHOS complexes inside the organelle within living human cells. It was estimated by electron microscopic studies that OXPHOS complexes represent 80% of the total intrinsic protein mass of the mitochondrial inner membranes (inner boundary + cristae) (75, 76), and covers up to 50% of the total membrane area (147). The estimated protein/lipid ratio is also very high in these membranes, around 75:25 (6, 7, 150). However, the distribution of mitochondrial proteins along the plane of this membrane still remains unknown. Schwerzmann *et al*. (147) combined bioenergetic measurements with stereological observations to study the molecular architecture of the inner membrane of rat liver mitochondria. They determined the density of each of the five OXPHOS complexes and found a value of 7,000/μM^2, which is in line with the study of Klingenberg (87). The statistical analysis of the mean nearest-neighbor distance between respiratory chain complexes give values of 200 Å for complexes I and II (dimeric forms), and 80 Å for complexes III (dimer) and IV (monomer). These short distances are consistent with an organization in supramolecular assemblies, as demonstrated by Schägger *et al*. (143). It is also in agreement with the existence of intermediate substrates channeling within the respiratory chain (16, 61, 62), or the measurement of rotational diffusion of complex IV, ANT, and cytochrome c in native inner membranes, indicated by the presence of immobilized populations (147). Using fluorescence anisotropy to measure mitochondrial membranes fluidity, we also observed values closer to rigidity in rat muscle and brain (1), or human fibroblasts (12). This suggests a model where the components of the OXPHOS system could be packed in the inner membranes to form molecular aggregates. This could be advantageous for the channeling of respiratory chain intermediates, conferring a higher efficiency of the system and a safety against the generation of free radicals species. Different analyses further demonstrate that the quinone

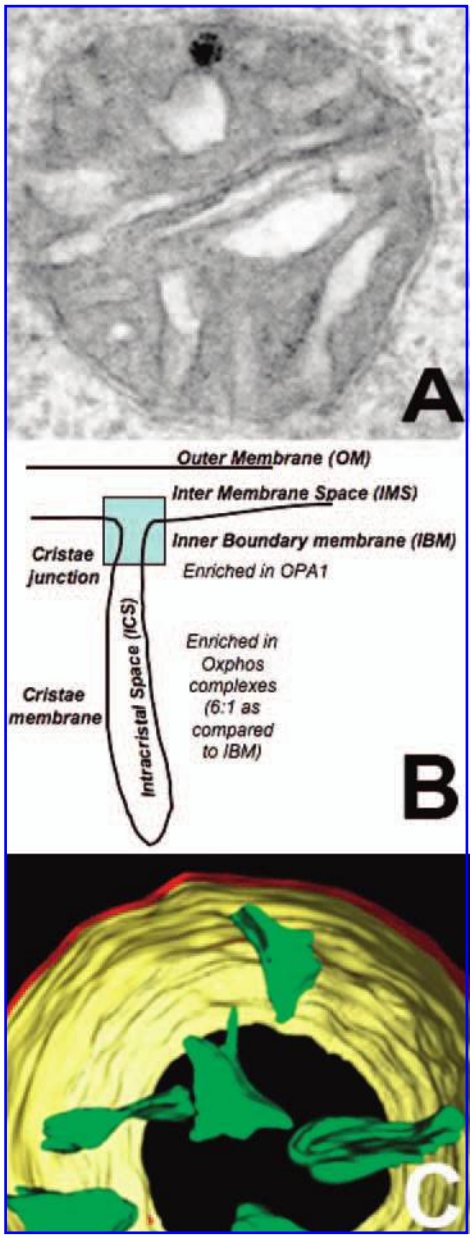

FIG. 6. Mitochondrial cristae junctions. (**A**) Sectional view of a mitochondrial tubule taken from HeLa cells grown in glucose-deprived medium, obtained by EM by Robert Gilkerson. Note the condensed conformation with the dense matrix and the large intracristal volume. (**B**) Different membrane compartments of the mitochondrial ultrastructure. (**C**) Tomographic view of the mitochondrial network (kindly provided by Dr. Luca Scorrano). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

pool is kinetically compartmentalized (16, 19, 100) and studies of cytochrome c also make a distinction between an active form bound to the membrane and a soluble form that contributes little to the electron transport rate (19, 57). Moreover, the respiratory chain is organized in supercomplexes, as demonstrated by the group of Herman Schägger (143, 144, 148), by using blue native electrophoresis on mitochondrial membranes from yeast or human tissues gently solubilized with digitonin. This was also called the "respirasome," and by analogy the group of Pedersen evidenced the existence of the mitochondrial "ATP synthasome," which includes the ATP synthase in complex formation with carriers for Pi and ADP/ATP (30). The mechanisms of formation of these different supramolecular assemblies still remain unknown; recently the interaction between cytochrome c reductase (complex III) and cytochrome c oxidase (complex IV) was investigated in *Saccharomyces cerevisiae* (77). The authors proposed a pseudo-atomic model of this interaction based on observations by electron microscopy and the comparison with atomic x-ray structures for complexes III and IV. It was concluded that the prime function of the supercomplex III(2) + IV(2) could be to serve as a scaffold for effective electron transport between complexes III and IV. The reader can refer to the review article by Boekema and Braun for more details on these supercomplexes (17). Another particularity of the mitochondrial oxidative phosphorylation system concerns F1F0 ATP synthase, the oligomeric state of which determines cristae morphology (130). The group of Jean Velours even proposed that the assembly of ATP synthase dimers could control of the biogenesis of the inner mitochondrial membrane (130).

Recent analyses show a dynamic subcompartmentalization of the inner membrane (161) which is organized in two distinct domains, the inner boundary membrane (IBM) and the criste membrane (CM). They are thought to be connected by narrow structures called the cristae junctions (Fig. 6B and C), as observed by electron tomography (54). Moreover, the topological analysis of OXPHOS complexes distribution on mitochondrial sections by electron microscopy revealed a preferential localization in the cristae as compared to the inner boundary membrane, with a ratio of 6:1 (65). In this study, the authors examined the distribution of PDH and complex III by immunolabeling and electron microscopy (EM) using specific mAbs and secondary antibodies with 5 nm gold particles attached. In sections of heart tissue, complex III was evenly distributed within the organelle, predominantly on cristae membranes, while PDH was clearly in the matrix space between cristae. The fact that the ratio of PDH to complex III determined by immunogold labeling is 273:1, similar to the 202:1 determined in Western blots, validated the methods. The authors calculated a distance between PDH complexes of 0.15 μM. The study by Vogel *et al*. (161) further developed such quantitative analysis of mitochondrial protein distribution in different membranous compartments by using immunoelectron microscopy in intact yeast cells. They showed that the distribution of seven subunits of the OXPHOS complexes revealed an enrichment ranging between 55% in the CM as compared to the total (IBM + OM + CM) for the ATP synthase subunit e, and 67% for the complex III core I. In addition, the length of the cristae membrane (CM) was on average 1.5-fold longer than that of the inner boundary membrane (IBM), which makes it the largest respiratory domain. The IBM and the CM also differ in their distribution of intermediate substrates of the respiratory chain. Studies of coenzyme Q and cytochrome c localization between the mitochondrial interior revealed the existence of two physical pools with possible differential utilization. Structural analyses demonstrated the physical compartmentalization of cytochrome c and, to a lesser extent, coenzyme Q (34, 56, 61, 123, 149, 162). In the view of Luca Scorrano, cristae could

serve to sequestrate a part of the cytochrome c that would be released at the level of the pore junctions during apoptosis. His group showed that the opening of cristae junctions is controlled by changes in OPA1 oligomeric state, further regulated by proteolytic cleavage (34, 56). Such possible existence of different pools of cytochrome c and coenzyme Q, from a kinetic and structural point of view, could influence the modalities of respiratory chain substrate utilization at different energy states, and explain in part the complex relationships between mutual changes in mitochondrial form and function. A correlated three-dimensional light and electron microscopy analysis of mitochondrial morphology during apoptosis revealed a large remodeling of the inner membrane into separate vesicular matrix compartments (155). According to the authors, such changes are not required for the efficient release of cytochrome c, and may have another function of relevance, such as the preparation to mitochondrial network fragmentation. However, fragmentation is not restricted to apoptosis and can be seen during the cell cycle (107), although it is reversible in this case. This reorganization of the mitochondrial interior, from cristae to vesicles, must also impact on the bioenergetic processes and play a role in physiology. Hence, the mitochondrial interior appears more like a compartmentalized system with active and inactive compounds sitting next to each other, which potentially can be recruited in situations of high energy demand via fusion and fission of the membranous system.

B. Four hierarchical levels of the mitochondrial network

The observation of mitochondrial membrane potential in a living cell indicates a strong heterogeneity with regions of higher potential lying underneath the plasma membrane (Fig. 7A). This brings us to reconsider chemiosmosis in terms of proteins and substrate topology, as well as compartmentalization. The interior of a mitochondrial tubule is illustrated in Fig. 6B and detailed in Fig. 7B, where the IBM is distinguished from the CM. The proton extrusion by the respiratory chain during the oxidation of NADH or $FADH_2$ occurs both in the CM and the IBM, thus generating two local electrochemical gradients. Depending on the physical continuity between the intracristae space (ICS) and the inter-membrane space (IMS), these two gradients could be different. The notion of $\Delta\Psi_B$ and $\Delta\Psi_C$ is introduced to designate the electrical gradient across the inner boundary membrane and the cristae membrane, respectively (Fig. 7). Differences in the distribution of carrier proteins that consume these gradients will also participate to create a difference between these two compartments. The question that remains to be answered is whether the cristal space can be considered equivalent to the IMS. Do they have the same viscosity, ionic composition, pH, and protein content? Are they separated by a cristal junction that may act as a diffusive barrier to protons? Even though cristal junctions have a diameter of 20 nm, which is wide especially when compared to the diameter of protons or even cytochrome c, they could present a kinetic barrier when completely closed. A difference in lipid composition between the CM and the IBM could also modify their permeability to protons, hereby changing the extent of proton leakage, and consequently, the electrochemical gradient. The fission of mitochondrial tubules into separate fragments could also modify the length of the IBM as compared to the CM and possibly permit the maintenance of a high $\Delta\Psi_B$ that would be compromised when energy substrate concentrations decreases in the matrix. However, the situation is not clear in the matrix space, as some studies indicate the existence of segmentation along the tubules that sequester the matrix into successive domains within the same tubule (Fig. 7B). This segmentation was observed in different analyses of mitochondrial interior physical continuity, using either JC_1 (see Fig. 6A), or photoactivable GFP, as well as FRAP experiments (36, 128, 159). The use of rosGFP1, or GFPpH also reveals zones of variable intensity in the mt-network of a same cell (see Fig. 7C). In this case, the lipid nature of the intratubular barriers remains to be clarified, as well as their protein composition. The apoptotic sequence proposed by Sun *et al*. (155) indicates that mitochondrial fragments no longer contain cristae, but present with large vesicular matrix compartments separated from each other. Again, the existence of internal barriers inside the tubule has important repercussions for the modalities of energy production, as they could abrogate the diffusion of substrates and metabolites required for oxidative phosphorylation (Fig. 7B). Different pre-fragmented domains could exist next to each other along the tubules, with differences in energy states, determined by the composition of OXPHOS complexes, the local concentration of substrates and activators of the energetic machinery, the variable volume of the different compartments (ICS and IMS), and the surrounding cytoplasm needs. Yet, the idea of mitochondrial tubules operating as long uninterrupted electric cables might be revisited. Furthermore, it is also possible that the internal heterogeneity of mitochondrial tubule, in regard to OXPHOS activity, could be dictated by the local needs for ATP and the demand for oxygen (112, 113), but more studies are needed to validate this view. Mitochondrial networking could also play a role in genetic inter-mitochondrial particles complementation (118), but this requires complete fusion and physical continuity. An alternative could be the existence of partial and transient subnetworks that will define larger functional domain. Twig *et al*. validated this view by using a matrix targeted photoactivable GFP (159). They showed that different subnetworks are coexisting and can undergo internal fission or fusion, without loss of their global architecture. Each network is defined by luminal continuity, equipotentiality, and boundaries which cannot be predicted without consideration of these parameters. This idea of subnetwork was previously proposed by the group of Dimitri Zorov that described the existence of "*Clusters Formed by Chains of Mitochondria that were called Streptio mitochondriale* (9). Taken together, these different observations permit us to define four hierarchical levels of organization of the mitochondrion: the segment, the tubule, the subnetwork, and the global network (Fig. 8A–D). Each of these structures could play different roles in the synthesis and delivery of the vital ATP to various cellular areas, as well as to coordinate the different functions of the mitochondrion.

IV. THE MITOCHONDRIAL NETWORK AS A MULTICELLULAR ORGANISM

A. Biological networks

To better understand the functional and structural dynamic compartmentalization of the mt-network requires a more thor-

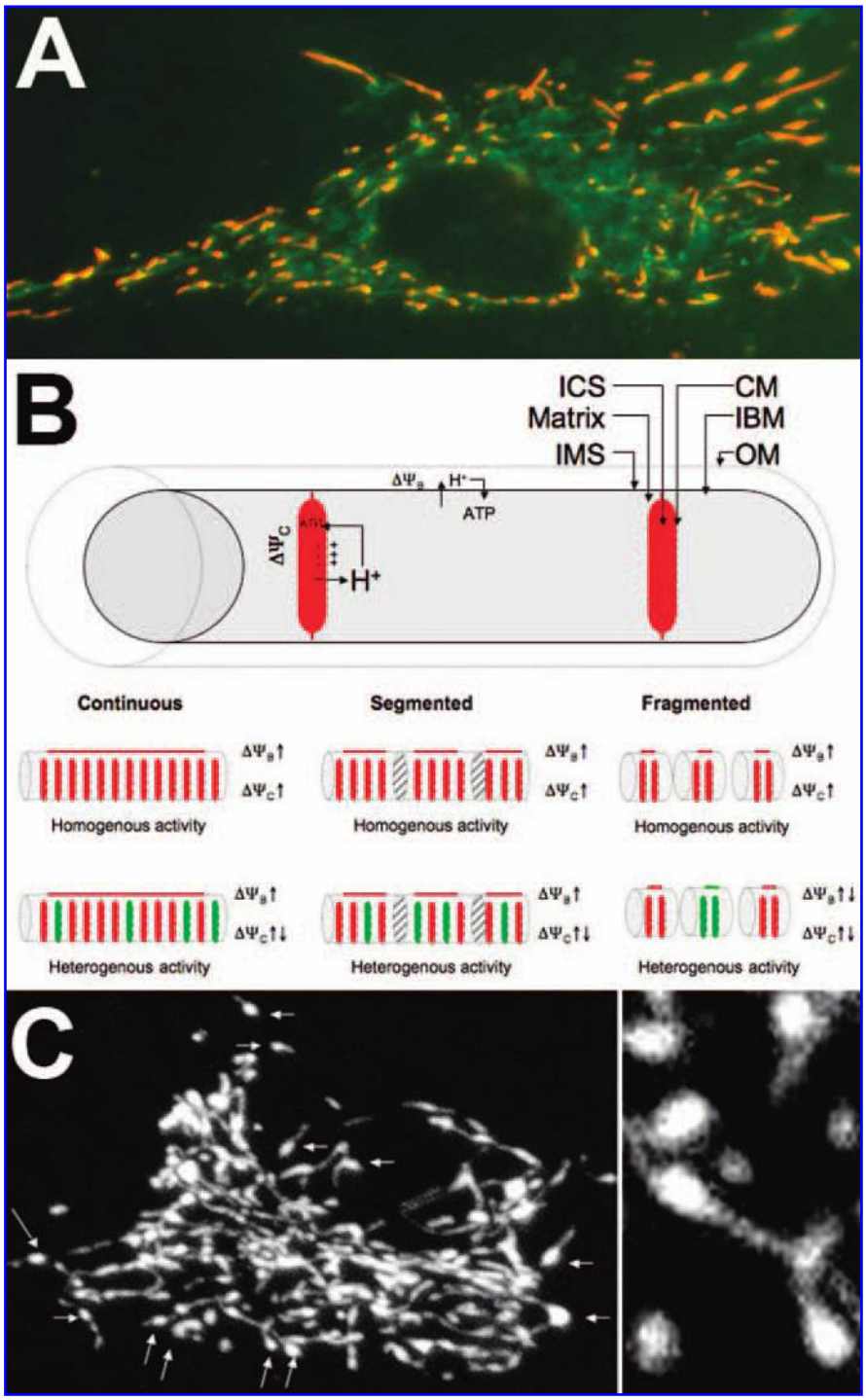

FIG. 7. Heterogeneity and compartmentalization of energy production in mitochondrial tubules. (**A**) Mitochondrial membrane potential was determined in human skeletal myoblasts cells (HSMM), using the dye JC1 (image obtained by Katarìna Smolková). Only the extremity of the tubules is *red*, suggesting a higher $\Delta\Psi$ in these regions. (**B**) Hypothetic localization of the $\Delta\Psi$ inside the mitochondrial tubules. The different situations of mitochondrial tubules physical continuity, segmentation, or fragmentation were envisaged. (**C**) Mitochondrial redox potential was measured with a redox-sensitive GFP (rosGFP1). The ratio image of the fluorescence signal determined at two different wavelengths of excitation shows regions of more reduced state. They correspond to budding area at the extremity of the tubules, indicating an intense biogenesis area or the beginning of fragmentation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

ough investigation of the heterogeneous composition of the four hierarchical levels described above (Fig. 8A–D). The concept of mitochondrial network is advantageous to stress the notion of linkage between different mitochondrial particles, and their possible function as a whole unit in certain situations. A network is defined as an extended arrangement of individual elements connected by conducting wires, used to distribute data or material widely and efficiently, over a large area. It precludes the idea of a complex system designed to function in a specific manner. In biology, one can find such networks at different levels that include the whole organism, with some bacteria that associate in "bunch of grapes" clusters (*Staphylococcus*) or in filaments (*Nocardia*), the tissue with the brain neuronal system, or the immune system, the cell with the biochemical reactions of the metabolism, or the networks of gene regulation and signal transduction, and the intracellular components with the mitochondrion. Such conserved network organization might provide a higher efficiency of the biological systems, and the dynamics of the mitochondrion could thereby permit adaptation to environmental constraints. Likewise, when starved of

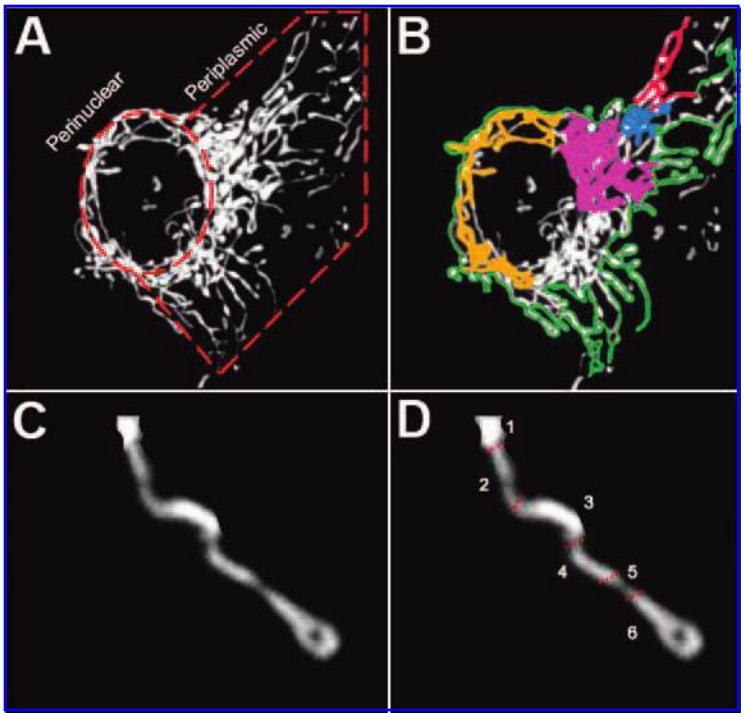

FIG. 8. The four hierarchical levels of the mitochondrial network organization. (**A**) Mitochondrial network of a HeLa cell was stained with a matrix targeted redox-sensitive GFP. It can be divided in two main regions, the perinuclear and the periplasmic, according to the observed heterogeneity in $\Delta\Psi$. (**B**) Different subnetworks are illustrated in different colors. They correspond to regions of physical continuity inside the tubular system. (**C**) The mitochondrial tubule makes the link between the perinuclear region and the cytosol. In neurons, it is moving along cytoskeletal tracks, and could carry the energy to regions of higher needs. (**D**) The redox state is heterogeneous along one single tubule, evidencing the possible existence of segments (six are shown here) of variable energetic activity. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

amino acids, *Myxobacteria* detects surrounding cells in a process known as quorum sensing (45), during which they migrate towards each other and aggregate to form fruiting bodies up to 500 μm long that contain ~100,000 bacterial cells. In these fruiting bodies, the bacteria perform separate tasks. By analogy, and given the possible ancestral bacterial origin of the mitochondrion, it is tempting to hypothesize that the fusion and fission phenomena could serve to modulate mitochondrial function, either as a collection of individual elements or as a (heterogeneous) multicellular organelle. However, our phylogenetic analysis of the proteins involved in fusion or fission indicates that they are currently not present in the a-proteobacterial ancestor of mitochondria, *R. prowazekii*, suggesting that they could have been in existence in the ancestral eukaryotic host.

Along these lines, the question of mitochondrial functional units can be posed. Can we consider the mitochondrial fragments as originating from the cleavage of pre-formed segments in the tubules? Are they functional, and what is their outcome after fragmentation?

B. Spatial distribution of the OXPHOS system

It can be proposed that some mitochondrial fragments remain inactive and contain "old" elements (protein, mtDNA) that are eventually captured by autophagosomes for degradation. However, the turnover of mitochondrial elements is not yet fully understood, and more investigations are required to study the link between mitochondrial biogenesis, autophagy, and mt-network organization. In addition, there might not be only one mechanism of fragmentation, but different types controlled by either metabolic activity, intracellular trafficking, or apoptosis. To address these questions requires more compositional and functional analyses of the mitochondrial tubules, segments, and particles. Current knowledge on the OXPHOS system intratubular organization essentially comes from immunocytological analyses performed on human cultured fibroblasts. First, by labeling mitochondrial DNA with diamidino-4′,6-phenylindol-2 dichlorhydrate (DAPI) or fluorescent *in situ* hybridization (FISH) probes (Fig. 9A), it can be seen a punctuate distribution along the tubules and throughout the mt-network. Likewise, staining mitochondrial respiratory chain complexes, such as complex I or complex IV, using specific monoclonal antibodies, indicates a quasi-homogenous distribution that can be seen along the tubules (Fig. 9B). Interestingly, after fragmentation, these different elements are evenly dispersed between the numerous mt-particles. It was estimated from microscopic studies that fragmentation of a single mt-network can result in thousands of mt-particles as observed in human cultured fibroblasts (26). In yeast, the mitochondrial tubules fragment into fewer (30–50) organelles (115, 152). The immuno-histochemical analysis of mt-particles in human fibroblasts further showed that each particle contains a sufficient amount of elementary components required for energy production such as the OXPHOS machinery and mitochondrial DNA ((26); Fig. 9). This is also true for the pyruvate dehydrogenase complex, as fragmentation of the mitochondrial reticulum generates a large number of mitochondrial particles with at least one, and usually several, PDH complexes in each (117).

This led us to propose the hypothesis of autonomous mitochondrial functional units (that we called "mito-units"), which could work either individually or connected as a network (26). It was further shown that each mt-particles can have a normal $\Delta\Psi$ value, as compared to that measured in tubules (157). Moreover, the physiological shift between mt-network and mt-

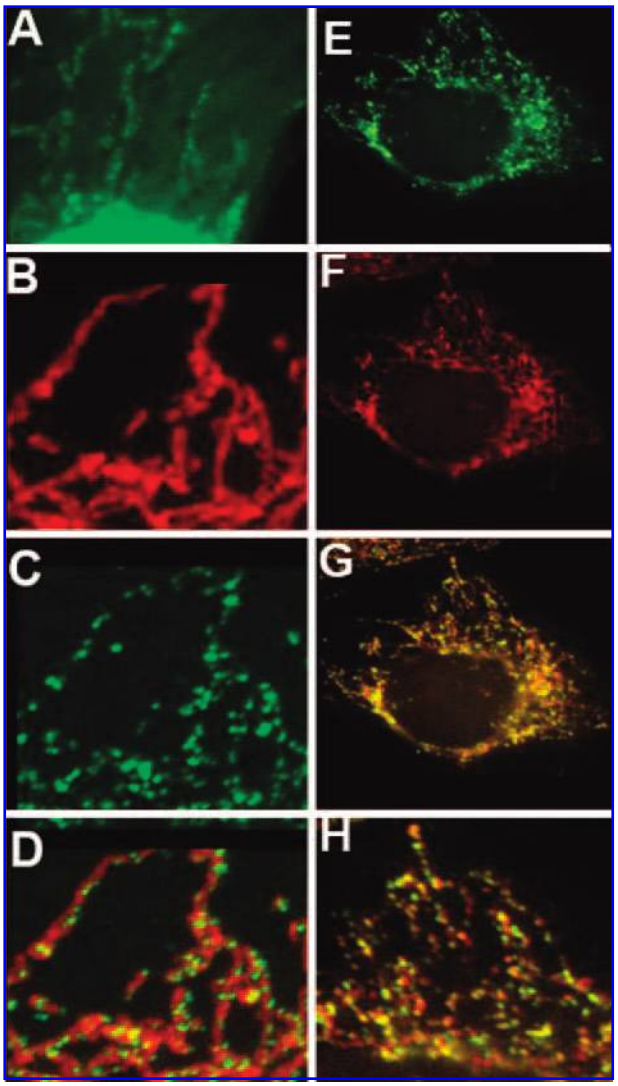

FIG. 9. Homogenous distribution of mitochondrial DNA and respiratory chain proteins in the mitochondrial network of HeLa cells. (**A–D**) The mt-DNA was stained with DAPI or a FISH probe (-amino-allyl dUTP) against COXI gene, conjugated to Alexa Fluor 488 dye (**C**). The mt-network was stained with mitotracker red (**B**), and (**D**) shows the merge of panels (**B**) and (**C**). It indicates a homogenous and punctuate distribution of mt-DNA within the tubules. (**E–H**) The mitochondrial respiratory chain complex I subunit 20 kD was stained with monoclonal antibodies obtained from Mitosciences (Eugene, OR), conjugated to a green Alexa dye 488 (**E**). The mitochondrial respiratory chain complex VI subunit I was stained with monoclonal antibodies obtained from Mitosciences, conjugated to red Alexa dye 594 (**F**). The merge panel of (**E**) and (**F**) is shown in (**G**). A magnification of the mt-network in (**H**) shows the colocalization of complex I and complex IV in most mt-particles. These images were obtained by Daciana Margineantu. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

particles closely relates to the needs for organelle trafficking, since the blocking of mitochondrial fission abolishes their motion along actin cables (160). Efficient fission of mitochondria facilitates the transport of these suborganelles by anterograde motors including kinesin, while the disruption of dynein–dynactin leads to the loss of Drp1 from mitochondria, which results in more interconnected, and consequently, poorer anterograde transport. Additional observations indicate that mt-fragments could be "pre-fragmented" in the tubule, as the fission protein DRP1 locates punctually at regions with reduced diameters that seem to correlate with sites of separation (84). The binding of GTP to DRP1 could activate the mechanical cleavage of tubules, and differentiate mitochondrial fragments from the rest of the tubule. We looked at the effect of DRP1 downregulation in human fibroblasts by using siRNA technology and observed that in absence of fission the mt-network looked albeit tubular, but abnormally interconnected with intense budding areas. To our surprise, and despite the reticular shape of the mt-network, the OXPHOS system was poorly efficient and ATP synthesis strongly decreased, as compared to the control (13). We observed that DRP1 absence led to an essential increase in mt-membranes fluidity, which could explain the difficulty for ATP synthase to phosphorylate ADP, or cytochrome c oxidase to consume reduced cytochrome c (see model of Fig. 10). In this view, the membrane fluidity is a determinant for the maintenance of oxidative phosphorylation and can be altered by alterations in fusion or fission of the mitochondrial network. The lipid composition of mitochondrial inner membrane is also important for the proper OXPHOS function, as well as for maintaining the mitochondrial network's configuration. For instance, a study of Choi *et al*. (33) showed that mitochondrial phospholipase (MitoPLD) is present on the external face of the organelle to promote trans-mitochondrial membrane adherence in a Mfn-dependent manner by hydrolyzing cardiolipin to generate phosphatidic acid, a fusogenic lipid. Likewise, the inhibition of mitochondrial phosphatidyl-serine decarboxylase (PISD) in transgenic mice triggers the fragmentation of the mt-network and causes embryonic lethality with mitochondrial deficiency (155). This highlights the importance of mitochondrial network architecture and dynamics for the diverse functions of this organelle. Taken together, these observations might allow one to consider the mitochondrial network as a collection of interconnected individual segments or pre-fragments.

V. REMODELING OF THE MITOCHONDRIAL NETWORK BY ENERGY SUBSTRATES

The question of whether the energy substrates control or regulate the process of energy production is a matter of debate in cellular bioenergetics (102, 136). Studies in the 1970s revealed the classic transition between the so-called orthodox *versus* condensed configuration of the mitochondrial internal structure. This was first reported by Hackenbrock who observed the condensation of mitochondrial profiles on tissue sections treated with ADP, an activator of energy production (70–72). The correspondence between the changes observed at the level of the mitochondrial network (external) and the cristae (internal) are illustrated in Fig. 2B. The mechanisms involved in this fast constriction are likely to involve osmotic processes consecutive to the entry of K^+ in the matrix, following the depolarization of

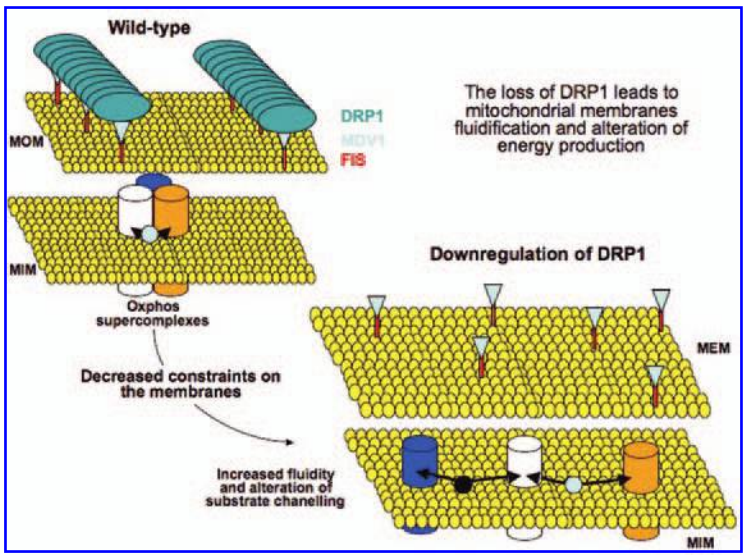

FIG. 10. Mitochondrial membrane fluidity is increased in HeLa cells where DRP1 levels were decreased. Mitochondrial membrane fluidity changes were reported by measuring fluorescence anisotropy changes of the lipophilic probe 1, 6-diphenyl-1, 3, 5-hexatriene (DPH). The fluorescence anisotropy was calculated from emission intensities through a polarizer whose polarization axis was oriented parallel (I_{VV}) and perpendicular (I_{VH}) to the vertical polarization of excitation light as explained in ref. 12. It indicates an increase in organelle membrane fluidity in HeLa cells where DRP1 silencing was induced. This could explain in part why mutations in the DRP1 gene lead to severe mitochondrial diseases. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

mitochondrial inner membrane caused by ADP phosphorylation. Hence, the orthodox condensed shift may not necessitate the intervention of fusion or fission proteins. In contrast, the long-term adaptations of mitochondrial morphology that follows OXPHOS preferential substrate utilization could be explained by a division of mitochondrial tubules, involving the fusion–fission machinery (83, 139). Even though numerous methods of microscopy are now available for deciphering changes in mt-network organization in living human cells (82), few reliable probes are available to simultaneously monitor changes in mitochondrial function and shape. In order to study mitochondrial form and function *in vivo*, and in a noninvasive manner, it is possible to use green fluorescent proteins sensitive to pH and redox state, which can be targeted to the matrix space (139). A large number of these proteins were created by the group of Jim Remington, who characterized RFP and several GFP biosensors described below (47, 74). In previous work, we introduced the leader sequence of the $E_1\alpha$ subunit of PDH into the plasmid coding for GFP-pH (Fig. 11A) or rosGFP1, and created stable lines of HeLa and 143B cells expressing these probes. It must be noted that these GFPs are unique in that they are ratiometric (Fig. 11A and B), that is, they allow determination of pH or redox potential within cells or between groups of cells, independent of photobleaching, indicator concentration, variable cell thickness, illumination stability, excitation path length, and nonuniform indicator distribution. In addition, these two ratiometric dual-excitation GFP biosensors allow high fluorescence, protease resistance, and stability throughout a wide range of pH and solvent conditions. After transfection, we adapted the GFP-expressing cells to alternative energy substrates such as glutamine, and induced the process called glutaminolysis, by removing glucose from the culture medium. This process requires an active oxidative phosphorylation for ATP production and was accompanied by a decrease in steady-state mitochondrial matrix pH (pHm) of 0.4 units, as compared to cells grown in presence of glucose. We further looked at the effect of this change of energy substrate usage on mitochondrial structure by electron microscopy (139). When compared to cells grown in high glucose medium, galactose-grown cells showed a consistent increase in matrix density and expansion of the cristal spaces, that is, the classical "orthodox" to "condensed" transformation which occurs with activation of OXPHOS as described earlier by Hackenbrock (see above). As a result, at low magnification and by using the staining procedures commonly used for EM analysis, mitochondria were difficult to identify in the cytosol of cells grown in glucose medium, whereas in galactose they were clearly apparent. Moreover, image analysis of high magnification TEM micrographs showed consistent increases in the amount of cristal membranes when cells were grown in absence of glucose without a notable difference in the overall mitochondrial content. In order to examine in parallel the mitochondrial network, cells were examined by fluorescence confocal microscopy using the matrix targeted GFP-pH as a mitochondrial marker. In HeLa cells grown in glucose medium, it appeared mostly clustered in the perinuclear region, and not typically extending far out into the cell (Fig. 11C). By contrast, in galactose-grown cells, the mitochondrial reticulum extended outward much more into further peripheral processes of the cell, appearing to be more elaborately interconnected and ramified. In addition, the mitochondrial reticulum was made up of narrower tubules in galactose-grown cells than in glucose grown cells, 0.48 *vs.* 0.32 microns in HeLa cells. Moreover, galactose-grown cells frequently displayed "rings" joined with one another by short filaments that revealed a restructuring of the mitochondrial reticulum. These observations demonstrate that significant changes in the ramification and interconnection of the mitochondrial network occur when OXPHOS energy production is increased by glucose deprivation.

Similar changes were observed in yeast forced to use OXPHOS to derive energy (83). This was obtained by growing the cells on medium containing glycerol as the only carbon source, as opposed to glucose-grown cells. In the oxidative conditions, the mt-network looked strongly branched and highly tubular. However, in this work, the authors observed an increase in the average tubular diameter from 339 ± 5 to 360 ± 4 nm when changing from glucose to glycerol. This was associated with a 2.8-fold increase in reticulum surface, resulting in an average increase in volume of the mitochondrial compartment by a factor of 3.0 ± 0.2. Taken together, these two studies demonstrate large changes in mt-network organization induced by the activation of OXPHOS, possibly leading to a higher efficiency of the system. In yeast, mitochondrial biogenesis was activated, while in our study it was

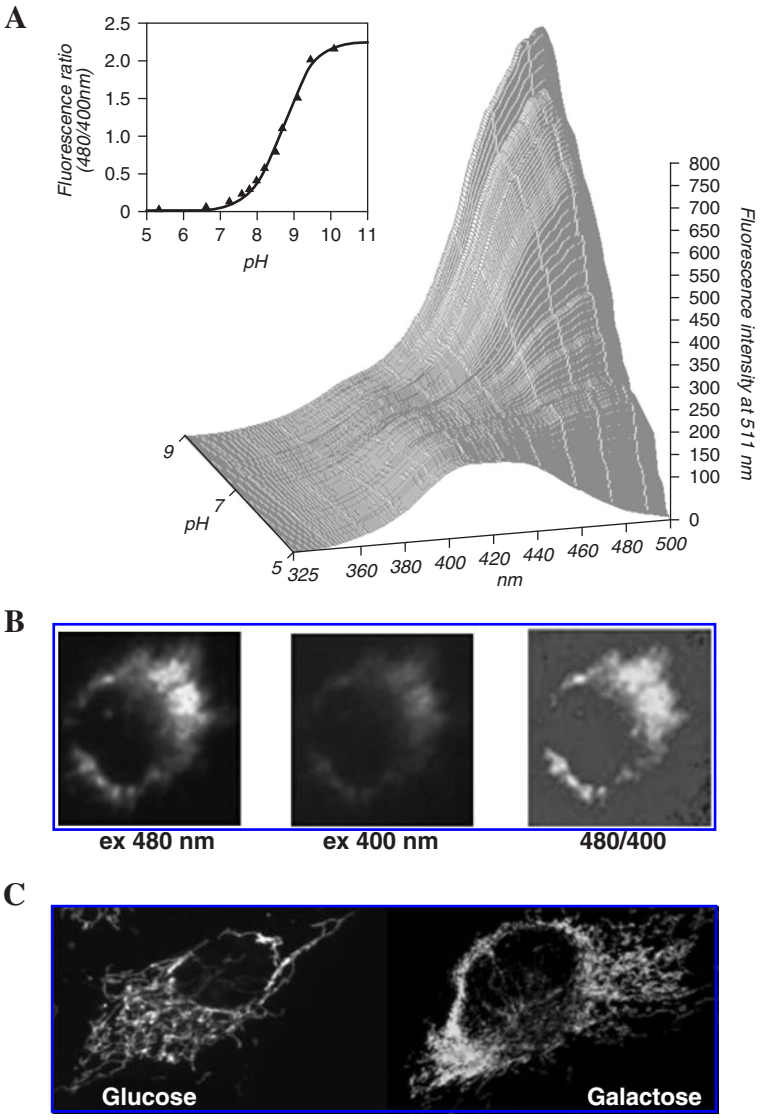

FIG. 11. Use of ratiometric GFP-pH to monitor OXPHOS activity and mt-network morphology *in vivo*. Mitochondrial matrix pH can be measured in living human cells using a green fluorescent protein that acts as a pH indicator (GFP pH - S65T/H148D). This GFP exhibits two pH-dependent excitation peaks with an isosbestic point that allows ratiometric pH measurements (**A**). HeLa cells were stably transfected with this GFP-pH targeted to the matrix space, as confirmed by fluorescence microscopy. To calibrate the signal *in situ*, the fluorescence ratio (480/400 nm) was measured in individual cells placed in various biological buffers (pH ranging from 5 to 11) in the presence of monensin 10 μM to allow equilibration of the matrix pH with that of the buffer. The calibration curve obtained on individual HeLa is shown in the insert of panel (**A**). To determine mitochondrial matrix pH, two images are acquired (**B**), and the ratio intensity is calculated to obtain the pH. In (**C**), the difference of mt-network organization between HeLa cells grown in glucose or galactose (glucose-deprived) media are shown. In galactose, cells are forced to use OXPHOS to derive energy, and a drop of 0.4 pH units was observed.

not. When pushed to the extreme situation where mitochondrial energy demand is dramatically increased due to a functional deficiency in complex I, the changes in mt-network organization can become excessive and lead to an outgrowth shape (91, 92) (cf. Fig. 3B). The signaling pathways by which this is accomplished must include transcription factors that alter expression of OXPHOS components. Lastly, in the study of Weber *et al*. (166), the cultivation of hepatoma cells in glucose-deprived medium led to an increase of mtTFA levels, suggesting the induction of mitochondrial biogenesis. No differences for the ATP/ADP ratio were observed, indicating that this is not the primary signal initiating these adaptative processes. Interestingly, the changes in mitochondrial matrix redox state measured in our study with the ros-GFP (139) could be responsible, in part, for the observed induction of mitochondrial protein expression in galactose medium. Such increased protein synthesis starts a complicated process of structural remodeling, the mechanism of which remains to be solved.

VI. INTRACELLULAR DISTRIBUTION OF MITOCHONDRIAL TUBULES

A. Mitochondrial tubules remain at sites of high energy needs

The mitochondrial network of cultured cell occupies a vast portion of the cytosol. Indeed, the predominant location of mitochondrial tubules is near the nucleus or the plasma membrane. Moreover, mitochondrial tubules are tethered at each end and bound laterally along microtubules (Fig. 12A). Accordingly, they can be extended and retracted when attached to the tips of elongating or shortening microtubules, respectively (169). This could permit tubules to accumulate in cell areas with high energetic needs. Basically, we can distinguish two main cytoplasm regions, that is, the periplasmic area that may correspond to the point of entry of oxygen, and the perinuclear zone which is closer to the delivery of nuclear-encoded mitochondrial pro-

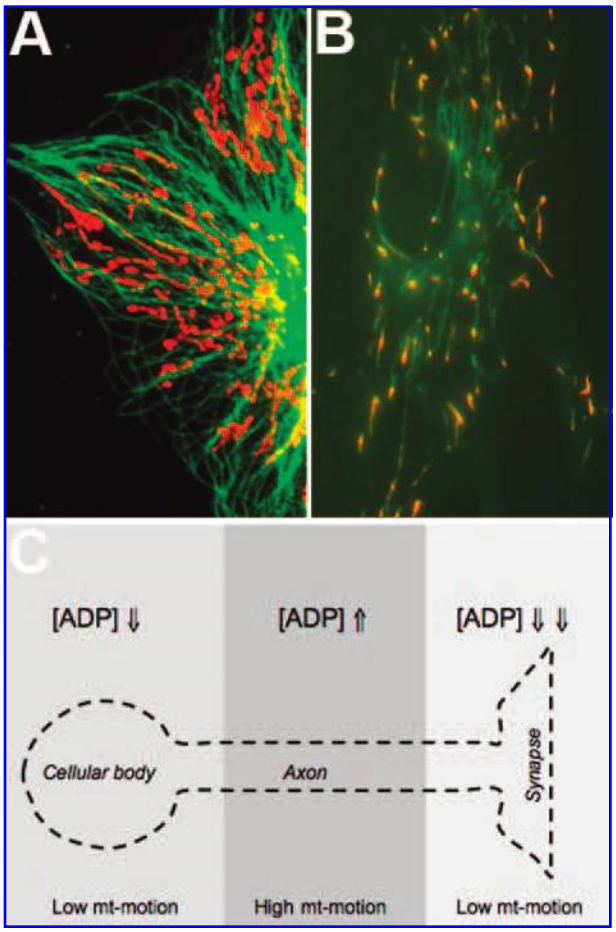

FIG. 12. Mitochondrial traffic according to energy needs. (**A**) The mitochondrial network stained with mitotracker red colocalizes with the microtubules inside the cell. (**B**) Staining of the mitochondrial $\Delta\Psi$ by Katarìna Smolková evidences regions of variable energetic activity. (**C**) The model of Mironov suggests that mitochondrial tubules stop at regions of low cytosolic [ADP], and thereby remain at regions of higher energy needs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

teins. In Fig. 12B, the periplamsic region of the mitochondrial network presents with the highest membrane potential value. A number of questions remain about the signals and mechanisms that control the intracellular distribution of mitochondrial tubules as a function of energy demand. Sergei L. Mironov examined these mechanisms in cultured respiratory neurons taken from neonatal mice (112). The local ATP concentration was measured by expressing the fusion protein EGFP-luciferase in the neurons, and the monitoring of luminescence by real-time microscopy, normalized by the fluorescence signal of EGFP. He proposed that local cytosolic ADP concentration could act as a chemical signal that would slow down the traffic of mitochondria, and thereby maintain the tubules in regions of energy needs (Fig. 12C). In neurons, this is observed in the vicinity of the synapses (113). These observations might allow one to distinguish two large regions of the mitochondrial network, with different energetic properties: the perinuclear region and the periplasmic area (Fig. 8A–D). This is also consistent with the work of Collins (36) that revealed a higher mitochondrial $\Delta\Psi$ in the periplasmic region, although more bioenergetic analyses are required to fully characterize the energy state. Indeed, a higher steady-state $\Delta\Psi$ value does not indicate a higher rate of ATP synthesis, as observed when comparing state 4 (resting state, high $\Delta\Psi$) and state 3 (intense ATP synthesis, lower $\Delta\Psi$) respectively. Under physiological conditions, essentially only substrate deprivation can limit the generation of the protonmotive force by the respiratory chain, and give a very low value of $\Delta\Psi$. The actual coupling degree of OXPHOS must be assessed in cultured cells, alternatively by using blockers of the OXPHOS system and monitoring their effect on cell respiration (12).

B. Periplasmic and perinuclear regions

To study the existence of cellular domains of different energy levels (59), luciferase was expressed in human fibroblasts taken from patients with various mitochondrial diseases, and targeted to different cell compartments: the cytosol, the subplasma membrane region, the mitochondrial matrix, and the nucleus of cells containing either wild-type or mutant mtDNA. It was observed that in presence of glycolytic substrates, both wild-type and mutant cells were able to maintain adequate ATP supplies in all compartments. Conversely, with the OXPHOS substrate pyruvate, ATP levels collapsed in all of these cell compartments of the mutant cells. In this case, no difference between the perinuclear and the periplasmic region were observed. However, in wild-type cells, normal levels of ATP were maintained with pyruvate in the cytosol and in the subplasma membrane region. Surprisingly, they were reduced in the mitochondria and, to a greater extent, in the nucleus (59). Hence, three zones of the mt-network could be considered for bioenergetic study on living cells (Fig. 8A–D). They might correspond to different subnetworks, as evidenced by Twig *et al*. (159). However, the situation is complicated by the fact that under situations of OXPHOS impairment, the mt-network can change its morphology and rearrange these subnetworks. For instance, we looked at the effect of a respiratory chain inhibitor on mitochondrial energy production and the subsequent changes on the network organization (12). We observed that the mitochondrial network maintains a tubular shape in the physiological range, while it changes when OXPHOS is inhibited. No matter how OXPHOS is impaired, the changes in mt-network organization seem to always lead to fragmentation (Fig. 13A–C). In situations of OXPHOS impairment, the local ADP concentration is high in most areas of the cell, and the energy-linked trafficking of mitochondrial tubules could be activated in multiple directions, leading to variegated and abnormal morphology. The motion of mitochondria could also play a role in supplying energy at regions of higher demand during embryogenesis. This was proposed by Dedov *et al*. who analyzed developing dorsal root ganglion neurons isolated from neonatal rats in culture (40). They observed that axonal extension and neuronal network formation coincided with a redistribution of mitochondrial clusters. The authors concluded that the clusters of mitochondria may be storage pools of mobile mitochondrial particles able to be mobilized to provide energy for axonal transport.

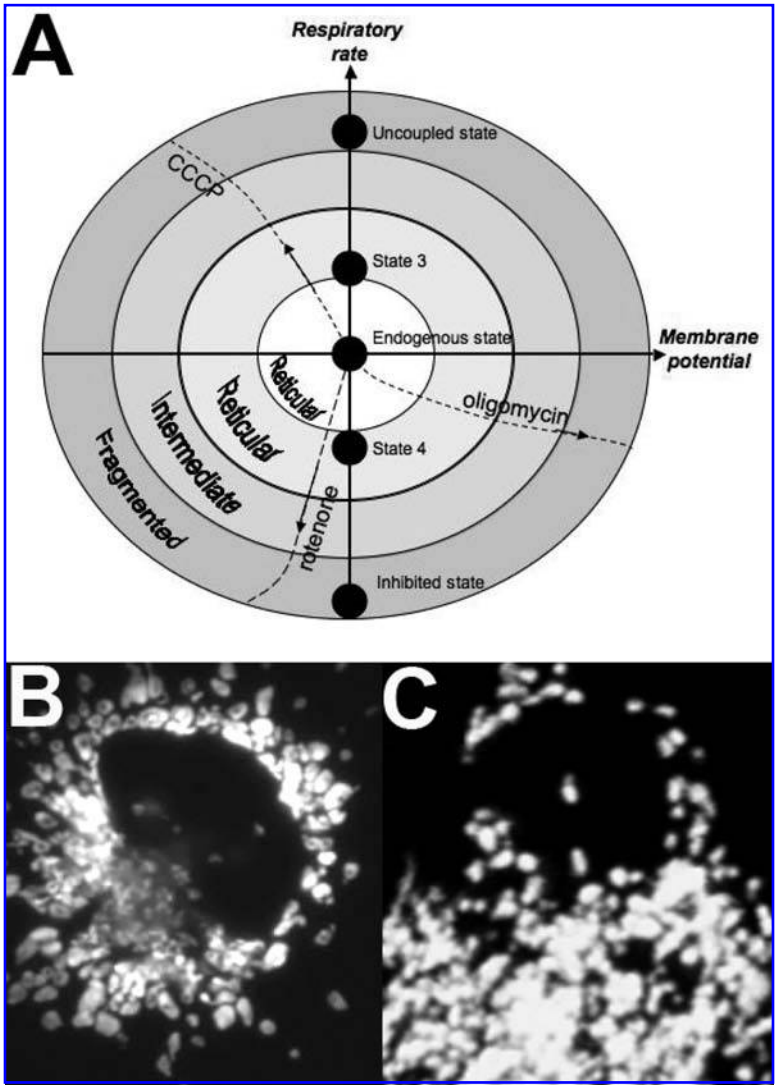

FIG. 13. Mitochondrial "flux-force-structure diagram" [inspired from Benard *et al.* (12)]. (**A**) The level of mitochondrial energy production was modulated in living cells grown in a galactose medium by three different means which include (a) respiratory chain inhibition with rotenone, (b) OXPHOS uncoupling with CCCP, and (c) inhibition of mitochondrial ATP synthesis with oligomycin. The diagram shows the variation of mitochondrial respiration, as a function of the $\Delta\Psi$, expressed as a percentage of the control value, measured on MRC5 cells grown in galactose medium. The corresponding changes in mt-network organization show three "zones" illustrated by concentric circles of different grey intensities. In (**B**), rotenone at 9.6 ng/ml was added for 4 h in the culture medium and led to the apparition of "donuts" in the mitochondrial network. In (**C**), rotenone 12 ng/ml, added for 4 h in the culture medium, triggered fragmentation.

Thus, mitochondrial trafficking inside the cells can be considered as an important determinant of mt-network morphology and may aid in compensating for OXPHOS defects. Moreover, intermediary profiles of the mt-network have been observed when either an uncoupler, a blocker of the phosphorylation or an inhibitor of the respiratory chain is used (Fig. 13B–C). For instance, we observed the formation of donuts (Fig. 13B) in cells treated with rotenone. This can be regarded as a direct consequence of OXPHOS impairment, or as a compensatory mechanism against the toxic compound (138). In cells treated with the highest doses (>6 ng/ml), the network fragmented further, and the donuts transformed into vesicles. This "fragmentation" could reflect the activation of apoptosis, possibly triggered by a rotenone-induced increase in ROS concentration, the drastic reduction of ATP concentration, or the significant drop in $\Delta\Psi$. We proposed that mt-network "circularization" (donut-formation) is a sign of metabolic stress in living cells, while "fragmentation" indicates more an irreversible engagement to cell death (12).

C. Intracellular oxygen gradients and mitochondrial network distribution

Inside mammalian cells, oxygen concentration follows a gradient, so that the local oxygen partial pressure can participate in the control of mitochondrial respiration. The significance of such metabolic regulation was revealed by the work of Erich Gnaiger, using high resolution polarography. He showed, for instance, that the oxygen affinity of the enzyme system involved in mitochondrial respiration indicates a significant role of oxygen supply in limiting ATP production (66). Accordingly, the distribution of mitochondrial tubules inside the cell could also impact on the production of ATP by controlling the disponibility for oxygen, with the richest zones located underneath the plasma membrane. The work of Mironov (112) (see above) suggests that ADP gradients could control the motion and distribution of mitochondrial tubules inside neurons, but the oxygen distribution could also play an important role. To our knowledge, there is yet no study that performed a comparative anal-

ysis of mitochondrial distribution and activity, as function of the intracellular oxygen concentration. This could help to understand the heterogenity of mitochondrial membrane potential, redox potential, and pH (see Fig. 7A, 11C, and 12B).

VII. MUTUAL CONTROL OF BIOENERGETICS AND MITOCHONDRIAL NETWORK ORGANIZATION

A. Mitochondrial shape and energy state

Yet it remains unknown whether mitochondrial ATP production is identical when the organelle harbors a network or fragmented configuration. More crucial, no clear demonstration has shown that mitochondria truly function as a global integrated network for synthesizing vital ATP through the oxidative phosphorylation (OXPHOS) process. What we are sure of is that mitochondrial shape is affected by variations in the state of energy production induced by (a) energy substrates, (b) pharmaceutical or genetic inhibition of OXPHOS, (c) pathological mutations, or (d) cell physiological state. We summarized some of these analyses in Table 1. Since the pioneering observations of Hackenbrock (70–72), few studies have analyzed the link between energetics and morphology of the mitochondrion. As seen above, the orthodox-condensed transition was confirmed at the level of the mitochondrial network, with the thinning and higher branching of tubules, upon a change in energy substrate from glycolytic towards oxidative (108, 139). In contrast, fragmentation of the mitochondrial network has been observed in fibroblasts taken from patients with a reduction of mitochondrial energy production caused by a genetic defect in respiratory chain subunits (27, 92, 131). The reciprocal is also true, since pathological mutations in genes responsible for mitochondrial fusion or fission have been associated with a defective organization of the mitochondrial network, and the inhibition of energy metabolism (2, 132, 165).

Hence, even though a relationship between mitochondrial form and function is suggested by these observations, it remains unclear whether different forms of the mitochondrial network can present with variable bioenergetic properties, and whether the shift between different configurations could be controlled by variations in mitochondrial energy production. The role of mitochondrial transmembrane electric potential ($\Delta\Psi$) is also unclear, as fragmentation of the mt-network was observed in situations where it was either abolished (80, 99, 105, 108, 109), increased (20, 39, 64, 105, 171), or decreased (20, 39, 105), respectively. For instance, the ρ^0 cells, that exhibit a slightly lower $|\Delta\Psi|$ as compared to the original cells (4, 104), contain mitochondria that present a more fragmented network organization (64). This suggests that changes in $\Delta\Psi$ are not sufficient to explain the full spectrum of the metabolic dependent modulations of mt-network organization. Alternatively, the role of mitochondrial ATP synthesis could be determinant since the depletion of NTPs by apyrase abolished fusion, leading to a fragmented mt-network (108). In addition, variations of mitochondrial energy states are associated with variable levels of free radical generation by the respiratory chain (85), and reactive oxygen species (ROS) have also been suggested as possible modulator of mt-network organization (133). In contrast, it was shown that the utilization of oligomycin, a specific inhibitor of the F_1-F_0 ATP synthase, or hydroxycinamic acid, an inhibitor of the phosphate carrier, also lead to mt-network fragmentation (39). However, in this case there is an increase in $|\Delta\Psi|$ (hyperpolarization), and a return to state 4 respiration (171). Further, the addition of CCCP diminished the extent of the fragmentation induced by oligomycin (39), suggesting that a high $|\Delta\Psi|$ could trigger fragmentation. Accordingly, fragmentation of the mt-network can be induced by cyclosporine A, an inhibitor of the mPTP that lead to an increase in $\Delta\Psi$. Other studies demonstrate that the inhibition of respiratory chain complexes I and III, using various drugs, lead to mt-network fragmentation (39,105). In this case, the $|\Delta\Psi|$ is decreased (20). Furthermore, the CCCP-induced fragmentation was shown to occur more efficiently in presence of a respiratory chain inhibitor (39). Hence, the sole changes in $\Delta\Psi$ value are not sufficient to explain the changes in mt-network organization. Moreover, the utilization of FCCP, DNP, or CCCP for the demonstration of the importance of the $\Delta\Psi$ in the fusion-fission process must be taken with caution. Indeed, bioenergetic studies have long shown the versatility of these compounds, characterized by a dual action of uncoupler and inhibitor of OXPHOS, depending on their concentration. Unfortunately, the limit between these two modes of action is very minute, as defined by a sharp bell-shaped curve of titration of mitochondrial respiration. Hence, the actual uncoupling effect of CCCP, FCCP, and DNP, must be verified by polarography and ATP measurement, as exemplified in (42). Another difficulty in the above listed studies concerns the utilization of tumor-derived cell lines grown in high-glucose medium, that derive ATP essentially from glycolysis (135), and where the OXPHOS system is less active (139). Worse, it can even work in reverse mode (32). In this case, the ATP produced by glycolysis is hydrolyzed at the level of the F_1F_0-ATP synthase, and the $\Delta\Psi$ maintained without activity of the respiratory chain, as demonstrated in the Rho ^{0} (104).

B. Flux-force-structure relationships

To clarify this situation, we analyzed the relationships between mitochondrial form and function on primary fibroblasts, nontumor derived, grown in a culture medium where the cells derive energy actively by the OXPHOS-linked glutaminolytic pathway (13). In this study, we combined three different approaches. First, we determined the bioenergetic features of HeLa cells impaired for fission of the mitochondrial network, by silencing the protein DRP1. This revealed the importance of mitochondrial membrane fluidity in the control of bioenergetics (Fig. 10). Second, we followed the changes in mitochondrial network organization, consecutive to the treatment of MRC5 primary fibroblasts with rotenone, a specific inhibitor of the respiratory chain complex I. It demonstrated that OXPHOS inhibition triggered modulation of the mt-network profile. Long-term treatment also evidenced compositional and ultrastructural adaptations of the mitochondrion. Third, we followed the changes in mt-network configuration in a variety of energetic situations, on human cell lines treated with different OXPHOS modulators, or in cells taken from two patients with a mito-

Table 1. Variation of Mitochondrial Network Organization in Physiology and Pathology

	Action	*Methodology*	*MT-network*	*Physiology*	*Reference*
Cell cycle	Synchronization of human cells in culture (143B)	Mitotracker labeling	Fragmentation before S phase, reticular gain in G1	Mt-network could be a check point of cell cycle	107
	Analysis in budding yeast	Destabilization of actin cables or the mitochore	Three classes of mitochondrial motility	Mitochondrial movement requires actin cables	51
	Mitotic yeast	Fluorescence microscopy, or spinning-disk confocal microscopy	Tethered to ends and bound laterally along the sides of microtubules	Tethered to the spindle-pole bodies and moved to the cellular ends	169
Apoptosis	Induction of apoptosis and co-expression of Bax	Confocal analysis and electron microscopy	Destabilization of the mitochondrial network	Fragmentation and redistribution of the mt-network during apoptosis	53
	Overexpression of Bax	Transfection of human cells	Condensed, fragmented, then clustered around the nucleus		41
	Fzo or Bc12 overexpression and induction of apoptosis	Confocal fluorescence microscopy	Elongated mitochondria with FZO, while fragmented with BCL2 overexpression	Sensitivity to apoptosis depends on the mt-network fusion-fission balance	154
	Induction of apoptosis by staurosporin, and silencing of fusion or fission proteins	RNAi OPA1 Confocal analysis	Fragmented, vesicular, cytochrome c release	Loss of $\Delta\Psi$, higher sensitivity to apoptosis	122
		-RNAi DRP1 (fission) -RNAi Fis1 (Fission)	Elongated	Resistance to apoptosis	98
Pathology	Oxphos diseases of various genetic origin	Monoclonal antibodies immunofluorescence	Abnormal: peripheral, ragged, fragmented	Alteration of mt-network in Oxphos deficient cells	73, 21
		Real Time Microscope Imaging	Swollen filamentous forms, nodal filaments, and ovoid forms	Mitochondrial morphology strongly associated with energy metabolism	131
	Study of mfn2 gene in Charcot–Marie-Tooth type 2A disease	Genetic analysis	Non-determined	Peripheral neuropathies	172
	Charcot–Marie-Tooth type 2A disease (CMT)	Mfn2 antisense	Non-determined	Reduced glucose oxidation, $\Delta\Psi$ and cell respiration related to CMT	132
	Overexpression and RNAi against GDAP 1	Immunofluorescence	Fragmented and vesicular	Alteration of mitochondrial dynamics related to CMT	119
	Autosomal dominant optic atrophy (Opa1 mutation)	Mitotracker labeling	Fragmented and vesicular	Decrease of $\Delta\Psi$ and ATP synthesis	2
	Patient with complex I deficiency	Mitochondrial staining, and confocal imaging	Alteration of mitochondrial shape	ROS-related outgrowth mt-network	90
	Patient with abnormal brain development	Mitotracer labeling	Defect of fission	Impaired dynamics leads to severe diseases	165

(*continued*)

Table 1. Variation of Mitochondrial Network Organization in Physiology and Pathology (Cont'd)

	Action	*Methodology*	*MT-network*	*Physiology*	*Reference*
Oxphos	Change in the type of cellular energy substrate: oxidative versus glycolytic	Ratiometric GFPs, pH and redox sensitive, TEM and confocal analysis	*In oxidative mode:* thin tubular, branched. *In glycolytic mode:* larger tubules, peri-nuclear	Adaptation of met-network to the carbon source.	139 83
	Rotenone treatment of Helas and MRC5s	Ratiometric GFP pH and GFP redox	Vesicular with "donuts"	Model of the flux-force-structure relationships	12
	CCCP treatment of Helas and MRC5s	Immunofluorescence	Fragmented	$\Delta\Psi$ is necessary for fusion	12, 80
	Disarrangement of F1F0-ATPsynthase (yeast)	Crosslinking of ATP synthase	Fragmented	Abnormal cell division and mt-DNA transmission to buds	60
	Repression of fusion protein MFN2	Immunofluorescence Anti-sense mRNA	Fragmented and clustering	Reduced glucose oxidation, $\Delta\Psi$ and cell respiration	8
	Repression of fusion protein MFN2, OPA1, and mfn-double mutant	Immunofluorescence shRNA, gene mutation, and respiratory assays	Largely fragmented	Growth defects, $\Delta\Psi$, and cell respiration loss	31
Dynamics	Overexpression of fusion protein Mfm1	Immunofluorescence Fluorescence microscopy	Super connectivity	Mixing of the whole mitochondria content	99
	Overexpression of fusion protein Mfn2	Immunofluorescence	Clustering around the nucleus	Mfn2 GTPase regulates or mediates mitochondrial fusion	140
	Silencing of fusion proteins Mfn 1 and 2	RNAi Mfn 1 and 2 cell fusion, and GFP	Immixing of mitochondrial populations from two cells	Mixing of whole mitochondria content	80
	Overexpression of the fission protein Drp 1	Overexpression	Fragmented	Alteration of calcium signaling	156

chondrial disease. Taken together, this allows the proposition of a model where the flux-force relationship of mitochondrial oxidative phosphorylation is governed by the changes in mt-network organization. Three OXPHOS parameters candidate for the signaling of mitochondrial energetic changes, towards the modulation of organelle dynamics and morphology: mitochondrial respiratory rate, $\Delta\Psi$, and H_2O_2 generation. These parameters are linked by nonlinear relationships dictated by the thermodynamic features of the system, and further vary with kinetic constraints relative to the steady-state under consideration. Basically, the $\Delta\Psi$ produced by the respiratory chain is consumed to produce ATP, ROS, and heat (leak). Accordingly, mitochondrial respiration and ATP synthesis (the fluxes) depend on $\Delta\Psi$ (the force), according to the so-called "flux-force" relationship (124). On isolated mitochondria, this relationship is nonlinear when $\Delta\Psi$ is modulated from zero (using CCCP), to its maximal value (using oligomycin) (see diagram of Fig. 13A). This was demonstrated in isolated mitochondria (124, 171), or in permeabilized cells (18), where the delivery of respiratory substrates and ADP can be controlled. In contrast, the utilization of OXPHOS effectors in entire cells, or even perfused tissues, does not permit the modulation of $\Delta\Psi$ in its entire range, as it is not exclusively produced by the respiratory chain nor entirely consumed for ATP synthesis or proton leak. In our study, ~60% of the endogenous mitochondrial membrane potential was maintained when mitochondrial respiration was fully inhibited. This indicates that $\Delta\Psi$ was created during respiratory chain inhibition by ions other than protons pumped by this chain. Similar observations were reported by other authors (11, 104). A nonprotonic generation of $\Delta\Psi$ was also suggested by our study through the utilization of CCCP, which could not decrease the membrane potential >70% of its endogenous value. The addition of oligomycin induced an increase in $\Delta\Psi$ of ~40%, as compared to the endogenous conditions. It indicates that respiration can be coupled in galactose medium, and that 40% of the $\Delta\Psi$ was used to synthesize ATP. The last parameter (*i.e.*, H_2O_2 production) also varies with $\Delta\Psi$ in a hyperbolic manner. Thus, the changes in mt-network organization must be discussed in regard of this flux-force relationship, as illustrated in Fig. 13A–C.

VIII. STORAGE OF INACTIVE OXPHOS COMPONENTS IN THE MITOCHONDRIAL NETWORK

A. Compartmentalization of mitochondrial proteins and substrates

The notion of intramitochondrial complementation is discernible from the observed compensation of OXPHOS defects, as visualized on the so-called "threshold plots" (138) of Fig. 14A. These graphic representations describe the effect of respiratory chain complexes inhibition on the energy fluxes, or the effect of respiratory rate deficiencies on cell proliferation. They show that the repercussion of respiratory chain deficiencies on global mitochondrial energy production (*i.e.*, the fluxes of oxygen consumption or ATP synthesis) is highly variable and not proportional. For instance, the analysis of the quantitative relationships between the decrease in a given respiratory chain complex activity and energy fluxes have suggested the existence of a "Biochemical Threshold Effect" (101). Below this threshold, even a strong inhibition in the activity of respiratory complexes does not affect the overall rate of mitochondrial respiration and subsequent ATP synthesis (37, 38, 97, 101), whereas above it, a sudden drop in energy fluxes arises. This phenomenon can be explained by the interplay of two different fundamental mechanisms: mobilization and attenuation. The former concerns the enzymes and involves the utilization of additional capacity by either molecular recruitment [as demonstrated for the adenine nucleotide translocator (49)] or by direct activation [as demonstrated for the glycogen synthase (142)] to accommodate an increase in flux. The latter concerns the substrates and constitutes a compensatory mechanism inherent to network systems, that derives originally from the Metabolic Control Analysis (MCA) (78, 86). It can be called "Metabolic Network Attenuation" (MNA), and considers that the inhibition of a local step within a metabolic pathway working at steady-state can be reduced at the level of the flux, via compensatory changes in intermediate metabolites concentrations. According to Thierry Letellier (101), both mechanisms rely on the existence of mobilizable activity, in the form of inactive respiratory chain complexes or reduced intermediate substrate. Interestingly, the advances in the definition of mitochondrial network organization indicate that a fraction of inactive OXPHOS complexes might be stored in specific regions, whereas excess intermediate substrate (cytochrome c and coenzyme Q) would also be sequestrated in an inactive (unutilized) pool. Although largely debated, this notion of molecular reserve is consistent with demonstration of a physical compartmentalization of proteins and metabolites within the mitochondrial interior. For what concerns the OXPHOS complexes, Faustin *et al*. (50) showed that a fraction of the ANT can be kept inactive by adjustment of its oligomeric state. Interestingly, Vogel *et al*. (161) recently proposed that mitochondrial cristae could act as a reservoir for proteins not needed for activity in the inner boundary membrane (IBM). Likewise, the proteins of the OXPHOS system could be recruited in situations of respiratory chain deficiency and serve as molecular reserve, as proposed by Faustin *et al*. (50).

B. Modalities of respiratory chain substrates utilization

Little is known about the utilization and compartmentalization of respiratory chain intermediate substrates under physiological conditions. The pioneering studies of Kroger and Klingenberg (95, 96) concluded for the existence of an homogenous pool of coenzyme Q, the redox level of which depends essentially on the activity ratio of the corresponding electron donors and acceptors. Thermodynamic analyses of ubiquinone oxidoreduction also considered this intermediate substrate as homogenous (48). However, more recent analyses demonstrate that the quinone pool is kinetically compartmentalized (16, 19, 100). The group of Lenaz showed that 84% of coenzyme Q is free in the inner membrane. Studies on cytochrome c also show a kinetic compartmentalization with the active form bound to the membrane, and the soluble form that contributes only little to the electron transport rate (19, 57). Moreover, structural analyses indicate the existence of a physical compartmentalization

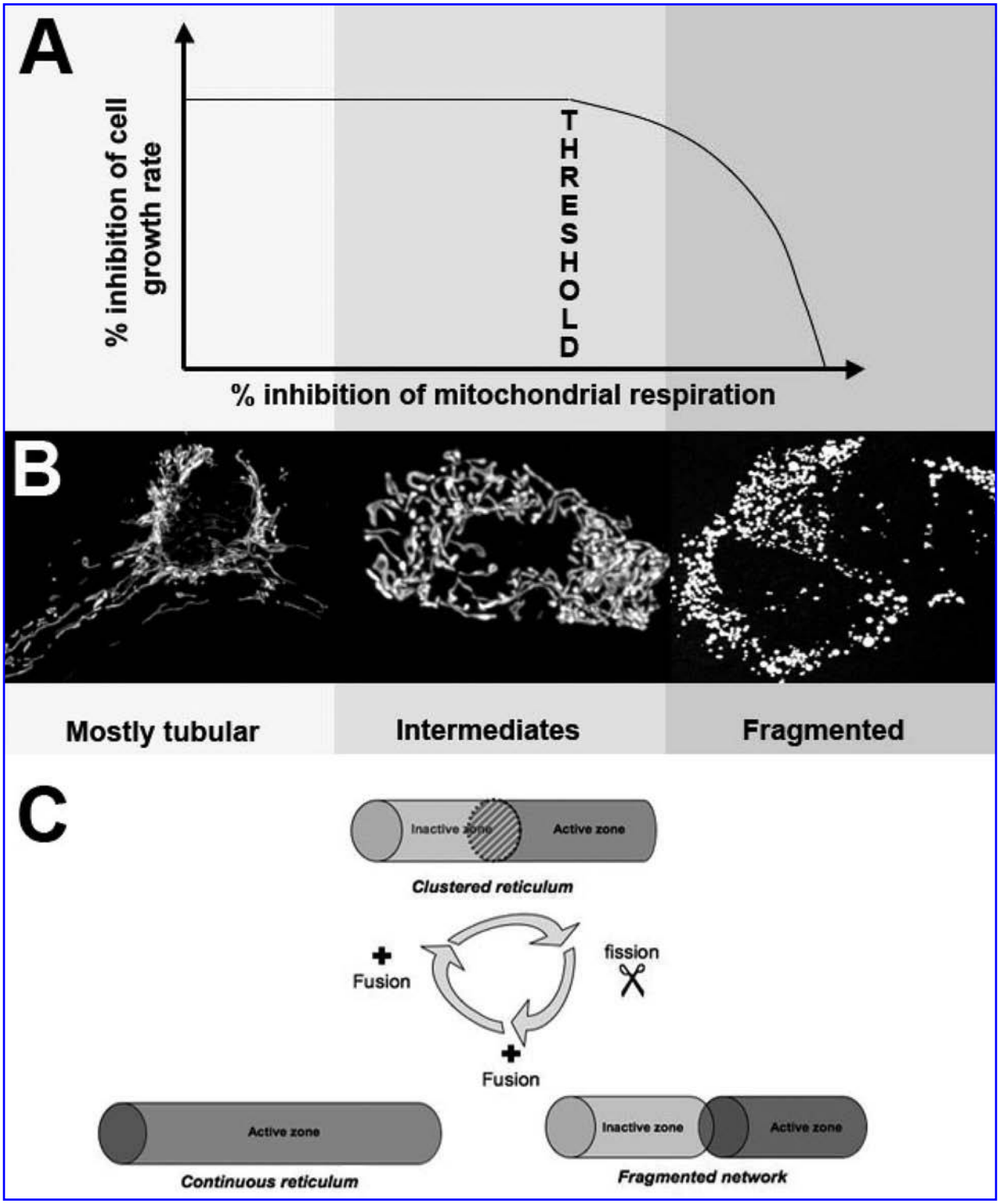

FIG. 14. The mitochondrial tubular system allows internal genetic and functional complementation. (**A**) Inhibition of mitochondrial respiratory chain impairs cellular activity only when a threshold is reached. The compensatory mechanisms that permit to attenuate the defective respiration might include the exchange of material, metabolites, proteins, and mtDNA inside the tubules. (**B**) Depending on the morphological state of the mt-network, the internal complementation is variable. (**C**) In the fragmented state, no exchange can occur between the different particles, creating an heterogeneous situation inside the cells. Reactions of fusion and fission can modulate the network organization and impact on the capacity for compensating OXPHOS defects. This might explain the presence of outgrowth in the mt-network of cells treated with rotenone for a long time.

of cytochrome c and, to a lesser extent, of coenzyme Q (34, 56, 61, 123, 149, 162). The possibility of the existence of different pools of cytochrome c and coenzyme Q, from a kinetic and structural point of view, could influence the modalities of respiratory chain substrate utilization at different energy states. However, most studies on the variation of coenzyme Q and cytochrome c redox state were performed on uncoupled submitochondrial particles (95, 96) or reconstituted systems (57) that do not retain substrate compartmentalization. Recently, we performed the analysis of coenzyme Q and cytochrome c redox state on intact mitochondria isolated from rat muscle or liver, respiring at state 3, and demonstrated the existence of a mobilizable pool of cytochrome c or coenzyme Q that was not initially reduced at state 3, and that was used during the compensation. The magnitude of the attenuation of pharmacologically-induced respiratory chain defects appeared to be determined by the size of this mobilizable pool.

Rearrangements of the mitochondrial network and changes of the cristae morphology could modify the distribution of enzyme and substrates from inactive to active compartments, thereby modulating the capacity for OXPHOS and attenuation of energy defects. Taken together, these different mechanisms could add up to permit an efficient compensation of OXPHOS defects and protect the cell against an energy crisis. We proposed previously a model of "hierarchical threshold effects," where the attenuation of OXPHOS defects is based on additive mechanism (138). Such compartmentalization of active and inactive parts of the mitochondrial network that could be recruited upon morphogenetic changes could take part in the global compensatory response.

IX. FUSING MITOCHONDRIAL PARTICLES TO COMPENSATE FOR OXPHOS DEFECTS

A. High tolerance of human cells towards OXPHOS defects

The different proteins located in the mitochondrial network can be rapidly degraded by the high local concentration of reactive oxygen species (ROS), precluding the need for an intense recycling. This creates a heterogeneous situation where active complexes could coexist with damaged ones along the tubules. In this situation, the exchange of proteins along the tubule interior could serve to rescue the damaged regions. The constant fusion and fission could permit mixing the mitochondrial content and maintaining a widespread activity throughout the cell (Fig. 14B and C). This could also serve to evenly distribute mt-DNA molecules, and manage OXPHOS deficiency in situations of adverse heteroplasmy. Measurements of the diffusion rate of GFP inside the mitochondrial matrix suggest that many of the matrix proteins could be organized peripherally in membrane-associated macromolecular complexes (128). A central region with relatively low protein density and low viscosity might thus be created in which solute diffusion is rapid and unrestricted. However, do the components of individual mt-particles mix after fusion, and can this really help to protect the overall function of the organelle? First, the artificial fusion of two different cells containing their mitochondrial network labeled with green or red matrix-targeted GFPs, respectively, shows that mixing effectively occurs after fusion (106). These so-called mitochondrial fusion assays were performed on human cultured cells, by using PEG or viruses for cell membranes fusion. In yeast, cell fusion occurs following mating, and the mixing of mtDNA and proteins has been observed [see review article by Jensen and Sesaki (84)]. To demonstrate the mixing of genetic content between fused mitochondrial particle, Nakada *et al*. created cybrid cell lines, and a transgenic mice that contained two types of mitochondrial DNA, the wild-type molecule and a deleted molecule (ΔmtDNA4696) (118). The study of the cybrid clones revealed first that heteroplasmy had to reach >60% of mutated mt-DNA before an impairment of complex IV (COX) activity and structural alteration of mitochondrial structure could be observed. This phenomenon is called the phenotypic threshold effect, and was confirmed in several studies (for review, see ref. 138). This high tolerance of the cybrids towards the presence of deleted mtDNA (loss of six tRNA genes essential for mitochondrial proteins) could be explained by an internal genetic complementation within the mitochondrial network, owing to the exchange of mtDNA, mRNAs, and peptides in the matrix, as well as potential metabolic complementation due to other mechanisms. The study of Nakada *et al*. (118) was the first to show genetic complementation in vivo by analyzing the skeletal muscle of different transgenic mice containing various degrees of heteroplasmy. They never observed the coexistence of COX active and inactive enzymes, by histochemical staining, within single muscle fibers, despite the presence of 89% heteroplasmy. This might indicate that genetic complementation was attained in the muscle fibers, possibly via the intermitochondrial particles fusion and mixing of their content. However, the molecular control of the mechanical activity responsible for mt-fission in response to an increase in energy demand is not clearly understood.

B. Quality control of mitochondrial fusion

Interestingly, the work of Duvezin *et al*. (46) recently described a mechanism by which the proteolytic processing of OPA1 could induce the fragmentation of energetically compromised mitochondrial segments, thereby preventing the fusion of dysfunctional mitochondrial particles with the rest of the mitochondrial network. Alternatively, for what concerns the proteins, Vogel *et al*. (161) proposed that mitochondrial cristae could act as a reservoir for proteins not needed for activity in the IBM. Again, the rearrangement of mitochondrial interior could permit the recruitment of such proteins in situations of energy needs. Moreover, the mitochondrial network is physically associated with other cellular compartments such as the endoplasmic reticulum, or the Golgi apparatus. Yet, it is not known how the different mt-network configurations interact with those cellular compartments, and whether changes in shape could regulate the associated signaling and transport processes. For instance, the endoplasmic reticulum interacts closely with the mitochondrial network at specific regions designated as Mitochondrial Associated Membranes (MAM). Only in those ar-

eas can some enzymes specialized in the synthesis of particular phospholipids be found. It is intriguing how these regions can reform after reversible mitochondrial fragmentation, to promote specific contact with the Golgi. Similar questions can be posed for all the other organelles. Hence, mitochondrial fission could select between active and inactive parts of the mitochondrial network, while fusion would be more involved in the compensatory responses. Both processes could maintain and potentially recruit some energetic activity when needed.

X. MITOCHONDRIAL NETWORK AND ORGANELLAR BIOGENESIS

The organization of mitochondria in most cultured human primary (noncancer) cells looks tubular and networked. However, different tissues present with specialized functions and variable energy needs, as well as differences in the type of energy substrate they consume. This may help shape the mitochondrial system in a tissue-specific manner, as observed for the expression of different genes and related bioenergetic parameters (12). It is estimated from different studies performed in human heart (158), mouse (116), or rat (52), that mitochondria contain around 600–700 identified proteins. However, a striking diversity in their expression levels was reported across tissues, suggesting a large regulatory and composition diversity of the mitochondrial proteome (52, 116). This could explain, in part, the morphologic (36, 63, 163) and functional differences (23) observed in mitochondrial sections observed by electron microscopy in different tissues (12). Such differences could also be observed at the level of the mitochondrial network organization, with possible variations in the expression levels of the different proteins involved in morphogenesis and dynamics. Moreover, one could ask whether the expression of mitochondrial morphogenetic genes is orchestrated by the regulators of mitochondrial biogenesis. Yet, a large body of data indicates that mitochondrial content depends on the cellular energy demand that is variable between tissues (13), or during development (137). It was also demonstrated that chronic exercise training increases the amount of mitochondrial protein, as well as organelle volume in skeletal muscle (35, 79), thereby improving endurance and exercise tolerance. Lastly, the type of energy substrate is also a key determinant of the cellular amount of respiratory chain content (44, 139). At the genetic level, recent data suggest that PGC1α (PPARγ co-activator-1α) is involved in the transcriptional control of mitochondrial biogenesis (5). It is further proposed that nitric oxide (NO) could act as the main regulator of this transcriptional regulatory network, as NO was shown to stimulate mitochondrial biogenesis under a large number of situations (10, 120, 121). However, it remains unclear how mitochondrial biogenesis interacts with mitochondrial dynamics. The study of Cartoni *et al*. (28) considered this problem, and analyzed the expression of mitofusins $^1/_2$, as well as ERRalpha expression in human skeletal muscle after physical exercise (28). They observed that Mfn1, Mfn2, NRF-2, and COX IV mRNA were increased 24 h post-exercise, while PGC-1alpha and ERRalpha mRNA increased 2 h post-exercise. These results provide evidence that PGC-1alpha not only mediates the increased expression of oxidative phosphorylation genes but also mediates changes in mitochondrial architecture in response to aerobic exercise in humans. The link between mitochondrial energy state and the induction of organelle biogenesis still remains unknown, and this will need to be understood for a complete deciphering of how the expression of dynamic proteins is regulated.

XI. MITOCHONDRIAL NETWORK AND PATHOLOGIES

A. Phenotypic and etiologic variability of mitochondrial diseases

The impairment of mitochondrial function has been associated with the emergence of severe human disorders regrouped under the name of mitochondrial cytopathies, even though this terminology was usually used only for defects restricted to the respiratory chain. The phenotypic presentation of mitochondrial diseases includes a wide spectrum of clinical manifestations that can affect all tissues of the organism, and the onset of these pathologies ranges from the new-born stage to adult age. The first mitochondrial disorder was reported in 1962; today, >120 syndromes caused by various mutations in mitochondrial DNA (mtDNA) or in nuclear DNA have been described (164). However, the pathogenesis of these diseases is generally not understood and the relationship between the presence of a given *a priori* pathogenic mutation in mtDNA and the occurrence of clinical signs in tissues (phenotype) remains problematic. Indeed, the study of the genotype–phenotype relationships in mitochondrial diseases has shown that, (a) the same mitochondrial pathology (same clinical features) can be caused by different mutations in mtDNA, and (b) the same genetic defect in mtDNA can lead to different clinical manifestations. This variability can be observed between different patients (who present different clinical manifestations but possess the same mutation in mtDNA), but also between different tissues in a given individual (tissue specificity). In addition, diagnostic studies showed that the presence of a defective respiratory chain complex in tissues does not always lead to pathology. All these observations have underlined the complexity of the clinical expression of mitochondrial deficiencies and several mechanisms could play a role in this phenomenon. The recent discovery of pathogenic mutations in genes essentials for fusion and fission of the mitochondrial network have complicated the physiopathological mechanisms of these diseases (43), and permit to question the similarities between enzymatic defects in respiratory chain complexes versus the perturbation of mt-network dynamics. Why do they lead to similar clinical consequences, how do they interact, and what are the molecular and signaling bases that connect each other? Recent hypotheses in the field of mitochondrial physiopathology even consider that defects of the mitochondrial network could be the final cause of the disease, consequently to mutations affecting primarily the respiratory chain complexes. For instance, the work of Duvezin *et al*. (46) analyzed the fusion protein OPA1 in different cells models with a genetic primary defect in the respiratory chain complexes. These included cybrids created from a patient with myoclonus epilepsy and ragged-red fibers (MERRF) syndrome,

mouse embryonic fibroblasts harboring an error-prone mitochondrial mtDNA polymerase gamma, heart tissue derived from heart-specific TFAM knock-out mice suffering from mitochondrial cardiomyopathy, and skeletal muscles from patients suffering from mitochondrial myopathies such as myopathy encephalopathy lactic acidosis and stroke-like episodes. They observed that dissipation of the mitochondrial membrane potential led to fast induction of proteolytic processing of OPA1 and concomitant fragmentation of mitochondria. Moreover, recovery of mitochondrial fusion depended on protein synthesis and was accompanied by resynthesis of large isoforms of OPA1. This could designate a novel mechanism by which the proteolytic processing of OPA1 could induce the fragmentation of energetically compromised mitochondrial segments. This could prevent the fusion of dysfunctional mitochondrial particle with the functional mitochondrial network. As discussed above, the relationships between mitochondrial energy production and mt-network organization are mutual (12). Hence, pathological mutations affecting either the respiratory chain, or the dynamic machinery, can both lead to OXPHOS deficiency and abnormal shaping of the mitochondrion.

B. Mitochondrial network architecture in patient's fibroblasts

The observations of mt-network architecture in cells from patients with a genetic defect in respiratory chain show inconsistent abnormalities of the mt-network. The patterns of these changes are not consistent, and can vary from one patient to another, as clinical signs typically do. Most studies looking at these aspects concerned the complex I deficiencies in human skin fibroblasts, since it is the most common cause of mitochondrial diseases. The group of Robinson (131) analyzed the mitochondrial structure and motion dynamics in living cells with energy metabolism defects by real time microscope imaging. They concluded that skin fibroblasts from patients with mitochondrial complex I deficiency and normal fibroblasts treated with rotenone, or antimycin A, contained higher proportions of mitochondria in the swollen filamentous forms, nodal filaments, and ovoid forms rather than the slender filamentous forms found in normal cells. They also reported a decreased motility with more ovoid mitochondrial forms compared to the filamentous forms. Likewise, it was shown that when CI activity was chronically reduced by 80% in human skin fibroblasts, using rotenone treatment, the percentage of moving mitochondria and their velocity decreased by 30% (88). It was proposed that ROS generated by the ETC in pathological situations could be responsible for such observed changes in mt-network organization. To suggest this hypothesis, Werner Koopman and Sjoerd Verkaart developed a fine and reliable method for the simultaneous quantification of oxidant levels and cell spreading (89, 90, 92), based the monitoring of CM-H_2DCF conversion into DCF upon intracellular oxidation, by video-rate confocal microscopy. After an extensive validation of their protocol, these authors looked at the ROS steady-state levels in fibroblasts taken from patients with a complex I deficiency and demonstrated a 2.5-fold higher oxidative stress in these cells. In another study, the same group demonstrated that superoxide production is increased in complex I deficient cell lines, in proportion to the enzymatic activity decrease. Interestingly, the redox state of these cells remained unaffected, even though oxidative stress was higher (92). Lastly, they analyzed the consequence of complex I deficiency of the mitochondrial network organization and concluded about a higher branching and elongation of the tubules, qualified as "mitochondrial outgrowth" (90).

The questions arise about the importance of mt-network adaptations for the diagnosis of mitochondrial diseases. This could allow a first check as an easy test on blood cells, with a rapid mitochondrial staining and direct visualization on a fluorescent microscope. Of course, all OXPHOS defects will not be identified this way, but large deficiencies could be observed at the level of the mt-network, owing that the cell type under consideration expresses sufficiently the metabolic defect. Recently, it was reported that an heterozygous, dominant-negative mutation in the dynamin-like protein 1 gene (DLP1) could cause a pathological condition associating microcephaly, abnormal brain development, optic atrophy and hypoplasia, persistent lactic acidemia, and a mildly elevated plasma concentration of very-long-chain fatty acids. The DLP1 protein is involved in the fission mt-network tubules, and we showed that its absence can impair the synthesis of ATP by the mitochondrion (12). Thus, the mitochondrial network organization is a new parameter that must be taken into account for physiopathological analyses of mitochondrial diseases.

XII. CONCLUSIONS

The recent field of mitochondrial dynamics has led to the reconsidering of bioenergetics in spatial terms. Likewise, the energy state appears to control, in part, the processes of fusion and fission of the mitochondrial network. This intimate and mutual relationship between mitochondrial form and function opens new perspectives for the understanding of what regulates energy metabolism and how cell physiology interacts with organelle functions. In this review, we proposed a hypothetical model of tubular internal compartmentalization which entails important implications for the sequestration of $\Delta\Psi$ and the delivery of ATP to cell areas, in the different configuration of the mt-network. This resurrects the old bioenergetic controversy about a localized or delocalized electrochemical gradient. The advances in the description of mitochondrial interior and the topological organization of respiratory chain supercomplexes also bring up to date the debate on the free-diffusive *versus* immobilized respiratory system. The discovery of intermediate substrates compartmentalization, along with the description of distinct physical pools, have implications for both the control of energy production and apoptosis. Accordingly, morphological changes of the mitochondrial compartments could permit to regulate the utilization of these different pools for the diverse functions of the mitochondrion. Moreover, we envisaged the fact that the fusion of inter-mitochondrial particles could lead the assembly of partial subnetworks involved in the genetic and functional compensation of local energy defects. The existence of intratubular domains of heterogeneous $\Delta\Psi$ and redox potential further demonstrates the existence of mitochondrial heterogeneity that must be maintained by mechanochemical processes yet undiscovered. Hence, the link between mitochondrial form and function can no longer be omitted in studies of en-

ergy metabolism or apoptosis, as well as the physical connection between these processes. The mitochondrial network and its fantastic dynamics could act as a physical connector between the different mitochondrial functions, and orchestrate most processes via changes in membrane organization. The aging process is also accompanied by alterations in mt-network morphology, as alteration of tubular fission retards aging in fungal systems (145). Hence, more fundamental studies on the exploration of the relationships between the different mt-network configurations and organelle functions will help to unravel the importance of the mitochondrion in the control of cell life and death.

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ABBREVIATIONS

ADP, adenosine diphosphate; ANT, adenine nucleotide translocator; ATP, adenosine triphosphate; CCCP, carbonyl cyanide m-chlorophenylhydrazone; COX, cytochrome *c* oxidase; CM, cristae membrane; CoQ, coenzyme Q; Cyt c, cytochrome c; DAPI, diamidino-4′,6-phénylindol-2 dichlorhydrate; DY, mitochondrial membrane electric potential; DNP, 2,4-dinitrophenol; DRP1, dynamin-related protein 1; EGFP, enhanced GFP; EM, electron microscopy; ETC, electron transfer chain; $FADH_2$, Flavin adenine dinucleotide reduced form; FCCP, carbonylcyanide-p-trifluoromethoxyphenylhydrazone; FISH, fluorescent *in situ* hybridization; FMN, flavin mononucleotide; 4Pi MICROSCOPE, confocal microscope with two opposing lenses used for high resolution imaging of fluorescence; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; GTP, guanidin triphosphate; H_2O_2, hydrogen peroxyde; IBM, inner boundary membrane; ICS, intra cristae space; IM, inner membrane; IMS, intermembrane space; JO_2, respiratory rate; MCA, metabolic control analysis; mPTP, mitochondrial permeability transition pore; mt-NETWORK, mitochondrial network; NADH, nicotinamide adenine dinucleotide reduced form; OM, outer membrane; OPA1, gene encoding a dynamin-related mitochondrial protein causing autosomal dominant optic atrophy; OXPHOS, oxidative phosphorylation ; PDH, pyruvate dehydrogenase complex; PLD, phospholipase D; RCR, respiratory control ratio; RFP, red fluorescent protein; ROS, reactive oxygen species, SDH, succinate dehydrogenase; STED MICROSCOPY, stimulated emission depletion microscopy; TMRM, tetramethyl rhodamine methyl ester.

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