Changes in mitochondrial surface charge mediate recruitment of signaling molecules during apoptosis

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Heit B, Yeung T, Grinstein S. Changes in mitochondrial surface charge mediate recruitment of signalling molecules during apoptosis. *Am J Physiol Cell Physiol* 300: C33–C41, 2011. First published October 6, 2010; doi:10.1152/ajpcell.00139.2010.—Electrostatic interactions with negatively charged lipids contribute to the subcellular localization of polycationic proteins. In situ measurements using cytosolic probes of surface charge indicate that normal mitochondria are not noticeably electronegative. However, during apoptosis mitochondria accrete negative charge and acquire the ability to attract cationic proteins, including K-Ras. The marked increase in the surface charge of mitochondria occurs early in apoptosis, preceding depolarization of their inner membrane, cytochrome c release, and flipping of phosphatidylinerine across the plasmalemma. Using novel biosensors, we determined that the increased electronegativity of the mitochondria coincided with and was likely attributable to increased exposure of cardiolipin, which is dianionic. Ectopic (over)expression of cardiolipin-binding proteins precluded the increase in surface charge and inhibited apoptosis, implying that mitochondrial exposure of negatively charged lipids is required for progression of programmed cell death.

**Mitochondria** are the final arbitrators between survival and programmed cell death. The release of cytochrome c is the first irreversible step in apoptosis, reflecting the balance between anti- and pro-apoptotic signals at the mitochondrial surface (16). Whereas the Bcl2 family proteins that participate in the initiation and progression of apoptosis have been studied extensively (reviewed in Ref. 32), much less is known about the lipid changes that accompany programmed cell death. During the early stages of apoptosis phosphatidylinerine (PS) appears on the outer leaflet of the plasma membrane, in part by flipping across the bilayer (31), and the phosphoinositol content drops noticeably (30). The accessibility and distribution of mitochondrial lipids also changes, in part, by delivery of plasmalemmal glycosphingolipids to their outer membrane (8). The functional significance of these changes is largely obscure.

It has recently become apparent that the accumulation of anionic lipids in certain cellular membranes serves to target and retain proteins with polycationic motifs (37). It is therefore conceivable that, by altering the surface charge, the lipid changes that accompany apoptosis may cause redistribution of cationic proteins, potentially contributing to the execution of programmed cell death. To investigate this possibility we used a recently developed probe, R-Pre, to quantify surface charge in cells undergoing apoptosis. R-Pre is a coincidence detector directed to membranes of high negative charge by the combination of a hydrophobic prenyl moiety and a polycationic motif (Fig. 1A; see Ref. 37 for details). Using this probe, we demonstrate that the mitochondria of apoptotic cells undergo a drastic increase in their surface charge, through the exposure of cardiolipin (CL) to the cytosol, and that this increase in surface charge is necessary for the progression of apoptosis.

**EXPERIMENTAL PROCEDURES**

**Materials.** All reagents were purchased from Sigma-Aldrich with the following exceptions: RPMI, PBS, Annexin V, tetramethyl rhodamine methyl ester (TMRM), 10-N-nonyl acridine orange (NAO), and DMEM were purchased from Invitrogen. Anti-FAS was purchased from Immunotect. Anti-CL serum was purchased from US Biologicals. DRAQ5, anti-Bid, and anti-caspase 8 were purchased from Cell Signaling Technologies. Fluorescent secondary antibodies were purchased from Jackson ImmunoResearch. Caspase 8-cleaved Bid and tumor necrosis factor-related apoptosis-inducing liquid (TRAIL) were purchased from R&D Systems. Purified lipids were purchased from Avanti Lipids. Restriction enzymes and ligase were purchased from New England Biolabs. pEGFP-N1 was purchased from BD Biosciences. pET28a was from Novagen. cDNA for human cytochrome c and CKMT1b were acquired from the NIH mammalian genome collection. Human cDNA for caspase 8, isoform B was purchased from AddGene. Poly-His protein isolation kit was from Pierce.

**Constructs.** The surface charge probes R-Pre-GFP and K-EGFP were described previously (36, 37). The probes for PS, phosphoinositol-3-phosphate (PI(3)P), phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2], and phosphatidylinositol 3,4,5-trisphosphate (PIP3) were defined previously (35, 36). The CL-binding probe (CLBD-GFP) was constructed by PCR-amplifying amino acids 39–74 of CKMT1b, using the primers CKMTFwd (CTT CGA ATT CGC CAC CAT GGC CAG TGA ACG AGC GAG GAC ATC CGG GTC GCA GAG CCG TGC ATA GAC TG). The resulting product was digested with BamHI and SalI and ligated into pEGFP-N1. To create recombinant CLBD-GFP-His6, the above construct was amplified using the primers pET28FWD (TAT ATC ATG ACC AGT GCG GCA GCT GCG GCT TC) and IMSREV (TCG ACT GCA GAA GGG AAG ACA GAC TTC AGC AGA AAT CTT TAT GAT ATT GGG G) and digested with BspHI and NotI. This was ligated into the pET28a vector cut with NcoI and NotI. The resulting protein was expressed in Escherichia coli and purified. Green fluorescent protein (GFP) bearing the mitochondrial import signal (IMS-GFP) was generated by PCR-amplifying the mitochondrial import signal of cytochrome c (amino acids 1–84) with the primers IMSFWD (CCG GAC TCA AGA TCT ATG GCG GCA GCT GCC GCT TC) and IMSREV (TGC ACT GCA GAA TTC TTA GGT CAC TGG CAC TCA CAG CCC). The resulting product was digested with BglII and EcoRI and ligated into pEGFP-N1. Caspase 8-GFP was generated by PCR amplifying caspase 8 isoform B with the primers Casp8FWD (TIT AAA GTC GAC ATG GAC TTC AGC AGA AAT CTT TAT GAT ATT GGG G) and Casp8REV (ACC GGT GGA TCC TTA TCA GAA GGA AAG ACA AGT TTT TCT ATG GTA AAA). The resulting product was digested with SalI and BamHI and ligated into pEGFP-N1.

**Induction of apoptosis and microscopy.** HeLa cells were grown in DMEM + 5% FBS and transfected using FuGene6 and the desired constructs. When high rates of transfection (>90%) were required, as...
in the case of samples used for immunoblotting, the cells were electroporated using the Amaxa system. The cells were treated with 5 μM blebbistatin before the induction of apoptosis to reduce membrane blebbing. Apoptosis was induced through the addition of anti-FAS IgM (1.5 μg/ml), recombinant TRAIL (1 μg/ml), staurosporine (1 μM) for 3–4 h, by the addition of 50 μg/ml etoposide for 24–48 h, or by 8 min of ultraviolet irradiation on a Fisher 312 nm transilluminator, followed by 3–4 h incubation. Cells were either imaged at the end point, or using time-lapse microscopy, with a Zeiss Axiovert 200/Quorum spinning-disc confocal microscope equipped with a ×63/1.4 NA and ×100/1.4 NA oil immersion objectives and a heated stage. A minimum of 10 cells were imaged per experiment, with each experiment repeated at least three times. Scoring the percentage of apoptotic cells was performed using either AnnexinV-488 staining to detect phosphatidylserine externalization or DRAQ5 staining to detect DNA condensation, depending on compatibility with other fluorophores used in the experiment.

Mitochondrial isolation and induction of apoptosis. Mitochondria were isolated using the procedure of Gottlieb and Adachi (11). HeLa cells were trypsinized, washed with MA (100 mM sucrose, 1 mM EGTA, 10 mM HEPES, 1 g/l BSA, 1 × mammalian protease inhibitor), suspended in MA + 10 mM triethanolamine and 5% Percoll, and lysed using nitrogen cavitation (4°C, 420 PSI, 20 min). Sucrose was added to the lysate to a final concentration of 250 mM, and the lysate centrifuged at 2,500 g for 5 min to remove nuclei and intact cells. The lysate was transferred to a clean tube and spun at 10,000 g for 15 min to pellet the mitochondria. The mitochondria were washed two times in MA + 200 mM sucrose and suspended in tBid-release buffer (10 mM HEPES, 125 mM KCl, 0.5 mM MgCl2, 3 mM glutamic acid, 3 mM succinic acid). If mitochondrial membrane potential was being assessed, cells were cultured with 500 nM TMRM for 30 min before mitochondria isolation. Apoptosis was induced in the isolated mitochondria through the addition of 200 nM caspase 8-cleaved human Bid (tBid) followed by incubation at 37°C for 2.5–30 min. The release of the potential-sensitive dye TMRM and/or the accumulation of charge (50 μM bimane-K-) and/or CL exposure (0.5 mM CLBD-GFP or 1/50 human anti-CL serum, followed by 1:2,000 anti-human Cy5 secondary) was monitored using a Hitachi F-2500 spectrophotometer or the microscope described above. The release of cytochrome c from these mitochondria was monitored by immunoblotting.

CL exposure in live cells. To assess CL exposure, HeLa cells were either left untreated or exposed to anti-FAS IgM to induce apoptosis, as above. Thirty minutes before imaging, cells were stained with 35 μM of the CL-specific dye NAO. NAO forms complexes with CL, resulting in the formation of excimers with an emission wavelength of ~640 nm (25). The cells were then transferred to an imaging chamber containing permeabilization buffer (in mM: 10 NaCl, 20 HEPES, 50 KCl, 2 K2HPO4, 90 K-glutamate, 1 MgCl2, 4 EGTA, and 2 CaCl2). The CL-induced NAO excimers were visualized with the microscopy system described above, using a 491-nm laser for excitation and detecting the emission using a Texas Red long-pass filter (>590 nm). Cells were labeled with MitoTracker deep red to determine the location of mitochondria when using NAO staining. Quenching by Trypan blue was used to determine the fraction of CL exposed to the cytoplasm. Because intact, live cells are impermeant to Trypan blue, the addition of the dye was followed immediately by 1 μg/ml of streptolysin-O, which selectively permeabilizes the plasma membrane.
(33). The degree of NAO quenching resulting from entry of Trypan blue (100 µg/ml) into the cytosol was quantified as a measure of the fraction of CL-induced NAO eximers exposed to the cytosol. Cells that underwent quenching by Trypan blue before the addition of streptolysin-O were deemed unhealthy and were not considered for subsequent analysis. In live cells NAO eximer fluorescence was observed virtually only in mitochondria. Regardless, any contribution of nonmitochondrial NAO eximers was excluded from analysis by using a region of interest (ROI) defined by MitoTracker deep-red staining.

Image analysis and statistics. Images were cropped and ROIs defined using imageJ. The resulting images and ROIs were then imported into Mathworks Matlab and colocalization and signal intensity analysis preformed using customized scripts. Statistical analysis was preformed using Graphpad Prism. All graphs are plotted as means ± SE. Unless otherwise noted, data were analyzed using an ANOVA with Bonferroni correction, with statistical significance set at \( P < 0.05 \).

RESULTS AND DISCUSSION

GFP-tagged R-Pre (Fig. 1A) was expressed in HeLa cells and visualized by spinning disc microscopy before and during apoptosis induced by cross-linking FAS. In untreated cells R-Pre localized almost exclusively to the inner aspect of the plasma membrane, revealing its uniquely negative charge conferred by the concentration of polyphosphoinositides and PS (Fig. 1B). Remarkably, R-Pre underwent an extensive redistribution following FAS activation, displaying a punctate pattern with reduced plasmalemmal staining (Fig. 1B). Dual-labeling experiments revealed that the intracellular structures that accumulated R-Pre in cells undergoing apoptosis correspond to mitochondria, which were not labeled by the probe in untreated cells (Fig. 1C). The mitochondrial accumulation of R-Pre, which attained levels similar to those at the plasma membrane, was also observed in cells where apoptosis was initiated by stimulation of TRAIL (Fig. 1D), by addition of staurosporine or etoposide, serum starvation, or exposure to ultraviolet light (see supplemental Fig. S1, A and B at the AJP-Cell Physiol website), and required caspase activity (Fig. 1E).

To ascertain that R-Pre is recruited to the mitochondria by electrostatic attraction, apoptotic cells were treated with lipophilic cations that integrate into anionic membranes, thereby neutralizing their charge. As documented in Fig. 2A, dibucaine, sphingosine, and squaleamine all displaced R-Pre from the mitochondria of apoptotic cells. Moreover, apoptotic mitochondria also accumulated a different surface charge probe, Kψ (Fig. 2, B and C), which was similarly displaced by lipophilic cations (Fig. 2D). Like R-Pre, Kψ was shown to be an effective indicator of negative surface charge (37) and was similarly found lining the membrane of untreated cells (Fig. 2C). However, Kψ lacks the prenyl moiety found in R-Pre and owes its amphiphilic character to hydrophobic amino acids (Fig. 2B). Therefore, targeting to apoptotic mitochondria is not unique to R-Pre and cannot be attributed solely to its prenyl moiety but is instead a reflection of changes in surface charge. Accordingly, shielding of charges by transiently (<30 s) increasing the ionic strength detached R-Pre from the membrane of untreated cells (Fig. 2, E and F) and from mitochondria in apoptotic cells (Fig. 2F).

The redistribution of R-Pre from the plasma membrane to mitochondria suggests that the surface negativity of the plasma membrane may have decreased, or that of the mitochondria increased, or a combination of these events. The loss of phosphoinositides, together with flipping of PS reported during apoptosis, are anticipated to reduce the negativity of the inner plasmalemmal leaflet. This should result in detachment of the surface charge probes, which may then relocate to mitochondria. To test whether release of plasmalemmal R-Pre caused its redistribution to mitochondria, nonapoptotic cells were exposed to ionomycin, a calcium ionophore. This manipulation is known to depress plasmalemmal surface charge by scrambling of PS and/or hydrolysis of phosphoinositides (4, 34) (see supplemental Fig. S2, A and B), recapitulating the changes observed during apoptosis. As shown in Fig. 2, G and H, while R-Pre was released from the plasma membrane, it did not relocalize to mitochondria under these conditions. Thus reduction of plasmalemmal surface charge is not sufficient to account for the redistribution of the probes to mitochondria, and a concomitant increase in the negativity of the exposed mitochondrial membranes must be invoked.

That mitochondrial surface charge increases during programmed cell death was validated using isolated mitochondria and recombinant tBid in an ex vivo model of apoptosis. Synthetic, bimane-labeled Kψ associated modestly with isolated, untreated mitochondria. This binding was likely hydrophobic in nature, inasmuch as it was not affected by lipophilic cations or by increasing the ionic strength (Fig. 2I). Addition of tBid markedly increased the association of the probe to the mitochondria, and the increase was obliterated by dibucaine and high ionic strength, implying an electrostatic mode of association. Together, these results demonstrate that the apoptosis-induced recruitment of the surface charge probes is due to an increase in mitochondrial surface charge, and that tBid is sufficient to induce this increase in surface charge.

Comparison of the kinetics of probe association with mitochondria in live cells revealed that while the lag between the addition of anti-FAS \( (T = 0) \) and the onset of probe accumulation varied among cells, the subsequent dynamics of appearance of surface charge was similar (Fig. 3A). By aligning the onset across multiple cells, it was therefore possible to compare the development of the change in surface charge with other events triggered by apoptosis. As shown in Fig. 3B, the increase in the surface charge of the mitochondria preceded the exposure of PS on the outer leaflet of the plasmalemma, assessed with annexin-V, and also preceded outer mitochondrial membrane (OMM) permeabilization as measured by the release of IMS-GFP, a GFP construct targeted to the intermembrane space by a mitochondrial import sequence. We also compared the course of accumulation of bimane-Kψ with the release of TMRM and cytochrome c in isolated mitochondria treated with recombinant tBid. The accumulation of Kψ preceded both cytochrome c release and inner mitochondrial membrane (IMM) permeabilization, as measured by TMRM release (Fig. 3C), confirming that mitochondrial charge presentation occurs early in apoptosis.

Several mechanisms could account for the increase in mitochondrial surface charge. Increased negative charge could result from the delivery to mitochondria of anionic glycosphingolipids from the surface membrane, as suggested by an earlier study (8). However, we failed to detect the appearance of plasmalemmal markers in apoptotic mitochondria (data not shown). Alternatively, negative charge may increase in apoptotic mitochondria by accumulation of PS, if its processing to
Fig. 2. Translocation of charge probes to apoptotic mitochondria is charge dependent. A: lipophilic cationic compounds displace the R-Pre charge probe from apoptotic mitochondria. B: structure of the Kφ surface charge probe. C: translocation of Kφ to mitochondria, identified by mito-RFP, during FAS-mediated apoptosis. In A and C, a control for photobleaching due to repeated image acquisition is also shown. D: lipophilic cationic compounds displace the Kφ probe from apoptotic mitochondria. E: a 30-s elevation of the ionic strength displaces R-Pre from the PM in nonapoptotic cells. F: quantification of the displacement of R-Pre from the PM and mitochondria of apoptotic cells by a 30 sec exposure to elevated ionic strength. G: effect of ionomycin-mediated reduction of plasmalemmal surface charge on R-Pre localization in nonapoptotic cells. H: quantification of R-Pre localization to the PM and mitochondria during ionomycin treatment of nonapoptotic cells. I: effects of dibucaine and high ionic strength (1 M NaCl) on bimane-Kφ binding to isolated but otherwise untreated (UT) or tBid-treated mitochondria. *P < 0.05 compared with isotonic (F) or control (I); †P < 0.05 compared with untreated control (UT), n = 5 (A, D, F, H) or 3 (I). Images are representative of a minimum of 30 cells per condition.
zwitterionic phosphatidylethanolamine is impaired, or by phosphorylation of phosphatidylinositol into more negatively charged polyphosphoinositides. However, direct assessment of PS accumulation, using the C2 domain of lactadherin, or of the presence of PI(3)P, PI(4,5)P2, or PI(3,4,5)P3, using FYVE- or PH-domain probes, failed to detect any increases in these lipids (supplemental Fig. S3A).

In contrast to other cellular membranes, where PS and phosphoinositides are thought to contribute most of the negative charge, mitochondria are richly endowed with another anionic lipid, CL, which bears one to two negative charges at physiological pH (14, 25). CL makes up ~20% of the IMM but is present only at low levels in the OMM (1). Transport of CL to the OMM during apoptosis (7, 30), or permeabilization of the OMM allowing access to the CL-rich IMM (17), could account for the observed changes in surface charge. Accordingly, recombinant bimane-Kφ bound much more avidly to liposomes containing 20% CL:80% PC than to liposomes consisting only of PC, and the association was blocked by polyvalent cations (Fig. 3D). To more directly test the involvement of CL we used NAO, a cationic dye that preferentially binds CL (13). NAO is a fluorescence resonance energy transfer (FRET) acceptor for bimane-labeled Kφ, and its quenching of bimane fluorescence can be used to probe the interaction between CL and Kφ. As shown in Fig. 3E, the fluorescence of bimane-labeled Kφ associated with untreated or tBid-treated isolated mitochondria. F: percent quenching of CL-NAO excimers by Trypan blue in SLO-permeabilized control and FAS-treated cells. G: accumulation of anti-CL antibody and release of IMS-GFP from isolated mitochondria treated with recombinant tBid. Inset: anti-CL and IMS-GFP in mitochondria incubated in tBid-free media. H: binding of CLBD-GFP to untreated (UT) or tBid-treated (tBid) mitochondria. *P < 0.05 compared with UT mitochondria; †P < 0.05 compared with tBid-treated mitochondria, n = 5 experiments (A and B) or 3 (C–H).

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contrast, 18–25% of NAO fluorescence was quenched in FAS-treated cells (Fig. 3F), demonstrating that there is a significant increase in cytoplasmic exposure of CL during apoptosis.

Although NAO is frequently used for the detection of CL in biological membranes, there is some controversy as to the specificity of this reagent (9, 13, 22, 24, 25). To ensure that we were specifically detecting CL, we took advantage of an anti-CL antibody. Treatment with tBid of mitochondria isolated from cells expressing IMS-GFP induced binding of anti-CL antibody and release of IMS-GFP with a time course resembling that observed in cells treated with anti-FAS (Fig. 3G). A similar pattern of CL exposure and mitochondrial permeabilization was observed using cytochrome c (supplemental Fig. S3, B–D), which is known to bind CL (27). A small fraction of the mitochondrial CL is known to be exposed to the cytoplasm in resting cells, where it is thought to play a role in the initiation of apoptosis (21).

Therefore, we hypothesized that masking cytoplasmically exposed CL should impair the initiation of apoptosis. This prediction was tested by heterologous (over)expression of CLBD, which will bind CL and shield its charge. CLBD expression inhibited apoptosis in anti-FAS-treated cells and inhibited tBid-mediated depolarization of isolated mitochondria (Fig. 4, A and B).

A small fraction of the mitochondrial CL is known to be exposed to the cytoplasm in resting cells, where it is thought to play a role in the initiation of apoptosis (21). Upon activation by FAS, caspase 8 is cleaved and its p43/41 cleavage product associates with mitochondria in a CL-dependent manner (10). This mitochondrion-associated caspase 8 then cleaves Bid to form tBid, which must also bind to cytoplasmically exposed CL to engage in its pro-apoptotic effects (10, 15, 20). Therefore, we hypothesized that masking cytoplasmically exposed CL should impair the initiation of apoptosis. This prediction was tested by heterologous (over)expression of CLBD, which will bind CL and shield its charge. CLBD expression inhibited apoptosis in anti-FAS-treated cells and inhibited tBid-mediated depolarization of isolated mitochondria (Fig. 4, A and B). Ectopic expression of CLBD-GFP inhibited the translocation of caspase 8 and Bid to mitochondria and also inhibited the
recruitment of R-Pre (Fig. 4, C and D). This impaired recruitment of caspase 8 and Bid was concurrent with impaired cleavage (activation) of Bid and partially also of caspase 8, which undergoes autocatalytic cleavage on the surface of apoptotic mitochondria (10) (Fig. 4, E and F). Combined with our previous observation that tBid was sufficient to induce charge exposure (Fig. 3, C, G, and H), these findings suggest that cytoplasmically exposed CL acts as a platform for caspase 8

Fig. 5. Cardiolipin-mediated charge exposure recruits charged proteins to mitochondria. A: recruitment of signaling molecules of varying charge to mitochondria in untreated (top) and FAS-treated (bottom) cells. Images are representative of a minimum of 15 cells per condition. B: quantitation of data from 4 experiments like that illustrated in A. *P < 0.05 compared with mitochondria recruitment of the same probe in nonapoptotic cells. C: displacement of K-Ras bound to mitochondria in apoptotic cells by lipophilic cationic compounds.

Fig. 6. Model of cardiolipin-mediated charge accumulation during apoptosis. FAS signaling induces translocation of caspase 8 to the small amount of cardiolipin normally found on the cytoplasmic face of the OMM (i), where it becomes activated and cleaves Bid to tBid (ii). tBid also binds to the small pool of exposed cardiolipin, inducing a signal which results in the translocation of additional cardiolipin to the cytoplasmic face of the OMM (iii). This creates a charged surface that accumulates pro-apoptotic proteins (BH2/3) and polycationic proteins such as K-Ras (iv).
activation. The subsequent accumulation of caspase 8-cleaved Bid (tBid) on these platforms induces the exposure of additional CL to the cytosol, leading to the acquisition of mitochondrial surface charge.

What is the functional significance of the CL-associated increase in mitochondrial surface charge? Because it is an early event, we hypothesized that the increased charge may drive the recruitment of proteins important for the completion of apoptosis. Indeed, masking CL was sufficient to block the accumulation of caspase 8 and Bid on apoptotic mitochondria (Fig. 4). However, any protein containing a polycationic domain has the potential to be recruited to apoptotic mitochondria via electrostatic interactions with cytoplasmically exposed CL. The ability of apoptotic mitochondria to attract proteins electrostatically was tested by comparing the recruitment of various GFP-tagged signaling molecules. K-Ras, which has a highly cationic motif and is pro-apoptotic (2, 26), did not associate with normal mitochondria but was markedly recruited to apoptotic mitochondria in a charge-dependent manner (Fig. 5, A–C). Rac1 and c-Src, which are somewhat less charged than K-Ras, were also attracted to apoptotic mitochondria, though to a lesser extent. H-Ras, which is highly homologous to K-Ras yet lacks the polycationic motif in the hypervariable region, was not found on apoptotic mitochondria (Fig. 5A).

Our data supports a model in which activated caspase 8 is recruited to the small amount of CL normally found on the cytosolic surface of mitochondria, where it mediates cleavage of Bid into tBid (Fig. 6). This tBid also binds to this small pool of CL, where it then induces the translocation of additional CL to the mitochondrial surface. This secondary exposure of CL precedes OMM permeabilization and serves to recruit pro-apoptotic members of the Bcl-2 protein family, as well as proteins containing polybasic domains (Fig. 6). This exposure of CL is absolutely required for the progression of apoptosis, since strong apoptotic stimuli failed to induce cell death when exposed CL was masked and its charge shielded (Fig. 4A). This exposure of CL likely recruits other Bcl-2 family proteins to the mitochondria. Indeed, several Bcl2-family members have been found to associate with mitochondrial membranes through an unidentified mechanism, and two of these (Bid and Bax) are known to interact directly with CL (18, 28). Furthermore, the additional CL exposed during early apoptosis would drive the formation of the Bax/Bak pores, which permeabilize the OMM during apoptosis (17). Whether these interactions involve specific recognition of the CL head group or are primarily due to electrostatic interactions remains to be defined. To date no stereospecific CL-recognition domains have been identified, raising the possibility that electrostatic interactions may be involved instead. Indeed, CL is known to cluster, allowing for the formation of regions of high-charge density even when only small increases in the amounts of exposed CL occur (6, 22). These zones of electrostatic interaction could be further enhanced by accumulation of phosphatidic acid, another lipid suggested to accumulate in mitochondria during apoptosis (1, 23). The specificity of such interactions would necessarily be limited. However, it is becoming increasingly clear that proteins are directed to their biological targets not by one but by multiple determinants acting synergistically. Such is the case for myristoylated alanine-rich C kinase substrate (MARCKS), K-Ras, and other proteins that accumulate in the plasma membrane by virtue of their cationic charge, in combination with a hydrophobic moiety (5, 12). It is noteworthy that K-Ras migrates from the membrane to mitochondria during apoptosis, indicating the appearance of an even greater surface charge on the exposed surface of mitochondria. Thus, while not sufficient, electrostatic interaction may be necessary to drive the association and retention of pro-apoptotic proteins with the mitochondria.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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