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Regulation of Anti-Apoptotic BCL2-Proteins by Non-Canonical Interactions: *The Next Step Forward or Two Steps Back?*

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ABSTRACT
All aspects of cellular biology affect the process of regulated cell death, or apoptosis, and disruption of this process is a causative event in many diseases. Therefore, a comprehensive understanding of all pathways that regulate apoptosis would increase our knowledge of basic cellular functions, as well as the etiologies of many diseases. In turn, we may be able to use this knowledge to better treat patients with diseases, including cancer. Although the basic signaling pathway that regulates apoptosis has been known for over 10 years, we still have much to learn about the upstream signaling components that can directly regulate the core apoptosis machinery. The focus of this review will be to direct attention to non-canonical regulators of the BCL2-family of proteins, especially our void of understanding of such interactions, and the controversy that surrounds some such interactions. J. Cell. Biochem. 113: 3–12, 2012. © 2011 Wiley Periodicals, Inc.

KEY WORDS: BCL2; BH3; APOPTOSIS; MITOCHONDRIA; MOMP

REVIEW

Arguably, the most well studied regulators of apoptosis are the BCL2-family proteins [Horvitz, 1999; Cory et al., 2003; Youle and Strasser, 2008]. Members of this family are defined by the presence of short amino acid motifs known as BCL homology (BH) domains [Lanave et al., 2004]. More than 20 members of this family have been identified within the genome of higher eukaryotes, and the family can be divided into two categories based on whether they inhibit or promote mitochondrial outer-membrane permeabilization (MOMP), which is the first commitment step in the demise of the cell [Adams and Cory, 1998]. Most important, for the purpose of this review are the six anti-apoptotic BCL2 (aaBCL2) proteins (BCL2, BCLxl, BCLw, MCL1, BFL1, and BCLb), which directly antagonize the function of pro-apoptotic multi-BH domain-containing proteins BAX and BAK, the proteins responsible for triggering MOMP [Tsujimoto, 1998; Beverly and Varmus, 2009]. Additional pro-apoptotic BCL2-family members, the so-called BH3-only proteins, act as agonists of BAK and BAX on mitochondrial membranes, antagonists of the aaBCL2 proteins, or both [Huang and Strasser, 2000].

Under normal homeostatic conditions, individual cells make the decision to live or die based upon the balance of pro-versus anti-apoptotic molecules present and functioning within the cell. Following an apoptotic stimulus the balance is shifted toward death by an increase in the activity of pro-apoptotic signals, usually by transcriptional or post-translational increases in the levels or activity of BH3-only proteins [Huang and Strasser, 2000]. Numerous review articles describe how BCL2-family proteins are regulated by BH3-containing proteins and how the process of apoptosis is executed. However, the role that non-BH3 domain-containing proteins play in regulating the functions of the aaBCL2-proteins has been under represented. Multiple examples of non-BH3-containing proteins interacting with and regulating BCL2 and/or BCLxl are found in the literature. Meanwhile, descriptions of analogous interactions for the remaining four aaBCL2-family members have been comparatively limited. The regulation of MOMP by the BCL2-family of proteins is widely thought to be the sole downstream function of the family, however there are functions of the aaBCL2-proteins that occur upstream of the mitochondria, and perhaps in parallel to the regulation of MOMP, that remain largely mysterious.

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Overall, the experimental evidence available to date argues that: (i) The six aaBCL2 proteins are regulated by (and can regulate) different signaling pathways, (ii) multiple cellular processes have a means by which they can activate and/or inhibit apoptosis, and (iii) Our understanding of the signals that regulate the aaBCL2-family of proteins, upstream of the mitochondria, is limited, especially for the aaBCL2-genes that have been more recently discovered. Altogether, at least 55 non-BH3/non-BCL2-family proteins have been previously reported to interact with at least one of the six aaBCL2 family members (Table I).

CONTROVERSY

It should be noted that a select few of the interactions described in this review have been contested, either by a simple lack of confirmation by multiple groups or by outright refutation by others. Although formal retractions have not been issued for any of the works discussed, some of the most extreme cases have resulted in opposing groups publishing articles that directly contest previously published findings. In most cases, however, the critics have remained much more subdued and only those investigators intimately familiar with the field are aware of such controversies. Rather than supporting or refuting any previously published work, any potential aaBCL2-family interactions that has been challenged or not well validated are clearly indicated in Table I. Therefore, if the reader is interested in any of these particular interactions, they are encouraged to further investigate that respective interaction in more detail.

ALL ROADS LEAD TO THE MITOCHONDRIA

The most obvious mechanism by which proteins could potentially regulate the function of aaBCL2 proteins is to alter the ability of the aaBCL2-protein to directly interact with BAX and/or BAK at the mitochondria (Table II, Class I; mitochondrial interaction) [Iwahashi et al., 1997; Komatsu et al., 2000; Tagami et al., 2000; Zhang et al., 2000; Rebollo et al., 2001; Mihara et al., 2003; Shirane and Nakayama, 2003; Yanagisawa et al., 2003; Pasinelli et al., 2004; Cheng et al., 2007; Mancini et al., 2009]. In fact, multiple mechanisms by which a protein interaction partner can either potentiate or inhibit the ability of aaBCL2-proteins to interact with BAX and/or BAK are found in the literature. The first mechanism is the sequestration of the aaBCL2-protein from the mitochondria. This is observed, for example, for RAD9A, in the context of DNA damage, such that RAD9A interacts with BCL2 and BCLxl leading to dissociation of the aaBCL2-protein from the mitochondria [Komatsu et al., 2000]. Thus, DNA damage is an example of an apoptotic stimulus that directly affects the localization of aaBCL2-proteins, therefore skewing the balance of life versus death signals within the cell. Similarly, the expression of either RTN1 or RTN4 can lead to the sequestration of aaBCL2-proteins to the endoplasmic reticulum promoting MOMP [Tagami et al., 2000]. It is not clear, however, whether there is a reciprocal regulation of RAD9A, RTN1, or RTN4 by the aaBCL2-proteins.

One of the more intriguing examples of an aaBCL2 protein being sequestered from the mitochondria is the interaction of Aiolos with BCLxl [Rebollo et al., 2001]. Aiolos is an Ikaros-family transcription factor known to play an important role in hematopoiesis. In a murine T-cell line deprived of IL-4, Aiolos is bound to BCLxl, inhibiting BCLxl from interacting with mitochondria, in turn poising the cell for death by activation of BAX/BAK. Following stimulation of the cell with IL-4, Aiolos becomes phosphorylated on tyrosine residues and this phosphorylation leads to the dissociation of Aiolos from BCLxl, restoring the ability of BCLxl to interact with mitochondria and block apoptosis. It is possible that this mode of regulation couples transcriptional regulation by Aiolos on target genes with direct regulation of apoptotsis following stimulation/deprivation of IL-4.

In contrast to the examples cited above, the literature also contains reports of proteins binding aaBCL2-proteins potentiating the interaction with mitochondria, thereby leading to a more potent inhibition of apoptosis. Two such examples are the interaction of either SMN1 or FKBP8 with BCL2 and BCLxl [Iwahashi et al., 1997; Shirane and Nakayama, 2003]. Interaction of FKBP8 with aaBCL2-proteins somehow leads to a dramatic relocalization of steady-state aaBCL2-proteins to the outer mitochondrial membrane, leading to more impressive protection following apoptotic stimuli [Iwahashi et al., 1997]. The mechanism responsible for the interaction of FKBP8 and aaBCL2 has not been elucidated, but it would be interesting to know the physiological stimuli that trigger this interaction. Similarly, SMN1 was found to localize with BCL2 and BCLxl on the mitochondrial outer membrane, leading to an increased resistance to apoptotic stimuli. Again, the physiological relevance of this event is not totally clear, particularly given that SMN1 is a putative ribonuclear protein (also, discussed in more detail below) [Shirane and Nakayama, 2003].

From the examples provided it is obvious that affecting the localization and mitochondrial interaction of aaBCL2-proteins is a potential mechanism for which therapeutics could be designed in order to effectively modulate apoptotic responses, at least in some cases. For instance, over-expression of FKBP8 led to a decrease in the ability of cells to respond following a variety of apoptotic stimuli, including etoposide, UV, and staurosporine [Iwahashi et al., 1997]. Normal sensitivity to apoptotic stimuli was restored by either co-expression of a dominant-negative version of FKBP8 or by RNAi-mediated loss of FKBP8. This experiment suggests a potential novel therapeutic strategy for normalizing homeostasis to tumor cells that express aberrant levels of FKBP8.

INTERACTION LEADS TO MODIFICATION

Modulation of protein function by post-translational modification is another potential outcome for the interaction of aaBCL2-proteins with non-canonical interaction partners (Table II, Class II; Protein modification) [Cheng et al., 1997; Deng et al., 1998; Yamamoto et al., 1999; Nakagawa and Yuan, 2000; Ueno et al., 2000; Pathan et al., 2001; Gil-Parrado et al., 2002; Lin et al., 2004; Tamura et al., 2004; Liu et al., 2005; Weng et al., 2005; Yang et al., 2005; Zhong et al., 2005; Li et al., 2007; Luciano et al., 2007; Guillemin et al., 2009;

TABLE I. List of Non-Canonical Proteins That Interact With BCL2-Family Members

Symbol	Name	Alternative names	Interaction identified	Function	References
BCL2					
MYC	MYC	c-MYC	IP, co-localization wb, func.	Anti-apop./prolif.	Jin et al. [2004]
CASP8	Caspase8	FLICE	Co-ip, co-local	Apop.	Ng et al. [1997]
SMN1 [Coovert et al., 2000]	Survival of motor neuron 1	SMA	Y2h, co-ip, co-local, func.	Anti-apop.	Iwahashi et al. [1997]
SOD1 [Gould et al., 2006]	Superoxide dismutase 1	SOD	Co-ip, crosslinking, func.	Anti-apop.	Pasinelli et al. [2004]
RRAS	Related RAS	p23	Y2h, co-ip	n.d.	Fernandez-Sarabia and Bischoff [1993]
MAPK8	Mitogen-activated protein kinase 8	JNK1	Co-ip, functional	Apop.	Yamamoto et al. [1999]
RAF1 [Olivier et al., 1997]	RAF1	c-RAF	Co-ip	Anti-apop.	Wang et al. [1994]
ITPR1	Nositol 1,4,5-triphosphate receptor 1	IP3R1, InsP3R	Co-ip, functional	Apop.	Chen et al. [2004]
PRNP	Prion Protein	PrP, PrPc	Y2h	n.d.	Kurschner and Morgan [1995]
MDM4	Transformed mouse 3T3 cell double minute 4	MDMX	Co-ip, co-local, functional	Apop.	Mancini et al. [2009]
TP53BP2	Tumor protein p53 binding protein, 2	53BP2, ASPP2, PPP1R13A	Y2h, co-ip, co-local	Apop.	Naumovski and Cleary [1996]
IRS2	Insulin receptor substrate 2	IRS-2	Co-ip	Anti-apop.	Ueno et al. [2000]
PPP2CA	Protein phosphatase 2, catalytic subunit, alpha	PP2Ac, PP2CA	Co-ip, functional	n.d.	Deng et al. [1998]
PARP1	Poly(ADP-ribose) polymerase 1	ADPRT1, PARP	Co-ip	Apop.	Song et al. [2002]
TP53AIP1	P53 regulated apoptosis inducing protein 1	P53AIP1	Co-ip, co-local, functional	Apop.	Matsuda et al. [2002]
MAPK1	Mitogen-activated protein kinase 1	ERK, P42MAPK	Co-ip, functional	Apop.	Tamura et al. [2004]
PXN	Paxillin	FLJ16691	Co-ip	n.d.	Sorenson and Sheibani [1999]
RPS3A	Ribosomal protein S3A	FTE1, MFTL	Co-ip	Anti-apop.	Hu et al. [2000]
CDK2	Cyclin-dependent kinase 2	p33	Co-ip	Prolif.	Schmitt et al. [2007]
CASP3	Caspase 3	CPP32, CPP32B	In vitro cleavage assay	Apop.	Cheng et al. [1997]
RAD9A	RAD9 homolog A	Rad9	Y2h, co-ip, co-local, func.	Apop.	Komatsu et al. [2000]
RTN4	Reticulon 4	RTN-x	Y2h, co-ip, co-local, func.	Apop.	Tagami et al. [2000]
PSEN1	Presenilin1	AD3, FAD, PS1	Y2h, co-ip, cross-link, fuc.	Apop.	Alberici et al. [1999]
SPNS1	Spinster homologue 1	Hspin1	Co-ip, co-local	Autophagy	Yanagisawa et al. [2003]
CDK1	Cyclin-dependent kinase 1	Cdc2	Co-ip	Cell cycle	Pathan et al. [2001]
FKBP8	FK506 binding protein 8, 38 kDa	FKBP38	Y2h, co-ip, co-local, func.	Anti-apop.	Shirane and Nakayama [2003]
NR4A1	Nuclear receptor subfamily 4, group A, member 1	Nur77, TR3	M2h, co-ip, co-local, func.	Apop.	Lin et al. [2004]
IRS1	Insulin receptor substrate 1	HIRS-1	Co-ip, co-local, functional	Anti-apop.	Ueno et al. [2000]
NLRP1	NLR family, pyrin domain containing 1	Card7, NALP1	Co-ip, fuctional	Apop.	Bruey et al. [2007]
TP53	Tumor protein p53	p53, TRP53	Co-ip, functional	Apop.	Mihara et al. [2003]
PPP1CA	Protein phosphatase 1, catalytic subunit, alpha	PP-1A, PPP1A	Y2h, co-ip, co-local	Apop.	Ayllón et al. [2001]
CAPN2	Calpain 2	CANP2, CANPL2	Cleavage assay	Apop.	Gil-Parrado et al. [2002]
Pin1	Peptidylprolyl cis/trans isomerase	DOD, UBL5	Co-ip	n.d.	Pathan et al. [2001]
AVEN	Apoptosis, caspase activation inhibitor	PDCD12	Y2h, co-ip	Anti-apop.	Chau et al. [2000]
BCAP31	B-cell receptor-associated protein 31	Bap31, p28	Far western/seq., co-ip, func.	Apop.	Ng et al. [1997]
BFAR	Bifunctional apoptosis inhibitor	BAR, RNF47	Y2h, co-ip, functional	Anti-apop.	Zhang et al. [2000]
BCLxl					
RTN1	Reticuon 1	NSP-C	Y2h, co-ip, functional	Apop.	Tagami et al. [2000]
PGAM5	Phosphoglycerate mutase family member 5	BXLBV68	Co-ip	n.d.	Lo and Hannink [2006]
IKZF3	IKAROS family zinc finger 3	Aiolos, ZNFN1A3	Y2h, co-ip, co-local	Apop.	Rebollo et al. [2001]
PSEN2	Presenilin 2	AD4, PS2, STM2	Y2h, co-IP, co-local	Apop.	Passer et al. [1999]
APAF1 [Moriishi et al., 1999]	Apoptotic peptidase activating factor 1	APAF-1, CED4	Co-ip	Apop.	Hu et al. [1998]
MYO5A	Myosin VA (heavy chain 12, myoxin)	MYH12, MYO5, MYR12	Co-ip	n.d.	Du et al. [2007]
PLK1	Polo-like kinase 1	PLK, STPK13	Co-local, functional	n.d.	Tamura et al. [2009]
CASP9	Caspase 9, apoptosis-related cysteine peptidase	APAF3	Co-ip	Apop.	Hu et al. [1998]
OLFM1	Olfactomedin 1	pancortin 1, AMY, NOE1	Co-ip, functional	Anti-apop.	Cheng et al. [2007]
RTN4	Reticulon 4	RTN-x	Y2h, co-ip, co-local, func.	Apop.	Tagami et al. [2000]
CDK1	Cyclin-dependent kinase 1	Cdc2	Co-ip, co-local, functional	Prolif.	Schmitt et al. [2007]
RAD9A	RAD9 homolog A	Rad9	Y2h, co-IP, functional	Apop.	Komatsu et al. [2000]
PPP1CA	Protein phosphatase 1, catalytic subunit, alpha	PP-1A, PPP1A	Co-ip	Apop.	Ayllón et al. [2002]
VDAC1	Voltage-dependent anion channel 1	PORIN, PORIN-31-HL	Co-ip, functional	Apop.	Shimizu et al. [2000]
SPNS1	SPINSTER homologue 1	Hspin1	Co-ip, co-local, functional	Autophagy	Yanagisawa et al. [2003]
PSEN1	Presenilin1	AD3, FAD, PS1	Y2h, functional	Apop.	Yamamoto et al. [1999]
FKBP8	FK506 binding protein 8, 38 kDa	FKBP38	Y2h, co-ip, co-local, func.	Anti-apop.	Tagami et al. [2000]
CAPN2	Calpain 2	CANP2, CANPL2, m-calpain	Cleavage assay	Apop.	Nakagawa and Yuan [2000]
AVEN	Tosis, caspase activation inhibitor	PDCD12	Y2h, co-ip, co-local	Anti-apop.	Chau et al. [2000]
BCAP31	B-cell receptor-associated protein 31	Bap31, p28	Co-ip	Apop.	Ng et al. [1997]
TP53	Tumor protein p53	p53, TRP53	Co-ip, functional	Apop.	Mihara et al. [2003]

(Continued)

TABLE I. (*Continued*)

Symbol	Name	Alternative names	Interaction identified	Function	References
IRS1	Insulin receptor substrate 1	HIRS-1	Co-ip	Anti-apop.	Ueno et al. [2000]
NLRP1	NLR family, pyrin domain containing 1	Card7, NALP1	Co-ip, fuctional	Apop.	Bruey et al. [2007]
TPT1	Tumor protein, translationally-controlled 1	TCTP, HRF	Co-ip, co-local, functional	Anti-apop.	Liu et al. [2005]
BFAR	Bifunctional apoptosis inhibitor	BAR, RNF47	Y2h, co-ip, functional	Anti-apop.	Zhang et al. [2000]
MCL1					
PCNA	Proliferating cell nuclear antigen	MGC8367	Y2h, co-ip, functional	Prolif.	Fujise et al. [2000]
DAD1	Defender against cell death 1	OST2	Y2h, co-ip, co-local	n.d.	Makishima et al. [2000]
TNKS	Tankyrase	PARP5A, PARPL, TINF1	Y2h, co-ip, functional	Apop.	Bae et al. [2003]
HUWE1	HECT, UBA and WWE domain containing 1	MULE, ARF-BP1	Co-ip, functional	Apop.	Zhong et al. [2005]
MAPK10	Mitogen-activated protein kinase 10	JNK3	Co-ip, functional	Apop.	Li et al. [2007]
USP9X	Ubiquitin specific peptidase 9, X-linked	DFFRX, FAF, FAM	Co-ip, functional	Anti-apop.	Schwickart et al. [2010]
IER3	Immediate early response 3	DIF2, GLY96, IEX1, PRG1	Y2h, co-ip	Apop.	Yoon et al. [2009]
TPT1	Tumor protein, translationally-controlled 1	TCTP, HRF	Co-ip, co-local, functional	Anti-apop.	Yang et al. [2005]
VDAC1	Voltage-dependent anion channel 1	PORIN, PORIN-31-HL	Co-ip (with cleaved MCL1)	Apop.	Weng et al. [2005]
PIN1	Peptidylprolyl cis/trans isomerase	DOD, UBL5	Co-ip, functional	Anti-apop.	Li et al. [2007]
CASP3	Caspase 3	CPP32, CPP32B	In vitro cleavage assay	Apop.	Weng et al. [2005]
CDK1	Cyclin-dependent kinase 1	Cdc2	Co-ip, kinase assay	Apop.	Harley et al. [2010]
BCLw					
PPP1CA	Protein phosphatase 1, catalytic subunit, alpha	PP-1A, PPP1A	Co-ip	Apop.	Ayllón et al. [2002]
BCLb					
NR4A1	Nuclear receptor subfamily 4, group A, member 1	Nur77, TR3	Co-ip, functional	Apop.	Luciano et al. [2007]
TPT1	Tumor protein, translationally-controlled 1	TCTP, HRF	Y2h, co-ip, co-local	Anti-apop.	Guillemin et al. [2009]
BFL1					
ING1	Inhibitor of growth family, member 1	p33; p47; p47ING1a	Y2h, co-ip	Apop.	Ha et al. [2002]
NR4A1	Nuclear receptor subfamily 4, group A, member 1	Nur77, TR3	Co-ip, functional	Apop.	Luciano et al. [2007]

Literature search of web databases including; STRING, Pubmed, Wikipedia, BioGrid, IntAct, and google scholar identified 55 different proteins as being capable of interacting with at least one of the BCL2-proteins. Columns: *Symbol*, Gene symbol for the encoded protein (symbols in bold represent that the interaction was only reported with a single BCL2-protein); *Name*, common protein name; *Alternative names*, other names that have been used for the protein; *Interaction identified*, technique used to identify the protein as a bona-fide interaction partner (*co-ip*, co-immunoprecipitation of the proteins from cell extracts or using purified proteins; *co-local*, co-localization experiments, either by immuno-flouresence, or biochemical fractionation of cell lysates; *y2h*, Yeast-two hybrid; *m2h*, Mammalian-2 hybrid; *functional*, experiments that demonstrate either the two proteins potentiate a phenotype when combined or that one of the two proteins is necessary for the observed phenotype of the other protein; *cleavage assay*, in vitro assay to demonstrate that incubation of the interacting protein causes cleavage of substrate); *Function*, how the interacting partner affects cellular function when introduced into the cell model of interest (n.d., function of protein was not determined); *References*, the publication in which the interaction was first reported. Symbols in the first column that are underlined are those interactions that have been refuted in the literature, reference for the refuted interaction is given immediately to the right of the symbol.

TABLE II. Classification of How the Interacting Proteins Affect, or are Affected by, the aaBCL2-Proteins

Class I	Mitochondrial interaction
SMN1	Localizes to mitochondria with BCL2 to block BAX
SOD1	SOD1 causes aggregates of BCL2/SOD
MDM4	Potentiates interaction of p53 phosphorylated on Ser46 with BCL2 on mitochondria
Rad9a	RAD9A interacts with BCL2/BCLxl in a BH3-dependent manner.
RTN4	Sequesters BCL2 to ER, blocking its activity at the mitochondria
OLFM1	Interacts with BCLxl and WASF1 to increase interaction with BAX on mitochondria
SPNS1	Binds BCL2 to block its anti-autophagic function
FKBP8	Promotes mitochondrial localization of BCL2/BCLxl
TP53	Binds and inhibits BCL2/BCLxl on mitochondria
BFAR	Scaffolds BCL2 to pro-CASP8
RTN1	Sequesters BCLxl to ER, blocking it activity at mitochondria
IKZF3	Un-phosphorylated Aiolos binds and sequesters BCLxl from mitochondria
Class II	Protein modification
MAPK8	Leads to phosphorylation of Ser70, Ser87, Thr69 on BCL2, leading to inactivation of BCL2
IRS1	Suppresses the phosphorylation of BCL2 by insulin signaling, and increases cell survival
IRS2	n.d. (likely same as IRS1)
PPP2CA	De-phoshorylates Ser70 of BCL2
MAPK1	Phoshporylates Ser87 of BCL2 leading to dissociation from mitochondria
Casp3	Cleaves BCL2 at Asp34
CDK1	Phosphorylates Ser70/Ser87 of BCL2 after microtubule destabilizing drug treatment
NR4A1	Interaction with BCL2/BCLb/BFL1 exposes BH3 domain, converting it to pro-apop.
CAPN2	Cleaves BCL2 at Gln73
PLK1	Phosphorylates BCL2 on 13 different ser/thr residues
TPT1	Leads to MCL1 protein stabilization by blocking Ubiquitination of MCL1
HUWE1	Ubiquitinates MCL1 leading to proteolytic degradation
MAPK10	Phosphorylates Ser121 of MCL1 to cause release of Pin1
USP9X	Stabilizes MCL1 by de-ubiquitination
Class III	Reverse regulation
MYC	BCL2 is recruited to nucleus, leads to increase in MYC half-life
CDK2	BCL2 associates with CDK2 to inhibit CDK2 kinase activity
Casp8	BCL2 sequesters Casp8 and keeps it in an inactive form
ITPR1	BCL2 inhibits the ability of ITPR1 to cause release of calcium from ER
TP53BP2	BCL2 blocks ability of 53BP2 to bind p53
PARP1	BCL2/S3a form complex with PARP1 to block PARP1 activity
TP53AIP1	TP53AIP leads to loss of MOMP, BCL2 blocks this function
NLRP1	BCL2 interaction blocks NLRP1 activation of CASP1
PPP1CA	BCL2/PP1/BAD form complex; BCL2 is the scaffold to bring PPP1CA to BAD
BCAP31	Binds BCL2 and pro-CASP8 on ER; after BCAP31 is cleaved to p20 it becomes apop.
APAF1	BCLxl blocks ability of APAF1 to interact and process CASP9
CASP9	BCLxl binds CASP9 and blocks it from interacting with APAF1
VDAC1	BCLxl BH4 domain leads to closing of VDAC1 channel in mitochondria
PCNA	Only an MCL1 protein capable of binding to PCNA blocks cell cycle
TNKS	MCL1 blocks ADP-ribosylation activity of TNKS
Class IV	Undefined/not determined
RRAS	n.d
RAF1	Phosphorylation of BCL2 not necessary for interaction
PRNP	n.d.
PXN	n.d
RPS3A	n.d.
PSEN1	BCL2 sequesters PSEN1; following apop. stimulus proteins dissociate
PIN1	Interaction occurs after BCL2 phosphorylated on Ser70 and Ser87
AVEN	Binds BCL2 to potentiate anti-apop., but mechanism unknown.
PGAM5	n.d.
PSEN2	Presence of PSEN2 sensitizes cells to apop.
MYO5A	n.d.
DAD1	n.d.
IER3	n.d.
ING1	n.d.

Each interacting protein has been classified into one of four broad categories based on the reported functional outcome and a brief summary of the interaction is provided.

Tamura et al., 2009; Harley et al., 2010; Schwickart et al., 2010]. Interaction of aaBCL2-proteins with proteins in this category leads to a wide variety of outcomes, including cleavage of the aaBCL2-proteins, alterations in the tertiary structure of aaBCL2-proteins, and alterations in the function aaBCL2-proteins by phosphorylation or ubiquitination. In all cases, interaction of an aaBCL2-protein with Class II proteins leads to dramatic alterations in aaBCL2-protein function.

With respect to protein cleavage, at least two proteins, CASP3 and CAPN2, have been identified that interact with aaBCL2-proteins and cleave them at residues near the N-terminus [Cheng et al., 1997; Gil-Parrado et al., 2002]. The cleavage promotes apoptosis by one of two mechanisms: (i) reduced levels of the affected aaBCL2-protein or (ii) by removal the N-terminal BH4 domain, thereby altering the conformation of cleaved aaBCL2-proteins, causing direct activation of BAX/BAK and MOMP [Cheng et al., 1997; Gil-Parrado et al., 2002].

Some of the aaBCL2-proteins can also be converted from anti-apoptotic to pro-apoptotic following interaction with the nuclear orphan receptor NR4A1 [Lin et al., 2004; Luciano et al., 2007]. The mechanism for how interaction of NR4A1 with aaBCL2-proteins leads to a phenotypic conversion of function is not completely understood, but likely involves alteration of the tertiary structure of the aaBCL2-protein leading to exposure of the internal BH3 domain and activation of BAX/BAK on mitochondrial membranes. This mechanism is even more intriguing given the fact that a nine amino acid peptide, corresponding to the interaction domain of NR4A1, was able to confer the phenotypic conversion of BCLb, leading to BCLb-dependent MOMP [Luciano et al., 2007]. The identification of additional aaBCL2-interacting proteins that possess this function would increase the potential pool of therapeutic targets capable of killing malignant cells with high levels of aaBCL2-proteins.

Interaction with kinases and phosphatases, which leads to changes in the phosphorylation status of proteins, is a common mechanism to alter protein function. A number of kinases have been identified as aaBCL2-interacting partners, including MAP kinases (MAPK8, MAPK1, MAPK10), CDKs (CDK1 and CDK2) and PLK1 [Yamamoto et al., 1999; Pathan et al., 2001; Tamura et al., 2004; Li et al., 2007; Schmitt et al., 2007; Tamura et al., 2009; Harley et al., 2010]. Many of these kinases phosphorylate overlapping Serine residues on BCL2, while some kinases seem to have specificity for individual residues. For example, MAPK8 (Ser70, Ser87, Thr69 of BCL2), MAPK1 (Ser87 of BCL2), and CDK1 (Ser70, Ser87 of BCL2) are all capable of phosphorylating BCL2 on Ser87, which reportedly causes dissociation of BCL2 from the mitochondria [Yamamoto et al., 1999; Tamura et al., 2004; Schmitt et al., 2007]. Meanwhile, PLK1 appears to phosphorylate BCLxl on as many as 13 different serine/threonine residues [Tamura et al., 2009]. An additional layer of specificity for the kinases is provided by the fact that each kinase can be activated by specific upstream signaling cascades. Also, phosphatases have been identified as aaBC2-interacting proteins and individual phosphatases can de-phosphorylate certain residues of the cognate interacting aaBCL2-protein. For example, PPP2CA has been identified as a BCL2-interacting proteins and PPP2CA was shown to de-phosphorylate BCL2 at Ser70 [Deng et al., 1998]. In theory, this would restore the ability of BCL2 to interact with

mitochondria thereby potentiating the anti-apoptotic functions of BCL2.

Finally, regulation of protein stability is an important mechanism for controlling protein function. The MCL1 protein has a short half-life, but dramatic increases in protein levels are often seen in human cancers. Recently, two proteins involved in ubiquitin-mediated proteolysis were described as MCL1 interacting proteins. The HECT-domain containing protein, HUWE1, is a ubiquitin ligase and interaction of HUWE1 with MCL1 results in the ubiquitination and subsequent proteolytic degradation of MCL1 [Zhong et al., 2005]. Conversely, USP9X was identified as an MCL1 interacting protein that possesses de-ubiquitinating activity [Schwickart et al., 2010]. As opposed to the interaction with HUWE1, the interaction of MCL1 with USP9X leads to dramatic stabilization of MCL1 protein resulting in increased cell survival. The expression of USP9X correlates with levels of MCL1 protein in some cancer patient samples, suggesting that USP9X and other similarly functioning proteins may be prognostic biomarkers for potential therapeutic response [Schwickart et al., 2010].

DO ALL ROADS LEAD TO THE MITOCHONDRIA?

Despite the number of publications describing the intricate details of how the aaBCL2-proteins regulate apoptosis, confusion exists about the capability of aaBCL2-proteins to perform BAX/BAK-independent functions. Of course, there are well documented examples of BCL2 regulating alternative forms of cell death, such as autophagy, but if/how aaBCL2-proteins can regulate the function of non-BH3 containing proteins is not clear [Levine et al., 2008]. In the literature there are examples of proteins that interact with aaBCL2 proteins and this interaction does not alter aaBCL2 function, but rather the aaBCL2 leads to an alteration of function of the interacting protein (Table II, Class III; reverse regulation) [Naumovski and Cleary, 1996; Ng et al., 1997; Hu et al., 1998; Fujise et al., 2000; Shimizu et al., 2000; Ayllón et al., 2001; Poulaki et al., 2001; Ayllón et al., 2002; Matsuda et al., 2002; Song et al., 2002; Bae et al., 2003; Chen et al., 2004; Jin et al., 2004; Weng et al., 2005; Bruey et al., 2007; Schmitt et al., 2007]. Interestingly, a number of these proteins are caspases, or are directly involved in the activation of caspase activity.

Unlike the Class I example above, where an active caspase cleaves an aaBCL2-protein, interaction of aaBCL2 with Class III proteins leads to inhibition of maturation of a pro-caspase or steric inhibition of caspase activation. For example, BCL2 interacts with pro-CASP8 keeping it in an inactive form and BCLxl interacts with APAF1 and CASP9 blocking the ability of these proteins to form an active apoptosome [Hu et al., 1998; Poulaki et al., 2001].

Another example, demonstrating a role for aaBCL2-proteins in inhibiting the activation of a caspase, has been observed for the regulation of the inflammasome by BCL2 and BCLxl [Bruey et al., 2007]. The inflammasome is a CASP1 containing complex that responds to pathogens resulting in the activation of CASP1 and production of IL1b. Interaction of BCL2 or BCLxl with NLRP1 blocks the ability of this complex to activate CASP1, which in turn decreases the production of IL1b, leading to an inferior inflammatory response. This is a curious example of how an aaBCL2 protein can play a role in regulating the immune response. One might speculate that a cell that has received prior damage would be a cell that is both more capable of dying (a decrease in the interaction of aaBCL2 with BAX/BAK) and less capable of responding to a pathogenic stimulus (increase interaction between aaBCL2 and NLRP1).

Interaction of aaBCL2-members with some proteins can also lead to potentiation of the function of the interacting protein. The proto-oncogene MYC has been identified as a BCL2-interacting partner and it appears as though this interaction leads to a dramatic stabilization of the steady-state levels of the MYC protein [Jin et al., 2004]. Increase in total MYC levels is a major mechanism by which the MYC protein is thought to lead to cellular transformation. An unexpected observation that occurs following this interaction is the presence of BCL2 in the nucleus. Although this is the compartment that MYC is thought to perform its main function, a role for BCL2 in the nucleus has not been well established. It may be that BCL2 is simply entering the nucleus as a MYC-client protein, but this finding requires further examination.

Interaction of aaBCL2-proteins with membrane-associated proteins has also been observed and the functional outcome is dependent on the particular interacting protein. For example, interaction of either BCLxl or MCL1 with VDAC1 leads to a closing of the channel and attenuation in the subsequent release of cytochrome c [Shimizu et al., 2000; Weng et al., 2005]. Similarly, interaction of BCL2 with ITPR1 leads to a decrease in the amount of calcium released from the ER in an ITPR1-dependent manner [Chen et al., 2004]. Both of these interaction would lead to an increase in the general health and survival of the cell, and therefore these could be mechanisms employed by the aaBCL2-proteins to facilitate their own functional interests.

At least two independent examples have been reported that suggest aaBCL2-proteins can regulate enzymatic activity of poly(ADP-ribose) polymerases. BCL2 has been found to inhibit PARP1 function and MCL1 was described as an inhibitor of TNKS activity [Song et al., 2002; Bae et al., 2003]. Interaction of BCL2 with PARP1 was not enough for activity inhibition, but rather the presence of RPS3A was somehow required to block the ability of PARP1 to perform its poly(ADP-ribose) polymerase functions [Song et al., 2002]. This finding is interesting because it is thought that PARP1 activity has a role in both the early and late stages of apoptosis and blocking, therefore it might be inferred that the ability of BCL2 to block PARP1 activity would be an additional mechanism by which BCL2 could delay execution of cell death.

aaBCL2-proteins can also behave as scaffolds to either bring together two additional interacting proteins. We have already seen how phosphatases can target critical phosphorylation events on aaBCL2-proteins, but additionally aaBCL2-proteins can interact with PPP1CA and act as a scaffold for interaction with the BH3-only protein, BAD [Ayllón et al., 2001, 2002]. Following the formation of this trimeric complex, PPP1CA de-phosphorylates BAD altering the ability of BAD to antagonize the aaBCL2-family members. This finding is a bit counter-intuitive because unphosphorylated BAD is thought to be the functional, pro-apoptotic molecule. So why would an aaBCL2 protein want to act as a scaffold between PPP1CA and

BAD, presumable leading to activation of BAD and inactivation of the aaBCL2-proteins?

THE ROAD LESS TRAVELED

The interest in identifying any protein that can modify the apoptotic cascade is especially evident in the literature by the number of proteins shown to interact with aaBCL2-proteins, but the functional outcome of the interaction is either not described or is not clear (Table II, Class IV; not determined/not defined) [Fernandez-Sarabia and Bischoff, 1993; Wang et al., 1994; Kurschner and Morgan, 1995; Alberici et al., 1999; Passer et al., 1999; Sorenson and Sheibani, 1999; Chau et al., 2000; Hu et al., 2000; Makishima et al., 2000; Pathan et al., 2001; Ha et al., 2002; Lo and Hannink, 2006; Du et al., 2007; Li et al., 2007; Yoon et al., 2009]. In many of these examples there is a demonstration of interaction, sometimes by multiple biochemical techniques, and a description of a phenotype that changes when the two proteins are expressed together, but no mechanistic insights into the interaction. This list includes some very interesting proteins that have been suggested to have roles in tumorigenesis; RRAS and RAF1 [Fernandez-Sarabia and Bischoff, 1993; Wang et al., 1994] and proteins involved in extracellular matrix/cytoskeletal signaling, PXN and MYO5A [Du et al., 2007; Sorenson and Sheibani, 1999]. Some of these initial studies have been followed-up and more details are available for the outcome of the described interactions, whereas some of the Class IV interacting proteins have not been further studied in the context of the aaBCL2-family. In many of these cases, we are left to speculate the possibilities of the interactions.

ARE THESE ALL "NON-BH3" INTERACTIONS?

In this review I have compiled a list of 55 "non-canonical" interaction partners for the aaBCL2-family (Table I). As part of the criteria for compiling the list I chose to omit proteins that are considered to be part of the BH3-only, or multi-BH3 containing families of proteins that have been previously characterized as having roles in regulating aaBCL2-proteins. Included in the list provided were two proteins, RAD9A and HUWE1, previously shown to contain a sequence that closely resembles that of the canonical BH3 motif. There are many variations of the BH3 motif but at its most basic consensus it is an amino acid sequence approximately ten residues in length, [LIVAT]-X-X-X-L-X-X-X-[GSAC]-D. X can be any amino acid, [LIVAT] is almost always a hydrophobic residue, usually Leucine, Isoleucine, Valine, Alanine, but can sometimes be Threonine; [GSAC] is either Glycine, Serine, Alanin, or Cysteine, and residues 5 and 10 are strictly conserved Leucine and Aspartic acid, respectively.

The relative simplicity of the BH3 motif and the fact that at least two of the proteins on our list were known to encode BH3 domains prompted me to examine the amino acid sequences of all 55 proteins on our list to determine if any of the other proteins contain putative BH3-motifs [Combet et al., 2000]. To our surprise I identified 15 proteins that encode at least one BH3 consensus sequence, in addition to the two previously described protein (Fig. 1). Interestingly, many of the proteins encode more than one such sequence. For instance, TNKS has five different sequences that fit the consensus BH3 motif. Of note, the five putative BH3 domains encoded by TNKS were within the ankyrin repeats, however when similar analysis was done with two other ankyrin-domain containing proteins, NOTCH1 and ANK1, no such BH3 domains were identified within the ankyrin motifs. Obviously, this is a simple

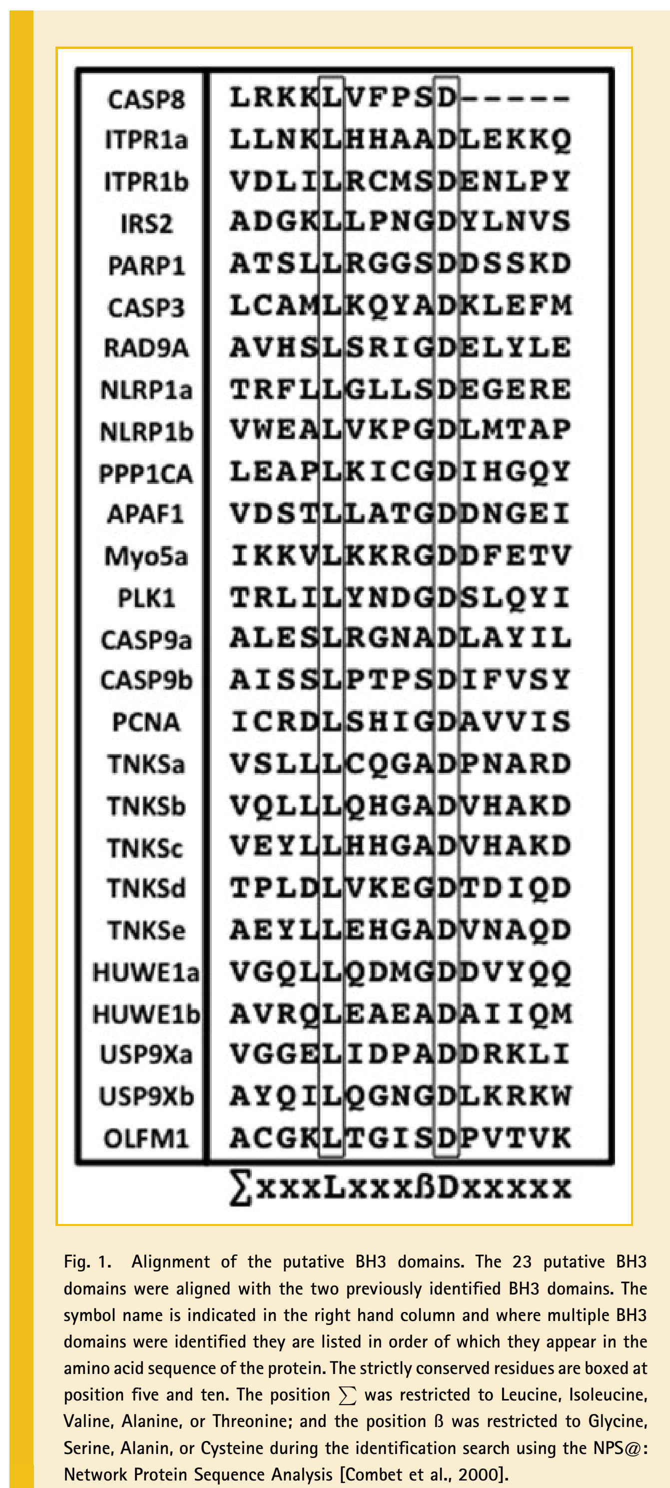

Fig. 1. Alignment of the putative BH3 domains. The 23 putative BH3 domains were aligned with the two previously identified BH3 domains. The symbol name is indicated in the right hand column and where multiple BH3 domains were identified they are listed in order of which they appear in the amino acid sequence of the protein. The strictly conserved residues are boxed at position five and ten. The position ∑ was restricted to Leucine, Isoleucine, Valine, Alanine, or Threonine; and the position ß was restricted to Glycine, Serine, Alanin, or Cysteine during the identification search using the NPS@: Network Protein Sequence Analysis [Combet et al., 2000].

bioinformatics based approach to identify sequence motif and experiments would have to be performed to prove, or disprove, that any of these motifs actually play a role in the interaction with the cognate aaBCL2 protein(s).

One major caveat to our bioinformatics approach to identifying a simple amino acid motif is the potential that the sequence is represented randomly at a high enough frequency to account for its presence in the proteins in our list. From our calculations (taking into consideration codon frequency of each amino acid and codon frequency within the genome) it appears that the defined consensus would be expected to be identified once every approx. 2800 amino acids. The 55 proteins on our list are comprised of nearly 38,000 total amino acids. Therefore, at random we would expect to find the BH3 motif approx. 13 times, however I actually identify the motif a total of 26 times. Again, this is a tantalizing suggestion that merits further work on these proteins.

CONCLUSIONS

It has been clear for many years that the processes that regulate apoptosis are altered in many diseases, including cancer. In fact, it is thought that the ability to undergo a normal apoptotic response is always lost during the transformation of a normal cell to a fully malignant cancer. Therefore, intense research has been focused on ways to restore the ability of cancer cells to respond to apoptotic stimuli. To this end, there are currently many clinical trials with multiple therapeutics designed to overcome the blockade in apoptosis. Most of these therapeutics inhibit the function of the aaBCL2-proteins, resulting in cell death of tumor cells. The inherent flaw in these types of approaches is that the therapeutic only inhibits one, or a few, of the aaBCL2-proteins. For example, a small molecule, ABT-737 (Abbott), which inhibits BCL2, BCLxl, and BCLw, has shown promise as a single agent therapeutic in many cancer types [Oltersdorf et al., 2005; Konopleva et al., 2006; Reed, 2006; Kohl et al., 2007; Shoemaker et al., 2008]. However, the downfall of this treatment is the possibility of either primary or acquired resistance caused by over-expression of MCL1 (which is not inhibited by the drug) [Konopleva et al., 2006]. One potential response to this scenario is to develop a drug that has a broader specificity and would also block the function of MCL1, which another company (GeminX) has claimed to do with a compound called Obatoclax [Nguyen et al., 2007]. Whether or not this drug will also be effective, or whether resistance will arise due to increased expression of BFL1 or BCLb remains to be determined. Instead, I propose that rather than designing multiple molecules that inhibit different combinations of aaBCL2-proteins, we understand completely the upstream signals that are capable regulating each of the aaBCL2-proteins. If we better understand all of the upstream signals that are unique for each aaBCL2-protein and all of the upstream signals that are shared by each of the aaBCL2-proteins we will have more targets to which we can design our therapeutics. In turn, these therapeutics could be used in combination with those molecules that already directly target aaBCL2-proteins or in combination with additional cytotoxic drugs already used in patients. However, it is obvious that even with this knowledge in hand, it will be a challenge to design potent inhibitors of any non-canonical regulators of aaBCL2-protein function.

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As far as I can tell this list of interacting proteins presented in this article represents a complete list of such proteins. Undoubtedly I have omitted some proteins that fit the criteria set forth within this article, if so this has not been intentional and I am sincerely apologetic to those investigators who performed the work. I also realize the speed at which this field is moving and acknowledge that this list will undoubtedly need to be updated in the not-so-distant future. Regardless, I hope this article will be particularly interesting to those focused on the regulation of aaBCL2 protein and that this article leads to some discussions and perhaps even re-visitation of the potential biochemical mechanisms by which the proteins on the list are capable of functioning. If nothing else, this article should serve as a resource for anyone interested in non-canonical regulation of the aaBCL2 signaling pathway.

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