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

the C terminus of Milton is an important regulator of the mitochondria associated motors and is involved in conferring the calcium sensitivity from Miro to the motors. Milton 847-1116 is sufficient for the mitochondrial binding, whereas the 750-847 amino acids are critical for the control of calcium sensitivity of mitochondrial motility.

1966-Pos

Biophysical Properties of Mitochondria Undergoing Fusion

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Emerging evidence shows the importance of genes controlling mitochondrial fusion in physiology and their deregulation in neurodegenerative and metabolic disorders. However, apart from $\Delta\Psi_m$, the biophysical properties of the fusion-competent mitochondria remain elusive. To evaluate the conditions of contact formation and fusion, we used organelle-targeted fluorescent proteins, including photoactivatable GFP, which allow tracking of individual mitochondria. In H9c2 cells, almost every mitochondrion is aligned with microtubules that provide the primary tracks for mitochondrial movement. Our results show that ~90% of fusion events involve moving mitochondria. However, fusion occurs irrespective of mitochondrial speed. Furthermore, ~80% of fusion events involve the tip portion of the mitochondrion, whereas only ~50% of these events involve organelle side. Nocodazol, a microtubule disrupting agent that inhibits mitochondrial movements decreases the fusion frequency and changes mitochondrial fusion sites. Strikingly, 80-90% of the physical contacts between adjacent mitochondria did not result in fusion events. To evaluate whether the fusion efficacy depends on the spacing between the outer and inner mitochondrial membranes we used drugs that alter the matrix volume. Valinomycin, a K^+ ionophore that induces matrix swelling evoked a decrease in mitochondrial motility leading to fewer contacts among mitochondria but the number of fusion events was maintained, indicating an increase in fusion efficacy. This change occurred at a low valinomycin concentration (0.25nM) that did not affect $\Delta\Psi_m$ or Opa1 cleavage. Nigericin (0.5μM), a K^+/H^+ ionophore that induces shrinkage of the matrix elicited fusion inhibition and mitochondrial aggregation. Importantly, no motility inhibition or Opa1 cleavage occurred at the same time and the $\Delta\Psi_m$ was increased. These results suggest that mitochondrial fusion is facilitated by mitochondrial motility, the key determinant of the inter-mitochondrial encounter numbers and preferentially involves the front-tip of the moving organelle. In addition, fusion efficacy depends on the mitochondrial matrix volume.

1967-Pos

Mitochondrial Fusion Dynamics in Human Skeletal Muscle-Derived Cells

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Mitochondria have a fundamental role in both muscle physiology and pathology. Mitochondrial fusion and fission are important for energy metabolism, calcium homeostasis and cell death. However, the mechanisms underlying mitochondrial dynamics are poorly understood, especially in physiological models such as skeletal muscle. Here we evaluated mitochondrial fusion dynamics in human skeletal muscle cells (HUSMC). Skeletal muscle satellite cells were isolated from human muscle biopsies and were maintained and differentiated in cell culture. Mitochondrial fusion events were evaluated by confocal imaging of cells expressing mitochondria matrix targeted DsRed and matrix targeted or outer mitochondrial membrane (OMM) targeted photoactivatable-GFP. When we tagged the mitochondria in ~20% of total cellular area with photoactivated-GFP, we found those mitochondria undergoing matrix fusion with a frequency of 1.3 ± 0.1 events/min/cell (n=70). Among the fusion events, 40% led to complete fusion and only 10% was followed by separation at the apparent fusion site within 20 to 40 seconds. Both complete and transient fusion events resulted mostly from longitudinal mergers, involving end to end interaction or from mergers of adjacent mitochondria in side to side orientation. Furthermore, we found that OMM and matrix fusion are sequential and separable steps, displaying 5.8 ± 1 seconds gap (n=10). Finally, we evaluated the mitochondrial fusion dynamics in HUSMC derived from both normal and malignant hyperthermia susceptible individuals. At resting state, no significant differences were found in the number of events or in their characteristics. Thus, mitochondrial fusion commonly occurs in HUSMC, and enables mixing of both soluble and integral membrane factors. This process would help to maintain the stability of mitochondrial metabolism. The relatively low frequency of the transient fusion is probably due to the parallel organization of the cytoskeletal tracks for mitochondria and to the limited mitochondrial motility.

1968-Pos

Imaging Interorganelle Contacts and Local Calcium Dynamics at the ER-Mitochondrial Interface

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The local coupling between ER and mitochondria is essential for proper cell function. A main role of the ER-mitochondrial junctions is to provide a local calcium signaling domain that is important both for keeping energy production in line with demand and for the control of apoptotic mechanisms. So far it has not been possible to visualize the tiny ER-mitochondrial contact points in living cells or to monitor the localized $[Ca^{2+}]$ changes in the narrow space between ER and mitochondria ($[Ca^{2+}]_{ER\text{-}mt}$).

Here, we exploited rapamycin-mediated heterodimerization of FKBP12 and FRB domains of fluorescent protein constructs respectively targeted to the outer mitochondrial membrane and the ER as drug-inducible inter-organelle linkers to identify ER-mitochondrial contacts and to measure the $[Ca^{2+}]_{ER\text{-}mt}$. High-resolution fluorescence imaging and 3D reconstruction revealed rapamycin-induced clustering of the ER-targeted fluorescent linker-half to the contact areas with the mitochondria without major changes in the spatial arrangement of the ER. Essentially all mitochondria displayed contacts with the ER in both RBL-2H3 and H9c2 cells. Plasma membrane-mitochondrial contacts were less frequent with ER stacks being inserted between the two organelles. Single mitochondria display discrete patches of ER contacts as well as continuous associations. Cytoplasmic and mitochondrial matrix $[Ca^{2+}]$ showed robust ER-mitochondrial Ca^{2+} transfer with considerable heterogeneity even among adjacent mitochondria. Pericam-containing linkers revealed IP_3-induced $[Ca^{2+}]_{ER\text{-}mt}$ signals that were resistant to buffering bulk cytosolic $[Ca^{2+}]_c$ increases and exceeded 9 uM. The largest $[Ca^{2+}]_{ER\text{-}mt}$ signals did not occur at the tightest associations, indicating space requirements for the Ca^{2+} transfer machinery and functional diversity among ER-mitochondrial junctions.

These studies provide direct evidence for the existence of high Ca^{2+} microdomains between the ER and mitochondria in living cells, and open new possibilities to probe the functional importance of this specialized compartment.

1969-Pos

Dependence of ER-Mitochondria Calcium Transfer on Different IP3 Receptor Isoforms

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IP3 receptors (IP3Rs) release Ca2+ from the ER, which is locally relayed to the mitochondria to control several aspects of mitochondrial function. Recent studies have suggested that type 3 IP3Rs (IP3R3) are particularly important for mediating the Ca2+ transfer at the ER-mitochondrial interface. We set out to systematically evaluate the respective role of each IP3R isoform in chicken DT40 cell lines that express only one IP3R isoform (double-knockout, DKO1, DKO2 and DKO3) or provide a null-background (triple-knockout, TKO) for analysis of mammalian IP3Rs and their mutants.

Simultaneous imaging of cytoplasmic and mitochondrial matrix [Ca2+] ([Ca2+]c and [Ca2+]m) was performed in either permeabilized cells challenged with IP3 or in muscarinic receptor overexpressing intact cells stimulated with carbachol (Cch), an IP3-linked agonist. Saturating IP3 evoked complete discharge of ER calcium and resulted in comparable [Ca2+]m increases in each DKO. Furthermore, the Cch-induced [Ca2+]c spike was closely followed by a [Ca2+]m rise in each DKO. When TKO cells were rescued with rat IP3R1 or IP3R3, the latter mediated a larger [Ca2+]c transient but the [Ca2+]m increases were very similar for both isoforms. The relationship between the [Ca2+]c peak and the corresponding [Ca2+]m response was also very similar for IP3R1 and IP3R3. To assess the impact of the release kinetic through IP3R1 in the mitochondrial Ca2+ transfer we used two point mutants of the IP3R1, which display either enhanced inhibition by Ca2+ (D426N) or are relatively insensitive to Ca2+ inhibition (D442N). D426N showed dampened [Ca2+]m signal and [Ca2+]m vs. [Ca2+]c relationship, whereas D442N displayed a steeper [Ca2+]m vs. [Ca2+]c relationship than the wild type IP3R1. Thus, each IP3R isoform can support local ER-mitochondrial Ca2+ transfer and their competence to activate mitochondrial Ca2+ uptake depends on their deactivation kinetic.