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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 32, pp. 23003–23012, August 11, 2006

PP2A Regulates BCL-2 Phosphorylation and Proteasome-mediated Degradation at the Endoplasmic Reticulum*[S]

Received for publication, March 21, 2006 Published, JBC Papers in Press, May 22, 2006, DOI 10.1074/jbc.M602648200

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Anti-apoptotic activity of BCL-2 is mediated by phosphorylation at the endoplasmic reticulum (ER), but how this phosphorylation is regulated and the mechanism(s) by which it regulates apoptosis are unknown. We purified macromolecular complexes containing BCL-2 from ER membranes and found that BCL-2 co-purified with the main two subunits of the serine/threonine phosphatase, PP2A. The association of endogenous PP2A and BCL-2 at the ER was verified by co-immunoprecipitation and microcystin affinity purification. Knock down or pharmacological inhibition of PP2A caused degradation of phosphorylated BCL-2 and led to an overall reduction in BCL-2 levels. We found that this degradation was due to the action of the proteasome acting selectively at the ER. Conversely, overexpression of PP2A caused elevation in endogenous BCL-2. Most importantly, we found that PP2A knock down sensitized cells to several classes of death stimuli (including ER stress), but this effect was abolished in a genetic background featuring knock in of a non-phosphorylatable BCL-2 allele. These studies support the hypothesis that PP2A-mediated dephosphorylation of BCL-2 is required to protect BCL-2 from proteasome-dependent degradation, affecting resistance to ER stress.

Apoptosis is an orderly process of cell demise with distinct morphological and biochemical features (1). The BCL-2 family plays a critical role in the regulation of apoptosis. There are both pro- and anti-apoptotic BCL-2 family members that respond to specific death signals to promote or inhibit cell death (2). In general, the anti-apoptotic members like BCL-2 display sequence conservation throughout four BCL-2 homology domains (BH1–4). Pro-apoptotic BCL-2 members can be further subdivided into more fully conserved, "multidomain" members possessing homology in BH1–3 domains (*e.g.* BAX and BAK) or "BH3-only" members that display only ~9 amino acids of sequence homology within their BH3 domains (*e.g.* BID, BIM, and BAD) (2).

Evidence suggests that the intrinsic pathway of apoptosis operates at the surface of subcellular organelles, of which the mitochondrion has been extensively studied (3). Several BCL-2 family members have also been shown to operate at the endoplasmic reticulum (ER).[4] The ER is the major storage site for intracellular calcium and is also vital for the modification and secretion of proteins. Consequently, agents that perturb ER function, including those that induce Ca^{2+} release from the ER (*e.g.* H_2O_2) or those that block protein modification/secretion and trigger an unfolded protein response (UPR) (*e.g.* tunicamycin and brefeldin A), can induce apoptosis (4, 5).

BAX, BAK, and BCL-2 localize to the ER. Deletion of BAX and BAK, or overexpression of BCL-2, causes resistance to death stimuli that operate both through ER Ca^{2+} and the UPR (6–8). Resistance of these cells to Ca^{2+}-dependent death stimuli is due to reductions of resting Ca^{2+} in the ER (9, 10). There is evidence that BCL-2 regulates ER Ca^{2+} in a mechanism involving the type 1 inositol trisphosphate receptor (IP3R-1) (11, 12). However, the mechanism by which BCL-2 protects against UPR-mediated death is unclear. In general, regulators of BCL-2 activity at the ER are unknown.

To this end, we took an unbiased approach to identify BCL-2-interacting protein partners at the ER membrane. We identified the main two subunits of PP2A, a tripartite serine/threonine phosphatase composed of one catalytic C subunit and one A subunit acting largely as a scaffold, and one of four classes of regulatory B subunits (13). The activity of several BCL-2 family proteins is regulated by post-translational modifications, and a number of groups have found that BCL-2 phosphorylation results in its inactivation (14–18). Phosphorylated BCL-2 has recently been shown to be predominantly localized to the ER, and mutation of the phosphorylation sites leads to protection against cell death from both classes of ER-dependent death stimuli and affects Ca^{2+} levels in the ER (19). Therefore, the coordination of phosphorylation and dephosphorylation of

* This work was supported in part by National Institutes of Health Grants P01 CA92625 and R01 CA50239 (to S. J. K.) and by P01 CA050661 (to T. M. R. and W. C. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

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[4] The abbreviations used are: ER, endoplasmic reticulum; PP2A, protein phosphatase 2A; HEK, human embryonic kidney; MEF, mouse embryonic fibroblast; OA, okadaic acid; shRNA, small hairpin ribonucleic acid; IP3R-1, type I inositol trisphosphate receptor; UPR, unfolded protein response; LM, light membrane; HM, heavy membrane; IP, immunoprecipitation; WT, wild type; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

BCL-2 by kinases and phosphatases is predicted to regulate BCL-2 activity at the ER.

We found that PP2A regulates BCL-2 phosphorylation and subsequent anti-apoptotic activity in an unexpected manner. Modulation of PP2A activity by pharmacological inhibition, small hairpin RNA (shRNA) knock down, or protein overexpression revealed that PP2A surprisingly influences proteasome-dependent degradation of phosphorylated BCL-2 at the ER. In summary, here we report that PP2A is a critical regulator of BCL-2 stability and hence the apoptotic threshold of the cell.

EXPERIMENTAL PROCEDURES

Purification and Mass Spectrometry—Whole brain was dissected from >30 C57/BL6 mice and homogenized using a glass dounce in isotonic buffer (200 mM sucrose, 10 mM Tris, 0.1 mM Ca^{2+}, 0.5 mM sodium fluoride, 0.2 mM sodium orthovanadate, protease inhibitor mixture; Sigma). The homogenate was fractionated by a series of centrifugation steps, first at 600 × *g* for 10 min (1×) to pellet nuclei and unbroken tissue. Second, the supernatant was centrifuged at 7000 × *g* for 10 min (≥3×) to pellet the heavy membrane (HM). Third, the supernatant was centrifuged 280,000 × *g* (1×) to pellet ER-rich light membrane (LM). The LM pellet was solubilized in 1% CHAPS-containing buffer (5 mM sodium phosphate, pH 7.4, 2.5 mM EDTA, 100 mM NaCl, 0.5 mM NaF, 0.2 mM sodium orthovanadate, protease inhibitor mixture (Sigma), 10% glycerol). To purify BCL-2, the solubilized LM fraction was precipitated with ammonium sulfate to 40%. The precipitate was resuspended and dialyzed into 1% CHAPS buffer. Proteins were bound to a MonoQ column and eluted off the column with an increasing linear gradient of 0.1 M^{-1} to M NaCl. Fractions that contained BCL-2 were dialyzed into 1% CHAPS buffer and separated by a Superose 6 column. Positive fractions were dialyzed into 4 mM CHAPS, 800 mM ammonium sulfate. The dialyzed proteins were bound to a TSK-Phenyl column and eluted with a decreasing linear gradient of 0.8 – 0 M ammonium sulfate. After a final dialysis into 1% CHAPS buffer, the BCL-2 fractions were separated through a Superdex 200 column. Proteins were identified by silver staining (Bio-Rad) and subsequent liquid chromatography/mass spectrometry/mass spectrometry sequencing using a Deca XP instrument (Thermo Finnigan). PP2A peptides were identified using XCorr cut-off values of 1.8, 2.1, and 2.8 for 1+, 2+, and 3+ peptides, respectively, and a dCN value of 0.08.

Cell Lines—Jurkat T cells were transfected with hBCL-2^{WT} or hBCL-2 T69A,S70A,S87A = hBCL-2^{AAA} (17, 19). Mouse embryonic fibroblasts (MEFs) from C57/BL6 wild-type or mBCL-2^{AAA} knock-in mice were transformed with SV40 genomic DNA, and clones of similar growth and morphology were selected.[5] Wild-type or BCL-2^{AAA} knock-in MEFs were transduced by lentivirus with shRNA directed at $mPP2A_C$ (1-CGGCAGATCACACAAGTGTAT; 2- GCTGCTATCATGGAATTAGAT; 3- GCCCAATGTGTGATCTCTTAT) or $mPP1_C$ (1- GCTGTTTGAGTATGGTGGCTT; 2- CGTGGTGTCTCCTTTACCTTT). $shPP2A_C$ and $shPP1_C$ vectors were courtesy of Harvard/MIT Broad Institute RNAi Consortium. Transduced MEFs were selected in 2 μg/ml of puromycin. SV40-transformed BCL-2^{null} MEFs expressing hBCL-2 were transduced by lentivirus with $shPP2A_C$ or $shPP1_C$. Human embryonic kidney (HEK) cells immortalized with hTERT, H-Ras, and large T antigen (20) were transduced by retrovirus with shB56γ or B56γ and selected for green fluorescent protein expression by flow cytometry (21). Jurkat T cells were grown in RPMI medium 1640 supplanted with β-mercaptoethanol, minimal amino acids, L-glutamine, penicillin/streptomycin, and 10% fetal bovine serum. MEFs were grown in Iscove's modified Dulbecco's medium supplanted with minimal amino acids, L-glutamine, penicillin/streptomycin, and 10% fetal bovine serum. HEK cells were grown in minimal Eagle's medium α containing 10% fetal bovine serum.

Immunoprecipitation—Brain ER was solubilized in 1% CHAPS buffer, precipitated with 40% ammonium sulfate, and dialyzed back into 1% CHAPS buffer. To immunoprecipitate BCL-2, 1 mg of ER protein was incubated with 10 μg of IgG and 10 μl of protein A/G beads for 2 h. The beads were spun down, and supernatant lysate was transferred to a fresh tube. Concurrently, 20 μg of BCL-2 N-19 antibody (Santa Cruz Biotechnology) was incubated with 30 μl of protein A/G beads (Santa Cruz) for 2 h. The beads were washed with 1 ml of 1% CHAPS buffer, and the precleared lysate was incubated with antibody-bound beads overnight. Following overnight incubation, beads were washed three times with 1% CHAPS buffer and boiled in SDS sample buffer. To immunoprecipitate PP2A and avoid co-migration of the Ig light chain with BCL-2, 1 mg of ER protein was incubated with PP2A A subunit 6F9 antibody (Covance) covalently coupled to a gel matrix using a ProFound co-immunoprecipitation kit (Pierce). To immunoprecipitate B56γ, mouse monoclonal antibodies were generated from full-length B56γ3 (distributed by Novus or Zymed Laboratories Inc.). Western blotting antibodies were used at 1 μg/ml.

Subcellular Fractionation—1.5 liters of hBCL-2-expressing Jurkat T cells were incubated with 10 nM okadaic acid or vehicle for 24 h. Subcellular fractionation was performed as described previously (19). 3 ml of Jurkat cells were lysed in radioimmune precipitation buffer (1% Nonidet P-40, 1% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM HEPES, pH 7.5, 2 mM EDTA, 0.5 mM NaF, 0.2 mM sodium orthovanadate, protease inhibitor mixture; Sigma) to create whole cell lysate. The remaining cells were suspended in HIM buffer (200 mM mannitol, 70 mM sucrose, 10 mM HEPES, pH 7.5, 1 mM EGTA, 0.5 mM NaF, 0.2 mM sodium orthovanadate, protease inhibitor mixture; Sigma). Cells were disrupted using a glass dounce, followed by passage through 27- and 30-gauge needles (10 times for each). The crude fraction containing nuclei and unbroken cells was removed at 600 × *g*. The supernatant was centrifuged at 7000 × *g* to pellet the HM fraction. The subsequent supernatant was centrifuged two more times to pellet the HM pellet, followed by centrifugation at 280,000 × *g* to bring down the LM fraction. The HM pellet was resuspended in HIM buffer and spun down once more at 600 × *g* and then 7000 × *g*. The resulting HM pellet was resuspended in HIM buffer, where an aliquot was lysed in radioimmune precipitation buffer. The remaining HM pellet was further purified from contaminating LM using a Percoll gradient (22). Samples were run on Tris-glycine SDS-PAGE

[5] M. C. Bassik, unpublished results.

containing 10% acrylamide to assess BCL-2 phosphorylation (19).

Cell Death Assay—Wild-type or BCL-2^{AAA} knock-in MEFs transduced with shPP2A_C 1 or control vector were treated with tunicamycin, brefeldin A, thapsigargin, H_20_2, taxol, etoposide, and staurosporine (all purchased from Sigma) as indicated in the figure legends. Annexin-V-Cy3 (Biovision) was used according to the manufacturer and quantified by fluorescence-activated cell sorter.

Real-time PCR—RNA was isolated from MEFs using an RNeasy kit (Qiagen), and cDNA was synthesized with random hexamer primers using Superscript III. Real-time PCR was performed for *bcl-2* (forward primer, 5′-AGTACCTGAACCGGCATCTG-3′; reverse primer, 5′-GCTGAGCAGGGTCTTCAGAG-3′) as previously described using Ribosome protein L4 for normalization (forward primer, 5′-AAGATGATGAACACCGACCTTAGC-3′; reverse primer, 5′-CCTTCTCTGGAACAACCTTCTCG-3′) (23).

RESULTS

Purification of BCL-2-interacting Partners at the Endoplasmic Reticulum—The ER-enriched light membrane fraction from mouse brain was solubilized in 1% CHAPS, a zwitterionic detergent previously shown to preserve the conformation of the proapoptotic BCL-2 family members (24). CHAPS concentrations up to 1% yielded BCL-2 in high molecular weight complexes as assessed by native gels (data not shown). BCL-2 was first purified by ammonium sulfate precipitation, followed by four sequential chromatography steps of MonoQ, Superose 6, TSK-Phenyl, and Superdex 200 (Fig. 1, *A* and *B*).

BCL-2-containing column fractions from the final Superdex 200 separation were subjected to SDS-PAGE and sequenced by liquid chromatography/mass spectrometry (Fig. 1*C*). The proteins enriched in these fractions were subunits of the vacuolar ATPase, SNAP-α, SNAP-γ, synaptogyrin, and α-contractin (actin homolog). These band assignments had either weak mass spectrometry coverage or their associations with BCL-2 could not be verified by co-immunoprecipitation (IP). Calcineurin, a well characterized BCL-2 protein partner (25), was also identified, validating our purification scheme (Fig. 1*C*). This association was verified by co-IP of endogenous ER proteins (Fig. 1*D*). Among the protein species identified, two that had the largest coverage by mass spectrometry corresponded to 65 and 36 kDa and were identified as components of the same enzyme complex. 12 peptide sequences covering 32% of the 65-kDa band identified the A subunit of protein phosphatase 2A (PP2A), and 6 peptide sequences covering 26% of the 36-kDa band identified the C subunit of PP2A (Fig. 1*C*). The anti-apoptotic activity of BCL-2 is known to be influenced by phosphorylation specifically at the ER (19); however, the phosphatase involved has never been identified. Although there are reports that PP2A associates with BCL-2, the ER had never been considered to host an interaction between the two proteins (26, 27). More importantly, mechanisms by which dephosphorylation regulates the anti-apoptotic activity of BCL-2 have remained unclear. Therefore, we decided to examine in detail the interaction of PP2A and BCL-2 at this organelle.

We first verified a previous finding that PP2A is capable of dephosphorylating BCL-2 *in vitro* (26) (supplemental Fig. S1). Because PP2A has never been thought to interact with BCL-2 at the ER, we next used IP of endogenous brain ER proteins to confirm that PP2A co-precipitated with BCL-2 at this organelle (Fig. 1*D*). Additional evidence for a PP2A-BCL-2 ER complex comes from purification from ER fractions with beads coupled to microcystin, an approach previously used to purify PP1 and PP2A enzyme complexes (28) (supplemental Fig. S2).

PP2A Inhibition Using Okadaic Acid Leads to Degradation of Phosphorylated BCL-2—To examine the effect of PP2A-mediated BCL-2 dephosphorylation, we treated Jurkat T cells that stably express hBCL-2 with okadaic acid (OA), another PP1/PP2A inhibitor. These cells have been previously used to follow the phosphorylation status of BCL-2 by molecular weight shift and by using an antibody to phospho-serine 70 (S70P) (17, 19), although additional sites are also most likely phosphorylated. We treated the cells with several concentrations of OA. Low concentrations of OA up to 10 nM led to an increase of hyperphosphorylated BCL-2, consistent with previous reports (26, 29) (Fig. 2*A*, *bracket 1*). To determine where in the cell PP2A affects BCL-2 phosphorylation, we incubated cells with 10 nM OA and fractionated them into mitochondria-containing heavy membrane and ER-containing light membrane (Fig. 2*B*). We found that the HM fraction contained significant LM contamination until being further purified through a Percoll gradient (Fig. 2*B*). OA caused a phosphorylation shift of BCL-2 in contaminated HM, but this phosphorylation shift disappeared in pure mitochondrial HM (Fig. 2*B*). In contrast, the phosphorylation shift was readily apparent in the LM, which is free of mitochondrial contamination and argues that PP2A principally regulates BCL-2 at the ER.

Hyperphosphorylated BCL-2 levels were reduced as OA levels were increased. (Fig. 2*A*, *bracket 2*). The biphasic nature of this response suggested that OA might enhance levels of phosphorylation at lower concentrations and enhance the degradation of the phosphorylated species at higher concentrations. Co-incubation of cells with MG132 stabilized the phosphorylated form of BCL-2 at high OA concentrations, arguing that phospho-BCL-2 is specifically targeted for proteasome degradation and influences endogenous BCL-2 levels (Fig. 2*C*). BCL-2 is phosphorylated in response to microtubule-damaging agents such as taxol at Thr-69, Ser-70, and Ser-87 (17). Mutation of these sites (BCL-2^{AAA}) abolishes the ability of taxol to induce BCL-2 hyperphosphorylation (17). To determine whether PP2A-mediated dephosphorylation specifically targets Thr-69, Ser-70, and Ser-87, hBCL-2^{AAA}-expressing Jurkat cells were treated with 30 nM OA and MG132. In contrast to wild-type (WT) hBCL-2, OA and MG132 incubation of hBCL-2^{AAA} cells resulted in no phosphorylation shift (Fig. 2*D*).

Knock Down of PP2A Catalytic Subunit Causes a Decrease in Endogenous BCL-2 That Is Dependent on BCL-2 Phosphorylation—Because OA is a general PP1/PP2A inhibitor, we wanted to investigate the role of PP2A in BCL-2 regulation more specifically. Therefore, we obtained shRNAs against the C β catalytic subunit of mPP2A (shPP2A_C). Partial knock down of PP2A_C caused a reduction in the level of endogenous BCL-2 without a corresponding decrease in BCL-2 mRNA levels

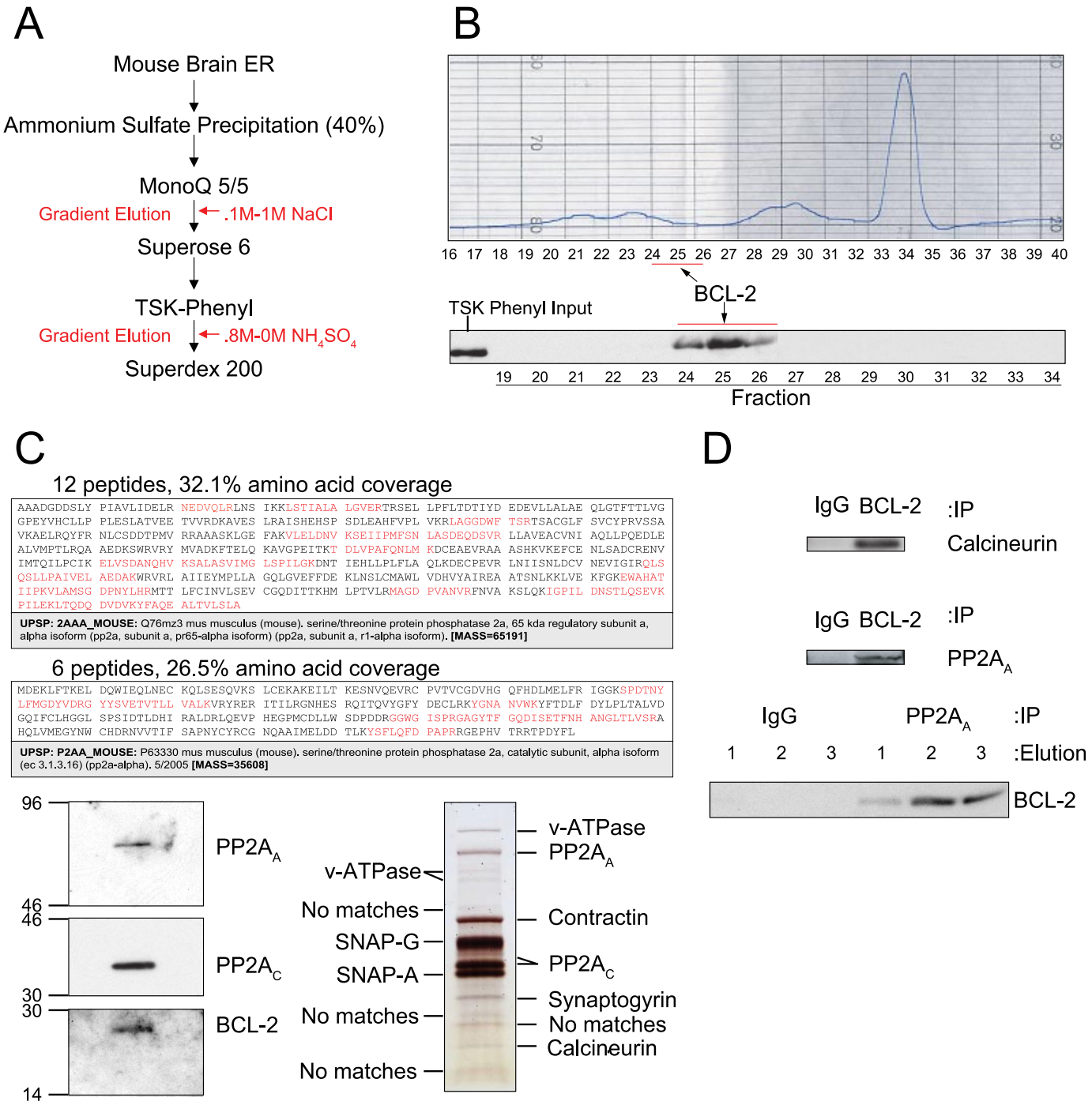


FIGURE 1. **Purification and validation of BCL-2-containing complexes at the endoplasmic reticulum (ER).** *A*, outline of the purification strategy developed to isolate BCL-2-containing complexes from mouse brain endoplasmic reticulum. *B*, the chromatogram of protein content from the final Superdex 200 step, along with BCL-2 Western blot of fractions, revealed that BCL-2 was concentrated to a small area separate from the largest protein peaks. *C*, SDS-PAGE and silver stain of pooled Superdex fractions 24, 25, and 26 with band assignments. Liquid chromatography/mass spectrometry/mass spectrometry sequences are shown of peptides derived from 36 and 65 kDa bands that identify PP2A subunits. Western blots of A and C subunits of PP2A, plus BCL-2 from pooled fractions, are shown. *D*, IPs using anti-BCL-2 antibody or IgG antibody were done using brain ER membranes solubilized in 1% CHAPS. Proteins isolated by IP were probed for calcineurin B by Western blot. The BCL-2 IP was also probed for the A subunit of PP2A. The PP2A A subunit was isolated by IP using beads covalently linked with antibody. The IP was used to probe for BCL-2 by Western blot. Beads were eluted three times to recover all antibody-bound proteins.

(Fig. 3, *A* and *B*). To determine whether phosphorylation is a prerequisite for PP2A-mediated degradation, we expressed shPP2A$_C$ in MEFs derived from BCL-2^{AAA} knock-in mice. Knock-in MEFs have similar protein levels of BCL-2 as WT MEFs in the whole cell lysate and the ER fraction.[5] Although PP2A$_C$ was reduced to a similar degree in BCL-2^{AAA} MEFs as in WT, endogenous BCL-2 protein levels were preserved in the BCL-2^{AAA} setting (Fig. 3*C*). This finding further supports our hypothesis that phosphorylation of BCL-2 is required for its degradation *in vivo*. It was interesting that the differences in BCL-2 protein levels were still apparent even though shPP2A$_C$ appeared to give an incomplete knockdown. PP2A$_C$ levels may

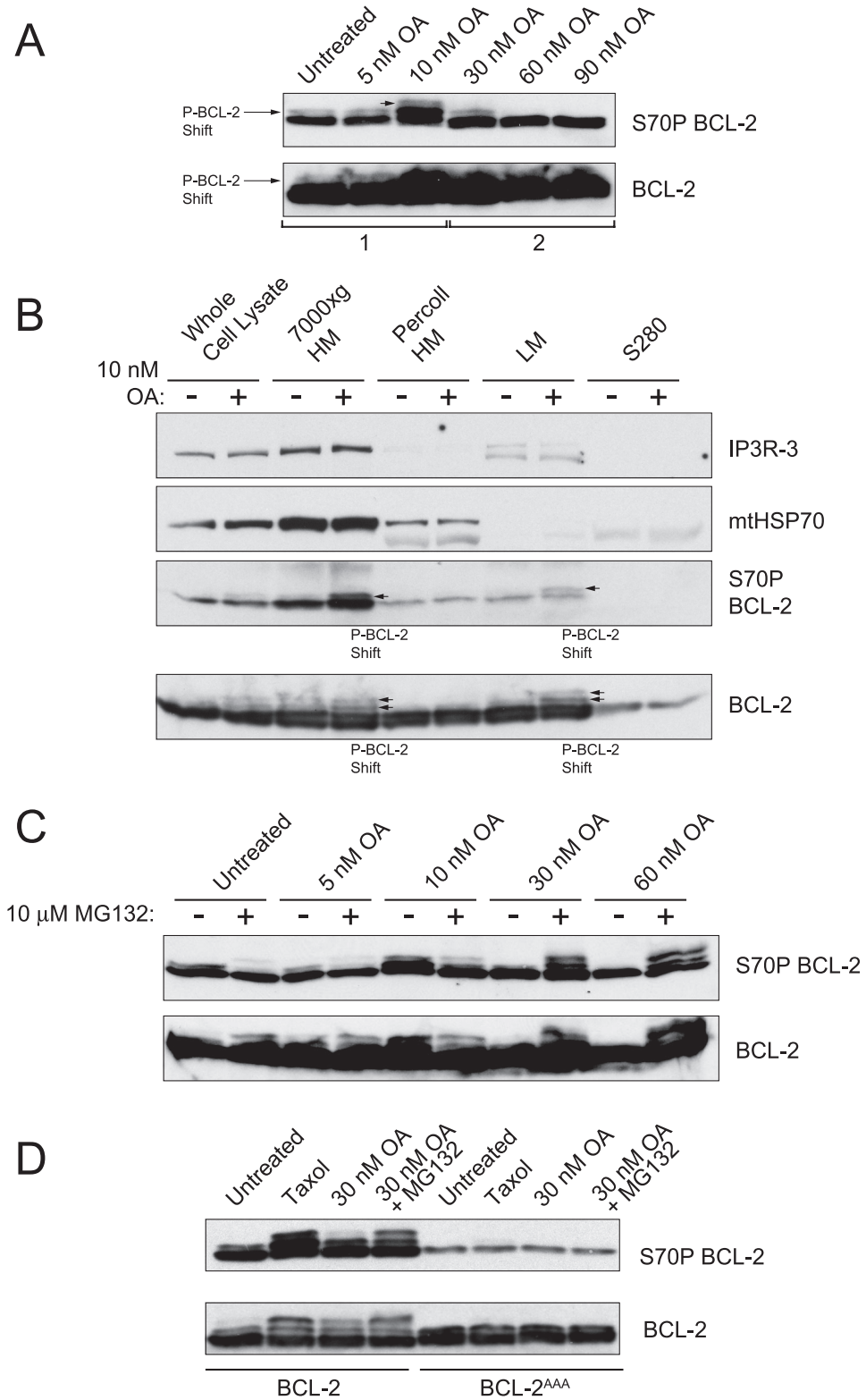
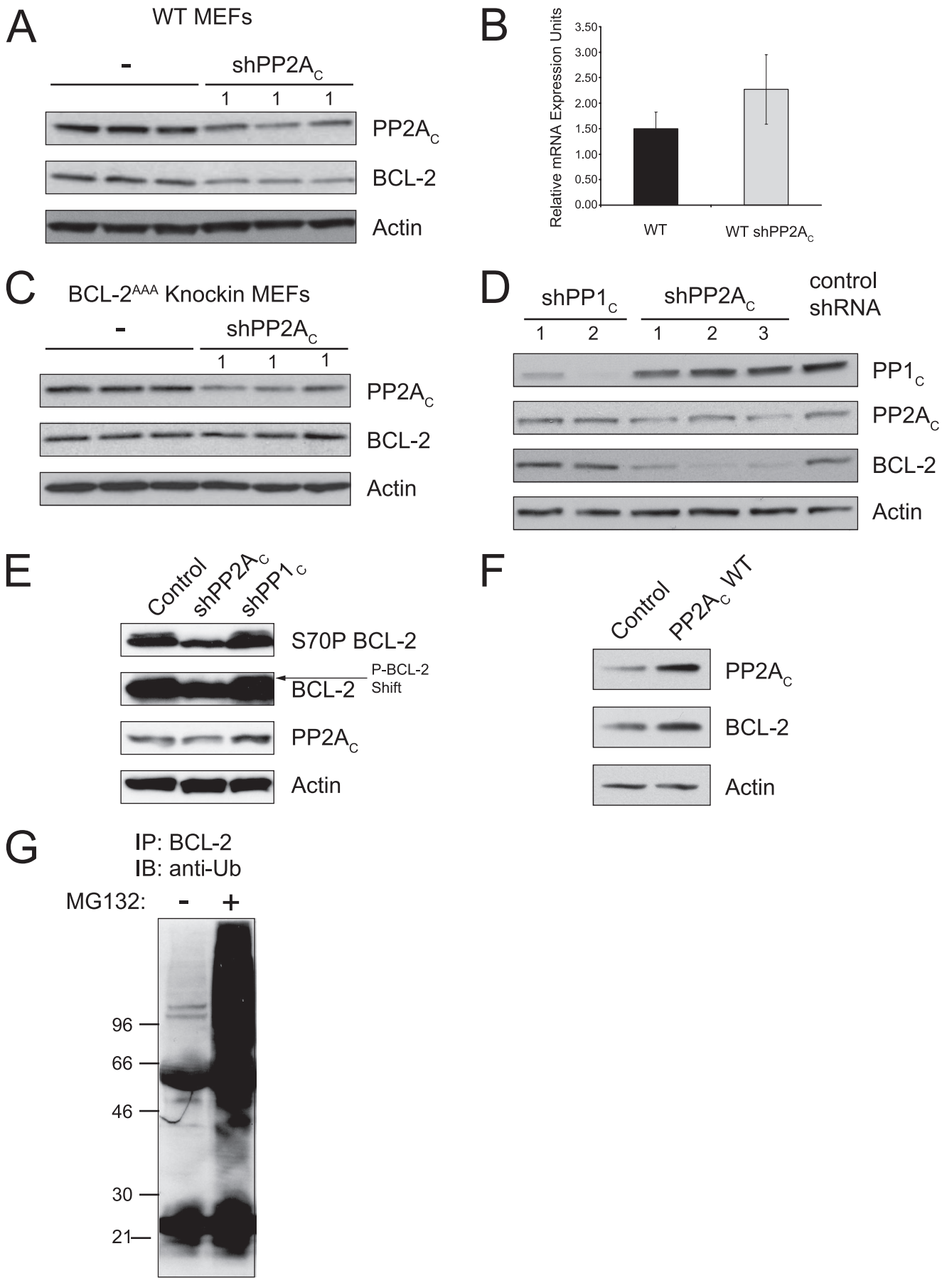


FIGURE 2. **Assessment of BCL-2 phosphorylation in Jurkat T cells using okadaic acid.** *A*, Jurkat T cells stably expressing hBCL-2 were treated with various concentrations of OA for 24 h. Whole cell lysates were probed with a BCL-2 antibody to visualize the phosphorylation shift or antibody specific for S70P BCL-2. Untreated, 5 nM, and 10 nM OA are covered by *bracket 1*; 30, 60, and 90 nM OA are covered by *bracket 2*. *B*, to examine subcellular localization of OA-mediated hyperphosphorylation, cells were treated with 10 nM OA or vehicle. After 24 h, cells were fractionated to isolate heavy membrane (*HM*), light membrane (*LM*), and S280 cytosol. Unpurified HM was isolated by centrifugation at 7000 × *g* alone (labeled 7000 × *g* HM), and mitochondrial HM was further separated from contaminating LM by Percoll gradient (labeled *Percoll HM*). Fractions, along with whole cell lysate, were probed with anti-BCL-2 antibody or S70P BCL-2 antibody. IP3R-3 was used as an ER marker, whereas mtHSP70 was used as a mitochondrial marker. *IP3R-3*, type 3 inositol trisphosphate receptor; *mtHSP70*, mitochondrial heat shock protein 70. *C*, Jurkat cells were treated with the indicated concentrations of OA. For each concentration of OA, a duplicate sample was co-incubated with 10 μM MG132. *D*, Jurkat cells expressing hBCL-2^{WT} or hBCL-2^{AAA} were treated with taxol, 30 nM OA, or 30 nM OA + 10 μM MG132. BCL-2 phosphorylation was assessed by Western blots with total BCL-2 and S70P BCL-2 antibodies.



reflect the fact that the antibody used sees both the C α and β isoforms of the catalytic subunit or the fact that substantial loss of $PP2A_C$ is lethal and could select out cells with complete $PP2A_C$ knockdown (30).

The other major phosphatase affected by OA, PP1, was also examined in comparison with PP2A. We observed almost complete knock down of the PP1 catalytic subunit ($PP1_C$) using two separate shRNA vectors with no effect on BCL-2 levels (Fig. 3*D*). In contrast, three unique shRNA vectors to $PP2A_C$ all caused significant reductions in BCL-2 (Fig. 3*D*). Because the S70P antibody specifically recognizes overexpressed human phospho-BCL-2 (19), we transduced $shPP2A_C$ and $shPP1_C$ into *bcl-2*$^{-/-}$ MEFs that express hBCL-2. BCL-2 phosphorylation was significantly reduced by $shPP2A_C$ with no change in non-phosphorylated protein, in agreement with our Jurkat cell data (Fig. 3*E*). $shPP1_C$ did not influence BCL-2 phosphorylation (Fig. 3*E*). We attempted to further demonstrate that PP2A affects BCL-2 levels by overexpressing $PP2A_C$ in WT MEFs. In contrast to inhibition, PP2A overexpression resulted in enhancement of BCL-2 protein levels (Fig. 3*F*). Together, these findings argue that PP2A influences total BCL-2 protein levels by modulating its phosphorylation.

Proteasome-dependent degradation involves the modification of protein substrates by ubiquitin. To determine whether BCL-2 is ubiquitinated, hBCL-2 MEFs were lysed and hBCL-2 was isolated by IP and probed for ubiquitin. Multiple high molecular weight ubiquitin bands were observed and enhanced when proteasome activity was inhibited by MG132, suggesting that BCL-2 is modified by ubiquitination (Fig. 3*G*).

Knock Down of PP2A Regulatory Subunit B56γ *Mimics Knock Down of the Catalytic Subunit*—As an alternative approach to inhibit PP2A activity without deleterious effects to cells, we knocked down one of the regulatory B subunits of PP2A. Knock down of regulatory subunit B56γ has been demonstrated to reduce intracellular phosphatase activity of PP2A, and B56 has been reported to associate with BCL-2 (21, 31). To determine whether B56 influences BCL-2 degradation, we obtained immortalized HEK epithelial cells that express shRNA against B56γ. Like $shPP2A_C$, shB56γ caused a significant decrease in BCL-2 protein levels in HEK cells with no effect on BAX (Fig. 4*A*). The reduction of BCL-2 most likely reflects a degradation of the phosphorylated species, although our S70P antibody does not detect endogenous BCL-2 phosphorylation. We assessed whether B56 regulates BCL-2 degradation via proteasome activity by adding MG132 to the medium. MG132 treatment led to an elevation of BCL-2 protein in shB56γ HEK cells, suggesting that BCL-2 is degraded by the proteasome in these cells (Fig. 4*B*). In contrast, BCL-X_L protein levels were not affected by MG132 in shB56γ HEK cells (Fig. 4*B*). Finally, overexpression of B56γ elevated the protein levels of BCL-2 (Fig. 4*C*). Therefore, modulation of B56γ expression levels can phenocopy modulation of $PP2A_C$ with respect to BCL-2 protein levels. Together, these data support the hypothesis that hyperphosphorylated BCL-2 is targeted for degradation through the proteasome.

Endogenous BCL-2 Phosphorylation Affects the Sensitivity of the Cells to Death Stimuli when PP2A$_C$ *Is Knocked Down*—To investigate whether cell death is affected by PP2A-mediated phosphorylation/degradation of BCL-2, we examined several death stimuli in $shPP2A_C$ MEFs. WT MEFs that express $shPP2A_C$ (Fig. 3*A*) were significantly more sensitive to both tunicamycin and brefeldin A, two death stimuli that trigger UPR-mediated death by blocking protein modification/secretion at the ER (Fig. 5, *A* and *C*). To determine whether this enhanced sensitivity to apoptosis was due to BCL-2 phosphorylation or other cellular targets of PP2A, we examined the same stimuli in BCL-2^{AAA} knock-in MEFs expressing $shPP2A_C$ (Fig. 3*C*). $shPP2A_C$ had no effect on tunicamycin- or brefeldin A-induced cell death in BCL-2^{AAA} MEFs (Fig. 5, *B* and *D*). These data suggest that BCL-2 dephosphorylation status is the major mechanism through which PP2A regulates apoptosis driven by ER/UPR stress, arguing against a pleiotropic effect of $PP2A_C$ knock down on cellular survival. Thapsigargin induces UPR-mediated death by blocking ER Ca^{2+} uptake, and $shPP2A_C$ again sensitized WT cells to this death stimulus (Fig. 5*E*). BCL-2^{AAA} knock-in MEFs were also sensitized by $shPP2A_C$ but to a lesser extent than in the WT setting (Fig. 5*F*). This suggests that BCL-2 phosphorylation is not as important for PP2A to regulate thapsigargin-induced death as it is for tunicamycin- or brefeldin A-induced death.

We next examined other classes of cell death inducers. H_2O_2 generates oxidative stress that triggers ER Ca^{2+} release. Of three H_2O_2 concentrations tested, only one led to differences between WT and BCL-2^{AAA} MEFs. WT $shPP2A_C$ MEFs were significantly more sensitive to 400 μM H_2O_2 than vector control cells but significantly less so in the BCL-2^{AAA} setting (Fig. 5, *G* and *H*). However, there was minimal difference in sensitivity between WT and BCL-2^{AAA} using 200 and 600 μM H_2O_2, suggesting that, like thapsigargin, BCL-2 phosphorylation has a far lesser role for PP2A to regulate H_2O_2-induced death. Death stimuli that are known to be affected by BCL-2^{AAA}, but do not signal directly through the ER, were also tested (19). Taxol triggered significantly more cell death in WT $shPP2A_C$ cells than those with control, but the difference was reduced in the BCL-2^{AAA} setting (Fig. 5, *I* and *J*). Treatment of WT $shPP2A_C$ MEFs with etoposide, a topoisomerase II inhibitor, resulted in significantly more cell death than treatment of WT control MEFs, but there was no difference in the BCL-2^{AAA} setting (Fig. 5, *K* and *L*). Therefore, PP2A and BCL-2 phosphorylation can affect death stimuli not specific to the ER and is consistent with the

FIGURE 3. **Assessment of BCL-2 phosphorylation and levels in MEFs by shRNA knock down of $PP2A_C$.** *A*, shRNA knock down of the catalytic subunit of PP2A (*shPP2A*$_C$) in WT MEFs. Three independent plates were transduced with the same $shPP2A_C$ hairpin (labeled *1*) to demonstrate reproducibility of $PP2A_C$ knock down and effect on BCL-2. Levels of the indicated proteins from whole cell lysate were assessed by Western blot. *B*, real-time PCR assessment of *bcl-2* mRNA levels in WT MEFs with and without $shPP2A_C$ (three separate transductions). *C*, $shPP2A_C$ was expressed in BCL-2^{AAA} knock-in MEFs in the same manner as *panel A*. *D*, knock down of PP1 catalytic subunit (*shPP1*$_C$). Two RNA hairpins directed at $PP1_C$ (labeled *1* and *2*) and three $PP2A_C$ RNA hairpins (labeled *1*, *2*, and *3*), along with control shRNA, were expressed in WT MEFs. BCL-2 protein levels were assessed by Western blot. *E*, overexpression of $PP2A_C$ in WT MEFs. *F*, $shPP2A_C$ (hairpin 1) and $shPP1_C$ (hairpin 1) were expressed in *bcl-2*$^{-/-}$ MEFs stably expressing hBCL-2. *G*, BCL-2 IP and ubiquitin Western blot. hBCL-2-expressing MEFs were incubated in vehicle or 10 μM MG132. Cells were lysed and solubilized in strong radioimmune precipitation buffer detergent to maximize dissociation of protein partners, and hBCL-2 was subsequently isolated by immunoprecipitation. The BCL-2 IP was probed for ubiquitin.

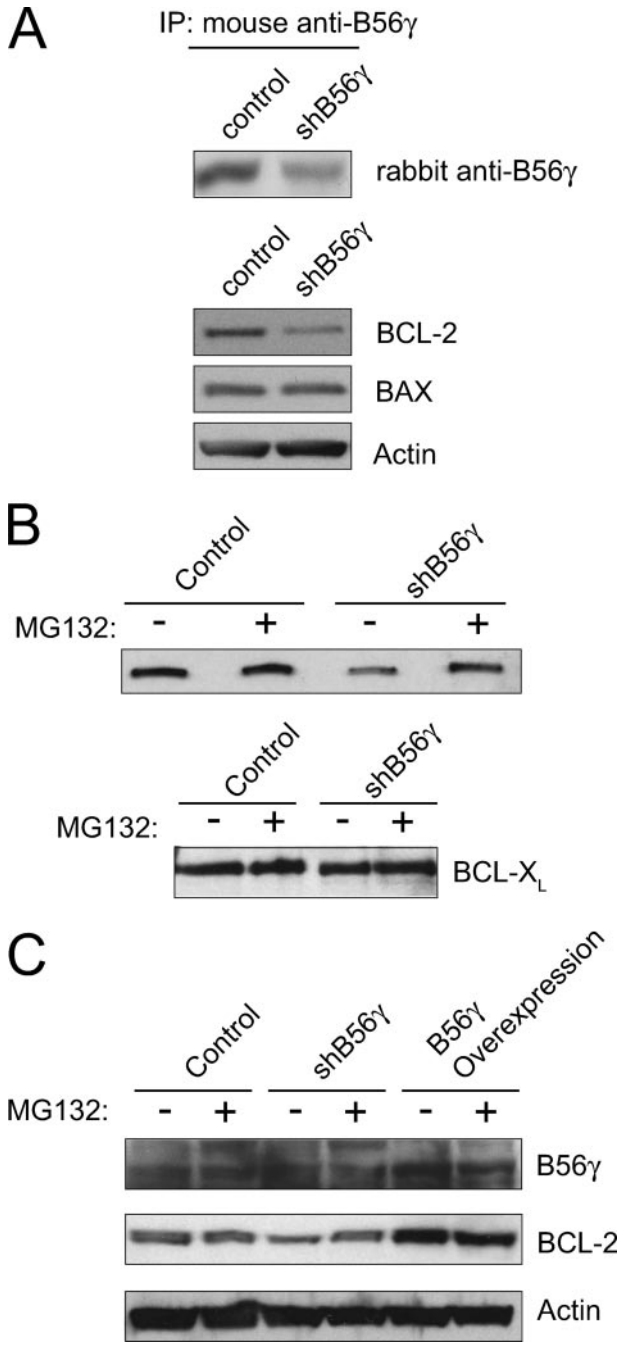


FIGURE 4. **Knock down of PP2A regulatory B subunit B56γ affects BCL-2 protein levels.** *A*, small hairpin RNA knock down of B56γ (*shB56γ*) in immortalized HEK cells. To assess knock down, B56γ was immunoprecipitated from 1 mg of lysate using a mouse monoclonal antibody, and a Western blot for B56γ was done with a rabbit polyclonal antibody (21). Protein levels of BCL-2, BAX, and actin were assessed from whole cell lysate. *B* and *C*, control and shB56γ cells were incubated with and without MG132 for 8 h. BCL-2 and BCL-X_L protein levels were assessed by Western blot from whole cell lysate. *B*, B56γ-overexpressing cells were also incubated with MG132. *C*, B56γ overexpression levels were assessed from whole cell lysate using the mouse monoclonal antibody.

ability of BCL-2 and PP2A to associate elsewhere in the cell. We also used staurosporine, a pan-kinase inhibitor that triggers classic mitochondrial apoptosis. WT shPP2A_C MEFs were slightly more sensitive to staurosporine compared with control, but to a lesser degree than to the other death stimuli tested (Fig. 5*M*). In addition, staurosporine sensitized BCL-2^{AAA} shPP2A_C MEFs to approximately the same degree as in the WT setting (Fig. 5*N*). Thus, PP2A does not require BCL-2 phosphorylation for sensitizing cells to staurosporine.

DISCUSSION

A number of groups have observed that phosphorylation attenuates the anti-apoptotic activity of BCL-2 (14–16, 19), but the mechanism underlying this effect has largely remained incompletely understood. In this study we describe a model whereby phosphorylation regulates BCL-2 activity by triggering its degradation. Using mouse brain endoplasmic reticulum, we purified the A and C subunits of PP2A in a macromolecular complex with BCL-2. Of note, we did not identify any B subunits of PP2A from our purification. It is possible that a B subunit associates with BCL-2 but was lost in earlier purification steps. This appears to be the case with IP3R-1, an ER resident that associates with BCL-2 (11, 12) (data not shown).

This is the first report that PP2A can regulate BCL-2 protein levels. Our observations suggest that the phosphatase activity of PP2A protects BCL-2 from proteasome degradation. To examine BCL-2 phosphorylation *in vivo*, we used cells that overexpress the human protein (19). Inhibiting PP2A with OA selectively degraded the phosphorylated species of overexpressed BCL-2, and blocking proteasome activity with MG132 could rescue this degradation. We have been unable to directly track the phosphorylation of endogenous BCL-2, but our genetic studies support the hypothesis that BCL-2 phosphorylation is requisite for PP2A-mediated degradation. Knocking down $PP2A_C$ led to a substantial reduction in total endogenous BCL-2 protein levels in WT MEFs but did not reduce the protein levels of MEFs containing non-phosphorylatable BCL-2^{AAA}. Moreover, HEK cells with shRNA knock down of the PP2A regulatory subunit B56γ also reduced endogenous BCL-2 protein levels. MG132 elevated BCL-2 in these cells and not in control HEK cells, suggesting that BCL-2 was degraded because its phosphorylation was enhanced. We cannot distinguish whether B56γ influences BCL-2 protein levels by specifically targeting to BCL-2 or non-specifically regulating total PP2A activity.

We observed an increase in phospho-BCL-2 levels with low concentrations of OA and proteasome degradation at higher concentrations, suggesting that we were measuring its effects on BCL-2 and on the degradation machinery itself with OA. BCL-2 degradation may not depend on its phosphorylation status alone. For example, we observed a linear increase in BCL-2 phosphorylation with no sign of degradation when cells were incubated with taxol (data not shown). PP2A subunit levels appear to be regulated by degradation (32), suggesting there might be a relationship between PP2A activity and proteasome mechanics. In support of this interpretation, genetic knock down of two different PP2A subunits resulted in BCL-2 degradation, whereas overexpression of those subunits elevated BCL-2 protein levels.

Most importantly, we found that $PP2A_C$ knock down sensitized MEFs to ER stress-induced apoptosis but had minimal effect in BCL-2^{AAA} knock-in MEFs. The same was true with selected other death stimuli tested. These findings strongly sug-

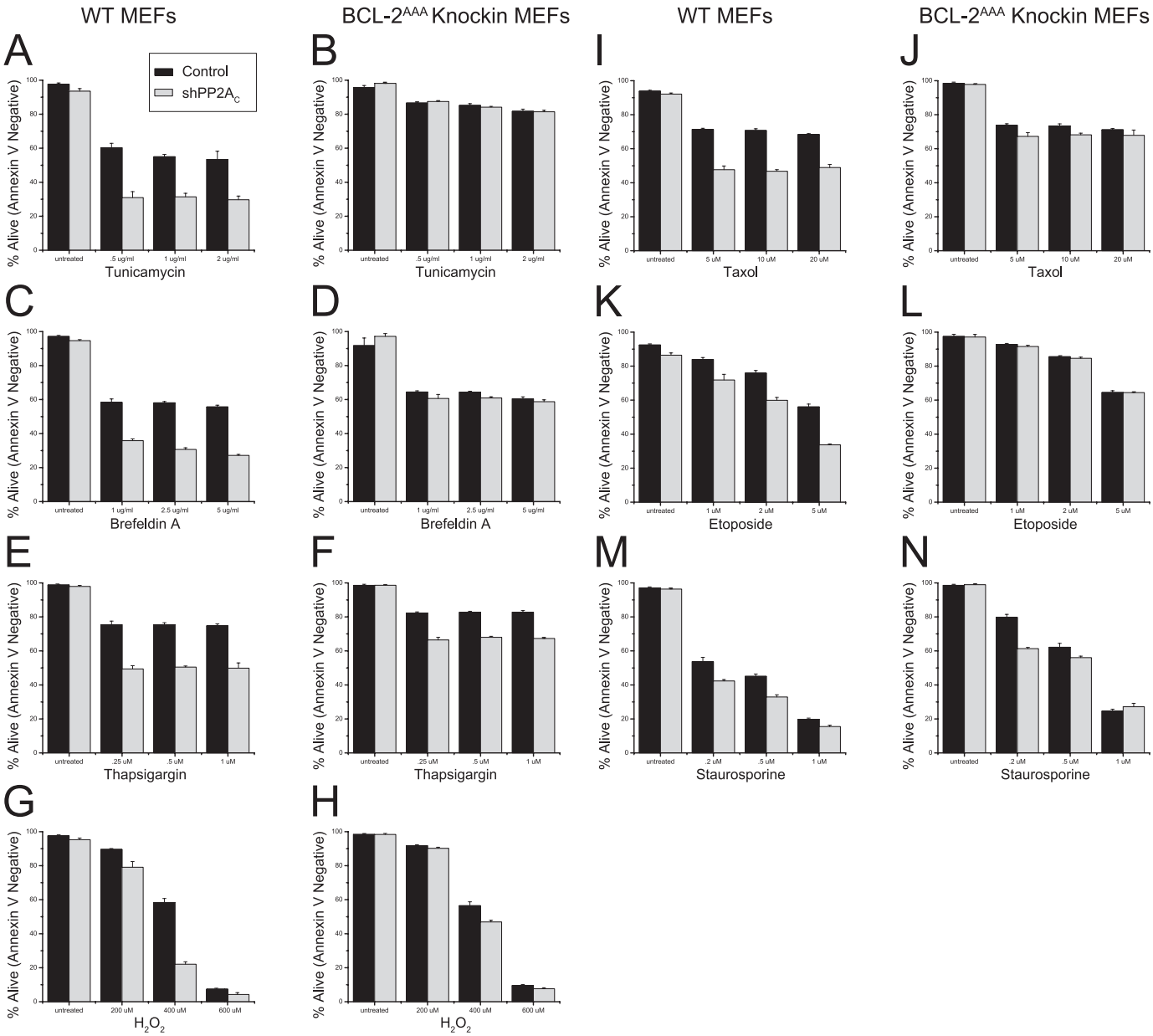


FIGURE 5. **PP2A$_C$ knock down affects sensitivity to ER-specific death stimuli ($n = 2–4$ experiments for each death stimulus, genotype).** *A–N*, WT MEFs containing shPP2A$_C$ or control were treated with the indicated concentrations of tunicamycin (*A*), brefeldin A (*C*), thapsigargin (*E*), H_2O_2 (*G*), taxol (*I*), etoposide (*K*), or staurosporine (*M*). BCL-2^{AAA} knock-in MEFs containing shPP2A$_C$ or vector were treated with the indicated concentrations of tunicamycin (*B*), brefeldin A (*D*), thapsigargin (*F*), H_2O_2 (*H*), taxol (*J*), etoposide (*L*), or staurosporine (*N*).

gest that PP2A can regulate BCL-2 levels and thus influence the ability of the cell to resist death stimuli. It appears that apoptotic sensitization by PP2A$_C$ knock down is more dependent on BCL-2 phosphorylation for certain classes of death stimuli than others. Specifically, non-phosphorylatable BCL-2^{AAA} protected cells from shPP2A$_C$ in response to inhibitors of protein modification/secretion at the ER (*i.e.* tunicamycin and brefeldin A) and inhibitors of cell cycle progression (*i.e.* taxol and etoposide), settings in which phosphorylation of BCL-2 is thought to be important (17, 19). BCL-2^{AAA} MEFs were significantly less protected or not protected from Ca^{2+}-associated death stimuli (*i.e.* thapsigargin and H_2O_2) and not protected from pan-kinase inhibition (*i.e.* staurosporine), suggesting PP2A mediates the effects of cell survival under select settings.

It is significant that specific PP2A complexes can act as tumor suppressors, such as those with B56γ. The shB56γ HEK cells that have reduced BCL-2 protein levels also grow anchorage independently in culture and cause tumor formation in mice (21). These studies imply apoptosis resistance may actually be reduced in certain tumorigenic cells, perhaps a sensitive target for therapy. On the other hand, a PP2A-based approach could be used to degrade BCL-2 in BCL-2-dependent tumors.

In summary, we have isolated PP2A as part of a macromolecular complex with BCL-2 at the endoplasmic reticulum. Together, our functional studies in three cell systems strongly suggest that dephosphorylation of BCL-2 by PP2A protects BCL-2 from proteasome-dependent degradation at the endo-

plasmic reticulum and fine-tunes the sensitivity of the cell to several cellular stresses, especially stresses at the ER.

Acknowledgments—We thank S. Oakes, C. Hetz, and N. Danial for helpful discussions and critical reading of this manuscript and E. Smith for editorial assistance. C. Denison and S. Gygi were instrumental for mass spectrometry analysis. R. Drapkin provided invaluable suggestions for protein purification.

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PP2A Regulates BCL-2 Phosphorylation and Proteasome-mediated Degradation at the Endoplasmic Reticulum

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J. Biol. Chem. 2006, 281:23003-23012.
doi: 10.1074/jbc.M602648200 originally published online May 22, 2006
