Identification of a Novel Protein MICS1 that is Involved in Maintenance of Mitochondrial Morphology and Apoptotic Release of Cytochrome c

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Mitochondrial morphology dynamically changes in a balance of membrane fusion and fission in response to the environment, cell cycle, and apoptotic stimuli. Here, we report that a novel mitochondrial protein, MICS1, is involved in mitochondrial morphology in specific cristae structures and the apoptotic release of cytochrome c from the mitochondria. MICS1 is an inner membrane protein with a cleavable presequence and multiple transmembrane segments and belongs to the Bi-1 super family. MICS1 down-regulation causes mitochondrial fragmentation and cristae disorganization and stimulates the release of proapoptotic proteins. Expression of the anti-apoptotic protein Bcl-XL does not prevent morphological changes of mitochondria caused by MICS1 down-regulation, indicating that MICS1 plays a role in maintaining mitochondrial morphology separately from the function in apoptotic pathways. MICS1 overproduction induces mitochondrial aggregation and partially inhibits cytochrome c release during apoptosis, regardless of the occurrence of Bax targeting. MICS1 is cross-linked to cytochrome c without disrupting membrane integrity. Thus, MICS1 facilitates the tight association of cytochrome c with the inner membrane. Furthermore, under low-serum condition, the delay in apoptotic release of cytochrome c correlates with MICS1 up-regulation without significant changes in mitochondrial morphology, suggesting that MICS1 individually functions in mitochondrial morphology and cytochrome c release.

INTRODUCTION

In eukaryotic cells, mitochondria continuously divide and fuse, resulting in the formation of tubular network structures (Yaffe, 1999; Griparic and van der Bliek, 2001; Mozdy and Shaw, 2003). Various and diverse cellular events give rise to dynamically changing mitochondrial networks (Youle and Karbowski, 2005; Chan, 2006; McBride *et al.*, 2006). Recent studies in yeast and mammalian cells revealed that the behaviors of these dynamic networks are controlled by distinct proteins, including OPA1/Mgm1, Drp1/Dnm1, Fis1, and Mfn1&2/Fzo1 (Yaffe, 1999; Jensen *et al.*, 2000; Karbowski and Youle, 2003; Westermann, 2003; Zhang and Chan, 2007). In addition, a variety of factors, including DAP3, MTP18, MIB, and endophilin B1, directly or indirectly regulate mitochondrial morphology in mammals (Karbowski *et al.*, 2004; Mukamel and Kimchi, 2004; Tondera *et al.*, 2005; Eura *et al.*, 2006). The mitochondrion is an organelle with two different membranes: the outer and inner

membranes. The outer membrane forms an envelope, and the inner membrane forms the cristae structure, comprising a narrow membrane that invaginates into the matrix. The cristae membrane is thought to be morphologically and functionally different from the inner boundary membrane along the outer membrane. For example, respiratory chains and ATP synthase preferentially distribute in the cristae membrane (Vogel *et al.*, 2006). In certain yeast ATP synthase mutants, intramitochondrial onion-like structures are observed (Paumard *et al.*, 2002). In mammals, mitofilin and OPA1 are involved in cristae morphology (Olichon *et al.*, 2003; John *et al.*, 2005). How the cristae structure is regulated and coordinated with the mitochondrial tubular network, however, remains unclear.

Mitochondria play a central role in the initial processes of apoptosis. The release of proapoptotic proteins from the mitochondria results in the processing and activation of downstream caspases, leading to the biochemical and morphological features of apoptosis (Green and Reed, 1998; Wang, 2001; Danial and Korsmeyer, 2004; Jiang and Wang, 2004). Among proapoptotic proteins, the efflux of cytochrome c is thought to be a key event in the subsequent apoptotic processes. A subclass of the Bcl-2 family proteins that includes Bax and Bak regulates the efflux by redistributing to the mitochondria, followed by outer membrane permeabilization (Sharpe *et al.*, 2004; Bouchier-Hayes *et al.*, 2005; Er *et al.*, 2006). The release of cytochrome c appears to involve two distinct pathways (Ott *et al.*, 2002; Scorrano *et al.*, 2002). One mediates the release depending on the outer

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Abbreviations used: Bi-1, Bax inhibitor-1; DSP, dithiobis (succinimidyl propionate); GFP, green fluorescent protein; LFG, life guard; OPA1, optic atrophy 1; RNAi, RNA interference.

membrane permeabilization by Bax, and the other additionally requires mobilization of cytochrome c in the cristae, which is induced by tBid. OPA1, a dynamin-related mitochondrial protein, controls mitochondrial membrane fusion through its proteolytic processing (Olichon *et al.*, 2003; Cipolat *et al.*, 2004; Ishihara *et al.*, 2006). In addition, OPA1 is involved in remodeling the cristae structure, leading to the efflux of cytochrome c during apoptosis (Frezza *et al.*, 2006).

In the present study, we characterized a new mitochondrial protein, MICS1, which localizes in the inner membrane. MICS1 is proposed to belong to the Bax inhibitor-1 (Bi-1) super family, which is comprised of at least four members, including the anti-apoptotic proteins Bi-1 and LFG (life guard; Somia *et al.*, 1999; Chae *et al.*, 2003, 2004). MICS1 down-regulation induced a failure to maintain the normal mitochondrial network and disorganization of the cristae. Furthermore, the proapoptotic proteins were rapidly released from the mitochondria of MICS1 knockdown cells during apoptosis. In contrast, an increase in the MICS1 gene dosage stabilized cytochrome c in the inner membrane independently from Bax targeting and permeabilization of the outer membrane during apoptosis. In addition, the retention of cytochrome c on the inner membrane correlated with MICS1 up-regulation in the low-serum conditions. Our findings suggest that MICS1 plays distinct roles in mitochondrial morphology and cytochrome c release.

MATERIALS AND METHODS

Cell Culture and DNA Transfection

HeLa cells were maintained and incubated for experiments at 37°C in DMEM supplemented with 10% fetal bovine serum, 4.5 g/l glucose, 50 U/ml penicillin, and 50 µg/ml streptomycin (medium A). Stable cell lines expressing a mitochondria-targeted Su9-DsRed (Taguchi *et al.*, 2007) or Bcl-XL (Otera *et al.*, 2005) were maintained in the medium A including 1 µg/ml puromycin. DNA transfection was carried out using FuGENE6 (Roche Diagnostics, Alameda, CA) according to the manufacturer's instructions. To obtain cells stably expressing cytochrome c-3FLAG, cells transfected with pTRE-puro-hcyc-3FLAG (see below) were grown in the medium A including 1 µg/ml puromycin, and individual colonies were harvested and grown to mass culture. The relative levels of expression of the protein were determined using immunoblotting, and those cell lines expressing cytochrome c-3FLAG were used in subsequent experiments.

Construction of Plasmids

The 1.0-kb PCR product containing the entire open reading frame for MICS1 was obtained by amplification with the primers (5'-ctggtgcaccgtgcacatgttgct3' and 5'-gaagctgagctcctcgagttctttctg3') using the human EST clone [IMAGE clone 25077 (accession number AF131820); Invitrogen, Carlsbad, CA] as a template and was cloned into pEF1-3HA, in which the inserted gene is expressed as a fusion protein tagged with three tandem HA by the EF1 promoter, to construct pEF1-MICS1-3HA. The same DNA fragment was also cloned into pNucScrIII, a vector containing humanized renilla green fluorescent protein (hrGFP) (Stratagene, La Jolla, CA) with three tandem SV40 nuclear localization signals the downstream of IRES (Internal Ribosomal Entry Site) sequence to simultaneously express both hrGFP and the inserted gene, to construct pNucScrIII-MICS1. The 1.2-kb DNA fragment including the coding region for MICS1-3HA from pEF1-MICS1-3HA was inserted into pcDNA3.1(-; Invitrogen) to construct pcDNA3.1-MICS1-3HA for in vitro translation. The DNA fragment coding for the carboxy-terminal region (amino acids 53-345) of MICS1 was cloned into pET28c (Novagen, Madison, WI) to construct pET28-MICS1 for preparation of recombinant protein as antigens. The human cytochrome c cDNA was amplified by reverse-transcriptase PCR using the primers (5'-aggatccaatatgggtgatgttgagaaaggca3' and 5'-ctcgagctcattagtagctttttgagata3') and cloned into p3FLAG-CMV14 (Sigma-Aldrich, St. Louis, MO), and then the 0.5-kb PCR fragment of the coding region for cytochrome c-3FLAG generated from the resulting plasmid was inserted into pTREpur3 (Clontech, Palo Alto, CA) to construct pTRE-puro-hcyc-3FLAG.

Preparation of Antibody

The MICS1 recombinant protein was expressed in the *Escherichia coli* BL21 (DE3) cells carrying pET28-MICS1, purified by SDS gel electrophoresis, eluted from the gels, and used for raising antibodies in rabbits. The anti-MICS1

serum was subjected to precipitation with ammonium sulfate, followed by dialysis to phosphate-buffered saline (PBS). The resultant fraction was then affinity-purified by incubation with nitrocellulose membrane strips to which the same recombinant protein was bound, followed by elution with 0.1 M glycine (pH 2.0). After dialysis, the eluate was used for immunoblotting.

Small Interference RNA Transfection

A small interference RNA (siRNA) duplex for MICS1 (sense, uagcaaccaag-caagaugcuuuggg; antisense, cccaagcaucuuugcuugguugcua; Invitrogen) or for OPA1 (Ishihara *et al.*, 2006) was transfected into HeLa cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After incubation for 48–72 h, cells were harvested and used for cell fractionation and immunoblotting. For morphological analyses, cells were transfected twice in a 48-h interval.

Subcellular Localization Analysis

Subcellular fractionation was performed as described previously (Nakamura *et al.*, 2004) with minor modifications. HeLa cells were washed with PBS, scraped off, and suspended in the homogenization buffer (10 mM HEPES-KOH, pH 7.4, 70 mM sucrose, and 0.22 M mannitol) containing protease inhibitor cocktail Complete EDTA-free (Roche Diagnostics). The cell suspension was passed 30 times through 27-gauge needle and then homogenized in Dounce homogenizer. After brief centrifugation (600 × g, 5 min), the supernatant was homogenized and then centrifuged at 5000 × g for 10 min to obtain the mitochondria fraction. The resultant supernatant was further centrifuged at 100,000 × g for 60 min to separate the microsomal and cytosolic fractions.

To examine submitochondrial localization, the isolated mitochondria fraction (30 µg) was treated with 50 µg/ml trypsin at 4°C for 30 min under either isotonic or hypotonic condition. After termination by addition of 10% TCA, proteins were precipitated and analyzed by SDS-PAGE and immunoblotting.

In Vitro Protein Import into Isolated Mitochondria

Cell-free protein synthesis was carried out using pcDNA3.1-MICS1-3HA as a template and TNT Quick Coupled Transcription/Translation System (Promega, Madison, WI) as described previously (Setoguchi *et al.*, 2006). Import of MICS1 into mitochondria was performed essentially as described previously (Kanaji *et al.*, 2000). In vitro-synthesized MICS1 was incubated at 30°C for 30 min in the buffer (50 mM sodium succinate, 5 mM Mg(OAc)₂, 1 mM ATP, and 0.5 mM NADH) with the mitochondria fraction (50 µg) prepared from HeLa cells in the presence of protease inhibitor cocktail Complete EDTA-free (Roche Diagnostics). If necessary, 0.1% Triton X-100 (Tx-100) was added to disrupt membranes after import reaction. After being treated at 4°C for 20 min with 50 µg/ml proteinase K, the reaction mixtures were subjected to analysis by SDS-PAGE and immunoblotting.

Cell Fractionation

To induce apoptosis, HeLa cells were incubated with 20 µM actinomycin D (Sigma-Aldrich) or 1 µM staurosporine (Sigma-Aldrich) in the presence or absence of 100 µM zVAD-fmk (Peptide Institute, Osaka, Japan). After incubation, cells were analyzed by immunofluorescence microscopy or cell fractionation. Cell fractionation using 0.2 mg/ml digitonin was carried out as described previously (Otera *et al.*, 2005).

Immunoblotting and Immunofluorescence Microscopic Analysis

Immunoblotting was performed as described previously (Oka *et al.*, 2004).

Immunofluorescence microscopy was carried out essentially as described previously (Ungar *et al.*, 2002) with minor modification as follows: cells grown on glass coverslips were treated for 8 h with 20 µM actinomycin D (Sigma-Aldrich) in the presence or absence of 100 µM zVAD-fmk (Peptide Institute) if necessary. Cells were fixed with 4% paraformaldehyde, permeabilized by incubation with 0.1% Tx-100 for 10 min, and incubated with primary antibodies. After incubation with Alexa-conjugated secondary antibodies (Invitrogen), images were acquired using a confocal microscope (Radiance 2000; Bio-Rad, Richmond, CA). Values (average ± SEM) of the efflux of cytochrome c and Smac and changes in mitochondrial morphology were calculated from data of at least three independent experiments, and 100–200 individual cells were counted in each experiment.

Antibodies used for immunoblotting and immunofluorescence microscopy were as follows: rabbit polyclonal antibodies: anti-Tim17 (Ishihara and Mihara, 1998), anti-Tim23 (Ishihara and Mihara, 1998), anti-Tim44 (Ishihara and Mihara, 1998), anti-Sec61β (Upstate Biotechnology, Lake Placid, NY), anti-Bax, NT (Upstate Biotechnology), anti-H450 (Ishihara *et al.*, 1990), anti-Tom20 (Iwahashi *et al.*, 1997), anti-HtrA2 (R&D Systems, Minneapolis, MN), anti-mitofilin (Eura *et al.*, 2006); mouse monoclonal antibodies: anti-cytochrome c (BD Biosciences, San Jose, CA), anti-Smac (BD Biosciences), anti-OPA1 (BD Biosciences), anti-Dlp1/Drp1 (BD Biosciences), anti-Tom20 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-GAPDH (Ambion, Austin, TX), anti-mHSP70 (Stressgen, San Diego, CA), anti-HA (16B12; Covance Laboratories,

Madison, WI), anti-FLAG (M2; Sigma); and anti-HA rat mAb (Roche Diagnostics).

Analysis of Cytochrome *c* Release in Semi-intact Cells

The preparation of semi-intact cells was performed essentially as described previously (Setoguchi *et al.*, 2006). After treatment for 8 h with dimethylsulfoxide (DMSO) or actinomycin D, MICS1-3HA-transfected cells on glass coverslips were washed, incubated at room temperature (RT) for 5 min with buffer A (20 mM HEPES-KOH, pH 7.4, 25 mM KCl, 2.5 mM Mg(OAc)₂, 0.25 M sucrose, and 1 μ M Taxol) containing 25 μ g/ml digitonin, and gently washed three times with the same buffer. If necessary, cells were incubated for 20 min at RT with 30 nM recombinant tBid (R&D Systems) in buffer A to induce cytochrome *c* release. The samples were washed and divided for two different purposes: 1) to confirm the induction of cytochrome *c* release, cells were fixed with 4% paraformaldehyde, completely permeabilized by incubating for 10 min with 0.1% Tx-100, and subjected to immunofluorescence analysis with antibodies to HA tag, cytochrome *c*, and Tim17, and 2) cells were incubated at RT for 60 min with both anti-mitofilin and anti-Tom20 antibodies in buffer A, washed, and fixed. After complete permeabilization, cells were incubated with anti-HA tag antibody and analyzed by immunofluorescence microscopy.

Chemical Cross-Linking and Immunoprecipitation

Cells were harvested, washed with PBS, and incubated with 0, 0.1, 0.25, or 0.5 mg/ml dithiobis (succinimidyl propionate) (DSP; Pierce, Rockford, IL) at 4 or 25°C for 15–30 min. After termination by addition of 50 mM Tris-HCl (pH 8.0), cells were solubilized at 4°C for 30 min in the buffer (50 mM Tris-HCl, pH 8.0, 0.1% SDS, 1% Tx-100, and 150 mM NaCl) and then centrifuged to remove insoluble materials. The resultant supernatant was incubated with anti-FLAG (M2) agarose (Sigma-Aldrich) for 3 h at 4°C, washed twice with PBS containing 1% Tx-100, and then washed once with PBS alone. The precipitates were analyzed by SDS-PAGE and immunoblotting.

Electron Microscopy

Electron microscopy immunoelectron microscopy were carried out as described previously (Eura *et al.*, 2003).

RESULTS

MICS1 Is a Mitochondrial Inner Membrane Protein with a Presequence

To find mitochondrial proteins that were poorly characterized or uncharacterized, we surveyed the database of the GFP-cDNA Localization Project (Simpson *et al.*, 2000), which provides information on putative intracellular localization of human proteins. Of ~1000 proteins for which information was available, one membrane protein with multiple transmembrane segments (UniGene Hs.352656) was selected. Because the protein was involved in maintaining the mitochondrial network and inner membranes (see below), it was named MICS1 (for *mitochondrial morphology and cristae* structure). To confirm the intracellular localization of MICS1, a carboxy-terminally HA-tagged MICS1 (MICS1-3HA) was introduced into HeLa cells. Immunofluorescence analysis revealed that MICS1 was found in the intracellular tubular network structures and colocalized with Tim17, a mitochondrial protein (Figure 1A). In subcellular fractionation, MICS1 was recovered in the mitochondrial fraction and was undetectable in the microsomal and cytosolic fractions (Figure 1C). These results indicate that MICS1 localizes in the mitochondria.

An anti-MICS1 antibody recognized a single band with a molecular size of 23 kDa in HEK293 and HeLa cell lysates (Figure 1B, solid arrowhead). When a plasmid carrying MICS1 was introduced into these cells, the same band was highlighted and an additional band with a molecular size of 26 kDa was detected (lane 3), suggesting that MICS1 is synthesized as a precursor. Similarly, MICS1-3HA expressed in HeLa cells migrated as a faster band compared with MICS1-3HA synthesized *in vitro* (Figure 1B, lanes 4 and 5). Therefore, *in vitro* mitochondrial import of MICS1 was examined (Figure 1D). When *in vitro*-synthesized MICS1 was

incubated with isolated mitochondria, a smaller band was detected (lane 2). The smaller band was resistant to proteinase K treatment (lane 3) and had the same molecular size as that of MICS1-3HA expressed in HeLa cells (lane 7). Furthermore, this processing was inhibited by CCCP, a protonophore that disrupts the membrane potential (lane 4). These results indicate that MICS1 is synthesized as a precursor with a cleavable presequence. In the amino-terminal region before the first hydrophobic segment, there were three putative sites recognized by the mitochondrial processing peptidase. To determine the presequence of MICS1, three truncated MICS1 proteins were synthesized *in vitro*. The MICS1 protein with a deletion (Δ 1–44) comigrated with the protein expressed in HeLa cells (Figure 1D, lanes 8 and 9), indicating that MICS1 has a cleavable presequence of 44 amino acid residues.

To examine the submitochondrial compartments in which MICS1 localizes, the mitochondrial fraction prepared from cells expressing carboxy-terminally 3FLAG-tagged MICS1 (MICS1-3FLAG) was treated with trypsin under isotonic and hypotonic conditions (Figure 1E). In both conditions, an outer membrane protein, Tom20, was sensitive to trypsin treatment, whereas a matrix protein, mtHSP70, was resistant. The inner membrane protein Tim17 was degraded only under hypotonic conditions that disrupt the outer membrane. Similarly, endogenous MICS1 was digested only under hypotonic conditions and converted to a 16-kDa protein. Under the same conditions, MICS1-3FLAG disappeared and smaller bands were not detected. These results indicate that MICS1 localizes in the inner membrane and its carboxy-terminal region faces the intermembrane space. In addition, alkaline extraction analysis indicated that MICS1 is an integral membrane protein (Figure 1F). Based on the deduced amino acid sequence, MICS1 is comprised mainly of seven hydrophobic segments and a carboxy-terminal hydrophilic domain with 50 amino acid residues. These findings led us to propose the topology for MICS1 shown in Figure 1G.

MICS1 Is Required for the Mitochondrial Tubular Network and Cristae Organization

Mitochondria are morphologically dynamic organelles that undergo continuous fission and fusion for maintenance of the tubular network structures (Yaffe, 1999; Griparic and van der Bliek, 2001). To examine the role of MICS1 in mitochondrial morphology, gene expression was repressed using RNA interference (RNAi). More than 80% of MICS1 was reduced by 48-h incubation with siRNA specific to MICS1 (Figure 2A). Because the mitochondrial tubular network is fragile, we used HeLa cells stably expressing Su9-DsRed, a mitochondria-targeted DsRed, to visualize the morphology without requiring cell fixation (Taguchi *et al.*, 2007). As shown in Figure 2B, MICS1 RNAi induced the appearance of moderately fragmented mitochondria (panel b, arrowhead) and lump-like structures near winding mitochondrial tubules (panel c). The siRNA for green fluorescent protein (GFP) did not induce these effects on the mitochondrial morphology (panel a). Furthermore, to examine the intramitochondrial ultrastructure, MICS1-knockdown cells were analyzed by conventional electron microscopy. The number of invaginations of the inner membranes, namely cristae, were clearly reduced in the MICS1-siRNA-transfected cells compared with control cells (Figure 2C). In addition, the cristae became curved and ring like structures of the inner membranes were commonly found in the MICS1-knockdown cells. Thus, MICS1 is crucial for mitochondrial networks and cristae organization.

Figure 1. MICS1 is a mitochondrial inner membrane protein with a presequence. (A) HeLa cells expressing MICS1-3HA were fixed, permeabilized, and stained with antibodies to HA tag and the mitochondrial protein Tim17. Images were obtained using confocal microscopy. Scale bar, 10 μ m. (B) Total cell lysates prepared from HEK293 (lane 1) and HeLa cells either untransfected (lane 2), transfected with MICS1 (lane 3) or with MICS1-3HA (lane 4), and MICS1-3HA synthesized in vitro using reticulocyte lysate (lane 5) were subjected to immunoblotting with an affinity-purified antibody to MICS1. Gray and solid arrowheads indicate precursor and mature forms of MICS1, respectively. (C) Total lysate (T) from HeLa cells was fractionated and separated into mitochondria-enriched (Mt), microsomal (Ms), and cytosolic (Cyto) fractions. The resultant fractions were subjected to immunoblotting with antibodies to MICS1, Tim17, the ER protein Sec61 β , and the cytosolic protein H450. (D) In vitro-synthesized MICS1-3HA was incubated at 30°C for 30 min with the mitochondria fraction with (lanes 4, 5) or without 0.2 mM CCCP (lanes 2, 3, and 6). After adding Tx-100 (lane 6), the reaction mixtures were treated with proteinase K (lanes 3, 5, and 6). In addition to the mixtures, 25% input of MICS1-3HA (lane 1), cell lysate from MICS1-3HA-expressing cells (lanes 7 and 8) and in vitro-synthesized MICS1 with an amino-terminal deletion of either 44 (lane 9), 56 (lane 10), or 83 amino acid residues (lane 11) were subjected to immunoblotting with anti-HA antibody. The asterisk indicates MICS1 synthesized from an internal methionine. p, precursor; m, mature. (E) Mitochondria fraction prepared from cells expressing MICS1-3FLAG was treated at 4°C for 30 min with or without 50 μ g/ml trypsin under isotonic (Mt) or hypotonic conditions (MP) and analyzed by immunoblotting with antibodies to FLAG tag, MICS1, the outer membrane protein Tom20, the inner membrane protein Tim17, and the matrix protein mtHSP70. Solid and gray arrowheads indicate

entire and truncated proteins, respectively. (F) Mitochondria fraction was incubated with or without 0.1 M Na₂CO₃ (pH 11.5), centrifuged to separate the supernatant (S) and pellet fractions (P), and analyzed by immunoblotting with antibodies to MICS1, mtHSP70, the integral membrane protein Tom40. (G) Topologic model of MICS1. IM, inner membrane. IMS, intermembrane space.

When MICS1 was overexpressed in wild-type HeLa cells, the mitochondrial morphology was drastically affected. To further investigate the effects of exogenous expression of MICS1 on mitochondrial morphology without fixing the cells, we used the Su9-DsRed-expressing HeLa cells and a plasmid coexpressing MICS1 and a nuclear targeted GFP under a single promoter. Immunofluorescence analysis showed that the GFP intensity was proportionally related to the MICS1 expression level. As a result, GFP was a good indicator of the degree of MICS1 expression. The degree of the morphological changes was correlated with the expression level of MICS1. More than 80% of the MICS1-expressing cells exhibited mitochondrial fragmentation and/or aggregation (Figure 2D). When MICS1 was highly expressed, mitochondria aggregated around the nucleus (panels c and d, white arrowheads). It is likely that the aggregated mitochondria in the MICS1-overexpressing cells exhibited a perinuclear localization after cell fixation (see Figures 2E and 4A). To further examine the mitochondrial structure, cells expressing MICS1-3HA were analyzed by immunoelectron microscopy (Figure S1). In the MICS1-overexpressing cells

(panel b), mitochondria that were heavily labeled with gold particles were vacuolated and found near the nucleus. In contrast, no such mitochondria were observed when MICS1-3HA was slightly expressed (panel a). In addition, overexpression of MICS1 influenced not only the mitochondrial morphology but also membrane potential. Staining with Mitotracker, a membrane potential-dependent mitochondrial dye, completely vanished in the MICS1-overexpressing cells, but not in the cells overproducing Tim23, an inner membrane protein with multiple hydrophobic segments (Figure 2E). Cells overexpressing MICS1 were not stained with propidium iodide, indicating that loss of mitochondrial membrane potential is not due to cell death.

MICS1 Down-Regulation Stimulates the Release of Cytochrome c during Apoptosis

MICS1 is predicted to belong to the Bi-1 super family, which includes Bi-1 and LFG (Chae *et al.*, 2003). The Bi-1 knockout cells are sensitive to reagents that induce ER stress, suggesting that Bi-1 controls ER stress-induced apoptosis (Chae *et al.*, 2004). Overexpression of LFG protects cells from Fas-

Figure 2. MICS1 is crucial for mitochondrial morphology and cristae organization. (A) Total cell lysates from HeLa cells transfected with an siRNA for either GFP or MICS1 were analyzed by immunoblotting with antibodies to MICS1 or GAPDH as a loading control. (B) HeLa cells stably expressing a mitochondria-targeted Su9-DsRed were transfected with a siRNA for either GFP (control) or MICS1. Live images were acquired by confocal microscopy. Arrowhead indicates mitochondrial fragmentation. A typical image including short and winding tubules and lump-structures was superimposed. Scale bar, 10 μ m. Data represent average $\pm$ SEM of three independent experiments; 100–200 individual cells were counted. (C) Cells transfected with a siRNA for either GFP (control) or MICS1 were fixed, and thin sections were visualized by electron microscopy. White and black arrowheads, normal and MICS1-knocked down mitochondria, respectively. Scale bar, 400 nm. (D) HeLa cells stably expressing Su9-DsRed were transfected with the plasmid coexpressing MICS1 and a nuclear targeted GFP (a and b), and analyzed without fixation by confocal microscopy as described in (B). High magnification of the images is shown (panels c and d). White and black arrowheads indicate cells with aggregated and fragmented mitochondria, respectively. Scale bar, 10 μ m. (E) Cells transfected with the plasmid carrying MICS1-3HA (a–c) or Tim23-3FLAG (d–f) were stained with Mitotracker, washed, fixed, and then incubated with anti-tag antibody, followed by immunofluorescence analysis. Scale bar, 10 μ m.

mediated apoptosis (Somia *et al.*, 1999). To assess the roles of MICS1 in apoptosis, the MICS1 siRNA-transfected cells were treated with apoptosis-inducing reagents. In the MICS1 knockdown cells, most of the cytochrome c was maintained in the mitochondria when treated with DMSO (Figure 3Ag), indicating that MICS1 down-regulation alone does not influence the targeting and localization of cytochrome c. When treated with actinomycin D in the presence of zVAD-fmk, a pan-caspase inhibitor, the release of cytochrome c into the cytoplasm was clearly enhanced by MICS1 RNAi (Figure 3A, compared with j). A similar effect was observed with staurosporine treatment (Figure 3B, left panel). The stimulatory effect of MICS1 RNAi on cytochrome c release was blocked by the exogenous expression of MICS1, but not that of Tim23 (Figure 3B, right panel), indicating that the increased release of cytochrome c is due to MICS1 down-regulation. The remarkable effect of MICS1 RNAi on cytochrome c release was observed in an early phase (Figure 3C). With 4-h actinomycin D incubation, 40.3% of the MICS1-knockdown cells exhibited cytoplasmic localization of cytochrome c, whereas 6.3% of the control

cells exhibited this localization. In addition to the microscopic analyses, cellular fractionation showed that MICS1 knockdown increased the amount of cytochrome c recovered from the supernatant fraction when incubated for 4 h with actinomycin D (Figure 3D). These results demonstrate that MICS1 down-regulation stimulates the apoptotic release of cytochrome c from the mitochondria.

In addition to cytochrome c, mitochondrial proapoptotic proteins are released into the cytoplasm during apoptosis (Saelens *et al.*, 2004). To investigate the effects of MICS1 knockdown on the release of the proapoptotic proteins, Smac (also named DIABLO) was examined. The release of Smac into the cytoplasm was accelerated by MICS1 RNAi (Figure 3E). The time course of Smac release was similar to that of cytochrome c release. Similar results were obtained with regard to the cytoplasmic release of another proapoptotic protein, HtrA2 (also referred to as Omi), suggesting that MICS1 down-regulation facilitates the release of the proapoptotic proteins as well as cytochrome c.

MICS1 down-regulation caused abnormal mitochondrial morphology and rapid apoptotic release of cytochrome c. To

Figure 3. MICS1 down-regulation induces the apoptotic release of cytochrome c and Smac. (A) HeLa cells transfected with a siRNA for either GFP (a–f) or MICS1 (g–l) were incubated for 8 h with DMSO or actinomycin D, then fixed, and immunostained with antibodies to cytochrome c and Tim17. Scale bar, 10 μ m. (B) Left panel, after transfection with the siRNA for either GFP (white) or MICS1 (blue), cells were incubated for 8 h with DMSO, staurosporine (STS), or actinomycin D (ActD) and analyzed by immunofluorescence microscopy as described in A. Right panel, MICS1 siRNA-transfected cells were further transfected with plasmid carrying either MICS1 or Tim23, treated with actinomycin D, and analyzed by immunofluorescence microscopy. Data represent average $\pm$ SEM of three independent experiments; 100–200 individual cells were counted. (C) After transfection with the siRNA for either GFP (white) or MICS1 (blue), cells were treated for 0, 4, 8, or 12 h with actinomycin D and analyzed by immunofluorescence microscopy as described in B. (D) Cells transfected with the siRNA for GFP or MICS1 were treated with DMSO (–) or actinomycin D (+), fractionated into the membrane (ppt) and cytosolic fractions (sup), and subjected to immunoblotting with antibodies to cytochrome c (cyt. c), the cytosolic protein H450 and the mitochondrial membrane protein Tim17. (E) Experiments were performed as described in C, except for immunofluorescence analysis using the anti-Smac antibody. (F) HeLa cells stably expressing Bcl-XL were transfected with the siRNA for GFP or MICS1. Left panel, the cells were incubated with DMSO or ActD, and apoptotic release of cytochrome c was analyzed as described in B. Right panel, the cells were further transfected with the Su9-DsRed expression plasmid, and mitochondrial morphology was analyzed by confocal microscopy. Data represent average $\pm$ SEM of three independent experiments; 100–200 individual cells were counted.

distinguish contribution of MICS1 to mitochondrial morphology from its role in apoptotic pathways, HeLa cells stably expressing an anti-apoptotic protein Bcl-XL (Otera *et al.*, 2005) were used. In the Bcl-XL stable cell lines, most of the cytochrome c (more than 85%) was retained in the mitochondria during apoptosis even when the siRNA for MICS1 was transfected (Figure 3F, left panel), indicating that the increased expression of Bcl-XL suppresses the apoptotic processes. In contrast, MICS1 knockdown clearly induced mitochondrial fragmentation (Figure 3F, right panel). It should be noted that mitochondria in the Bcl-XL stable cell lines seemed to be easily fragmented even when the GFP siRNA was transfected, presumably because of exogenous expression of the carboxy terminal tail-anchored protein, which induces changes in mitochondrial morphology (Horie *et al.*, 2002). Thus, MICS1 regulates mitochondrial morphology independently of the apoptotic processes.

MICS1 Overexpression Partially Inhibits Cytochrome c Release during Apoptosis

To examine the effects of MICS1 overexpression on cytochrome c release, cells transfected with the plasmid carrying MICS1-3HA were treated with actinomycin D. Even when incubated with actinomycin D, most cytochrome c in the MICS1-3HA-expressing cells was still retained in a perinu-

clear compartment (Figure 4A, arrowhead), overlapping with the localization of Tim17, which is consistent with the observation that mitochondria in the MICS1-transfected cells aggregated around the nucleus (Figure 2D). Only 15.6% of MICS1-3HA-transfected cells exhibited obvious cytoplasmic staining of cytochrome c after 8-h treatment with actinomycin D (Figure 4B), whereas there were no significant effects in vector or Tim23 expression plasmid-transfected cells (45.3 or 45.2% of cells with cytochrome c release, respectively). These results indicate that cytochrome c is retained in the mitochondria of MICS1-overproducing cells. Similar results were obtained when MICS1 lacking the carboxy-terminal hydrophilic domain was overexpressed, indicating that the flanking region extruding into the intermembrane space is not necessary for the retention of cytochrome c. Likewise, the number of abnormal nuclei induced during apoptosis was reduced in the MICS1-overexpressing cells compared with control cells (Figure 4C). There were no significant differences in the numbers of surviving cells, however, between the control and MICS1-transfected cell populations during apoptosis. These findings suggest that MICS1 overexpression delays cytochrome c release and subsequent processes, including nuclear fragmentation, but fails to protect cells from apoptotic death. To biochemically analyze cytochrome c release in the MICS1-overexpressing cells,

Figure 4. MICS1 overproduction partially inhibits the apoptotic release of cytochrome c. (A) Cells transfected with the plasmid carrying MICS1-3HA were treated for 8 h with DMSO (a–f) or actinomycin D (g–i), fixed, and immunostained with antibodies to HA tag and cytochrome c. Arrowheads indicate MICS1-3HA transfected cells with cytochrome c retained. Scale bar, 10 μ m. (B) Cells transfected with a vector or the plasmid carrying MICS1-3HA were treated with DMSO or actinomycin D, immunostained, and analyzed as described in A. Data represented average $\pm$ SEM of three independent experiments; 100–200 individual cells were counted. (C) Experiments were performed as described in B except for staining with DAPI. (D) Cells transfected with the plasmid expressing Tom20(N33)-GFP or GFP-OMP25 were treated with actinomycin D, fixed, and analyzed by immunofluorescence with antibodies to GFP and cytochrome c. Arrowheads indicate the transfected cells with cytochrome c released. Scale bar, 10 μ m. (E) MICS1-3HA transfected cells were analyzed as described in A except for immunostaining with antibodies to HA tag, Smac, or HtrA2. Arrowheads, the transfected cells with either Smac or HtrA2 released. Scale bar, 10 μ m. (F) Cells transfected with the siRNA for GFP (control) or OPA1 were further transfected with a vector or the plasmid carrying MICS1-3HA, treated for 8 h with actinomycin D, immunostained, and analyzed as described in B.

we attempted to isolate cell lines stably expressing MICS1-3HA because the transfection efficiency of the MICS1 plasmid was low. Despite several efforts using three different expression plasmids with strong promoters, we did not obtain cell lines that overproduced MICS1-3HA. All cell lines we isolated expressed MICS1-3HA levels $\sim$ 10-fold lower than the endogenous protein, suggesting that continuous overproduction of MICS1 is cytotoxic.

Apoptotic release of cytochrome c depends on the mitochondrial morphology. In particular, down-regulation of Drp1 or Fis1, responsible for mitochondrial membrane fission, prevents cytochrome c from being released from the mitochondria (Karbowski and Youle 2003; Lee *et al.*, 2004). To examine the possibility that mitochondrial aggregation inhibits cytochrome c release, we used two mitochondria-targeted GFP proteins, Tom20(N33)-GFP and GFP-OMP25. Overexpression of either of these GFP fusion proteins frequently induces mitochondrial aggregation (Kanaji *et al.*, 2000; Horie *et al.*, 2002). As expected, mitochondria aggregated around the nucleus after transfection of these GFP fusion proteins. None of the GFP fusion proteins, however, influenced the release of cytochrome c during apoptosis (Figure 4D, arrowheads), indicating that the inhibition of cytochrome c release is not due to mitochondrial aggregation.

Similar to cytochrome c, proapoptotic proteins, including Smac and HtrA2, were rapidly released into the cytoplasm in the MICS1-knockdown cells (Figure 3E). We therefore examined the effects of MICS1 overexpression on the release of proapoptotic proteins. Surprisingly, both Smac and HtrA2 were normally released into the cytoplasm, regardless of the expression of MICS1-3HA (Figure 4E). There were no significant differences in the release efficiencies of Smac and HtrA2 between untransfected and MICS1-transfected cells, indicating that the release of Smac and HtrA2 is not affected by MICS1 overproduction.

OPA1 is thought to be involved in cristae remodeling and its inactivation stimulates apoptosis-induced release of cytochrome c (Olichon *et al.*, 2003; Lee *et al.*, 2004; Frezza *et al.*, 2006). To examine whether OPA1 is required for the inhibition of cytochrome c release induced by MICS1 overexpression, OPA1 was knocked down in the MICS1-overexpressing cells. In control cells, apoptotic release of cytochrome c was stimulated by OPA1 RNAi as reported previously (Olichon *et al.*, 2003; Lee *et al.*, 2004). OPA1 knockdown enhanced cytochrome c release even when MICS1 was overexpressed (Figure 4F), indicating that OPA1 contributes to MICS1-mediated retention of cytochrome c in the mitochondria during apoptosis. It should be noted that OPA1 down-regulation did not completely cancel the effect of MICS1

Figure 5. MICS1 does not influence Bax targeting and outer membrane permeabilization. (A) Cells transfected with the plasmids carrying either MICS1-3HA (a–j), Bi-1-3HA (k–o), or LFG-3HA (p–t) were treated for 8 h with DMSO or actinomycin D, fixed, and subjected to immunofluorescence analysis using antibodies to HA tag, Bax, and cytochrome c. Higher magnification images of the Bax staining are shown (b, g, l, and q). Arrowheads indicate Bax-positive cells expressing either MICS1-3HA, Bi-1-3HA, or LFG-3HA. Scale bar, 10 μ m. Data represent average $\pm$ SEM of three independent experiments; 100–200 individual cells were counted. (B) MICS1-3HA-transfected cells were treated for 8 h with DMSO or actinomycin D, digitonin-permeabilized, incubated with antibodies to an outer membrane protein Tom20 and an inner membrane protein mitofilin, and analyzed as described in *Materials and Methods*. Arrowhead indicates the MICS1-3HA transfected cell stained by anti-mitofilin antibody. Scale bar, 10 μ m. (C) Top panels, cells were digitonin-

permeabilized and incubated with a recombinant tBid, and the cytochrome c release was analyzed by immunofluorescence microscopy with antibodies to HA tag, Tim17, and cytochrome c. Arrowheads indicate the MICS1-3HA expressing cells with cytochrome c retained. Bottom panels, semi-intact cells were incubated with a recombinant tBid, washed, further incubated with antibodies to Tom20 and mitofilin, and analyzed as described in B. Arrowheads indicate transfected cells stained by anti-mitofilin antibody. Scale bar, 10 μ m.

overexpression on cytochrome c release, suggesting that MICS1 play a distinct role in the apoptotic release of cytochrome c.

MICS1 Does Not Influence Bax Targeting and Mitochondrial Outer Membrane Permeabilization during Apoptosis

Bax, a cytoplasmic protein under healthy conditions, translocates to the mitochondria after proapoptotic stimulation and then oligomerizes on the outer membrane. The oligomerized form is considered to be crucial for mitochondrial outer membrane permeabilization to facilitate the efflux of proapoptotic proteins (Sharpe *et al.*, 2004; Er *et al.*, 2006). Therefore, mitochondrial targeting of Bax was examined in the MICS1-3HA-transfected cells. Immunofluorescence analysis revealed Bax as dot-like staining on the mitochondria after treatment with actinomycin D, whereas the signal was undetectable when incubated with DMSO (Figure 5A, a and e). In the MICS1-transfected cells in which cytochrome c was retained, Bax clearly targeted the mitochondria (Figure 5Ae, arrowheads). The ratio of Bax-positive cells was similar between control and MICS1-transfected cells (22.8 ± 1.2 and $23.7 \pm 1.6\%$, respectively). Bax was not easily detected because of its dot-like staining and mitochondrial fragmentation in apoptotic cells, likely resulting in underestimation of the number of Bax-positive cells. These results indicate that although Bax targeted the mitochondria normally, cytochrome c was still retained in the MICS1-transfected cells.

Bi-1 and LFG, two Bi-1 super family members, are anti-apoptotic proteins located in the ER and endomembrane compartments, respectively (Somia *et al.*, 1999; Chae *et al.*, 2004). We examined the effects of these proteins on Bax targeting and cytochrome c release. Neither protein influenced the mitochondrial targeting of Bax (Figure 5A, i and m, arrowheads). Moreover, the release of cytochrome c was usually observed in Bax-targeted cells in which these proteins were overexpressed. Thus, Bi-1 and LFG are not implicated in an apoptotic pathway via mitochondria.

After targeting the mitochondria, Bax conformationally changes and oligomerizes on the outer membrane, thereby inducing outer membrane permeabilization (Antonsson *et al.*, 2000). To examine whether outer membrane permeabilization was induced in the MICS1-transfected cells, we assessed the accessibility of an antibody to an inner membrane protein in semi-intact cells. Treatment with a low concentration of digitonin disrupts the plasma membrane without affecting the integrity of the mitochondrial outer membrane (Setoguchi *et al.*, 2006). After transfecting the cells with an MICS1-3HA expression plasmid, cells were treated with either DMSO or actinomycin D, permeabilized with digitonin, and incubated with antibodies to an outer membrane protein, Tom20, and an inner membrane protein, mitofilin, exposing a large hydrophilic domain to the intermembrane space (John *et al.*, 2005). When incubated with DMSO, mitochondria were immunostained by the anti-Tom20 antibody, but not the anti-mitofilin antibody (Figure 5B, a and b), indicating that treatment with digitonin induces perforation of only the plasma membrane; the outer membrane remains intact. After actinomycin D treatment, the mitochondria were clearly immunostained by the anti-mitofilin antibody, and the staining overlapped with staining by the anti-Tom20 antibody (Figure 5B, e–h). Thus, the anti-mitofilin antibody could access the inner membrane if the outer membrane was permeabilized. In the MICS1-transfected cells, mitochondrial staining by the anti-mitofilin antibody was observed only when incubated with actinomycin D (Figure 5Be, arrowhead), indicating that outer membrane permeabilization occurs in MICS1-overproducing cells. In addition to the apoptosis-inducing reagent, we attempted tBid-mediated cytochrome c release using the semi-intact cells. The MICS1-transfected cells were digitonin-permeabilized, incubated with a recombinant tBid, and subjected to immunofluorescence analysis. On treatment with tBid, cytochrome c was released into the cytoplasm of the untransfected cells, whereas cytochrome c was retained in the mitochondria of MICS1-overexpressing cells (Figure 5Ce, arrowheads). Under the same conditions, as well as under treatment with

Figure 6. Coprecipitation of MICS1 with cytochrome c. (A) Cells stably expressing cytochrome c-3FLAG were incubated at 4 or 25°C with DSP at the indicated concentrations, solubilized, and subjected to immunoprecipitation with anti-FLAG antibody, followed by immunoblotting with antibodies to FLAG tag, MICS1, and inner membrane proteins (Tim23, Tim44). Input, 10% of solubilized cell lysates. (B) Cells transfected with the plasmids carrying MICS1-3HA, Bi-1-3HA, or LFG-3HA were incubated with or without DSP and analyzed by immunoprecipitation and immunoblotting. Input, 10% of solubilized cell lysates. L.C., light chains of immunoglobulins.

actinomycin D, the anti-mitofilin antibody recognized the inner membrane of the semi-intact cells in which MICS1 was overproduced (Figure 5Cm, arrowheads). These results demonstrate that MICS1 overexpression does not influence Bax targeting and outer membrane permeabilization during apoptosis.

MICS1 Coprecipitates with Cytochrome c

Despite permeabilization of the outer membrane, cytochrome c was retained in the mitochondria of MICS1-overproducing cells. To examine the possibility that MICS1 interacts with cytochrome c in the inner membrane, immunoprecipitation with cytochrome c was examined. Because commercially available anti-cytochrome c antibodies were not to sufficiently specific for efficient immunoprecipitation, a carboxy-terminally three FLAG-tagged cytochrome c (cytochrome c-3FLAG) was stably expressed in HeLa cells and confirmed to have the same behavior as an endogenous protein with regard to the MICS1-mediated inhibition of release from the mitochondria. Without cross-linkers, MICS1 was not coprecipitated by pull-down of cytochrome c-3FLAG. Therefore, the membrane-permeable, thiol-cleavable cross-linker DSP was used before membrane solubilization. Cells were harvested, incubated with DSP, solubilized with detergents, and subjected to immunoprecipitation with an anti-FLAG antibody. MICS1 was clearly coprecipitated with cytochrome c-3FLAG and the amount was DSP concentration-dependent (Figure 6A). The inner membrane

proteins Tim23 and Tim44, however, were not detected, indicating that MICS1 specifically interacts with cytochrome c. The interaction of exogenously expressed MICS1 with cytochrome c was examined. After transfection with the plasmid for either MICS1-3HA, Bi-1-3HA, or LFG-3HA, cells were incubated with DSP, solubilized, and immunoprecipitated with anti-FLAG antibody. MICS1-3HA, but not Bi-1-3HA and LFG-3HA, was significantly coprecipitated with cytochrome c-3FLAG in a DSP-dependent manner (Figure 6B). Thus, cytochrome c interacts with both endogenous and exogenous MICS1, but not with Bi-1 and LFG.

Low Serum Conditions Up-Regulate MICS1 Expression and Delay Apoptotic Release of Cytochrome c

MICS1 overproduction not only delayed cytochrome c release, but also induced abnormal mitochondrial morphology. Bcl-XL expression in the MICS1 knockdown cells showed that MICS1 functions in maintaining the tubular networks independent of the apoptotic processes (Figure 3F). Conversely, to assess the role of MICS1 in the cytochrome c release separately from the effects of morphological changes, we surveyed cell culture conditions in which MICS1 expression was increased but caused no or fewer changes in mitochondrial morphology. We found that MICS1 expression was up-regulated when HeLa cells were cultured in low-serum medium. After transferring to medium containing 0.5% serum, the amount of MICS1 gradually increased, and at the 72-h incubation it was threefold

Figure 7. Low-serum conditions induce MICS1 up-regulation and delay the apoptotic release of cytochrome c. (A) HeLa cells were cultured in medium containing 0.5% serum for the indicated times and analyzed by immunoblotting with antibodies to MICS1 and Tom40. (B) Cells expressing Su9-DsRed were cultured for 72 h in the medium containing either 10% (a) or 0.5% FBS (b). Live images were collected by confocal microscopy. Scale bar, 10 μ m. (C) Cells cultured for 72 h in medium containing either 10% or 0.5% serum were treated for 0, 6, 9, or 12 h with actinomycin D. Total cell lysates were analyzed by immunoblotting with antibodies to OPA1 or Drp1 as a loading control. (D) Cells cultured for 72 h in medium containing either 10% or 0.5% serum were treated for 8 h with actinomycin D, fractionated using digitonin into the membrane (ppt) and cytosolic fractions (sup), and analyzed by immunoblotting with antibodies to the indicated proteins. Values (%) of cytochrome c released in the cytosolic fraction were shown. cyt. c, cytochrome c. (E and F) Cells were transfected for 72 h with the siRNA for either MICS1 (E) or OPA1 (F) in the 0.5% serum medium. Cell fractionation and immunoblotting were performed as described in D.

higher than the initial amount (Figure 7A). In contrast, levels of Tom40, a mitochondrial membrane protein, did not change after the shift to the low-serum medium. In the 0.5% serum medium, cells grew slowly but looked normal presumably because of a high concentration of glucose for providing enough energy. Under these conditions, mitochondria still maintained their tubular structures and did not aggregate (Figure 7B). Therefore, to examine the cytochrome c release under the low-serum condition, cells grown in medium containing 10 or 0.5% serum were treated with actinomycin D, fractionated, and separated into cytosolic and membrane fractions. In the 10% serum condition, most of the cytochrome c was recovered in the supernatant fraction after actinomycin D treatment. In contrast, in the 0.5% serum medium, half of the cytochrome c was retained in the membrane fraction after 6-h actinomycin D treatment (Figure 7D). Eventually, most of the cytochrome c was released, even in the low-serum condition. As expected, MICS1 increased under the low-serum condition even in the presence of actinomycin D. Thus, the low-serum condition not only induces MICS1 expression but also delays cytochrome c release. To test whether the delay in cytochrome c release requires MICS1, MICS1 was knocked down under the low-serum condition. In the MICS1 knockdown cells, most of the cytochrome c was recovered in the supernatant fraction after 6-h actinomycin D treatment. Under the same treatment, half of the cytochrome c was still present in the membrane fraction of the GFP siRNA-transfected cells (Figure 7E), indicating that MICS1 is crucial for the delay of the cytochrome c release under the low-serum condition. Together with the finding that overexpression of exogenous MICS1 delayed the apoptotic release of cytochrome c in normal-serum level medium (Figure 4A), these results suggest that MICS1 helps to retain cytochrome c in the inner membrane, apart from the morphological changes.

OPA1 was involved in the retention of cytochrome c induced by MICS1 overexpression (Figure 4F). The proteolytic processing of OPA1 is crucial for regulation of mitochondrial fusion, and treatment with actinomycin D or CCCP stimulates the processing (Ishihara *et al.*, 2006; Duvezin-Caubet *et al.*, 2007). Therefore, the OPA1 amount and processing were examined in the low-serum condition. OPA1 expression was unaffected when cultured in the 0.5% serum medium, and the time course of the OPA1 processing induced by actinomycin D treatment was unchanged (Figure 7C). Thus, the delay in the cytochrome c release is not caused by changes in the OPA1 processing. Because OPA1 contributed to the retention of cytochrome c caused by MICS1 overproduction in normal-serum level medium (Figure 4F), the effect of OPA1 RNAi was then examined under the low-serum condition. In the low-serum condition, more than 90% of OPA1 was knocked down by the OPA1-specific siRNA. After actinomycin D treatment, most of the cytochrome c was released into the supernatant fractions of the OPA1 knockdown cells, whereas more than half of the cytochrome c was still present in the membrane fractions of the GFP siRNA-transfected cells (Figure 7F). In addition, OPA1 knockdown did not influence MICS1 up-regulation. These results indicate that OPA1 itself, but not its processing, is responsible for the delay in the cytochrome c release under the low-serum condition, as well as MICS1. It should be noted that actinomycin D-dependent processing of OPA1 was enhanced by MICS1 knockdown (Figure 7E), suggesting that MICS1 may participate in OPA1 processing.

DISCUSSION

In this study, we found a new mitochondrial inner membrane protein, MICS1, that is involved in maintenance of mitochondrial morphology and cristae structures and regulates the apoptotic release of cytochrome c from the mitochondria. A cDNA coding for MICS1 was originally isolated using the subtraction approach as a gene that is repressed in transgenic mice expressing a growth hormone antagonist (Li *et al.*, 2001), suggesting that MICS1 is a growth hormone-inducible protein. Unfortunately, we failed to detect human growth hormone-induced MICS1 protein expression in human lymphocyte IM-9 cells, but MICS1 was ubiquitously expressed in all cell lines tested (unpublished data). MICS1 belongs to the Bi-1 super family, members of which share conserved transmembrane domains containing six or seven hydrophobic segments (Chae *et al.*, 2003). Only two of the members, Bi-1 and LFG, have been characterized to function as anti-apoptotic proteins in ER stress- and Fas-mediated pathways, respectively (Somia *et al.*, 1999; Chae *et al.*, 2004). Because MICS1 locates in the mitochondria and negatively regulates the apoptotic release of cytochrome c, it is likely that MICS1 acts as a unique regulator of apoptotic pathways via mitochondria in the Bi-1 super family.

MICS1 down-regulation induced abnormal mitochondrial morphology; that is, mild fragmentation, lump-like structures, and short curved tubules. Such changes in mitochondrial morphology were also observed in the Bcl-XL-expressing cells when MICS1 was repressed, indicating that MICS1 is involved in maintaining mitochondrial tubular networks separately from the role in apoptotic pathways. A similar mitochondrial morphology was observed in the body wall muscle cells of nematode when the *Caenorhabditis elegans* homologue (K11H12.8) of MICS1 was knocked down by RNAi (R. Ichishita, T. Oka, and K. Mihara, unpublished data), suggesting that the roles of MICS1 in mitochondrial morphology are conserved. Loss of Drp1, Fis1, OPA1, or Mfn1/2 governing membrane fusion and fission induces drastic changes in mitochondrial morphology; completely fragmented and elongated mitochondria result from the down-regulation of proteins required for fusion and fission machineries, respectively (Chen *et al.*, 2003; Ishihara *et al.*, 2003; Olichon *et al.*, 2003; Lee *et al.*, 2004; Jofuku *et al.*, 2005). MICS1 RNAi induced clear changes in mitochondrial morphology, but the effect was moderate compared with the phenotypes induced by the down-regulation of Drp1, Fis1, OPA1, or Mfn1/2, suggesting that MICS1 is dispensable directly for machineries controlling membrane fusion and/or fission. Nonetheless, MICS1 down-regulation induced cristae disorganization. It is possible that abnormal mitochondrial morphology results in a failure to maintain cristae integrity. Although the loss of OPA1 can be due to cristae disorganization, mitochondria in the MICS1 knockdown cells did not exhibit expanded cristae, which are a characteristic feature of cells lacking OPA1 (Olichon *et al.*, 2003; Frezza *et al.*, 2006). These findings, together with the lack of evidence for a physical interaction between MICS1 and OPA1, suggest that MICS1 has a distinct role in cristae organization, which is presumably separate from that of OPA1.

The release of proapoptotic proteins, including cytochrome c, Smac, and HtrA2, was enhanced by MICS1 knockdown. Increasing the MICS1 gene dosage, however, did not influence the apoptotic release of Smac and HtrA2. Only cytochrome c was retained in the mitochondria by MICS1 overproduction. Stimulation of the apoptotic release of Smac and HtrA2 might be due to disorganized cristae structures.

Bax targeting and subsequent outer membrane permeabilization occurred normally in MICS1-overexpressing cells, indicating that cytochrome c release is inhibited downstream of outer membrane perforation. Cytochrome c is thought to be present in pools loosely and tightly bound to the inner membrane. Although outer membrane permeabilization is sufficient to trigger the release of a certain population of cytochrome c, detachment of a major pool that is tightly bound to the inner membrane is proposed to require the remodeling of cristae structures (Ott *et al.*, 2002; Scorrano *et al.*, 2002). Although tBid induces a striking remodeling of cristae structures (Scorrano *et al.*, 2002), cytochrome c was clearly retained in the mitochondria of the cells overexpressing MICS1 after incubation with tBid. Furthermore, the retention of cytochrome c mediated by MICS1 overexpression was not completely canceled by OPA1 RNAi, which induces the remodeling of cristae structures (Frezza *et al.*, 2006). These results suggest that the inhibition of cytochrome c release by MICS1 is mediated by protein-protein interactions, but not membrane organization. Indeed, MICS1 coprecipitated with cytochrome c. Nonetheless, the possibility that other proteins and/or lipids facilitated the association of cytochrome c with MICS1 could not be ruled out because of inefficient cross-linking with MICS1.

Recently, Drp1 down-regulation was reported to partially inhibit the apoptotic release of cytochrome c, but not other proapoptotic proteins, including Smac and HtrA2 (Parone *et al.*, 2006; Estaquier and Arnoult, 2007). This inhibition is thought to account for alterations of the OPA1 processing by Drp1 RNAi. Neither MICS1 overproduction nor knockdown influenced the total amount of Drp1 (unpublished data). The OPA1 processing and Drp1 amount did not change under the low-serum condition. Furthermore, MICS1 overexpression induced mitochondrial aggregation, whereas Drp1 down-regulation induced mitochondrial elongation. It is unlikely that MICS1 contributes to the inhibition of cytochrome c release induced by Drp1 down-regulation.

Our studies suggest that MICS1 has the two individual functions as follows: First, MICS1 is necessary for maintenance of mitochondrial morphology in specific cristae structures. MICS1 down-regulation decreased the number of invaginated inner membranes. So far, mitofilin and OPA1 are reported to be responsible for cristae morphology (Olichon *et al.*, 2003; John *et al.*, 2005). Different from the effects of RNAi for mitofilin or OPA1, there were no mitochondria with expanded cristae or with onion-like inner membrane structures in the MICS1 knockdown cells, suggesting that MICS1 has a distinct function in controlling the invagination of inner membranes. In addition, MICS1 overexpression caused mitochondrial aggregation and vacuolation, eventually inducing cytotoxicity. Vacuoles found in mitochondria of the MICS1-overexpressing cells were low electron dense, suggesting that the intermembrane space or cristae structure, but not matrix, was expanded. Although it is still unclear why MICS1 overexpression is cytotoxic, MICS1 may contribute to an association between the invaginated membranes. Second, MICS1 facilitates the tight association of cytochrome c with the inner membranes. In the low-serum condition, the delayed apoptotic release of cytochrome c correlated with MICS1 up-regulation. Serum depletion causes a decrease in the mitochondrial membrane potential, triggering cytochrome c release in Rat-1 cells (Annis *et al.*, 2001). Under the low-serum condition, an increase in MICS1 may counteract this effect, thereby preventing the easy entry into the apoptotic pathway. Conversely, in the normal serum condition MICS1 gradually decreased in response to the progress of apoptosis (Figure 7D), which may facilitate rapid

release of cytochrome c. In contrast to Bi-1 and LFG, MICS1 overproduction could not completely protect cells from apoptotic death. Thus, MICS1 may temporarily interrupt an apoptotic pathway via the mitochondria to allow cells to survive under unhealthy conditions.

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