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LATERAL MOBILITY OF PHOSPHOLIPIDS IN THE EXTERNAL AND INTERNAL LEAFLETS OF NORMAL AND HEREDITARY SPHEROCYTIC HUMAN ERYTHROCYTES

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The lateral diffusion coefficients (D) and the mobile fractions of the fluorescent phospholipid *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (NBD-PE) and of membrane proteins labelled with fluorescein isothiocyanate, were measured by fluorescence photobleaching recovery on erythrocytes from healthy persons and from a hereditary spherocytosis patient. Measurements of lipid probe mobility were performed on ghosts labelled by NBD-PE exclusively at the external monolayer, or at both sides of the membrane. Our results indicate the following: (1) The mean values and the temperature dependence of D are different at the external and internal membrane leaflets. (2) In both normal and HS ghosts the mobile fraction of NBD-PE in the external monolayer does not depend significantly on temperature. On the other hand, the mobile fraction in the internal monolayer is reduced as the temperature is decreased. (3) At low temperatures, the mobile fraction of NBD-PE in the internal monolayer of spherocytic ghosts is significantly lower than the mobile fraction in the internal monolayer of normal ghosts. (4) No differences were observed between the mobilities of membrane proteins in normal and in spherocytic ghosts. However, differences were observed between the two cell populations in the temperature-dependence of the intrinsic fluorescence of unlabelled membrane proteins. The implications of these results for membrane phospholipid asymmetry and for cytoskeletal interactions with the internal lipid monolayer are discussed.

Introduction

Several lines of evidence indicate that the external and internal leaflets in erythrocyte membranes differ in their phospholipid composition and physical properties. Vertical asymmetry in phospholipid head-group distribution in human erythrocytes has been reported by several groups [1–4], indicating that sphingomyelin and phosphatidylcholine are located mostly in the outer monolayer, while phosphatidylethanolamine and phosphatidylserine are found largely in the internal monolayer. The two leaflets in erythrocytes and in other mammalian cells display physically distinct characteristics, as shown by ESR and fluorescence depolarization studies [5–7]. Recently, we have employed fluorescence photobleaching recovery to demonstrate differences between the lateral mobility of lipids in the external and internal monolayers in turkey erythrocyte ghosts [8]. Measurements of the lateral mobility of the fluorescent lipid N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (NBD-PE) in-

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Abbreviations: NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; FITC, fluorescein isothiocyanate.

corporated into the external leaflet or into both sides of the membrane showed temperature-dependent immobilization (reduction in the mobile fraction with decreasing temperatures) in the internal monolayer but not in the external one. Interestingly, the fraction of activatable adenylate cyclase catalytic units in this system shows a parallel decrease with temperature, with similar discontinuities [9], indicating that the changes in the properties of the internal monolayer are reflected in the biological activity of membrane-associated systems.

Interactions of lipids in the inner leaflet with the cytoskeleton could provide a basis for maintaining phospholipid vertical asymmetry, as well as for the different biophysical properties of the two monolayers. Evidence for the involvement of interactions between cytoskeletal components and phospholipids in maintaining the lipid asymmetry of erythrocyte membranes was reported recently [10–12]. In view of the demonstration that cytoskeleton-membrane protein interactions retard the lateral mobility of the latter in erythrocytes [13–15] and in other cell types [16,17], such interactions could also lead to the different temperature-dependence of lipid lateral mobility in the external and internal monolayers.

Some insight into this question may be gained by studying the lateral mobility of membrane components in normal erythrocytes and in erythrocytes from patients who suffer from hereditary spherocytosis, a disease which is caused by defects in the erythrocyte cytoskeletal structure [19–22]. In this communication we report mobility measurements, which demonstrate differences between the lateral motion of the lipid probe NBD-PE in the internal leaflets of normal and hereditary spherocytic (HS) ghosts.

Materials and Methods

Reagents. NBD-PE was obtained from Avanti (Birmingham, AL); its ratio of saturated to unsaturated fatty acids [8] suggests that it partitions equally into fluid and gel lipid phases. Fluorescein isothiocyanate (FITC) was purchased from Molecular Probes (Junction City, OR). All other chemicals were of the highest purity available.

Preparation and labelling of cells. Normal and HS ghosts were prepared from fresh blood taken into a tube containing sodium citrate, and used within 12 h in the experiments. Blood was obtained from healthy donors and from a 24-year-old female hereditary spherocytosis patient twelve years after splenectomy. The patient had moderate hemolytic anemia since childhood, which was gone after the splenectomy. However, the erythrocytes remained abnormal, and revealed the characteristics typical of hereditary spherocytosis: increased osmotic fragility, high specific gravity values [23] and elevated glycerol permeability [24,25]. Resealed ghosts were prepared according to Steck et al. [26]. Briefly, washed erythrocytes were hemolysed in cold phosphate buffer (5 mM sodium phosphate, pH 8). After washing three times by centrifugation, resealing was induced by suspending the ghosts in phosphate-buffered saline (150 mM NaCl/5 mM sodium phosphate (pH 8)) 40 min at 37 °C. This method of ghost preparation does not remove the cytoskeletal proteins [27,28].

For labelling only the external erythrocyte monolayer with NBD-PE, 0.1 ml packed whole erythrocytes suspended in 4 ml phosphate-buffered saline were incubated (15 min, 37 °C) with a 1 : 100 dilution of 1 mg/ml NBD-PE in ethanol, and excess NBD-PE was removed by successive washings of the labelled cells. Labelled cells were kept on ice, and taken for experiments within 5 h after labelling. The slow rate of phospholipid flip-flop in erythrocyte membranes (over 4–5 h at 37 °C, and much slower at low temperatures [4,29,30]) ensures that the probe remains in the outer monolayer during the experiment. NBD-PE labelling from both sides of the membrane was achieved employing an identical procedure, except that unsealed ghosts were labelled prior to the resealing.

Labelling of erythrocyte membrane proteins with FITC was performed by a modification [31] of the procedure of Fowler and Branton [32]. Whole erythrocytes (20% suspensions in phosphate-buffered saline) were incubated (30 min, 4 °C) with an equal volume of 2 mg/ml FITC in 0.2 M sodium carbonate buffer (pH 9.4). The cells were washed by centrifugation twice in the sodium carbonate buffer,and twice in the phosphate-buffered saline (1 : 20 dilution each time). This procedure results in about 60% of the labelling

being in band 3, and about 30% in glycophorin [31,32].

Fluorescence photobleaching recovery. Lateral diffusion of NBD-PE and of fluorescein-labelled membrane proteins was measured by the FPR technique [33,34], employing an apparatus described earlier [35] and built according to the design of Koppel [36]. Recently, it has been shown that the bleaching conditions employed in FPR experiments do not alter the diffusion measurements [37–39]. The lateral diffusion measurements were performed on resealed ghosts which were wet-mounted in the phosphate-buffered saline between two coverslips, and placed on a temperature-controlled microscope stage [8]. The monitoring laser beam (488 nm, 0.02 μW, from an argon ion laser) was focused on the cell surface to a spot of 0.85 μm Gaussian radius with an X100 oil immersion lens. A brief, intense pulse (40 ms, 0.2 mW) bleached 60–70% of the fluorescence in the illuminated region, and the fluorescence recovery was monitored with the attenuated beam. D and the mobile fraction were determined from the fluorescence recovery curves [34].

Fluorescence measurements. The intrinsic fluorescence of unlabelled membrane proteins in normal and spherocytic ghosts (white ghosts devoid of haemoglobin, prepared as described according to Steck et al. [26]) was measured with a Hitachi-Perkin-Elmer MPF-4 spectrofluorimeter equipped with a temperature-controlled cuvette holder. 10 μl ghost suspension (containing 3 μg membrane protein) were diluted in 2 ml phosphate-buffered saline. The sample was excited at 280 nm, and the emitted fluorescence was continuously monitored at 340 nm as the temperature of the sample was changed from 6 °C to 40 °C.

Results

Lipid mobility in ghost membranes

The lateral mobility of NBD-PE was measured by fluorescence photobleaching recovery in ghosts labelled either at the external monolayer or from both sides of the membrane. Four parameters were followed in these experiments: the average values of the diffusion coefficients, the distribution of the D values, the mobile fraction, and the distribution of the mobile fraction values.

At 35 °C or at 20 °C, small differences were observed between the mean D values for labelling at the external monolayer or from both sides, the latter values being somewhat higher. This is consistent with earlier findings [5,40] which indicated that the inner monolayer of erythrocyte membranes is more fluid. The differences between the two types of labelling were more pronounced at low temperatures (6 °C), where the situation was reversed and D for labelling at the external monolayer became larger (Table I). This pattern

TABLE I

LATERAL MOBILITY OF NBD-PE INCORPORATED INTO THE EXTERNAL OR INTO BOTH THE EXTERNAL AND INTERNAL MONOLAYERS OF SPHEROCYTIC AND NORMAL ERYTHROCYTE GHOSTS

Cells were labelled with NBD-PE exclusively at the external leaflet or from both sides of the membrane, as described under Materials and Methods. Diffusion coefficients and mobile fraction values were determined by fluorescence photobleaching recovery on 30–40 ghosts in each case. The results are given as the mean ± S.E.

Cell type	Labelling method	6 °C		20 °C		35 °C	
		D (cm^2/s) ($\times 10^9$)	Mobile fraction	D (cm^2/s) ($\times 10^9$)	Mobile fraction	D (cm^2/s) ($\times 10^9$)	Mobile fraction
Normal ghosts	External monolayer	1.30 ± 0.04	0.86 ± 0.02	2.00 ± 0.06	0.81 ± 0.03	4.2 ± 0.2	0.82 ± 0.02
Normal ghosts	External + internal	0.80 ± 0.03	0.68 ± 0.03	2.7 ± 0.1	0.79 ± 0.01	5.5 ± 0.2	0.78 ± 0.03
Spherocyte ghosts	External monolayer	1.20 ± 0.04	0.85 ± 0.03	2.3 ± 0.1	0.83 ± 0.02	6.3 ± 0.3	0.86 ± 0.02
Spherocyte ghosts	External + internal	0.90 ± 0.06	0.53 ± 0.03	3.0 ± 0.1	0.71 ± 0.02	7.7 ± 0.3	0.86 ± 0.02

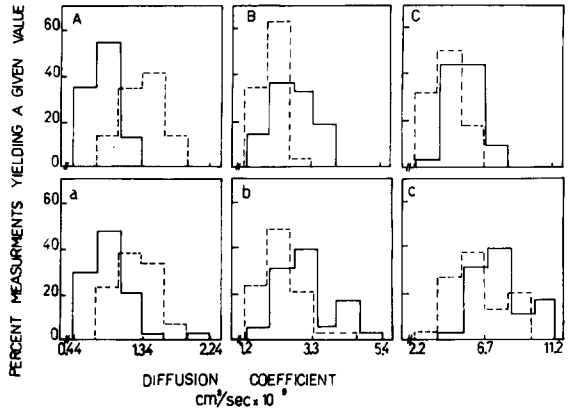

Fig. 1. Histograms of the distribution of D values in normal and spherocytic ghost populations labelled by NBD-PE at both sides of the membrane or exclusively in the external monolayer. (Capital letters) Distribution of D values in the normal ghost population at 6 °C (A), 20 °C (B) and 35 °C (C). (a, b, c) Distribution in spherocytic ghosts at the same temperatures. (– – –) D values obtained by exclusively labelling the external monolayer. (———) D values obtained by labelling both the external and internal ghost monolayers. 30–40 ghosts were measured in each category.

was observed both with spherocytic and normal ghosts. Further insight into the differences in the mobile lipid population was obtained by following the distribution of the D values among the population of ghosts, rather than the mean values of D (Fig. 1). The histograms of the D values for the two types of labelling (from the outside, or from both sides of the membrane) were different both in normal and in spherocytic ghosts. The differences in the histogram pattern are statistically significant ($P < 0.05$), as determined by the Kruskal-Wallis ranking sum test [41]. On the other hand, an analogous analysis does not show significant differences between the histograms of the D values of normal and spherocytic ghosts, for both labelling methods (Fig. 1). A similar situation was observed with the mean D values, except at 35 °C, where somewhat higher values were observed on spherocytic ghosts (Table I). Thus, while one can detect differences in the lateral phospholipid diffusion process between the external and internal monolayers at all temperatures, such differences are observed between normal and spherocytic ghosts only at 35 °C.

A different situation is observed with the mobile fraction of NBD-PE (the fraction of the marker which is laterally mobile on the time scale of the experiment). The mean value of the mobile fraction of NBD-PE in the external monolayer of both normal and spherocytic ghosts does not change with temperature, while the mobile fraction of NBD-PE in both external and internal leaflets shows a marked reduction at low temperatures (Table I). However, the reduction in the mobile fraction in spherocytic ghosts (for two-sides labelling) was considerably more pronounced than in normal ghosts (Table I). An analogous picture is revealed when the histograms of the mobile fractions are compared: no differences are observed in the distribution of this parameter in the external

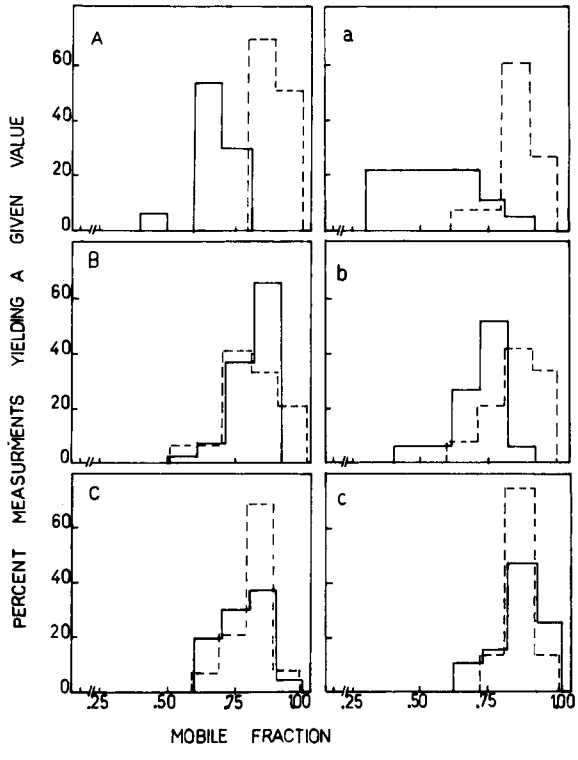

Fig. 2. Histograms of the mobile fractions in normal and in spherocytic ghost populations labelled by NBD-PE at both sides of the membrane or exclusively in the external monolayer. (Capital letters) Distribution of the mobile fraction values in the normal ghost population at 6 °C (A), 20 °C (B) and 35 °C (C). (a, b, c) Distribution of the mobile fractions in spherocytic ghosts at the same temperatures. (– – –) mobile fraction values obtained by labelling the external monolayer. (———) mobile fraction values obtained by labelling both the external and the internal monolayers. 30–40 ghosts were measured in each category.

monolayer (either as a function of temperature, or when normal and spherocytic ghosts are compared). However, for two-sides labelling, the pattern of the mobile-fraction histograms varies with temperature, and significant differences are observed between normal and spherocytic ghosts (Fig. 2). The largest differences between normal and spherocytic ghosts are observed at 6 °C, where 82% of the mobile-fraction measurements on normal ghosts yield values in the range of 0.65–0.85, while in spherocytic ghosts 85% of the measurements are equally distributed around 0.30–0.75. Statistical analysis by the Kruskal-Wallis ranking sum test [41] indicates that the differences between the histogram patterns of normal and spherocytic ghosts labelled from both sides are significant ($P < 0.05$) not only at 6 °C, but also at 20 °C and at 35 °C (Fig. 2).

Mobility and fluorescence of membrane proteins

Since interactions with cytoskeletal elements were reported to be involved in retarding the lateral mobility of erythrocyte membrane proteins [13–15], it was of interest to determine whether spherocytic and normal ghosts exhibit different membrane protein mobilities.

The results of photobleaching studies on the mobility of FITC-labelled membrane proteins (mostly band 3 and glycophorin, see Materials and Methods) are depicted in Table II. No differences are observed between spherocytic and normal ghosts at 20 °C either in the mean values of D or of the mobile fraction. The similarity between the two preparations holds also at 6 °C, where marked differences were observed between spherocytic and normal ghosts in the dynamic behavior of the lipid probe NBD-PE at the internal monolayer. The D values at 20 °C are rather close to those observed by Schindler et al. [31] in fresh-fused whole erythrocytes at 30 °C, and about 2-fold higher than the values reported by Golan and Veatch [15] for ghosts labelled with eosin maleimide at 21 °C (approx. 80% of the label in band 3).

The failure to detect differences in the lateral mobility of membrane proteins between normal and spherocytic ghosts does not necessarily mean that the interactions of membrane proteins with the cytoskeleton in the two groups of erythrocytes are identical. A more likely possibility is that the difference in these interactions is not sufficient to induce a measurable effect on membrane protein mobility in the specific type of spherocytic ghosts studied in the present experiments. In order to investigate this possibility, we have studied the temperature dependence of the intrinsic fluorescence of membrane proteins (contributed by both integral and peripheral membrane proteins) in unlabelled spherocytic and normal ghosts (Fig. 3). The results reveal that normal and spherocytic ghosts share a common inflection temperature (34–35 °C), but at the lower temperature range the protein fluorescence of spherocytic ghosts displays an inflection at 25 °C, while in normal ghosts an inflection occurs at 16 °C. The occurrence of this difference in the lower temperature range is in accord with the finding that the most distinct differences between the properties of the internal monolayer in spherocytic and normal ghosts are observed at low temperatures (Figs. 1 and 2).

TABLE II

LATERAL MOBILITY OF MEMBRANE PROTEINS ON SPHEROCYTIC AND NORMAL HUMAN ERYTHROCYTE GHOSTS

Cells were labelled with fluorescein isothiocyanate as described under Materials and Methods. Diffusion coefficients and mobile fractions were determined by fluorescence photobleaching recovery on 10–15 ghosts in each case. The values shown are the mean ± S.E.

Cell type	6 °C		20 °C	
	D (cm^2/s) ($\times 10^{10}$)	Mobile fraction	D (cm^2/s) ($\times 10^{10}$)	Mobile fraction
Normal ghosts	0.36 ± 0.04	0.21 ± 0.05	1.1 ± 0.1	0.26 ± 0.04
Spherocytic ghosts	0.40 ± 0.05	0.21 ± 0.04	1.0 ± 0.1	0.31 ± 0.04

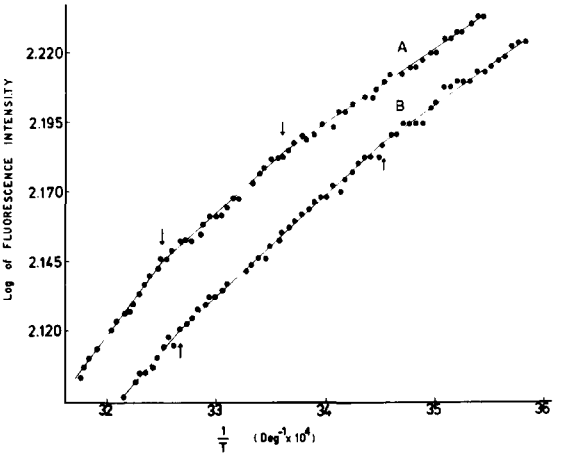

Fig. 3. Dependence of the fluorescence intensity of the total membrane proteins in normal and in spherocytic ghosts on temperature. (A) Fluorescence measurements on spherocytic ghosts. (B) Fluorescence measurements on normal ghosts. The arrows indicate inflection temperatures. The fluorescence intensity was sampled in steps of 0.5 deg. C between 6 °C and 40 °C.

Discussion

The results of lipid-diffusion measurements (Table I; Figs. 1, 2) indicate that the dynamic properties of the phospholipids in the external and internal membrane leaflets of both normal and spherocytic erythrocytes can change independently. The temperature dependence of NBD-PE diffusion in the two monolayers is different, and the dissimilarity of the two monolayers increases at lower temperatures. These differences apply both to the *D* values (which are lower for labelling at the external monolayer at 35 °C and 20 °C, and lower for labelling from both sides at 6 °C) and to the mobile fraction. The latter parameter does not change with temperature for labelling at the external leaflet, while a marked reduction is observed at low temperatures for two-sides labelling. These findings are in accord with our former results on turkey erythrocyte ghosts [8], which demonstrated different temperature dependence for the lateral diffusion of NBD-PE in the external and the internal monolayers. It is therefore possible that this phenomenon is a general property of erythrocytes from various species.

Studies performed by several groups have indicated differences between the external and internal monolayers of erythrocyte membranes in phospholipid headgroup distribution [1–4], and in the cholesterol level, which is much higher in the external monolayer [42,43]. The different lipid composition of the two leaflets could account for the different dynamic properties of NBD-PE incorporated in them. However, since the vertical asymmetry of lipids in erythrocyte membranes appear to be strongly dependent on cytoskeletal properties [10–12], interactions between cytoskeletal components and specific lipids are likely to be involved in maintaining this asymmetry. Such interactions could thus contribute at least indirectly to the different dynamic properties of the two monolayers.

Since the dynamic properties of the two membrane leaflets can change independently (Table I; Figs. 1, 2), alterations in the cytoskeletal structure are expected to have a much more pronounced effect on the lipids in the internal monolayer, which can be in direct interaction with the cytoskeleton. Such a change in the properties of the cytoskeleton is encountered in hereditary spherocytosis, which is caused by several different molecular defects in cytoskeletal components [19–21]. In accord with the prediction that altered cytoskeletal interactions would have stronger effects on the internal monolayer, our results demonstrate that the differences in phospholipid lateral diffusion between normal and spherocytic erythrocytes occur mainly in the internal monolayer while no significant differences are observed between the two cell populations in the external monolayer (except for *D* at 35 °C, which is somewhat higher on spherocytic ghosts).

An interesting mechanism by which the erythrocyte cytoskeleton could affect the physical and dynamic properties of the inner membrane leaflet is through stabilization of the lipid bilayer against lateral phase separation, or other rearrangements (e.g., in the headgroup regions) which lead to the appearance of laterally immobile lipid regions at low temperatures [8,9]. In such a case, the altered cytoskeletal structure in spherocytic ghosts will have a weaker stabilizing effect as compared with normal ghosts, thus leading to a lower mobile fraction of NBD-PE in the internal leaflet of spherocytic ghosts at low temperatures. This notion is in accord with earlier findings on

the interaction of cytoskeletal proteins with model membranes [44], which demonstrated that direct interaction of spectrin and actin with phosphatidylserine/phosphatidylcholine vesicles prevented lateral phase separation in this system, through reduction of the temperature in which it occurred.

An alternative possibility is that the differences between spherocytic and normal ghosts in the dynamic properties of the internal monolayer is due to different phospholipid compositions. However, this possibility is much less likely, since it has been demonstrated that vertical asymmetry in phospholipid distribution is conserved in spherocytic erythrocytes [12], and that the phospholipid composition in normal and spherocytic ghosts is similar [45].

Drastic modifications of the erythrocyte cytoskeleton can result in a loss of the phospholipid vertical asymmetry, as demonstrated by the finding that reaction of human erythrocyte spectrin SH groups with oxidizing agents (tetrathionate and diamide) leads to the appearance of internal phospholipids in the external monolayer [10]. Thus, the conservation of phospholipid vertical asymmetry in human spherocytic ghosts indicates that the defects in their cytoskeletal structure are not drastic, at least as far as interactions involved in maintaining the asymmetry are concerned. This notion is supported by the reports that the differences between spherocytic and normal human erythrocytes are expressed generally in rather mild alterations in the cytoskeletal structure (for a review, see Ref. 46), and not in acute deficiency in spectrin or other major cytoskeletal components. This hypothesis gains further support from the finding that the lateral mobility of membrane proteins on spherocytic ghosts is as restricted as in normal ghosts (Table II), a restriction which is believed to occur through interactions with the cytoskeleton [13–15,47]. The similar lateral mobility of membrane proteins on spherocytic and normal human ghosts differs from the reports on membrane protein mobility in a line of spherocytic mice ($WBB6F_1$, sph/sph), which was found to be much higher than in their normal counterparts [14]. Unlike in human spherocytosis types, the mouse spherocytic erythrocyte almost completely lacks the major components of the normal erythrocyte cytoskeletal matrix [48]. It is reasonable to conclude that the much more severe nature of the cytoskeletal defects in mouse spherocytes (as compared with human spherocytic erythrocytes) are responsible for the high lateral mobility of their membrane proteins.

Although the lateral mobility of membrane proteins on spherocytic human ghosts is retarded to the same extent as on normal ghosts, the temperature dependence of the intrinsic fluorescence of the total membrane proteins (both integral and peripheral) was different in the two cell populations at the lower temperature range (Fig. 3). Although the physical basis for the differences in fluorescence is not yet understood, this observation is in accord with the notion that there are temperature-sensitive differences between spherocytic and normal erythrocytes in the organisation or conformation of these proteins, and these differences are reflected in their fluorescence at low temperatures. Such a phenomenon is not surprising in view of the fact that spectrin and actin, together with other proteins associated with them through ankyrin (e.g. band 3, band 4.1 and 4.9), comprise about half of the total membrane-associated proteins in erythrocytes [49]. However, our experiments cannot exclude the possibility that the differences between normal and spherocytic ghosts in membrane protein fluorescence are caused by different patterns of local lipid rearrangements, although the similar lipid composition in normal and spherocytic ghosts [45] reduces the likelihood of this possibility.

In conclusion, our results suggest distinct dynamic properties of the phospholipids in the external and internal leaflets of human erythrocytes. In addition, the differences observed between spherocytic and normal erythrocyte ghosts indicate that normal interactions between cytoskeletal proteins and the internal monolayer play a role in stabilizing the latter against structural rearrangements, which result in lower mobile fractions at low temperatures.

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