

GRP78/BiP Inhibits Endoplasmic Reticulum BIK and Protects Human Breast Cancer Cells against Estrogen Starvation–Induced Apoptosis

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Abstract

The recent development of hormonal therapy that blocks estrogen synthesis represents a major advance in the treatment of estrogen receptor–positive breast cancer. However, cancer cells often acquire adaptations resulting in resistance. A recent report reveals that estrogen starvation–induced apoptosis of breast cancer cells requires BIK, an apoptotic BH3-only protein located primarily at the endoplasmic reticulum (ER). Searching for novel partners that interact with BIK at the ER, we discovered that BIK selectively forms complex with the glucose-regulated protein GRP78/BiP, a major ER chaperone with prosurvival properties naturally induced in the tumor microenvironment. GRP78 overexpression decreases apoptosis of 293T cells induced by ER-targeted BIK. For estrogen-dependent MCF-7/BUS breast cancer cells, overexpression of GRP78 inhibits estrogen starvation–induced BAX activation, mitochondrial permeability transition, and consequent apoptosis. Further, knockdown of endogenous GRP78 by small interfering RNA (siRNA) sensitizes MCF-7/BUS cells to estrogen starvation–induced apoptosis. This effect was substantially reduced when the expression of BIK was also reduced by siRNA. Our results provide the first evidence that GRP78 confers resistance to estrogen starvation–induced apoptosis in human breast cancer cells via a novel mechanism mediated by BIK. These results further suggest that GRP78 expression level in the tumor cells may serve as a prognostic marker for responsiveness to hormonal therapy based on estrogen starvation and that combination therapy targeting GRP78 may enhance efficacy and reduce resistance. [Cancer Res 2007;67(8):3734–40]

Introduction

The estrogen receptor is a key regulator and therapeutic target in breast cancer etiology and progression. Endocrine therapy, which blocks the estrogen receptor signaling pathways, is one of the most important systemic therapies in breast cancer treatment (1). Antiestrogens such as tamoxifen have been widely used as adjuvant therapy for women with estrogen receptor–positive breast carcinoma because of its effectiveness and low toxicities compared with systemic chemotherapy (2). Fulvestrant (Faslodex), a newer estrogen receptor antagonist in clinical use in metastatic hormone receptor positive breast cancer, has no agonist activity and causes degradation of the estrogen receptor, thus eliminating estrogen-sensitive gene transcription (3). In addition, third-generation

aromatase inhibitors (e.g., anastrozole, letrozole, and exemestane), which block the conversion of adrenally derived androgens to estrogen in postmenopausal women, provide even better efficacy and tolerability (4). Despite these significant advances, *de novo* or acquired resistance is frequently observed, and this remains a critical clinical problem. Thus, understanding the molecular mechanisms responsible for endocrine resistance is of primary importance toward improving the treatment of breast cancer.

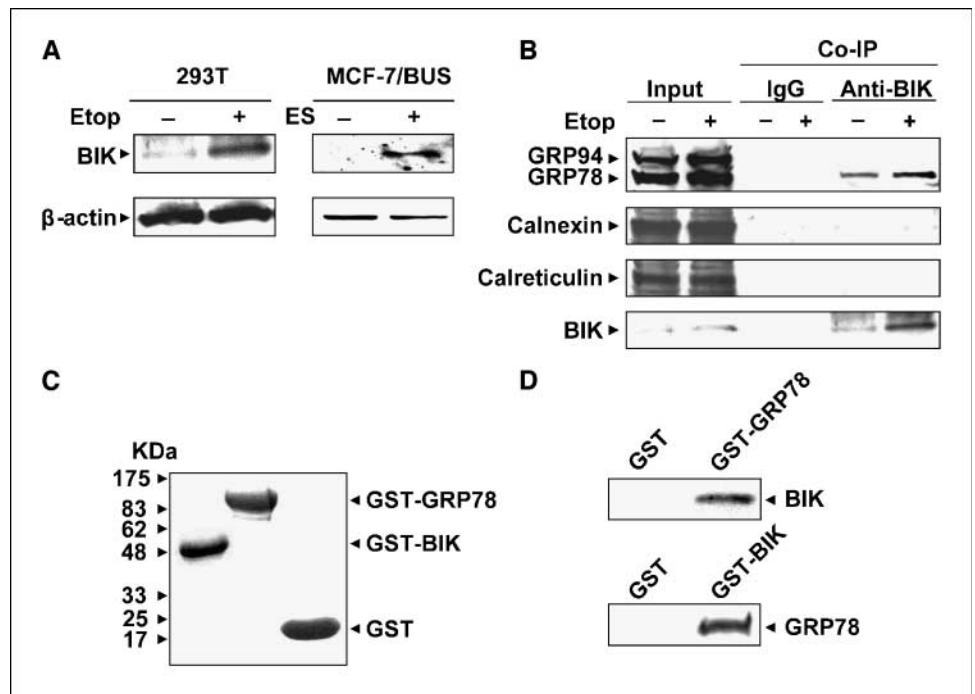
It has been widely accepted that estrogen is required for the proliferation of estrogen receptor–positive human breast cancer cells, and recent evidence shows that estrogen is also essential for the survival of breast cancer cells (5). When subjected to estrogen starvation, which mimics the effect of aromatase inhibitors, or exposed to antiestrogens, significant apoptosis of breast cancer cells is observed. The BCL-2 family proteins are key regulators of apoptosis. The antiapoptotic members of the BCL-2 family, such as BCL-2, share three or four conserved domains known as BCL-2 homology (BH) regions. The proapoptotic members such as BAX share two or three BH domains. Whereas the proapoptotic members facilitate the release of cytochrome *c* from the mitochondria, resulting in Apaf-1 activation and subsequent caspase activation, the antiapoptotic members suppress this pathway (6). A third group of apoptosis regulators, referred to as BH3-only proteins, only share the nine-amino-acid BH3 region. In their active conformation, BH3-only BCL-2 members regulate the ability of BAX and BAK to oligomerize in the mitochondrial outer membrane and release intermediate proteins, including cytochrome *c*, to the cytosol (7). BH3-only proteins can also bind directly to the antiapoptotic members of the BCL-2 family through the BH3 domain and inhibit their activity. Previous studies showed that antiestrogens have no effect on the expression of proapoptotic protein BAX but suppress antiapoptotic BCL-2 expression, correlating with induction of apoptosis (8). Nonetheless, the molecular mechanisms whereby the BCL-2 protein family members regulate estrogen starvation–mediated apoptosis are not well understood.

A recent report reveals that BIK, an apoptotic BH3-only protein, plays a critical role in promoting estrogen starvation or antiestrogen-induced apoptosis of human breast cancer cells (9). Using, as a model system, a human breast carcinoma MCF-7 subline referred to as MCF-7/BUS, which has been vigorously characterized as growing in an estrogen dose–dependent manner (10), BIK mRNA and protein are found to be strongly induced by estrogen starvation or antiestrogen treatment, and knockdown of BIK by small interfering RNA (siRNA) significantly inhibits apoptosis caused by antiestrogen treatment. BIK induction has been reported in human cells in response to p53 overexpression and genotoxic agents such as doxorubicin. Interestingly, BIK contains a single transmembrane segment at its extreme COOH terminus, but in contrast to most BH3-only proteins, which target primarily the mitochondria with some also localizing in the

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Figure 1. Selective association of endogenous BIK with GRP78. **A**, 293T cells were either nontreated or treated with 50 $\mu\text{mol/L}$ etoposide (*Etop*) for 6 h and were harvested 24 h later. MCF-7/BUS cells were cultured either in regular DMEM or in estrogen-free DMEM for 48 h. Western blots of total protein lysates from these cells were done with antibodies against BIK and β -actin. **B**, cell lysates prepared from control and *Etop*-treated 293T cells were immunoprecipitated with anti-BIK or normal IgG. The immunoprecipitates were applied in parallel with input lysates to SDS-PAGE and Western blotted with antibodies against GRP78, GRP94, calnexin, calreticulin, and BIK. **C**, Coomassie blue staining of GST-GRP78, GST-BIK, and GST resolved by SDS-PAGE. **D**, lysates of 293T cells were incubated with GST-GRP78, GST-BIK, or GST-linked beads. The bound proteins were resolved by SDS-PAGE and probed for GRP78 or BIK by Western blotting.



endoplasmic reticulum (ER), BIK is integrated almost exclusively in the membrane of the ER (11). Immunofluorescence confocal microscopy shows that BIK colocalizes with calnexin, an ER transmembrane protein, and subcellular fractionation shows that BIK codistributes with ER proteins calnexin and GRP78/BiP (11, 12). Although BIK does not interact directly with proapoptotic BAX and BAK, it regulates a BAX/BAK–dependent release of Ca^{2+} from the ER stores and operates with other BH3-only proteins to cause rapid release of cytochrome *c* from the mitochondria and the activation of caspases (11, 12). The discovery that BIK is a key mediator for estrogen starvation and antiestrogen-induced apoptosis implies that inhibition of BIK expression or activity at the ER site may represent a novel molecular mechanism for endocrine resistance in human breast cancer.

The glucose-regulated protein GRP78, also referred to as BiP, is a major molecular chaperone at the ER (13, 14). GRP78, a multifunctional protein with antiapoptotic properties, is a key prosurvival component of the unfolded protein response, an evolutionarily conserved adaptive measure for ER stress (15–17). In a variety of cancer cell lines, solid tumors, and biopsy specimens from human cancer, including human breast cancer, the level of GRP78 is highly elevated, correlating with malignancy, metastasis, and drug resistance (18–20). GRP78 is overexpressed in malignant but not benign human breast lesions, and associates with resistance to chemotherapy in breast cancer patients (21, 22). The strong, natural induction of GRP78 in solid tumors can be attributed to glucose starvation stress in poorly vascularized tumors and altered metabolism of cancer cells such that they exhibit a much higher glucose utilization rate than normal cells (23). Through direct or indirect interactions with specific caspases and other upstream components of the proapoptotic pathways initiating from the ER, GRP78 is postulated to regulate the balance between cell survival and apoptosis (19, 24–27). Here, we report that GRP78, but not other ER chaperones, forms a complex with BIK. Whereas GRP78 overexpression inhibits BIK and estrogen starvation–induced BAX activation and apoptosis, suppression of

endogenous GRP78 by siRNA sensitizes human breast cancer cells to estrogen starvation–induced apoptosis. Our findings provide the first evidence that a major ER chaperone protein, GRP78, confers resistance to estrogen starvation–induced apoptosis in human breast cancer cells via a novel mechanism mediated by the BH3-only protein BIK. These results further suggest that combination therapy targeting GRP78 may enhance efficacy and reduce resistance to hormonal therapy based on estrogen starvation of breast cancer cells.

Materials and Methods

Cell lines and culture conditions. The estrogen-dependent cell line MCF-7/BUS was provided by A.M. Soto (Tufts University, Medford, MA) and has been described (28). The human embryonic kidney 293T cells and MCF-7/BUS cells were maintained in DMEM supplemented with 10% fetal bovine serum. Estrogen starvation of MCF-7/BUS cells was done as described (9). Briefly, the cells were washed thrice with phenol red–free DMEM and incubated in washing medium at 37°C for 60 min. The MCF-7/BUS cells were then cultured in phenol red–free DMEM supplemented with 5% charcoal/dextran–stripped fetal bovine serum for 24 to 72 h as indicated. For etoposide treatment, the cells were incubated with 50 $\mu\text{mol/L}$ etoposide for 6 h and cultured for another 24 h before harvest.

Expression vectors. The plasmids pcDNA3-Flag-BIK-b5TM and pcDNA3-Flag-BIK were provided by G.C. Shore (McGill University, Montreal, Canada) and their construction has been described (11). In pcDNA3-Flag-BIK-b5TM, the COOH-terminal transmembrane domain of BIK was replaced by the transmembrane domain of cytochrome *b₅*, which targets the protein to the ER. The construction of pcDNA3-His-GRP78 has been described (29).

Transient transfections and adenovirus infections. 293T cells were grown to 60% to 80% confluence. Two micrograms of pcDNA3-Flag-BIK-b5TM plasmid were cotransfected with 2 μg of His-GRP78 or empty vector by using Polyfect (Qiagen) as described (30). The green fluorescent protein (*GFP*) gene driven by cytomegalovirus promoter was added to monitor for transfection efficiency. Empty vector was added to adjust the total amount of plasmids to be the same. Forty-eight hours later, the transfected cells were subjected to cell death assays, Western blot, or coimmunoprecipitation.

For construction of the adenovirus expression vectors, either GFP or a His-tagged full-length hamster Grp78 cDNA was subcloned into an adenoviral vector and its expression was driven by the cytomegalovirus promoter. The sequence in the final construct was confirmed by DNA sequencing. MCF-7/BUS cells were infected at 100 plaque-forming units/cell with adenovirus vectors expressing GFP or GRP78. For mitochondrial membrane potential staining, because GFP interferes with the green fluorescence of this assay, the adenovirus empty vector was used as the negative control. After 24 h, the infected cells were subjected to estrogen starvation for 48 h. Each transfection or infection was done in duplicate and was repeated two to three times.

Western blots and quantitation. The Western blots were done as described (30). The primary antibodies were goat anti-BIK (N-19, Santa Cruz Biotechnology, Santa Cruz, CA), rat anti-GRP78 (76-E6, Santa Cruz Biotechnology), rat anti-GRP94, rabbit anti-calnexin, rabbit anti-calreticulin (Stressgen), mouse anti-Flag M2, mouse anti-poly(ADP-ribose) polymerase (PARP; F-2, Santa Cruz Biotechnology), and mouse anti- β -actin (Sigma-Aldrich). Anti- β -actin was diluted at 1:2,000; anti-BIK at 1:500; and other antibodies at 1:1,000. Respective horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) at 1:1,000 dilution were used. The Western blots were quantitated by Fluor-S MultiImager (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. All quantitations were normalized against β -actin.

Coimmunoprecipitation assays. The coimmunoprecipitation assays were done as described (25). Briefly, 500 μ g of total protein extract from each sample were pretreated with protein G-Sepharose beads (Upstate), followed by incubation with 5 μ g of goat anti-BIK antibody (N-19, Santa Cruz Biotechnology) or mouse anti-Flag M2 antibody (Sigma-Aldrich). For negative controls, the respective goat or mouse immunoglobulin G (IgG; Santa Cruz Biotechnology) was used.

Glutathione S-transferase pull-down assays. Glutathione S-transferase (GST)-GRP78 and GST-BIK were constructed by subcloning full-length hamster Grp78 cDNA and human BIK into the *Bam*H1/*Xho*I and *Bam*H1/*Sal*I sites of pGEX 4T1, respectively (Pharmacia Biotech). Conditions for the GST pull-down assays have been described (31) with the following modifications. Five micrograms of GST-BIK, GST-GRP78, and GST bound to glutathione-Sepharose beads (Sigma-Aldrich) were incubated with 500 μ g of total protein extract on a rotating shaker at 4°C for 16 h. The beads were collected by centrifugation at 2,000 rpm for 5 min and washed thrice with

extraction buffer. The bound proteins were eluted in SDS-PAGE sample loading buffer and subjected to SDS-PAGE and Western blotting.

Cell death and apoptotic assays. The cell death trypan blue exclusion assay was done as described (23). For mitochondrial membrane potential staining, the Mitochondrial Permeability Transition Detection Kit (Immunocytochemistry, Bloomington, MN) was used following the manufacturer's protocol. The cell cultures were then washed with PBS and examined under a fluorescence microscope. Each assay was done in triplicate.

Flow cytometric analysis of BAX-associated immunofluorescence. On initiation of apoptosis, BAX undergoes conformational change that exposes an otherwise inaccessible NH₂-terminal epitope (32). A mouse monoclonal antibody against amino acids 12 to 24 (clone 6A7, PharMingen) was used to detect the BAX with proapoptotic conformational change. MCF-7/BUS cells were harvested and fixed in 0.25% paraformaldehyde in PBS for 5 min. BAX staining and fluorescence-activated cell sorting (FACS) analysis of BAX activation were done as described (32).

Small interfering RNA. The siRNA against Grp78 is 5'-ggagcgcgaugaucauagadTdT-3' as described (33). The siRNA against Bik is 5'-aagaccuccucagagacau-3' (9). The control siRNA is Silencer Negative Control #3 siRNA (Ambion) composed of a 19-bp scrambled sequence without significant homology to any known gene sequences from mouse, rat, or human. MCF-7/BUS cells were grown to 50% confluence and transfected with control siRNA or siRNA against Grp78 or Bik using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. The experiments were repeated two to three times.

Results

Endogenous BIK selectively forms complex with GRP78.

First, we determined the inducibility of BIK protein by different stress conditions. In the human embryonic kidney cell line 293T, BIK protein was present at a low basal level under normal culture conditions. On treatment with etoposide, a topoisomerase I inhibitor, the level of BIK protein was substantially elevated (Fig. 1A). In the human breast carcinoma MCF-7/BUS cells, the level of BIK protein was dramatically induced by estrogen starvation (Fig. 1A). In contrast, ER stress inducers such as thapsigargin or tunicamycin do not induce BIK (data not shown).

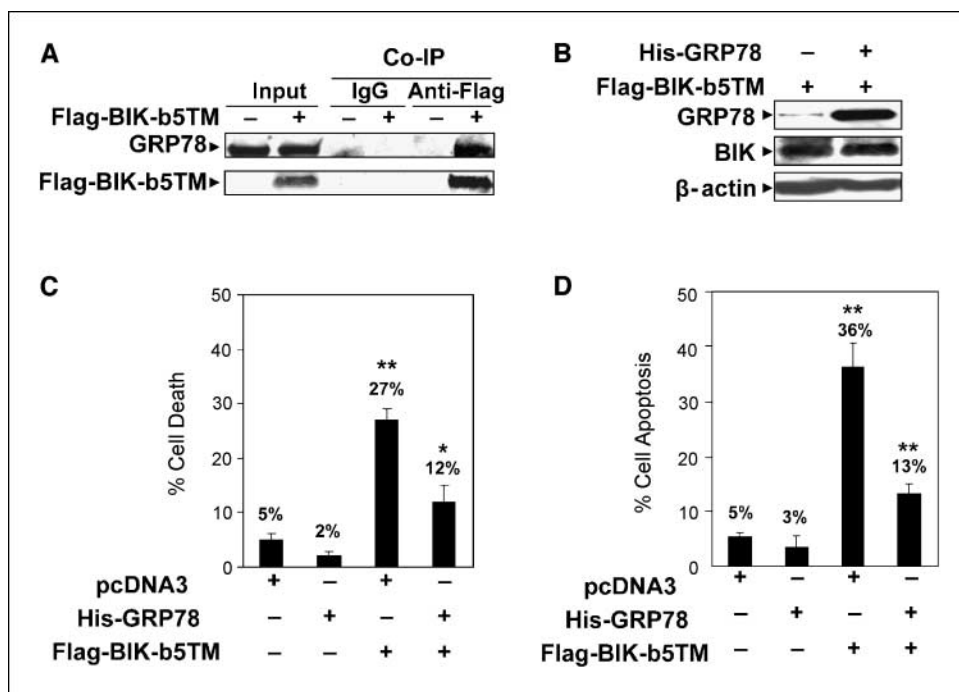
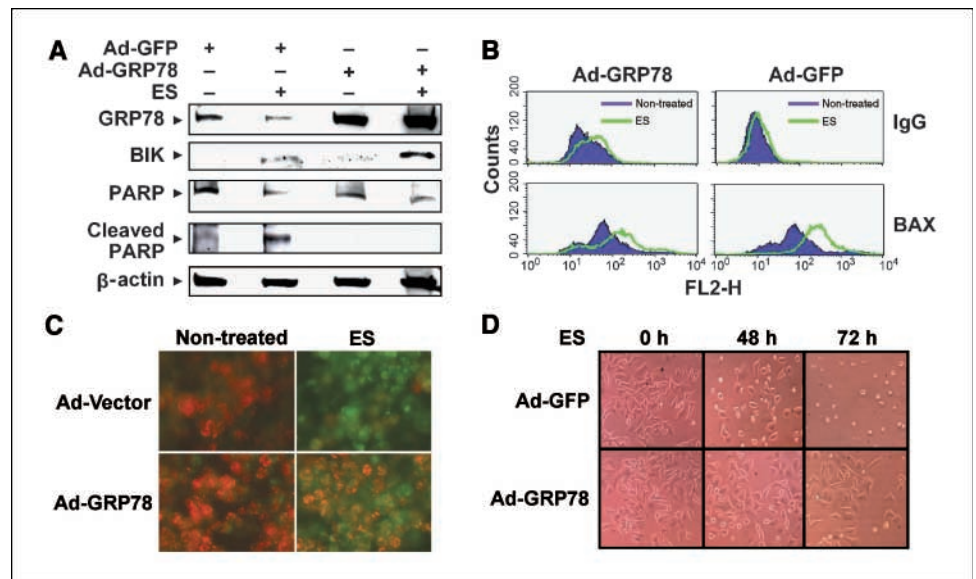


Figure 2. Binding of GRP78 to ER-targeted BIK and suppression of its proapoptotic activity. *A*, cell lysates prepared from 293T cells transfected with either empty vector pcDNA3(-) or vector expressing Flag-BIK-b5TM(+) were immunoprecipitated with either anti-Flag antibody or normal IgG as a control. The immunoprecipitates were resolved by SDS-PAGE and Western blotted with anti-GRP78 and anti-Flag antibodies. *B* to *D*, 293T cells were transfected with empty vector pcDNA3(-), pcDNA3-Flag-BIK-b5TM, or pcDNA3-His-GRP78, alone or in combination as indicated. *B*, the expression level of each protein was determined by Western blot. *C*, the percent cell death in each transfection was assessed by trypan blue exclusion assay. *D*, the percent of apoptotic cells was assessed by mitochondrial membrane potential staining. *C* and *D*, columns, mean from three experiments, each of which assayed at least 400 cells for every group; bars, SE. *, $P < 0.05$; **, $P < 0.01$.

Figure 3. Overexpression of GRP78 rescues MCF-7/BUS cells from estrogen starvation–induced apoptosis. **A**, cell lysates from MCF-7/BUS cells infected with Ad-GFP or Ad-GRP78 cultured either in regular medium or in estrogen-free medium for 48 h were subjected to SDS-PAGE and Western blots. The levels of GRP78, BIK, β -actin, cleaved PARP (a signature of apoptosis), and uncleaved PARP are indicated. **B**, FACS analysis of the same samples in (A) using mouse anti-BAX and phycoerythrin-labeled antimouse antibodies. **C**, mitochondrial membrane potential staining of MCF-7/BUS cell cultures either in regular medium or in estrogen-free medium after infection of adenovirus empty vector (Ad-Vector) or Ad-GRP78. Red fluorescence, normal mitochondrial membrane potential; green fluorescence, collapsed mitochondrial membrane potential and early apoptosis. **D**, general morphology under a light microscope of MCF-7/BUS cells at 0, 48, and 72 h after estrogen starvation.



Thus, the induction of BIK occurs under selective stress conditions in human cells.

As a first step toward understanding how BIK is regulated at the ER, we searched for its interactive partners by coimmunoprecipitation followed by Western blot with known ER proteins. We discovered that BIK selectively interacts with GRP78. In coimmunoprecipitation assays, BIK complexed with GRP78 in both untreated cells and cells where BIK level was elevated by etoposide treatment (Fig. 1B). The interaction between endogenous GRP78 and BIK is specific because this complex was not observed using control IgG as the precipitating antibody, and other abundant ER proteins such as GRP94, calnexin, and calreticulin were not detected in the BIK immunoprecipitate (Fig. 1B). To confirm the physical interaction between GRP78 and BIK, they were both expressed as bacterial GST-fusion proteins. The yield and purity of the GST-proteins were confirmed by Coomassie blue staining (Fig. 1C). In pull-down assays, GST-GRP78, but not the GST protein, was able to bind BIK from total cell extract, and reversely, GST-BIK, but not the GST protein, was able to bind GRP78 (Fig. 1D). Thus, BIK and GRP78 form a complex both *in vivo* and *in vitro*.

GRP78 binds ER-targeted BIK and blocks its apoptotic activity. To determine the functional interaction between GRP78 and BIK in the ER, 293T cells were transfected with a vector expressing Flag-tagged BIK, selectively targeted to the ER by using the cytochrome b_5 transmembrane domain (b5TM). Western blot analysis confirmed expression of the Flag-tagged BIK-b5TM in the transfected cells and coimmunoprecipitation using anti-Flag antibody confirmed complex formation between GRP78 and the ER-targeted BIK *in vivo* (Fig. 2A). To test for the effects of GRP78 on BIK activity, the expression vector for ER-targeted BIK was cotransfected into 293T cells with either the expression vector for His-tagged GRP78 or the empty vector pcDNA3. Coexpression of the His-tagged GRP78 and Flag-tagged BIK in the transfected cells was confirmed by Western blot (Fig. 2B). Cell death determined by trypan blue exclusion reveals that cells expressing ER-targeted BIK exhibited a 5-fold increase in the percent of cell death compared with cells transfected with pcDNA3 (Fig. 2C). This increase was reduced by half in cells overexpressing GRP78, providing the first evidence that GRP78 is able to counteract cell death mediated by

BIK. To determine whether the cell death observed was due to apoptosis, identical transfection experiments were done and the extent of apoptosis was determined by lipophilic cation fluorescent staining that detects changes in mitochondrial membrane potential. As summarized in Fig. 2D, ER-targeted BIK expression induced apoptosis in the transfected cells and GRP78 overexpression reduced ER-targeted BIK-induced apoptosis by 3-fold.

GRP78 overexpression inhibits estrogen starvation–induced BAX activation and apoptosis. Because BIK is an upstream regulator of BAX and estrogen starvation–induced apoptosis, inhibition of BIK activity by GRP78 overexpression should suppress these downstream pathways. To test this in the context of estrogen-dependent human cancer cells, MCF-7/BUS cells were infected with adenovirus vectors expressing either GRP78 (Ad-GRP78) or, as a control, GFP (Ad-GFP). Overexpression of GRP78 in the Ad-GRP78–infected cells was confirmed by Western blot (Fig. 3A). On estrogen starvation, BIK was induced, correlating with BAX activation (Fig. 3A and B). Estrogen starvation resulted in fluorescent histogram curve shift with the mean fluorescence value increased from 77 to 313 when compared with the nontreated cells, indicating an increase of the active form of BAX as recognized by the BAX conformation specific antibody (Fig. 3B). In agreement with GRP78 counteracting BIK activity, the activation of BAX by estrogen starvation was suppressed in cells overexpressing GRP78 as compared with cells expressing GFP, with the mean fluorescence value decreased from 183 for cells expressing GFP to 70 for cells overexpressing GRP78 (~48% suppression; Fig. 3B).

To test independently the protective effect of GRP78 in estrogen starvation–induced apoptosis, the same cells were subjected to the mitochondrial permeability transition assay. In this assay, the lipophilic MitoPT reagent penetrates the healthy mitochondria in nonapoptotic cells, aggregates, and produces red fluorescence in the negatively charged mitochondria. In early apoptotic cells, on collapse of the mitochondrial membrane potential, the MitoPT reagent distributes throughout the cell and fluoresces green. As shown in Fig. 3C, MCF-7/BUS cells overexpressing GRP78 showed substantial reduction in mitochondrial membrane potential change on 48 h of estrogen starvation, as compared with cells infected with the empty vector. Further, because MCF-7/BUS cells are devoid of

caspase-3, a useful indicator of apoptosis in these cells is estrogen starvation-induced cleavage of endogenous PARP (9). In non-apoptotic cells, PARP exists in its uncleaved form (116 kDa), whereas in apoptotic cells, PARP is cleaved by activated caspases into an 85-kDa fragment. As shown in Fig. 3A, the cleaved form of PARP was evident in estrogen-starved cells infected with Ad-GFP but was not observed in cells infected with Ad-GRP78. Finally, as shown by light microscopy, cells transfected with Ad-GFP gradually lost viability on estrogen starvation treatment, and by 72 h, most cells exhibited rounded morphology, whereas ~50% the GRP78 overexpressing cells were still viable (Fig. 3D). Collectively, these results provide several lines of evidence that GRP78 protects human breast cancer against estrogen starvation-induced apoptosis.

Knockdown of endogenous GRP78 sensitizes human breast cancer cells to estrogen starvation-induced apoptosis. To test directly whether the down-regulation of endogenous GRP78 protein level will sensitize human breast cancer to estrogen starvation-induced apoptosis, we used siRNA to knockdown expression of GRP78 in MCF-7/BUS cells. As shown in Fig. 4A, transient transfection of a Grp78-suppressing siRNA substantially reduced the level of GRP78 as compared with control siRNA. The siRNA against Grp78 is specific because it has no effect on the expression of another major ER chaperone protein, GRP94, or on the expression of β -actin. In cells growing in normal culture medium, siRNA against Grp78 and control siRNAs had little effect

on the mitochondrial membrane potential (Fig. 4B). In contrast, in cells undergoing estrogen starvation for 24 h, there was a marked increase in apoptosis in cells transfected with the siRNA against GRP78 as compared with cells transfected with the control siRNA (Fig. 4B). Thus, GRP78 protects human breast cancer cells against estrogen starvation-induced apoptosis.

To test further whether this protective effect acts through BIK directly, we used siRNA to knock down GRP78 and BIK, either alone or in combination, in MCF-7/BUS cells subjected to estrogen starvation. To complement the measurement of apoptotic cells, the amount of apoptosis induced by estrogen starvation was determined by quantitation of PARP cleavage. As shown in Fig. 4C, the expression of GRP78 and BIK protein was substantially reduced by their specific siRNA as compared with control siRNA. Knockdown of BIK by siRNA decreased PARP cleavage as compared with cells transfected with control siRNA whereas knockdown of GRP78 increased PARP cleavage (Fig. 4D). Further, knockdown of BIK substantially reduced the enhanced PARP cleavage mediated by knockdown of GRP78 (Fig. 4D). The reduction was more than BIK knockdown alone. These results confirmed that BIK mediates estrogen starvation-induced apoptosis in MCF-7/BUS cells and further showed that GRP78 inhibits apoptosis in estrogen-starved breast cancer cells, in part, through suppression of BIK.

Discussion

Aromatase inhibitors represent a major advance in the treatment of estrogen receptor-positive breast cancer; however, cancer cells frequently acquire adaptations to allow them to develop resistance (1, 2). In this study, we explored the relationship between BIK, a proapoptotic BH3-only protein that facilitates estrogen starvation and antiestrogen-induced apoptosis (9), and GRP78, a major ER chaperone with antiapoptotic properties naturally induced in the tumor microenvironment. Our results support a new role for GRP78 as an inhibitor of BIK-mediated apoptosis via physical and functional interactions, and that GRP78 confers resistance to estrogen starvation-induced apoptosis in human breast cancer cells. Because both BIK and GRP78 are localized to the ER, this study also provides direct evidence that the ER is a novel regulatory site for estrogen starvation-induced apoptosis as well as resistance, and establishes GRP78 as an upstream regulator of the apoptosis signaling cascade through targeting BIK.

BIK was first discovered by DNA microarray analysis as the only BH3-only protein among the 13 other protein members being evaluated that is strongly induced by the presence or absence of estrogens or antiestrogens in human breast cancer cells (9). BIK is also unique in that, unlike the other BH3-only proteins, it is primarily localized to the ER (11, 12). Importantly, BIK targeted to the ER is capable of activating BAX indirectly and provokes cytochrome *c* release from the mitochondria (11). Whereas the mitochondria has been well established as a major player in apoptosis, the ER has emerged as another key site for the regulation of apoptosis and initiates parallel apoptotic pathways in response to a variety of stress conditions (34–36). Further, there is cross talk between the mitochondria and the ER, and BIK represents an exciting new link whereby a protein localized in the ER can initiate cytochrome *c* release from the mitochondria (11, 37). There are reports that the *BIK* gene contains missense mutations and alterations within the intronic regions in human

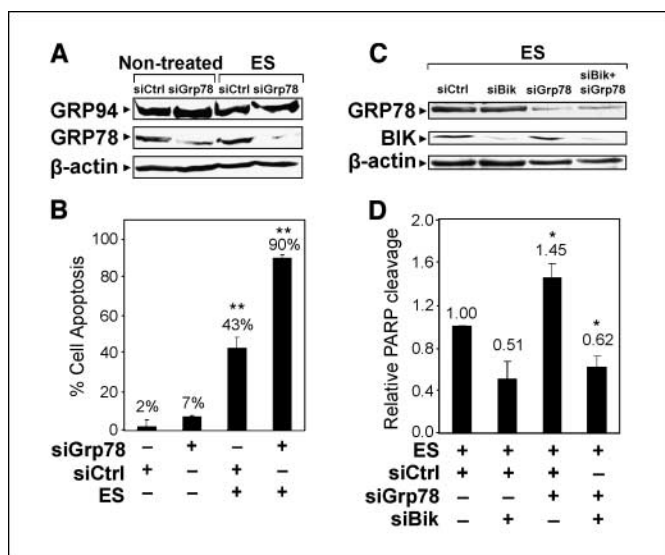


Figure 4. Knockdown of GRP78 sensitizes MCF-7/BUS cells to estrogen starvation-induced apoptosis. *A*, cell lysates from MCF-7/BUS cells transfected with siGrp78 oligomers or control siRNA (*siCtrl*) for 24 h and subsequently cultured in regular or estrogen-free medium (*ES*) for 24 h were subjected to SDS-PAGE and Western blotting to probe for levels of GRP78, GRP94, and β -actin. *B*, MCF-7/BUS cells were cultured in either regular or estrogen-free medium for 24 h after transfection of siGrp78 or control siRNA as indicated. The percent of apoptotic cells was assessed by mitochondrial membrane potential staining. *Columns*, mean from three experiments; *bars*, SE. **, $P < 0.01$. *C*, MCF-7/BUS cells were transfected with control siRNA, siGrp78, or siBik, alone or in combination as indicated for 24 h and then cultured in estrogen-free medium for 24 h. The total amount of siRNA in each condition was adjusted to be the same by addition of control siRNA. Cell lysates were collected and subjected to SDS-PAGE and probed for levels of GRP78, BIK, and β -actin by Western blotting. *D*, cell lysates from (*C*) were subjected to SDS-PAGE and Western blotted with anti-PARP antibody. The Western signal of full-length PARP and apoptosis signature fragment were quantitated with Fluor-S Multimager. The relative PARP cleavages are shown with the PARP cleavage in cells transfected with control siRNA set as 1. *Columns*, mean from two experiments; *bars*, SE. *, $P < 0.05$.

peripheral B-cell lymphomas, which could potentially give rise to isoforms with altered structure and/or function (38). However, in human breast cancer cells, there is no evidence of sequence mutations of Bik cDNA (9).

In searching for partners interacting with BIK at the ER, we discovered that BIK selectively forms complexes with GRP78, but not with GRP94, calnexin, or calreticulin, which are also major ER proteins. GRP78 is a central regulator of ER function due to its role in protein folding and assembly, targeting misfolded protein for degradation, ER calcium binding, and controlling the activation of transmembrane ER stress inducers (13–15, 19). In a variety of experimental systems, the cytoprotective function of GRP78 is well established (19, 23–27, 30). Whereas GRP78 is an ER lumen protein, the recent discovery that a subpopulation of GRP78 can exist as a ER transmembrane protein implies that it can potentially interact directly with the cytosolic components of the apoptotic pathway and regulate their activity (24, 25). For example, GRP78 has been reported to form complexes with procaspases, such as caspase-7 and mouse caspase-12, both of which associate with the outer ER membrane. GRP78 overexpression blocks cleavage of procaspase-7 to its active form (25). Conversely, inhibition of the formation of the GRP78-caspase-7 complex results in caspase-7 activation, resulting in increase in apoptosis (27, 39).

The protective function of GRP78 against a wide variety of stresses suggests that GRP78 may also interfere with the activity of key upstream regulators of apoptosis. Here, we show that GRP78 overexpression inhibits the apoptotic activity of BIK, as well as BAX activation and apoptosis mediated by estrogen starvation. A recent report shows that in epidermoid carcinoma cells, knockdown of GRP78 by siRNA leads to BAX activation, cytochrome *c* release, and increased sensitivity to doxorubicin; however, the mechanism whereby GRP78 suppresses BAX activation is not known (26). Because both etoposide and doxorubicin are strong inducers of BIK and BAX is a downstream target of BIK, our discovery that GRP78 is an interactive partner of BIK and that GRP78 can block BIK activity provides an explanation why GRP78 suppression sensitizes cells to BAX activation and apoptosis induced by doxorubicin as well as etoposide. How might GRP78 suppress BIK activity? Binding to client proteins is a general feature of molecular chaperones, which enables them to serve as buffering agents by masking the functional domain or altering the conformation of the client protein (40). One scenario is that GRP78 binding to BIK may alter its conformation or interfere with its heterodimerization with other interactive partners essential for its proapoptotic activity. For

example, it has been reported that BIK can cooperate with the weak BH3-only protein NOXA to activate BAX, resulting in rapid cytochrome *c* and caspase activation (37). Disruption of this interaction by GRP78 may impair the ability of BIK to induce apoptosis. Future studies mapping the interactive domains of BIK and GRP78 will provide further insight and resolve these issues.

In summary, whereas the development of resistance against estrogen starvation is likely to be complex and involve multiple pathways, our results identify the ER as a key cellular organelle for apoptosis mediated by estrogen starvation. The two major players are BIK, a prodeath molecule, and GRP78, a prosurvival protein. Because about two thirds of breast cancer patients showed elevated level of GRP78 (21, 22), our results predict that in this subset of human breast cancer, high elevation of GRP78 will block the ability of BIK to cause cell death resulting from estrogen starvation. If our hypothesis is correct, our findings may lead to the development of two novel clinical applications of GRP78. First, for patients with hormone receptor–positive breast cancer, GRP78 overexpression may be a prognostic marker for resistance to hormonal therapy based on estrogen starvation. In such instances, upfront treatment options may be individualized and refined based on the expression pattern of particular breast cancers. Second, GRP78 might be used as a novel therapeutic target to overcome resistance of hormone receptor–positive breast cancers to hormonal therapy based on estrogen starvation. In this regard, drugs that target GRP78 have been developed and show promise in preclinical studies where they have been shown to prevent tumor progression and sensitize tumors to chemotherapy treatment (27, 39, 41–43). Finally, we recently showed that antiangiogenesis therapy can lead to induction of GRP78 in human breast tumors (23). These preclinical data suggest that such therapy, when administered in combination with therapy based on estrogen starvation, could enhance resistance and disease recurrence.

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