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Bilayer/Cytoskeleton Interactions in Lipid-Symmetric Erythrocytes Assessed by a Photoactivable Phospholipid Analogue†

Deepti Pradhan,‡,§ Patrick Williamson,‖ and Robert A. Schlegel*,‡
Department of Molecular and Cell Biology, The Pennsylvania State University, University Park, Pennsylvania 16802, and Department of Biology, Amherst College, Amherst, Massachusetts 01002
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ABSTRACT: Two mechanisms have been proposed for maintenance of transbilayer phospholipid asymmetry in the erythrocyte plasma membrane, one involving specific interactions between the aminophospholipids of the inner leaflet of the bilayer and the cytoskeleton, particularly spectrin, and the other involving the aminophospholipid translocase. If the former mechanism is correct, then erythrocytes which have lost their asymmetric distribution of phospholipids should display altered bilayer/cytoskeleton interactions. To test this possibility, normal erythrocytes, erythrocytes from patients with chronic myelogenous leukemia or sickle disease, and lipid-symmetric and -asymmetric erythrocyte ghosts were labeled with the radioactive photoactivable analogue of phosphatidylethanolamine, 2-(2-azido-4-nitrobenzoyl)-1-acyl-*sn*-glycero-3-phospho[^{14}C]ethanolamine ([^{14}C]AzPE), previously shown to label cytoskeletal proteins from the bilayer. The labeling pattern of cytoskeletal proteins in pathologic erythrocytes and lipid-asymmetric erythrocyte ghosts was indistinguishable from normal erythrocytes, indicating that the probe detects no differences in bilayer/cytoskeleton interactions in these cells. In contrast, in lipid-symmetric erythrocyte ghosts, labeling of bands 4.1 and 4.2 and actin, and to a lesser extent ankyrin, by [^{14}C]AzPE was considerably reduced. Significantly, however, labeling of spectrin was unaltered in the lipid-symmetric ghosts, suggesting that its relationship with the bilayer is normal in these lipid-symmetric cells. These results do not support a model in which spectrin is involved in the maintenance of an asymmetric distribution of phospholipids in erythrocytes.

The phospholipids of the erythrocyte plasma membrane are nonrandomly distributed between the inner and outer leaflets of the bilayer, with the aminophospholipids phosphatidylserine (PS)[1] and phosphatidylethanolamine (PE) concentrated in the inner leaflet and the choline phospholipids phosphatidylcholine (PC) and sphingomyelin (Sph) concentrated in the outer leaflet (Op den Kamp, 1979). Since the rate of transbilayer diffusion of the phospholipids is fast in comparison to the lifetime of the cell (Middlekoop et al., 1986), some mechanism must maintain lipid asymmetry, and two have been proposed. One postulates that the aminophospholipids are concentrated in the inner leaflet by the action of an ATP-dependent aminophospholipid translocase (Seigneuret & Devaux, 1984; Morrot et al., 1990). The other proposes that the aminophospholipids are trapped in the inner leaflet by interactions with the proteins of the cytoskeleton (Haest & Deuticke, 1976; Haest et al., 1978; Williamson et al., 1982). Several authors have suggested that both mechanisms operate cooperatively to maintain the normal lipid distribution (Middlekoop et al., 1988; Connor & Schroit, 1990; Kumar et al., 1990).

If cytoskeleton/lipid interactions maintain lipid asymmetry, no single protein, binding at a 1:1 molar ratio, is present in sufficient quantity to bind all of the aminophospholipids in the inner leaflet, since the inner leaflet contains about 3×10^7 molecules of PS and 6×10^7 molecules of PE, only 1×10^5 molecules each of the spectrin chains, band 4.1, band 2.1, and perhaps 5×10^5 molecules of actin (Goodman & Shiffer, 1983). However, the large size of the spectrin molecules and

† This work was supported by U.S. Public Health Service Grant CA28921.
* Author to whom correspondence should be addressed.
‡ The Pennsylvania State University.
§ Present address: Department of Pathology, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853.
‖ Amherst College.

[1] Abbreviations: BSA, bovine serum albumin; CML, chronic myelogenous leukemia; [^{14}C]AzPE, 2-(2-azido-4-nitrobenzoyl)-1-acyl-*sn*-glycero-3-phospho[^{14}C]ethanolamine; PC, phosphatidylcholine; PDA, pyridyldithioethylamine; PE, phosphatidylethanolamine; PMSF, phenylmethanesulfonyl fluoride; PS, phosphatidylserine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Sph, sphingomyelin.

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their extended conformation raise the possibility that spectrin/lipid interactions, at a lipid:protein binding ratio of about 100:1, could provide the number of necessary binding sites for the aminophospholipids, although these would likely have a relatively low binding affinity. Accordingly, several investigators have searched in vitro for spectrin/aminophospholipid interactions, with mixed results (Mombers et al., 1980; Bitbol et al., 1989). On the other hand, evidence for band 4.1/aminophospholipid interactions in vitro is considerable (Sato & Ohnishi, 1983; Shiffer et al., 1988; Rybicki et al., 1988; Cohen et al., 1988). Whether these interactions occur in vivo is a separate question.

A more subtle question is raised by the observation that phospholipid asymmetry can be lost, for example, in erythrocytes or ghosts exposed to elevated levels of cytoplasmic Ca^{2+} (Williamson et al., 1985; Chandra et al., 1987; Connor et al., 1990). Although loss of lipid asymmetry over short times (<1–2 h) implies that the rate of lipid diffusion from one leaflet to the other must increase, such an increase by itself will not alter lipid distribution if this distribution is maintained by binding interactions with the cytoskeleton: these interactions will bias the *equilibrium* distribution of the aminophospholipids in the inner leaflet, irrespective of the rates of transverse diffusion (Williamson et al., 1987). If binding interactions between aminophospholipids and spectrin are responsible for maintaining lipid asymmetry, then in lipid-symmetric Ca^{2+}-treated erythrocyte membranes, these interactions must be abrogated. Indeed, such a view is inherent in proposals such as those of Verhallen et al. (1988) that Ca^{2+}-induced cleavage of cytoskeletal ankyrin by calpain could account for loss of lipid asymmetry in platelets.

To analyze phospholipid/cytoskeleton interactions in vivo, we synthesized a photoactivable derivative of the aminophospholipid PE and applied it to erythrocytes (Pradhan et al., 1989). Our results suggested that aminophospholipid/cytoskeleton interactions do indeed occur in vivo, most predominantly with band 4.1 In this report, we apply the probe to normal and pathologic erythrocytes, and to lipid-asymmetric and -symmetric ghosts to determine whether alterations in cytoskeleton/phospholipid interactions can be detected in cases where phospholipid distribution is altered. We find that loss of lipid asymmetry is accompanied by changes in the interaction between the membrane bilayer and several cytoskeletal proteins, particularly band 4.1 and actin. In contrast, however, spectrin/aminophospholipid interactions are not detectably different in lipid-symmetric and -asymmetric cells, suggesting that such interactions do not contribute to the maintenance of lipid asymmetry in erythrocytes.

Materials and Methods

Chemical Synthesis. All chemicals used for syntheses were of reagent grade. 2-(2-Azido-4-nitrobenzoyl)-1-myristoyl-*sn*-glycero-3-phospho[^{14}C]ethanolamine ([^{14}C]AzPE) was synthesized as reported (Pradhan et al., 1989). Pyridyldithioethylamine (PDA) was synthesized as described by Connor and Schroit (1988).

Cells. Blood samples from patients and volunteers at the M. D. Anderson Cancer Center (Houston, TX), Mt. Sinai Hospital (Chicago, IL), and The Pennsylvania State University (University Park, PA) were collected by peripheral venipuncture into heparin according to institutional guidelines after consent was obtained. Blood was centrifuged, and the serum and buffy coat were removed. Erythrocytes were washed 3 times in phosphate-buffered saline (PBS: 137 mM NaCl, 10 mM KCl, 7.4 mM Na_2HPO_4, 2.6 mM NaH_2PO_4, pH 7.4, and 5 mM dextrose). Lipid-symmetric and -asymmetric ghosts were prepared by using the preswell lysis and resealing method (Williamson et al., 1985; Schlegel & McEvoy, 1987). Briefly, to 0.1 mL of packed preswelled normal erythrocytes was added 0.4 mL of lysis buffer [7.4 mM Na_2HPO_4, 2.6 mM NaH_2PO_4, 1 mM $MgCl_2$, 0.1 mM EGTA, pH 7.4, 0.1% (w/v) bovine serum albumin (BSA), and 1 mM phenylmethanesulfonyl fluoride (PMSF)] with (lipid-symmetric) or without (lipid-asymmetric) 1.0 mM $CaCl_2$, while vortex-mixing. The lysate was kept on ice for 2 min after which 40 μL of reseal buffer (10X PBS, 1 mM $MgCl_2$, and 1 mM PMSF) was added to restore isotonicity. Following incubation at 37 °C in a water shaker bath for 30 min, cells were pelleted and washed with cold PBS. Lipid distribution was verified by staining with merocyanine 540 and assessing dye binding by fluorescence microscopy as described previously (Schlegel et al., 1987).

PDA Treatment. Erythrocytes at 2×10^8 cells/mL were incubated with 1 mM PDA for 30 min at 4 °C; ghosts were treated with PDA by having it present at 1 mM during lysis and resealing.

Labeling with [^{14}C]AzPE. [^{14}C]AzPE (1.2 nmol) dissolved in 10 μL of ethanol was added to 10^9 cells suspended in 1 mL of PBS. After vortex-mixing, the suspensions were incubated in the dark at 37 °C for 2.5 h. Photolysis was accomplished by irradiation for 10 min in a Rayonet Mini Reactor, RMR-500, equipped with four medium-pressure mercury lamps (300 nm).

Analysis of Probe Location. Following labeling, cells were pelleted, and an aliquot of the supernatant was counted for radioactivity. The cell pellet was washed with 10 volumes of PBS containing 0.5% BSA (w/v) and lysed in 10 volumes of hypotonic buffer (4.66 mM Na_2HPO_4 and 0.34 mM NaH_2PO_4, pH 8.0, containing 1 mM EGTA and 1 mM PMSF). The membranes were pelleted and washed 5 times with the same buffer. Both the membrane pellet and the combined supernatants were extracted 4 times with 1.5 volumes of solvent ($CHCl_3/CH_3OH$ 2:1 v/v), and aliquots were counted. The combined aqueous fractions from the membrane pellet were concentrated with Centricon-10 microconcentrators prior to analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

SDS–PAGE. Membrane fractions were solubilized in SDS sample buffer and analyzed on either 5.6 or 7.5% gels using the Laemmli system (1970). Gels were stained with Coomassie blue and scanned by using a laser densitometer (LKB 2202 Ultro Scan). Gels were then cut into 2-mm slices which were solubilized overnight in 3% Protosol (Du Pont) at 70 °C and counted in 10 mL of EcoScint (National Diagnostics).

Results

If EGTA is used to chelate Ca^{2+} during lysis and resealing of erythrocytes, the resulting ghosts maintain their asymmetric distribution of phospholipids, providing controls for studies of lipid-symmetric ghosts prepared by lysing and resealing in the presence of Ca^{2+} (Williamson et al., 1985). Besides these experimentally produced lipid-symmetric erythrocytes, several types of pathologic erythrocytes have been found to have an altered transbilayer distribution of phospholipids. Erythrocytes from patients with chronic myelogenous leukemia (CML) have more aminophospholipids exposed on their exterior than normal erythrocytes (Kumar & Gupta, 1983). The same is true for sickle erythrocytes in their deoxygenated state, although when oxygenated the distribution is normal (Lubin et al., 1981; Choe et al., 1986). In the present study, normal erythrocytes, lipid-symmetric and -asymmetric ghosts, CML erythrocytes, and oxygenated sickle erythrocytes (as pathologic negative controls) were compared.

Table I: Amounts of Membrane Proteins Relative to Band 3[a]

protein (band no.)	relative peak area				
	normal	LAG	LSG	CML	Sickle
1	78 (77)	76 (76)	57 (76)	73	73
2	63 (63)	59 (62)	58 (62)	61	61
2.1	37 (36)	35 (36)	28 (35)	32	32
2.2	21 (20)	21 (20)	21 (20)	21	21
2.3			27		
3	100 (100)	100 (100)	100 (100)	100	100
4.1	27 (23)	24 (22)	23 (23)	23	22
4.2	35 (31)	31 (31)	31 (31)	31	30
4.5	31 (30)	31 (30)	30 (31)	31	31
5	22 (21)	22 (21)	23 (21)	22	21
6	22 (22)	22 (22)	22 (22)	22	21

[a] Values in parentheses are for cells pretreated with PDA. LAG, lipid-asymmetric ghosts; LSG, lipid-symmetric ghosts.

Incubating [^{14}C]AzPE with normal erythrocytes results in spontaneous incorporation of about 90% of the probe into the cells (Pradhan et al., 1989). Similar values were obtained with ghosts, sickle erythrocytes, and CML erythrocytes. In all cases, >80% of added label was recovered in the membrane fraction after lysis and centrifugation, with approximately 10% recovered in the cytoplasmic fraction. In all cases, photolysis of cells labeled with probe resulted in two significant changes in the extractibility of probe with chloroform. First, the cytoplasmic probe was quantitatively converted from a chloroform-soluble form to a chloroform-insoluble form, as might be expected if it became covalently linked to cytoplasmic protein. Second, of the membrane-associated probe, virtually all of which was chloroform-soluble prior to photolysis, a small but significant fraction (3–4%) became chloroform-insoluble, corresponding to label linked to membrane cytoskeletal proteins (Pradhan et al., 1989). The extent of conversion was again similar in all cases except for lipid-symmetric ghosts, where conversion was significantly reduced ($P \leq 0.01$) by about a third compared to other cells.

When membrane fractions of photolysed cells were analyzed by SDS–PAGE, recovery of both membrane and cytoskeletal proteins was essentially identical in all cell types (Table I) except lipid-symmetric ghosts, where the recovery of α-spectrin (band 1) and ankyrin (band 2.1) was significantly reduced by about 25%, with the concomitant appearance of band 2.3, an ankyrin proteolytic fragment (Siegel et al., 1980) resulting from the activation of erythrocyte calpain by cytoplasmic Ca^{2+} (Hall & Bennett, 1987). The pattern of labeling of proteins by [^{14}C]AzPE is shown in Figure 1A. As observed originally in normal cells (Pradhan et al., 1989), band 4.1 was the most prominently labeled protein in all cell types, with lesser but significant amounts of label appearing in spectrin, ankyrin, band 4.2, and band 5 (actin). In all cell types, this efficient labeling of cytoskeletal proteins was in marked contrast to the poor labeling of integral membrane proteins, most notably band 3 (the anion transporter).

Only in lipid-symmetric ghosts was a reduction in labeling of cytoskeletal proteins observed. This effect is most easily seen by normalizing labeling to normal cells. As shown in Figure 1B, normalized values indicate that in lipid-asymmetric ghosts, sickle erythrocytes, and CML erythrocytes, labeling of all proteins (except band 6, the peripheral protein glyceraldehyde-3-phosphate dehydrogenase) was generally within about 10% of the levels observed in normal cells, while in lipid-symmetric ghosts, labeling of spectrin, ankyrin, actin, and bands 4.1 and 4.2 was only about half as great as in normal erythrocytes.

The differences in labeling of cytoskeletal proteins in lipid-symmetric ghosts compared to normal erythrocytes could

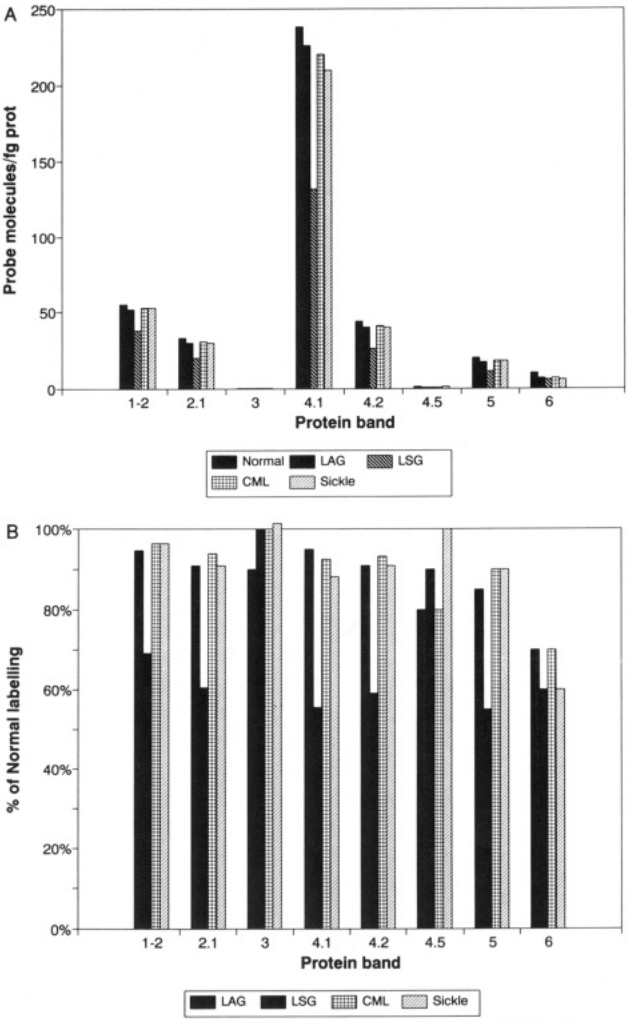

FIGURE 1: [^{14}C]AzPE labeling of membrane and cytoskeletal proteins. Delipidated membranes from photolysed cells were analyzed by SDS–PAGE, and gels were sliced and counted for radioactivity. (A) On the basis of the amount of each protein in the sample and the amount of probe associated with each protein, the number of probe molecules per femtogram of protein was calculated. (B) Data normalized to normal erythrocytes. LAG, lipid-asymmetric ghosts; LSG, lipid-symmetric ghosts.

derive from several sources. The cytoplasmic Ca^{2+} used to produce the lipid-symmetric ghosts not only activates calpain activity (Kuboki et al., 1990), as seen in Table I, but also inhibits the aminophospholipid translocase (Bitbol et al., 1987). To eliminate any differences in probe distribution resulting from inhibition of the translocase by Ca^{2+}, normal cells and ghosts were treated with PDA, a sulfhydryl-reactive inhibitor of the translocase (Connor & Schroit, 1988). Since the translocase is not involved in the mechanism by which Ca^{2+} induces loss of asymmetry (B. Verhoven, R. A. Schlegel, & P. Williamson, unpublished results), lysis and resealing in the presence of Ca^{2+} and PDA still produced lipid-symmetric ghosts, and the asymmetric distribution of phospholipids in normal cells and ghosts prepared without Ca^{2+} was unaffected by PDA (data not shown).

As shown in Table I, besides being an inhibitor of the translocase, PDA is also a very efficient inhibitor of calpain: in its presence, the recovery of spectrin and ankyrin (as well as other membrane proteins) was essentially identical in normal erythrocytes and lipid-symmetric and -asymmetric ghosts. Although overall labeling of cytoskeletal proteins (but not the integral membrane protein bands 3 and 4.5) in all cell types was somewhat reduced by PDA (Figure 2), much of the re-

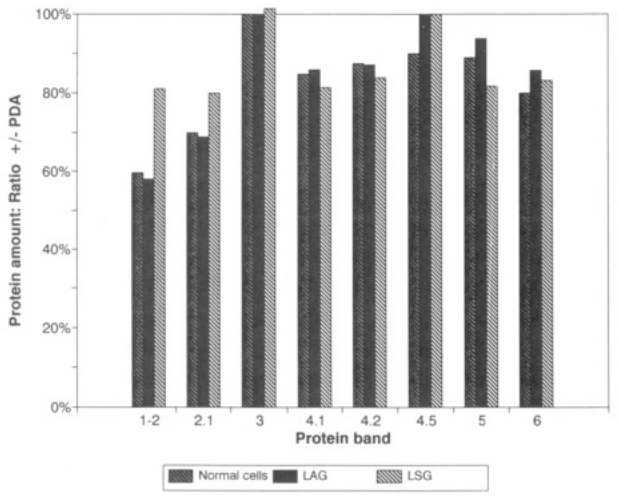

FIGURE 2: [^{14}C]AzPE labeling of membrane and cytoskeletal proteins in PDA-treated cells relative to untreated cells. Cells were treated with PDA and then labeled with [^{14}C]AzPE. Following photolysis, delipidiated membranes were analyzed by SDS–PAGE, and gels were sliced and counted for radioactivity. On the basis of the amount of each protein in the sample and the amount of probe associated with each protein, the number of probe molecules per femtogram of protein was calculated. Values are expressed as the ratio of probe labeling in cells treated with PDA versus untreated cells. LAG, lipid-asymmetric ghosts; LSG, lipid-symmetric ghosts.

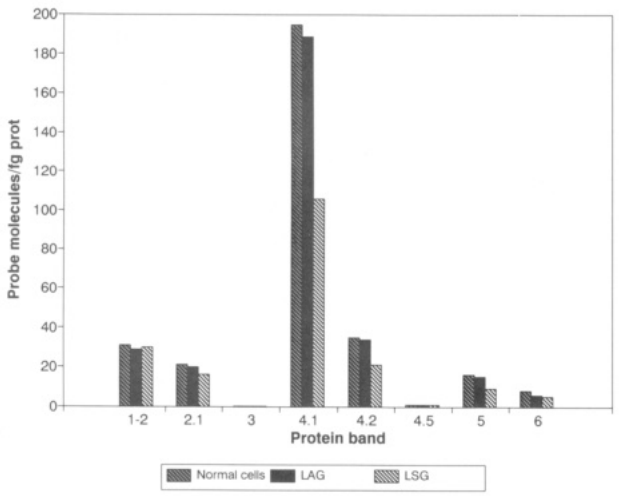

FIGURE 3: [^{14}C]AzPE labeling of membrane and cytoskeletal proteins in PDA-treated cells. Values are probe molecules per femtogram of protein calculated for PDA-treated cells in Figure 2. LAG, lipid-asymmetric ghosts; LSG, lipid-symmetric ghosts.

duction in labeling of ankyrin and all of the reduction in labeling of spectrin seen in untreated lipid-symmetric ghosts, relative to normal erythrocytes and lipid-asymmetric ghosts, were eliminated in PDA-treated cells (Figure 3). However, the reduction in labeling of actin and bands 4.1 and 4.2 in lipid-symmetric ghosts, relative to normal erythrocytes and lipid-asymmetric ghosts, in cells treated with PDA was still observed, and labeling of band 6 was reduced irrespective of the presence of Ca^{2+}.

Discussion

The studies presented here were designed to determine whether bilayer/cytoskeleton interactions, as assessed by labeling of cytoskeletal proteins by the phospholipid analogue [^{14}C]AzPE, are altered in pathologic erythrocytes or model cells in which lipid asymmetry is distorted. As shown previously, [^{14}C]AzPE incorporated into the bilayer effectively labels membrane cytoskeletal proteins, but different proteins are labeled to different extents: while spectrin is readily labeled, its specific activity is exceeded by that of band 4.1. In principle, differential labeling could result either from differences in the affinity of various proteins for lipid or from differences in the proximity of various proteins to the bilayer, or both. Irrespective of the extent to which a protein is labeled relative to others, however, differences in labeling of that protein in different types of cells reflect differences in bilayer/protein interactions. To the extent that the affinity of a particular protein for lipids is a function of its structure, these differences among cell types in bilayer/protein interaction, detected by differences in protein labeling, should primarily reflect differences in proximity of the protein to the bilayer.

The results presented here indicate unambiguously that any differences in lipid distribution or flip rates in oxygenated sickle erythrocytes and CML erythrocytes are not accompanied by differences in bilayer/cytoskeleton interactions detectable by [^{14}C]AzPE labeling. This generalization applies to all the cytoskeletal proteins labeled by the probe, and provides no evidence that changes in these interactions can account for the altered lipid distribution previously reported in CML erythrocytes.

In contrast to these results with pathologic erythrocytes, bilayer/cytoskeleton interactions appear to be rather dramatically altered in ghosts where lipid asymmetry has been completely lost. In one instance, band 6, labeling by [^{14}C]-AzPE is reduced in both lipid-symmetric and -asymmetric ghosts, suggesting that lysis and resealing are responsible for reduced labeling, rather than any difference in lipid-symmetric and -asymmetric cells. However, reduction in labeling of other cytoskeletal proteins in lipid-symmetric ghosts was not mirrored in lipid-asymmetric ghosts, suggesting that differences in the interaction of these proteins with the bilayer were specific to lipid-symmetric ghosts.

When PDA was used to inhibit the aminophospholipid translocase, a reduction of 20–40% in cytoskeletal protein labeling was observed in both lipid-symmetric and -asymmetric ghosts, as expected from a reduction in [^{14}C]AzPE transport; labeling of band 3 was unaffected. Importantly, however, the difference in cytoskeletal labeling between lipid-symmetric and -asymmetric ghosts persisted. Because PDA also inhibited calpain, labeling of spectrin and ankyrin could be assessed in the absence of their proteolysis. Under these conditions, labeling of spectrin was similar in normal erythrocytes, lipid-asymmetric ghosts, and lipid-symmetric ghosts, suggesting that reduced labeling of these proteins in the absence of PDA was a consequence of proteolysis. In contrast, the difference in labeling of other cytoskeletal proteins in lipid-symmetric vs lipid-asymmetric ghosts persisted.

The overall conclusion from these studies is that, when differences due to cell lysis and proteolysis are eliminated, loss of lipid asymmetry in erythrocyte ghosts is associated with reduction in [^{14}C]AzPE labeling of bands 4.1 and 4.2 and actin, and to a lesser extent ankyrin, suggesting that interactions of these cytoskeletal proteins with the bilayer are altered in lipid-symmetric ghosts. Significantly, however, labeling of spectrin is unaltered in lipid-symmetric ghosts, relative to either normal erythrocytes or lipid-asymmetric ghost controls, suggesting that changes in the relationship of this protein and the lipid bilayer are not detectably altered in these lipid-symmetric cells. As stoichiometric considerations suggest that the spectrin/lipid interaction is the most plausible component of an equilibrium binding mechanism for maintenance of lipid asymmetry, these results suggest that alterations in such interactions do not account for the loss of lipid asymmetry in these cells.

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