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Sigma-1 Receptors Regulate Bcl-2 Expression by Reactive Oxygen Species-Dependent Transcriptional Regulation of Nuclear Factor κB

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ABSTRACT

The expression of Bcl-2, the major antiapoptotic member of the Bcl-2 family, is under complex controls of several factors, including reactive oxygen species (ROS). The σ-1 receptor (Sig-1R), which was recently identified as a novel molecular chaperone at the mitochondria-associated endoplasmic reticulum membrane (MAM), has been shown to exert robust cellular protective actions. However, mechanisms underlying the antiapoptotic action of the Sig-1R remain to be clarified. Here, we found that the Sig-1R promotes cellular survival by regulating the Bcl-2 expression in Chinese hamster ovary cells. Although both Sig-1Rs and Bcl-2 are highly enriched at the MAM, Sig-1Rs neither associate physically with Bcl-2 nor regulate stability of Bcl-2 proteins. However, Sig-1Rs tonically regulate the expression of Bcl-2 proteins. Knockdown of Sig-1Rs down-regulates whereas overexpression of Sig-1Rs up-regulates bcl-2 mRNA, indicating that the Sig-1R transcriptionally regulates the expression of Bcl-2. The effect of Sig-1R small interfering RNA down-regulating Bcl-2 was blocked by ROS scavengers and by the inhibitor of the ROS-inducible transcription factor nuclear factor κB (NF-κB). Knockdown of Sig-1Rs up-regulates p105, the precursor of NF-κB, while concomitantly decreasing inhibitor of nuclear factor-κBα. Sig-1R knockdown also accelerates the conversion of p105 to the active form p50. Lastly, we showed that knockdown of Sig-1Rs potentiates H_2O_2-induced apoptosis; the action is blocked by either the NF-κB inhibitor oridonin or overexpression of Bcl-2. Thus, these findings suggest that Sig-1Rs promote cell survival, at least in part, by transcriptionally regulating Bcl-2 expression via the ROS/NF-κB pathway.

Bcl-2 is a key molecule regulating "apoptosis," a deliberate life relinquishment of the cell. Bcl-2 has been implicated in pathophysiology of several human diseases such as Alzheimer's disease and cancer, thus providing a clue that Bcl-2 may serve as a potential target for treating human diseases (Danial and Korsmeyer, 2004; Youle and Strasser, 2008). Bcl-2 prevents apoptosis caused by a variety of cellular stresses such as oxidative stress, heat shock, and cytokine deprivation (Reed, 1994; Danial and Korsmeyer, 2004; Youle and Strasser, 2008). Bcl-2 localizes at mitochondrial outer membranes, as well as at the endoplasmic reticulum (ER) (Krajewski et al., 1993). One of the primary actions of Bcl-2 is to block homodimerization of proapoptotic Bax at mitochondria (Oltvai et al., 1993). Bcl-2 also regulates the Ca^{2+} mobilization at the ER (Pinton and Rizzuto, 2006). However, the precise localization and function of Bcl-2 at the ER remain to be clarified.

Regulating Bcl-2 expression is an important element in promoting cellular survival. The *bcl-2* gene possesses three exons and two major promoters (i.e., P1 and P2) (Seto et al., 1988). The P1 site is the major transcriptional promoter containing the Sp1-binding site and cyclic AMP response element (CRE), which are under control of transcription factors such as c-Jun and nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) (Heckman et al., 2002). Recent evidence indicates that reactive oxygen species (ROS) are the

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ABBREVIATIONS: ER, endoplasmic reticulum; CRE, cyclic AMP response element; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; ROS, reactive oxygen species; Sig-1R, σ-1 receptor; MAM, mitochondria-associated endoplasmic reticulum membrane; IP3R3, 1,4,5-trisphosphate receptor type III; PPBP, 4-phenyl-1-(4-phenylbutyl) piperidine; SKF-10,047, *N*-allylnormetazocine; CHO, Chinese hamster ovary; FBS, fetal bovine serum; NAC, *N*-acetyl cysteine; NLA, nitro-L-arginine; ERK, extracellular signal-regulated kinase; IκBα, inhibitor of nuclear factor-κBα; OxyR, cytochrome *c* oxidoreductase; siRNA, small interfering RNA; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; RT, reverse transcription; ANOVA, analysis of variance.

potent regulators of the Bcl-2 expression (Hildeman et al., 2003; Li et al., 2004). ROS decrease the expression of bcl-2 mRNA by promoting CRE-binding protein or NF-κB to CRE and κB sequences on the bcl-2 promoter (Sohur et al., 1999; Pugazhenthi et al., 2003).

The σ-1 receptor (Sig-1R) is an ER protein that shares no homology with any mammalian proteins (Hanner et al., 1996). The Sig-1R was recently identified as a novel molecular chaperone targeting the ER subdomain associated with mitochondria [i.e., the mitochondria-associated ER membrane (MAM)] (Hayashi and Su, 2007). Sig-1Rs stabilize the conformation of the proteins at the MAM, such as inositol 1,4,5-trisphosphate receptor type III (IP3R3) (Hayashi and Su, 2007). The Sig-1R has been implicated in several human diseases, including neurodegenerative diseases, drug abuse, and cancer (Snyder and Largent, 1989; Maurice and Lockhart, 1997; Matsumoto et al., 2007; Palmer et al., 2007; Fontanilla et al., 2009). Of the most prominent actions of Sig-1Rs or those ligands is their robust cellular protective effect (Maurice and Lockhart, 1997; Bowen, 2000). Sig-1R agonists have been shown to promote cellular survival by preventing oxidative stress caused by ischemia (Schetz et al., 2007), diabetes (Smith et al., 2008), inflammation (Wang et al., 2008), and β-amyloid toxicity (Meunier et al., 2006).

A few studies have recently suggested a possible relationship between Sig-1Rs and the Bcl-2 family in neuronal survival. Sig-1R agonists prevent up-regulation of Bax in cortical neurons exposed to β-amyloid 25 to 35 (Marrazzo et al., 2005). The Sig-1R agonist 4-phenyl-1-(4-phenylbutyl) piperidine (PPBP) was shown to prevent down-regulation of bcl-2 mRNA caused by ischemic conditions (Yang et al., 2007). The action of PPBP is fully abolished by the Sig-1R antagonist rimcazole (Yang et al., 2007). In contrast, dehydroepiandrosterone sulfate, a neurosteroid activating Sig-1Rs, promotes cellular survival in PC-12 cells by inducing Bcl-2, but the action is insensitive to both Sig-1R agonist SKF-10,047 and antagonist haloperidol (Charalampopoulos et al., 2004). Therefore, although recent findings support the notion that regulating the Bcl-2 family may be of potential elements in the cellular protective action of Sig-1Rs, discrepancies (possibly a result of variance with selectivity and potency of Sig-1R ligands) remain to be solved. In this study, we sought to provide direct evidence to prove the involvement of the ROS/Bcl-2 pathway in the cellular protection induced by Sig-1Rs. Instead of using Sig-1R ligands, we used molecular biological approaches (e.g., gene transfer or silencing) to control Sig-1R's activity. Although the physical interaction of Sig-1Rs with Bcl-2 proteins was originally expected in light of nature of the Sig-1R as a molecular chaperone, the data provided evidence that Sig-1Rs transcriptionally control expression of Bcl-2 by regulating the ROS/NF-κB pathway.

Materials and Methods

Materials. Chinese hamster ovary (CHO) cells were from American Type Culture Collection (Manassas, VA). Minimal essential medium-α + Glutamax, heat-inactivated fetal bovine serum (FBS), Lipofectamine-2000, Hoechst 33342, and Alexa-conjugated secondary antibodies were purchased from Invitrogen (Carlsbad, CA). Cycloheximide, H_2O_2, *N*-acetyl cysteine (NAC), nitro-L-arginine (NLA), and Tempol were from Sigma-Aldrich (St. Louis, MO). Oridonin was from EMD Biosciences (San Diego, CA). Specific antibodies were purchased as follows: anti-FLAG from Sigma-Aldrich; monoclonal Bcl-2, NF-κB p105/50 subunit, extracellular signal-regulated kinase (ERK), and calnexin from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); polyclonal Bcl-2, monoclonal Bcl-2 (SPM117), ERp57, and inhibitor of nuclear factor-κBα (IκBα) from Abcam Inc. (Cambridge, MA); cytochrome *c* oxidoreductase (OxyR) from Invitrogen; cytochrome *c*, monoclonal IP3R3, Nucleoporin p63, and BiP from BD Biosciences (San Jose, CA); and polyclonal IP3R3 from Millipore Corporation (Billerica, MA). Anti-Sig-1Rs were raised as described previously (Hayashi and Su, 2003).

Cell Culture and Vector Transfection. CHO cells were cultured in minimal essential medium-α containing 10% heat-inactivated FBS and 2 mM Glutamax at 37°C with 5% CO_2. Cells were treated with compounds or transfected in six-well plates. The cDNA encoding small interfering RNA (siRNA) was constructed in the pSIREN vector (Clontech, Mountain View, CA) as described previously (Hayashi and Su, 2004). The expression vector for FLAG-tagged Sig-1Rs was constructed by ligating the polymerase chain reaction (PCR)-amplified rat Sig-1R cDNA (Hayashi and Su, 2001) in the pFLAG vector (Sigma-Aldrich). The Bcl-2 pCIneo vector was donated by Drs. Xingming Deng and W. Stratford May, Jr. (University of Florida Shands Cancer Center). Cells were transfected 1 day before experiments by applying the DNA/Lipofectamine-2000 mixture (1 μg/2 μl) to the culture medium. Concentrations of vectors in medium are 1 μg/ml for the FLAG-tagged Sig-1R, 0.20 to 0.50 μg/ml for Bcl-2, 0.33 μg/ml for the wild-type Sig-1R, 1 μg/ml for ER-DsRed, 0.3 μg/ml for Mito-DsRed, and 1 μg/ml for Sig-1R siRNA/control siRNA. After 6 h, transfected cells were harvested and spread on a new plate with or without ROS inhibitors, H_2O_2, or oridonin. The membrane fractionation and immunocytochemistry verified that Bcl-2 expressed by the optimized transfection condition shows the identical subcellular distribution as that of endogenous Bcl-2 in CHO cells.

Immunocytochemistry and Nuclear Staining for Apoptosis. CHO cells were grown on a round coverslip coated with poly-D-lysine. After fixation with 4% paraformaldehyde in 0.1 M phosphate buffer (10 min at room temperature), cells were permeabilized with 0.2% Triton X-100 for 5 min followed by blocking with 10% nonfat dry milk. Fixed cells were incubated with primary antibodies in phosphate-buffered saline (PBS) containing 4% bovine serum albumin, 0.1% Triton X-100, and 5% FBS. Primary antibodies used were monoclonal Bcl-2 (1:50), polyclonal Bcl-2 (1:100), monoclonal IP3R3 (1:50), polyclonal IP3R3 (1:100), monoclonal ERp57 (1:100), and monoclonal OxyR (1:100). After incubation with Alexa-conjugated secondary antibodies, images were captured by using the UltraView confocal system (PerkinElmer Life and Analytical Sciences, Waltham, MA). The monoclonal anti-Bcl-2 recognizes the amino acids 1 to 205, whereas the polyclonal Bcl-2 recognizes the amino acids 1 to 18 of human Bcl-2 with cross-reactivity to endogenous Bcl-2 in CHO cells. Because the expression level of endogenous Bcl-2 is considerably low in CHO cells, immunocytochemistry was performed basically in CHO cells transfected with Bcl-2 cDNA under the optimized overexpression condition (see above). For nuclear staining, fixed cells were incubated with Hoechst 33342 (10 μg/ml) for 15 min followed by a brief washing with PBS. Nuclear images were captured by fluorescence microscopy. Five fields were captured randomly from single wells of a 12-well plate. Six wells were analyzed in each group.

MAM Preparation. The MAM fraction was prepared as described previously (Rusiñol et al., 1994). In brief, CHO cells grown on two 15-cm dishes were homogenized by a glass Dounce homogenizer in the homogenization buffer (0.25 M sucrose, 10 mM HEPES/KOH, pH 7.4). The homogenate was centrifuged at 500*g* to yield the P1 nuclear fraction. The supernatant was centrifuged at 10,300*g* for 20 min to yield the crude mitochondrial fraction. The supernatant was centrifuged at 100,000*g* for 1 h to obtain P3 microsomal and cytosolic fractions. The crude mitochondrial fraction in 0.5 ml of isolation medium (250 mM mannitol, 5 mM HEPES/KOH at pH 7.4, and 0.5 mM EGTA/KOH) was layered on a Percoll solution [225 mM mannitol, 25 mM HEPES/KOH at pH 7.4, 1 mM EGTA/KOH, and 30% (v/v) Percoll] followed by centrifugation at 95,000*g* for 30 min. The

purified mitochondrial fraction and the MAM fraction were collected followed by washings with the isolation medium.

Immunoprecipitation. Cells were suspended in PBS followed by cross-linking with dithiobis (succinimidyl propionate) (Thermo Fisher Scientific, Waltham, MA) at 150 μg/ml (15 min at 4°C). The reaction was stopped by adding Tris-HCl (pH 8.8, 30 mM). Cell lysates were prepared by suspending the cross-linked cells in the buffer containing 50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.2% SDS, 0.5% sodium deoxycholate, 150 mM NaCl, and the protease inhibitor mixture (Sigma-Aldrich). After centrifugation at 12,000*g*, the supernatant was incubated overnight with primary anti-FLAG or anti-Bcl-2 antibodies. The cell lysate was incubated with Sepharose protein A (GE Healthcare, Little Chalfont, Buckinghamshire, UK) or with protein A/G Plus-agarose beads (Santa Cruz Biotechnology, Inc.) for 90 min. After washing with lysis buffer, immunoprecipitants were boiled in 2× sample buffer and applied to Western blotting.

Nuclear Preparation. Cells were incubated with Nuclei EZ lysis buffer (Sigma-Aldrich) for the purification of nuclei. Cell lysates were centrifuged at 500*g* for 5 min. The supernatant was kept as a post-nuclear cell lysate. The pellet was washed twice with the same lysis buffer and kept at −80°C until used in Western blotting.

Total RNA Extraction and Reverse-Transcription PCR. Total RNA in CHO cells was extracted with Total RNA Isolation NucloeSpin RNA II (Macherey-Nagel, Inc., Bethlehem, PA). The reverse transcription (RT)-PCR for bcl-2 mRNA was performed by using Titanium one-step RT-PCR kit (Clontech) with 0.5 μg of total RNA and 24.5 μl of the reaction mixture under the following thermal cycle: 50°C for 60 min, 94°C for 5 min, 40 cycles of 94°C (30 s), 57°C (30 s), 68°C (45 s), followed by 72°C for 2 min and 4°C (GeneAmp PCR System 9700; Applied Biosystems, Foster City, CA). Sig-1R mRNA was amplified by the same RT-PCR condition but with 25 cycles. The following primers were used: the bcl-2 antisense primer 5′-CTACTGCTTTAGTGAACC-3′, the bcl-2 sense primer 5′-GGAAG-GATGGCGCAAGCCGGGAG-3′, the Sig-1R sense primer 5′-CCAG-GCTGCCCGCT-3′, and the Sig-1R antisense primer 5′-TGAGTC-CCAGCGAGTAGAGAAATGG-3′. PCR products were analyzed by 2% agarose electrophoresis followed by imaging with Image Station 440CF (Kodak IBI, New Haven, CT) under UV light.

Western Blotting. Cells were briefly washed and harvested in ice-cold PBS. Cell pellets after a centrifuge at 3000*g* for 10 min were suspended into 2× sample buffer (0.13 M Tris-HCl, pH 6.8, 4.2% SDS, and 20% glycerol). After a brief sonication, samples were centrifuged at 16,000*g* (10 min), and the supernatants were kept at −20°C. Protein assays were performed by using the Micro BCA Assay (Thermo Fisher Scientific). After SDS-polyacrylamide electrophoresis, proteins were transferred onto a polyvinylidene fluoride membrane (Bio-Rad Laboratories, Hercules, CA) by Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories). The membrane was blocked with 10% nonfat dry milk in Tris-based saline buffer with Tween 20 (20 mM Tris base, 500 mM NaCl, 0.005% Tween 20, pH 7.5) followed by incubation overnight with primary antibodies. After washing with Tris-based saline buffer with Tween 20, the membrane was incubated for 1 h with secondary antibodies conjugated with horseradish peroxidase (Thermo Fisher Scientific). Protein bands were visualized with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and Image Station 440CF (Kodak IBI). Data were analyzed using Prism 3.0cx (GraphPad Software Inc., San Diego, CA).

Statistical Analysis. All the quantifications for Western blotting and RT-PCR were performed by 1D Image Analysis Software (Eastman Kodak, Rochester, NY). Data were submitted to statistical analyses for the Prism 3.0cx software (GraphPad Software Inc.). Data were presented as percentage of control with S.E.M. The level of statistical significance is $p < 0.05$. For comparison of two groups, Student's *t* test was used. For data with multiple groups, two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test was used.

Results

Sig-1Rs Regulate the Bcl-2 Protein Expression. Sig-1R ligands have been shown to block the alterations of Bax or Bcl-2 expression induced by pathological conditions (Marrazzo et al., 2005; Yang et al., 2007). To clarify whether regulating the expression of Bcl-2 family is the bona fide action of Sig-1Rs, we first examined whether knockdown or overexpression of Sig-1Rs per se may affect the expression of two Bcl-2 family proteins in CHO cells. Western blotting using whole CHO cell lysates found that siRNA against Sig-1Rs significantly decrease the protein level of Bcl-2 but not that of Bax (Fig. 1). Conversely, overexpression of Sig-1Rs significantly up-regulated Bcl-2 (Fig. 1).

MAM Localization of Bcl-2 and Sig-1R. Because both Sig-1Rs and Bcl-2 are known to regulate Ca^{2+} transmission from ER to mitochondria (Pinton and Rizzuto, 2006; Hayashi et al., 2009), we speculated that these proteins may localize at the same ER locus (i.e., MAM), and thus may physically interact with each other. The association might regulate the protein stability/degradation of Bcl-2 via the chaperone activity of Sig-1Rs. To address the possibility, we first examined the cellular localization of Bcl-2 in CHO cells. The differential centrifugation combined with a Percoll gradient centrifugation showed that Bcl-2 is present in P1 nuclear and mitochondrial fractions with a marginal level in the P3 fraction. However, Bcl-2 was also present in the MAM fraction (Fig. 2A). Under our optimized condition for transfection of Bcl-2 (see under *Materials and Methods*), transiently trans-

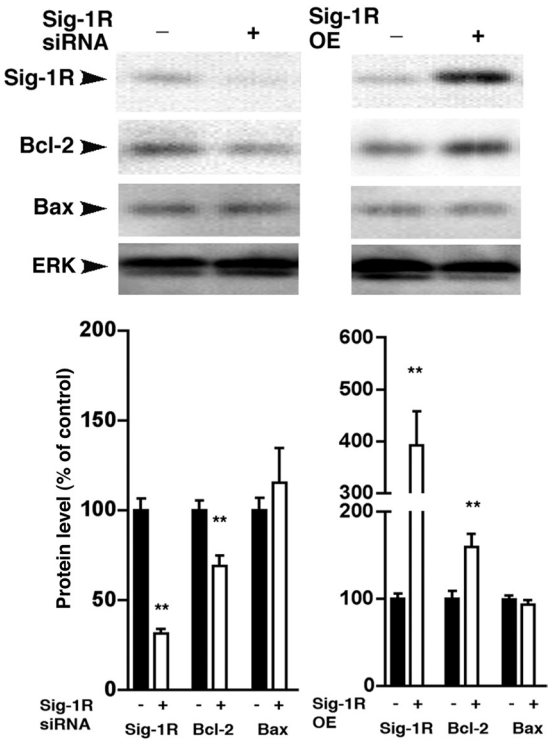

Fig. 1. Sig-1Rs tonically regulate the expression of Bcl-2 in CHO cells. Sig-1Rs were knocked down or overexpressed (OE) by transfecting vectors in CHO cells as described under *Materials and Methods*. Total cell lysates (30 μg/lane) were analyzed by Western blotting. As controls, the pSIREN vector carrying an inactive siRNA (control siRNA) or the empty pCR3.0 vector was transfected. ERK is for the loading controls. The graphs represent mean ± S.E.M. from six separate samples. *, $p < 0.05$; **, $p < 0.01$ by Student's *t* test.

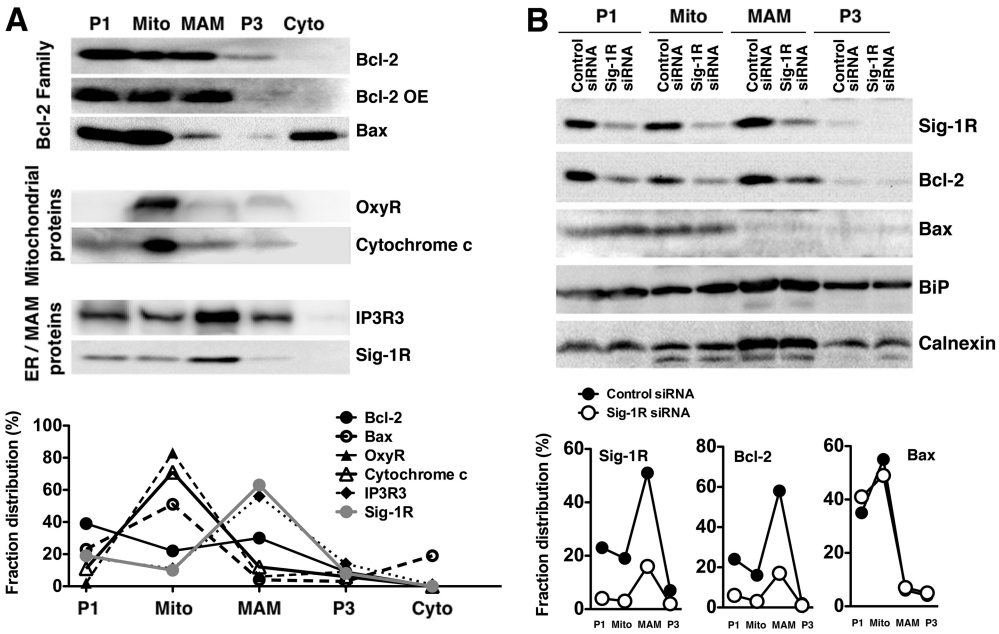

Fig. 2. Subcellular distribution of Sig-1Rs and Bcl-2. A, enrichment of Sig-1Rs and Bcl-2 in the MAM fraction. The differential centrifugation combined with a Percoll gradient centrifugation was performed to obtain five different fractions (see *Materials and Methods*). Ten micrograms of proteins from each fraction was analyzed by Western blotting. Thirty micrograms per lane was used for Bax immunoblotting. The second panel from the top represents the distribution of transfected Bcl-2 (OE) with the similar distribution as that of endogenous Bcl-2 (top). Transfected Bcl-2 was selectively detected by the monoclonal Bcl-2 antibody clone SPM117. Marker proteins of mitochondria or ER/MAM were also measured by Western blotting. The graph represents fraction distributions of proteins where the sum of five fractions was taken as 100% for each protein. B, no effect of Sig-1R knockdown on the subcellular distribution of Bcl-2. Membrane fractions were prepared from CHO cells transfected with Sig-1R siRNA or control siRNA as described in A. The images represent the result from three independent experiments. The graphs show fraction distributions of Sig-1Rs, Bcl-2, and Bax, where the sum of four fractions was taken as 100% for each protein. Note that Sig-1R siRNA decrease Bcl-2 in all the fractions without affecting the subcellular distribution.

fected Bcl-2 also showed the same cellular distribution as that of endogenous Bcl-2 (Fig. 2A). On the other hand, Bax was present mostly in P1, mitochondrial, and cytosolic fractions but was much lower in the MAM and P3 fractions (Fig. 2A). Sig-1R and IP3R3, the well characterized MAM proteins (Hayashi and Su, 2007), were most enriched in the MAM fraction (Fig. 2A). OxyR and cytochrome *c* were present exclusively in the mitochondrial fraction (Fig. 2A), indicating few contaminations of mitochondrial membranes in the MAM fraction.

We examined whether down-regulation of Bcl-2 caused by Sig-1R siRNA in the total lysate (Fig. 1) is attributed to organelle-specific down-regulation of Bcl-2, particularly that at the MAM. As shown in Fig. 2B, knockdown of Sig-1Rs decreased Bcl-2 proteins similarly in any fractions containing Bcl-2 (Fig. 2B). Thus, Sig-1R knockdown decreases the total Bcl-2 proteins without affecting the subcellular distribution.

To further confirm the MAM localization of Bcl-2, immunocytochemistry using the monoclonal anti-Bcl-2 antibody was performed. Fluorescence confocal microscopy showed that a portion of Bcl-2 colocalizes with Mito-DsRed expressed in mitochondria (yellow in Fig. 3, A and B). However, the higher magnification revealed that a significant amount of Bcl-2 is also present in close vicinity to mitochondria (green in Fig. 3A, bottom). The polyclonal antibody recognizing the different domain of Bcl-2 also showed exactly the same pattern: presence of Bcl-2 in both mitochondria and their vicinity (Fig. 3B). The same distribution of Bcl-2 was also observed when endogenous mitochondrial protein OxyR was immunocytochemically visualized (Fig. 3C).

Next, the localization of Bcl-2 was compared with that of ER-specific proteins. ER-DsRed, similar to ERp57, predominantly expressed at reticular structures of the ER (Fig. 3D), whereas MAM-specific IP3R3 localized dominantly at punctate structures inside the cell (Fig. 3D). Confocal microscopy showed that a number of IP3R3-containing punctates appose mitochondria that are visualized by expressing Mito-DsRed (Fig. 3E, top). It is noteworthy that Bcl-2 showed its partial colocalization with IP3R3 (Fig. F), supporting the notion that a portion of Bcl-2 is present at the MAM.

Bcl-2 Is Not a Substrate of Sig-1R Chaperones. The Sig-1R is a molecular chaperone that regulates stability of proteins at the MAM via direct protein-protein interactions (Hayashi and Su, 2007). To examine whether Sig-1Rs directly associate with Bcl-2, thus stabilizing the latter, we performed immunoprecipitation in CHO cells overexpressing Sig-1R-FLAG and Bcl-2. The same immunoprecipitation protocol successfully detected the physical association of Sig-1Rs with BiP in CHO cells (Hayashi and Su, 2007). Although FLAG antibodies efficiently immunoprecipitated Sig-1R-FLAG, the antibodies failed to coimmunoprecipitate Bcl-2 (Fig. 4A). Conversely, Bcl-2 antibodies, although efficiently immunoprecipitated Bcl-2, also failed to coimmunoprecipitate Sig-1R-FLAG (Fig. 4B), suggesting no potential interaction between the two proteins.

We next examined whether knockdown of Sig-1Rs may regulate stability of Bcl-2. Degradation of Bcl-2 was monitored in CHO cells treated with cycloheximide, a protein synthesis inhibitor. Although Sig-1R siRNA causes down-regulation of Bcl-2 (Fig. 1), the degree of the Bcl-2 degrada-

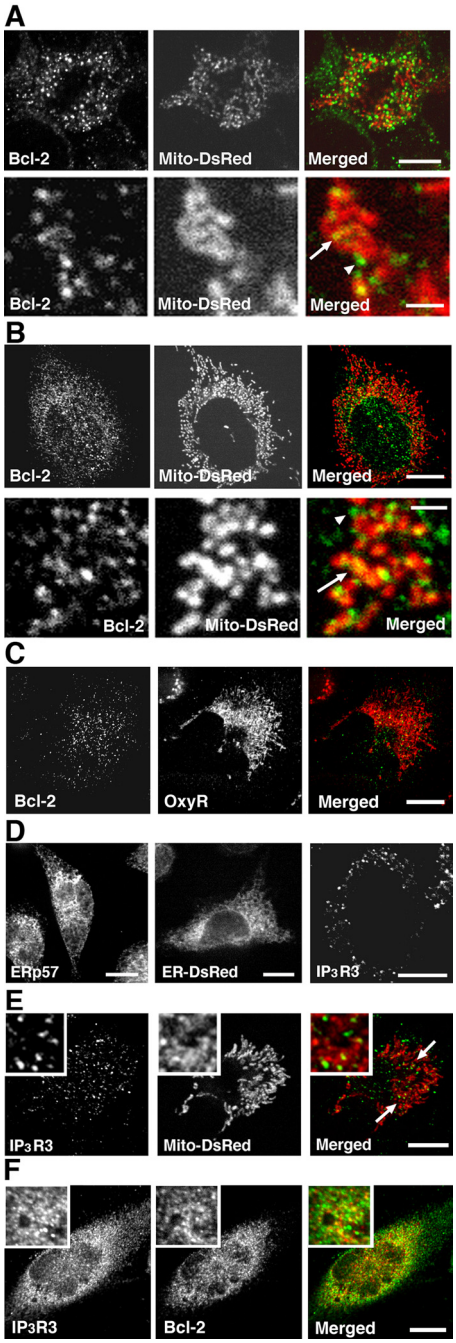

Fig. 3. Confocal microscopy for the intracellular distribution of Bcl-2. A, cellular localization of Bcl-2 and mitochondria in CHO cells. Mitochondria were visualized by expressing Mito-DsRed fluorescent proteins (red). Bcl-2 (green) was labeled with monoclonal Bcl-2 (1:50) primary antibodies and Alexa-480 secondary antibodies (bar = 10 μm). The second row is at a higher magnification (bar = 2 μm). The arrow indicates the mitochondrial localization of Bcl-2, whereas the arrowhead indicates Bcl-2 in close vicinity to mitochondria. B, spatial distribution of Mito-DsRed and Bcl-2 (in green) labeled with polyclonal Bcl-2 (1:100) primary antibodies (bar = 10 μm). The second row is at a higher magnification (bar = 2 μm). Arrow, mitochondrial localization of Bcl-2; arrowhead, Bcl-2 in close vicinity to mitochondria. C, spatial distribution of Bcl-2 and OxyR. Polyclonal Bcl-2 (1:100) and monoclonal OxyR (1:100) antibodies were used (bar = 10 μm). Note that a portion of Bcl-2 (in green) is not colocalized with the mitochondrial protein OxyR. D, cellular localization of ER proteins in CHO cells. Note: ERp57 and ER-DsRed distribute on reticular network structures of the ER, whereas IP3R3 specifically targets punctate structures (bar = 10 μm). E, IP3R3 localize at the MAM. Monoclonal IP3R3 (1:50) primary antibodies and Alexa-480 secondary antibodies were used. IP3R3-containing MAMs appose mitochondria expressing Mito-DsRed (arrows and inset; bar = 10 μm). F, partial colocalization between IP3R3 (in green) and Bcl-2 (in red). Monoclonal Bcl-2 (1:50) and polyclonal IP3R3 (1:100) were the primary antibodies used; bar = 10 μm.

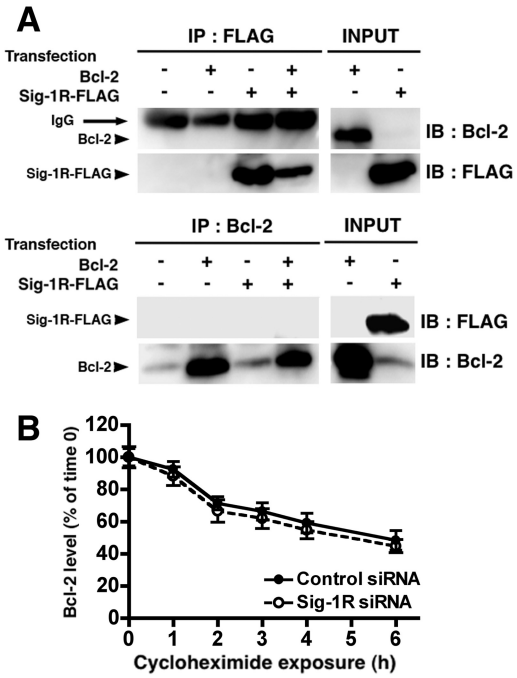

Fig. 4. Bcl-2 is not a substrate of Sig-1R chaperones. A, no interaction between Sig-1Rs and Bcl-2 detected in immunoprecipitation. Protein lysates from CHO cells overexpressing Bcl-2 and/or Sig-1R-FLAG were prepared as described under *Materials and Methods*. For immunoprecipitation, either monoclonal Bcl-2 antibodies or FLAG antibodies were used. Immunoprecipitants and total cell lysates (INPUT) were analyzed by Western blotting. Two top panels represent the successful immunoprecipitation of Sig-1R-FLAG with FLAG antibodies (IB: FLAG) but without coimmunoprecipitation of Bcl-2 (IB: Bcl-2). IgG, the Ig light chain of monoclonal FLAG antibodies used for immunoprecipitation. Bottom two panels represent efficient immunoprecipitation of Bcl-2 with monoclonal Bcl-2 antibodies (IB: Bcl-2) but without coimmunoprecipitation of Sig-1R-FLAG (IB: FLAG). Bcl-2 bands seen in samples without the Bcl-2 transfection (bottom) represent endogenous Bcl-2. B, kinetics of Bcl-2 degradation in CHO cells transfected with control (closed circle) or Sig-1R siRNA (open circle with dotted lines). Twenty-four hours after transfection, CHO cells were treated with cycloheximide (50 μg/ml) for different periods to prevent the protein synthesis. Levels of Bcl-2 were measured by Western blotting. The protein level at each time point is shown as percentage of time 0 h of respective groups. At the 0-h time point, the absolute level of Bcl-2 in CHO cells transfected with Sig-1R siRNA is 54.6% of the level in cells transfected with control siRNA (data not shown).

tion remained the same between control siRNA and Sig-1R siRNA-transfected CHO cells (Fig. 4B).

Sig-1Rs Regulate the mRNA Level of bcl-2. We next examined whether knockdown of Sig-1Rs might promote down-regulation of Bcl-2 at the mRNA level. The RT-PCR found that bcl-2 mRNA were significantly decreased by siRNA against Sig-1Rs (Fig. 5, A and B). Conversely, overexpression of Sig-1Rs increased the mRNA level of bcl-2 (Fig. 5C), suggesting that Sig-1Rs tonically regulate the transcription of bcl-2 mRNA or stability of bcl-2 mRNA. Because Sig-1R siRNA failed to alter the expression level of transiently transfected Bcl-2 (data not shown), where the transcription is driven by the exogenous promoter on the expression vector, Sig-1Rs likely regulate the transcription of bcl-2 processed at the endogenous promoter of the *bcl-2* gene.

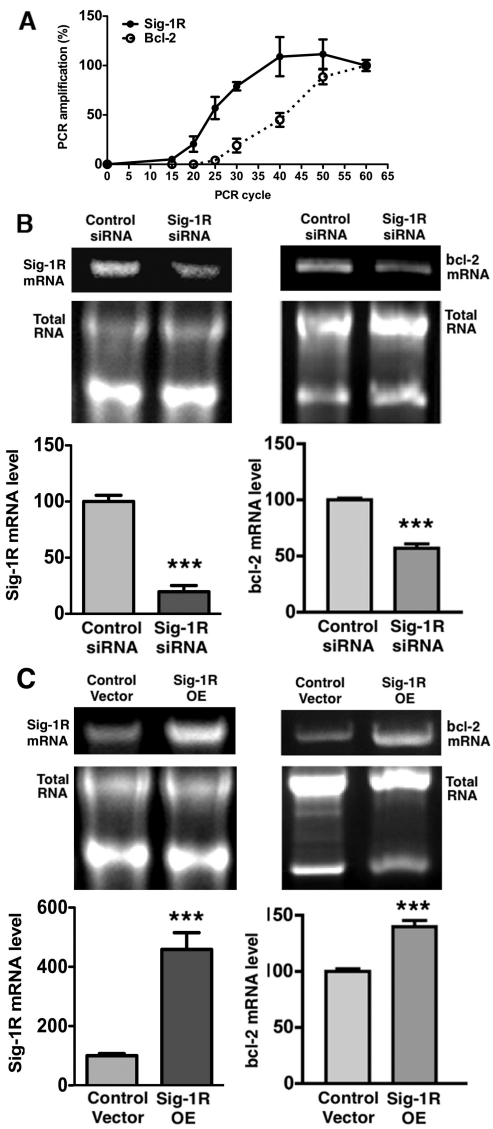

Fig. 5. Sig-1Rs regulate the expression of the bcl-2 mRNA. A, amplifications of Sig-1R mRNA and bcl-2 mRNA by RT-PCR. Total mRNA from CHO cells (0.5 μg) was amplified with specific primer sets for Sig-1R and bcl-2 (see *Materials and Methods*) at different cycles. Amplified cDNA was measured by agarose electrophoresis ($n = 2$). B, effect of Sig-1R knockdown on the mRNA level of bcl-2. The level of the bcl-2 or Sig-1R mRNA was measured by RT-PCR by using total RNA extracts (0.5 μg for each) from CHO cells transfected with either control siRNA or Sig-1R siRNA. Amplified cDNA and total RNA were visualized in the agarose gel. PCR cycles are 40 cycles for bcl-2 and 25 cycles for Sig-1Rs. See under *Materials and Methods* for details of RT-PCR. The graphs represent mean ± S.E.M. ($n = 5$). ***, $p < 0.001$ by Student's t test. C, effect of Sig-1R overexpression on the mRNA level of bcl-2. Total RNA was extracted from CHO cells transfected with either control vectors or Sig-1R overexpression (OE) vectors. The mRNA level of bcl-2 or Sig-1Rs was measured by RT-PCR. The graphs represent mean ± S.E.M. ($n = 4$). ***, $p < 0.001$ by Student's t test.

Sig-1Rs Regulate Transcription of bcl-2 by Inhibiting the NF-κB Pathway. As previously reported (Sohur et al., 1999; Pugazhenthi et al., 2003), our sequence analyses using TFsearch (Heinemeyer et al., 1998) found multiple consensus κB sequences in the *bcl-2* promoter (base pairs at −14 to −23, −1788 to −1797, −1792 to −2001, or −2088 to −2097 of the human *bcl-2* DNA; GenBank accession number NG_009361). Because Sig-1Rs have been implicated in neuroprotection against oxidative stress (Meunier et al., 2006), we hypothesized that Sig-1Rs transcriptionally regulate the Bcl-2 expression via the pathway involving the ROS-responsive transcription factor NF-κB.

First, we examined whether free radical scavengers could prevent Sig-1R siRNA-induced down-regulation of Bcl-2. Down-regulation of Bcl-2 caused by Sig-1R knockdown was completely abolished by Tempol (the superoxide anion scavenger) or NAC (the scavenger of both superoxide and H_2O_2) but not by NLA (the nitric oxide synthase inhibitor; Fig. 6A), indicating that ROS, particularly O_2^- and/or H_2O_2, but not nitric oxide, are involved in down-regulation of Bcl-2 caused by Sig-1R siRNA. Next, we examined whether knockdown of Sig-1Rs may alter the activation and/or expression of NF-κB. Knockdown of Sig-1Rs promoted the moderate increase of p105, the precursor of the NF-κB complex, and the large increase of p50, the active component in the NF-κB complex (Fig. 6B). KCl-induced up-regulation of p105 and the induction of p50 were accelerated by knocking down Sig-1Rs (Fig. 6C). The increase of p50 caused by Sig-1R knockdown was abolished by Tempol or NAC but not significantly by NLA (Fig. 6D). It is known that the NF-κB complex containing p50 translocates to the nucleus on activation of the complex (Karin, 2006). In fact, Sig-1R siRNA increased p50 in the nuclear fraction (Fig. 6E). Sig-1R siRNA also caused the decrease of IκBα, the key inhibitor of NF-κB (Fig. 6F). On the other hand, overexpression of Sig-1Rs promoted suppression of p105 accompanied by nearly complete abolishment of p50 (Fig. 6G). However, overexpression of Sig-1Rs did not significantly affect the level of IκBα (Fig. 6G).

NF-κB Pathway Is Involved in Sig-1R siRNA-Induced Bcl-2 Down-Regulation and Apoptosis. Oridonin is a selective inhibitor of NF-κB that disrupts the interaction between the active form of the NF-κB complex and DNA (Ikezoe et al., 2005). To confirm whether NF-κB is involved in down-regulation of Bcl-2 caused by Sig-1R knockdown, CHO cells transfected with either control or Sig-1R siRNA were treated with oridonin at different concentrations. As shown in Fig. 7A, oridonin itself promoted slight up-regulation of Bcl-2 in control cells. Oridonin blocked down-regulation of Bcl-2 caused by Sig-1R siRNA in a dose-dependent manner (Fig. 7A). The effect of Sig-1R overexpression to up-regulate Bcl-2 was also abolished by oridonin (Fig. 7B).

It has been shown that knockdown of Sig-1Rs increases vulnerability of cells to proapoptotic stimuli (Hayashi and Su, 2007). To examine whether the identified pathway plays a role in the cellular protective action of Sig-1Rs, we tested oridonin and Bcl-2 overexpression in apoptosis promoted by Sig-1R siRNA. Apoptosis was initiated in CHO cells by challenging 50 μM H_2O_2 for up to 48 h. Control cells showed an increase in apoptosis 8 h after the H_2O_2 exposure, and the proportion of apoptotic cells continuously increased up to 48 h (Fig. 7C). Sig-1R siRNA accelerated H_2O_2-induced apoptosis as shown in increased apoptotic cells particularly 8 h after the H_2O_2 exposure (Fig. 7C). Cells overexpressing Bcl-2 showed slightly higher resistance to H_2O_2 compared with control cells, but the difference was not statistically significant, suggesting that endogenously expressed Bcl-2 may provide the nearly maximum antiapoptotic action in control cells. On the other hand, overexpression of Bcl-2 markedly inhibited apoptosis potentiated by Sig-1R siRNA (Fig. 7C).

We also tested whether the inhibition of NF-κB by oridonin may prevent the action of Sig-1R siRNA potentiating

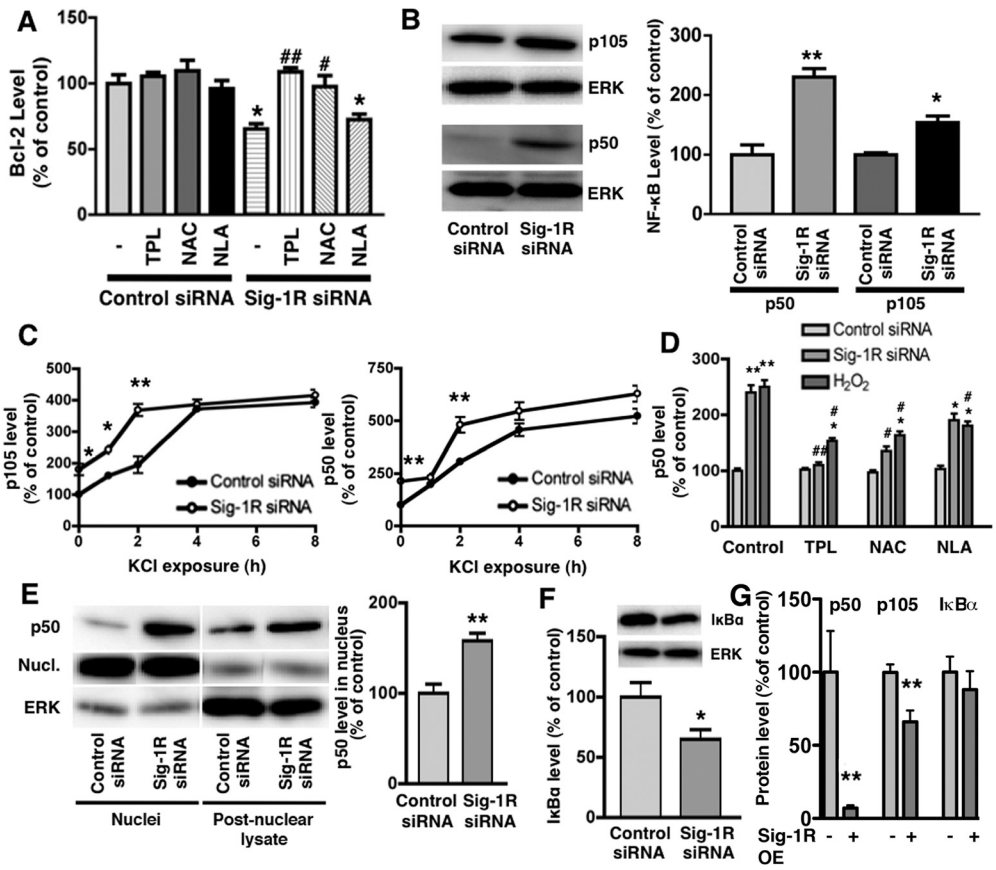

Fig. 6. Sig-1R knockdown promotes activation of NF-κB. A, Sig-1R siRNA promotes down-regulation of Bcl-2 in a ROS-dependent manner. Free radical scavenger Tempol (TPL, 0.5 mM), NAC (5 mM), and NLA (0.5 mM) were applied to culture medium 6 h after the transfection of CHO cells with control or Sig-1R siRNA. After further incubation for 18 h, cell lysates were analyzed by Western blotting (30 μg/lane). Blc-2 levels were normalized to the internal controls (total ERK) and presented as percentage of control. Two-way ANOVA of effect of siRNA transfection or scavenger treatments on the Bcl-2 level is $F(2,29;$ transfection$) = 16.23$, $p = 0,0006$; $F(4,29;$ treatment$) = 9.578$, $p = 0.0003$. *, $p < 0.05$ compared with control siRNA without scavenger, ##, $p < 0.01$, #, $p < 0.05$ compared with Sig-1R siRNA without scavenger (Bonferroni post hoc test; $n = 4$). B, Sig-1R siRNA increases levels of p50 and p105 subunits of the NF-κB complex. Levels of p50 or p105 analyzed by Western blotting were normalized to ERK and shown as percentage of control in the graph ($n = 10$). *, $p < 0.05$, **, $p < 0.001$ compared with control siRNA by Student's t test. C, increased KCl induced induction of p50 and p105 in CHO cells knocking down Sig-1Rs. KCl at 50 mM was applied to culture medium for indicated periods. Protein levels of p50 and p105 were measured by Western blotting followed by normalization to ERK. Two-way ANOVA of effect of time or KCl treatment on p105 level is $F(4,40;$ time$) = 102.1$, $p < 0,0001$; $F(4,40;$ treatment$) = 52.92$, $p < 0.0001$; $F(4,40;$ interaction$) = 7.754$, $p = 0.0014$. *, $p < 0.05$, **, $p < 0.01$ compared with control siRNA by Bonferroni post hoc tests. Two-way ANOVA of effect of time or KCl treatment on p50 level is $F(4,40;$ time$) = 84.54$, $p < 0,0001$; $F(4,40;$ treatment$) = 34.81$, $p < 0.0001$. **, $p < 0.01$ compared with control siRNA by Bonferroni post hoc test ($n = 4$). D, Effect of free radical scavengers on Sig-1R siRNA/H_2O_2-induced induction of p50. After transfection with either control siRNA or Sig-1R siRNA, CHO cells were treated with free radical scavengers for 18 h at the same concentrations used in A or with H_2O_2 (50 μM) for 4 h. Twenty-four hours after transfection, cells were harvested. Levels of p50 in total cell lysates were analyzed by Western blotting followed by normalization to ERK. Two-way ANOVA of effect of siRNA transfection or scavenger treatments on the Bcl-2 level is $F(3,72;$ transfection$) = 130.0$, $p < 0,0001$; $F(4,72;$ treatment$) = 52.99$, $p < 0.0001$. *, $p < 0.05$, **, $p < 0.01$ compared with control siRNA; #, $p < 0.05$, ##, $p < 0.01$ compared with Control (no scavenger) with the same siRNA transfection or H_2O_2 treatment (Bonferroni post hoc test, $n = 4$). E, nuclear p50 is increased by Sig-1R siRNA. Nuclei and postnuclear lysate were prepared from CHO cells transfected with control or Sig-1R siRNA as described under *Materials and Methods*. In Western blotting, 30 μg/lane of lysates was loaded. Nucl, nucleoporin p63. In the graph, nuclear p50 was normalized to nucleoporin p63 ($n = 6$). **, $p < 0.01$ compared with control siRNA by Student's t test. F, decreased IκBα in CHO cells transfected with Sig-1R siRNA. IκBα levels were measured by Western blotting (20 μg/lane) followed by normalization to ERK. *, $p < 0.01$ compared with control siRNA by Student's t test ($n = 6$). G, effect of Sig-1R overexpression on levels of p105, p50, and IκBα. Twenty-four hours after transfection of wild-type Sig-1Rs, the levels of each protein were measured by Western blotting (20 μg/lane) followed by normalization to ERK. **, $p < 0.05$ compared with control vector by Student's t test; $n = 5$.

H_2O_2-induced apoptosis. Although oridonin showed a marginal effect on H_2O_2-induced apoptosis in control cells, it inhibited the action of Sig-1R siRNA to potentiate H_2O_2-induced apoptosis (Fig. 7D).

Discussion

In the present study we showed that 1) Bcl-2 localizes at the MAM but does not serve as a substrate protein for Sig-1R chaperones; 2) Sig-1Rs transcriptionally and tonically regulate the expression of Bcl-2 via the ROS/NF-κB pathway; and 3) blocking NF-κB or up-regulating Bcl-2 abolishes the potentiation of H_2O_2-induced apoptosis caused by Sig-1R knockdown. These findings imply the significant importance of the ROS/NF-κB pathway in the cellular protective action promoted by Sig-1Rs.

Regulating the expression of Bcl-2, thus maintaining the Bcl-2/Bax ratio at the high level, is important for cellular survival (Reed, 1994; Danial and Korsmeyer, 2004; Youle and Strasser, 2008). The cell possessing multifunctional regulatory systems can control the expression of Bcl-2 at various steps, including transcription, translation, and protein deg-

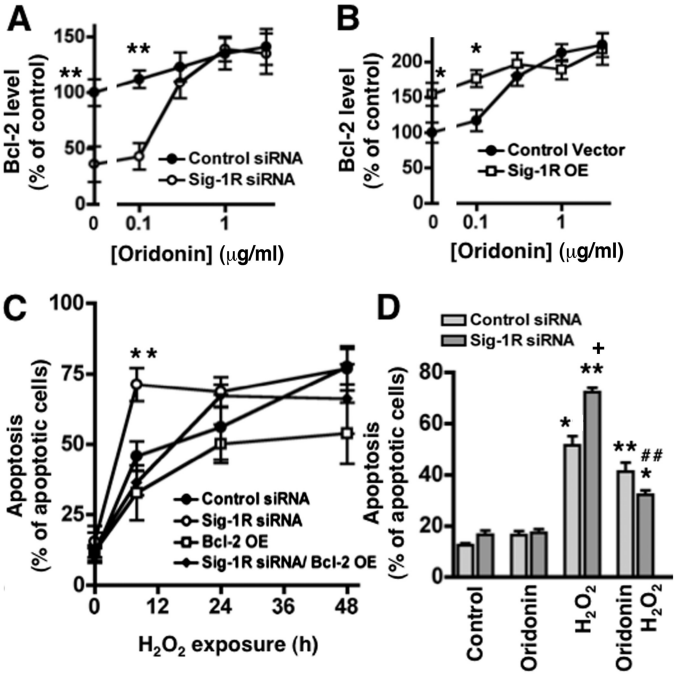

Fig. 7. Involvement of the NF-κB/Bcl-2 pathway in cell protection of Sig-1Rs. A, dose-dependent inhibition by oridonin of Bcl-2 down-regulation caused by Sig-1R siRNA. Six hours after transfection with control siRNA or Sig-1R siRNA, cells were cultured with oridonin for the following 18 h. Bcl-2 protein levels were measured by Western blotting with normalization to internal controls (total ERK). The graph represents mean ± S.E.M. ($n = 6$). Two-way ANOVA of effect of siRNA or concentrations of oridonin is $F(4,60; \text{siRNA}) = 11.87$, $p = 0.0012$; $F(4,60; [\text{oridonin}]) = 32.61$, $p < 0.0001$. **, $p < 0.01$ compared with control siRNA at the same dose of oridonin (Bonferroni post hoc test). B, dose-dependent inhibition by oridonin of Bcl-2 up-regulation caused by Sig-1R overexpression (OE). Six hours after transfection with empty vectors or Sig-1R overexpression vectors, cells were cultured with oridonin for the following 18 h. Bcl-2 levels were measured as described in A. Two-way ANOVA of effect of vector transfection or concentrations of oridonin is $F(4,60; \text{OE}) = 4.324$, $p = 0.0427$; $F(4,60; [\text{oridonin}]) = 12.85$, $p < 0.0001$. *, $p < 0.05$ compared with control vector at the same dose of oridonin (Bonferroni post hoc test; $n = 6$). C, effect of Sig-1R siRNA and/or Bcl-2 overexpression on H_2O_2-induced apoptosis in CHO cells. Twenty-four hours after transfection with siRNA and/or Bcl-2 expression vectors, CHO cells were treated with H_2O_2 (50 μM) for indicated periods. Apoptotic cells were counted as described under *Materials and Methods*. Two-way ANOVA of effect of transfection or exposure time of H_2O_2 is $F(4,80; \text{transfection}) = 5.586$, $p = 0.0016$; $F(4,80; \text{exposure time}) = 46.02$, $p < 0.0001$. **, $p < 0.01$ compared with control siRNA at 8 h (Bonferroni post hoc test; $n = 6$). D, effect of oridonin on H_2O_2-induced apoptosis. CHO cells were transfected with control siRNA or Sig-1R siRNA. Six hours after transfection, oridonin (2 μg/ml) was applied to culture medium for the following 18 h. Twenty-four hours after transfection, H_2O_2 (50 μM) was challenged for 8 h with or without oridonin. Apoptotic cells were counted as described in *Materials and Methods*. Two-way ANOVA of effect of drug treatment or transfection is $F(4,48; \text{treatment}) = 32.61$, $p < 0.0001$; $F(2,48; \text{transfection}) = 1.155$, $p = 0.2889$. *, $p < 0.05$, **, $p < 0.001$ compared with control (no drug treatment) with the same siRNA transfection. ##, $p < 0.01$ compared with H_2O_2 alone transfected with Sig-1R siRNA. +, $p < 0.05$ compared with H_2O_2 alone transfected with control siRNA (Bonferroni post hoc test; $n = 6$).

radation. As a potent regulator of the Bcl-2, ROS regulate the expression of Bcl-2 via both transcription and protein degradation (Hildeman et al., 2003; Li et al., 2004). ROS-activated transcription factors such as NF-κB often negatively regulate transcription of the *bcl-2* gene (Sohur et al., 1999; Pugazhenthi et al., 2003). Under certain conditions ROS decrease a half-life of Bcl-2 protein by facilitating protein degradation (Li et al., 2004). In squamous cell carcinoma OSC-4, knocking down Mn-superoxide dismutase activates ubiquitination and proteasomal degradation of Bcl-2 but suppresses degradation of Bax without activating NF-κB (Li et al., 2004). It is interesting to note that, in our system of knocking down Sig-1Rs, down-regulation of Bcl-2 is caused mainly by activation of NF-κB. Knockdown of Sig-1Rs affected neither the degradation of Bcl-2 proteins nor the Bax protein level. This discrepancy might be in part the result of the difference in the cell types used in these studies. However, it would be worth noting that the Sig-1R agonist PPBP is also shown to selectively prevent down-regulation of the bcl-2 mRNA, but not the bax mRNA, in primary cortical neurons under hypoxia and glucose deprivation (Yang et al., 2007). Thus, the action of Sig-1Rs regulating the bcl-2 mRNA level could be seen in a wide variety of cell types. Sig-1R might regulate the generation of specific type(s) of ROS (e.g., H_2O_2), and/or controlling sensitivity of NF-κB to ROS thus might be more specifically involved in the process activating the ROS/NF-κB pathway. It is also important to point out that the up-/down-regulation of Bcl-2 caused by manipulations of the Sig-1R expression in our study was induced without any stressors; the mode of action was different from that in previous reports in which Sig-1R ligands exert the action on the bcl-2 mRNA level only in the presence of proapoptotic stimuli (Yang et al., 2007). Thus, our study shows the novel action of Sig-1Rs that tonically and intrinsically regulates the expression of Bcl-2 proteins, but not merely by ameliorating stress caused by pathological insults.

The tight link between Sig-1Rs and NF-κB is observed particularly in results shown in Figs. 6 and 7. We showed that knockdown of Sig-1Rs not only increases the expression of p105 precursors but also the formation of the active form p50 and its nuclear localization, all leading to activation of the NF-κB complex (Karin, 2006). Furthermore, we found that the NF-κB inhibitor oridonin completely inhibits both down-regulation of Bcl-2 caused by Sig-1R siRNA and up-regulation of Bcl-2 caused by overexpression of Sig-1Rs. Although oridonin showed only a marginal effect on apoptosis induced by H_2O_2 in our system, indicating the presence of NF-κB-independent cell death pathways activated by H_2O_2, it selectively abolished the effect of Sig-1R siRNA potentiating H_2O_2-induced apoptosis. Thus, our findings clearly indicate that the ROS/NF-κB/Bcl-2 pathway is a crucial element in constituting the cellular protective action of the Sig-1R against oxidative stress.

The regulation of the Ca^{2+} transmission between ER and mitochondria plays an important role in controlling apoptosis and cellular survival (Pinton and Rizzuto, 2006). Previous studies showed that Bcl-2 interacts with the regulatory domain of IP3R to inhibit the channel opening of IP3R, thus Ca^{2+}-overloading in mitochondria as well (Rong et al., 2008). On the other hand, some studies showed that Bcl-2 activates the release of Ca^{2+} from ER pools, leading to the decrease of the Ca^{2+}content in ER Ca^{2+} pools (Lam et al., 1994). The lowered Ca^{2+} content is postulated to prevent overloading of mitochondrial Ca^{2+} under cellular stress (Pinton and Rizzuto, 2006). In light of the functional similarity, we originally speculated that Sig-1R chaperones might associate with Bcl-2, thus stabilizing Bcl-2 at the MAM to regulate Ca^{2+} signaling. However, immunoprecipitation failed to show the physical interaction of Bcl-2 with Sig-1Rs. Although results from immunoprecipitation may not be sufficient to totally negate the potential physical interaction, the data showing

that Sig-1R knockdown failed to alter stability of Bcl-2 strongly indicate that Bcl-2 does not serve as a substrate protein for Sig-1R chaperones; thus, the physical interaction is unlikely present. It is shown that the almost entire sequence of the Bcl-2 polypeptide is either anchored to the cytoplasmic surface of mitochondria or embedded in the outer mitochondrial membrane (Youle and Strasser, 2008). On the other hand, the chaperone domain of the Sig-1R is shown to reside in the lumen of the ER (Hayashi and Su, 2007). Therefore, it is likely that Bcl-2 may take the same membrane topology at the ER (i.e., cytoplasmic localization) as shown previously (Kim et al., 2004); thus, the physical association of these two proteins may not be achieved in vivo.

Although a number of studies postulated that Bcl-2 at ER membranes may localize in close vicinity to mitochondria based on the function of Bcl-2 regulating the Ca^{2+} transport from ER to mitochondria (Pinton and Rizzuto, 2006), it is worth noting that few studies have examined the MAM localization of Bcl-2. Thus, our findings provide direct evidence to support the notion that Bcl-2 is highly concentrated at the MAM.

In this study, we presented a molecular mechanism by which Sig-1Rs promote cellular survival under oxidative stress. Data indicate that Sig-1Rs transcriptionally regulate the expression of Bcl-2 via the ROS/NF-κB pathway, which may partly explain the robust neuroprotective action of Sig-1Rs seen in in vitro systems and in animal models of neurodegeneration (Meunier et al., 2006; Yang et al., 2007). Emerging questions that should be answered in the future may include 1) how does the Sig-1Rs' innate chaperone activity at the MAM promote the suppression of ROS generation and thus prevent activation of NF-κB; and 2) are there any molecules/signaling pathways that are involved in linking Sig-1Rs at the MAM to ROS generation? Sig-1Rs are shown to inhibit cell death induced by thapsigargin (Hayashi and Su, 2007), a typical ER stress inducer promoting accumulation of protein aggregates in the ER. However, Sig-1Rs also prevent cell death caused by a variety of stressors, including glucose deprivation (Hayashi and Su, 2007) and H_2O_2 (Fig. 7), that may promote protein aggregation and activation of cell death signals more predominantly in the cytoplasm or in mitochondria. Because Sig-1Rs regulate the Ca^{2+} influx from the MAM to mitochondria that leads to activation of mitochondrial metabolisms and generation of ROS (Pinton and Rizzuto, 2006; Hayashi and Su, 2007; Hayashi et al., 2009), dysregulation of the mitochondrial Ca^{2+} signaling might be involved in underlying mechanisms by which Sig-1Rs regulate ROS accumulation/scavenging. Alternatively, because Sig-1Rs are implicated in regulation of lipid transport/metabolisms (Hayashi and Su, 2003) and because the MAM plays a crucial role in regulating metabolisms of lipids and glucose, the major determinant of cellular redox (Rusiñol et al., 1994; Voelker, 2005), Sig-1Rs might regulate the redox state of the cell by regulating lipid/glucose metabolisms.

In conclusion, we showed that the ROS/NF-κB pathway plays a pivotal role in the Sig-1R's regulation of the Bcl-2 expression. The findings suggest that Sig-1R ligands that activate or up-regulate Sig-1Rs may serve as a new class of cellular protective agents that promote up-regulation of Bcl-2.

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