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Electron tomography of mitochondria after the arrest of protein import associated with Tom19 depletion

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In a mutant form of *Neurospora crassa*, in which sheltered RIP (repeat induced point mutation) was used to deplete Tom19, protein transport through the TOM/TIM pathway is arrested by the addition of p-fluorophenylalanine (FPA). Using intermediate-voltage electron tomography, we have generated three-dimensional reconstructions of 28 FPA-treated mitochondria at four time points (0–32 h) after the addition of FPA. We determined that the cristae surface area and volume were lost in a roughly linear manner. A decrease in mitochondrial volume was not observed until after 16 h of FPA treatment. The inner boundary membrane did not appear to shrink or contract away from the outer membrane. Interestingly, the close apposition of these membranes remained over the entire periphery, even after all of the cristae had disappeared. The different dynamics of the shrinkage of cristae membrane and inner boundary membrane has implications for compartmentalization of electron transport proteins. Two structurally distinct types of contact sites were observed, consistent with recently published work. We determined that the cristae in the untreated (control) mitochondria are all lamellar. The cristae of FPA-treated mitochondria retain the lamellar morphology as they reduce in size and do not adopt tubular shapes. Importantly, the crista junctions exhibit tubular as well as slot-like connections to the inner boundary membrane, persisting until the cristae disappear, indicating that their stability is not dependent on continuous protein import through the complex containing Tom19.

Introduction

The process of protein import into mitochondria from the cytoplasm is necessary for proper function as only thirteen of the thousands of proteins present in mitochondria are mitochondrially synthesized. The rest are synthesized in the cytosol and imported by a translocation process, which in many instances must cross both the outer and inner boundary membranes. Most translocation processes are driven by the protein import machinery in the outer and inner membrane known as TOM and TIM, respectively.

Using a technique called “sheltered RIP (repeat induced point mutation)”, Harkness et al. (1994) introduced a nonfunctional mutant gene for Tom19 (known in older nomenclature as MOM19), a protein import receptor in *Neurospora crassa*. The mutation is “sheltered” in a heterokaryon strain, 28.17, containing an unaffected nucleus that under permissive conditions provides a functional Tom19 protein. Upon growth in the presence of FPA (p-fluorophenylalanine), however, the nucleus containing the defective gene is favored and the culture gradually becomes depleted in Tom19.

Tom19 performs an essential function in the maintenance of mitochondrial function (Harkness et al., 1994). Deficiency in Tom19 produced a marked growth defect of *N. crassa*; but the hyphae remained viable. It was discovered that mutant mitochondria became highly deficient in certain cytochromes, cristae membranes, oxidative phosphorylation, and protein synthesis over the course of 32 h of continuous FPA treatment. Protein import into isolated mutant mitochondria was diminished by 6 to 30-fold for most proteins. For the majority of precursor proteins tested from all submitochondrial compartments, Tom19 was required for effective transport into

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mitochondria. These results argued strongly that Tom19 plays a major role for mitochondrial entry of precursor proteins. The structural effects of Tom19 depletion were demonstrated by thin-section electron microscopy of fixed and embedded specimens of 28.17 grown in the presence of FPA for 8, 16, and 32 hours; after 32 hours the outer and inner boundary membranes did not appear diminished, yet almost no cristae were noted.

In order to answer questions about the stability of membrane architectural components as the cristae are depleted, three-dimensional (3-D) visualization is needed. Electron tomography provides the needed visualization tools to produce 3-D reconstructions (Perkins et al., 1999). Because electron tomography captures a large portion of the volume of in situ mitochondria, it can reveal complex membrane topographies commonly found with the inner membrane and can elucidate the structural characteristics of relatively small features, such as tubular crista junctions and contact sites (Perkins and Frey, 2000). Recently, cryo-electron tomography was used to investigate membrane structures in isolated *N. crassa* mitochondria (Nicastro et al., 2000). An interesting tripartite morphology of cristae membranes that was part of a 3-D network of interconnected lamellar cristae was revealed in mitochondria preserved in vitreous ice. Crista junctions were observed, but with openings larger than previously reported for mammalian mitochondria imaged in situ (Mannella et al., 1994; 1997; Perkins et al., 1997a; 1998).

We analyzed tomographic reconstructions of in situ mitochondria from control and FPA-treated *N. crassa* hyphae. This analysis: (1) defined the nature of *N. crassa* cristae viewed in situ, (2) determined that slot-like crista junctions as well as tubular junctions exist in these fungal mitochondria in contrast to mammalian mitochondria in which only tubular crista junctions were observed, and (3) determined that these junctions persist as the cells become deficient in Tom19 until the cristae finally disappear, at which point the junctions also disappear. Mitochondrial volume decreased but only after 16 h of FPA treatment. We also found two different contact site structures. One is similar to the contact sites first described by Hackenbrock (1966) in which the two membranes come close together with no space in between. In the other type of contact site, the outer membrane and the inner boundary membrane maintain a constant separation with bridging structures connecting the two.

Materials and methods

Thin-section electron microscopy

Hyphae of *N. crassa* strain 28.17 were prepared for electron microscopy as described previously (Harkness et al., 1994). Briefly, hyphae were fixed in 1.5% potassium permanganate for 20 min at room temperature followed by washing with water until the suspension became colorless. In order to prepare hyphae so as to visualize mitochondria, extraction of cytoplasmic components was required. Permanganate was chosen as the primary fixative because many cytoplasmic (and mitochondrial matrix) components are extracted with this fixative, whereas membrane components are more strongly fixed. The result is greater contrast of the mitochondrial membranes. Subsequent fixation was accomplished with 3% (vol/vol) glutaraldehyde in 0.1 M cacodylate buffer on ice for 30 min followed by 1% (wt/vol) osmium tetroxide and 1.5% (wt/vol) potassium chromate mixed together in water. En bloc staining was with 1% (wt/vol) uranyl acetate for 16 h at room temperature. The cells were dehydrated in a graded ethanol series and embedded in Epon 812 plastic.

For the volumetric measurements shown in Fig. 6a, sections from these samples were cut with a thickness ≈ 50 nm. These sections were stained for 10 min in 2% aqueous uranyl acetate, followed by 2 min in lead salts. Images of nominally untitled sections were collected with a JEOL 2000FX electron microscope operated at 180 kV. The negatives from the series were digitized using a 1024×1024 pixel cooled CCD camera (Photometrics) with 14-bit precision. The program, NIH Image (National Institutes of Health, Bethesda, MD), was used to measure area profiles from which volume ratios were calculated.

Electron microscope tomography

Sections from the same samples used for thin-section electron microscopy were cut with a thickness ≈ 500 nm. These sections were stained for 1 h in 2% aqueous uranyl acetate, followed by 1 h in lead salts. Fiducial cues consisting of 20 nm colloidal gold particles were deposited on one side of the specimen. Three-dimensional reconstructions of in situ mitochondria were created using electron tomography (Perkins et al., 1999). For each reconstruction, a series of images was collected with a JEOL 4000EX intermediate-voltage electron microscope operated at 400 kV. The specimen was irradiated before initiating a tilt series in order to limit anisotropic specimen thinning during image collection. Preirradiation in this manner subjected the specimen to the steepest portion of the nonlinear shrinkage profile before images were collected. The specimen was tilted in equal angular increments about an axis perpendicular to the optical axis of the microscope using a computer-controlled goniometer to increment accurately the angular steps. These single-axis tilt series were recorded on film at 40000 magnification at angular increments of 2° from -60° to $+60^\circ$. Each image represents a projection of density in the specimen from a particular angle of view. The illumination was held to near parallel beam conditions and film density maintained constant by varying the exposure time in order to minimize distortions in the projected image (Fan et al., 1995). The negatives from the series were digitized with the Photometrics CCD camera. The sampling varied slightly from tilt series to tilt series and was between 1.79 and 2.03 nm per pixel.

3-D reconstruction and analysis

Image processing was performed with the SUPRIM software suite (Schroeter and Bretau diere, 1996). Translational and rotational alignment of each tilt series was done first by cross-correlational alignment and subsequently by fine adjustments through the use of fiducial gold marks. The positions of the selected fiducial marks were used to perform a least-squares alignment across the images of the tilt series. After alignment, reconstructions were computed by an R-weighted back-projection algorithm (Frank, 1992). A typical volume size was $\approx 0.1 \mu\text{m}^3$. Volume segmentation of the mitochondrial structures was performed by manual tracing in the planes of highest resolution with the program XVOTRACE (National Center for Microscopy and Imaging Research, San Diego, CA). The mitochondrial reconstructions were visualized using ANALYZE (Mayo Foundation, Rochester, MN) or the surface-rendering graphics of SYNU (National Center for Microscopy and Imaging Research, San Diego, CA). These programs allow one to step through slices of the reconstruction in any orientation and to track or model features of interest in three dimensions. Measurements of structural features were made from planes within the reconstructed volume with the program NIH Image or within segmented volumes by the program XDEND (National Center for Microscopy and Imaging Research, San Diego, CA).

The mitochondrial thickness in the reconstructed volumes ranged from 80 to 390 nm. Using the derivation provided by Woodcock (1992) we calculated the x, y-resolution, which ranges from 6 nm in our thinnest reconstruction to 27 nm in our thickest. However, the resolution in our reconstructions is better than calculated because the heavily stained features, the membranes, occupied a relatively small volume (Perkins et al., 1997a). With similarly prepared specimens, others have found that the observed resolution is greater than the calculated resolution by a factor of ≈ 3 (Mannella et al., 1994; Woodcock, 1992) or 2–9 nm in this case. Because the tilt angles ranged from $+/-60^\circ$, rather than $+/-90^\circ$, the z-resolution is $1.8 \times$ poorer than the x, y-resolution (Radermacher,

1988) or 3.6–16 nm for the strongly stained features. Increases in z-resolution can be obtained with finer angular increments when collecting tilt series (Woodcock, 1992), double-tilt tomography (Penczek et al., 1995), and possibly by collecting higher tilt angles. By observing the variation of strong features in successive x-y slices, we estimated the z-resolution in our reconstructions to be no worse than 10 nm, which was adequate for the analysis conducted on membranes reported here. Additional details of the tomographic methods used can be found in Perkins et al. (1997b), Martone et al. (1999), and Lenzi et al. (1999).

Visual determination of tilt series alignment

Frank and McEwen (1992) reported that the commonly used visual evaluation of tilt series alignment could detect errors in rotational alignment of 0.5° and errors in translational alignment of 2 pixels. The procedure involves viewing the tilt series in rapid succession, producing a “movie” effect (animation mode), and evaluating how smoothly features in the images rotate about the tilt axis without in-plane rotations or jumps. Even though a rotational misalignment of 0.5° and a translational misalignment of 2 pixels seem small, relatively small errors in alignment can affect significantly the resolution of the reconstruction. In general, the alignment error must be substantially smaller than the resolution limit to prevent the attenuation of information close to this limit. Moreover, rotational misalignment produces distortions that increase in magnitude at the image periphery. The use of fiducial marks far from the tilt axis can propagate these peripheral errors to more central regions of the image (Fan et al., 1995). Even with the potential for propagating peripheral errors, fiducial mark alignment is still needed because although cross-correlational methods perform translational alignment excellently, they do not usually provide adequate rotational alignment (Frank and McEwen, 1992).

It is important to know the magnitude of tilt-series misalignment and the extent to which it degrades the resolution in our tomographic reconstructions. Because a rotational misalignment of 0.5° produces a registration error of 4.5 pixels at the edge of a 1024×1024 pixel² image, a blur over 4–5 pixels results, which degrades the resolution to a level below that needed to accurately map mitochondrial membranes. Based on previous evaluations of resolution (Perkins et al., 1997a; 1998), we believed that we could align significantly better than the 0.5° previously reported (Frank and McEwen, 1992), but we had not quantified it. Table I provides the results of a semi-quantitative evaluation of our typical efforts at translational and rotational tilt-series alignments. If the alignment shows obvious jumps or wobbles in animation mode, we redo it. On the basis of what degree of misalignment is visually detectable in animation mode, we report that our alignments are better than 1 pixel in translation and $0.^\circ$ in rotation. Because we digitized at ≈ 2 nm/pixel, our translational misalignment is no worse than 2 nm and our rotational misalignment is no worse than 4 nm at the periphery, both below the estimated z-resolution of 7–10 nm in our reconstructions.

Method for testing tilt series alignment

After fiducial alignment of one of our tilt series, one tilt image, chosen at random, was file-format converted to TIFF format and in Adobe Photoshop either translated or rotated by known, small values,

representing typical translational or rotational misalignment, respectively. Noticeable borders produced by the translational or rotational processes were removed by pasting matching regions into these areas so they were no longer detectable during animation of the tilt series. After back converting to SUPRIM format, these images were inserted into the proper place in the tilt series. Without knowing which image was misaligned, we viewed the entire tilt series on a graphics terminal using the program XFIDO in “animation mode”, which displays each image of the series in rapid succession. The ability to correctly identify the misaligned image by eye was confirmed a posteriori.

Results

Tomographic reconstructions

Harkness et al. (1994) reported that Tom19 depletion greatly inhibited protein import into mitochondria and decreased the amount of inner membrane to the point that at longer times of growth on FPA (32 h) no cristae were observed, although mitochondria with outer and inner boundary membranes were still present without a change in size. Electron tomography was used to examine the membrane architecture of mitochondria in *N. crassa* depleted of Tom19, and in control cells that were not depleted to extend the earlier work in the context of a new paradigm of mitochondrial structure (Mannella et al., 1994; 1997; Perkins et al., 1997a; 1998). Electron tomography is currently the imaging technique that provides the highest 3-D resolution of the internal structure from thick sections of mitochondria (Frank, 1995). Intermediate voltage (400 kV) was needed in order to reduce multiple and inelastic scattering of electrons as they passed through the specimen, degrading the image quality. We collected two tilt series from separate hyphae for each of the time points resulting in the reconstruction of 28 mitochondria – four from 0 h, 2 from 8 h, 8 from 16 h, and 14 from 32 h time points. More mitochondria were reconstructed for the 16 h and 32 h time points because the close packing of mitochondria resulted in multiple reconstructions from a given tilt series.

Each reconstruction was a 3-D solid composed of cubic voxels that usually were viewed as stacks of planes, each one voxel thick, sliced through the volume oriented in a user-defined direction (Fig. 1). It should be emphasized that the cross-sections through the tomographic volumes more clearly define the cristae than do the electron micrographs of the untitled sections. This clarity is a result of the tomographic method and illustrates the need for a 3-D technique that separates membrane features that are otherwise obscured in micrographs because they overlap one another in projection images through relatively thick sections of cells. The insets in

Tab. I. Visual test for tilt series alignment.

Translational misalignment ^a	Observations ^b	Rotational misalignment ^a	Registration error at edge ^c	Observations ^b
0.5 pixels	barely detectable	0.05°	0.45 pixels	undetectable
1	obvious	0.1	0.90	barely detectable
2	very obvious	0.2	1.8	obvious

^a After fiducial alignment, one image in the tilt series was replaced by the same image either translated or rotated by the indicated values, representing translational or rotational misalignment, respectively. Without knowing which image was misaligned, we viewed the entire tilt series on a graphics terminal in “animation mode”, which rasters through the series in rapid succession.

^b If the tilt series is well aligned, features in the images will rotate smoothly about the tilt axis without jumps or in-plane wobbles. By visual inspection, one can detect translational or rotational misalignment in a given image, or series of images. If the misalignment is obvious, then the fiducial alignment is reworked until the misalignment is undetectable or barely detectable.

^c The maximum registration error between images, found at the edges of a 1024×1024 pixel² image, due to the given rotational misalignment.

the 16-h reconstruction identify two distinct classes of contact sites. Mitochondrial contact sites are defined as sites where the outer and inner boundary membranes come close together with no discernible space between them (Hackenbrock, 1966). To clearly identify contact sites, a resolution of at least 10 nm is required. Thus, it is important that a z-resolution of 7–10 nm was attained in our mitochondrial reconstructions. This is considerably better than can be achieved by serial section electron microscopy, and allowed unambiguous tracking of contact sites in three dimensions. In *N. crassa* mitochondria, contact sites were easily visualized because the outer and inner boundary membranes were separated by a ≈ 8 nm space of density lighter than the membranes, except at these sites. The upper inset provides an example of a classical contact site wherein the outer and inner boundary membranes are “pinched together”. The lower inset shows a different class of contact site with three closely spaced electron dense “bridges” that span the intermembrane space without the outer and inner membranes coming together.

Resectioning the tomographic volumes along various axes confirmed the lamellar character of cristae and the punctate nature of contact sites. An example of resectioning is provided in Figure 2. By blocking smaller volumes, different faces sectioned through the mitochondrion are presented. Perpendicular faces show the lamellar aspect of the cristae. The insets display mutually perpendicular views of (different) contact sites and emphasize their punctate profiles. Coupled with previous reports (Mannella et al., 1997; Perkins et al., 1997a; 1998), the results reported here add to the growing body of work suggesting that for in situ mitochondria, contact site dimensions are relatively uniform across cell types, even those as different as mammalian and fungus. Volume resectioning helped us to discover that the cristae in the untreated (control) mitochondria are all large, lamellar compartments. It also allowed us to determine that the cristae of FPA-treated mitochondria retain their lamellar morphology as they become smaller and that they do not adopt tubular shapes.

Tubular crista junctions similar to those observed in mammalian mitochondria (Mannella et al., 1997; Perkins et al., 1997a; 1998) were observed in *N. crassa* mitochondria (Fig. 3)

for all time points after FPA treatment. However, a surprising discovery was the existence of slot-like crista junction openings in addition to the typical tubular junctions. These slot-like openings are elongated along the axis of closest proximity of the cristal lamellar face to the inner boundary membrane. There does not appear to be two classes of junctions, but rather a continuous distribution of opening sizes within a certain range (24–100 nm). Figure 3 displays slices through a tomographic reconstruction orientated to cut perpendicular to the cristal lamellae and shows that the tubular junction appears in only a small number of consecutive slices, as do both classical and bridge contact sites. These thin slices demonstrate the power of electron tomography to document the 3-D extent of small features inside mitochondria.

Three-dimensional tomographic reconstruction allowed us to probe for substructural alterations inside intact mitochondria at 8, 16, and 32 h time points (Fig. 4). The dimensions of substructures, such as contact site width and extent, crista width, and crista junction opening were tracked by stepping serially through the slices (each ≈ 2 nm thick) of the reconstructed volumes using the computational tool, NIH Image. We found that the extensive lamellar cristae in control mitochondria connected to the inner boundary membrane via crista junctions. Crista junctions are the membranous connections between the crista membrane and the inner boundary membrane and encircle openings of various sizes. A detailed examination of five classes of measurements – outer-inner membrane widths, cristae widths, crista junction opening, contact site widths, and contact site diameters (consisting of several hundred measurements per class) – found that very little difference in dimensions were apparent at the different time points (data not shown). Hence, all time points were averaged together for a comparison (Fig. 4) with other types of mitochondria (neuronal and brown fat). The mean values for *N. crassa* mitochondria for a given class of measurements fell within significantly less than one standard deviation of measurement of the other mitochondrial types, indicating little deviation between mitochondria of very different cells. We found that the crista junction opening has a mean of 40 nm with a much larger variance than found for mammalian junctions reflecting the observation that *N. crassa* mitochondria possess slot-like openings elongated along the lamellar face as well as the commonly described tubular openings. The inner boundary membrane lies close to the outer membrane with a mean total thickness across both membranes of 20 nm at places other than classical contact sites. The mean value for the measured diameter (10 nm) of bridge contacts indicates that these are stalks connecting the outer and inner membranes.

Volume segmentation and analysis

Segmentation is a visual tool that defines and dissects volume components and thus facilitates interpretation, measurement and understanding of interrelationships of complex structures (Perkins et al., 1997b). Once the membranes in the reconstruction were segmented, surface-rendered volumes were displayed and rotated to study membrane topology and crista junction distribution (Fig. 5). A wealth of information can be quickly taken in by rotating the segmented volumes. For example, when observing the edges of the cristae, it became apparent that there are many places where the crista membrane abuts the inner boundary membrane without forming a crista junction. Segmenting eight volumes (two from each time point) confirmed that the inner mitochondrial membrane invaginates

◀ **Fig. 1.** Projection images and slices through the tomographic volumes of semi-thick sections ($\approx 0.5 \mu\text{m}$) of *N. crassa* taken with an intermediate-voltage electron microscope (400 kV). (a) 0° images from representative tilt series used to generate 3-D reconstructions of mitochondria taken from control, 8, 16, and 32 h time points after the addition of FPA. The black dots seen rarely in a few of the images are 20 nm colloidal gold particles that were used as fiducial marks for aligning the tilt series. The large stain granules, most evident in the 16-h and 32-h images, originated from the longer poststaining times used to enhance the contrast of the membranes. Longer poststaining was needed because of extraction during primary fixation that was advantageous for preparing hyphae (see Materials and methods). (b) A single slice through each of the mitochondria shown in (a) and reconstructed by electron tomography. The tomogram has the advantage of visualizing the entire outer and inner membrane topologies, usually through stacks of slices ≈ 2 nm thick, at relatively high resolution. The greater membrane clarity in the slices compared to the projection images is due to the tomographic method. The insets in the 16-h reconstruction identify two distinct classes of contact sites. The upper inset highlights a classical contact site showing the outer and inner boundary membranes “pinched together”. The lower inset shows three closely spaced electron-dense “bridges” that span the intermembrane space without a change in distance between the outer and inner membranes. The scale bar applies to all panels.

Fig. 2. Portions of control and 32-h mitochondrial tomographic volumes, sliced in perpendicular planes. Three-dimensional perspectives allowed us to track features, such as contact sites, along perpendicular faces (insets) that confirmed their punctate nature. Resectioning of tomographic volumes along user-defined axes emphasizes the lamellar nature of cristae and the better z-resolution compared to other 3-D techniques, such as serial-section reconstruction. Arrowheads point to perpendicular perspectives of lamellar cristae. In the 32-h volume, the only crista in the blocked mitochondrion shown in the foreground retained its lamellar profile (did not convert to a tubular topology) even though it did not extend very far into the matrix.

to form cristae only through crista junctions (one segmented volume for each time point is shown in Fig. 5). The good z-axis resolution compared with the section thickness was important for visualizing the extent of crista junctions and contact sites. For example, multiple junctions connecting a crista to the same side of the inner boundary membrane were visualized (see the control and 8 h volumes in Fig. 5). Even though *N. crassa* cristae are large and lamellar, similar to those in brown fat mitochondria, they differ from these in that slot-like junctions are just as prevalent as tubular crista junctions and the cristae do not extend across the volume in stacks as do brown fat cristae, but rather have a more radial extension. We suggest that the lamellar cristae in *N. crassa* mitochondria do not form ordered stacks as they do in brown fat because the relative amount of crista membrane in *N. crassa* mitochondria is smaller. The stacking of lamellar cristae in brown fat mitochondria may be

driven by the need to pack crista membrane efficiently. Quantitative results, such as surface area and volume ratios, were extracted from these volumes (Figure 6). As is hinted by the rendered volumes shown in Figure 5, the mitochondrial volume appears to have lessened after 32 h of FPA treatment.

Volumetric and ratio measurements were made using thin sections, instead of tomography, as this technique allows one to sample many more mitochondria. Observations of bridge and classical contact sites indicated that no statistically significant change in the relative numbers of these two types of contacts occurred over the time course of FPA treatment (data not shown). The program, NIH Image was used to measure area profiles from which mean volume ratios were calculated (Fig. 6a). Ratios were calculated with the mean value of the control measurements in the denominator for each time point. We conducted a Student's t-test to test the null hypothesis that

Fig. 3. Serial x-y slices through a control mitochondrial tomographic volume providing examples of the limited extent of crista junctions and contact sites along the direction of the lamellar face. **(a)** Tubular crista junction. The crista membrane is shown detached from the inner boundary membrane in the *first* row of images. The *second* row shows the crista junction opening connecting the intracristal and intermembrane spaces. The *third* row again shows the crista detached. In the second panel of the first row, the outer membrane (OM), inner boundary membrane (IBM) and the membranes for one crista (CM) are outlined for clarification. *Arrows* in the first and third rows point to

the stricture separating the intracristal space from the intermembrane space. Each slice has a width of 4.4 nm in the first and second rows and 2.2 nm in the third row. **(b)** Classical contact site. The outer and inner boundary membranes come close together in a “pinched” fashion (*arrows*). These punctate sites are about 14–16 nm in all dimensions. Each slice is 2.2 nm in width. **(c)** Bridge contact site. Instead of pinching, the outer and inner boundary membranes are connected by a “bridge” of density (*arrowheads*). The slice width is 2.2 nm. The scale bar applies to all panels.

the 32 h mean volume would be reasonably likely to be obtained from sampling randomly a population with the mean volume recorded at 0 h (control). The probability of this test is shown in the Figure and is 2.8%. Thus, at a significance level of 95%, the null hypothesis is rejected and we conclude that the volumes at 32 h are different (smaller)

than those in the control. The biological significance of this result is that even while cristae shrinkage is predominant, shrinkage of the inner boundary and/or outer membrane may also occur, but apparently not at the rate of cristae shrinkage. Another factor to consider is the finding that mutant cells grown in the presence of FPA displayed a drastic reduction in

Fig. 4. Histograms summarizing and comparing the means and standard deviations (error bars) of the distributions of substructure dimensions of *N. crassa*, brown fat (BAT), and neuronal mitochondria. Measurements were conducted on the tomographic reconstructions of 28 *N. crassa* mitochondria using the program NIH Image on equally spaced slices through the volumes, usually 40–90 slices per volume. Because little difference in dimensions was apparent between the different time points for the *N. crassa* mitochondria, all the data were averaged together for comparison with the other mitochondrial types. Values for the brown fat and neuronal mitochondria were taken from Perkins et al. (1998). The “contact width” was measured perpendicular to the membranes, from the outer edge of the outer membrane through to the inner edge of the inner membrane. The “contact diameter” was measured parallel to the membranes. For *N. crassa* mitochondria, contact sites were divided into two classes – classical and bridge. “Outer-inner membrane” was measured perpendicular to the membranes, from the outer edge of the outer membrane through to the inner edge of the inner membrane at randomly selected points along the periphery that were not contact sites. “Crista junction” is the largest opening measured across the bounding membranes at sites where the inner boundary membrane involutes into the interior. The larger mean and variance of *N. crassa* mitochondria compared to those of neuronal and brown fat reflects the greater variation of opening sizes, some being tubular, whereas others are more elongated. The *N. crassa* tubular crista junctions are also shown separately to compare with the dimensions of other tubular junctions (BAT and neuronal). “Crista width” is the width across the bounding membranes at randomly selected points of cristae at sites other than junctions.

growth rate after a time lag of ≈ 20 h (Harkness et al., 1994). Our observation that mitochondrial volumes did not decrease until after 16 h may be related to the reported time lag in cellular growth rate reduction. This delayed dynamic is strikingly different from the roughly linear rate of decrease in crista volume and membrane surface area described next.

The ratios of cristae volume to total mitochondria volume and cristae surface area to inner boundary membrane surface area are shown in Figures 6b and 6c, respectively. These measurements were made on the eight segmented volumes using the program XDEND. This method has the advantage of accurate volume and surface area measurements as long as the segmentation was accurate. In the mutant mitochondria, the relative volume and membrane surface area of cristae decreased roughly linearly with time as the cells were treated with FPA (Fig. 6b, c). The architecture, however, remained the same, lamellar (but smaller) with both tubular and slot-like crista

junctions (Fig. 5). Straight lines were fitted to the data points in Figure 6b, c using least squares and their equations shown boxed in the figure. From these equations, the rates of cristae volume loss and surface area loss were calculated. To comprehend what the loss of membrane surface area is equivalent to, a simple illustration is provided. A rate of $51,000 \text{ nm}^2/\text{h}$ is equivalent to losing the membrane surface area occupied by roughly 800 cytochrome oxidase (Cox) molecules, based on structural studies conducted by Frey (1994) showing a Cox dimer occupying $\approx 62 \text{ nm}^2$ inner membrane surface area. We chose not to perform a similar illustration for cristae volume loss because of the possibility that the volume loss may not correspond to loss of intracristal components, but rather to a displacement of these components, most likely into the intermembrane space, as the cristae membrane is lost. This is consistent with the findings that all of the proteins analyzed from the intracristal and intermembrane spaces, including cytochrome c, were not altered significantly in their levels, irrespective of the amount of Tom19 present (Harkness et al., 1994).

How do crista junctions correlate with disappearing cristae? Figure 7 displays the mean number of cristae and crista junctions as a function of FPA exposure. The plots show the same trend indicating that cristae disappearance correlates with the removal of crista junctions. In addition, the z-axis resolution allowed us to probe for suggestive openings in the inner membrane that might be remnants of crista junctions left behind as cristae disappear; we found none. These observations provide evidence that as the membrane of a crista is finally completely depleted, the crista junction that connected the crista to the inner boundary membrane is also lost.

Discussion

In order to answer questions about *N. crassa* mitochondrial membrane architecture as the cristae are depleted, a 3-D characterization of fine structure was needed. The electron tomography analysis reported here complements and extends the earlier work of Harkness et al. (1994) by identifying features of these mitochondria that were previously unknown. Electron tomography has a history of contributing to the understanding of spatial relationships in complex, 3-D biological structures (Bullitt et al., 1997; Horowitz et al., 1997; Koster et al., 1997; McEwen and Marko, 1999; Mehlin et al., 1995; O'Toole et al., 1999). It proved to be a valuable tool for probing which membrane components were stable and which were altered after cessation of protein import from the cytosol through Tom19. It aided the investigation of cristae topology under these conditions, and importantly, revealed that crista junctions persist until complete depletion of cristae membrane upon which the junctions close. The method enabled us to examine volumes at a resolution an order of magnitude better than attainable by serial section reconstruction or stereo imaging. Another advantage is the capability to slice through objects in many orientations, permitting multiple views of fine structure. This allowed us to determine that *N. crassa* cristae are lamellar without exception and to confirm the punctate nature of contact sites.

Our previous work with electron tomography of mitochondria led to the recognition of a mitochondrial structure, the crista junction (Perkins et al., 1997a), that helped to establish a

Fig. 5. Surface-rendered depictions of the 3-D reconstructions shown in Figure 1 after the volumes were segmented by contouring the regions bounded by the outer, inner, and cristae membranes. Note that the inner boundary and cristae membranes are continuous surfaces but were segmented independently to highlight their separate topographies. Distinct cristae were segmented separately to examine membrane morphology and connectivity. Some of the rendered cristae were omitted for clarity. The outer membrane is shown in blue, the inner boundary membrane in grey, and the cristae membranes in yellow. The

views arrayed on the bottom (side views) are perpendicular views to those on top. For the bottom views, the peripheral membranes were made translucent to be able to fully visualize the cristae. It can be seen that all of the cristae are lamellar. Crista junctions (CJ) are labeled and the variable size of their openings is evident in these side views. Multiple junctions connecting a crista to the same side of the inner boundary membrane are visible in the control and 8-h volumes. The 3-D axes next to the 32-h views show the relative orientation of top and bottom panels. The scale bar applies to all tomographic volumes.

Fig. 6. Surface area and volume measurements of segmented mitochondrial reconstructions. (a) Volumetric measurements from thin sections ≈ 50 nm thick. Relative volumes were calculated from area profiles from which volumes were estimated. Ratios of mean volumes are provided scaled to the control mean value. The numbers of mitochondria measured are displayed above each data point. A Student's t-test was conducted with the volumetric data from the control and 32-h measurements with the probability of 2.8% that they come from populations with equivalent mean volumes. The conclusion is that the volumes at 32 h are different (smaller) than those in the control. (b) Ratio of cristae volume to mitochondria volume. Measure-

ments were made on eight segmented volumes, two from each time point. (c) Ratio of cristae surface area to inner boundary membrane (IBM) surface area for the same eight volumes as in (b). The relative volume and membrane surface area of cristae decreased approximately linearly with time. Thus, using a least squares fit, straight lines were superimposed on the data points and their equations shown boxed. From these equations, the rates of cristae volume loss and membrane surface area loss were calculated and displayed in their respective boxes. An example of the equivalent loss of Cox molecules illustrates the magnitude of turnover in the cristae membranes upon Tom19 inhibition.

new paradigm of cristae architecture (Mannella et al., 1997; Frey and Mannella, 2000). Because crista junctions persist during the shrinkage of *N. crassa* cristae, we have learned that they are stable under two extremes of cristae morphology, brown fat mitochondria with a large area of crista membranes (Perkins et al., 1998) and *N. crassa* mitochondria depleted of crista membranes. It was hypothesized that the crista junction architecture may act as a diffusion barrier for metabolites and/or soluble proteins between the intracristal space and the intermembrane space (Mannella et al., 1997; 1999). The existence of slot-like crista junctions with their elongated openings challenges this hypothesis, at least in *N. crassa* mitochondria. However, even slot junctions do not extend across the entire width of mitochondria, as in the baffle model (Perkins and Frey, 2000), so restricted diffusion may still be

possible. The lamellar geometry of *N. crassa* and brown fat mitochondria differs from that found in neuronal mitochondria. Perkins and coworkers (1997a) discovered that many neuronal cristae remained tubular throughout their length, whereas other tubular sections of cristae merged to form lamellar compartments. The observation of slot-like crista junctions in *N. crassa* mitochondria is the first time that we have noted a non-tubular architecture.

Figure 6c shows that the rate of depletion of cristae membrane is much higher than that of the inner boundary membrane. One hypothesis derived from the different depletion dynamics of these two components of the inner membrane, connected by crista junctions, is that the relative stability of the inner boundary membrane is due to movement of lipids and/or proteins from the cristae to compensate for losses in the former.

Fig. 7. Mean numbers of cristae and crista junctions as a function of time of FPA exposure. Measurements were collected from all 28 tomographic reconstructions. The plots show the same trend providing evidence that crista junctions disappear with the cristae and do not remain behind as a stable structure.

We have noticed that in situ, the inner boundary membrane is closely apposed to the outer membrane. This apposition may be a sufficiently strong structural motif to draw membranous material from the cristae to the periphery. Another hypothesis is that even though the inner boundary and cristae membranes are contiguous, the composition of the two differs. It was proposed that crista junctions segregate inner membrane proteins between the inner boundary membrane and crista membrane (Perkins et al., 1997a; 1998). Could partitioning occur with electron transport chain proteins, e.g., Cox? Depletion of Cox by more than 10-fold as a result of Tom19 knockout (Harkness et al., 1994) coupled with the high rate of cristae loss might indicate that this protein is primarily in the cristae membrane. Localization of this protein within the inner membrane has been ambiguous, however. Different groups have obtained different results, with one report of Cox activity in both inner boundary and cristae membranes (Hiraoka and Hirai, 1992) and two reports of activity essentially only in cristae membranes (Perotti et al., 1983; Werner and Neupert, 1972). Why would segregation of membrane proteins be required or advantageous? One possibility is that the functions of different classes of inner membrane proteins are more suited for one location compared to another. For example, it is probably advantageous for metabolite transport proteins and protein import machinery to be located within the inner boundary membrane where they can participate in facilitated transport across both outer and inner membranes at the same site. On the other hand, electron transport proteins, e.g. Cox, and the ATP synthetase could be localized to the crista membrane as their function is not enhanced by being near the outer membrane. Because both metabolite transport proteins and those required for ATP synthesis are abundant in the inner membrane, segregation among the two inner membrane components, inner boundary membrane and cristae membrane, is a viable hypothesis. Furthermore, import of the ADP/ATP carrier protein is not blocked by loss of TOM19 (Harkness et al., 1994). It is also possible that cristae components have an advantage being on the cristae side of crista junctions. One

advantage might be that the ordered arrangement of tricarboxylic acid cycle and respiratory chain enzymes into segregated domains involving the entire cristae may aid the requirement for fast reaction rates and controlled catalysis of these enzymes (Sjostrand et al., 1988; Sjostrand, 1991).

On the basis that crista junctions appear to be a uniform structural principle across mammalian tissue types, as seen in electron tomographic reconstructions of liver (Mannella et al., 1994; 1997), neuronal (Perkins et al., 1997a; b), and brown fat mitochondria (Perkins et al., 1998), we hypothesized that the crista junction architecture might be present in non-mammalian cells as well. Recently, Nicastro and coworkers (2000) used cryo-electron tomography to show that this architecture exists in isolated *N. crassa* mitochondria preserved in vitreous ice. In agreement with our in situ work, the crista junction openings in these mitochondria were elongated (up to 200 nm across the largest dimension) compared to those found in mammalian tissues that are circular and ≈ 28 nm in diameter. Electron tomography on mitochondria embedded in vitreous ice is a new development (Mannella et al., 1999) that has as one of its goals the identification of macromolecules in mitochondria that have undergone a minimal amount of structural perturbation from specimen preservation (Nicastro et al., 2000). While this technique is promising for isolated mitochondria, it is not yet feasible for tissue samples. Even though isolated organelles can be frozen in physiological buffers, they are still not in their natural environment; they can and do undergo structural perturbation from the isolation procedure and from surface tension forces prior to freezing. On the other hand chemical fixation followed by dehydration and plastic embedment of specimens can also introduce significant structural changes. Hence, it is important to compare information from complementary techniques while striving to minimize sample preparation artifacts in each. We reported that cryofixed and freeze-substituted mitochondria display structural features very similar to those observed in chemically fixed and embedded specimens (Perkins et al., 1998). Furthermore, Nicastro and coworkers report that the basic 3-D structure of ice-embedded mitochondria is consistent with mitochondria prepared by chemical fixation. Indeed, many of the measurements they report confirm our earlier work, including the outer to inner membrane distance of ≈ 20 nm, an 8 nm gap between these two membranes, 13 nm contact site width, and narrow intracristal spaces (≈ 30 nm across). Both studies, Nicastro and coworkers' of purified mitochondria and this study of mitochondria in situ, found only lamellar cristae in *N. crassa* mitochondria, and no tubular cristae similar to those observed in neuronal and liver mitochondria were observed. Finally mitochondria in both studies were in the orthodox conformation with a relatively large matrix compartment, and neither study found condensed mitochondria (Hackenbrock, 1968) arguing that this conformation does not exist under normal, unperturbed conditions.

The results obtained by Nicastro et al. (2000) differed from ours in some respects. As expected the matrix of ice-embedded mitochondria is considerably more dense than in our chemical fixed organelles because we chose a fixation procedure that extracts some of the matrix (and cytoplasmic) components in order to better visualize the membranes. More significantly, however, tripartite cristae were found in ice-embedded purified mitochondria as part of a network of interconnected lamellar cristae. This network differs remarkably from our observations in thin sections and tomographic volumes, as well as published

figures (Harkness et al., 1994) showing in situ mitochondria having no tripartite nor interconnected cristae, similar to the cristae in brown fat mitochondria (Perkins et al., 1998). Whereas we cannot rule out that the cristae structures we observe are not influenced by preparation artifacts, we point out the possibility that the cristae network is produced by the isolation procedure and/or the reported compression (Nicastro et al., 2000) during preparation for freezing. For example, crista junctions in isolated mitochondria can change in size in response to changes in matrix volume (von Ahsen et al., 2000). The ellipsoidal mitochondria, typically 600–1500 nm in size, were reported by Nicastro and coworkers to be compressed to 200–450 nm. It is conceivable that this compression could force stretching and fusion of membranes. It was reported that cytoplasmic components, and to a lesser extent intramembranous components, often appeared to be stretched to form fibrils after rapid freezing of unfixed and uncryoprotected tissue (Dempsey and Bullivant, 1976). Further work on the effect of compression due to surface tension is needed to determine if it may be sufficient to cause fusion of cristae. In addition, the frequent presence of inside-out inner membrane vesicles in the samples prepared by Nicastro and coworkers, while offering an opportunity to study membrane components at higher resolution, indicates substantial disruption of the inner membrane during the isolation steps.

The type of contact site that forms a bridge spanning the intermembrane space has been recently identified in other cell types. Mannella et al. (1999) report that liver mitochondria preserved in vitreous ice have densities about 15 nm in size appearing to bridge the two membranes at numerous points along the mitochondrial periphery. Using quick freeze, deep-etch electron microscopy, Senda and Yoshinaga-Hirabayashi (1998) showed in rat anterior pituitary and adrenal cortex cells that three kinds of intermembrane bridges exist within mitochondria that may play a role in the structural integrity of the two membrane systems. One type of bridge connects the outer and inner boundary membranes and appears similar to the bridges found in liver mitochondria and those reported here. It was proposed that the bridges across the outer and inner boundary membranes might keep these membranes apart and thus maintain the intermembrane space, or perhaps prevent its dilation in situ.

The existence of crista junctions among the types of mitochondria studied by us and others suggests that they have an integral role in efficient cristae functioning and that there may be some components that cause their formation and stabilize their structure. This led to the hypothesis that cristae begin by a budding of the inner boundary membrane at these membranous junctions and grow by the addition of proteins and phospholipids, perhaps at the junction site, causing tubular cristae to grow longer (Perkins et al., 1997a; 1998). Crista architecture varies from totally tubular, to small lamellar compartments formed where tubular cristae have merged, to large lamellar crista filling a mitochondrial cross section with many connections to the inner boundary membrane via crista junctions. Because of this observation, we proposed a model wherein larger cristae are formed when many crista, growing from crista junctions, contact and fuse within the mitochondrial matrix. Future work will test this model by first depleting the cristae membranes, as with the work reported here, and allowing growth of Tom19-deficient cells in the absence of FFA. This will restore activity of the nucleus containing the unaffected Tom19 gene, stimulating protein import and the

growth of inner membrane and cristae (Harkness et al., 1994). Thus, using electron tomography, we will be able to follow the growth of cristae from a starting point with very little cristae through to a normal control condition.

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