Mitochondria are important participants in apoptosis, releasing cytochrome c into the cytoplasm and undergoing extensive fragmentation. However, mechanisms underlying these processes remain unclear. Here, we demonstrate that cytochrome c release during apoptosis precedes mitochondrial fragmentation. Unexpectedly, OPA1, a dynamin-like GTPase of the mitochondrial intermembrane space important for maintaining cristae structure, is co-released with cytochrome c. To mimic the loss of OPA1 occurring after its release, we knocked down OPA1 expression using RNA interference. This triggered structural changes in the mitochondrial cristae and caused increased fragmentation by blocking mitochondrial fusion. Because cytochrome c is mostly sequestered within cristae folds but released rapidly and completely during apoptosis, we examined the effect of OPA1 loss on cytochrome c release, demonstrating that it is accelerated. Thus, our results suggest that an initial mitochondrial leak of OPA1 leads to cristae structural alterations and exposure of previously sequestered protein pools, permitting continued release in a feed-forward manner to completion. Moreover, our findings indicate that the resulting OPA1 depletion causes a block in mitochondrial fusion, providing a compelling mechanism for the prominent increase in mitochondrial fragmentation seen during apoptosis.

Programmed cell death (PCD), along with its main phenotype apoptosis, comprises a cellular suicide program essential for development and tissue homeostasis in all metazoan animals (1). Mitochondria are important participants in PCD resulting from a variety of death stimuli via the release after Bax/Bak-mediated mitochondrial outer membrane permeabilization (MOMP) of cytochrome c and other apoptogenic factors into the cytoplasm, where they trigger activation of caspases and subsequently promote cell death (2, 3). During PCD mitochondria also undergo extensive fragmentation, which recent studies have indicated may promote apoptotic cell death in the nematode Caenorhabditis elegans as well (4).

The morphology of mitochondria within a cell is normally controlled by a precise balance of fusion and fission (5), but the mechanisms whereby mitochondria fragment extensively during apoptosis remain controversial. One possibility is that the release of proteins after MOMP stimulates mitochondrial division by activating fission and/or inhibiting fusion. An alternative hypothesis is that mitochondrial division itself is necessary, directly or indirectly, for the release of apoptogenic factors such as cytochrome c (6). Another area of contention centers on the release of cytochrome c itself during PCD. Although most cytochrome c (>80%) is normally sequestered within mitochondrial cristae folds, release is known to be rapid and complete during apoptosis (7). Although the underlying mechanism remains unclear, it has been suggested to occur through changes such as mitochondrial fragmentation or inner membrane cristae reorganization (8, 9). Here, we sought to clarify the mechanisms and temporal relationships of mitochondrial fragmentation and ultrastructural cristae changes in the context of MOMP and the resulting cytochrome c release during apoptosis.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—HeLa cells and HeLa cells stably overexpressing Bcl-2 were cultured under standard conditions in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 IU of penicillin, and 50 μg/ml streptomycin. Transient transfection of HeLa cells with Mito-DsRed2 (BD Biosciences Clontech) was performed using FuGENE 6 (Roche Applied Science) to identify mitochondria. Transfection with photoactivatable Mito-PAGFP was performed as described previously (10). zVAD-fmk and MitoTracker Red CMXRos were purchased from Calbiochem and Molecular Probes, respectively. Actinomycin D and staurosporine were purchased from Sigma-Aldrich.

Subcellular Fractionation and Immunoblotting—HeLa cells were harvested in isotonic mitochondrial buffer (MB; 210 mM mannitol, 70 mM sucrose, 1 mM EDTA, and 10 mM HEPES (pH 7.5)) supplemented with the protease inhibitor mixture Complete (Roche Molecular Biochemicals) and homogenized for 30–40 strokes with a Dounce homogenizer. Samples were transferred to Eppendorf centrifuge tubes and centrifuged (500 × g, 5 min, 4 °C) to remove nuclei and unbroken cells. The resulting supernatant was then centrifuged (10,000 × g, 30 min, 4 °C) to obtain the heavy membrane fraction enriched for mitochondria; the resulting supernatant was collected as the cytosolic fraction. The resulting supernatant was then centrifuged (500 × g, 5 min, 4 °C) to obtain the heavy membrane fraction enriched for mitochondria; the resulting supernatant was collected as the cytosolic fraction. Cytosolic and heavy membranes fractions (30 and 10 μg of protein, respectively) were resolved by SDS-PAGE (10–20% Tricine gels; Novex) and transferred to nitrocellulose membranes (Amersham Biosciences). After blocking nonspecific sites for 1 h at room temperature with 5% nonfat milk and 0.2% Tween 20 in phosphate-buffered saline (pH 7.4), the membrane was incubated with anti-cytochrome c mouse mono-
clonal (BD Biosciences Pharmingen, clone 7H8.2C12) (1:2000 dilution) and anti-OPA1 rabbit polyclonal (3 μg/ml) antibodies as described previously (11). To confirm equal protein loading and transfer, membranes were subsequently reprobed with anti-actin (Sigma-Aldrich, clone AC-40) (1:5000) and anti-Cox IV monoclonal antibodies (Molecular Probes, clone 10G8) (1:3000) as indicated. After treatment with horse-radish peroxidase-linked goat anti-mouse or anti-rabbit secondary antibodies (1:2000; Amersham Biosciences), immunoreactive proteins were detected using enhanced chemiluminescence (ECL; Amersham Biosciences).

**Immunofluorescence Microscopy**—Cells grown in LabTek chambers were fixed for 5 min at −20 °C in methanol followed by permeabilization with 0.15% Triton X-100 in phosphate-buffered saline for 15 min. The cells were then incubated for 1 h in blocking buffer (2% bovine serum albumin in phosphate-buffered saline) followed by incubation overnight with either a rabbit polyclonal anti-OPA1 (11) (1.5 μg/ml) or a mouse monoclonal anti-cytochrome c (BD Biosciences Pharmingen, clone 6H2.B4) (1:800) antibody. Cells were washed 3 times for 10 min each in blocking buffer then incubated for 2 h with Alexa Fluor secondary anti-mouse and anti-rabbit antibodies (Molecular Probes). Images were acquired using a Zeiss LSM 510 confocal microscope through a 63× oil fluorescence objective (Carl Zeiss, Inc.).

**Isolation of Mitochondria and in Vitro Assays for the Release of Mitochondrial Factors**—Mitochondria were isolated intact from HeLa cells by sucrose density gradient centrifugation. Briefly, cells were harvested in phosphate-buffered saline containing 5 mM EDTA, centrifuged at 750 × g for 10 min, washed, and resuspended in isotonic MB supplemented with protease inhibitors. Cells were broken by five passages through a 25-gauge needle fitted onto a 5-ml syringe, and the suspension was then centrifuged at 2000 × g at 4 °C. This procedure was repeated until nearly all of the cells were broken. Supernatants from each step were pooled before centrifugation (13,000 × g, 10 min, 4 °C). The resulting pellet containing mitochondria was resuspended in 1 ml of MB and layered on top of a discontinuous sucrose gradient consisting of 19 ml of 1.2 M sucrose, 1 mM EDTA, and 0.1% bovine serum albumin in 10 mM HEPES (pH 7.5) over 16 ml of 1.6 M sucrose, 1 mM EDTA, and 0.1% bovine serum albumin in 10 mM HEPES (pH 7.5). Samples were centrifuged (27,000 rpm, 2 h, 4 °C) in a Beckman SW28 rotor. Mitochondria were recovered at the 1.6 M/1.2 M sucrose buffer interface, washed, and resuspended in MB.

Isolated mitochondria (30 μg) were incubated in the presence or absence of various concentrations of caspase-8-cleaved recombinant human Bid (tBid; R&D Systems) in 200 μl of KCl buffer (125 mM KCl, 4 mM MgCl₂, 5 mM NaHPO₄, 5 mM succinate, 0.5 mM EGTA, 15 mM HEPES-KOH (pH 7.4), and 5 μM rotenone) for 20 min at 30 °C, and the mitochondria were recovered by centrifugation (13,000 × g, 5 min, 4 °C). In other experiments, 10 mM tBid was used for various time points, and the mitochondria were recovered by centrifugation as above. Aliquots of mitochondrial pellets (5 μg protein) and the corresponding volume of the supernatant fractions were subjected to SDS-PAGE using 10–20% Tricine gels (Novex) and their respective contents of cytochrome c and OPA1 were estimated by immunoblotting using mouse monoclonal anti-cytochrome c (BD Sciences Pharmingen) (1:2000; clone 7H8.2C12) or polyclonal rabbit anti-OPA1 (11) (3 μg/ml) antibodies. Consistent loading of the mitochondrial pellet was verified using anti-voltage-dependent anion channel antibodies (Calbiochem; clone 31H1L) (1:6000). Cytochrome c release was also quantitated using a colorimetric enzyme-linked immunosorbent assay (Quantikine DCTC0; R&D Systems) as previously described (9), according to the manufacturer’s instructions.

**Proteinase K, Carbonate, and KCl Treatments of Isolated Mitochondria**—Isolated mitochondria prepared as described above were incubated for 20 min on ice with 200 μg/ml proteinase K in 500 μl of MB or 500 μl of 20 mM HEPES-KOH buffer for mitochondrial swelling. The pellet was recovered by centrifugation (130,000 × g, 30 min, 4 °C). For carbonate extraction, isolated mitochondria were resuspended in a 0.1 M Na₂CO₃ buffer (pH 11.0) and incubated on ice for 30 min; the pellet was then recovered by centrifugation (130,000 × g, 30 min, 4 °C). For salt wash experiments, mitochondria were diluted 10-fold in buffers consisting of either 30 mM NaCl or 500 mM NaCl in 3 mM Tris-HCl (pH 7.4) and sonicated at the highest level 10 times for 10 s per cycle. The pellet was recovered by centrifugation (130,000 × g, 30 min, 4 °C). Proteins from the resulting supernatants were concentrated by precipitation with 12% (w/v) trichloroacetic acid. Cytochrome c, OPA1, and VDAC levels were then assessed in both fractions by immunoblotting.

**RNA Interference**—Silencing of OPA1 and Mfn1 expression in HeLa cells was achieved using small interfering RNAs (siRNAs). Synthetic oligonucleotides representing sequences from positions 1810 to 1831 of OPA1 (5′-GUUAUCAGUCAGGCAGGTTT-3′; GenBank™ accession number AB011139) and 186 to 207 of Mfn1 (5′-GGAGACACAUUUUGUAAGATT-3′; GenBank™ accession number AF29637) were produced by Ambion. A Silencer Negative Control siRNA (Ambion) was used for control experiments. HeLa cells grown to 30–40% confluency were transfected 3 times sequentially with annealed siRNA duplexes using Oligofectamine (Invitrogen). Levels of OPA1 and Mfn1 were assessed by immunoblotting. For detection of Mfn1, a polyclonal rabbit antisemur (12) (a gift of Dr. K. Mihara, Kyushu University, Fukuoka, Japan) was used (1:500 dilution). Equal protein loading was assessed using an anti-actin monoclonal antibody (Sigma-Aldrich, clone AC-40) (1:5000).

**Transmission Electron Microscopy**—HeLa cells were fixed with a solution of 2.5% paraformaldehyde, 0.5% glutaraldehyde buffered with 0.1 M sodium phosphate (pH 7.4) for 24 h at 4 °C, dehydrated with ethanol at 4 °C, and immersed in a 1:1 mixture of propylene oxide and Epon and finally embedded in Epon by polymerization at 60 °C for 48 h. Ultrastructural analyses were performed on a JEOL1010 electron microscope.

**Time-lapse Confocal Fluorescence Microscopy and Kinetics of Cytochrome c-GFP Release**—HeLa cells stably expressing cytochrome c-GFP (13) were cultured in LabTek chambers under standard conditions and maintained in Dulbecco’s modified Eagle’s medium without phenol red (Invitrogen), supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 IU penicillin, and 50 μg/ml streptomycin. Six hours after apoptosis induction, cytochrome c-GFP release was analyzed every 30 s through a 63× oil fluorescence objective using a PerkinElmer Life Sciences UltraView R5 Nipkow-disk confocal microscope equipped with a 488-nm argon laser and a temperature controller to maintain the temperature at 37 °C. GFP excitation was kept as low as possible to avoid photo-destruction of the cell. Quantitative analysis of the fluorescence images was performed using MetaMorph software. The release kinetics of cytochrome c-GFP are depicted as the S.D. of the average pixel intensity across individual cells, as previously described (13). Compartmentalized cytochrome c-GFP within mitochondria contributes to a high S.D., and more homogeneously distributed cytochrome c-GFP in the cytoplasm gives rise to a low S.D. For direct comparisons and statistical analyses, initial S.D. values were normalized, and single cell standard deviation traces were scaled from 100 (base line before the release) to 0 (base line after completion of the release). Data were exported to Microsoft Excel and converted into graphical displays.
Mitochondrial Fragmentation Occurs Downstream of Bax/Bak-mediated Mitochondrial Outer Membrane Permeabilization—It is generally accepted that PCD occurs downstream of Bax/Bak-mediated MOMP (2, 14). Because previous reports have also shown that Bax and Bak promote mitochondrial fragmentation (15), we sought to determine whether this fragmentation also occurs after MOMP. We investigated HeLa cells stably expressing cytochrome c-GFP (13) to determine the temporal relationship between MOMP (assessed by cytochrome c-GFP release) and mitochondrial fragmentation. Cells stably expressing cytochrome c-GFP were transiently transfected with Mito-DsRed2 to label mitochondria and then treated with actinomycin D to induce Bax/Bak-mediated MOMP (14) in the presence of the broad caspase inhibitor zVAD-fmk to prevent apoptosis. Mitochondrial morphology and cytochrome c-GFP release were then assessed in living cells every 5 min. In agreement with a previous report (16), we observed that cytochrome c-GFP was released before mitochondrial fragmentation; minutes later mitochondria started to fragment in a caspase-independent manner (Fig. 1A). Analysis of multiple different cells revealed that mitochondrial fragmentation typically occurred ~10 min after cytochrome c-GFP was completely released (Fig. 1B), similar to the findings of Gao et al. (16). Essentially the same results were obtained substituting staurosporine, another pro-apoptotic drug, for actinomycin D (data not shown). Experiments performed in the absence of zVAD-fmk similarly demonstrated mitochondrial fragmentation on average ~10 min after the complete release of cytochrome c-GFP (data not shown).

Because the timing of mitochondrial fragmentation relative to release of cytochrome c after MOMP is an important issue for clarifying mechanisms of mitochondrial fragmentation during PCD, our results are particularly noteworthy in light of a recent study reporting that expression of the pro-apoptotic protein Bax in the protozoan Trypanosoma brucei, which has only a single mitochondrion, causes the release of cytochrome c followed by mitochondrial fragmentation, with these events temporally well separated (17). These findings conceptually support the data we present in Fig. 1, as well as those of Gao et al. (16). Furthermore, in mammalian cells, Bax (and Bak) are known to form clusters at scission sites of mitochondria (18), but by that time cytochrome c has already been released into the cytoplasm, consistent with our assertion that cytochrome c release precedes mitochondrial fragmentation.

OPA1 Is a Membrane-associated Protein Released After Bax/Bak-mediated MOMP—Recently, Youle and co-workers (10) reported that a block of mitochondrial fusion occurs close in time to Bax/Bak-mediated MOMP, implicating the inhibition of the mitochondrial fusion machinery in mitochondrial fragmentation during apoptosis. In mammalian cells mitochondrial fusion requires the activity of three dynamin-related, large GTPases: Mfn1, Mfn2, and OPA1 (5, 8). Although Bax co-localizes with Mfn2 in the same foci during PCD (18), no direct or indirect interactions among any of these proteins have been reported, and the mechanism of this mitochondrial fusion arrest remains unclear.

To explain the block of mitochondrial fusion during PCD, we considered that levels of Mfn1, Mfn2, and/or OPA1 in mitochondria might be decreased, and we compared them in mitochondrial fractions from both control and apoptotic cells. Although we did not observe any significant differences in levels of Mfn1 or Mfn2, both integral membrane proteins of the mitochondrial outer membrane (8), a marked reduction of OPA1 protein was found in the mitochondria of apoptotic cells (data not shown). Because OPA1 has been identified as a mitochondrial inter-membrane space protein tightly associated with the inner mitochon-

\[ \text{Statistical Analyses—Data were compared using Student’s t test. Differences were considered to be significant if } p < 0.05. \]

\[ \text{RESULTS} \]

\[ \text{Mitochondrial Fragmentation Occurs Downstream of Bax/Bak-mediated Mitochondrial Outer Membrane Permeabilization—It is generally accepted that PCD occurs downstream of Bax/Bak-mediated MOMP (2, 14). Because previous reports have also shown that Bax and Bak promote mitochondrial fragmentation (15), we sought to determine whether this fragmentation also occurs after MOMP. We investigated HeLa cells stably expressing cytochrome c-GFP (13) to determine the temporal relationship between MOMP (assessed by cytochrome c-GFP release) and mitochondrial fragmentation. Cells stably expressing cytochrome c-GFP were transiently transfected with Mito-DsRed2 to label mitochondria and then treated with actinomycin D to induce Bax/Bak-mediated MOMP (14) in the presence of the broad caspase inhibitor zVAD-fmk to prevent apoptosis. Mitochondrial morphology and cytochrome c-GFP release were then assessed in living cells every 5 min. In agreement with a previous report (16), we observed that cytochrome c-GFP was released before mitochondrial fragmentation; minutes later mitochondria started to fragment in a caspase-independent manner (Fig. 1A). Analysis of multiple different cells revealed that mitochondrial fragmentation typically occurred ~10 min after cytochrome c-GFP was completely released (Fig. 1B), similar to the findings of Gao et al. (16). Essentially the same results were obtained substituting staurosporine, another pro-apoptotic drug, for actinomycin D (data not shown). Experiments performed in the absence of zVAD-fmk similarly demonstrated mitochondrial fragmentation on average ~10 min after the complete release of cytochrome c-GFP (data not shown). Because the timing of mitochondrial fragmentation relative to release of cytochrome c after MOMP is an important issue for clarifying mechanisms of mitochondrial fragmentation during PCD, our results are particularly noteworthy in light of a recent study reporting that expression of the pro-apoptotic protein Bax in the protozoan Trypanosoma brucei, which has only a single mitochondrion, causes the release of cytochrome c followed by mitochondrial fragmentation, with these events temporally well separated (17). These findings conceptually support the data we present in Fig. 1, as well as those of Gao et al. (16). Furthermore, in mammalian cells, Bax (and Bak) are known to form clusters at scission sites of mitochondria (18), but by that time cytochrome c has already been released into the cytoplasm, consistent with our assertion that cytochrome c release precedes mitochondrial fragmentation. OPA1 Is a Membrane-associated Protein Released After Bax/Bak-mediated MOMP—Recently, Youle and co-workers (10) reported that a block of mitochondrial fusion occurs close in time to Bax/Bak-mediated MOMP, implicating the inhibition of the mitochondrial fusion machinery in mitochondrial fragmentation during apoptosis. In mammalian cells mitochondrial fusion requires the activity of three dynamin-related, large GTPases: Mfn1, Mfn2, and OPA1 (5, 8). Although Bax co-localizes with Mfn2 in the same foci during PCD (18), no direct or indirect interactions among any of these proteins have been reported, and the mechanism of this mitochondrial fusion arrest remains unclear. To explain the block of mitochondrial fusion during PCD, we considered that levels of Mfn1, Mfn2, and/or OPA1 in mitochondria might be decreased, and we compared them in mitochondrial fractions from both control and apoptotic cells. Although we did not observe any significant differences in levels of Mfn1 or Mfn2, both integral membrane proteins of the mitochondrial outer membrane (8), a marked reduction of OPA1 protein was found in the mitochondria of apoptotic cells (data not shown). Because OPA1 has been identified as a mitochondrial inter-membrane space protein tightly associated with the inner mitochon-}
We hypothesized that its association with the inner mitochondrial membrane might be disrupted as a consequence of Bax/Bak-mediated MOMP, and soluble OPA1 would then be available for release from mitochondria. This was confirmed when we compared cellular fractions from untreated and apoptotic cells. Indeed, the OPA1 protein, like cytochrome c, not only disappeared from the mitochondrial fraction of cells treated with pro-apoptotic drugs, but this disappearance also coincided with its appearance in the cytosolic fraction (Fig. 2A). OPA1 release during PCD was similarly revealed by immunocytochemistry (Fig. 2B) and as for cytochrome c (7), its release was markedly inhibited in cells stably overexpressing the anti-apoptotic protein Bcl-2 (Fig. 2A). Next, mitochondria isolated from HeLa cells were incubated in vitro with recombinant tBid, a BH3-only member of the Bcl-2 family that requires either Bax or Bak to trigger MOMP (14). Both dose-escalation and kinetic studies with tBid demonstrated that OPA1 and cytochrome c are co-released after MOMP (Fig. 2C). Nevertheless, although cytochrome c release was essentially complete, a slightly greater fraction of OPA1 remained in the mitochondria after MOMP, a result confirmed by both immunocytochemistry and immunoblotting using isolated mitochondria (Fig. 2B and C). Most likely, the OPA1 remaining in mitochondria after MOMP represents protein still associated with the inner mitochondrial membrane through tight protein-protein or protein-lipid interactions (20). However, this is an area of some controversy, since recent reports have suggested that whereas mammalian OPA1 is a protein tightly bound to the inner membrane (19), its yeast ortholog Mgm1p may be a transmembrane protein anchored in this membrane (8). Here, we have found, in agreement with Griparic et al. (19), that nearly all of the OPA1 protein was released from sonicated mitochondrial membranes after high pH carbonate buffer extraction as well as after low or high salt washes (Fig. 2D), confirming that it is membrane-associated. By contrast VDAC, an integral membranous but not an integral membrane protein (19), we hypothesized that its association with the inner mitochondrial membrane might be disrupted as a consequence of Bax/Bak-mediated MOMP, and soluble OPA1 would then be available for release from mitochondria. This was confirmed when we compared cellular fractions from untreated and apoptotic cells. Indeed, the OPA1 protein, like cytochrome c, not only disappeared from the mitochondrial fraction of cells treated with pro-apoptotic drugs, but this disappearance also coincided with its appearance in the cytosolic fraction (Fig. 2A). OPA1 release during PCD was similarly revealed by immunocytochemistry (Fig. 2B) and as for cytochrome c (7), its release was markedly inhibited in cells stably overexpressing the anti-apoptotic protein Bcl-2 (Fig. 2A).
membrane protein, was not extracted by these same treatments (Fig. 2D).

**OPA1 Loss Is Associated with Changes in Cristae Structure and Mitochondrial Fragmentation Due to Mitochondrial Fusion Block**—In cells undergoing PCD, the complete release of cytochrome c occurs rapidly over ~5 min (13). Because we observed that OPA1 is co-released from mitochondria with cytochrome c after MOMP (Fig. 2), release of OPA1 likely occurs within a similar time frame. To mimic the loss of mitochondrial OPA1 that occurs during PCD, we transfected siRNAs to knock down OPA1 protein expression in HeLa cells (Fig. 3A).

Reminiscent of previous reports investigating OPA1 (19, 21) and yeast Mgm1p (22), we observed that OPA1 loss results in extensive mitochondrial fragmentation (Fig. 3, B and C) as well as perturbation of the mitochondrial inner membrane, with prominent disorganization of the cristae (Fig. 3D). Using a cell culture mitochondrial fusion assay employing a photoactivable Mito-PAGFP construct (10), we further demonstrated that loss of OPA1 is associated with a block of mitochondrial fusion; in contrast, fusion was normal both in cells treated with control siRNA and in untreated cells (Fig. 3E). Thus, although OPA1 is localized to the mitochondrial intermembrane space, it is required for proper mitochondrial fusion, and loss of OPA1 from mitochondria after MOMP likely participates in the

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**FIGURE 3.** Knock down of OPA1 expression in cells triggers cristae structural alterations and mitochondrial fragmentation associated with a block of mitochondrial fusion. A, HeLa cells were transfected sequentially 3 times with OPA1 siRNA or control siRNA. OPA1 protein levels were then assessed by immunoblotting of extracts from untransfected, control siRNA-transfected, and OPA1 siRNA-transfected cells. Actin was used as a protein loading control. An asterisk (*) indicates a probable nonspecific protein band. B, cells were transfected as in A, and the percentage of cells with fragmented mitochondria was determined after mitochondrial staining with MitoTracker Red CMXRos. Data represent the means ± S.D. of 4 independent experiments, with 300 cells per condition. Representative images are shown in C, with the boxed areas enlarged in the lower panels. D, cells were transfected as in A, and the mitochondrial ultrastructure was investigated by transmission electron microscopy. Scale bars represent 500 nm. The bracketed regions in the left panels are enlarged in the right panels. E, HeLa cells were co-transfected with Mito-DsRed2 and Mito-PAGFP (10). To photoactivate PAGFP, regions demarcated with white circles were irradiated with λ ~ 413 nm light. A time-lapse confocal acquisition (using λ ~ 488-nm laser activation) of a series of z sections spanning the entire cell thickness was then performed to study Mito-PAGFP diffusion as a result of mitochondrial fusion over time. Images shown are pseudo-colored projections of the stacked z-series (upper panels). Mito-PAGFP fluorescence was quantitated using MetaMorph software, and data represent the means ± S.D. of 15 single cell time-lapse measurements per condition (lower panel).
fragmentation of mitochondria during PCD through an inhibition of mitochondrial fusion.

Loss of OPA1 Facilitates and Accelerates the Release of Cytochrome c From Mitochondria—Recently, it was proposed that a reorganization of mitochondrial cristae is required for the redistribution of cytochrome c normally sequestered from the intermembrane space proper within cristae folds, thus allowing complete release of cytochrome c (9). Because we and others have demonstrated that OPA1 is required for maintenance of cristae structure and given that OPA1 co-released with cytochrome c (Fig. 2), the leak of an initial pool of cytochrome c might provide a basis for the hypothesis that OPA1 might require OPA1 mitochondrial cristae remodeling, thus permitting the release of additional pools.

To test this hypothesis, we studied the release of cytochrome c in vitro using mitochondria isolated from cells in which OPA1 protein expression was knocked down using siRNA, OPA1(−) mitochondria as well as mitochondria derived from control cells. Incubation for 20 min with increasing concentrations of recombinant tBid demonstrated that OPA1(−) mitochondria required about 10-fold lower tBid concentrations than control mitochondria to exhibit complete release of cytochrome c (Fig. 4, A and B). Next, in kinetic studies in vitro using 10 nM tBid for various times, we observed that release of cytochrome c was complete within 20 min in control mitochondria, whereas it was complete within only 15 min in OPA1(−) mitochondria (Fig. 4, C and D). Moreover, release of cytochrome c from OPA1(−) mitochondria was detected as early as 5 min after the addition of 10 nM tBid, whereas at the same time point release was not yet observed in mitochondria from control cells (Fig. 4, C and D). Similar results were obtained in all cases when oligomeric Bax was substituted for tBid (data not shown).

Recently, it was reported that OPA1 requires Mfn1 to promote mitochondrial fusion (23). Thus, mitochondria isolated from cells in which Mfn1 expression had been knocked down using siRNA were also investigated. Although mitochondria from Mfn1 knock down cells, Mfn1(−) mitochondria, were also fragmented as expected (24) (Fig. 5), Mfn1(−) mitochondria did not exhibit a complete release of cytochrome c at lower concentrations of tBid and did not show any differences in release as compared with control cells (Fig. 4). Thus, the effects of loss of OPA1 on cytochrome c release are not due to a general effect of increased mitochondrial fragmentation.

We studied the role of OPA1 loss on the release kinetics of cytochrome c more precisely using HeLa cells stably expressing cytochrome c-GFP (13). To assess the duration of cytochrome c release, the S.D. of the average brightness of all the pixels in the image of individual cells was measured. In this paradigm the S.D. is high under conditions of punctate fluorescence (e.g. when cytochrome c is within mitochondria) and low when fluorescence is diffuse, as occurs after cytochrome c release into the cytoplasm (13). As in OPA1(−) HeLa cells, knock down of OPA1 expression using siRNA in HeLa cells stably expressing cytochrome c-GFP also led to mitochondrial fragmentation and alteration of the cristae structure in comparison to control siRNA-transfected cells (Fig. 6A). Kinetic studies performed by analyzing OPA1 siRNA cells every 30 s revealed that the release of cytochrome c-GFP from all mitochondria occurred over a period of ~3.5 min (Fig. 6, B and C; see also Supplemental Video S1) regardless of the cell death signal used (actinomycin D or staurosporine), whereas complete release took ~5 min in control cells (Fig. 6, B and C; see also Supplemental Video S2), with the latter result similar to that previously reported by Goldstein et al. (13). In contrast, although siRNA-mediated knock down of Mfn1 in HeLa cells

![Figure 4](http://www.jbc.org/content/280/42/35747/F4.large.jpg)
stably expressing cytochrome c-GFP triggered mitochondrial fragmentation without modifying the internal cristae structure (Fig. 6A), it did not increase the rate of cytochrome c-GFP release, which occurred over ~5 min (Fig. 6, B and C; Supplemental Video S3), similar to that in control cells. In fact, the OPA1-dependent changes in cytochrome c release kinetics presented in Fig. 6 are probably underestimated, since in both control and Mfn1(−) cells the endogenous OPA1 is still being released. Overall, these findings are consistent with our in vitro results (Fig. 4), indicating that loss of OPA1, but not of Mfn1, from mitochondria facilitates the rapid and complete release of cytochrome c. Thus, this effect is likely due specifically to effects of OPA1 on cristae structure and not on its effect of promoting mitochondrial fusion.

**DISCUSSION**

**Insights into Mechanisms of Rapid and Complete Cytochrome c Release and Mitochondrial Fragmentation during Apoptosis—**Mitochondrial fusion is blocked close in time downstream to Bax/Bak-mediated MOMP, suggesting that inhibition of the fusion machinery is involved in mitochondrial fragmentation during PCD, in concert with an increase in Drp1-mediated mitochondrial fission (13). Our data demonstrating that OPA1, a dynamin-like GTPase required for mitochondrial fusion (5, 8), is released from mitochondria during PCD before mitochondrial fragmentation provides a compelling mechanism for these observed changes in fusion.

Another significant finding in this study is that loss of OPA1 accelerates cytochrome c release, which might account for the rapid and complete release seen during PCD. In fact, it has been estimated that only ~15% of the total mitochondrial cytochrome c is typically available in the intermembrane space (25), with the remaining cytochrome c sequestered across narrow junctions within mitochondrial cristae. However, it is well known that the complete release of cytochrome c is a rapid event during PCD (13). To explain this dichotomy, it has been suggested that mitochondrial cristae remodeling and exposure of these pools occurs but through an unknown mechanism (9). In fact, more recent studies have reported that OPA1 is involved in maintaining proper mitochondrial cristae structure (19, 21). When we studied the effects of OPA1 loss on cytochrome c release, we observed in OPA1(−) mitochondria that, once started, release reached completion more rapidly. These findings suggest that mitochondrial cristae rearrangements mediated by the loss of OPA1 participate in the release of cytochrome c. Last, in both intact cells and in vitro, OPA1 was co-released with cytochrome c. Thus, we propose a model whereby the first leak of OPA1 (along with cytochrome c) as a consequence of MOMP results in alterations in cristae structure and exposure of additional pools of OPA1 and cytochrome c, permitting subsequent release in a feed-forward reaction to completion. A very recent study has reported that mouse embryonic fibroblasts deficient in OPA1 through RNA-mediated interference had impaired mitochondrial fusion (26), in agreement with our results. These fibroblasts also exhibited severe cellular defects, including poor cell growth, widespread heterogeneity of mitochondrial membrane potential (∆ψm), and decreased cellular respiration (26), suggesting that mitochondrial fusion per se is important for mitochondrial function. In the context of PCD, the mitochondrial deficiency of OPA1 after its release into the cytoplasm may, thus, participate in the ∆ψm loss and decreased respiration that are observed during PCD (7).

Although our model supports the notion that mitochondrial fragmentation can occur from the release of OPA1 after MOMP, data from some other studies initially appear inconsistent. For example, overexpression of a dominant-negative form of Drp1, a dynamin-like GTPase involved in mitochondrial fission and fragmentation (5, 8), inhibits cytochrome c release during PCD (15). Nevertheless, in agreement with Gao et al. (16) and Esseiva et al. (17), we directly observed that mitochondrial morphology changes occur several minutes after the complete release of cytochrome c, suggesting that mitochondrial fragmentation may in fact not be involved in the release of cytochrome c and that the observed effects of dominant-negative Drp1 on release are due perhaps to other alterations in mitochondrial structure or function. Indeed, Drp1-dependent mitochondrial fission occurs continually in cells under normal conditions, without evidence of cytochrome c release (6). Moreover, the mitochondrial network in cells overexpressing the dominant-negative of Drp1 is very long and interconnected, and since cristae remodeling is important for the complete release of cytochrome c (9), impairment in such remodeling in the elongated, fused mitochondria might prevent the complete release of cytochrome c.

Concordant with the notion that mitochondrial fragmentation itself does not seem to be involved in the release of cytochrome c (whereas it may sensitize to cell death as we have recently reported (27)), we did not observe any effects of knock down of Mfn1 expression on its release, although the mitochondria were similarly fragmented as in OPA1(−) cells. In contrast to mitochondria from OPA1(−) cells, however, the cristae of mitochondria from Mfn1(−) cells do not show any obvious structural changes as compared with control mitochondria, supporting our proposal that cristae remod-
FIGURE 6. Knock down of OPA1 expression accelerates the kinetics of cytochrome c release in cells. A, HeLa cells stably expressing cytochrome c-GFP (13) were transfected sequentially 3 times with control, OPA1, or Mfn1 siRNAs as indicated. Mitochondrial morphology and ultrastructure were assessed as described in Fig. 3. C and D, scale bars represent 500 nm. B, left panel, HeLa cells stably expressing cytochrome c-GFP were transfected sequentially 3 times with control, OPA1, or Mfn1 siRNAs as indicated, and then cell death was induced by actinomycin D (10 μM) in the presence of the caspase inhibitor ZVAD-fmk (100 μM). The time (min) after exposure to actinomycin D is shown at the top or bottom of each frame; images were taken every 30 s. For each transfection condition, two cells showing a redistribution of the fluorescence signal in response to actinomycin D are presented. Right panel, for each transfection condition, individual traces plotted as % of initial S.D. versus time are shown for the two cells in the corresponding left panel. The release of cytochrome c-GFP was detected as a reduction in the S.D. of the GFP pixel intensity. Cell 1 is the upper cell and Cell 2 is the lower cell in each panel. Note that the slope of release is steeper in the OPA1 siRNA-transfected than in the control siRNA- or Mfn1 siRNA-transfected cells. C, the time required for complete release of cytochrome c-GFP was assessed during apoptosis induced by actinomycin D (ActD; 10 μM) or staurosporine (STS; 1 μM) in control-, OPA1-, and Mfn1-siRNA transfected cells. Statistical significance was assessed using Student’s t test (ns, no statistically significant difference). *, p < 0.05. Error bars represent S.D. For each condition, n = 25 cells.
eling may be necessary and sufficient for the complete release of cytochrome c.

In summary, our findings support a model in which MOMP and cytochrome c release precede mitochondrial fragmentation during PCD. The mitochondrial release of OPA1, a mitochondrial intermembrane space protein involved in maintaining cristae structure and required for mitochondrial fusion, facilitates the release of cytochrome c and itself through cristae remodeling in a feed-forward manner. The resultant loss of OPA1 from mitochondria as a consequence of its release inhibits mitochondrial fusion, thus promoting the characteristic mitochondrial fragmentation observed during PCD.

Acknowledgments—We thank Dr. R. J. Youle (NINDS, National Institutes of Health, Bethesda, MD) for helpful discussions and support, Dr. D. R. Green (La Jolla Institute for Allergy and Immunology, San Diego, CA) for providing HeLa cells stably expressing cytochrome c-GFP and for helpful discussions, Dr. A. van der Bliek (David Geffen School of Medicine, UCLA) and Dr. K. Mihara (Kyushu University, Fukuoka, Japan) for providing antibodies against OPA1 and Mfn1, respectively, and Plateforme Imagerie Dynamique (Institut Pasteur, Paris, France) and Dr. C. Smith (NINDS Light Imaging Facility, National Institutes of Health) for assistance with confocal microscopy.

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Release of OPA1 during Apoptosis Participates in the Rapid and Complete Release of Cytochrome c and Subsequent Mitochondrial Fragmentation
Damien Arnoult, Alain Grodet, Yang-Ja Lee, Jérôme Estaquier and Craig Blackstone

J. Biol. Chem. 2005, 280:35742-35750.
doi: 10.1074/jbc.M505970200 originally published online August 22, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M505970200

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