GRIM-19 Is Essential for Maintenance of Mitochondrial Membrane Potential

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Submitted July 19, 2007; Revised January 28, 2008; Accepted February 7, 2008
Monitoring Editor: Donald Newmeyer

GRIM-19 was found to copurify with complex I of mitochondrial respiratory chain and subsequently was demonstrated to be involved in complex I assembly and activity. To further understand its function in complex I, we dissected its functional domains by generating a number of deletion, truncation, and point mutants. The mitochondrial localization sequences were located at the N-terminus. Strikingly, deletion of residues 70–80, 90–100, or the whole C-terminal region (70–144) led to a loss of mitochondrial transmembrane potential ($\Delta\Psi_m$). However, similar deletions of another two complex I subunits, NDUFA9 and NDUFS3, did not show such effect. We also found that deletion of the last 10 residues affected GRIM-19's ability to be assembled to complex I. We constructed a dominant-negative mutant containing the N-terminal 60 and the last C-terminal 10 residues, which could be assembled into complex I, but failed to maintain normal $\Delta\Psi_m$. Cells overexpressing this mutant did not spontaneously undergo cell death, but were sensitized to apoptosis induced by cell death agents. Our results demonstrate that GRIM-19 is required for electron transfer activity of complex I, and disruption of $\Delta\Psi_m$ by GRIM-19 mutants enhances the cells' sensitivity to apoptotic stimuli.

INTRODUCTION

Mitochondria are subcellular organelles that are essential in the regulation of cellular bioenergetics. In most eukaryotic cells, mitochondria are the major source of ATP, which is produced through oxidative phosphorylation (OXPHOS) by the mitochondrial respiratory chain (RC). The RC is localized in the inner membrane of the mitochondria and consists of four multisubunit complexes (complexes I–IV) and two additional electron carriers, coenzyme Q10 and cytochrome *c* (Hatefi, 1985; Schägger and Pfeiffer, 2000). The RC catalyzes OXPHOS by transferring two electrons from reducing substrates (NADH-FADH₂) to molecular oxygen. The electron transport generates an electrochemical proton gradient across the inner membrane, measured as mitochondrial transmembrane potential ($\Delta\Psi_m$), which drives ATP synthesis by complex V, the ATP synthase (Mitchell, 1961; Schultz and Chan, 2001). Among five complexes, complex I, which consists of 45 subunits in the bovine heart, is the largest (Carroll *et al.*, 2006). Besides respiration, mitochondria also play important roles in the regulation of apoptosis. The mitochondrial pathway of apoptosis is initiated by the proapoptotic Bcl-2 family proteins, such as Bax and Bak, which form pores and induce mitochondrial outer membrane permeabilization. This leads to a release of cytochrome *c*, loss of

$\Delta\Psi_m$, and activation of various caspases. These caspases cleave specific substrates within the cell to produce changes associated with apoptosis (Danial and Korsmeyer, 2004; Wei *et al.*, 2001). The central role of mitochondria in the regulation of both metabolism and apoptosis suggests a possible link between these two crucial cellular processes (Newmeyer and Ferguson-Miller, 2003; Hammerman *et al.*, 2004). $\Delta\Psi_m$ is important for ATP production and mitochondrial protein transport. On the other hand, disruption of $\Delta\Psi_m$ is also implicated in various apoptotic phenomena (Ly *et al.*, 2003). However, the precise role of $\Delta\Psi_m$ in the apoptotic process is not clear.

GRIM-19 (Genes associated with Retinoid-IFN-induced Mortality-19) was originally identified as an interferon (IFN)- β and retinoic acid (RA)-inducible gene with apoptotic effects in human cancer cell lines (Angell *et al.*, 2000). However, it was subsequently found to copurify with mitochondrial NADH:ubiquinone oxidoreductase (complex I) in the bovine heart (Fearnley *et al.*, 2001). By gene targeting in mice, we further demonstrated that GRIM-19 is localized in complex I and is essential for complex I assembly and electron transfer activity (Huang *et al.*, 2004). Various biological functions of GRIM-19 have been revealed. Knockout of GRIM-19 in mice caused early embryonic lethality, indicating its essential role in the embryonic development (Huang *et al.*, 2004). Further studies in *Xenopus* demonstrated that GRIM-19 is necessary for early heart development by regulating Ca²⁺ homeostasis and the Ca²⁺-dependent NFAT signaling pathway (Chen *et al.*, 2007). Furthermore, the mechanism of the GRIM-19/RC-mediated regulation in IFN/RA-induced cell death has also been uncovered (Huang *et al.*, 2007). RC complex I has recently been reported to be involved in viral infections (Reeves *et al.*, 2007). A viral RNA product, β 2.7 RNA, encoded by human cytomegalovirus interacts with GRIM-19 and complex I. This interaction stabilizes $\Delta\Psi_m$, sustains ATP production and protects infected cells from rotenone-induced apoptosis. Most recently, GRIM-19 was reported to act as a

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E07-07-0683>) on February 20, 2008.

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Abbreviations used: aa, amino acids; $\Delta\Psi_m$, mitochondrial transmembrane potential; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; IFN, interferon; GRIM, gene associated with retinoid-interferon-induced mortality; OXPHOS, oxidative phosphorylation; RA, all-*trans*-retinoic acid; RC, respiratory chain.

tumor suppressor to inhibit cell transformation induced by constitutively activated Stat3 (Kalakonda *et al.*, 2007).

Although the biological functions of GRIM-19 have been widely investigated, the molecular mechanism of its functions, is not well defined. Although the genetic evidence showed its effect on mitochondrial complex I assembly and activity, it is unknown whether GRIM-19 is a structural subunit or a functional subunit for the complex I activity. Therefore, a detailed characterization of GRIM-19 itself is warranted. In this study, we dissected the functional domains of GRIM-19 by systematic generation of a series of internal and truncation mutants. By doing so, we defined its mitochondrial localization sequences and domains for the complex I assembly and activity. Furthermore, a unique role of GRIM-19 in the maintenance of $\Delta\psi_m$ and protection of cells from apoptosis induced by classical death stimuli were demonstrated. These data therefore establish the primary functions of GRIM-19 in mitochondria.

MATERIALS AND METHODS

Cell Culture, Chemicals, and Reagents

MCF-7 cells and HEK 293T cells were maintained in RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS). HeLa cells were maintained in eagle's minimum essential medium (MEM) supplemented with 10% FBS. $\rho 0$ cells were generated as described (Appleby *et al.*, 1999). Briefly, human 143B osteosarcoma cells' mtDNA was depleted by continuously culturing the cells in DMEM with ethidium bromide (100 $\mu\text{g}/\text{ml}$) and 10% FBS. Generated $\rho 0$ cells were confirmed by the absence of COX I and COX III with Western blot analysis. $\rho 0$ cells were maintained in DMEM medium supplemented with uridine and pyruvate. Tetramethylrhodamine ethyl ester (TMRE), MitoTracker Red CMXRos, TOPRO-3, ionomycin, and antibodies against NDUFA9, NDUFS3, and complex II 70-kDa subunit were purchased from Molecular Probes (Eugene, OR). VDAC antibody was purchased from Calbiochem (San Diego, CA), and HA antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). TNF- α , IFN- β /RA, etoposide, and rotenone were from Sigma. Cytochrome *c* antibody was from BD Pharmingen (San Diego, CA) and GRIM-19 antibody was generated as described (Lufei *et al.*, 2003).

Plasmid Construction and DNA Transfection

Human GRIM-19 sequence was amplified by RT-PCR from HeLa cells. WT-GRIM-19 with complete open reading frame was cloned into HindIII and XhoI sites of mammalian expression vector pXJ40-HA. Internal and truncation mutants of GRIM-19 were generated using overlapping extension PCR and cloned into pXJ40-HA in the same way as that of WT-GRIM-19. Point mutations were introduced in the GRIM-19-containing plasmid by the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), using a set of primers encompassing the inserted point mutations, to generate GRIM-19 G139R, G139V, Y143A, and Y143D, in which Gly139 or Tyr143 was substituted by Arg, Val, Ala, and Asp, respectively. The hemagglutinin (HA) tag was added at the C-termini of the GRIM-19 sequences in all constructs. NDUFA9 and NDUFS3 genes were amplified from 293T cells. The open reading frames of NDUFA9 and NDUFS3 were cloned into HindIII/XhoI and XhoI/NotI sites of pXJ40-HA vector, respectively. Constructs were sequenced to verify the deletions and point mutations. Transfection of plasmids to cells was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Western Blot Analysis

Western blot analysis was performed as described previously (Huang *et al.*, 2007). Briefly, cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% deoxycholic acid, 1% Triton X-100, 0.1% SDS, 0.25 mM EDTA) with the protease inhibitor cocktail (Roche, Basel, Switzerland). The lysate was separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with phosphate-buffered saline (PBS) containing 0.1% Tween 20 and 1% bovine serum albumin before it was incubated with the appropriate primary and secondary antibodies.

Immunofluorescence

Cells (5×10^5) were grown on glass coverslips in six-well plate. After incubation with 50 nM MitoTracker CMXRos for 30 min, cells were fixed with culture medium containing 3.7% formaldehyde for 15 min at 37°C. The fixed cells were permeabilized with PBS containing 0.2% Triton X-100 for 15 min and blocked with FDB (5% normal goat serum, 2% FBS, and 2% bovine serum albumin in PBS) for 1 h. The primary antibody (polyclonal anti-HA or

monoclonal anti-cytochrome *c*) was incubated for 1 h and then washed three times with PBS containing 0.1% Triton X-100. Fluorescein isothiocyanate (FITC)-conjugated anti-rabbit secondary antibody was incubated to recognize the HA epitope, whereas CY3-conjugated anti-mouse secondary antibody was used to detect cytochrome *c*. After washing, cells were incubated with TOPRO-3 (1:2000 dilution) for 10 min before mounting. The cells were examined with confocal microscopy (Radiance 2000, Bio-Rad, Hercules, CA).

Measurement of $\Delta\psi_m$

Cells (1×10^6) were incubated with culture medium containing 50 nM TMRE for 30 min at 37°C in the dark, harvested, and washed in PBS. Cells were resuspended in buffer A (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl_2 , 1 mM sodium EGTA, 1 mM dithiothreitol, and 250 mM sucrose) and analyzed by FACS. The reading of TMRE staining intensity in FCCP (carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone)-treated cells was set to be zero in fluorescence-activated cell sorting (FACS) measurement.

Cytochrome *c* Release Assay

Cells (1×10^6) were harvested and lysed on ice for 15 min with buffer containing 50 mM KCl, 5 mM MgCl_2 , 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, 0.025% digitonin, and 20 mM HEPES (pH 7.2) with protease inhibitor cocktail (Roche, Basel, Switzerland). Lysates were centrifuged at 13,200 rpm ($16,100 \times g$) for 30 min to remove debris. Twenty micrograms of protein in the supernatant was subjected to Western blot analysis and probed with anti-cytochrome *c* antibody.

Blue-Native PAGE and In-Gel Activity Assay

Blue-Native PAGE (BN-PAGE) was performed in a mini-gel format using the Bio-Rad Mini Protean system. Mitochondria were isolated as described previously (Huang *et al.*, 2004). Freshly isolated mitochondria containing 500 μg proteins were resuspended in 80 μl of extraction buffer (750 mM of 6-aminocaproic acid, 50 mM of bis-Tris, pH 7.0, and 10% dodecylmaltoside). The mitochondrial proteins were solubilized by frequent pipetting on ice for 30 min. After the removal of debris by centrifuging at 100,000 rpm for 10 min, 5 μl of 5% Serva blue G in 500 mM caproic acid was added into supernatant. The supernatant was separated by a polyacrylamide native gradient gel ranging from 5 to 18% to detect the individual RC complexes. After BN-PAGE, the proteins were transferred to PVDF membrane in transfer buffer (25 mM Tris, 192 mM glycine, 0.05% SDS, and 20% methanol). The membrane was blocked with 5% milk in PBS for at least 4 h, and normal Western blot assay was carried out. In-gel colorimetric reaction for oxidative phosphorylation of complex I and II was performed on the BN-PAGE as described previously (Huang *et al.*, 2004).

Complex I Spectrophotometric Enzyme Assay

Mitochondria were isolated from 5×10^6 HEK 293T cells as described previously (Huang *et al.*, 2004) and subjected to NADH oxidation assay described previously with minor modifications (Rouslin, 1983). Briefly, isolated mitochondria were resuspended in 0.1 M Tris-HCl buffer (pH 7.0) and sonicated for 10 s on ice before measurement. Mitochondrial homogenates, 100 μl , were added into 900 μl of prewarmed (30°C) reaction buffer (0.1 M Tris-HCl, pH 7.0, 0.3 mM NADH, 0.1 mM coenzyme Q1, 1 mM KCN, and 2 mM NaN_3). For measurement of the rotenone insensitive NADH oxidation activity, 5 μl of 0.5 mM rotenone was added to the mixture. The mixture with or without rotenone was transferred to a prewarmed (30°C) quartz cuvette and immediately put into spectrophotometer. The absorbance of reaction mixture was measured at every 10-s interval for 100 s at 340 nm.

Apoptosis Assay (sub-G1 Assay)

Apoptosis assay was performed as described previously by Jenny *et al.* (2005). Briefly, HEK 293T cells (5×10^5 cells) were resuspended in 200 μl of PBS and 1 ml of ice-cold 70% ethanol. The suspension was kept at -20°C for at least 30 min. The cell pellet was then resuspended in 100 μl of ribonuclease A buffer (100 $\mu\text{g}/\text{ml}$) for 5 min at room temperature followed by further staining with 300 μl of 50 $\mu\text{g}/\text{ml}$ propidium iodide (PI) for 30 min. The DNA content was analyzed by FACS.

RESULTS

Residues 20–30 and 40–60 Are Required for Mitochondrial Localization of GRIM-19

GRIM-19 contains 144 amino acids (aa) with no conserved motifs/domains identified. To further study its function in mitochondrial complex I, we generated a series of internal deletion and truncation mutants of human GRIM-19 by deleting ~ 10 aa for each mutant and attaching an HA tag to their C-termini (Figure 1A). First we examined the cellular localization of the mutant proteins by immunofluorescence

Figure 1. GRIM-19 aa 20–30 and 40–60 are mitochondrial localization signals. (A) Schematic diagram of GRIM-19 internal deletion and C-terminal deletion mutants. For internal deletion mutants, ~ 10 aa was deleted in each mutant from the N- to the C-terminus. Larger deletion mutants $\Delta 96-124$ and $\Delta 96-134$ were also included. Numbers indicate aa in GRIM-19. (B) WT and the internal deletion mutants of GRIM-19 were transfected into MCF-7 cells. Subcellular localization of GRIM-19 proteins was detected by anti-HA primary antibody and FITC-conjugated secondary antibody (green). Mitochondria were labeled with Mito-Tracker Red CMXRos (red). Cells were mounted and examined with a confocal microscopy (Radiance 2000, Bio-Rad). Merged images are shown. Scale bar, 10 μm . (C) A series of GRIM-19 internal deletion mutants were transfected into HEK 293T cells. Subcellular localization of different GRIM-19 deletion mutants were determined by cellular fractionation. The mitochondrial and cytosolic fractions were collected and subjected to Western blot analysis probed with antibodies as indicated. The expression levels of the WT and mutant GRIM-19 in total cell lysate are also shown.

assay. Staining for wild-type (WT) GRIM-19 showed specific mitochondrial filament or rod-like staining which was colocalized with Mito-Tracker Red CMXRos. Among all the internal deletion mutants, three mutants, $\Delta 20-30$, $\Delta 40-50$, and $\Delta 50-60$, lost the typical punctated mitochondrial staining and exhibited diffused staining pattern (Figure 1B). These results were further verified by cellular fractionation. In contrast to the WT GRIM-19 and the other mutants which were detected only in the mitochondrial fraction, $\Delta 20-30$, $\Delta 40-50$, and $\Delta 50-60$ mutants were absent in the mitochondrial fraction and mislocalized in the cytosol (Figure 1C). This indicates that aa 20–30 and 40–60 of GRIM-19 harbor mitochondrial signal sequences that determine its mitochon-

drial localization. In agreement with this, all the C-terminal truncation mutants shown in Figure 1A were localized in the mitochondria, except for mutant 1–50, which lost its mitochondrial localization (data not shown).

Residues 134–144 Affects GRIM-19 Insertion to Complex I

Knockout of GRIM-19 in mouse blastocysts leads to disruption of the RC complex I assembly (Huang *et al.*, 2004). We further investigated how GRIM-19 is assembled into complex I. We checked the assembly ability of different GRIM-19 deletion mutants by comparing their amounts present in complex I. HEK 293T cells were transfected with equal amount of the WT or mutant GRIM-19 and harvested 24 h

Figure 2. Aa 134–144 of GRIM-19 affects its assembly ability to complex I. (A and B) HA-tagged GRIM-19 mutants were transfected into 293T cells. BN-PAGE was used to separate mitochondrial RC complexes and subjected to Western blot analysis. Anti-HA antibody was used to detect transfected WT or mutant GRIM-19 presented in complex I (top panels). GRIM-19 antibody was used to detect both transfected and endogenous GRIM-19 (second panels). Mitochondrial complex II was detected by antibody against the 70-kDa subunit of complex II, as a control (third panels). Expression levels of transfected GRIM-19 were monitored by SDS-PAGE and Western blot analysis using anti-HA antibody (bottom panels). (C) Sequence comparison of the last 10 aa of GRIM-19 among different species. Highly conserved Gly (G) and Tyr (Y) are highlighted. (D) GRIM-19 point mutants (Y139A, Y139D, G143R, and G143V) were transfected into 293T cells. The experiments were performed as described in A and B. WT and mutant 1–96 were included as controls.

later. The RC complexes were resolved with BN-PAGE, and the protein amounts of different truncation mutants in complex I were measured by Western blot analysis. Our data showed that the assembly ability of mutants 1–134, 1–124, 1–114, and 1–96 was drastically decreased in comparison with WT-GRIM-19 (Figure 2A). This suggested that the C-terminal 10 aa (134–144) could affect the GRIM-19 assembly to complex I. In support of this, internal deletion mutants with intact C-terminal 10 residues such as $\Delta 96$ –134 and $\Delta 96$ –124 (Figure 1A) showed restored assembly abilities (Figure 2B). Comparison of the GRIM-19 protein sequences among different species revealed two highly conserved aa (Gly139 and Tyr143) in this region (Figure 2C). Point mutants Y143A, Y143D, and G139R, in which the original aa residues were substituted by residues with very different structures and characteristics, displayed greatly decreased GRIM-19 assembly ability (Figure 2D). In contrast, G139V, in which Gly was replaced by a similar aa, Val, displayed normal assembly. Because HA epitope is highly charged which could change the property of the mutant proteins, we replaced it with Myc-tag in four point mutants described above. These Myc-tagged mutants behave similarly to their HA-tagged counterparts (Supplementary Figure 1). These results further support that the C-terminus affects GRIM-19 assembly to complex I.

Aa 70–80 and 90–100 Are Required for Maintenance of $\Delta\Psi_m$

In GRIM-19 knockout blastocysts, complex I almost totally lost its enzymatic activity due to failure of complex I holoenzyme assembly. However, this observation did not answer the question of whether GRIM-19 is just a structural subunit for complex I architecture or is a crucial enzymatic subunit. To address this, we tested whether GRIM-19 contains a domain that affects complex I electron transfer activity by assessing the changes of $\Delta\Psi_m$. GRIM-19 mutants were transfected into MCF-7 cells and costained with the Mito-Tracker Red CMXRos. Mito-Tracker Red CMXRos is a dye that accumulates only in the active mitochondria and is used to detect $\Delta\Psi_m$. Although expressions of the mutants $\Delta 70$ –80, $\Delta 80$ –90, and $\Delta 90$ –100 were equally strong, the cells transfected with mutant $\Delta 70$ –80 or $\Delta 90$ –100 showed much weaker or undetectable mitotracker staining, whereas the mutant $\Delta 80$ –90 was stained normally (Figure 3A, left panel).

Consistent with these results, the cells transfected with the C-terminal truncation mutants (1–90, 1–80, and 1–70) also showed less mitotracker staining (middle panel). The percentage of depolarized cells in each group of transfected cells is shown in the right panel.

To confirm that the lack of mitotracker staining was due to a loss of $\Delta\Psi_m$, we quantitatively measured $\Delta\Psi_m$ with TMRE, a positive-charged fluorescence indicator of $\Delta\Psi_m$. WT GRIM-19 and GRIM-19 deletion mutants ($\Delta 70$ –80 or $\Delta 90$ –100) were transfected into MCF-7 cells. After TMRE staining, the total cells were subjected to FACS analysis. In cells transfected with WT GRIM-19, only 3% of cells showed low $\Delta\Psi_m$. In contrast, 13.91 and 25.86% of cells transfected with $\Delta 70$ –80 and $\Delta 90$ –100 deletion mutants, respectively, displayed low $\Delta\Psi_m$. As negative controls, FCCP, a potent uncoupler of oxidative phosphorylation, led to complete disruption of mitochondrial membrane potential (100%), whereas rotenone, a classical complex I inhibitor, resulted in 98% of cells with low levels or loss of $\Delta\Psi_m$ (Figure 3B). This further confirmed that deletion of aa 70–80 or 90–100 affect mitochondrial membrane potential.

To find out whether other RC complex I subunits also affect $\Delta\Psi_m$, we cloned two complex I subunits, NDUFA9 and NDUF53, and generated truncation mutants containing the N-terminal 1–100 aa that include their mitochondrial signal peptides (Walker, 1992) followed by short sequences. Although these deletion mutants were able to insert to complex I (Supplementary Figure 2), they did not overtly affect $\Delta\Psi_m$ (Figure 3, C and D). These data suggest that GRIM-19 might be a subunit with special functions in complex I.

Generation of a Dominant Negative GRIM-19 Mutant That Disrupts $\Delta\Psi_m$

On the basis of our data, we generated a potent dominant-negative mutant (DN-GRIM-19) containing the N-terminal 1–60 aa and the C-terminal 10 aa (134–144), as shown in Figure 4A. This mutant strongly reduced $\Delta\Psi_m$, as shown by a loss of mitotracker staining (Figure 4B). Although only 5% of cells expressing WT-GRIM-19 or transfected with control vector showed less mitotracker staining, 76% of the cells expressing DN-GRIM-19 showed significantly less or almost nondetectable mitotracker staining (Figure 4C). Compared with deletion mutant 1–60, which caused loss of $\Delta\Psi_m$ in 52% of transfected cells, the DN-GRIM-19 had a stronger

Figure 3. Aa 70–80 and 90–100 of GRIM-19 are required for maintenance of $\Delta\Psi_m$. (A) HA-tagged GRIM-19 internal deletion mutants ($\Delta 70-80$, $\Delta 80-90$, and $\Delta 90-100$) and truncation mutants (1–90, 1–80, and 1–70) were transfected into MCF-7 cells. GRIM-19 proteins were detected with anti-HA antibody and FITC-conjugated secondary antibody (green). Mitochondria were labeled with Mito-Tracker Red CMXRos (red). $\Delta\Psi_m$ was detected by the staining of Mito-Tracker CMXRos. Cells were mounted and examined with a confocal microscope (left and middle panels). One hundred transfected cells from each group were randomly selected, and the number of the cells that lost $\Delta\Psi_m$ was counted and indicated as percentage of the total cells. The numbers represent the mean of three independent experiments, with SD shown as error bars (right panel). (B) Vector, WT-GRIM-19, GRIM-19 internal deletion mutants ($\Delta 70-80$, $\Delta 90-100$) and DN-GRIM-19 were transfected into MCF-7 cells. After transfection for 24 h, cells were stained with 50 nM TMRE and subjected to FACS analysis. Events ($n = 30,000$) were counted for each group. As controls, untransfected cells were either treated with 10 μM of FCCP for 2 h (for complete depolarization) or 4 μM of rotenone for 4 h (for inhibition of complex I) before harvesting for FACS. The reading of TMRE staining intensity in FCCP-treated cells was set to 0 in FACS measurement. Percentage of total cells with low $\Delta\Psi_m$ is indicated. (C and D) Two complex I subunits, NDUFA9 (C) and NDUF3 (D) and the mutants are illustrated on top of the figures. Close boxes at the N-termini indicate the mitochondrial localization signal sequences predicted using MITOPROT server. The numbers represent aa contained in the WT and mutant constructs. The indirect immunofluorescence experiment was performed as described in A. Scale bar, 10 μm .

effect. This could be due to the presence of the C-terminal 10 aa in DN-GRIM-19, which confers better assembly ability. Its effect was further verified by FACS analysis in which 51.73% of total cells showed low $\Delta\Psi_m$ (see Figure 3B).

We also checked whether loss of mitotracker staining was due to impaired mitochondrial integrity by staining the cells with antibody against COX IV, a subunit of RC complex IV. As shown in Figure 4D, although the mitotracker staining was

absent in DN-GRIM-19-expressing cells, COX IV staining in the same cells was intact. This confirmed that loss of $\Delta\Psi_m$ by the DN-GRIM-19 was not due to mitochondrial damage.

DN-GRIM-19 Compromises Complex I Activity without Affecting Its Assembly

Because $\Delta\Psi_m$ was reduced in cells transfected with DN-GRIM-19, we further tested whether this was due to a de-

Figure 4. DN-GRIM-19 strongly reduces $\Delta\Psi_m$. (A) Schematic diagram of DN-GRIM-19. The numbers indicate aa. (B) WT and DN-GRIM-19 were transfected into MCF-7 cells. GRIM-19 proteins were detected by anti-HA primary antibody and FITC-conjugated secondary antibody (green). $\Delta\Psi_m$ was detected with Mito-Tracker CMXRos (red). Nuclei were stained with TOPRO-3 (blue). The merged images are shown. (C) Vector, WT-GRIM-19, deletion mutant 1–60, and DN-GRIM-19 were transfected into MCF-7 cells. Immunostaining was performed as described above. Transfected cells ($n = 100$) were randomly picked. The number of cells showing low $\Delta\Psi_m$ was counted, and the percentage of such cells was calculated and presented in bar graphs as the mean of three experiments with SD shown as error bars in the right panel. (D) Experiments were performed as described in B, except that instead of TOPRO-3 staining, cells were stained with anti-COX IV antibody and CY5-conjugated secondary antibody (blue).

Figure 5. DN-GRIM-19 decreases mitochondrial complex I electron transfer activity. (A) WT, DN, and deletion mutant 1–60 of GRIM-19 were transfected into HEK 293T cells. After transfection for 24 h, cells were harvested, and mitochondria from cells were isolated. Mitochondrial proteins were solubilized and separated by BN-PAGE. Enzymatic activity of complex I and II were measured in gel respectively as described in *Materials and Methods*. (B and C) Transfection and BN-PAGE were performed as described in A, followed by Western blot analysis. (B) Anti-GRIM-19 antibody was used to detect the total GRIM-19 proteins in mitochondrial complex I, and anti-complex II 70-kDa subunit antibody was used to detect the complex II. (C) Anti-HA antibody was used to detect the amount of transfected proteins (WT, DN and 1–60 of GRIM-19) which were assembled in mitochondrial complex I. (D) HEK 293T cells were transfected with vector, WT, or DN-GRIM-19. Mitochondrial fraction was isolated, and NADH oxidation was measured in the absence or presence of rotenone by a change of OD reading at 340-nm wavelength per 10 s as described in *Materials and Methods*. The rate of NADH oxidation of each cell population is listed in Table 1. The same amount of mitochondria from cells transfected with vector (lane 1), WT- (lane 2), or DN-GRIM-19 (lane 3) was lysed and subjected to Western blot analysis shown in the bottom panels. NDUF9 antibody was used to monitor the amount of complex I, and the expression levels of transfected plasmids are shown by probing with anti-HA antibody. (E) The rotenone sensitive complex I activity was quantified from data shown in Table 1 and is indicated with a bar graph. (F) DN-GRIM-19 was transfected into

human 143B cells or $\rho 0$ cells. DN-GRIM-19 proteins were detected by anti-HA polyclonal primary antibody and FITC-conjugated secondary antibody (green). $\Delta\Psi_m$ was detected with Mito-Tracker CMXRos (red). Scale bar, 10 μm . (G) The same samples for BN-PAGE described in A were denatured by boiling in the equal volume of 2 $\times$ protein loading buffer, separated with SDS-PAGE, and subjected to Western blotting with anti-complex II 70-kDa subunit, anti-NDUF9, anti-NDUFS3, anti-VDAC, anti-GRIM-19, and anti-HA antibodies. Anti-GRIM-19 antibody was used to detect the endogenous GRIM-19, whereas anti-HA antibody was used to detect the transfected WT-, DN-, and GRIM-19 1–60.

crease of mitochondrial complex I enzymatic activity. HEK 293T cells were transfected with WT, deletion mutant 1–60, or DN-GRIM-19. BN-PAGE was used to separate the RC complexes, and the complex I enzymatic activity was tested directly in gel. The results showed that in the DN-GRIM-19-transfected cells, complex I activity was much lower than that in the WT-GRIM-19-transfected cells (Figure 5A), although the total mitochondrial complex I amount was not decreased when monitored by Western blotting with GRIM-19 antibody (Figure 5B). The GRIM-19 deletion mutant 1–60 also showed decreased complex I activity but not as potent as DN-GRIM-19. This could be due to the higher amount of DN-GRIM-19 inserted into complex I compared with the deletion mutant 1–60 (Figure 5C).

We also quantitatively determined mitochondrial complex I enzymatic activity by measuring the NADH oxidation rate using a spectrophotometer. The rate of NADH oxidation is proportional to the slope of the kinetic curve. As shown in Figure 5D, the overall NADH oxidation rate in the DN-GRIM-19-transfected cells was much slower than that in the vector or WT GRIM-19-transfected cells (left panel). However, in the presence of rotenone, the NADH oxidation rates decreased to a similar level in all three groups. The experiments were repeated three times, and the statistical

data are shown in Table 1. The rotenone-sensitive NADH oxidation rate (proportional to complex I activity) was calculated by subtracting NADH oxidation rate in the presence of rotenone from the total NADH oxidation rate in the absence of rotenone (Figure 5E). The results showed that vector and WT GRIM-19-transfected cells recorded complex I activity of 2.32 and 2.35 $\mu\text{mol}/\text{min}/\text{mg}$, respectively, whereas DN-GRIM-19-transfected cells showed low activity of 0.57 $\mu\text{mol}/\text{min}/\text{mg}$, which accounted to only 25% of activity obtained in the vector-transfected cells.

$\rho 0$ cells lack functional RC because of depletion of the mitochondrial DNA. However, in these cells, mitochondria are shown to be capable in regaining their $\Delta\Psi_m$ in an RC-independent pathway, as reported previously (Appleby

Table 1. The rate of NADH oxidation

Rotenone	Vector ($\mu\text{mol}/\text{min}/\text{mg}$)	GRIM-19 ($\mu\text{mol}/\text{min}/\text{mg}$)	DN-GRIM-19 ($\mu\text{mol}/\text{min}/\text{mg}$)
– (n = 3)	3.86 $\pm$ 0.7	3.96 $\pm$ 0.2	1.93 $\pm$ 0.4
+ (n = 3)	1.54 $\pm$ 0.2	1.61 $\pm$ 0.2	1.36 $\pm$ 0.5

et al., 1999). If DN-GRIM-19 disrupted $\Delta\Psi_m$ by decreasing the complex I activity, it was predicted that this mutant would not affect $\Delta\Psi_m$ in $\rho 0$ cells. It appears to be the case as DN-GRIM-19 did not change $\Delta\Psi_m$ in $\rho 0$ cells, but was able to disrupt $\Delta\Psi_m$ in parental 143B cells (Figure 5F). These results indicate that a loss of $\Delta\Psi_m$ in cells expressing DN-GRIM-19 is correlated to the decrease of the complex I enzymatic activity.

In the GRIM-19 knockout blastocysts, complex I cannot be assembled, and the amounts of complexes II, III, IV, and V were also decreased. In agreement with these observations, the protein levels of some individual subunits of the RC complexes were also down-regulated (Huang *et al.*, 2004). However, we did not observe a similar effect from DN-GRIM-19 (Figure 5, A and B, for complex II assembly and activity). Furthermore, expression levels of various RC complex subunits remained unaffected (Figure 5G). These results suggest that DN-GRIM-19 does not affect mitochondrial complex I assembly. Altogether, these data suggest that GRIM-19 is not only required for the complex I assembly, but also critical for its electron transfer activity and maintenance of $\Delta\Psi_m$. The functional domain for this activity resides in the region of aa 70–100, which is distinct from that responsible for the complex I assembly (aa 1–60 and 134–144).

Loss of $\Delta\Psi_m$ Caused by DN-GRIM-19 Does Not Induce Cytochrome *c* Release But Sensitizes Cells to Undergo Apoptosis

Because DN-GRIM-19 specifically decreased $\Delta\Psi_m$, we further examined the possible apoptosis in DN-GRIM-19-transfected cells. A special MCF-7 cell line (Yang *et al.*, 2001), which had been stably transfected with caspase 3 and thus had the ability to undergo apoptosis upon death stimulation, was used in the following experiments. Although DN-GRIM-19 disrupted $\Delta\Psi_m$, there was an absence of cytochrome *c* release from mitochondria (Figure 6).

Although cells transfected with DN-GRIM-19 did not undergo apoptosis under normal growth conditions, we tested whether these cells were sensitized to undergo cell death when challenged with death stimuli. Transfected cells were treated with staurosporine for various periods, and cytochrome *c* release was examined by Western blot analysis. There were higher amounts of cytochrome *c* release in the DN-GRIM-19-expressing cells at early time points (8 and 16 h) after staurosporine treatment. However, in cells expressing WT-GRIM-19, cytochrome *c* release was delayed as

shown by high level of cytochrome *c* release only after 24 h of treatment (Figure 7A). The kinetic analysis was also carried out in the transfected cells treated with TNF- α . At 3 h, cytochrome *c* release was apparent in the cells expressing DN-GRIM-19 but not WT-GRIM-19 (Figure 7B). Consistent with the Western blotting results, the immunofluorescence results showed that the WT-GRIM-19-expressing cells displayed normal cytochrome *c* staining in the mitochondria, whereas the DN-GRIM-19-transfected cells showed diffused cytochrome *c* staining in the cytoplasm after TNF- α treatment (Figure 7C). The statistical data for cell death were obtained in the cells transfected with vector, WT-, or DN-GRIM-19 after treatment with TNF- α , as well as rotenone, ionomycin, or IFN/RA. As shown in Figure 7, D–F, rotenone, ionomycin, or TNF- α induced higher rates of cell death in the transfected cells than untreated cells. However, a much higher percentage of cell death (about twofold) was observed in cells expressing DN-GRIM-19 than in those expressing WT-GRIM-19 or vector. IFN/RA treatment caused prolonged cell death through different mechanisms (Huang *et al.*, 2007). In these cells, there was no obvious difference between cells transfected with WT- and DN-GRIM-19 (Figure 7G). Apoptosis was also analyzed by PI staining followed by flow cytometry. The apoptotic cells were characterized by sub-G1 population due to DNA fragmentation and consequent loss of DNA content. As shown in Figure 7H, ~24% of the DN-GRIM-19-transfected cells underwent apoptosis after treatment with etoposide, in comparison with 11% of the vector- or WT-transfected cells. This suggests that DN-GRIM-19-transfected cells are more sensitive to death stimuli.

DISCUSSION

GRIM-19 Is Localized in Mitochondria

The cellular location of a protein is a key factor in determining its basic biological function. GRIM-19 was found to associate with mitochondrial complex I from bovine heart by protein purification and sequencing analysis, which provided the first clue to its mitochondrial localization (Fearnley *et al.*, 2001). Subsequently, its mitochondrial localization was demonstrated by indirect immunofluorescence and cellular fractionation in various cell types and species (Lufei *et al.*, 2003; Huang *et al.*, 2004). GRIM-19 as a component of complex I was also confirmed by immunopurification of complex I and proteomics approach (Carroll *et al.*, 2003; Murray *et al.*, 2003). However, GRIM-19 was originally reported as a “nuclear” protein and later was reported to be localized in the perinuclear region and cytoplasm by the same group (Angell *et al.*, 2000; Kalvakolanu, 2004). These controversial data have made its exact localization in the cell elusive. In this study, by measuring the cellular localization of more than 24 GRIM-19 deletion mutants, we found that all the mutants are exclusively localized in the mitochondria, except three that displayed diffused staining patterns, primarily in the cytoplasm (Figure 1 and data not shown). Our results confirm the localization of GRIM-19 in mitochondria and suggest that its biological roles are likely to be based on its function in mitochondria.

Most mitochondrial proteins are encoded by nuclear genome, synthesized in the cytosol, and then transported to mitochondria. Two types of mitochondrial targeting signal have been described to mediate the import process: presequences and internal signals (Truscott *et al.*, 2003). Bovine complex I contains 45 subunits in which 7 are encoded by mitochondrial DNA and 38 are encoded by nuclear genes

Figure 6. Loss of $\Delta\Psi_m$ caused by DN-GRIM-19 does not induce cytochrome *c* release. WT and DN-GRIM-19 were transfected into MCF-7 cells that stably express caspase-3. The transfected GRIM-19 were detected by anti-HA primary antibody and FITC-conjugated secondary antibody (green). $\Delta\Psi_m$ was detected by Mito-Tracker Red CMXRos staining (red). Cytochrome *c* was detected with anti-cytochrome *c* antibody (blue). The merged images are shown.

Figure 7. DN-GRIM-19 sensitizes cells to cytochrome *c* release and apoptosis. (A) WT- and DN-GRIM-19 were transfected into HeLa cells. Twenty-four hours after transfection, cells were incubated with 1 μ M staurosporine for various times. Cytochrome *c* release assay was conducted as described in *Material and Methods*. (B) WT and DN-GRIM-19 were transfected into MCF-7 cell line stably expressing caspase 3. Twenty-four hours after transfection, cells were either untreated (un) or treated with 4 ng/ml TNF- α for the period as indicated. Cytochrome *c* release assay was performed as described in A. (C) WT and DN-GRIM-19 were transfected into MCF-7 cells as described in B, and cells were treated with TNF- α for 3 h. Immunofluorescence experiments were conducted. GRIM-19 proteins were detected with polyclonal anti-HA antibody and Cy5-conjugated secondary antibody (blue). Cytochrome *c* was detected with anti-cytochrome *c* mAb and FITC-conjugated secondary antibody (green). Scale bar, 10 μ m. (D) MCF-7 cells stably expressing caspase 3 were transfected with vector, WT, or DN-GRIM-19. Twenty-four hours after transfection, cells were treated with 4 ng/ml TNF- α for 8 h. Immunofluorescence experiments were conducted as described in C. One hundred transfected cells were randomly picked. The number of cells showing cytochrome *c* release was counted, and the percentage of such cells was calculated and presented in bar graphs as the mean of three experiments with SD shown as error bars. (E–G) HeLa cells were transfected with vector, WT, or DN-GRIM-19 and treated with rotenone (5 μ M) for 10 h, ionomycin (200 μ M) for 24 h, or IFN- β (1000 u/ml) and RA (2 μ M) for 48 h. The experiments were performed as described in D. (H) HEK 293T cells were transfected with vector, WT, or DN-GRIM-19. Cells were either untreated or treated with etoposide for 24 h. DNA content was analyzed by flow cytometry after staining with PI. The percentage of sub-G1 cells is indicated. The data show a representative of three independent experiments.

(Carroll *et al.*, 2006). Among them, 18 subunits have N-terminal mitochondrial import sequences that are removed during the import process, whereas the rest do not have the precursor sequences (Carroll *et al.*, 2005). GRIM-19 belongs to the latter group. Our previous observation suggested a sequence for mitochondrial localization at the N-terminal portion of GRIM-19 (Lufei *et al.*, 2003). In agreement to that, in this study, we further mapped its mitochondrial targeting sequences at aa 20–30 and 40–60, and both regions are

required for its mitochondrial targeting. Interestingly, a potential transmembrane α -helical domain between residues 29 and 47 in GRIM-19 has been predicted (Fearnley *et al.*, 2001), which overlaps with our identified mitochondrial targeting sequences. Therefore, the transmembrane domain and its surrounding sequences serve as mitochondrial targeting sequence for GRIM-19 import, as reported in the other mitochondrial proteins (Waizenegger *et al.*, 2003).

Figure 8. Functional domains of GRIM-19. The mitochondrial targeting sequences and functional domains are summarized in a schematic diagram. The numbers indicate aa of GRIM-19. The red, yellow, and green boxes represent sequences for mitochondrial targeting, maintenance of $\Delta\Psi_m$, and enhancing assembly, respectively. TM, predicted transmembrane domain.

GRIM-19 Is a Critical Subunit of RC Complex I

GRIM-19 is associated with complex I in both bovine and human heart (Fearnley *et al.*, 2001; Murray *et al.*, 2003). However, it was unclear whether GRIM-19 was a bona fide subunit or an associated impurity. Although genetic evidence indicates that knockout of GRIM-19 in mouse blastocysts or knockdown of GRIM-19 in *Xenopus* embryos impairs complex I assembly and activity (Huang *et al.*, 2004; Chen *et al.*, 2007), these results do not reveal the mechanism of how it functions in the complex I. In other words, it is unclear whether loss of complex I enzymatic activity in the GRIM-19 knockout cells was due to a failure of complex I assembly or activity, or both. In this study, we defined its functional domains. As summarized in Figure 8, the N-terminal domain (aa 1–60) is essential for the mitochondrial localization of GRIM-19 and also for its incorporation to complex I, and the middle region (aa 70–100) is required for the electron transfer activity of complex I. The last C-terminal 10 aa promote GRIM-19 assembly to complex I. These data clearly demonstrate that GRIM-19 is a functional subunit of complex I that is not only important for complex I assembly but also essential for its electron transfer activity. In addition, we show that GRIM-19 has a unique role in the maintenance of $\Delta\Psi_m$, which is overlapped with its electron transfer activity. GRIM-19 is the first complex I protein that is studied in such detail. These data and the various mutants we have generated in this study will facilitate further investigation for the mechanisms of complex I assembly and function.

DN-GRIM-19 as a Novel Tool for Functional Study of $\Delta\Psi_m$ in Apoptosis

The role of $\Delta\Psi_m$ in apoptosis has been widely studied, with contradictory results reported in different systems. It remains unclear whether loss of $\Delta\Psi_m$ is an initiator or an effect of apoptosis and whether it is necessary for the induction of apoptosis at all (Ly *et al.*, 2003). A decrease of $\Delta\Psi_m$ was reported in early apoptotic process in many systems, and cells with low $\Delta\Psi_m$ underwent spontaneous apoptosis (Zamzami *et al.*, 1995; Cohen, 1997). However, contrary evidence showed that disruption of $\Delta\Psi_m$ did not induce rapid apoptosis and was therefore not a prerequisite for cell death (Finucane *et al.*, 1999). Other evidence suggested that loss of $\Delta\Psi_m$ might be a late and subsequent event in apoptotic pathways (Ly *et al.*, 2003). The relationship between loss of $\Delta\Psi_m$ and cytochrome *c* release is also controversial. It has been reported that loss of $\Delta\Psi_m$ is subsequent to cytochrome *c* release (Goldstein *et al.*, 2000), and a two-step process required for cytochrome *c* release has been proposed (Ott *et al.*, 2002) as opposed to the “all-or-nothing” release model

(Martinou *et al.*, 2000). In our study, surprisingly, we found that the C-terminal region of GRIM-19 is essential for the maintenance of $\Delta\Psi_m$ (Figures 3 and 4). By using the DN-GRIM-19 mutant, we show that permanent loss of $\Delta\Psi_m$ itself is not sufficient to trigger the release of cytochrome *c* (Figure 6). However, it sensitizes cells to undergo cell death when stimulated by the classical death signals (Figure 7). Our results support the idea that normal $\Delta\Psi_m$ and/or complex I activity protects against the initiation of apoptosis triggered by death stimuli. Therefore, this mutant, which is more specific and less toxic in comparison to $\rho 0$ cells or the complex I inhibitors, provides a novel model for further studies of complex I/ $\Delta\Psi_m$ functions in apoptosis.

On the other hand, why DN-GRIM-19 sensitizes cells to death induced by rotenone is currently unclear because both rotenone and DN-GRIM-19 disrupt mitochondrial membrane potential by inhibiting complex I activity. The exact mechanism for this requires further investigation. One possibility is that the time course of their effects may be different. For example, although 4–5 μM of rotenone can inhibit complex I activity rapidly and completely in isolated mitochondria, it takes 2 h for us to observe the obvious loss of $\Delta\Psi_m$ after rotenone treatment in intact cells (data not shown). The DN-GRIM-19 transfection, however, may have resulted in low membrane potential after their expression and therefore renders the cells more sensitive to the rotenone-induced cell death. Alternatively, the possibility of DN-GRIM-19 causing cytochrome *c* release by other effects independent of the membrane potential loss cannot be excluded. In fact, we observed certain degree of mitochondrial fragmentation in the DN-GRIM-19-transfected cells, which may also facilitate the cytochrome *c* release. Nevertheless, we have noticed that although ~98% of control cells lost their $\Delta\Psi_m$ after 4 h of rotenone treatment (Figure 3B), only 15% of cells underwent cytochrome *c* release after rotenone treatment for 10 h (Figure 7D). Again, this indicates that loss of $\Delta\Psi_m$ alone does not efficiently lead to cytochrome *c* release.

RC/Complex I Regulates Cell Death via Different Mechanisms

Combination treatment of IFN/RA causes cancer cell death in a prolonged kinetics (3–5 d). Previously, we demonstrated that this treatment induces expression of GRIM-19, as well as other subunits of mitochondrial complexes I, III, IV, and V. We also showed that the RC regulates IFN/RA-induced cell death by affecting target gene expression and ROS production. However, RC does not exert similar effects on cell death stimulated by the classical death reagents such as UV and staurosporine, which induce cell death more rapidly (Huang *et al.*, 2007). In our current study, we demonstrated that the complex I activity, more specifically, an intact $\Delta\Psi_m$, is important for protecting cells from apoptosis triggered by the classical death reagents. This agrees with our previous report and further reveals that, in addition to energy production, GRIM-19 is widely involved in protecting or promoting cell death induced by different death agents via distinct mechanisms.

ACKNOWLEDGMENTS

We thank F. Zhou (Yale University) for establishing of a $\rho 0$ cell line and K. F. Wan and C. P. Lim for critical reading of the manuscript. This work was supported by the Agency for Science, Technology and Research of Singapore. X.C. is an adjunct staff in the Department of Biochemistry, National University of Singapore.

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