Quantifying the Impact of Membrane Microtopology on Effective Two-dimensional Affinity*§

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Just as interactions of soluble proteins are affected by the solvent, membrane protein binding is influenced by the surface environment. This is particularly true for adhesion receptors because their function requires tightly apposed membranes. We sought to demonstrate, and further, to quantify the possible scale of this phenomenon by comparing the effective affinity and kinetic rates of an adhesion receptor (CD16b) placed in three distinct environments: red blood cells (RBCs), detached Chinese hamster ovary (CHO) cells, and K562 cells. Effective affinity reflects both the intrinsic receptor-ligand kinetics and the effectiveness of their presentation by the host membranes. Expression of CD16b, a low affinity Fcy receptor, was established by either transfection or spontaneous insertion via its glycosylphosphatidylinositol anchor. Binding to IgG-coated RBCs, measured using a micropipette method, indicated a 50-fold increase in effective affinity for receptors on RBCs over CHO and K562 cells, whereas the off rates were similar for all three. Electron microscopy confirmed that specific tight contacts were broad in RBC-RBC conjugates but sparse in CHO-RBC conjugates. We suggest that through modulation of surface roughness the cytoskeleton can greatly impact the effectiveness of adhesion molecules, even those with no cytoplasmic structures. Implications for locomotion and static adhesion are discussed.

Cell–cell bond formation must be preceded by the creation of a contact zone, a region in which surface-bound receptors and ligands are able to bridge narrow gaps, properly aligning by lateral and rotational diffusion (1). Rough cells initially may form isolated point contacts that over time might be broadened and connected through active membrane processes (2). Alternatively, the cells may simply possess inherently smooth surfaces capable of broad initial contacts. A smooth membrane will not enhance binding to a soluble ligand because the soluble protein can diffuse freely into the membrane folds of rough cells. However, in adhering to immobilized ligand, the ability to form an expansive tight contact area is a distinct advantage. Therefore, cell-cell adhesion depends not only on the intrinsic kinetic rates of the receptor-ligand interaction but also on how effectively the host membranes present these molecules (3).

In this study we quantified the effects of various microtopological presentations on cell adhesion by comparing the effective affinity of the same receptor (CD16b) in three distinct host environments: erythrocytes (RBCs),1 Chinese hamster ovary (CHO) cells, and erythroleukemic K562 cells. Although all other Fc receptors have intracellular domains, CD16b (FcRIIb) terminates at the lipid bilayer with a glycosylphosphatidylinositol (GPI) moiety and so has no direct cytoskeletal interface. Currently there are no data to suggest the existence of multiple activation states in CD16b. These simplifications permit a more focused investigation into the role of the membrane environment in the functionality of this receptor.

The adhesive character of CD16b in each environment was quantified by using a recently developed micropipette method (4). This assay is unique in its yield of estimates for the effective kinetic rate constants for binding of membrane-bound ligand, so-called two-dimensional parameters. Earlier results from the application of this assay to the binding of CD16b-transfected CHO and K562 cells to IgG-coated RBCs produced similar two-dimensional binding parameters for both transfectants (5, 6). The objective of the present study was to examine an additional environment, the RBC membrane, that has a markedly smoother morphology and to compare these results with those from the earlier CHO and K562 studies. We also visually compared the structure of the different cell to cell contacts with scanning electron microscopy (SEM). In this manner we were able to demonstrate a major role for membrane microtopology in cell adhesion and suggest an expanded role for the cytoskeleton in the regulation of adhesion.

**EXPERIMENTAL PROCEDURES**

**Cells and Proteins**—Erythrocytes were isolated from whole blood and stored in EA545 RBC additive solution (5). Transfected cell lines and murine monoclonal antibodies were produced in house except for UPC-10 (IgG2a, Sigma). CD16b (NA2 allotype) was detergent-extracted from transfected K562 cells and purified by affinity chromatography (7) with the addition of 1,10-phenanthroline (5 mM) to the lysis buffer. Purity was confirmed by SDS-polyacrylamide gel electrophoresis.

Reconstitution and Chromium-Chloride Coupling—Reconstitution, or transfer of purified CD16b to target membranes, was accomplished by coincubation at 37 °C according to Nagarajan et al. (7). Reconstituted RBCs were kept in ice-cold EA545 for up to 2 weeks (5). Covalent coupling of human IgG to the surfaces of RBCs (CrCl2-coupled IgG or reconstituted CD16b) were determined by quanitative indirect fluorescent immunoassay using LFA-3 expression as in Ref. 5. Site densities of CD16b on K562 and CHO cells were quantified by radioimmunoassay using 125I-CLBFcgran1 Fab fragments.

1 The abbreviations used are: RBC, red blood cell; CHO, Chinese hamster ovary; GPI, glycosylphosphatidylinositol; SEM, scanning electron microscopy.

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Membrane Microtopology and Effective Two-dimensional Affinity

The Micropipette Adhesion Frequency Assay—Details of the micropipette system and the adhesion frequency assay have been described (4). Briefly, the assay involves using a computer-controlled piezoelectric actuator to move a pair of cells (one receptor-bearing and one ligand-bearing) into contact, hold them for a prescribed duration, and then quickly retract one. Direct observation of any deflection in the flexible RBC membrane indicates the presence of at least one bond at the moment of retraction. A video demonstration of the micropipette assay is available online (Video 1). For each cell line 40–80 cell pairs were examined over a range of contact durations and receptor-ligand expression levels, with at least 100 consecutive adhesion cycles performed for each cell pair. Adhesion frequencies were either directly compared or used to generate characteristic binding parameters. In the latter cases, estimates for the effective affinity and kinetics were determined (4, 8) by fitting the adhesion frequency data to a probabilistic model using a nonlinear regression method based on the Levenberg-Marquardt algorithm (5).

Scanning Electron Microscopy—Samples intended for CD16b localization studies were labeled with CLBFcgran1 followed by goat anti-mouse IgG polyclonal antibody conjugated to 12-nm gold particles (Jackson Immunologicals; Ref. 3). Conjugated samples were prepared by mixing equal amounts of receptor and ligand-bearing cells, centrifuging (5 min, 200 × g), incubating on ice for 30 min, and gently resuspending with a wide mouth pipette. Normal RBCs replaced the reconstituted RBCs in negative controls. Samples were examined for conjugate formation under light microscopy prior to fixation. Subsequent sample processing and imaging were performed by R. Apkarian (Emory University Integrated SEM Facility). Specimens were staged in the lens of an IS1 DS-130 SEM operated at 5 kV. Localization images were obtained below the lens using a 4-mm working distance.

RESULTS

CD16b and IgG Expression Levels on the Host Cell Surfaces—CD16b-expressing K562 and CHO cells, transfected from the same vectors, were used as independent cellular hosts for the adhesion receptor. Because CD16b utilizes a hydrophobic GPI anchor, it can be reinserted, or reconstituted, into the lipid bilayer of any available membrane (7). Accordingly, CD16b was purified from the K562 transfected membranes and reconstituted into RBCs and wild-type CHO cells. The ligand, human IgG, was covalently coupled to RBCs by a chromium-chloride method. Expression levels for all cells are shown in Fig. 2: Flow cytometry before and after each experiment series confirmed expression stability.

RBC-reconstituted CD16b Binds Specifically to RBC-bound IgG—Specificity was established with the micropipette assay (Fig. 3a). RBCs that underwent the sham reconstitution (i.e. without CD16b present) showed minimal binding to IgG+ RBCs. Preincubation of CD16b+ RBCs with the function-blocking monoclonal antibody CLBFcgran1 (10 µg/ml, 30 min, 4 °C) suppressed the binding seen when preincubating these cells with an irrelevant mlgG2a. Similarly, preincubation of IgG+ RBCs with soluble dimeric CD16a, a moderate affinity Fcγ receptor, partially blocked binding, whereas soluble dimeric B7, a non-IgG-associating protein, had no effect.

The Time-dependent Adhesion Frequency Provides Estimates for the Two-dimensional Kinetic Rates $A_k$ and $k_r$ and the Effective Affinity $A_K$—The micropipette assay was used to generate time-dependent adhesion frequency curves from three sets of RBC pairs expressing various levels of CD16b and IgG. An additional series of experiments was performed with cells coated with ovalbumin rather than IgG. Results are shown in Fig. 3b. All data were fit simultaneously to a previously described probabilistic adhesion model (4) to determine the effective affinity and kinetic rates for the RBC-RBC system.

The adhesion model (and its generalized extension (8)) is premised on the idea that, once membranes become close enough for receptors and ligands to bridge the gap, the actual event of forming or breaking each incremental bond is probabilistic. The kinetic rates influence this probability in a predictable way. The initial slope of the adhesion frequency curve is proportional to the forward kinetic rate $k_f$, whereas the equilibrium frequency reflects the affinity $K$. The equilibrium adhesion frequency and initial slope also depend on the product of the receptor and ligand population densities (as is evident in Fig. 3b) and the contact area $A_c$. Because acceptable contact gaps must be on the order of tens of nanometers, the true contact area is not determinable in the micropipette assay (although a constant apparent contact area, as seen under light microscopy, is maintained by the piezo throughout all experiments). The contact area therefore is embedded with $K$, to form what we have termed the effective affinity $A_K$. Because it includes information regarding both the intrinsic reaction kinetics of the molecules and the effectiveness of their presentation, effective affinity will determine the membrane-specific level of bond formation for any given level of receptor-ligand expression. Note that these are two-dimensional binding parameters describing the association of membrane-bound receptors and ligands. The commonly used three-dimensional parameters, governing the binding of soluble ligand to membrane-bound receptors, do not necessarily predict the two-dimensional behavior (5).

Best fits of the data to the model produced estimates for the effective affinity as well as the reverse kinetic rate constant for IgG+ RBC to CD16b+ RBC adhesion. The effective forward rate constant is derivable from $A_k = A_{K}k_r$ and $k_r$ Using this single pair of parameters, all three curves shown in Fig. 3b were generated, illustrating the high quality of the estimates.

RBC Effective Affinity Is 50-fold Higher Than That of CHO or K562 Cells, but There Are No Differences in Reverse Kinetic Rates—The effective affinity and reverse rate for CD16b+ RBC are compared in Fig. 4c with those for CHO cells (5) and K562 cells (6). When hosted by RBCs, the receptor displayed a tremendous 50-fold increase in effective affinity over the CHO and K562 cell hosts. However, no significant differences in reverse kinetic rates were found, indicating that the affinity enhancement was due solely to a much increased effective forward rate on the RBC host.

Increased Binding Efficiency Is Not Due to Reconstitution—Because the receptor was expressed on RBCs via reconstitution (protein transfer) and by transfection (gene transfer) in the other hosts, we investigated whether reconstitution itself could account for the enhanced binding. Using the same aliquot of purified protein, CD16b was reconstituted onto both RBCs and wild-type CHO cells. With a fixed contact time of 5 s, cells were assayed for binding frequency against IgG-coated RBCs. Sham-reconstituted cells were negative controls. Results are shown in

Fig. 1. a, RBCs bearing CD16b (left cell) and IgG (right cell) are repeatedly brought into contact (b) for 1–16 s using a computer-driven piezoelectric actuator. A transient deflection of the weakly aspirated RBC indicates the presence of an adhesion at the moment of retraction. c, no adhesion; d, adhesion. A supplemental video is available online.
Fig. 4b. Just as with the transfected CHO cells, the reconstituted CHO cells exhibited a low level of binding relative to the RBCs. In fact, using the parameters obtained with the transfected CHO cells (Fig. 4a), a predicted adhesion frequency was calculated and found to be quite similar to the adhesion frequency actually observed here using reconstituted CHO cells, thus establishing the equivalency of the reconstitution and transfection modes of expression. It should be noted also that the excellent correspondence between the predictions based on earlier experiments and results from new experiments with a different CD16b preparation suggests a high degree of reproducibility for all reagents and protocols employed.

Enhanced Binding to RBCs Is Not Evident in the Three-dimensional Association of Soluble Antibody to the F/G Loop of Membrane-bound CD16b—Unlike two-dimensional interactions, microtopological differences should not impact three-dimensional binding because a soluble ligand can easily access both peaks and valleys on the cell surface. To test this in our system we used Scatchard analysis to measure the three-dimensional affinity of the monoclonal antibody CLBFεgran1 for RBC-reconstituted CD16b. CLBFεgran1 binds CD16b in the area of the F/G loop on the membrane-proximal globular domain of the receptor (9). The F/G loop also hosts critical epitopes for IgG binding (10). The CD16b-CLBFεgran1 dissociation constant, $K_d = 1/K_a$, was found to be 8 ± 2 nM with reconstituted RBCs and 12 ± 5 nM with transfected CHO cells (11), indicating the lack of any significant binding enhancement for RBCs with an antibody in solution. It is also further evidence of the equivalency of the two expression modes.

RBC Doublets Form Broad Tight Contacts, Whereas CHO-RBC Conjugates Do Not—The surface microtopology of RBCs, CHO cells, and K562 cells was examined by scanning electron microscopy (Figs. 2 and 5). Although the RBC surfaces were quite smooth, CHO cells (detached with EDTA) were densely covered with long microvilli, and K562 cells displayed bulbous extensions averaging 200–400 nm in height. Still, even on the red cell intermembrane gaps greater than about 50 nm are in effect infinite, precluding any possibility for receptor-ligand interaction. Quantifying true contact area formation by direct means has proven challenging (12), although Dustin et al. (13) has demonstrated a promising approach. In this paper we have presented effective affinity as an indirect measure when intrinsic affinity can be controlled. For a more direct examination,
CD16b-IgG-mediated RBC-RBC and CHO-RBC conjugates were prepared and viewed under SEM (Fig. 5, a and b). High magnification imaging of the region of contact between RBC-RBC conjugates revealed broad areas in which the membrane gap would likely accommodate receptor-ligand interactions. In contrast, the CHO-RBC conjugates were characterized by widely distributed point contacts between CHO microvilli and the RBC membrane. This was consistent with Mege et al. (12), who presented transmission electron microscopy images of RBCs showing sparse small contacts with rough-surfaced macrophages.

**DISCUSSION**

The effective forward kinetic rate, $A_k$, was found to be about 50-fold higher when the receptor CD16b was hosted by RBCs rather than CHO or K562 cell membranes. Purification and reconstitution of the protein did not create any alterations leading to increased binding. The evidence points toward the host cell environment as the source of this effect rather than any variation in the protein itself.

These results suggest the presence of a 50-fold greater true contact area in RBC-RBC pairings over those in CHO-RBC or K562-RBC pairings, all with similar apparent contact areas. A larger true contact area will increase the rate at which bonds form but will have no influence on their reverse rates because the latter governs the dissociation of preexisting bonds. This pattern is evident in the data presented here. The enhanced RBC binding was isolated to the forward kinetic rate and two-dimensional assays only, consistent with our hypothesis.
that microtopology was the underlying mechanism for the enhancement.

**Cell-dependent Differences in Lateral Diffusion Should Not Affect the Outcomes of Short Duration Low Affinity Contacts**—There has been considerable recent discussion regarding the effects of lateral diffusion on receptor-ligand interactions. Although the lateral diffusion of lipid-anchored proteins on RBCs may differ from that on K562 or CHO cells, such differences are unlikely to play a significant part in the micropipette assay employed here. There are two elements of higher lateral diffusion that may affect adhesion: (i) faster depletion-driven diffusion of free molecules into the contact area as bonds form, and (ii) more frequent free receptor-ligand interactions within the contact. In the first case, bond formation lowers the concentration of free molecules in the contact area relative to the noncontact surfaces, therefore driving the flux of additional free molecules into the contact area (13). In high affinity cases this initial flow might be rapid. However, in very low affinity (and very brief) interactions such as those described here, only a few bonds are expected to form despite the presence of hundreds to thousands of free receptors and ligands in the contact area. (This can be seen in Fig. 3b, in which the frequency of having no adhesion at all is considerable.) Depletion therefore is so limited that even if bound molecules were immediately replaced the increase in population density (and hence, adhesion frequency) would be negligibly small. This is in contrast with long duration, actively spreading contacts, where lateral diffusion has been demonstrated to have a role in the rate of adhesion strengthening (14).

Note that the mechanism described above can potentially affect bond formation rate but not the ultimate equilibrium adhesion level. This is also true for the second rapid diffusion feature, increased molecular interaction frequency. In a fixed amount of time, a highly mobile receptor may encounter a larger number of free ligands than a less mobile molecule. In a high affinity system this may result in a noticeable acceleration of bond formation and, hence, an earlier equilibrium, but it will also cause an increase in the dissociation rate. Diffusion is fundamentally a process that can alter the rate at which a system reaches equilibrium but cannot alter the final equilibrium state. Therefore the 50-fold equilibrium affinity difference described in this paper cannot be explained by dissimilar diffusivities.

It is interesting to note that even the kinetic rates will be independent of diffusivity if the adhesion is reaction-limited rather than diffusion-limited. This, in fact, is the case here, again because of very low affinity. With \( k_e = 0.5/s \) and \( k_f = 3 \times 10^{-6} \mu \text{m}^2/\text{s} \) (assuming at least half of the apparent contact area is true contact in RBC-RBC adhesions) and a very slow diffusion coefficient of \( 0.01 \mu \text{m}^2/\text{s} \), the procedure of Lauffenburger and Linderman (15) decomposes \( k_e \) into a diffusion-driven \( k_e^d \) of \( 2 \times 10^{-2} \mu \text{m}^2/\text{s} \) and a reaction-driven \( k_e^r \) of \( 3 \times 10^{-6} \mu \text{m}^2/\text{s} \). So the diffusion step is conservatively four orders faster than the reaction step, strongly suggesting reaction-limited diffusion-independent kinetic rate constants and accounting for the nearly identical \( k_e \) values in Fig. 4a. We have used fluorescence recovery after photobleaching to estimate the actual diffusion coefficient of CD16b on CHO cells to be \( 0.16 \mu \text{m}^2/\text{s} \), an even more reaction-limiting value than above.\(^2\)

**Cell-specific Localization or Clustering Patterns Could Contribute**—Although the simplest explanation for the 50-fold difference in the CD16b effective affinity appears to be the distinct microtopology of the RBCs as compared with CHO and K562 cells, there may be other cell-specific factors playing a contributory role that will require additional studies to clarify. For example, localization of molecules into regions of low or high adhesive potential has been observed in several other cases. On neutrophils, L-selectin is localized preferentially on microvilli tips, whereas integrin \( \alpha_\text{M} \beta_{2} \) is predominantly on the cell body (3). In this study, a surface distribution that localized the CD16b near the cell body and away from promontories on both CHO and K562 cells would contribute to the diminished binding seen relative to RBCs. Because GPI proteins lack a cytoplasmic domain, nonrandom distributions would rely on either an association with a transmembrane protein linked to the cytoskeleton or lipid interactions.

Clustering (distinct from localization) of receptors could increase their effective affinity by lowering the entropic barrier to ligand binding. GPI-rich lipid microdomains, termed rafts, have been proposed, although they remain controversial (16, 17). At present their cellular distribution patterns and the proportion of GPI molecules likely associated with them is unclear. Our preliminary SEM studies have shown a seemingly random distribution of CD16b on RBC membranes (Fig. 5c). To be consistent with the binding data presented in this paper, receptor clustering would have to be extensive on RBCs while being nearly absent on both K562 and CHO cell membranes. This state would have to prevail despite very low CD16b densities on the RBCs and quite high CD16b densities on the transfected K562 and CHO cells. Although such a state cannot be excluded, the evidence suggests it is unlikely to be a factor in the present studies.

**An Expanded Role for the Cytoskeleton in Adhesion Regulation**—Modifications to protein expression, conformation, and localization are well recognized mechanisms of adhesion regulation. This study suggests that modulation of surface smoothness also may be an important regulatory mechanism, implying an expanded role for the cytoskeleton in cell adhesion. There is a growing body of evidence supporting this hypothesis. Treatment with cytochalasin increases the contact area in lectin-mediated adhesion of rat hepatocytes (18) and adherent erythroleukemia cells (19). Although similar treatment disrupts integrin-mediated focal contacts, it appears to enhance weaker nonfocal adhesions. Cytochalasin treatment leads to the arrest of neutrophils undergoing P-selectin-mediated rolling (20) and increases the strength of rolling adhesion on both E- and P-selectin (21). In the latter study, hypo-osmotic swelling of neutrophils produced similar results on P-selectin surfaces, and electron microscopy showed that microvilli had been nearly abolished, strongly implicating surface smoothing in the promotion of adhesion.

Localized close contact formation is beneficial in several important cases. Reflection interference contrast microscopy reveals close contacts on the upstream side of endothelial cells exposed to flow (22), thereby maximizing their stability. Total internal reflection and reflection interference contrast microscopy of rapidly moving keratocytes displayed a rim of tight contact at the leading edge, with speed closely correlated with uniformity of this contact region (23). The common model for locomotion involves the transport of adhesion molecules from posterior to anterior in superposition with cycles of leading edge extension/whole-cell retraction. The performance of the transport process is expected to be reflected in the cell crawling speed. Through microtopological manipulation alone, the cytoskeleton may be able to shift the effective affinity of the local receptor population (even those with no cytoskeletal associations) by as much as 50-fold. This would lower the transport threshold required for locomotion substantially or might even replace the transport process altogether.

\(^2\)S. Chelsa and C. Zhu, unpublished data.
intriguing cases of normal motility despite suppression of integrin transport (24).

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