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Modulation of membrane protein lateral mobility by polyphosphates and polyamines

[fluorescence redistribution after fusion (FRAF)/glycoprotein mobility/cytoskeleton/spermine/2,3-diphosphoglycerate]

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ABSTRACT The lateral mobility of fluorescein-labeled membrane glycoproteins was measured in whole unlysed erythrocytes and erythrocyte ghosts by the technique of "fluorescence redistribution after fusion." Measurements were made on polyethylene glycol-fused cell pairs in which only one member of the couplet was initially fluorescently labeled. Diffusion coefficients were estimated from the rate of fluorescence redistribution determined from successive scans with a focused laser beam across individual fused pairs. This technique allows for the analysis of diffusion within cell membranes without the possible damaging photochemical events caused by photobleaching. It was found that lateral mobility of erythrocyte proteins can be increased by the addition of polyphosphates (i.e., ATP and 2,3-diphosphoglycerate) and decreased by the addition of organic polyamines (i.e., neomycin and spermine). This control is exerted by these molecules only when they contact the cytoplasmic side of the membrane and is not dependent upon high-energy phosphates. Microviscosity experiments employing diphenylhexatriene demonstrated no changes in membrane lipid state as a function of these reagents. Our results, in conjunction with data on the physical interactions of cytoskeletal proteins, suggest that the diffusion effector molecules alter the lateral mobility of erythrocyte membrane proteins through modifications of interactions in the shell, which is composed of spectrin, actin, and component 4.1.

Recent studies have demonstrated the possible importance of lateral mobility of membrane components in mediating certain cellular processes (1–4). For example, the lateral movement of the β-adrenergic receptor relative to the adenylate cyclase appears to be required for activation of the cyclase by isoproterenol (5, 6). Such enzymatic processes may, therefore, be controlled by regulating the lateral mobility of proteins. Numerous diffusion measurements of membrane glycoproteins have established that the lateral mobility of proteins is restricted. This restriction is widely believed to be caused by interactions with the cell cytoskeleton (7, 8).

In the human erythrocyte, proteins of the cell cytoskeleton have been shown to control the lateral distribution of membrane glycoproteins. When cytoskeletal elements on the cytoplasmic membrane face are aggregated by antibodies (9) or changes in pH (10), the membrane-spanning glycoproteins are aggregated in the same region of the membrane (11). Likewise, the lateral mobility of erythrocyte glycoproteins is believed to be inhibited by cytoskeletal components (12). *In vitro* studies of the Triton cytoskeleton of erythrocytes have indicated that cytoskeletal structures are dissociated or destabilized by treatment with DNase I (13) or polyphosphates (unpublished results). To explore the nature of integral membrane protein lateral mobility, we have devised a method to assess quantitatively the diffusion of labeled membrane glycoproteins and determine the effects of cytoskeletal disrupting agents on that mobility. In this paper, we will describe the method and show that the lateral mobility of membrane glycoproteins is increased by polyphosphate compounds and decreased by organic amines in a manner consistent with the observed cytoskeleton stability.

MATERIALS AND METHODS

Materials. ATP, 2,3-diphosphoglycerate (2,3-P_2-glycerate), spermine, neomycin, diphenylhexatriene (DPH), and diisopropyl fluorophosphate were obtained from Sigma. The polyethylene glycol was from Baker (6000–10,000 daltons). Research Organics (Cleveland, OH) supplied the dichlorotriazinyl aminofluorescein. All other chemicals were reagent grade or better.

Erythrocyte Preparation. Human blood was drawn from healthy donors with 10 mM citrate as an anticoagulant. Blood was centrifuged (500 × *g* for 10 min), the supernatant and buffy coat were aspirated, and the erythrocytes were resuspended in phosphate-buffered saline [P_i/NaCl (10 mM Na_2HPO_4/0.81% NaCl, pH 7.4)]. These steps were repeated twice with the final erythrocyte pellet resuspended in an equal volume of a Hepes–Ringer's solution (120 mM NaCl/4.8 mM KCl/1.3 mM $CaCl_2$/1.0 mM $MgSO_4$/11.0 mM glucose/18 mM Hepes/1 mM Na_2HPO_4, pH 7.4). To inhibit proteolysis, 9 vol of the erythrocyte suspension was mixed with 1 vol of 20 mM diisopropyl fluorophosphate in Hepes–Ringer's solution and incubated at 37°C for 30 min. After diisopropyl fluorophosphate treatment, cells were centrifuged (500 × *g* for 10 min), the supernatant was aspirated, and the erythrocyte pellet was resuspended in 4 vol of P_i/NaCl. This procedure was repeated once.

Fluorescence Labeling. A 20% suspension of diisopropyl fluorophosphate-treated erythrocytes in P_i/NaCl was mixed with an equal volume of 0.2 M NaH_2BO_3 (pH 10.1) containing (per ml) 2 mg of dichlorotriazinyl aminofluorescein, a derivative of fluorescein that forms a covalent bond with amino groups. After 30 min on ice, the mixture was diluted with 15 vol of 0.2 M NaH_2BO_3 (pH 10.1) and centrifuged (500 × *g* for 10 min). The supernatant was aspirated, and the cells were resuspended in 15 vol of P_i/NaCl. This procedure was repeated twice except that the final cell pellet was resuspended in an equal volume of P_i/NaCl. Unlabeled cells were treated with the same buffers and washes in the absence of dichlorotriazinyl aminofluorescein.

Fusion Procedure. A 1:1 mixture of fluorescent and unlabeled cells (50% cell suspension in P_i/NaCl) was incubated at 37°C for 0.5 hr before mixing with an equal volume of 50% polyethylene glycol in P_i/NaCl also at 37°C. After the cell

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Abbreviations: 2,3-P_2-glycerol, 2,3-diphosphoglycerol; DPH, diphenylhexatriene; P_i/NaCl, phosphate-buffered saline; FRAF, fluorescence redistribution after fusion.

mixture was incubated for 1–2 min at 37°C, 1 vol of the mixture was added to 15 vol of diluted P_i/NaCl (8.5 vol of P_i/NaCl was mixed with 6.5 vol of distilled H_2O) at room temperature (22–24°C) with vigorous mixing. Samples were taken within 10 min for slides. EDTA (5.0 mM) was included to inhibit transglutaminase, lipase, and other metal-requiring enzymes that may affect mobility (14, 15).

Slide Preparation. Prewashed microscope slides were placed in 0.1 M Na_2HPO_4 (pH 7.5) for at least 1 min, rinsed with distilled water, and dried prior to use. Coverslips were rinsed with distilled water and dried. Reagents were added to the fused cells on the slides at the final concentrations noted and diluted with distilled water to maintain a low ionic strength. The final mixtures were covered with coverslips and sealed with warm paraffin to prevent evaporation.

DPH Rotational Mobility. The fluorescence polarization of DPH was measured in erythrocyte membranes at room temperature. The intact cells (50% suspension) were mixed before lysis with DPH at 2 μg/ml (DPH was added as a 1 mg/ml solution in acetone). Membranes were prepared by lysis of diisopropyl fluorophosphate-treated cells (1:50) in 5 mM Na_2HPO_4 (pH 7.4) and subsequent centrifugation (35,000 × *g* for 10 min). The cell pellet was then resuspended in 5 mM Na_2HPO_4 (pH 7.4). This procedure was repeated twice. Measurements were carried out according to the procedure of Shinitzky and Barenholz (16).

Fluorescence Redistribution After Fusion (FRAF). Lateral diffusion coefficients of fluorescein-labeled membrane proteins were determined from the measured redistribution of fluorophore on the surface of individual fused erythrocyte pairs. The distribution of fluorescence was recorded as a function of time in successive high-resolution scans of a focused laser beam along a linear scan axis. A detailed description of the experimental apparatus is presented elsewhere (17). Briefly, a standard fluorescence microscope, equipped for incident illumination, is used to focus the laser beam onto the sample and collect the fluorescence for detection and subsequent processing by photon-counting electronics. The angular orientation of the incident laser beam, and hence the location on the sample of the focused spot along the scan axis, is controlled by a servo-activated galvanometric optical scanning mirror. Fluorescein emission was monitored with an incident wavelength of 4765 Å and a combination of Leitz dichroic mirror TK510 and barrier filter K510. All fluorescence measurements were performed with a ×40/0.65 NA dry objective. Suitable fused combinations of labeled and unlabeled cells were identified under darkfield and diffuse expanded laser beam illumination.

The upper part of Fig. 1 shows schematically the redistribution of labeled molecules on the surface of a fused pair. Actual scans (0.5 μm per point) along the long axis of such a pair are reproduced below. Note the geometrical edge effect, characteristic of a rounded surface-labeled cell.

Each recorded fluorescence distribution is characterized by a calculated concentration "polarization," defined as:

$$p(t) = \frac{c_+(t) - c_-(t)}{c_+(t) + c_-(t)}, \qquad [1]$$

where, following the notation of Peters *et al.* (18), $c_+(t)$ and $c_-(t)$ are the concentrations of fluorophore averaged over the areas originally labeled and unlabeled, respectively. $c_+(t)$ and $c_-(t)$ are computed by numerical integration over the appropriate portions of the scan data (see Fig. 1). This complex geometry of a fused pair is not amenable to exact analysis. For simplicity, we have analyzed the data assuming all labeled molecules moved with diffusion coefficient D on the surface of a sphere of radius r, uniformly labeled over one hemisphere at $t = 0$. This problem has been solved (18, 19) giving:

$$p(t) = \sum_{k=0}^{\infty} P_k \exp[-(2k+1)(2k+2)\tau] \qquad [2]$$

where

$$P_k = (4k+3)\left[\frac{(2k)}{2^{2k+1}k!(K+1)!}\right]^2 \qquad [3]$$

and τ is the dimensionless parameter

$$\tau = Dt/r^2. \qquad [4]$$

Values of D were computed from experimental traces of $p(t)$ by determining the apparent range of τ covered in a known period of time. Distances of half the long axis of each fused pair,

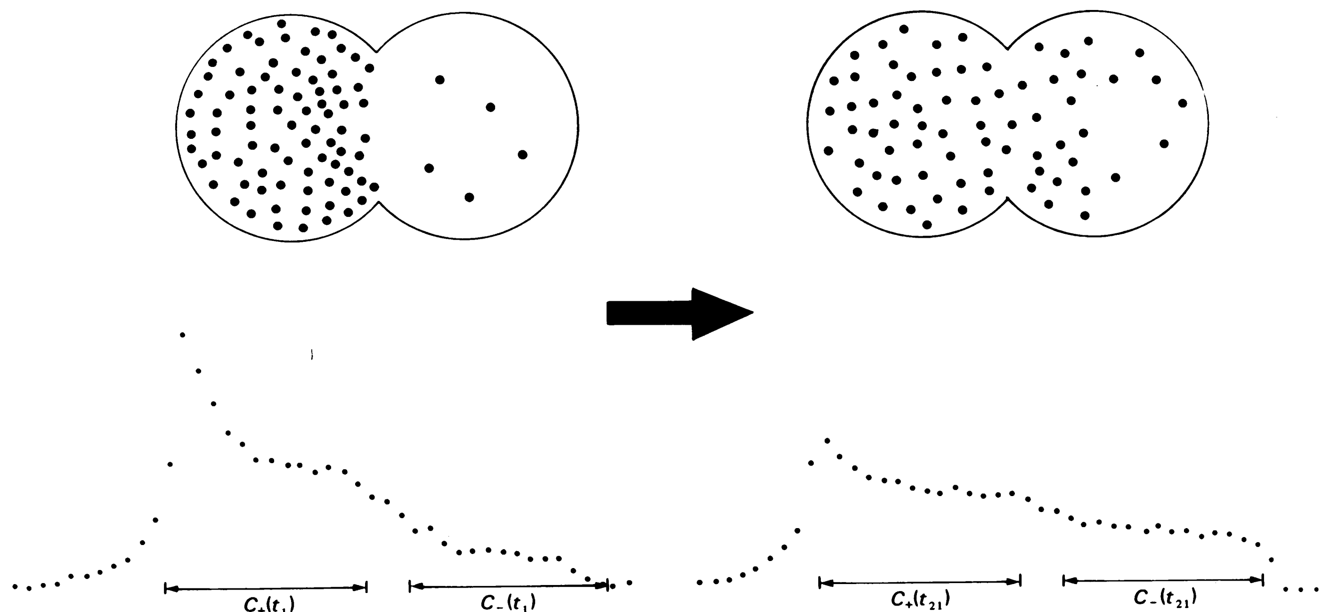

FIG. 1. Schematic diagram of two fused erythrocytes, one fluorescently labeled (dots) and the other unlabeled. The diagrams appear above actual scan traces. Redistribution occurred after 2 hr. The areas chosen for integration are listed as C_+ and C_-.

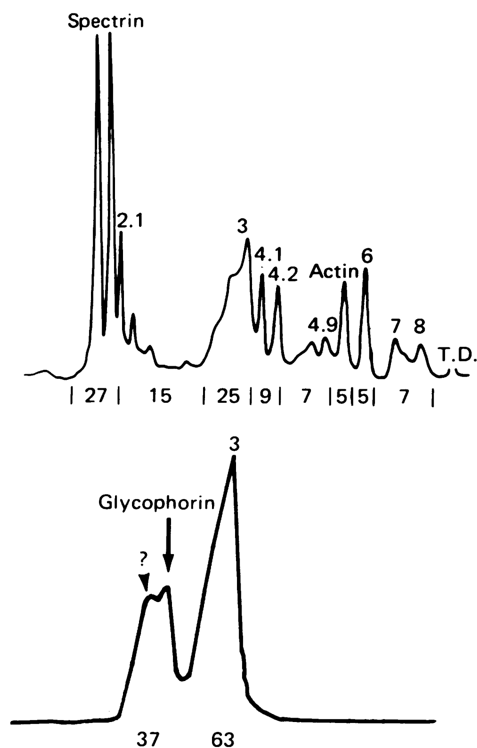

FIG. 2. Densitometer scans of sodium dodecyl sulfate/polyacrylamide gels (3.25% acrylamide) of DTAF-labeled erythrocyte membranes stained with Coomassie blue (*Upper*) and photographed under laser illumination (488 nm) with a 510-nm barrier filter (*Lower*) (the negative was scanned). The % of total areas of the peaks are noted below the scans.

derived from the fluorescence scan data, were used as effective values of r.

RESULTS

Distribution of Fluorescent Label. Less than 1% of the cellular fluorescence of labeled cells was found in the cell lysate. This provided strong evidence that the label resided predominantly in membrane components. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of the labeled erythrocytes demonstrated that glycophorin and band 3 were the prime labeling sites on the membrane (Fig. 2). No fluorescence was observed near the dye front, indicating negligible lipid labeling.

Fusion Procedure. The fusion procedure employed caused no detectable alteration of membrane components as judged by the Coomassie blue staining patterns of sodium dodecyl sulfate/polyacrylamide gels. It was found that hypotonic solutions promoted fusion and were essential for the fusion of ATP-depleted cells. Although this treatment produced significant lysis, enough fused cells remained intact for the duration of the experiment to allow occasional whole cell measurements. Typically, 1–10% of the cells formed fused complexes.

Lateral Mobility. Over the relatively short periods of time of these experiments (0.5 hr), measured traces of $p(t)$ followed that predicted, assuming all labeled components can be characterized with a single diffusion coefficient (see Fig. 3). Observation of several samples overnight, however, indicated that the fluorescence redistribution was not monophasic and that perhaps as much as 20% of the labeled proteins diffused at a considerably slower rate. Values of D reported here should thus be considered as an average over the population of molecules.

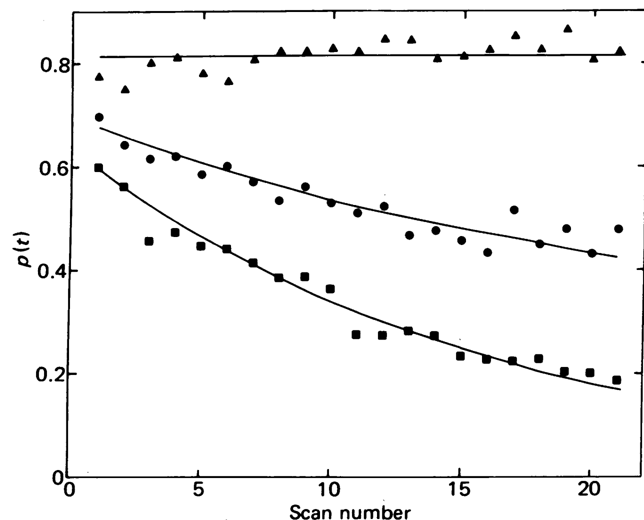

FIG. 3. Experimental values of $p(t)$ as a function of scan number for ATP-depleted ghosts at 30°C with: no reagents added (●), 12.5 mM ATP added (■), and 0.6 mM neomycin added (▲). In each case, 48 point scans of 9.6 s duration were initiated every 96 s. The solid curves are theoretical values corresponding to $D/r^2 = 1.06 \times 10^{-4}\ s^{-1}$ for the untreated sample ($D = 2.12 \times 10^{-11}\ cm^2/s$) and $D/r^2 = 3.39 \times 10^{-4}\ s^{-1}$ for the ghosts treated with 12.5 mM ATP ($D = 11.2 \times 10^{-11}\ cm^2/s$). The neomycin data is set against a horizontal straight line.

Diffusion coefficients were determined relative to control samples consisting of ghosts of fused ATP-depleted cells, which were significantly less mobile than fresh fused whole cells ($D = 2.2 \times 10^{-11}\ cm^2/s$ and $9.2 \times 10^{-11}\ cm^2/s$, respectively). Because phosphorylated compounds cause a dramatic disruptive effect on isolated erythrocyte cytoskeletons (unpublished results), we added a number of these agents to fused erythrocyte ghosts and determined their effect on the apparent diffusion constant of the fluorescein-labeled membrane proteins. At the concentrations indicated, these reagents significantly increased the measured diffusion coefficients approximately back up to the level of fresh whole cells (see Table 1 and Fig. 3).

Organic polyamines, on the other hand, had profound inhibitory effects on lateral mobility (see Table 1 and Fig. 3). These compounds, specifically neomycin and spermine, affected the membrane protein mobilities at concentrations (<1 mM) that did not appear to damage the membranes. In addition, 60 mM Tris blocked protein mobility (data not shown).

In all instances, changes in protein mobility appeared only after cell lysis in the presence of reagents. Protein mobilities in fused ATP-depleted whole cells remain unaffected by these compounds (data not shown).

Membrane Fluidity as Measured by Rotational Diffusion of DPH. To investigate the possibility that changes in lateral diffusion occurred due to alterations in lipid state (20, 21) we determined the "microviscosity" of the erythrocyte membrane lipids with and without addition of polyphosphates. DPH was incorporated into erythrocyte membranes and measurements were carried out. Table 2 is a summation of those results. No significant changes were observed from the control. This result is in marked contrast to the changes in lateral mobility observed with lower concentrations of ATP and 2,3-P_2-glycerol (Table 1).

DISCUSSION

By using a quantitative FRAF analysis, we have found that the lateral mobility of membrane glycoproteins can be dramatically altered by the addition of small molecules. Previous fusion studies have employed visual examination of the fluorophore

Table 1. Effect of polyphosphates and polyamines on relative lateral diffusion

Treatment*	$D/D_{control}$†
ATP (12.5 mM)	4.0
2,3-P_2-glycerol (12.5 mM)	2.5
Neomycin (0.6 mM)	<0.01‡
Spermine (0.6 mM)	<0.01‡

* To ensure a homogeneous population of cells for more accurate comparison, the cells were depleted of ATP by incubation at 37°C for 24 hr in P_i/NaCl with penicillin and streptomycin at 0.1 mg/ml added to inhibit bacterial growth.

† ATP-depleted ghosts were used as controls. The ratios, $D/D_{control}$, were determined from comparisons of data taken the same day with the same cell preparation. $D_{control} = 2.2 \times 10^{-11}$ cm²/s. All ratios are based on comparisons between groups of at least three measurements. Experiments were performed at 30°C.

‡ No detectable redistribution over a 30-min period (see Fig. 3).

distribution (21, 22). In addition to the subjective factors involved in that method, it is incapable of separating the slow and fast components of diffusion that have been found in other systems and are indeed observed by the FRAF method. Polyethylene glycol was the fusogen of choice because it did not affect the membrane protein patterns in sodium dodecyl sulfate/polyacrylamide gels and left the membrane barrier of the cells intact with regard to hemoglobin permeability. On the other hand, the Sendai virus fusogen has been found to stimulate hydrolytic activity and to cause lysis (23). Our preliminary results from fluorescence redistribution after photobleaching experiments, performed under conditions that inhibit photodamage (24), indicate that polyethylene glycol treatment and fusion do not produce drastic qualitative or quantitative changes of glycoprotein mobility in erythrocytes. Glycoproteins appear to be free to diffuse over micrometer distances at rates that are not significantly changed by the polyethylene glycol-induced fusion procedure.

The mathematical analysis of the FRAF data that we have employed assumes a simplified geometry and thus is expected to lead to systematic deviations in the calculated values of D. We have every reason to expect, however, that it will give us reliable relative values. To ensure that this is the case, comparisons between different samples were made whenever possible over overlapping ranges of $p(t)$—i.e., over identical extents of redistribution. The FRAF data will allow more detailed analysis of the diffusion process once the diffusion equation has been solved for the particular geometry of the fused cell pair.

Recently, one of us (M. P. S., unpublished results) has demonstrated that Triton-derived cytoskeletons from intact erythrocytes are disrupted by polyphosphates such as ATP, 2,3-P_2-glycerol, GTP, and inositol hexaphosphate. Because many of these compounds are present normally in fresh erythrocytes, fused ATP-depleted cells were employed in these studies as a control. Our intention was to develop a system that

Table 2. Polarization of fluorescence measurements

Reagent	P*	η, poise†
Buffer	0.313	4.26
ATP (30 mM)	0.323	4.72
2,3-P_2-glycerol (30 mM)	0.308	4.05

* Average of three values. $P = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$, where $I_{\parallel}$ and $I_{\perp}$ are the intensities of the fluorescence with polarizations parallel and perpendicular, respectively, to the incident polarization, corrected for instrumental anisotropies.

† Apparent microviscosity calculated according to the approximate formula (6): $\eta = [I_{\parallel}/(I_{\perp} - 1)/(0.73 - 0.27I_{\parallel})]/I_{\perp}$

would allow quantitative addition of polyphosphate components to the erythrocyte. This reconstitive approach in fact indicated that the addition of polyphosphates to fused ATP-depleted erythrocyte ghosts increased the diffusion coefficients to approximately that of whole cells. The increases in glycoprotein mobility paralleled the observed decrease in cytoskeleton stability. Because the compounds altered glycoprotein mobility only in ghosts and not in impermeable whole cells, they appear to act on the cytoplasmic membrane face where the cytoskeletal components are found. The fact that low energy polyphosphates such as 2,3-P_2-glycerol cause increased mobility in the presence of EDTA supports the view that phosphate hydrolysis is not required to initiate changes in mobility in erythrocyte ghosts.

It has been found (25, 26) that, when erythrocytes are oxygenated, thereby increasing the level of 2,3-P_2-glycerol and ATP, the membranes, at the same time, become more "flexible." This flexibility in all likelihood facilitates transport through capillaries. Our results indicate another possible influence of metabolism on the state of the cell membrane. The regulation of cell metabolism can also result in the regulation of glycoprotein mobility via direct interactions of metabolic intermediates with submembranous cytoskeletal components.

Our results demonstrate significant changes in glycoprotein diffusion coefficients in the absence of variation in the lipid microviscosity. This is consistent with the idea that glycoprotein lateral mobility is governed largely by protein–protein interactions. It has recently been shown (27) that even protein rotational diffusion appears to be insensitive to changes in lipid microviscosity. No significant changes in the rotational diffusion of band 3 in the human erythrocyte membrane were observed over a wide range of the cholesterol-to-phospholipid mole ratio.

Given our observation that polyphosphate compounds increase the diffusion rates, one might predict that agents that complex or otherwise remove endogenous polyphosphates from membrane binding sites would decrease glycoprotein mobility. Neomycin, for example, is known to bind to triphosphoinositide (28), a polyphosphate that is present at high concentration at the membrane surface (29). Indeed, neomycin did prove to be a potent inhibitor of glycoprotein mobility, but other polyamines such as spermine are also quite effective inhibitors. Even the Tris cation at high concentration (60 mM) inhibits mobility. Because basic proteins affect the state of spectrin aggregation (11) and polyamines have been shown to have an effect on the interactions of structural proteins such as clathrin (30), it is not unlikely that a direct cytoskeletal interaction is involved in the decreases in membrane protein mobilities we observed with polyamines.

Previous studies of glycoprotein distribution in erythrocytes have shown that aggregation of spectrin, the major cytoskeletal protein in the erythrocyte, results in the aggregation of all glycoproteins (9, 10). Recent studies of glycoprotein rotational diffusion (26, 31) and of Triton cytoskeletons of intact erythrocytes (13), however, do not find direct attachments of the majority of membrane glycoproteins to spectrin. It is thus believed that the spectrin–actin lattice or shell can act as a barrier to lateral movement of the glycoproteins (12). Currently, two models for this type of control exist; either glycoproteins attach and detach from generally rigid cytoskeletal frames (7, 32) or the actual physical state of the cytoskeleton changes, resulting in a change of diffusion rate "through" the network (7, 32, 33). Our results in conjunction with other data (ref. 13; unpublished results) support the latter mechanism for the control of membrane protein mobility in erythrocytes. We believe these findings may have general implications for a number of bio-

logical processes wherein the diffusion of membrane proteins is implicated—e.g., receptor stimulation of adenylate cyclase, endocytosis, mitogenesis, and membrane differentiation.

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