family proteins, the cytotoxic effects of these BH3 peptides can be reduced in certain cancer cells. We recently found that the amphipathic tail-anchoring peptide (ATAP) from Bfl-1, a bifunctional Bcl-2 family member, displayed strong pro-apoptotic activity by permeabilizing the mitochondrial outer membrane (MOM). Here we tested if the activity of ATAP requires other cellular factors and whether ATAP has an advantage over the BH3 peptides in targeting cancer cells. We reconstituted the membrane permeabilizing activity of ATAP in liposomes and found that ATAP rapidly released fluorescent molecules of the size of cytochrome *c*, suggesting that ATAP membrane permeabilizing activity is independent of other protein factors. Confocal microscopic imaging revealed specific targeting of ATAP to MOM, whereas BH3-peptides showed diffuse cytosolic distribution. While the pro-apoptotic activity of BH3 peptides was largely inhibited by either overexpression of Bcl-2 or Bcl-x_L or nullification of Bax and Bak in cells, the apoptotic function of ATAP was not affected by these cellular factors. Since ATAP can target to mitochondria membrane and its potent apoptotic activity does not dependent on the content of Bcl-2 family proteins, it represents a promising lead for a new class of anti-cancer drugs that can potentially overcome the intrinsic apoptosis-resistant nature of cancer cells.

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Biophysical Characterization of Peptide Membrane Interactions and Membrane Permeabilization

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Certain membrane-active amphipathic peptides, such as antimicrobial peptides, possess a characteristic feature of having several cationic amino acid residues at either N- or C-terminus. In an attempt to determine the functional significance of C-terminal cationic residues, we have studied the membrane binding mode and membrane pore formation by a 20-amino acid synthetic amphipathic peptide. Two cationic residues were replaced to Leu or Glu to see how the membrane binding and permeabilization activities of the peptide are modulated by charge neutralization and charge reversal mutations. The depth of membrane insertion of the wild type and mutant peptides was determined by measuring the quenching of the fluorescence of a single, C-terminally located tryptophan by dibromo-phosphatidylcholines brominated at various positions of the acyl chains, using pure POPC and 70% POPC + 30% POPG in lipid vesicles. In case of pure POPC membranes, Trp in all three peptides was located at around 9 Å from the membrane center, while for 30% POPG, Trp was inserted much deeper. Studies on peptide-mediated calcein release from phospholipid vesicles were conducted to see if the peptides are able to cause membrane pore formation and content release. Peptides with positively charged residues showed maximum calcein release for both lipid compositions whereas negative or hydrophobic residues were less effective in vesicle permeabilization. Preliminary ATR-FTIR experiments using both POPC and POPG have been performed to identify the orientation of the peptides within the lipid bilayer and their effect of the lipid order.

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Regulation of Apoptosis at the Mitochondrial Level by Bcl-2 Proteins

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During the life-time of higher organisms the turnover of the cell mass has to be highly regulated by apoptosis, one of the main types of programmed cell death. The anti-apoptotic integral membrane protein Bcl-2 belongs to the Bcl-2 protein family, which functions as a major gatekeeper in the mitochondrial apoptotic pathway. This protein has been found to be highly over-expressed in breast cancer tumors and also been shown to be involved in the inherent resistance to anti-cancer drugs. Our aim is to work with the full-length Bcl-2 protein and study its interplay with both, its mitochondrial membrane environment and the pro-apoptotic Bcl-2 family protein Bax. Bax is the counter-player of Bcl-2 and is upon activation translocated from the cytosol to the mitochondrial outer membrane where it forms oligomers. These oligomers function as pores which enable the release of cytochrome c to the cytosol, followed by initiation of the caspase activation cascade and subsequently cell death.So far we have been working on the expression and purification of Bax and Bcl-2, and managed to isolate full-length Bax using E. coli as expression system. For Bcl-2 we have performed cell free expression, and have succeeded in the purification of the full-length protein, solubilized with detergent. The next step is to characterize the proteins when they are reconstituted into model membrane, using spectroscopic methods such as CD and 31P solid state NMR spectroscopy. We are especially interested in how the presence of oxidized lipids influences the protein-protein and protein-membrane interactions, mimicking the apoptotic conditions triggered by reactive oxygen species.

Our ultimate goal is to provide structural information of the membrane-mediated mechanism underlying the action of Bcl-2 as a potent inhibitor of cell death, information crucial for the development of new anti-cancer drugs.

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Interactions Between Bcl-2, Bcl-xl, and Bax at the Mitochondria: Keep Your Friends Close but Your Enemies Closer

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Cytochrome c release, the commitment step of apoptosis, is regulated at the mitochondria through protein-protein interactions between the Bcl-2 family proteins. An imbalance of this interaction network due to the up-regulation of the proto-oncogenes *Bcl-2* and/or *Bcl-xL* lead to a resistance to apoptosis and is associated with tumor formation. Bcl-2 or Bcl-xL overexpression act at the level of the mitochondrial outer membrane (MOM) by inhibiting Bax-mediated apoptosis; more particularly MAC formation and cytochrome c release. However, the molecular mechanisms through which Bcl-2 or Bcl-xL affect earlier steps of Bax-mediated apoptosis are not fully understood. We found that Bax insertion into the MOM was the earliest apoptotic step inhibited by Bcl-2 overexpression. However, Bax insertion was not modified if Bcl-xL was overexpressed instead of Bcl-2. This suggested Bcl-2 and Bcl-xL have different functional roles in inhibiting MAC formation. Surprisingly, we also found that mitochondrial Bax redistribution and change of conformation were not inhibited but rather spontaneously increased in response of Bcl-2/xL overexpression. This increase in mitochondrial associated Bax required a physical interaction between Bax and Bcl2 or Bcl-xL. We therefore propose that, at least when up-regulated, Bcl-2 and Bcl-xL behave as Bax receptors which stabilize Bax binding to the MOM. This sequestration of Bax by Bcl-2/xL would then consequently increase Bax mitochondrial relocalization, but further inhibit Bax-mediated MOM permeabilization and later steps of apoptosis.

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Demise of the Umbrella Model in BAK-Mediated Mitochondrial Membrane Permeabilization During Apoptosis

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BCL-2 proteins, including BAX and BAK, play critical roles in apoptosis. Upon activation by cell death signals, BAX or BAK forms large oligomeric pores in the mitochondrial outer membrane, permeabilizing mitochondria. This allows the escape of the cell death factors such as cytochrome *c* from the intermembrane space into the cytoplasm, where they execute downstream cell death events. Using a proteo-liposomal system that recapitulates the pore-forming processes by BAX or BAK (Oh et al. *JBC* 2010; 285, 28924-28937), we previously demonstrated that spin labels attached in the BH3 (Bcl-2 homology domain 3) domain were juxtaposed within 5-10 Å distance in the oligomeric form in the membrane, providing direct evidence for the existence of the BH3:BH3 contact interface between nearby monomers. We have further determined the conformational changes in BAK upon membrane insertion by applying the site-directed spin labeling method of EPR. Pairs of spin labels introduced at various positions in BAK allowed us to measure the inter-domain distances before and after the pore-formation. The results show that BAK unfolds and inserts into the membrane in an unexpected way, contrary to the predictions by the ''umbrella model'' in which the sandwiching outer layers of the BAK protein open up to expose the core α5-α6 helical hairpin for membrane insertion. Our results also indicate that residue 83 in the BH3 domain is in close proximity to the central region of α5 helix (*i.e.*, residue 135) but is removed from its amino-terminus (*i.e.*, residue 122) within a monomer in the membrane-inserted oligomeric pore. These results identify α5 helix as the previously unknown domain in contact with the BH3 domain in the membrane bound state of BAK (*ibid*), providing further insights into the mechanism of BAK (or BAX) dimerization and oligomerization.

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HAX-1: A Multifaceted Family of Apoptotic Regulators

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HAX-1 was first identified as a binding partner of HS1, a protein involved in the maturation of T cells. To date, studies on HAX-1 have mainly focused on the prototypical variant I protein, demonstrating that it has anti-apoptotic properties. Recent evidence, however, has indicated that the HAX-1 gene is heavily spliced, producing a number of isoforms with distinct molecular compositions and presumably varied functional activities. In light of this observation, we sought to characterize the different HAX-1 isoforms expressed in rat striated muscles.

Using 2D gel electrophoresis and RT-PCR analysis, we determined the existence of at least seven distinct HAX-1 proteins, and further identified five novel mRNA variants, in addition to the seven known ones, in developing and adult rat myocardium. Our analysis of the various isoforms revealed profound differences in their subcellular localization and anti-apoptotic activities. Certain