Archives of Biochemistry and Biophysics
Vol. 381, No. 2, September 15, pp. 295–301, 2000
doi:10.1006/abbi.2000.1986, available online at http://www.idealibrary.com on IDEAL®

Stimulated Nonspecific Transport of Phospholipids Results in Elevated External Appearance of Phosphatidylserine in *ras*-Transformed Fibroblasts

Albena Momchilova,* Lidia Ivanova,† Tania Markovska,* and Roumen Pankov‡,§,[1]

**Institute of Biophysics, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria; †Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, Missouri 63110; ‡Faculty of Biology, Sofia University, 1421 Sofia, Bulgaria; and §National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, Maryland 20892*

Received April 21, 2000, and in revised form June 20, 2000

The content of phosphatidylserine (PS) was found to be increased three times in the plasma membrane outer leaflet of *ras*-transformed fibroblasts compared to their nontransformed counterparts. In an attempt to determine the mechanisms responsible for the enhanced external appearance of PS, we investigated the activities of aminophospholipid translocase and the nonspecific lipid scramblase. Both transport systems could separately or in combination contribute to PS accumulation in the extracellular leaflet. Aminophospholipid transfer was assessed by measuring the rate of NBD-PS internalization, and scramblase activity was estimated from the internalization of NBD-PC. The results showed that the aminophospholipid transport was inhibited and the nonspecific transport was stimulated in *ras*-transformed cells. To assess which of these two transport systems was related to elevation of PS external appearance, each of them was submitted to reversible alterations and the content of PS was measured simultaneously. Aminophospholipid translocase activity was inhibited by pyridyldithioethylamine treatment and reversed by reduction with dithiothreitol. Scramblase activity was modulated by a calcium repletion–depletion procedure. Calcium depletion was performed by cell incubation with BAPTA-AM and EGTA as Ca^{2+} intracellular and extracellular chelators. Restoration of the intracellular Ca^{2+} was achieved by cell incubation with Ca^{2+} and Ca^{2+}-ionophore A23187. The results showed that the changes in PS outer appearance did not correlate with the uptake of NBD-PS but were closely related to NBD-PC internalization, suggesting that the nonspecific bidirectional lipid transfer was the major transport system translocating PS to the outer leaflet in *ras*-transformed cells.

Key Words: **phosphatidylserine; membrane asymmetry; aminophospholipid translocase; lipid scramblase; *ras* transformation.**

The asymmetric distribution of the major phospholipid classes between the two membrane leaflets is well documented (1–3). Under normal conditions, the aminophospholipids phosphatidylserine (PS)[2] and phosphatidylethanolamine (PE) are concentrated predominantly in the inner membrane leaflet whereas the choline-containing phospholipids phosphatidylcholine (PC) and sphingomyelin are sequestered mostly in the extracellular leaflet. The nonrandom distribution of PS between the two membrane monolayers is maintained by an ATP-dependent aminophospholipid translocase (4) which transfers PS and PE to the inner leaflet. This asymmetry can be abolished by another transport mechanism performing nonspecific movement of phospholipid molecules both inward and outward, referred to as scramblase (5). Activation of phospholipid scrambling has been reported for erythrocytes (6), platelets (7), and lymphocytes (8). This process can be induced by elevation of the intracellular calcium concentration

[1] To whom correspondence should be addressed. Fax: (301) 402-0897. E-mail: RPankov@dir.nidcr.nih.gov.

[2] Abbreviations used: PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; NBD-PC, 1-oleoyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine; NBD-PS, 1-oleoyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphoserine; PDA, pyridyldithioethylamine; HBS, Hepes-buffered saline; DTT, dithiothreitol.

0003-9861/00 $35.00

(6, 9), which inhibits the aminophospholipid inward translocation in human erythrocytes (10). Stimulation of the nonspecific lipid scrambling and/or inactivation of the aminophospholipid inward transport can abrogate the normal transmembrane lipid distribution resulting in some cells in accumulation of PS in the extracellular leaflet. Enhanced surface exposure of PS has been reported for apoptotic (11) and tumorigenic (12) cells, as well as platelets and erythrocytes upon activation (13, 14). Cells expressing PS in the outer monolayer become susceptible to recognition and clearance by the reticuloendothelial system (15).

In this study, we have investigated the external exposure and transbilayer movement of PS in *ras*-transformed NIH 3T3 fibroblasts in comparison with their nontransformed counterparts. Our recent studies revealed some major alterations in the lipid metabolism and especially the aminophospholipid metabolism in *ras*-transformed cells (16). Since *ras* oncogenes are associated with a variety of human cancers (17), studies on the mechanisms which would make *ras*-expressing cells susceptible to destruction are of special interest. Our initial observations showed that the content of externally exposed PS was about three times higher in transformed compared to nontransformed cells. To discriminate between the possible mechanisms underlying these observations, we investigated the activities of aminophospholipid translocase and the nonspecific lipid scramblase, which could either separately or in combination induce enhancement of PS content in the outer membrane leaflet. The results showed that aminophospholipid translocase was inhibited and scrambling activity was stimulated in the transformed cells. To determine the relative contribution of the two tested transport mechanisms in PS external appearance, both of them were submitted to reversible alterations and the level of PS was measured simultaneously. Aminophospholipid translocase, which is susceptible to oxidative injury, was inhibited by treatment with an oxidizing agent, this inhibition being abolished by subsequent reduction, and scramblase activity was modulated by a Ca^{2+} depletion–repletion procedure. The results showed that the alterations in PS outer appearance correlated closely with the changes in scramblase activity and not with the changes observed for aminophospholipid translocase. Taken together, these data suggest that the nonspecific bidirectional lipid transfer was the major transport system responsible for elevation of PS in the extracellular membrane leaflet of *ras*-transformed fibroblasts.

MATERIALS AND METHODS

Materials. 1-Oleoyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine (NBD-PC) and the corresponding phosphatidylserine analog (NBD-PS) were obtained from Avanti Polar Lipids (Alabaster, AL). Pyridyldithioethylamine (PDA) was synthesized according to the procedure of Connor and Schroit (18). Culture medium, antibiotic/antimycotic solution, and serum were from GIBCO BRL Life Technologies. BAPTA-AM was from Calbiochem (San Diego, CA). The thrombin-sensitive chromogen S2238 was from Kabi Laboratories (Franklin, OH). Bovine serum albumin, dithiothreitol, prothrombin, factor Xa, Ca^{2+}-ionophore A23187, Fura 2-AM, EDTA, EGTA, and all other chemicals (except where otherwise stated) were from Sigma Chemical Co. (St. Louis, MO).

Cell culture. NIH 3T3 fibroblasts transfected with activated human Ha-*ras* gene (19) and the parental cell line were a kind gift from Dr. Channing J. Der (The University of North Carolina at Chapel Hill, Chapel Hill, NC). The cells were cultured as described elsewhere (20, 21). In brief, fibroblasts were grown in Dulbecco's modified Eagle's medium containing 400 μg/ml G418 (Geneticin), supplemented with 9% (v/v) fetal calf serum at 37°C in a humidified 5% CO_2 atmosphere. To obtain cell suspension, the monolayers were harvested by treatment with 0.05% trypsin and resuspended by pipetting in Hepes-buffered saline (HBS), 136 mM NaCl, 2 mM KCl, 0.5 mM $MgCl_2$, 5 mM glucose, 10 mM Hepes (pH 7.4). HBS contained 1 mM $CaCl_2$ in all experiments except in Ca^{2+} depletion–repletion procedures, in which Ca^{2+} concentration is specified for each assay (see below). For inhibition of aminophospholipid transferase activity, fibroblasts were incubated in the presence of 2 mM PDA at 4°C as described by Connor and Schroit (22). To reverse PDA-induced inhibition, 10 mM dithiothreitol (DTT) was added during labeling with fluorescent lipid analogs (see below). For calcium-depletion experiments, the fibroblasts were incubated in Ca^{2+},Mg^{2+}-free HBS containing 20 μM BAPTA-AM and 1 mM EGTA for 30 min. Calcium repletion was performed by incubation of cells in the presence of 500 μM $CaCl_2$ and 2 μM Ca^{2+} ionophore A23187 for 15 min. Prior to labeling, the cells were incubated with 500 μM PMSF for 30 min at 37°C to prevent degradation of the inserted NBD lipid and cooled to 2°C to suppress endocytosis.

Labeling with fluorescent lipid analogs. Fluorescent labeling was performed as described by Kok *et al.* (23), with modifications. Briefly, C12-NBD-PS and C12-NBD-PC were dried under nitrogen, solubilized in ethanol, and added to the cell suspension (10 nmol of fluorescent lipid per 10^6 cells) in HBS under stirring. After the incubation, the fibroblasts were washed with ice-cold HBS to remove the noninserted lipid analogs and incubated at 20°C for 45 min (results in Table II and Fig. 2) or as indicated.

Back-exchange. The internalization of the phospholipid fluorescent analogs into the inner membrane leaflet was assessed by back-exchange to serum albumin as described by Pomorski *et al.* (24), with modifications. Exchangeable fluorescent lipids located in the outer membrane leaflet can be removed, allowing quantitative determination of the lipid internalized into the inner monolayer, i.e., the lipid fraction, which could not be back-exchanged. In short, cells were incubated twice with 5% fatty acid free BSA on ice for 10 min, followed by extensive washing with HBS and centrifugation at 12,000*g*. The pellets were solubilized in 2% Triton X-100 and the amount of internalized phospholipid was determined by comparing the fluorescence intensity before and after back-exchange to albumin. The fluorescence of the NBD-labeled lipids was measured at a wavelength of 535 nm (excitation wavelength 468 nm) on a Perkin Elmer spectrofluorimeter using 10-nm slit widths.

Prothrombin-converting activity assay. Prothrombinase activity of fibroblasts was measured as described by Utsugi *et al.* (12), with modifications. Briefly, fibroblasts were diluted in HBS to a final concentration of 2×10^5 cells/0.1 ml and incubated at 37°C in the presence of 6 mM $CaCl_2$, 6 nM factor Xa, 10 nM factor Va, and 5 μM prothrombin at a final volume of 600 μl. The reaction was stopped after 3 min by addition of 15 mM EDTA in 50 mM Tris–HCl. The thrombin-dependent chromogenic substrate S2238 (0.5 mM) was added and the rate of thrombin formation was calculated from the

TABLE I

Content of Phosphatidylserine in the Outer Membrane Leaflet of Control and *ras*-Transformed Fibroblasts Treated with PDA and DTT (ng/10^6 cells)

Treatment	Control	*ras*
None	17 ± 2	52 ± 4
PDA	19 ± 3	55 ± 5
PDA + DTT	19 ± 3	54 ± 4

Note. Data are expressed as means ± SE of three experiments. PDA, pyridyldithioethylamine; DTT, dithiothreitol.

rate of chromogen formation monitored at 405 nm on a Pharmacia LKB 4054 spectrophotometer. The rate of thrombin formation was determined using a calibration curve obtained from measuring definite amounts of thrombin. The initial rate of thrombin conversion activity is directly proportional to the content of PS present on the extracellular surface (25, 26).

Lipid analysis. Lipid extraction was performed with chloroform/methanol according to the method of Bligh and Dyer (27). The organic phase obtained after extraction was concentrated and analyzed by thin-layer chromatography. The phospholipid fractions were separated on silica gel G60 plates in a solvent system containing chloroform/methanol/2-propanol/triethylamine/0.25% KCl (30:9:25:18:6 v/v) (28). The location of the separate fractions was visualized by fluorescence or iodine staining. The spots were scraped and quantified by determination of the inorganic phosphorus (29).

Other procedures. The intracellular calcium concentration was determined by the Fura-2-AM method exactly as described by Lennon *et al.* (30). The protein concentration was estimated according to the method of Lowry (31). Data are expressed as means ± SE for two or three independent experiments as indicated. Differences between the means were analyzed by Student's *t* test.

RESULTS

In this work, we investigated the differences in the transmembrane distribution of PS and the mechanisms underlying these differences in plasma membranes of control and *ras*-transformed NIH 3T3 fibroblasts. The content of PS in the outer membrane leaflet of *ras*-transformed cells was about three times the control level as calculated by the prothrombin-converting assay (Table I). Since membrane-bound aminophospholipid translocase generally transfers aminophospholipids from the outer to the inner membrane leaflet (4), we measured the degree of internalization of NBD-labeled PS into the plasma membrane to assess whether inhibition of this activity could be the reason for the enhanced external appearance of PS. As evident from Fig. 1, the internalization of NBD-PS into the inner leaflet reached 80% in control cells and 70% in *ras* cells after 1 h of incubation. In contrast, the inward translocation of NBD-PC, which was used to measure the nonspecific transmembrane translocation of phospholipids other than aminophospholipids, was higher by 12% in the transformed cells (Fig. 1). So the elevated external appearance of PS in *ras* cells could be due either to the observed partial loss of aminophospholipid inward transport or to the stimulated nonspecific bidirectional transfer of lipid molecules. To discriminate between these two mechanisms, the aminophospholipid translocase was inhibited by incubation with PDA before labeling (Table II). PDA is an agent initiating thiol–disulfide exchange (18), which inhibits the inward transport of aminophospholipids in erythrocytes (22). Our preliminary studies showed that PDA inhibited efficiently the internalization of PS in fibroblasts as well. Such inhibition induced by oxidizing agents can be reversed upon reduction with DTT (22). PDA treatment reduced the inward translocation of NBD-PS in both control and transformed cells (Table II) but did not significantly alter the content of externally exposed PS in *ras* cells (Table I). NBD-PC internalization into fibroblast membranes did not seem affected by PDA treatment (Table II) although PDA-induced inhibition of scramblase has been reported in platelets (14). Subsequent incubation of PDA-treated fibroblasts with DTT recovered about 90% of the initial inward translocation of NBD-PS (Table II). Apparently, these manipulations of aminophospholipid

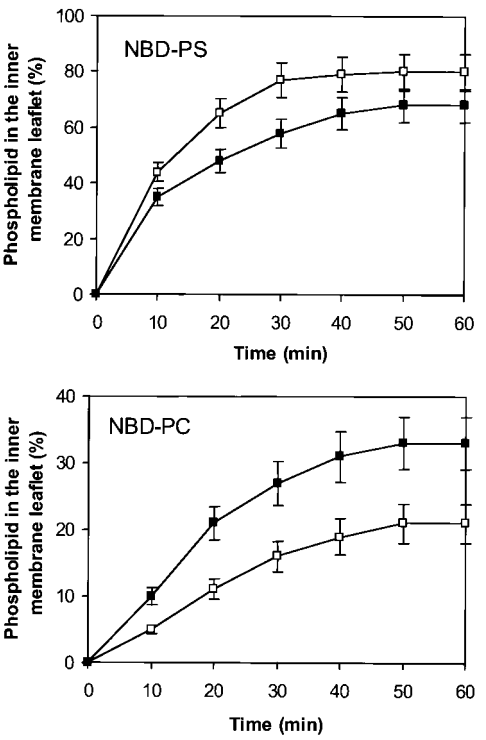

FIG. 1. Inward translocation of NBD-PS (upper panel) and NBD-PC (lower panel) in control (open symbols) and *ras*-transformed (filled symbols) fibroblasts. Cells in suspension were labeled with NBD-PS and NBD-PC at 2°C, washed with HBS, and incubated at 20°C for the indicated time. The fraction of fluorescent phospholipid in the inner leaflet was determined by back-exchange to serum albumin as described under Materials and Methods. The data represent the means ± SE of three separate experiments.

TABLE II

Translocation of NBD-PS and NBD-PC to the Inner Membrane Leaflet of Control and *ras*-Transformed Fibroblasts Treated with PDA and DTT (%)

	Control		*ras*	
Treatment	PS	PC	PS	PC
None	81	22	68	34
PDA	23[a]	21	24[a]	32
PDA + DTT	74[b]	22	61[b]	33

Note. PS, phosphatidylserine; PC, phosphatidylcholine. NBD-PS and NBD-PC are fluorescent analogs of PS and PC. Data are means of three separate experiments. Statistical significance was assessed by comparing nontreated to PDA-treated ($^aP < 0.001$) and PDA-treated to (PDA + DTT)-treated ($^bP < 0.001$) cells.

TABLE III

Content of Phosphatidylserine in the Outer Membrane Leaflet of Control and *ras*-Transformed Fibroblasts with Altered Level of Intracellular Ca^{2+} (ng/10^6 cells)

Ca^{2+} modulation	Control	*ras*
None	17 ± 2	52 ± 4
Depletion	16 ± 2	36 ± 3[a]
Repletion	17 ± 2	45 ± 4[b]

Note. Results are means ± SD of three separate experiments. Statistical significance was assessed by comparing untreated to Ca^{2+}-depleted cells ($^aP < 0.001$) and Ca^{2+}-depleted to Ca^{2+}-repleted cells ($^bP < 0.01$).

translocase activity were not accompanied by corresponding changes in the content of PS in the outer leaflet of *ras* fibroblasts (Table I), indicating lack of correlation between aminophospholipid inward transfer and PS external appearance.

The other transport mechanism which could contribute to enhancement of the PS level in the external membrane leaflet is the nonspecific inward–outward lipid transfer referred to as scrambling. The movement of PC between the monolayers is usually due to this scrambling, which is why NBD-PC was used to measure the nonspecific transfer activity (32). Our interest was focused on scramblase also because of the elevated degree of PC internalization observed in *ras*-transformed cells (Fig. 1). Since it is well documented that the nonspecific bidirectional lipid movement is largely calcium dependent in different cell lines (10, 12), modulation of the intracellular calcium level seems a reliable way to assess the participation of phospholipid scrambling in PS outward translocation. To test whether calcium was essential for PS external appearance, fibroblasts were incubated for 30 min in calcium-free HBS containing BAPTA-AM and EGTA as intracellular and extracellular Ca^{2+} chelators, respectively, and then submitted to measurement of PS in the outer leaflet. The internalization of NBD-PS and NBD-PC to the inner leaflet was measured simultaneously. The intracellular Ca^{2+} concentration in *ras* cells was 112 nM before depletion and 23 nM after incubation with BAPTA-AM and EGTA as measured by the fura-2 method (30). Reduction of the intracellular calcium resulted in a marked reduction of PS external expression in the transformed cells (Table III). Furthermore, calcium repletion restored, although incompletely, the content of PS in the outer leaflet of *ras* cells (Table III). PS uptake was altered insignificantly (66 versus 71%) upon calcium reduction in *ras* cells, demonstrating that calcium was not essential for PS uptake in both control and transformed cells (Fig. 2). Control cells were incubated in calcium-containing HBS since no significant differences were observed in PS internalization upon calcium omission (Fig. 2). However, PC translocation to the inner leaflet showed a marked calcium dependence in both control and *ras* cells (Fig. 3). In transformed cells, calcium depletion was accompanied by a 57% reduction of NBD-PC uptake (Fig. 3), illustrating that the elevated rate of NBD-PC internalization (Fig. 1) was due to stimulated calcium-dependent lipid transport. Calcium repletion induced a complete recovery of PC uptake in *ras* cells as compared to *ras* cells not submitted to calcium depletion–repletion procedures (Fig. 3).

We also investigated the metabolism of the inserted phospholipid analogs after being internalized into the

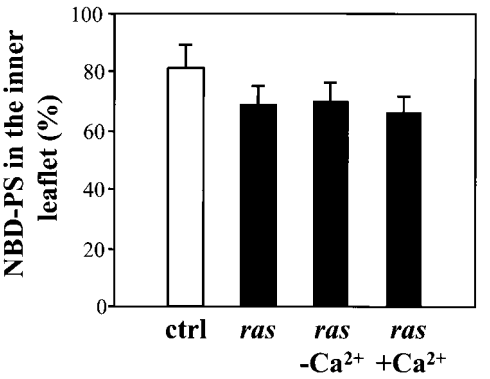

FIG. 2. Internalization of NBD-PS in control (open bar) and *ras*-transformed (solid bars) fibroblasts submitted to a calcium depletion ($-Ca^{2+}$) and calcium repletion ($+Ca^{2+}$) procedure. Ca^{2+} depletion was performed by cell incubation with BAPTA-AM and EGTA and Ca^{2+} repletion was carried out by incubation in the presence of Ca^{2+} and Ca^{2+}-ionophore A23187 as described under Materials and Methods. Internalization of NBD-PS was assessed after fibroblast incubation at 20°C for 45 min. The data represent the means ± SE of three separate experiments. Differences were significant ($P < 0.001$) between values measured in control cells (open bar) and nontreated *ras* cells (first solid bar). Differences between values observed for nontreated *ras* cells and *ras* cells submitted to Ca^{2+} depletion and Ca^{2+} repletion were insignificant.

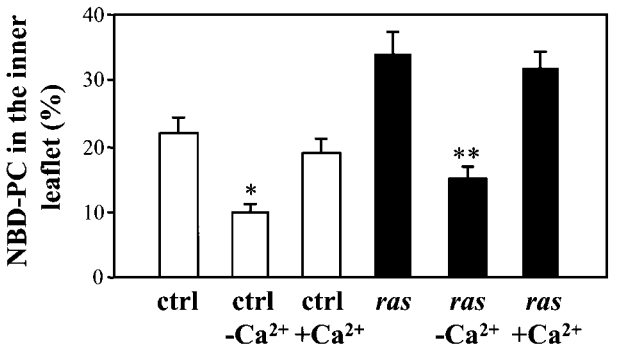

FIG. 3. Internalization of NBD-PC in control (open bars) and *ras*-transformed (solid bars) fibroblasts submitted to a calcium depletion ($-Ca^{2+}$) and calcium repletion ($+Ca^{2+}$) procedure. Incubation conditions are as in Fig. 2. The data represent the means ± SE of three separate experiments. * $P < 0.001$ compared to control nontreated cells. ** $P < 0.001$ compared to *ras*-transformed nontreated cells.

cells to determine whether they were hydrolyzed by membrane-bound phospholipases (data not shown). The results indicated that about 86% of the introduced lipid remained unhydrolyzed throughout the period of investigation, indicating that the fluorescent probes measured in the inner membrane leaflet were mostly phospholipids and not their hydrolytic products.

DISCUSSION

Nucleated cells are a complicated model to study membrane asymmetry because the lipid analogs used in such studies become metabolized or internalized to the subcellular organelles, which hinders prolonged measurements at physiological temperatures. Yet many recent reports have been devoted to investigation of lipid asymmetry on nucleated cells (24, 32). In the present work, we studied the differences in PS transmembrane distribution and the transport mechanisms responsible for these differences in control and *ras*-transformed NIH 3T3 fibroblasts. Our previous investigations demonstrated significant alterations in the aminophospholipid metabolism in *ras* cells (16, 21). Preliminary studies showed a 10% increase in the PE level present in the extracellular monolayer of transformed cells as measured with exogenous NBD-PE. However, the content of PS in the outer leaflet or *ras* cells was about three times the content in control cells. Ordinarily, PS is located almost entirely in the cytoplasmic membrane monolayer and its outward translocation makes cells susceptible to macrophage recognition and destruction (15). PS external appearance has been observed in cells undergoing apoptosis (11), in tumorigenic cells (12), or upon cell activation (13, 14). The predominant location of PS in the inner membrane monolayer is maintained by an ATP-requiring aminophospholipid translocase (4), but the mechanisms responsible for its appearance in the outer leaflet remain unclear. It has been suggested that PS outward translocation is accompanied by both loss of aminophospholipid translocase activity and elevation of the nonspecific transbilayer movement of phospholipids (32). In an attempt to clarify the mechanisms underlying the accumulation of PS in the outer leaflet, we investigated the relative contribution of two membrane transport systems—the specific aminophospholipid translocase and the nonspecific lipid scramblase. The internalization of NBD-labeled PS was measured to assess the aminophospholipid translocase activity and NBD-PC was used as a marker of the nonspecific transport between the two membrane leaflets. As mentioned above, the higher PS appearance in the outer monolayer could be due either to a loss of aminophospholipid translocase activity, which would result in a reduced transfer of PS to the inner monolayer, or to an increase of the nonspecific bidirectional lipid transport, which would relocate some PS molecules back to the outer leaflet. Our results showed that the aminophospholipid transport was inhibited and the nonspecific bidirectional translocation was stimulated in the transformed cells, implying that each of them or both taken together could induce accumulation of PS in the outer leaflet. To test which of these transport mechanisms was related to PS accumulation in the outer leaflet of *ras* cells, both activities were submitted to reversible alterations and the corresponding changes in PS external appearance were monitored. To induce aminophospholipid transfer inhibition, the cells were pretreated with PDA, which inhibits the inward transport of aminophospholipids in erythrocytes (18). Our preliminary studies proved that PDA was an effective inhibitor of PS inward movement in fibroblasts as well. Thus, if loss of aminophospholipid translocase activity was the major reason for the altered PS asymmetry in *ras*-transformed cells, this inhibitor should increase additionally the appearance of PS in the external membrane monolayer. The data indicated that although PDA treatment reduced the rate of NBD-PS internalization in both control and *ras*-transformed fibroblasts, the content of PS in the external leaflet was not significantly enhanced in PDA-treated *ras* cells compared to nontreated ones. We chose PDA to inhibit aminophospholipid translocase because it triggers initiation of thiol–disulfide exchange, which can be reversed upon reduction of the disulfide bridges by DTT. The aim of these studies was to determine whether opposite alterations of aminophospholipid translocase activity were accompanied by corresponding changes in PS external appearance. The results demonstrated that the inhibition and restoration of aminophospholipid translocase activity in *ras* cells were not followed by corresponding elevation and reduction of PS content in the outer membrane leaflet (Tables I and II). These data suggest that the

reduction of aminophospholipid inward translocation observed in *ras* cells was not the major reason for PS external appearance, implying the participation of a different transport mechanism.

The other tested lipid transport system which could contribute to PS appearance in the extracellular leaflet is the nonspecific scramblase, which transports phospholipid molecules in both directions between the two membrane leaflets (5). There are many studies documenting that scramblase is activated upon elevation of the intracellular Ca^{2+}concentration in different cells (6, 32). Our preliminary investigations showed a similar Ca^{2+} concentration dependence of the nonspecific lipid transport in fibroblasts as measured by internalization of NBD-PC (data not shown). Hence, the stimulation of the nonspecific lipid transport by calcium seems to be a common feature of various cell types. Calcium has been reported to inhibit aminophospholipid translocase in erythrocytes (10), although data seem to be controversial on this issue as far as other cell types are concerned (32). To test the effect of intracellular calcium on the nonspecific lipid transport and PS outer appearance, transformed and nontransformed fibroblasts were submitted to a calcium depletion–repletion procedure. Incubation of *ras* cells with BAPTA-AM and EGTA as intracellular and extracellular Ca^{2+} chelators, respectively, did not affect significantly PS internalization (Fig. 2), but induced a decrease of PS appearance in the outer leaflet (Table III). In addition, calcium depletion resulted in reduction of PC internalization, illustrating that the nonspecific calcium-dependent lipid transfer was suppressed. Restoration of the intracellular calcium by incubation in the presence of Ca^{2+} and Ca^{2+}-ionophore A23187 was able to induce PS reappearance in the outer leaflet, indicating clearly that the outward translocation of PS was a calcium-dependent process and was most likely performed by the nonspecific lipid scramblase. This hypothesis is strongly supported by the correlation between PC internalization and the changes in PS outer appearance (Fig. 3, Table III). Taken together, these data suggest that the calcium-activated nonspecific lipid transport is responsible for the outward translocation of PS in *ras*-transformed cells. That loss of aminophospholipid translocase activity was not the major reason for PS external appearance in *ras* cells can be seen by a simple comparison of Tables I and II. However, certain contribution of the reduced aminophospholipid inward transfer observed in *ras* cells should not be completely ruled out.

In this work, we investigated the role of two membrane-bound transport systems, which could be responsible for the enhanced PS level in the outer membrane leaflet of *ras*-transformed fibroblasts—the aminophospholipid translocase and the nonspecific bidirectional lipid scramblase. We suggest that the nonspecific calcium-dependent inward–outward lipid transport is predominantly responsible for the enhanced appearance of PS in the external leaflet of *ras*-transformed fibroblasts. The higher expression of PS in the outer membrane leaflet of *ras*-transformed cells might as well be related to higher susceptibility to macrophage recognition and subsequent destruction, which makes studies on the mechanisms responsible for PS transmembrane translocation particularly important.

ACKNOWLEDGMENT

The authors thank Sonia Levi for technical assistance in preparation of the manuscript.

REFERENCES

1. Williamson, P., and Schlegel, R. A. (1994) *Mol. Membr. Biol.* **11,** 199–216.
2. Zwaal, R. F. A., and Schroit, A. J. (1997) *Blood* **89,** 1121–1132.
3. Bevers, E. M., Comfurius, P., Dekkers, D. W. C., and Zwaal, R. F. A. (1998) *Biochim. Biophys. Acta* **1439,** 317–330.
4. Seigneuret, M., and Devaux, P. F. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 3751–3755.
5. Connor, J., Pak, C. H., Zwaal, R. F. A., and Schroit, A. J. (1992) *J. Biol. Chem.* **267,** 19412–19417.
6. Williamson, P., Kulick, A., Zachowski, A., Schlegel, R. A., and Devaux, P. F. (1992) *Biochemistry* **31,** 6355–6360.
7. Smets, E., Comfurius, P., Bevers, E., and Zwaal, R. F. A. (1994) *Biochim. Biophys. Acta* **1195,** 281–286.
8. Schlegel, R. A., Stevens, M., Lumley-Sapinski, K., and Williamson, P. (1993) *Immunol. Lett.* **36,** 283–288.
9. Zwaal, R. F. A., Comfurius, P., and Bevers, E. M. (1993) *Biochem. Soc. Trans.* **21,** 241–253.
10. Bitbol, M., Feldman, P., Zachowski, A., and Devaux, P. F. (1987) *Biochim. Biophys. Acta* **904,** 268–282.
11. Fadok, V. A., Voelker, D. R., Campbell, P. A., Cohen, J. J., Bratton, D. L., and Henson, P. M. (1992) *J. Immunol.* **148,** 2207–2216.
12. Utsugi, T., Schroit, A. J., Connor, J., Bucana, C. D., and Fidler, I. J. (1991) *Cancer Res.* **51,** 3062–3066.
13. Bevers, E. M., Comfurius, P., and Zwaal, R. F. A. (1983) *Biochim. Biophys. Acta* **736,** 57–66.
14. Williamson, P., Bevers, E. M., Smeets, E. F., Comfurius, P., Schlegel, R. A., and Zwaal, R. F. A. (1995) *Biochemistry* **34,** 10448–10455.
15. Allen, T. M., Williamson, P., and Schlegel, R. A. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 8067–8071.
16. Momchilova, A., and Markovska, T. (1999) *Int. J. Biochem. Cell Biol.* **31,** 311–318.
17. Lacal, J., and Carnero, A. (1994) *Oncol. Rep.* **1,** 677–693.
18. Connor, J., and Schroit, A. J. (1988) *Biochemistry* **27,** 848–851.
19. Der, C. J., Finkel, T., and Cooper, J. M. (1986) *Cell* **44,** 167–176.
20. Momchilova, A., Markovska, T., and Pankov, R. (1998) *Biochimie* **80,** 1055–1062.
21. Momchilova, A., Markovska, T., and Pankov, R. (1999) *Biochem. Mol. Biol. Int.* **47,** 555–561.
22. Connor, J., and Schroit, A. J. (1989) *Biochemistry* **28,** 9680–9685.

23. Kok, J. W., Eskelinen, S., Hoekstra, K., and Hoekstra, D. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 9896–9900.
24. Pomorski, T., Muller, P., Zimmermann, B., Burger, K., and Devaux, P. F. (1996) *J. Cell. Sci.* **109,** 687–698.
25. Connor, J., Bucana, C., Fidler, I., and Schroit, A. J. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 3184–3188.
26. Comfurius, P., Bevers, E. M., and Zwaal, R. F. A. (1994) *in* Biomembrane Protocols. Architecture and Function (Graham, J. M., and Higgins, J. A., Eds.), pp. 131–142, Humana Press, Clifton, NJ.
27. Bligh, E. G., and Dyer, W. F. (1959) *Can. J. Physiol.* **37,** 911–917.
28. Touchstone, J. C., Chen, J. C., and Beaver, K. M. (1980) *Lipids* **15,** 61–62.
29. Kahovcova, J., and Odavic, R. (1969) *J. Chromatogr.* **40,** 90–95.
30. Lennon, S. V., Kilfeather, S. A., Hallett, M. B., Campbell, A. K., and Cotter, T. G. (1992) *Clin. Exp. Immunol.* **87,** 465–471.
31. Lowry, R. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
32. Bratton, D. L., Fadok, V. A., Richter, D. A., Kailey, J. M., Guthrie, L. A., and Henson, P. M. (1997) *J. Biol. Chem.* **272,** 26159–26165.