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Signal Transduction

Molecular Cancer Research

SGK Kinase Activity in Multiple Myeloma Cells Protects against ER Stress Apoptosis via a SEK-Dependent Mechanism

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Abstract

To assess the role of the serum and glucocorticoid-regulated kinase (SGK) kinase in multiple myeloma, we ectopically expressed wild type or a phosphomimetic version of SGK into multiple myeloma cell lines. These cells were specifically resistant to the ER stress inducers tunicamycin, thapsigargin, and bortezomib. In contrast, there was no alteration of sensitivity to dexamethasone, serum starvation, or mTORC inhibitors. Mining of genomic data from a public database indicated that low baseline SGK expression in multiple myeloma patients correlated with enhanced ability to undergo a complete response to subsequent bortezomib treatment and a longer time to progression and overall survival following treatment. SGK overexpressing multiple myeloma cells were also relatively resistant to bortezomib in a murine xenograft model. Parental/control multiple myeloma cells demonstrated a rapid upregulation of SGK expression and activity (phosphorylation of NDRG-1) during exposure to bortezomib and an SGK inhibitor significantly enhanced bortezomib-induced apoptosis in cell lines and primary multiple myeloma cells. In addition, a multiple myeloma cell line selected for bortezomib resistance demonstrated enhanced SGK expression and SGK activity. Mechanistically, SGK overexpression constrained an ER stress–induced JNK proapoptotic pathway and experiments with a SEK mutant supported the notion that SGK's protection against bortezomib was mediated via its phosphorylation of SEK (MAP2K4) which abated SEK/JNK signaling. These data support a role for SGK inhibitors in the clinical setting for myeloma patients receiving treatment with ER stress inducers like bortezomib.

Implications: Enhanced SGK expression and activity in multiple myeloma cells contributes to resistance to ER stress, including bortezomib challenge. *Mol Cancer Res; 14(4); 397–407. ©2016 AACR.*

Introduction

The serum and glucocorticoid-regulated kinase (SGK) is in the AGC family of kinases as is AKT. These kinases are activated by several phosphorylation events. Phosphorylation in the activation loop is mediated by PI3K/PDK1 stimulation and additional phosphorylation in the C-terminal turn and hydrophobic motifs are mediated by TORC2 (1–3). Both SGK and AKT have many similar substrates but their activities are considered complementary rather than redundant. For example, the influence of both these AGC kinases is to inhibit the forkhead transcription factor family member FKHRL1 activity through phosphorylation but they selectively phosphorylate different residues on FKHRL1 (4). SGK is induced transcriptionally by serum (5, 6), glucocorticoids (GC; refs.5, 6), and stress stimuli (7). GC-induced expression is mediated by a glucocorticoid response element (GRE) in the SGK promoter (8) whereas stress stimuli utilize a p38MAPK cascade for induction (7).

Although many studies have documented an important role for AKT in the biology of multiple myeloma malignant plasma cells (9–12), relatively little is known about SGK in this tumor model. However, a recent publication (13) demonstrated the ability of the multiple myeloma growth factor IL-6 and bone marrow stromal cells to induce SGK in several multiple myeloma cell lines (MMCL) as well as primary cells. Induction by these environmental signals was mediated via a JAK/STAT pathway. Furthermore, short hairpin RNA (shRNA) silencing of SGK1 was cytotoxic to MMCLs. The fact that GCs are such rapid and intense stimulators of SGK expression and are used so frequently in chemotherapy of multiple myeloma patients, suggests another key role for SGK. We, thus, initiated the current project to test whether SGK protects multiple myeloma cells against certain forms of cytotoxic injury. We identified a specific protection against ER stress inducers including bortezomib. Further work indicated SGK protection is mediated by inhibition of JNK activation. The protective role of SGK during ER stress suggests future therapeutic targeting of SGK would be most advantageous when combined with ER stress inducers like bortezomib.

Materials and Methods

Cell lines, reagents, plasmids, and transfections

MMCLs were obtained from ATCC. The bortezomib-resistant (BR) 8226 line was a gracious gift from Dr. R. Orlowski, MD

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

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Anderson Cancer Center. This latter cell line has been described previously (14, 15). Bortezomib was obtained from Millennium Pharmaceuticals. Tunicamycin and thapsigargin were purchased from Sigma-Aldrich. The SGK inhibitor GSK65309 was purchased from Tocris (Tocris Bioscience). The HA-AKT gene was amplified by PCR from plasmid pDONR223-AKT (Addgene plasmid 23752) with inclusion of the HA sequence in the 5′ to generate HA-AKT, and HA-AKT was subsequently subcloned into pLenti6. Similarly, pDONR223-SGK1 (Addgene plasmid 23708) was used as the DNA template for subcloning HA-SGK1 into pLenti6 plasmid. pLenti6 HA-AKTS473D and pLenti6 HA-SGK1S422D were created from pLenti6 HA-AKT and pLenti6 HA-SGK1 plasmids respectively, with QuickChange XL Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer's protocol. The kinase dead (KD) double SGK mutant S422A/T256A was likewise created with the Mutagenesis Kit. The SEK1S80A construct was generated from pReceiver SEK1 (GeneCopoeia EX-A0727-Lv105) with Agilent's QuickChange XL Site-Directed Mutagenesis Kit. pReceiver-Lv105 (GeneCopoeia EX-NEG-Lv105) was used as the empty vector (EV) control. Lentivirus was produced by the UCLA Vector Core facility and stable cell lines were made by transducing cells with lentivirus and selecting with antibiotics. Lentivirus-expressing shRNA targeted to SGK was purchased from Sigma.

Evaluation of protein and RNA expression

Western blot analysis was performed as described previously (9, 10). Real-time PCR for SGK RNA and GAPDH RNA was performed as described previously (16). All real-time PCR samples were run in triplicate.

Xenograft model

EV-transfected or SGK-transfected 8226 cells were mixed with Matrigel and injected subcutaneously into the flank of NOD/SCID mice (10^7 cells/mouse). When tumors became palpable (200–400 mm^3), mice were randomized to control (vehicle-injected) or low-dose (0.5 mg/kg) or high-dose (1 mg/kg) bortezomib treatment groups (10 mice/group). Tumor growth was measured every other day as previously described. Vehicle or bortezomib injections were given intraperitoneally, twice per week. Mice were sacrificed when tumor volume reached >2,000 mm^3.

Cytotoxicity, apoptosis, and protein stability assays

The activated caspase-3 flow cytometric assay for apoptosis and MTT cytotoxicity assay were performed as described previously (9, 10). Stability of SGK protein was determined as described previously (17).

Analysis of Millennium gene expression data

The Millennium Pharmaceuticals dataset was accessed from the Broad Institute Multiple Myeloma Genomics Portal and analyze with GENE-E. Sgk expression values were extracted and along with sample information, copied onto Microsoft Excel spreadsheet. All data was sorted and analyzed in Excel. Gene Expression Omnibus (GEO) accession GSE9782 was also used for data analysis.

Statistical analysis

The Student *t* test was used to calculate *P* value. Combinatorial indices (CI) were calculated as described (18) using Calcusyn software version 1.1.1 (Biosoft). CI values were calculated from mean results of four independent apoptosis assays.

Results

SGK protects against bortezomib, tunicamycin, and thapsigargin but not against serum starvation, dexamethasone, or mTORC inhibitors

To test for protective effects of SGK in multiple myeloma cells, we ectopically expressed HA-tagged wild-type (WT) SGK,or a phosphomimetic version (SGK422D) into MMCLs. As AKT overexpression is a well-known antiapoptosis protective effect in multiple myeloma cells (11, 12), we also tested whether SGK transfection had secondary effects on AKT activity and whether AKT transfection had any effects on SGK. Figure 1A demonstrates successful expression of the WT and phosphomimetic SGK transgenes and documents their kinase activity. SGK kinase activity in transfected 8226 and MM1.S MMCLs was demonstrated as an increase in phosphorylation of the SGK substrate NDRG-1 on T346. Supplementary Fig. S1 demonstrates a similar increase in SGK activity in transfected OPM-2 multiple myeloma cells. In all three cell lines (Fig. 1A and Supplementary Fig. S1), expression of the HA-tagged phosphomimetic SGK was significantly lower than the HA-WT SGK. Nevertheless, both transfections resulted in NDRG-1 phosphorylation without any effects on AKT phosphorylation (Fig. 1A). Conversely, expression of WT or phosphomimetic AKT resulted in enhanced AKT phosphorylation but had little effect on NDRG-1 phosphorylation (Fig. 1A). Ectopic expression of WT or phosphomimetic SGK had no consistent effect on multiple myeloma cell growth over 24 to 72 hours (not shown). We also ectopically expressed a KD SGK mutant into 8226 and MM1.S cells (Fig. 1B). The KD mutant had no effect on NDRG-1 phosphorylation.

The SGK-transfected cells were then challenged with increasing concentrations of the mTORC1/mTORC2 inhibitors pp242 or torin, the ER stress inducers bortezomib, thapsigargin, or tunicamycin, as well as dexamethasone or serum starvation. Apoptosis was then measured by flow cytometric analysis of activated caspase-3 expression. As shown in Fig. 1C and D, ectopic expression of WT or phosphomimetic SGK in 8226 or MM1.S MM cells protected against bortezomib and both versions of SGK (WT and phosphomimetic) were comparable. The 8226 assay reports data from 48-hour exposures. A similar protection was seen in 8226 cells at 24 hours (not shown). In contrast, there was no effect of expression of the KD SGK on bortezomib-induced apoptosis (black bars). WT SGK overexpression also protected OPM-2 multiple myeloma cells against bortezomib (Supplementary Fig. S1B). Similar experiments suggested protection against additional ER stress inducers. This is shown for 8226 cells (Fig. 1E) challenged with thapsigargin or tunicamycin as well as in OPM-2 cells (Supplementary Fig. S1C). In contrast, SGK overexpression had no protective effect against the second-generation mTORC1/2 inhibitors pp242 and torin (Supplementary Fig. S2), serum starvation (Supplementary Fig. S3A), or dexamethasone (Supplementary Fig. S3B). In contrast, overexpression of AKT clearly prevented apoptosis induced by mTORC inhibition (Supplementary Fig. S2). Clearly, there are substrates of AKT phosphorylation that are unaffected by SGK which are relevant to apoptosis induced by TORC2 inhibition.

Effects of SGK overexpression on responses to bortezomib were tested in a murine xenograft model. Immunodeficient mice were first challenged subcutaneously with either 10^7 EV-transfected or SGK-transfected 8226 cells and carefully monitored for the earliest detection of palpable tumors. All challenged mice developed

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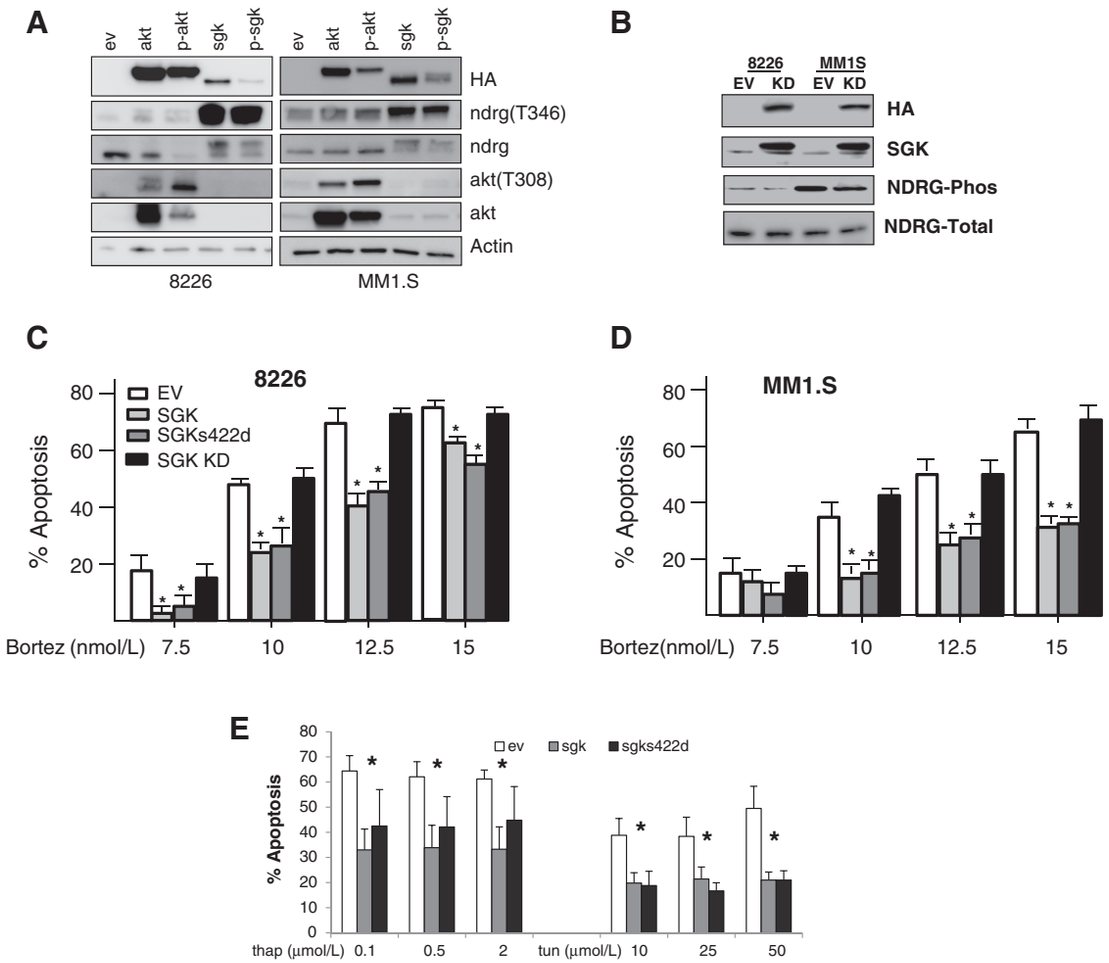

Figure 1.

A, lysates from 8226 and MM1S EV control cells or cells expressing HA-akt, HA-aktS473D [p-akt (phosphomimetic)], HA-sgk, or HA-SGKS422D [p-sgk (phosphomimetic)] were immunoblotted for HA, phospho-ndrg(T346), total ndrg, phospho-akt(T308), akt, and actin. B, lysates from 8226 and MM1.S cells transfected with HA-tagged EV or a KD SGK mutant were immunoblotted. C and D, transfected 8226 and MM1.S cell lines were treated with indicated concentrations of bortezomib for 24 hours (MM1.S) and 48 hours (8226). Apoptosis was then assessed by active caspase-3 flow cytometry. Data represent % apoptosis above nontreated controls, mean ± SE of five experiments. Asterisk (*) denotes apoptosis in SGK (WT) and SGK S422D-expressing cells significantly less than in EV control cells or cells transfected with KD SGK (SGK-KD), $P < 0.05$. E, transfected 8226 cells were similarly treated with increasing concentrations of thapsigargin (thap) or tunicamycin (tun) for 24 hours and apoptosis assessed. Data report % apoptosis above controls, mean ± SE, $n = 3$. Asterisk (*) denotes significantly ($P < 0.05$) decreased apoptosis in both SGK-transfected lines versus EV control.

tumors with a slightly more rapid onset and growth of SGK-transfected tumors versus EV-control tumors (Fig. 2A). When tumors first became palpable, mice were randomized to receive vehicle (control) or bortezomib at concentrations of 0.5 mg/kg (low dose) or 1 mg/kg (high dose). Injections were given twice per week. Bortezomib injections were initiated when tumor volume was identical in the two groups of mice (240 ± 25 mm^3 (mean ± SD) in SGK-transfected tumors and 260 ± 40 mm^3 in EV-transfected tumors). As shown in Fig. 2B, SGK-transfected tumors were relatively resistant to bortezomib. Both high-dose and low-dose bortezomib significantly induced antitumor effects in EV-transfected cells (left) whereas only high-dose bortezomib demonstrated a statistically significant slowing of tumor growth in the SGK-overexpressing cells (right). To ensure that the decreased sensitivity to bortezomib in mice with SGK-transfected tumors was not due to an unexpected effect altering the pharmacodynamics of the drug, we tested tumor lysates for induction of ubiquitination of IKB-α. Bortezomib-induced inhibition of proteasomal function should be seen as an accumulation of ubiquitinated proteins. As shown in Supplementary Fig. S4, lysate from SGK-transfected tumors demonstrated a bortezomib-induced increase in IKB ubiquitination indicating that the relative resistance to tumor cytoreduction was not due to an inability of bortezomib to block the proteasome.

Impact of SGK expression on clinical response to bortezomib

The selective SGK-mediated protection against ER stress inducers indicated a specific cross-talk between an SGK-dependent pathway and ER-stress cascades. As the ER stress induced by bortezomib is a clinically relevant therapeutic modality, we assessed the possible clinical relevance of the SGK-mediated resistance to bortezomib in MMCLs by investigating SGK gene

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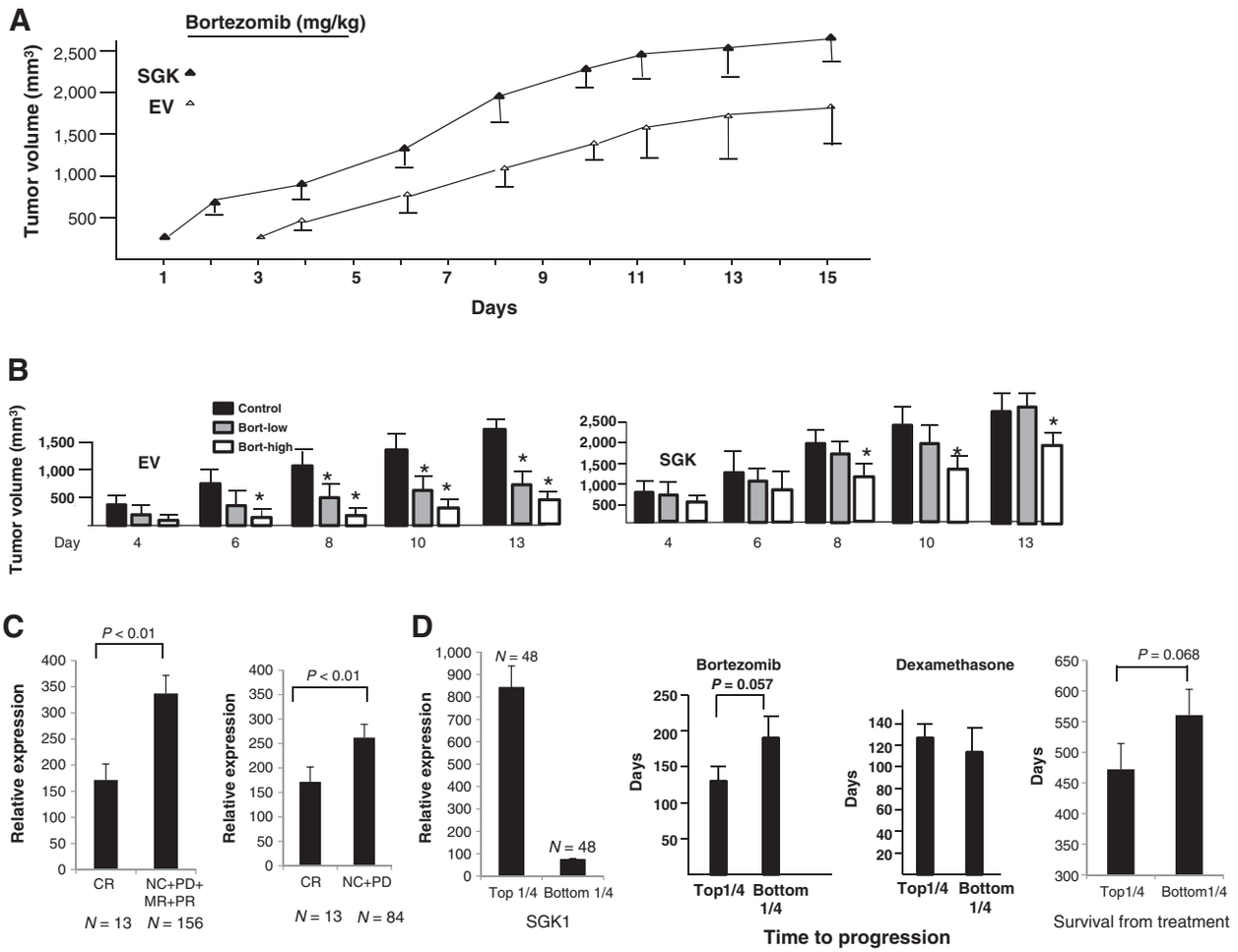

Figure 2.

A, mice challenged with EV- or SGK-transfected 8226 cells (10^7/mouse). Tumor volume represents mean ± SE, $n = 8$ mice/group. B, mice challenged with EV- or SGK-transfected cells and treated with bortezomib. Bortezomib administered intraperitoneally twice per week at 0.5 (low dose) or 1 mg/kg (high dose). Treatment began at time of first detection of tumors. Data are tumor volume (mean ± SE, $n = 10$ mice group). Asterisk (*) represents significantly ($P < 0.05$) decreased tumor volume versus control. C, relative expression values for sgk in bortezomib-treated patients with CR versus no response (NR) + progressive disease (PD) + minimal response (MR) + partial response (PR; left graph) or CR versus NC + PD (right graph). D, expression values for patients were sorted according to increasing sgk expression and divided into quartiles. Left graph indicates SGK expression values for top $^1/_4$ (highest) and bottom $^1/_4$ (lowest) sgk-expressing quartiles. Average time to progression (middle graphs) for patients treated with either bortezomib or dexamethasone comparing those in top quartile versus those in bottom quartile. Data are expressed as mean ± SE. Right graph shows similar data (mean ± SE) for OS.

expression in patients prior to initiation of treatment with bortezomib from three clinical trials [the APEX phase III trial, its companion phase II 040 trial, and the SUMMIT phase II trial (19)], utilizing a public database (GEO). A baseline low SGK expression highly correlated ($P < 0.01$) with the likelihood of obtaining a complete response (CR) to subsequent bortezomib treatment. As shown in Fig. 2C, this is true whether we compared CR patients with all others [NC (no change) + PD (progressive disease) + PR (partial response) + MR (minimal response)] or only to patients not achieving any response (NC or PD). In contrast, there was no significant difference in SGK expression when comparing patients receiving a PR to those with no response or progression (not shown). To further test for an effect of SGK expression on outcome, we divided bortezomib-treated patients into quartiles corresponding to the highest and lowest SGK expression levels (Fig. 2D, left). As shown in Fig. 2D (middle), the median time to progression (TTP) in the quartile with the highest SGK expression was less than the quartile with the lowest expression ($P = 0.057$). In one of these three clinical trials (APEX trial), patients were randomized to bortezomib versus dexamethasone treatment arms. When SGK expression was tested versus TTP in dexamethasone-treated patients, high SGK expression did not associate with decreased time to progression (Fig. 2D, middle). As shown in Fig. 2D (right), the median overall survival (OS) in the quartile with the highest SGK expression was also less in bortezomib-treated patients.

Regulation of bortezomib sensitivity by SGK

The above results in transfected multiple myeloma cells (Fig. 1) suggested SGK activity regulated sensitivity of multiple myeloma cells to ER stress inducers like bortezomib. However, it could be argued that the high SGK expression levels in ectopic transfection

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models were nonphysiologic. We, thus, investigated a role for SGK in nontransfected multiple myeloma cells exposed to bortezomib. Figure 3A demonstrates that SGK protein expression and activity (i.e., NDRG-1 phosphorylation) was significantly upregulated by exposure of parental 8226, MM1.S, and OPM-2 cells to bortezomib. A time course experiment (Fig. 3B) demonstrated enhanced bortezomib-induced SGK expression by 3 hours which lasted at least up to 6 hours of exposure. These data are reminiscent of previous work (11) demonstrating a stimulation of AKT activity as an adaptive response to bortezomib in multiple myeloma cells. However, while SGK protein expression was significantly increased by bortezomib, AKT expression is unaffected (11). The relatively short half-life of SGK (17) and ubiquitin/proteasome mode of degradation probably accounts for some of the bortezomib-induced upregulation of protein expression. Protein stability assays support this notion (Supplementary Fig. S5). Exposure to thapsigargin also upregulated SGK expression (Fig. 3C).

We next investigated whether this upregulated SGK expression is a protective response to bortezomib in untransfected cells. SGK knockdown by itself is toxic to multiple myeloma cells (13) so we utilized an SGK inhibitor where kinase activity could be reduced

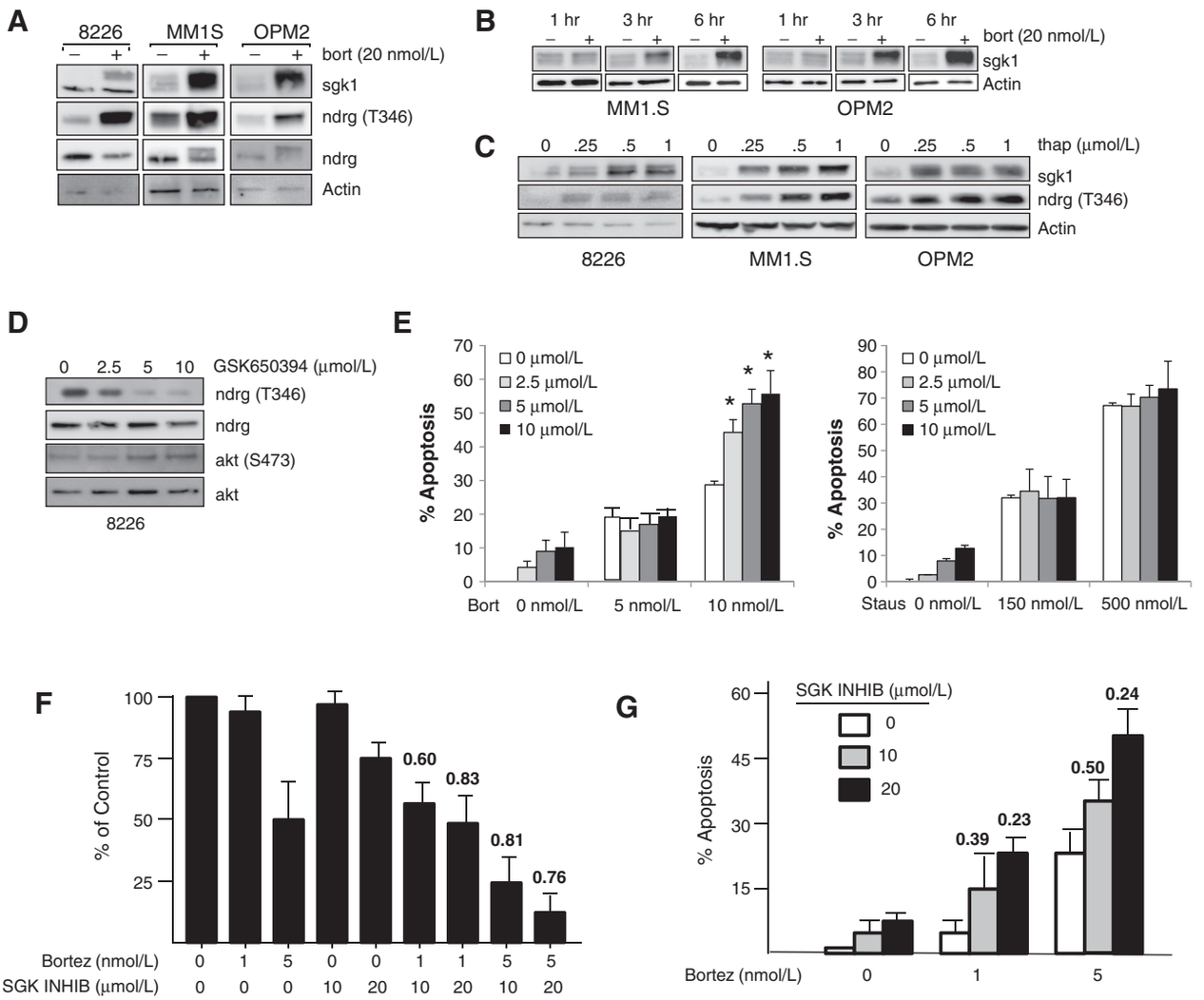

Figure 3.

A, parental 8226, MM1.S, or OPM-2 lines treated ± bortezomib (bort) at 20 nmol/L for 8 hours followed by immunoblot. B, parental MM1.S and OPM-2 cell lines treated ± bortezomib (20 nmol/L) for 1, 3, or 6 hours followed by immunoblot assay. C, 8226, MM1.S, and OPM-2 lines treated with increasing concentrations of thapsigargin (μmol/L) for 7 hours, followed by immunoblot assay. D, 8226 cells treated with increasing concentrations of SGK inhibitor (GSK650394) for 3 hours, followed by immunoblot assay. E, percent apoptosis (flow cytometry for activated caspase-3, mean ± SE, $n = 5$) of 8226 cells treated with increasing concentrations of SGK inhibitor [0, 2.5, 5, or 10 μmol/L (different colored bars)] in combination with bortezomib (bort; 0, 5, or 10 nmol/L; left) or staurosporine (staus; 0, 150, or 500 nmol/L; right). Asterisk (*) denotes % apoptosis in combined treatment groups that is significantly ($P < 0.05$) greater than the arithmetic sum of the individual treatments. F, primary CD138-isolated multiple myeloma cells incubated with increasing concentrations of bortezomib ± SGK inhibitor and cell survival assayed 48 hours later. Data are viable cell recovery versus untreated cells (% of control), mean ± SD, $n = 4$. CI values for combinations are shown above the bars. G, same primary cell preparations as in "F" ($n = 4$) were assayed for apoptosis (flow cytometry for activated caspase 3). Results are % mean apoptosis ± SD. CI values shown above bars.

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but not completely ablated. As shown in Fig. 3D, the inhibitor (GSK 650394) prevented SGK phosphorylation of NDRG-1. Inhibition was specific for SGK as AKT phosphorylation was maintained. In fact, AKT phosphorylation even slightly increased at high concentrations of the inhibitor. The SGK inhibitor is modestly cytotoxic, by itself, at 72 and 96 hours but has little effect at 24 hours. At 24 hours, the inhibitor, when used alone at 2.5, 5, or 10 μmol/L, induced minimal apoptosis above control (3%, 8%, and 10% apoptosis, respectively) in 8226 cells (Fig. 3E). When combined with 10 nmol/L bortezomib for 24 hours, 2.5 μmol/L of inhibitor increased apoptosis from 29% to 44%, 5 μmol/L increased it to 53% and 10 μmol/L increased it to 56%. These values (means ± SD of four separate experiments as shown in Fig. 3E) resulting from combined treatment are significantly ($P < 0.05$) greater than the sums of individual treatments (shown by asterisks). Combinatorial indices <1 (Supplementary Fig. S6) were also identified indicating a synergistic interaction. The fact that AKT activity slightly increased with use of the SGK inhibitor (Fig. 3D), possibly due to a yet explained negative feedback relationship between AKT and SGK activity, makes the synergistic increase in anti-multiple myeloma cytotoxicity even more impressive. As shown in Fig. 3E (right), when identical concentrations of the SGK inhibitor were combined with a nonspecific inducer of apoptosis (staurosporine), there was no enhancement and all CI values were >1 (Supplementary Fig. S6). The lack of a nonspecific enhancement of multiple myeloma cell apoptosis (e.g., with staurosporine) strengthens the notion of a specific SGK-dependent regulation of bortezomib-induced multiple myeloma cell death.

The SGK inhibitor also synergizes with bortezomib for anti-multiple myeloma effects in primary specimens. As shown in Fig. 3F, the inhibitor used alone has minimal effect (none at 10 μmol/L and 25% cytotoxicity at 20 μmol/L). When the nontoxic 10 μmol/L inhibitor is added to 1 nmol/L bortezomib, cytotoxicity increases from 7% to 40% and, when added to 5 nmol/L bortezomib, cytotoxicity increases from 50% to 75%. A similar increase in cytotoxicity is seen when 20 μmol/L inhibitor is used. Combinatorial indices are shown above the bars in Fig. 3F and demonstrate a synergistic interaction. When 1° cells were assayed by flow cytometry for apoptosis induction, a similar synergy was identified with CIs of 0.23–0.5 (Fig. 3G).

SGK mediates protection against bortezomib in BR multiple myeloma cells

A previous study (14) has shown that in 8226 cells selected for resistant to bortezomib, the IGF-1 pathway was upregulated and this upregulation served as a mechanism for bortezomib resistance. As IGF-1 theoretically would activate SGK as well as AKT, we assessed whether these BR 8226 cells demonstrated activated SGK, AKT, or both kinases. These resistant cells did not significantly downregulate synthesis of monoclonal lambda light chains which could have restrained ER front-loading (86% and 94% Ig lambda synthesis vs. control 8226 cells after 24 and 48 hours of culture). However, as shown in Fig. 4A, nontreated BR 8226 cells (demonstrated significantly increased levels of SGK expression, SGK activity (NDRG phosphorylation) as well as AKT phosphorylation relative to parental WT cells. As expected from above Fig. 3, these activities are further increased by bortezomib exposure. However, it is notable that total SGK protein expression is also markedly increased in BR cells whereas total AKT expression is relatively unaffected. The enhanced SGK expression in 8226 BR cells versus WT cells ($7.5 \pm 1.5 \times$ fold increase by densitometric ratio of SGK/actin, mean ± SE, $n = 3$) is comparable with that achieved by transfection of 8226 cells with the WT SGK vector (example in Fig. 4A, right side, $8.7 \pm 2 \times$ fold increase, $n = 5$ separate experiments). The increase in total SGK expression in resistant BR cells is also present at the RNA level (Fig. 4B).

To determine whether SGK1 participates in mediating bortezomib resistance in this cell model, the SGK1 inhibitor was once again used in combination with bortezomib to examine whether inhibiting SGK1 would sensitize resistant BR 8226 cells to bortezomib. First, to ensure GSK650394 was effective in inhibiting SGK in BR cells, they were treated with increasing concentrations of GSK65039 and assessment of ndrgT346 phosphorylation was performed. As shown in Fig. 4C, the concentration required to inhibit NDRG phosphorylation was greater in BR cells than in parental 8226 cells (see above, Fig. 3), probably due to the fact that increased SGK expression is found in the resistant cells. In fact, only 40 μmol/L consistently inhibited SGK activity. Once again, at the highest concentration of the SGK inhibitor, an increase in AKT phosphorylation was seen. Next, in an MTT assay, resistant BR and WT 8226 cells were challenged with different combinations of GSK65309 and bortezomib concentrations for 24 hours (Fig. 4D). When compared with 8226wt cells, BR cells treated with bortezomib retained resistance up to a concentration of 20 nmol/L of GSK65309. However, the SGK inhibitor at a concentration of 40 μmol/L significantly reversed 8226BR resistance to bortezomib at concentrations of 15 and 20 nmol/L. This result implicates SGK1 as one of the mediators of bortezomib resistance in 8226BR cells.

Additional experiments demonstrated a reversal of bortezomib resistance in BR cells following shRNA knockdown of SGK (Fig. 4E). SGK knockdown itself decreased multiple myeloma cell survival by 25%. The addition of bortezomib further induced cytoreduction whereas control BR cells infected with an shRNA targeting a scrambled sequence (SCR) remained completely resistant to bortezomib. Although these latter experiments are a little more difficult to interpret because of the anti-multiple myeloma effect of shRNA used alone (as described in ref.13), they demonstrate the ability of SGK knockdown to significantly sensitize BR cells to bortezomib.

Molecular mechanism of SGK's regulatory effect

Enhanced ER stress stimulates the unfolded protein response (UPR) which consists of three arms that initially protect against proteotoxicity but which induce apoptotic death if ER stress is not sufficiently relieved (20). One of these is stimulated by IRE-1 (20). IRE-1, an ER transmembrane protein activated by increased malfolded protein loads, contains a cytosolic domain with both kinase and endoribonuclease activities (21). The latter mediates splicing and activation of the protective XBP-1 transcription factor whereas IRE-1 kinase activity results in activation of ASK-1 with subsequent stimulation downstream through SEK and JNK1. It has been reported that a JNK inhibitor prevents bortezomib-induced multiple myeloma cell death (22), indicating that the IRE-1/ASK-1/JNK pathway is an effector apoptotic pathway in bortezomib-treated multiple myeloma cells. We confirmed that finding in our cells utilizing a JNK inhibitor as shown in Fig. 5A. We then tested whether SGK-overexpressing multiple myeloma cells are altered in stimulation of this apoptosis-inducing pathway. Results shown in Fig. 5B support this notion. As shown, bortezomib treatment of EV control MM1.S and 8226 cells

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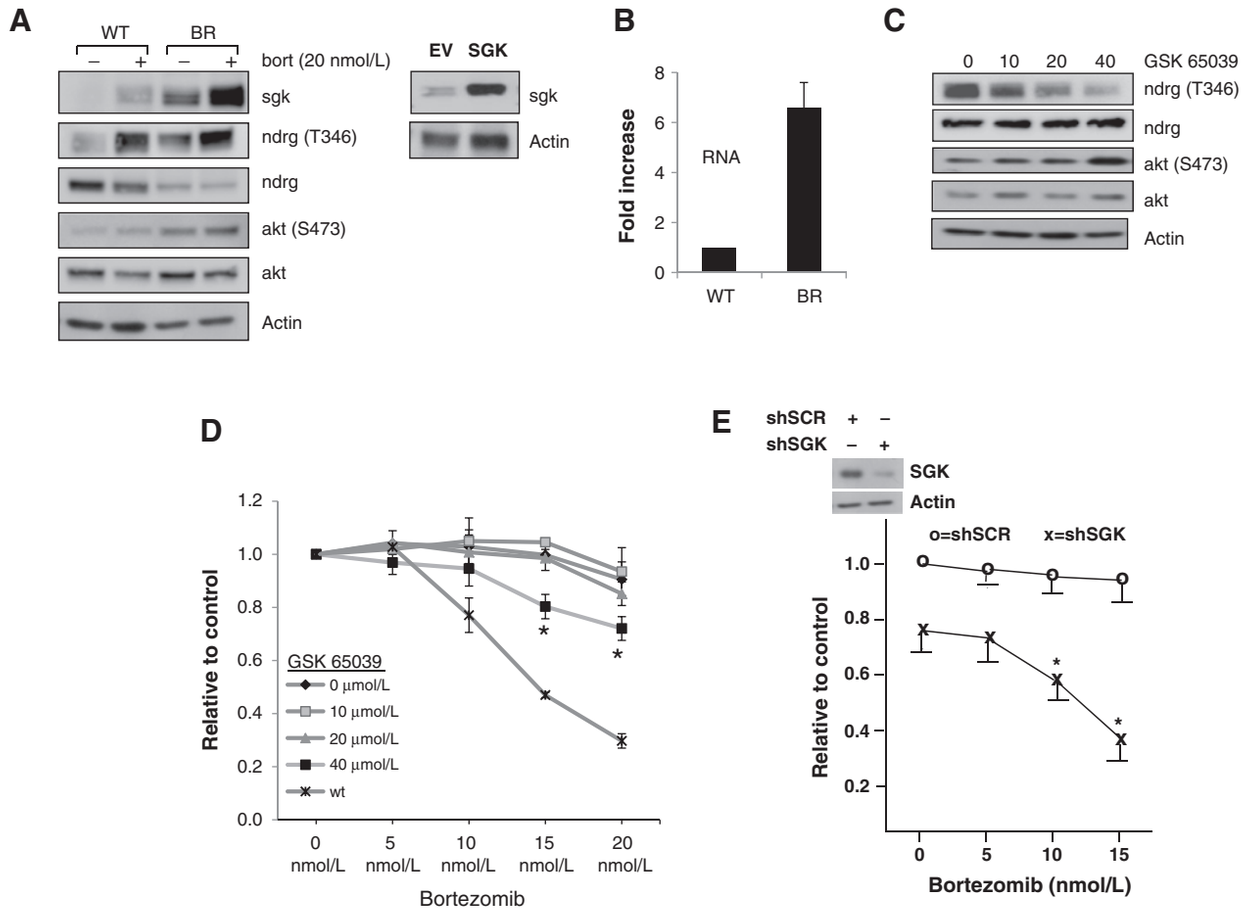

Figure 4.

A, control (WT) or BR 8226 lines, treated ± bortezomib (20 nmol/L) for 8 hours, followed by immunoblot assay. Right: example of increased SGK expression in 8226 cells transfected with WT SGK versus EV. B, qt-PCR assessment of SGK RNA expression in control (WT) or resistant (BR) lines. C, BR cells treated with SGK inhibitor for 3 hours, followed by immunoblot assay. D, MTT assay of control (WT) or BR cells treated with bortezomib for 24 hours. BR cells also exposed to increasing concentrations of the SGK inhibitor GSK 65039. Data represent mean ± SE, $n = 4$. * denotes significantly ($P < 0.05$) decreased cell survival compared with BR cells exposed to 0 μmol/L of SGK inhibitor. E, survival assay of BR cells infected with lentivirus expressing shRNA for SGK (shSGK) or control targeting a scrambled sequence (shSCR). Cell lines treated with an increasing concentration of bortezomib for 24 hours. Data are mean ± SE, $n = 3$ with * denoting significant decrease in bortezomib-induced survival versus no bortezomib ($P < 0.05$).

induces phosphorylation of IRE-1, JNK, and the c-jun JNK substrate. This is best seen following 20 nmol/L of bortezomib. However, in SGK-transfected cell lines, the induction of JNK and c-jun phosphorylation is abated. In contrast, bortezomib-stimulated phosphorylation of IRE-1 is unaffected by SGK overexpression indicating that SGK's inhibitory effect on JNK occurs downstream of IRE-1 phosphorylation. Additional support for the relevance of both pathways is found in BR 8226 multiple myeloma cells. As shown in Fig. 5C, these SGK-activated cells are relatively deficient in activation of JNK phosphorylation when exposed to bortezomib.

An additional UPR pathway resulting in CHOP induction has also been identified as an effector of ER stress–induced apoptosis (23). CHOP may accomplish this through its transcriptional induction of the DR5 death receptor (23). In multiple myeloma cells, SGK overexpression constrains bortezomib-induced expression of CHOP and DR5 (Supplementary Fig. S7). However, DR5 silencing in untransfected multiple myeloma cells had no effect on bortezomib-induced apoptosis (Supplementary Figs. S7B and S7C) so the significance of this SGK-dependent regulation is unclear.

The ability of SGK to prevent JNK activation might be explained by effects on SEK. As described above, JNK is activated during ER stress following upstream signaling through an IRE-1/ASK-1/SEK pathway. SGK phosphorylates SEK on S78 in murine cells which corresponds to S80 in human cells (24) and, in so doing, inhibits its ability to phosphorylate and activate JNK. Enhanced phosphorylation of SEK on S80 is shown in 8226 and MM1.S MM cells transfected with SGK and exposed to bortezomib (Fig. 6A). In addition, exposure of two preparations of primary multiple myeloma cells to bortezomib also resulted in enhanced SEK phosphorylation on S80 (Fig. 6B). To test whether this phosphorylation of SEK contributed to SGK-mediated resistance to bortezomib in cell lines, we overexpressed a SEK phospho-mutant [serine-to-alanine at position 80 (S80A)] which is resistant to SGK phosphorylation and regulation. As shown in Fig. 6C, the SEK1

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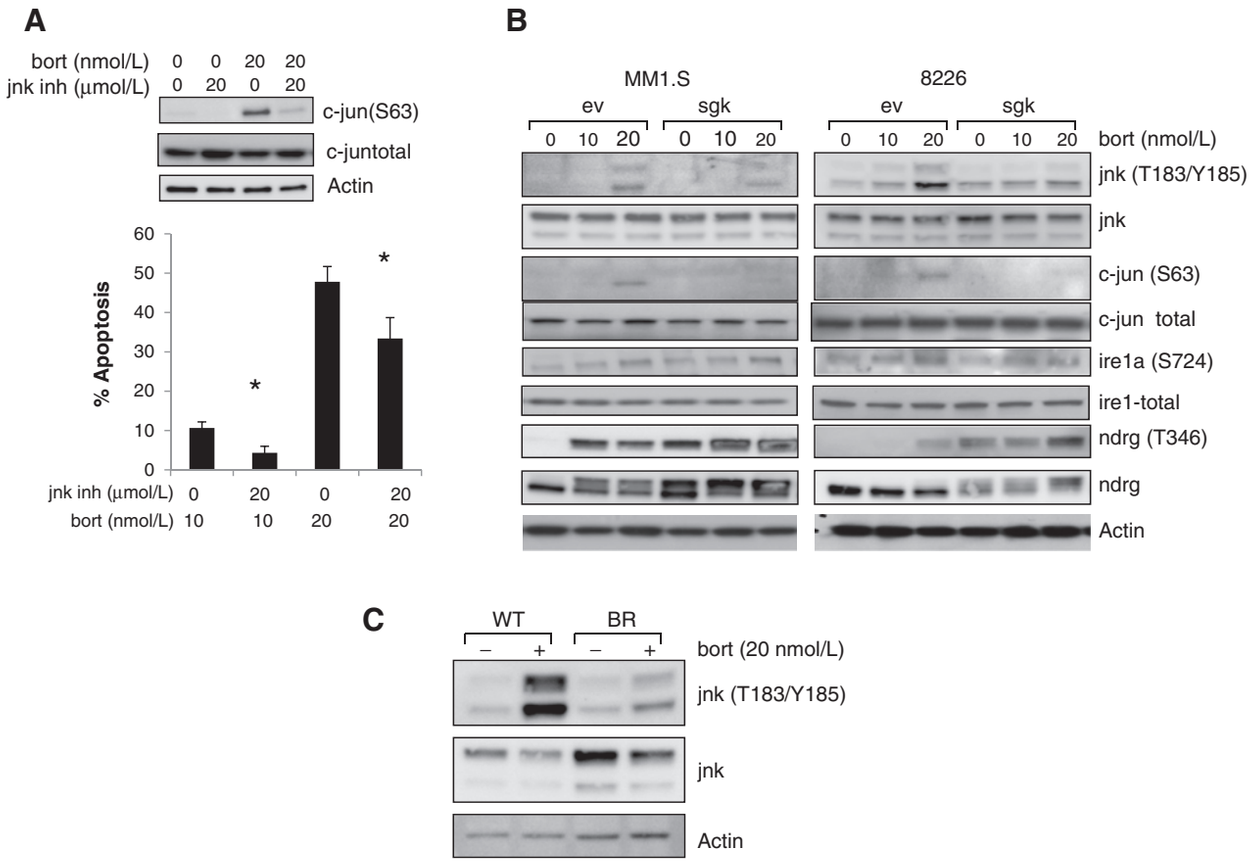

Figure 5.
A, 8226 cells treated with bortezomib ± a JNK inhibitor followed by immunoblot assay for phospho-c-jun and an apoptosis assay. Apoptosis data are mean ± SE, $n = 3$. Bortezomib-induced apoptosis was significantly ($P < 0.05$) inhibited by the JNK inhibitor. Asterisk (*) denotes significant decreased apoptosis, $P < 0.05$, versus bortezomib treatment without the JNK inhibitor. B, MM1.S (left) and 8226 cells (right) transfected with EV or WT SGK (sgk), treated ± bortezomib at 10 or 20 nmol/L for 8 hours, followed by immunoblot assay for phosphorylated JNK (T183/Y185), total JNK, phosphorylated c-jun (S63), phosphorylated IRE-1 (S724), phosphorylated NDRG (T346), or total NDRG. C, BR cells or control WT cells treated ± bortezomib for 8 hours followed by immunoblot assay.

S80A mutant was abundantly expressed versus endogenous SEK1, and was able to enhance bortezomib-induced JNK kinase activity assessed by c-jun phosphorylation. To evaluate whether SEK1 S80A could overcome SGK protection against bortezomib, EV control and SEK1 S80A-expressing 8226 SGK and SGKS422D cells were challenged with bortezomib for 24 hours and cell survival (cytotoxicity) and apoptosis was then assessed. As shown previously, when compared with control cells, SGK overexpressing multiple myeloma cells were resistant to cytotoxicity (Fig. 6D) and apoptosis (Fig. 6E). However, expression of SEK1S80A in SGK-overexpressing cells moderately but significantly ($P < 0.05$, denoted by asterisks) overcame this protection (Fig. 6D andE). These results indicate that bortezomib resistance mediated by SGK activity is partly dependent upon SGK's phosphorylation and inactivation of SEK.

Discussion

The results of this study indicate that SGK levels and activity regulate sensitivity of multiple myeloma cells to ER stress–induced cytotoxicity. SGK overexpressing MMCLs were specifically resistant *in vitro* to thapsigargin, tunicamycin, and bortezomib but were not altered in sensitivity to mTOR inhibitors, dexamethasone, or serum starvation. They were also relatively resistant to bortezomib used in mice. In addition, a MMCL *in vitro* selected for bortezomib resistance demonstrated upregulated SGK expression and activity. Furthermore, low SGK expression levels in patients significantly correlated with attainment of CR following bortezomib treatment and high SGK levels were associated with a shorter TTP and OS. As heightened signaling in multiple myeloma cells through the IGF-1 axis has been previously correlated with poor outcome in bortezomib-treated patients (14), it is possible that the association of SGK expression with poor outcome is due to the fact that expression is simply a marker for IGF-1 signaling. However, prior work (13, 25) did not identify IGF-1–mediated induction of SGK expression suggesting that SGK expression is an independent predictor of outcome. Mechanistically, SGK over activity protected bortezomib-treated multiple myeloma cells via its ability to phosphorylate SEK which downregulated subsequent JNK phosphorylation/activation. SGK also inhibited induction of the proapoptotic CHOP/DR5 pathway.

In addition to ER stress, SGK expression is enhanced in cells exposed to other stress stimuli (7) and to GCs (5, 6). In results not

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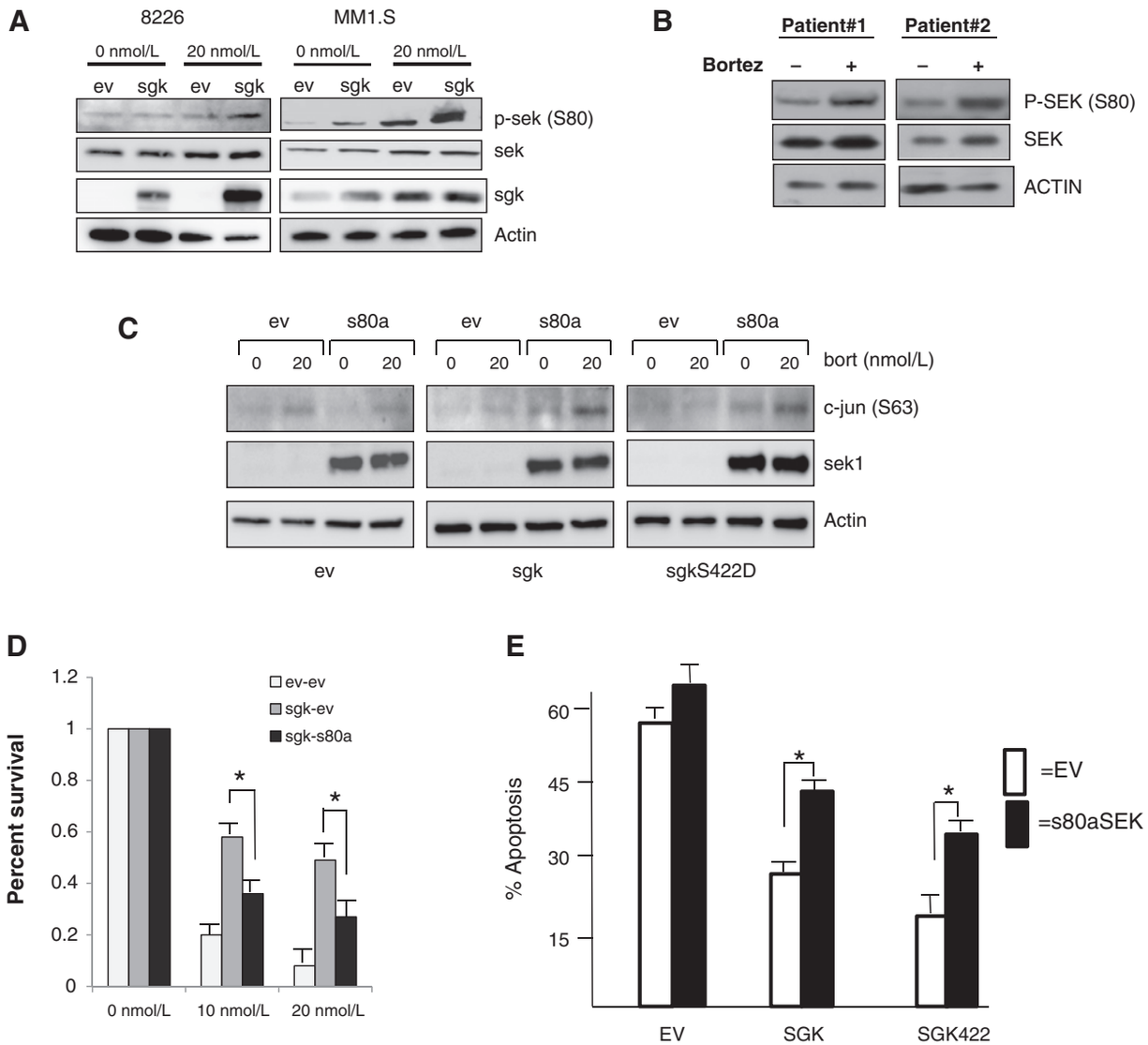

Figure 6.

A, EV- or SGK-transfected 8226 and MM1.S cells treated ± bortezomib (20 nmol/L) for 8 hours, followed by immunoblot for phospho-SEK, total SEK, SGK, and actin. B, two separate CD-138–purified primary cell preparations harvested from patient bone marrow and treated ± bortezomib (20 nmol/L for 6 hours) followed by immunoblot. C, EV-, WT SGK (sgk)-, or phosphomimetic SGK (sgkS422D)-transfected 8226 cells were stably transfected with lentivirus expressing an empty insert (EV) or the SEK (S80A) phospho-mutant. Following treatment ± bortezomib at 20 nmol/L (4 hours), immunoblot assay was performed for phosphorylated c-jun (S63), SEK1, or actin. D, EV-transfected 8226 cells, secondarily transfected with lentivirus expressing an empty insert (EV-EV), SGK overexpressing cells secondarily transfected with the same EV (SGK-EV) or SGK overexpressing cells secondarily transfected with the SEK mutant (SGK-S80A), treated ± bortezomib at 0, 10, or 20 nmol/L for 24 hours, followed by cytotoxicity assay. Results are percent cells surviving versus untreated groups (bortezomib = 0 nmol/L), mean ± SE, $n = 3$. Asterisk (*) denotes significant ($P < 0.05$) difference in cytotoxicity between SGK overexpressing cells transfected with EV versus phospho-mutant (s80a) SEK. E, EV-, WT SGK (SGK) or phosphomimetic SGK (SGK422)-overexpressing multiple myeloma cells secondarily transfected with EV (white bars) or SEK S80A mutant (black bars), treated ± 10 nmol/L bortezomib, followed by apoptosis assay. Results are percent apoptosis above control (no bortezomib), mean ± SE, $n = 4$; Asterisk (*) denotes significant ($P < 0.05$) difference in apoptosis between EV versus phospho-mutant (s80a) SEK.

shown, we confirmed this SGK activation in our MMCLs treated with dexamethasone. Although preexposure to dexamethasone protected against bortezomib, this protection could not be negated by SGK inhibition, indicating resistance resulted from SGK-independent effects. The data suggest that SGK enhancement during ER stress provides a significant antiapoptotic protection whereas similar induction occurring subsequent to dexamethasone challenge does not. This is reminiscent of a prior study (26) which showed that SGK plays a protective antiapoptotic role when induced by stress stimuli whereas GC-induced SGK does not alter apoptosis. IL6 and bone marrow stromal cells also induce SGK expression in multiple myeloma cells (13) and this may also function to protect them from apoptosis.

A previous publication (27) demonstrated that multiple myeloma cells had heightened basal, presumably protective, UPR function, and were rapidly induced into a terminal lethal phase

when exposed to bortezomib. The ability of a JNK inhibitor to prevent bortezomib-induced multiple myeloma cell death in this report confirms prior work by the Dana-Farber group and implicates the IRE-1/ASK-1/SEK/JNK UPR pathway. As recently demonstrated (28, 29), JNK, activated during ER stress, translocates to and binds the mitochondrial protein Sab, followed by disrupted mitochondrial respiration, increased mitochondrial ROS generation and apoptosis. SGK overexpression restrained this apoptotic pathway suggesting a mechanism of resistance. These engineered resistant cell lines became resensitized by expression of a SEK phospho-mutant which further suggests SGK protection functions via phosphorylation and inactivation of SEK.

Although a SEK-dependent mechanism is supported in studies of SGK-transfected cells, it is not completely clear whether SEK phosphorylation is relevant to the clinical setting or relevant to non-SGK hyperactivation models. SEK S80 phosphorylation is enhanced by bortezomib exposure in EV-transfected control MMCLs (Fig. 6A, most clearly seen in MM1.S cells) as well as in primary specimens (Fig. 6B) providing some support for the relevance of SGK phosphorylation of SEK. It is also clear that the SGK inhibitor synergizes with bortezomib in nontransfected MMCLs and primary cells (Fig. 3E–G). However, the S80a mutant did not significantly increase c-jun phosphorylation (Fig. 6C) or apoptosis (Fig. 6E) in bortezomib-treated EV control cells. Quite possibly, an additional SEK-independent result of SGK activity, such as prevention of CHOP induction (i.e., as in Supplementary Fig. S7A) or inhibition of a different stress activated kinase located between IRE-1 and SEK, plays a greater role in sensitivity of cells that do not contain hyperactivated SGK. A recent report (23) identified a DR5-dependent pathway as an additional mediator of UPR-stimulated apoptosis. SGK overexpression also restrained DR5 induction although the significance of this is unclear.

A previous study (14) supported the notion that signaling through the IGF-receptor (IGF-R) axis regulates sensitivity of multiple myeloma cells to bortezomib and this has prompted the development of clinical trials testing the combination of bortezomib with IGF-R inhibitors. Although the IGF/IGF-R axis does not necessarily induce expression of SGK, once SGK expression is upregulated, it would be primed for rapid activation by enhanced IGF-R-mediated signaling. Thus, enhanced SGK expression may synergize with activated IGF-R signaling for overwhelming resistance. Presumably, an IGF-R inhibitor would downregulate SGK signaling and resensitize multiple myeloma cells to bortezomib.

The other key AGC kinase, AKT, also protects against bortezomib-induced multiple myeloma cell death (11) and is a potential target for AKT inhibitors to be combined with proteasome inhibitors. In similar fashion to our study with SGK, the effect of AKT was to regulate the downstream ER stress–induced pathways that presumably result in multiple myeloma cell death. An additional similarity was the activation of AKT upon bortezomib exposure [i.e., S473 phosphorylation (Fig. 5C and E inref. 11)], comparable with SGK activation (i.e., NDRG-1 phosphorylation) in similarly treated cells. One difference was the finding that SGK protein expression was significantly increased by bortezomib (Fig. 3A) whereas AKT expression is unaffected (11). Although results in Supplementary Fig. S5 support the notion that part of the upregulated SGK expression is due to proteasome inhibition and increased SGK protein stability, bortezomib exposure also resulted in a modest increase in SGK RNA (1.3 times control, not shown). In addition, the finding that thapsigargin, an independent ER stress inducer without proteasome inhibition, also enhanced SGK expression (Fig. 3C) suggests additional mechanisms of upregulated SGK protein expression.

In summary, our results indicate a regulatory role for SGK in the multiple myeloma cell response to ER stress. The data provide a rationale for future development of SGK inhibitors in this disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: B. Hoang, A. Lichtenstein
Development of methodology: B. Hoang, Y. Shi, P.J. Frost, V. Mysore, A. Lichtenstein
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B. Hoang, P.J. Frost, V. Mysore
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B. Hoang, P.J. Frost, A. Lichtenstein
Writing, review, and/or revision of the manuscript: B. Hoang, A. Lichtenstein
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B. Hoang, Y. Shi, C. Bardeleben
Study supervision: B. Hoang, A. Lichtenstein

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SGK Kinase Activity in Multiple Myeloma Cells Protects against ER Stress Apoptosis via a SEK-Dependent Mechanism

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