Mobilization of Lysosomal Calcium Regulates the Externalization of Phosphatidylserine during Apoptosis*

Banafsheh Mirnikjoo, Krishnakumar Balasubramanian, and Alan J. Schroit

From the Department of Cancer Biology, University of Texas, M. D. Anderson Cancer Center, Houston, Texas 77030

A hallmark of apoptotic cells is the Ca\(^{2+}\)-dependent appearance of phosphatidylserine (PS) at the cell surface as a result of its redistribution from the inner-to-outter plasma membrane leaflet. Although endoplasmic reticulum and mitochondrial Ca\(^{2+}\) are known to participate in apoptosis, their role in PS externalization has not been established. In this study, several organelle-specific fluorescent markers and Ca\(^{2+}\)-sensitive probes were used to identify the source of Ca\(^{2+}\) critical to PS externalization. By employing Rhod-2AM, fluorescein-labeled high molecular weight dextran, and Calcium Green 1, we provide evidence that lysosomes respond to apoptotic stimuli by releasing their luminal Ca\(^{2+}\) to the cytosol. Cells treated with the cytosolic phospholipase A\(_2\) inhibitor, cPLA2\(_{2\alpha}\), had no effect on caspase activation but exhibited a significant decrease in lysosomal Ca\(^{2+}\) release and externalization of PS in response to apoptotic stimuli. Similarly, cells depleted of lysosomal Ca\(^{2+}\) underwent programmed cell death yet failed to externalize PS. These data indicate that although Ca\(^{2+}\) release from other intracellular organelles to the cytosol is adequate for apoptosis, the release of Ca\(^{2+}\) from lysosomes is critical for PS externalization.

The regulated transbilayer redistribution of phosphatidylserine (PS)\(^{2\rightarrow}\) from the inner-to-outter bilayer leaflet of cells is a physiologically significant event critical to apoptosis and many other processes (1–3). Once translocated from the cytoplasmic to the external membrane leaflet, PS provides a platform for the assembly of enzyme complexes that regulate the propagation of the coagulation cascade (4) and serves as a key recognition ligand for the ultimate elimination of the cell from the host by phagocytic reticuloendothelial cells (5–7). Despite tremendous progress in deciphering apoptotic pathways, however, the mechanism(s) responsible for the appearance of PS at the cell surface remains elusive (8–10).

Numerous studies have revealed the involvement of several lipid transporters in the regulation of transbilayer lipid distributions in eukaryotic cells (11, 12). The concerted action of an inward-directed aminophospholipid translocase and a less specific outward-directed floppase are considered to be responsible for maintaining the dynamic equilibrium distribution of phospholipids between the plasma membrane bilayer leaflets of quiescent cells (13). Activation of a putative protein, coined phospholipid “scramblase,” dissipates normal membrane lipid asymmetry by facilitating the bidirectional transbilayer movement of all phospholipid classes irrespective of the composition of the polar head group of the lipid (14, 15). The most pronounced effect of perturbed lipid asymmetry is the appearance of PS in the external bilayer leaflet. Although the activation pathways for scramblase activity may differ between different cell types, a key upstream event seems to be a sustained elevation in intracellular [Ca\(^{2+}\)] that occurs through its release from intracellular stores and by capacitive entry across the plasma membrane.

The major stores of intracellular Ca\(^{2+}\) are the mitochondria (16) and ER (17, 18). Although the regulation of Ca\(^{2+}\) transients through these stores clearly represent critical check points in the regulation of apoptosis, the role of other intracellular Ca\(^{2+}\) stores in the externalization of PS has not been determined. In this study, we provide evidence that lysosomes respond to apoptotic stimuli by releasing their luminal Ca\(^{2+}\) to the cytosol. Our data indicate that Ca\(^{2+}\) released from these vesicles is critical to apoptosis-dependent expression of PS at the cell surface.

EXPERIMENTAL PROCEDURES

Cells, Cell Lines, and Reagents—Murine embryonic fibroblasts (MEF) were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 15% fetal bovine serum, 50 μg/ml uridine, 2 mM glutamine, and 100 units/ml leukemia inhibitory factor. All of the fluorescent probes used for Ca\(^{2+}\) measurements and intracellular organelle identification were purchased from Invitrogen. The phospholipase A\(_2\) inhibitor, cPLA2\(_{2\alpha}\) inhibitor (catalog number 525143), was purchased from Calbiochem.

Apoptosis and Measurement of Apoptotic Markers—Cells (~10\(^{6}\)/2.5 cm coverslip) were triggered into apoptosis by treatment with tumor necrosis factor α (50 ng/ml)/cyclohexamide (1 μg/ml) or staurosporine (STS, 0.5 μM) for the indicated times at 37 °C. Cells on coverslips or in suspension (10\(^6\)/ml) were analyzed for PS externalization by incubating the cells with FITC-labeled annexin V for 10 min at 20 °C in 0.5 ml of Tris-buffered saline containing CaCl\(_2\) (2 mM) and propidium iodide (1.0 μg/ml) followed by fluorescence-activated cell sorter analysis. For DNA fragmentation (propidium iodide/cell cycle anal-
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**RESULTS**

**Sustained Elevation in Cytosolic Ca^{2+} Is Required for PS Externalization**—Previous results from this laboratory have shown that incubation of Jurkat cells with thiol-disulfide exchange reagents results in the immediate and sustained release of Ca^{2+} from intracellular stores to the cytosol and redistribution of PS from the inner-to-outer membrane leaflet of the cell (3). This occurs in the absence of cytochrome c release, caspase activation, and DNA fragmentation, suggesting that independent mechanisms regulate PS externalization and apoptosis. Indeed, although apoptosis is not reversible (once initiated), it can be enforced through the release of Ca^{2+} from endogenous stores to the cytosol with thiol reagents (3) or by incubation of the cells in the presence of Ca^{2+} ionophore/Ca^{2+} (Fig. 1A). Because thapsigargin triggers release of Ca^{2+} from ER stores, we deter-

**FIGURE 1. Thapsigargin-dependent ER Ca^{2+} fluxes and PS externalization.** A, MEF were incubated with N-ethylmaleimide (NEM) (0.5 μM for 30 min at 4 °C) or A23187 (5 μM) in the presence of Ca^{2+} (1 mM) for 20 min at 37 °C. Cell surface PS was determined by flow cytometry analysis of FITC-annexin V-labeled cells. B, MEF were labeled with Fura-2AM for 30 min. The cells were then washed, and Ca^{2+} levels were estimated by assessing the fluorescence intensities at 540 nm (λ_{ex} 340 and 380 nm). Relative cytosolic Ca^{2+} levels were monitored following the addition of TG at 3 min. A, control cells; B, 50 nM TG; C, 200 nM TG; and D, 50 nM TG in the presence of Ca^{2+} (2 mM). The table inset shows the fraction of FITC-annexin V-labeled cells assessed 20 min after the indicated treatments.

**FIGURE 2. Ca^{2+} homeostasis during apoptosis.** A, MEF were incubated with STS for 0, 2, 4, and 6 h. The cells were then labeled with Fura-2AM for 30 min at 37 °C. A, bars show the increase in basal cytosolic Ca^{2+} as was assessed by fluorescence at 540 nm (λ_{ex} 340 and 380 nm). At the indicated times, cells were assessed for externalized PS with FITC-annexin V (O). B and C show confocal images of MEF with Rhod-2-labeled mitochondria incubated for 4 h in the absence (B) or presence (C) of STS (0.5 mM).

(continued)
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Aptosis-dependent Increase in Cytosolic Ca\(^{2+}\) Levels Is Associated with PS Exposure—The data presented above suggest a direct relationship between the levels of cytosolic Ca\(^{2+}\) and the propensity of cells to externalize PS. To determine whether PS externalization during apoptosis is also related to cytosolic Ca\(^{2+}\) levels, apoptosis was initiated in MEF with STS. At the indicated times, the cells were washed and labeled with Fura-2AM or Rhod-2AM, and cytosolic Ca\(^{2+}\) levels were estimated by fluorescence spectroscopy and confocal microscopy, respectively (Fig. 2). Cells incubated with STS demonstrated a progressive increase in cytosolic Ca\(^{2+}\) levels and a concomitant increase in the fraction of annexin V-positive, PS-expressing, cells. Consistent with these observations, the cytosol of Rhod-2-labeled control cells did not fluoresce, indicating very low preapoptosis Ca\(^{2+}\) levels (Fig. 2B). Upon apoptosis, however, a dramatic increase in the intensity of cytosolic fluorescence was observed (Fig. 2C), indicating a significant increase in the concentration of cytosolic Ca\(^{2+}\). Taken together, with the TG results shown in Fig. 1, these data suggest that the propensity of cells to externalize PS is directly dependent on sustained increases in cytosolic Ca\(^{2+}\).

Rhod-2AM Labeling of Endosomes and Lysosomes—In contrast to the typical mitochondrial staining pattern obtained after incubation of the cells with Rhod-2AM at 4°C (Figs. 2B and 3A), cells exhibited prominent staining of relatively large vacuole-like organelles when incubated with the probe at 37°C (Fig. 3B). To identify these organelles, the cells were labeled with known organelle tracking dyes. Double labeling of the cells with MitoTracker green (mitochondria label), NBD-C6-ceramide (Golgi and ER label), or ER tracker green (endoplasmic reticulum label) together with Rhod-2AM at 37°C indicated that these organelles were distinct from mitochondria, Golgi, and ER, respectively (Fig. 3, C–E).

Because the morphology and size of the Rhod-2-labeled organelles were consistent with endocytic vesicles, we determined whether these structures were pinocytotic Ca\(^{2+}\)-containing vesicles by incubating cells with Rhod-2AM in the presence of the aqueous space marker FITC-labeled dextran or the lysosome tracer, LysoTracker Green. Both the probes co-localized to the Rhod-2AM-labeled organelles, indicating that these structures were indeed endosomes (Fig. 3F) and/or lysosomes.
in cells incubated in the absence of Ca\textsuperscript{2+} did not fluoresce, suggesting that these vesicles were indeed Ca\textsuperscript{2+}-free (Fig. 5A). Upon apoptosis (Fig. 5E and analysis of DNA fragmentation, not shown), however, both populations exhibited cytosolic Rhod-2 fluorescence (Fig. 5, B and D). Because the Mg\textsuperscript{2+}-incubated cells did not fluoresce before the initiation of apoptosis (Fig. 5A), the source of Rhod-2 fluorescence was likely from increased cytosolic Ca\textsuperscript{2+} that originated from organelles other than endocytotic vesicles. Importantly, although the Mg\textsuperscript{2+}-incubated cells were apoptotic, as assessed by activation of caspases (Fig. 5E), PS externalization was inhibited (Fig. 5B). On the other hand, the Ca\textsuperscript{2+}-incubated vesicles redistributed both Ca\textsuperscript{2+} from the endocytotic vesicles to the cytosol (Fig. 5, C versus D, red fluorescence) and PS from the inner-to-outer membrane leaflet (annexin V-positive; Fig. 5D). As expected, assessment of cytosolic Ca\textsuperscript{2+} with Fura2 revealed that lysosomes contributed a significant amount of imported exogenous Ca\textsuperscript{2+} to the cytosol upon apoptosis (Fig. 5F).

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**Role of Endocytotic Vesicle Ca\textsuperscript{2+} in PS Externalization**—To determine the fate of vesicular Ca\textsuperscript{2+} in apoptosis-induced PS externalization, endosomes were specifically labeled by incubating MEF in the presence of the cell-impermeable Ca\textsuperscript{2+} probe, CG1, and the aqueous space marker dextran red. Confocal microscopy of control and apoptotic cells showed that both probes redistributed in an independent manner (Fig. 4). In control cells, the vesicles adopted a preferential perinuclear distribution, and essentially all of them were labeled with both fluorescent probes (Fig. 4A). In apoptotic cells, however, the CG1 fluorescence appeared to be cytosolic, suggesting that Ca\textsuperscript{2+} entrapped within the vesicles redistributed to the cytosol in an apoptosis-dependent manner (Fig. 4B). The average intensity of CG1 fluorescence in the cytosol of apoptotic cells was \( \sim \)1.7-fold higher than in control cells (Fig. 4, center panels).

Although these data raise the possibility that vesicular Ca\textsuperscript{2+} plays a role in apoptosis-dependent PS externalization, it is not clear whether this is an obligatory source of Ca\textsuperscript{2+}. To unequivocally determine whether vesicular Ca\textsuperscript{2+} is required for PS externalization, MEF were incubated in Ca\textsuperscript{2+}-free medium that was substituted with 2 mM Mg\textsuperscript{2+} during the time course of the experiment. The rationale for this experimental design was to generate cells that contained Ca\textsuperscript{2+}-free endocytotic vesicles and trigger apoptosis and to determine the propensity of the apoptotic cells to express PS on their outer membrane leaflet. MEF were labeled with Rhod-2AM at 37°C for 1 h, and the distribution of fluorescence was monitored before and after triggering apoptosis. The data presented in Fig. 5 indicate that, in contrast to control cells incubated in the presence of extracellular Ca\textsuperscript{2+} (Fig. 5C), the Rhod-2-containing vesicles

**DISCUSSION**

Apoptosis is a highly regulated process that involves multiple upstream checkpoints that control a variety of sequentially activated pathways. For example, the release of Ca\textsuperscript{2+} from the ER
and its subsequent uptake into mitochondria initiate organelle remodeling that results in loss of mitochondrial membrane potential (16–18, 29), opening of the permeability transition pore, and release of factors that trigger apoptosis. Clearly, Ca²⁺ homeostasis and alterations in amplitude and its spatial and temporal patterns play a central role in regulating cell death.

Ca²⁺ has also been shown to play a critical role in the movement of PS from the inner-to-outer bilayer leaflet of the cells. Unlike the complex Ca²⁺ transients involved in apoptosis, PS externalization only requires temporal elevations in cytosolic [Ca²⁺]. Because PS externalization is one of the earliest consequences of apoptosis that precedes cytochrome c release and caspase activation, we initiated an investigation into the source of Ca²⁺ that might regulate the redistribution of PS between bilayer leaflets.

The data presented in Fig. 1 indicate that PS externalization is associated with elevation of cytosolic Ca²⁺. However, the TG data indicate that PS externalization requires not only elevated cytosolic Ca²⁺ levels but that these levels need to persist to initiate the transbilayer redistribution of PS. Similarly, treatment of cells with apoptosis-inducing agents resulted in concomitant increases in both cytosolic Ca²⁺ and cell surface PS (Fig. 2).

Preliminary experiments to determine optimal labeling of MEF mitochondria with Rhod-2AM revealed that organelle specificity was lost when the probe was incubated with the cells at 37 °C (Fig. 3A). Organelle-specific probes showed that the Rhod-2 colocalized with both endosomes and lysosomes (Fig. 3, F and G). Because the fluorescence of Rhod-2 is directly proportional to [Ca²⁺], these organelles likely contained relatively high [Ca²⁺]. Indeed, endocytotic pathways can provide a significant amount of cellular calcium because uptake of the ion takes place through pinocytosis of the extracellular medium (30). Using rhodamine-labeled dextran in combination with the cell-impermeable Ca²⁺ probe, CG1, we showed that upon apoptosis, both probes redistributed independently (Fig. 4). Although the rhodamine-dextran was retained within the vesicles, the redistribution of CG1 fluorescence indicated that both Ca²⁺ and CG1 were transferred from the vesicles to the cytosol.
Taken together, these data are consistent with endocytosed Ca\(^{2+}\) being released from the lumen of lysosomes and used to mediate membrane events that regulate PS externalization.

To directly determine the potential contribution of vesicular Ca\(^{2+}\) to PS externalization, the propensity of MEF containing Ca\(^{2+}\)-free and Ca\(^{2+}\)-containing vesicles to undergo cell death and externalize PS was determined. This key experiment demonstrated that although the Ca\(^{2+}\) present in intracellular organelles (ER and mitochondria) is sufficient for apoptosis, the spatial redistribution of vesicular Ca\(^{2+}\) to the cytosol was critical for PS externalization (Fig. 5).

Because cytosolic PLA2 is known to affect lysosome stability (27, 28), it is possible that activated PLA2 promotes the release of lysosomal Ca\(^{2+}\). To test this, MEF were triggered into apoptosis in the absence and presence of the PLA2 inhibitor cPLA2. The data presented in Fig. 6 indicate that inhibition of PLA2 reduced the level of cytosolic Ca\(^{2+}\) by ∼40% but completely inhibited PS externalization. Because cytosolic Ca\(^{2+}\) levels were not completely inhibited (Fig. 6F) and the Rhod-2-labeled lysosomes remained intensely fluorescent (Fig. 6D), it is possible that the increase observed was due to release from other organelles. Because PLA2 is activated with Ca\(^{2+}\), these data raise the possibility that Ca\(^{2+}\) released from other organelles during apoptosis activates the enzyme, which in turn destabilizes the lysosomes to release additional Ca\(^{2+}\) to levels that are sufficient for PS externalization.

In summary, the data presented here indicate that although apoptosis and PS externalization are both regulated through a common cytosolic Ca\(^{2+}\)-dependent pathway, PS externalization requires higher cytosolic [Ca\(^{2+}\)] that is only achievable through the contribution of imported vesicular Ca\(^{2+}\), which is released to the cytosol in an apoptosis-dependent manner. Although these studies addressed the source of Ca\(^{2+}\), the actual mechanism by which increased cytosolic Ca\(^{2+}\) redistributes PS between bilayer leaflets remains elusive. Our data do, however, establish the concept that a vesicular Ca\(^{2+}\)-dependent signaling mechanism is primarily responsible for the regulation of PS externalization during apoptosis.

Acknowledgments—We thank Johanna Ramoth for technical assistance and Drs. Andreas Bergman and Joya Chandra for suggestions and continued support.

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