The Stability and Expression Level of Bok Are Governed by Binding to Inositol 1,4,5-Trisphosphate Receptors*

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Bok is a member of the Bcl-2 protein family that governs the intrinsic apoptosis pathway, although the role that Bok plays in this pathway is unclear. We have shown previously in cultured cell lines that Bok interacts strongly with inositol 1,4,5-trisphosphate receptors (IP3Rs), suggesting that it may contribute to the structural integrity or stability of IP3R tetramers. Here we report that Bok is similarly IP3R-associated in mouse tissues, that essentially all cellular Bok is IP3R bound, that it is the helical nature of the Bok BH4 domain, rather than specific amino acids, that mediates binding to IP3Rs, that Bok is dramatically stabilized by binding to IP3Rs, that unbound Bok is ubiquitinylated and degraded by the proteasome, and that binding to IP3Rs limits the pro-apoptotic effect of overexpressed Bok. Agents that stimulate IP3 activity, apoptosis, phosphorylation, and endoplasmic reticulum stress did not trigger the dissociation of mature Bok from IP3Rs or Bok degradation, indicating that the role of proteasome-mediated Bok degradation is to destroy newly synthesized Bok that is not IP3R associated. The existence of this unexpected proteolytic mechanism that is geared toward restricting Bok to that which is bound to IP3Rs, implies that unbound Bok is deleterious to cell viability and helps explain the current uncertainty regarding the cellular role of Bok.

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2 The abbreviations used are: BH, Bcl-2 homology; IP3, inositol 1,4,5-trisphosphate; IP3R, inositol 1,4,5-trisphosphate receptor; ER, endoplasmic reticulum; PARP, poly (ADP-ribose) polymerase; UPP, ubiquitin-proteasome pathway; TM, transmembrane; CHX, cycloheximide; GnRH, gonadotropin-releasing hormone; IP, immunoprecipitation; PII, polyethyleneimine; MD, molecular dynamics.

Materials—αT3 cells and HEK 293T cells were cultured as described (14). Antibodies raised in rabbits were: anti-IP3R1, anti-IP3R2, anti-IP3R3, anti-IP3R4, anti-Bak, anti-Bax, anti-Bok, anti-Mcl-1, anti-Bcl-xL, anti-Bcl-2, anti-caspase-3, anti-caspase-9, anti- PARP, anti-poly (ADP-ribose) polymerase (PARP) and the Carol M. Baldwin Breast Cancer Research Fund. The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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raised against amino acids 19–32 of mouse Bok (4, 7). Mouse monoclonal antibodies were: anti-ubiquitin clone FK2 (BioMol International), anti-HA epitope clone HA11 (Covance), anti-FLAG epitope clone M2 (Sigma), and anti-p97 (Research Diagnostics Inc.). Horseradish peroxidase-conjugated secondary antibodies, gonadotropin-releasing hormone (GnRH), N-ethylmaleimide, protease inhibitors, Triton X-100, CHAPS, cycloheximide (CHX), thapsigargin, and forskolin were purchased from Sigma. DTT, Precision Plus™ Protein Standards, and SDS-PAGE reagents were from Bio-Rad. Protein A-Sepharose CL-4B was from GE Healthcare. MG132 was from Biomol. Staurosorpine was from Enzo Life Sciences. Linear, MW~25,000 polyethyleneimine (PEI) was from Polysciences Inc.

**Cell Lysis, Immunoprecipitation (IP), and SDS-PAGE**—To prepare lysates, cells or brain tissues were harvested at ice-cold lysis buffer containing either 1% CHAPS or 1% Triton X-100 (14). CHAPS was used for all experiments involving IP, except when exogenous Bok ubiquitination was assessed, when cells were harvested with DTT-free Triton X-100 lysis buffer supplemented with 5 mM N-ethylmaleimide, followed by addition of 5 mM DTT 30 min later (25). Lysates were incubated on ice for 30 min and clarified by centrifugation at 16,000 × g for 10 min at 4 °C. For IP, clarified lysates were incubated with antisera and Protein A-Sepharose CL-4B for ~16 h at 4 °C, and IPs were washed thoroughly with lysis buffer, were resuspended in gel-loading buffer, were incubated at 37 °C for 30 min or 100 °C for 3 min, were subjected to SDS-PAGE, and proteins were transferred to nitrocellulose for probing as described (14).

**Analysis of Exogenous Bok in HEK and HeLa Cells**—Vectors encoding mouse Bok tagged at the N terminus with a triple FLAG epitope (3F-Bok) and mouse IP3R1 tagged at the C terminus of Bok WT and BokL34G were then performed using the RosettaBackrub (30) was first used to create a model of BokL34G, which revealed little effect on the overall structure of the residue 24–42 α-helical region, but significant shifts in the position of some amino acid side chains. MD simulations of this region of Bok WT and BokL34G were then performed using the Gromacs 4.5.5 package (31) and Amber99SB force field (32). Each peptide was solvated in an octahedral box filled with TIP3P water and the net charges were neutralized with 0.15 M NaCl. The PME method was used to treat long-range electrostatic interactions, a cutoff of 10 Å was used for the Coulomb energy and Van Der Waals interactions, the system was energy minimized until reaching 1000 kJ/mol/nm, and then equilibrated for 100 ps using the v-rescale method at 300 K, followed by pressure equilibration for 100 ps using the Parrinello-Rahman method at 300 K, and the LINCS algorithm was applied to constrain all bond lengths. In the final 10–ns MD simulation, the time step was 2 fs, and structures were saved every 2 ps.

**Data Analysis**—All experiments were repeated at least once (n = the number of independent experiments) and representative images of gels and traces are shown. Quantitated data are graphed as mean ± S.E. when n ≥ 3 or mean ± range when n = 2.

**Results**

**Bok-IP3R1 Binding in Vivo and the Origin of Bok Variants**—We have shown previously, via IP, that Bok binds tightly to IP3Rs in various cultured cell lines (14). Examination of mouse brain shows that such binding also occurs in vivo (Fig. 1), since IP of IP3R1 from brain cerebral cortex and hippocampus lysates specifically isolates Bok (lanes 2 and 3). Further, anti-IP3R1 depleted the vast majority of both IP3R1 and Bok from the

**Measurement of mRNA Levels**—RNA was extracted using the RNeasy Mini Kit (Qiagen) and was reverse transcribed into cDNA using the Reverse Transcription System (Promega). qRT-PCR was performed using the forward primer GATGGAGCGGATGTCCCTCAAG and the reverse primer TCTCTGCCAACACAGGAA, a Taq master mix developed in-house containing SYTO-82 (Molecular Probes), 3 mM MgCl2 and TaqDNA Polymerase (Thermo Fisher), a Stratagene MX3000P real time qPCR thermal cycler, and the following PCR cycling parameters: 10 min at 95 °C, followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. Results were analyzed by normalizing to the housekeeping genes ribosomal protein S18, peptidylprolyl isomerase A, and hypoxanthine phosphoribosyltransferase 1, using the 2−ΔΔCt method (28).
**Binding to I$_3$ Receptors Stabilizes Bok**

![FIGURE 1. Co-IP of Bok with IP$_3$R1 in mouse brain tissues.](image)

Mouse brain cerebral cortex (C) and hippocampus (H) lysates were incubated without or with anti-IP$_3$R1, and IPs (lanes 1–3) and lysates (either pre- or post-IP; lanes 4–7) were subjected to SDS-PAGE and probed for the proteins indicated. p97 and Bcl-2 served as negative controls that did not co-IP and show that Bok co-IP is specific. Co-migrating IgG light chain seen in the Bcl-2 probe is indicated by the asterisk.

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lysates (compare lanes 4 and 5 with 6 and 7), indicating that essentially all Bok is IP$_3$R1-associated.

It is noteworthy that in mouse tissues and cell lines (Figs. 1 and 2, lanes 1–2) (14), anti-Bok, which is raised against amino acids 19–32 of mouse Bok (Fig. 3A), recognizes two species (at 21 and 23 kDa). Bands at 21 and 23 kDa were also seen after expression of exogenous Bok from mouse Bok cDNA in HEK cells (Fig. 2, lane 4). The existence of 21- and 23-kDa bands appears to be due to alternative translation initiation (33, 34), since mutation of the second AUG codon in the Bok coding region (that encodes Met$^{15}$) blocks formation of the 21-kDa band (lane 5). Interestingly, the 3F-Bok construct (Fig. 3A) used previously to map the Bok-IP$_3$R interaction (14) generated anti-Bok-immunoreactive bands at 27, 23, and 21 kDa (lane 6), with the anti-FLAG immunoreactive band at 27 kDa corresponding to full-length 3F-Bok, and the bands at 23 and 21 kDa corresponding to untagged Bok starting at Met$^{1}$ or Met$^{15}$. Again, evidence for alternative translation initiation comes from the observation that 3F-Bok$^{M15A}$ does not generate the 21kDa band (lane 7) and that 3F-Bok$^{A1–14}$ (Fig. 3A) does not generate the 23kDa band. Thus, Bok mRNA appears to be translated using either Met$^{1}$ or Met$^{12}$ as the initiation codon, perhaps because of “leaky scanning” (33, 34) during the initiation of translation.

**Resolution of the Determinants of Bok Binding**—To resolve the determinants of Bok binding to IP$_3$R1 we made a series of truncations and point mutations in the vicinity of the BH4 domain (Fig. 3A), analyzed the ability of these constructs to interact with IP$_3$R1HA (Fig. 3D), and generated molecular models to gain insight into the effects of these modifications on the structure of Bok (Fig. 3, B and C). The Bok model (Fig. 3B), which is based on Bax and Bak, indicates that the BH4 domain (bright pink) is located within the first $a$-helical region of Bok (residues 24–42), which lies in an accessible cleft. Sequential truncations within this $a$-helical region (Fig. 3, A and C) confirmed the role for the BH4 domain in mediating binding, since 3F-Bok$^{A1–27}$ and 3F-Bok$^{A1–32}$ bound well to IP$_3$R1HA, but 3F-Bok$^{A1–39}$ did not (Fig. 3D, lanes 2–5). Surprisingly, it does not appear that it is specific residues in the BH4 domain that mediate binding, but rather the overall structure of the domain, as 3F-Bok$^{A34–36}$ and 3F-Bok$^{A37–38}$ bind well, but 3F-Bok$^{A34–36}$ does not (Fig. 3D, lanes 6–8). To confirm this idea, we mutated Leu$^{24}$ to Gly, since adjacent Gly residues destabilize $a$-helices (35) and a previous study has shown that a di-Gly mutation in the BH4 domain of Bcl-2 blocks binding to IP$_3$Rs (36). This mutation distorts the BH4 domain of Bok, as indicated by MD simulation (Fig. 3C), and indeed, the 3F-Bok$^{A34–36}$ mutant does not bind to IP$_3$R1HA (Fig. 3D, lane 9).

**Exogenous Bok Expression Is Dependent upon Binding to IP$_3$R1**—We noted in certain experiments using the co-transfection approach shown in Fig. 3D, that binding-deficient Bok mutants did not express as well as constructs that bound strongly to IP$_3$R1HA. To examine this more carefully we monitored the effect of IP$_3$R1HA on 3F-Bok expressed from a range of cDNA amounts (Fig. 4A). This revealed that IP$_3$R1HA dramatically enhanced 3F-Bok expression, particularly when the lowest amounts of 3F-Bok cDNA were used (lanes 1–6). This was truly because of binding to IP$_3$R1HA, because IP$_3$R1HA$^{A1–1903}$, which does not bind Bok (14), did not affect 3F-Bok expression, while IP$_3$R1HA$^{A1–1884}$, which does bind to Bok (14), enhanced 3F-Bok expression (Fig. 4A, lanes 7–12). The effect of binding to IP$_3$R1 on Bok expression was confirmed by the fact that the expression level of binding-deficient mutants (3F-Bok$^{A34–36}$ and 3F-Bok$^{A34–38}$) was essentially unaffected by co-expression of IP$_3$R1HA (Fig. 4B, lanes 4 and 8), while 3F-Bok$^{WT}$ and 3F-Bok$^{A34–36}$ expression was greatly enhanced (lanes 2 and 6).

CHX-chase experiments showed that 3F-Bok was turned over more rapidly in the absence of IP$_3$R1HA than in the presence of IP$_3$R1HA (Fig. 4C), indicating that Bok is unstable in the absence of IP$_3$R1, which in turn accounts for the low expression level. To probe the apparent degradation mechanism, cells were incubated with MG132, an inhibitor of the proteasome (Fig. 4D). This markedly increased 3F-Bok levels in the absence of IP$_3$R1HA (lanes 3–6), but had no effect on 3F-Bok levels when IP$_3$R1HA was present (lanes 1 and 2), indicating that “free” 3F-Bok is degraded by the proteasome. Conversely, the levels of 3F-Bak were unaffected by MG132 (lanes 7 and 8), showing that the increase seen for 3F-Bak is protein specific and not due to a generic effect on expression from the plasmid. Finally, the relatively rapid decline in 3F-Bok levels in the presence of CHX was partially blocked by MG132 (lanes 9–13), again implicating the proteasome in the turnover of free 3F-Bok.

To establish whether Bok is ubiquitinated, 3F-Bok-HA was immunoprecipitated from control and MG132-treated HEK
cells and probed for ubiquitin. This revealed the presence of high molecular mass ubiquitinated species after MG132 treatment (Fig. 5, lane 3), that was not seen in control samples (lanes 1 and 5). Thus, ubiquitination of exogenous Bok appears to mediate its degradation.

**Endogenous Bok Levels Are Dramatically Reduced by IP$_3$R1 Deletion**—To determine whether endogenous Bok levels are also IP$_3$R1-dependent, we examined αT3 cells, the cell type in which we originally discovered that endogenous Bok and IP$_3$R1 interact strongly (14), and in which IP$_3$R1 constitutes ~99% of total IP$_3$R content (22). Remarkably, CRISPR/Cas9-mediated deletion of IP$_3$R1 caused a dramatic decline in Bok levels (Fig. 6A), to ~2% of that seen in control cells (Fig. 6B), since the Bok immunoreactivity seen with 30 μg of IP$_3$R1KO cell lysate (lane 1) was equivalent to that seen with 0.5–1 μg of control cell lysate (lanes 2 and 3). The decline in Bok expression was totally specific, as the levels of other pertinent proteins were unaffected (Fig. 6A), and was not due to loss of Bok mRNA, the levels of which were not substantially different in control αT3 and αT3IP$_3$R1KO cells (Fig. 6C). Further, reintroduction of IP$_3$R1HA and IP$_3$R1HA$_{A1–1884}$, which bind Bok (14), into αT3IP$_3$R1KO cells caused a partial recovery of Bok expression, not seen with the binding-deficient construct IP$_3$R1HA$_{A1–1903}$ (Fig. 6D); it is likely that the recovery is only partial because of the relatively low transfection efficiency seen in αT3 cells (24, 25). This confirms that the Bok gene and mRNA are normal in αT3IP$_3$R1KO cells and indicates that Bok is degraded in the absence of IP$_3$R1. The degradation mechanism seems identical to that seen for exogenous 3F-Bok, since in αT3IP$_3$R1KO cells, endogenous Bok levels were enhanced by MG132 (Fig. 6E, lanes 1–4) and CHX-chase showed that endogenous Bok is rapidly degraded (lanes 5–7) in a MG132-sensitive manner (lanes 8 and 9); none of these changes were seen in control αT3 cells (Fig. 6E, lanes 10–18). It is perhaps surprising that treatment with MG132 only doubled the level of Bok in αT3IP$_3$R1KO cells (Fig. 6E, lanes 1–4) and that Bok levels did not approach the amount seen in control αT3 cells (Fig. 5, lanes 11–18). This may be because MG132 only doubled the level of Bok in αT3IP$_3$R1KO cells (Fig. 5, lanes 11–18), since the Bok expression triggers apoptosis (7–10), we examined whether binding to IP$_3$Rs is important for this effect by comparing the pro-apoptotic effects of overexpressed BokWT and BokL$_{34G}$ (which does not bind to IP$_3$Rs). Interestingly, we found that both BokWT and BokL$_{34G}$ trigger apoptosis, as indicated by enhanced caspase-3 and PARP cleavage, and remarkably, that BokL$_{34G}$ is more effective (Fig. 7, lanes 2 and 3). Under these conditions, some BokWT co-immunoprecipitates with endoge-
nous IP3Rs, while BokL34G remains completely free (lower panels; lanes 2 and 3). This indicates that it is not necessary for exogenous Bok constructs to bind IP3Rs to be pro-apoptotic and, in fact, that the binding of Bok to IP3Rs limits apoptosis, apparently by limiting the amount of free Bok. Thus, free Bok, rather than IP3R-bound Bok, seems to be the trigger for apoptosis.

**Stimuli Do Not Cause Release of Bok from IP3R1**—Various agents were added to αT3 cells to determine whether Bok release from IP3R1 can be triggered, since that might cause Bok degradation and a decrease in cellular Bok levels. However, Fig. 8 (lanes 5–13) shows that neither the amount of Bok that co-IPs with IP3R1, nor cellular Bok levels, were substantially altered by staurosporine, a kinase inhibitor that triggers apoptosis and causes caspase-3-mediated IP3R1 cleavage (14–16), by forskolin, which triggers cAMP-dependent protein kinase-mediated phosphorylation of IP3R1 (15) and Bok (37), or by thapsigargin, which inhibits ER Ca2+/H11001-ATPase, depletes Ca2+/H11001 stores and leads to ER stress (12, 13). A slight but consistent decrease in Bok levels and co-IP was seen in response to GnRH (lanes 1–4), which triggers a robust increase in IP3 levels, IP3R1 activation, and proteasome-mediated IP3R1 degradation (14, 24, 25). However, that decrease is most likely because Bok bound to IP3R1 is degraded in unison with IP3R1 (14), rather than because it is released from IP3R1.

**Discussion**

Our data indicate that the stability and thus the expression level of Bok is highly dependent upon binding to IP3Rs. This
was demonstrated by focusing on the interaction of both exogenous and endogenous Bok with IP₃R1, the most widely expressed and best understood IP₃R type (15, 16). When IP₃R1 is absent and, thus, the predominant binding site for Bok is also absent, “free” Bok appears to be degraded by the ubiquitin-proteasome pathway (UPP) (38). The extent to which Bok levels are dependent upon IP₃R1 is quite remarkable, with the steady-state level of Bok in H9251T3IP3R1KO cells being 2% of that seen in control H9251T3 cells. Using pharmacological agents and GnRH, we were unable to trigger dissociation of the Bok-IP3R1 complex and reduce endogenous Bok levels, suggesting that the extreme sensitivity of free Bok to the UPP is not a mechanism to control the levels of mature Bok that was once IP3R-associated. Rather, it likely represents a cellular mechanism to disallow newly synthesized Bok from existing in a free form. It is important to note that Bok, like several other Bcl-2 protein family members, has a TM domain at the very C terminus (1–3, 7) and is thus a “tail anchored protein” (39–42). It is currently unclear how newly synthesized TA proteins are inserted into membranes, but for at least some it appears that the BAG6/Ubl4A/TRC35 complex acts to chaperone the proteins from the ribosome to the ER membrane insertion machinery (42, 43). It seems plausible that when IP₃R1 is absent, the lack of an appropriate docking site for newly synthesized Bok at the ER membrane means that its insertion into the ER membrane is impaired and that it is diverted to the UPP. Such a mechanism that causes the destruction of free Bok is entirely consistent with the observation that essentially all cellular Bok is bound to IP₃R1. Other Bcl-2 protein family members are also targeted by the UPP (44–47), and is absent and, thus, the predominant binding site for Bok is also absent, “free” Bok appears to be degraded by the ubiquitin-proteasome pathway (UPP) (38).

The extent to which Bok levels are dependent upon IP₃R1 is quite remarkable, with the steady-state level of Bok in αT3IP₃R1KO cells being ~2% of that seen in control αT3 cells. Using pharmacological agents and GnRH, we were unable to trigger dissociation of the Bok-IP₃R1 complex and reduce endogenous Bok levels, suggesting that the extreme sensitivity of free Bok to the UPP is not a mechanism to control the levels of mature Bok that was once IP₃R-associated. Rather, it likely represents a cellular mechanism to disallow newly synthesized Bok from existing in a free form. It is important to note that Bok, like several other Bcl-2 protein family members, has a TM domain at the very C terminus (1–3, 7) and is thus a “tail anchored protein” (39–42). It is currently unclear how newly synthesized TA proteins are inserted into membranes, but for at least some it appears that the BAG6/Ubl4A/TRC35 complex acts to chaperone the proteins from the ribosome to the ER membrane insertion machinery (42, 43). It seems plausible that when IP₃R1 is absent, the lack of an appropriate docking site for newly synthesized Bok at the ER membrane means that its insertion into the ER membrane is impaired and that it is diverted to the UPP. Such a mechanism that causes the destruction of free Bok is entirely consistent with the observation that essentially all cellular Bok is bound to IP₃R1. Other Bcl-2 protein family members are also targeted by the UPP (44–47), and is absent and, thus, the predominant binding site for Bok is also absent, “free” Bok appears to be degraded by the ubiquitin-proteasome pathway (UPP) (38).
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it will be interesting to see if they too are subject to a similar kind of “quality control.”

It is intriguing that the 23- and 21-kDa Bok variants appear to originate from alternative translational initiation at Met¹ and Met¹⁵. It remains possible that proteolysis could account for the 21-kDa variant, but that seems unlikely because mutation of Met¹⁵ to Ala completely blocks formation of the 21-kDa band and highly Met-specific endoproteases are not known. Alternative translational initiation could result from “leaky scanning” (33, 34), although why this should occur for Bok mRNA is unclear, since the Kozak consensus sequence around Met¹ (GCCAUGG) is ideal (33, 34). It is equally puzzling that exogenous 3F-Bok generates the 21- and 23-kDa variants, as well as the full-length construct at 27 kDa, since this indicates that “leaky scanning” also occurs at the initiation codon at the start of the 3X FLAG region, again despite an ideal Kozak sequence (ACCAUGG). Perhaps secondary structure of Bok mRNA disrupts the scanning process (33, 34). Whatever their origin, both forms of Bok bind to IP₃R1 (e.g. Fig. 1), which is to be expected given that the binding is mediated by the BH4 domain (residues 34–39). However, it is likely that the two forms will have different properties, since full-length (23 kDa) Bok can be phosphorylated at Ser⁸ (37), which is absent from the 21-kDa form.

The region to which Bok binds appears to be a flexible, unstructured, surface-exposed loop within the ARM3 domain of IP₃Rs (14, 16). The ARM3 domain is composed of an ensemble of 6 armadillo repeats, and together with the ARM 1 and 2 domains, is thought to form a flexible, solenoid-like structure that facilitates propagation of ligand-evoked signals to the channel pore (16). The ARM3 domain is also a regulatory hotspot, containing sites for phosphorylation, ATP binding, Ca²⁺ binding, caspase-3 cleavage, and ubiquitination (15, 16, 48). Proteolysis within the ARM3 domain has recently been suggested to provide a novel mode of IP₃R regulation (49) and it is intriguing that such proteolysis is inhibited by Bok, most likely because of steric hindrance (14). In view of the ability of Bcl-2 to regulate IP₃R channel activity (17–20), and the recently described findings that Bcl-xL activates channel gating by interacting with BH4 domain-like helices in the IP₃R C-terminal tail (50), it is somewhat surprising that initial experiments with control and Bok⁻/⁻ mouse embryonic fibroblasts did not reveal a major effect of Bok on the Ca²⁺-mobilizing function of IP₃Rs (14). However, a caveat with this study is that the Bok⁻/⁻ cells have adapted in terms of IP₃R1–3 expression (14, 16). The ARM3 domain is also a regulatory hotspot, containing sites for phosphorylation, ATP binding, Ca²⁺ binding, caspase-3 cleavage, and ubiquitination (15, 16, 48). Proteolysis within the ARM3 domain has recently been suggested to provide a novel mode of IP₃R regulation (49) and it is intriguing that such proteolysis is inhibited by Bok, most likely because of steric hindrance (14). In view of the ability of Bcl-2 to regulate IP₃R channel activity (17–20), and the recently described findings that Bcl-xL activates channel gating by interacting with BH4 domain-like helices in the IP₃R C-terminal tail (50), it is somewhat surprising that initial experiments with control and Bok⁻/⁻ mouse embryonic fibroblasts did not reveal a major effect of Bok on the Ca²⁺-mobilizing function of IP₃Rs (14). However, a caveat with this study is that the Bok⁻/⁻ cells have adapted in terms of IP₃R1–3 expression (14) and thus, whether Bok binding to the ARM3 domain directly regulates the Ca²⁺ mobilizing function of IP₃Rs remains to be determined. Another possibility, given the strength of the Bok-IP₃R interaction and that Bok has a TM domain, is that Bok contributes to the structural integrity or stability of IP₃R tetramers. Interestingly, Bok is well expressed in the brain (4, 7, 12) and is enriched in the CA3 region of the hippocampus (51, 52). Our data indicate that in cortical cortex and hippocampus, Bok is bound to IP₃R1, and in this context, Bok binding should, at minimum, serve to protect IP₃R1 from caspase-3-mediated proteolysis (14).

Bok is widely and highly expressed in mammalian tissues and is well conserved across mammalian species, yet attempts to define its biological role, and in particular the role it is sus-pected to play in apoptosis, have not led to a clear consensus view (4, 7, 11, 12, 53, 54). This may, in part, be because of the tight binding between Bok and IP₃Rs and the degradation of free Bok by the UPP; in experiments in which exogenous Bok is introduced into cells, the amount of Bok expressed will be limited by the UPP and will vary between cell types because of differences in IP₃R expression (14). Further, it is tempting to speculate that the existence of a cellular mechanism that efficiently destroys free Bok indicates that free Bok is harmful to the cell, and that in experiments in which Bok is overexpressed, apoptosis occurs because UPP-mediated Bok degradation is overwhelmed and free Bok is formed (7–10). This view is supported by our finding that BokL⁹⁴⁶G is more pro-apoptotic than BokWT. Certainly, if the apoptosis seen in Bok overexpression experiments (7–10) is considered to be “non-physiological,” it would help to rationalize data showing that there are no major apoptotic deficiencies attributable to Bok in Bok⁻/⁻ mice (4, 11, 12), or in mice in which Bok is deleted in combination with Bak and Bax (11, 55).

Author Contributions—R. J. H. W. conceived and coordinated the study and wrote the paper with substantial editorial input from J. J. S. J. J. S. performed and analyzed all of the experiments shown, with input from E. J. Z. (Fig. 1) and L. M. S. (Fig. 6). X. H. performed the molecular modeling and MD simulations. F. A. W. created the cell lines used in Fig. 6. J. J. S. and F. A. W. both helped guide the study. All authors reviewed the results and approved the final version of the manuscript.

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