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Diffusion of Glycophorin A in Human Erythrocytes

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Abstract

Several lines of evidence suggest that glycophorin A (GPA) interacts with band 3 in human erythrocyte membranes including: i) the existence of an epitope shared between band 3 and GPA in the Wright b blood group antigen, ii) the fact that antibodies to GPA inhibit the diffusion of band 3, iii) the observation that expression of GPA facilitates trafficking of band 3 from the endoplasmic reticulum to the plasma membrane, and iv) the observation that GPA is diminished in band 3 null erythrocytes. Surprisingly, there is also evidence that GPA does not interact with band 3, including data showing that: i) band 3 diffusion increases upon erythrocyte deoxygenation whereas GPA diffusion does not, ii) band 3 diffusion is greatly restricted in erythrocytes containing the Southeast Asian Ovalocytosis mutation whereas GPA diffusion is not, and iii) most anti-GPA or anti-band 3 antibodies do not co-immunoprecipitate both proteins. To try to resolve these apparently conflicting observations, we have selectively labeled band 3 and GPA with fluorescent quantum dots in intact erythrocytes and followed their diffusion by single particle tracking. We report here that band 3 and GPA display somewhat similar macroscopic and microscopic diffusion coefficients in unmodified cells, however perturbations of band 3 diffusion do not cause perturbations of GPA diffusion. Taken together the collective data to date suggest that while weak interactions between GPA and band 3 undoubtedly exist, GPA and band 3 must have separate interactions in the membrane that control their lateral mobility.

Graphical abstract

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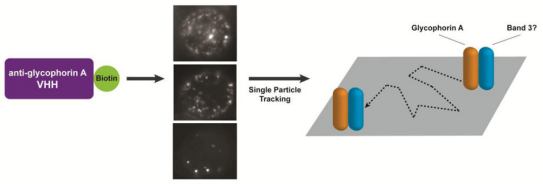

Keywords

erythrocyte membrane structure; glycophorin A; band 3; V_HH; single particle tracking

1. Introduction

The human erythrocyte membrane is comprised of a lipid bilayer containing multiple membrane-spanning proteins supported by an underlying cytoskeleton of spectrin oligomers extending like spokes of a wheel from central actin protofilament hub. Two major bridges connect the spectrin-actin cytoskeleton to the lipid bilayer, both of which involve the major membrane-spanning protein, band 3 (a.k.a. the anion transporter, AE1, SLC4A1). The adducin-to-band 3 bridge connects the junctional complex at the spectrin-actin hub to the lipid bilayer, while the ankyrin-to-band 3 bridge tethers the ankyrin complex (located near the middle of the spectrin tetramer) to the bilayer [1]. Both bridges appear to be essential to membrane stability [2-6].

Based on the above model of the erythrocyte membrane, three populations of band 3 are believed to exist. Approximately 40% of band 3 molecules are thought to associate with glycophorin A, Rh proteins, CD47, and several other polypeptides [7] in a complex that is tethered to the cytoskeleton via the above ankyrin bridge. Another ~35% of band 3 molecules are likely anchored via the adducin bridge to the aforementioned junctional complex that also contains glycophorin C, Rh proteins, GLUT1 and the peripheral proteins, actin, protein 4.1, p55, spectrin and dematin [7]. The remaining ~25% of band 3 molecules are believed to diffuse freely in the lipid bilayer, unencumbered by attachment to the cytoskeleton but constrained by the boundaries of spectrin corrals [7].

Glycophorin A (GPA) is the major transmembrane sialoglycoprotein in erythrocytes, and although its molecular function remains incompletely understood, several lines of evidence demonstrate that it interacts with band 3. Thus, expression of the Wright B blood group antigen requires the interaction of GPA and band 3 [8, 9] and anti-Wr^b antibodies immunoprecipitate both band 3 and GPA [10, 11]. In addition, binding of anti-GPA antibodies has been shown to immobilize both GPA and band 3 while simultaneously increasing membrane rigidity [12-14]. This is likely mediated by a ligand-induced conformational change in the cytoplasmic domain of GPA which in some manner increases its association with the cytoskeletal network [15]. Other studies have shown that GPA facilitates trafficking of band 3 from the endoplasmic reticulum to the cell surface [16-18], and still other publications report that band 3 null red blood cells lack GPA due to rapid degradation of the protein in the cytoplasm [19], i.e. implying that GPA and band 3 associate

during posttranslational processing and/or intracellular trafficking processes. Based on these and other data, it is widely assumed that GPA associates with band 3 at the ankyrin complex.

Although an interaction between GPA and band 3 appears to be established, it remains unknown what fraction of GPA is linked to band 3. To answer this question, we have developed a biotinylated glycophorin-A specific camel V_HH fragment that can be used to monitor the diffusion of GPA in intact erythrocytes. After confirming that binding of the camel V_HH fragment does not perturb erythrocyte structure, we labeled the V_HH fragment with a quantum dot and utilized single particle tracking to characterize the diffusion properties of glycophorin A in relation to the diffusion of band 3. Our diffusion data suggest that at least a fraction of GPA has a distinct anchor to the red cell cytoskeleton that is totally independent of band 3.

2. Materials and Methods

2.1 Materials

Isolation and characterization of recombinant camel anti-human glycophorin A V_HH has been described previously [20]. R-10 monoclonal antibody was a kind gift from Dr. David Anstee (Bristol Institute for Transfusion Sciences, NHS Blood and Transplant). Sulfo-NHS-LC-Biotin was purchased from ThermoFisher Scientific. All other materials were from Sigma-Aldrich unless otherwise stated.

2.2 Collection of Blood and Preparation of Cells

Blood samples were collected from individuals following informed consent in accordance with the Declaration of Helsinki and approval from the Purdue University Institutional Review Board. Whole blood was collected by venipuncture into acid-citrate-dextrose-containing tubes and pelleted at 1000 × g. After removal of the buffy coat, erythrocytes were washed 3x with PBS, pH 7.4, containing 5 mM glucose. Packed cells were then used as prescribed for the specific experiment. Due to the propensity for donor to donor variation, an effort was made to use multiple donors for each experiment or to use different donors when repeating an experiment.

2.3 Biotinylation of Camel Anti-human Glycophorin A V_HH

Sulfo-NHS-LC-Biotin from Thermo Scientific (#21335) was used per the manufacturer's directions. Stoichiometric ratios of camel V_HH fragment (IH4) and biotinylation reagent were adjusted to give no more than 1 biotin/IH4 molecule. Following reaction for 2 h at 4°C, the solution was dialyzed against PBS in a 10,000 MWCO micro-dialyzer to remove unreacted biotinylation reagent and return the solution to its original volume and concentration. This stock is referred to as IH4-biotin and was used without further purification. Successful biotinylation was confirmed by immunoblotting as well as immunofluorescence.

2.4 SDS-PAGE and Western Blotting

Washed, intact erythrocytes were prepared at 4% hematocrit in SDS sample buffer containing 5% 2-mercaptoethanol and run on a 10% SDS-PAGE gel. The proteins were

transferred to a nitrocellulose membrane, which was then blocked overnight in a solution of 5% milk in Tris-buffered saline containing 0.05% Tween 20. The membrane was cut, and identical pieces were probed with either the IH4 antibody, detected with murine anti-HA followed by anti-mouse-HRP, or with biotinylated IH4 detected with anti-biotin-HRP.

2.5 Immunofluorescence Microscopy

Washed, packed erythrocytes were suspended at 5% hematocrit in PBS and incubated with either IH4 or IH4-biotin for 1 h with shaking at room temperature. For saturating conditions, 4 μM IH4 was used. For competition, cells were first incubated for 1 h with unlabeled IH4, followed by the desired concentration of IH4-biotin. Following labeling with IH4, cells were washed 2x in PBS containing 0.1% BSA. Anti-biotin FITC diluted 1:100 was added to the cells for 30 min at room temperature, followed again by washing 2x with PBS containing 0.1% BSA. Cells were diluted to 0.1-0.2% in PBS containing 0.1% BSA and allowed to settle on a polylysine-coated coverslip for 10 min prior to imaging with an Olympus FV-1000 confocal microscope.

2.6 Ektacytometry

Cells were suspended at 5% hematocrit in 800 μL of PBS and incubated for 1 h at room temperature with or without IH4-biotin (4 μM) or the anti-GPA monoclonal antibody termed R-10 (10 μg/ml). The incubated cells were pelleted and resuspended in 100 μL of PBS, which was then added to 4 mL of polyvinylpyrrolidone sample buffer and loaded on to the ektacytometer. Samples were subjected to increasing shear stress by running the cylinder from 0-250 rpm at an acceleration rate of 2 rpm/sec. The change in elongation index (EI) with shear stress was recorded by a computer running a LabView program designed to collect the data.

2.7 Single Particle Tracking

2.7.1. Measurement of Band 3 Diffusion on IH4-treated Erythrocytes—The design and labeling of erythrocytes with DIDS-biotin has been described previously [21]. Briefly, DIDS-biotin was constructed from DIDS (4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid; i.e. a covalent band 3-specific inhibitor) by linking it via a short peptide spacer to biotin. Specific labeling of only one or two band 3 molecules per cell was then achieved by incubating RBCs at 5% hematocrit in PBS with ~10^{-11} M DIDS-biotin at 37°C for 1.5 h. After washing with PBS containing 0.1% BSA, cells were resuspended to 5% hematocrit and incubated with streptavidin-conjugated Q-dot 525 (Invitrogen) for 30 min at room temperature with shaking. After washing, cells were then incubated with 1:100 dilution (4 μM) of unlabeled IH4, after which cells were diluted and placed on a coverslip for imaging. A second sample was incubated with IH4-biotin in the same manner, but labeled instead with streptavidin-AlexaFluor 488 for 30 min, followed by washing with PBS containing 0.1% BSA. Cells were deposited onto a polylysine-coated coverslip and video recordings were collected at room temperature for 1000 frames at 120 frames/s and analyzed as described previously [21].

2.7.2 Measurement of GPA Diffusion with IH4—To label single GPA molecules with IH4-biotin, red cells at 5% hematocrit were incubated with 10^{-9} M IH4-biotin in PBS at

room temperature for 1 h followed by washing with PBS containing 0.1% BSA. Cells were resuspended to 5% hematocrit and incubated with streptavidin-conjugated Q-dot 525 for 30 min at room temperature with shaking. After washing, cells were deposited onto a polylysine-coated coverslip and videos were recorded for 1000 frames at 120 frames/s. Treatment of cells with orthovanadate for single particle tracking measurements was done as previously described [22], where 2 mM orthovanadate was added to the coverslips and videos were collected from 30 to 60 min after addition (when phosphorylation of band 3 was maximal).

3. Results

3.1 Biotinylation of IH4

The recombinant camel antibody IH4 was modified by derivatization with biotin for subsequent fluorescence labeling. Previous mapping studies have shown that IH4 recognizes the sequence $Y_{52}PPE_{55}$ in GPA which is N-terminal of the Wright b antigen site, and binding studies with fresh erythrocytes have revealed that saturable binding is reached at IH4 concentrations >2 μM [20]. Because sequence analysis of IH4 showed five exposed lysine residues, none of which appeared to be in the complementarity determining region, a lysine-reactive NHS-biotin was chosen to functionalize IH4 for use in single particle tracking.

Following biotinylation, IH4-biotin was tested for its specificity for GPA. As shown in Fig. 1, biotinylated IH4 retained its ability to label GPA in human RBCs as determined by immunoblot analysis (Fig. 1A) and immunofluorescence imaging of whole cells (Fig. 1B). Immunoblot analysis (Fig. 1A) demonstrated that IH4 and IH4-biotin label three major protein bands, consistent with the anticipated recognition of the GPA dimer, GPA/GPB heterodimer, and GPA monomer [23]. Fluorescent labeling of cells with IH4-biotin and anti-biotin FITC was found to be dose-dependent in the nanomolar concentration range, resulting in labeling of only a few GPA molecules per cell at very low nM concentrations (Fig. 1C).

3.2 IH4 does not alter cell deformability

Because work by other labs has shown that several anti-GPA antibodies can induce erythrocyte membrane rigidification [13, 14], we wished to evaluate whether IH4 might alter RBC membrane rigidity before proceeding to use the antibody fragment to analyze GPA diffusion. In order to determine whether the monovalent camel antibody fragment might perturb membrane stiffness, ektacytometry was performed on fresh RBCs in the presence of both IH4 and IH4-biotin. As shown in Fig. 2, addition of either IH4 or IH4-biotin did not have any significant effect red cell deformability, whereas the monoclonal antibody R-10 caused reduced cell deformability, consistent with previous reports in the literature [13, 14].

3.3 Diffusion of band 3 in the presence of IH4

In addition to inducing membrane rigidification, anti-GPA antibodies such as R-10 have been previously shown to restrict the lateral mobility of band 3 [13, 24, 25]. To determine whether IH4 might similarly perturb band 3 lateral diffusion, diffusion of band 3 was measured in the presence of near saturating levels of IH4 using single particle tracking

methodologies. For this purpose, band 3 was labeled in intact fresh erythrocytes with a DIDS-biotin conjugate and its diffusion was followed by single particle tracking as described previously [21]. Both microscopic diffusion coefficients (D_μ; corresponding to band 3 diffusion over short time intervals where its movement may be largely determined by its interactions with binding partners) and macroscopic diffusion coefficients (D_M; corresponding to band 3 diffusion over longer time intervals where band 3 mobility may be mainly restricted by the presence/absence of physical barriers such as spectrin) were determined. As previously reported by Kodipilli et al. [21], band 3 mobility in normal human erythrocytes is characterized by a bimodal distribution of microscopic diffusion coefficients (D_μ) and a single Gaussian distribution of macroscopic diffusion coefficients (D_M). As shown in Fig. 3A, band 3 in untreated control cells exhibited an average D_μ of 1.8×10^{-11} cm^2/s [78%] and 1.1×10^{-10} cm^2/s [22%], largely similar to previously reported values [21], and there were only minor changes in D_μ and D_M upon addition of IH4 (Fig. 3B), with the majority of band 3 molecules diffusing with an average D_μ of 1.7×10^{-11} cm^2/s [66%] and a smaller more mobile fraction exhibiting a mean D_μ of 5.0×10^{-11} cm^2/s [34%]. Moreover, for both control and IH4-treated samples, the compartment size distributions were best fit by a single Gaussian distribution with a mean of 32 nm and 34 nm, respectively (Fig. 3). These data suggest that labeling of erythrocytes with IH4 does not significantly affect the lateral diffusion of band 3.

3.4 GPA diffusion in normal intact erythrocytes

With no remarkable effects of a saturating concentration (4 μM) of IH4 on either red cell deformability or band 3 mobility, a 4000-fold lower concentration of IH4-biotin (1 nM) was used to track GPA diffusion in intact normal human erythrocytes. Diffusion data were collected from a total of six healthy donors. Because some donor to donor variation was observed in individual data sets (Appendix A), pooled data were used for the final analyses. As shown in Fig. 4A, GPA exhibited a bimodal distribution of microscopic diffusion coefficients, where approximately 75% of the GPA diffused with a mean D_μ of 5×10^{-11} cm^2/s, and the remaining 25% was more restricted, with a mean D_μ of 1.0×10^{-11} cm^2/s. Since the mean diffusion coefficient of even the fastest GPA population (5×10^{-11} cm^2/s) was at least an order of magnitude slower than what has been observed for freely diffusing membrane proteins in intact RBCs [26] or in cytoskeleton-free membranes [27], it is likely that the majority of GPA molecules measured in this study are restricted by an interaction of some kind. While this restricted mobility could derive from either an interaction with the spectrin-based cytoskeleton or an association with other transmembrane proteins, it is also conceivable that GPA's diffusion may be constrained by entanglement of its elaborate carbohydrate side chains with other oligosaccharide chains in the red cell's glycocalyx (i.e. GPA has 16 oligosaccharide side chains attached to the extracellular domain of the polypeptide [28]).

Analyses of the macroscopic diffusion coefficient of GPA yielded a single Gaussian distribution with a mean of 1.67×10^{-12} cm^2/s. Since an unrestricted protein should exhibit diffusion which is independent of the timescale of measurement (i.e. D_M close to D_μ), this ~10-fold reduction in diffusion at long time scales further suggests some degree of confinement but not complete immobilization. The compartment sizes measured from

confined and hop trajectories yielded a single Gaussian distribution of sizes with a mean of 36 nm, i.e. similar to the compartment size in which the diffusion of band 3 is confined.

3.5 Effect of band 3 phosphorylation on GPA diffusion

Orthovanadate (OV) has been previously shown to promote the tyrosine phosphorylation of band 3, leading to rupture of the band 3-ankyrin interaction and the consequent increase in band 3 mobility [22]. Given the proposed association between GPA and band 3, we next investigated the effect of band 3 tyrosine phosphorylation on GPA mobility. GPA diffusion was analyzed using the same OV concentration (2 mM) and treatment time (30-60 min) employed in our previous study of band 3 diffusion [22]. As expected, cells treated with OV exhibited the same morphological changes, including membrane ruffling and vesiculation, seen in previous experiments with OV [22]. Moreover, analysis of band 3 mobility showed the same 10-fold increase in D_μ reported previously by Ferru et al. (data not shown; [22]).

Surprisingly, treatment with 2 mM OV caused no significant change in the diffusion of GPA (Fig. 5). Thus, the mean value of D_μ for the predominant population of GPA in OV-treated cells was 6.7×10^{-11} cm^2/s [79%] compared to 5.0×10^{-11} cm^2/s [75%] in untreated erythrocytes. Similarly, D_M values for OV-treated and untreated control RBCs were 1.67×10^{-12} cm^2/s and 1.5×10^{-12} cm^2/s, respectively. The compartment sizes in which GPA is free to diffuse were also largely unaffected by OV administration, increasing only from 38 to 43 nm. This absence of any major effect of OV treatment on GPA diffusion was unexpected, especially in view of the 10-fold increase in band 3 mobility.

4. Discussion

Consistent with previous studies using fluorescence recovery after photobleaching (FRAP) and single particle tracking [24, 29, 30], we have found that GPA diffusion is both highly retarded and locally confined in intact erythrocytes, suggesting an interaction with other proteins in the membrane or cytoskeleton. Because considerable data demonstrate an interaction between GPA and band 3 [8-19], including observations showing i) GPA and band 3 together form the Wright b blood group antigen [8-11], ii) some antibodies to GPA induce membrane rigidification and band 3 immobilization [12-14], iii) the absence of GPA can impact the rate of anion transport through band 3 [31], iv) GPA can form a complex with band 3 in the endoplasmic reticulum [32, 33], and v) GPA can facilitate movement of band 3 from the endoplasmic reticulum to the cell surface [16-18], it was important to determine which of the three known populations of band 3 might be associating with GPA. Thus, we have previously found that ~25% of band 3 molecules exhibit free diffusion on short timescales, indicating no direct attachment to the cytoskeleton [7]. Because this unrestricted motion and corresponding larger compartment size (71 nm) was not seen for GPA, we can conclude that GPA is not associated with the freely diffusing population of band 3 (Fig. 4B, [21]). A second and third population of band 3 are attached to the junctional and ankyrin complexes in the cytoskeleton where they give rise to an overlapping distributions of D_μ ranging from 10^{-12} cm^2/s to 10^{-10} cm^2/s, with a mean of ~10^{-11} cm^2/s (Fig. 4B, [21]). The ankyrin-bound population, which is tethered on just two sides, is believed to have slightly greater freedom of movement than the junctional complex population, which is tethered by

spectrin on 5-7 sides. At this same time scale of measurement, the majority of GPA (75%) contributes to a single population with an average D_μ of 5×10^{-11} cm^2/s; i.e. similar to the previously observed D_μ for the ankyrin-bound band 3 population and consistent with the majority of GPA having some association with band 3 or another component at the ankyrin complex. The slower GPA population, which has an average D_μ of 1×10^{-11} cm^2/s and represents only 25% of the total GPA, could arise from another source such as: 1) a population that is bound to band 3 at the junctional complex (although there is little evidence for this), or 2) a fraction that independently associates with a distinct, more immobilized component of the cytoskeleton. At longer time scales, GPA diffusion was very similar to band 3, although the distribution of macroscopic diffusion coefficients for GPA was much narrower than that for band 3, suggesting less heterogeneity in its long range mobility. The single distribution of relatively slow D_M compared to the D_μ suggests that GPA diffusion is restricted even at longer time scales, possibly by some interaction with the cytoskeleton or another transmembrane protein in the membrane. While it is possible that the sampling size or fitting of the data to the Gaussian curves could be inaccurate, each of the studies involved measurement of the diffusion of GPA and band 3 in 150 to 600 different erythrocytes from at least six different donors which should reduce the probability of these types of errors.

Surprisingly, treatment with orthovanadate, which is known to disrupt the band 3-ankyrin association and cause release of band 3 from the cytoskeleton [22], leading to a 10-fold increase in band 3 diffusion coefficient, does not significantly affect GPA diffusion. Although this was unexpected, one can envision several possible explanations. First, the established association of GPA with band 3 could either be weak or highly transient. Data supporting this hypothesis include the fact that: i) the mobility of band 3 increases upon erythrocyte deoxygenation whereas the mobility of GPA does not [29], ii) the diffusion coefficients of band 3 decrease dramatically in erythrocytes containing the Southeast Asian Ovalocytosis mutation whereas the diffusion coefficients of GPA do not [34, 35], and iii) most anti-GPA or anti-band 3 antibodies do not co-immunoprecipitate both proteins from erythrocyte membranes [10]. Second, it is also possible that another, yet unknown interaction still restricts GPA diffusion, even when band 3 is released from its cytoskeletal anchor. At least a fraction (as much as 20%) of GPA is present as a GPA/GPB heterodimer and GPB has been shown to have an independent interaction with RhAG during biosynthesis [36]. In fact, the association of GPA with GPB has been proposed to tether the Rh complex (which consists of RhD, RhCE, RhAG, GPB, LW, and CD47) to band 3 at the ankyrin complex, creating a macrocomplex [36]. Independent associations with other membrane proteins such as protein 4.2 or ankyrin could also confine GPA. A third possibility is that GPA could also associate with band 3 at the junctional complex. However, as noted above, this interpretation seems unlikely as mutations in ankyrin produce concomitant deficiencies in band 3 and GPA [37-39], suggesting that these proteins reside in the same macrocomplex. Additionally, disruption of the band 3-adducin-spectrin bridge of the junctional complex results in reduced protein 4.1 and glycophorin C content, but no alterations in spectrin, ankyrin, or glycophorin A [6].

4.1 Conclusions

Analysis of the lateral diffusion coefficients of band 3 and GPA in unmodified intact erythrocytes suggest that the two major erythrocyte membrane proteins diffuse at somewhat similar rates, suggesting a possible weak or transient interaction between the two polypeptides. However, the fact that the dramatic increases in band 3 diffusion that occur upon treatment with ortho-vanadate (see above) or erythrocyte deoxygenation [29] are not accompanied by a change in GPA diffusion demonstrates that any association between the two proteins cannot be permanent. Moreover, the observation that GPA diffusion remains restricted when band 3 is induced to diffuse more rapidly suggests that GPA must have a distinct anchor to the cytoskeleton that can confine its movement independent of band 3.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Highlights

- Glycophorin A and band 3 are abundant integral proteins in the erythrocyte membrane
- Evidence of a glycophorin A - band 3 interaction in erythrocytes remains equivocal
- Diffusion of glycophorin A appears to be restricted and locally confined
- Glycophorin A likely has an anchor to the cytoskeleton independent of band 3

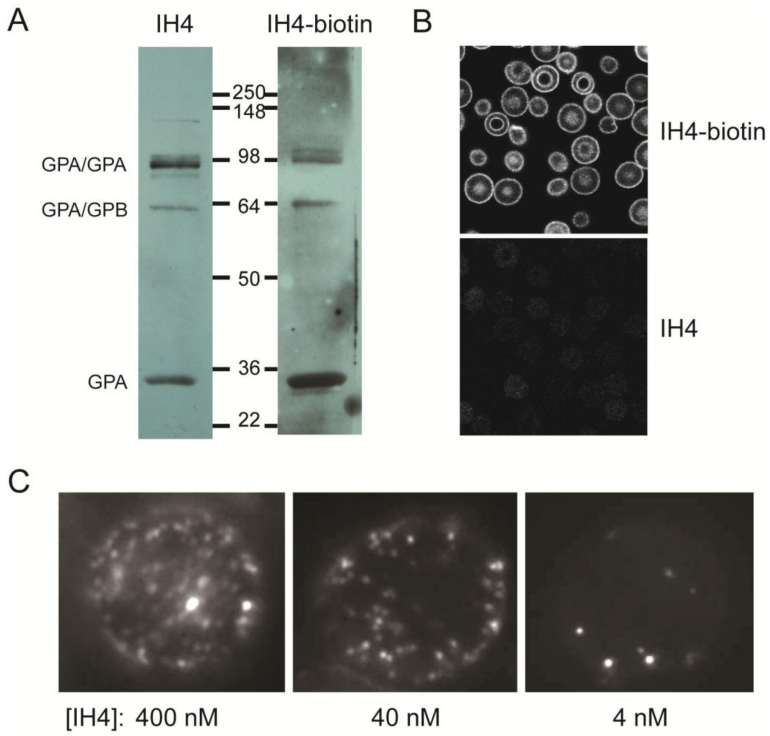

Fig. 1. Recombinant biotinylated camel V_HH fragment IH4 specifically labels glycophorin A

(A) Immunoblot analysis of protein bands from whole human RBCs labeled with IH4 (left) or IH4-biotin (right), and then detected with murine anti-HA followed by anti-mouse-HRP (left) or anti-biotin-HRP (right). **(B)** Image of RBCs labeled first with IH4-biotin (upper panel) or IH4 (lower panel) and then detected with anti-biotin-FITC. **(C)** Titration of the antibody to estimate a concentration appropriate for single molecule tracking. Erythrocytes were incubated with the indicated concentrations of IH4-biotin, followed by anti-biotin-FITC and viewed under a confocal microscope.

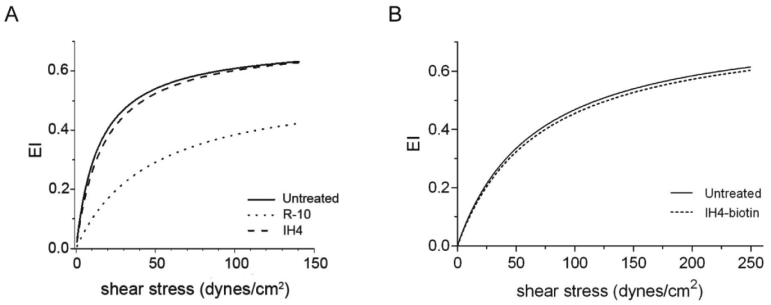

Fig. 2. Recombinant monovalent camel antibody IH4 and IH4-biotin do not alter RBC deformability

(A) Representative deformability curves are shown for fresh RBCs treated with no antibody (solid), unlabeled IH4 (dashed), or the monoclonal anti-GPA antibody (R-10) known to increase erythrocyte rigidity (dotted). EI = elongation index **(B)** Comparison of the deformability of cells treated with or without IH4-biotin. To assure that IH4-biotin was indeed binding to the treated RBCs, binding was confirmed by agglutination with anti-biotin antibody after each sample was run.

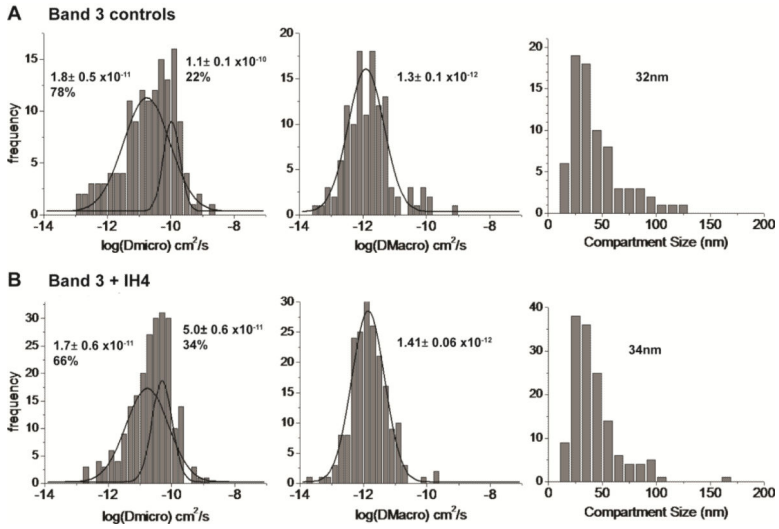

Fig. 3. Recombinant camel IH4 antibody does not perturb band 3 mobility

Distributions of microscopic diffusion coefficients, macroscopic diffusion coefficients, and compartment sizes were measured in **(A)** untreated control cells, and **(B)** cells treated with IH4 at a concentration (4 μM) that is 4000-fold higher than the concentration used to measure glycophorin A diffusion.

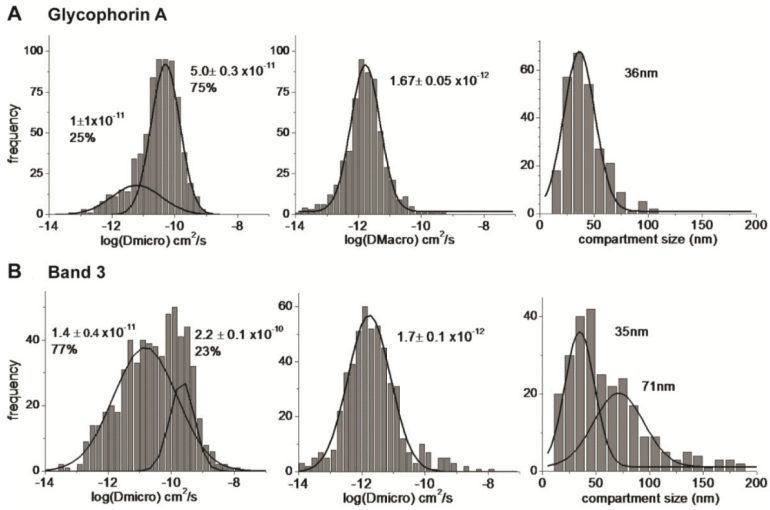

Fig. 4. Comparison of glycophorin A and band 3 diffusion in unmodified (control) human erythrocytes

(A) GPA diffusion data collected on samples from multiple donors, and **(B)** band 3 diffusion data as previously published by Kodipilli et al. (21).

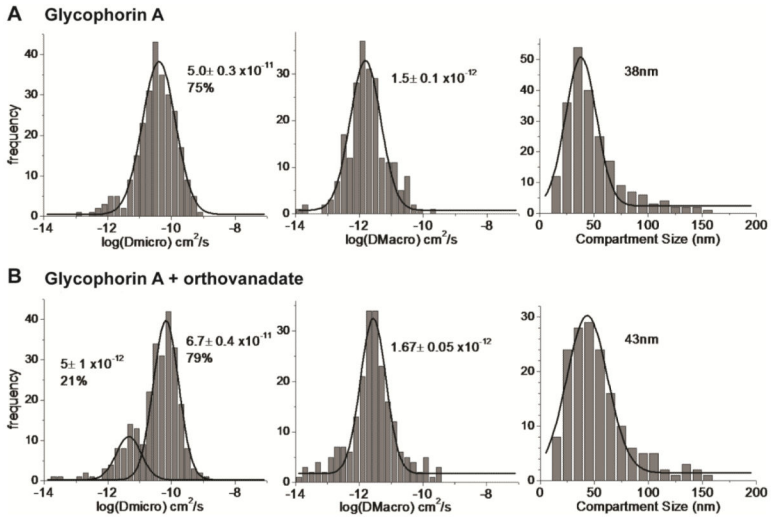

Fig. 5. Glycophorin A mobility is independent of the interaction of band 3 with the cytoskeleton

Distribution of GPA diffusion coefficients obtained from **(A)** control and **(B)** OV-treated red blood cells. Cells were incubated with 2 mM OV for 30-60 min, and GPA diffusion was monitored by single particle tracking. The distributions of GPA diffusion coefficients on untreated cells were measured the same day and on the same blood samples used for the controls.