

## Level of Expression of Phospholipid Scramblase Regulates Induced Movement of Phosphatidylserine to the Cell Surface\*

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We recently identified a 35-kDa erythrocyte membrane protein, phospholipid scramblase, that promotes  $\text{Ca}^{2+}$ -dependent transbilayer movement of phosphatidylserine (PS) and other phospholipids (PL) in reconstituted proteoliposomes (Zhou, Q., Zhao, J., Stout, J. G., Luhm, R. A., Wiedmer, T., and Sims, P. J. (1997) *J. Biol. Chem.* 272, 18240–18244). To determine whether this same protein is responsible for the rapid movement of PS from inner-to-outer plasma membrane leaflets in other cells exposed to elevated cytosolic calcium concentration ( $[\text{Ca}^{2+}]_c$ ), we analyzed how induced movement of PS to the cell surface related to expression of PL scramblase. Exposure to  $\text{Ca}^{2+}$  ionophore A23187 resulted in rapid PS exposure in those cell lines constitutively high in PL scramblase (HEL, Epstein-Barr virus-transformed B-lymphocytes, and Jurkat), whereas this response was markedly attenuated in cells expressing low amounts of this protein (Raji, HL60, and Dami). To confirm this apparent correlation between PL scramblase expression and PS egress at elevated  $[\text{Ca}^{2+}]_c$ , Raji cells were transfected with PL scramblase cDNA in pEGFP-C2, and stable transformants expressing various amounts of GFP-PL scramblase fusion protein were obtained. Clones expressing GFP-PL scramblase showed distinctly plasma membrane-localized fluorescence. When compared either with untransfected Raji cells or with transformants expressing GFP alone, clones expressing GFP-PL scramblase fusion protein showed increased exposure of PS at the cell surface in response to elevated  $[\text{Ca}^{2+}]_c$ , accompanied by increased expression of membrane catalytic function for the prothrombinase enzyme complex. These data indicate that transfection with PL scramblase cDNA promotes movement of PS to cell surfaces and suggest that this protein normally mediates redistribution of plasma membrane phospholipids in activated, injured, or apoptotic cells.

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The plasma membrane phospholipids (PL)<sup>1</sup> are normally asymmetrically distributed, with phosphatidylcholine (PC) and sphingomyelin located primarily in the outer leaflet and the aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine restricted to the cytoplasmic leaflet (1, 2). An increase in intracellular  $\text{Ca}^{2+}$  due to cell activation, cell injury, or apoptosis causes a rapid bidirectional movement of the plasma membrane PL between leaflets, resulting in exposure of PS and phosphatidylethanolamine at the cell surface (1, 3–5). This exposure of the plasma membrane aminophospholipids has been shown to promote assembly and activation of several key enzymes of the coagulation and complement systems, as well as to accelerate the clearance of injured or apoptotic cells by the reticuloendothelial system, suggesting that  $\text{Ca}^{2+}$ -induced remodeling of plasma membrane PL is central to both vascular hemostatic and cellular clearance mechanisms (1, 6–10).

We recently identified an integral membrane protein from human erythrocytes that when reconstituted into liposomes mediated a  $\text{Ca}^{2+}$ -dependent, bidirectional scrambling of PL between membrane leaflets, mimicking the action of  $\text{Ca}^{2+}$  at the endofacial surface of the erythrocyte membrane (11–13). Here we show that when cell lines are compared, the level of expressed PL scramblase generally reflects the extent to which PS migrates to the plasma membrane outer leaflet in response to elevated  $[\text{Ca}^{2+}]_c$ . We also demonstrate that the propensity for PS to become exposed at cell surfaces can be manipulated by altering the level of expression of PL scramblase through plasmid transfection.

### EXPERIMENTAL PROCEDURES

**Materials**—All restriction enzymes were from New England BioLabs, Inc. (Beverly, MA). KlenTaq polymerase and pEGFP-C2 vector were from CLONTECH Laboratories (Palo Alto, CA). OPTI-MEM and geneticin were from Life Technologies, Inc. Fetal bovine serum, RPMI 1640, cell dissociation solution, Hanks' balanced salt solution, protein A-Sepharose-CL4B, leupeptin, and bovine serum albumin were from Sigma. UltraLink Iodoacetyl resin and SuperSignal ULTRA Chemiluminescence Kit were from Pierce. All other chemicals were of reagent grade.

**Cell Culture**—Human cancer cell lines erythroleukemic HEL, promyelocytic leukemia HL-60, chronic myelogenous leukemia K562, lymphoblastic leukemia MOLT-4, acute T-cell leukemia Jurkat, Burkitt's lymphoma Raji, and megakaryocytic DAMI were from American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 containing 10% fetal bovine serum. Epstein-Barr virus-transformed cell line W9 established from peripheral B-lymphocytes of a normal donor was maintained as described previously (14).

**Antibodies**—Anti-GFP, a murine monoclonal antibody against GFP, was from CLONTECH Laboratories. Anti-FVa, a murine monoclonal antibody V237 reactive against human or bovine factor Va light chain, was the generous gift of Dr. Charles T. Esmon (Oklahoma Medical Research Foundation, Oklahoma City, OK). Anti-PL scramblase-E306-W318, a rabbit antibody raised against the carboxyl-terminal peptide sequence E306-W318 of human PL scramblase, has previously been described (13). The IgG fraction was isolated on protein A-Sepharose-CL4B, and the peptide-reactive antibody was purified by affinity chromatography on peptide [Cys]-ESTGSQEQKSGVW coupled to Ultra-Link Iodoacetyl resin.

**Plasmid Construction**—Human PL scramblase cDNA insert was released from plasmid pMAL-C2-PL scramblase (13) by double cutting with *Eco*R1 and *Sal*I, respectively, and then ligated into pEGFP-C2

<sup>1</sup> The abbreviations used: PL, phospholipid(s); PS, phosphatidylserine;  $[\text{Ca}^{2+}]_c$ , cytosolic calcium concentration; GFP, green fluorescent protein; FVa, coagulation factor Va.

vector using the same restriction site. The pEGFP-C2-PL scramblase plasmid was amplified from single clones in *Escherichia coli* strain Top 10, and the orientation and reading frame of the insert were confirmed by sequencing on an ABI DNA Sequencer Model 373 Stretch (Perkin Elmer-Applied Biosystems, Foster City, CA).

**Transfection of Raji Cells with pEGFP-PL Scramblase**— $1.6 \times 10^7$  Raji cells were electroporated with 160  $\mu$ g of plasmid DNA (pEGFP-C2-PL scramblase or pEGFP-C2) in a total volume of 0.8 ml of OPTI-MEM, selected with 1.5 mg/ml geneticin, sorted by flow cytometry (FACStar, Becton-Dickinson Immunocytometry Systems, San José, CA), and dilutionally cloned. pEGFP-PL scramblase transformants expressing the 62-kDa GFP-PL scramblase fusion protein were identified by Western blotting with anti-GFP and with anti-PL-306-W318 antibodies. Comparable GFP-expressing clones served as controls.

**Fluorescence Microscopy**—Phase contrast and fluorescence microscopy were performed with a ZEISS AXIOSKOP microscope (Carl Zeiss, Inc., Thornwood, NY) equipped for epifluorescence, and images were recorded with a MC100 camera system.

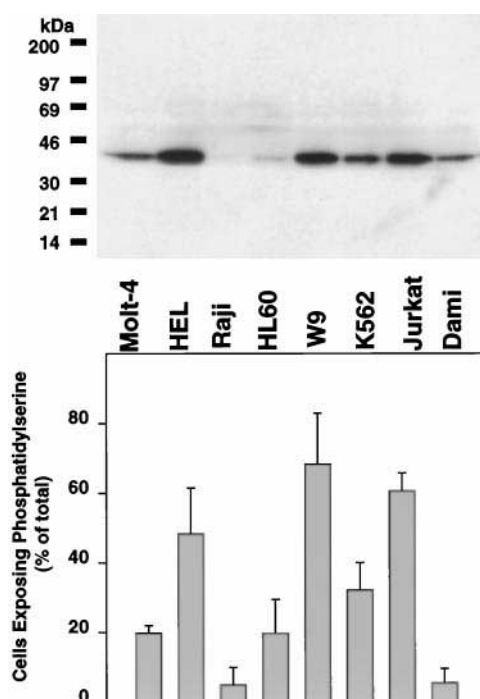
**Western Blot Analysis**—Western blotting of GFP and PL scramblase antigens was performed using  $1.5 \times 10^6$  cells/lane. Cell pellets were extracted with 2% (v/v) Nonidet P-40 in 5 mM EDTA, 50 mM benzamide, 50 mM *N*-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin in Hanks' balanced salt solution. Following SDS-polyacrylamide gel electrophoresis and transfer to nitrocellulose, the blocked membrane was incubated with either 10  $\mu$ g/ml of rabbit anti-PL scramblase-E306-W318, or 1:10,000 dilution of mouse anti-GFP. The blots were developed using SuperSignal ULTRA chemiluminescence.

**Measurement of Cell Surface PS in Response to Elevated  $[Ca^{2+}]_c$** —Calcium ionophore-induced exposure of PS on the surface of all cell lines analyzed was detected by the specific binding of coagulation factor Va (light chain) as described previously (7, 14). Briefly, cells were washed and suspended ( $2 \times 10^6$  cells/ml) at 37 °C in RPMI 1640 supplemented with 0.1% bovine serum albumin, 20 mM HEPES, and adjusted to 1.2 mM free  $[Ca^{2+}]_c$ . At time 0, A23187 (final concentration, 0 or 2  $\mu$ M) was added, and at times indicated, 6 mM EGTA was added. PS exposed at each time point was detected by incubating (10 min, room temperature) 50  $\mu$ l of the cell suspension with 10  $\mu$ g/ml FVa, followed by 10  $\mu$ g/ml anti-FVa, to detect the cell-bound FVa light chain. After staining with 10  $\mu$ g/ml Tri-Color conjugated goat anti-mouse IgG (CALTAG Laboratories, Burlingame, CA), single cell fluorescence was quantified by flow cytometry (FL3 channel, FACScan, Becton Dickinson Immunocytometry Systems). In experiments in which cell lysis was monitored by uptake of propidium iodide, cells were stained for bound FVa with fluorescein isothiocyanate conjugate of goat anti-mouse IgG (FL1 channel) substituting for Tri-Color conjugate, and propidium iodide was detected in FL3 channel. Propidium iodide (0.5  $\mu$ g/ml) was added immediately before dilution for flow cytometry.

**Prothrombinase Assay**—Prothrombinase activity of Raji cells was determined by modification of methods previously described for platelets, using the chromogenic thrombin substrate S2238 (7).

## RESULTS

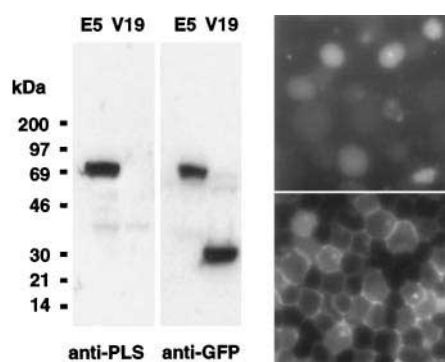
**Analysis of PL Scramblase in Various Human Cell Lines**—Proteoliposomes reconstituted with erythrocyte PL scramblase exhibit accelerated transbilayer movement of fluorescent phospholipids in response to added  $Ca^{2+}$ , similar to the observed effect of calcium on the endofacial surface of the red cell membrane (11–13). To determine whether this same protein is responsible for mediating the accelerated egress of plasma membrane PS that is observed under conditions of elevated cytosolic  $[Ca^{2+}]_c$ , we examined whether the level of expression of PL scramblase in various human cell lines correlated to the induced movement of PS to the surface of these cells. In these experiments, the cell surface exposure of PS was monitored by the binding of the light chain of coagulation factor Va, a PS-specific binding protein whose affinity for the PS headgroup is independent of  $Ca^{2+}$  concentration (7, 14). As illustrated in Fig. 1, when challenged with ionophore, human cell lines exhibit considerable differences in the extent to which PS is mobilized to the cell surface. Among the cells tested, Raji, HL60, and Dami were notably unresponsive to A23187, whereas HEL, W9, and Jurkat showed notably robust responses. This apparent cell type-specific variability in response to induced elevation of  $[Ca^{2+}]_c$  was consistently maintained through many months of



**FIG. 1. Analysis of PL scramblase in various human cell lines.** Constitutive expression of PL scramblase was analyzed in the human cell lines indicated. *Upper panel*, Western blots. Shown are results obtained by Western blotting with antibody specific for PL scramblase carboxyl-terminal residues E306-W318 (see "Experimental Procedures"). Each lane contains the total protein extract of  $1.5 \times 10^6$  cells. *Lower panel*, cell surface exposure of PS in various human cell lines. The cells indicated were washed and suspended at 37 °C in the presence of 1.2 mM free  $Ca^{2+}$ , and 2  $\mu$ M A23187 was added. After 5 min, EGTA was added, and cells were analyzed for surface exposed PS as detected by cell-bound FVa light chain (see "Experimental Procedures"). Data plotted represent the mean increase ( $\pm$  S.D.) in the number of cells that stained positive for surface PS after 5 min of incubation with ionophore, after correction for initial background of PS-positive cells before addition of ionophore (time 0).

passage in culture. As also shown in Fig. 1, we observed considerable differences in the content of PL scramblase protein among these various cell lines, and their sensitivity to induced exposure of plasma membrane PS (*lower panel*) generally reflected the amount of cellular PL scramblase protein detected by Western blotting (*upper panel*). Those cell lines that were most responsive to induced elevation of  $[Ca^{2+}]_c$  (HEL, W9, and Jurkat) also expressed greatest amounts of PL scramblase antigen, whereas cell lines with attenuated response to  $[Ca^{2+}]_c$  (Raji, HL60, and Dami) contained relatively little of this protein. Cell lines Molt-4 and K562 showed intermediate responses to elevated  $[Ca^{2+}]_c$  and expressed intermediate levels of PL scramblase antigen. These relatively large differences in cell line-specific expression of this protein was found to correspond to marked differences in level of specific mRNA as detected by Northern blotting with PL scramblase cDNA (Ref. 13 and data not shown). Whereas the  $Ca^{2+}$ -dependent movement of PS generally reflected the level of expression of PL scramblase antigen per cell, some exceptions were noted, most particularly when results for HL60 and Dami are compared (Fig. 1). We also noted that those cell lines with the highest content of PL scramblase exhibited a higher background of PS exposed in the absence of ionophore. This was most notable for HEL, for which 15–30% of cells exposed PS *prior* to the addition of A23187, resulting in an underestimation of the ionophore-dependent response.

**Membrane Changes Underlying Ionophore Response**—To determine whether the increase in PS exposure in calcium ionophore



**FIG. 2. Expression of GFP-PL scramblase fusion protein in transformed Raji cell lines.** Western blotting for either GFP (*anti-GFP*) or PL scramblase (*anti-PLS*) was performed on stably transformed Raji clones (*left panel*). Clone E5 was transfected with pEGFP-C2-PL scramblase; clone V19 was transfected with pEGFP-C2 (vector control). Similar results were obtained for all transformed clones analyzed, including 23 clones transformed with pEGF-C2-PL scramblase and 23 clones transformed with pEGFP-C2. *Right-hand panels* show fluorescence photomicrographs of GFP fluorescence expressed in the transformed Raji clones. *Upper right panel* shows fluorescence of cells expressing GFP (vector only control); *lower right panel* shows cells transfected with pEGFP-C2-PL scramblase plasmid and expressing GFP-PL scramblase fusion protein. Data are shown of a single experiment representative of results obtained for all clones transfected with either pEGFP-C2 or pEGFP-C2-PL scramblase.

phore-treated cells reflected actual transbilayer movement of PS or increased sensitivity of the plasma membrane to lytic disruption, FVa binding to the cell surface was monitored simultaneously with uptake of propidium iodide, as a measure of cell lysis. In the B-lymphocyte line W9 and other cells expressing high level PL scramblase, addition of ionophore resulted in a marked increase in cell surface receptors for FVa that distinctly preceded the later uptake of propidium iodide (data not shown). This confirmed that in this PL scramblase-expressing cell line, elevation of  $[Ca^{2+}]_c$  induces a rapid movement of PS to the cell surface *before* onset of cell lysis is observed. By contrast, Raji cells that are virtually devoid of PL scramblase (Fig. 1), exhibited a general insensitivity of the plasma membrane to elevated  $[Ca^{2+}]_c$ , as reflected either by *de novo* appearance of PS at the cell surface or by cellular uptake of propidium iodide.

**Transfection of the Raji Cell Line with pEGFP-C2-PL Scramblase**—To confirm that the extent to which PS moves to the cell surface with elevation of  $[Ca^{2+}]_c$  actually depends upon plasma membrane content of PL scramblase, we stably transformed Raji, a cell line exhibiting low endogenous PL scramblase expression (Fig. 1) by transfection with plasmid pEGFP-C2-PL scramblase. This plasmid expresses PL scramblase as a fusion protein with GFP, facilitating flow cytometric sorting of transformants for subsequent cloning and enabling quantitation by fluorescence of the content of recombinant protein in select clones. Expression of full-length GFP-PL scramblase fusion protein in transformed clones was confirmed by Western blotting with antibody against GFP and with antibody against the carboxyl terminus of human PL scramblase (Fig. 2). As is also illustrated by Fig. 2, clones that expressed the GFP-PL scramblase fusion protein showed a distinct rim appearing pattern of fluorescence, consistent with trafficking of GFP-PL scramblase to the plasma membrane.

**Analysis of PS Mobilizing Function in GFP-PL Scramblase Transformants**—Clonal populations of transformed Raji cells expressing comparable levels of either GFP-PL scramblase or GFP (transformed with pEGFP-C2 lacking insert) were analyzed for their capacity to mobilize PS to the cell surface (Fig. 3). In response to an A23187-induced elevation of  $[Ca^{2+}]_c$ , transformants expressing the GFP-PL scramblase fusion con-

struct showed a marked increase in both the rate and extent that PS became exposed on the cell surface, when compared with either the identically treated parental Raji cell line or to GFP-expressing clones transformed with pEGFP-C2 vector alone (Fig. 3). We noted a small but reproducible increase in the background level of PS exposure in transformants expressing GFP-PL scramblase protein when compared either with the parental Raji cell lines or with GFP-expressing clones transformed with vector alone. The increase in FVa binding seen for the GFP-PLS transformed cell lines was accompanied by equivalent increase in membrane coagulant function as measured by prothrombinase assay (data not shown).

**Level of Expression of PL Scramblase Regulates Capacity to Mobilize PS to the Cell Surface**—To confirm the apparent correlation between endogenous cell content of PL scramblase and plasma membrane sensitivity to elevated  $[Ca^{2+}]_c$  that is evident when different human cell lines are compared (Fig. 1), we analyzed multiple Raji clones that were stably transfected with either GFP-PL scramblase or with GFP vector alone (Fig. 4). These experiments confirmed that a cell's capacity to mobilize PS to the surface can be increased through expression of GFP-PL scramblase fusion protein, whereas this response is unaffected by cell content of GFP.

## DISCUSSION

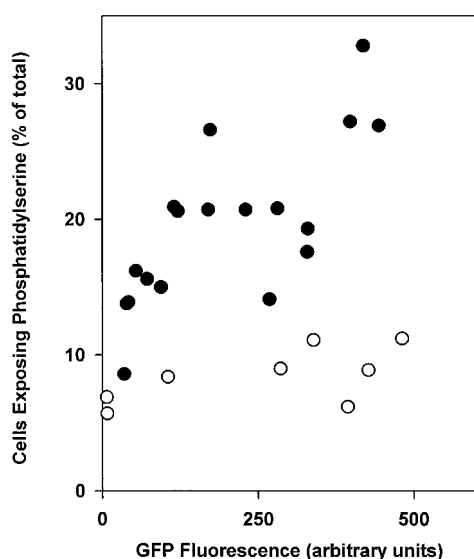
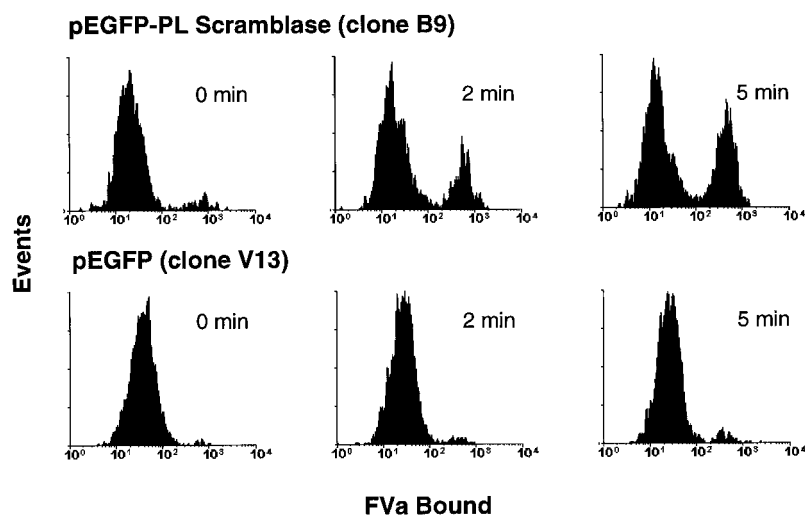
These results provide the first evidence that the PL scramblase protein identified in the erythrocyte membrane and implicated in  $[Ca^{2+}]_c$ -induced remodeling of membrane phospholipids actually functions to induce accelerated transbilayer movement of plasma membrane phospholipid in human cells that express this protein. Our results also confirm that the level of expression of PL scramblase can determine the extent to which PS is mobilized to the cell surface upon elevation of  $[Ca^{2+}]_c$  and suggest that this protein normally functions to mediate the redistribution of plasma membrane phospholipids in response to the entry of calcium into the cytosol. Furthermore, these data provide the first indication that the movement of PS and other procoagulant aminophospholipids from plasma membrane inner leaflet to the cell surface can be manipulated by selectively altering the level of expression of a particular cellular protein, either through direct transfection with the PL scramblase cDNA or potentially by another intervention affecting cell expression of this protein.

In addition to conferring increased sensitivity of the plasma membrane to ionophore-induced elevation of  $[Ca^{2+}]_c$ , we generally observed a higher background of PS exposure (in the absence of ionophore) in those transfected cell clones expressing large amounts of the GFP-PL scramblase fusion protein. This elevated background PS exposure was also observed in the case of untreated HEL, the cell line containing the highest endogenous content of PL scramblase. Although we suspect that this increased background reflects the enhanced sensitivity of the plasma membrane of these cells to any adventitious elevation of  $[Ca^{2+}]_c$  during cell processing for assay, we cannot exclude the possibility that these cells are also inherently more fragile due to the large amounts of PL scramblase that is inserted into the plasma membrane.

Although the movement of plasma membrane PS to the cell surface at elevated  $[Ca^{2+}]_c$  can be demonstrated in a variety of cells and tissues (2, 15), we detect marked differences in the levels of PL scramblase mRNA and protein among different human cell types, which is generally reflected by corresponding differences in sensitivity to this  $[Ca^{2+}]_c$ -induced collapse of plasma membrane PL asymmetry (Fig. 1 and Ref. 13). In particular, we note that the content of PL scramblase in human platelet is approximately 10-fold greater than that of the erythrocyte, which is consistent with the respective PS-mobilizing



**FIG. 3. Transfection with PL scramblase cDNA confers increased sensitivity of the plasma membrane to intracellular calcium.** Surface exposure of PS was measured in individual Raji clones that were stably transfected with either pEGFP-C2 or with pEGFP-C2-PL scramblase. Experiments were performed under the conditions described for Fig. 1, except that analysis was gated to include only those cells distinctly positive for GFP fluorescence (FL1 channel), and cell-bound FVa was stained with Tri-color conjugate and detected in FL3 channel (see "Experimental Procedures"). Time indicated represents the time elapsed after addition of ionophore. Results are shown for pEGFP-C2-PL scramblase-transformed Raji clone B9 and pEGFP-C2-transformed control V13.



**FIG. 4. Level of expression of PL scramblase determines plasma membrane sensitivity to intracellular  $Ca^{2+}$ .** The relationship between cell content of recombinant protein (GFP fluorescence detected in FL1 channel; *abscissa*) and numbers of cells that expose PS after 2 min of incubation with A23187 (*ordinate*) is plotted for multiple transformed Raji clones. The experiment was performed and the data were analyzed as described for Fig. 3. *Open symbols* indicate individual clones stably transfected with pEGFP-C2 (correlation coefficient,  $r = 0.3$ ); *closed symbols* indicate individual clones stably transfected with pEGFP-PL scramblase (correlation coefficient,  $r = 0.7$ ). Data of single experiment representative of three so performed are shown.

potential and different roles of these two cells in contributing procoagulant membrane surface for thrombin generation during blood clotting (13). In addition to the relatively high levels of PL scramblase identified in circulating human platelets, this protein was also most abundant in the cell line HEL, whereas only small amounts of this protein (and low PL scramblase activity) was detected for Dami (Fig. 1), two human cancer cell lines exhibiting partial megakaryocytic-like properties. It is also noteworthy that several of the lymphoma-derived cell lines

(e.g. Raji and MOLT-4) express considerably reduced levels of PL scramblase and also show distinctly attenuated PS exposure in response to elevated  $[Ca^{2+}]_i$  when compared either with peripheral blood leukocytes or with Epstein-Barr virus transforms of normal lymphocytes. The collapse of plasma membrane phospholipid asymmetry is a relatively early event in apoptosis of lymphocytes and other cells, and the consequent exposure of PS on the cell surface is thought to contribute to phagocytic removal of such cells by scavenger macrophages (9, 10, 16, 17). It is therefore of interest to consider whether the apparent resistance of certain lymphoma-derived cell lines to such remodeling of plasma membrane phospholipids due to a decrease in expression of PL scramblase in the plasma membrane might contribute to the proliferative potential and *in vivo* survival of these or other transformed cells.

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