Apoptosis contributes to the regulation of cell growth and regeneration and to the development of neoplasia. Mcl-1 is an anti-apoptotic protein that is particularly important for the development of hematological and biliary malignancies, but the mechanism of action of Mcl-1 is unknown. A number of pro- and anti-apoptotic proteins exhibit their effects by modulating Ca\(^{2+}\) signals, so we examined the effects of Mcl-1 on components of the Ca\(^{2+}\) signaling pathways that are known to regulate apoptosis. Expression of Mcl-1 did not affect expression of the inositol 1,4,5-trisphosphate (InsP\(_3\)) receptor (InsP\(_3\)R) (12), which is the principal ER Ca\(^{2+}\) release channel in most types of cells. Mcl-1 (myeloid cell leukemia-1) was first identified because this gene is up-regulated early in the differentiation of the ML-1 human myeloid leukemia cell line and was found to be a member of the emerging Bcl-2 gene family as well (13). However, the mechanism by which Mcl-1 inhibits apoptosis is not entirely understood. Because Mcl-1 is a member of the Bcl-2 family, we examined the effects of this protein on Ca\(^{2+}\) signaling pathways.

**EXPERIMENTAL PROCEDURES**

**Materials, Reagents, and Cell Lines**—The Ca\(^{2+}\) dyes fluo-4, mag-fluo-4, and rhod-2, the mitochondrial dye MitoTracker green, and the nuclear stain TO-PRO-3. Secondary antibodies were Alexa 488 anti-rabbit (1:500), Alexa 568 anti-mouse, and Alexa 647 anti-rabbit IgG (Molecular Probes). Mz-Cha-1 cells derived from a human biliary adenocarcinoma (15) were kindly provided by Dr. Greg Fitz (University of Texas Southwestern). Cells were maintained at 37 °C with 5% CO\(_2\). Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum plus antibiotics.

**Immunofluorescence**—Immunofluorescence was performed on Mz-Cha-1 cells and paraffin-embedded sections of human liver biopsy specimens as described previously (16). Bcl-2, Bcl-xL, and Mcl-1 were detected with mouse monoclonal (1:100) and anti-Mcl-1 rabbit polyclonal (1:100). Cells were also labeled with the nuclear stain TO-PRO-3. Negative controls were performed under each experimental condition by incubating tissue with buffer at 100 °C. Primary antibodies used were anti-type III IP3R-3 (1:100) and anti-Cam (1:100).

**Immunoprecipitation**—Immunoprecipitation was performed with the antibody against Bcl-2 and Bcl-xL (1:100) from Santa Cruz Biotechnology (Santa Cruz, CA) and the polyclonal antibody against Mcl-1 (1:200). Immunoprecipitations were performed as described previously (17).

**Western Blot**—Western blots were performed as described previously (17). Membranes were probed with mouse monoclonal (1:100) and anti-human polyclonal (1:100) antibodies. After incubation with horseradish peroxidase-conjugated secondary antibodies (1:1000), blots were stained with ECL (Amersham Biosciences) and exposed to X-ray film (Eastman Kodak). Immunoprecipitated samples were resolved by SDS-PAGE (10% gel for Mcl-1 and Bcl-2 and 12% gel for Bcl-xL) and transferred to nitrocellulose membranes. Membranes were probed with mouse monoclonal (1:200) and anti-human polyclonal (1:100) antibodies. After incubation with horseradish peroxidase-conjugated secondary antibodies (1:1000), blots were stained with ECL (Amersham Biosciences) and exposed to X-ray film (Eastman Kodak). Enzymes were measured with a luciferase assay kit.

**Immunohistochemistry**—Immunohistochemistry was performed on paraffin sections using anti-human Bcl-2, Bcl-xL, and Mcl-1 (1:100) from Santa Cruz Biotechnology (Santa Cruz, CA) and the polyclonal antibody against Mcl-1 (1:200). Sections were treated with 1 mM citrate buffer at 100 °C. Primary antibodies used were anti-type III IP3R-3 (1:100) and anti-Cam (1:100).

**Immunofluorescence**—Immunofluorescence was performed on paraffin sections using anti-human Bcl-2, Bcl-xL, and Mcl-1 (1:100) from Santa Cruz Biotechnology (Santa Cruz, CA) and the polyclonal antibody against Mcl-1 (1:200). Immunofluorescence was performed as described previously (17).
obtained using machine settings at which no fluorescence was detectable in negative control samples. Immunofluorescence images were obtained by excitation at 488 nm with observation at 505–550 nm to detect Alexa 488 or GFP, by excitation at 543 nm and observed at >585 nm to detect Alexa 568 or DsRed, then by excitation at 633 nm with observation at >650 nm to detect Alexa 647 or TO-PRO-3. Mcl-1 or InsP₃R immunofluorescence was quantified in transfected Mz-Cha-1 cells by normalizing the fluorescence relative to the fluorescence detected in nearby non-transfected cells. To perform this calculation, transfected cells in each microscopic field were identified by the expression of DsRed or GFP. Mean fluorescence in each transfected cell then was divided by mean fluorescence measured in at least five non-transfected cells in the same microscopic field, and the ratio was multiplied by 100%.

Plasmids and Transfections—The plasmids for Mcl-1 cDNA and siRNA have been described previously (17). In particular, a specific double-stranded 21-nucleotide RNA sequence homologous to the target message was used to silence Mcl-1 (17). Cells were co-transfected with enhanced green fluorescent protein (GFP) or DsRed (both from Clontech) to identify transfected cells. Transfections were carried out using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. Transfection efficiency was typically 5–10%. Cells were used 48 h after transfection.

Mitochondrial Preparation and Cytosolic Extracts—Mitochondria were isolated using a commercially available mitochondria isolation kit (Sigma) according to the manufacturer’s instructions. Briefly, mitochondria were prepared from cells by homogenization followed by low speed (600 × g) and then high speed (1100 × g) centrifugation. The final pellet contained the crude mitochondrial fraction that was used for immunoblots. Purity of the mitochondrial fraction was validated by a fluorometric assay that measures uptake of the cationic carbocyanine dye JC-1 by intact mitochondria (18). In selected experiments, JC-1 was used to measure mitochondrial membrane potential directly in intact Mz-Cha-1 cells by exciting the specimens at 543 nm and observing at 515 nm to detect mag-fluo-4 emission signals. Cells were incubated for 30 min at 37 °C with JC-1 (10 μg/ml), and then examined by confocal microscopy. Fluorescence images were obtained by excitation at 488 nm with observation at 505–550 nm and at >585 nm to detect potential-sensitive color shifts.

Immunoblot Analysis—Immunoblots were performed as described previously (16). Briefly, cells were lysed in lysis buffer (20 mM Tris–HCl pH 7.4, 5 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin, and 1 mM leupeptin), and then protein content was determined by the Bradford assay. Proteins were separated by SDS-PAGE using a 12% polyacrylamide gel, and then transferred to protein nitrocellulose membranes (Bio-Rad). Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS-T, 25 mM Tris-HCl, pH 7.5, 150 mM NaCl) plus 0.05% Tween 20 or 1 h, and incubated with rabbit anti-Mcl-1 antibody (1:500) in 5% nonfat dry milk in TBS-T at 4 °C overnight, followed by incubation (1 h) with peroxidase-conjugated anti-rabbit immunoglobulin G secondary antibody (Bio-Rad, 1:4000) in TBS-T. Blots were visualized by enhanced chemiluminescence using the ECL plus kit (Amersham Biosciences). A Bio-Rad GS-700 imaging densitometer was used for quantitative analysis of the blots.

Calcium Measurements—Free mitochondrial Ca²⁺ (Ca₂⁺ₘ) was measured in wild type Mz-Cha-1 cells and in Mz-Cha-1 cells transiently transfected with Mcl-1 cDNA or Mcl-1-specific siRNA using the mitochondrial Ca²⁺ dye rhod-2 and time lapse confocal microscopy. rhod-2 can sometimes detect cytosolic rather than mitochondrial Ca²⁺, but rhod-2 labeling in Mz-Cha-1 cells was punctuate, and Ca²⁺ signals detected by rhod-2 were different from those detected by the cytosolic Ca²⁺ dye fluo-4 (not shown), suggesting that rhod-2 fluorescence reflected Ca₂⁺ₘ in these cells. Cells were incubated for 30 min at 37 °C with rhod-2/AM (10 μM). Coverslips containing the cells were transferred to a custom-built perfusion chamber on the stage of a Bio-Rad MRC-1024 confocal microscope (Hercules, CA) (14, 16, 19). Ca₂⁺ₘ was monitored in these cells by exciting the specimens at 543 nm and observing at >585 nm to detect rhod-2 emission signals. Cells were stimulated with ATP (10 or 100 μM) or staurosporine (1 μM), and images were acquired every 150–600 ms. Increases in Ca₂⁺ₘ were expressed as percent increases in fluorescence intensity of rhod-2. In separate studies, ER Ca²⁺ was measured using the low affinity Ca²⁺ dye mag-fluo-4 (Kₚ,22 μM) and examined by confocal microscopy (22). Cells were incubated for 30 min at 37 °C with mag-fluo-4/AM (6 μM) then observed using the same confocal imaging system used to detect rhod-2. ER Ca²⁺ was monitored in these cells by exciting the specimens at 488 nm and observing at >515 nm to detect mag-fluo-4 emission signals. Cells were stimulated with thapsigargin (2 μM) to deplete ER Ca²⁺ stores, and images were acquired every 1–2 s. Changes in ER Ca²⁺ were expressed as percent increases or decreases in fluorescence intensity of mag-fluo-4. rhod-2 and mag-fluo-4 fluorescence was quantified in transfected Mz-Cha-1 cells by normalizing the fluorescence relative to...
Mcl-1 and Mitochondrial Ca$^{2+}$

Figure 2. Distribution of Mcl-1 and the InsP$_3$ receptor in Mz-ChA-1 cells. Confocal immunofluorescence images were obtained from Mz-ChA-1 cells. Cells were triple-labeled with antibodies against Mcl-1 (green), type III InsP$_3$R (red), and the nuclear stain TO-PRO-3 (blue). Mcl-1 is distributed diffusely in cytosol, whereas the InsP$_3$R is more concentrated in the perinuclear region. As in primary cholangiocytes, no co-localization is seen. Scale bar, 5 μm.

Figure 3. Up-regulation and down-regulation of Mcl-1 expression. Western blots demonstrating expression of Mcl-1 in proteins extracted from HEK239 cells that were untreated or treated with siRNA or cDNA for Mcl-1. siRNA inhibited the expression of Mcl-1 in HEK239 cells, whereas cDNA increased the expression. Data were normalized by β-actin expression determined in the same blots. Amount of protein used was 10 μg in each lane. Mz-ChA-1 cells are used as a positive control.

Figure 4. Identification of single Mz-ChA-1 cells that over- or underexpress Mcl-1. Each confocal immunofluorescence image is triple-labeled to reveal Mcl-1 (blue), the type III InsP$_3$R (green), and DsRed (red), which is used as a marker of transfection. A, Mz-ChA-1 cells transfected with DsRed alone. Labeling for Mcl-1 and the InsP$_3$R are similar to what is observed in nearby non-transfected cells. B, Mz-ChA-1 cells transfected with DsRed and Mcl-1 siRNA. Mcl-1 labeling is decreased compared with non-transfected cells, but InsP$_3$R labeling is similar to what is seen in non-transfected cells. C, Mz-ChA-1 cells transfected with DsRed plus cDNA for Mcl-1. Mcl-1 labeling is increased compared with non-transfected cells, but InsP$_3$R labeling is similar among all cells. Scale bar, 20 μm.

Figure 5. Mcl-1 does not affect expression of the InsP$_3$ receptor. A, for each experimental group, immunofluorescence for Mcl-1 and the type III InsP$_3$R was quantified in individual cells and was normalized by immunofluorescence detected in at least 10 nearby non-transfected cells. Mcl-1 labeling decreased significantly in Mz-ChA-1 cells treated with siRNA and increased significantly in cells transfected with Mcl-1 cDNA (*, p < 0.001 relative to control cells transfected with DsRed alone). In contrast, labeling for the InsP$_3$R did not change in either of these groups. Results are mean ± S.E. (n = 10 cells in each group). B, Mcl-1 expression and InsP$_3$R expression are uncorrelated (r = 0.22; p > 0.2). Solid line, linear regression curve; dotted lines, 95% confidence intervals for regression curve.

RESULTS

Expression of Mcl-1 and Type III InsP$_3$R in Primary Cholangiocytes and in a Cholangiocarcinoma Cell Line—Mcl-1 is thought to play a role in development of cholangiocarcinoma (17), but expression of this protein has not been investigated in primary biliary tissue. Therefore we used confocal immunofluorescence to examine expression of Mcl-1 in liver biopsies from eight patients with normal bile ducts, five patients with biliary obstruction due to stone disease (n = 4) or malignancy (n = 1), and five patients with cholangiocarcinoma (Fig. 1). Specimens were co-labeled for the type III InsP$_3$R (InsP$_3$R-3). This isoform accounts for >80% of InsP$_3$R$s$ in bile ducts and is known to be concentrated in the apical region (16, 20). Mcl-1 labeling was present in normal cholangiocytes and was distributed diffusely throughout the cytosol, whereas InsP$_3$R-3 was concentrated apically as shown previously (Fig. 1A). Both Mcl-1 and InsP$_3$R-3 were detected in cholangiocarcinoma as well (Fig. 1B). Quantitative immunofluorescence suggested that Mcl-1 expression was increased in cholangiocarcinoma, because fluorescence increased from 86 ± 1 pixel values in normal cholangiocytes to 124 ± 2...
Mcl-1 expression was decreased to 58 ± 1 pixel values, and InsP3R-3 expression was nearly absent in patients with biliary obstruction (p < 0.0001 relative to normal tissue, Fig. 1C). Interpretation of these results is limited, because there was insufficient tissue from these patient specimens to perform quantitative immunoblots. However, these results demonstrate that Mcl-1 is expressed in primary human cholangiocytes and suggest that expression is increased in cholangiocarcinoma. Next, we examined the expression and distribution of Mcl-1 and InsP3R-3 in Mz-ChA-1 cells, because this is a well characterized human cholangiocarcinoma cell line (21) and InsP3R-3 accounts for nearly all of the InsP3R in these cells (data not shown). Mcl-1 was distributed throughout the cytosol, whereas InsP3R-3 was distributed in a perinuclear pattern. The two proteins did not co-localize (Fig. 2). Based on these findings, Mz-ChA-1 cells were used as the model cell line for the remaining studies.

Mcl-1 Does Not Alter Expression of the InsP3 Receptor—Bcl-xL has been shown to inhibit apoptosis in part by decreasing expression of the InsP3R (12), so we examined whether Mcl-1 similarly alters InsP3R expression. Cells were loaded with the low affinity Ca2+ dye mag-fluo-4 to label ER Ca2+ stores, and then examined by confocal microscopy. A, confocal image of an Mz-ChA-1 cell loaded with the low affinity Ca2+ dye mag-fluo-4. Fluorescence is distributed in a reticular pattern, consistent with labeling of the ER Ca2+ pool. B, ER Ca2+ is labeled with mag-fluo-4 (green), and transfected cells are labeled with DsRed in addition. Top row: transfection with DsRed alone. Middle row: DsRed plus Mcl-1 siRNA. Bottom row: DsRed plus Mcl-1 cDNA. Scale bar, 20 μm. C, quantification of mag-fluo-4 fluorescence. For each group, mag-fluo-4 fluorescence in transfected cells was normalized by fluorescence in at least 30 nearby non-transfected cells. Labeling for ER Ca2+ was not significantly increased or decreased in any group of transfected cells. Results are mean ± S.E. (n = 18 cells in each group). D, change in mag-fluo-4 fluorescence during stimulation with the SERCA inhibitor thapsigargin (TG; 2 μM). The decrease in fluorescence reflects TG-induced depletion of ER Ca2+. E, summary of TG experiments. TG-induced depletion of ER Ca2+ stores is not altered by decreased (siRNA) or increased (cDNA) expression of Mcl-1 (p > 0.8 by analysis of variance).
expression. To investigate this, cells were transfected with either Mcl-1 cDNA or siRNA for Mcl-1, to increase or decrease Mcl-1 expression, respectively (Fig. 3). HEK293 cells were used to validate the Mcl-1 cDNA and siRNA constructs (Fig. 3), and all subsequent transfections were performed in Mz-ChA-1 cells. Cells were co-transfected with DsRed as a marker of successful transfection. Control cells were transfected with DsRed alone. Expression of Mcl-1 and InsP$_3$R in each group was evaluated simultaneously by quantitative confocal immunofluorescence (20) and was normalized by expression of these proteins in non-transfected cells on the same coverslips (Figs. 4 and 5). Mcl-1 fluorescence in the DsRed control group was 100%, whereas that of InsP$_3$R-3 was 102% of the fluorescence in non-transfected cells in co-culture (Fig. 3), whereas that of InsP$_3$R-3 was 102% of that seen in non-transfected cells in co-culture (Fig. 3, lanes 3 and 5). Mcl-1 fluorescence in siRNA-treated cells was reduced to 54% of that of non-transfected cells in co-culture (p < 0.005 versus control), whereas that of InsP$_3$R-3 in these cells was 92% ±6% (p > 0.1 versus control). The fluorescence intensity of Mcl-1 labeling in cells transfected to overexpress this protein was 193% ± 21% of that seen in non-transfected cells in co-culture (p < 0.005 versus control), whereas that of InsP$_3$R-3 was 102% ± 12% (p > 0.1 versus control). Thus, neither increased nor decreased expression of Mcl-1 altered the expression of the InsP$_3$R. To analyze these results further, we examined the relationship between normalized Mcl-1 fluorescence and normalized InsP$_3$R fluorescence over the range of values for Mcl-1 (Fig. 5B). There was no correlation between Mcl-1 and InsP$_3$R fluorescence (r = 0.22; p > 0.2), providing further evidence that Mcl-1 does not regulate expression of InsP$_3$R.

Mcl-1 Does Not Affect ER Calcium Stores—Bcl-2 inhibits apoptosis in part by decreasing ER Ca$^{2+}$ stores (11), so we examined whether Mcl-1 similarly alters ER Ca$^{2+}$. To determine whether Mcl-1 influences ER Ca$^{2+}$ stores, Mz-ChA-1 cells were loaded with the low-affinity Ca$^{2+}$ dye mag-fluo-4 (K$_{D}$ 22 µM) and examined by confocal microscopy (22). mag-fluo-4 fluorescence was distributed in a reticular fashion (Fig. 6A), consistent with selective labeling of ER Ca$^{2+}$ stores. Cells were transfected with either Mcl-1 cDNA or siRNA for Mcl-1, along with DsRed as a marker of successful transfection. Control cells were transfected with DsRed alone. As an additional control, mag-fluo-4 fluorescence in all transfected cells was compared with fluorescence signals observed in co-cultured non-transfected cells on the same coverslips (Fig. 6B). The mag-fluo-4 fluorescence intensity of cells in each group was determined to quantify these observations. The ratio of fluorescence in transfected/non-transfected cells was 1.03 ± 0.08 (n = 38) in control cells (DsRed only), 1.10 ± 0.14 (n = 18) in cells transfected with siRNA to decrease Mcl-1 expression, and 0.99 ± 0.15 (n = 23) in cells transfected with Mcl-1 cDNA to increase Mcl-1 expression (p > 0.25 for each group, Fig. 6C). Stimulation with the SERCA inhibitor thapsigargin (2 µM) induced a progressive decrease in mag-fluo-4 fluorescence (Fig. 6D), providing further evidence of selective labeling of ER Ca$^{2+}$ stores. Treatment with thapsigargin induced a similar decrease in fluorescence in all (control, siRNA, and cDNA) transfected cells as well as in non-transfected cells (p > 0.8 by analysis of variance, Fig. 6E). These results provide evidence that, unlike Bcl-2, Mcl-1 does not affect the size of ER calcium stores.

Mcl-1 Inhibits Mitochondrial Calcium Signaling—Immunoblot analysis of mitochondrial and non-mitochondrial fractions of Mz-ChA-1 cells demonstrated that Mcl-1 is localized to the mitochondria (Fig. 7A), consistent with previous reports (23). Confocal immunofluorescence of Mcl-1 was performed to further confirm this observation (Fig. 7B). Mcl-1 co-localized with oxidative phosphorylation complex I, which is expressed only in mitochondria. This provides additional evidence that Mcl-1 is mitochondrial, so we examined the effects of Mcl-1 on mitochondrial Ca$^{2+}$ signals. Excessive increases in free mitochondrial Ca$^{2+}$ can lead to formation of the permeability transition pore (25) and can also release cytochrome c and induce apoptosis (26). To determine whether Mcl-1 influences free mitochondrial Ca$^{2+}$ signals, Mz-ChA-1...
cells were loaded with the mitochondrial \( \text{Ca}^{2+} \) dye rhod-2 (27, 28) and examined by confocal microscopy. To confirm the subcellular distribution of rhod-2, a subset of cells were loaded with both rhod-2 and the mitochondrial dye MitoTracker green (Fig. 8). Confocal imaging demonstrated that the two dyes co-localized, which demonstrates that rhod-2 preferentially labels mitochondria in this cell type. Cells were then transfected with either Mcl-1 cDNA or siRNA for Mcl-1, along with GFP as a marker of successful transfection. Control cells were transfected with GFP alone. As an additional control, mitochondrial \( \text{Ca}^{2+} \) signals in all transfected cells were compared with signals observed in co-cultured non-transfected cells on the same coverslips. Cells were stimulated with staurosporine (1 \( \mu \)M) to induce apoptosis. Staurosporine increased mitochondrial \( \text{Ca}^{2+} \) in 5 of 6 cells in which Mcl-1 expression was reduced (Fig. 10A), and in none of 5 cells in which Mcl-1 was overexpressed (Fig. 10B). For comparison, staurosporine increased mitochondrial \( \text{Ca}^{2+} \) in 2 of 6 cells transfected with GFP-cytochrome c alone, which is similar to the frequency with which \( \text{Ca}^{2+} \) signals were observed in non-transfected cells (26 of 109 cells, or 24%). Next, the subcellular localization of rhod-2 and GFP-cytochrome c was monitored in transfected cells before and 3 h after treatment with staurosporine. rhod-2 and GFP-cytochrome c were co-localized prior to treatment (not shown). The two labels no longer were co-localized after treatment in cells in which Mcl-1 expression was decreased (Fig. 10C), indicating the loss of cytochrome c from mitochondria that is associated with development of apoptosis. In contrast, co-localization persisted in cells in which Mcl-1 expression was increased (Fig. 10C), consistent with protection against apoptosis. Because of the specific mitochondrial actions of Mcl-1, we examined the effects of Mcl-1 expression on mitochondrial membrane potential using the cationic potential-sensitive dye JC-1. Cells were transfected with either Mcl-1 cDNA or siRNA for Mcl-1, along with GFP as a marker of successful transfection. Control cells were transfected with GFP alone. As an additional control, JC-1 fluorescence in all transfected cells was compared with fluorescence signals detected in co-cultured non-transfected cells on the same coverslips. JC-1 fluorescence in non-transfected cells was no different from what was detected in cells transfected with Mcl-1 cDNA (102 ± 3% of JC-1

![Figure 9](http://www.jbc.org/)
Mcl-1 and Mitochondrial Ca$^{2+}$

**FIGURE 10.** Mcl-1 inhibits mitochondrial Ca$^{2+}$ signals associated with apoptosis. Mz-ChA-1 cells were loaded with rhod-2 and stimulated with staurosporine while mitochondrial Ca$^{2+}$ signals were monitored by confocal microscopy. A, staurosporine increases mitochondrial Ca$^{2+}$ in a cell transfected with siRNA for Mcl-1 plus GFP-cytochrome c. Result is representative of what was observed in 5 of 6 cells. B, staurosporine does not increase mitochondrial Ca$^{2+}$ in a cell transfected with Mcl-1 cDNA plus GFP-cytochrome c. A modest Ca$^{2+}$ increase is elicited by subsequent stimulation with a maximal concentration of ATP. Similar results were observed in each of five transfected cells. C, staurosporine induces loss of cytochrome c from mitochondria in cells in which Mcl-1 expression is reduced (top row) but not in cells in which Mcl-1 expression is increased (bottom row). These confocal images were obtained 3 h after treatment with staurosporine.

**FIGURE 11.** Inhibition of Ca$^{2+}$ signaling pathways by Bcl-2 family members. Bcl-2 decreases the size of ER Ca$^{2+}$ stores, whereas Bcl-XL indirectly inhibits release of Ca$^{2+}$ from the ER into the cytosol by inhibiting expression of the InsP$_3$R. Cytosolic Ca$^{2+}$ signals are transmitted into the mitochondria, and repeated or excessive increases in mitochondrial Ca$^{2+}$ result in formation of the permeability transition pore (PTP), which releases cytochrome c and promotes apoptosis. Mcl-1 inhibits mitochondrial Ca$^{2+}$ signals.

flourescence in non-transfected cells, $p > 0.3$), siRNA (98 ± 7% of non-transfected cells, $p > 0.4$), or GFP alone (106 ± 7%, $p > 0.25$). These findings demonstrate that Mcl-1 inhibits the mitochondrial Ca$^{2+}$ signaling that is associated with development of apoptosis and suggest that this effect is not due to changes in mitochondrial membrane potential.

**DISCUSSION**

Mitochondria play an integral role in Ca$^{2+}$ signaling pathways and patterns. Mitochondria are in close apposition to InsP$_3$Rs (30) and thus are exposed to more abrupt and intense increases in Ca$^{2+}$ than occurs in most regions of the cytosol (30). Because mitochondria take up Ca$^{2+}$ via a potential-driven unipor (31), this spatial arrangement enables mitochondria to modulate increases in cytosolic Ca$^{2+}$. For example, oxidizable substrates that energize mitochondria increase the ampli-

Ca$^{2+}$ is involved in several critical steps in the induction of apoptosis, including activation of caspases (4) plus PTP formation as noted above (37). Ca$^{2+}$ release from the InsP$_3$R is a particularly important step for inducing apoptosis. Initial evidence suggested that the type III InsP$_3$R is more effective than the type I isoform in inducing apoptosis (38), although subsequent work suggested the type I InsP$_3$R can mediate apoptosis as well (39). The ability of the InsP$_3$R to modulate apoptosis is related to its actions as a Ca$^{2+}$ release channel. Ca$^{2+}$ released from the InsP$_3$R can be taken up by neighboring mitochondria and lead to cytochrome c release. Cytochrome c that has leaked from mitochondria then binds directly to the InsP$_3$R, which blocks the Ca$^{2+}$-induced inhibition of the receptor that occurs at high cytosolic Ca$^{2+}$ concentrations (26).

This leads to a positive feedback loop whereby enhanced InsP$_3$-medi-
Mcl-1 and Mitochondrial Ca$^{2+}$

ated Ca$^{2+}$ release causes further Ca$^{2+}$ overload of mitochondria, leading to further leakage of cytochrome c and then further enhancement of InsP$_3$-mediated Ca$^{2+}$ release (26). Conversely, factors that inhibit InsP$_3$-mediated Ca$^{2+}$ release lead to inhibition of apoptosis. For example, the mitochondrial stress pathway leading to apoptosis activates nuclear factor of activated T cells, which in turn binds to the InsP$_3$R promoter and increases expression of the InsP$_3$R. The anti-apoptotic protein Bcl-x$_L$ reduces binding of nuclear factor of activated T cells to DNA, resulting in decreased InsP$_3$R expression (12). This in turn reduces InsP$_3$-mediated Ca$^{2+}$ release to protect against apoptosis (12). In contrast to Bcl-x$_L$, Bcl-2 instead increases leakage of Ca$^{2+}$ from the ER, but this also results in decreased Ca$^{2+}$ signals in both the cytosol and mitochondria (11). Recent evidence suggests that the pro-apoptotic protein Bak exhibits an effect opposite to that of Bcl-2; Bak increases Ca$^{2+}$ loading in the ER, which enhances transmission of Ca$^{2+}$ signals to the mitochondria (40). Mcl-1 is thought to be the principal Bcl-2 family member in cholangiocytes (17), but little is known about the mechanism by which it inhibits apoptosis. Both apoptosis (41) and InsP$_3$R expression (16) are decreased in cholangiocarcinoma as well (17), and the current work provides evidence that Mcl-1, like Bcl-x$_L$, indirectly affects Cam$^2+$

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