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Wheat germ agglutinin stabilization of erythrocyte shape: role of bilayer balance and the membrane skeleton

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Abstract

The effects of wheat germ agglutinin (WGA), *Limulus* lectin, and concanavalin A on cell shape changes were examined in human erythrocytes. These agents inhibited echinocytosis in cells having elevated cytosolic Ca^{2+} or incorporated foreign phosphatidylcholine, but had no effect on cell stomatocytosis in response to incorporated phosphatidylserine. The role of the membrane skeleton in this selective membrane fixation was examined. WGA inhibited echinocytosis in cells previously depleted of polyphosphoinositides to reduce membrane skeleton binding to transmembrane proteins, treated with phorbol ester to enhance protein 4.1 phosphorylation, heat-treated to denature spectrin, alkylated with *p*-chloromercuribenzoate to dissociate glycophorin from the membrane skeleton, or subjected to elevated cell 2,3-diphosphoglycerate to alter organization of the spectrin-actin-protein 4.1 complex. *Limulus* lectin and increased concentrations of WGA also stabilized discoid shape in pronase-digested cells containing no detectable intact glycophorin. In contrast, cell digestion with sialidase abolished the shape-stabilizing effect of WGA. The results suggest that the membrane skeleton is not involved in WGA shape stabilization. Rather, they suggest that glycoproteins and glycolipids interact with the lectin to stabilize cell surface molecular associations, forming a superficial calyx that inhibits outward, but not inward, membrane bending.

Keywords: Lectin; Cell surface curvature; Membrane skeleton; Bilayer balance model; Phospholipid

1. Introduction

The normal biconcave discoid shape of the human erythrocyte can be transformed into a spiked echinocytic shape by cellular ATP depletion [1], Ca^{2+} loading [2] or addition of amphipathic agents [3]. The biconcave disc also can be driven to invaginated stomatocytes by addition of cationic amphipaths such as chlorpromazine [4] or by incorporation of exogenous phosphatidylserine [5,6]. Control of normal red cell shape has been attributed to the protein membrane skeleton that laminates the cytoplasmic face of the lipid bilayer [7,8]. One model invokes a complex of the lipid bilayer, the membrane skeleton, and associated integral membrane proteins acting as a 'trilayer couple' as the cell undergoes shape transformations [9]. This model is based on in vitro studies of membrane components that revealed binding interactions between the spectrin-protein 4.1-actin complex and the cytoplasmic domain of the major transmembrane protein glycophorin (GP), an interaction mediated by the phosphoinositide lipid cofactors phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphatidylinositol 4-phosphate (PIP) [10,11]. The populations of these inositides decrease dramatically in response to metabolic depletion or increased cytosolic Ca^{2+} [12–14]. The model suggests that the shape change produced by these conditions reflects the resulting disruption of normal GP–protein 4.1 interaction.

Abbreviations: BSA, bovine serine albumin; 2,3-DPG, 2,3-diphosphoglycerate; Con A, concanavalin A; CPZ, chlorpromazine; DLPC, dilauroylphosphatidylcholine; DLPE, dilauroylphosphatidylethanolamine; DLPS, dilauroylphosphatidylserine; EPR, electron paramagnetic resonance; GP, glycophorin; HCT, hematocrit; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; lysoPC, lysophosphatidylcholine; Mes, 2-(*N*-morpholino)]ethanesulfonic acid; NAG, *N*-acetylglucosamine; PBS, phosphate-buffered saline; PCMB, *p*-chloromercuribenzoate; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PMA, phorbol 12-myristate 13-acetate; spin PC, 1-palmitoyl-2-stearoyl-(10-doxyl)-*sn*-glycero-3-phosphocholine; supplemented buffer, (additional 5 mM glucose, 10 mM inosine, and 1 mM adenosine were added to the corresponding buffer); WGA, wheat germ agglutinin.

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Another model for control of red cell shape is the bilayer couple hypothesis, which attributes cell shape changes to alterations in the relative areas of the inner and outer lipid monolayers [15]. According to this model, any event or amphipathic agent that causes the outer monolayer to expand relative to the inner monolayer produces echinocytes. Conversely, preferential expansion of the inner leaflet results in the formation of stomatocytes. This model can account for the disc-to-echinocyte shape change seen in metabolically depleted or Ca^{2+}-elevated cells without invoking membrane skeleton or GP involvement: phosphoinositide breakdown shrinks the area of the inner lipid monolayer to an extent consistent with the observed shape change [13].

The lectin wheat germ agglutinin (WGA), which binds selectively to GP and mediates GP–membrane skeleton interactions [16], has effects on cell morphology that may bear on the intrinsic control mechanisms. WGA strongly inhibits echinocytosis induced by amphipaths, Ca^{2+} loading, or metabolic depletion [7,9,17,18], but does not inhibit primaquine-induced stomatocytosis [18]. Such inhibition of cell shape changes apparently entails multivalent binding of the lectin to GP [7].

The morphological constraints imposed by WGA have been attributed to interaction of the lectin-bound GP with the membrane skeleton proteins [7,9]. If a change in the structure or association of the GP-protein 4.1 complex is instrumental in (or required for) the membrane deformations of shape changes, possibly lectin binding to GP might inhibit such changes by stabilizing the preexisting GP state. Through its binding to 4.1, the GP might in turn predispose the membrane skeleton to resist alteration [9].

An aspect of WGA/GP shape control that is not readily explained, either by the membrane skeleton hypothesis or by simple bilayer balance arguments, is its lack of symmetry. WGA imposes a membrane constraint that evidently restricts bilayer evagination, but has no effect on the inward bending attendant to stomatocytosis. This perturbation of normal bilayer cooperativity may signal a change in lipid dynamics or lipid–protein interactions that sheds light on mechanisms of shape control.

This contribution describes the effects of WGA and other cell surface-reactive agents on the dynamics of red cell shape transformations, and explores the role of the membrane skeleton in WGA-mediated shape fixation. Using phospholipids as echinogenic and stomatogenic agents, lipid uptake and transbilayer disposition are examined in the presence and absence of the lectin. The limits of the WGA effect are examined and its asymmetry is explored. Protein 4.1 and other components of the membrane skeleton are perturbed selectively, and the subsequent morphological effects of WGA are examined. Finally, similar selective stabilization of discoid shape is demonstrated using concanavalin A, *Limulus* lectin, and limited glutaraldehyde fixation.

2. Materials and methods

Wheat germ agglutinin (WGA, from *Triticum vulgaris*), Con A (from *Canavalia ensiformis*), lectin from *Limulus polyphemus*, *N*-acetylglucosamine (NAG), dilauroylphosphatidylcholine (DLPC), dilauroylphosphatidylethanolamine (DLPE), dilauroylphosphatidylserine (DLPS), neuraminidase (from *Cl. perfringens*, Type X), proteinase (Pronase E, Type XXV), phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma (St. Louis, MO). Spin PC (1-palmitoyl-2-stearoyl-(10-doxyl)-*sn*-glycero-3-phosphocholine) was purchased from Avanti (Birmingham, AL). A23187 was purchased from Calbiochem (San Diego, CA). [^{14}C]DLPC was synthesized from DLPE and methyl iodide plus [^{14}C]methyl iodide as described previously [19,21].

2.1. Cells

Blood was obtained by venipuncture from healthy adults. Erythrocytes were isolated by centrifugation and washed three times with four volumes of 150 mM NaCl and once with 138 mM NaCl, 5 mM KCl, 6.1 mM Na_2HPO_4, 1.4 mM NaH_2PO_4,1 mM $MgSO_4$, 5 mM glucose, pH 7.4 (PBS). Cells were used within 10 h of being drawn. For incubations longer than 4 h at 37°C, penicillin G (100 μg/ml) and streptomycin (100 μg/ml) were added to retard bacterial growth.

To determine cell morphology, cells were fixed in PBS containing 0.1% glutaraldehyde, and examined by bright field microscopy (500 ×). Echinocytes were assigned a morphology score of +1 to +5 based on Bessis's nomenclature [13,30]. Discocytes were assigned a score of 0, and stomatocytes, a score of −1 to −4 [5,6]. The average score for a field of 100 cells was defined as morphological index [MI] [31].

2.2. General procedures involving lectin treatment

To prevent cell agglutination, erythrocytes were diluted to 0.2% before adding equal volumes of lectin solutions [7]. The final concentration of WGA was 1–2 μg/ml at 0.1% cell suspension, unless otherwise specified. The concentrations of other lectins are indicated in figure legends. If not specified, all incubations involving lectins were carried out at room temperature in PBS buffer.

2.3. Lipid vesicle preparation and cell-vesicle incubation

Small unilamellar vesicles were prepared by suspending lipids (DLPC or DLPS) in PBS and sonicating the suspension to clarity in a bath sonicator [20]. The conditions of cell-vesicle incubations (length of incubation, temperature, and concentration of lipids) are specified in the figure legends.

Electron paramagnetic resonance (EPR) spectra of spin-labeled cells. Pure spin PC vesicles (15 μM) were incubated for 2 h with cells pretreated with WGA or buffer. After incubation, cells were separated from the supernatant and EPR spectra of cell pellets were taken at room temperature, 9.52 GHz, 100 mW, with a Varian E-112 spectrometer [21].

Back extraction of incorporated lipids. Sonicated DLPC vesicles containing a trace amount of [^{14}C]DLPC were incubated with cells (0.1%) pretreated with or without WGA. After incubation at room temperature for 15 min, BSA was added to half of the cell suspension to yield final concentration of up to 33 mg/ml, and incubation was continued for 10 min. Cells treated with or without BSA were separated from the supernatant [21], and residual [^{14}C]DLPC in the cell pellets was measured by scintillation counting [6]. Extracted [^{14}C]DLPC was calculated from the difference in radioactivity in PBS and BSA-treated cell samples.

2.4. Altering native lipid membrane and membrane skeleton

Intact cells were treated by the following strategies to alter native membrane structure. After such treatment, cells were then incubated with DLPC vesicles in the presence and absence of WGA.

Increasing intracellular 2,3-diphosphoglycerate (2,3-DPG). 2,3-DPG was increased by incubating cells in Deuticke's IPP buffer (50 mM P_i, 10 mM PP_i, 10 mM inosine, 80 mM NaCl, pH 7.4) for 2 h at 37°C, a condition known to induce no detectable hemolysis [23]. After incubation, cells were washed three times in hypotonic NaCl/Hepes (90 mM NaCl, 20 mM Hepes (pH 7.4)) to restore cell volume, since cells shrink as 2,3-DPG increases [24]. Aliquots of cell samples were reserved to measure pH_i and 2,3-DPG. The latter was determined using a Sigma 2,3-DPG assay kit.

Heat denaturation of spectrin. Cells were diluted to 20% with PBS and subjected to heating at 48°C for 10 min. Aliquots of heated cells were cooled on ice before making ghosts. Ghost membranes were made from heat-treated and control cells by lysing them in 5 mM phosphate buffer (pH 8) and pelleting the membranes at 22 000 $\times g$ for 10 min. Membrane proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described [33].

Phosphorylation of protein 4.1. Cells (30%) were prelabeled with ^{32}P by incubation in supplemented NaCl/Hepes buffer (189.4 mM NaCl, 20 mM Hepes, 10 mM inosine, 10 mM glucose and 1 mM adenosine (pH 7.4)) containing 200 μCi/ml [^{32}P]P_i. After 20 h incubation at 37°C, cells were washed to remove [^{32}P]P_i. Phosphorylation of protein 4.1 was initiated by incubating ^{32}P-labeled cells (20%) with 0.5 μM phorbol 12-myristate 13-acetate (PMA) at 37°C for 30 min [25]. Excess PMA was washed away after the incubation. Aliquots of cell suspensions were prepared as membrane ghosts and their proteins were analyzed by gel electrophoresis. Autoradiograms of Coomassie blue stained and dried gels were obtained by exposing gels to X-ray films.

p-Chloromercuribenzoate (PCMB) treatment of cells. Cells (10–50%) were incubated with various concentrations of PCMB (0–3.2 mM) at 37°C for 30 min. Cells treated with PCMB were mechanically fragile, tending to lyse during washing and resuspension [26]; thus washing was omitted. To fix PCMB-treated cells, dithiothreitol was included in fixative to react with excess PCMB. Red cell membranes and Triton shells (Triton-insoluble residues) from control and PCMB-treated cells were made following the published protocol [26,27], and their proteins were separated by SDS-PAGE, followed by periodic acid-Schiff stain to monitor glycophorin [34].

Depletion of polyphosphoinositides (PIP_2/PIP). The content of PIP_2/PIP was monitored by prelabeling cells with ^{32}P. To deplete PIP_2/PIP, ^{32}P-labeled cells were treated with various concentrations of Ca^{2+} (0–1 mM) and A23187 for 15–70 min at 37°C. A23187 was added in ethanol/dimethyl sulfoxide (95:5, v/v). The final concentration of the ionophore was 5 μM and ethanol was less than 1% (v/v). Ca^{2+} loading experiments were carried out in KCl/Hepes buffer (130 mM KCl, 20 mM Hepes, 5 mM glucose, 1 mM $MgSO_4$ (pH 7.4)) to prevent K^+ efflux and cell shrinkage [28].

To restore discoid shape to Ca^{2+}-loaded cells (repletion), cells were washed with EGTA (4 mM) and BSA (0.5%), and then incubated for 15 min at 37°C in supplemented KCl/Hepes [29].

Aliquots of cell samples from ^{32}P-labeled cells, Ca^{2+}-crenated, and repleted cells were extracted and separated by TLC as described previously [13,32]. ^{32}P-labeled phos-

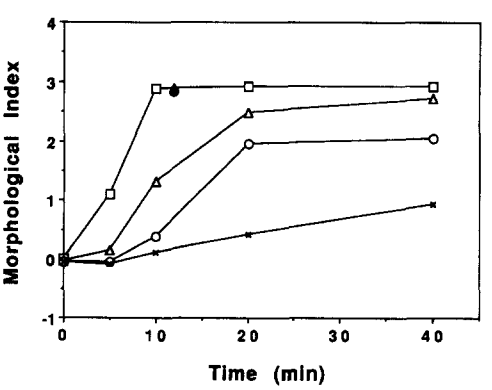

Fig. 1. WGA inhibition of DLPC-induced echinocytosis, and the release of inhibition by NAG. Cells were treated with PBS or WGA, and then incubated with 0.1 μM DLPC vesicles. At the times indicated, aliquots of cells were removed and fixed. Morphological indices were calculated as described in Materials and methods. Control (□); 1 μg/ml WGA (△); 1.5 μg/ml WGA (○); 2 μg/ml WGA (×). At $t = 10$ min, 5 mM NAG was added and incubated for 2 min; NAG added to cells containing 1 μg/ml WGA (▲), and 1.5 μg/ml WGA (●).

pholipids were quantified by AMBIS Radioanalytic Imaging System (AMBIS Systems, San Diego, CA).

2.5. Enzyme treatment of cells

Cells were treated with neuraminidase or pronase. Neuraminidase (0.1 unit/ml) was incubated with cells (10%) for 1 h at 37°C [7]. Pronase (0.25 mg/ml) was incubated with cells (25%) for 8.5 h at 37°C. The reaction was stopped by 15 mM EDTA, and placed on ice for 5 min [22]. Under these conditions, the enzyme treatments induced no detectable hemolysis. Aliquots of enzymatically modified cells were prepared as membrane ghosts and their proteins were analyzed by gel electrophoresis, the rest were incubated with amphipaths in the presence or absence of lectins.

3. Results

3.1. Effects of WGA on red cell shape transformations

Echinocytosis

Discocyte-to-echinocyte transitions: Red cell echinocytosis was induced by incubating cells with DLPC vesicles in the presence or absence of WGA. The rate of cell shape change and the equilibrium state of echinocytosis were dependent on the concentrations of PC and the lectin. In the absence of WGA, cells exposed to 0.1 μM DLPC crenated extensively (Fig. 1). WGA inhibited the extent of this shape change in a dose-dependent manner: 2 μg/ml WGA largely abolished the echinocytosis, particularly upon brief exposure to PC. Treatment with NAG, which competes with GP for the lectin, reversed the WGA effect and rapidly converted the DLPC-treated cells to echinocytes (Fig. 1, closed symbols). These observations are consistent with earlier reports [7,17,18].

Echinocyte-to-discocyte transitions. Amphipath induced discocyte-to-echinocyte shape changes are reversible; BSA extraction can remove an echinogen from the membrane outer monolayer and restore discoid shape. Additionally, stomatocytic agents can revert echinocytes to discs by compensatory expansion of the inner lipid leaflet. Earlier workers reported that WGA prevented such reversal: SDS-induced echinocytes subsequently treated with WGA were not restored to discoid shape when the SDS was extracted quantitatively with BSA [7]. Thus WGA was thought to fix echinocytic as well as discoid morphology. In the present work, several approaches were found to restore WGA-treated echinocytes to discoid shape. WGA-treated echinocytes exposed to the inner monolayer intercalator DLPS or the stomatogenic agent CPZ reverted to discs and stomatocytic forms, depending on the concentrations employed (Figs. 2a, 2b). Additionally, prolonged incubation with BSA was found to restore WGA-treated echinocytes to mostly discoid morphology (Fig. 2c). Thus,

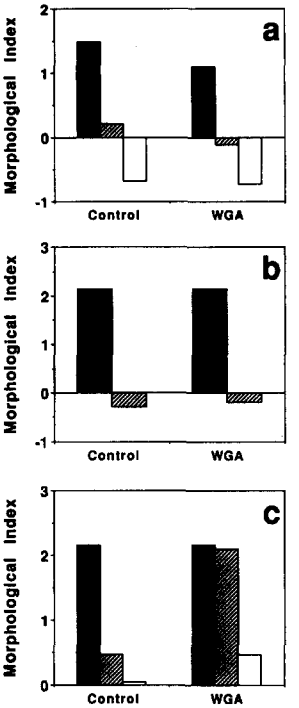

Fig. 2. WGA cannot prevent shape reversion of echinocytes to discocytes or stomatocytes. Echinocytes were generated by incubating cells with different concentrations of DLPC vesicles. After treatment without (control) or with WGA (1.5 μg/ml), cell morphology was monitored after echinocytes were (a) incubated with 0 μM (closed bar), 0.5 μM (shaded bar), or 1 μM (open bar) DLPS vesicles for 60 min; (b) incubated with 42 μM CPZ for 0 min (closed bar), and 15 min (shaded bar); (c) extracted with 1 mg/ml BSA for 0 min (closed bar), 5 min (shaded bar), and 30 min (open bar).

direct expansion of the membrane inner monolayer or extraction of outer monolayer amphipaths restored discoid morphology to WGA-treated echinocytes, although the second process was slower in WGA-treated cells than in controls.

Stomatocytosis

Discocyte-to-stomatocyte transitions. Cells incubated with DLPS undergo a biphasic shape transformation, with a transient echinocytic stage, followed by reversion to discs and eventually stomatocytes as the aminophospholipids are translocated from the outer to the inner membrane monolayer [5,6]. Preincubation of cells with WGA did not inhibit the steady state stomatocytosis induced by DLPS; cells treated with or without WGA attained similar stomatocytic shape (Fig. 3a). However, the transient echinocytosis was greatly inhibited in WGA-treated cells. The degree of this inhibition was WGA concentration-dependent (Fig. 3a). These observations are consistent with an earlier report [18] employing primaquine: WGA has no detectable effect on stomatocytic shape changes.

Stomatocyte-to-discocyte transitions. A shape change from stomatocytic to discoid form can be achieved by means similar to echinocytosis: in bilayer balance terms,

the net result in both events is an outer monolayer expansion relative to the inner monolayer. To test whether WGA affects the shape reversion from stomatocytes to discs, PC reversion of DLPS-induced stomatocytes was examined. PS-induced stomatocytes were treated with a concentration of DLPC sufficient to completely reverse stomatocytosis and yield echinocytes from control cells (Fig. 3b). On treatment with PC in the presence of WGA, these stomatocytes reverted rapidly to discoid form, but did not become echinocytic (Fig. 3b). Thus, WGA fixation does not inhibit membrane changes from invaginated to near planar configurations.

In summary, these studies showed that shape interconversions of discocytes and stomatocytes are not affected by WGA.

3.2. Localization of PC in cells pretreated with WGA

It is possible that WGA binding could stabilize discocytes by altering the intercalation of the echinogenic agent or its disposition in the bilayer. The mode of membrane association and distribution of echinogen were compared in WGA-treated and control cells, using spin- and radiolabeled PC. Control and WGA-treated cells were incubated with spin PC vesicles. At the end of 2 h incubation, control cells were slightly echinocytic (MI = +0.3); WGA-treated cells remained discoid (MI = +0.01). Cells were separated from supernatants, and EPR spectra were examined. The EPR spectra of WGA-treated and control cells exhibited the broad anisotropic triplet (as shown in Fig. 2, Ref. [21]), indicating the lipids are intercalated into the membrane bilayer.

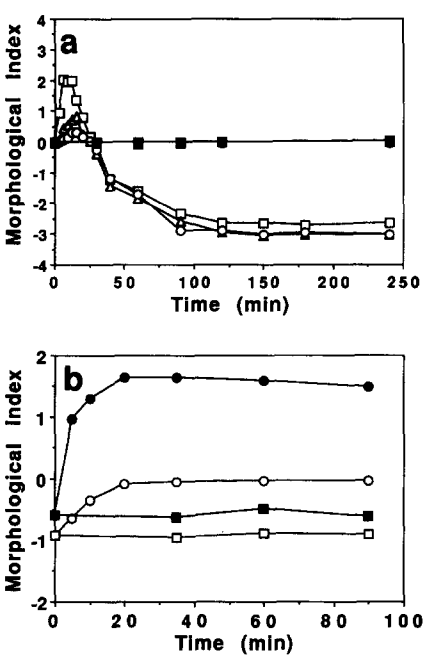

Fig. 3. (a) WGA cannot inhibit DLPS-induced stomatocytosis. Cells were incubated in PBS or WGA, and then treated with 0 (closed symbols) or 0.75 μM DLPS vesicles (open symbols). Control cells (□,■); 1 μg/ml WGA (△,▲); 1.5 μg/ml WGA (○,●). (b) WGA cannot inhibit shape reversion from stomatocytes to discocytes. Stomatocytes were generated by incubation of cells (10% HCT) with 15 μM DLPS for 45 min at 37°C. Cells were then diluted to 0.2% and treated with PBS (closed symbols) or WGA (open symbols), incubated further without (□,■) or with 0.16 μM DLPC vesicles (○,●).

Table 1
BSA extraction of [^{14}C]DLPC

Experiment	MI (−BSA/+BSA) [a]		% extracted [b]	
	(+)WGA	(−)WGA	(+)WGA	(−)WGA
I	0/−0.07	+2.17/−0.10	74.07	83.20
II	−0.02/−0.32	+1.54/−0.38	79.10	88.60

[a] Control and WGA-treated cells were incubated with 0.1 μM [^{14}C]DLPC for 15 min at room temperature. BSA (final concentration of 3.3 mg/ml) or PBS was then added to cells and incubation was continued for 10 min. Shown are cell morphologies (MI's) before BSA (−BSA) and after BSA (+BSA) extraction.

[b] Cells and DLPC vesicles were separated on a sucrose cushion. [^{14}C]DLPC remaining in the cell pellet was determined, and % extracted lipids was calculated as described in Materials and methods.

WGA might inhibit amphipath-dependent echinocytosis by inducing abnormal transbilayer lipid scrambling, equalizing the monolayer populations of the foreign lipid. The transbilayer distribution of incorporated echinogen (0.07 μM [^{14}C]DLPC) was investigated using BSA extraction, which removes relatively hydrophilic outer monolayer lipids [7]. Control cells crenated on exposure to [^{14}C]DLPC and recovered discoid shape on BSA extraction (Table 1). WGA-treated cells remained discoid throughout PC- and BSA treatment. The [^{14}C]DLPC retained in cell pellets after BSA extraction was similar for WGA-treated and control cells (Table 1). Higher concentrations of BSA extracted the labeled lipid more extensively; at the highest levels tested (33 mg/ml), control and WGA-treated cells retained only 3–7% of the DLPC. This result is inconsistent with wholesale scrambling of the foreign lipid in WGA-treated cells.

3.3. Membrane skeleton integrity and WGA function

Elevated intracellular 2,3-DPG. At concentrations higher than the normal 5 mM, the cellular metabolite 2,3-DPG dissociates the spectrin-actin-protein 4.1 complex, increases the lateral mobility of GP, and decreases cytosolic pH [24,35]. Cells were incubated in IPP buffer for 2 h at 37°C, producing a cytosolic pH of 7.02 and a 2,3-DPG concentration of 16.75 mM. Comparable values for control cells incubated in PBS were 7.18 and 4.62 mM, respectively. All are consistent with previously reported values [24].

Heat denaturation of spectrin. Cells were heated at 48°C for 10 min, a treatment reported to denature spectrin partially [36,37]. SDS-PAGE analysis of membranes from such heat-treated cells revealed low molecular weight proteins not found in control membranes, consistent with

Table 2
WGA inhibition of echinocytosis in membrane skeleton-perturbed cells [a]

	Morphological index (MI) [b]			
	DPG elevation	heat treatment	4.1 phosphorylation	PCMB alkylation
Control cells + DLPC	+2.05	+1.45	+1.85	+2.71
Treated cells + DLPC	+2.03	+0.65	+2.15	+2.87
Control cells + WGA + DLPC	+0.45	+0.08	+0.03	+0.84
Treated cells + WGA + DLPC	+0.32	+0.04	+0.02	+1.14

[a] Membrane skeleton–GP interactions were perturbed in treated cells by elevation of 2,3-DPG, heat denaturation of spectrin, 4.1 phosphorylation or PCMB alkylation as described in Materials and methods.
[b] Cell morphologies (MI's) were determined after control or treated cells were incubated with DLPC vesicles (0.075–0.15 μM) for 20 min in the presence or absence of WGA.

reported membrane association of cytosolic components in heat-damaged cells [38].

Protein 4.1 phosphorylation. Increased phosphorylation of protein 4.1 is reported to decrease its association with the spectrin-actin complex and to reduce its ability to promote the binding of spectrin to actin [41,42]. Cells prelabeled with ^{32}P were treated with PMA for 30 min. A sample of membranes analyzed by SDS-PAGE and autoradiography showed enhanced 4.1 phosphorylation as previously reported [25,39,40]. Whether this increased labeling represents a net increase in 4.1 phosphorylation or increased turnover is not clear.

Alkylation with p-chloromercuribenzoate (PCMB). Cells were treated with varying concentrations of alkylating agent PCMB, and their morphologies and the compositions of their Triton shells were analyzed. After treatment with 3.2 mM PCMB, cells were mostly intact and discoid, and their Triton shells contained no detectable GP, which is consistent with dissociation of the membrane skeleton from a major site of membrane association as has been reported [26].

Cells treated by each of these four perturbants maintained discoid morphology, and crenated on exposure to DLPC vesicles (Table 2). In all cases, WGA inhibited such crenation substantively (Table 2). Although heat-treated cells appeared less echinocytic than controls, they converted to comparable echinocytes at a slightly higher DLPC concentration (not shown). PCMB-alkylated cells crenated in a way subtly different from control echinocytes: spicules were finer and their distribution less even, with few developing in the cell concavity. However, this slightly anomalous crenation was inhibited significantly by WGA (Table 2).

Depletion of membrane polyphosphoinositides. PIP_2 is a cofactor that promotes GP-protein 4.1 association in vitro [11,14,43]. ^{32}P-labeled cells were incubated with Ca^{2+} and A23187 to reduce PIP_2 and PIP. TLC analysis of extracted phospholipids revealed significant decreases in radioactivity of both PIP_2 and PIP (Table 3); with repeated treatments with Ca^{2+} and the ionophore, or with a single exposure to a relatively high Ca^{2+} concentration, both inositides decreased to approx. 10% of control levels. In all cases, the Ca^{2+}-treated cells became echinocytic (Table 3).

Table 3
WGA inhibition of echinocytosis in polyphosphoinositide-depleted cells

Ca^{2+} (mM)	PIP (%)		PIP_2 (%)		Morphological index (MI)			
	crenated [a]	repleted [a]	crenated	repleted	crenated	repleted	repleted + PC [b]	repleted + WGA + PC [c]
0	100 [d]	100	100	100	+0.04	−0.05	+2.04	+0.05
0.2, 1 × [e]	37.3	n.d. [f]	51.5	n.d.	+0.99	+0.06	+2.13	+0.19
0.2, 2 × [e]	20.9	n.d.	25.5	n.d.	+1.05	+0.04	+2.09	+0.22
0.2, 3 × [e]	16.4	16.2	19.4	15.6	+1.07	+0.05	+2.05	+0.18
0.5	20.9	22.1	24.0	27.1	+2.81	−0.68	+1.90	−0.15
1.0	19.4	22.1	25.0	21.1	+3.22	−0.95	+1.68	−0.17
1.0 [g]	0	0	10.9	8.8	+3.80	−1.04	+1.48	−0.20

[a] ^{32}P-labeled cells (20%) were crenated by incubation with 5 μM A23187 and various concentrations of calcium for 15 min at 37°C; crenated cells were then repleted as described in Materials and methods.
[b] Repleted cells were diluted to 0.2% HCT, and then incubated with 0.15 μM DLPC for 10 min at room temperature.
[c] Repleted and diluted cells were incubated with 0.15 μM DLPC for 10 min in the presence of WGA.
[d] The radioactivity of PIP and PIP_2 from crenated and repleted cells was quantitated by AMBIS Radioanalytic Imaging System™, and normalized to control cells.
[e] Cells were crenated and repleted once (1 ×), twice (2 ×), and three (3 ×) times.
[f] n.d.: not determined.
[g] Cells were incubated with Ca^{2+} at 10% HCT for 70 min at 37°C.

The Ca^{2+}-induced morphology change is reversible: when Ca^{2+} and the ionophore were removed by EGTA and BSA, cells incubated with nutrients at 37°C recovered discoid morphology (Table 3). The most severely echinocytic samples eventually developed stomatocytic morphology, likely reflecting loss of outer monolayer area through membrane budding [12,44]. This shape recovery was not accompanied by regeneration of the phosphoinositide lipids (Table 3).

PIP_2-deficient discocytes prepared as described were incubated with DLPC in the presence or absence of WGA. In the absence of the lectin, all samples became echinocytic (Table 3). WGA inhibited this echinocytosis completely (Table 3) in both controls and PIP_2-depleted cells.

3.4. Glycophorin-independent shape stabilization

Lectin effects on enzyme-treated cell. To investigate the importance of glycophorin in mediating shape fixation by WGA, the cell surface was modified by incubating intact cells with pronase (to remove the extracellular domain of GP) or neuraminidase (to cleave all sialic acid residues from glycoproteins and glycolipids). SDS-PAGE and Schiff staining analysis of membrane proteins showed that both treatments left almost no intact GP-A dimer, consistent with prior reports [22,45].

After pronase or neuraminidase treatment, cells crenated normally on incubation with DLPC vesicles (Fig. 4, A, B, C). WGA inhibited the echinocytosis in pronase-treated cells significantly (Fig. 4, E), as did *Limulus* lectin (Fig. 4, F), another sialic acid binding lectin [7]. In contrast, the shape stabilization effect of WGA was abolished completely in desialated cells (Fig. 4, G).

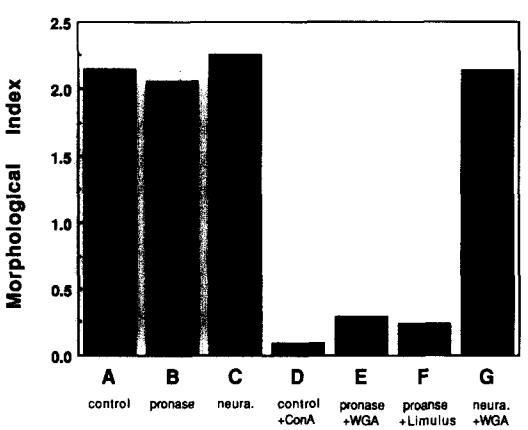

Fig. 4. Effect of lectins on control and enzyme treated cells. Control cells were incubated with 0.1 μM DLPC vesicles for 15 min at room temperature in the absence of any lectin (A), or in the presence of 2.5 mg/ml Con A (D). Pronase-treated cells were incubated with DLPC in the absence of any lectin (B), or in the presence of 30 μg/ml WGA (E) or 30 μg/ml *Limulus* lectin (F). Neuraminidase treated cells were incubated with DLPC in the absence of WGA (C), or in the presence of 30 μg/ml WGA (G).

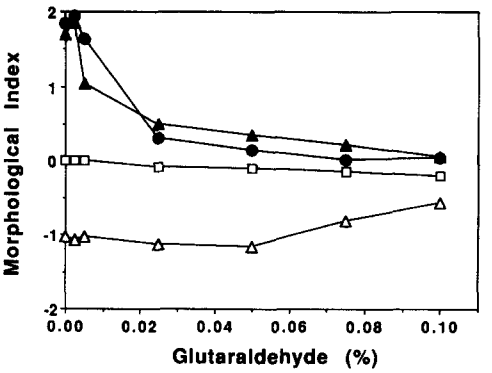

Fig. 5. Effect of glutaraldehyde on cell shape fixation. Cells (50%) were pretreated with glutaraldehyde (0–0.1%, v/v) for 10 min at 37°C. After removal of glutaraldehyde, cells were incubated at room temperature in PBS buffer (□), with 0.075 μM DLPC vesicles for 15 min (●), with 0.01 μM DLPS vesicles for 90 min (△), or with 0.25 mM Ca^{2+}/5 μM A23187 for 15 min (▲).

Shape fixative effect of Con A. Con A is a Band 3-specific lectin. At concentrations greater than 50 μg/ml, Con A inhibited DLPC-induced echinocytosis (Fig. 4, D) as well as Ca^{2+} crenation (data not shown). The shape fixative effect of Con A was reversed when competing sugars (glucose/mannose) were added to displace the bound lectin (data not shown).

Limited fixation by glutaraldehyde. Cells were preincubated with varying concentrations of glutaraldehyde, then washed to remove unreacted glutaraldehyde. Glutaraldehyde-treated cells were incubated with DLPC or DLPS vesicles, or loaded with 0.2 mM Ca^{2+}. Glutaraldehyde concentrations as low as 0.01% inhibited echinocytosis induced by DLPC or Ca^{2+} loading (Fig. 5). In contrast, stomatocytosis was not affected unless cells were prefixed with much higher concentrations of glutaraldehyde (> 0.07%) (Fig. 5, [46]). SDS-gel electrophoresis showed that inhibition of stomatocytosis correlated with crosslinking of membrane skeleton components; the lower glutaraldehyde concentrations, which inhibited echinocytosis selectively, produced no detectable protein crosslinking [46].

4. Discussion

The experiments described in this contribution were undertaken to examine further the molecular basis of WGA cell shape fixation. The phospholipids DLPC and DLPS were used to induce echinocytosis and stomatocytosis, respectively. DLPC is used in preference to SDS or lysoPC [7,18] because its passive transbilayer flip-flop is relatively slow; it remains confined to the outer monolayer for many hours [5,21], permitting examination of cell shape responses in extended time domains. Likewise, DLPS is a well defined inner monolayer intercalator [5,47] that has no reported secondary effects on cell metabolism, unlike cationic amphipaths such as primaquine [4].

4.1. Asymmetric effects of WGA on cell shape transformations

Consistent with earlier studies employing SDS, lysoPC, and primaquine, WGA control of phospholipid-induced cell shape changes is exerted in only one direction. Echinocytosis induced by DLPC is inhibited effectively (Fig. 1), but DLPS-induced stomatocytosis is not affected (Fig. 3).

WGA also inhibits the echinocyte-to-discocyte transformation observed when SDS-induced echinocytes are treated briefly with BSA [7]. However, WGA confers resistance to BSA reversion only for short time periods. DLPC-induced echinocytes, which can be monitored over extended times, are restored to near normal discoid shape by BSA extraction in the absence or presence of WGA (Fig. 2c).

One possible interpretation of WGA-induced resistance to BSA shape reversion may lie in a report [48] that BSA increases the membrane viscosity of WGA-treated cells. Micropipet deformation studies showed that cells treated with BSA and WGA in combination recovered from deformation 2–3-times more slowly than controls [48]. It is possible that the slower BSA reversion of WGA-treated echinocytes reflects the hysteresis of a more viscous membrane.

4.2. Mechanisms of WGA cell shape fixation

Lipid bilayer imbalance. WGA might stabilize discocytes by preventing a change in bilayer balance. Several lines of evidence negate this possibility. Earlier work showed that WGA does not inhibit echinogen binding to cells [7,18]. Conceivably, binding of WGA could create a surface lattice that permits superficial binding of intact amphipath vesicles but inhibits amphipath insertion into the bilayer [18]. However, the EPR spectrum of associated spin PC indicates that foreign lipid inserts into the membrane of WGA-treated cells; additionally, the rate and extent to which a radiolabeled PC can be extracted are consistent with stable intercalation in the membrane outer monolayer (Table 1). Thus, echinogen incorporation and monolayer distribution are not affected by WGA.

Membrane skeleton fixation. The possible role of membrane skeleton in WGA-mediated cell shape fixation was investigated in experiments designed to perturb the intact GP-membrane skeleton complex.

The in vitro interaction of GP with protein 4.1 is mediated by PIP/PIP_2 [11,14]. Ca^{2+} treatment led to a 60–90% reduction in radiolabeled PIP and PIP_2; concomitantly, cells were transformed to echinocytes (Table 3). Subsequent removal of Ca^{2+} restores the cells to discoid shape without regeneration of PIP/PIP_2 (Table 3). On incubation with DLPC, control and PIP/PIP_2-depleted discocytes crenate identically, and WGA inhibits this echinocytosis to equal extents in both (Table 3). These observations argue against a role for the inositides in regulation of cell shape, whether by modulation of GP–membrane skeleton interactions or by other mechanisms [1].

Thus, selective disruptions of spectrin (heat treatment) and of all reported associations between GP and the membrane skeleton complex (depletion of PIP/PIP_2, PCMB treatment, etc.) do not prevent WGA from stabilizing discocyte shape. Further, although spectrin denaturation and cell alkylation produce subtle alteration in the appearance of echinocytes, none of these protein skeleton perturbations affect normal discocyte shape or prevent echinocytic responses. These findings argue against a role of a GP-PIP_2 membrane skeleton complex in maintaining discocyte shape or actively participating in cell shape changes.

4.3. Glycophorin-independepent cell shape stabilization

The failure to abolish WGA fixation by disrupting GP-membrane skeleton associations suggests that the lectin may act by a less selective mechanism. Indeed, pronase digested cells contain no detectable intact glycophorin, yet resist crenation in the presence of WGA and *Limulus* lectin (Fig. 4). In contrast, sialidase treatment yields cells that crenate equally in the presence or absence of WGA (Fig. 4). These experiments demonstrate that GP is not the sole cell receptor to effect lectin shape fixation; lectins can exert shape fixation by binding to sialic acid residues on glycolipids.

Stabilization of discocytes is not unique to lectins that bind sialic acid. Con A, selective for mannose and glucose, also inhibits echinocytosis by DLPC (Fig. 4). Discocyte stabilization also is observed in cells subjected to limited covalent crosslinking with glutaraldehyde. At very low concentrations, glutaraldehyde produces no detectable crosslinking of membrane skeleton proteins; yet these minimally fixed cells are resistant to crenation by DLPC or Ca^{2+} loading. As with lectin-treated cells, cells minimally fixed with glutaraldehyde are not inhibited from cupping on treatment with DLPS (Fig. 5).

In summary, agents with diverse binding characters share common features with WGA: stabilization of discocytes against crenation, but not stomatocytosis.

4.4. Outer monolayer fixation by a 'kinetic calyx'

Conditions that alter membrane skeleton organization demonstrably (*N*-ethylmaleimide alkylation, diamide oxi-

[1] While the conditions of this experiment deplete the PIP/PIP_2 population significantly, a small fraction of PIP/PIP_2 (10–20%) persists. It is unlikely that this residual PIP_2 mediates the anchorage of membrane skeleton to GP: a recent study of the binding of protein 4.1 to membrane vesicles stripped of peripheral proteins demonstrated that only 65–70% of PIP_2, all of which is accessible to erythrocyte phosphoinositol phospholipase C, is involved in regulating 4.1 binding.

dation, extensive glutaraldehyde fixation) produce cells resistant to both echinocytosis and stomatocytosis [49]. The differential effects of lectins and glutaraldehyde on outward and inward membrane bending suggest a selective stabilization of outer monolayer. An alternative mechanism for lectin shape fixation is a localized change in protein–lipid or glycolipid–glycolipid interactions that stabilizes outer monolayer associations, but has limited influence on dislocations of the more remote inner monolayer/membrane skeleton components. WGA binding is known to increase erythrocyte membrane stiffness, detectable as resistance to deformations requiring positive curvature change [18,50,51]. The basis of this effect is not established, but while some workers attribute the diminished membrane flexibility to enhanced GP–membrane skeleton interactions [9,17], others propose that WGA bound near the termini of carbohydrate 'trees' directly impedes membrane flexion [50]. If multivalent lectin or glutaraldehyde complexes form crosslinks that are compressible but not expandable, a selective resistance to outward membrane bending (as seen both in diminished deformability and inhibited evagination) would be expected. Although the amount of WGA needed to fix discoid morphology is small, the on-and-off rate for binding to sialic acid residue is fast [7], so the stabilizing effect on the cell surface might reflect a time-averaged effect of many such associations. On the other hand, while an individual glutaraldehyde crosslink presumably affects a smaller region of the membrane than a lectin, the greater numbers of glutaraldehyde links evidently produce a similar effect.

5. Conclusion

These observations demonstrate that lectin shape stabilization is a generalized phenomenon which may act through a surface fixation mechanism that alters organization of the outer monolayer of the membrane without affecting transbilayer associations.

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