Activated Mitofusin 2 Signals Mitochondrial Fusion, Interferes with Bax Activation, and Reduces Susceptibility to Radical Induced Depolarization*S

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Mitochondrial fusion in higher eukaryotes requires at least two essential GTPases, Mitofusin 1 and Mitofusin 2 (Mfn2). We have created an activated mutant of Mfn2, which shows increased rates of nucleotide exchange and decreased rates of hydrolysis relative to wild type Mfn2. Mitochondrial fusion is stimulated dramatically within heterokaryons expressing this mutant, demonstrating that hydrolysis is not requisite for the fusion event, and supporting a role for Mfn2 as a signaling GTPase. Although steady-state mitochondrial fusion required the conserved intermembrane space tryptophan residue, this requirement was overcome within the context of the hydrolysis-deficient mutant. Furthermore, the punctate localization of Mfn2 is lost in the dominant active mutants, indicating that these sites are functionally controlled by changes in the nucleotide state of Mfn2. Upon staurosporine-stimulated cell death, activated Bax is recruited to the Mfn2-containing puncta; however, Bax activation and cytochrome c release are inhibited in the presence of the dominant active mutants of Mfn2. The dominant active form of Mfn2 also protected the mitochondria against free radical-induced permeability transition. In contrast to staurosporine-induced outer membrane permeability transition, pore opening induced through the introduction of free radicals was dependent upon the conserved intermembrane space loop, which contains a highly conserved tryptophan residue. In yeast, this region of the protein is required for mitochondrial fusion and has been shown to anchor Fzo1p to sites of membrane contact between the inner and outer membrane (8). Although Mfn1 and Mfn2 are 60% identical, recent evidence with both in vitro mitochondrial docking assays and in rescue experiments of Mfn1 knock-out cells has shown that Mfn1 appears to play a more direct role in mitochondrial docking (7, 9) and that it functions in cooperation with the intermembrane space dynamin like GTPase Opa1 (autosomal dominant optical atrophy 1) (10). The role of Mfn2 in mitochondrial fusion has remained elusive, although it is clearly required for fusion and can be found in heterodimeric complexes with Mfn1. Recent studies have shown that the nucleotide binding and hydrolysis properties of the two Mfn proteins are distinct (9), consistent with the idea that the two GTPases regulate different steps along the fusion pathway (5). These steps may include the processes that drive mitochondrial motility, tethering, assembly of a fusion pore to facilitate lipid bilayer mixing and eventually leading to inner mitochondrial membrane fusion. Given the complexity of these molecular requirements, the Mfn proteins do not act alone, and studies in yeast have identified a number of additional proteins required for mitochondrial fusion, including the outer membrane protein, Ugo1p (11–13), the inner membrane serine protease Rbd1p (14–16), an F-box-

* This work was supported in part by the Canadian Institutes of Health Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ The on-line version of this article (available at http://www.jbc.org) contains a supplemental figure and video.

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1 The abbreviations used are: Mfn1 and Mfn2, Mitofusin 1 and Mitofusin 2, respectively; CFP, cyan fluorescent protein; Δψ, mitochondrial electrochemical potential; DRP1, dynamin-related protein 1; ECFP, enhanced cyan fluorescent protein; fnk, fluoromethyl ketone; FP, fluorescent protein; Fzo1p, Fuzzy Onion 1 protein; GFP, green fluorescent protein; GST, glutathione S-transferase; His6, hexahistidine; HR, heptad repeat; IMS, intermembrane space; Mant GMP-PNP, N-methylthraniloyl guanosine 5′-[(β,γ-imino)triphosphate]; PBS, phosphate-buffered saline; PEG, polyethylene glycol; STS, staurosporine; YFP, yellow fluorescent protein; Z, benzylxoxycarbonyl.
containing protein Mdm30p (17), along with other candidates like Mdm35p, Mdm34p, and Mdm39p (18).

One of the outstanding questions in the field of mitochondrial dynamics remains the physiological importance of mitochondrial fission and fusion under steady-state conditions. Knock-outs of either Mfn1 or Mfn2 are embryonic lethal (6), demonstrating an essential role of mitochondrial fusion for viability. In addition, mutations within the Mfn2 gene have been found in patients suffering from Charcot-Marie-Tooth neuropathy type 2A, and six of seven of these mutations were found within the conserved GTPase region (19, 20). Interestingly, evidence that Mfn2 may exhibit intracellular signaling activity has come from one study that identified the rat Mfn2 (called hyperplasia suppressor gene HSG) as an important antiproliferative protein, which interferes with the Ras pathway and blocks signaling from growth factor receptors at the plasma membrane (21). Although the mechanism for this inhibition is unknown, these findings suggest that Mfn2 and/or the morphological state of the mitochondria is highly integrated into cellular signaling cascades.

Another example of how the mitochondrial morphology is integrated into cellular signaling events is the growing evidence for a role of mitochondrial dynamics in the progression of apoptosis. For example, two of the proteins required for mitochondrial fission, Fis1p and DRP1, are also essential for programmed cell death (22–25). Fis1p knock-down by small interfering RNA blocks recruitment and activation of Bax at the surface of mitochondria after a death stimulus, indicating an essential role for this small integral membrane protein in apoptosis (25). Similarly, although loss of DRP1 does not dramatically interfere with Bax activation, cytochrome c release is partially inhibited, and mitochondrial fission is blocked in these cells (25, 26). In addition, small interfering RNA knock-down of Opa1, a protein required for mitochondrial fusion, results in fragmented mitochondria, which are highly sensitized to the loss of electrochemical potential and cytochrome c release (25, 27). In contrast, overexpression of the two mitochondrial proteins together provides some protection against different apoptotic stimuli (28). These recent data highlight the dual nature of mitochondrial GTPases in the regulation of both mitochondrial dynamics and the mitochondrial contribution to programmed cell death.

Given the increasing evidence that Mfn1 plays a direct role in mitochondrial tethering (5, 9), we have specifically investigated the function of Mfn2 in the process of mitochondrial tethering (5, 9), we have specifically investigated cell death.

EXPERIMENTAL PROCEDURES

Construct Preparation and Reagents—The cDNA encoding human Mfn2 (KIAA0214) was graciously provided by Kazusa DNA Research Institute, Japan. Mfn2 cDNA was PCR amplified using standard protocols, for insertion into pECFP-C1 (Clontech) and pCDNA3.1 (Invitrogen) with BamHI and HindIII restriction sites. Mfn2RasG12V-CFP was prepared with QuikChange mutagenesis (Stratagene, La Jolla, CA) using pECFP-C1-Mfn2 as the template and a set of oligonucleotides designed to replace amino acids GTSNGKS with GAVGYGKS. The restriction site NarI was introduced into the primers for screening purposes. The pCDNA3-GST, Mfn2His6, and Mfn2RasG12V-His6 construct vectors for protein purification from transfected cell lysates were also prepared using subcloning techniques, and all sequences used in this work were confirmed. Mfn2ex11-CFP was prepared with QuikChange mutagenesis using pECFP-C1 Mfn2 as the template and a set of oligonucleotides designed to replace the tryptophan amino acid at position 631 with a proline. The Apal restriction site was introduced into the primers for screening purposes. Mfn2w631p-CFP was prepared by isolating the DNA fragment encoding amino acids 1–431 of Mfn2RasG12V-CFP by digestion with HindIII and SalI and subcloning this fragment containing the RasG12V mutation into the Mfn2ex11-CFP construct cut with the same enzymes, thereby replacing the wild type GTPase domain of Mfn2ex11-CFP with the Mfn2RasG12V mutation. The cDNA encoding DsRed2 was amplified from pDsRed2-C1 (Clontech) using primers designed for digestion with BamHI and XbaI and ligation into the pCDS3-pCCT vector (29). Tom7-CFP was prepared from Mike Ryan, La Trobe University, Melbourne Australia. Mfn2 mouse polyclonal antiserum was generated against a mixture containing both recombantly expressed Mfn2C179T7C-GST and a synthetic Mfn2NH2-terminal peptide CNSIVTVKKKRM1-0H (Dalton Chemical Laboratories, Toronto, Canada), conjugated to 5 mg of maleimide-activated keyhole limpet hemocyanin. Antiserum was generated following a 56-day standard immunization protocol. Polyclonal antibodies against fluorescent proteins (anti-FP) used for immunoelectron microscopy were purchased from Clontech. Dihydroethidium (Molecular Probes, Eugene, OR) was utilized to determine steady-state levels in transfected cells. Monoclonal 7H8.2C12 anti-cytochrome c antibodies were obtained from BD Biosciences, and rabbit polyclonal anti-Bax antibodies were obtained from Chemicon International. Cell signaling solutions. Alexa Fluor 350 and 594 goat anti-mouse or rabbit secondary antibodies from Molecular Probes were used for staurosporine (STS) experiments. Z-VAD was obtained from Enzyme Systems Products (Aurora, Canada), and STS was obtained from Sigma.

Transfection and Imaging of COS-7 Cells—Transfection and imaging methods were exactly as described in Ref. 29. For electrochemical potential determination, cells were incubated with 50 nM MitoFluor Red 589 (Molecular Probes) at 37 °C for 20 min. Whole cell images were acquired for untransfected and transfected cells by exciting at 589 nm with the CFP/YFP/DeRed triple pass filter (Chroma, Brattleboro, VT). The presence of transfected CFP-tagged protein was confirmed by exciting at 434 nm using the same filter set. Areas of interest were selected for each cell, and total fluorescence arbitrary units were calculated for each cell. The total fluorescence intensity/cell was quantified as the sum of the values of each pixel within the area of interest minus the average background signal obtained/pixel. The number of cells within each fluorescence distribution range was scored and tabulated as a percentage.

For MitoFluor Red 589 (Molecular Probes) flickering experiments, 50 μM dye was added to the chamber medium and incubated with the cells for 1 hr. After equilibration of the dye, images were collected by exciting at 589 nm with the CFP/YFP/DeRed triple pass filter (Chroma) for 1 s followed by a 2.5-s delay. The presence of transfected CFP-tagged protein was confirmed as indicated above. The time series were analyzed, and each frame where all mitochondrial dye was released from the whole cell was plotted.

To determine steady-state radical loads, cells were incubated with 5 μM dihydroethidium at 37 °C for 20 min. Dihydroethidium oxidation by superoxide to ethidium was visualized by excitation at 547 nm. Whole cell imaging and fluorescence quantification for untransfected and transfected cells were performed as indicated above.

Mant GMP-PNP Binding Assay—COS-7 cells were transfected with pOCT-CFP, Mfn2-CFP, Mfn2RasG12V-CFP, and Rab5-CFP fusion constructs 48 h after transfection. The cells were detergent treatment, washed with PBS and with nucleotide binding buffer (220 mM mannitol, 68 mM sucrose, 200 mM NaCl, 2 mM MgCl2, 0.5 mM EGTA, 2.5 mM KHPO4, 10 mM Hepes, pH 7.4, 1 mg/ml bovine serum albumin) containing protease inhibitors. Cells were then broken in a cell cracker, and the whole lysate was centrifuged at 10,000 rpm to concentrate heavy membrane fractions. Pellets were resuspended in binding buffer, and 50-μl aliquots were mixed with Mant GMP-PNP (1 μM final concentration, Molecular Probes) and incubated at 37 °C for different times. After incubation the aliquots were scanned for emission fluorescence of Mant nucleotides in a QuantaMaster 6000SE (Photonic Technology International, London, Canada) (excitation at 360 nm). The peak emission at 448 nm was recorded, and the background fluorescence of the nucleotide alone was subtracted. Each value was also normalized for total protein concentration in the sample (determined using the DC protein assay (Bio-Rad)) and for the level of recombinant protein expression by measuring the CFP signal obtained in the fluorometer upon excitation at 434 nm, emission at 477 nm.

For the purification of GST, Mfn2-GST, and Mfn2RasG12V-GST from transfected cell lysates, 10-cm dishes of COS-7 cells were transfected using Lipofectamine 2000 (Invitrogen), and after 12 h they were treated with trypsin, washed, and lysed with TNE buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM, EDTA, 1 mM dithiothreitol, 60 μg/ml chymotrypsin, 1 μM leupeptin, 25 μg/ml antipain, 2 μg/ml aprotinin, 40 μg/ml 4-aminophenylmethane-sulphonyl fluoride, 1 mM pepstatin A), containing 1% Triton X-100 for 2 h at 4 °C. Lysates were adjusted to 40% sucrose and centrifuged at 70,000 rpm for 1 h at 4 °C. Supernatants were diluted to 10% sucrose with TNE + Triton X-100 buffer and were
incubated overnight with glutathione-Sepharose beads at 4 °C. Beads were washed, and GST fusion proteins were eluted with 50 mM reduced glutathione in TNE buffer + 10% sucrose. Eluted aliquots (50-μl elution in duplicate with 0.2 μM GST-MPF-PNP for 15 min, at 37 °C) were assayed for fluorescence (excitation, 360 nm; emission, 448 nm). Normalization was done by assaying the enzymatic activity of GST within each aliquot, with 1-chloro-4-dinitrobenzene (Sigma) and glutathione as substrates, with formation of a product with absorbance at 340 nm (Amersham Biosciences), being the product formation rate proportional to the amount of GST in the aliquots. Known concentrations of bacterially purified GST were used as a standard in this assay. Bacterially expressed Rab5-GST was purified as described previously (30).

**GTP Hydrolysis Assay—** COS-7 cells were transfected with Mfn2-His, Mfn2RasG12V-His, or LacZ-His, and the following day cells were washed with PBS, harvested by scraping, and centrifuged. Cell pellets were resuspended in a small volume of TXN solubilization buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM β-mercaptoethanol, 2 mM MgCl2, protease inhibitor mixture) and incubated for 1 h at 4 °C. Lysates were then cleared at 70,000 g for 30 min at 4 °C, and supernatants were incubated with nickel-nitriilotriacetic acid-agarose beads for 1.5 h at 4 °C. Beads were then centrifuged and washed twice with TXN buffer containing 200 μM ATP (to remove chaperones and other nonspecific proteins of the lysate) followed by two more washes with TXN buffer containing 20 mM imidazole. Proteins were eluted at 100 and 200 mM imidazole in TXN buffer. Protein concentrations were determined by the Bio-Rad method. 10 μg of the different eluted proteins were preloaded with 1 μl of [γ-32P]GTP (2,000 Ci/mmol) in exchange buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 1% Triton X-100, 2 mM MgCl2, 1 mM dithiothreitol) by incubating for 30 s at 37 °C in a 140-μl volume. The reaction was then diluted 10 times to trap the nucleotide in place and begin γ-32P/GTP hydrolysis. From this mix, aliquots were taken, in triplicate, at different time points (0, 1, 2, 5, and 10 min), applied to nitrocellulose filters in a vacuum manifold, and flushed immediately with 1 ml of PBS (to remove unbound nucleotide and hydrolyzed 32P). Filters were then placed in vials and assayed for 32P scintillation counting.

**Electron Microscopy—** COS-7 cells were transfected with the appropriate cDNA in 10-cm dishes with L-glutamine 2000 for 16 h. Following this, fluorescence was first examined using the light microscope to ensure that transfection was at least 70% prior to trypsin treatment and washing of the cells in PBS. The washed cells were then pelleted and fixed in 1.6% glutaraldehyde in 0.1 M sodium cacodylate buffer prior to osmium tetroxide and uranyl acetate staining, spurr resin embedding, and final thin sectioning of the blocks, and the grids were stained en bloc. For immunochemical electron microscopy, the subfractionation procedure was followed. Cells were treated with trypsin, washed in PBS, fixed in 1.6% glutaraldehyde, and centrifuged at 3,000 g for 15 min. Cell pellets were embedded in LR white (Marivac, PQ, Canada), thin sections were cut with a Leica Ultracut E ultramicrotome, immunolabeled with polyclonal anti-FP antibodies and 10-nm gold-labeled secondary antibodies (Jackson Laboratories, Bar Harbor, ME) and counterstained with nickel-nitrotriacetic acid-agarose beads. Digital images were taken using a JEOL 1230 TEM at 60 kV equipped with two filters HQ520/20 and D600/40 to separate the GFP and cytoplasm emission signals, and images were captured as described above. The transfected CFP-tagged protein was imaged using the CFP/YFP dual pass filter (TillPhotronics) along with the beam splitter equipped with D465/30 and HQ535/30 filters (data not shown). The acquired images were saved as .tiff images and overlaid in Adobe Photoshop for image assembly.

**PEG Fusion Assay—** The whole cell fusion assay was performed as described previously (4). Given that the broad excitation spectra of DsRed overlaps with the GFP excitation wavelength, special precautions were taken to ensure that the images of fused mitochondria did not include any bleed-through between the channels. For this, live cells were imaged on the Olympus IX70 microscope with a 100 × objective U Plan Apochromat, NA 1.35–0.50 objective, exited at 488 nm (GFP) and 560 nm (DsRed) with the PolyChrome IV monochromator (TillPhotronics, Graefening, Germany) through a fluorescein isothiocyanate/Cy3/Cy5 triple pass filter (Chroma). The emitted light was filtered through an additional dual view beam splitter (Optical Insights, Santa Fe, NM) equipped with two filters HQ520/20 and D600/40 to separate the GFP and DsRed emission signals, and images were captured as described above. The transfected CFP-tagged protein was imaged using the CFP/YFP dual pass filter (TillPhotronics) along with the beam splitter equipped with D465/30 and HQ535/30 filters (data not shown). The acquired images were saved as .tiff images and overlaid in Adobe Photoshop for image assembly.

**RESULTS**

Creation of a Hydrolysis-deficient Mutant of Mfn2—To determine whether nucleotide hydrolysis of Mfn2 was essential for mitochondrial fusion, we constructed a RasG12V mutant. Our purpose in designing the mutant was to maintain nucleotide binding and exchange properties of Mfn2 and yet reduce the intrinsic rate of nucleotide hydrolysis. Therefore we replaced the residues within the P-loop with those from the activated RasG12V (31–33) by replacing amino acids GAVGAV with GAVGVGK (the consensus for GTP binding is GxxxGKS (34)). We first compared the nucleotide exchange properties of this mutation (Mfn2RasG12V) with wild type Mfn2 and controls by using crude extracts from cells transfected with CFP fusion proteins. COS-7 cells were transfected with matrix-targeted GST fusion proteins (negative control), wild type Mfn2-CFP, Mfn2RasG12V-CFP, or Rab5-CFP as a positive control. Rab5 is the best characterized of the Rab GTPases and is known to regulate early endosome fusion (35). Regulatory GTPases of the Ras family are all characterized by their low intrinsic rates of hydrolysis and high nucleotide affinities, which results in stable nucleotide states that require accessory proteins for their activity. Given the evidence that Mfn2 exhibits low rates of nucleotide hydrolysis and high affinity (9), it is relevant to use Rab5 as a positive control in these assays. After transfection with CFP-tagged constructs, the cells were harvested, broken, and the mitochondria-enriched heavy membrane fractions were incubated with the environment-sensitive nonhydrolyzable GTP analog Mant GMP-PNP (36, 37). Increased Mant nucleotide fluorescence is a direct measurement of nucleotide exchange. As expected, untransfected cells, or cells transfected with the matrix CFP control plasmid, bound a constant level of Mant GMP-PNP because of the endogenous GTPases present in the extracts. Interestingly, overexpression of Mfn2-CFP did not significantly alter the basal amount of nucleotide binding in the heavy membrane fraction (Fig. 1A). Overexpression of Rab5 provided a 4-fold increase in Mant GMP-PNP binding within the first 10 min after incubation at 37 °C (Fig. 1A). This signal is specific because addition of unlabelled GTP competes for the binding. Notably, although transfusion of Mfn2-CFP had no effect on basal nucleotide binding in this assay, transfection of Mfn2RasG12V-CFP demonstrated a significant increase in nucleotide binding (Fig. 1A). We next isolated Mfn2RasG12V-GST or Mfn2-GST from transfected mammalian cells using glutathione-Sepharose beads. We employed a quantitative GST enzyme assay with 1-chloro-2,4-dinitrobenzene as substrate and determined the total amount of GST fusion proteins purified by normalizing/mol of purified protein (Fig. 1B). GST-tagged protein was purified from transfected cell lysates, and, as in total cell lysates, the nucleotide binding experiments demonstrate a 2-fold increase in activity of Mfn2RasG12V-GST relative to the wild type protein (Fig. 1B). In addition, the stimulated exchange activity of the Mfn2RasG12V mutant reached levels similar to those obtained with molar equivalents of recombinant Rab5-GST (Fig. 1B). Although we could detect no significant differences in nucleotide binding to Mfn2-CFP within whole cell extracts (Fig. 1A), the GST-purified Mfn2 did demonstrate nucleotide binding/exchange relative to the GST control (Fig. 1B). These biochemical data indicate that purified Mfn2 has extremely low rates of nucleotide exchange, consistent with high affinity binding. These data are consistent with published data that have also shown much stronger binding of Mfn2 to nucleotide relative to Mfn1 (9). Our data now show that mutations within the P-loop of Mfn2 increase nucleotide exchange relative to the wild type protein, thereby bringing the rates closer to the intrinsic rates observed for the Rab GTPases.

We next examined the intrinsic rates of [γ-32P]GTP hydrolysis using the established filter-based assay quantifying the release of the labeled tertiary phosphate from the GTP-bound protein purified from transfected COS-7 cells (Fig. 1D) (38).
Mfn2 Is Localized in Specific Subdomains along Mitochondrial Tubules in a Nucleotide-dependent Manner—We examined the cellular consequences of the GTPase mutant by transfecting COS-7 cells with wild type Mfn2-CFP, GTPase mutant Mfn2RasG12V-CFP, or a truncation mutant completely lacking the GTPase domain Mfn2(430-757)CFP. Transfection of COS-7 cells with the wild type Mfn2 resulted in increased interconnectivity among the mitochondrial reticulum (Fig. 2), which upon high levels of expression appears as a cluster (1, 2, 4). As reported previously (40), Mfn2-CFP was localized in puncta along the mitochondrial tubules. Transfection of the GTPase mutant Mfn2RasG12V-CFP resulted in the clustering of mitochondria, which appeared to be fused even at the lowest levels of detectable expression (Fig. 2). Importantly, unlike Mfn2-CFP, Mfn2RasG12V-CFP was distributed evenly along the surface of these clusters (Fig. 2). In contrast, transfection of the Mfn2(430-757)CFP mutant resulted in mitochondria visibly fragmented into small, spherical units that cluster together in a stable manner (Fig. 2 and data not shown), similar to those found in the Mfn1 GTPase truncation mutant (7). These phenotypes are specific to Mfn2 because transfection of another mitochondrial outer membrane protein, Tom7 (24), did not cause significant mitochondrial clustering (Fig. 2). In addition, these phenotypes were not affected by the presence of the CFP tag because transfection of untagged constructs gave similar results (data not shown). We considered that the assembly of the Mfn2 puncta may depend upon the conserved IMS residues. Mammalian Mfn2 contains only 2–3 amino acids separating the two transmembrane domains, one of which is a highly conserved tryptophan residue that we replaced with a proline.
residue (Mfn2W631P). This proline residue is naturally occurring within the IMS loop in Drosophila melanogaster Fzo, indicating that this substitution should not alter the topology of Mfn2. Mfn2W631P-CFP remains sharply localized in distinct foci; however, the tubular morphology of the mitochondria is reduced, and the organelles eventually fragment (Fig. 1). Creation of a double mutant where both the Mfn2RasG12V and Mfn2W631P mutations are present (Mfn2RVWP-CFP) also resulted in a loss of Mfn2 puncta, indicating that the punctate localization does not depend upon the conserved IMS residues, rather it is determined by the nucleotide state (Fig. 2). Video analysis showed that the Mfn2-CFP puncta remained highly immobile and were not observed at sites of mitochondrial fusion, nor did they significantly colocalize with the cytoskeleton (actin filaments or microtubules), and they did not accumulate at sites of endoplasmic reticulum/mitochondrial contact (data not shown).

To ensure that the clustered mitochondria within cells transfected with Mfn2 and mutants maintained their electrochemical potential, we quantified the total fluorescence intensity within cells loaded with a ΔΨ-dependent dye and scored the results in a distribution profile as shown in Table I. In untransfected cells, the fluorescence intensity was between 1 and 3 \times 10^6 arbitrary units in 90% of the cells examined. Transfection of Mfn2W631P showed some loss in total dye uptake, with 61% of the cells examined exhibiting fluorescent arbitrary units of fewer than 1 \times 10^6. Transfection of the other constructs did not show significant loss in potential, but showed a broader distribution profile than untransfected cells, with many transfected cells exhibiting fluorescence units higher than normal. For example, transfection of Mfn2, Mfn2RasG12V, or Mfn2RVWP showed 15–20% of cells having greater than 4 \times 10^6 fluorescent arbitrary units. These data demonstrate that electrochemical potential is maintained, with a slight reduction in cells transfected with Mfn2W631P.

PEG-induced Cell Fusion Demonstrates That Mfn2RasG12V Is a Dominant Active Mutant—To assess directly the fusion competence of mitochondria in cells expressing these proteins, we employed a well characterized assay that induces fusion between whole cells transfected with different matrix marker proteins (4–6). Mitochondria from heterokaryons transfected with marker proteins alone fused completely within 8–12 h (Fig. 3, top left panels) (4–6). Mitochondria from donor cells expressing Mfn2-CFP also fused with acceptor mitochondria within a similar time course (Fig. 3, middle left, n = 29); however, ~20% of the heterokaryons expressing Mfn2-CFP scored complete content mixing within 2 h. Strikingly, fusion between mitochondria expressing Mfn2RasG12V-CFP with acceptor GFP organelles occurred within 30 min after the addition of PEG in >90% of the observed cell fusions (Fig. 3, bottom left, n = 20). Video analysis of the PEG fusion assay shows that the Mfn2RasG12V-CFP cluster remains immobile, whereas the GFP-containing acceptor mitochondria are motile within the heterokaryon (see supplemental video). This efficient fusion between mitochondria was highly significant because the migration of mitochondria from one cell into another is a slow process in mammalian cells. In control experiments very few mitochondria within the heterokaryon had migrated across the cell boundary in the 1st h (Fig. 3). In contrast, the rapid and/or directed migration of the GFP-expressing mitochondria toward the Mfn2RasG12V-CFP-expressing DsRed mitochondria allowed complete fusion of all mitochondria to occur within 30 min. This indicates that the presence of Mfn2RasG12V within one population of mitochondria initiates a cascade of events that lead to highly efficient mitochondrial fusion. Therefore, we consider the Mfn2RasG12V to be the first characterized dominant active, GTPase-deficient mutant that stimulates mitochondrial fusion.

The Mfn2<sub>430-757</sub>-CFP construct completely inhibited the fusion, confirming the requirement for the GTPase domain (Fig. 3, top right). In addition to a role in contact site formation, previous studies with yeast Fzo1p have demonstrated the requirement for the IMS loop of Fzo1p for mitochondrial fusion (8). Consistent with this, fusion was inhibited between mitochondria containing Mfn2W631P-CFP and acceptor GFP-containing mitochondria. Surprisingly, the inhibition of mitochondrial fusion conferred by the Mfn2W631P mutation (Fig. 3, middle right) was rescued by the Mfn2RasG12V mutation within the double mutant (Fig. 3, bottom right), indicating that the conserved IMS domain is not directly required for the fusion event, but likely plays a more regulatory role in the activation of Mfn2.
The fused clusters in Mfn2RasG12V-transfected cells were qualitatively similar to the wild type (Fig. 4A), indicating that the end point of the fused mitochondrial reticulum is similar, even though the PEG fusion assay demonstrated that kinetics of the fusion event are different (Fig. 3). Analysis of Mfn2W631P-transfected cells did not reveal any interconnecting membranes between the clustered organelles. Notably, some of the mitochondria in the Mfn2W631P-transfected cells appeared to “unravel,” with membranous material emanating beyond the clear boundaries of the outer membrane (Fig. 4A). This may be caused by the loss of contact site formation (8), leading to aberrant membrane architecture. Mitochondria in cells expressing Mfn2RVWP-CFP contained stacks of parallel membranes and extensive membrane whorls that were interconnected throughout the cluster. Immunolabeling of the fused mitochondrial clusters indicates that the Mfn2 protein is found within the interconnecting membranes, demonstrating that these membranes are derived, at least in part, from the outer mitochondrial membrane (Fig. 4B). Regardless of this massive alteration in membrane architecture, the cristae and electron dense matrix compartments appeared normal, consistent with their ability to maintain electrochemical potential (Table I) and stimulate mitochondrial fusion (Fig. 3). Mitochondria within cells expressing Mfn2(430–757)-CFP were docked together within a cluster, consistent with the proposed tethering function of the HR2 domain (7). However, there was no fused membrane material between the mitochondria (Fig. 4A), demonstrating that the fused membranes do not arise simply because of nonspecific mitochondrial clustering.

Activated Mfn2 Represses Bax Activation, Cytochrome c Release, and Permeability Transition—Given the increasing involvement of dynamic changes in mitochondrial morphology during the progression of apoptosis, we next examined the specific consequence of Mfn2 activation on the mitochondrial response to two different types of stimuli. Stimulation of programmed death by STS treatment resulted in the efficient recruitment and activation of Bax to the mitochondria, as revealed by an antibody that specifically recognizes the conformationally active form of Bax (41). As expected (42, 43), the amount of Bax activation correlated with the amount of cytochrome c release (∼50% by 3 h, Fig. 5, A and B, n = 379). As a negative control, we transfected cells with DRP1(K38E) (44–46), which has been shown to block mitochondrial fission and inhibit cytochrome c release from mitochondria (26, 47). As expected, only 3% of these cells showed cytochrome c release after 3 h of STS treatment, with ~10% of cells showing Bax activation (n = 66). By 5 h of treatment, this level of Bax activation increased without a release of cytochrome c (data not shown), as has been reported previously within cells transfected with DRP1(K38A) (40). Transfection of Mfn2-CFP

### Table I

| Fluorescence<sup>a</sup> | Distribution profile of intensities of ΔΨ-dependent dye uptake<sup>b</sup> |
|-------------------------|------------------------------------------------------------------|
| A.U. × 10<sup>4</sup>  | %       | %       | %       | %       | %       | %       |
| <1                     | 4.8     | 19      | 9       | 61.1    | 14.3    | 16.7    | 6.3     |
| 1–2                    | 57.1    | 28.6    | 25      | 33.3    | 38.1    | 33.3    | 18.8    |
| 2–3                    | 33.3    | 28.6    | 25      | 5.6     | 9.5     | 38.9    | 37.5    |
| 3–4                    | 4.8     | 9.5     | 19      | 0       | 23.8    | 11.1    | 37.5    |
| >4                     | 0       | 15      | 22      | 0       | 15      | 0       | 0       |

<sup>a</sup> A.U., arbitrary units.
<sup>b</sup> Percent of total cells.
<sup>c</sup> UTF, untransfected.
showed a reduction of STS-induced Bax activation and cytochrome c release (Fig. 5A), with only ~35% of cells showing susceptibility to STS treatment (Fig. 5B, n = 92). Interestingly, in the 35% of STS-sensitive cells, many of the Mfn2-CFP-containing puncta colocalized with activated Bax (insets, Fig. 5A). Mfn2RasG12V-CFP and Mfn2RVWP-CFP both repressed Bax activation and cytochrome c release, where only ~20% of cells were susceptible to STS treatment (Fig. 5B, n = 69 and 72, respectively). In contrast, the fusion-incompetent Mfn2W631P-CFP did not provide protection against Bax activation or cytochrome c release (Fig. 5A, n = 73). As with Mfn2-CFP, activated Bax often colocalized with Mfn2W631P-CFP-containing puncta (see insets, Fig. 5A). Cells transfected with the dominant negative mutant Mfn2460-757-CFP were highly sensitive to STS treatment, with 80% of cells showing complete cytochrome c release by 3 h (Fig. 5A, n = 95). Oddly, the amount of cytochrome c release in this condition (~80%) did not mirror the amount of Bax activation (~40%), which could be explained because cytochrome c was released in Mfn2430-757-CFP-transfected cells in a Bax-independent manner, without any death stimuli (Fig. 5A). As expected, transfection of the other Mfn2 constructs showed normal, mitochondrial cytochrome c staining in the absence of any death stimuli (data not shown). Taken together, the data show that activated Mfn2 is a repressor of Bax activation and outer membrane pore formation.

Given that activated Mfn2 can provide protection against Bax activation and cytochrome c release triggered by external apoptotic stimuli, we next wanted to investigate whether Mfn2 could also protect the mitochondria from damage induced by internal metabolic stress. We therefore adapted an assay that artificially produces free radicals within the matrix of the mitochondria, thereby triggering permeability transition and permeability transition pore opening in the absence of Bax activation (48–50). This approach allowed us to damage the mitochondria from the matrix side, simulating physiological systems where mitochondrial radical loads are increased because of excessive respiratory activity or other stresses. The ΔΨ-sensitive dye tetramethylrhodamine ester and its derivatives, such as MitoFluor Red 589, become photoactivated upon exposure to light and subsequently produce free radicals within the matrix of the mitochondria. With the increasing accumulation of free radicals, the permeability transition pore opens, and protons become equilibrated across the inner membrane (49, 50). Concomitant with the loss in ΔΨ, the potential-sensitive dye is redistributed within the cell, a process observed using time lapse video microscopy. The mitochondria then regain ΔΨ, which allows the reuptake of the potential-sensitive dye, and in time lapse they appear to “flicker” until they become terminally depolarized. 400 images were collected over 25 min by exciting MitoFluor Red 589 for 1,000 ms followed by a 2,500-ms delay. Fig. 6 shows that 84% of the untransfected cells incubated with 50 nM MitoFluor Red 589 reached terminal depolarization within the first frame category (frames 1–250, n = 56). Mfn2-CFP-expressing cells revealed a moderate, but
significant protection from free radical-induced damage, with a varied distribution of rates of dye loss across the four frame categories ($p < 0.003$ Fisher’s exact test, $n = 19$). Similar to the protection granted against STS treatment, 62% of the cells containing a fused mitochondrial reticulum induced by transfection of Mfn2RasG12V-CFP in the absence of death stimuli is shown in lower right panel. Insets show higher magnifications of the individual channels, as indicated. Note the colocalization of Bax with Mfn2-CFP proteins upon activation. The percentage of cells with cytochrome c release and Bax activation were scored at 3 h after STS treatment, and the data were plotted as a percentage distribution of total cells.

The specificity of this assay was further verified by creating a fused reticulum after transfection with Mfn2RasG12V-CFP but did not affect the loss of dye in these experiments because 82% ($n = 22$) of the mitochondria were terminally depolarized within the first frame category. These effects are not caused by differences in either MitoFluor dye loading or absolute free radical production because fluorescent quantification of initial MitoFluor dye uptake and the radical dye dihydroethidium shows no differences between the transfected versus untransfected controls (Tables I and II). The specificity of this assay was further verified by creating a fused reticulum after transfection with
FIG. 6. Activation of Mfn2 protects against permeability transition. COS-7 cells, as indicated, were incubated with 50 nM MitoFluor Red 589, exposed to light (see “Experimental Procedures”), and scored for terminal depolarization. The results were converted to a percent value and plotted as a frequency distribution for each transfection condition, as indicated in the graph.

Table II

| Fluorescence | A.U. × 10^6 |
|--------------|-------------|
| 💙 UTF       | 106 41.7 40 26.7 61.5 12.5 12 10 |
| 💙 Mfn2-CFP  | 1–2 25 50 40 38.5 62.5 59 34.5 |
| 💙 Mfn2RasG12V-CFP | 2–3 25 10 20 0 18.8 29 24.1 |
| 💙 Mfn2W631P-CFP | 3 8.3 0 13.3 0 6.3 0 31 |
| 💙 Mfn2RVWP-CFP | 430-757-CFP |
| 💙 DRP1(K38E)-CFP |

- 💙 A.U., arbitrary units.
- 💙 UTF, untransfected.

Activated Mfn2 Signals Mitochondrial Fusion

Cates a striking proliferation of interconnected membranes, shedding new light on the plasticity of the mitochondrial membranes. Furthermore, we have determined that the conserved tryptophan residue within the intermembrane space region of Mfn2 is not essential to form a fusion pore but is required to activate fusion within the context of the wild type GTPase. The mutational analysis also allowed us to determine that the punctate localization of Mfn2 (40) is regulated by the nucleotide state. The hydrolysis-deficient mutants of Mfn2 that stimulate mitochondrial fusion do not readily form puncta, whereas the mutants that inhibit mitochondrial fusion are found in these foci. Finally, the activated Mfn2 constructs significantly repress Bax activation, cytochrome c release, and free radical-induced permeability transition. Taken together, these data reveal a role for Mfn2 as a regulator of mitochondrial fusion and as a nucleotide-dependent modulator of the apoptotic response.

The PEG fusion assay demonstrates that Mfn2 is a regulatory GTPase, which, when in the activated form, is capable of signaling to neighboring mitochondria to fuse in an accelerated manner. Because Mfn2RasG12V-CFP is anchored within the mitochondrial outer membrane of the DsRed-containing mitochondria in Fig. 3, it follows that Mfn2_RasG12V-initiated cytosolic events resulting in the efficient recruitment and fusion of GFP-containing mitochondria with the Mfn2_RasG12V-CFP/DsRed reticulum. Clearly there are a number of molecular events that contribute to this dramatic increase in fusion, including increased motility events, as well as the activation of the fusion machinery. Our data therefore indicate that Mfn2 may play a dual role, both as a direct constituent of the tethering/fusion machinery (minimally through the coiled coil do-
interacting proteins, those both cytosolic and mitochondrial, to define better the molecular events that link the GTPase cycle of Mfn2 with mitochondrial fusion and the control of permeability transition.

Acknowledgments—We are extremely thankful to the anonymous reviewers, who have played an essential role in the development of this work. We are grateful to Marino Zerial (MPI-CBG Dresden, Germany) for the use of Rab5-CFP and Rab5-GST, Mike Ryan (La Trobe University, Australia) for Tom7-GFP, and to Kazuhisa Nakayama (University of Tsukuba, Japan) for the DRP1(K38A) constructs used in this study. We thank members of the laboratory, Gordon Shore, and Marta Miaczynska for critical comments on the manuscript.

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J. Biol. Chem. 2005, 280:25060-25070.
doi: 10.1074/jbc.M501599200 originally published online May 4, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M501599200

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