**IAPs**

The only known endogenous caspase inhibitors are members of a second family of proteins identified by apoptosis research, the IAP (inhibitor of apoptosis proteins) family. IAPs were originally described in insect viruses as viral proteins produced during cellular infection to block host cell apoptosis.

In addition to other cellular functions, IAPs bind to specific caspases (3,6,7,9) to block catalytic activity or maturation of procaspases. IAP proteins contain one to three BIR (baculovirus IAP repeat) domains that coordinate zinc, and one or more additional protein-interaction domains.

Structure of BIR2 Zn finger domain from XIAP
Individual IAP proteins use different domains to occlude the substrate grooves of specific caspases. The N-terminal sequence of the p10 small subunit of certain processed caspases fits a binding pocket on the BIR domain to strengthen the interaction. Similar proteins have been identified in yeast, flies and worms.

Model of BIR domain interacting with caspase-3 active site

IAPs also function as ubiquitin E3 ligases, labeling proteins for degradation by the 26S proteasome. The cIAP-1 and cIAP-2 proteins bind to Traf-1, an adaptor in TNF receptor signaling to NF-kB and Jun-N kinase pathways, cell survival responses. An apoptotic response is reinforced by cIAP-mediated Traf-1 degradation, triggered by TNF binding to the TNF-RII receptor.

**SMAC/Diablo and Omi/HtrA2**

Two proteins normally localized in the mitochondrial intermembrane space, SMAC/Diablo and Omi/HtrA2, can bind to the BIR domain of IAPs via an N-terminal sequence related to the N-terminal caspase small subunit and competitively displace bound caspases.

Structure of SMAC (Blue/Green) binding to pocket of BIR domain
The N-terminus of active SMAC/Diablo is generated by removal of a presequence during mitochondrial import, while Omi/HtrA2 is a stress-activated serine protease that is cleaved by autoprocessing. Cytoplasmic translocation of SMAC/Diablo and Omi/HtrA2 during apoptosis provides an additional mechanism for caspase activation. The reaper, grim, hid and sickle proteins in Drosophila function similarly with fly IAPs and have N-terminal homology to SMAC/Diablo and HtrA2.

IAPs can direct self-ubiquitination or ubiquitin ligation to caspases and reaper family proteins, suggesting that IAP complexes can flag a variety of proteins for destruction. This mechanism appears well-designed for detecting differences in protein conformation and aggregation state. While all known IAPs inhibit apoptosis, only a subset is known to bind caspases. It is likely that regulation of protein stability has multiple inputs to apoptotic pathways, as found for cell cycle control.

REGULATORS OF APOPTOSIS

The founding member of this family, Bcl-2, was discovered as the defining oncogene in follicular lymphomas, located at one reciprocal breakpoint of the t(14;18) (q32;q21) chromosomal translocation. Although lacking in transforming activity, cells transduced with Bcl-2 remained viable for extended periods in the absence of growth factors and, in combination with the c-myc oncogene, turned murine B-cell precursors tumorigenic. The first pro-apoptotic BH (Bcl-2 homology) protein to be identified, Bax, was co-immunoprecipitated in stoichiometric amounts with Bcl-2. Bax-transfected cells died faster in the absence of growth factor than control cells and, later, expression of Bax was shown to be capable of directly triggering apoptosis.
Activation of caspases is controlled by Bcl-2 proteins

Since the discovery of Bcl-2 and Bax, the BH family in mammalian cells has expanded by 17 members, with five acting principally as survival factors and twelve hastening cell death in various experimental systems. Homologs of BH proteins exist in all metazoans and several animal DNA viruses.

**Bcl-2-related survival proteins**

Early experiments showed that the relative steady state levels of anti-apoptotic BH members and death-promoting BH members correlated with the cellular sensitivity to a death stimulus, such as withdrawal of growth factors. Moreover, the relative amounts of pro- and anti-apoptotic members were manifested by differential associations between these factors. Both anti- and pro-apoptotic BH proteins form homo-dimers and some can associate as hetero-dimers.
Mutational studies have tended to support the functional importance of associations between pro- and anti-apoptotic family members. The BH family is recognized by conserved homology domains BH1-4. The BH1, 2 and 3 domains from anti-apoptotic proteins form a hydrophobic groove that binds to the hydrophobic face of an alpha helical BH3 domain from a pro-apoptotic binding partner. Certain mutations in the anti-apoptotic BH members such as the BH1 domain Gly145Ala in Bcl-2 and Gly138Ala in Bcl-xL, disrupt associations with pro-apoptotic family members and result in strong loss of survival function. The critical interactions may be between anti-apoptotic members and BH3-only proteins, not the multi-domain pro-apoptotic proteins Bax, Bak and Bok, since it has been suggested the latter interactions are induced by detergents during preparation of cell lysates, and thus artifactual (Hsu and Youle, J Biol Chem 272:13829-34, 1997). This is not a settled issue, however, since the lipid environment of these membrane-inserted proteins may promote similar interactions.

These studies support an agonist-antagonist relationship between Bcl-2 anti-apoptotic and pro-apoptotic family members. Cells deficient in two multidomain pro-apoptotic family members (Bax/-/Bak/- mouse embryonic fibroblasts) are completely resistant to multiple apoptotic stimuli (Wei MC et al, Science 292: 727-30, 2001), suggesting that they function downstream of the anti-apoptotic factors.

Expression of Bcl-2-related proteins in heterologous systems such as yeast has been undertaken to probe the intrinsic functions of these proteins in a model devoid of endogenous Bcl-2 homologues. Bcl-2 inhibits death triggered by Bax or Bak expression, as well as those associated with prolonged stationary phase, oxidative and NaCl stress and superoxide dismutase-deficient strains. While the relevance of these models to mammalian apoptosis is not proven, they suggest that Bcl-2 has intrinsic functions independent of its mammalian pro-apoptotic binding partners.
The Bcl-2-related survival proteins are found mostly as integral membrane proteins associated with the outer mitochondrial membrane, with lesser amounts in endoplasmic reticulum and nuclear envelope membranes. The COOH-terminal hydrophobic domain of Bcl-2 functions as a mitochondrial targeting sequence.

During apoptosis, increased permeability of the outer mitochondrial membrane has been demonstrated by the release of multiple proteins normally retained within the intermembrane space and by accessibility of the inner membrane electron transport complexes to exogenous cytochrome c. One approach to identifying anti-apoptotic functions of Bcl-2 has been to examine its effect on the mitochondrial events of apoptosis.

The permeability transition pore (PTP) is a calcium-activated high conductance channel in the inner mitochondrial membrane implicated in mitochondrial membrane depolarization and osmotic swelling of mitochondria. Loss of mitochondrial membrane potential ($\Delta \Psi_M$) and mitochondrial swelling have been observed in some (but not all) examples of apoptosis. Osmotic swelling of the mitochondrial matrix space causes secondary rupture of the less-expandable outer membrane and release of cytochrome c and other proteins in the intermembrane space.
A direct action of Bcl-2 and Bcl-xL on the PTP has been proposed. Bcl-xL associates with the outer membrane voltage-dependent anion channel (VDAC), a core component of the PTP. In particular, a peptide comprising the NH2-proximal BH4 domain of Bcl-2 or Bcl-xL inhibits non-specific membrane permeability associated with VDAC. Electrophysiological studies confirmed suppression of PTP activity in mitochondria with high levels of Bcl-2. However, against this mechanism of cytochrome c release are the results of cyclophilin D knockouts demonstrating preservation of apoptotic responses including cytochrome c release despite loss of Ca\(^{2+}\)-induced PTP swelling (Nakagama T et al., Nature 434: 652-8, 2005; Baines CP et al., Nature 434: 658-62, 2005).

An alternative hypothesis for mitochondrial functions of Bcl-2 involves maintenance of outer membrane conductivity (Vander Heiden MG et al., J Biol Chem 276: 19414-9, 2001). The exchange of mitochondrial ATP and cytoplasmic ADP is substantially impaired following growth factor-deprivation. Bcl-xL enhances mitochondrial ATP/ADP exchange in growth factor-deprived cells, as does disruption of the outer mitochondrial membrane. Bcl-xL inhibits voltage dependent closure of VDAC channels in artificial membranes. In this model, cytochrome c release would result indirectly from osmotic swelling secondary to the membrane hyperpolarization due to a shift from State III (+ADP) to State IV (-ADP) respiration.

**Bax-like killer proteins**

Faster progress has been made in understanding pro-apoptotic functions of Bax-like proteins (Reed JC. Cell Death Diff 13: 1378-86, 2006). Important developments along the way have included the recognition of an intrinsic pore-forming activity, initially suggested by the x-ray crystallographic and NMR-determined Bcl-xL structure (Muchmore et al., Nature 23: 335-41, 1996).
The tertiary structure of Bcl-xL is similar to known structures of the diphtheria toxin T domain and bacterial colicins, model pore-forming proteins. Similar pore-forming activities have been identified with the anti-apoptotic proteins Bcl-xL, and Bcl-2, and pro-apoptotic Bax, Bcl-xS and Bid (Minn et al. 1997; Schendel et al., 1997; Schlesinger et al., 1997; Schendel et al., 1999). Once an apoptotic stimulus is delivered, cytosolic Bax protein is delivered to its mitochondrial site of action (Wolter et al., 1997; Hsu et al., 1997).

Pores formed by homo-oligomers of Bax are primary candidates for the mechanism of cytochrome c escape from mitochondria. Recombinant Bax forms high conductance channels in artificial membranes with predominant conductances of 0.5 nS - 1.5 nS. Pore sizing using dextran molecules of different Stokes diameters indicate Bax pore diameters up to 22-30 Å, large enough for cytochrome c passage. Titration studies suggest that four Bax molecules were required for cytochrome c transport. Bax
complexes ranging from 41-260 kDa, consistent with homodimers and larger oligomers, are observed by crosslinking during apoptosis. Recently, patch-clamping studies identified a novel, high conductance channel in the mitochondrial outer membrane (mitochondrial apoptosis-induced channel or MAC) coincident with mitochondrial translocation of Bax. MAC activity was suppressed by Bcl-2. Bax has also been reported to decrease the stability of planar lipid bilayers through a decrease in linear tension, resulting in hydrophilic pores within the lipid membrane itself. This effect was not seen with Bcl-xL.

The BH3 homology region has special significance for pro-apoptotic functions. Initially identified in Bak as a domain required both for cytotoxicity and heterodimerization with Bcl-xL, synthetic BH3 peptides induce apoptosis and mitochondrial cytochrome c release. BH3 peptides bind to a hydrophobic groove that is identified in 3-D structures of both anti- and pro-apoptotic multidomain family members. This appears to have direct consequences for the function of these proteins. In the case of pro-apoptotic Bax and Bak, BH3 peptides induce a conformation change (exposing an N-terminal epitope and changing protease sensitivity), and promote a shift from monomers to dimers and higher order oligomers (Desagher et al., J Cell Bio 144: 891-901, 1999; Eskes et al., Mol Cell Biol 20: 929-35, 2000). These changes precede a change in membrane association from a loosely attached to fully inserted state. Bax also exists in a soluble cytosolic conformation, and BH3 peptides may trigger translocation to mitochondrial and other intracellular membranes by displacing the C-terminal membrane anchoring domain, which is folded back into the hydrophobic groove (Suzuki M et al., Cell 103: 645-54, 2000).
In the case of anti-apoptotic proteins (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1), they are usually regarded as passively sequestering BH3-only proteins, thus preventing BH3-only protein-dependent activation of Bax and Bak. There is evidence, however, that BH3-only proteins have similar effects on the subcellular localization, conformation and activity of anti-apoptotic proteins (e.g. Jeong et al., EMBO 23: 2146-55, 2004). An x-ray crystallographic structure of Bcl-xL homodimers was recently published, with an unexpected 3D domain swapping mechanism of dimerization (O'Neill et al., J Mol Biol. 356: 367-81, 2006).

For each monomer subunit, the anti-parallel arrangement of the central alpha-5 and 6 helices is re-oriented, with the turn as a hinge point for establishment of a continuous helical segment spanning alpha-5 and 6. Protein segments on either side of the hinge
region participate in separate “monomeric” folds, creating an extensive intermolecular interface. Binding of BH3 peptides to the hydrophobic groove alters the kinetics of monomer-dimer conversion (Denisov AY et al., Biochemistry 46:734-70, 2007), suggesting a mechanism for the observed effects of peptide binding on function of this class of proteins.

BH3 domains are present in anti- and pro-apoptotic multi-domain proteins (e.g. Bcl-2, Bcl-xL, Bax, Bak), in addition to the BH3-only family members. They are probably not functionally equivalent, since the amphipathic BH3 helical domain in the 3-D structures of the multi-domain proteins is oriented such that the hydrophobic face faces into the interior of the protein, and thus unable to bind to a second protein without a fairly drastic change in protein folding. BH3-only proteins appear to be intrinsically unstructured (i.e. floppy), with folding of the BH3 helix coupled to interacting with the hydrophobic groove of a binding partner (Hinds MG et al. Cell Death Differ 14: 128-36, 2006).

A recent development in this field is the discovery of small molecules that act as BH3 peptidomimetics and bind to the hydrophobic groove on Bcl-xL and Bcl-2 (Oltersdorf T et al., Nature 435: 677-81, 2005). The types of assays used for screening collections of chemical compounds for inhibitory activity include competitive binding assays with pro-apoptotic BH3 peptides, computational docking simulations, and cell-based assays for selective cytotoxicity based on Bcl-xL expression. These compounds appear to act by shifting BH3-only proteins from interactions with anti-apoptotic to pro-apoptotic family members, leading to activation of Bax and Bak. There is still some controversy around this idea (e.g. Willis SN et al., Science 315: 856-9, 2007).