Human Erythrocyte Membrane Lipid Asymmetry: Transbilayer Distribution of Rapidly Diffusing Phosphatidylserines†

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ABSTRACT: Human erythrocytes were incubated with sonicated vesicles composed of diheptanoyl-, dioctanoyl-, didecanoyl-, or dimyristoylphosphatidylserine, and the transbilayer distribution of the incorporated foreign lipid was examined by monitoring changes in cell morphology (Daleke & Huestis (1989) *J. Cell. Biol. 108*, 1375). Cells incubated with all phosphatidylserine homologs crenated initially and then reverted to discoid and stomatocytic morphology. Cells exposed to didecanoyl- or dimyristoylphosphatidylserine retained stable stomatocytic morphology during more than 10 h of incubation at 37 °C. Cells exposed to the diheptanoyl or dioctanoyl homologs reverted from stomatocytes to discocytes within 1–4 h. This reversion was more rapid for the shorter acyl chain diheptanoylphosphatidylserine. Reversion was accelerated in both cases by vanadate, an inhibitor of the aminophospholipid translocator. Heat denaturation of cytoskeletal proteins had no effect on phosphatidylserine-induced stomatocytosis or on the reversion to discoid shape of cells exposed to the short-chained homologs. These observations suggest that the aminophospholipid transporter rather than cytofacial lipid binding sites plays the primary role in maintenance of phosphatidylserine asymmetry in the erythrocyte membrane bilayer.

In the human erythrocyte membrane, the choline phospholipids (phosphatidylcholine (PC)[1] and sphingomyelin (SM)) are found predominantly in the outer leaflet of the bilayer, while phosphatidylserine (PS) and phosphatidylethanolamine are localized exclusively or preponderantly in the inner-membrane monolayer. Prior to discovery of the aminophospholipid translocator protein, the distribution of PS had been attributed to its interactions with cytoskeletal proteins, in particular spectrin (Haest et al., 1978). Studies of normal and sickled red cells and spectrin-poor vesicles suggested that this protein is a strong stabilizing factor in maintaining lipid asymmetry (Dressler et al. 1984; Middlekoop et al., 1988). Experiments by Sato and Onishi (1983) and Rybicki et al. (1988) revealed a high-affinity association between protein 4.1 and PS vesicles, and Cohen et al. (1988) identified a 30-kDa fragment of protein 4.1 that was necessary for such binding.

The discovery of a protein that transports PE and PS across the red cell membrane called into question the primacy of the cytoskeleton in controlling aminophospholipid distribution (Seigneuret & Devaux, 1984; Daleke & Huestis, 1985; Connor & Schroit, 1987; Zachowski et al., 1986; Zachowski & Devaux, 1987). Calvez et al. (1988), working with spectrin-poor vesicles, surmised that PS asymmetry results from the kinetic properties of the aminophospholipid translocator rather than from binding between lipid and cytoskeletal proteins. This conclusion supported calculations [Hermann & Muller, 1986; based on the experimental findings of Seigneuret and Devaux (1984)] indicating that PS asymmetry could be maintained by differing rates of inward and outward transport, catalyzed by a bidirectional translocator. In contrast, a later model based on the same experimental data (Williamson et al., 1987) concluded that the transporter acted only as a catalyst for the flip-flop motion of the aminophospholipids and that binding of inner-monolayer lipids to cytoskeletal proteins was necessary to maintain lipid asymmetry. However, spectrin–bilayer interactions appear normal in lipid-symmetric cells (Pradhan et al., 1991), and, at least in human cells, heat denaturation of spectrin does not lead to extensive aminophospholipid randomization (Kumar et al., 1990; Gudi et al., 1990). Some investigators invoke a tandem mechanism, in which both transport and cytofacial binding generate stable asymmetry (Connor & Schroit, 1987; Kumar et al., 1990).

This article describes a series of experiments designed to address aspects of both hypotheses. As Homan and Pownall (1988) demonstrated, the rate of passive transbilayer diffusion (flip-flop) of PC is significantly faster for lipids containing short ($<C_{10}$) acyl chains, as compared with longer chain homologs. Using cell morphology to monitor the disposition of incorporated lipids, we compared the behavior of PS homologs having 7–14-carbon acyl chains introduced into normal and variously compromised erythrocytes. If the distribution of PS is dictated by head group association with cytoskeletal proteins, alteration of its passive transbilayer diffusion rate should not affect its eventual distribution. Additionally, heat denaturation of cytoskeletal attachment sites should permit fast-diffusing PS to randomize between the monolayers, with clear morphological consequences. Alternatively, if the aminophospholipid translocator is solely responsible for PS localization in the inner monolayer, fast-diffusing PS molecules may challenge the ability of the translocator to maintain PS asymmetry. Addition of vanadate, a specific inhibitor of the Mg^{2+}-ATP-dependent translocator, should amplify this effect.

EXPERIMENTAL PROCEDURES

Materials. Blood was obtained from healthy human volunteers by venipuncture and collected into Vacutainer tubes

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[1] Abbreviations: DDPC, didecanoylphosphatidylcholine; DDPS didecanoylphosphatidylserine; DHPC, diheptanoylphosphatidylcholine; DHPS, diheptanoyphosphatidylserine; DMPC, dimyristoylphosphatidylcholine; DMPS, dimyristoylphosphatidylserine; DOPC, dioctanoylphosphatidylcholine; DOPS, dioctanoylphosphatidylserine; PC, phosphatidylcholine; PS, phosphatidylserine; PBS, phosphate-buffered saline; SM, sphingomyelin.

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(Becton-Dickenson) containing sodium citrate to yield 0.4%. Erythrocytes were isolated by resuspending the cell pellet in 3–4 vol of 150 mM NaCl, centrifuging at 3000*g* for 5 min, and aspirating the supernatant and top layer of cells. Three saline washes were followed by one wash in 4 vol of phosphate-buffered saline (PBS; 138 mM NaCl, 5 mM KCl, 6.1 mM NaH_2PO_4, 1 mM $MgSO_4$, 5 mM glucose, pH 7.4) and centrifugation at 5000*g* for 10 min. Cells not used immediately were stored at 4 °C.

Dimyristoyl PS (DMPS) was purchased from Avanti Polar Lipids, Inc. Diheptanoyl-, dioctanoyl-, didecanoyl-, and dimyristoylphosphatidylcholines (DHPC, DOPC, DDPC, and DMPC, respectively) were products of Sigma Chemical Company.

Synthesis of DHPS, DOPS, and DDPS. Short acyl chain PSs were prepared from the corresponding PCs by the method of Comfurius and Zwaal (1977). DHPC, DOPC, and DDPC were dissolved in distilled ethyl ether to a concentration of 25 mg/mL and combined with an equal volume of L-serine (4.8 M in 100 mM acetate buffer, pH 5.6) plus 5 IU of phospholipase D (cabbage; Sigma Chemical Co.). The mixture was stirred at 40 °C, and an additional 5 IU of the enzyme was added every 30 min. After 4 h, the reaction was quenched by addition of an equal volume of 200 mM EDTA. The ether was evaporated, 4.3 vol of $CHCl_3/CH_3OH$ (5:8) was added, and the mixture was stirred for 30 min at room temperature. The precipitated enzyme was removed by filtration through a medium frit, and the filtrate was extracted with 1 vol of H_2O and 3.7 vol of $CHCl_3$. The organic layer was concentrated, and the reaction products were separated on a Whatman CM-52 cation-exchange cellulose column.

Vesicle Preparaton. Unilamellar vesicles were prepared by sonicating lipid suspensions to clarity in 150 mM NaCl, 10 mM Na_2HPO_4, pH 7.4 (NaCl/P_i) under argon in a bath sonicator. Lipid concentrations are as given in the figure legends.

Cell–Vesicle Incubations. Cells at 37 °C in phosphate-buffered saline were suspended with an equal volume of sonicated lipid vesicles to yield a final hematocrit of 22. After 10 min at 37 °C, cells were pelleted by centrifugation (8800*g*, 3 min), and the supernatant, containing unincorporated lipid, was removed. Cells were resuspended in phosphate-buffered saline (hematocrit 22) and returned to 37 °C. After the times indicated in the figure legends, aliquots of cell suspensions were fixed and their morphology examined. In extended incubations, cells were supplemented every 5 h with penicillin (100 μg/mL), streptomycin (500 μg/mL), and sugars (100 mM inosine, 100 mM glucose, and 10 mM adenosine in NaCl/P_i, pH 7.4, 10% (v/v)).

Vanadate Treatment. After 4 h of incubation of the cell suspensions at 37 °C, Na_3VO_4 was added to control and lipid-treated cell suspensions to yield a final concentration of 100 μM Na_3VO_4. After 30 min of further incubation of the cell suspensions at 37 °C, EDTA was added to some samples to yield a concentration of 1 mM. Morphology of all samples was monitored at the times shown in the figure legends.

Heat Denaturation of Spectrin. After 2 h of incubation at 37 °C, control and lipid-treated cells were heated to 50 °C for 11 min and then returned to 37 °C for further incubation (Kumar et al., 1990).

Morphology Assay. At the time intervals given in the figure legends, aliquots of cell suspensions were fixed for 15 min at room temperature in 10 vol of glutaraldehyde solution (1% in 150 mM NaCl). Cell morphology was examined by bright field microscopy. Crenated cells were assigned scores of +1 to +5 according to their degree of crenation; discocytes were assigned scores of 0; and stomatocytes were scored −1 to −4 with increasingly severe stomatocytosis. The average score for a field of 100 cells was taken as the morphological index (MI). This indexing system, originated by Bessis et al. (1973), is described in detail in Daleke and Huestis (1985). Due to quantitative differences in the morphology responses of erythrocytes from different blood donors, it is not practical to represent shape change data as averages of multiple experiments. Data shown in the figures represent averages of replicate (3–5) samples taken in a single representative experiment; experimental error in evaluation of multiple fields is typically ≤0.1 MI unit. This is approximately the size of the figure symbols.

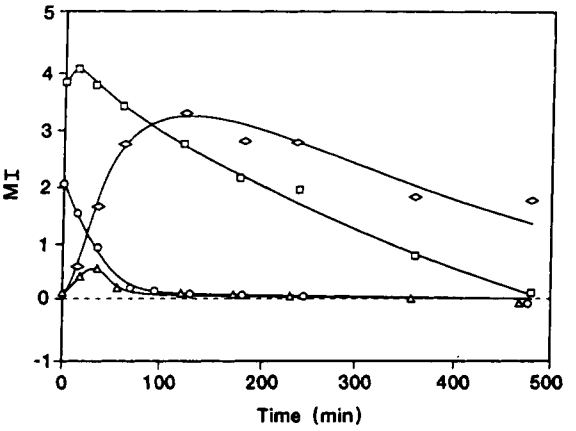

FIGURE 1: Morphological response of erythrocytes exposed to short acyl chain PC vesicles. Cells were incubated for 10 min at 37 °C with 0.1 mM DHPC (△), DOPC (○), or DDPC (□). Unincorporated lipid was removed (t = 0), and cells were incubated further in phosphate-buffered saline. Superimposed (◇) is the morphological response of cells incubated continuously with 1 mM DMPC.

RESULTS

Incorporation of Short-Chained PCs into Erythrocytes. Erythrocytes were incubated for 10 min with vesicles of DHPC, DOPC, or DDPC (0.1 mM) or DMPC (1 mM), and their morphological responses were examined (Figure 1). Cells exposed to DMPC, DDPC, or DOPC became echinocytic, a change evident in less than 1 min for DDPC and DOPC. Consistent with its slower transfer between membranes, DMPC induced echinocytosis more slowly, with its maximum effect apparent after 2 h. (Also due to kinetic differences in lipid diffusion, cells incubated with 0.1 mM DMPC did not crenate on the time scale examined (not shown).)

With continued incubation, the crenated cells reverted to discoid morphology but at different rates. Cells treated with DOPC, with eight-carbon acyl chains, regained discoid shape with a half-time of 30 min. DDPC-treated cells reverted to discocytes with a half-time of 4.5 h, and DMPC-treated cells showed only minimal reversion on this time scale. Cells treated with 0.1 mM DHPC, the shortest chain lipid, became only slightly echinocytic and reverted to discoid shape within 1 h.

Incorporation of Short-Chained PSs into Erythrocytes. Figure 2 shows the morphological response of red cells exposed to PS homologs containing 7- (DHPS), 8- (DOPS), or 10- (DDPS) carbon acyl chains. Cells were incubated with lipid vesicles for 10 min at 37 °C and then washed free of unincorporated lipid and incubated further at 37 °C. Immediately on addition of the lipid, cells crenated transiently but then reverted to stomatocytic morphology. The rate of echinocyte-to-stomatocyte reversion was rapid and, as reported

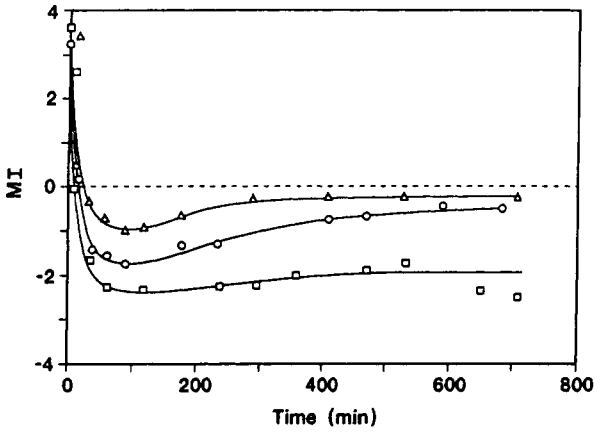

FIGURE 2: Morphological responses of erythrocytes exposed to short acyl chain PS vesicles. Cells were incubated for 10 min at 37 °C with 6.9 mM DHPS (△) or with 1 mM DOPS (○) or DDPS (□). Unincorporated lipid was removed (t = 0), and cells were incubated further in sugar-supplemented phosphate-buffered saline.

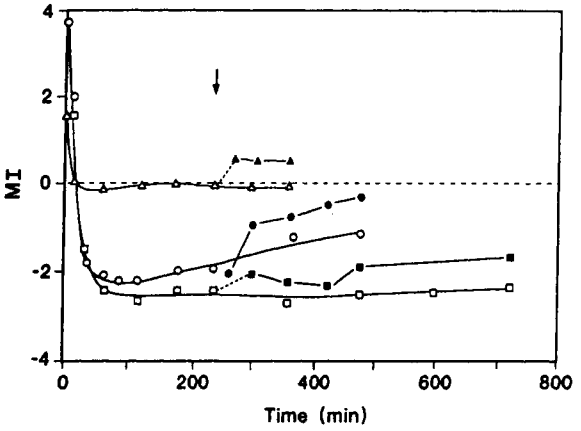

FIGURE 3: Effect of vanadate on morphology of erythrocytes exposed to short acyl chain PS. Cells were treated with 1.2 mM DDPS (□), 1 mM DOPS (○), or 1 mM DHPS (△), isolated from unincorporated lipid, and incubated in buffer at 37 °C. After 4 h, 0.1 mM Na_3VO_4 was added to an aliquot of each sample (closed symbols).

previously (Daleke & Huestis, 1985), was indistinguishable for the three PS homologs. With further incubation, cells treated with the two shorter acyl chain homologs (DHPS, DOPS) evolved further, from stomatocytic to discoid shape. This reversion occurred more rapidly for DHPS ($t_{1/2}$ 15 min) than for DOPS ($t_{1/2}$ 4.5 h). Cells treated with DDPS retained stable stomatocytic morphology over more than 12 h of incubation, consistent with the previously reported behavior of DMPS-treated cells (Daleke & Huestis, 1985).

Effect of Vanadate on PS-Treated Erythrocytes. After treatment with DHPS, DOPS, or DDPS, cells were incubated for 4 h at 37 °C and then exposed to 100 μM sodium orthovanadate (Figure 3). DHPS-treated cells, which had reverted to discoid morphology by 4 h, crenated slightly on exposure to vanadate (Figure 3, closed symbols). Vanadate accelerated the ongoing stomatocyte-to-discocyte reversion of DOPS-treated cells and produced a minor but reproducible reversion in DDPS-generated stomatocytes.

In a separate experiment, cells were treated with PS homologs and vanadate as in Figure 3. After 30 min of incubation with vanadate, cell suspensions were supplemented with EDTA (1 mM final concentration). This treatment diminished or reversed the vanadate effect (Figure 4). DHPS-treated cells regained discoid morphology, DOPS-treated cells maintained stomatocytic shape comparable to control DOPS stomatocytes (i.e., exposed to neither vanadate nor EDTA),

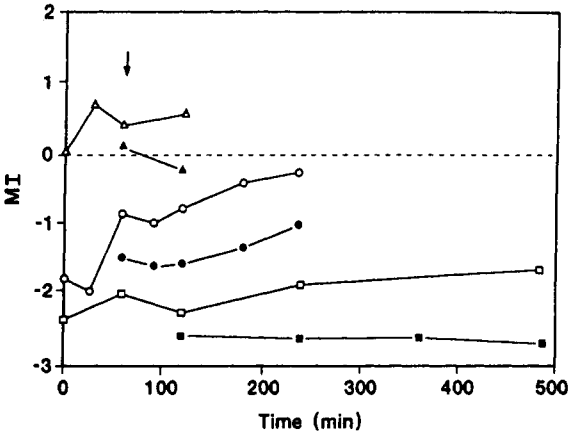

FIGURE 4: Effect of vanadate on PS-treated erythrocytes reversed by EDTA. Cells were treated with DHPS (△), DOPS (○), or DDPS (□) and then vanadate, as in Figure 3. After 30 min of incubation of the cells (arrow), 1 mM EDTA was added to half of each sample (closed symbols), and samples were incubated further.

and DDPS-treated cells retained stomatocytic shape.

Heat Denaturation of Spectrin. Cells were incubated at 37 °C for 2 h with DHPS, DOPS, or DMPS. One sample of each was heated to 50 °C for 11 min and then returned to 37 °C and monitored further (Figure 5). In each case, the morphology of heat-treated cells did not differ significantly from controls maintained at 37 °C: cells containing the shorter acyl chain homologs DHPS and DOPS continued slow reversion toward discoid shape, and cells containing DMPS (Figure 5B) retained stable stomatocytic morphology.

DISCUSSION

One mechanism that might generate the aminophospholipid asymmetry observed in many plasma membranes is head-group-specific lipid binding to components of the membrane protein skeleton. Heat denaturation of cytoskeleton components such as spectrin (Jackson et al., 1973; Rakow & Hochmuth, 1975; Kumar et al., 1990) might be expected to release lipids from such binding, leading to their randomization between membrane monolayers. However, such randomization is difficult to examine in native cells. The rates of passive transmembrane diffusion are slow for lipids having long acyl chains, and it is difficult to maintain metabolic competence in cells incubated in vitro for the extended periods required for such diffusion. ATP depletion per se could lead to membrane reorganization, complicating interpretation of the result.

The present studies address this question by examining the behavior of red cells exposed to PS homologs with short (C_7 to C_{14}) acyl chains. Shorter-than-native acyl chain PSs can insert into red cell membranes and are transported normally from the outer to the inner monolayer (Daleke & Huestis, 1985). By analogy with PCs (Homan & Pownall, 1988), the rate of passive transbilayer diffusion (flip-flop) would be expected to be acyl chain-dependent, increasing with decreasing acyl chain length. If head-group-specific binding sites exist, short acyl chain lipids should be sequestered like their longer homologs, despite their rapid passive flip rates, and the role of spectrin in such sequestration could be examined by heat denaturation.

As has been shown in reextraction studies of incorporated radiolabeled lipids, cell morphology is a reliable indicator of the incorporation and transbilayer distribution of exogenous lipids (Ferrell et al., 1985a; Daleke & Huestis, 1989). Outer-monolayer intercalators such as PCs crenate cells, while

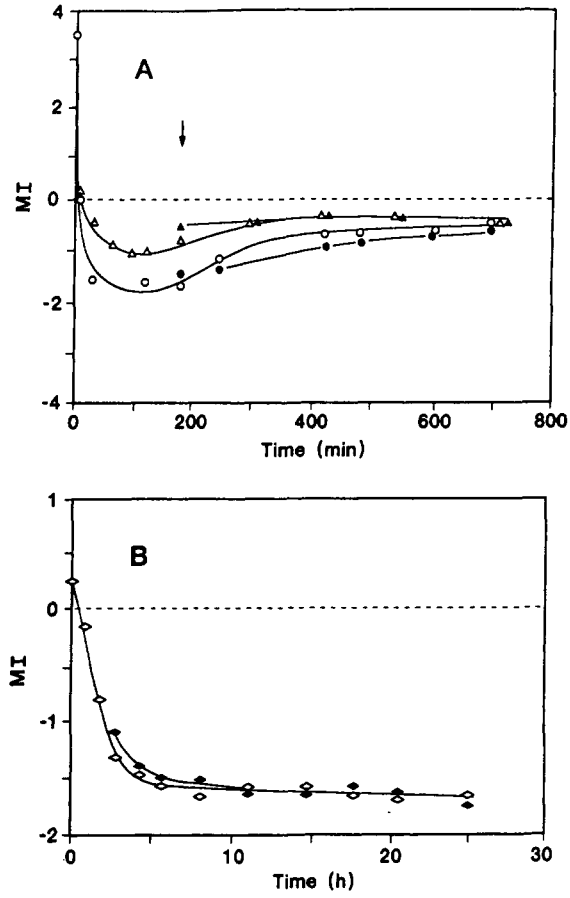

FIGURE 5: Effect of heat denaturation on erythrocytes treated with PS. (A) Cells were preincubated for 10 min with 1 mM DOPS (○) or 6.9 mM DHPS (△). After 2 h, some samples (closed symbols) were heated to 50 °C for 11 min and then returned to 37 °C and incubated further. (B) Cells were preincubated for 30 min with 1 mM DMPS, isolated, and incubated at 37 °C in supplemented buffer (◇). After 3 h, half of the sample (◆) was heated to 50 °C for 11 min and then returned to 37 °C and incubated further.

exogenous PS induces transient crenation followed by reversion to discoid and then stomatogenic forms. This study used cell shape transformations to monitor incorporation, transport, and transbilayer diffusion of the short acyl chain PC and PS homologs.

Cells were incubated with vesicles of the homologous series DHPC (C_7), DOPC (C_8), DDPC (C_{10}), and DMPC (C_{14}). DOPC and DDPC induced immediate crenation (Figure 1), consistent with rapid transfer and insertion into the membrane outer monolayer (Sheetz & Singer, 1974). The shape response was similar but slower for DMPC, as expected from its slower transfer between membranes (Fuji & Tamura, 1983; Ferrell et al., 1985b). Once crenation occurred, DMPC-treated cells remained echinocytic over 10 h of incubation at 37 °C. In contrast, DOPC- and DDPC-treated cells reverted gradually to discoid morphology, a process more rapid for DOPC ($t_{1/2}$ 30 min) than for DDPC ($t_{1/2}$ 4.5 h). Cells exposed to the shortest chain homolog, DHPC, showed little crenation even at early times and reverted to discoid morphology within 1 h. These responses are consistent with initial insertion of all PCs in the membrane outer monolayer, followed by redistribution between both monolayers through passive diffusion, at rates that increase with decreasing acyl chain length. Thus, the acyl chain dependence of passive PC flip-flop reported in vesicle studies (Homan & Pownall, 1988) also is found in erythrocyte membranes.

Cell morphological response to PS homologs also varied with lipid acyl chain composition (Figure 2). In all cases, treatment with PS vesicles induced rapid initial crenation. Subsequently, all PS-treated cells underwent rapid reversion to discoid and then stomatocytic morphology, reflecting acyl chain-independent active PS translocation to the membrane inner monolayer. In these initial phases, cell responses to short acyl chain PS were indistinguishable from the processes observed with DMPS and longer chain homologs (Daleke & Huestis, 1985).

On further incubation, DDPS(C_{10})-treated cells retained stable stomatocytic morphology (as is found with longer chain PS). In contrast, DHPS(C_7)-treated cells reverted to discoid shape, a process with a $t_{1/2}$ of 15 min. DOPS(C_8)-treated cells also reverted toward discs but more slowly ($t_{1/2}$ 4.5 h). Thus, rate and/or extent of stomatocyte-to-discocyte reversion varied inversely with lipid acyl chain length.

While the mechanisms of these slow changes could be more complex (see below), all are consistent with inner-to-outer leaflet flip-flop which, in the case of the shortest chain homologs, is rapid enough to challenge the capacity of the aminophospholipid translocator to sequester the lipid in the inner monolayer. Such rapid passive diffusion of PS homologs is not consistent with stable association between the lipid head group and cytoskeletal components.

To examine this inference further, the effects of spectrin denaturation were studied. If the cytoskeleton contributes to maintenance of PS asymmetry, heat denaturation of spectrin could increase the rate and/or extent to which rapidly diffusing PS returns to the outer monolayer. However, heating cells to 50 °C for 11 min [treatment which reduces the solubility of Triton shell proteins, the extractability of spectrin–actin complexes, and the bilayer association of that complex (Gudi et al., 1990)] did not alter the rate or extent of stomatocyte-to-discocyte reversion for DHPS- or DOPS-treated cells (Figure 6A), nor did such heating produce evident randomization of DMPS (Figure 6B). Thus, no evidence for spectrin's involvement was found. This result is in agreement with the earlier report (Gudi et al., 1990) that spectrin denaturation does not increase outer-monolayer accessibility of endogenous PS.

If the slow shape reversions observed with short-chain PS homologs reflect the relative rates of passive bidirectional flip-flop and active outside-to-inside aminophospholipid transport, such reversions should be accelerated by inhibition of the transporter by agents such as vanadate (Bergmann et al., 1984; Bitbol & Devaux, 1988). This was found: DHPS- and DOPS-treated cells reverted more rapidly in the presence of vanadate, and DDPS-treated cells showed slow reversion (Figure 3). Chelation of vanadate with EDTA (Amos & Sawyer, 1972) reversed these effects; cells became more stomatocytic (Figure 4), consistent with recovered translocator activity.

These studies did not address the possibility that short acyl chain PS homologs could alter red cell shape in complex secondary ways. These could involve alteration of the cytoskeleton, redistribution of other lipids in the membrane, or alteration of endogenous PS transport in the outward direction. However, any such effects would have to (1) respond to PS acyl chain length in the graduated manner observed and (2) be enhanced rapidly and reversibly by the PS transport inhibitor vanadate.

The simplest interpretation of these findings is that spectrin plays at best a secondary role in maintaining PS in the inner leaflet of the membrane bilayer. Instead, localization of PS

arises primarily from the activity of the aminophospholipid translocator.

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