Non-apoptotic Phosphatidylserine Externalization Induced by Engagement of Glycosylphosphatidylinositol-anchored Proteins*

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The exposure of phosphatidylserine (PS) on the cell surface is a general marker of apoptotic cells. Non-apoptotic PS externalization is induced by several activation stimuli, including engagement of immunoreceptors. Immune cells can also be activated by aggregation of glycosylphosphatidylinositol-anchored proteins (GPI-APs). However, it is unknown whether cell triggering through these proteins, lacking transmembrane and cytoplasmic domains, also leads to PS externalization. Here we show that engagement of GPI-APs in rodent mast cells induces a rapid and reversible externalization of PS by a non-apoptotic mechanism. PS externalization triggered by GPI-AP-specific monoclonal antibodies was dependent on the activity of H−/ATP synthase and several other enzymes involved in mast cell signaling but was independent of cell degranulation, free cytoplasmic calcium up-regulation, and a decrease in lipid packing as determined by merocyanine 540 binding. Surprisingly, disruption of actin cytoskeleton by latrunculin B or plasma membrane integrity by methyl-β-cyclodextrin had opposite effects on PS externalization triggered through GPI-AP or the high affinity IgE receptor. We further show that PS externalization mediated by GPI-APs was also observed in some other cells, and its extent varied with antibodies used. Interestingly, effects of different antibodies on PS externalization were additive, indicating that independent stimuli converge onto a signaling pathways leading to PS externalization. Our findings identify the cell surface PS exposure induced through GPI-AP as a distinct mechanism of cell signaling. Such a mechanism could contribute to “inside-out” signaling in response to pathogens and other external activators and/or to initiation of other functions associated with PS externalization.

The plasma membrane exhibits a marked asymmetry in transbilayer distribution of phospholipids. Aminophospholipids, including phosphatidylserine (PS), are usually restricted to the inner leaflet of the membrane. This phospholipid asymmetry is maintained by activity of energy-dependent flippases and floppases that mediate, respectively, inward-directed and outward-directed transfer of phospholipids (1–3). Furthermore, equilibration of phospholipids between the two plasma membrane leaflets seems to be regulated by lipid scramblase, which facilitates bi-directional migration of phospholipids across the bilayer (4, 5). In response to some stimuli, the phospholipid asymmetry is lost, and PS is translocated to the exoplasmic leaflet of plasma membrane (6). Externalized PS is observed in apoptotic, injured, infected, senescent, or necrotic cells and becomes a target for recognition by phagocytes (7–11). PS externalization is also detected at certain stages of cell development (12) and in the course of activation of immune cells by different stimuli, including engagement of immunoreceptors (13–16).

It has been described that PS externalization is triggered by stimuli enhancing the concentration of free cytoplasmic calcium, which regulates the activities of lipid translocases and promotes randomization of plasma membrane phospholipids (1, 17, 18). This notion was corroborated by experiments indicating that drugs which enhance calcium influx also enhance PS externalization (1). In mast cells, antigen or antibody-mediated aggregation of the high affinity IgE receptor (FcεRI) triggers signaling pathways leading to increased tyrosine phosphorylation of numerous proteins, enhanced concentration of free cytoplasmic calcium [Ca2+], and release of preformed granules (19). This process, called degranulation, is associated with rapid and transient externalization of PS (13, 14). A weak secretory response can also be induced through aggregation of glycosylphosphatidylinositol-anchored proteins (GPI-APs) by monoclonal antibodies (mAbs) (20, 21). However, the exact mechanism of cell activation through GPI-APs, which have no transmembrane and cytoplasmic domain, is unclear. Further...
more, it is not known whether engagement of GPI-AP could also lead to PS externalization. In this study we have analyzed externalization of PS in mast cells and some other cell types after GPI-AP triggering. Our data indicate for the first time that engagement of GPI-AP can induce PS externalization by a distinct mechanism of intracellular signaling.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—The following mAbs were used: MRCOX7 (OX7), specific for Thy-1.1 (22); 1aG4/C5, recognizing Thy-1.2 (23); anti-FcεRIα subunit, clone 5.14 (24); trinitrophenyl (TNP)-specific immunoglobulin (Ig) E (IGEL b4 1) (25); TEC-21, recognizing a GPI-AP TEC-21 (21); D6.17.7, specific for carcinoembryonic antigen (CEA; CD66e) (26); Phosphotyrosine-specific mAb (PY-20) conjugated to horseradish peroxidase was purchased from BD Biosciences. Rat CD48-specific mAb (CD48) was obtained as hybridoma supernatant from Serotec (Oxford, UK). Anti-mouse IgG-cyanine 3 conjugate was bought from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). F(ab) fragment of OX7 mAb was prepared using Immunopure Fab preparation kit (Pierce). The following reagents were used: annexin V—fluorescein isothiocyanate (FITC; BD Biosciences), benzoxycarbonyl-VAD-fluoroanilinoacetate (VAD-FMK) (QBIOGENE-Alexis, Grünberg, Germany), 4-amino-5-(4-chlorophenyl)-7-[(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) inhibitor, methyl-α-D-mannopyranoside (αMM), latrunculin B (Merck-Calbiochem), and Fura-2-AM (Molecular Probes, Eugene, OR). Phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus cereus*, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt dihydrate (DIDS) and all other chemicals were from Sigma-Aldrich.

**Cells**—The origin of rat basophilic leukemia (RBL) cells, clone 2H3, and their transfectant, RBL-gT1.2/1, expressing both the endogenous Thy-1.1 and the transfected Thy-1.2 gene, have been described (27). The cells were grown in complete culture medium consisting of a 1:1 mixture of RPMI 1640 and minimal essential medium supplemented with 10% (v/v) fetal calf serum (FCS), extra d-glucose (2.5 mg/ml), and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). RBL-2H3 cells expressing human CEA, clone RBL-CEA/2D1, were prepared by co-transfection of human CEA cDNA (28), subcloned into the p91023B expression vector (courtesy of R. Kaufman, Genetics Institute, Boston), and psTNeo B vector (29), conferring resistance to the neomycin analogue Genetin (G418). Permanent transfectants were isolated by growing the cells in selective medium containing G-418 (Invitrogen, 400 μg/ml estimated pure G418). Mouse myelomonocytes WEHI-3 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) FCS, antibiotics, 1% minimal essential medium nonessential amino acids, and 10 mM sodium pyruvate. Mouse 3T3 fibroblasts were cultured in complete culture medium. Human colon carcinoma cells, SW1417, were obtained from the American Type Culture Collection and cultured in RPMI 1640 with 10% (v/v) FCS and antibiotics. Isolation and culturing of bone marrow mast cells (BMMC) derived from wild type or linker for activation of T cells (LAT)-deficient mice have been previously described (30). Rat peritoneal mast cells (RPMC) were recovered by peritoneal cavity lavage with phosphate-buffered saline (PBS; 135 mM NaCl, 1.7 mM Na₂HPO₄, 2 H₂O, and 5 mM KH₂PO₄, pH 7.4) followed by density gradient centrifugation over Ficoll gradient (31). The suspensions contained >95% mast cells as determined by staining with 0.1% toluidine blue. Rat peritoneal exudate cells were elicited by intraperitoneal administration of 8 ml of 3% (w/v) thioglycolate in PBS into male rats (Wistar). Elicited peritoneal exudate cells were harvested 5 days later by peritoneal lavage with sterile RPMI 1640, 10% FCS, added to glass Petri dishes, and incubated at 37 °C in 5% CO₂. After 6 h the dishes were flushed to remove non-adherent cells, and those adherent (macrophages) were further incubated in fresh RPMI 1640, 10% FCS. Two days later the cells were collected by trypsinization. Peripheral blood from C57BL/6 mice was obtained by tail bleeding into 3.8% (w/v) sodium citrate. Mononuclear blood cells were obtained using Histopaque® 1119 and 1077 (Sigma-Aldrich) according to the manufacturer’s instructions. A fraction of mononuclear cells was collected, resuspended in RPMI 1640, 2% FCS, and incubated with Leuko-Pak® leukocyte filter (Fenwal Laboratories, Deerfield, IL) for 40 min at room temperature. The non-adherent cells, >85% T lymphocytes as inferred from the expression of Thy-1.2 glycoprotein (32), were collected and subjected to further analysis. *Escherichia coli* (strain M15) was grown in LB medium (33) at 37 °C for 12–16 h (A₆₀₀ ~ 3). The collected cells were washed, adjusted to ~10¹⁰/ml, and immediately used for activation studies.

**Cell Activation and PI-PLC Treatment**—Cells were washed with buffered saline solution (BSS; 20 mM HEPES, pH 7.4, 135 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 5.6 mM glucose) supplemented with 1.8 mM CaCl₂ and 0.1% bovine serum albumin (BSS/BSA). The cells were resuspended in BSS/BSA at a concentration 20 × 10⁶/ml. The suspension was mixed with twice-concentrated activators in BSS/BSA at a ratio 1:1, incubated at 37 °C for 20 min, and centrifuged at 150 × g for 5 min. The effect of inhibitory drugs was studied on cells pretreated with particular inhibitors at 37 °C as indicated under “Results.” The drugs were also present during cell activation and annexin V-FITC labeling. To remove GPI-AP, the cells (15 × 10⁶/ml) in BSS/BSA without calcium were incubated without (control) or with PI-PLC (1.5 units/ml) for 50 min at 37 °C.

**Determination of Cell Degranulation and Intracellular Free Calcium Concentration**—Degranulation of the cells was assessed by the amount of β-glucuronidase released into supernatant. 20-μl aliquots of the supernatant were mixed with 60 μl of 40 μM 4-methylumbelliferyl β-D-glucuronide. After 60 min of incubation at 37 °C, the reaction was stopped by adding 200 μl of ice-cold 0.2 M glycine buffer, pH 10.0, and fluorescence was determined in a microtiter plate reader Fluorostar (SLT Labinstruments GmbH, Grodd, Austria) with 365-nm excitation and 460-nm emission filters. Total cell content of the enzyme was evaluated in supernatants from cells lysed in 0.1% Triton X-100. Concentrations of intracellular free calcium [Ca²⁺], was measured using Fura-2 as a probe as previously described (34). Briefly, the cells were incubated at 37 °C for 30 min with 2 μM Fura-2-AM and 2.5 mM probenecid. Cells were then washed in BSS/BSA/probenecid and immediately before assessment washed once more in BSS/BSA. The samples were...
transferred into cuvettes and analyzed on luminescence spectrometer LS 50B (PerkinElmer Life Sciences) with excitation wavelengths at 340 and 380 nm and constant emission at 510 nm. Calcium concentrations were calculated by means of Winlab software (PerkinElmer).

**PS Externalization and Flow Cytometry Analysis**—Exposure of PS on the cell surface was detected by FITC-labeled annexin V (35, 36) using a modified manufacturer protocol (BD Biosciences). Briefly, cells were spun down and resuspended in annexin V binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) at a concentration of 2 × 10⁶ cells/ml. 38 µl of the cell suspension was mixed with 1 µl of annexin V-FITC and 2.5 µl of propidium iodide (PI, 50 µg/ml) and incubated for 15 min at room temperature in the dark. The reaction was stopped by adding 200 µl of annexin V binding buffer, and samples were immediately analyzed by flow cytometry on FACSCalibur with CellQuest software (BD Biosciences). Only PI-negative cells were further analyzed. In experiments using cells activated by co-culturing with *E. coli* or under conditions lacking extracellular calcium, the samples were washed with ice-cold annexin V binding buffer followed by incubation with annexin V-FITC and PI for additional 30 min on ice. Aliquots of 200 µl of ice-cold annexin V binding buffer were then added, and the samples were immediately subjected to flow cytometry; to get reproducible results, keeping the cells under ice-cold conditions during the whole procedure was essential. In experiments evaluating the kinetics of PS externalization, cells were incubated at room temperature for 15 min followed by washing with ice-cold annexin V binding buffer, and incubated with annexin V-FITC and PI on ice. After 30 min the cells were transferred to 37 °C, and the reaction was stopped by adding 200 µl of ice-cold annexin V binding buffer at various time intervals. The amount of annexin V-FITC bound was immediately quantified by flow cytometry. To determine antibody binding, cells were washed with ice-cold PBS supplemented with 1% BSA (PBS/BSA) and stained on ice for 30 min with anti-mouse IgG-FITC conjugate (10 µg/ml in PBS/BSA). The cells were washed 3 times with ice-cold PBS and analyzed by flow cytometry. To determine merocyanine 540 (MC540) binding, cells were washed and resuspended at a concentration of 5 × 10⁶/ml in BSS/BSA supplemented with 0.05 µM MC540 followed by incubation at 37 °C for 3 min before analysis by real time flow cytometry carried out at 37 °C.

**Confocal Microscopy**—Cells were spun down and resuspended in annexin V binding buffer at a concentration of 6 × 10⁶ cells/ml. Fifty µl of the cell suspension was mixed with 3 µl of annexin V-FITC and incubated at room temperature in the dark for 15 min. Then the cells were transferred on poly-L-lysine-coated coverslips (12 mm in diameter) placed in wells of a 24-well plate. Five min later 400 µl of 5% paraformaldehyde in annexin V binding buffer was added, and the samples were incubated at room temperature for 15 min followed by washing with PBS and blocking with PBS/BSA for 10 min at room temperature. The cells were then stained on ice for 20 min with anti-mouse IgG-cyanine 3 conjugate (10 µg/ml in PBS/BSA) and washed again with PBS. Images were acquired with a Leica TCS NT/SP confocal system in conjunction with a Leica DMR microscope (Leica Microsystems GmbH, Wetzlar, Germany) equipped with oil objective100×/1.4 numerical aperture.

**RESULTS**

**PS Externalization Induced in Activated Mast Cells in the Absence of the Secretory Response**—The study was initiated by analyzing the correlation between cell surface exposure of PS, assessed by annexin V-FITC binding, and secretory response of mast cells exposed to various stimuli. Experiments using TNP-specific IgE and antigen (TNP-BSA) confirmed previous reports (13, 14) showing that the engagement of FceRI in BMMC induced PS externalization which correlated with the secretory response determined by the release of β-glucuronidase (Fig. 1A). Interestingly, when the cells were exposed to lectin concanavalin A (Con A), PS externalization was comparable with that found in cells activated through FceRI even though Con A induced only minute degranulation. Similarly, exposure of the cells to bacterial strain *E. coli* induced PS externalization in the absence of secretory response. As expected, in BMMC from mice defective in LAT, an important transmembrane adaptor protein required for FceRI signaling (37), both the antigen-induced PS externalization and the secretory response were reduced. Surprisingly, however, PS exposure induced by Con A or *E. coli* in LAT⁻/⁻ cells was not diminished (Fig. 1A). PS externalization concomitant with weak or no degranulation was also observed in RBL-2H3 cells or in freshly isolated RPMC exposed to Con A or *E. coli* (Fig. 1, B and C). In the latter cells strong secretory response was observed after pretreatment of the cells with thapsigargin, an inhibitor of endoplasmic reticulum Ca²⁺ ATPase. Enhanced secretion from RPMC in response to thapsigargin was, however, not associated with any stronger increase in PS externalization. Thus, the extent of PS externalization in mast cells does not always correlate with the extent of degranulation.

PS externalization induced by Con A was completely inhibited in cells pretreated with αMM, a specific competitive inhibitor of Con A binding (Fig. 1D; αMM/Activ). Partial inhibition of PS externalization by αMM was also observed in cells stimulated with *E. coli*. Even when αMM was added after stimulation with Con A or *E. coli* (Activ/αMM), the amount of surface PS was still reduced, suggesting that the processes are reversible and depend on constant engagement of mannose-containing plasma membrane glycoconjugates. Pretreatment of the cells with PI-PLC, which cleaves GPI linkage, significantly (p < 0.005) reduced the PS externalization induced by both Con A and *E. coli* (Fig. 1D), implying that GPI-anchored proteins are involved in the process of PS externalization.

**PS Externalization Induced by Triggering through GPI-AP**—Next we tested whether PS externalization could be induced by mAb specific for GPI-AP. For these experiments we selected several antibodies recognizing GPI-AP expressed on mast cells: Thy-1.1 (recognized by OX7 mAb) (20, 27), CD48 (38, 39), and TEC-21 (21). The antibodies were used at such con-
centrations (5 μg/ml for OX7 and anti-TEC-21 and 1:10-diluted hybridoma supernatant for anti-CD48) that induce dimerization of the target antigens but no degranulation (see below). As shown in Fig. 2, A and B, all antibodies bound to RBL-2H3 cells and induced PS externalization. PS externalization was also observed in freshly isolated RPMC exposed to OX7 or anti-CD48 (Fig. 2, C and D). As expected, anti-TEC-21 induced no PS externalization in RPMC because TEC-21 is not expressed in these cells (not shown). These data indicate that the engagement of GPI-AP leads to PS externalization.

**PS Externalization Induced through GPI-AP Is Rapid and Reversible**—To further characterize the mechanism leading to PS externalization through engagement of GPI-AP, we used RBL-2H3 cells and Thy-1.1-specific mAb OX7. We found that PS externalization induced through Thy-1.1 is a rapid process reaching a plateau at ~20 min after triggering (Fig. 3A). PS externalization was not absent in cells incubated without antibody or with 1aG4/C5, an antibody that is specific for the Thy-1.2 isoform (Fig. 3A) and does not bind to RBL-2H3 cells (27). These findings confirmed the specificity of the reaction and proved that the binding of annexin V-FITC to nonactivated cells is not enhanced during the whole (40 min) incubation period at 37 °C. Pretreatment with PI-PLC abolished the binding of OX7 to the cells and PS externalization (Fig. 3B; PI-PLC/OX7). Incubation with OX7 to allow externalization of PS followed by PI-PLC treatment reduced not only the amount of OX7 bound but also PS exposure (Fig. 3B; OX7/PI-PLC); this indicates that the process of Thy-1.1-induced PS externalization is reversible and depends on a continuous Thy-1.1 engagement. When intact OX7 mAb or its F(ab) fragment was used, they bound to the RBL-2H3 cells at comparable levels, but only the intact (divalent) antibody

![Figure 1](https://example.com/figure1.png)  
**FIGURE 1.** Various stimuli induce PS externalization and degranulation in different mast cell types. A, BMMC were obtained from wild type (WT) or LAT deficient (LAT−/−) mice, sensitized with TNP-specific IgE (1 μg/ml), and stimulated for 30 min with antigen (TNP-BSA; 0.5 μg/ml). Alternatively, non-sensitized BMMC were activated for 30 min with Con A (20 μg/ml) or E. coli (10^10/ml). B–C, RBL-2H3 cells (B) or freshly isolated RPMC (C) were exposed to thapsigargin (Tn, 4 μM), Con A (20 μg/ml), or E. coli (10^10/ml) for 20 min. PS externalization was determined by flow cytometry after staining of the cells with annexin V-FITC. Thin and thick lines indicate annexin V-FITC binding to nonactivated (Control) and activated cells, respectively. Degranulation was determined by measurement of β-glucuronidase released into supernatant. D, RBL-2H3 cells were activated by adding Con A (50 μg/ml) or E. coli (10^10/ml) after (Activ/MMA) or before (MMA/Activ) treatment with MMA (50 mM, 30 min). Alternatively, the cells were pretreated with PI-PLC before activation. Data were normalized to Con A- or E. coli-activated cells treated with vehicle alone (BSS/BSA, Control). In A–C, typical flow cytometry profiles from at least three independent experiments are shown.
induced PS externalization (Fig. 3C). Thus, the minimum requirement for induction of PS externalization is Thy-1.1 dimerization.

**Distinct Mechanisms of PS Externalization in Cells Activated through GPI-AP**—To find out whether Thy-1.1-induced PS externalization is mediated by the same mechanism as the one effective in FcεRI-activated cells, we compared levels of annexin V–FITC binding in cells activated by the OX7 mAb (which dimerizes Thy-1.1) and cells activated by the 5.14 mAb (which dimerizes FcεRI) (40, 41). Data presented in Fig. 4A show that performance of OX7 was comparable with 5.14. As a control we also used calcium ionophore (ionomycin)-activated cells and confirmed previous data (13, 17, 42) that ionomycin also induces PS externalization. Recently, Elliott et al. (42) suggested a model in which calcium ionophore-induced PS redistribution is preceded by and dependent on a decrease in lipid packing, which was detected by a fluorescent probe MC540 that binds preferentially to the outer leaflet of the plasma membrane with relatively loosely packed lipids (43, 44). To determine whether engagement of Thy-1.1 is also accompanied with decreased lipid packing, we studied the kinetics of MC540 binding to control and activated RBL-2H3 cells. Data in Fig. 4B show a dramatic increase in MC540 binding in ionomycin-activated cells but no enhanced binding in cells activated by OX7 or 5.14. These data indicate that PS exposure in Thy-1.1- and FcεRI-activated cells is mediated by a mechanism independent of a decrease in lipid packing. Further experiments showed that dimerized Thy-1.1, unlike dimerized FcεRI, was ineffective in inducing any degranulation (Fig. 4C).
Phosphatidylserine Externalization via GPI-anchored Proteins

FIGURE 4. Molecular mechanisms leading to PS externalization are different in Thy-1.1- and FcɛRI-activated cells. A, PS externalization induced in RBL-2H3 cells stimulated with OX7 mAb (5 μg/ml; dashed red line), 5.14 mAb (5 μg/ml; dashed and dotted blue line), or ionomycin (4 μM; dashed and double-dotted green line) for 20 min and determined as described in Fig. 1. Control (black line) represents stimulation with vehicle alone (BSS/BSA). B, real time flow cytometric analysis of cells labeled with MC540 after stimulation (arrow) with OX7, 5.14, or ionomycin. For further explanation, see the legend to Fig. 4A. C, secretory response of the mAb-stimulated cells as determined by measuring the concentration of β-glucuronidase in supernatants. D, calcium response in mAb-stimulated cells. The cells were labeled with Fura-2 and stimulated by OX7 mAb (5 μg/ml; dashed red line), 5.14 mAb (5 μg/ml; dashed and dotted blue line), or vehicle alone (black line) added at the time point indicated by an arrow. E, tyrosine phosphorylation of the mAb-stimulated cells as determined by immunoblotting (IB) using lysates from the corresponding cells. Positions of molecular mass standards in kDa are shown on the left. F, PS externalization induced in mAb-stimulated cells pretreated for 10 min with the caspase inhibitor (zVAD-FMK, 200 μM), inhibitors of PS translocation (DIDS and glybenclamide), phosphatidylinositol-3 kinase (wortmannin), Src- and Syk-family kinases (PP2), and aurovertin B (Auro-/Glc, 2 μM) used in the absence of glucose, or stimulated in the absence of extracellular calcium (Ca2+). G, PS externalization induced in the mAb (black bars, OX7; white bars, 5.14)-stimulated cells pretreated for 15 min with Mg-ATP-synthase inhibitors, also exhibited reduced PS externalization, indicating that ATP-dependent mechanisms are involved. Impaired PS externalizations were also observed after exposure of cells to the mAb in the absence of extracellular Ca2+ followed by transfer of the cells on ice and the addition of annexin V binding buffer with Ca2+. A dramatic difference between Thy-1.1- and FcɛRI-mediated PS exposure was observed in cells pretreated with methyl-β-cyclodextrin (MβCD), which does not bind to the cells but effectively removes cellular cholesterol (45, 46). In OX7-activated cells PS externalization was inhibited by MβCD, whereas it was enhanced in 5.14-activated cells (Fig. 4G). Similarly, if the cells were pretreated with latrunculin B, an inhibitor of actin polymerization (47, 48), PS externalization was inhibited in OX7-activated but potentiated in cells treated with 5.14 (Fig. 4G).

Data on spatial distribution of externalized PS obtained by confocal microscopy show that externalized PS in cells activated through OX7-dimerized Thy-1.1 is distributed in spots and patches, whereas Thy-1.1 is distributed mostly homogeneously (Fig. 4H). A similar distribution pattern was observed in cells activated through 5.14-dimerized FcɛRI; externalized PS was present in spots and patches. Whereas FcɛRI showed a homogeneous distribution. Thus, the dramatic difference in degranulation between Thy-1.1- and FcɛRI-activated cells does not have its counterpart in a similarly different distribution of externalized PS.

Additive Effects of Different GPI-AP on PS Externalization—

In an attempt to better understand the mechanisms of PS externalization induced through various GPI-AP and/or FcɛRI, we studied the effect of triggering with various combinations of antibodies. We found that stimulation of RBL-2H3 cells with increasing concentrations of priming antibodies reached a plateau in PS externalization at 2 μg/ml of OX7 and 0.5 μg/ml of 5.14 (Fig. 5, A–B). Nevertheless, after simultaneous activation and aurovertin B (Auro-/Glc, 2 μM) used in the absence of glucose, or stimulated in the absence of extracellular calcium (Ca2+), PS externalization induced in the mAb (black bars, OX7; white bars, 5.14)-stimulated cells pretreated for 15 min with Mg-ATP-synthase inhibitors, also exhibited reduced PS externalization, indicating that ATP-dependent mechanisms are involved. Impaired PS externalizations were also observed after exposure of cells to the mAb in the absence of extracellular Ca2+ followed by transfer of the cells on ice and the addition of annexin V binding buffer with Ca2+. A dramatic difference between Thy-1.1- and FcɛRI-mediated PS exposure was observed in cells pretreated with methyl-β-cyclodextrin (MβCD), which does not bind to the cells but effectively removes cellular cholesterol (45, 46). In OX7-activated cells PS externalization was inhibited by MβCD, whereas it was enhanced in 5.14-activated cells (Fig. 4G). Similarly, if the cells were pretreated with latrunculin B, an inhibitor of actin polymerization (47, 48), PS externalization was inhibited in OX7-activated but potentiated in cells treated with 5.14 (Fig. 4G).

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Data on spatial distribution of externalized PS obtained by confocal microscopy show that externalized PS in cells activated through OX7-dimerized Thy-1.1 is distributed in spots and patches, whereas Thy-1.1 is distributed mostly homogeneously (Fig. 4H). A similar distribution pattern was observed in cells activated through 5.14-dimerized FcɛRI; externalized PS was present in spots and patches. Whereas FcɛRI showed a homogeneous distribution. Thus, the dramatic difference in degranulation between Thy-1.1- and FcɛRI-activated cells does not have its counterpart in a similarly different distribution of externalized PS.
of the cells by OX7 and 5.14 (both at 5 μg/ml; Fig. 5C), an additive effect on PS externalizations was observed. Interestingly, a similar additive effect on PS externalizations was also observed after simultaneous engagement of the target antigens with the following pairs of mAb: OX7 and TEC-21 (Fig. 5D), OX7 and CD48 (Fig. 5E), and CD48 and TEC-21 (Fig. 5F). These data suggest that different plasma membrane molecules, even those anchored to the plasma membrane via lipid tail, could utilize different signaling pathways leading to PS externalization.

**Prolonged Engagement of Thy-1.1 or FcγRI Has a Different Effect on PS Externalization but a Comparable Effect on Cell Proliferation**—PS externalization induced by engagement of GPI-AP or FcγRI depends on the enzymatic activity of several signaling molecules. In all experiments described so far cells were exposed to the activating mAb for a short time interval, 40 min or less. Next, we studied PS externalization and antibody binding in cells exposed to anti-Thy-1.1 or anti-FcγRI for longer time intervals. When cells were cultured with OX7 mAb for 0.5, 24, or 72 h and then analyzed for PS externalization and antibody binding, no dramatic difference in the levels of PS externalization was noticed. However, with 5.14 mAb, PS externalization reached its maximum 0.5 h after triggering and decreased 24 h later. In cells incubated with 5.14 for 72 h, no PS externalization was observed even though binding of 5.14 mAb was still detectable (Fig. 6, A–B). Thus, FcγRI triggering induces only a transient PS externalization, whereas engagement of Thy-1.1 leads to a sustained PS exposure. It should be noted that incubation of cells for up to 72 h with OX7 or 5.14 did not impair the cell viability, as determined by PI staining (not shown), and had little effect on cell proliferation (Fig. 6C).

**PS Externalization Induced through GPI-AP Is Cell Type- and Antibody-dependent**—To determine whether PS externalization induced through GPI-AP is confined to mast cells and antibodies actually used, we extended the studies to several other cell types and mAb. When RBL-gT12/1 cells, expressing both endogenous Thy-1.1 and the transfected mouse Thy-1.2 gene, were exposed to OX7 (Thy-1.1 specific) or 1aG4/C5 mAb (Thy-1.2 specific), PS externalization was evident after triggering with both mAb (Fig. 7, top row, two left panels). CEA is another GPI-AP expressed in a wide variety of epithelial malignancies, including colon cancer and colon cancer-derived cell lines such
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FIGURE 7. PS externalization induced by engagement of GPI-AP is dependent on the cell type and antibody used. Cells of different origin were exposed to anti-Thy-1.1 (OX7), anti-Thy-1.2 (1aG4/C5), anti-CEA (CEA), or anti-CD48 (CD48) mAb for 30 min, and annexin V-FITC and antibody binding were determined as described in Figs. 1 and 2. Typical flow cytometry profiles from at least three independent experiments are shown.

as SW1417. Antibody-mediated aggregation of CEA in SW1417 induced no PS externalization (not shown), suggesting that either the cells are unresponsive or the mAb is inefficient in inducing PS exposure in these cells. To get more details on CEA-mediated PS externalization in responsive cells, we transfected CEA cDNA into RBL-2H3 cells and analyzed their properties. As shown in Fig. 7 (top row, two right panels) CEA-specific mAb bound to the RBL-CEA/2D1 cells but failed to induce PS externalization. The same CEA-transfected cells were still capable of responding to OX7 triggering; this suggests that antibody-mediated dimerization of CEA is not sufficient to induce PS externalization even in RBL-derived cells.

Mouse thymocytes and peripheral T cells express large amount of Thy-1.2 detectable by 1aG4/C5 mAb, and this antibody induced PS externalization in both those cell types (Fig. 7, second row). Engagement of Thy-1.1 in rat thymocytes with OX7 mAb induced PS externalization as well (Fig. 7, third row, two left panels). Weak PS externalization was also observed in rat peritoneal macrophages exposed to anti-

CD48 mAb (Fig. 7, third row, two right panels). Thy-1.2 glycoprotein is also expressed in mouse myelomonocytic cell line WEHI-3 and mouse fibroblasts, 3T3. Although these two cell lines bound comparable amounts of Thy-1.2-specific mAb 1aG4/C5, PS externalization was only observed in WEHI-3 cells (Fig. 7, bottom panels). These data indicate that PS externalization induced through engagement of GPI-AP is cell type- and antibody-specific.

DISCUSSION

Here we show that the engagement of GPI-AP induces externalization of PS in living cells by a distinct mechanism of cell signaling. Most of the experiments were performed with rat mast cell line RBL-2H3 expressing large amounts of GPI-AP Thy-1.1 (27), CD48 (38), and TEC-21 (21). However, PS externalization was not confined to those cells but was also evident in some other cell types triggered through different GPI-AP. It should be noted that PS externalization after exposure to a limited panel of mAbs specific for GPI-APs was absent in several cell lines such as SW1417 and 3T3 fibroblast, indicating that it depends on cells and on antibodies actually used.

PS externalization is a characteristic feature of early apoptotic cells (1, 49) and seems to be phylogenetically conserved (50). Data presented in this study indicate, however, that in Thy-1.1-activated mast cells PS externalization occurs in the absence of apoptosis. Incubation of cells for prolonged time intervals with antibodies specific for GPI-AP had no effect on cell viability, and the proliferation rate was almost the same as that in control cells. Furthermore, PS externalization was dependent on steady dimerization of Thy-1.1 glycoprotein; treatment with PI-PLC of Thy-1.1-activated cells, already externalizing PS, resulted not only in removal of Thy-1.1 but also in decreased PS exposure. Finally, PS externalization could be reduced by several compounds known to specifically inhibit either the enzymes involved in lipid transfer (DIDS and glybenclamide), in the early stages of mast cell signaling (PP2, piceatannol and wortmannin), and/or in the production of ATP (oligomycin, aurovertin B). On the other hand, PS externalization was not inhibited by zVAD-FMK, an inhibitor of caspases, enzymes that play an important role in apoptosis. Thus, although non-apoptotic PS externalization has recently been described after activation of several cell types including B

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and T lymphocytes, granulocytes, and mast cells (13, 14, 16, 42, 51–53), our data are the first showing that non-apoptotic PS externalization can be induced through engagement of GPI-AP.

PS externalization in mast cells has previously been observed after FcRI triggering (13, 14). Because activation through this immunoreceptor leads to degranulation, it has been proposed that PS externalization could be a useful marker of degranulation in individual cells (13, 14, 54). Degranulation of mast cells can also be induced by an extensive aggregation of GPI-AP (20, 21, 27), and therefore, GPI-AP-induced PS externalization might also be expected to reflect GPI-AP-induced degranulation, utilizing similar signaling pathways as those used in FcRI-triggered cells. However, several lines of evidence presented in this study and summarized in Table 1 indicate that the mechanisms leading to the PS externalization induced in Thy-1.1- or FcRI-activated cells differ and that PS can be externalized in the absence of degranulation. First, the extent of PS externalization in Thy-1.1-activated cells was at least the same or higher as the one in FcRI-activated cells, although only the latter showed degranulation. For cell activation experiments we used IgG class antibodies recognizing FcRI or GPI-AP and dimerizing them. Dimerization of FcRI is sufficient to induce secretion (40, 41), but dimerization of Thy-1.1 is not (Fig. 4C). Second, a dramatic difference between cells activated through dimerized Thy-1.1 and FcRI was also observed at the level of calcium response; rapid and strong in FcRI-activated cells but undetectable in Thy-1.1-activated cells. Data obtained with Thy-1.1-activated cells corroborate the previous observations, suggesting that enhanced [Ca$^{2+}$], is not necessarily a prerequisite for PS externalization (53, 55). It should be noted, however, that extracellular calcium was required for full PS exposure induced by both Thy-1.1 and FcRI triggering. The combined data suggest that the role of Ca$^{2+}$ in PS externalization is confined to the plasma membrane; in Thy-1.1-activated cells the local increase of Ca$^{2+}$ may be sufficient to induce PS exposure but insufficient to induce further signal propagation and degranulation. Third, a dramatic difference between Thy-1.1- and FcRI-activated cells was also observed at the level of tyrosine phosphorylation. In Thy-1.1-activated cells, the spectrum of phosphorylated proteins was very limited yet still sufficient for PS externalization. An important role of tyrosine-phosphorylated proteins could be deduced from the fact that inhibitors of Src- and Syk-family kinases inhibited the PS externalization in both Thy-1.1 and FcRI-activated cells. Fourth, the process of Thy-1.1- and FcRI-induced PS externalization exhibited different sensitivity to several pharmacological inhibitors. The most dramatic difference was observed in cells pretreated with latrunculin B, which enhanced PS externalization in FcRI-activated cells but inhibited this process in Thy-1.1-activated cells. Latrunculin B inhibits actin polymerization by sequestering the monomeric actin (47) and has been reported to enhance degranulation in mast cells activated through both FcRI and extensively aggregated Thy-1.1 (48, 56). The observed differences in the sensitivity of PS externalization to latrunculin suggest that actin serves as a negative regulator in FcRI-mediated PS externalization but a positive regulator in Thy-1.1-mediated PS externalization. Effects of MβCD on PS externalization were also opposite; MβCD pretreatment caused an enhanced PS exposure in FcRI-activated cells and a reduced PS exposure in Thy-1.1-activated cells. MβCD is known to deplete cellular cholesterol and enhances in this way the degranulation in mast cells activated through both FcRI and extensively aggregated Thy-1.1 (34, 57). Inhibition of PS externalization in MβCD-pretreated and GPI-AP-activated cells could hypothetically be related to localization of GPI-anchored proteins in putative lipid rafts (58–61) and their involvement in PS externalization through GPI-AP proteins (see below). Our data with Thy-1.1-activated cells are in accordance with previous studies showing that PS externalization is inhibited by actin cytoskeleton inhibitor, cytochalasin D, and MβCD (17, 62). However, in FcRI-activated cells the same treatment led to opposite results, indicating that actin and cholesterol requirements for PS externalization depend on the activation pathway utilized. Fifth, the additive effect of simultaneous engagement of GPI-AP and FcRI on PS externalization suggests that signaling pathways induced through these different plasma membrane molecules initiate signals independently and converge on a common signaling pathway leading to PS externalization. Interestingly, this additive effect was also observed when two different GPI-anchored proteins were engaged, supporting the concept that GPI-AP could inhabit different membrane microdomains and could, thus, trigger different signaling pathways (63). Finally, prolonged incubation of cells with anti-FcRI or anti-Thy-1.1 mAb induced only a transient or sustained PS externalization, respectively. The sustained exposure of PS in cells cultured in the presence of anti-Thy-1.1 mAb had no effect on proliferation; cells cultured in the presence of anti-Thy-1.1 or anti-FcRI mAb proliferated almost at the same rate as did the control cells.

The exact molecular mechanism of PS externalization in cells activated through GPI-AP remains enigmatic. GPI-APs have been putatively localized in lipid rafts (58–61). Engagement of GPI-AP could be sensed by those signaling molecules located in the same raft, and in this way the signal could be transduced (60). This model is supported by studies documenting the formation of complexes of GPI-APs with Src family kinases (58, 59). Alternatively, GPI-APs might form complexes with transmembrane proteins, which in turn could create a bridge between GPI-APs and cytoplasmic signaling molecules.

### Table 1

| Parameter                                      | Activation mediated through dimerization of |
|-----------------------------------------------|-------------------------------------------|
|                                               | Thy-1 | FcRI |
| PS externalization                            | +     | +    |
| Degranulation                                 | −     | −    |
| Ca$^{2+}$ response                            | −     | +    |
| Tyrosine phosphorylation                      | ±     | ±    |
| PS externalization after treatment of the cells with Latrunculin MβCD | ↓     | ↑    |
| PS externalization after prolonged stimulation | +     | −    |

*Table 1 Summary of differences between Thy-1- and FcRI-mediated PS externalization in RBL-2H3 cells*

+, response; −, no response; ±, partial response; ↓, decreased activity; ↑, increased activity.
or could themselves have signaling properties as suggested by literature data (63–65). Our data are compatible with both of these models.

Recently, Elliott et al. (42) proposed a model assuming that PS externalization may occur by a translocase-independent mechanism at energetically favorable sites of membrane perturbations where lipid packing is decreased. These data were based in part on the finding that fluorescent dye MC540, which binds preferentially to the plasma membranes with relatively loosely packed lipids, showed enhanced binding to calcium ionophore-stimulated lymphocytes with enhanced PS exposure (42). Our finding that Thy-1.1- and FcεRI-activated cells exhibited MC540 binding comparable with nonactivated cells suggests, however, that other mechanisms are more likely to be involved.

The asymmetric distribution of PS in the plasma membrane of nonactivated cells could be due to the activity of ATP-dependent flippases that hydrolyze ATP to flip PS against a concentration gradient (1–3). Export of phospholipids from the inner to the outer plasma membrane leaflet is mediated by ATP-concentration gradient (1–3). Export of phospholipids from the inner to the outer plasma membrane leaflet is mediated by ATP-dependent flippases/flippases. In addition, Ca2+

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Acknowledgments—We thank Anna Koffer for critical reading of the manuscript and Hana Mrázová, Dana Lorenčíková, and Šárka Síhánková for expert technical assistance.
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