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Bcl-2 Antiapoptotic Proteins Inhibit Beclin 1-Dependent Autophagy

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Summary

Apoptosis and autophagy are both tightly regulated biological processes that play a central role in tissue homeostasis, development, and disease. The antiapoptotic protein, Bcl-2, interacts with the evolutionarily conserved autophagy protein, Beclin 1. However, little is known about the functional significance of this interaction. Here, we show that wild-type Bcl-2 antiapoptotic proteins, but not Beclin 1 binding defective mutants of Bcl-2, inhibit Beclin 1-dependent autophagy in yeast and mammalian cells and that cardiac Bcl-2 transgenic expression inhibits autophagy in mouse heart muscle. Furthermore, Beclin 1 mutants that cannot bind to Bcl-2 induce more autophagy than wild-type Beclin 1 and, unlike wild-type Beclin 1, promote cell death. Thus, Bcl-2 not only functions as an antiapoptotic protein, but also as an antiautophagy protein via its inhibitory interaction with Beclin 1. This antiautophagy function of Bcl-2 may help maintain autophagy at levels that are compatible with cell survival, rather than cell death.

Introduction

Autophagy is an evolutionarily conserved pathway that involves the sequestration and delivery of cytoplasmic material to the lysosome, where it is degraded and recycled. In theory, autophagy may help promote cell survival, either by purging the cell of damaged organelles, toxic metabolites, and intracellular pathogens or by generating the intracellular building blocks required to maintain vital functions during nutrient-limiting conditions. However, in theory, autophagy may also promote cell death through excessive self-digestion and degradation of essential cellular constituents. Despite recent advances in understanding its molecular mechanisms and biological functions (Levine and Klionsky, 2004), it is unclear whether autophagy acts fundamentally as a cell survival or cell death pathway—or both.

Morphologic and genetic studies have yielded conflicting results regarding the role of autophagy in cell survival and cell death. When intracellular nutrients are limited, as in the case of starvation or growth factor deprivation, autophagy genes are essential to maintain cellular ATP energy production, macromolecular synthesis, and cell survival (Lum et al., 2005). The absence of autophagy genes results in death in nitrogen-starved yeast, starvation-induced chlorosis in plants, death of starved *Dictyostelium*, defective dauer development in *C. elegans*, early neonatal lethality in mice following interruption of the placental food supply, accelerated death of growth factor-deprived, apoptosis-resistant fibroblasts, and apoptosis of starved HeLa cells (Boya et al., 2005; Kuma et al., 2004; Levine and Klionsky, 2004; Liu et al., 2005; Lum et al., 2005). Furthermore, the protective actions of autophagy against infection with intracellular pathogens, protein aggregation diseases, and aging in metazoan organisms (Levine and Yuan, 2005) are likely to represent a prosurvival function at the cellular level.

Paradoxically, autophagy may also represent a form of nonapoptotic cell death, which has been implicated in the demise of a variety of cell types during development, tissue homeostasis, and in response to toxic stimuli (Lockshin and Zakeri, 2004). In most studies, autophagic cell death has been defined by morphologic criteria, and it is unclear whether autophagy directly contributes to death or is a failed effort to preserve cell viability. However, RNAi directed against the autophagy genes, *atg7* or *beclin 1*, suppresses death in mouse L929 fibroblastic cells treated with the caspase inhibitor, ZVAD, and RNAi against *atg5* or *beclin 1* suppresses death in $bax^{-/-}$, $bak^{-/-}$ murine embryonic fibroblasts (MEFs) treated with etoposide or staurosporine (Shimizu et al., 2004; Yu et al., 2004). Downregulation of Atg5 expression or ectopic expression of a dominant-negative Atg5 mutant blocks IFN-γ-induced death in HeLa cells (Pyo et al., 2005). Thus, autophagy genes can be involved in the death execution process.

Moreover, complex interrelationships are likely to exist between autophagy and the apoptotic cell death pathway (Levine and Yuan, 2005). Regulators of apoptosis activation also function as regulators of autophagy activation, including the sphingolipid, ceramide, the death-receptor signaling molecules, TRAIL and FADD, and the serine/threonine death kinases, DAPk and DRP-1. Similarly, some signaling pathways, such as the

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A

GAL4 binding plasmid	β-galactosidase reaction with GAL4 activation domain plasmid						
	None	p53	Lamin	Beclin 1	Beclin 1 F123A	Beclin 1 I125A	Atg6
None	-	-	-	-	-	-	-
SV40	-	+	-	-	-	-	-
Bcl-2	-	-	-	+	-	+	-
KSHV v-Bcl-2	-	-	-	+	-	+	-
Bcl-2 G145A	-	-	-	-	-	-	-

B

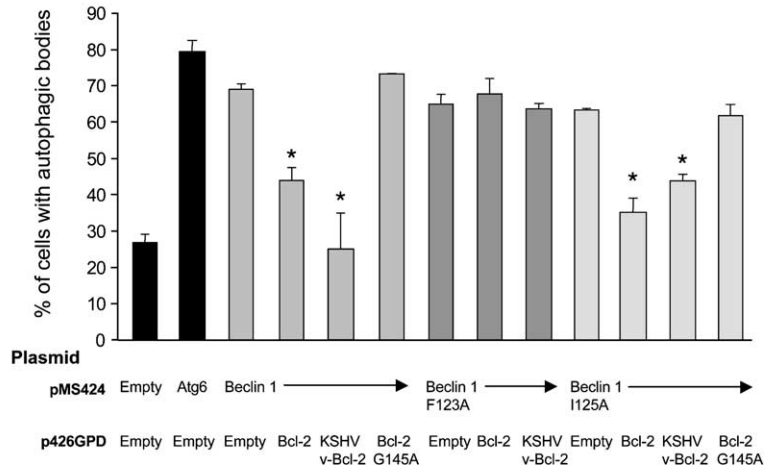

Figure 1. Human Bcl-2 and Viral Bcl-2 Interact with Beclin 1 and Inhibit Beclin 1-Dependent Autophagy in Starved *atg6*-Disrupted Yeast

(A) Yeast two-hybrid assay interactions between Beclin 1/Atg6 autophagy proteins and Bcl-2 antiapoptotic proteins. + indicates positive reaction at 15 min; – indicates negative reaction at 12 hr.

(B) Quantitation of autophagy in *atg6*Δ yeast cotransformed with the plasmids encoding the indicated gene products. Results shown represent mean ±SEM percentage of cells with autophagic bodies for combined data from six independent experiments. Asterisk denotes $p < 0.001$ when compared to yeast cells cotransformed with pMS424/Beclin 1 and empty p426GPD.

Class I PI3K/Akt signaling pathway, inhibit both apoptosis and autophagy. Morphologic features of both autophagic and apoptotic cell death can be observed in the same cell (e.g., insect glands during metamorphoses), and in the case of steroid-triggered salivary gland death in *Drosophila*, caspase inhibition blocks the death of cells exhibiting an autophagic morphology. Autophagy may activate apoptosis, and conversely, genetic inhibition of autophagy can activate apoptotic death in nutrient-starved mammalian cells.

The interaction between the antiapoptotic protein, Bcl-2, and the autophagy protein, Beclin 1, represents a potentially important point of convergence of the apoptotic and autophagic machinery. Beclin 1, the mammalian ortholog of yeast Atg6/Vps30, was first identified in a yeast two-hybrid screen as a Bcl-2-interacting protein (Liang et al., 1998) and is a haploinsufficient tumor-suppressor gene that is frequently monoallelically deleted in human sporadic breast, ovarian, and prostate cancer (Levine and Klionsky, 2004). Beclin 1 is part of a Class III PI3K complex that participates in autophagosome formation, mediating the localization of other autophagy proteins to the preautophagosomal membrane (Kihara et al., 2001). Although the Bcl-2 binding domain of Beclin 1 is required for the antiviral and prosurvival effects of Beclin 1 during lethal murine encephalitis (Liang et al., 1998), the biological significance of the interaction between Bcl-2 and Beclin 1 has not been explored.

In this study, we use yeast and mammalian cells to evaluate whether the interaction between Bcl-2 and Beclin 1 regulates starvation-induced autophagy. We found that cellular and virally encoded Bcl-2 proteins bind to Beclin 1 and disrupt its autophagy function. In the absence of Bcl-2 binding, Beclin 1 mutants induce excessive autophagy and promote cell death. Together, these findings suggest that the Beclin 1-Bcl-2 complex functions as a brake on levels of autophagy and autophagy gene-dependent cell death.

Results

Cellular and Viral Bcl-2 Inhibit Beclin 1-Dependent Autophagy in Yeast

Yeast disrupted of the *beclin 1* ortholog, *ATG6*, are deficient in starvation-induced autophagy, and this defect can be complemented by orthologs from higher eukaryotes (Liang et al., 1999; Melendez et al., 2003; Liu et al., 2005). In a yeast two-hybrid assay, we found that both cellular Bcl-2 and Bcl-2 encoded by Kaposi's sarcoma-associated herpesvirus (KSHV v-Bcl-2) bind strongly to Beclin 1, but not to yeast Atg6 (Figure 1A). Therefore, by measuring starvation-induced autophagy in *atg6*Δ yeast cotransformed with plasmids expressing either wild-type Beclin 1 and Bcl-2 or mutants of Beclin 1 and Bcl-2 that fail to bind to each other, we investigated whether the interaction between Bcl-2-like proteins and Beclin 1 alters the autophagy function of Beclin 1.

As previously shown, wild-type Beclin 1 restores star-

vation-induced autophagy in *atg6*Δ yeast (Figure 1B). Two Beclin 1 mutants containing substitution mutations within the Bcl-2 binding domain of Beclin 1 (amino acids 88–150; Liang et al., 1998) also rescue autophagy in *atg6*Δ yeast (Figure 1B). These include a mutant Beclin 1 protein (Beclin 1 F123A) which is defective in Bcl-2 binding and a control mutant Beclin 1 protein (Beclin 1 I125A) that does not affect binding to Bcl-2 (Figure 1A). Beclin 1 F123A and Beclin 1 I125A were expressed in yeast at similar levels as those observed for wild-type Beclin 1 (data not shown).

Cellular Bcl-2 and KSHV v-Bcl-2 both significantly inhibited the ability of either wild-type Beclin 1 or the control mutant Beclin I125A to rescue starvation-induced autophagy in *atg6*Δ yeast (Figure 1B) ($p < 0.001$, t test). In contrast, cellular Bcl-2 and KSHV v-Bcl-2 had no effect on autophagy rescue by the Bcl-2 binding defective mutant, Beclin 1 F123A, suggesting that a direct interaction with Beclin 1 is required for Bcl-2 and KSHV v-Bcl-2-mediated inhibition of Beclin 1-dependent autophagy. Furthermore, a mutation in the BH1 domain of Bcl-2, Bcl-2 G145A, that blocks the yeast two-hybrid interaction of Bcl-2 with Beclin 1 (Figure 1A), also blocks the ability of Bcl-2 to antagonize autophagy in *atg6*Δ yeast expressing either wild-type Beclin 1 or the control mutant Beclin 1 I125A (Figure 1B). This result, together with the failure of wild-type Bcl-2 and KSHV v-Bcl-2 to block autophagy mediated by the Bcl-2 binding defective mutant of Beclin 1, demonstrates a role for Bcl-2-Beclin 1 interactions in antagonizing the autophagy function of Beclin 1.

Cellular and Viral Bcl-2 Inhibit Beclin 1-Dependent Autophagy in Mammalian Cells

Previous FRET analysis showed that Bcl-2 and Beclin 1 can interact in mammalian cells (Liang et al., 1998). To confirm these findings biochemically and identify mutations that block the interaction, we performed coimmunoprecipitations of HEK293 cells transiently transfected with Beclin 1 and Bcl-2-like proteins (Figures 2A and 2B). Wild-type Bcl-2, but not a BH1 domain mutant of Bcl-2 (Bcl-2 G145A) or a BH2 domain mutant of Bcl-2 (Bcl-2 W188A), coimmunoprecipitated with Beclin 1 (Figure 2A). KSHV vBcl-2 also coimmunoprecipitated with Beclin 1 (Figure 2B).

To evaluate whether Bcl-2 and KSHV v-Bcl-2 inhibit Beclin 1-dependent autophagy in mammalian cells, we used human MCF7 breast carcinoma cells derived from a tumor with a monallelic deletion of *beclin 1* (Holt et al., 1996). Most MCF7 subclones express low or undetectable levels of endogenous Beclin 1 (Liang et al., 1999; Scarlatti et al., 2004; Figure 2C) and fail to increase autophagic activity in response to starvation unless Beclin 1 is ectopically expressed (Furuya et al., 2005; Liang et al., 1999, 2001). Therefore, we assessed the effects of cellular Bcl-2 and v-Bcl-2 expression on the ability of Beclin 1 to promote starvation-induced autophagy in MCF7 cells. To measure autophagy, we expressed the fluorescent autophagy marker, GFP-LC3, a highly specific marker of mammalian autophagy (Mizushima et al., 2004). When autophagy is stimulated, GFP-LC3 redistributes from a diffuse cytoplasmic pattern to form punctate structures that label preautophagosomal and autophagosomal membranes.

As expected, we found that autophagy is increased following nutrient deprivation in MCF7 cells expressing *beclin 1* (MCF7.*beclin 1* cells) but not in MCF7.control cells ($p < 0.001$; t test). Expression of Bcl-2 or KSHV v-Bcl-2 inhibited starvation-induced autophagy in MCF7.*beclin 1* cells (Figures 2D and 2E; $p < 0.001$ for Bcl-2 or KSHV v-Bcl-2 versus empty vector; t test). Neither Bcl-2 nor KSHV v-Bcl-2 altered autophagy levels in MCF7.control cells (data not shown). In contrast to the effects of wild-type Bcl-2, expression of Bcl-2 mutants that fail to bind to Beclin 1, Bcl-2 G145A or Bcl-2 W188A, did not inhibit autophagy in starved MCF7.*beclin 1* cells, despite similar levels of expression as wild-type Bcl-2 (Figure 2C). Thus, cellular and viral Bcl-2 block Beclin 1-dependent autophagy in both yeast and mammalian cells.

To evaluate the effects of Bcl-2 on autophagy in an independent cell line, we used human colon carcinoma cells that are stably transfected with Bcl-2 (HT-29 Bcl-2 cells; Lacour et al., 2003). HT-29 control cells express detectable levels of Beclin 1 but not Bcl-2 (Figure 3A) and can increase autophagic activity following amino acid deprivation (Pattingre et al., 2003). In HT-29 Bcl-2 cells, Bcl-2 coimmunoprecipitates with endogenous Beclin 1 (Figure 3A). Unlike HT-29 control cells, HT-29 Bcl-2 cells do not increase autophagic activity following amino acid starvation, as measured by either the quantitation of GFP-LC3-positive dots (Figure 3B) or by quantitative electron microscopy (Figures 3C and 3D). To verify that the reduced number of autophagosomes in starved HT-29 Bcl-2 versus HT-29 control cells represents decreased autophagy, rather than an accelerated disappearance of GFP-LC3-positive autophagosomes, we also measured the degradation of long-lived proteins (Figure 3E). In HT-29 control cells but not in HT-29 Bcl-2 cells, there was a significant increase in proteolysis of long-lived cellular proteins during starvation. Together, these results suggest that Bcl-2 blocks autophagy at a stage prior to association of LC3 with the preautophagosomal membrane.

Bcl-2 Inhibits the Formation of the Beclin 1/hVps34 PI3K Complex and Beclin 1-Associated Class III PI3K Activity

Beclin 1 functions in autophagy as part of a complex with hVps34/Class III PI3K (Kihara et al., 2001). Generation of PI3P by the Beclin 1/hVps34 complex is thought to be important in mediating the localization of other autophagy proteins to preautophagosomal membranes. Since Bcl-2 downregulates Beclin 1-dependent autophagy, we evaluated the effects of Bcl-2 expression on Beclin 1/hVps34 complex formation and activity in HT-29 cells.

HT-29 Bcl-2 cells expressed similar levels of both Beclin 1 and hVps34 as HT-29 control cells, but in HT-29 Bcl-2 cells, less hVps34 coimmunoprecipitated with Beclin 1 (Figure 3F). This suggested that Bcl-2 overexpression interferes with the formation of the Beclin 1/hVps34 complex. To confirm that the functional activity of this complex was decreased in HT-29 Bcl-2 cells, we labeled the Class III PI3K product, PI3P, with a GFP-2xFYVE domain plasmid (Viera et al., 2001). We observed significantly more colocalization of Beclin 1 with

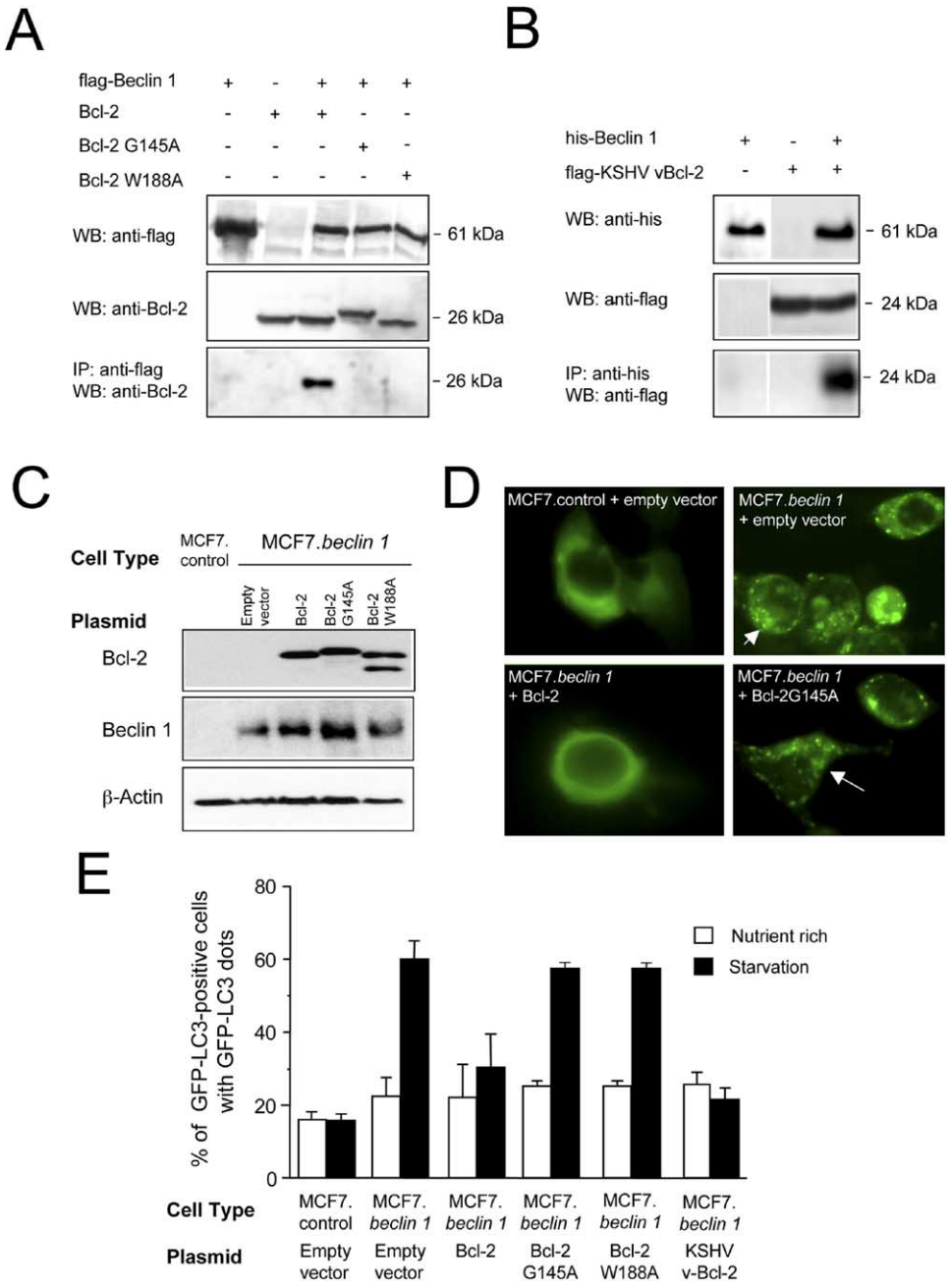

Figure 2. Human Bcl-2 and Viral Bcl-2 Inhibit Beclin 1-Dependent Autophagy in MCF7 Human Breast Carcinoma Cells

(A and B) Coimmunoprecipitation of Beclin 1 and Bcl-2 (A) or KSHV vBcl-2 (B) in HEK293 cells cotransfected with indicated plasmids.

(C) Western blot analyses of MCF7.control cells and MCF7.*beclin 1* cells transfected with the indicated Bcl-2 expression plasmids. The Western blot shown to detect Bcl-2 represents a 3 s exposure of the enhanced chemoluminescence. Bcl-2 is detectable in the MCF7.control cells and the MCF7.*beclin 1* cells transfected with empty vector with a longer blot exposure (data not shown).

(D) Representative images of GFP-LC3 staining in MCF7.control and MCF7.*beclin 1* cells cotransfected with GFP-LC3 and indicated plasmid. Arrows denote representative cells containing GFP-LC3 dots (i.e., autophagosomal structures).

(E) Light-microscopic quantitation of autophagy in MCF7.control cells and MCF7.*beclin 1* cells cotransfected with GFP-LC3 and plasmid indicated below x axis. Results shown represent mean ±SEM for combined data from five independent experiments.

GFP-2xFYVE in HT-29 control as compared to in HT-29 Bcl-2 cells (Figure 3G). These findings suggest that Bcl-2 inhibits the formation of the autophagy-promoting Beclin 1/hVps34 complex and decreases Beclin 1-associated Class III PI3K activity.

Endogenous Beclin 1 Localizes to the Mitochondria and Endoplasmic Reticulum (ER) and ER-targeted Bcl-2 Inhibits Autophagy

The Beclin 1 binding partner, hVps34/Class III PI3K, is predominantly found on endosomes and the trans-

Golgi network (TGN; Gillooly et al., 2000), whereas Bcl-2 is predominantly found on the outer mitochondrial and ER membranes (Germain and Shore, 2003). While overexpressed Beclin 1 colocalized with Bcl-2 in the mitochondria and ER in COS7 cells (Liang et al., 1998), endogenous Beclin 1 has been reported to localize predominantly to the TGN (Kihara et al., 2001). Since Bcl-2 is not found in the TGN, we hypothesized that endogenous Beclin 1 must also localize to other subcellular compartments where Bcl-2 is found. Using confocal microscopy, we found that endogenous Beclin 1 in HT-29 cells colocalizes both with dyes that label mitochondria and that label ER (Figure 4A). We also detected Beclin 1 in the mitochondria and ER in subcellular fractionation experiments in HT-29 cells (data not shown).

Next, we evaluated whether Bcl-2 exerts its antiautophagy activity at the mitochondria and/or ER. We used Bcl-2 mutants that have restricted subcellular localization, including a mutant in which the C-terminal hydrophobic sequence of Bcl-2 is exchanged for an equivalent sequence from ActA (resulting in outer mitochondrial membrane-specific localization) and a mutant in which the C-terminal sequence is exchanged for a sequence from cytochrome b5 (resulting in ER-specific localization; Zhu et al., 1996). In HT-29 cells, similar levels of Bcl-2 protein expression were observed in cells cotransfected with GFP-LC3 and wild-type Bcl-2, mitochondrial-targeted Bcl-2, or ER-targeted Bcl-2 (Figure 4B). However, mitochondrial-targeted Bcl-2 did not inhibit starvation-induced autophagy, whereas ER-targeted Bcl-2 inhibited starvation-induced autophagy as effectively as wild-type Bcl-2 (Figure 4C). Similar results were observed in MCF7.*beclin 1* cells expressing wild-type, mitochondrial-targeted, or ER-targeted Bcl-2 (Figures 4D and 4E). Thus, in two different cell types, Bcl-2 functions at the ER and not at the mitochondria to inhibit starvation-induced autophagy.

Endogenous Bcl-2 Binds to Endogenous Beclin 1 in a Nutrient-Dependent Manner and Downregulates Levels of Starvation-Induced Autophagy

Our data in MCF7 and HT-29 cells indicate that Bcl-2 and KSHV v-Bcl-2 enforced expression inhibit Beclin 1-dependent starvation-induced autophagy. To address whether endogenous Bcl-2 also interacts with Beclin 1 and regulates starvation-induced autophagy, we performed studies in an autophagy-competent cell line, HeLa cells, that expresses detectable endogenous levels of both Bcl-2 and Beclin 1 (Figure 5A). We found that endogenous Bcl-2 binds to endogenous Beclin 1 and that this interaction is regulated by cellular nutrient status (Figure 5A). During autophagy-inducing conditions (i.e., starvation), minimal levels of Bcl-2 coimmunoprecipitate with Beclin 1; during autophagy-inhibitory conditions (i.e., growth in nutrient-rich media), high levels of Bcl-2 coimmunoprecipitate with Beclin 1; and during growth in normal media, intermediate levels of Bcl-2 coimmunoprecipitate with Beclin 1. This pattern suggests that the differential binding of endogenous Bcl-2 and Beclin 1 may be a physiological mechanism to regulate autophagy induction in response to starvation.

To confirm that endogenous Bcl-2 negatively regulates starvation-induced autophagy in HeLa cells, we used a hairpin siRNA to knock down Bcl-2 expression. We confirmed that *bcl-2* siRNA, but not a control scrambled siRNA, results in a decrease in the levels of Bcl-2 expression (Figure 5B). This decrease was observed at 48 but not at 24 hr after transfection. At the 48 hr time point when endogenous Bcl-2 protein levels were reduced, cells cotransfected with GFP-LC3 and *bcl-2* siRNA had approximately twice as many autophagosomes per cell during starvation as cells cotransfected with GFP-LC3 and a control siRNA ($p = 0.007$; t test; Figure 5C). This increased autophagy in HeLa cells following *bcl-2* gene silencing demonstrates that endogenous Bcl-2 negatively regulates levels of starvation-induced autophagy.

Bcl-2 Inhibits Starvation-Induced Autophagy in Mouse Cardiac Muscle

We evaluated whether Bcl-2 can also regulate autophagy during starvation in vivo. Previously, Mizushima et al. (2004) showed that prolonged starvation is a potent activator of autophagy in the cardiac muscle of mice expressing the transgenic fluorescent autophagy marker, GFP-LC3. Therefore, we crossed GFP-LC3 transgenic mice with mice that transgenically express human Bcl-2 under the control of the αMHC promoter (Imahashi et al., 2004; Figure 5A). Under normal nutrient conditions, we did not detect autophagy (as measured by GFP-LC3 dots) in either control or Bcl-2 transgenic/GFP-LC3 mice (data not shown). Following prolonged starvation, we observed a significant reduction in the magnitude of autophagy in Bcl-2 transgenic/GFP-LC3 mice as compared to in non-Bcl-2 transgenic control/GFP-LC3 littermates (Figures 6E and 6F; $p = 0.008$; t test). Thus, transgenic Bcl-2 expression downregulates starvation-induced autophagy in cardiac muscle.

Beclin 1 Mutants that Cannot Bind to Bcl-2 Induce Excess Levels of Autophagy and Are Prodeath

To further address the role of Beclin 1-Bcl-2 interactions in autophagy regulation, we compared the levels of autophagy induction in MCF7 cells transfected with wild-type Beclin 1 or Bcl-2 binding defective mutants of Beclin 1. We used three mutants of Beclin 1, including Beclin 1 lacking amino acids 88–150 (Beclin 1ΔBcl-2BD), Beclin 1 F123A, and Beclin 1 I125A. Similar to our findings in yeast two-hybrid assays (Liang et al., 1998; Figure 1A), Beclin 1ΔBcl-2BD and Beclin 1 F123A were unable to coimmunoprecipitate Bcl-2 in transfected HEK293 cells, whereas Beclin 1 I125A coimmunoprecipitated Bcl-2 as efficiently as wild-type Beclin 1 (Figure 6A).

The number of autophagosomes per cell was approximately doubled in nutrient-starved MCF7 cells expressing Bcl-2 binding defective mutants of Beclin 1 (e.g., Beclin 1ΔBcl-2BD and Beclin 1 F123A) as compared to in nutrient-starved MCF7 cells expressing Beclin 1 proteins that can bind to Bcl-2 (e.g., wild-type Beclin 1 or Beclin 1 I125A) (Figure 6B; $p = 0.026$ for Beclin 1ΔBcl-2 BD versus wild-type Beclin 1; $p = 0.034$ for Beclin 1 F123A versus wild-type Beclin 1; t test). The number of autophagosomes per cell was also signifi-

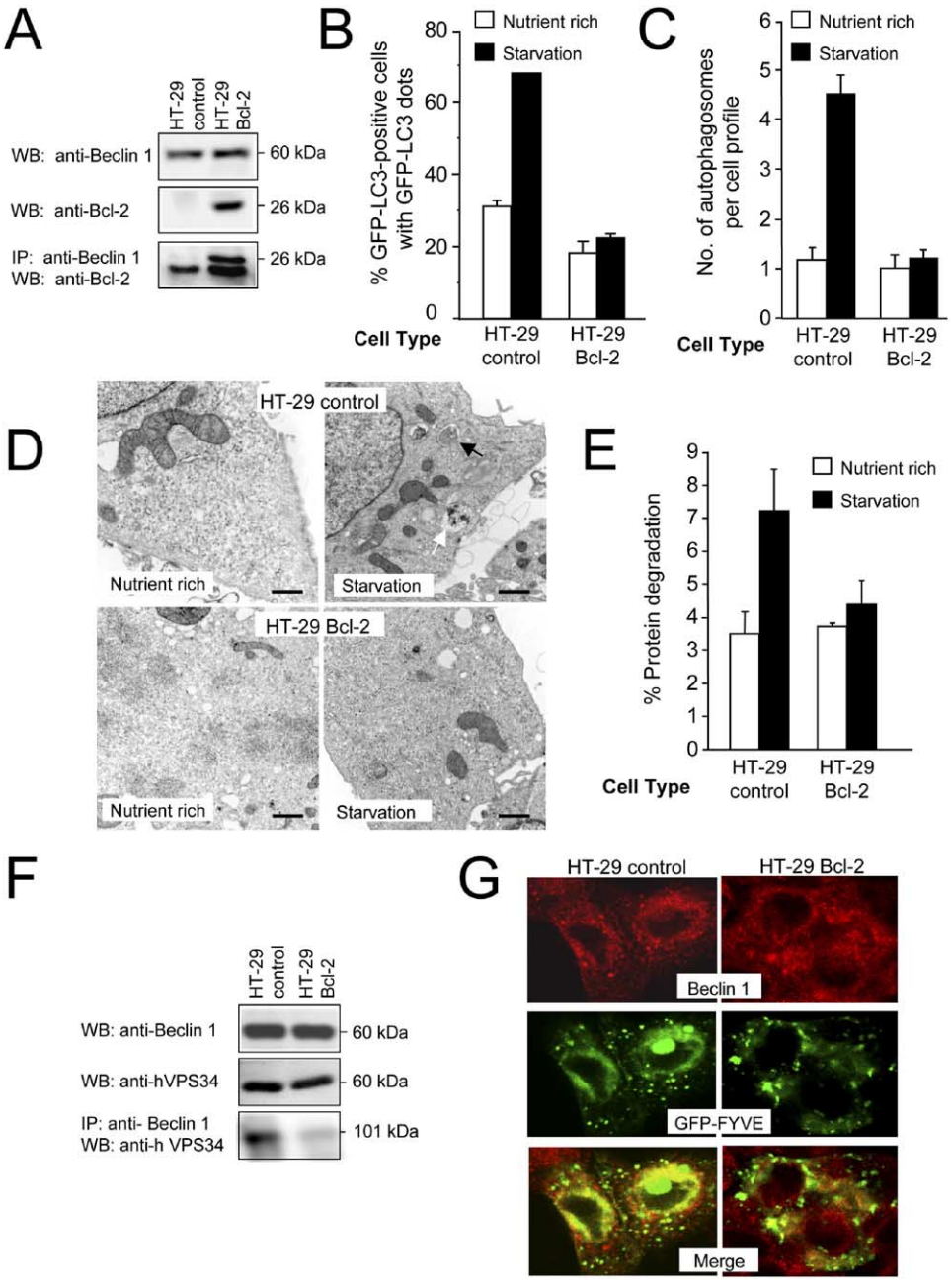

Figure 3. Bcl-2 Inhibits Autophagy in HT-29 Human Colon Carcinoma Cells, Partially Blocks the Interaction of Beclin 1 with hVps34, and Decreases Beclin 1-Associated Class III PI3K Activity

(A) Coimmunoprecipitation of Beclin 1 and Bcl-2 in HT-29 Bcl-2 cells.

(B) Light microscopic quantitation of autophagy in HT-29 control cells and HT-29 Bcl-2 cells transfected with GFP-LC3. Results shown represent mean ±SEM for combined data from three independent experiments.

(C) Electron microscopic quantitation of autophagy in HT-29 control cells and HT-29 Bcl-2 cells. Results shown represent the mean ±SEM number of autophagosomes per cell profile for 50 cell profiles per condition.

(D) Representative electron micrographs of HT-29 control and HT-29 Bcl-2 cells. Arrows denote autophagosomes (black, early autophagosome; white, late autophagosome). Scale bars, 1 μm.

(E) Autophagic degradation of long-lived cellular proteins in HT-29 control cells and HT-29 Bcl-2 cells. Results shown represent mean ±SEM for combined data from three independent experiments. The starvation-induced increase in protein degradation in HT-29 control cells was completely blocked by 10 mM 3-methyladenine (data not shown).

(F) Coimmunoprecipitation of hVps34 with anti-Beclin 1 antibody in HT-29 control and HT-29 Bcl-2 cells.

(G) Representative confocal microscopy images of Beclin 1 expression and GFP-FYVE domain expression in HT-29 and HT-29 Bcl-2 cells transfected with a plasmid encoding GFP-FYVE, a PI3P binding domain. Beclin 1 was detected using an anti-Beclin 1 primary antibody and a rhodamine-conjugated secondary antibody. Yellow color represents colocalization of Beclin 1 and GFP-FYVE, which reflects Beclin 1-associated Class III PI3K activity.

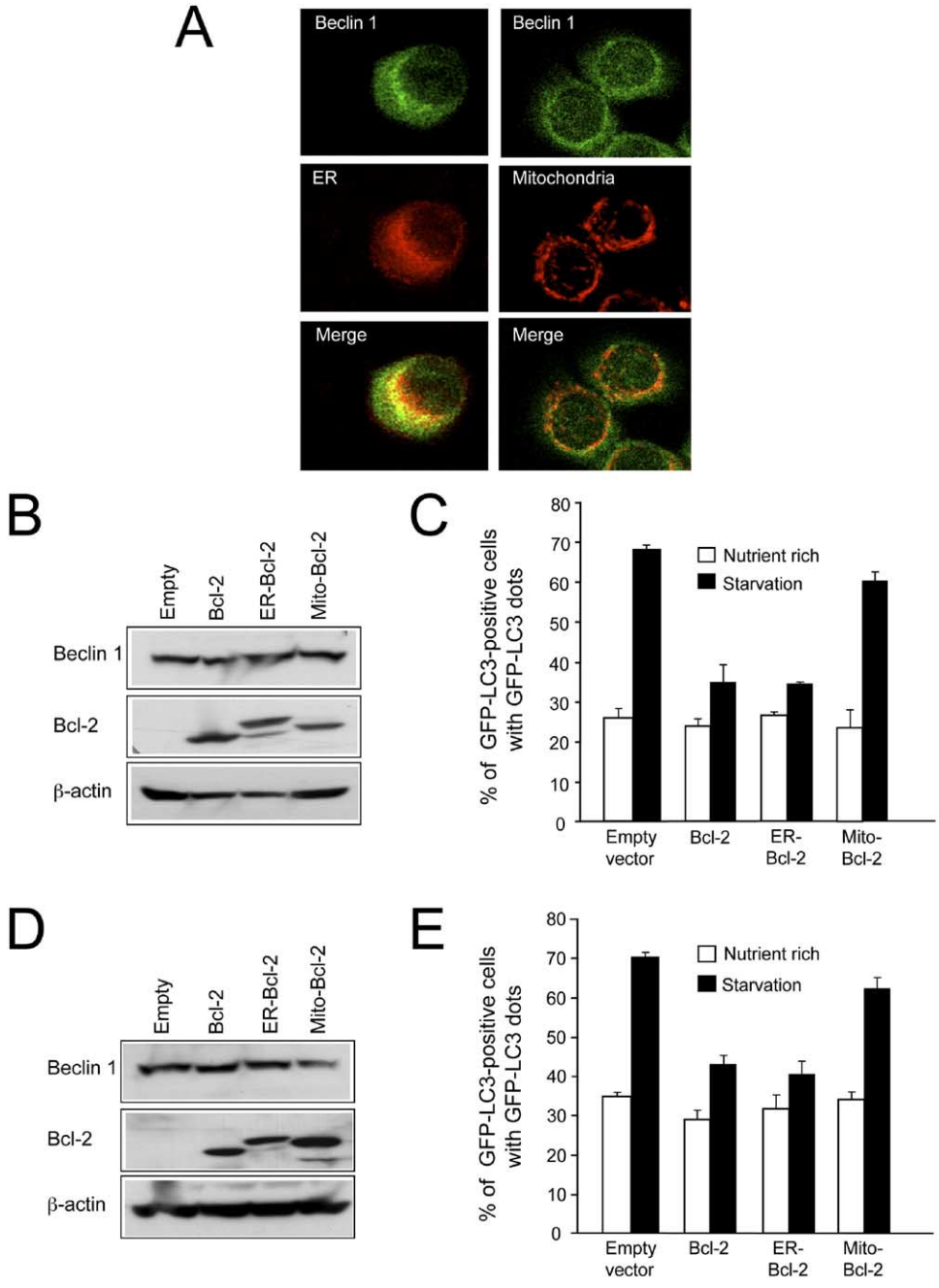

Figure 4. ER-Targeted Bcl-2 but not Mitochondrial-Targeted Bcl-2 Inhibits Autophagy in HT-29 and MCF7 Cells

(A) Representative confocal microscopy images of endogenous Beclin 1 colocalization with ER and mitochondria in HT-29 control cells. Beclin 1 was detected using an anti-Beclin 1 primary antibody and a FITC-conjugated secondary antibody. ER and mitochondria were visualized using ER-Tracker Red and MitoTracker Red, respectively. Yellow color represents colocalization of Beclin 1 and ER (left lower panel) or Beclin 1 and mitochondria (right lower panel).

(B and D) Western blot analyses of Beclin 1, Bcl-2, and β-actin expression in HT-29 control cells (B) and in MC7.*beclin 1* cells (D) transfected with the indicated Bcl-2 expression plasmids.

(C and E) Light microscopic quantitation of autophagy in HT-29 cells (C) and in MCF7.*beclin 1* cells (E) cotransfected with GFP-LC3 and plasmid indicated below x axis. For (C) and (E), the results shown represent mean ±SEM for combined data from three independent experiments. Abbreviations: ER-Bcl-2, ER-targeted Bcl-2; Mito-Bcl-2, mitochondrial-targeted Bcl-2.

cantly increased in MCF7 cells expressing Bcl-2 binding defective mutants of Beclin 1 during growth in normal culture media ($p = 0.003$ for Beclin 1ΔBcl-2 BD versus wild-type Beclin 1; $p = 0.003$ for Beclin 1 F123A versus wild-type Beclin 1; t test), indicating that these mutants increase levels of basal autophagy in the absence of starvation or treatment with a specific autophagy stimulus. Similar levels of Beclin 1 protein were

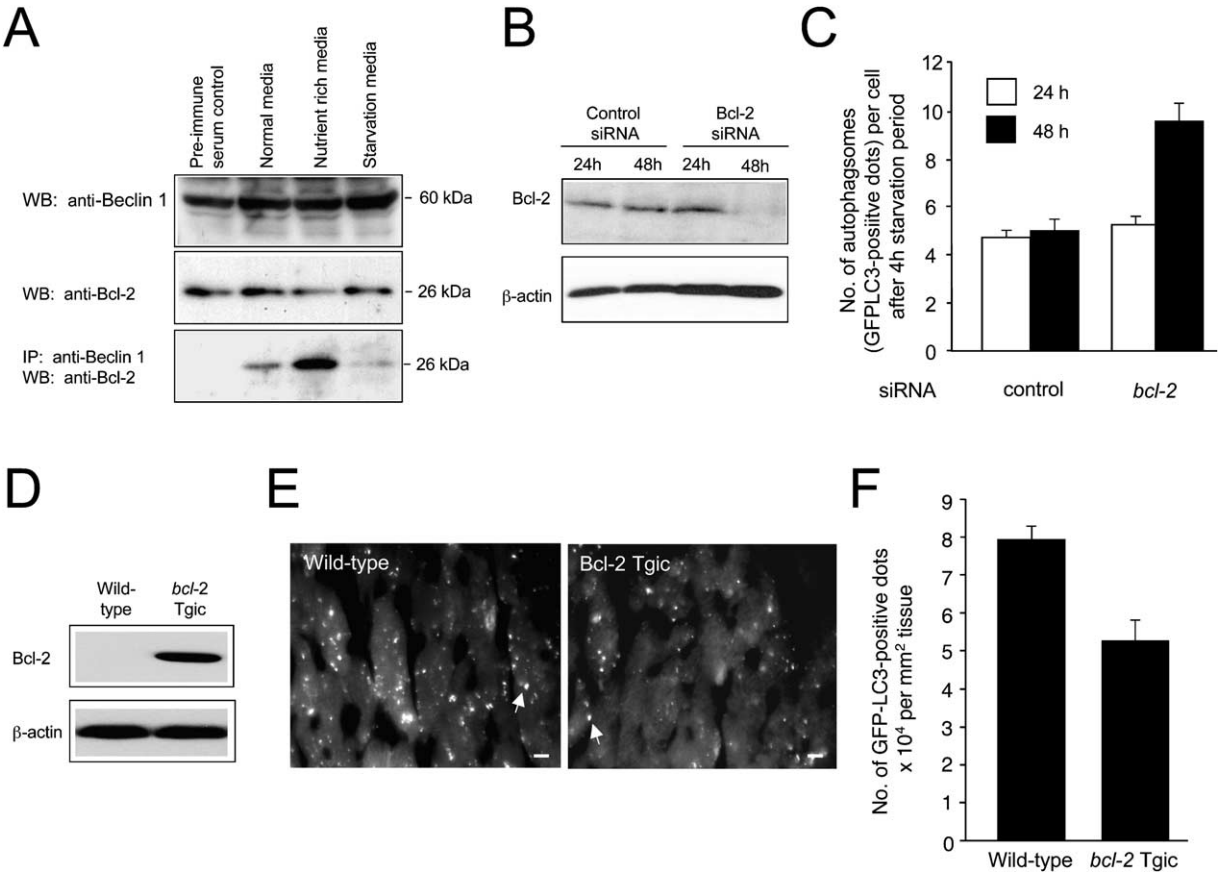

Figure 5. *bcl-2* Gene Silencing Increases Levels of Starvation-Induced Autophagy in HeLa Cells, and Bcl-2 Transgenic Expression Reduces Levels of Starvation-Induced Autophagy in Mouse Heart Muscle

(A) Coimmunoprecipitation of Beclin 1 and Bcl-2 in HeLa cells. Cells were either grown in normal media (lanes 1 and 2), nutrient-rich media (lane 3), or starvation media (lane 4) for 4 hr prior to coimmunoprecipitation with preimmune goat serum (lane 1) or a goat polyclonal anti-Beclin 1 antibody (lanes 2–4).

(B) Western blot analysis of Bcl-2 and β-actin expression in HeLa cells transfected with a plasmid encoding a control scrambled siRNA or siRNA against Bcl-2.

(C) Light microscopic quantitation of autophagy in HeLa cells co-transfected with GFP-LC3 and control siRNA or with GFP-LC3 and *bcl-2* siRNA. Results shown represent the mean ±SEM for combined data from three independent experiments.

(D) Western blot analyses of human Bcl-2 and mouse β-actin expression in wild-type and Bcl-2 transgenic mouse heart homogenates.

(E) Representative images of GFP-LC3 staining and (F) quantitation of GFP-LC3-positive dots in cardiac muscle of mice following 48 hr of starvation. Results shown in (F) represent the mean ±SEM for approximately 20 images obtained per mouse for eight mice of each genotype. Arrows in (E) denote representative GFP-LC3-positive punctate dots. Scale bars, 5 μM.

expressed in MCF7 cells transfected with wild-type and mutant forms of Beclin 1 (Figure 6C), suggesting that the increased level of autophagy in MCF7 cells transfected with Bcl-2 binding defective mutants of Beclin 1 could not be explained by higher levels of protein expression. Rather, the increased autophagy in these cells likely represents a failure of endogenous Bcl-2-like proteins to downregulate Beclin 1-dependent autophagy. No obvious differences were detected in the amount of hVps34 that coimmunoprecipitated with wild-type Beclin 1 versus Bcl-2 binding defective mutants of Beclin 1 (data not shown), suggesting that the increased autophagic activity of these mutants is not due to increased formation of Beclin 1/hVps34 complexes.

We investigated whether the increased levels of autophagy triggered by Bcl-2 binding defective Beclin 1 mutants during normal growth conditions affected the survival of MCF7 cells. The viability of MCF7 cells transfected with wild-type Beclin 1 or the control mutant protein Beclin 1 I125A was similar to that observed in MCF7 cells transfected with a control empty vector (Figure 6D). In contrast, the Bcl-2 binding defective mutants of Beclin 1, Beclin 1ΔBcl-2BD or Beclin 1 F123A, increased cell death both during growth in normal conditions ($p < 0.001$; t test) and when cells were starved for 4 hr ($p < 0.001$; t test). Of note, the Bcl-2 binding defective mutants also increased cell death when transfected into other cell types, including HEK293 cells, NIH3T3 cells, and COS7 cells (data not shown) indicating that this prodeath effect is not limited to cells such as MCF7 that have a mutation in caspase-3.

We examined whether the cell death induced by Bcl-2 binding defective Beclin 1 mutants could be

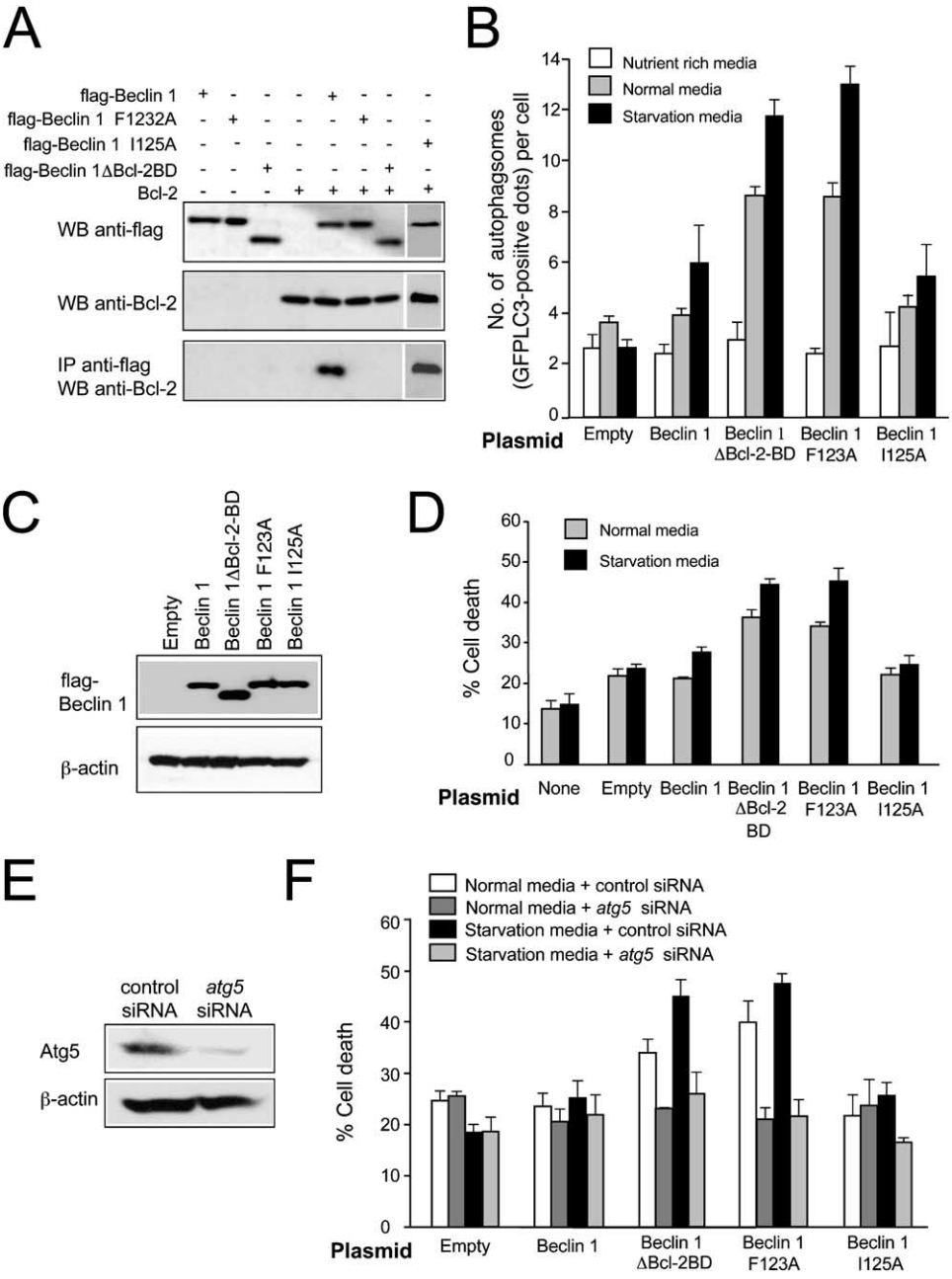

Figure 6. Beclin 1 Mutants that Cannot Bind to Bcl-2 Induce Higher Levels of Autophagy and Promote *atg5*-Dependent Cell Death in MCF7 Cells

(A) Coimmunoprecipitation of Beclin 1 mutants and Bcl-2 in HEK293 cells cotransfected with indicated plasmids.

(B) Light microscopic quantitation of autophagy in MCF7.control cells cotransfected with GFP-LC3 and *beclin 1* expression plasmid indicated below x axis. Results shown represent the mean ±SEM for combined data from three independent experiments.

(C) Western blot analysis of Flag-Beclin 1 and β-actin expression in MCF7 cells 30 hr after transfection with indicated plasmids.

(D) Quantitation of cell death by trypan blue exclusion 30 hr after transfection with the indicated plasmids in MCF7.control cells growing in normal media or subjected to 4 hr of starvation.

(E) Western blot analysis of Atg5 and β-actin expression in MCF7.control cells 30 hr after transfection with a plasmid encoding either a control siRNA or an siRNA against *atg5*.

(F) Quantitation of cell death by trypan blue exclusion in MCF7.control cells 30 hr after transfection with the indicated Beclin 1 expression plasmids and a plasmid containing either control siRNA or *atg5* siRNA. Cells were either maintained in normal media or subjected to 4 hr of starvation prior to cell death quantitation. For (D) and (F), results shown represent mean ±SEM for combined data from three independent experiments. For (C–F), assays were performed on total MCF7 cell populations; using a GFP expression plasmid to measure transfection efficiency, MCF7 cell transfection efficiency was estimated to range between 50% and 60%.

blocked by siRNA directed against a downstream autophagy gene, *atg5*, that functions in autophagosome formation (Mizushima et al., 2001). At 30 hr after transfection, at the time of cell death quantitation, Atg5 protein expression was significantly reduced in MCF7 cells transfected with a plasmid encoding a hairpin siRNA against *atg5* as compared to cells transfected with a plasmid encoding a control hairpin siRNA (Figure 6E). *Atg5* siRNA but not control siRNA blocked cell death induced by Beclin 1ΔBcl-2BD and Beclin 1 F123A both during growth in normal media and following a 4 hr starvation period (Figure 6F). Thus, the downstream autophagy gene, *atg5*, is required for cell death induced by mutant forms of Beclin 1 that cannot bind to Bcl-2.

Discussion

Bcl-2 Antiapoptotic Proteins Inhibit Autophagy In Vitro and In Vivo

Our results demonstrate that both cellular Bcl-2 and viral Bcl-2 encoded by Kaposi's sarcoma-associated herpesvirus inhibit the lysosomal degradation pathway of autophagy. The enforced expression of either Bcl-2 or KSHV vBcl-2 decreased levels of starvation-induced autophagy in yeast and mammalian cells. In addition, transgenic Bcl-2 expression reduced levels of starvation-induced autophagy in mouse cardiomyocytes, indicating that the autophagy inhibitory function of Bcl-2 occurs in vivo and not only in vitro. Furthermore, siRNA silencing of endogenous Bcl-2 in HeLa cells increased levels of starvation-induced autophagy, suggesting that basal levels of Bcl-2 play a role in the regulation of autophagy.

Our observation that Bcl-2 inhibits starvation-induced autophagy in multiple different cell types and both in vitro and in vivo suggests that it is a physiologically important regulator of autophagy. Previous studies have indirectly implicated a role for Bcl-2 in the negative regulation of autophagy (Levine and Yuan, 2005). Bcl-2 has been shown to block caspase-independent cell death and the degradation of mitochondria, which are two processes postulated to involve autophagy. However, direct evidence that Bcl-2 inhibits the autophagy pathway has been lacking.

The biological effects of Bcl-2 have been largely attributed to its effects on the apoptotic pathway, although it has been recognized that Bcl-2 family members are multifunctional proteins that can influence other cellular processes, including cell cycle progression, calcineurin signaling, glucose homeostasis, and transcriptional repression by p53 (Danial and Korsmeyer, 2004; Reed, 1998). Our results suggest that Bcl-2 antiapoptotic family members also target the autophagy pathway. This concept has important implications for diverse aspects of biology, since autophagy genes play a role in promoting survival during stress conditions, in differentiation and development, in lifespan extension, in tumor suppression, and in cell death in apoptosis-deficient cells (Levine and Klionsky, 2004; Levine and Yuan, 2005). Therefore, the inhibition of autophagy by cellular and virally-encoded Bcl-2 antiapoptotic family members may represent a novel mechanism by which Bcl-2 family members function as oncogenic and antideath molecules and by which they modulate various homeostatic, developmental, and disease processes.

In contrast to our findings that Bcl-2 functions as an inhibitor of autophagy, a recent study demonstrated an opposite role for Bcl-2 antiapoptotic family members in the regulation of autophagy. Shimizu et al. (2004) reported that Bcl-x_L or Bcl-2 overexpression increases autophagy in transformed MEFs treated with etoposide. The basis for the differences between these data and our findings is not yet clear. It is possible that Bcl-2 family members vary in their regulatory effects on autophagy depending on the cell type or stimulus. However, it is worth noting that etoposide is not a physiological inducer of autophagy and that etoposide induces apoptosis rather than autophagy gene-dependent death in most cell types. In our study, we focused on the role of Bcl-2 in starvation-induced autophagy, since starvation is an important physiologic inducer of autophagy across a broad range of eukaryotic species. Furthermore, autophagy plays a prosurvival rather than prodeath role during starvation, thereby allowing us to dissociate the inhibitory effects of Bcl-2 on cell death from its effects on autophagy. Our findings demonstrate that at least during starvation, Bcl-2 functions to inhibit, rather than promote, autophagy. Other studies using different physiological stimuli of autophagy are required to determine whether the inhibitory effects of Bcl-2 are restricted to nutrient deprivation or whether Bcl-2 plays a more general role in the negative regulation of autophagy.

Bcl-2 Inhibits Autophagy via an Interaction with Beclin 1

Our results suggest that the mechanism by which Bcl-2 inhibits autophagy is likely to involve its interaction with Beclin 1. We found that Bcl-2-mediated inhibition of Beclin 1-dependent autophagy is prevented by either mutations in Bcl-2 that block binding to Beclin 1 or by mutations in Beclin 1 that block binding to Bcl-2. Although the specific BH1-domain and BH2-domain Bcl-2 mutant proteins used in this study also fail to bind to proapoptotic Bcl-2 family members (Sedlak et al., 1995; Yin et al., 1994), we found that these mutants failed to inhibit Beclin 1-dependent autophagy in yeast cells that lack any endogenous pro- or antiapoptotic Bcl-2-like family members. This observation argues strongly for an independent role of Bcl-2-Beclin 1 interactions in the inhibition of autophagy.

In HT-29 cells, the inhibitory effects of Bcl-2 are associated with disruption of the Beclin 1/hVps34 complexes, which are thought to be essential for early stages of autophagosomal formation (Kihara et al., 2001). However, in MCF7 cells, the increased autophagy induced by Bcl-2 binding defective mutants of Beclin 1 are not associated with increased binding of hVps34 to the Beclin 1 mutants. Therefore, it is possible that the mechanism by which Bcl-2 inhibits the autophagy function of Beclin 1 involves either the disruption of Beclin 1/hVps34 complex formation or antagonism of other as-of-yet-defined actions of Beclin 1 that are necessary for autophagy. In either case, it is unlikely that there is direct competition between Bcl-2 and

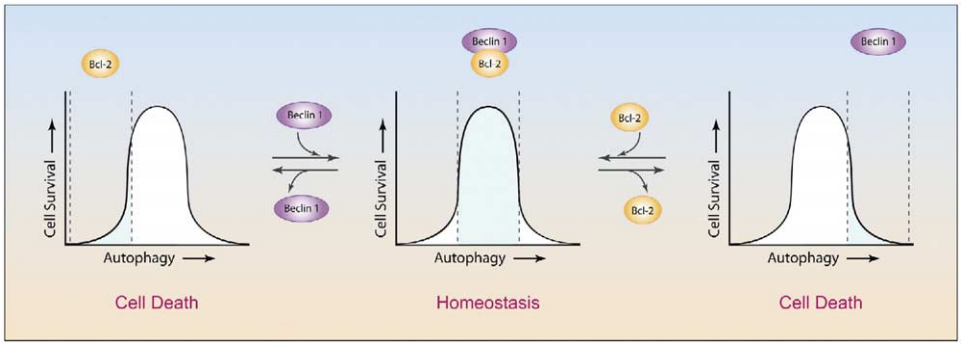

Figure 7. Model of a Rheostat Function of the Beclin 1-Bcl-2 Complex in Autophagy Regulation

Physiological levels of autophagy (shaded region of center graph) are essential for normal cellular homeostasis, and the absence of autophagy genes (shaded region of left graph) increases cell death during nutrient deprivation and other forms of cellular stress (reviewed in Levine and Klionsky [2004]). In contrast, excessive, nonphysiological levels of autophagy (shaded region of right graph) promote autophagy gene-dependent cell death. The relative amounts of Beclin 1 and Bcl-2 (or perhaps other Bcl-2 family members) complexed with each other within a cell govern the threshold for transition from cell homeostasis (center graph) to cell death (right graph).

hVps34 for binding to Beclin 1 since the subcellular localization of Bcl-2 and hVps34 are not known to overlap. In this study, we found that some endogenous Beclin 1 localizes to the ER and that ER-targeted Bcl-2 effectively inhibits autophagy in both HT-29 and MCF7 cells. It is possible that Bcl-2 results in a redistribution of Beclin 1 away from the TGN (where it is active in autophagy induction; Kihara et al., 2001) to the ER; however, we did not observe any differences in the amount of Beclin 1 localized to the ER in cells that express low or high levels of Bcl-2 (data not shown). More likely, the interaction between Bcl-2 and Beclin 1 in the ER blocks a signal that is essential for autophagy induction.

The ability of Bcl-2 to inhibit autophagy through a direct interaction with Beclin 1 is of particular interest with respect to cancer. Bcl-2 was originally discovered in the context of its translocation next to the immunoglobulin gene promoter at the chromosomal breakpoint of t(14;18) bearing human follicular lymphoma, and transgenic mice harboring a Bcl-2 immunoglobulin minigene develop follicular hyperplasia (Danial and Korsmeyer, 2004). Of note, deletion of one allele of *beclin 1* predisposes mice to developing spontaneous B cell lymphomas, suggesting that levels of Beclin 1-mediated autophagy influence the risk of malignancies arising from B lymphocytes (Qu et al., 2003; Yue et al., 2003). Thus, in addition to inhibitory effects on the canonical apoptosis pathway, the inhibitory effects of Bcl-2 on Beclin 1-dependent autophagy may contribute to the development of B cell lymphomas. Furthermore, given our findings that the viral Bcl-2 encoded by KSHV inhibits Beclin 1-dependent autophagy, it will also be important to determine the role of viral Bcl-2-Beclin 1 interactions in oncogenesis mediated by KSHV and other γherpesviruses.

The finding that Bcl-2 inhibits Beclin 1-dependent autophagy raises the question of how physiological autophagy occurs in normal cells that express both Bcl-2 and Beclin 1. Our results suggest that cellular nutrient conditions that regulate autophagy also regulate the interaction of Bcl-2 and Beclin 1. When autophagy is maximally induced by amino acid starvation, Bcl-2 binding to Beclin 1 is minimal, and when autophagy is inhibited by amino acid excess, Bcl-2 binding to Beclin 1 is maximal. Thus, the dissociation of Bcl-2 from Beclin 1 may be an important mechanism for activating autophagy in response to starvation and potentially other physiological stimuli. Conversely, the association of Bcl-2 and Beclin 1 during normal growth conditions may be a mechanism for preventing inappropriate activation of autophagy. This concept is consistent with our observation that Bcl-2 binding defective mutants of Beclin 1 promote increased levels of autophagy in the absence of starvation.

It is not yet known how nutrient conditions regulate the interaction between Bcl-2 and Beclin 1. Since we found that Bcl-2 functions at the ER to inhibit autophagy and previous studies have shown that phosphorylated Bcl-2 localizes predominantly to the ER (Bassik et al., 2004), one intriguing possibility is that Bcl-2 may be a target for autophagy-inhibitory signaling kinases involved in nutrient sensing. For example, Bcl-2 has been noted to be phosphorylated following addition of growth factors (Poommipanit et al., 1999) and dephosphorylated following treatment with rapamycin, a pharmacological inhibitor of the evolutionarily conserved nutrient sensor, TOR kinase, that functions downstream of growth factor-signaling molecules to suppress autophagy (Asnaghi et al., 2004). While yeast genetic studies have indicated that autophagy genes are downstream of TOR (Noda and Ohsumi, 1998), the relevant targets of TOR in autophagy suppression are not yet defined. Given our observation that cellular nutrient status regulates formation of the autophagy inhibitory Bcl-2-Beclin complex, it will be important to determine whether modulation of the Bcl-2-Beclin 1 interaction by TOR kinase or other nutrient signaling pathways is involved in autophagy suppression.

Bcl-2 Inhibition of Beclin 1 Autophagy Function May Prevent Autophagy Gene-Dependent Cell Death

We found that mutant proteins of Beclin 1 that cannot bind to Bcl-2 induce excess levels of cellular autophagy

in the absence of exposure to nutrient deprivation or other specific autophagy stimuli. These excess levels of autophagy are associated with the induction of a form of cell death that can be prevented by siRNA directed against *atg5*, a downstream autophagy gene, suggesting that at "nonphysiological" levels, the autophagy pathway can lead to cell death. These observations lead to the hypothesis that Bcl-2 downregulation of autophagy, through its interaction with Beclin 1, may prevent autophagy from occurring at elevated levels that promote cell death.

Based upon these findings and previous evidence that *ATG6/beclin 1* family members and other autophagy genes promote cellular survival during starvation (Boya et al., 2005; Levine and Klionsky, 2004), we propose a conceptual model for understanding the interrelationship between autophagy, cell survival, and cell death (Figure 7). According to this model, autophagy is necessary as an adaptive response to nutrient deprivation and other forms of cellular stress, and the absence of autophagy increases susceptibility to death when cells confront stressful stimuli. However, if levels of autophagy are induced beyond a physiological range, the autophagy pathway can contribute to death execution. Our results suggest that the Beclin 1-Bcl-2 complex may function as rheostat that ensures that autophagy levels remain within a homeostatic range rather than in a nonphysiological range that triggers cell death. When Beclin 1 is bound to Bcl-2 (or potentially other Bcl-2 family members), cells respond to autophagy-inducing stimuli in a regulated manner. However, when Beclin 1 function is "unchecked" by Bcl-2-like proteins, excessive levels of autophagy may promote cell death. Thus, similar to the theory that the relative concentrations of Bcl-2 pro- and antiapoptotic family members govern the threshold for activation of apoptosis in a given cell, we propose that the relative concentration (or binding) of Bcl-2 and Beclin 1 govern the threshold for activation of autophagy and autophagy-gene-dependent cell death.

Experimental Procedures

Please see Supplemental Experimental Procedures for information on yeast strains, plasmid constructions, siRNA suppression of gene expression, Western blot analyses, confocal microscopy, and more details on coimmunoprecipitation assays.

Cell Culture

HEK293 and HeLa cells were obtained from ATCC (American Type Culture Collection, Rockville, Maryland). MCF7.control cells and MCF7.*beclin 1* cells were previously described (Furuya et al., 2005). HT-29 cells stably transfected with a plasmid containing human *bcl-2* (HT-29 Bcl-2 cells) or empty vector (HT-29 control cells) were previously described (Lacour et al., 2003).

Mouse Strains

Cardiac-specific Bcl-2 transgenic mice (Imahashi et al., 2004) were crossed with GFP-LC3 transgenic mice (Mizushima et al., 2004). Two-month-old offspring of either sex were used for autophagy experiments.

Yeast Two-Hybrid Assays

pGBT9 plasmids containing cellular *bcl-2* or *KSHV v-bcl-2* were cotransformed with pGAD424 plasmids containing *beclin 1* in *S. cerevisiae* SFY526 cells, and transformants were screened for LacZ activity by a colony lift filter assay.

Autophagy Assays

Yeast autophagy was measured in cells subjected to 4 hr of starvation using differential interference contrast (DIC) microscopy as described (Talloczy et al., 2002).

Mammalian autophagy in MCF7 cells, HT-29 cells, and HeLa cells was measured by light microscopic quantitation of cells transfected with GFP-LC3 as described (Furuya et al., 2005). For most experiments, the percentage of GFP-LC3-positive cells with GFP-LC3 punctate dots was determined. However, in experiments requiring a precise assessment of levels of autophagy per cell, the number of GFP-LC3 punctate dots per cell in GFP-LC3-positive cells was determined. A minimum of 50–100 cells per sample was counted for triplicate samples per condition per experiment. Prior to analysis, cells were starved for 4 hr in Earle's balanced salt solution (EBSS; starvation medium), cultured in EBSS supplemented with 10% fetal calf serum and a 10× amino acid mixture (nutrient-rich medium), or maintained in DMEM with 10% fetal calf serum (normal media). In HT-29 control and HT-29 Bcl-2 cells, the degradation of radioactive ^{C14}valine-labeled long-lived proteins was measured as described (Pattingre et al., 2004). Electron microscopic (EM) analyses of autophagy in HT-29 control and HT-29 Bcl-2 cells were performed as described (Liang et al., 2001).

Autophagy was measured in the hearts of Bcl-2 trangenic/GFP-LC3 and non-Bcl-2 transgenic control/GFP-LC3 mice following 48 hr of starvation as described (Qu et al., 2003). All animal studies were performed in accordance with UT Southwestern Institutional Animal Care and Use Committee Guidelines.

Coimmunoprecipitation Assays

Beclin 1-Bcl-2 coimmunoprecipitations were performed in HEK293 cells using an anti-Flag antibody or an anti-His antibody to immunoprecipitate epitope-tagged Beclin 1. To immunoprecipitate endogenous Beclin 1, a rabbit polyclonal anti-Beclin 1 antibody (Novus Biologicals, Littleton, Colorado) was used in HT-29 control and HT-29 Bcl-2 cells, and a goat polyclonal antibody was used in HeLa cells (Santa Cruz Biologicals, Santa Cruz, California). Coimmunoprecipitation of Beclin 1 and hVps34 was performed as described (Furuya et al., 2005)

Cell Death Assays

MCF7.control cells were transfected using Lipofectamine 2000 (InVitrogen, Carlsbad, California) with pCR3.1 plasmids expressing wild-type or mutant forms of Beclin 1 alone (4 μg DNA) or in combination with control or *atg5* pSilencer 4.1-CMV puro vectors (2 μg DNA of each vector). Thirty hours after transfection, cell viability was measured by trypan blue exclusion. A minimum of 100 cells per sample for triplicate samples was counted per condition per experiment.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and can be found with this article online at http://www.cell.com/cgi/content/full/122/6/927/DC1/.

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