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Identification of cytochrome *c* oxidase subunit 6A1 as a suppressor of Bax-induced cell death by yeast-based functional screening

So Young Eun [a,1], Im Sun Woo [a,1], Han-Su Jang [b], Hana Jin [a], Min Young Kim [a], Hye Jung Kim [a], Jae Heun Lee [a], Ki Churl Chang [a], Jin-Hoi Kim [c], Han Geuk Seo [a,*]

[a] Department of Pharmacology, Gyeongsang Institute of Health Science, Gyeongsang National University School of Medicine, 92 Chilam-dong, Jinju 660-751, Republic of Korea
[b] Department of Research and Development, Gyeongbuk Institute for Bioindustry, 1319-84 Songcheon-dong, Andong 760-380, Republic of Korea
[c] Department of Animal Biotechnology, Kon-Kuk University, 1 Hwayang-dong, Seoul, Republic of Korea

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ABSTRACT

Human cytochrome *c* oxidase subunit VIa polypeptide 1 (COX6A1) was identified as a novel suppressor of Bcl-2-associated X protein (Bax)-mediated cell death using yeast-based functional screening of a mammalian cDNA library. The overexpression of COX6A1 significantly suppressed Bax- and *N*-(4-hydroxyphenyl)retinamide (4-HPR)-induced apoptosis in yeast and human glioblastoma-derived U373MG cells, respectively. The generation of reactive oxygen species (ROS) in response to Bax or 4-HPR was inhibited in yeast and U373MG cells that expressed COX6A1, indicating that COX6A1 exerts a protective effect against ROS-induced cell damage. 4-HPR-induced mitochondrial translocation of Bax, release of mitochondrial cytochrome *c*, and activation of caspase-3 were markedly attenuated in U373MG cells that stably expressed COX6A1. Our results demonstrate that yeast-based functional screening of human genes for inhibitors of Bax-sensitivity in yeast identified a protein that not only suppresses the toxicity of Bax in yeast, but also has a potential role in protecting mammalian cells from 4-HPR-induced apoptosis.

Cytochrome *c* oxidase (COX), the terminal enzyme of the mitochondrial respiratory chain, catalyzes the transfer of electrons from reduced cytochrome *c* to oxygen. COX is a large transmembrane protein located in the inner mitochondrial membrane of eukaryotic cells [1]. Mammalian COX consists of 13 subunits, with three mitochondrial-encoded subunits (COX1–COX3) forming the core enzyme. The remaining subunits are encoded by the nuclear genome and consist of small polypeptides that surround the catalytic core [1]. Defects in COX activity are associated with significant pathology, usually affecting highly metabolic tissues, including brain, muscle, and eyes [2]. The expression of rat *COX6A1* mRNA transcripts has been detected in cultured rat primary neurons by both *in situ* hybridization and RNase protection assay [3]. Murine *COX6A1* mRNA has also been detected in the mouse brain by *in situ* hybridization [4]. Of note, the levels of both rat and mouse *COX6A1* mRNA change in response to neuronal energy demand [3,4]. Recent reports have shown that the overexpression of COX in human hematopoietic cells suppresses the cytotoxic effects of TNF-α [5].

The heterologous expression of Bax in yeast results in cell death, and there is a growing body of evidence that Bax-induced yeast cell death is mechanistically similar to the process that occurs in mammalian cells [6–11]. Yeast cells that express Bax under the control of an inducible promoter exhibit a conditional lethal Bax-dependent phenotype [6–11]. Using this system, several groups have successfully identified a variety of proteins that are involved in Bax-induced cell death in yeast. These proteins include DNA-binding proteins, such as chromosomal high-mobility group box-1 (HMGB1); enzymes that are involved in the inhibition of free radicals, such as peroxidases; Bax-binding proteins, such as Bax inhibitor-1 (BI-1) and Ku70; a plant vesicle-associated membrane protein involved in vesicular trafficking; and a number of other proteins of unknown function [7–11].

In tumor cells, reduced sensitivity to apoptotic signals can result from either a decrease in the activity of pro-apoptotic proteins, or the acquisition of gain-of-function anti-apoptotic molecules [12]. The activation of anti-apoptotic genes in tumors is a compelling molecular target in the field of anti-cancer gene therapy [13], and the identification of novel anti-apoptotic genes that are expressed in tumor cells is expected to lead to new anti-cancer gene therapeutics. We have established a yeast-based system for the functional screening of anti-apoptotic mammalian genes from a tumor cell-derived cDNA library. In the current study, we identified COX6A1 as a novel inhibitor of Bax-induced cell death in *Saccharomyces cerevisiae*, and showed that the overexpression of COX6A1 in U373MG glioblastoma cells suppresses *N*-(4-hydroxyphenyl)retinamide (4-HPR)-induced cell death.

* Corresponding author. Fax: +82 55 759 0609.
E-mail address: hgseo@gnu.ac.kr (H.G. Seo).
[1] These authors should be considered co-first authors.

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Materials and methods

Materials. Mouse anti-Bax and -Heat shock protein 60 (HSP60) monoclonal antibodies (mAbs), and rabbit anti-cytochrome *c* and -caspase-3 polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit anti-Flag polyclonal antibody was obtained from Cell Signaling (Beverly, MA). 4-HPR was obtained from Calbiochem (La Jolla, CA).

Yeast strains and plasmid constructs. The yeast strain used in these studies, *S. cerevisiae* W303-1a (*MATa ade2-1 can1-100 his3-11.15 leu2-3.112 trp1-1 ura3-1*), was cultured using standard procedures [14]. The construction of pGilda-Bax has been previously described [15].

Yeast-based functional screening. A glioblastoma U373MG cDNA library was constructed using pADGAL4-2.1 (Eugentech, Daejeon, Korea). W303-1a/Bax, containing the inducible plasmid pGilda-Bax, was grown in glucose-based synthetic dropout (SD) medium (0.67% Yeast Nitrogen Base-w/o aminoacid, 2% glucose) supplemented with amino acids (L-arginine 20 mg/L, leucine 20 mg/L, lysine 30 mg/L, methionine 20 mg/L, tryptophan 20 mg/L, adenine hemisulfate 20 mg/L, uracil 20 mg/L) and deficient in histidine (SD-Glc/His^-). Cells were transformed with the U373MG cDNA library by the lithium acetate method [16]. The transformed cells (4.6×10^5) were plated onto galactose-based SD medium supplemented with amino acids and deficient in leucine and histidine (SD-Gal/His^-Leu^-). Plasmids were isolated from viable colonies and then reintroduced into W303-1a/Bax to confirm the suppression of Bax lethality phenotype.

Cell growth and viability assays. W303-1a/Bax carrying pADGAL4-2.1-COX6A1, pADGAL4-2.1-Bcl-2 or pADGAL4-2.1-Bcl-x_L was grown for 16 h at 30 °C in SD-glucose medium. For spot assays, cells were grown in SD-glucose medium for 1 day, and then the cultures were diluted to different concentrations. An aliquot (5 μl) of each culture dilution was spotted onto SD-glucose or -galactose plates and the plates were incubated for 2 days (glucose medium) or 3 days (galactose medium). For growth curves, cells were grown for 20 h at 30 °C in SD-glucose medium. The cells were diluted in fresh SD-galactose medium to an absorbance at 600 nm (OD_{600}) of 0.05, and then aliquots of the culture were removed at the indicated times and cell density was determined by OD_{600}.

Mammalian cell culture and construction of stable transfectants. Human glioblastoma-derived U373MG cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin G and 100 μg/ml streptomycin at 37 °C. Stable U373MG transfectants that expressed the Flag epitope alone, or a Flag epitope fusion protein of COX6A1 (Flag-COX6A1) were generated by transfection with Flag-pcDNA3.1 or Flag-pcDNA3.1-COX6A1, respectively.

Detection of ROS. Yeast or U373MG transfectants were incubated with 50 or 10 μM 2′,7′-dichlorofluorescein diacetate (H_2DCF-DA) for 120 or 30 min, respectively. After washing twice with ice-cold PBS, cell fluorescence was immediately analyzed using a FACScan flow cytometer (Becton–Dickinson Immunocytometry Systems, San Jose, CA).

Western blot analysis. Cell lysates were prepared from yeast or U373MG cells. Proteins were subjected to SDS–polyacrylamide gel electrophoresis and then transferred onto a Hybond-P^+ polyvinylidene difluoride membrane (Amersham Biosciences, Piscataway, NJ). Membranes were incubated with the indicated primary antibodies, and immunoreactive proteins were detected using WEST-ZOL Plus (iNtRON Biotechnology, Seoul, Korea).

MTT assay. U373MG cells were seeded into 24-well plates at a density of 2×10^4 cells/well. After incubation overnight, the cells were treated with the indicated concentrations of 4-HPR for 72 h, and then 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to the culture medium. After incubation for an additional 4 h, the formazan reaction product was solubilized in acidified isopropanol and measured spectrophotometrically.

DAPI staining and fluorescence-activated cell sorting. U373MG cells were cultured on coverslips and then treated with 20 μM 4-HPR for 48 h. Cells were fixed with 4% paraformaldehyde, and then stained with 4′,6-diamidino-2-phenylindole (DAPI) (2 μg/ml) for 30 min. Apoptotic cells were scored by an independent observer over four individual low-power fields. For the analysis of apoptosis using fluorescence-activated cell sorting, cells were plated at a density of 5×10^5 cells/100-mm culture dish, and then treated with 20 μM 4-HPR or dimethyl sulfoxide (DMSO) for 72 h. Cell cycle profiles were determined by staining with propidium iodide (PI) using a FACScan flow cytometer.

Fractionation of cell extracts. U373MG cells were seeded at a density of 5×10^5 cells/100-mm culture dishes and then treated with 4-HPR for the indicated periods of time. After homogenization with a glass dounce homogenizer, the homogenate was fractionated into cytosolic and mitochondrial fractions, as described previously [17].

Statistical analysis. Data is presented as the means ± standard error (SE). Statistical analysis was performed using the Student's *t*-test.

Results

Screening for anti-apoptotic genes that inhibit the Bax-sensitive phenotype in yeast

The yeast strain W303-1a/Bax carries the Bax-expression plasmid, pGilda-Bax, which encodes full-length mouse Bax under the control of the galactose-inducible yeast *GAL*1 promoter [9]. A human glioblastoma U373MG cDNA expression library was transformed into W303-1a/Bax, and we isolated 24 clones that were capable of suppressing the growth-inhibitory effect of mouse Bax from a total of 4×10^6 transformants. We determined the identity of the 24 Bax-resistant clones by sequence analysis and a search of the GenBank/EMBL nucleotide sequence databases. The strongest suppressor of Bax-sensitivity was a 330 base pair cDNA (GenBank Accession No. BC070186) that encoded a predicted open reading frame of 110-amino acid residues.

Protective effect of COX6A1on Bax-induced yeast cell death

We first examined whether the expression of COX6A1, or the apoptotic regulatory proteins Bcl-2 and Bcl-x_L, affected the expression levels of Bax in W303-1a/Bax. Bax was undetectable when cells were grown in glucose-based medium, and was readily detected in all of the transformants within 12 h of culture in galactose-based medium, which suggested that COX6A1, Bcl-2 and Bcl-x_L do not interfere with the expression of Bax protein in yeast (Fig. 1A). When W303-1a transformants were grown overnight in liquid glucose-containing medium and then streaked onto glucose- or galactose-containing SD plates, there were no differences in growth on glucose-containing SD plates among the four transformants (Fig. 1B, left panel). In contrast, W303-1a/Bax failed to grow on galactose-containing medium (Fig. 1B, right panel). Co-expression of COX6A1 with Bax resulted in a significant increase in growth on galactose, similar to that induced by the co-expression of Bcl-2 or Bcl-x_L (Fig. 1B, right panel). To determine whether COX6A1 affected the growth of Bax-expressing yeast, we monitored the growth rate of the various yeast transformants following inoculation into fresh galactose-containing medium. Yeast that expressed Bax alone exhibited a significantly slower rate of growth as

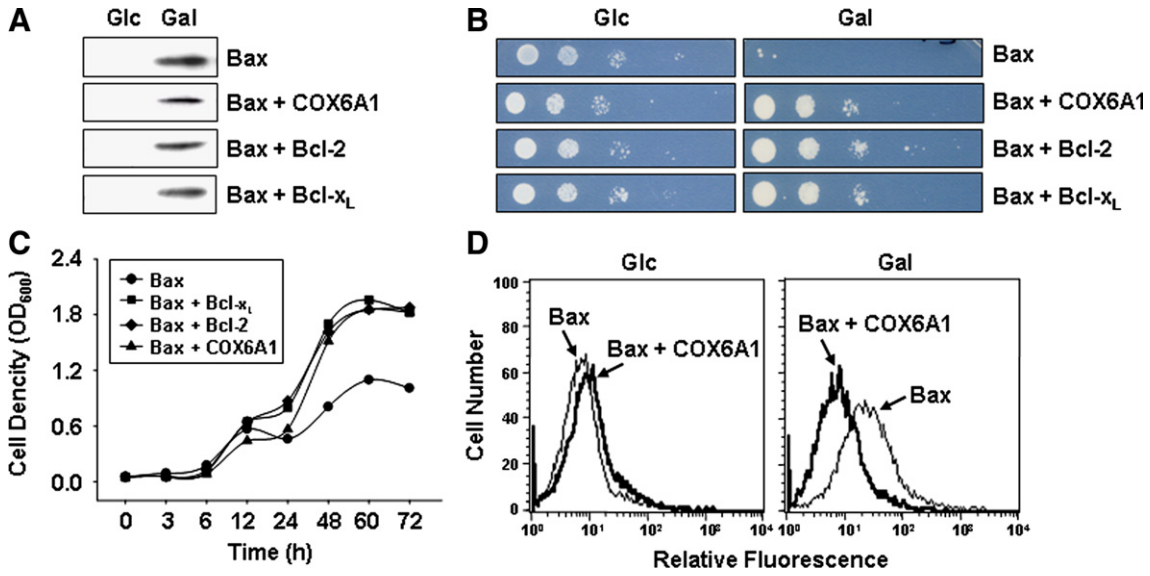

Fig. 1. COX6A1 suppresses Bax-induced yeast cell death. (A) W303-1a cells harboring the indicated mammalian genes were grown in SD-glucose medium (Glc) for 16 h at 30 °C. The cells were then transferred to fresh SD-galactose (Gal) or SD-glucose medium and incubated for an additional 12 h at 30 °C. Total cell lysates were analyzed by Western blot using an anti-Bax mAb. (B) Cells grown in SD-glucose medium for 1 day were adjusted to an OD_{600} of 0.1 and then diluted to the indicated concentrations (OD_{600} = 1, 0.1, 0.01, 0.001, and 0.0001). An aliquot (5 μl) of each dilution was spotted onto SD-glucose or -galactose plates and then the plates were incubated for 2 days or 3 days, respectively. (C) Cells grown in SD-glucose medium were inoculated into SD-galactose medium, and growth was monitored by measuring the OD_{600}. (D) The indicated yeast transformants were grown in glucose or galactose medium for 12 h, and then incubated with 50 μM H_2DCF-DA for 2 h at room temperature. The levels of ROS were quantified by flow cytometry. The results are representative of three independent experiments.

compared to yeast that co-expressed Bax and COX6A1, and the effect of COX6A1 on the growth of Bax-expressing cells was similar to that of Bcl-2 or Bcl-x_L.

Involvement of ROS in Bax-induced yeast cell death

Recently, it was demonstrated that ROS accumulate in cells that overexpress Bax, and function as effector molecules in Bax-induced apoptotic cell death in yeast [9]. To investigate whether ROS were involved in the suppression of Bax-induced yeast cell death by COX6A1, we examined the production of ROS during Bax-induced cell death using H_2DCF-DA. As shown in Fig. 1D, ROS production was similar in Bax and Bax/COX6A1 transformants that were grown in glucose-containing medium. In contrast, COX6A1 co-expression markedly inhibited the generation of ROS in Bax-expressing cells that were grown on galactose-containing medium. These results suggested that COX6A1 suppresses Bax-induced cell death by preventing the accumulation of intracellular ROS.

Effect of COX6A1 on 4-HPR-induced apoptosis in mammalian cells

The anti-neoplastic agent 4-HPR has been shown to be effective in inducing apoptotic cell death in mammalian cells [18]. To determine whether COX6A1 had a suppressive effect on 4-HPR-induced cell death, we generated stable U373MG transfectants that expressed Flag-COX6A1, or Flag alone as a control. As shown in Fig. 2A, treatment of the control cells with 4-HPR resulted in a decline in cell viability in a concentration-dependent manner (Fig. 2A), whereas 4-HPR-mediated cytotoxicity was significantly attenuated in cells that overexpressed Flag-COX6A1. Exposure to 4-HPR also resulted in dramatic morphological changes and a marked inhibition of growth in Flag-transfectants (Fig. 2B). In contrast, both the morphological and growth inhibitory effects of 4-HPR were suppressed in Flag-COX6A1 transfectants (Fig. 2B). When we examined apoptosis in these cells using DAPI staining, the incidence of apoptosis was significantly lower in Flag-COX6A1 transfectants than in the control cells (Fig. 2C). Furthermore, treatment with 4-HPR resulted in a marked accumulation of cells in sub-G_1 phase in Flag-transfected cells as compared to Flag-COX6A1 transfectants (Fig. 2D). Taken together, these results strongly suggested that the expression of COX6A1 alters the sensitivity of U373MG cells to apoptotic stimuli.

Effect of COX6A1 on 4-HPR-induced ROS generation

Because COX6A1 inhibited the production of ROS induced by Bax in yeast cells, we were interested in whether COX6A1 also effected the production of ROS in mammalian cells, since 4-HPR is known to induce mitochondria-derived oxidative stress in glioblastoma cells [19]. 4-HPR elicited a significant increase in intracellular ROS in control cells that expressed Flag alone, and the increase in intracellular peroxides was almost completely abolished by the overexpression of COX6A1 (Fig. 3A). The COX6A1-dependent inhibitory effect on ROS production occurred in a time- and dose-dependent manner (Fig. 3B–D).

Effect of COX6A1 on apoptotic signals induced by 4-HPR

Cells were treated with 4-HPR for different periods of time, and the translocation of Bax was examined by Western blot. Following treatment with 4-HPR, the mitochondrial translocation of Bax from Flag-COX6A1 transfectants attenuated over time, as compared to control cells. There was a reduction of cytochrome *c* in cytosolic fraction in Flag-COX6A1 cells in response to 4-HPR, accompanied by a sustained levels of cytochrome *c* in mitochondrial fractions (Fig. 4A), and the activation of caspase-3 was suppressed in Flag-COX6A1 transfectants (Fig. 4B). These results suggested that the overexpression of COX6A1 confers resistance to cell death through the regulation of key apoptotic targets.

Discussion

We have identified COX6A1 as a suppressor of apoptosis using yeast-based functional screening for suppressors of Bax-induced

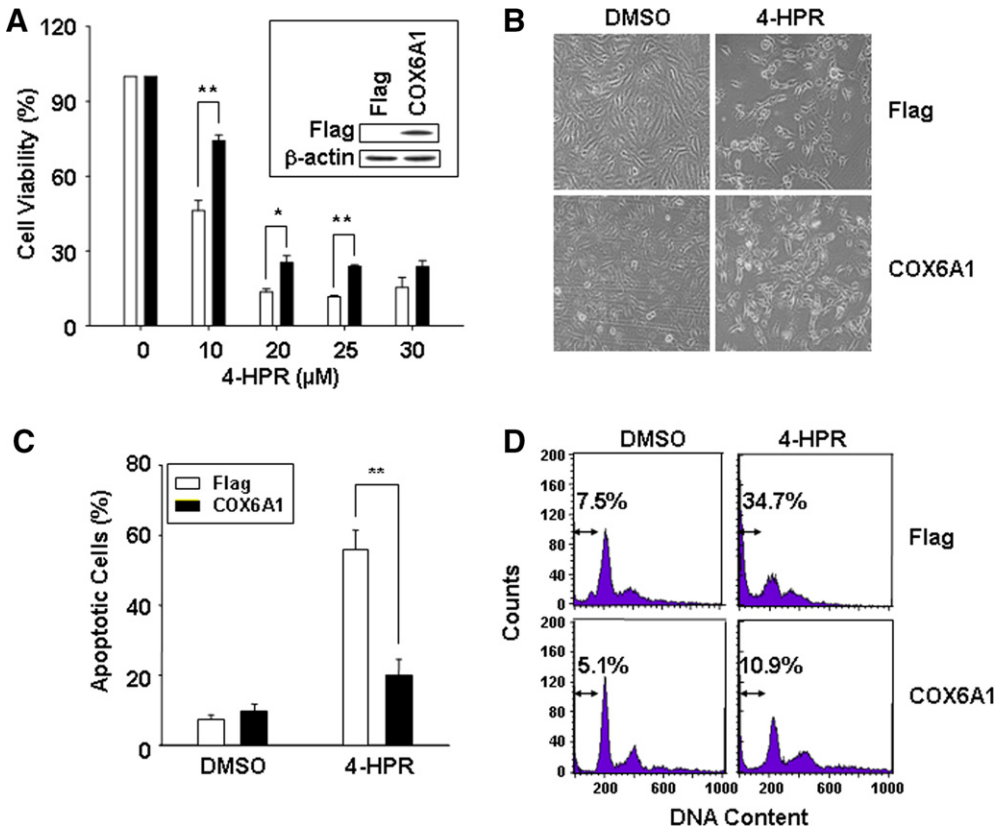

Fig. 2. Overexpression of COX6A1 attenuates 4-HPR-induced cell death in mammalian cells. (A) Cells that expressed Flag alone (open bars) or Flag-COX6A1 (closed bars) were incubated with the indicated concentrations of 4-HPR for 72 h. Cell viability was determined by MTT assay. The expression levels of COX6A1 were evaluated by Western blot (insert). Data represents the means ± SE ($n = 3$), $^{*}P < 0.05$, $^{**}P < 0.01$. (B) Cells were exposed to 20 μM 4-HPR for 48 h and then viewed by phase-contrast microscopy (magnification, 100×). (C) Cells were treated with 20 μM 4-HPR for 48 h and then apoptotic cells were visualized by DAPI staining. The number of apoptotic cells in at least four random fields were scored ($n = 50$ each). Data represents the means ± SE of three independent experiments, $^{**}P < 0.01$. (D) Cells were incubated with DMSO or 20 μM 4-HPR for 72 h and then stained with PI. The apoptotic cell fraction (sub-G_1, hypodiploid peak) was quantified by flow cytometry. Data is representative of three separate determinations.

apoptosis in yeast, and we demonstrated that COX6A1 also inhibits 4-HPR-induced apoptosis in mammalian cells. We presented data suggesting that the mechanism of inhibition of Bax- and 4-HPR-induced cell death by COX6A1 involves the regulation of ROS production. The overexpression of COX6A1 suppressed Bax- and 4-HPR-mediated cell death and inhibited ROS production, which suggests that the anti-apoptotic action of COX6A1 involves the suppression of ROS production.

Using yeast-based functional screening, we identified COX6A1 as a suppressor of apoptosis in Bax-mediated cell death in yeast. A number of recent studies have demonstrated the pivotal role of yeast-based functional screening in the identification of anti-apoptotic proteins, including BI-1, Ku70, HMGB1, sphingomyelin synthase 1, and ascorbate peroxidase [7,9–11,20]. COX6A1 is a terminal enzyme of the mitochondrial electron transport chain [1]. Although COX6A1 has been shown to play a role in energy metabolism, recent reports have suggested that it is also involved in stress-induced apoptosis and neurodegenerative diseases in organs with high-energy demand [21,22]. Our results suggest that COX6A1 functions as an inhibitor of Bax, and can suppress Bax- or 4-HPR-induced cell death.

We also demonstrated that the overexpression of COX6A1 effects the production of ROS in response to Bax or 4-HPR. COX6A1 expression resulted in a nearly complete inhibition of intracellular ROS production in response to Bax expression or 4-HPR treatment, which suggests that COX6A1 confers resistance to cell death through the inhibition of ROS production. Although the molecular mechanism of COX6A1-mediated inhibition of ROS generation and resistance to apoptotic cell death remains to be elucidated, it is worth noting that the anti-oxidants butylated hydroxyanisole or vitamin c have been shown to inhibit 4-HPR-induced apoptosis in head and neck squamous carcinoma cells (HNSCC) via the suppression of ROS production [23].

The suppression of 4-HPR-induced cell death in cells that overexpressed COX6A1 appeared to involve the regulation of Bax, cytochrome *c*, and caspase-3. In a previous report, the relocalization of Bax to the mitochondrial membrane was implicated in ROS-mediated apoptosis induced by 4-HPR [24]. In HNSCC cells, 4-HPR also induces apoptosis through the stimulation of ROS production, and it has been postulated that ROS mediate the apoptotic signals induced by 4-HPR through the relocalization of Bax and cytochrome *c* [23]. The overexpression of cytochrome *c* oxidase has been shown to suppress the cytotoxic effects of TNF-α in HL-60 cells [5]. It has also been shown that the inhibition of cytochrome *c* oxidase by low-concentrations of nitric oxide induces cell death through a marked increase in ROS production, implicating ROS in nitric oxide-mediated cell death [25]. Thus, the possibility remains that the suppression of ROS production is involved in the anti-apoptotic action of COX6A1. In support of this hypothesis, we showed that the generation of ROS and apoptosis were suppressed in U373MG cells that stably expressed COX6A1. However, additional studies are needed to clarify the precise molecular mechanism(s) of suppression of 4-HPR-induced apoptotic cell death by COX6A1.

In summary, we have developed a powerful tool for the isolation of novel anti-apoptotic genes. Using a yeast-based functional

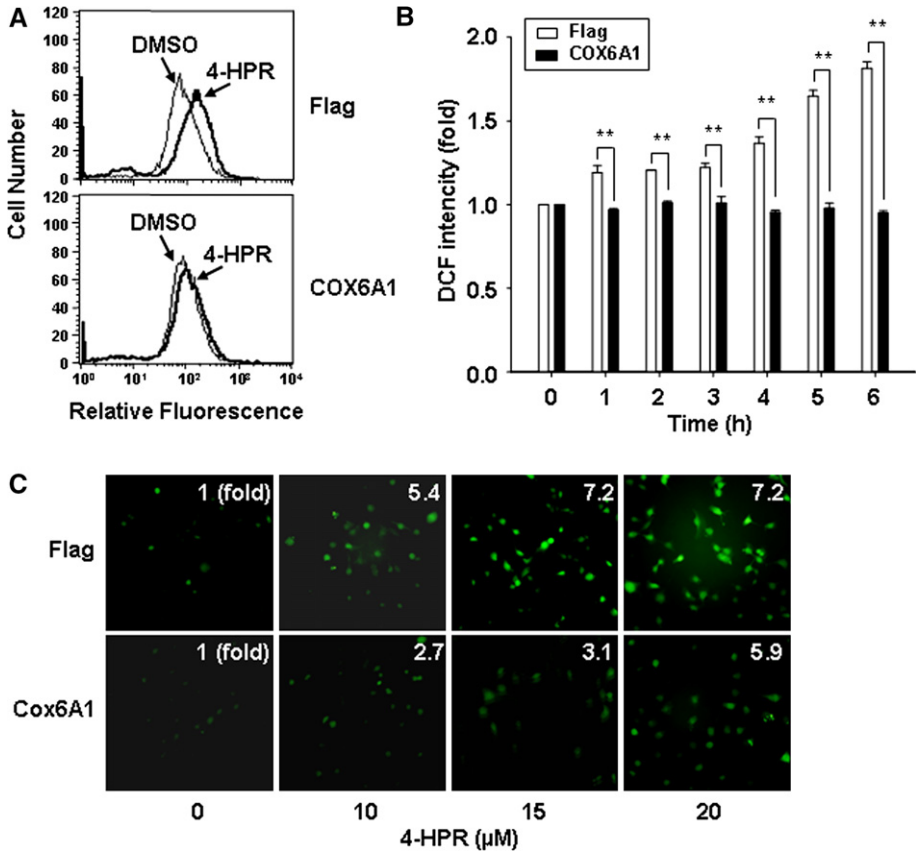

Fig. 3. Stable expression of COX6A1 nearly abolishes 4-HPR-induced ROS production. Cells that expressed Flag-COX6A1 or Flag alone were exposed to DMSO or 20 μM 4-HPR for 6 h (A,C), or for the indicated periods of time (B), and then treated with the oxidant-sensitive probe H_2DCF-DA (10 μM). The levels of ROS in the cells were quantified by flow cytometry (A), microplate reader (B), or fluorescence microscopy (C). Data in (B) represents the means ± SE ($n = 4$), $^{**}P < 0.01$. The results in (A,C) are representative of three independent experiments.

genetic screen, we have identified COX6A1 as a suppressor of Bax- and 4-HPR-induced apoptosis in yeast and mammalian cells, respectively, and shown that the suppression of apoptosis by COX6A1 involves the inhibition of ROS production.

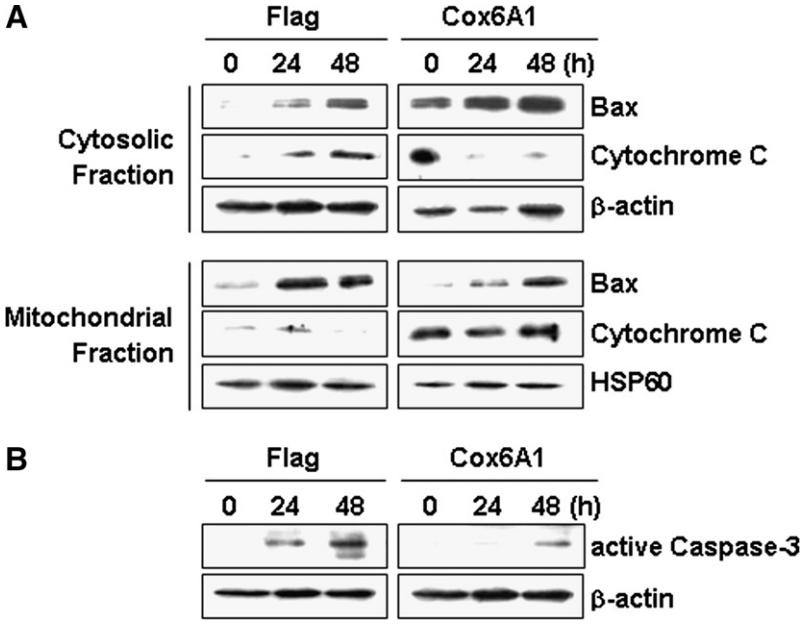

Fig. 4. Overexpression of COX6A1 suppresses the translocation of Bax, the release of cytochrome *c*, and the activation of caspase-3. (A) Cells that stably expressed Flag-COX6A1 or Flag alone were incubated for the indicated periods of time with 20 μM 4-HPR. The cells were then fractionated into cytosolic and mitochondrial fractions, and protein translocation was analyzed by Western blot using the indicated primary antibodies. (B) Total cell lysate was analyzed for the presence of activated caspase-3. Data is representative of three independent experiments.

Acknowledgments

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