Structural and functional features and significance of the physical linkage between ER and mitochondria

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The role of mitochondria in cell metabolism and survival is controlled by calcium signals that are commonly transmitted at the close associations between mitochondria and endoplasmic reticulum (ER). However, the physical linkage of the ER–mitochondria interface and its relevance for cell function remains elusive. We show by electron tomography that ER and mitochondria are adjoined by tethers that are ~10 nm at the smooth ER and ~25 nm at the rough ER. Limited proteolysis separates ER from mitochondria, whereas expression of a short “synthetic linker” (<5 nm) leads to tightening of the associations. Although normal connections are necessary and sufficient for proper propagation of ER-derived calcium signals to the mitochondria, tightened connections, synthetic or naturally observed under apoptosis-inducing conditions, make mitochondria prone to Ca2+ overloading and ensuing permeability transition. These results reveal an unexpected dependence of cell function and survival on the maintenance of proper spacing between the ER and mitochondria.

Introduction

Mitochondria have been recently established as both physiological targets and relay points in intracellular Ca2+ signaling, contributing to a spectrum of cellular events ranging from oxidative ATP generation (Hajnoczky et al., 1995; Robb-Gaspers et al., 1998; Jouaville et al., 1999) to apoptotic cell death (Ferri and Kroemer, 2001; Demaurex and Distelhorst, 2003). This versatile function of mitochondria depends on the generation of mitochondrial matrix [Ca2+]m signals. The [Ca2+]m signal results from activation of the uniporter-mediated Ca2+ uptake that shows a relatively low Ca2+ affinity (Kirichok et al., 2004; Nicholls, 2005). The mitochondrial Ca2+ uptake of the IP3 receptor (IP3R)–mediated Ca2+ release is facilitated locally by the high cytoplasmic [Ca2+]c/([Ca2+]m) microdomains around the IP3Rs at focal contact areas between the ER and mitochondria (Rizzuto et al., 1998). Notably, mitochondria exhibit structural and functional diversity (Collins et al., 2002), and subsets of mitochondria may interact locally with other organelles (Hoth et al., 2000; Dolman et al., 2005). The local [Ca2+]c control between IP3Rs and mitochondria seems to occur at stable sites between the ER and mitochondria (Filippin et al., 2003) and displays a “quasisynaptic” organization (Csordas et al., 1999). Mitochondria-associated ER membranes are also involved in multiple mechanisms of joint operation between the two organelles, in the synthesis of the mitochondrial cytochrome c oxidase (Parimoo et al., 1982) and phospho- and glycosphingolipids (Voelker, 2005).

The existence of physical links between ER and mitochondria have been suggested based on cosedimentation of ER particles with mitochondria and electron microscopic observations of close associations between mitochondria and ER vesicles (Shore and Tata, 1977; Meier et al., 1981; Mannella et al., 1998). Recently, several mitochondrial or ER bound proteins have been shown to be important for maintaining the spatial relationship between ER and mitochondria and, hence, have also been implicated as possible linking elements: DLP-1/DRP1-1 (Pitts et al., 1999; Varadi et al., 2004), tumor autocrine motility factor receptor (Wang et al., 2000), and PACS-2 and BAP31 (Simmen et al., 2005). IP3Rs have also been postulated to interact with the Voltage-dependent anion-selective channel to form an ER–mitochondria Ca2+ tunnel (Rapizzi et al., 2002). Heterogeneity in the distance between the interfacing ER and outer mitochondrial membranes (OMMs; Pacher et al., 2000) also indicates that the contact formation may depend on several factors and raises the intriguing possibility that the ER–mitochondria
distance may be controlled to affect ER and mitochondria function. However, despite the attention paid to the structural basis of the ER–mitochondria communication, the fundamental question of whether direct physical linkage between ER and mitochondria is required for the local [Ca\(^{2+}\)] coupling remains to be elucidated. Here, we visualize the ER–mitochondria tethers and show that the local Ca\(^{2+}\) coupling can be weakened and strengthened by demolition and enforcement of the interorganellar protein linkage, respectively. Furthermore, our data reveal a novel regulatory role of the ER–mitochondria gap width in Ca\(^{2+}\) signaling and in cell survival.

**Results and discussion**

To directly visualize the structures responsible for the physical association of the ER with mitochondria, we used electron tomography (ET), which can reveal fine structural details missed in conventional micrographs because of overlapping densities (Mannella et al., 1994). Tomographic analysis of isolated rat-liver mitochondria (conventionally fixed, plastic-embedded or unfixed, frozen-hydrated) show narrow particles connecting the OMM to putative ER vesicles (Fig. 1 A and Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200604016/DC1). These “tethers” tend to occur in clusters of six or more, spaced 13–22 nm apart, spanning intermembrane distances of 6–15 nm with indications of increments occurring in 5-nm steps (Table S1). Electron micrographs and tomograms of plastic-embedded liver mitochondria (conventionally fixed, plastic-embedded or unfixed, frozen-hydrated) show narrow particles connecting the OMM to putative ER vesicles (Fig. 1 A and Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200604016/DC1). These “tethers” tend to occur in clusters of six or more, spaced 13–22 nm apart, spanning intermembrane distances of 6–15 nm with indications of increments occurring in 5-nm steps (Table S1).

Electron micrographs and tomograms of plastic-embedded liver (not depicted) and DT40 cells (Fig. 1) indicate numerous ER–mitochondria associations similar to wild-type cells (Fig. S1, B–E), suggesting that an IP3R-independent linkage exists between ER and mitochondria.

To disrupt the ER–mitochondria physical coupling, limited proteolysis was used. Confocal images of isolated liver mitochondria preparations showed abundant overlapping immunoreactivity for both IP3R (type 1 and 2) and cytochrome c oxidase, an enzyme of the inner mitochondrial membrane (Fig. 2 A), indicating that the IP3Rs reside in mitochondria-associated ER. When this preparation was trypsinized (40 \(\mu\)g/ml for 150 s followed by addition of soybean trypsin inhibitor [SBI] at 250 \(\mu\)g/ml) and recentrifuged, the IP3R immunoreactivity disappeared (Fig. 2 B) and was recovered in the light membranes (not depicted). The IP3-sensitive Ca\(^{2+}\) store was also quantified by measurement of the IP3 + thapsigargin (Tg)-induced Ca\(^{2+}\) release in the 10,000-g pellet (ER–mitochondria complex) and supernatant (ER only) of both the control and trypsin-pretreated

Figure 1. **Tethering structures between ER and mitochondria visualized by ET.** (A, top) Slice (2 nm thick) from tomogram of a frozen-hydrated rat-liver mitochondrion (700-nm diameter), showing several attached putative ER vesicles. Bar, 100 nm. (bottom) Higher magnification (2\(\times\)) slices through vesicles 1–3 showing tethers (arrowheads). Bar, 50 nm. (B) Micrograph of a DT40 TKO cell (200-nm-thick plastic section) showing ER flanking a mitochondrion. Bar, 250 nm. (C) Slice (3 nm thick) from tomogram of this field. (D) Surface model of ER (yellow) and OMM (red). (E) Subfields (130-nm diameter) from ER–mitochondria interface regions (1–3 are from boxed region in D) with tethers indicated by arrowheads (black arrowheads indicate tethers that terminate on ribosomes). (F) 3D models of three subregions in E, showing isodensity surfaces that best visualize the tethers (gray), membrane surfaces (OMM, red; ER, yellow), and ribosomes (blue ellipsoids). The resolution is ~8 nm in the z (vertical or section thickness) direction.
liver mitochondria (Fig. 2 C). Trypsin pretreatment caused a twofold increase in Ca\(^{2+}\) release in the supernatants and a significant decrease in the pellets (Fig. 2 C). Similar findings were obtained in RBL-2H3 cells (Csordas et al., 1999). Suspensions of digitonin-permeabilized cells were treated with proteinase K, another serine protease (20 μg/ml for 150 s), and [Ca\(^{2+}\)] \(_{\text{i}}\) and [Ca\(^{2+}\)] \(_{\text{m}}\) were simultaneously monitored fluorometrically. In control cells, 8 μM IP\(_{3}\) evoked an abrupt increase in [Ca\(^{2+}\)] \(_{\text{i}}\) (Fig. 2 D, left). In the proteinase K–pretreated cells, the IP\(_{3}\)-induced [Ca\(^{2+}\)] \(_{\text{i}}\) increase was preserved, but the [Ca\(^{2+}\)] \(_{\text{m}}\) increase was practically eliminated (Fig. 2 D and Fig. S2 C). Because proteinase K treatment did not affect IP\(_{3}\)-induced Ca\(^{2+}\) release (either in the presence or absence of mitochondrial uncouplers) and failed to inhibit the mitochondrial uptake of directly added Ca\(^{2+}\) (Fig. 2 D and Fig. S2 C), it is likely that proteinase K inhibited the transfer of released Ca\(^{2+}\) from IP3Rs to the mitochondria.

Similar data were obtained when trypsin was used instead of proteinase K (Fig. 2 E). Consistent with earlier reports, trypsin-digested preparations retained IP\(_{3}\)-induced Ca\(^{2+}\) uptake in permeabilized RBL-2H3 cells (Csordas et al., 1999). Suspensions of digitonin-permeabilized cells were treated with proteinase K, another serine protease (20 μg/ml for 150 s), and [Ca\(^{2+}\)] \(_{\text{i}}\) and [Ca\(^{2+}\)] \(_{\text{m}}\) were simultaneously monitored fluorometrically. In control cells, 8 μM IP\(_{3}\) evoked an abrupt increase in [Ca\(^{2+}\)] \(_{\text{i}}\) (Fig. 2 D, left). In the proteinase K–pretreated cells, the IP\(_{3}\)-induced [Ca\(^{2+}\)] \(_{\text{i}}\) increase was preserved, but the [Ca\(^{2+}\)] \(_{\text{m}}\) increase was practically eliminated (Fig. 2 D and Fig. S2 C). Because proteinase K treatment did not affect IP\(_{3}\)-induced Ca\(^{2+}\) release (either in the presence or absence of mitochondrial uncouplers) and failed to inhibit the mitochondrial uptake of directly added Ca\(^{2+}\) (Fig. 2 D and Fig. S2 C), it is likely that proteinase K inhibited the transfer of released Ca\(^{2+}\) from IP3Rs to the mitochondria.

We next evaluated the effect of proteolytic treatment on mitochondrial Ca\(^{2+}\) signaling. IP3R-mediated Ca\(^{2+}\) release effectively supports mitochondrial Ca\(^{2+}\) uptake in permeabilized RBL-2H3 cells (Csordas et al., 1999). Suspensions of digitonin-permeabilized cells were treated with proteinase K, another serine protease (20 μg/ml for 150 s), and [Ca\(^{2+}\)] \(_{\text{i}}\) and [Ca\(^{2+}\)] \(_{\text{m}}\) were simultaneously monitored fluorometrically. In control cells, 8 μM IP\(_{3}\) evoked an abrupt increase in [Ca\(^{2+}\)] \(_{\text{i}}\) (Fig. 2 D, left). In the proteinase K–pretreated cells, the IP\(_{3}\)-induced [Ca\(^{2+}\)] \(_{\text{i}}\) increase was preserved, but the [Ca\(^{2+}\)] \(_{\text{m}}\) increase was practically eliminated (Fig. 2 D and Fig. S2 C). Because proteinase K treatment did not affect IP\(_{3}\)-induced Ca\(^{2+}\) release (either in the presence or absence of mitochondrial uncouplers) and failed to inhibit the mitochondrial uptake of directly added Ca\(^{2+}\) (Fig. 2 D and Fig. S2 C), it is likely that proteinase K inhibited the transfer of released Ca\(^{2+}\) from IP3Rs to the mitochondria.

Similar data were obtained when trypsin was used instead of proteinase K (Fig. 2 E). Consistent with earlier reports, trypsin-digested preparations retained IP\(_{3}\)-induced Ca\(^{2+}\) release (Yoshikawa et al., 1999) but almost completely suppressed the mitochondrial Ca\(^{2+}\) uptake caused by addition of 10 μM CaCl\(_{2}\) (Fig. 2 E; initial rates were 0.96 ± 0.08 for control and 1.25 ± 0.17 μM/s for trypsin, respectively; n = 3). The trypsin dose dependence and time course data (Fig. S2 C) further illustrate that the Ca\(^{2+}\) transfer from IP3Rs to the mitochondrial matrix is very sensitive to trypsinolysis, whereas the Ca\(^{2+}\) release or mitochondrial Ca\(^{2+}\) uptake by itself is hardly inhibited. Thus, limited proteolysis disrupts the link between ER and mitochondria and suppresses the propagation of the IP3R-mediated Ca\(^{2+}\) release to the mitochondria.

To tighten the physical coupling between ER and mitochondria, we created a construct that encodes monomeric red fluorescent protein (mRFP) fused to the OMM targeting sequence of mAKAP1 at the N terminus and fused to the ER targeting sequence of yUBC6 at the C terminus (mAKAP1[34–63])mRFP-yUBC6, OMM–ER linker). Based on the size of the fluorescent protein (4.2 × 2.4 nm), the maximal length of this construct is <5 nm. As a control, the above construct was also prepared without the ER targeting sequence (mAKAP1[34–63])mRFP). Cells expressing the constructs showed red fluorescence localized to the mitochondria and displayed mitochondrial aggregation in some cells (unpublished data). To analyze the ER–mitochondria interface, the transfected cells were sorted and prepared for transmission EM (TEM). The mAKAP1(34–63)mRFP transfected cells displayed numerous associations between ER and mitochondria, but the interface area only involved a small fraction of the mitochondrial perim-eter (Fig. 3 A, left) similar to the situation in nontransfected cells (Fig. 4 B). The ER–OMM distance showed bimodal distribution, having the most frequent values at 10–15 and 25–30 nm, similar to the results of the ET analysis. In contrast, in the OMM–ER linker transfected cells, the ER formed a cap over large mitochondrial areas and the cleft between the
ER membrane and the OMM was extremely narrow (Fig. 3 A, right). On average, the ER–mitochondria distance at these sites decreased from 24 ± 3 to 6 ± 1 nm and the interface area increased fourfold in the presence of the OMM–ER linker (Fig. 3 B). Thus, expression of the OMM–ER linker caused the associations to become tighter and the interface area to increase.

To evaluate the effect of the enhanced physical coupling between the organelles on the Ca²⁺ transport, we conducted imaging of [Ca²⁺]ₘ, [Ca²⁺]ₘ, and [Ca²⁺]ₘ by ratiometric imaging with Fluo-4 and Pericam targeted to the mitochondrial matrix (Bird et al., 1999; Csordas and Hajnoczky, 2001). The nuclear matrix [Ca²⁺]ₘ signals were unaltered by the OMM–ER linker (as a control, either the mitochondria- or the ER-targeted part of the linker was overexpressed). In the cells expressing the OMM–ER linker, the [Ca²⁺]ₘ signal showed an initial elevation followed by a partial decay to a plateau. After a longer period of time, a gradual elevation appeared turning to a steep and robust [Ca²⁺]ₘ increase (Fig. 4 A, red). This second [Ca²⁺]ₘ rise began at different time points in the individual cells, causing a more gradual rise in the mean response (Fig. 4 A, bottom). In control cells, the [Ca²⁺]ₘ elevation was similar to those in the OMM–ER linker cells; however, the second [Ca²⁺]ₘ rise developed much more slowly (Fig. 4 A, black). Analysis of [Ca²⁺]ₘ signals in single cells showed an early onset of both the gradual [Ca²⁺]ₘ elevation and the steep and robust [Ca²⁺]ₘ increase (Fig. 4 A, right) in the cells expressing the OMM–ER linker. The second [Ca²⁺]ₘ elevation was prevented by the addition of either 5 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone and 2.5 μM EGTA or by 5 μM cyclosporin A, a drug interfering with the Ca²⁺-dependent activation of the permeability transition pore, suggesting that it depended on mitochondrial Ca²⁺ uptake and was a result of Ca²⁺ release from Ca²⁺-overloaded mitochondria (unpublished data). Hence, mitochondria were susceptible to Ca²⁺ overloading and permeabilization in cells where the ER–mitochondria coupling was tightened by the OMM–ER linker.

The mitochondrial Ca²⁺ dysregulation was regularly followed by detachment of the cells, indicating the loss of viability. Because Ca²⁺ transfer to the mitochondria is a key step in induction of many forms of cell death, we reasoned that tightening of the ER–mitochondria coupling may contribute to the execution of apoptosis.
of the cells induced by certain proapoptotic stimuli. To this end, RBL-2H3 cells were exposed to apoptotic conditions (serum starvation and tunicamycin treatment) and were fixed for EM at 24 h, before the onset of cell detachment. Analysis of the dimensions of the ER–mitochondria interface showed shortening of the mean distance between ER and mitochondria in both the serum-starved and tunicamycin-treated cells and an increase in the frequency of tight associations (<6 nm distance; Fig. 4 B).

The high incidence of the tight associations could not be attributed to the condensation of the apoptotic cells because the perimeter or area of the cell cross sections has not been altered yet. These results suggest that narrowing of the ER–mitochondria gap occurs in intact cells and may be an important step in the execution of some apoptotic mechanisms.

The scheme in Fig. 4 C illustrates the novel aspects of the ER–mitochondria signaling uncovered in the present work. The association between ER and mitochondria is due to the presence of tethers that link both smooth and rough ER to the mitochondria. The length of the tethers displays some diversity, giving rise to distances between ER and mitochondria.

In response to apoptotic agents the ER–mitochondria gap narrows, indicating dynamic regulation of the interorganellar junction. In healthy cells, the ER–mitochondria tethering ensures the propagation of IP3R-linked Ca2+ signals to the mitochondria to coordinate ATP production with the stimulated state of the cell and to enable the mitochondrial Ca2+ buffering. However, the gap between the organelles is sufficiently wide to isolate mitochondria from the slow Ca2+ leakage from the ER. Relaxing the ER–mitochondria coupling suppresses the Ca2+ signal propagation to the mitochondria, putting at risk the Ca2+-dependent control of mitochondrial metabolism. In contrast, tightening of the coupling invokes mitochondria in the handling of Ca2+.
under resting conditions, sensitizing mitochondria to Ca\(^{2+}\) overloading and leading to permeabilization and committing the cells to a cell death pathway. Tightening of the connections seems to be relevant for several mechanisms of cell death. Thus, these results reveal an unexpected dependence of cell function and survival on the maintenance of a proper spacing between the ER and mitochondria.

**Materials and methods**

**DNA constructs**

To construct the OMM-ER linker, mRFP was targeted to the ER by using the C-terminal ER localization sequence of the yeast UBC6 protein (K73234, residues 233–250: MYVIGIAIIFVUGEMK), through the linker (SGLEBRQACSNSRV; Varnai et al., 2005). This construct was complemented with the N-terminal mitochondrial localization sequence of the mouse AKAP1 protein (V84389, residues 34–63: MAIQLRSLFLAPGLALLGWFWKSK). The ratio of pericam targeted to the mitochondrial or nuclear matrix was provided by A. Miyawaki (Institute of Physical and Chemical Research, Wako-city, Japan).

**Cells**

RBL-2H3 cells were cultured as described previously (Csordas et al., 1999). Cells were transfected with cDNA by means of electroporation in suspensions (4.5 × 10⁶ cells + 20 μg of each cDNA in 250 μl medium). Electroporation was performed in a BTX-830 square-pulse generator in a 4-mm gap cuvette using a single 230 V, 13-ms pulse. For FACS sorting and to FACS sorter (DaKocyTomation) equipped with a 488-nm laser, 8.5–12.5 × 10⁹ cells transfected with a construct of interest and EGFP were cultured for 24 h.

DT40 (wild type and iP3R knockouts alike were a gift from T. Kuroaki, Kansai Medical University, Hirakuchi, Japan) cells were cultured in suspension, in RPMI 1640 with glutamine supplemented with penicillin-streptomycin, 2 mM g-glutamine, 10% heat inactivated FCS, and 1% chicken serum (Invitrogen) in 5% CO₂ and 95% air at 37°C.

**Fluorometric measurements of \(\left[\text{Ca}^{2+}\right]_\text{c}\) and \(\left[\text{Ca}^{2+}\right]_\text{m}\) in suspensions of permeabilized RBL-2H3 cells**

Experiments were performed as described earlier (Csordas and Hajnoczky, 2001).

**Fractionation of RBL-2H3 cells**

Cells grown overconfluent in tissue culture flasks (7–10 × 10⁶ cells) were loaded with fura2FF/AM, harvested using trypsin/versene, and washed with NaHepes/EGTA. All further steps were performed at 4°C. The cells were exposed to hypotonicity for 10 min (1.4 ml intracellular medium [ICM]; 120 mM KCl, 10 mM NaCl, 1 mM K2HPO4, 20 mM Tris-Hepes, 2 mM MgATP, and 1 μM each of antipain, leupeptin, and pepstatin; pH 7.2) diluted fivefold with dH2O and supplemented with 200 μM EGTA and 5 mM MgCl2. Subsequently, the cells were homogenized in a dounce glass/glass homogenizer (30–35 strokes, tight pestle). To restore osmolarity, 3 vol of 100% ICM supplemented with 125 mM sucrose, 200 mM EGTA, and 5 mM MgCl2 was added. To eliminate unbroken cells and nuclei, the homogenate was centrifuged at 1,000 g for 10 min. The supernatant was further centrifuged at 10,000 g for 15 min, and the pellet (mitochondrial fraction) was resuspended in 400–500 μl ICM plus protease inhibitors and 10 μM EGTA and stored on ice. Attachment to CellTak (BD Biosciences) coated coverslips was performed at room temperature for 5 min in the presence of 2 mM Mg²⁺ ATP in 25–50 μl vol.

**Fractionation of rat liver**

The protocol was adapted from Meier et al. (1981). The liver of a 150–300-g male Sprague-Dawley rat was perfused with ~200 ml NaHepes/EGTA and was removed. All the further steps were done at 4°C. The liver was cut up to small pieces with scissors and washed with ICM. After determination of the wet weight, a 1:4 homogenate was prepared in 350 mM sucrose containing 2.5 mM magnesium acetate and 10 mM Tris maleate, pH 7.4. Homogenization was performed in a 60 ml glass-Teflon homogenizer (11 strokes at 900 rev/min). The homogenate was filtered through two layers of sterile gauze and once more through one layer of Miracloth (Calbiochem). The mitochondrial fraction was obtained by centrifuging the supernatant of the 900-g (10 min) fraction at 8,000 g for 15 min.

**Particles**

Particles were attached to CellTak-coated coverslips as described above in the previous paragraph.

**Immunostaining of the mitochondrial fractions**

The membrane fractions attached to coverslips were fixed in 3% paraformaldehyde. A monoclonal anti-human cytochrome c oxidase complex IV subunit 1 antibody (Invitrogen) was used to visualize mitochondria and polyclonal anti-IP3R1- and 2 antibodies (Affinity BioReagents, Inc.) were used to visualize the IP3Rs. The secondary antibodies were fluorescently labeled (Alexa Fluor 488 and 568). Images were acquired using a confocal system (Radiance 2001; Bio-Rad Laboratories), and colocalization was evaluated using Lasersharp software (Bio-Rad Laboratories; Csordas and Hajnoczky, 2001).

**Fluorometric monitoring of extravascular \(\left[\text{Ca}^{2+}\right]_\text{c}\) in suspension of rat-liver mitochondrial fraction**

A 20–25-μl aliquot of the crude mitochondrial fraction was transferred to 800 μl ICM supplemented with 1.5 μM fura2/FA, 2 mM Mg-ATP, 2 mM succinate, and protease inhibitors in a stirred cuvette at 35°C. Ratiometric recording of fura2 fluorescence was performed as described for the permeabilized cells.

**Fluorescence imaging of \(\left[\text{Ca}^{2+}\right]_\text{c}\) in single cells and in adherent subcellular particles**

RBL-2H3 cells or mitochondrial fractions attached to coverslips were placed in 1 ml buffer to the heated stage (35°C) of a microscope (IX70 [Olympus]; 40×; UApo360) connected to a cooled charge-coupled device camera (PXL; Photometrics). Ratiometric imaging of fura2FF and pericam was used to monitor \(\left[\text{Ca}^{2+}\right]_\text{c}\) and \(\left[\text{Ca}^{2+}\right]_\text{m}\) as described previously (Csordas and Hajnoczky, 2001; Yi et al., 2004).

**TEM and ET**

For embedding, a standard protocol was used (Pacher et al., 2000). Ultrathin sections for TEM were poststained with UA and sodium bismuth (Pacher et al., 2000). The sections were examined with either a scanning transmission electron microscope (model 7000; Hitachi) or a digital transmission electron microscope (lecnai 12; Philips) driven by Gatan software.

For ET of DT40 cells, 150–300-nm-thick sections were cut from epoxy blocks, and 15-nm colloidal gold particles were applied to one side as alignment markers. Tilt series were collected on an AEl EM7 high-voltage electron microscope operated at an accelerating voltage of 1,000 kV. Images were serially recorded around two orthogonal tilt axes, over angular ranges of ±60° at 2° intervals.

**Cryo-EM of isolated mitochondria**

To cryo-EM of isolated mitochondria, 3–5-μl aliquots of mitochondrial suspensions (10–20 mg/ml in 0.225 M manitol and 0.075 M sucrose) containing 10-nm colloidal gold particles were deposited on freshly glow-discharged 300-mesh copper grids with holey carbon films. Grids were blotted with filter paper and immediately plunged into liquid ethane cooled by liquid nitrogen. Tilt series were collected over an angular range of ±60° at 2° intervals (total dose ~24 electrons/A²) using a transmission electron microscope (JEM-600FX; JEOL) equipped with Gatan cryo-transfer unit and a TVIPS 1024 × 1024 charge-coupled device camera.

Images were aligned and tomographic reconstructions calculated as previously described (Penczek et al., 1995), using the weighted back-projection method as implemented in the SPIDER image processing system (Frank et al., 1996). 3D models were generated by density thresholding using Iris Explorer (Numerical Algorithms Group) or surface rendering in Iris Explorer after manual membrane tracing in Sterecon (Marko and Leith, 1996). In the case of plastic sections, the z dimension (section thickness) of the final models was increased by 20% to compensate for radiation-induced thinning of the plastic section. Lengths of tethers connecting mitochondrial outer membranes and ER membranes were determined using NIH Image.

**Online supplemental material**

Table S1 shows the dimensions of the ER–mitochondria interaction areas. Fig. S1 shows tight ER–mitochondria associations in quick frozen and chemically fixed isolated liver mitochondria and in wild-type and iP3R1KO DT40 cells. Fig. S2 demonstrates protein linkage between ER and mitochondria in RBL-2H3 cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200604016/DC1.

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