mechanisms through which upregulation of Bcl2 affects 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 Bcl2 overexpression. Paradoxically, we also found that BAX translocation to the mitochondria was not inhibited but rather spontaneously increased in this same genetic context. This increase in mitochondrial associated BAX required a physical interaction between BAX and Bcl2. We therefore propose that, at least when upregulated, Bcl2 behaves as a 'decoy receptor' which sequestrates BAX at the mitochondria but inhibits its insertion into the MOM, committing the cell to survive.
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1961-Pos
Compartmentalization of BCL2 Family Proteins Mediated by Organelle Lipid Membranes
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Cancer is defined by a pronounced inhibition of cell death. The BCL2 family of proteins tightly regulates the delicate balance between life and death. One method of regulation is the compartmentalization of antagonistic members. For example, Bax, a pro-apoptotic member of this family, acts as the penultimate factor in the apoptotic cascade by releasing apoptogenic factors such as Cytochrome C from the mitochondrial lumen. The normally cytosolic protein translocates from one internal compartment to another through an elusive mechanism. Individual organelles are defined not only by function (mediated by specific membrane bound proteins), but by the unique composition of their phospholipid membranes. In this work, we have evaluated the contribution of organelle lipids to the localization of of BCL2 proteins.

1962-Pos
In Search of the Structure of MAC in the Mitochondrial Outer Membrane
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Several groups have tried to determine the structure of the channel (MAC) formed in mitochondrial outer membranes (OM) of apoptotic cells or in synthetic membranes by Bax and related proteins/peptides, using electron microscopy (EM), atomic force microscopy and x-ray diffraction. Here, pore-like structures ~3-10 nm were handpicked from transmission EM images of uranyl-acetate-stained OMs isolated from control and apoptotic (IL3-deprived) FL5.12 cells. These ''candidate pores'' were aligned by correlation procedures, and class averages defined by principal component and K-means analyses. Main differences in the class averages were (1) the presence of one or more dark (stain-filled) pores, and (2) the nature of white (stain excluding) features around the pores. A class average consisting of a single 3-nm pore with pronounced white rim was rotationally averaged and used as a reference for cross-correlation searches of 50 OM images from control and apoptotic cells. Searches using this 3-nm ''donut'' motif and the same motif doubled in size (6-nm ''donut'') yielded thousands of ''hits'' in both control and apoptotic membranes, which were subsequently aligned and classified as before. The predominant stain-filled structures found with both motifs were not circular but elongated (up to ~4x6 nm), extending away from stain-excluding crescent-shaped features. The radial anisotropy ruled out reference bias and was inconsistent with pores formed by rings of evenly spaced protein subunits. We hypothesize that the different classes of structures detected represent stages in formation of MAC as an increasingly large membrane bilayer defect (or ''cleft'') induced by successive aggregation or clustering of Bax/Bak molecules. A progressive assembly mechanism for MAC has been recently suggested by real-time monitoring of MAC conductances in isolated mitochondria by patch clamping (Martinez-Caballero et al. J Biol Chem 284: 12235-45). Supported by NIH grant GM57249.

1963-Pos
Effects of MAC Formation on Mitochondrial Morphology
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Accumulating literature associate mitochondrial dynamics with apoptosis, since regulation of either process has reciprocal effects. These processes seem to converge in formation of the mitochondrial apoptosis induced channel, MAC, which releases cytochrome c and triggers the degradation phase of apoptosis. While Bax and Bak, core components of MAC, were shown to interact with fusion and fission proteins, some studies also suggest proteins from the intermembrane space could leak to the cytosol and further promote mitochondrial fission during apoptosis. The temporal relationship between apoptosis induction, MAC formation and mitochondrial fragmentation was investigated by time lapse microscopy. MAC function was induced through staurosporine treatment and microinjection of tBid or cytochrome c. MAC formation and mitochondrial dynamics under these conditions were monitored in Hela cells (clone 10) that stably express low levels of GFP-Bax and were transiently transfected with a pDsRed-2 plasmid. GFP-Bax relocation to mitochondria only during apoptosis signals MAC formation, while pDsRed-2 expression shows mitochondrial structure as red fluorescence. Treatment with staurosporine and microinjection with tBid or cytochrome c induced relocation of Bax and collapse of the mitochondrial network. The temporal relationship between these two events was further analyzed. Interestingly, pretreatment with iMAC2, a specific MAC blocker, protected against cell death and prevented mitochondrial fragmentation after tBid injection. Our results suggest a link exists between MAC formation and collapse of the mitochondrial network during apoptosis. Supported by NIH grant GM57249.

1964-Pos
Mechanism of the Mitochondrial Cytochrome C Release Wave in Bid-Induced Apoptosis
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Bid, a BH3-only Bcl2 family protein, plays a central role in apoptosis. Bid is cleaved by caspase-8 and other enzymes forming tBid that induce mitochondrial outer membrane (OMM) permeabilization and cytochrome c (cyto-c) release. However a mystery remains how Bid synchronizes the function of a large number of discrete organelles, particularly in mitochondria-rich liver or muscle cells. Here we showed that tBid (0.5-50nM) elicited progressive OMM permeabilization and complete cyto-c release with a dose-dependent lag time and rate in H9c2 cell populations. Once started, the OMM permeabilization was not prevented by tBid washout. In contrast, the dose-response for digitonin-induced OMM permeabilization displayed quantal behavior. In single cell imaging studies, permeabilized H9c2 and primary human cardiac cells transfected with cyto-c-GFP showed complete tBid-induced cyto-c-GFP release closely followed by mitochondrial depolarization. The cyto-c-GFP release started at discrete sites and propagated through the mitochondria with a constant velocity and a relatively stable kinetic of release in each organelle. Similar tBid-induced cyto-c-GFP release wave was documented in intact H9c2 cells transfected with tBid. The waves were not dependent on Ca^{2+}, caspase activation or permeability transition pore opening. However, treatment with MnTMPyP, a ROS scavenger or overexpression of mitochondrial superoxide dismutase suppressed the coordinated cyto-c release and also inhibited tBid-induced cell death. On the other hand, both superoxide and hydrogen peroxide sensitized mitochondria to the tBid-induced permeabilization. Thus, tBid engages a ROS-dependent inter-mitochondrial signaling mechanism for spatial amplification of the apoptotic signal by mitochondrial waves.

1965-Pos
Role of Milton Domains in the Calcium-Dependent Regulation of Mitochondrial Motility
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The mammalian GRIF1 and OIP106, and the drosophila Milton are kinesin-binding proteins that form a complex with the Miro GTPase, an outer mitochondrial membrane EF-hand protein, to support the movement of mitochondria along the microtubules. Our study demonstrates that in H9c2 cells overexpressing OIP106 or GRIF1, the basal motility of mitochondria is increased, whereas the sensitivity to calcium-induced movement inhibition is decreased. To dissect the interaction between Milton, kinesin and Miro, three different Milton constructs were tested: Milton (1-450), the soluble domain of Milton; Milton (750-1116), lacking the kinesin heavy chain binding site and Milton (847-1116) that lacks additional ~100 amino acid presumably containing part of the Miro binding site. Immunohistochemistry revealed that the overexpressed Milton (1-450) was cytoplasmic, whereas the other two Milton constructs showed mitochondrial localization. The basal mitochondrial motility was increased by Milton (750-1116) but was not altered by Milton (847-1116) or Milton (1-450). A plot of mitochondrial motility against slowly rising cytoplasmic [Ca2+] induced by thapsigargin (2μM), shows that overexpression of Milton (750-1116) significantly reduced the calcium sensitivity of mitochondrial motility reminiscent of OIP106 and GRIF1. By contrast, Milton (847-1116) or Milton (1-450) did not have any effect. The thapsigargin-induced cytoplasmic calcium signal was not affected by any of the Milton constructs. These data indicate that