THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 35, pp. 25340–25349, August 30, 2013

The Bcl-2 Protein Family Member Bok Binds to the Coupling Domain of Inositol 1,4,5-Trisphosphate Receptors and Protects Them from Proteolytic Cleavage*

Received for publication, June 27, 2013 Published, JBC Papers in Press, July 24, 2013, DOI 10.1074/jbc.M113.496570

Jacqualyn J. Schulman[‡], Forrest A. Wright[‡], Thomas Kaufmann[§], and Richard J. H. Wojcikiewicz[‡1]

From the [‡]Department of Pharmacology, SUNY Upstate Medical University, Syracuse, New York 13210 and [§]University of Bern, Medical Faculty, Institute of Pharmacology, Friedbuehlstrasse 49, CH-3010 Bern, Switzerland

Background: Bok is a Bcl-2 protein family member, with largely unknown properties.
Results: Bok binds constitutively to IP_3 receptors and protects them from proteolysis, but does not appear to alter their Ca^{2+} channel activity.
Conclusion: Bok is novel component of IP_3 receptor complexes.
Significance: A new locus for studies on IP_3 receptor and apoptosis regulation has been defined.

Bok is a member of the Bcl-2 protein family that controls intrinsic apoptosis. Bok is most closely related to the pro-apoptotic proteins Bak and Bax, but in contrast to Bak and Bax, very little is known about its cellular role. Here we report that Bok binds strongly and constitutively to inositol 1,4,5-trisphosphate receptors (IP_3Rs), proteins that form tetrameric calcium channels in the endoplasmic reticulum (ER) membrane and govern the release of ER calcium stores. Bok binds most strongly to IP_3R1 and IP_3R2, and barely to IP_3R3, and essentially all cellular Bok is IP_3R bound in cells that express substantial amounts of IP_3Rs. Binding to IP_3Rs appears to be mediated by the putative BH4 domain of Bok and the docking site localizes to a small region within the coupling domain of IP_3Rs (amino acids 1895–1903 of IP_3R1) that is adjacent to numerous regulatory sites, including sites for proteolysis. With regard to the possible role of Bok-IP_3R binding, the following was observed: (i) Bok does not appear to control the ability of IP_3Rs to release ER calcium stores, (ii) Bok regulates IP_3R expression, (iii) persistent activation of inositol 1,4,5-trisphosphate-dependent cell signaling causes Bok degradation by the ubiquitin-proteasome pathway, in a manner that parallels IP_3R degradation, and (iv) Bok protects IP_3Rs from proteolysis, either by chymotrypsin *in vitro* or by caspase-3 *in vivo* during apoptosis. Overall, these data show that Bok binds strongly and constitutively to IP_3Rs and that the most significant consequence of this binding appears to be protection of IP_3Rs from proteolysis. Thus, Bok may govern IP_3R cleavage and activity during apoptosis.

The Bcl-2 protein family governs the intrinsic (mitochondrial) apoptotic pathway, and contains members that are anti-apoptotic (*e.g.* Bcl-2 and Mcl-1), and also members that are pro-apoptotic (*e.g.* Bax and Bak); complex interactions between these proteins control apoptosis and cell viability (1, 2). These proteins contain conserved Bcl-2 homology (BH)[2] domains, and it has been generally thought that pro-apoptotic proteins like Bax and Bak contain three BH domains (BH1, BH2, and BH3), whereas anti-apoptotic proteins like Bcl-2 and Mcl-1 contain four (BH1, BH2, BH3, and BH4), with the BH4 domain conferring anti-apoptotic activity (1, 2). An additional group of pro-apoptotic BH3-only proteins (*e.g.* Bim and Bad) completes the family (1, 2). This canonical view is not settled, however, as it appears that pro-apoptotic proteins like Bax and Bak may also contain a BH4 domain (2, 3).

Bax and Bak are closely related proteins that, when active, induce permeabilization of the outer mitochondrial membrane, cytochrome *c* release, caspase activation, and ultimately, apoptosis (1–3). Interestingly, while knock-out of either Bax or Bak has little effect on mouse physiology (although $Bax^{-/-}$ males are sterile), Bax/Bak knock-out mice exhibit various severe defects, indicating redundancy of function for Bax and Bak (1). Bok, which was originally identified as an Mcl-1-binding protein, is similar in sequence to Bax and Bak and has the same arrangement of domains (4–6), but has been investigated much less thoroughly (1–3). Bok is expressed in many, and perhaps all, mammalian cell types (7, 8) and, like Bax and Bak (3, 5, 6), its overexpression in cultured cells causes apoptosis (4–9). Mice lacking Bok are phenotypically normal and hematopoietic cells derived from these mice respond normally to apoptotic stimuli (7), again indicative of redundancy among the Bok/Bax/Bak group. However, it is important to note that the existence of severe defects in Bax/Bak knock-out mice shows that Bok cannot compensate for the absence of Bak and Bax (1), and suggest that it has unique properties. Indeed, the pro-apoptotic effect of Bok is not seen in cells lacking Bak and Bax, indicating that it acts in concert with or upstream of Bak and Bax (8). Further, the atypical transmembrane domain of Bok appears to direct it

* This work was supported in whole or in part by National Institutes of Health Grant DK049194.

[1] To whom correspondence should be addressed: Department of Pharmacology, SUNY Upstate Medical University, 750 E. Adams St., Syracuse, NY 13210. Tel.: 315-464-7956; Fax: 315-464-8014; E-mail: wojcikir@upstate.edu.

[2] The abbreviations used are: BH, Bcl-2 homology; IP_3, inositol 1,4,5-trisphosphate; IP_3R, inositol 1,4,5-trisphosphate receptor; ER, endoplasmic reticulum; UPP, ubiquitin-proteasome pathway; GnRH, gonadotropin-releasing hormone; LPA, oleoyl-L-α-lysophosphatidic acid; MEF, mouse embryonic fibroblast; $[Ca^{2+}]_c$, cytosolic Ca^{2+} concentration.

toward the endoplasmic reticulum (ER) and Golgi, rather than to the mitochondrial outer membrane, which is the more typical location for many other Bcl-2 family members (8). How this distribution contributes to the cellular role of Bok remains unclear, however (8). Finally, the Bok gene region is frequently deleted in cancer, suggesting that it might be a tumor suppressor (10).

An extensive, but confusing, literature exists regarding the regulation of inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3Rs) by Bcl-2 family proteins (11, 12). IP_3Rs are ~300 kDa proteins that form tetrameric, IP_3- and Ca^{2+}-gated Ca^{2+} channels in ER membranes of mammalian cells and play a key role in cell signaling (13, 14). There are three highly homologous IP_3R types in mammals, IP_3R1, IP_3R2, and IP_3R3, and although their tissue distribution varies, they have similar properties, are often co-expressed, and form heteromers (13–15). IP_3, in concert with Ca^{2+} binding, induces yet to be defined conformational changes in the tetrameric channel that allow Ca^{2+} to flow from stores within the ER lumen into the cytosol to increase cytosolic Ca^{2+} concentration (13, 14). IP_3Rs also play a role in controlling mitochondrial Ca^{2+} uptake and metabolism, and intrinsic apoptosis, and myriad effects of Bcl-2 family members on IP_3R activity and ER Ca^{2+} content have been described (11, 12, 16). Perhaps most significantly, it has been reported that Bcl-2 promotes prosurvival Ca^{2+} signals, while inhibiting proapoptotic Ca^{2+} signals (17–20, 11, 12), and similar effects have been reported for Bcl-xL and Mcl-1 (19, 21, 11, 12); it remains controversial, however, whether this is due to a direct effect of Bcl-2, etc. on the Ca^{2+} releasing properties of IP_3Rs, or because they also reduce ER Ca^{2+} content (11, 12, 16). Conversely, it has been reported that Bax and Bak increase ER Ca^{2+} content (22, 23), and it has been proposed that this reflects the fact that Bcl-2 family members regulate IP_3R phosphorylation and thus Ca^{2+} leak from the ER (11, 16, 22). Physical interactions between anti-apoptotic Bcl-2 family proteins and IP_3Rs have been described (18–21), and, at least for Bcl-2, this is mediated by the BH4 domain (20, 24–26). In contrast, to date, there is no clear evidence for direct binding of pro-apoptotic proteins to IP_3Rs (22, 23, 11).

Here we report that Bok interacts robustly with IP_3Rs, that this interaction is by far the strongest among the Bcl-2 family, that the binding site is in the coupling domain of IP_3Rs adjacent to other regulatory sites, and that the most significant consequence of this binding appears to be protection of IP_3Rs from proteolysis.

EXPERIMENTAL PROCEDURES

Materials—αT3 cells, HeLa cells, and HEK 293T cells were cultured as described (15, 27, 28). SV40 large T antigen-immortalized wild-type (C57BL/6) and $Bok^{-/-}$ (C57BL/6) mouse embryonic fibroblasts (MEFs) (8) were cultured in DMEM plus 5% fetal bovine serum and antibiotics (100 units penicillin and 100 μg of streptomycin/ml). Antibodies raised in rabbits were: anti-IP_3R1, raised against amino acids 2731–2749 of IP_3R1 (15); anti-$IP_3R1^{326-341}$, a kind gift from Dr. Suresh Joseph, Thomas Jefferson University, Philadelphia, PA; anti-$IP_3R1^{1829-1848}$ (Affinity Bioreagents, Inc.); anti-IP_3R2 and anti-IP_3R3 (15); anti-erlin2 (28); anti-HA epitope (30): anti-Mcl-1 D35A5, anti-Bcl-xL 54H6, anti-caspase-3 9662, and anti-Bcl-2 50E3 (Cell Signaling Technology); anti-Bak 06–536 (Millipore); anti-Bax N-20 (Santa Cruz Biotechnology Inc.); and anti-Bok, raised against amino acids 19–32 of mouse Bok (7, 8). Mouse monoclonal antibodies were: anti-IP_3R3, raised against amino acids 22–230 of human IP_3R3 (BD Transduction Labs); anti-ubiquitin clone FK2 (BioMol International); anti-HA epitope clone HA11 (Covance), and anti-FLAG epitope clone M2 (Sigma). Rat monoclonal anti-$IP_3R1^{679-727}$ clone 4C11 was a kind gift from Dr Katsuhiko Mikoshiba, RIKEN, Japan. Horseradish peroxidase-conjugated secondary antibodies, raised in goat, were obtained from Sigma. Gonadotropin-releasing hormone (GnRH), oleoyl-L-α-lysophosphatidic acid (LPA), *N*-ethylmaleimide, ATP, IP_3, protease inhibitors, Triton X-100, CHAPS, chymotrypsin type I-S, and digitonin were purchased from Sigma. DTT, Precision Plus™ Protein Standards, and SDS-PAGE reagents were from Bio-Rad. Protein A-Sepharose CL-4B was from Amersham Biosciences. MG132 was from Biomol. Staurosporine was from Enzo Life Sciences.

Cell Lysis, Immunoprecipitation, SDS-PAGE, and Mass Spectrometry—To prepare cell lysates for SDS-PAGE or for immunoprecipitation, cells were harvested with ice-cold lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, 1% CHAPS, or 1% Triton X-100, 10 μM pepstatin, 0.2 mM phenylmethylsulfonyl fluoride, and 0.2 μM soybean trypsin inhibitor, pH 8.0) usually supplemented with 1 mM DTT. When IP_3 receptor polyubiquitination was assessed in MEFs, cells were harvested with DTT-free lysis buffer, then 5 mM *N*-ethylmaleimide was added to the lysates for 30 min, followed by 5 mM DTT. Lysates were incubated on ice for 30 min and clarified by centrifugation at 16,000 × *g* for 10 min at 4 °C. To immunoprecipitate specific proteins, clarified lysates were incubated with antisera and protein A-Sepharose CL-4B for 4–16 h at 4 °C, and immunoprecipitates were washed thoroughly with lysis buffer, resuspended in gel-loading buffer (29), incubated at 37 °C for 30 min, subjected to SDS-PAGE, and either transferred to nitrocellulose for immunoblotting, or silver stained as described (30). Immunoreactivity was detected, and mass spectrometry was performed as described (30).

Analysis of Exogenous Bok and IP_3Rs—A vector encoding mouse Bok tagged at the N terminus with a triple FLAG epitope (3F-Bok) was created by inserting the mouse Bok cDNA sequence (8) into the KpnI/BamHI sites of p3xFLAG-CMV-10 (Sigma). This provided the template for the creation by PCR (primers available upon request) of vectors encoding 3F-Bok with N-terminal deletions and point mutations (Fig. 4*A*). Likewise, a vector encoding mouse IP_3R1 tagged at the C terminus with an HA epitope (27) was the origin of N-terminal deletion mutants (Fig. 4*C*). Vectors encoding IP_3R1–3 have been described (31). The authenticity of all cDNAs was confirmed by DNA sequencing. HEK 293T cells seeded at 3–4 × 10^5/9.6 cm^2 well, were transfected ~24 h later with 1–2 μg of cDNAs and 6–9 μl of Superfect (Qiagen), and ~24 h later were harvested with ~0.4 ml/well 1% CHAPS lysis buffer. Lysates were then incubated without or with anti-IP_3R1, anti-IP_3R2, anti-IP_3R3, anti-HA, or anti-FLAG, and immunoprecipitates and input lysates were subjected to SDS-PAGE.

Ca^{2+} Mobilization—Cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) was measured essentially as described (29). Cells were harvested with HBSE (29), centrifuged (500 × *g* for 5 min), resuspended in DMEM, recentrifuged, and finally resuspended at 2 mg of protein/ml in DMEM plus 10% fetal bovine serum and 10 μM Fura 2-AM. After 1 h at 37 °C, cells were centrifuged (500 × *g* for 1 min), were washed once with KHB (29), were resuspended in KHB at 1 mg protein/ml and $[Ca^{2+}]_c$ was measured at either 25 or 37 °C (29). To prepare permeabilized cells, cells were harvested with HBSE, were centrifuged (1500 × *g* for 5 min), were resuspended and incubated at 37 °C for 10 min in DMEM plus 10% fetal bovine serum, were recentrifuged, were resuspended in ice-cold cytosol buffer (120 mM KCl, 2 mM KH_2PO_4, 2 mM $MgCl_2$, 2 mM ATP, 20 mM HEPES, pH 7.3), were recentrifuged, and finally were incubated at ~6 mg protein/ml in cytosol buffer with 100 μg/ml digitonin for 10 min at 4 °C. The permeabilized cells were then centrifuged (2000 × *g* for 2 min at 4 °C), were resuspended in cytosol buffer, 1 μM Fura-2 was added, and $[Ca^{2+}]$ in the suspension was measured (29).

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.

RESULTS

Bok Associates with IP_3 Receptors—Several proteins that regulate IP_3Rs have been identified by co-immunoprecipitation; for example, 3 proteins (erlin1, erlin2, and RNF170) that mediate the degradation of activated IP_3Rs by the ubiquitin-proteasome pathway (UPP) were discovered on the basis of their association with IP_3R1 immunoprecipitated from GnRH-stimulated αT3 mouse pituitary cells (28, 30, 32–34). Additional analysis of anti-IP_3R1 immunoprecipitates from αT3 cells revealed the presence of a 23 kDa co-immunoprecipitating protein that was shown by mass spectral analysis to be Bok (Fig. 1*A*).

αT3 cells respond to GnRH with a robust increase in IP_3 formation (35) and IP_3R degradation by the UPP (27, 28, 32–35), mediated by the erlin1/2 complex and RNF170, which rapidly associate with activated IP_3R1 (30, 32–34). However, in contrast to erlin2, Bok co-immunoprecipitation with IP_3R1 was unaltered by acute exposure to GnRH, indicating that Bok is constitutively associated with IP_3R1, irrespective of the activation state of IP_3R1 (Fig. 1*B*). Since, in principle, the presence of Bok in anti-IP_3R1 immunoprecipitates could be an artifact of unanticipated cross-reactivity of Bok with anti-IP_3R1 (the epitope for which is amino acids 2731–2749 of IP_3R1), we also examined whether other antibodies raised against different IP_3R1 epitopes could recover Bok. This was indeed the case, as antibodies raised against amino acids 326–341 and 1829–1848 of IP_3R1 also efficiently recovered Bok (data not shown). Furthermore, in MEFs, which express substantial amounts of both IP_3R1 and IP_3R3 (Fig. 7), immunoprecipitation with either anti-IP_3R1 or anti-IP_3R3 recovered Bok (Fig. 1*C*). Thus, Bok association with IP_3Rs is not dependent upon how IP_3Rs are immunoprecipitated and is seen in multiple cell types. Interestingly, and somewhat surprisingly given the extensive literature on the topic (11, 12, 16–21, 24–26), other members of the Bcl-2 family did not detectably co-immunoprecipitate with IP_3R1 (Fig. 1*D*);

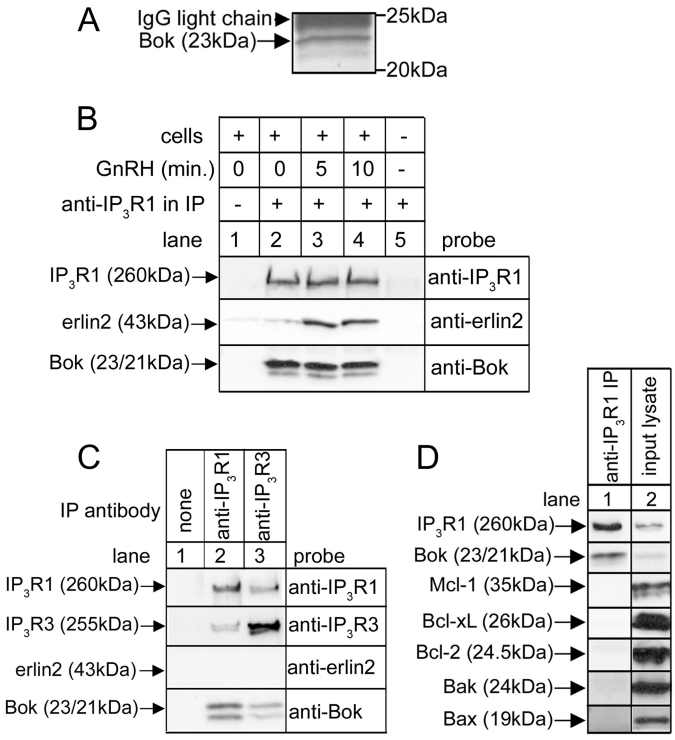

FIGURE 1. **Bok co-immunoprecipitates with IP_3Rs.** Cells were harvested with 1% CHAPS lysis buffer, and immunoprecipitates were prepared and subjected to SDS-PAGE (n = 2). *A*, 20–25-kDa region of a silver-stained gel of an anti-IP_3R1 immunoprecipitate from αT3 cells is shown and the 23 kDa band marked with an *arrow* was identified by mass spectrometry as Bok. *B*, αT3 cells were treated for the indicated times with 100 nM GnRH, and anti-IP_3R1 immunoprecipitates (*lanes 2–4*) and controls (*lanes 1* and *5*) were probed in immunoblots for IP_3R1, erlin2 and Bok. *C*, using MEFs, anti-IP_3R1 and anti-IP_3R3 immunoprecipitates (*lanes 2* and *3*), and a control (*lane 1*) were probed in immunoblots for IP_3R1, IP_3R3, erlin2 (negative control) and Bok. Note that because of IP_3R heterotetramerization (13, 15), anti-IP_3R1 recovers some IP_3R3, and anti-IP_3R3 recovers some IP_3R1. Mouse monoclonal anti-IP_3R3 was used for these experiments. *D*, using αT3 cells, anti-IP_3R1 immunoprecipitates and input cell lysates were probed in immunoblots for the proteins indicated.

this was the case for all of the family members examined, whether anti-apoptotic (Mcl-1, Bcl-2, and Bcl-xL), or pro-apoptotic (Bak and Bax). Overall, these data indicate that among the Bcl-2 family, Bok exhibits by far the strongest interaction with IP_3Rs.

The Majority of Bok Is IP_3R-associated—In addition to demonstrating that Bok associates with IP_3R1, the data in Fig. 1*D* also suggest that a large proportion of cellular Bok is IP_3R-associated, since the relatively weak Bok signal seen in cell lysates is enhanced in IP_3R1 immunoprecipitates to an extent similar to that seen for IP_3R1. The extent to which Bok is IP_3R associated was more carefully assessed in αT3 cells, and also in HeLa cells and MEFs (Fig. 2). In all cell types anti-IP_3R1 recovered Bok, and interestingly HeLa cell Bok migrated as a single band at 22 kDa, rather than as the doublet at 23/21 kDa seen in αT3 cells and MEFs (*upper panels*, *lanes 2*, *4*, and *6*). Importantly, anti-IP_3R1 depleted the vast majority of IP_3R1 immunoreactivity from αT3 and HeLa cell lysates and caused a similar decrease in Bok immunoreactivity (*lower panels*, *lanes 1–4*), indicating that in these cell types most, and perhaps all, Bok is IP_3R associated. A similar situation exists in MEFs, in which IP_3R immunoprecipitation was less efficient (for a reason that remains unclear), but in which IP_3R1 immunodeple-

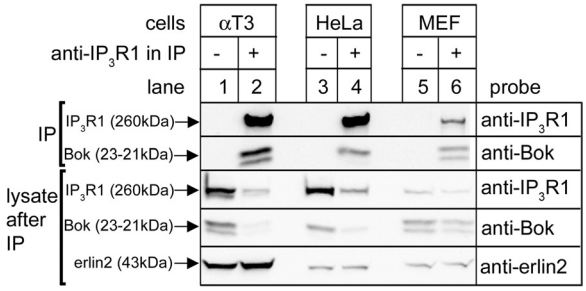

FIGURE 2. **Immunodepletion of IP$_3$R1 causes equivalent immunodepletion of Bok.** Cells were harvested with 1% CHAPS lysis buffer, lysates were incubated without or with anti-IP$_3$R1, and immunoprecipitates and post-immunoprecipitation lysates were subjected to SDS-PAGE. Immunoblots were then probed for IP$_3$R1 and Bok, and for erlin2, which served as a loading control for cell lysates ($n = 2$).

tion was again equivalent to that seen for Bok (*lower panels*, *lanes 5* and *6*).

Bok Binds Preferentially to IP$_3$R1 and IP$_3$R2—To examine whether Bok binds equally well or differentially to IP$_3$R1, IP$_3$R2, and IP$_3$R3, HEK 293T cells were co-transfected to express 3F-Bok and each receptor type, followed by assessment of interactions by co-immunoprecipitation (Fig. 3). Previous studies have shown that exogenous IP$_3$Rs are expressed at high levels in HEK 293T cells, such that the contribution of endogenous IP$_3$Rs to biochemical measurements (*e.g.* IP$_3$R phosphorylation) is negligible or undetectable (31). Immunoprecipitation of IP$_3$R1 and IP$_3$R2 recovered a considerable amount of 3F-Bok (Fig. 3*A*, *lanes 2* and *4*), while much less was recovered when IP$_3$R3 was immunoprecipitated (*lane 6*). Note that lane 6 also shows that Bok does not bind nonspecifically to IgG (also shown in Fig. 4*D*, *lane 1*). Importantly, conditions were arranged such that similar amounts of each receptor type were expressed and immunoprecipitated, as demonstrated by Coomassie staining of duplicate gels (Fig. 3*A*, *top panel*). Further, the observed interaction between IP$_3$Rs and 3F-Bok represents a physiological phenomenon, rather than an event that occurs after cell lysis, since mixing lysates from cells expressing either IP$_3$R1, or 3F-Bok did not result in co-immunoprecipitation of 3F-Bok (Fig. 3*B*, *lane 2*). The preferential binding of Bok to IP$_3$R1 and IP$_3$R2 was confirmed by immunoprecipitating 3F-Bok from the co-transfected cells (Fig. 3*C*); this led to the recovery of a substantial amount of IP$_3$R1 and IP$_3$R2 (*lanes 2* and *6*), but much less IP$_3$R3 (*lane 10*). Thus, Bok binds selectively to IP$_3$R1 and IP$_3$R2.

Determinants of Bok-IP$_3$R Binding—To define the region of Bok that binds to IP$_3$Rs, Bok was initially truncated from the N terminus to remove specific regions (Fig. 4*A*) and binding to IP$_3$R1 was assessed by co-immunoprecipitation (Fig. 4*B*). That 3F-Bok$^{\Delta 1-14}$ co-immunoprecipitates well, but 3F-Bok$^{\Delta 1-45}$ does not (Fig. 4*B*, *lanes 3* and *4*), indicates that the region between amino acids 15–45 of Bok mediates binding to IP$_3$Rs, and suggests that the putative BH4 domain might be responsible. This notion was examined further with a mutant in which residues of the putative BH4 domain were replaced with alanine (Fig. 4*A*); that 3F-Bok^{A34-38} does not co-immunoprecipitate (Fig. 4*B*, *lane 6*) provides additional evidence that the BH4 domain mediates binding to IP$_3$Rs, although the possibility remains that deletion/disruption of the BH4 domain perturbs another region of Bok that is the true mediator of binding.

Conversely, to define the IP$_3$R region that binds to Bok, IP$_3$R1 was truncated from the N terminus to remove specific regions, guided by knowledge of the domain structure of IP$_3$Rs (13, 14, 32) (Fig. 4*C*). That 3F-Bok co-immunoprecipitates with IP$_3$R1HA$^{\Delta 1-1581}$ as well as it does with IP$_3$R1HA^{WT} (Fig. 4*D*, *lanes 3* and *4*), but not with IP$_3$R1HA$^{\Delta 1-2268}$ (*lane 8*), initially indicated that Bok binds between residues 1582–2268 (*i.e.* to the C-terminal part of the coupling domain). More subtle truncations were used to locate the exact binding site; 3F-Bok bound strongly to both IP$_3$R1HA$^{\Delta 1-1884}$ and IP$_3$R1HA$^{\Delta 1-1894}$ (*lanes 5* and *6*), but not to IP$_3$R1HA$^{\Delta 1-1903}$ (*lane 7*), indicating that Bok binds between residues 1895–1903 (amino acid sequence PSRKKAKEP). Interestingly, this highly charged sequence is completely conserved in IP$_3$R1 from other species (*e.g.* rat and human), but not in IP$_3$R2 and IP$_3$R3. However, a sequence with a very similar charge distribution is found in the corresponding region of IP$_3$R2 (PRMRVRDS), and is completely conserved in mouse, rat, and human.

The Significance of Bok Binding to IP$_3$Rs—To examine the significance of the Bok-IP$_3$R interaction, we initially compared the properties of SV40 large T antigen-immortalized wild-type (WT) and Bok$^{-/-}$ MEFs. Analysis of lysates showed that the absence of Bok did not alter the levels of other Bcl-2 family proteins (Fig. 5*A*), and analysis anti-IP$_3$R1 immunoprecipitates showed that the absence of Bok did not facilitate the binding of other Bcl-2 family proteins to IP$_3$R1 (Fig. 5*B*). Interestingly, however, IP$_3$R levels were altered by the absence of Bok, with IP$_3$R1 levels increasing, and IP$_3$R2 and IP$_3$R3 levels decreasing (Fig. 5*A*; also see Fig. 7*A*).

Examination of $[Ca^{2+}]_c$ showed that LPA, which has previously been reported to stimulate IP$_3$ formation in fibroblasts (36), induced essentially identical responses in WT and Bok$^{-/-}$ MEFs (Fig. 6*A*). In these experiments, EGTA was added just prior to LPA to chelate extracellular Ca^{2+} and block Ca^{2+} entry (37), such that changes in $[Ca^{2+}]_c$ are a measure of the release of intracellular Ca^{2+} stores. Thus, Bok does not appear to regulate IP$_3$R-mediated Ca^{2+} store release in intact cells under these conditions. To more directly assess IP$_3$R function, IP$_3$-induced Ca^{2+} mobilization was assessed in suspensions of permeabilized cells, in which exogenous IP$_3$ can gain access to IP$_3$Rs and release ER Ca^{2+} stores. Again, however, Ca^{2+} mobilization was the same in WT and Bok$^{-/-}$ MEFs (Fig. 6*B*), indicating that the Ca^{2+} mobilizing function of IP$_3$Rs is not governed by Bok. Further, re-uptake of the Ca^{2+} released by IP$_3$ was identical in WT and Bok$^{-/-}$ MEFs, indicating that ER Ca^{2+} storage is also not regulated by Bok (Fig. 6*B*).

Prolonged activation of IP$_3$Rs triggers their down-regulation by the UPP; they are first polyubiquitinated, and then transferred to the proteasome for degradation (32, 33, 38). To examine whether these events are influenced by Bok, MEFs were exposed to LPA, and IP$_3$R levels were monitored. LPA caused a decline in IP$_3$R1–3 levels in WT MEFs in a manner that was partially blocked by the proteasome inhibitor MG132 (Fig. 7*A*, *lanes 1–6*), indicating that IP$_3$Rs are down-regulated by the UPP under these conditions (27, 32). However, very similar down-regulation was seen in Bok$^{-/-}$ MEFs (*lanes 7–12*), indi-

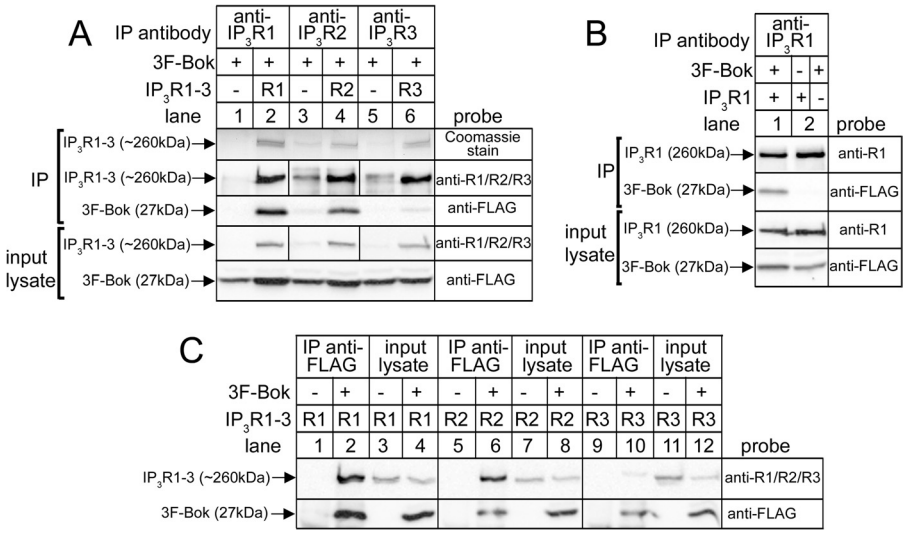

FIGURE 3. **Bok binds selectively to IP$_3$R1 and IP$_3$R2.** HEK 293T cells were transfected with cDNAs encoding 3F-Bok and/or each IP$_3$R type and 24 h later were harvested with 1% CHAPS lysis buffer, lysates were incubated with anti-IP$_3$R1, anti-IP$_3$R2, anti-IP$_3$R3, or anti-FLAG, and immunoprecipitates and input lysates were subjected to SDS-PAGE ($n = 3$). Gels were then either stained with Coomassie blue, or immunoblots were probed for IP$_3$R1–3 (with anti-IP$_3$R1–3) or 3F-Bok (with anti-FLAG). *A*, assessment of 3F-Bok binding to immunoprecipitated IP$_3$R1, IP$_3$R2, and IP$_3$R3. *B*, demonstration that 3F-Bok and IP$_3$R1 do not co-immunoprecipitate when lysates from cells expressing either IP$_3$R1 or 3F-Bok are mixed and then incubated with anti-IP$_3$R1 to immunoprecipitate IP$_3$R1. *C*, assessment of IP$_3$R binding to immunoprecipitated 3F-Bok.

cating that Bok is not required for this process (this was true for IP$_3$R1 and IP$_3$R3, whereas IP$_3$R2 was undetectable). Consistent with this conclusion, IP$_3$R polyubiquitination, the other hallmark of IP$_3$R processing by the UPP (27, 32), was not inhibited by the absence of Bok (Fig. 7*B*). In fact, IP$_3$R polyubiquitination was slightly greater in the Bok$^{-/-}$ MEFs, but this is most likely due to the increased expression of IP$_3$R1 and its presence in immunoprecipitates (compare *lanes 4 versus 2*, and *8 versus 6*).

Interestingly, Bok expression was reduced in parallel to IP$_3$Rs expression in stimulated cells. This was apparent in LPA-stimulated WT MEFs and, again, was partially blocked by MG132 (Fig. 7*A*, *lanes 1–6*), suggesting that it is via the UPP. Additional experiments with αT3 cells confirmed that Bok down-regulation occurs as IP$_3$R levels decline (Fig. 7*C*, *upper two panels*), and also that as Bok levels decline, the amounts of other Bcl-2 family proteins and erlin2 are unaffected (*lower panels*). Analysis of the time-dependence of GnRH effects showed that IP$_3$R1 and Bok decline at similar rates, and, again, that MG132 partially blocks the declines (Fig. 7*D*). Further, GnRH induced the formation of high molecular mass Bok immunoreactivity (*e.g.* at 26–27 kDa) that was enhanced by MG132, suggesting that Bok might be ubiquitinated (Fig. 7*D*, *lane 2* and *lanes 7–10*). Further evidence that Bok is ubiquitinated in stimulated αT3 cells came from analysis of anti-IP$_3$R1 immunoprecipitates (Fig. 7*E*). As expected, Bok co-immunoprecipitates strongly with IP$_3$R1 (*lanes 4* and *5*), and the high molecular mass anti-Bok immunoreactive bands seen in the presence of GnRH at 26–27 kDa and above (*lane 5*), were also found to be anti-ubiquitin immunoreactive (*lane 2*). This selective ubiquitination and down-regulation of Bok and IP$_3$Rs, together with the finding that a large proportion of cellular Bok is IP$_3$R associated, suggests that they may be processed by the UPP as a combined unit.

Finally, we examined whether Bok might regulate IP$_3$R1 proteolysis, since its binding site is adjacent to the site cleaved by caspase-3 during apoptosis (13, 39, 40) and to one of the chymotrypsin-sensitive sites that is thought to be a flexible, exposed loop (13, 32, 41, 42) (Fig. 4*C*). Interestingly, we found that Bok markedly inhibited chymotrypsin-induced IP$_3$R1 proteolysis *in vitro*, as gauged by the production of fragments IV and V (Fig. 8). Exposure of IP$_3$R1 immunopurified from transfected cells to chymotrypsin generated free fragments V and IV at ~90 and ~40 kDa, respectively (Fig. 8*A*, *lanes 2* and *6*), and this was almost completely blocked when 3F-Bok was co-expressed with IP$_3$R1 (*lanes 4* and *8*). Instead, an ~130 kDa fragment was formed that was recognized by both fragment IV- and V-specific antisera, and thus appears to be a cleavage product composed of fragments IV+V (*lanes 4* and *8*). An analogous result was obtained when IP$_3$R1 was immunopurified from WT and Bok$^{-/-}$ MEFs; in this case, the generation of free fragments V and IV was much more efficient for material immunopurified from Bok$^{-/-}$ MEFs (Fig. 8*B*, *lanes 4* and *8*), than for material immunopurified from WT MEFs, in which a band corresponding to fragments IV+V was again observed (*lanes 2* and *6*). Remarkably, then, it appears that the association of Bok with IP$_3$Rs inhibits chymotrypsin-induced cleavage at the fragment IV-V junction.

We also examined the sensitivity of IP$_3$R1 to caspase-3, by monitoring IP$_3$R1 cleavage in cells undergoing apoptosis (Fig. 8*C*). In response to the kinase inhibitor staurosporine and other pro-apoptotic agents, caspase-3 is activated and IP$_3$R1 is cleaved into two major fragments, a C-terminal, ~95 kDa fragment that may form "leaky" channels, and an ~170 kDa fragment that accounts for the remainder of the protein (39, 40, 43, 44). In the presence of staurosporine, both of these fragments were much more readily visible in Bok$^{-/-}$ MEFs than in WT MEFs, while caspase-3 activation was approximately the same in both cell types (Fig. 8*C*, *lanes 3* and *4*). Thus, Bok appears to protect IP$_3$R1 from the proteolytic activity of caspase-3.

Overall, these data indicate that despite binding strongly to IP$_3$Rs and regulating their expression, Bok does not appear to

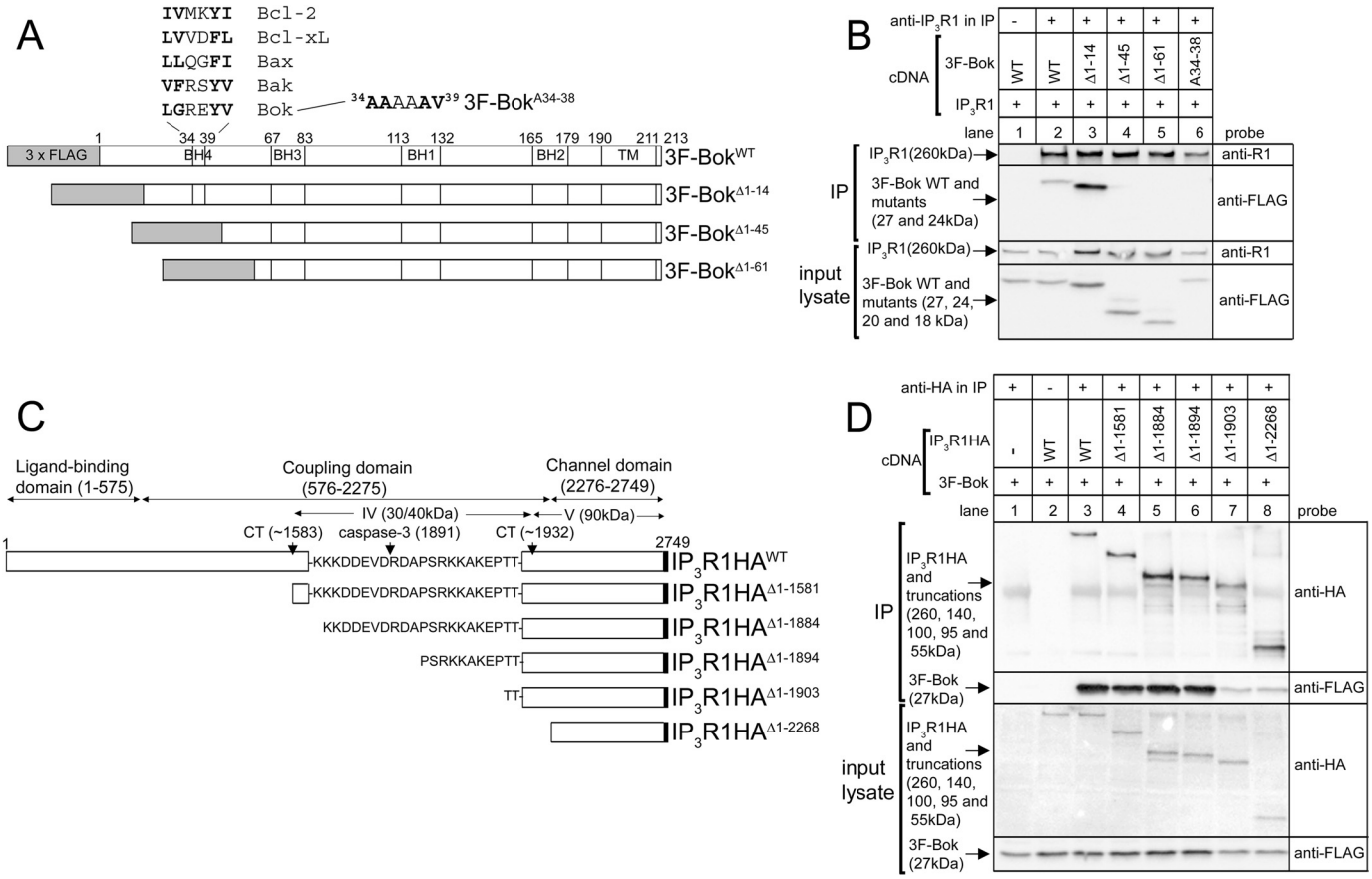

FIGURE 4. **Analysis of the determinants of Bok-IP_3R binding.** *A*, 3F-Bok constructs used to map the region that binds to IP_3Rs. Shown are the N-terminal triple FLAG tag (*gray box*), the position of the BH1–4 domains (5, 6), the transmembrane (*TM*) region, and the sequence of the putative BH4 domain of mouse Bok, together with that for Bcl-2, Bcl-xl, Bax, and Bak. These approximate to the consensus sequence $\Phi_1\Phi_2X_1X_2\Phi_3\Phi_4$, where X is any residue, Φ is a hydrophobic residue, and Φ_3 is an aromatic residue (3, 47). Also shown is the point mutant used to further resolve the binding site. *B*, HEK 293T cells were transfected with cDNAs encoding 3F-Bok constructs and IP_3R1, and 24 h later were harvested with 1% CHAPS lysis buffer, lysates were incubated without or with anti-IP_3R1, immunoprecipitates and input lysates were subjected to SDS-PAGE, and immunoblots were probed for IP_3R1 or the 3F-Bok constructs ($n = 3$). *C*, IP_3R1HA constructs used to map the region that binds to Bok. Shown are the C-terminal HA tag (*thick black line*), the positions of the 3 functional domains (the ligand-binding domain, the coupling domain, and the channel domain) (13, 14, 32), two of the sites (at ~1583 and ~1932) susceptible to proteolysis by chymotrypsin (*CT*) and two of the proteolytic fragments (IV and V) (13, 32, 41, 42), and the caspase-3 cleavage site (at 1891) (13, 39, 40). Numbering is according to the mouse sequence. *D*, HEK 293T cells were transfected with cDNAs encoding IP_3R1HA constructs and 3F-Bok, and 24h later were harvested with 1% CHAPS lysis buffer, lysates were incubated without or with rabbit polyclonal anti-HA, immunoprecipitates and input lysates were subjected to SDS-PAGE, and immunoblots were probed for 3F-Bok or the IP_3R1HA constructs with mouse monoclonal anti-FLAG or anti-HA ($n = 3$).

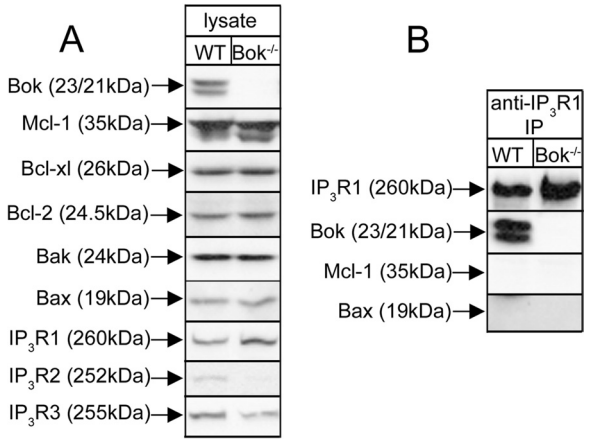

FIGURE 5. **Bcl-2 family members in WT and Bok$^{-/-}$ MEFs.** Cells were harvested with 1% Triton lysis buffer, lysates (*A*), and anti-IP_3R1 immunoprecipitates (*B*) were prepared, samples were subjected to SDS-PAGE, and immunoblots were probed for the proteins indicated ($n = 3$). Additional probing of immunoprecipitates did not reveal any binding of Bcl-xL, Bcl-2, or Bak to IP_3R1 in WT or Bok$^{-/-}$ MEFs (data not shown)

regulate the Ca^{2+} gating function of IP_3Rs, or the ability of IP_3Rs to be degraded by the UPP. However, Bok is selectively degraded by the UPP in parallel to IP_3Rs and, most importantly, protects IP_3Rs from the proteolytic effects of chymotrypsin *in vitro* and caspase-3 *in vivo* during apoptosis.

DISCUSSION

In summary, we find (i) that Bok binds constitutively to IP_3Rs, (ii) that the determinants of binding appear to be the BH4 domain of Bok and a small peptide in a highly regulated region of the coupling domain of IP_3Rs, (iii) that the binding of Bok does not seem to regulate the Ca^{2+} mobilizing function of the channels formed by IP_3Rs, (iv) that Bok is degraded by the UPP in parallel to the degradation of IP_3Rs that occurs when IP_3-dependent cell signaling is persistently activated, and, most importantly, (v) that Bok protects IP_3Rs from proteolysis *in vitro* and *in vivo* during apoptosis. Overall, these data show that Bok is tightly associated with IP_3Rs and that one of its cellular roles is to control IP_3R proteolysis.

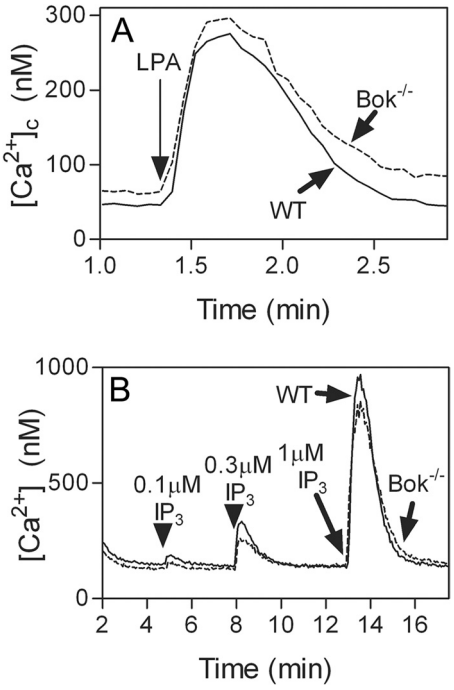

FIGURE 6. **Ca^{2+} signaling in WT and Bok$^{-/-}$ MEFs.** *A*, cells in suspension were loaded with Fura 2, were exposed to 1 μM LPA (*arrow*) and $[Ca^{2+}]_c$ was calculated from the 340/380 nm fluorescence ratio. EGTA (3 mM) was added 1 min prior to LPA to reduce extracellular $[Ca^{2+}]$ to ~100 nM (37) and eliminate Ca^{2+} entry (n>10). *B*, suspensions of permeabilized cells were exposed to increasing IP_3 concentrations in the presence of Fura-2, and $[Ca^{2+}]$ was calculated from the 340/380 nm fluorescence ratio ($n = 2$).

The strength of Bok binding to IP_3Rs is surprising. Clear co-immunoprecipitation of Bok with IP_3Rs was seen using either CHAPS or Triton X-100 as detergents, and remarkably, most, if not all, cellular Bok was found to be IP_3R bound, since Bok was immunodepleted in parallel to IP_3Rs when IP_3Rs were immunoprecipitated. Further, under conditions that allowed for robust Bok binding, the binding of other Bcl-2 family proteins was not detectable. This is perplexing in view of the extensive literature indicating that anti-apoptotic Bcl-2 family members bind to IP_3Rs (11, 12, 16–21, 24–26). However, evaluation of that literature suggests only relatively weak binding of endogenous pro-apoptotic Bcl-2 family members to endogenous IP_3Rs (18, 21, 22, 45, 46), and reveals that many of the studies were conducted with overexpressed proteins, or with purified recombinant protein fragments (18–21, 24, 26), which may not faithfully mimic physiological interactions. Such relatively weak binding may be beyond the limits of detection under the experimental conditions used herein, which were geared toward the assessment of the particularly strong Bok-IP_3R interaction.

Our studies with Bok and IP_3R mutants indicate that the putative BH4 domain of Bok (3, 47) mediates binding to IP_3Rs and that the Bok binding site is located within a small sequence (PSRKKAKEP) found in a highly regulated region of IP_3Rs, that is likely a surface-exposed loop (32, 33, 41). Interestingly, the BH4 domain of Bcl-2 also appears to mediate its binding to IP_3Rs (20, 24–26), although the Bcl-2 binding site is located within a completely different IP_3R region (amino acids 1389–1408 of IP_3R1) (25, 26). It has also been shown that the BH4 domain of Bcl-xL binds much more weakly to IP_3Rs than does the Bcl-2 BH4 domain, because it contains an aspartic acid residue instead of a lysine at position X_2 of the BH4 consensus sequence (20) (Fig. 4*A*). Overall, this information indicates that the BH4 domain of Bok and some other Bcl-2 family proteins is critical for mediating binding to IP_3Rs, but that different modes of interaction are possible. Since Bax and Bak also contain a very similar putative BH4 domain (Fig. 4*A*), it is puzzling that they too do not bind to IP_3Rs. The explanation may be that Bok is more localized to the ER and Golgi than Bax and Bak, apparently because of differences in their TM regions (8). This would place Bok in the proximity of IP_3R receptors, which are also predominantly localized to the ER (13, 14), but are also present in the Golgi (48).

The nature of the Bok binding region in IP_3R1 is intriguing. It is obvious that the sequence is highly polar (5 of 9 residues are charged), and it is tempting to speculate that this could drive the interaction with the Bok BH4 domain, which also contains charged residues (Fig. 4*A*). A similar sequence is found in the corresponding region of IP_3R2 (4 of 8 residues are charged), but no such charge density is found in the corresponding region of IP_3R3, which is very different from IP_3R1 and IP_3R2 in this area. This diversity may well account for the ability of Bok to bind strongly to IP_3R1 and IP_3R2, but not to IP_3R3.

The challenge now is to determine the significance of the Bok-IP_3R interaction. The data from MEFs, either replete with or lacking Bok, indicate that Bok does not regulate the ability of IP_3Rs to act as Ca^{2+} channels, or the ability of the ER to sequester Ca^{2+}, at least in unperturbed cells. Further, the processing of activated IP_3Rs by the UPP was not dependent upon the presence of Bok, indicating that it does not play a role in this homeostatic phenomenon (32, 33). Bok did, however, regulate IP_3R expression. This was seen in MEFs, in which lack of Bok increased IP_3R1 expression, but decreased IP_3R2 and IP_3R3 expression. Effects of exogenous Bcl-2 family proteins on IP_3R expression have been seen in other cell types (46, 49), although not always (45). While the basis for these expression changes remains to be defined, overall our results indicate that Bok is not critical to the central function of IP_3Rs, which is to act as IP_3-gated Ca^{2+} channels.

Interestingly, in cells in which IP_3-dependent signaling was persistently activated, Bok was degraded by the UPP, while the levels of other Bcl-2 family protein remained unaltered. It is well known that IP_3Rs are degraded by the UPP under these conditions (32, 33) and the finding that Bok is degraded in parallel is consistent with the notion that endogenous Bok and IP_3Rs are constitutively associated. Bok and IP_3Rs may well be extracted from the ER membrane, ubiquitinated and degraded as a unit (38), and it will be interesting to see whether the proteins that mediate IP_3R ubiquitination (the erlin1/2 complex and the ubiquitin ligase RNF170 (30, 34)) also mediate Bok ubiquitination. IP_3R degradation by the UPP is seen to be a homeostatic response to persistent cell stimulation that allows cells to reduce the sensitivity of their Ca^{2+} stores to IP_3 and thus suppress Ca^{2+} mobilization (32, 33). That Bok should be degraded in parallel is currently of unknown significance, but given that Bok is generally considered to be pro-apoptotic, loss of Bok could well influence the viability of the stimulated cells.

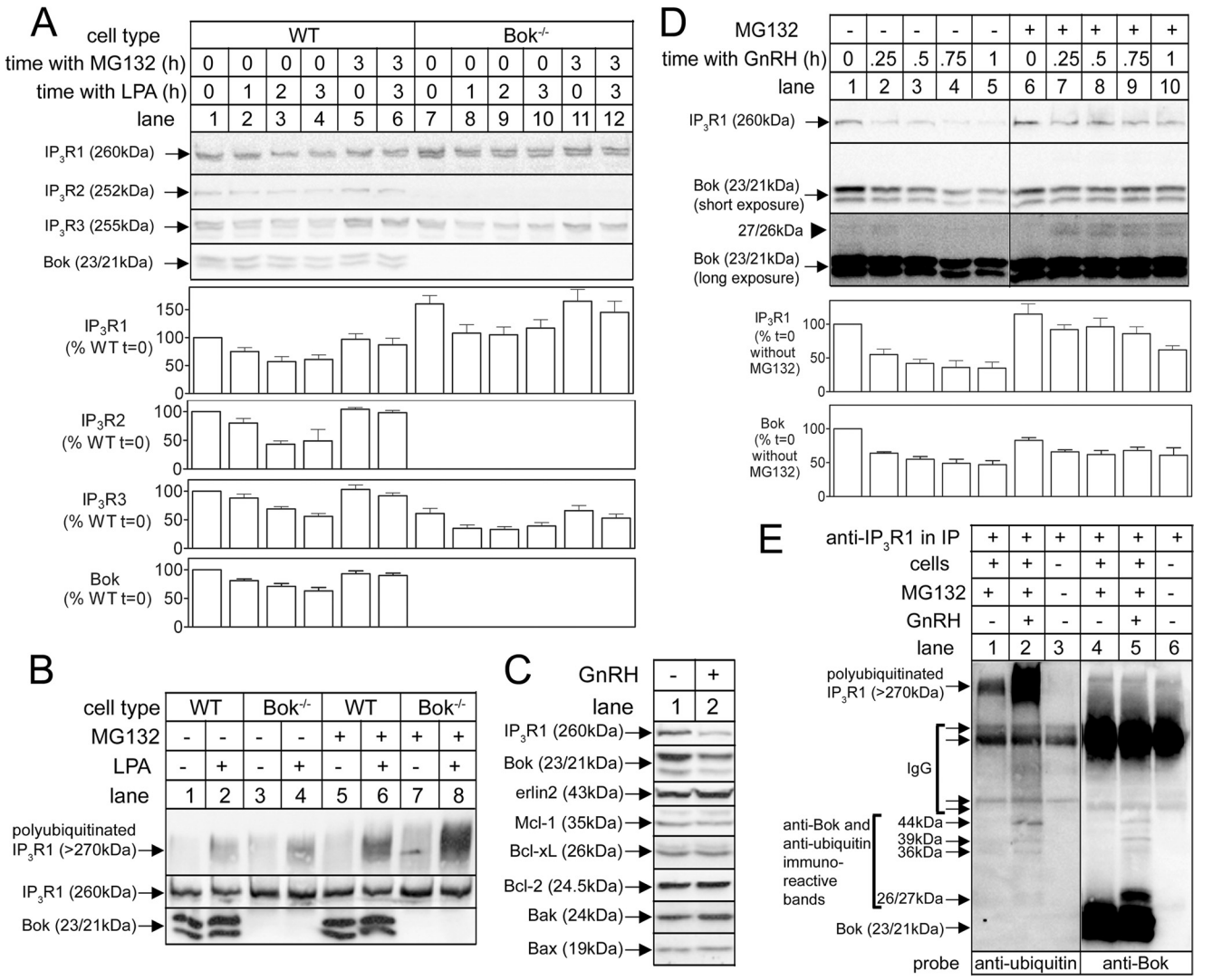

FIGURE 7. **Degradation of IP_3Rs and Bok by the UPP in MEFs and αT3 cells.** *A*, MEFs were incubated with 10 μM LPA and 10 μM MG132 for the times indicated, were harvested with 1% Triton lysis buffer, lysates were prepared, samples were subjected to SDS-PAGE, and immunoblots were probed with anti-IP_3R1–3 and anti-Bok as indicated. The histograms show combined quantitated IP_3R1–3 and Bok immunoreactivity normalized to WT $t = 0$ values from multiple independent experiments ($n \geq 3$). *B*, MEFs were incubated without or with 10 μM LPA for 50 min, in the absence or presence of 10 μM MG132 (which was added 10 min prior to LPA), were harvested with 1% Triton lysis buffer, anti-IP_3R1 immunoprecipitates were prepared, samples were subjected to SDS-PAGE, and immunoblots were probed with anti-ubiquitin, anti-IP_3R1 and anti-Bok as indicated ($n = 2$). *C*, αT3 cells were incubated without or with 1 μM GnRH for 2 h, were harvested with 1% CHAPS lysis buffer, lysates were prepared, samples were subjected to SDS-PAGE, and immunoblots were probed for the proteins indicated ($n = 2$). *D*, αT3 cells were incubated with 1 μM GnRH for the times indicated in the absence or presence of MG132 (which was added 30 min prior to GnRH), and were processed as in *C*. The histograms show combined quantitated IP_3R1 or Bok immunoreactivity from multiple independent experiments ($n = 3$). *E*, αT3 cells were incubated without or with 1 μM GnRH for 15 min, in the presence of 10 μM MG132 (which was added 30 min prior to GnRH), were harvested with 1% CHAPS lysis buffer, anti-IP_3R1 immunoprecipitates were prepared, samples and controls (*lanes 3* and *6*) were subjected to SDS-PAGE, and immunoblots were probed with anti-ubiquitin and anti-Bok as indicated ($n = 2$).

The most significant effect of Bok binding appears to be inhibition of IP_3R proteolysis by chymotrypsin *in vitro* at the fragment IV-V junction (at amino acid ~1932), and by caspase-3 *in vivo* during apoptosis (at amino acid 1891). It seems entirely plausible that this could result from steric hindrance by Bok of the ability of chymotrypsin and caspase-3 to access their cleavage sites. This inhibitory effect of Bok may well be biologically relevant, as the C-terminal IP_3R1 fragment resulting from caspase-3 cleavage has been proposed to form an IP_3-insensitive, "leaky" channel that contributes toward the apoptotic cascade (40, 43), although this notion is controversial (44, 50, 51). Indeed, a very recent study shows that the IP_3R1 fragments generated by caspase-3 remain associated and form functional channels fully capable of responding to IP_3 and contributing toward apoptosis (44). Thus, the significance of the inhibitory effect of Bok on IP_3R proteolysis will be better understood when the significance of IP_3R cleavage by caspase-3 is fully elucidated.

The cellular role of Bok has been an enigma since its discovery (1, 2, 7). Based on its similarity to Bax and Bak and overexpression studies, it has been assumed that Bok is pro-apoptotic (4–9) and, indeed, very recent evidence from $Bax^{-/-}$ $Bok^{-/-}$ mice indicates that it can act pro-apoptotically in ovaries (52). Interestingly, however, Bok can also act anti-apoptotically; $Bok^{-/-}$ cells undergo apoptosis more readily than control cells in response to brefeldin A, a drug that inhibits ER to Golgi transport and causes ER stress (8). Determining the role played

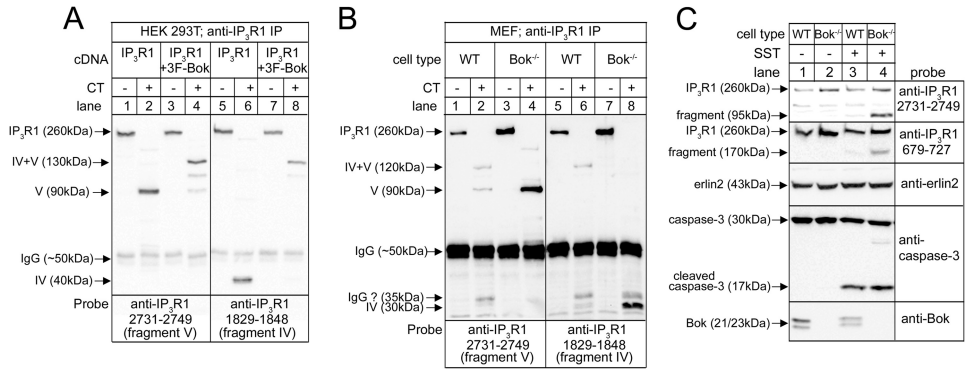

FIGURE 8. **Effects of Bok on IP$_3$R1 proteolysis.** *A* and *B*, anti-IP$_3$R1 immunoprecipitates, prepared from transfected HEK 293T cells or MEFs with 1% CHAPS lysis buffer, were washed with ice-cold PBS, were incubated with 1 μg/ml chymotrypsin (*CT*) for 15 min at 25 °C, were boiled for 5 min in gel-loading buffer (29), samples were subjected to SDS-PAGE, and immunoblots were probed for fragment V with anti-IP$_3$R1$^{2731-2749}$ and fragment IV with anti-IP$_3$R1$^{1829-1848}$ ($n = 2$). IP$_3$R1-derived immunoreactive bands are indicated with arrows, as is cross-reacting IgG heavy chain at ~50 kDa, and a probable IgG-derived fragment at ~35 kDa. *C*, cells were incubated with 1 μM staurosporine (SST) for 14 h, were harvested with 1% Triton lysis buffer, lysates were subjected to SDS-PAGE, and immunoblots were probed with anti-IP$_3$R1$^{2731-2749}$ and anti-IP$_3$R1$^{679-727}$ to detect C- and N-terminal IP$_3$R1 fragments, respectively, and for the other proteins indicated. Erlin2 served as a loading control ($n = 2$).

by IP$_3$R binding in these effects of Bok will be a fascinating topic for future studies and should help unravel the subtleties of Bok action.

Acknowledgments—We thank Danielle Sliter and Jennifer Vella for assisting with the initial identification of Bok, Danielle Sliter for helpful suggestions and for providing the Bax and Bak antibodies and the IP$_3$R1HA$^{\Delta 1-2268}$ construct and Andreas Strasser for providing the Bok$^{-/-}$ MEFs.

REFERENCES

1. Youle, R. J., and Strasser, A. (2008) The BCL-2 protein family: opposing activities that mediate cell death. *Nat. Rev. Mol. Cell Biol.* **9,** 47–59
2. Kelly, P. N., and Strasser, A. (2011) The role of Bcl-2 and its pro-survival relatives in tumourigenesis and cancer therapy. *Cell Death Differ.* **18,** 1414–1424
3. Westphal, D., Dewson, G., Czabotar, P. E., and Kluck, R. M. (2011) Molecular biology of Bax and Bak activation and action. *Biochim. Biophys. Acta* **1813,** 521–531
4. Hsu, S. Y., Kaipia, A., McGee, E., Lomeli, M., and Hsueh, A. J. W. (1997) Bok is a pro-apoptotic Bcl-2 protein with restricted expression in reproductive tissues and heterodimerizes with selective anti-apoptotic Bcl-2 family members. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 12401–12406
5. Hsu, S. Y., and Hsueh, A. J. W. (1998) A splicing variant of the Bcl-2 member Bok with a truncated BH3 domain induces apoptosis but does not dimerize with antiapoptotic Bcl-2 proteins *in vitro*. *J. Biol. Chem.* **273,** 30139–30146
6. Inohara, N., Ekhterae, D., Garcia, I., Carrio, R., Merino, J., Merry, A., Chen, S., and Nuñez, G. (1998) Mtd, a novel Bcl-2 family member activates apoptosis in the absence of heterodimerization with Bcl-2 and Bcl-xL. *J. Biol. Chem.* **273,** 8705–8710
7. Ke, F., Voss, A., Kerr, J. B., O'Reilly, L. A., Tai, L., Echeverry, N., Bouillet, P., Strasser, A., and Kaufmann, T. (2012) BCL-2 family member BOK is widely expressed but its loss has only minimal impact in mice. *Cell Death Differ.* **19,** 915–925
8. Echeverry, N., Bachmann, D., Ke, F., Strasser, A., Simon, H-U., and Kaufmann, T. (2013) Intracellular localization of the BCL-2 family member BOK and functional implications. *Cell Death Differ.* **20,** 785–799
9. Bartholomeusz, G., Wu, Y., Ali Seyed, M., Xia, W., Kwong, K-Y., Hortobagyi, G., and Hung, M-C. (2006) Nuclear translocation of the pro-apoptotic Bcl-2 family member Bok induces apoptosis. *Molecular Carcinogenesis* **45,** 73–83
10. Beroukhim, R., Mermel, C. H., Porter, D., Wei, G., Raychaudhuri, S., Donovan, J., Barretina, J., Boehm, J. S., Dobson, J., Urashima, M., Mc Henry, K. T., Pinchback, R. M., Ligon, A. H., Cho, Y. J., Haery, L., Greulich, H., Reich, M., Winckler, W., Lawrence, M. S., Weir, B. A., Tanaka, K. E., Chiang, D. Y., Bass, A. J., Loo, A., Hoffman, C., Prensner, J., Liefeld, T., Gao, Q., Yecies, D., Signoretti, S., Maher, E., Kaye, F. J., Sasaki, H., Tepper, J. E., Fletcher. J. A., Tabernero, J., Baselga, J., Tsao, M. S., Demichelis, F., Rubin, M. A., Janne, P. A., Daly, M. J., Nucera, C., Levine, R. L., Ebert, B. L., Gabriel, S., Rustgi, A. K., Antonescu, C. R., Ladanyi, M., Letai, A., Garraway, L. A., Loda, M., Beer, D. G., True, L. D., Okamoto, A., Pomeroy, S. L., Singer, S., Golub, T. R., Lander, E. S., Getz, G., Sellers, W. R., and Meyerson, M. (2010) The landscape of somatic copy-number alteration across human cancers. *Nature* **463,** 899–905
11. Distelhorst, C. W., and Bootman, M. D. (2011) Bcl-2 interaction with the inositol 1,4,5-trisphosphate receptor: Role in Ca^{2+} signaling and disease. *Cell Calcium* **50,** 234–241
12. Akl, H., and Bultynck, G. (2013) Altered Ca^{2+} signaling in cancer cells targeting IP3 receptors: Proto-oncogenes and tumor suppressors targeting IP3 receptors. *Biochim. Biophys. Acta* **1835,** 180–193
13. Foskett, J. K., White, C., Cheung, K. H., and Mak, D. O. (2007) Inositol trisphosphate receptor Ca^{2+} release channels. *Physiol. Rev.* **87,** 593–658
14. Seo, M. D., Velamakanni, S., Ishiyama, N., Stathopulos, P. B., Rossi, A. M., Khan, S. A., Dale, P., Li, C., Ames, J. B., Ikura, M., and Taylor, C. W. (2012) Structural and functional conservation of key domains in $InsP_3$ and ryanodine receptors. *Nature* **483,** 108–112
15. Wojcikiewicz, R. J. H. (1995) Type I, II and III inositol 1,4,5-trisphosphate receptors are unequally susceptible to down-regulation and are expressed in markedly different proportions in different cell types. *J. Biol. Chem.* **270,** 11678–11683
16. Rizzuto, R., Marchi, S., Bonora, M., Aguiari, P., Bononi, A., De Stefani, D., Giorgi, C., Leo, S., Rimessi, A., Siviero, R., Zecchini, E., and Pinton, P. (2009) Ca^{2+} transfer from ER to mitochondria: When, how and why. *Biochim. Biophys. Acta* **1787,** 1342–1351
17. Zhong, F., Davis, M. C., McColl, K. S., and Distelhorst, C. W. (2006) Bcl-2 differentially regulates Ca^{2+} signals according to the strength of T cell receptor activation. *J. Cell Biol.* **172,** 127–137
18. Rong, Y-P., Aromolaran, A. S., Bultynck, G., Zhong, F., Li, X., McColl, K. S., Matsuyama, S., Herlitze, S., Roderick, H. L., Bootman, M. D., Mignery, G. A., Parys, J. B., De Smedt, H., and Distelhorst, C. W. (2008) Targeting Bcl-2-IP3 receptor interaction to reverse Bcl-2's inhibition of apoptotic calcium signals. *Mol. Cell* **31,** 255–265
19. Eckenrode, E. F., Yang, J., Velmurugan, G. V., Foskett, J. K., and White, C. (2010) Apoptosis protection by Mcl-1 and Bcl-2 modulation of inositol 1,4,5-trisphosphate receptor-dependent Ca^{2+} signaling. *J. Biol. Chem.* **285,** 13678–13684
20. Monaco, G., Decrock, E., Akl, H., Ponsaerts, R., Vervliet, T., Luyten, T., De Maeyer, M., Missiaen, L., Distelhorst, C. W., De Smedt, H., Parys, J. B., Leybaert, L., and Bultynck, G. (2012) Selective regulation of IP_3-receptor-

mediated Ca^{2+} signaling and apoptosis by the BH4 domain of Bcl-2 *versus* Bcl-xL. *Cell Death Differ.* **19,** 295–309
21. White, C., Li, C., Yang, J., Petrenko, N. B., Madesh, M., Thompson, C. B., and Foskett, J. K. (2005) The endoplasmic reticulum gateway to apoptosis by Bcl-xL modulation of the $InsP_3R$. *Nat. Cell Biol.* **7,** 1021–1028
22. Oakes, S. A., Scorrano, L., Opferman, J. T., Bassik, M. C., Nishino, M., Pozzan, T., and Korsmeyer, S. J. (2005) Proapoptotic Bax and Bak regulate the type 1 inositol trisphosphate receptor and calcium leak from the endoplasmic reticulum. *Proc. Natl. Acad. Sci. U.S.A.* **102,** 105–110
23. Jones, R. G., Bui, T., White, C., Madesh, M., Krawczyk, C. M., Lindsten, T., Hawkins, B. J., Kubek, S., Frauwirth, K. A., Wang, Y. L., Conway, S. J., Roderick, H. L., Bootman, M. D., Shen, H., Foskett, J. K., and Thompson, C. B. (2007) The proapoptotic factors Bax and Bak regulate T cell proliferation through control of endoplasmic reticulum Ca^{2+} homeostasis. *Immunity* **27,** 268–280
24. Monaco, G., Beckers, M., Ivanova, H., Missiaen, L., Parys, J. B., De Smedt, H., and Bultynck, G. (2012) Profiling of the Bcl-2/Bcl-xL-binding sites on type 1 IP_3 receptor. *Biochem. Biophys. Res. Commun.* **428,** 31–35
25. Rong, Y-P., Bultynck, G., Aromolaran, A. S., Zhong, F., Parys, J. B., De Smedt, H., Mignery, G. A., Roderick, H. L., Bootman, M. D., and Distelhorst, C. W. (2009) The BH4 domain of Bcl-2 inhibits ER calcium release and apoptosis by binding the regulatory and coupling domain of the IP3 receptor. *Proc. Natl. Acad. Sci. U.S.A.* **106,** 14397–14402
26. Zhong, F., Harr, M. W., Bultynck, G., Monaco, G., Parys, J. B., De Smedt, H., Rong, Y-P., Molitoris, J. K., Lam, M., Ryder, C., Matsuyama, S., and Distelhorst, C. W. (2011) Induction of Ca^{2+}-driven apoptosis in chronic lymphocytic leukemia cells by peptide-mediated disruption of Bcl-2-IP_3 receptor interaction. *Blood* **117,** 2924–2934
27. Wojcikiewicz, R. J. H., Xu, Q., Webster, J. M., Alzayady, K., and Gao, C. (2003) Ubiquitination and proteasomal degradation of endogenous and exogenous inositol 1,4,5 trisphosphate receptors in αT3–1 anterior pituitary cells. *J. Biol. Chem.* **278,** 940–947
28. Pearce, M. M., Wang, Y., Kelley, G. G., and Wojcikiewicz, R. J. (2007) SPFH2 mediates the endoplasmic reticulum-associated degradation of inositol 1,4,5-trisphosphate receptors and other substrates in mammalian cells. *J. Biol. Chem.* **282,** 20104–20115
29. Oberdorf, J., Webster, J. M., Zhu, C. C., Luo, S. G., and Wojcikiewicz, R. J. (1999) Down-regulation of types I, II and III inositol 1,4,5 trisphosphate receptors is mediated by the ubiquitin / proteasome pathway. *Biochem. J.* **339,** 453–461
30. Lu, J. P., Wang, Y., Sliter, D. A., Pearce, M. M., and Wojcikiewicz, R. J. (2011) RNF170, an endoplasmic reticulum membrane ubiquitin ligase, mediates inositol 1,4,5-trisphosphate receptor ubiquitination and degradation. *J. Biol. Chem.* **286,** 24426–24433
31. Soulsby, M. D., and Wojcikiewicz, R. J. (2007) Calcium mobilization via type III inositol 1,4,5-trisphosphate receptors is not altered by PKA-mediated phosphorylation of serines 916, 934 and 1832. *Cell Calcium* **42,** 261–270
32. Wojcikiewicz, R. J., Pearce, M. M., Sliter, D. A., and Wang, Y. (2009) When worlds collide: IP_3 receptors and the ERAD pathway. *Cell Calcium* **46,** 147–153
33. Wojcikiewicz, R. J. H. (2012) Inositol 1,4,5-trisphosphate receptor degradation pathways. *WIREs Membr. Transp. Signal.* **1,** 126–135
34. Pearce, M. M., Wormer, D. B., Wilkens, S., and Wojcikiewicz, R. J. (2009) An endoplasmic reticulum (ER) membrane complex composed of SPFH1 and SPFH2 mediates the ER-associated degradation of inositol 1,4,5-trisphosphate receptors. *J. Biol. Chem.* **284,** 10433–10445
35. Xu, Q., Farah, M., Webster, J. M., and Wojcikiewicz, R. J. (2004) Bortezomib rapidly suppresses ubiquitin thiolesterification to ubiquitin-conjugating enzymes and inhibits ubiquitination of histones and type I inositol 1,4,5-trisphosphate receptors. *Mol. Cancer Ther.* **3,** 1263–1269
36. Kelley, G. G., Kaproth-Joslin, K. A., Reks, S. E., Smrcka, A. V., and Wojcikiewicz, R. J. (2006) G-protein-coupled receptor agonists activate endogenous phospholipase Cε and phospholipase Cβ3 in a temporally distinct manner. *J. Biol. Chem.* **281,** 2639–2648
37. Wojcikiewicz, R. J., Tobin, A. B., and Nahorski, S. R. (1994) Muscarinic receptor-mediated inositol 1,4,5-trisphosphate formation in SH-SY5Y neuroblastoma cells is regulated acutely by cytosolic Ca^{2+} and by rapid desensitization. *J. Neurochem.* **63,** 177–185
38. Vembar, S. S., and Brodsky, J. L. (2008) One step at a time: endoplasmic reticulum-associated degradation. *Nat. Rev. Mol. Cell Biol.* **9,** 944–957
39. Hirota, J., Furuichi, T., and Mikoshiba, K. (1999) Inositol 1,4,5-trisphosphate receptor type 1 is a substrate for caspase-3 and is cleaved during apoptosis in a caspase-3-dependent manner. *J. Biol. Chem.* **274,** 34433–34437
40. Assefa, Z., Bultynck, G., Szlufcik, K., Nadif Kasri, N., Vermassen, E., Goris, J., Missiaen, L., Callewaert, G., Parys, J. B., and De Smedt, H. (2004) Caspase-3-induced truncation of type 1 inositol trisphosphate receptor type 1 accelerates apoptotic cell death and induces inositol trisphosphate-independent calcium release during apoptosis. *J. Biol. Chem.* **279,** 43227–43236
41. Yoshikawa, F., Iwasaki, H., Michikawa, T., Furuichi, T., and Mikoshiba, K. (1999) Trypsinized cerebellar inositol 1,4,5-trisphosphate receptor. *J. Biol. Chem.* **274,** 316–327
42. Maes, K., Missiaen, L., Parys, J. B., De Smet, P., Sienaert, I., Waelkens, E., Callewaert, G., and De Smedt, H. (2001) Mapping of the ATP-binding sites on inositol 1,4,5-trisphosphate receptor type 1 and type 3 homotetramers by controlled proteolysis and photoaffinity labeling. *J. Biol. Chem.* **276,** 3492–3497
43. Verbert, L., Lee, B., Kocks, S. L., Assefa, Z., Parys, J. B., Missiaen, L., Callewaert, G., Fissore, R. A., De Smedt, H., and Bultynck, G. (2008) Caspase-3-truncated type 1 inositol 1,4,5- trisphosphate receptor enhances intracellular Ca^{2+} leak and disturbs Ca^{2+} signalling. *Biol. Cell* **100,** 39–49
44. Alzayady, K., Chandrasekhar, R., and Yule, D. I. (2013) Fragmented inositol 1,4,5-trisphosphate receptors retain tetrameric architecture and form functional Ca^{2+} release channels. *J. Biol. Chem.* **288,** 11122–11134
45. Chen, R., Valencia, I., Zhong, F., McColl, K. S., Roderick, H. L., Bootman, M. D., Berridge, M. J., Conway, S. J., Holmes, A. B., Mignery, G. A., Velez, P., and Distelhorst, C. W. (2004) Bcl-2 functionally interacts with inositol 1,4,5 trisphosphate receptors to regulate calcium release from the ER in response to inositol 1,4,5 trisphosphate. *J. Cell Biol.* **166,** 193–203
46. Hanson, C. J., Bootman, M. D., Distelhorst, C. W., Wojcikiewicz, R. J., and Roderick, H. L. (2008) Bcl-2 suppresses Ca^{2+} release through inositol 1,4,5-trisphosphate receptors and inhibits Ca^{2+} uptake by mitochondria without affecting ER calcium store content. *Cell Calcium* **44,** 324–338
47. Kvansakul, M., Yang, H., Fairlie, W. D., Czabotar, P. E., Fischer, S. F., Perugini, M. A., Huang, D. C., and Colman, P. M. (2008) Vaccinia virus anti-apoptotic F1L is a novel Bcl-2-like domain-swapped dimer that binds a highly selective subset of BH3-containing death ligands. *Cell Death Differ.* **15,** 1564–1571
48. Pizzo, P., Lissandron, V., and Pozzan, T. (2010) The trans-golgi compartment: A new distinct intracellular Ca^{2+} store. *Commun. Integr. Biol.* **3,** 462–464
49. Li, C., Fox, C. J., Master, S. R., Bindokas, V. P., Chodosh, L. A., and Thompson, C. B. (2002) Bcl-xL affects Ca^{2+} homeostasis by altering expression of inositol 1,4,5-trisphosphate receptors. *Proc. Natl. Acad. Sci. U.S.A.* **99,** 9830–9835
50. Elkoreh, G., Blais, V., Béliveau, E., Guillemette, G., and Denault, J-B. (2012) Type 1 inositol 1,4,5-trisphosphate receptor is a late substrate of caspases during apoptosis. *J. Cell. Biochem.* **113,** 2775–2784
51. Akimzhanov, A. M., Barral, J. M., and Boehning, D. (2013) Caspase 3 cleavage of the inositol 1,4,5-trisphosphate receptor does not contribute to apoptotic calcium release. *Cell Calcium* **53,** 152–158
52. Ke, F., Bouillet, P., Kaufmann, T., Strasser, A., Kerr, J., and Voss, A. K. (2013) Consequences of the combined loss of BOK and BAK or BOK and BAX. *Cell Death Dis.* **4,** e650