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Mitochondria play a pivotal role as an ATP generator in aerobically growing cells, and their defects have long been implicated in the cellular aging process, although its detailed underlying mechanisms remain unclear. Recently, we found that, in the cellular senescent process of Chang cells induced by desferoxamine mesylate, an iron chelator, a significant decrease of intracellular ATP level was accompanied by decline in complex II activity, which preceded acquisition of the senescent phenotype. In the present study, we investigated the mechanism of how the mitochondrial ATP productivity was damaged by iron chelation and how complex II defect was involved in the senescent arrest. The ATP loss was irreversible and accompanied by sustained collapse of mitochondrial membrane potential (ΔΨm), but the ATP loss itself did not seem to be essential in progression to the senescent arrest. The ΔΨm disruption was due to decreased mitochondrial respiration, which was primarily associated with the defective complex II activity. Furthermore, we found that the declined activity of complex II was mainly due to down-regulation of protein expression of the iron-sulfur subunit, which was associated with the irreversibility of the arrest. Finally, we demonstrated that specific inhibition of complex II with 2-thenoyltrifluoroacetone induced overall delay of the cell cycle, suggesting that the delayed arrest by desferoxamine mesylate might be in part due to inhibition of complex II activity. Taken together, our results suggest that complex II might be considered as one of the primary factors to regulate mitochondrial respiratory function by responding to the cellular iron level, thereby influencing cellular growth.

Maintenance of energy in a cell is critical to the cellular growth and proliferation. In aerobically growing cells, most of cellular energy is primarily generated through mitochondrial oxidative phosphorylation (OXPHOS) in the form of ATP. The OXPHOS is composed of the four respiratory chain complexes and F1F0-ATP synthase (complex V). The respiratory chain comprises NADH-ubiquinone reductase (NQR or complex I), succinate-ubiquinone reductase (SQR; complex II), ubiquinol-cytochrome c reductase (complex III), and cytochrome c oxidase (complex IV). Accompanied by an electron transfer reaction through the respiratory complexes, the proton gradient is generated across the mitochondrial inner membrane, and the resulting ΔΨm is dissipated via complex V to synthesize ATP (1, 2). When mitochondrial respiration is disrupted by limited oxygen supply (hypoxic) or by any other reason, the cells often adopt a metabolic switch from aerobic to fermentative ATP production to keep the ATP level in order to survive against the stringent aerobic energy production (3). However, when overall energy supply is insufficient regardless of various causes, growth of cells is impeded, and they are destined to death through apoptotic or necrotic process, depending on the cellular ATP level (4, 5). On the other hand, the loss of mitochondrial respiratory control also contributes to release of apoptogenic factors from mitochondria in addition to overproduction of reactive oxygen species (ROS) in stressed or growth factor-deprived cells (6–9), further implying that mitochondrial respiratory defect is involved in cellular destination. To date, however, it has not been clearly understood whether the ATP level itself or a certain mitochondrial defect is critical to cajole cellular fate to apoptosis, necrosis, senescence, or even to cancerous transformation.

The mitochondrial damage has long been implicated in the aging process, because the mitochondrion is particularly vulnerable to oxidative stress in addition to its function as a major generator of free radicals, which have been firmly recognized as a causal actor of the aging process (10–14). Several studies have shown age-linked decrease in the activity of respiratory chain enzyme complexes (15, 16) and F0F1-ATP synthase (17). In addition, in intact hepatocytes, an age-dependent decrease of the mitochondrial membrane potential was also shown, together with increased mitochondrial ROS generation (18, 19). Although overall mitochondrial defect and mitochondrial ROS generation have generally been observed in aging and cellular senescent process, there has yet been no study to show direct involvement of a defect of specific respiratory complex, except recent reports that a mutation in the cytochrome b subunit of complex II caused oxidative stress, resulting in premature aging in nematode (20, 21).
Iron is an essential element in mitochondrial respiration, because the electron carriers within the respiratory complexes such as cytochromes and iron-sulfur clusters utilize iron as direct electron acceptors and donors (2, 22). Therefore, the mitochondrial respiratory activities are predicted to be sensitive to cellular iron levels. We recently reported that DFO induced Chang cells, an immortalized normal human hepatocyte cell line, to senescence-like irreversible growth arrest. During the arrest, a specific induction of TGF-β1 and p27kip1 was accompanied, implying its associated role in arresting the cell cycle progression at G1 phase (23). However, their induction was observed after only 3 days of exposure to DFO, and neutralization of TGF-β1 could not reverse the arrest, suggesting that other mechanisms are involved in the cellular commitment to the irreversible senescent arrest at the earlier stage. In the present study, we have elucidated that during the DFO-induced senescent arrest, complex II activity was disrupted at the earlier stage through down-regulation of iron-sulfur protein, its second largest subunit, and was accompanied with the irreversible ΔΨm disruption and concomitant decline of ATP level. Furthermore, we found that a specific inhibitor of complex II activity, 2-thienyltrifluoroacetone (TTFA), delayed the overall cell cycle progression, as in the case of DFO. These observations led us to conclude that complex II could be considered as one of the primary factors to regulate mitochondrial function by responding to the intracellular iron level, thus influencing cellular growth rate by controlling the overall progression of the cell cycle.

EXPERIMENTAL PROCEDURES

Cell Culture, DFO Treatment, and Transfection Condition—Chang cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) or DMEM (high glucose) supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen) in a 37 °C incubator with 5% CO2 in air. Cells (5 × 10^5) were seeded into 100-mm culture plates, cultured in DMEM containing 10% FBS for 24 h, and treated with 500 μM DFO for the indicated periods. The cells were continuously cultured by refreshing every 3 days with the medium containing DFO, and, if necessary, the cells were split to maintain subconfluence. Transfection was performed using LipofectAMINE reagent according to the protocol provided by the manufacturer (Invitrogen).

Determination of Cellular ATP Level—Cellular ATP levels were measured by the bioluminescence assay according to the protocol provided with an ATP determination kit (Molecular Probes, Inc., Eugene, OR). In brief, cells cultured in the absence or presence of DFO for the indicated periods were lysed with passive lysis buffer (Promega), and protein concentration was determined by the Bio-Rad protein assay. Cell lysate (5 μg) was mixed with 100 μl of luciferase reagent, and luminescence was measured with a Bio-Tek spectrophotometer.
DFO irreversibly induced ΔΨm disruption and cellular ATP loss. Chang cells were cultured in DMEM containing 10% FBS and treated with 1 mM DFO for a certain period of time (nd), and the media were refreshed without the media without DFO for the remaining days (+). A, cellular ATP level after release from DFO treatment. On the eighth day, the cells were harvested, and the lysates were assayed for total cellular ATP level. Results are mean ± S.D. of triplicate assays of two independent experiments. B, ΔΨm of the cells was visualized by staining them with JC-1 fluorescence dye (a), and the cells were stained for SA-β-gal activities (b) at the indicated times.

**Table I**

Effect of DFO on cellular ATP level and ΔΨm in Huh7 and Hep3B cells

<table>
<thead>
<tr>
<th></th>
<th>Huh7</th>
<th>Hep3B</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP pmol/μg lysate</td>
<td>ΔΨm (mean values)</td>
<td>ATP pmol/μg lysate</td>
</tr>
<tr>
<td>C</td>
<td>6.84 ± 0.97</td>
<td>124.8</td>
</tr>
<tr>
<td>1d</td>
<td>1.89 ± 0.32</td>
<td>92.8</td>
</tr>
<tr>
<td>2d</td>
<td>1.14 ± 0.08</td>
<td>86.4</td>
</tr>
<tr>
<td>3d</td>
<td>1.36 ± 0.17</td>
<td>79.5</td>
</tr>
</tbody>
</table>

*Huh7 or Hep3B cell lysates were assayed for total cellular ATP level after treatment without or with 0.5 mM DFO for the indicated periods. The values represent mean ± S.D. of triplicates.*

*ΔΨm was analyzed by flow cytometry after staining cells with TMRM fluorescence dye. The higher value indicates the more active mitochondria.*

**Fig. 2.** DFO irreversibly induced ΔΨm disruption and cellular ATP loss. Chang cells were cultured in DMEM containing 10% FBS and treated with 1 mM DFO for a certain period of time (nd), and the media were refreshed without the media without DFO for the remaining days (+). A, cellular ATP level after release from DFO treatment. On the eighth day, the cells were harvested, and the lysates were assayed for total cellular ATP level. Results are mean ± S.D. of triplicate assays of two independent experiments. B, ΔΨm of the cells was visualized by staining them with JC-1 fluorescence dye (a), and the cells were stained for SA-β-gal activities (b) at the indicated times.

**Senescence-associated β-Galactosidase Assay**—Senescence-associated β-galactosidase (SA-β-gal) was assayed at pH 6.0 as described by Dimri et al. (24) with a slight modification. Briefly, cells were washed twice with PBS, fixed to plates by 3% formaldehyde for 5 min, washed with PBS, and then incubated overnight in freshly prepared staining solution (40 mM citrate-phosphate buffer, pH 6.0, containing 1 mg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; Sigma), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM MgCl₂). The stain was visible 12 h after incubation at 37 °C.

**Assays for Mitochondrial Respiratory Chain Activities**—The activities of NADH-cytochrome c reductase (NCR), the integrated activity of complexes I and III and succinate-cytochrome c reductase (SCR), the integrated activity of complex II and III, and SQR alone in cell lysate were measured as described previously (26) with slight modification. Briefly, cell pellets were lysed in PBS buffer, and a portion (50 μg) of lysate was used for each assay. To determine NCR activities, the initial rate to reduce cytochrome c in the presence of NADH was monitored at 550 nm with SCR mixture (50 mM sodium/potassium phosphate, pH 7.4; 0.25 mM EDTA, 20 μM cytochrome c) and 0.25 mM KCN. The reaction was started by the addition of 100 μM NADH with a rapid mix to detect the rotenone-sensitive initial rate. For SCR activities, TFFA-sensitive initial rate to reduce cytochrome c in the presence of succinate was monitored at 550 nm with SCR mixture (50 mM sodium/potassium phosphate, pH 7.4; 0.25 mM EDTA, 20 μM cytochrome c, 20 mM sodium succinate) and 2 mM KCN. The specific activities of NCR and SCR are expressed as pmol of reduced cytochrome c/μg of lysate protein for 1 min by spectrophotometrically (Ultrospec 3000; Amersham Biosciences) following the increase of absorbance of reduced cytochrome c at room temperature. Succinate-ubiquinone reductase activity was assayed through the succinate-ubiquinone-dichlorophenolindophenol activity. A portion (50 μg) of lysate was added to the reaction mixture (20 mM sodium succinate, 25 mM sodium/potassium phosphate buffer, pH 7.2, 2 μg/ml antimycin, 2 μg/ml rotenone, 2 mM KCN, 50 μM dichlorophenolindophenol), and the reaction was started by adding 65 μM coenzyme Q10 (Sigma). The initial rate of dichlorophenolindophenol reduction was measured at 600 nm in a Ultrospec 3000.

**Differential Spectrum of Cytochrome c**—1 ml of 100 μM cytochrome c (Sigma) was incubated at the indicated temperature for a certain period of time in the absence or presence of DFO. Differential spectrum of reduced minus oxidized cytochrome c between 500 and 600 nm was obtained from the dithionite minus potassium ferricyanide-treated...
The absence or presence of DFO, washed with TD buffer (0.137 M NaCl, 51580
A
GAAGGCTGGGG-3
used in this study were as follows: for human GAPDH, 5
-CCATGGA-
/H11032
illuminator (Gel Doc 2000; Bio-Rad). The sequences of the primer sets
each subconfluent culture by using TRIzol reagent (Invitrogen). 1
/H9262
0.5
PCR products were electrophoresed on 1.5% agarose gel, stained with
one-tenth volume of cDNA synthesis reaction mixture. The final RT-
Shiga, Japan). Subsequently, PCR cycles were performed by using
blastosis virus RT XL using RNA PCR kit version 2.1 (Takara Inc.,
total RNA was used as template for cDNA synthesis with avian myelo-
5m M KCl, 0.7 mM Na2HPO4, 2 5m M Tris-HCl, pH 7.4), and collected by
trypsinization. After resuspending the cells in 0.3 ml of complete me-
the maximum respiration rate, and its specificity for mitochondrial
respiration was confirmed by adding 5 mM KCN. Maximum cellular
respiration rates were expressed as the ratio of DNP-uncoupled
versus

FIG. 3.

Fig. 3. Significant decrease of complex II activity preceded the ΔΨm disruption and the ATP loss. Chang cells were cultured in
DMEM containing 10% FBS and treated with 1 mM DFO for the indicated periods. A, the cell lysates were assayed for NCR (●) and SCR (■) activities. Results are expressed as μmol of reduced cytochrome c/min/mg of lyase protein. B, complex II (SQR) activity of the lysates was analyzed and expressed as a percentage of control from the initial velocity. Results of A and B are mean ± S.D. of triplicate assays of two independent experiments. C, the degree of ΔΨm disruption was analyzed by counting fluoresced green cells (inactive mitochondria) and comparing the intensity
using flow cytometry after staining the cells with JC-1 fluorescence dye. The percentages of cell number in M2 region are presented in the lower panel. The cell lysates were also assayed for the total cellular ATP level at the indicated times.

spectrum using a spectrophotometer (Ultraspex 4300; Amersham Biosciences). The absorbance differences between 550 and at 580 nm of the spectra were represented for redox capacity of cytochrome c.

Endogenous Cellular Oxygen Consumption—Endogenous respiration was measured as described previously (27) with slight modification. Briefly, exponentially growing Chang cells (5 × 10⁶) were cultured in the absence or presence of DFO, washed with TD buffer (0.137 M NaCl, 5 mM KCl, 0.7 mM Na2HPO4, 2 5 mM Tris-HCl, pH 7.4), and collected by trypsinization. After resuspending the cells in 0.3 ml of complete medium without phenol red, the cells were transferred to the chamber of Mitocell equipped with a Clark oxygen electrode (782 Oxygen Meter; Strathkelvin Instruments, Glasgow, UK). Oxygen consumption rates were measured after adding 30 μM 2,4-dinitrophenol (DNP) to obtain the maximum respiration rate, and its specificity for mitochondrial respiration was confirmed by adding 5 mM KCN. Maximum cellular respiration rates were expressed as the ratio of DNP-uncoupled versus KCN-inhibited O2 consumption rate.

Reverse Transcriptase (RT)-PCR—Total RNA was prepared from each subconfluent culture by using TRIzol reagent (Invitrogen). 1 μg of total RNA was used as template for cDNA synthesis with avian myeloblastosis virus RT XL using RNA PCR kit version 2.1 (Takara Inc., Shiga, Japan). Subsequently, PCR cycles were performed by using one-tenth volume of cDNA synthesis reaction mixture. The final RT-PCR products were electrophoresed on 1.5% agarose gel, stained with 0.5 μg/ml ethidium bromide solution, and visualized on a UV trans-

Western Blot Analysis and Antibodies—Cells were washed twice with PBS and lysed with lysis buffer (50 mM Tris-Cl, pH 7.5, 0.1 mM EDTA, 1% (v/v) Triton X-100, 10 μg/ml each aprotinin and leupeptin, and 1 mM phenylmethylsulfonyl fluoride). A portion (50 μg) of the lysate protein was separated by SDS-PAGE, and the proteins were electrically transferred onto nitrocellulose membrane (Protran; Schleicher and Schuell). The membrane was blocked for 1 h at 24 °C with blocking solution (PBS containing 0.05% Tween 20 and 5% (w/v) nonfat milk). Subsequently, the membranes were incubated in blocking solution at 4 °C with the 1 μg/ml of available monoclonal antibodies against subunits of OXPHOS complexes (complex II-Fp, A11142; complex II-Ip, A11141; complex I, A11140; complex III, A11143; complex IV subunit I, A6403; complex IV subunit IV-A6431, complex V, A11144; Molecular Probes), α-actin (sc-1615, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or α-tubulin (Ab-1; Oncogene, Boston, MA). Membranes were then washed twice with PBS containing 0.05% Tween 20 and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG antibodies (Amersham Biosciences) for 1 h at 24 °C. Detection was carried out by treating the membranes with ECL reagents (Amersham Biosciences) and exposing them to x-ray film.

Cell Synchronization and Cell Cycle Analysis—Chang cells were synchronized at late G1 phase by thymidine double treatment (0.5 mg/ml) for 18 h with an 8-h interval. The cells were released from the synchronization by refreshing the medium in the absence or presence of 0.4 mM TTFA or 0.5 mM DFO and collected at the indicated times. Cell cycle analysis was performed by propidium iodide staining according to the protocol provided with CycleTESTPLUS DNA reagent kit (Becton Dickinson). Briefly, cells were trypsinized, collected by centrifuga-
tion, and washed with and resuspended in PBS before fixing in 70% ethanol at a density of 1 × 10^6 cells/ml. According to the protocol, the fixed cells were stained with 50 μg/ml propidium iodide for at least 10 min before flow cytometric analysis (Becton-Dickinson FACsorter). The instrument was set to collect 1 × 10^4 cells, and the cell cycle profile was analyzed using CELLQuest software. For analyzing the effect of TTFA on cell cycle patterns of asynchronous cells, subconfluent cells were treated with or without 0.4 mM TTFA for the indicated periods and then collected.

RESULTS

Total Cellular ATP Level Decreased Significantly with Concurrent ∆Ψm Disruption in DFO-induced Senescent Arrest—In order to address the possibility that mitochondrial activity could be a primary target of iron depletion in the senescent arrest induced by DFO, total cellular ATP level was determined. As shown in Fig. 1A, Chang cells contained about 16 pmol of ATP/1 μg of lysate protein when they were cultured in DMEM medium containing 10% FBS. When treated with DFO, the total cellular ATP level significantly decreased to 8.7 pmol/μg of lysate (53.8% of control) within 1 day but did not decrease further thereafter. This finding implied that elucidation of mechanisms involved in the ATP loss within 24 h could greatly help in finding the specific target of iron depletion. We next monitored ∆Ψm by staining cells with JC-1 fluorescence dye in order to investigate the mechanism of how the cellular ATP level decreased. As the exposure time to DFO increased, green fluorescence-stained cells, which represented the cells harboring inactive mitochondria with low ∆Ψm, continuously increased, whereas the cells with active red fluorescence-stained mitochondria decreased (Fig. 1B). This result suggested that the ATP loss was mainly due to the ∆Ψm disruption. However, it should be noted that the total cellular ATP level was still maintained at half the control level until the end of experiment (up to the 7th day), despite the continuously aggravated disruption of ∆Ψm. Eventually, we found that the ATP level after ∆Ψm disruption was partially complemented by activated glycolytic ATP production (data not shown).

Next, we attempted to restore the DFO-induced cellular ATP loss with medium supplemented with high glucose in order to explore whether the ATP loss itself is critical to destine cells to the senescent arrest. As shown in Fig. 1C, with high glucose medium, the total cellular ATP level was maintained 1.6-fold higher than the control. During 3-day treatment of DFO in high glucose medium, the ATP level dropped to 51.8% of the control (no DFO treatment in high glucose), which was still comparable with that of the control cells (no DFO treatment) with normal medium. However, the ∆Ψm of the cells was disrupted, and the cells gained SA-β-gal activity (Fig. 1C). These data suggested that the ATP loss itself was not important in the progression to senescent arrest; however, some other mitochondrial dysfunction related to mitochondrial OXPHOS might be involved. Similar ∆Ψm disruption and cellular ATP loss by DFO were also observed in other hepatocyte cell lines such as Huh7 and Hep3B cells (Table I), in which gain of the senescent phenotype was previously confirmed (23).

The Irreversible ∆Ψm Disruption and ATP Loss Were Associated with the DFO-induced Senescent Arrest—In order to examine whether the disruption of mitochondrial ATP productivity was related to the irreversibility of the senescent arrest, Chang cells were treated with 1 mM DFO for various periods of time and then released by replacing the medium with fresh medium without DFO. Transient exposure of the cells to DFO for 1 day partially recovered the ATP level, but exposure for longer than 2 days induced irreversible ATP loss (Fig. 2A). This result was well correlated with the irreversible ∆Ψm disruption and gain of SA-β-gal activity, as shown in Fig. 2B.
Significant Inhibition of Complex II Activity Was Correlated with the \( \Delta \Psi_{m} \) Disruption and ATP Loss—To elucidate how \( \Delta \Psi_{m} \) was disrupted, we monitored the respiratory chain activities within 48 h of the exposure to DFO by assessing SCR (complex II-III-coupled) and NCR (complex I-III-coupled) activities. Whereas no changes were observed in NCR activities, except a slight increase at 48 h, SCR activity was decreased to 48% of the control at 6 h and to 22% at 12 and 24 h (Fig. 3A). The difference between NCR and SCR activity profiles represents the difference between complex I and II activities, because complex III is a common factor in both activities. Therefore, SCR activity alone was further examined, and we confirmed the significant decrease in SCR activity within 12 h (Fig. 3B). The inhibition of complex II activity was followed by \( \Delta \Psi_{m} \) disruption and ATP loss (Fig. 3C), implying that the disruption of mitochondrial activities could have been initiated by the inhibition of complex II activity.

DFO Could Chelate Iron from Cytochrome c at 37°C in Vitro—Next, a question arises how SCR activity could be inhibited by DFO. To address this question, we first examined whether the NQR or SCR activity was directly inhibited by DFO. However, no direct inhibition of the NCR and SCR activities was observed by the addition of DFO up to 2 mM (data not shown). Nevertheless, we could not exclude the possibility of the different experimental conditions between the above \textit{in vitro} assay and cell culture system.

SQR contains 10 irons in the form of intracomplex electron carriers such as \( b_{560}- \) heme and iron-sulfur clusters (2Fe-2S, 3Fe-4S, and 4Fe-4S). In the case of iron-sulfur clusters, irons are associated with inorganic sulfurs, or the sulfur atoms of cysteine reside in the protein; however, the iron embedded within the heme structure is coordinated to two histidine residues (28). To understand the effect of DFO on direct chelation of iron from iron-containing prosthetic groups, the effect of DFO on redox capacity of cytochrome c was analyzed, because of the difficulty in isolating intact iron sulfur protein or cytochrome \( b_{560} \) subunits from SQR. The redox capacity was expressed as the difference of absorbances at 550 and 580 nm of the dithionite-reduced minus ferricyanide-oxidized differential spectrum of cytochrome c. When cytochrome c was incubated with 1 mM DFO at 4°C, the redox capacity was not changed (data not shown); however, the redox capacity was continuously reduced, as the incubation temperature was increased up to 50°C (Fig. 4A). When treated with DFO at 37°C, the redox capacity of cytochrome c kept decreasing in dose- and time-dependent manners, showing 60% inhibition at 24 h with 1 mM DFO (Fig. 4B and C). And the inhibited redox capacity was slowly restored up to 80% by the addition of 2.5 mM FeCl3 (Fig. 4C), suggesting that the decrease of the redox capacity was due to iron chelation but not due to other effects such as conformational change. These data implied that direct chelation of Fe\(^{3+}\) from cytochromes by DFO was possible in the culture system but with a very slow rate. However, it should be mentioned that cytochrome c is a unique soluble cytochrome acting between respiratory chain complexes III and IV. Other cytochromes and iron-sulfur proteins of respiratory complexes are all complexed with several other subunits and embedded within membrane, implying that chelation of iron from the complex might be more difficult. This idea could be further supported by our earlier finding that the NCR activity was not affected up to 48 h (Fig. 3).

We further examined the effect of DFO on endogenous KCN-sensitive mitochondrial respiration by monitoring cellular oxygen consumption rate with a Clark oxygen electrode. As shown in Fig. 5A, DFO did not inhibit directly both coupled and
DNP-uncoupled cellular respiration; however, the maximal cellular respiration rate of DFO-treated cells, represented as a ratio of DNP-uncoupled versus KCN-inhibited O$_2$ consumption, decreased to 59.7% at 1 day and 55.2% at 2 days after exposure to DFO (Fig. 5B), which corresponded well with the complex II activity profile.

Decrease of Complex II Activity Was Due to Down-regulation of Its Iron-Sulfur Subunit by Iron Chelation—It has generally been accepted that treatment of cells with iron chelator affects cellular iron level through transferrin and ferritin, the iron transporting and storing system, and in turn regulates gene expression through interaction of iron regulatory protein (IRP) and iron-responsive element (IRE) of the target genes (29). We investigated whether iron chelation had an impact on SQR expression using Western blot and RT-PCR analyses: SQR is composed of four subunits, including flavoprotein (Fp), iron-sulfur protein (Ip), and two membrane-anchoring ubiquinone-binding proteins (QPs1 and QPs2; Fig. 6A), which are all encoded by nuclear DNA. As shown in Fig. 6A, the expression level of the flavoprotein, which is the largest subunit (70 kDa) and contains FAD, did not show any change up to 5 days, whereas Ip expression abruptly decreased from the first day (Fig. 6B). mRNA levels of two smaller subunits, which contain cytochrome b$_{560}$ and a quinone binding site, started to decrease 3 days after the treatment (Fig. 6C). In order to evaluate in detail the relationship between the complex II activity and Ip expres-
Complex II Defect during Iron Chelation-induced Senescence

Figure 7. Down-regulation of complex II-Ip protein was involved in the irreversible ∆Ym disruption. A, Chang cells were cultured in DMEM containing 10% FBS and treated with 0.5 mM DFO for 3 days (3d), and the media were refreshed with the media without DFO for another 3 days (+3). The expression level of Ip subunit of complex II was examined by Western blot analysis and compared with that of Fp subunit. B, Chang cells were transfected with complex II/Ip antisense plasmid (AS) or pcDNA3 (vector) and cultured for another 2 days, and the expression level of the Ip protein was analyzed by Western blot analysis. The disrupted extent of ∆Ym (lower panel) was analyzed by monitoring the intensity of green fluorescence after staining the cells with JC-1 using flow cytometry.

Discussion

The involvement of mitochondrial genetic and functional defect in the aging process has long been documented, despite much controversy on detailed molecular mechanism due to its diverse effect. Much attention has been focused on overproduction of mitochondrial ROS accompanied by a decrease of mitochondrial respiratory activities, which has been suggested to result from a vicious cycle of random oxidative damage on the naked mitochondrial DNA. In order to delineate involvement of any specific mitochondrial defect, we analyzed mitochondrial activities in senescence-associated growth arrest of Chang cells induced by iron chelation. An irreversible decrease of intracellular ATP level was found to be associated with the arrest. Although several studies have also shown age-associated decrease of the ATP/ADP ratio, accompanied by mitochondrial defects (15–17, 30, 31), the involvement and role of the decreased ATP level in the aging process remain unclear. In our present system, however, the loss of ATP itself was not the critical factor, although the mitochondrial damage might be (Fig. 1). Interestingly, the ATP loss was accompanied by ∆Ym disruption and correlated well with specifically decreased complex II activity (Fig. 3), implying that complex II defect might contribute to the ∆Ym collapse, because the proton pumping process does not occur through complex II. A few possibilities can be offered to explain that the processes may be mediated by the complex II defect. First, the ∆Ym disruption is the consequence of an overall decrease of reduction rate of ubiquinone to ubiquinol, a substrate of complex III. Second, the citric acid cycle blocked by inhibition of the complex II may diminish cellular NADH level and subsequently decrease electron transfer through complex I-III-IV. Third, ROS produced by the defective complex II (28) can damage other mitochondrial complex activities. Among the above possibilities, the last scenario appears to be quite unlikely, since the enzymatic turnover rates of complexes I and III were not affected in this DFO-induced arrest. However, the possibility that ROS disrupt overall mitochondrial activity through an unknown mechanism could not be excluded. Nevertheless, our present data demonstrated biochemical connection existing between the molecular defect of the complex II, sustained mitochondrial dysfunction, and finally cellular commitment to the irreversible senescent arrest. Recently, the complex II defect by the mev-1(kn1) mutation has been reported to lead the nematode Caenorhabditis elegans to premature senescence (20, 21). Although the cause of induction of the complex II deficiency in the nematode is different from that of...
our iron chelation system, the resulting complex II defect and its primary action are the same, thus emphasizing involvement of the complex II defect in the senescent process.

In the present study, the defect of the complex II activity was found to be due to down-regulation of the Ip subunit by iron depletion, and its irreversible down-regulation was well correlated with the collapse of respiration rate and ΔΨm, ATP loss and even with appearance of SA-β-gal activity (Figs. 2 and 7), implicating its involvement in the arrest. However, there arises a question of how iron depletion specifically repressed the complex II-Ip expression. A few studies have described that iron chelation down-regulated the Ip subunit of complex II in Drosophila (32, 33). These researchers found that the IRE sequence existing within the 5′-untranslated region of the Ip gene of complex II was structurally similar to that of ferritin, and the IRE of the complex II-Ip gene (SDHb) had similar affinity for IRP-1 to the ferritin H chain IRE, thereby showing comparable sensitivity to iron depletion (32). Although these studies support our data well, specific inhibition of the Ip subunit of complex II cannot be explained, because complex I and III have several iron-sulfur proteins. We therefore examined whether gene expression of Ip of the complex II was more sensitive or specific to iron depletion than those of other complexes. When examined by RT-PCR, the mRNA levels of the Ips of complex I, complex II, and complex III were all found to be reduced; however, a much faster decline of the Ip mRNA level of complex II was observed (see Supplemental Material). The faster decrease of the mRNA level could be explained by the existence of two AUUUA sequences on the 3′-UTR mRNA destabilizing elements (see Supplementary Material and Ref. 34).

It has been well established that the regulation of gene expression via the IRP-IRE complex occurs at translational machinery. Unfortunately, protein levels of Ips of complex I and III (Ip-I, Ip-III) could not be detected at the present stage because of unavailability of antibodies for those proteins. However, by analyzing the existence of putative IRE sequences, one may be able to deduce whether these expressions can be post-transcriptionally regulated. Since the IRE sequence within the Ip gene of complex II has been reported to exist in 5′-UTR and to be structurally similar to that of ferritin (see Supplemental Material), we searched and compared the 5′-UTR sequence of one of the Ip-Is and Ip-III with that of Ip-II. There was no putative IRE sequence found in 5′-UTR of both Ip-I and Ip-III. These results obtained indicated that the down-regulation of complex II-Ip was due to both fast mRNA destabilization and post-transcriptional repression through IRE-IRP complex formation. Taken together, among mitochondrial respiratory proteins, the complex II could be considered to be the most sensitive component to respond to the cellular iron level.

What would be the most substantial impact of the complex II defect on cellular growth? The recent finding that mutation of a component of complex II induced premature aging in nema-
tode (20, 21) could be an epoch-making event, because abnormalities in complex II are relatively rare except for a few genetic mutations found in paraganglioma and pheochromocytoma (35–37). From these reports, possible contribution of a specific mitochondrial defect to premature aging could be delineated. Although mitochondrial ROS generated by a complex II defect has been implicated in the aging process (20, 21, 28, 38), no clear effect of the defect on cell cycle or cellular growth has been shown. We analyzed the direct effect of the defect of the complex II activity on the cell cycle by employing the specific complex II inhibitor, TTFA. In asynchronous Chang cells, TTFA did not arrest the cells at any specific phase of cell cycle, despite decreased cellular growth (data not shown). The reduced cellular growth was due to sluggish progression of overall cell cycle, despite decreased cellular growth (data not shown). The slow G1 arrest by DFO resulted from concerted sequential action on the dNTP level by both double-thymidine block (DTB) and G1 arrest, and the disruption was resulted from the specific inhibition of complex II activity through down-regulation of Ip protein of complex II. The loss of ATP itself was not critical to induce senescent arrest; however, some mitochondrial dysfunction might be critical. The most important significance of our findings appears to be the involvement of a complex II defect in the cellular senescence by sensing the cellular iron level.

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