

# Early Redistribution of Plasma Membrane Phosphatidylserine Is a General Feature of Apoptosis Regardless of the Initiating Stimulus: Inhibition by Overexpression of Bcl-2 and Abl

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## Summary

A critical event during programmed cell death (PCD) appears to be the acquisition of plasma membrane (PM) changes that allows phagocytes to recognize and engulf these cells before they rupture. The majority of PCD seen in higher organisms exhibits strikingly similar morphological features, and this form of PCD has been termed apoptosis. The nature of the PM changes that occur on apoptotic cells remains poorly defined. In this study, we have used a phosphatidylserine (PS)-binding protein (annexin V) as a specific probe to detect redistribution of this phospholipid, which is normally confined to the inner PM leaflet, during apoptosis. Here we show that PS externalization is an early and widespread event during apoptosis of a variety of murine and human cell types, regardless of the initiating stimulus, and precedes several other events normally associated with this mode of cell death. We also report that, under conditions in which the morphological features of apoptosis were prevented (macromolecular synthesis inhibition, overexpression of Bcl-2 or Abl), the appearance of PS on the external leaflet of the PM was similarly prevented. These data are compatible with the notion that activation of an inside-outside PS translocase is an early and widespread event during apoptosis.

Programmed cell death (PCD)<sup>1</sup> is a fundamental feature of many important biological processes (1–4). Mammalian cells, as well as many other cell types, typically undergo a series of distinct morphological changes as they die; this form of PCD has been termed apoptosis (5). This subset of PCD appears to account for the majority of PCD in higher organisms.

A critical stage of apoptosis involves the acquisition of surface changes by the dying cell that result in the recognition and uptake of these cells by phagocytes (6–9). The nature and kinetics of these surface changes are the subject of active investigation but are still largely unknown (10). A series of studies has shown that apoptotic human neutrophils, as well as some other cell types, are recognized by bone marrow- and blood-derived macrophages by means of a

thrombospondin (TSP)/ $\alpha_v\beta_3$  (vitronectin receptor [VnR])/CD36 complex, which interacts with an as yet unidentified “charge-sensitive” moiety on the apoptotic cell (9–14).

There is also evidence to suggest that phosphatidylserine (PS), which is normally almost totally confined to the inner leaflet of the plasma membrane (PM), may act as a membrane “flag” on apoptotic cells (15–17). Macrophages elicited from the peritoneal cavities of mice specifically recognized apoptotic cells in a manner that was inhibited by liposomes containing PS, but not by liposomes containing other aminophospholipids (15, 16). However, because of the lack of a specific probe for PS, changes in the distribution of this aminophospholipid during apoptosis have yet to be demonstrated directly.

A recently discovered family of proteins, the annexins, has been found to have high affinity for aminophospholipids in the presence of  $\text{Ca}^{2+}$  ions (18–20). A member of this family, annexin V, has been shown by several groups to preferentially bind PS (18, 19, 21) and to inhibit PS-dependent procoagulant reactions (22). Annexin V has also been used successfully as a probe of PS exposure during platelet

<sup>1</sup>Abbreviations used in this paper: Act D, actinomycin D; CHX, cycloheximide; Dex, dexamethasone; PA, phosphatidic acid; PC, phosphatidylcholine; PCD, programmed cell death; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PM, plasma membrane; PS, phosphatidylserine; SM, sphingomyelin; TSP, thrombospondin; VnR, vitronectin receptor; VP-16, etoposide.

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activation, a major source of procoagulant activity (21, 23). Thus, annexin V provides a convenient tool with which to determine directly whether changes in membrane PS distribution occur as a general feature of apoptosis and to measure the kinetics of these changes on a single-cell basis. In this regard, a recent report demonstrated increased annexin V binding during serum withdrawal-induced apoptosis of murine germinal center B cells as well as a B cell line (24).

Here we show that apoptosis, induced by a variety of agents, was accompanied by dramatic changes in PS distribution at the PM, as assessed by the increased annexin V-binding properties of these cells. Notably, changes in PS asymmetry were detected before the morphological changes associated with apoptosis had occurred; the changes preceded loss of membrane integrity by several hours. PS redistribution was detected during apoptosis of all cells of human and mouse origin tested and in response to all stimuli that resulted in apoptosis. Significantly, repressors of apoptosis such as Bcl-2 and Abl were also found to inhibit PS externalization, providing further evidence that this event is tightly coupled to apoptosis.

## Materials and Methods

**Cell Culture and Reagents.** All cell lines were cultured in RPMI 1640 containing 5% FCS and were routinely subcultured every 2–3 d. Cells were grown at 37°C in a humidified 5% CO<sub>2</sub> atmosphere and were seeded at a density of 0.5–1 × 10<sup>6</sup> cells/ml in most experiments. Cycloheximide (CHX), actinomycin D (Act D), dexamethasone (Dex), etoposide (VP-16), staurosporine, hydrogen peroxide, purified phospholipids, and Percoll were all purchased from Sigma Chemical Co. (St Louis, MO). Anti-Fas IgM mAb (CH-11) was purchased from Kamiya Biomedical Co. (Thousand Oaks, CA). N-acetyl sphingosine (C<sub>2</sub>-ceramide) was purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA).

**Isolation of Human Neutrophils.** Human neutrophils were purified from heparinized blood drawn from normal healthy volunteers, according to standard procedures (9).

**Isolation of Murine Thymocytes.** Thymi were removed from 6–16-wk-old mice and were disrupted between sterile glass slides to liberate thymocytes. Thymocytes were then washed three times in HBSS. Thymocytes prepared in this way were routinely 99% viable, as assessed by trypan blue exclusion assay.

**Bcl-2 and Abl-transfected Cell Lines.** CEM<sub>neo</sub> and CEM<sub>bcl-2</sub> cells, stably transfected with the retroviral vectors pZipNeo and pZip-Bcl-2, respectively, were kindly provided by Dr. John Reed (La Jolla Cancer Research Foundation, La Jolla, CA). Transfectants were recloned by limiting dilution and were screened for Bcl-2 expression by Western blot (data not shown). HL-60 cells were transfected with a plasmid containing the Abl temperature-sensitive mutant, pRK-160, or a control plasmid (pRPP4), the kind gift of Dr. Jean Wang (University of California, San Diego, CA). Cells were then selected for 5 d in G418 and cloned by limiting dilution, followed by screening for Abl expression by Western blot (McGahon, A. J., S. J. Martin, R. P. Bissonnette, T. G. Cotter, and D. R. Green, manuscript submitted for publication).

**Propidium Iodide Uptake Assay.** Cell membrane permeability was assessed by determining the ability of cells to exclude the

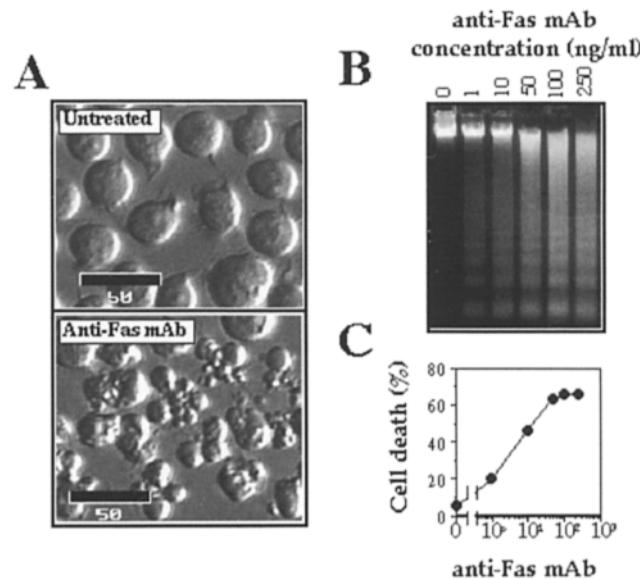
DNA-binding fluorescent dye propidium iodide as previously described (25).

**Cell Cycle Analysis.** The decreased binding of DNA-binding dyes to apoptotic cells (these cells appear as a distinct peak below the G<sub>0</sub>/G<sub>1</sub> peak) allows the discrimination of apoptotic cells from their healthy counterparts by conventional cell cycle analysis (26). Cell cycle analysis was performed as previously described (25).

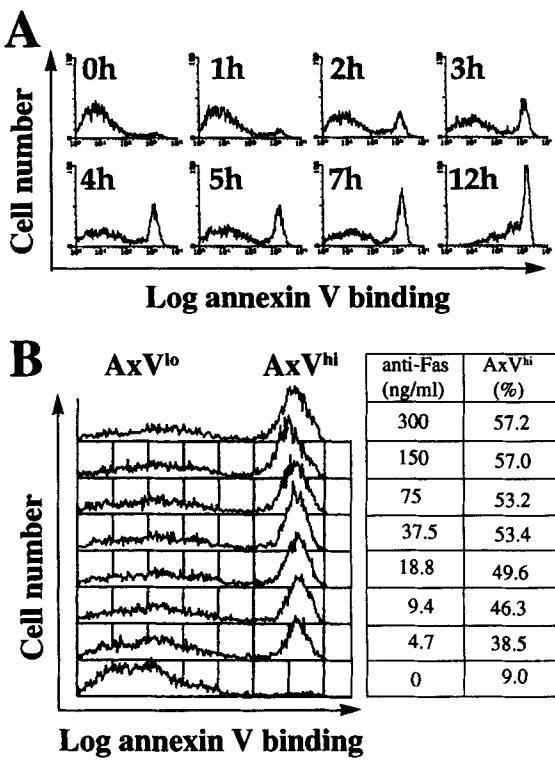
**Scoring of Apoptosis.** Apoptotic cells were identified using previously published criteria (5). Briefly, cytocentr preparations of cells were stained with Leukostat (Fisher Scientific Co., NY) to reveal nuclear morphology. Apoptotic cells were discriminated from healthy cells by the condensed and fragmented appearance of their nuclei under light microscopy.

**Annexin V-FITC Preparation and Binding Assays.** Recombinant annexin V was produced and purified as previously described (27). Cells were assessed for binding of annexin V by incubating for 5 min in 0.1–1 µg/ml FITC-conjugated annexin V (1:1 stoichiometric complex) in Hepes buffer (10 mM Hepes-NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>), as described (24), followed by flow cytometry. To distinguish cells that had lost membrane integrity, propidium iodide dye was added to a final concentration of 10 µg/ml before analysis.

For inhibition experiments, liposomes composed of 70% phosphatidylcholine (PC) and 30% of each of the phospholipids PS, phosphatidylinositol (PI), phosphatidic acid (PA), sphingomyelin (SM), and phosphatidylethanolamine (PE), or 100% PC, were prepared as follows. Pure preparations (10 mg/ml) of lipids in chloroform/methanol (90:10) were added to glass tubes in appropriate combinations and were evaporated to dryness under N<sub>2</sub> gas. Lipid mixtures were then resuspended in Hepes buffer, to a final concentration of 10<sup>-3</sup> M, by vortexing. Liposomes were then prepared by sonicating the lipid mixtures on ice for 3–5 min



**Figure 1.** Induction of apoptosis by ligation of Fas (CD95) on Jurkat cells. (A) Cells were cultured in either medium alone or medium containing 250 ng/ml of anti-Fas IgM for 7 h and viewed under phase contrast microscopy. The scale bar is in arbitrary units. (B) Detection of internucleosomal DNA fragmentation in Jurkat cells treated with the indicated concentrations of anti-Fas IgM for 7 h. (C) Cell death, as assessed by propidium iodide uptake, after a 24-h exposure to the indicated concentrations of anti-Fas IgM. Each data point was derived from an analysis of 10,000 cells from a representative experiment.



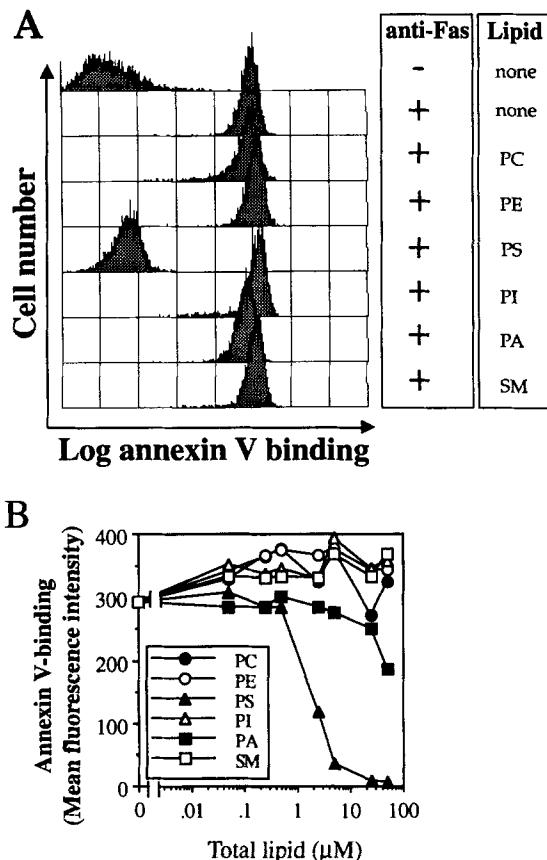
**Figure 2.** Changes in plasma membrane PS distribution during apoptosis induced by Fas ligation. (A) Time course of PS externalization in Jurkat cells exposed to 200 ng/ml anti-Fas IgM, as assessed by the binding of annexin V–FITC. (B) Jurkat cells were exposed to the indicated concentrations of anti-Fas IgM for 7 h. Annexin V binding was assessed by flow cytometry, as described in Materials and Methods. 5,000 cells were analyzed in each condition. Results are representative of six independent experiments.

in a sonifier (model 250; Branson Ultrasonics Corp., Danbury, CT) on low output. Liposomes were used within 24 h of preparation. Before staining cells with annexin V–FITC, liposomes were added to the annexin V–FITC solution at various final concentrations (see Results), followed by incubation for 10 min at room temperature to allow binding of the annexin V. Cells were then stained with the annexin V–liposome mixture in the usual manner, and binding was assessed by flow cytometry.

**DNA Isolation and Electrophoresis.** DNA was isolated and subjected to electrophoresis as previously described (28).

## Results

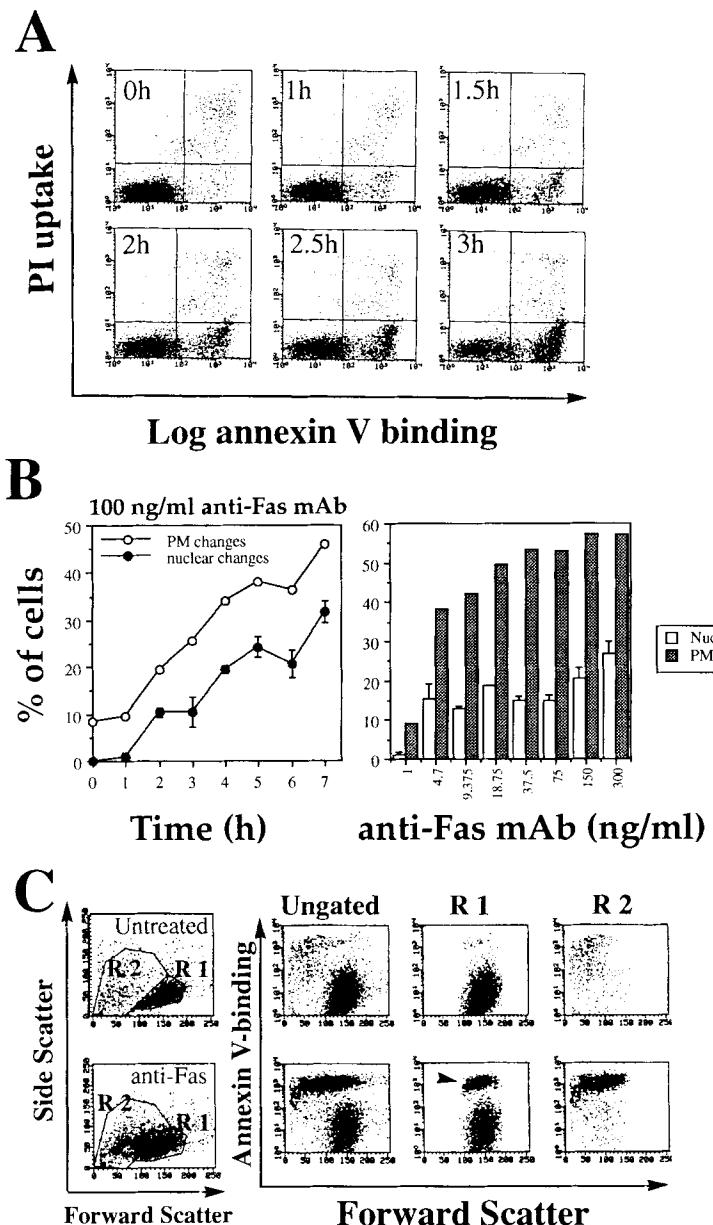
**Induction of Apoptosis in Jurkat Cells by Fas Ligation.** Fas (CD95/Apo-1) is a cell-surface molecule belonging to the TNF/nerve growth factor receptor family, which has previously been shown to transduce a death signal upon ligation by either anti-Fas mAbs (29) or its specific ligand (FasL; 30). Fas–FasL interactions are likely to be important physiological triggers of cell death in the immune system (31) and possibly elsewhere (32). Jurkat, a T lymphocytic leukemia cell line, constitutively expresses Fas and is rapidly killed upon treatment of these cells with anti-Fas antibody (Fig. 1). As in previous reports (29), cells killed under these conditions were found to exhibit all of the characteristic



**Figure 3.** Inhibition of annexin V–FITC binding to apoptotic cells by PS liposomes. Jurkat cells were cultured in medium alone or medium containing 100 ng/ml anti-Fas IgM for 6 h, followed by staining with annexin V–FITC in the presence or absence of the indicated liposomes, prepared as described in Materials and Methods. (A) Annexin V binding to untreated versus anti-Fas IgM-treated Jurkat cells in the presence or absence of 5  $\mu$ M of the indicated liposomes. 10,000 cells were analyzed in each condition. (B) annexin V binding to anti-Fas IgM-treated Jurkat cells in the presence or absence of the indicated concentrations of liposomes. 10,000 cells were analyzed in each case. Data are representative of two separate experiments.

features of apoptosis, including dramatic PM blebbing and collapse of cells into numerous intact vesicles (apoptotic bodies; Fig. 1 A), cleavage of DNA into nucleosomal multiples (Fig. 1 B), and, ultimately, uptake of vital dyes, such as propidium iodide (Fig. 1 C).

**Aminophospholipid Redistribution at the PM Is Rapidly Detected upon Ligation of Fas on Jurkat Cells.** The PM of a healthy cell generally exhibits an asymmetric distribution of its major phospholipids. Typically, the inner PM leaflet contains essentially all PS, most PE and PI, with SM largely confined to the outer leaflet, and PC distributed equally between both leaflets (33). As outlined above, because of its high affinity for PS, annexin V is a convenient probe for monitoring changes in the distribution of this phospholipid in the PM during apoptosis. To determine whether PS externalization occurred during apoptosis induced by Fas ligation, Jurkat cells were treated with anti-Fas antibody and probed for changes in surface PS expression at various times



**Figure 4.** PS exposure on the external PM leaflet precedes some of the other features of apoptosis. (A) Kinetics of PS externalization, as assessed by annexin V–FITC binding, versus loss of PM integrity, as assessed by propidium iodide uptake, in Jurkat cells treated with 1  $\mu$ g/ml of anti-Fas IgM. 10,000 cells were analyzed at each time point. (B) Correlation between the appearance of PS surface-positive cells and cells with morphological features of apoptosis after exposure to anti-Fas mAb. In the dose-response experiment (right), cells were exposed to anti-Fas IgM for 7 h. Quantitation of annexin V binding was performed on 5,000 cells at each time point. The proportion of cells with apoptotic nuclei was assessed on cytopsins of cells made at the same time points. Results are the mean of four randomly selected fields, with a minimum of 100 cells scored per field. (C) Detection of annexin V binding before cell shrinkage, as assessed by changes in light scattering properties of the cells, during Fas-induced apoptosis. Jurkat cells were either left untreated (top) or were exposed to 300 ng/ml of anti-Fas IgM (bottom) for 5 h. Apoptotic cells (R2) can be discriminated from normal cells (R1) on the basis of their light scattering properties (left), as indicated.

thereafter. As shown in Fig. 2 A, before treatment with anti-Fas antibody, only one population of cells was detected that displayed a uniform low binding to annexin V–FITC ( $AxV^{lo}$ ). However, within 1 h of Fas ligation, a population of cells with dramatically increased annexin V-binding properties ( $AxV^{hi}$ ) began to emerge in these cultures and increased rapidly thereafter. Similar time-dependent increases in  $AxV^{hi}$  cells were observed over a wide range of anti-Fas antibody concentrations (Fig. 2 B).

**Annexin V Binding to Apoptotic Cells Is Inhibited by PS but not Other Phospholipids.** To confirm that annexin V binding to apoptotic cells reflected the exposure of PS on these cells, anti-Fas IgM-treated Jurkat cells were stained with annexin V–FITC in the presence or absence of liposomes prepared from a panel of phospholipids (see Materials and Methods). Fig. 3 A demonstrates that annexin V binding to

anti-Fas mAb-treated cells was almost completely inhibited in the presence of 5  $\mu$ M PS liposomes, but remained unchanged in the presence of equal amounts of liposomes prepared from PC, PE, PI, PA, and SM. Half-maximal inhibition of annexin V binding was observed with 2  $\mu$ M PS liposomes (Fig. 3 B). Partial inhibition of annexin V binding was observed with high concentrations of PA liposomes, but not other phospholipids (Fig. 3 B). These data indicate that annexin V binding is highly specific for PS.

**PS Exposure on Apoptotic Cells Precedes Increases in Membrane Permeability and Nuclear Condensation.** Although our initial experiments revealed that externalization of PS accompanied Fas-induced apoptosis of Jurkat cells, it remained a possibility that the appearance of  $AxV^{hi}$  cells coincided with the appearance of cells with “leaky” membranes in these cultures, thereby allowing annexin V to gain access to

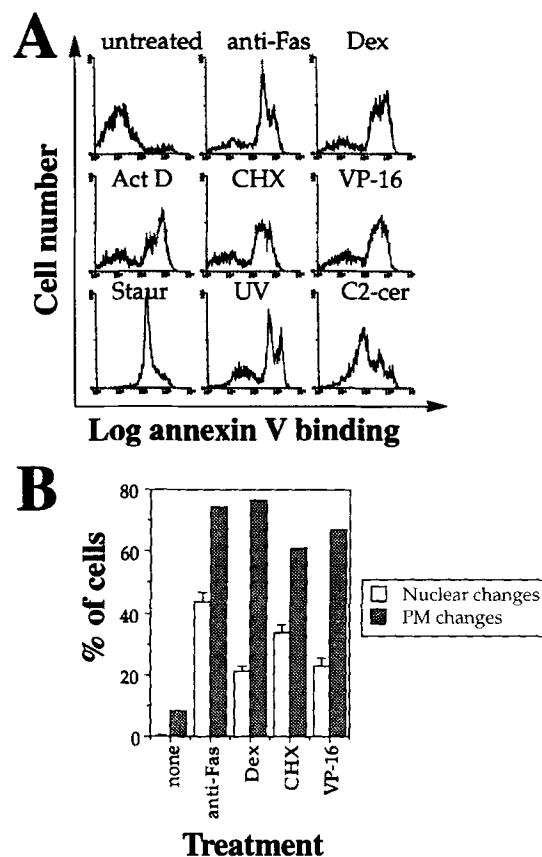
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PS inside the cell. We considered this unlikely since increases in PM permeability typically occur late during apoptosis. However, to exclude this possibility, Jurkat cells treated with anti-Fas IgM were stained with annexin V–FITC along with propidium iodide dye, to reveal cells that bound annexin V and have PM damage. These experiments (Fig. 4 A) demonstrated that a major population of AxV<sup>hi</sup>/propidium iodide<sup>+</sup> cells appeared in anti-Fas antibody-treated cultures (*lower right quadrants*), well before any increases in cell permeability occurred, thus confirming that annexin V was indeed binding to the external PM leaflet of these cells.

To determine whether apoptosis-associated changes in PM lipid asymmetry occurred coincident with or before the classical morphological features of apoptosis, Jurkat cells were induced to undergo apoptosis by Fas ligation, and the appearance of cells with morphological features of apoptosis in these cultures was monitored over time by directly enumerating these cells on stained cytospun cell preparations. At each time point, the proportion of AxV<sup>hi</sup> cells in the same cultures was assessed by flow cytometry. As shown in Fig. 4 B, AxV<sup>hi</sup> cells in anti-Fas IgM-treated Jurkat cultures were found in greater abundance at each time point than cells that were apoptotic on morphological criteria. Furthermore, using another parameter of apoptotic cell death—cell shrinkage (which can be measured by flow cytometry as a change in the light scattering properties of these cells), AxV<sup>hi</sup> cells were also found in abundance in the cell population that had not yet undergone any reduction in cell size in anti-Fas IgM-treated cultures (Fig. 4 C; *region 1, arrow*). Taken together, these data suggest that redistribution of PS during apoptosis may precede the nuclear condensation and cell shrinkage events also seen during this process.

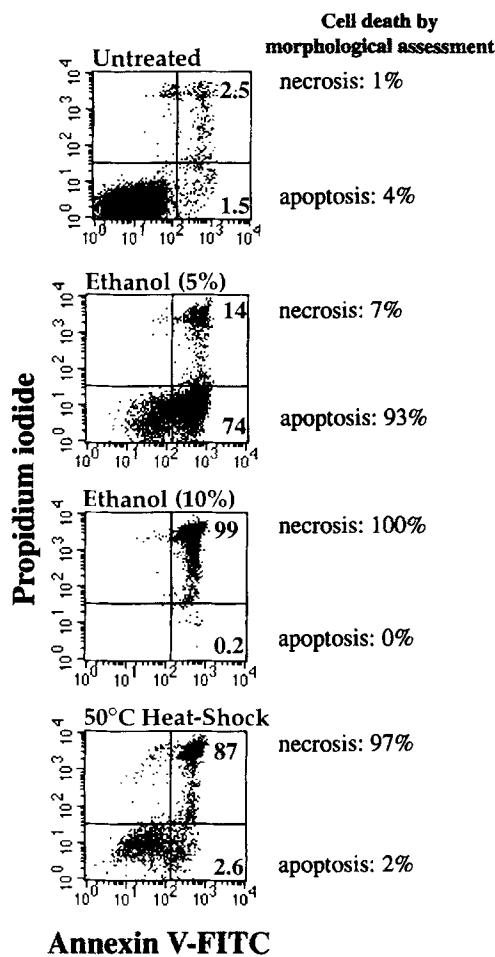
**PS Exposure during Apoptosis Is Stimulus Independent.** Because membrane changes are fundamental features of apoptosis, these changes should occur regardless of the initiating stimulus. To determine whether this was true with respect to PS externalization, we exposed Jurkat cells to a range of apoptosis-inducing agents (34–37). Fig. 5 A demonstrates that PS exposure accompanied apoptosis of Jurkat cells in response to all of the agents tested. Correlating the proportion of apoptotic cells, based on morphological criteria, with the proportion of AxV<sup>hi</sup> cells in each culture at the same time point, it was again found that changes in PM phospholipid distribution consistently preceded the nuclear changes associated with apoptosis (Fig. 5 B). The appearance of AxV<sup>hi</sup> cells in these cultures also preceded propidium iodide dye uptake, as in previous experiments (data not shown).

**Annexin V Binding to Necrotic Cells Occurs Only as a Consequence of Membrane Rupture.** An essential difference between apoptosis and necrosis is the kinetics of loss of membrane integrity during these forms of cell death. Whereas apoptotic cells retain the ability to exclude vital dyes for several hours, necrotic cells undergo rapid swelling and lysis within minutes (1, 5, 36). To confirm that increases in annexin V binding occur during necrosis coincident with loss of membrane integrity, Jurkat cells were exposed to apop-



**Figure 5.** PS externalization on Jurkat cells during apoptosis induced by diverse stimuli. (A) Cells were cultured for 7 h in either medium alone or in the presence of 300 µg/ml anti-Fas IgM, 10 µM Dex, 10 µM Act D, 100 µM CHX, 100 µM etoposide (VP-16), and 2.5 µM staurosporine (Staur) after irradiation for 15 min on a UVB transilluminator (UV), or 50 µM C<sub>2</sub>-ceramide (C<sub>2</sub>-cer), as indicated. 5,000 cells were analyzed in each condition. (B) Correlation between the appearance of cells with changes in PM phospholipid distribution and cells with morphological features of apoptosis (assessed on stained cytospun cell preparations) in Jurkat cultures exposed to the indicated stimuli at the concentrations described in A. Morphological data represent the means of four randomly selected fields, with a minimum of 100 cells counted per field.

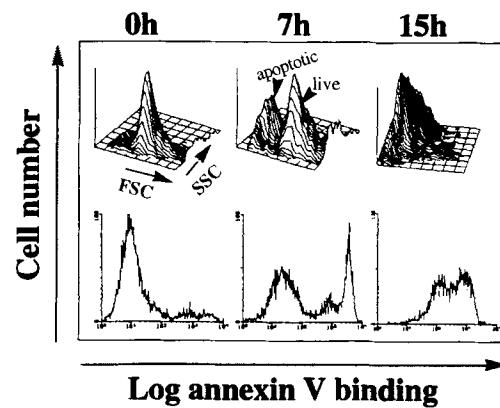
tosis-inducing concentrations of ethanol as well as necrosis-inducing concentrations of ethanol or heat shock, as previously described for a variety of cell types (38). Fig. 6 illustrates that Jurkat cells undergoing apoptosis in response to low-dose ethanol treatment bound annexin V–FITC well before any increase in membrane permeability, as before (Fig. 4 A). In contrast, Jurkat cells undergoing necrosis at a higher dose of ethanol, or because of 50°C heat shock, exhibited increases in annexin V–FITC binding only when membrane integrity was lost (Fig. 6). Very few cells in cultures undergoing necrosis exhibited increased annexin V-binding properties in the absence of vital dye uptake, which implies that increased annexin V binding to necrotic cells can be attributed solely to entry of annexin V into the cell and binding to PS on the inner leaflet of the plasma membrane. Alternatively, because PM disruption during necrosis is extensive, membrane lipids are likely to redis-

**Annexin V-FITC**

**Figure 6.** Annexin V binding and propidium iodide uptake of Jurkat cells undergoing apoptosis versus necrosis. Jurkat cells were induced to undergo apoptosis by exposure to 5% ethanol for 90 min or induced to undergo necrosis by exposure to 10% ethanol or 50°C heat shock, for a similar period, followed by staining with annexin V-FITC and propidium iodide. Cells (10,000 per treatment) were then analyzed by flow cytometry. Numbers within the dot plots represent the percentage of cells in each quadrant. The percentage of apoptotic or necrotic cells was also assessed at the same time point by direct morphological assessment on cytospin preparations of cells from each culture. Results are derived from the means of four randomly selected fields, with a minimum of 100 cells counted per field.

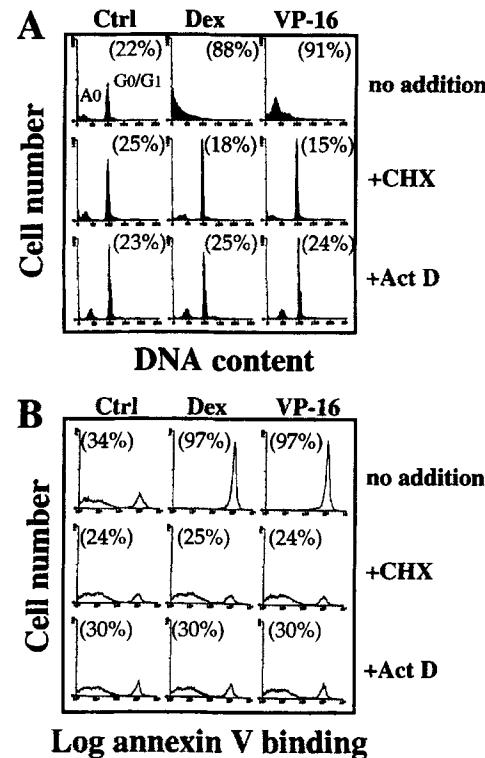
tribute rapidly to both sides of the PM by simply flowing around rupture points.

**PS Externalization Is a Widespread Phenomenon during Apoptosis of Cells of Human and Murine Origin.** To explore further whether loss of PS asymmetry is a general accompaniment to apoptosis, we extended our studies to other cell types. The A1.1 murine T cell hybridoma has previously been found to undergo apoptosis as a consequence of activation via the TCR-CD3 complex (39). Activation-induced cell death of A1.1 cells has also recently been shown to be a Fas-dependent phenomenon (40). Using this system, we observed that A1.1 cells also externalized PS coincident with their entry into apoptosis (Fig. 7). As in the previous experiments with Jurkat cells, there was a dra-



**Figure 7.** Changes in PS asymmetry during activation-induced cell death of a murine T cell hybridoma. A1.1 cells were cultured for the indicated times in the presence of plate-immobilized anti-CD3 mAb (145-2C11) and were then analyzed for apoptosis-associated changes in light scattering properties (*top*), and PS redistribution (*bottom*).

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**Figure 8.** Redistribution of PS during apoptosis of murine thymocytes is protein and RNA synthesis dependent. Freshly isolated thymocytes were cultured for 16 h in medium alone (Ctrl) or medium containing 1  $\mu$ M Dex or 10  $\mu$ M VP-16, in the presence or absence of 50  $\mu$ M CHX or 10  $\mu$ M Act D, as indicated. (*A*) Assessment of apoptosis by cell cycle analysis. Apoptotic cells (A<sub>0</sub>) appear below the G<sub>0</sub>/G<sub>1</sub> peak of cells, as shown. Numbers in parentheses refer to the percentage of apoptotic cells detected in each condition. (*B*) Detection of cells with PS redistribution during exposure to the same treatments as indicated in *A*. Numbers in parentheses refer to the percentage of cells exhibiting increased annexin V binding in each condition. Results are representative of six separate experiments. 10,000 cells were analyzed under each condition.

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matic and rapid increase in AxV<sup>hi</sup> cells concomitant with the appearance of apoptotic cells in A1.1 cultures exposed to immobilized anti-CD3 antibody.

Several other cell types that have been extensively studied in relation to apoptosis were also examined: the HL-60 human promyelocytic leukemia cell line (35, 38); CEM, a T lymphoblastoid line (28); normal murine thymocytes (34); and human peripheral blood neutrophils (9, 11–13). Cells were induced to die by treatment with a variety of stimuli known to trigger apoptosis in these cells (see legends to Figs. 8–10). In each case, the induction of apoptosis in all of these cell types correlated with the rapid appearance of AxV<sup>hi</sup> cells in these cultures (Figs. 8 and 9 and data not shown). Annexin V binding in cultures undergoing apoptosis preceded propidium iodide uptake in all cases (data not shown).

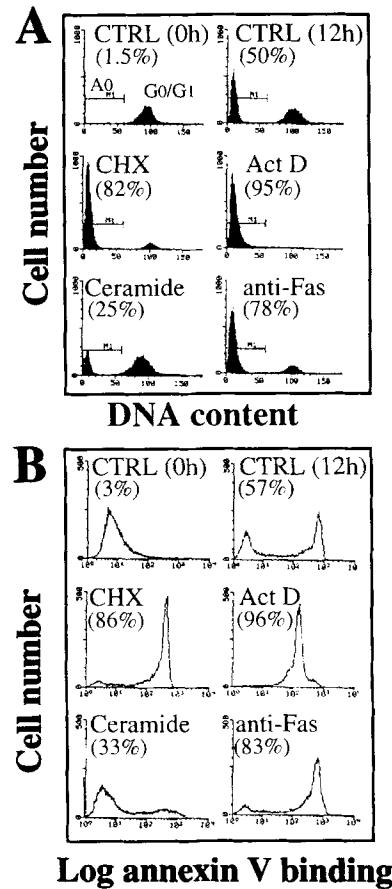
Because thymocyte apoptosis is one case where apoptosis can be blocked by protein or RNA synthesis inhibitors (34), we tested whether these inhibitors could also effectively block the appearance of AxV<sup>hi</sup> cells in thymocyte cultures treated with dexamethasone or VP-16. Fig. 8 A shows that Dex- or VP-16-induced thymocyte apoptosis, as assessed by the appearance of cells with sub-G<sub>0</sub>/G<sub>1</sub> DNA content (25), was blocked by macromolecular synthesis inhibition, as was the appearance of AxV<sup>hi</sup> cells in the same cultures (Fig. 8 B). Under all conditions, the majority of thymocytes that exhibited increased annexin V-binding characteristics excluded propidium iodide dye (data not shown).

Freshly isolated peripheral blood neutrophil populations contained few sub-G<sub>0</sub>/G<sub>1</sub> (apoptotic) cells (Fig. 9 A) or AxV<sup>hi</sup> cells (Fig. 9 B). As with thymocytes, agents that either delayed (C<sub>2</sub>-ceramide) or accelerated (Fas ligation, Act D, CHX) the spontaneous apoptosis of neutrophils induced concomitant decreases or increases in the proportion of AxV<sup>hi</sup> cells in these cultures (Fig. 9).

These data provide compelling evidence for a tight association between apoptosis and exposure of PS on the external PM leaflet, regardless of the cell type or apoptosis-inducing stimulus.

**Inhibition of Apoptosis and Membrane Phospholipid Redistribution by Bcl-2 and Abl.** Several genes with the ability to repress apoptosis have been described in recent years. Thus far, apoptosis repressor proteins fall into two main groups: the *bcl-2* and *abl* gene families. Bcl-2 and Abl have both been shown to protect against apoptosis induced by a wide range of stimuli (41, 42). Because the protection from death afforded by Bcl-2 and Abl is generally assessed by either enumerating morphologically apoptotic cells or conducting vital dye assays, we sought to determine whether overexpression of these gene products blocked apoptosis-associated membrane changes as effectively as they blocked the morphological features of apoptosis.

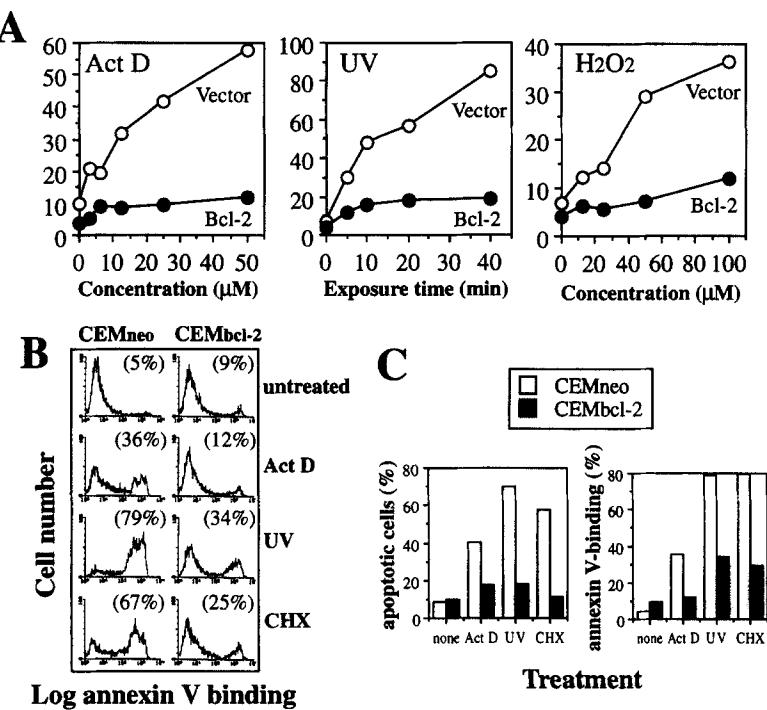
To address this question, cell lines stably transfected with *bcl-2* or *abl* were compared with their vector-transfected counterparts for their ability to resist changes in PM phospholipid distribution during treatment with various apoptosis-inducing stimuli. As shown in Fig. 10 A, CEM<sub>neo</sub>



**Figure 9.** PS externalization during spontaneous as well as Fas- or drug-induced apoptosis of human peripheral blood neutrophils. Freshly isolated peripheral blood neutrophils were incubated for 12 h in either medium alone (untreated) or medium containing 50  $\mu$ M CHX, 10  $\mu$ M Act D, 50  $\mu$ M C<sub>2</sub>-ceramide, or 500 ng/ml anti-Fas IgM. (A) Assessment of apoptosis by cell cycle analysis. (B) Detection of cells with externalized PS during exposure to the same treatments as indicated in A. Results are representative of four separate experiments. 10,000 cells were analyzed under each condition.

(vector-transfected) cells readily underwent apoptosis in response to several stimuli, whereas CEM<sub>bcl-2</sub> (*bcl-2*-transfected) cells were protected under the same conditions. Significantly, CEM<sub>bcl-2</sub> cells were also found to be protected from externalizing PS under conditions in which PS externalization was readily detected in CEM<sub>neo</sub> cells (Fig. 10, B and C), suggesting that Bcl-2 acts upstream of the effector elements that induce these changes.

To determine whether overexpression of Abl also blocked membrane changes during apoptosis, HL-60 cells were transfected with a temperature-sensitive *v-abl* construct (HL-60<sub>abl</sub><sup>ts</sup>) which is active at 32°C but inactive at 39°C. These cells were found to be resistant to the induction of apoptosis at the permissive temperature for Abl but readily died at the restrictive temperature (Fig. 11 A). Similarly, PS externalization on HL-60<sub>abl</sub><sup>ts</sup> cells in response to CHX treatment was blocked at 32°C but not at 39°C (Fig. 11 B), whereas PS externalization in response to CHX treatment



**Figure 10.** Inhibition of apoptosis-associated PM changes by Bcl-2. (A) Death of CEM<sub>neo</sub> (vector-transfected) compared with CEM<sub>bcl-2</sub> cells after exposure to the indicated treatments for 24 h. Cell death was assessed by uptake of propidium iodide. Each data point was derived from an analysis of 5,000 cells. Results shown are representative of six separate experiments. (B) PS externalization detected in CEM<sub>neo</sub> versus CEM<sub>bcl-2</sub> cells cultured for 8 h in either medium alone (untreated), 25  $\mu\text{M}$  Act D, after a 10 min exposure to UV light, or 50  $\mu\text{M}$  CHX. Numbers in parentheses represent the percentage of AxV<sup>hi</sup> cells detected under each condition. Results are from a representative experiment. 5,000 cells were analyzed in each condition. (C) Bcl-2 inhibits apoptotic morphology, as well as apoptosis-associated PS externalization, as assessed by annexin V binding. Cells were exposed for 8 h to the same treatments as indicated in B.

occurred on HL-60<sub>vector</sub> cells at both temperatures (data not shown).

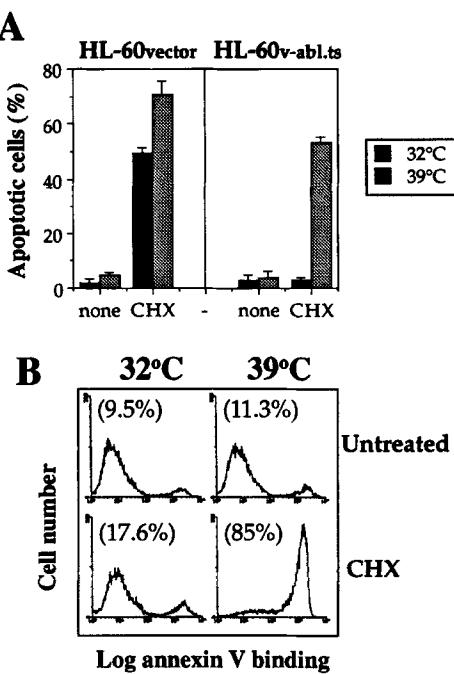
## Discussion

In this study we have shown that PS, normally confined to the internal leaflet of the PM, is externalized during apoptosis in a stimulus-independent manner. Our results also suggest that PS exposure precedes the nuclear changes that define apoptosis and also precedes the loss of membrane integrity by several hours. PS externalization was efficiently blocked under conditions (macromolecular synthesis inhibition, overexpression of Bcl-2 or Abl) in which the morphological features of apoptosis were prevented, suggesting that this event is tightly coupled to apoptosis.

Because increased annexin V binding was detected as an early event in every model of apoptosis tested, this protein should prove to be a very useful general probe for apoptosis, particularly because annexin V appears to detect apoptosis-associated membrane changes on live cells before the nuclear condensation events that occur during this process.

Although this study provides direct evidence that redistribution of PM phospholipids is a widespread event during apoptosis, it remains likely that macrophages "see" apoptotic cells using a variety of receptors. To date, reports in the literature have described at least five separate membrane changes that may lead to recognition of apoptotic cells by phagocytes (Fig. 12). (a) Early studies indicated that changes in membrane carbohydrate structures accompanied apoptosis of murine thymocytes, insofar as macrophage uptake of these cells could be inhibited by N-acetyl glucosamine or N-acetyl galactosamine, implicating a sugar lectin-type interaction in this process (7, 8). (b) Studies by Savill and col-

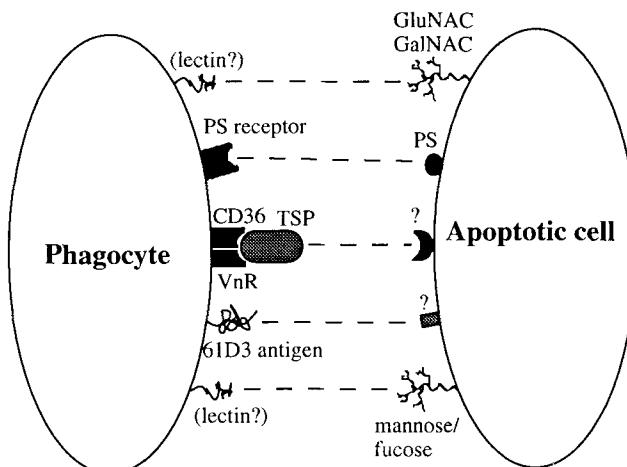
leagues have shown that apoptotic human neutrophils are recognized by blood- or bone marrow-derived macrophages because of the appearance of a "charge-sensitive" TSP-binding moiety on these cells (9–13). The nature of this moiety is still unknown, however. (c) Of particular relevance to this study, Fadok and colleagues (15, 16) have provided convincing evidence for the involvement of PS in the recognition of apoptotic cells by certain macrophage populations. Not all macrophage populations recognized apoptotic cells in a PS-dependent manner; rather, macrophages derived from the peritoneal cavity of mice (an inflammatory site) used a putative PS receptor to bind apoptotic cells, whereas bone marrow- or blood-derived macrophages used the TSP/VnR/CD36 system (15, 16). Interestingly, acquisition of the ability to recognize PS on apoptotic cells was associated with a loss of the ability to recognize cells via the VnR, suggesting that these are mutually exclusive recognition mechanisms (17). (d) Human fibroblasts have also been found to recognize a TSP-binding moiety on apoptotic cells (43). However, these cells also appear to bind apoptotic cells using a mechanism that can be inhibited by mannose or fucose sugars, suggesting that fibroblasts use the VnR as well as a mannose/fucose-specific lectin to bind apoptotic cells (43). (e) Other workers have recently implicated a 75-kD cell-surface receptor, defined by the 61D3 antibody, in the recognition of apoptotic neutrophils and lymphocytes by macrophages (44). In addition, recent studies have found that thymocytes undergoing spontaneous as well as steroid-induced apoptosis up-regulate TCR- $\beta$ /CD3, CD69, and CD25, and down-regulate CD4 and CD8 on their surface (45). It is not yet known whether these changes have any role in the process of thymocyte deletion/recognition in vivo.



**Figure 11.** Inhibition of apoptosis-associated PM changes by Abl. HL-60 cells expressing a temperature-sensitive *v-abl* (*abl.ts*), which is active at 32°C and inactive at 39°C, were cultured in medium alone (untreated) or medium containing 50  $\mu$ M CHX, as indicated. (A) Induction of apoptosis in HL-60<sub>vector</sub> versus HL-60<sub>abl.ts</sub> cells after culture for 6 h under the indicated conditions. Apoptosis was assessed by enumerating apoptotic cells on cytopsized cell preparations, as described in Materials and Methods. Results shown are the means of three randomly selected fields with a minimum of 100 cells counted per field. (B) PS externalization on HL-60<sub>abl.ts</sub> cells treated as indicated in A. 10,000 cells were analyzed in each condition. The numbers in parentheses refer to the percentage of cells exhibiting increased annexin V-binding properties.

The reasons for the apparent use of such a diverse array of receptors for the recognition and uptake of apoptotic cells are not yet understood. It is possible that some of the membrane changes that stimulate macrophage recognition occur only on particular cell types and are not in widespread use. However, it is also likely that there is some redundancy in the macrophage–apoptotic cell interaction, given the critical nature of this interaction in the PCD process. Information from the *Caenorhabditis elegans* model of PCD has already suggested that the recognition and engulfment phases of this process are likely to be complex (2). Of the 11 genes known to be required for normal cell death in this worm, 7 of these (*ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7*, *ced-8*, and *ced-10*) are involved in the engulfment phase (2). It is plausible that this group of genes form several receptor-ligand pairings, much like the scheme depicted in Fig. 12.

The nonfacilitated passage of charged lipids with polar head groups, such as PS, across the PM is extremely slow, on the order of many hours or even days (46). Evidence suggests that transport (flip-flop) of phospholipids from the internal to the external leaflet of the PM is an active process, facilitated by an ATP-dependent membrane translocase. Although elusive for several years, one such translo-



**Figure 12.** Schematic representation of phagocyte–apoptotic cell interactions that have been described to date (see text for details). GluNAC, N-acetyl glucosamine; GalNAC, N-acetyl galactosamine.

case, which appears to be responsible for transporting PC across the PM, has now been identified (47). Rather surprisingly, this PC translocase is a member of the *mdr* (multi-drug resistance) gene family, members of which (*mdr1*; *p-glycoprotein*) have been previously implicated in the ATP-dependent export of a diverse array of hydrophobic chemotherapeutic drugs from malignant cells. The existence of a PC translocase (Mdr2) suggests that a family of similar flippases, specific for other phospholipids, may also exist (46). Therefore, one explanation for the loss of PM phospholipid asymmetry during apoptosis could be the activation of a specific inside–outside PS translocase, which would explain the extremely rapid kinetics of PS externalization on apoptotic cells (Figs. 2, A and B, and 4, A and B, and 6).

Previously, Fadok and colleagues (15) suggested that the hypothetical exposure of PS on the outer PM leaflet of apoptotic cells may be due to the failure of an outside–inside PS translocase that has been implicated in the maintenance of normal PM asymmetry in erythrocytes and platelet membranes (48, 49). However, this would require the spontaneous flip of PS from the inner to the outer PM leaflet, a process that would require several hours. In view of the rapid externalization of PS observed on apoptotic cells in this study (Figs. 2 A, 4 A, and 6), we tend to favor an active model of PS transport during apoptosis.

Studies have also suggested that PM-associated phospholipid-binding cytoskeletal proteins, such as spectrin, may play a role in limiting the movement of phospholipids across the PM under normal circumstances (50). Furthermore, disruption of these cytoskeletal components has been shown to facilitate redistribution of membrane phospholipids (50). In this context, we have recently found that fodrin, the nonerythroid form of spectrin, becomes proteolytically cleaved during many, if not all, forms of apoptosis (37). We are currently investigating the possibility that PS exposure and fodrin proteolysis during apoptosis are linked phenomena.

PS translocation was found to be inhibited under conditions in which apoptosis was inhibited, either because of macromolecular synthesis inhibition (Fig. 8B) or overexpression of Bcl-2 or Abl (Figs. 10B and 11B). Presumably, none of these agents interfered with the activity of the putative PS translocase directly, because these agents also blocked all other apoptosis-associated changes. Thus, the effector element(s) responsible for activating PS translocase activity is likely to be downstream Bcl-2 and Abl in the apoptosis pathway. However, a recent study by Lagasse and Weissman (51) has suggested that not all PM changes that stimulate uptake of senescent cells by macrophages are inhibited by Bcl-2. These workers found that neutrophils from *bcl-2*-transgenic mice were protected from undergoing apoptosis during *in vitro* culture, as compared with neutrophils from nontransgenic control littermates. However, neutrophil numbers in *bcl-2*-transgenic mice remained normal, and Bcl-2-expressing neutrophils were still recognized and engulfed by peritoneal macrophages, despite being morphologically normal in appearance. This suggests that Bcl-2-expressing neutrophils exhibit cell surface changes that allow macrophage recognition and engulfment independent of apoptotic changes.

These data are provocative, particularly given the tight correlation that has been reported for neutrophils exhibiting apoptotic features with the ability of macrophages to bind and engulf these cells (9, 11). One possible explanation for these observations is that, because neutrophils do not normally express Bcl-2, the component(s) of the cell death machinery responsible for triggering membrane changes is regulated in a Bcl-2-independent fashion in these cells. However, it may also be the case that some membrane changes associated with neutrophil senescence are indeed uncoupled from the apoptotic changes (Fig. 12). This possibility remains to be tested.

In conclusion, we have shown that PS transport to the external PM leaflet is a widespread event during apoptosis. We propose that this may be mediated by the early activation of a PS inside–outside translocase, similar to the recently discovered PC translocase Mdr2 (47). Future studies will focus on understanding the intracellular events that result in externalization of PS during apoptosis and determining whether proteolytic cleavage of membrane-associated cytoskeletal proteins and/or a PS-specific translocase plays a role in this process.

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