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The large conductance calcium-activated potassium channel affects extrinsic and intrinsic mechanisms of apoptosis

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Abstract

The large conductance calcium-activated K⁺ or BK channel underlies electrical signals in a number of different cell types. Studies show that BK activity can also serve to regulate cellular homeostasis by protecting cells from apoptosis due to events such as ischemia. Recent coimmunoprecipitation studies combined with mass spectrometry suggest putative protein partners that interact with BK to regulate intrinsic and extrinsic apoptotic pathways. Here, we test two of those partners to determine the effects on these two signaling pathways. Through reciprocal coimmunoprecipitation (coIP) experiments, we show that BK interacts with p53 and FADD both in mouse brain and when overexpressed in a heterologous expression system, such as HEK293 cells. Moreover, coIP experiments using N- and C-terminal fragments reveal that FADD interacts with the C-terminus of BK, whereas p53 interacts with either the N- or C-terminus. Immunolocalization studies show that BK colocalizes with p53 and FADD in the mitochondrion and plasmalemma, respectively. HEK293 cells that stably express BK are more resistant to apoptosis when p53 or FADD are overexpressed or when their intrinsic and extrinsic pathways are stimulated via Mitomycin C or TNF-related apoptosis-inducing ligand (TRAIL), respectively. Moreover, when stimulating with TRAIL, caspase 8 activation decreases in BK expressing cells. These data suggest that BK may be part of a larger complex of proteins that protect against apoptosis by interacting with pro-apoptotic proteins such as p53 and FADD.

Keywords

BK channel; p53; FADD; protein-protein interactions; apoptosis

INTRODUCTION

The various stages of the cell cycle are regulated by numerous proteins, which intricately interact with each other in the process of cell proliferation and apoptosis. Among these are p53 and FADD. Localized to the nucleus, p53, has a principal role in repairing DNA lesions for cell survival, by activating DNA damage checkpoints during the cell cycle or to induce apoptosis (Roos & Kaina, 2006). P53 also translocates to the mitochondria and increases the permeabilization of the mitochondrial membrane by inducing the pro-apoptotic proteins,

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Bax and Bak of the Bcl-2 family (Lindenboim et al., 2011). While p53 mediates the intrinsic pathway of apoptosis through the mitochondria (Vaseva & Moll, 2013), the Fas Associated Protein with Death Domain (FADD) is an important adaptor protein that mediates apoptosis initiated via extrinsic pathways such as death receptors in the plasmalemma. FADD is composed of two protein interaction domains: a death domain and a death effector domain (DED) (Chinnaiyan et al., 1995). The death domain of FADD binds directly to the death domain of death receptors, such as Fas and TRAIL-RI and -RII, whereas its DED interacts with procaspase 8, triggering the activation of apoptosis (Fischer et al., 2003).

BK is a potassium channel that is ubiquitously expressed with a highly conserved amino acid sequence. It underlies physiological functions such as muscle contraction, synaptic transmission, and hearing sensitivity. BK can be described in three parts: a small extracellular N-terminus, seven transmembrane domains containing a pore for the efflux of K^+ , and a large cytosolic tail with a Ca^{2+} sensor. BK is found in the plasmalemma and inner mitochondrial membrane (Skalska et al., 2009) and interacts with numerous proteins such as actin (Zou et al., 2008), cortactin (Tian et al., 2006), Rab11b (Sokolowski et al., 2012), Akt, and GSK among others (Kathiresan et al., 2009; Bian et al., 2011; Sokolowski et al., 2011). Recent evidence shows that BK can save cells from death during cerebral ischemia (Liao et al., 2010), while bioinformatic analyses suggest that this channel may be part of a larger interactome that regulates cell apoptosis (Kathire et al., 2009; Sokolowski 2011). This phenomenon likely entails a number of protein interactions that give BK a biological role in cell viability/apoptosis. In the present study, we examined such an interaction to test this hypothesis, by determining if BK interacts directly with proapoptotic proteins, p53 and FADD.

MATERIALS AND METHODS

Cloning

Two tandem hemagglutinins (HA) were ligated to the C-terminus of BK using a previously cloned DEC-type α -subunit (aa residues M1-C1195) (Kathiresan et al., 2009) as a template for a forward primer: 5'-CCG CTC GAG GCT CTA ATA GCT GAG GA -3' with a *XhoI* sequence at 5' that was set at the *XhoI* site in BK. The reverse primer with an *XbaI* site at 5': 5'-TGC TCT AGA CTA AGC GTA ATC AGG AAC GTC GTA AGG GTA AGC GTA ATC AGG AAC GTC GTA AGG GTA ACA TTC ATC TTC AAC TTC TCT GAT TGG A -3' contained a tandem HA sequence. PCR conditions were: denaturation at 94°C for 2 min, then 35 cycles at 94°C for 30 s, annealing at 55°C for 30 s, extension at 68°C for 2 min and final extension at 72°C for 10 min. The fragment was gel-purified and ligated to pcDNA3.1-BK, which was cut at *XhoI* and *XbaI*.

Clonal p53 with a V5 tag was constructed by amplifying p53 from total RNA obtained from mouse cochlea with primers designed from a mouse p53 sequence (Accession No. NM_011640). The forward primer for RT-PCR consisted of: 5'-CGC GGA TCC ACC ATG GGT AAG CCT ATC CCT AAC CCT CTC CTC GGT CTC GAT TCT ACG ACT GCC ATG GAG GAG TCA CA -3' followed by a *BamHI* site at 5' with a V5 tag and the reverse primer: 5'-ACG CGT CGA CAG TCA GTC TGA GTC AGG CCC CAC T-3' with a *SalI*

site at 5'. The gel-purified PCR product of p53 was cloned to the sites of *Bam*HI at 5' and *Xho*I at 3' in pcDNA3.1 (V5-p53).

FADD was also cloned from mouse cochlea by RT-PCR using a primer pair designed from mouse FADD (Accession No. NM_010175). The forward primer: 5'-CGC GGA TCC ACC ATG GGT AAG CCT ATC CCT AAC CCT CTC CTC GGT CTC GAT TCT ACG GAC CCA TTC CTG GTG CTG CT-3' was followed by a *Bam*HI site at 5' with V5 tag and the reverse primer: 5'-CCG CTC GAG AGG CTT GTC AGG GTG TTT CTG AGG A -3' was attached at a *Xho*I site at 5'. The insert of FADD was gel-purified and ligated to a *Bam*HI site at 5' and a *Xho*I site at 3' of pcDNA3.1 (V5-FADD). RT conditions for p53 and FADD were 50°C for 30 min, followed by PCR conditions as before. Primers were synthesized (Integrated DNA Technologies) and clones confirmed by sequencing (University of Florida, Gainesville, FL).

To determine potential interaction sites between BK and p53 or FADD, three BK cDNA constructs were used to cotransfect HEK293 cells with either p53 or FADD. Full-length BK was made as mentioned previously. Truncated BK variants missing either the N- or C-terminus were cloned using the parameters described above, but with different primer pairs. BK, inclusive of the N-terminus and 7 transmembrane domains (aa residues M1-M314), was cloned into pcDNA3.1 at restriction sites *Bam*HI and *Xho*I using forward primers, as noted above with a double HA tag and reverse primer: 5'-CCG CTC GAG TTA CAT GGC CAG TCC CCC GAG GA -3' with an *Xho*I site attached at the 5' end. BK, inclusive of the C-terminus only, contained aa residues M314-C1195. This construct was cloned into pcDNA3.1 at restriction sites *Bam*HI and *Xho*I using forward primer: 5'-CGC GGA TCC ACC ATG TTT GCC AGC TAC GTC CCT GA -3' with a *Bam*HI site attached at the 5' end and reverse primer: 5'-TGC TCT AGA CTA AGC GTA ATC AGG AAC GTC GTA AGG GTA AGC GTA ATC AGG AAC GTC GTA AGG GTA ACA TTC ATC TTC AAC TTC TCT GAT TGG A -3' with an *Xba*I site and a tandem HA sequence attached at the 5' end.

Coimmunoprecipitation

All procedures for procuring tissue were accomplished using NIH guidelines and approved by the USF IACUC. Whole brain was dissected from a postnatal day (PD) 21 CBA/J mouse, rinsed in cold PBS and sonicated in RIPA buffer, then centrifuged at 20,800 x g, 4°C for 20 min. Immunoprecipitations were accomplished using the immunocomplex-capture technique. Lysates were pre-cleared with Protein G-Sepharose beads (GE Healthcare) followed by incubation with 3 µg of either anti-p53 monoclonal antibody (Santa Cruz), or anti-FADD polyclonal antibody (Santa Cruz). Immunocomplexes were permitted to bind to a 50 µl packed volume of beads O/N, after which the beads were washed and eluted in sample buffer (Sigma), by heating at 85°C for 5 min. The same procedure was employed for reciprocal coimmunoprecipitations (coIPs) by using 6 µg of anti-BK polyclonal antibody (Chemicon). Samples were fractionated on an 8% or 15% SDS-PAGE gel, transferred, and blots blocked in 5% milk/0.05% Tween20 (Bio-Rad) in PBS for 1 h. Blots were probed with a primary antibody at 4°C O/N consisting of monoclonal anti-p53 antibody, polyclonal anti-FADD antibody (Santa Cruz), or monoclonal anti-BK antibody (BD Transduction), each diluted 1:100. To visualize p53 and FADD, HRP-conjugated secondary antibodies were

used consisting of goat anti-mouse IgG, Lc-specific (1:5000) and goat anti-rabbit IgG, Fc-specific, respectively (1:10,000; Jackson ImmunoResearch). The secondary antibodies used to visualize BK as a partner for p53 and FADD consisted of HRP-conjugated goat anti-mouse IgG, Lc-specific and goat anti-rabbit IgG, Fc-specific, respectively (both at 1:10,000). Immunoreactive bands were developed using ECL (Amersham) and Magic Mark XP (Invitrogen) was used as the protein standard to estimate relative mobilities.

To study BK/p53 or BK/FADD interactions *in vitro*, HEK cells were cotransfected with 2 µg of pcDNA3.1 containing one of the following BK variants: tandem HA-tagged full-length BK (aa M1-C1195), N-term BK (M1-M314), or C-term BK (M314-C1195) plus either V5-tagged p53 or FADD, and 10 µl of Lipofectamine2000 (Invitrogen). Transfection was accomplished when the cells were 80% confluent in a 35 mm dish. Cells were cultured in DMEM containing 10% FBS for 48 h with one medium change after 24 h. Cell preparation and immunoprecipitations were accomplished as before except monoclonal anti-V5 (Invitrogen) and anti-HA (Sigma) antibodies were used for reciprocal experiments. Following fractionation and blocking, blots were probed with anti-HA or -V5 antibody (1:5000) at 4°C O/N followed by HRP-conjugated secondary antibodies of goat anti-mouse IgG (Upstate, 1:1500), goat anti-mouse IgG-Lc specific, and rabbit anti-mouse IgG-Fc specific (Jackson ImmunoResearch Laboratories, 1:12000), for BK, p53, and FADD, respectively. Immunoreactive bands were developed as before. For BK variants that included either the C- or N-terminus, transfections and coIPs were performed as described above.

Immunocytochemistry

A HEK cell suspension was added to a 35mm dish containing a collagen-coated coverslip and cotransfected with BK-HA and p53-V5 or FADD-V5 using Lipofectamine2000. Cotransfected cells were cultured for 24 h (p53) or 39 h (FADD) in DMEM/10% FBS. Cells were fixed using 4% paraformaldehyde and 0.1 M PB, and permeabilized with 0.2% TritonX-100/10% goat serum. Cells were blocked with 10% goat serum, then incubated with primary monoclonal anti-V5 and -HA antibodies (1:200) for 1 h at RT. Alexa 594 red (V5) and Alexa 488 green (HA) conjugated isotype specific secondary antibodies at 1:500 (Molecular Probes) were used for visualization. To identify mitochondrial colocalization of BK and p53 in MitoTracker (red) CMX-ROS (Invitrogen) -treated cells, detection was performed using Alexa 350 blue (V5, 1:250) (Invitrogen) and Alexa 488 green (HA, 1:500). Cells were mounted in Vectashield with DAPI (Vector Laboratories) and imaged with a Leica SP5 AOBS tandem scanning inverted confocal microscope.

2.2 Stable HEK-BK cell line—To obtain HEK293 (HEK) cells that stably express BK, cells were transfected with 4 µg of BK-HA and 10 µl of Lipofectamine2000 in a 60 mm dish. Cells were incubated in DMEM/10% FBS at 37°C in 5% CO₂ for 48 h, then passaged at 1:100 into fresh medium. G418 (Cellgro) was added to the culture at a concentration of 250 µg/ml the following day and incubated until positive cells colonized to a 1–2 mm diameter. Each colony was isolated and cultured in selective medium with 250 µg/ml of G418 and stable cells collected and stored in DMSO in liquid N₂.

p53- and FADD Overexpression

Experiments were performed to determine the apoptotic effects of overexpressing p53 and FADD in the presence or absence of BK. HEK cells without BK or HEK cells stably expressing BK (HEK-BK) at 10×10^4 cells/well for p53 experiments, or 20×10^4 cells/well for FADD experiments, were added to each well of a 24-well plate and cultured for 24 h. P53/pcDNA3.1 or FADD/pcDNA3.1 at 0.5 μ g was transfected into each cell line using 2 μ l of Lipofectamine 2000, while HEK-BK and HEK cells transfected with empty vector served as controls. After 24 h of incubation, cells were fixed in 4% paraformaldehyde/0.1 M PB, pH 7.5, for 20 min at RT, then washed in PBS 2X and mounted as before. Apoptotic DAPI-stained nuclei were counted (Guelen et al., 2004) in five fields per well using a 40x objective on an EVOS digital inverted microscope with DAPI filter (Fisher Scientific). The ratio of apoptotic cells to total cells was determined for each group of HEK and HEK-BK cell lines with and without p53 or FADD. Each treatment was performed in triplicate and the experiment repeated three times.

Stimulation of Intrinsic and Extrinsic Apoptotic Pathways

To stimulate the p53 (intrinsic) and FADD (extrinsic) pathways, HEK and HEK-BK cells were split at 5×10^4 cells/well in a 96-well plate and cultured at 37°C, 5% CO₂ for 24 h. Mitomycin C at 10 μ g/ml of (MMC, Sigma) or recombinant human TRAIL (R&D systems) at 250 ng/ml was added to each well and incubated for another 24 h. Treated and untreated cells were analyzed for apoptosis using a photometric enzyme-immunoassay kit (Roche) that detects cytoplasmic histone-associated DNA fragments. Cells were harvested and treated with lysis buffer. One tenth of each cell lysate was aliquoted to a well containing anti-histone-coated MP-modules in a 96-well plate. After rinsing wells, substrate solution was added to each well and the plate incubated at RT to reach the sufficient color for a photometric analysis. Solution color in each well was measured at 405 nm with a reference wavelength of 490 nm. Each treatment was performed in triplicate and the experiment repeated three times.

Caspase-8 Detection

HEK and HEK-BK cells were split at 150×10^4 cells into 35mm dishes and cultured for 24 h. Cells were treated with 250 ng/ml of recombinant human TRAIL for another 24 h, then harvested in RIPA buffer to obtain a lysate that was fractionated on a 12% SDS-PAGE gel and prepared for Western analysis. Blots were probed with an anti-caspase-8 mouse monoclonal antibody (1:150; Cell Signaling) followed by an HRP-conjugated goat anti-mouse secondary antibody (1:1000; Upstate). Immunoreactive bands were developed as before to visualize full-length and cleaved caspase-8. Experiments were repeated three times and band densitometries measured for statistical analyses.

Statistics

Band densitometry and (OD) measurements were normalized by calculating the percentage ratio of each measurement relative to the highest measurement obtained, which was set at 100%. One-way ANOVAs and a MANOVA were performed for these experiments using GraphPad InStat 3.0 (GraphPad Software, Inc.) and STATISTICA (Statsoft, Inc.)

respectively. Multiple *post hoc* comparisons were accomplished using a Tukey test with significance set at $p = 0.05$.

RESULTS

BK Interacts with p53 and FADD *in vivo*

Reciprocal coIPs were used to verify BK and p53 or FADD interactions using whole brain lysates obtained from P21 mice (Fig. 1). Products of immunoprecipitation, using anti-p53 or FADD antibody-complexed Sepharose beads, revealed that both proteins coprecipitate a peptide species at ~100 kDa, which approximates the molecular weight of BK alone (Kathiresan et al., 2009) (Figs. 1A, C). Reciprocal coIPs, using anti-BK antibody bound beads, revealed the coprecipitation of a peptide species at ~53 kDa and ~27 kDa, molecular weights equivalent to p53 and FADD, respectively (Figs 1B, D).

BK interacts with p53 and FADD *in vitro*

Proteins p53 and FADD initiate apoptosis at the mitochondrial membrane and plasmalemma, respectively (Broughton et al., 2009). Consequently, the potential for BK to interact with these proteins at these subcellular regions may alter cell survival. Before exploring the BK effect via intrinsic (p53) and extrinsic (FADD) mechanisms of apoptosis, we determined if these interactions could be replicated *in vitro* using a heterologous expression system. Reciprocal coIPs were performed using HEK cells cotransfected with full-length, as well as N- and C-terminal ends of HA-tagged BK (BK-HA) (Fig. 2). Results show that V5-tagged p53 interacts with all three BK variants, including both the N- and C-termini (Fig. 2A). Reciprocal experiments were performed in which each BK variant was used to coimmunoprecipitate V5-tagged p53. Again, the results show that BK interacts with all three variants, that is, full-length BK as well as the N- and C-termini of BK (Fig. 2B). The next series of experiments determined which region of BK interacts with FADD-V5. The results show that V5-tagged FADD associates with both full-length BK as well as its C-terminus (Fig. 2C). Again, the reciprocal experiment verified these results in that only full-length BK or its C-terminus coprecipitated FADD (Fig. 2D).

Colocalization of BK with p53 and FADD

To further ascertain where these interactions take place *in vitro*, we sought to colocalize p53-V5 and FADD-V5 with BK-HA by cotransfecting HEK cells and immunolocalizing these proteins using confocal microscopy. P53-V5 was detected extensively in the nucleus with some immunostaining in the cytoplasm (Fig. 3A). BK was found at the membrane as well as in cytoplasmic regions (Fig. 3B). Merged images reveal BK/p53 colocalization in distinct cytoplasmic regions (Fig. 3C). A similar experiment revealed BK/FADD colocalization at the plasmalemma (Figs. 3D–F). To determine if BK/p53 colocalization occurs in the mitochondrion, we used a red mitochondrial marker and fluorescently labeled BK (green) and p53 (blue) for visualization (Figs. 4A–D). Merged images reveal their colocalization in mitochondria as white (Fig. 4E).

BK protects against p53- and FADD-overexpression

To ascertain the effect of BK on p53 and FADD-induced apoptosis, apoptotic nuclei were quantified in normal HEK cells and in a HEK-BK cell line, both of which were transfected to overexpress either p53 or FADD. A one-way ANOVA showed a BK and p53 treatment effect ($F = 273.44$, $p < 0.0001$) (Fig. 5A). Pairwise comparisons revealed that p53 overexpression significantly increased apoptosis by ~20% ($p < 0.001$) relative to non-treated cells, whereas the presence of BK significantly decreased p53-induced apoptosis by ~15%, relative to non-BK expressing cells ($p < 0.001$). Similarly, a one-way ANOVA showed a BK and FADD treatment effect ($F = 540.77$ and $p < 0.0001$) (Fig. 5B). Pairwise comparisons showed that FADD overexpression significantly increased apoptosis by ~20% ($p < 0.001$). However, this effect decreased by ~16% ($p < 0.001$), in HEK cells that stably expressed BK.

BK Protects Against Intrinsic and Extrinsic-Initiated Apoptosis

Mitomycin C initiates the translocation of p53 to the mitochondria (Li et al., 2010), thereby inducing apoptosis, whereas TRAIL initiates apoptosis by binding to TRAIL receptors I and II. To determine if BK alters these effects, Mitomycin C or TRAIL were used to stimulate endogenous p53 or FADD pathways, respectively, in HEK-BK and HEK cells. A one-way ANOVA showed that there was a statistically significant treatment effect of Mitomycin C and BK ($F = 132.51$, $p < 0.0001$) (Fig. 6A). Pairwise comparisons revealed that apoptosis significantly decreased by ~40% in Mitomycin C-treated HEK-BK versus HEK cells ($p < 0.001$), suggesting that BK protected against an intrinsic apoptotic pathway linked to p53. Similarly, a one-way ANOVA showed a statistically significant treatment effect of TRAIL and BK ($F = 297.56$, $p < 0.0001$) (Fig. 6B). Pairwise comparisons showed that apoptosis significantly decreased by ~40% in HEK-BK versus HEK cells ($p < 0.001$), suggesting that BK could also alter an apoptotic pathway that was initiated extrinsically by FADD.

To further measure the BK effect, we examined the activation of cleaved caspase-8 expression in HEK-BK and HEK cells stimulated with the ligand TRAIL. A one-way MANOVA with two dependent variables (i.e., procaspase-8 and cleaved caspase-8) showed that there was a statistically significant treatment effect resulting from BK and TRAIL ($F_{\text{Procaspase}} = 43.49$, $p < 0.001$; $F_{\text{CleavedCaspase}} = 1085.36$, $p < 0.001$) (Fig. 7). Pairwise comparisons *post hoc* showed a statistically significant decrease in procaspase of 32% and 27% ($p < 0.001$) in TRAIL-treated HEK and HEK-BK, respectively, relative to their respective controls. Concomitant with this decrease was an increase in cleaved caspase 8 in TRAIL-treated cells. However, this increase was 21% less when comparing TRAIL-treated HEK-BK versus HEK cells ($p < 0.001$). This outcome suggests that the BK/FADD interaction decreases the expression of cleaved caspase-8, which is an active apoptotic caspase initiated by the DED of FADD.

DISCUSSION

Evidence shows that K^+ channels play a central role in cellular homeostasis (Yu, 2003) to the extent that they may be useful as a potential drug target to prevent diseases tied to cellular apoptosis (Leung, 2010). Our data, both *in vivo* and *in vitro*, suggest BK/p53 and BK/FADD interactions, linking this channel to proteins involved in both intrinsic and

extrinsic pathways of apoptosis. P53 is a key protein that associates with pro- and anti-apoptotic proteins both in the nucleus and the mitochondria. FADD can localize to the nucleus as a result of nuclear localization signals in its DED, thereby protecting cells from apoptosis (Gómez-Angelats & Cidlowski). It can also translocate to the cytoplasm to interact with death receptors during apoptosis.

Extensive evidence now shows that the BK channel is relatively ubiquitous and found in different subcellular compartments, including the inner mitochondrial membrane (Singh et al., 2012 for review). There are several scenarios to explain how p53 might interact with BKmito, by examining multiprotein complexes in this subcellular compartment. Previously, we showed BKmito expression in hair cells of the mouse cochlea, a finding that was determined using coimmunoprecipitation, 2-D gels and mass spectrometry, followed by bioinformatic analyses (Kathiresan et al., 2009). Moreover, we showed that a transfected BK-DEC variant could express in the mitochondria of CHO cells. A more recent experiment, from this lab using similar techniques, suggests that BKmito may interact with a larger multiprotein complex that forms the permeability transition pore (PTP) of mitochondria (Sokolowski et al., 2011). The PTP is a high conductance channel that, when opened, leads to cell death. Both BKmito and K_{ATP} channels are thought to prevent ischemia by inhibiting the PTP, either by physical interaction or inhibition of factors that open the PTP (O'Rourke et al., 2005 for review).

Interestingly, p53 can form a complex with cyclophilin D, a key regulator of the PTP, to open this pore and initiate necrosis (Vaseva et al., 2012). Thus, BK may be part of this larger multiprotein complex that includes p53 during times of stress. A second scenario would include the protein prohibitin. A high-throughput study using mass spectrometry shows that BK coimmunoprecipitates prohibitin when using cochlear tissues (Sokolowski et al., 2011). While p53 and BK are in the outer and inner mitochondrial membranes, respectively, evidence suggests that p53 can interact with inner mitochondrial membrane proteins. An example is prohibitin, which modulates p53-induced apoptosis (Kathiria et al., 2012), while stabilizing membrane integrity (Merkwirth et al., 2012). Thus, BK may be part of a BK/prohibitin/p53 complex. Finally, p53 can also interact with mitochondrial channels, as demonstrated for the voltage-dependent ion channel (VDAC) during times of non-stress and cell proliferation (Ferecatu et al., 2009). A similar scenario might underlie the BKmito/p53 interaction. Further studies are needed to determine the function of these BK multiprotein complexes in light of the many functions of p53 in maintaining mitochondrial homeostasis through its pro-survival and pro-death actions.

Interestingly, our data show that both the N- and C-terminus of BK can interact with p53. These sites of interaction are not too surprising, given that p53 has a considerable amount of intrinsic disorder (Xue et al., 2013), as determined by its amino acid sequence. Additionally, a recent study suggests that there is also disorder in the N- and C-terminal ends of BK as well as in regions between the two RCK domains of the C-terminus (Peng et al., 2014). Within intrinsic disordered regions lie molecular recognition features (MorFs) that consist of short disordered regions of five to 25 amino acids that transition to order, upon binding protein partners that function in regulation and signaling. Intrinsic regional disorder resists crystallization in its unbound state and allows for flexibility (Wu et al., 2010; Yuan et al.,

2010). Moreover, regions of intrinsic disorder have properties amenable to protein-protein interactions that can occur with increased speed as well as variability and adaptability to structural conformations (Uversky, 2013).

Unlike p53, our results suggest that FADD interacts only with the C-terminus of BK. FADD is prominently found during cell death or apoptosis. However, FADD can also have a role in cell survival and proliferation as well as development and cell cycle progression (Newton et al., 1998; Walsh et al., 1998; Zhang et al. 1998). Whether FADD is pro-apoptotic or pro-survival is dependent on FADD's location in either the cytoplasm or nucleus, respectively (Tourneur & Chiocchia, 2010 for review). During apoptosis, cells begin to condense, a characteristic that is a function of apoptotic volume decrease. Regulation of volume decrease can be dependent on KCa channels such as IK and BK. Evidence shows, for example, that BK mediates TRAIL induced apoptosis in glioma cells, a mechanism that is dependent on intracellular calcium (McFerrin et al., 2010). Our results show that the presence of BK can alter TRAIL induced cell death, potentially by not only maintaining K⁺ homeostasis but by directly interacting with FADD.

To further elucidate this relationship, we need to consider the BK interactome. Previous evidence shows that BK is part of an interactome that includes nucleoside diphosphate kinase (NDK) and Fas-associated factor 1 (FAF1), using coimmunoprecipitation, 2-D gels, mass spectroscopy, and bioinformatics (Sokolowski et al. 2011). The interactome suggests that FAF1, which binds FADD to facilitate apoptosis (Ryu et al., 2003), is a secondary partner of BK via the primary partner valosin-containing protein (VCP/p97). Recent evidence also shows that p97 and FAF1 can form a complex (Ewens et al., 2014) and that p97 recognizes KCa members KCa2.3 and 3.1 in a ubiquitination process (Gao et al., 2008). Thus, BK may have a role in this complex not only by mediating the effects of calcium but also by regulating FADD. Interestingly, within this interactome is a primary partner of BK, nucleoside-diphosphate kinase (NDK), whose interaction is also underscored by previous evidence showing that NDK interacts with KCa family member, KCa3.1, by increasing the activity of this channel. In contrast to FAF1, NDK can play a neuroprotective role (Brust et al., 2008), thereby further mediating the effects of FADD at the plasmalemma.

In conclusion, to the best of our knowledge, this is the first evidence of BK interacting with mitochondrial p53 and FADD. It has been known for some time that neuronal apoptosis is mediated by outward potassium current (Yu 2003; Leung, 2010, see for review). Given the cytoprotective role of mitochondrial BK, and the pro-survival and pro-death functions of p-53, our evidence suggests that BK mediates these effects through a physical interaction. Similarly, there was no previous implication for a BK/FADD interaction. Whether this interaction is part of a larger complex is presently unknown, but our data suggest a direct BK/FADD interaction that may inhibit the apoptotic actions of the TRAIL-initiated pathway.. These data along with previous bioinformatic findings provide a guide to further test the complex interactions that maintain cellular homeostasis through the BK channel.

Acknowledgments

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References

- Bian S, Bai JP, Chapin H, Le Moellic C, Dong H, Caplan M, Sigworth FJ, Navaratnam DS. Interactions between β -catenin and the HSlo potassium channel regulates HSlo surface expression. *PLoS One*. 2011; 6:e28264. [PubMed: 22194818]
- Broughton BR, Reutens DC, Sobey CG. Apoptotic mechanisms after cerebral ischemia. *Stroke*. 2009; 40:e331–339. [PubMed: 19182083]
- Brust AK, Ulbrich HK, Seigel GM, Pfeiffer N, Grus FH. Effects of cyclooxygenase inhibitors on apoptotic neuroretinal cells. *Biomark Insights*. 2008; 3:387–402. [PubMed: 19578520]
- Chinnaiyan UM, O'Rourke K, Tewari M, Dixit VM. FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell*. 1995; 81:505–512. [PubMed: 7538907]
- Ewens CA, Panico S, Kloppsteck P, McKeown C, Ebong IO, Robinson C, Zhang X, Freemont PS. The p97-FAF1 protein complex reveals a common mode of p97 adaptor binding. *J Biol Chem*. 2014; 289:12077–12084. [PubMed: 24619421]
- Ferecatu I, Bergeaud M, Rodríguez-Enfedaque A, Le Floch N, Oliver L, Rincheval V, Renaud F, Vallette FM, Mignotte B, Vayssière JL. Mitochondrial localization of the low-level p53 protein in proliferative cells. *Biochem Biophys Res Commun*. 2009; 387:772–777. [PubMed: 19635458]
- Fischer U, Janicke RU, Schulze-Osthoff K. Many cuts to ruin: a comprehensive update of caspase substrates. *Cell Death Differ*. 2003; 10:76–100. [PubMed: 12655297]
- Gao Y, Chotoo CK, Balut CM, Sun F, Bailey MA, Devor DC. Role of S3 and S4 transmembrane domain charged amino acids in channel biogenesis and gating of KCa2.3 and KCa3.1. *J Biol Chem*. 2008; 283:9049–9059. [PubMed: 18227067]
- Gómez-Angelats M, Cidlowski JA. Molecular evidence for the nuclear localization of FADD. *Cell Death Differ*. 2003; 10:791–797. [PubMed: 12815462]
- Guelen L, Paterson H, Gaken J, Meyers M, Farzaneh F, Tavassoli M. TAT-apoptin is efficiently delivered and induces apoptosis in cancer cells. *Oncogene*. 2004; 23:1153–1165. [PubMed: 14691460]
- Kathiria AS, Neumann WL, Rhee J, Hotchkiss E, Cheng Y, Genta RM, Meltzer SJ, Souza RF, Theiss AL. Prohibitin attenuates colitis-associated tumorigenesis in mice by modulating p53 and STAT3 apoptotic responses. *Cell*. 2012; 150:5778–5789.
- Kathiresan T, Harvey M, Orchard S, Sakai Y, Sokolowski B. A protein interaction network for the large conductance Ca(2+)-activated K(+) channel in the mouse cochlea. *Mol Cell Proteomics*. 2009; 8:1972–1987. [PubMed: 19423573]
- Leung YM. Voltage-gated K⁺ channel modulators as neuroprotective agents. *Life Sci*. 2010; 86:775–780. [PubMed: 20385147]
- Li CH, Cheng YW, Liao PL, Kang JJ. Translocation of p53 to mitochondria is regulated by its lipid binding property to anionic phospholipids and it participates in cell death control. *Neoplasia*. 2010; 12:150–160. [PubMed: 20126473]
- Liao Y, Kristiansen AM, Oksvold CP, Tuvnes FA, Gu N, Rundén-Pran E, Ruth P, Sausbier M, Storm JF. Neuronal Ca²⁺-activated K⁺ channels limit brain infarction and promote survival. *PLoS One*. 2010; 5:e15601. [PubMed: 21209897]
- Lindenboim L, Borner C, Stein R. Nuclear proteins acting on mitochondria. *Biochim Biophys Acta*. 2011; 1813:584–596. [PubMed: 21130123]
- McFerrin MB, Turner KL, Cuddapah VA, Sontheimer H. Differential role of IK and BK potassium channels as mediators of intrinsic and extrinsic apoptotic cell death. *Am J Physiol Cell Physiol*. 2012; 303:C1070–1078. [PubMed: 22992678]
- Merkwirth C, Martinelli P, Korwitz A, Morbin M, Brönnek HS, Jordan SD, Rugarli EI, Langer T. Loss of prohibitin membrane scaffolds impairs mitochondrial architecture and leads to tau hyperphosphorylation and neurodegeneration. *PLoS Genet*. 2012; 8:e1003021. [PubMed: 23144624]
- O'Rourke B, Cortassa S, Aon MA. Mitochondrial ion channels: gatekeepers of life and death. *Physiology*. 2005; 20:303–315. [PubMed: 16174870]

- Peng Z, Sakai Y, Kurgan L, Sokolowski B, Uversky V. Intrinsic disorder in the BK channel and its interactome. *PLoS One*. 2014; 9:e94331. [PubMed: 24727949]
- Roos WP, Kaina B. DNA damage-induced cell death by apoptosis. *Trends Mol Med*. 2006; 12:440–450. [PubMed: 16899408]
- Ryu SW, Lee SJ, Park MY, Jun JI, Jung YK, Kim E. Fas-associated factor 1, FAF1, is a member of Fas death-inducing signaling complex. *J Biol Chem*. 2003; 278:24003–24010. [PubMed: 12702723]
- Singh H, Stefani E, Toro L. Intracellular BK_(Ca) (iBK_(Ca)) channels. *J Physiol*. 2012; 590:5937–5947. [PubMed: 22930268]
- Skalska J, Bednarczyk P, Piwońska M, Kulawiak B, Wilczynski G, Dołowy K, Kudin AP, Kunz WS, Szewczyk A. Calcium ions regulate K(+) uptake into brain mitochondria: The evidence for a novel potassium channel. *Int J Mol Sci*. 2009; 10:1104–1120. [PubMed: 19399240]
- Sokolowski S, Harvey M, Sakai Y, Jordan A, Sokolowski B. The large conductance calcium-activated K(+) channel interacts with the small GTPase Rab11b. *Biochem Biophys Res Commun*. 2012; 21:221–225. [PubMed: 22935415]
- Sokolowski B, Orchard S, Harvey M, Sridhar S, Sakai Y. Conserved BK channel-protein interactions reveal signals relevant to cell death and survival. *PLoS One*. 2011; 6:e28532. [PubMed: 22174833]
- Tian L, Chen L, McClafferty H, Sailer CA, Ruth P, Knaus HG, Shipston MJ. A noncanonical SH3 domain binding motif links BK channels to the actin cytoskeleton via the SH3 adapter cortactin. *FASEB J*. 2006; 20:2588–2590. [PubMed: 17065230]
- Tourneur L, Chiocchia G. FADD: a regulator of life and death. *Trends Immunol*. 2010; 7:260–269. [PubMed: 20576468]
- Uversky VN. A decade and a half of protein intrinsic disorder: biology still waits for physics. *Protein Sci*. 2013; 22:693–724. [PubMed: 23553817]
- Vaseva AV, Marchenko ND, Ji K, Tsirka SE, Holzmann S, Moll UM. P53 opens the mitochondrial permeability transition pore to trigger necrosis. *Cell*. 2012; 149:1536–1548. [PubMed: 22726440]
- Vaseva AV, Moll UM. Identification of p53 in mitochondria. *Methods Mol Biol*. 2013; 962:75–84. [PubMed: 23150438]
- Walsh CM, Wen BG, Chinnaiyan AM, O'Rourke K, Dixit VM, Hedrick SM. A role for FADD in T cell activation and development. *Immunity*. 1998; 8:439–449. [PubMed: 9586634]
- Wu Y, Yang Y, Ye S, Jiang Y. Structure of the gating ring from the human large-conductance Ca(2+)-gated K(+) channel. *Nature*. 2010; 466:393–397. [PubMed: 20574420]
- Xue B, Brown CJ, Dunker AK, Uversky VN. Intrinsically disordered regions of p53 family are highly diversified in evolution. *Biochim Biophys Acta*. 2013; 1834:725–738. [PubMed: 23352836]
- Yu SP. Regulation and critical role of potassium homeostasis in apoptosis. *Prog Neurobiol*. 2003; 70:363–386. [PubMed: 12963093]
- Yuan P, Leonetti MD, Pico AR, Hsiung Y, MacKinnon R. Structure of the human BK channel Ca²⁺-activation apparatus at 3.0 Å resolution. *Science*. 2010; 329:182–186. [PubMed: 20508092]
- Zhang J, Cado D, Chen A, Kabra NH, Winoto A. Fas-mediated apoptosis and activation-induced T-cell proliferation are defective in mice lacking FADD / Mort1. *Nature*. 1998; 392:296–300. [PubMed: 9521326]
- Zou S, Jha S, Kim EY, Dryer SE. A novel actin-binding domain on Slo1 calcium-activated potassium channels is necessary for their expression in the plasma membrane. *Mol Pharmacol*. 2008; 73:359–368. [PubMed: 17989352]

Fig. 1.

BK interacts with p53 and FADD as demonstrated by reciprocal coIPs using 21 day-old mouse brain. **A:** A blot probed with anti-BK monoclonal antibody recognizes a polypeptide species of ~100 kDa in precipitations using either a BK (lane 1) or p53 (lane 3) antibody complexed to Protein G Sepharose beads. **B:** A reciprocal experiment shows that when the blot is probed with an anti-p53 monoclonal antibody, it recognizes a polypeptide of ~53 kDa in precipitations using either p53 (lane 1) or BK (lane 3) antibody complexed to beads. **C:** A blot probed with anti-BK antibody shows a polypeptide species of ~100 kDa in precipitations using either BK (lane 1) or FADD (lane 3) antibody complexed to beads. **(D)** A reciprocal experiment shows that when the blot is probed with an anti-FADD polyclonal antibody, it recognizes a polypeptide of ~27 kDa in precipitations using either FADD (lane 1) or BK (lane 3) antibody complexed to beads. A bead control was used in all immunoprecipitation experiments (lanes 2 and 4), consisting of lysate mixed with IgG beads in the absence of antibody. Lane "M" represents the molecular weight marker to estimate relative mobilities. Antibodies used to immunoprecipitate and to probe blots were of either the same or different clonalities in order to resolve the target proteins and secondary antibodies. The latter were Lc- or Fc- specific and used where appropriate. Heavy chain (HC) IgG is denoted in panels.

Fig. 2.

Results of reciprocal CoIPs following HEK cell transfection with HA-tagged constructs of full-length BK, BK N- or C-terminus only (as marked above each panel), plus either V5-tagged p53 or V5-tagged FADD. **A:** Results of V5-tagged p53 CoIP experiments (*lanes 1, 4, 7*) demonstrate that p53 coprecipitates peptide species of ~130, 35, and 100 kDa, respectively. These are the expected weights of full-length BK, BK N-terminus only, and BK C-terminus only. **B:** The reciprocal experiments show that the three expressed constructs of BK (Full, C- and N-term) precipitate a peptide species of ~56 kDa, the expected weight of p53 (*lanes 1, 4, 7*). An Lc-specific secondary antibody was used to probe this blot. **C:** Results of FADD CoIP experiments demonstrate that FADD precipitates peptide species of ~130 and 100 kDa (*lanes 1 and 7*), respectively. These are the expected weights of full-length BK and the C-term of BK. FADD does not precipitate the shorter (~35 kDa) N terminus BK (*lane 4*). **D:** The reciprocal experiments show that two expressed constructs of BK (Full and C-term) precipitate a peptide species of ~27 kDa, the expected weight of FADD (*lanes 1, 7*). Controls are shown in all four panels and consist of IgG-coated beads mixed with lysate in the absence of antibody (*lanes 2, 5, 8*), and Western blots of lysate alone (*lanes 3, 6, 9*). Both light (LC) and heavy chain (HC) IgGs are marked appropriately. Designations below each panel designate the primary antibody used to probe the blot.

Fig. 3.

BK colocalizes with p53 and FADD *in vitro*. **A–C:** HEK cells transfected with BK-HA and p53-V5 and visualized respectively as fluorescent green and red. P53 is immunoreactive in the nucleus and as punctate staining in the cytoplasm. BK appears at the plasmalemma and in regions of the cytoplasm. Overlap reveals orange punctate staining in the cytoplasm (arrows). **D–F:** HEK cells transfected with BK-HA and FADD-V5 and visualized respectively as fluorescent green and red. Colocalization is seen as yellow at the membrane.

Fig. 4.

Colocalization of BK and p53 in mitochondria. **A:** Alexa 488 green was used to visualize HA-tagged BK in HEK 293 cells at both the membrane and the mitochondria. An enlarged view of panel A (white rectangle) is shown in panels B-E. **B:** Enlarged view of BK expression. **C:** Enlarged view of Alexa 350 blue was used to visualize V5-tagged p53, whereas, **D:** MitoTracker red shows the mitochondria in the marked region of panel A. **E:** Immunostaining overlap of red, green and blue is visualized as white. Scale bars: (A) 8 μm , (BE) 4 μm .

Fig. 5. HEK-BK cells are protected from cell death while overexpressing p53 and FADD. **A:** HEK-BK cells are protected from the overexpression of p53 and **B:** FADD compared to controls consisting of HEK and HEK-BK cells transfected with empty vector. OD readings were normalized as before. A one-way ANOVA with pairwise comparisons, to the control group consisting of HEK cells containing empty construct, was calculated to obtain $*p < 0.05$, $**p < 0.001$. Error bars represent the standard deviation from the mean.

Fig. 6.

BK enhances HEK cell survival while stimulating intrinsic and extrinsic apoptotic pathways.

A: HEK-BK cells treated with Mitomycin C, a stimulator of endogenous p53, show less cell death relative to Mitomycin C-treated HEK cells without BK. **B:** HEK-BK cells treated with human TRAIL ligand show a similar resistance to apoptosis. Densitometry measurements were averaged across enzyme immuno-assays accomplished in triplicate for each treatment, then normalized (100%) to the highest OD value across three separate experiments. A one-way ANOVA with pairwise comparisons, to the control group consisting of HEK cells containing empty construct, was calculated to obtain $**p < 0.001$.

Fig. 7.

BK decreases activation of cleaved caspases. TRAIL activation of cleaved caspase-8 is significantly reduced in HEK cells that stably express BK (*lane 4*) compared to TRAIL-treated HEK cells lacking BK. Apoptosis is slightly higher relative to HEK (*lane 1*) and HEK-BK (*lane 2*) cells without TRAIL treatment. Densitometry measurements were normalized as before. Statistical significance was determined using a one-way MANOVA with two dependent variables and *post hoc* paired comparisons to obtain $**p < 0.001$. Error bars represent the standard deviation.