Inhibition of MEK Sensitizes Human Melanoma Cells to Endoplasmic Reticulum Stress-Induced Apoptosis

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Abstract

Past studies have shown that activation of mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK is a common cause for resistance of melanoma cells to death receptor–mediated or mitochondria-mediated apoptosis. We report in this study that inhibition of the MEK/ERK pathway also sensitizes melanoma cells to endoplasmic reticulum (ER) stress-induced apoptosis, and this is mediated, at least in part, by caspase-4 activation and is associated with inhibition of the ER chaperon glucose-regulated protein 78 (GRP78) expression. Treatment with the ER stress inducer tunicamycin or thapsigargin did not induce significant apoptosis in the majority of melanoma cell lines, but resistance to these agents was reversed by the MEK inhibitor U0126 or MEK1 small interfering RNA (siRNA). Induction of apoptosis by ER stress when MEK was inhibited was caspase dependent with caspase-4, caspase-9, and caspase-3 being involved. Caspase-4 seemed to be the apical caspase in that caspase-4 activation occurred before activation of caspase-9 and caspase-3 and that inhibition of caspase-4 by a specific inhibitor or siRNA blocked activation of caspase-9 and caspase-3, whereas inhibition of caspase-9 or caspase-3 did not inhibit caspase-4 activation. Moreover, overexpression of Bcl-2 inhibited activation of caspase-9 and caspase-3 but had minimal effect on caspase-4 activation. Inhibition of MEK/ERK also resulted in down-regulation of GRP78, which was physically associated with caspase-4, before and after treatment with tunicamycin or thapsigargin. In addition, siRNA knockdown of GRP78 increased ER stress-induced caspase-4 activation and apoptosis. Taken together, these results seem to have important implications for new treatment strategies in melanoma by combinations of agents that induce ER stress and inhibitors of the MEK/ERK pathway. [Cancer Res 2007;67(20):9750–61]

Introduction

Several cellular stress conditions, such as nutrient deprivation, hypoxia, alterations in glycosylation status, and disturbances of calcium flux, lead to accumulation and aggregation of unfolded and/or misfolded proteins in the endoplasmic reticulum (ER) lumen and cause so-called ER stress (1–3). The ER responds to the stress conditions by activation of a range of stress response signaling pathways to alter transcriptional and translational programs, which couples the ER protein folding load with the ER protein folding capacity and is termed the unfolded protein response (UPR; refs. 1–3).

The UPR of mammalian cells is initiated by three ER transmembrane proteins, activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 (IRE1), and double-stranded RNA-activated protein kinase-like ER kinase (PERK), which act as proximal sensors of ER stress. Under unstressed conditions, the luminal domains of these sensors are occupied by the ER chaperon glucose-regulated protein 78 (GRP78; refs. 1–3). On ER stress, sequestration of GRP78 by unfolded proteins activates these sensors by inducing phosphorylation and homodimerization of IRE1 and PERK and relocalization of ATF6 to the Golgi where it is cleaved by site 1 and site 2 proteases (S1P and S2P) leading to its activation as a transcriptional factor (1–3).

The UPR is fundamentally a cytoprotective response. However, excessive or prolonged UPR results in apoptotic cell death. Processing of caspase-2, caspase-3, caspase-4, caspase-7, caspase-8, caspase-9, and caspase-12 has been observed in ER stress-induced apoptosis (4–7). Among them, caspase-12 is thought to be a key mediator as caspase-12–deficient mouse cells are partially resistant to ER stress-induced apoptosis (8). However, caspase-12 is expressed only in rodents. Its human homologue is silenced by several mutations during evolution (9). Human caspase-4 has been shown to fulfill the function of caspase-12 and plays an important role in ER stress-induced apoptosis of human neuroblastoma and HeLa cells (7). Moreover, the c-Jun NH_2-terminal kinase (JNK), the transcription factor CAAT/enhancer binding protein homologous protein (CHOP), and deregulation of Bcl-2 and inhibitor of apoptosis protein family members have all been suggested to play roles in ER stress-induced apoptosis (5, 6, 10–13).

There is increasing evidence to show that the UPR is activated in various solid tumors, perhaps due to nutrient deprivation and hypoxia [e.g., elevated expression of GRP78 has been reported in several cancers, such as breast cancer and prostate cancer (14–17)]. Moreover, GRP78 expression has been shown in some cases to be associated with tumor development and growth and correlated with resistance to certain forms of chemotherapy. It seems that some cancer cells may have adapted to ER stress by activation of the UPR without resulting in apoptosis (15, 16, 18). The central feature of this adaptive response has been suggested to be maintenance of expression of proteins that facilitate survival, such as GRP78, without persistence of proapoptotic proteins, such as CHOP (19). In addition, other survival signaling pathways, such as the phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK pathways, may also

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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play roles in counteracting the apoptosis-inducing potential of ER stress (14, 20).

Previous studies have shown that constitutive activation of the MEK/ERK pathway is a common cause for resistance of melanoma cells to apoptosis mediated by the death receptor and mitochondrial apoptotic pathways (21–24). In view of this, we examined the potential interaction between the UPR and the MEK/ERK pathway in regulation sensitivity of melanoma cells to ER stress-induced apoptosis. We show in this report that inhibition of the MEK/ERK signaling pathway also sensitizes cultured human melanoma cells to ER stress-induced apoptotic cell death. This is mediated, at least in part, by activation of caspase-4 that in turn leads to mitochondria-dependent apoptosis. We demonstrate, for the first time, that inhibition of MEK/ERK down-regulates GRP78 expression and blocks its induction by the UPR. This may play a role in activation of caspase-4 and enhancement of sensitivity of the cells to ER stress-induced apoptosis.

Materials and Methods

Cell lines. Human melanoma cell lines Mel-RM, MM200, IgR3, Mel-CV, Me4405, Sk-Mel-28, Mel-FH, Sk-Mel-110, and Me1007 have been described previously (21, 25). They were cultured in DMEM containing 5% FCS (Commonwealth Serum Laboratories). Melanocytes were kindly provided by Dr. P. Parsons (Queensland Institute of Medical Research, Brisbane, Queensland, Australia) and cultured in medium supplied by Clonetics. The cell line of human embryonic fibroblasts, FLOW 2000, was cultured in DMEM containing 5% FCS as described previously (26).

Antibodies, recombinant proteins, and other reagents. Tunicamycin and thapsigargin were purchased from Sigma Chemical Co. They were dissolved in DMSO and made up in stock solutions of 1 mmol/L, respectively. The cell-permeable general caspase inhibitor Z-Val-Ala-Asp(OMe)-CH_2F (z-VAD-fmk), the caspase-3–specific inhibitor Z-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-CH_2F (z-DEVD-fmk), the caspase-9–specific inhibitor Z-Leu-Glu(Ome)-His-Asp(Ome)-CH_2F (z-LEHD-fmk), the caspase-8–specific inhibitor Z-lle-Glu(Ome)-Thr-Asp(Ome)-CH_2F (z-IETD-fmk), and the caspase-2–specific inhibitor Z-Val-Ala-Asp(OMe)-Val-Ala-Asp(OMe)-CH_2F (z-VDVAD-fmk) were purchased from Calbiochem. The caspase-4–specific inhibitor Z-Leu-Glu-Val-Asp-FMK (z-LEVD-fmk) was from BioVision. The rabbit polyclonal antibodies against caspase-3, caspase-8, caspase-2, and caspase-9 were from Stressgen. The mouse monoclonal antibody (mAb) against caspase-4 was from Abcam. The mouse mAb against poly(ADP-ribose) polymerase (PARP) was from PharMingen. The rabbit mAb against GRP78 was purchased from Santa Cruz Biotechnology. Isotype control antibodies used were the ID4.5 (mouse IgG2a) mAb against *Salmonella typhi* supplied by Dr. L. Ashman (Institute for Medical and Veterinary Science, Adelaide, South Australia, Australia), the 107.3 mouse IgG1 mAb purchased from PharMingen, and rabbit IgG from Sigma Chemical.

Apoptosis. Quantitation of apoptotic cells by measurement of sub-G_1 DNA content using the propidium iodide method was carried out as described elsewhere (21, 25). 4′,6-Diamidino-2-phenylindole (DAPI) staining was done according to the manufacturer's instructions (Molecular Probes) as described elsewhere (25).

Western blot analysis. Western blot analysis was carried out as described previously (21, 25). Labeled bands were detected by Immun-Star HRP Chemiluminescent kit, and images were captured and the intensity of the bands was quantitated with the Bio-Rad VersaDoc image system.

Detection of XBP1 mRNA splicing. The method used for detection of unspliced and spliced XBP1 mRNAs was as described previously (27). Briefly, reverse transcription-PCR (RT-PCR) products of XBP1 mRNA were obtained from total RNA extracted using primers 5′-cggtgcgcggtgcg-tagtctgga-3′ (sense) and 5′-tgaggggctgagaggtgcttcct-3′ (antisense). Because a 26-bp fragment containing an *Apa*LI site is spliced on activation of XBP1 mRNA, the RT-PCR products were digested with *Apa*LI to distinguish the active spliced form from the inactive unspliced form. Subsequent electrophoresis revealed the inactive form as two cleaved fragments and the active form as a noncleaved fragment.

Caspase activity assay. Measurement of caspase activities by fluorometric assays was done as described previously (28). The specific substrates z-DEVD-AFC, Ac-LEVD-AFC, and z-LEHD-AFC were used to measure caspase-3, caspase-4, and caspase-9 activities, respectively (Calbiochem). The generation of free AFC was determined using FLUOstar OPTIMA (Labtech) set at an excitation wavelength of 400 nm and an emission wavelength of 505 nm.

Immunoprecipitation. Methods used were as described previously with minor modification (29). Briefly, 100 μL of lysates were precleared by incubation with 20 μL of a mixture of protein A-Sepharose and protein G-Sepharose packed beads (Santa Cruz Biotechnology) in a rotator at 4°C for 2 h and then with 20 μL of fresh packed beads in a rotator at 4°C overnight. Twenty micrograms of anti-GRP78 antibody or control immunoglobulin were then added to the lysate and rotated at 4°C for 2 h. The beads were then pelleted by centrifugation and washed five times with ice-cold lysate buffer before elution of the proteins from the beads in lysate buffer at room temperature for 1 h. The resulted immunoprecipitates were then subjected to SDS-PAGE and Western blot analysis.

Plasmid vector and transfection. Stable Mel-RM transfectants of *Bcl-2* were established by electroporation of the PEF-puro vector carrying human *Bcl-2* provided by Dr. David Vaux (Walter and Eliza Hall Institute, Melbourne, Victoria, Australia) and described elsewhere (21, 29).

Real-time PCR. Total RNA was isolated with SV total RNA isolation system (Promega). RT-PCR was carried out using Moloney murine leukemia virus transcriptase and oligo(dT), and the resulting cDNA products were used as templates for real-time PCR assays. Real-time RT-PCR was done using the ABI Prism 7700 sequence detection system (PE Applied Biosystems). Mixture (25 μL) was used for reaction, which contains 5 μL cDNA sample (0.5–1 μg/μL) and 9 mmol/L $MgCl_2$. Primers and probes for GRP78 were from the real-time PCR assay kit, Assay-on-Demand for GRP78 (assay ID: Hs99999174-m1), and were added according to the manufacturer's protocol (Applied Biosystems). Analysis of cDNA for β-actin was included as a control. After incubation at 50°C for 2 min followed by 95°C for 10 min, the reaction was carried out for 40 cycles of the following: 95°C for 15 s and 60°C for 1 min. The threshold cycle value (*C*t) was normalized against β-actin cycle numbers. The relative abundance of mRNA expression of a control sample was arbitrarily designated as 1, and the values of the relative abundance of mRNA of other samples were calculated accordingly.

Small interfering RNA. Melanoma cells were seeded at 4×10^4 per well in 24-well plates and allowed to reach ~50% confluence on the day of transfection. The small interfering RNA (siRNA) constructs used were obtained as the siGENOME SMARTpool reagents (Dharmacon), the siGENOME SMARTpool MEK1 (M-003571-01-0010), the siGENOME SMARTpool GRP78 (M-008198-01), the siGENOME SMARTpool caspase-4 (M-004404-00), and the siGENOME SMARTpool caspase-9 (M-003309-00). The nontargeting siRNA control, SiConTRolNon-targeting siRNA pool (D-001206-13-20), was also obtained from Dharmacon. Cells were transfected with 50 to 100 nmol/L siRNA in Opti-MEM medium (Invitrogen) with 5% FCS using LipofectAMINE reagent (Invitrogen) according to the manufacturer's transfection protocol. Twenty-four hours after transfection, the cells were switched into medium containing 5% FCS and treated as designed before quantitation of apoptotic cells by measurement of sub-G_1 DNA content using the propidium iodide method in flow cytometry. Efficiency of siRNA was measured by Western blot analysis.

Results

Melanoma cells are relatively resistant to ER stress-induced apoptosis. To study the apoptosis-inducing potential of ER stress in melanoma, we treated Mel-RM and MM200 cells with tunicamycin and thapsigargin, respectively, at a range of concentrations for 48 h. Figure 1*A* shows that tunicamycin and thapsigargin markedly up-regulated GRP78 and induced splicing

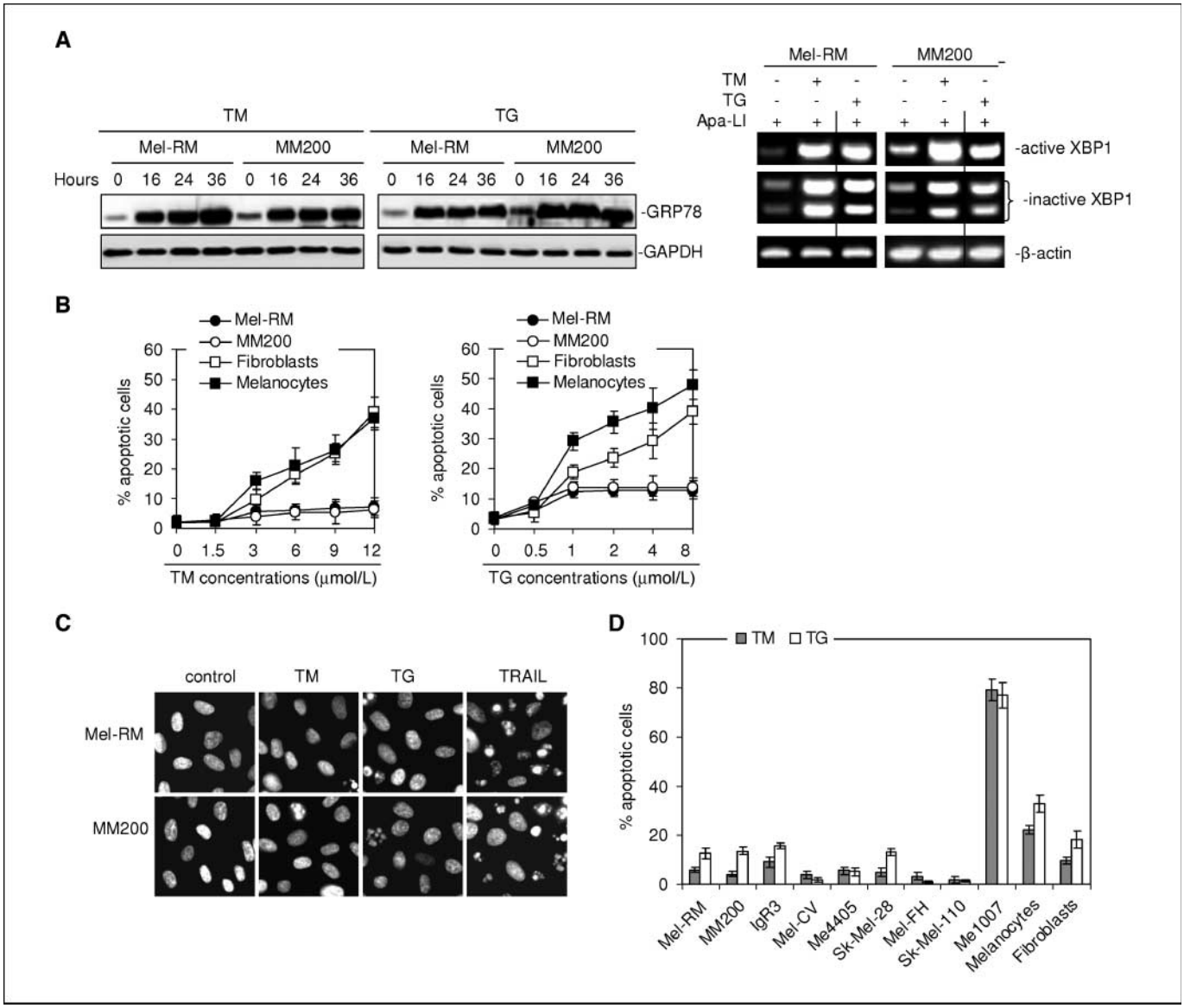


Figure 1. The majority of melanoma cell lines are relatively resistant to ER stress-induced apoptosis. *A,* tunicamycin (*TM*) and thapsigargin (*TG*) induce activation of the UPR in melanoma cells. *Left,* whole-cell lysates from Mel-RM and MM200 cells with or without treatment with tunicamycin (3 μmol/L) or thapsigargin (1 μmol/L) for indicated periods were subjected to Western blot analysis of GRP78 expression; *right,* RT-PCR products of XBP1 mRNA from Mel-RM and MM200 cells with or without treatment with tunicamycin (3 μmol/L) or thapsigargin (1 μmol/L) for 16 h were digested with *Apa*LI for 90 min followed by electrophoresis. The longer fragment derived from the active form of XBP1 mRNA and two shorter bands derived from the inactive form are indicated. Data are representative of three individual experiments. *GAPDH,* glyceraldehyde-3-phosphate dehydrogenase. *B,* Mel-RM and MM200 cells are relatively resistant to ER stress-induced apoptosis. Mel-RM and MM200 cells, melanocytes, and fibroblasts with or without treatment with tunicamycin (*left*) or thapsigargin (*right*) at indicated concentrations for 48 h were subjected to measurement of apoptosis by the propidium iodide method using flow cytometry. *Points,* mean of three individual experiments; *bars,* SE. *C,* representative microphotographs of DAPI staining. Mel-RM and MM200 cells were treated with tunicamycin (3 μmol/L), thapsigargin (1 μmol/L), or TRAIL (200 ng/mL) for 48 h followed by visualization of DNA staining with DAPI using a fluorescence microscope. Data are representative of three individual experiments. *D,* a summary of studies on induction of apoptosis by ER stress in a panel of melanoma cell lines and normal cells (melanocytes and fibroblasts). The cells treated with tunicamycin (3 μmol/L) or thapsigargin (1 μmol/L) for 48 h were subjected to measurement of apoptosis by the propidium iodide method using flow cytometry. *Columns,* mean of three individual experiments; *bars,* SE.

of XBP1 mRNA, indicative of activation of the UPR. However, both of them induced only minimal to moderate levels of apoptosis (<20% apoptotic cells), even when used at relatively high doses (tunicamycin at 12 μmol/L and thapsigargin at 8 μmol/L), which efficiently killed fibroblasts and melanocytes by induction of apoptosis (Fig. 1*B*). Relative resistance of Mel-RM and MM200 cells to ER stress-induced apoptosis was confirmed in assays with DAPI staining. As shown in Fig. 1*C*, the majority of the cells displayed normal nuclear morphology after exposure to tunicamycin or thapsigargin, whereas DNA fragmentation and/or chromatin condensation was frequently observed in cells treated with tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) that is known to induce apoptosis in Mel-RM and MM200 cells and was used as a control. Studies on a panel of melanoma cell lines revealed that neither tunicamycin nor thapsigargin induced significant apoptosis in all but Me1007 (Fig. 1*D*).

Inhibition of MEK/ERK sensitizes melanoma cells to ER stress-induced apoptosis. We studied if ER stress induces

activation of the MEK/ERK pathway by examining phosphorylation (activation) of ERK1/2 in whole-cell lysates from Mel-RM and MM200 cells with or without exposure to tunicamycin or thapsigargin for varying periods. Whole-cell lysates from untreated melanocytes that are known not to express phosphorylated ERK1/2 were included as negative controls (22). As shown in Fig. 2*A*, ERK1/2 was constitutively activated in both cell lines, but the levels of activation were not increased by treatment with either tunicamycin or thapsigargin. This indicates that ER stress does not induce further MEK/ERK activation in melanoma cells.

We next studied the effect of inhibition of MEK on apoptosis-inducing potential of ER stress in melanoma cells by treating Mel-RM and MM200 cells with the MEK inhibitor U0126 1 h before the addition of tunicamycin or thapsigargin for another 24 h. Figure 2*B* shows that, although U0126 inhibited activation of ERK1/2 in the presence or absence of tunicamycin or thapsigargin, it significantly sensitized the cells to tunicamycin- or thapsigargin-induced apoptosis ($P < 0.05$, two-tailed Student's t test). The MEK inhibitor alone did not cause appreciable apoptotic cell death in both cell lines.

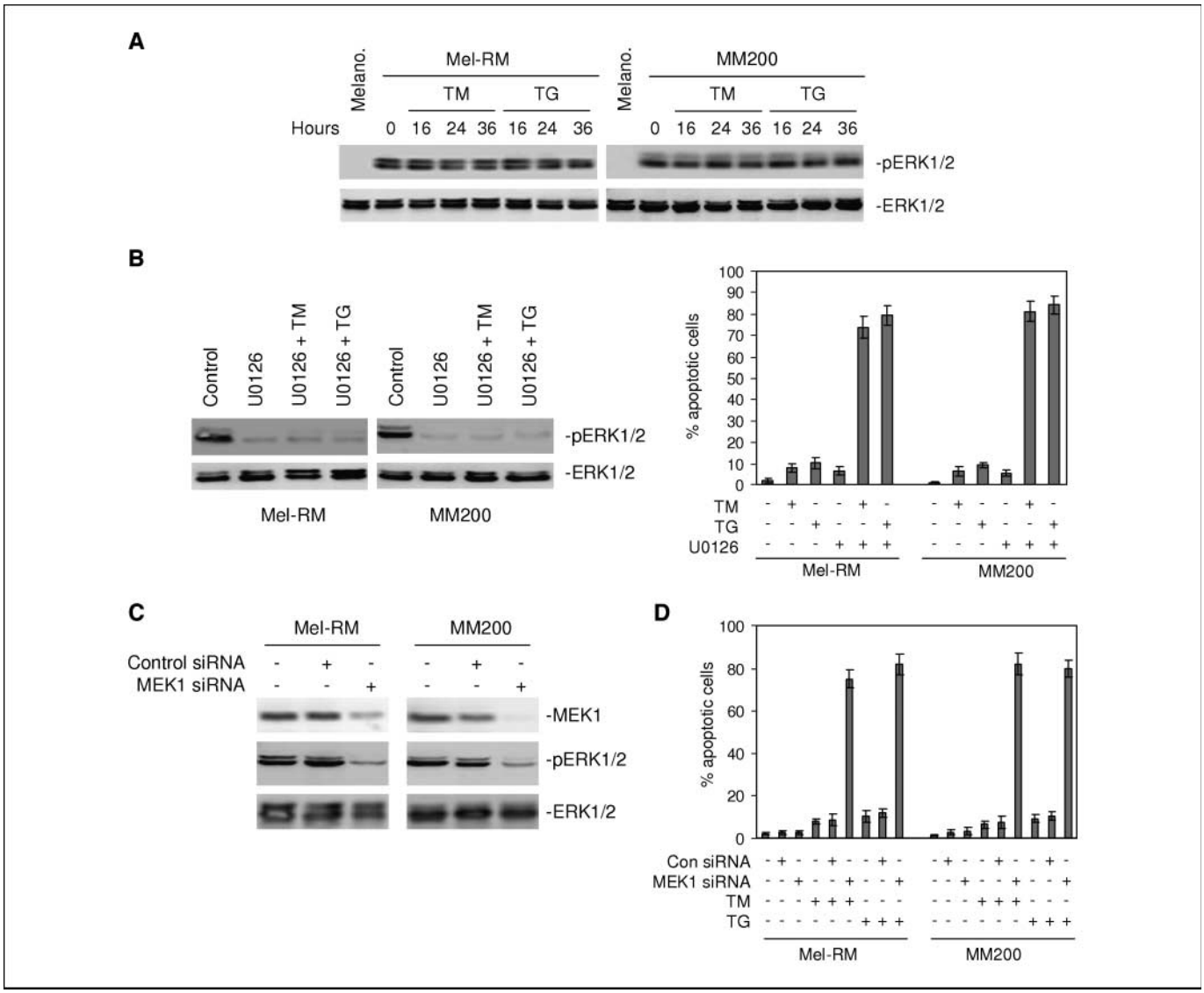


Figure 2. Inhibition of MEK sensitizes melanoma cells to ER stress-induced apoptosis. *A,* tunicamycin and thapsigargin do not induce further activation of ERK1/2 in melanoma cells. Whole-cell lysates from Mel-RM and MM200 cells with or without treatment with tunicamycin (3 μmol/L) or thapsigargin (1 μmol/L) for indicated periods were subjected to Western blot analysis. Whole-cell lysates from untreated melanocytes (*Melano.*) were included as negative controls for phosphorylated ERK1/2 (*pERK1/2*). Data are representative of three individual experiments. *B,* U0126 sensitizes melanoma cells to ER stress-induced apoptosis. *Left,* whole-cell lysates from Mel-RM and MM200 cells treated with U0126 (20 μmol/L) for 1 h with or without the addition of tunicamycin (3 μmol/L) or thapsigargin (1 μmol/L) for another 24 h were subjected to Western blot analysis. Data are representative of three individual experiments. *Right,* Mel-RM and MM200 cells treated with U0126 (20 μmol/L), tunicamycin (3 μmol/L), or thapsigargin (1 μmol/L) for 48 h or U0126 (20 μmol/L) for 1 h before the addition of tunicamycin (3 μmol/L) or thapsigargin (1 μmol/L) for another 48 h were subjected to measurement of apoptosis by the propidium iodide method using flow cytometry. *Columns,* mean of three individual experiments; *bars,* SE. *C,* siRNA knockdown of MEK1 inhibits activation/phosphorylation of ERK. Mel-RM and MM200 cells were transfected with the control or MEK1 siRNA. Twenty-four hours later, whole-cell lysates were subjected to Western blot analysis of MEK1, phosphorylated ERK1/2, and ERK1/2 expression. Data are representative of three individual experiments. *D,* MEK1 siRNA sensitizes melanoma cells to ER stress-induced apoptosis. Mel-RM and MM200 cells were transfected with the control or MEK1 siRNA. Twenty-four hours later, the cells were treated with tunicamycin (3 μmol/L) or thapsigargin (1 μmol/L) for 48 h. Apoptosis was measured by the propidium iodide method using flow cytometry. *Columns,* mean of three individual experiments; *bars,* SE.

To confirm that U0126-mediated sensitization of melanoma cells to ER stress-induced apoptosis was due to inhibition of MEK/ERK signaling, we silenced MEK1 expression in Mel-RM and MM200 cells by transfecting the cells with a MEK1 siRNA pool. As shown in Fig. 2*C*, siRNA knockdown of MEK1 inhibited its expression by 85% and 90% in Mel-RM and MM200 cells, respectively. Consequently, the levels of ERK1/2 activation were substantially blocked in both cell lines. Figure 2*D* shows that inhibition of MEK1 by siRNA resulted in a marked increase in apoptotic cell death induced by tunicamycin or thapsigargin in comparison with cells transfected with the control siRNA ($P < 0.05$, two-tailed Student's t test).

Sensitization of melanoma cells to ER stress-induced apoptosis is caspase dependent. We examined if sensitization of melanoma cells to ER stress-induced apoptosis by inhibition of MEK/ERK depends on the caspase cascade by treating Mel-RM and MM200 cells with the general caspase inhibitor z-VAD-fmk 1 h before the addition of U0126 followed by tunicamycin or thapsigargin. Figure 3*A* shows that z-VAD-fmk markedly inhibited apoptosis induced by tunicamycin or thapsigargin in the presence of U0126 in both cell lines ($P < 0.05$, two-tailed Student's t test), suggesting that the caspase cascade plays a determining role in sensitization of melanoma cells to ER stress-induced apoptosis.

To further elucidate the caspases involved, we studied the effects of specific inhibitors against caspase-2, z-VDVAD-fmk, caspase-3, z-DEVD-fmk, caspase-4, z-LEVD-fmk, caspase-8, z-IETD-fmk, and caspase-9, z-LEHD-fmk, on U0126-mediated sensitization of Mel-RM and MM200 cells to tunicamycin- and thapsigargin-induced apoptosis, respectively. As shown in Fig. 3*A*, the inhibitor against caspase-4, caspase-9, or caspase-3 inhibited apoptosis induced by tunicamycin or thapsigargin in the presence of U0126 to varying degrees. In contrast, the inhibitor against caspase-2 or caspase-8 exhibited only minimal to moderate inhibitory effects on tunicamycin- or thapsigargin-induced apoptosis (Fig. 3*A*) but blocked apoptosis induced by TRAIL to varying degrees (Supplementary Fig. S1). The specificity of the caspase-4 inhibitor z-LEVD-fmk on ER stress-induced apoptosis was evidenced by failure of the caspase-4 inhibitor to block staurosporine-induced apoptosis that is independent of caspase-4 (Supplementary Fig. S2; ref. 7). These observations suggest that caspase-4, caspase-9, and caspase-3 are involved in sensitization of melanoma cells to ER stress-induced apoptosis by U0126.

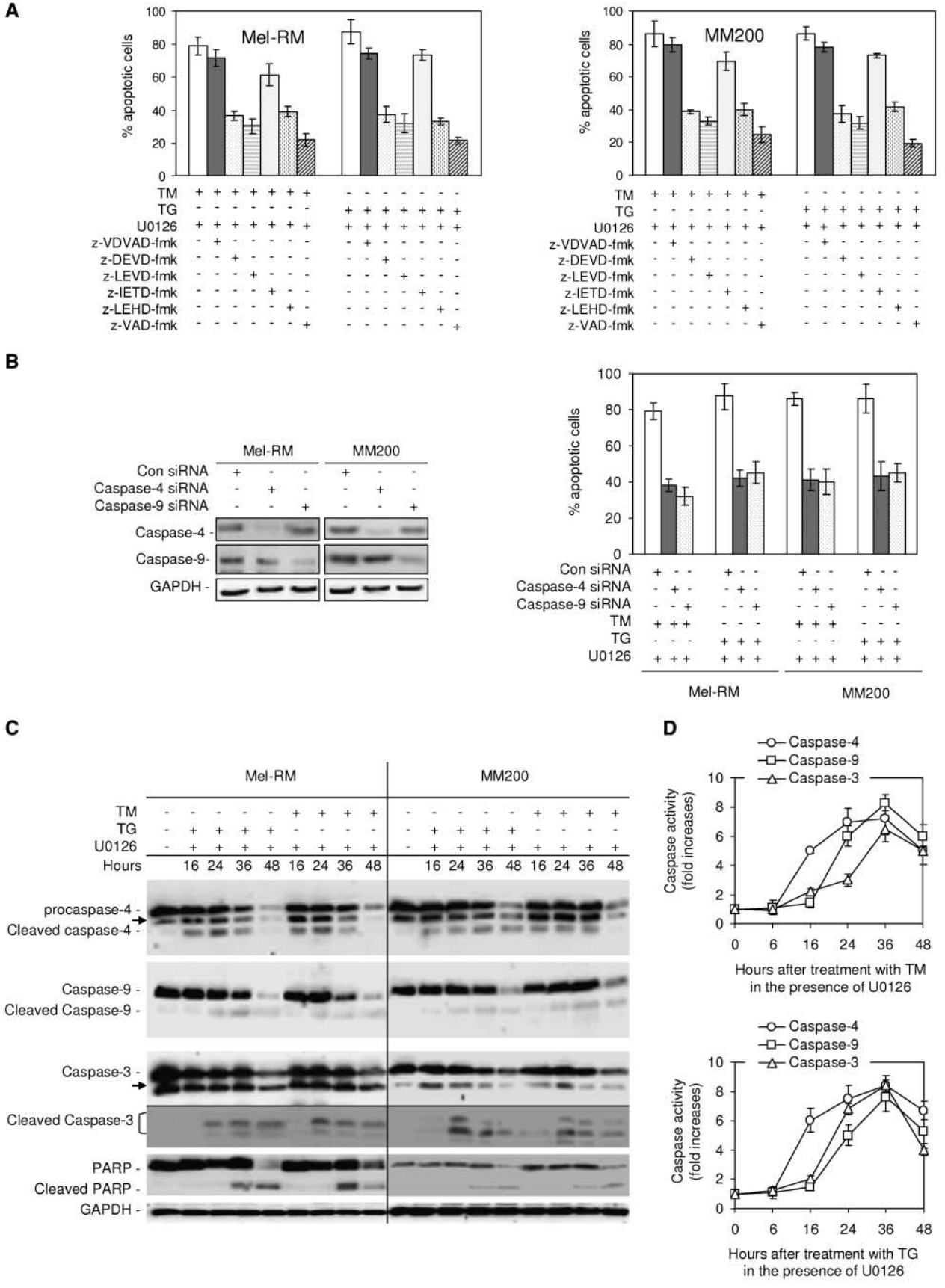
To confirm the role of caspase-4 and caspase-9 in sensitization of melanoma cells to ER stress-induced apoptosis by U0126, we silenced caspase-4 and caspase-9 by specific siRNA pools in Mel-RM and MM200 cells, respectively. Figure 3*B* shows that, whereas the caspase-4 siRNA significantly reduced the levels of caspase-4 but not caspase-9 expression, the caspase-9 siRNA similarly decreased the levels of caspase-9 but not caspase-4 expression. The levels of apoptosis induced by tunicamycin or thapsigargin in the presence of U0126 were reduced by >50% in cells transfected with either the caspase-4 or caspase-9 siRNA in comparison with those transfected with the control siRNA (Fig. 3*B*).

Consistent with results in assays with the caspase inhibitors, Western blot analysis showed that caspase-4, caspase-9, and caspase-3 were activated, and the caspase-3 substrate PARP was cleaved by tunicamycin or thapsigargin in the presence of U0126 (Fig. 3*C*). There was no activation of caspase-8 and caspase-2 after the same treatment (data not shown). Activation of caspase-4, caspase-9, and caspase-3 was also confirmed in fluorometric assays detecting activities of the caspases by specific substrates in whole-cell lysates as shown for Mel-RM (Fig. 3*D*). Similar results were also obtained for MM200 (data not shown). Whereas caspase-4 activation could be detected as early as 16 h, caspase-9 or caspase-3 activities could only be observed at or after 24 h after the addition of tunicamycin or thapsigargin in both Western blot analysis and caspase activity assays. Notably, activities of caspase-4 or caspase-9 measured in fluorometric assays remained relatively high even at 48 h after treatment when the protein levels of these caspases decreased markedly as shown in Western blot analysis (Fig. 3*C* and *D*). This is most likely due to that the caspases were further processed to even smaller forms, which could not be detected by the antibodies used in Western blot analysis, but still possessed activities to process their substrates. Tunicamycin, thapsigargin, or U0126 alone did not induce notable activation of caspase-4, caspase-9, or caspase-3 (data not shown).

Overexpression of Bcl-2 inhibits apoptosis, but not activation of caspase-4, induced by tunicamycin or thapsigargin in the presence of U0126. We examined the effect of Bcl-2 on sensitization of melanoma cells to ER stress-induced apoptosis by inhibition of MEK/ERK in Mel-RM and MM200 cells transfected with cDNA encoding Bcl-2. As shown in Fig. 4*A*, overexpression of Bcl-2 markedly blocked tunicamycin- and thapsigargin-induced apoptosis in the presence of U0126, with the percentages of inhibition being >80% for tunicamycin and thapsigargin in both Mel-RM and MM200 cells, respectively. Figure 4*B* shows that overexpression of Bcl-2 inhibited activation of caspase-9 and caspase-3 but not activation of caspase-4. This suggests that activation of caspase-4 occurred upstream of caspase-9 and caspase-3 and is not inhibitable by Bcl-2.

To confirm that caspase-4 is an apical caspase in induction of apoptosis by tunicamycin or thapsigargin in the presence of U0126,

Figure 3. Sensitization of melanoma cells to ER stress-induced apoptosis is caspase dependent. *A,* Mel-RM and MM200 cells were treated with the general caspase inhibitor z-VAD-fmk (20 μmol/L), the caspase-2 inhibitor z-VDVAD-fmk (50 μmol/L), the caspase-3 inhibitor z-DEVD-fmk (20 μmol/L), the caspase-4 inhibitor z-LEVD-fmk (30 μmol/L), the caspase-8 inhibitor z-IETD-fmk (20 μmol/L), or the caspase-9 inhibitor z-LEHD-fmk (20 μmol/L) for 1 h before adding U0126 (20 μmol/L) for another 1 h followed by tunicamycin (3 μmol/L) or thapsigargin (1 μmol/L) for a further 48 h. Apoptosis was measured by the propidium iodide method using flow cytometry. *Columns,* mean of three individual experiments; *bars,* SE. *B,* siRNA knockdown of caspase-4 or caspase-9 inhibits sensitization of melanoma cells to ER stress-induced apoptosis by U0126. *Left,* Mel-RM and MM200 cells were transfected with the control siRNA, caspase-4 siRNA, or caspase-9 siRNA. Twenty-four hours later, whole-cell lysates were subjected to Western blot analysis. Data are representative of three individual experiments. *Right,* 24 h after transfection with the control siRNA, caspase-4 siRNA, or caspase-9 siRNA, Mel-RM and MM200 cells were treated with U0126 (20 μmol/L) for 1 h before the addition of tunicamycin (3 μmol/L) or thapsigargin (1 μmol/L) for a further 48 h. Apoptosis was measured by the propidium iodide method using flow cytometry. *Columns,* mean of three individual experiments; *bars,* SE. *C,* tunicamycin and thapsigargin induce activation of caspase-4, caspase-9, and caspase-3 in the presence of U0126. Mel-RM and MM200 cells were treated with U0126 (20 μmol/L) for 1 h before the addition of tunicamycin (3 μmol/L) or thapsigargin (1 μmol/L) for indicated periods. Whole-cell lysates were subjected to Western blot analysis of caspase-3, caspase-4, caspase-9, and PARP. The lower part of the caspase-3 graph was obtained from the same membrane with longer exposure to better visualize the cleaved forms of caspase-3. The arrow-pointed bands in figures for caspase-4 and caspase-3 are presumably either nonspecific bands or intermediately cleaved caspase-4 and caspase-3, respectively. Data are representative of three individual experiments. *D,* Mel-RM cells were treated with U0126 (20 μmol/L) for 1 h before the addition of tunicamycin (3 μmol/L; *top*) or thapsigargin (1 μmol/L; *bottom*) for indicated periods. Whole-cell lysates were harvested and caspase activities were measured with specific substrates for caspase-3, caspase-4, and caspase-9, respectively, in fluorometric assays. The values of activity in the cells without treatment were arbitrarily designated as 1. The values of activity in cells treated with tunicamycin or thapsigargin in the presence of U0126 were compared with those in cells without treatment and are expressed as the fold increase. *Points,* mean of three individual experiments; *bars,* SE.



we monitored activation of caspase-9 and caspase-3 in whole-cell lysates from Mel-RM and MM200 cells treated with the caspase-4 inhibitor z-LEVD-fmk before the addition of U0126 and tunicamycin or thapsigargin. Figure 4*C* shows that the caspase-4 inhibitor blocked activation of caspase-9 and caspase-3 induced by tunicamycin or thapsigargin in the presence of U0126. In contrast, the caspase-9 inhibitor z-LEHD-fmk or the caspase-3 inhibitor z-DEVD-fmk had only a minimal effect on induction of the caspase-4 activity by the same treatment (data not shown). Thus, activation of caspase-4 seemed to be at least one of the initiating factors in sensitization of melanoma cells to ER stress-induced apoptosis by inhibition of MEK.

Sensitization of melanoma cells to ER stress-induced apoptosis by inhibition of MEK/ERK is associated with blockage of induction of GRP78. GRP78 is believed to play an essential role in protection of cells from ER stress-induced apoptosis (14, 19). We therefore studied if sensitization of melanoma cells to ER stress-induced apoptosis by inhibition of MEK/ERK is related to regulation of GRP78. Figure 5*A* and *B* shows that exposure to U0126 resulted in reduction in the levels of GRP78 expression and attenuated its induction by tunicamycin or thapsigargin in both Mel-RM and MM200 cells. Similarly, inhibition of MEK/ERK by siRNA knockdown of MEK1 decreased GRP78 expression in both cell lines (Fig. 5*C*).

To examine if inhibition of MEK blocks transcription of GRP78 induced by ER stress, we monitored mRNA levels of GRP78 by real-time PCR in Mel-RM and MM200 cells treated with U0126 1 h before the addition of tunicamycin or thapsigargin. As shown in Fig. 5*D*, GRP78 mRNA levels in cells treated with tunicamycin or thapsigargin in the presence of U0126 were markedly lower than those in cells treated with tunicamycin or thapsigargin alone. Taken together, these results indicate that activation of the MEK/ERK signaling pathway may play a role in regulation of GRP78 expression.

GRP78 is associated with caspase-4 and prevents its activation in melanoma cells. We also studied if there is a physical association between caspase-4 and GRP78 in human melanoma cells. As shown in Fig. 6*A*, the endogenous caspase-4 could be coimmunoprecipitated with endogenous GRP78. The amount of caspase-4 that was coprecipitated with GRP78 increased after exposure of the cells to tunicamycin or thapsigargin. This was associated with increased amount of GRP78 in the precipitates, presumably due to increased cellular contents of the GRP78 protein induced by tunicamycin or thapsigargin (Fig. 1*A*). This result indicates that GRP78 is physically associated with caspase-4 in human melanoma cells.

To confirm the role of GRP78 in protection of melanoma cells from ER-stress induced apoptosis, we transfected a GRP78-specific siRNA pool into Mel-RM and MM200 cells. Figure 6*B* shows that the GRP78 siRNA markedly inhibited GRP78 induction in both cell lines. Figure 6*C* shows that inhibition of GRP78 by siRNA resulted in substantial increases in sensitivity of melanoma cells to apoptosis induced by tunicamycin or thapsigargin in the absence of U0126. Consistently, tunicamycin- or thapsigargin-induced activities of caspase-4 were increased when GRP78 was inhibited by siRNA (Fig. 6*D*). It is of note that U0126 alone induced apoptosis but not caspase-4 activation in Mel-RM and MM200 cells, with GRP78 being knocked down by siRNA (Fig. 6*C* and *D*).

Discussion

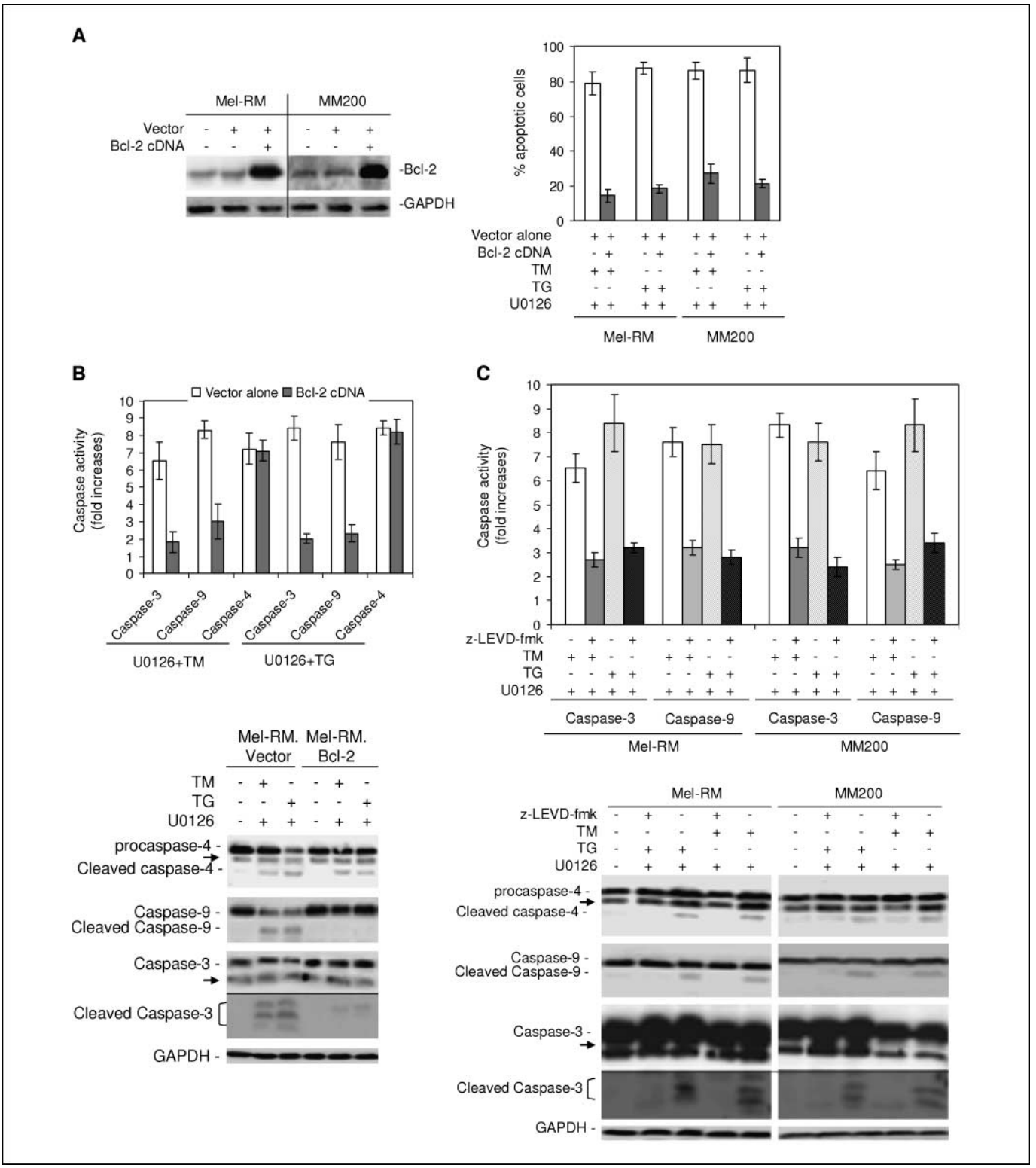
The results above seem to provide several new insights into the apoptosis resistance mechanisms that inhibition of MEK/ERK may target in melanoma. They show that cultured melanoma cell lines, with few exceptions, do not undergo significant apoptosis when submitted to extreme degrees of ER stress induced by tunicamycin or thapsigargin. However, ER stress-induced apoptosis is readily triggered when the MEK/ERK pathway is inhibited by either a MEK inhibitor or MEK1 siRNA. This is mediated, at least in part, by activation of caspase-4 that leads to subsequent apoptosis through the mitochondrial apoptotic pathway. Furthermore, the ER chaperon GRP78 seems to be a target of the MEK/ERK pathway responsible for the inhibition of ER stress-induced apoptosis.

The MEK/ERK signaling pathway is constitutively activated in virtually all melanomas (23, 24, 30, 31), which is a common cause for resistance of melanoma cells to induction of apoptosis (21–24). Although the UPR induced by tunicamycin or thapsigargin did not result in further activation of ERK in melanoma cells, in contrast to previous reports in other cellular systems (14, 20), the constitutively activated MEK/ERK pathway seemed to play an important role in protection of melanoma cells from ER stress-induced apoptosis,

Figure 4. Overexpression of Bcl-2 inhibits sensitization of melanoma cells to ER stress-induced apoptosis by U0126 but not caspase-4 activation. *A,* overexpression of Bcl-2 inhibits sensitization of melanoma cells to tunicamycin- or thapsigargin-induced apoptosis by U0126. *Left,* Bcl-2 was overexpressed in Mel-RM and MM200 cells transfected with cDNA encoding Bcl-2 but not in the cells transfected with the vector alone. Whole-cell lysates were subjected to Western blot analysis. *Right,* Mel-RM and MM200 cells transfected with cDNA for Bcl-2 or vector alone were treated with U0126 (20 μmol/L) for 1 h before the addition of tunicamycin (3 μmol/L) or thapsigargin (1 μmol/L) for another 48 h. Apoptosis was measured by the propidium iodide method using flow cytometry. *Columns,* mean of three individual experiments; *bars,* SE. *B,* overexpression of Bcl-2 inhibited activation of caspase-9 and caspase-3 but not caspase-4 induced by tunicamycin or thapsigargin in the presence of U0126. *Top,* Mel-RM cells transfected with cDNA for Bcl-2 or vector alone were treated with U0126 (20 μmol/L) for 1 h before the addition of tunicamycin (3 μmol/L) or thapsigargin (1 μmol/L) for another 36 h. Whole-cell lysates were harvested and caspase activities were measured with specific substrates for caspase-3, caspase-4, and caspase-9, respectively, in fluorometric assays. The values in the cells without treatment were arbitrarily designated as 1 (data not shown). The values of activity in cells treated with tunicamycin or thapsigargin in the presence of U0126 were compared with those in cells without treatment and are expressed as the fold increase. *Columns,* mean of three individual experiments; *bars,* SE. *Bottom,* Mel-RM cells transfected with vector alone or cDNA for Bcl-2 were treated with U0126 (20 μmol/L) for 1 h before the addition of tunicamycin (3 μmol/L) or thapsigargin (1 μmol/L) for 36 h. Whole-cell lysates were harvested and subjected to Western blot analysis. The lower part of the caspase-3 graph was obtained from the same membrane with longer exposure to better visualize if caspase-3 is cleaved. The arrow-pointed bands in figures for caspase-4 and caspase-3 are presumably either nonspecific bands or intermediately cleaved caspase-4 and caspase-3, respectively. Data are representative of three individual experiments. *C,* the caspase-4 inhibitor z-LEVD-fmk inhibited activation of caspase-9 and caspase-3 induced by tunicamycin or thapsigargin in the presence of U0126. *Top,* Mel-RM and MM200 cells were treated with z-LEVD-fmk (30 μmol/L) for 1 h before adding U0126 (20 μmol/L) for another 1 h followed by tunicamycin (3 μmol/L) or thapsigargin (1 μmol/L) for a further 36 h. Whole-cell lysates were harvested and caspase activities were measured with specific substrates for caspase-9 and caspase-3, respectively, in fluorometric assays. The values in the cells without treatment were arbitrarily designated as 1 (data not shown). The values of activity in cells treated with tunicamycin or thapsigargin in the presence of U0126 were compared with those in cells without treatment and are expressed as the fold increase. *Columns,* mean of three individual experiments; *bars,* SE. *Bottom,* Mel-RM and MM200 cells were treated with z-LEVD-fmk (30 μmol/L) for 1 h before adding U0126 (20 μmol/L) for another 1 h followed by tunicamycin (3 μmol/L) or thapsigargin (1 μmol/L) for a further 36 h. Whole-cell lysates were harvested and subjected to Western blot analysis. The lower part of the caspase-3 graph was obtained from the same membrane with longer exposure to better visualize the cleaved forms of caspase-3. The arrow-pointed bands in figures for caspase-4 and caspase-3 are presumably either nonspecific bands or intermediately cleaved caspase-4 and caspase-3, respectively. Data are representative of three individual experiments.

as inhibition of MEK by either the chemical inhibitor U0126 or MEK1 siRNA markedly sensitized melanoma cells to tunicamycin- or thapsigargin-induced apoptosis. This result is of particular interest in that it raises the possibility that constitutive activation of the MEK/ERK pathway in melanoma may lay a basis for adaptation of the cells to ER stress conditions.

Various mechanisms have been implicated in the induction of apoptosis by ER stress, such as CHOP-mediated up-regulation of TRAIL-R2 and/or down-regulation of Bcl-2 (5, 10), up-regulation of the BH3-only protein PUMA (13, 32), activation of the JNK (6, 33), and activation of caspase-12 in murine systems (6, 8) and its counterpart caspase-4 in human cells (7). In the present study,



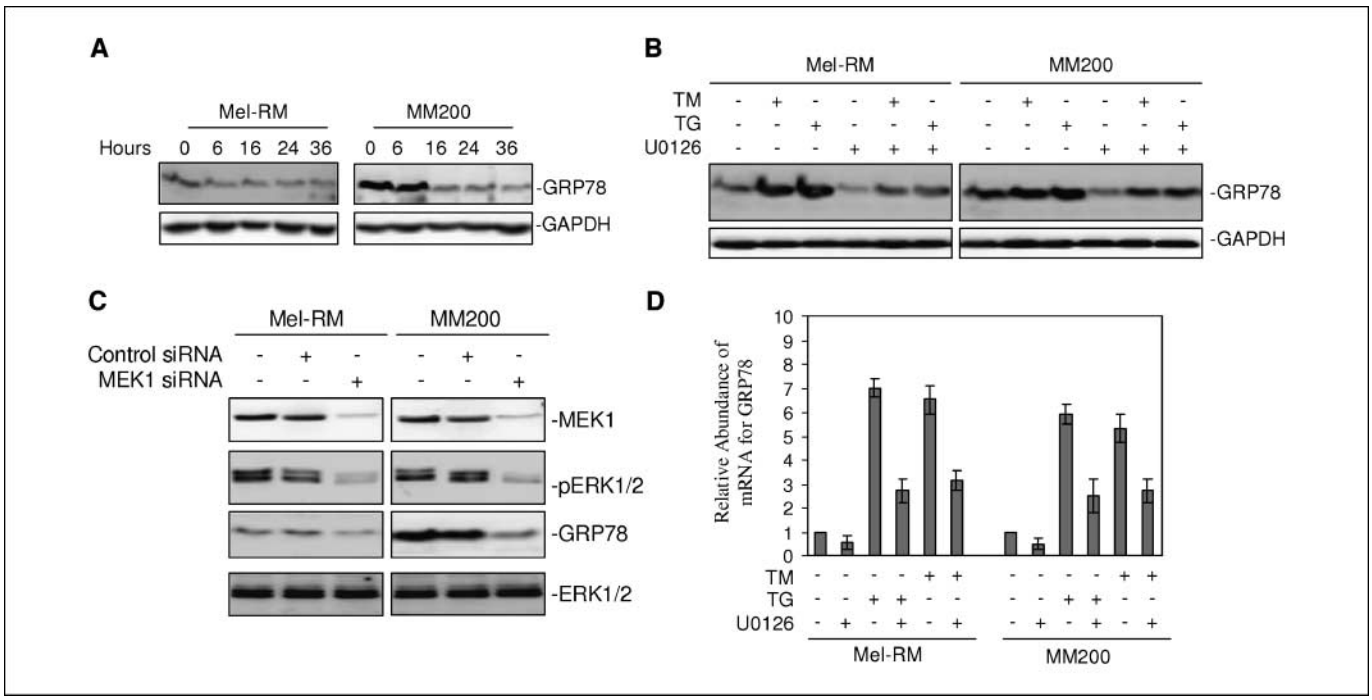


Figure 5. Inhibition of MEK down-regulates GRP78 expression and partially blocks its up-regulation by tunicamycin or thapsigargin. *A,* Mel-RM and MM200 cells were treated with U0126 (20 μmol/L) for indicated periods. Whole-cell lysates were subjected to Western blot analysis of GRP78 expression. Data are representative of three individual experiments. *B,* U0126 blocks up-regulation of GRP78 by tunicamycin or thapsigargin. Mel-RM and MM200 cells were treated with U0126 (20 μmol/L) for 1 h before the addition of tunicamycin (3 μmol/L) or thapsigargin (1 μmol/L) for a further 24 h. Whole-cell lysates were subjected to Western blot analysis of GRP78 expression. Data are representative of three individual experiments. *C,* GRP78 expression is decreased in Mel-RM and MM200 cells with MEK1 being knocked down by siRNA. Mel-RM and MM200 cells were transfected with the control or MEK1 siRNA. Twenty-four hours later, whole-cell lysates were subjected to Western blot analysis of MEK1, phosphorylated ERK1/2, GRP78, and ERK1/2 expression. Data are representative of three individual experiments. *D,* treatment with U0126 reduces the levels of GRP78 mRNA expression. Mel-RM and MM200 cells were treated with U0126 (20 μmol/L), tunicamycin (3 μmol/L), or thapsigargin (1 μmol/L) for 24 h or U0126 for 1 h before the addition of tunicamycin or thapsigargin for another 24 h. Total RNA was isolated and subjected to real-time PCR analysis for GRP78 mRNA expression. The relative abundance of mRNA expression before treatment was arbitrarily designated as 1. *Columns,* mean of three individual experiments; *bars,* SE.

ER stress-induced apoptosis triggered by inhibition of MEK was caspase dependent and involved caspase-4, caspase-9, and caspase-3, as shown by specific inhibitors and siRNA knockdown of caspase-4 and caspase-9. Caspase-4 activation occurred before activation of caspase-9 and caspase-3, suggesting that caspase-4 may be the upstream caspase in the caspase cascade. This was supported by the finding that overexpression of Bcl-2 blocked activation of caspase-9 and caspase-3, and apoptosis, but had minimal effect on caspase-4 activation. Moreover, the caspase-4 inhibitor z-LEVD-fmk blocked activation of caspase-9 and caspase-3, but neither the caspase-9 inhibitor, z-LEHD-fmk, nor the caspase-3 inhibitor, z-DEVD-fmk, had significant effect on caspase-4 activation induced by tunicamycin or thapsigargin in the presence of U0126. Taken together, these observations placed caspase-4 as an apical caspase in sensitization of melanoma cells to ER stress-induced apoptosis by inhibition of MEK/ERK.

How caspase-4 is activated by tunicamycin or thapsigargin when the MEK/ERK pathway is inhibited in melanoma cells is not clear. In the murine system, several mechanisms have been suggested to be responsible for ER stress-induced caspase-12 activation (6, 8, 34). For instance, the protease calpain, on activation by calcium released from ER, can activate caspase-12 (8). In addition, caspase-12 has also been reported to be activated by a direct association with the ER stress transducer IRE1α and the adaptor protein TRAF2 (6, 34). Our investigation of the mechanism involved in the MEK/ERK-mediated inhibition of ER stress-induced apoptosis led to a focus on GRP78, which was found to be physically associated with caspase-4, as reported for caspase-12 in the murine system (35–37). GRP78 was decreased and its up-regulation by ER stress was blocked, albeit partially, by inhibition of MEK in melanoma cells. Moreover, inhibition of GRP78 by siRNA enhanced activation of caspase-4 induced by tunicamycin or thapsigargin. These results indicate that GRP78 may participate in controlling the activation of caspase-4 in human melanoma cells. The physical association between GRP78 and caspase-4 also suggests that caspase-4 may be present in the ER. This was supported by the punctate staining pattern of caspase-4, indicative of organelle localization, observed in immunofluorescence microscopy studies (Supplementary Fig. S3). Caspase-4 is localized to the ER membrane and mitochondria in human neuroblastoma cells (7).

Our finding that U0126 alone did not induce caspase-4 activation in melanoma cells when GRP78 was knocked down suggests that release of caspase-4 itself may not be sufficient to cause its activation. It seems that down-regulation of GRP78 plays a role in facilitating activation of caspase-4. Other factors, such as activation of proteases by ER stress, may be directly responsible for driving caspase-4 activation when GRP78 is down-regulated by inhibition of MEK/ERK (6, 34, 38). It remains possible that GRP78 also protects melanoma cells from ER stress-induced apoptosis by other mechanisms, such as binding to the unfolded proteins and/or calcium, thus alleviating ER stress conditions (6, 14, 39).

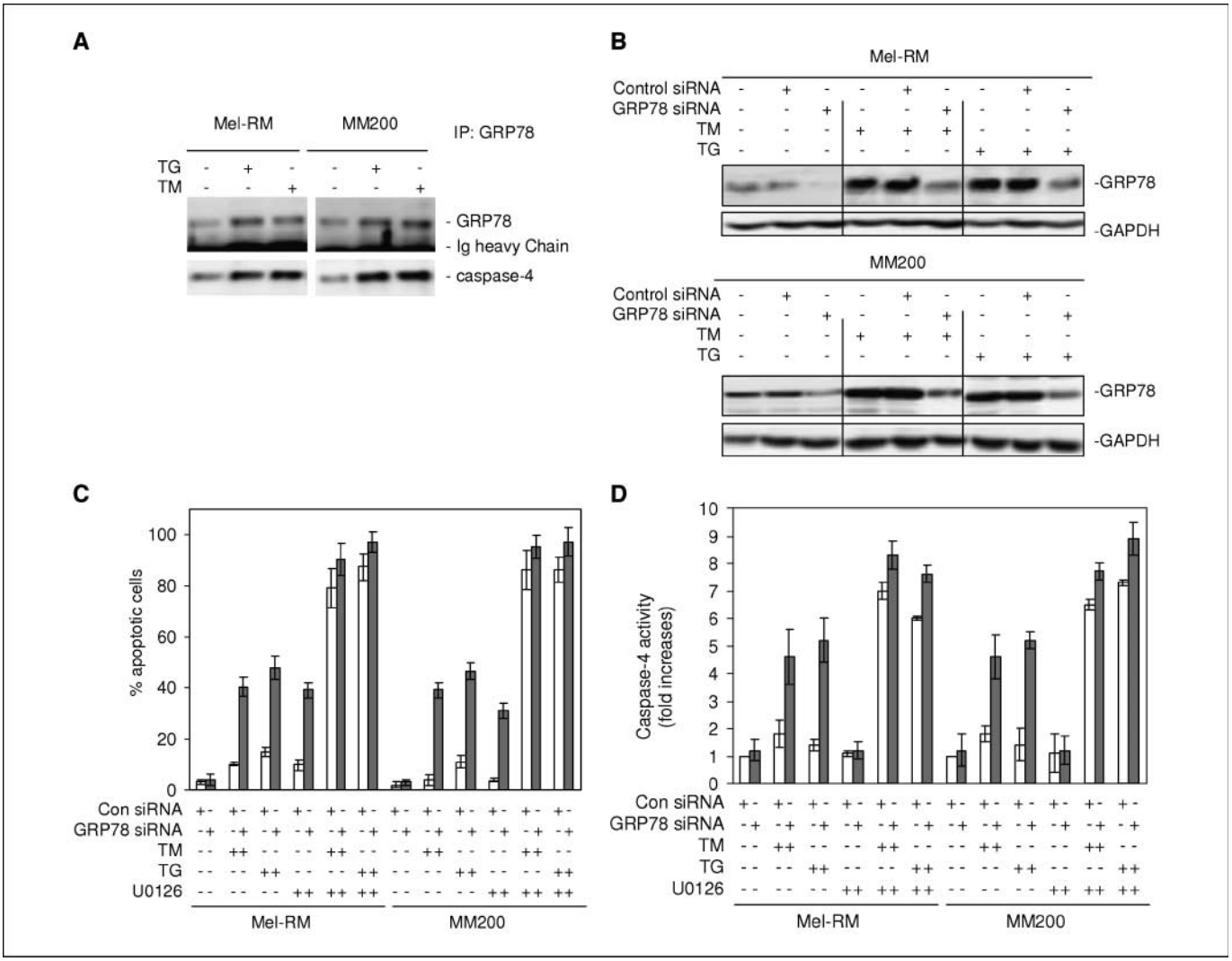


Figure 6. Down-regulation of GRP78 may contribute to tunicamycin- or thapsigargin-induced caspase-4 activation and apoptosis in the presence of U0126. *A,* GRP78 is physically associated with caspase-4. Whole-cell lysates from Mel-RM and MM200 cells with or without treatment with tunicamycin (3 μmol/L) or thapsigargin (1 μmol/L) for 24 h were subjected to immunoprecipitation with an antibody against GRP78. The precipitates were subjected to SDS-PAGE and probed with antibodies against GRP78 and caspase-4, respectively. Data are representative of three individual experiments. *B,* siRNA knockdown of GRP78 inhibits its expression and up-regulation by tunicamycin or thapsigargin. Mel-RM (*top*) and MM200 (*bottom*) cells were transfected with the control or GRP78 siRNA. Twenty-four hours later, whole-cell lysates were subjected to Western blot analysis of GRP78 expression. Data are representative of three individual experiments. *C,* siRNA knockdown of GRP78 sensitizes melanoma cells to tunicamycin- or thapsigargin-induced apoptosis. Mel-RM and MM200 cells were transfected with the control or GRP78 siRNA. Twenty-four hours later, the cells were treated with U0126 (20 μmol/L), tunicamycin (3 μmol/L), or thapsigargin (1 μmol/L) for 48 h or U0126 for 1 h before the addition of tunicamycin (3 μmol/L) or thapsigargin (1 μmol/L) for a further 48 h. Apoptosis was measured by the propidium iodide method using flow cytometry. *Columns,* mean of three individual experiments; *bars,* SE. *D,* siRNA knockdown of GRP78 enhances tunicamycin- or thapsigargin-induced caspase-4 activity. Mel-RM and MM200 cells transfected with the control or GRP78 siRNA were treated with tunicamycin (3 μmol/L) or thapsigargin (1 μmol/L) for 36 h. Whole-cell lysates were harvested and caspase-4 activity was measured with the specific caspase-4 substrate z-LEHD-AFC in fluorometric assays. The values of caspase-4 activity in cells transfected with the control siRNA without treatment were arbitrarily normalized as 1. The values of activity in the cells treated as indicated were compared with those in cells transfected with the control siRNA without treatment and are expressed as the fold increase. *Columns,* mean of three individual experiments; *bars,* SE.

The inhibitory effect of either the caspase-4 inhibitor or caspase-4 siRNA on sensitization of melanoma cells to ER stress-induced apoptosis was incomplete. This is most likely due to simultaneous operation of other apoptotic mechanisms when the UPR is induced whereas MEK is inhibited. CHOP did not seem to play a role in that siRNA knockdown of CHOP had no effect on sensitization of melanoma cells to apoptosis induced by tunicamycin or thapsigargin (data not shown). Bcl-2 family members play essential roles in regulating apoptosis (40–42). Although they are thought to function primarily on mitochondria, recent reports suggest that they can also act on the ER to which they are also located (43, 44). We have found in a separate study that inhibition of MEK results in up-regulation of the BH3-only proteins PUMA and Bim but down-regulation of Mcl-1 in melanoma cells (data not shown). If these changes play roles in sensitization of melanoma cells to ER stress-induced apoptosis is not clear, we have found, however, that both tunicamycin and thapsigargin up-regulated Mcl-1 expression, which was substantially attenuated in the presence of U0126 (Supplementary Fig. S4).

GRP78 expression is primarily regulated at the transcriptional level, mediated by multiple copies of the ER stress response element within the GRP78 promoter region (45, 46). We show by real-time PCR that GRP78 mRNA levels were decreased by inhibition of MEK/ERK in the presence or absence of tunicamycin

or thapsigargin, indicating that the MEK/ERK pathway may participate in regulation of GRP78 transcription. All three arms of the UPR can activate transcription of GRP78 (46, 47). Among them, the ATF6 signaling pathway is the most potent activator for GRP78 induction (46, 47). ATF6 has been reported to undergo post-translational modifications, such as phosphorylation by the p38 MAPK, further enhancing its efficacy as an activator of GRP78 transcription (48). Given that MEK/ERK is constitutively activated in melanoma cells (23, 24, 30, 31), it is conceivable that the MEK/ERK signaling pathway may directly or indirectly interact with the UPR signaling pathways, most likely through post-translational regulation of the UPR sensors and/or effectors as p38 does, thus regulating GRP78 expression. As far as we are aware, this is the first demonstration that GRP78 is a target of the MEK/ERK pathway, but we are unable to conclude if this is a direct or indirect effect from other targets in the MEK/ERK pathway.

It is unclear why the melanoma cell line Me1007, in contrast to all the other lines included in this study, is highly sensitive to ER stress-induced apoptosis. This did not seem to be associated with the constitutive levels of expression of GRP78 and activation of ERK1/2 because both GRP78 and phosphorylated ERK1/2 were expressed in this cell line at levels comparable with those in the resistant Mel-RM and MM200 lines (data not shown). This suggests that regulation of ER stress-induced apoptosis in melanoma is more complicated than just GRP78 and the MEK/ERK pathway. Moreover, caspase-4 did not seem to be the apical caspase in ER stress-induced apoptosis in this cell line (data not shown). Examination of the mechanism(s) by which ER stress induces apoptosis in Me1007 would be a useful means for understanding more clearly how the majority of melanoma cell lines are resistant to apoptosis induced by ER stress.

In summary, we show in this report that inhibition of MEK/ERK sensitizes cultured human melanoma cells to ER stress-induced apoptosis. This is mediated, at least in part, by activation of caspase-4, which is otherwise suppressed by the ER chaperone GRP78. These results suggest that combinations of agents that induce ER stress and those inhibiting the MEK/ERK pathway may be effective strategies against melanoma. However, normal melanocytes and fibroblasts seemed to be relatively sensitive to ER stress-induced apoptosis, indicating that careful evaluation of clinically relevant ER stress-inducing agents in combination with inhibitors targeting the MEK/ERK pathway is required before *in vivo* investigations are carried out. Of interest, GRP78 was found to be expressed at relatively high levels in cultured melanoma cells and on melanoma tissue sections but was hardly detectable in melanocytes and fibroblasts (data not shown). This suggests that targeting GRP78 may have tumor-specific selectivity and thus being useful in treatment of melanoma.

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Inhibition of MEK Sensitizes Human Melanoma Cells to Endoplasmic Reticulum Stress-Induced Apoptosis

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