Influence of Receptor Lateral Mobility on Adhesion Strengthening between Membranes Containing LFA-3 and CD2

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Abstract. We have used an in vitro model system of glass-supported planar membranes to study the effects of lateral mobility of membrane-bound receptors on cell adhesion. Egg phosphatidylcholine (PC) bilayers were reconstituted with two anchorage isoforms of the adhesion molecule lymphocyte function-associated antigen 3 (LFA-3). The diffusion coefficient of glycosyl phosphatidylinositol (GPI)-anchored LFA-3 approached that of phospholipids in the bilayers, whereas the transmembrane (TM)-anchored isoform of LFA-3 was immobile. Both static and laminar flow assays were used to quantify the strength of adherence to the lipid bilayers of the T lymphoma cell line Jurkat that expresses the counter-receptor CD2. Cell adhesion was dependent on LFA-3 density and was more efficient on membranes containing the GPI isoform than the TM isoform. Kinetic measurements demonstrated an influence of contact time on the strength of adhesion to the GPI isoform at lower site densities (25–50 sites/μm^2), showing that the mobility of LFA-3 is important in adhesion strengthening. At higher site densities (1,500 sites/μm^2) and longer contact times (20 min), Jurkat cell binding to the TM and GPI isoforms of LFA-3 showed equivalent adhesion strengths, although adhesion strength of the GPI isoform developed twofold more rapidly than the TM isoform. Reduction of CD2 mobility on Jurkat cells at 5°C greatly decreased the rate of adhesion strengthening with the TM isoform of LFA-3, resulting in a 30-fold difference between the two LFA-3 isoforms. Our results demonstrate that the ability of a membrane receptor and its membrane-bound counter-receptor to diffuse laterally enhances cell adhesion both by allowing accumulation of ligands in the cell contact area and by increasing the rate of receptor–ligand bond formation.

CELL–CELL interactions are involved in a diverse array of biological functions ranging from morphogenesis to the generation of immune responses. Specific cell surface glycoproteins are known to mediate cell adhesion by ligation with specific counter-receptors. Binding of adhesion molecules allows close apposition of two or more cells, during which time the same adhesion molecules or other receptors in the contact area may mediate responses such as signal transduction and cell locomotion (reviewed by Springer, 1990).

Bond formation between two adhesion molecules requires that the cell membranes containing these molecules come into close contact. The frequency of random collision of receptor and counter-receptor molecules is likely to be low during the initial cell–cell contact given the low densities of adhesion molecules on the cell surfaces. A substantial number of adhesive bonds may be required to establish a stable cell–cell interaction, depending on the affinity between the receptor and counter-receptor. It has been shown previously that Fc_ϵ receptors can redistribute to the contact area at which the specific ligand is presented on the apposing cell surface, liposomes, or latex beads (Michl et al., 1983; McCloskey and Poo, 1986). This contact-induced receptor redistribution occurs as a passive phenomenon and the strength of conjugation is found to correlate with the extent of Fc_ϵ receptor redistribution (McCloskey and Poo, 1986). Receptor redistribution may otherwise correlate with the colocalization of cytoskeletal elements, such as that of lymphocyte function-associated antigen 1 (LFA-1)[1] and fibronectin receptor with talin (Burridge, 1987; Kupfer et al., 1989).

Redistribution of adhesive receptors has been postulated to be important for efficient cell adhesion (reviewed in Singer and Kupfer, 1988). The ability of adhesive receptors to diffuse laterally is predicted to be critical for the formation of intercellular adhesion, and the adhesion strength should increase with time. These predictions have not been experimentally tested or quantified, however. Since the lateral mobility of cell surface proteins may differ by three orders of magnitude (10^{-8}–10^{-11} cm^2/s) and may be regulated by as-

1. *Abbreviations used in this paper*: FRAP, fluorescence recovery after photobleaching; LFA, lymphocyte function-associated antigen; OG, octyl-β-D-glucopyranoside; TM, transmembrane.

sociated membrane, submembrane, or extracellular components (Pollerberg et al., 1986; Wier and Edidin, 1986, 1988; Wade et al., 1989), the lateral mobility of adhesion molecules may play an important role in determining the strength and kinetics of cell adhesion.

In this study, we examine the effects of adhesion receptor mobility on CD2-mediated T cell adhesion. CD2 is expressed on human T lymphocytes. Its counter-receptor, lymphocyte function-associated antigen 3 (LFA-3), is a broadly distributed adhesion glycoprotein that exists in two membrane anchorage isoforms, one with a glycosyl-phosphatidylinositol (GPI) moiety and the other with a transmembrane (TM) polypeptide domain (Dustin et al., 1987*b*; Seed, 1987; Wallner et al., 1987). CD2/LFA-3 mediated adhesion is one of the pathways required for the interaction of T lymphocytes with antigen-presenting cells and target cells (Springer, 1990), and is involved in the interaction of thymocytes with thymic epithelial cells in the thymus (Denning et al., 1988; Yang et al., 1988). We have incorporated the two isoforms of LFA-3 into glass-supported planar membranes; in this system the GPI isoform is laterally mobile whereas the TM isoform is immobile. We applied controlled detachment forces to compare the relative adhesive strengths of Jurkat T lymphoma cells binding through CD2 to the mobile and immobile LFA-3 isoforms in planar membranes. Our results show that the ability of receptors to diffuse laterally enhances adhesion markedly, both by allowing accumulation of receptors in the cell contact area and by increasing the rate of bond formation.

Materials and Methods

mAbs and Cell Lines

The anti-LFA-3 mAb TS2/9 is a mouse IgG1 antibody (Sanchez-Madrid et al., 1982). It was affinity purified from ascites using protein A-coupled Sepharose (Sigma Chemical Co., St. Louis, MO). The Jurkat T lymphoma cell line was maintained in RPMI 1640 medium containing 10% FCS, 5 mM glutamine, and 50 μg/ml gentamycin. Jurkat cells in the log phase of growth (6–9 × 10^5 cells/ml) were used in experiments. As determined by flow cytometry, these cells express uniform levels of CD2 on the cell surface.

Purification of LFA-3

The affinity purification of LFA-3 has been described previously (Dustin et al., 1987*a*). The GPI isoform was purified from erythrocytes (Dustin et al., 1987*a*) and the TM isoform from the GPI anchor-deficient mutant JY B lymphoblastoid cell clone 33 (Hollander et al., 1988). LFA-3 was eluted in buffer containing 1% (wt/vol) octyl-β-D-glucopyranoside (OG) for the preparation of liposomes. Purified LFA-3 was quantified by Bradford's protein assay (Bio-Rad Laboratories, Richmond, CA).

Preparation of Liposomes

Unilamellar liposomes were prepared by the method of OG dialysis (Mimms et al., 1981). Egg PC (Avanti Polar Lipids, Pelham, AL) was diluted in chloroform, dried under an argon stream, and then placed under vacuum (20 μm) for 2 h. The lipid film was redissolved at 0.4 mM in 25 mM Tris-HCl (pH 8.0)/150 mM NaCl (TS) containing 2% (wt/vol) OG, and was mixed with an equal volume of known amounts of LFA-3 in TS/1% OG. Typically, 6 μg of the GPI isoform (mol wt 50–55 kD) and 7 μg of the TM isoform (mol wt 60–70 kD) of LFA-3 in a final volume of 1 ml yielded ~1,000 sites/μm^2 in the planar membranes, and the number of sites/μm^2 was linear with LFA-3 concentration in the range used in this report. The lipid/OG/LFA-3 mixture was dialyzed against TS at 4°C over 36 h with three buffer changes. The liposome suspension was stored at 4°C under argon to minimize lipid oxidation.

Preparation of Planar Membranes

Planar membranes were formed by fusion of liposomes in contact with a glass surface (hydrophilic support) (McConnell et al., 1986). Before use, all glass surfaces were boiled in detergent (Linbro 7× solution; Flow Laboratories, McLean, VA) for 15 min, rinsed extensively in deionized distilled water for at least 24 h, and then stored in ethanol. Liposomes were deposited as a droplet on a glass coverslip or slide at 22°C for 30 min. For fluorescence recover after photobleaching (FRAP) measurements, glass coverslips (no. 1 thickness; Fisher Scientific Instruments, Pittsburgh, PA) cut to 1 × 1-cm squares were placed onto 50-μl liposome droplets at the bottom of 24-well tissue culture plates. After incubation the coverslips were inverted under buffer to expose the membranes that were formed. For plate inversion assays, glass coverslips (5 mm diam, no. 1 thickness; Bellco, Vineland, NJ) were attached to the bottom of 96-well microtiter plates (Linbro Titertek; Flow Laboratories) by silicone household glue (General Electric Co., Waterford, NY) and 20-μl liposome droplets were placed on top. For laminar flow assays, glass slides (45 × 60 mm, no. 2 thickness; Corning Glass Works, Corning, VA) were placed in 10-cm petri dishes. A 20-μl liposome droplet was placed in a demarcated area (1 cm diam) and a glass coverslip (1 cm diam; Bellco, Vineland, NJ) was then used to cover the liposomes to reduce evaporation during the incubation time. After the planar membranes were formed, excess liposomes were removed by several changes of binding medium. Planar membranes were never exposed to air. Planar membranes thus formed were uniform and continuous as shown by incorporation of a fluorescent lipid analogue, NBD-PE, and examination under the interactive laser cytometer (ACAS 570; Meridian Instruments, Okemos, MI) (see FRAP below).

Determination of Site Densities by Radioimmunoassays

Radioimmunoassays were used to determine the site densities of LFA-3 in the upper leaflet of the planar membranes. For each batch of liposomes reconstituted with different quantities of LFA-3, planar membranes were formed on 5-mm-diam round glass coverslips glued to the bottom of 96-well microtiter plates in triplicate as described above. ^{125}I-labeled TS2/9 mAb (5 μCi/μg specific activity) was incubated with the membranes at a saturating concentration of 10 μg/ml in 50 μl binding medium (RPMI, 10% FCS, 25 mM Hepes, pH 7.4) at 4°C for 1 h. Unbound mAb was removed by 12 aspiration washes with ice-cold medium using a cut-off 21-gauge needle, such that the membranes were always covered by 50 μl of binding medium and were never exposed to air. Bound mAb was then removed by 0.1 M NaOH for gamma counting. Only the LFA-3 molecules incorporated in the upper leaflet of the membrane bilayer which are available for cell binding were quantified. For experiments using LFA-3 coated on plastic in the presence or absence of OG, 96-well microtiter plates were used as described above without glass coverslips. Proteins were coated at 4°C for 12 h, and other adsorption sites on plastic were blocked by incubation with TS containing 1% heat-treated (80°C, 20 min) bovine serum albumin (BSA) and 0.02% sodium azide at 22°C for 1 h. Radioimmunoassays were performed as described for planar membranes except that unbound mAb was removed by 8 aspiration washes using an uncut 21-gauge needle.

The site densities of LFA-3 were calculated based on a monovalent binding of the anti-LFA-3 mAb TS2/9 at its saturating concentration. Standard deviations of site densities determined were shown in x-axis error bars for plate inversion assays. For laminar flow assays, the nominal site densities of LFA-3 in planar membranes mentioned in the text and figures correspond to actual measured values in sites/squared microns as follows: for the GPI isoform, 1,500: 1,452 ± 126; 1,000: 994 ± 31; 250: 259 ± 9; 50: 59 ± 21; 25: 27 ± 4; for the TM isoform, 1,500: 1,491 ± 13; 1,000: 1,069 ± 46; 350: 370 ± 35; 50: 56 ± 7.

Scatchard Analysis of the TS2/9 mAb Binding to LFA-3 Isoforms

LFA-3 isoforms were coated on plastic in 96-well microtiter plates in the presence of 0.1% OG as described above, and other adsorption sites on plastic were blocked by incubating with TS containing 1% heat-treated BSA and 0.02% sodium azide at 37°C for 4 h, followed by 4°C for 16 h. ^{125}I-labeled TS2/9 mAb was added in a serial dilution of 0.09 to 10 μg/ml in binding medium and was incubated at 4°C for 16 h before washing. Nonspecific binding of ^{125}I-labeled TS2/9 was determined in the presence of 100-fold higher concentrations of unlabeled mAb and was subtracted from the total counts bound. The derived specific binding was used for Scatchard analysis.

Scatchard analysis was also carried out on lipid bilayers but there was more scatter in the values, probably due to the glue used for securing the glass coverslips. However, K_d values were similar for the TM and GPI isoforms of LFA-3 in lipid bilayers (8.8 and 6.3 × 10^{-9} M).

Fluorescence Recovery after Photobleaching (FRAP)

Conjugation of TS2/9 mAb to fluorescein isothiocyanate (FITC) (Sigma Chemical Co.) was carried out using standard methods (Goldman, 1968), except that FITC was dissolved in dimethyl formamide instead of DMSO. Free uncoupled FITC was removed by G25 Sephadex gel filtration, and protein aggregates were removed by centrifugation at 20,000 *g* for 20 min. Planar membranes containing LFA-3 were incubated with FITC-conjugated TS2/9 at 22°C for 30 min and then washed. The washed membranes on glass coverslips were transferred to a glass slide under buffer. FRAP measurements were taken within 120 min of slide preparation. N-(-7-nitrobenz-2-oxa-1,3-diazol-4-yl)dipalmitoyl-L-α-phosphatidylethanolamine (NBD-PE) (Avanti Polar Lipids) was incorporated into egg PC liposomes (2 mol%) to determine the lateral mobility of phospholipids in the planar membranes.

FRAP measurements were performed on a Meridian ACAS 570 interactive laser cytometer at 22°C and all data were analyzed with a modified nonlinear least squares algorithm (Bevington, 1969; Axelrod et al., 1976; Golan et al., 1986). The operational parameters for the Meridian cytometer are described elsewhere (Ferguson, L. M., et al., manuscript in preparation). Measuring intensities and amplification settings were adjusted such that the signal from unlabeled bilayers was <1% of the fluorescence signal from the labeled bilayers. Fluorescence recovery of NBD-PE, the GPI, and TM isoforms of LFA-3 was typically monitored for 20, 40, and 85 s, respectively.

Determination of Isoelectric Points

LFA-3 proteins were separated by isoelectric focusing in tube gels (Bio-Rad Laboratories) using pI 2.5–5.0 ampholytes (Pharmacia, Uppsala, Sweden). The pI gradient was determined by slicing the tube gels into 5-mm-long pieces, equilibrating with degased distilled water and measuring the pH (O'Farrell, 1975). Proteins were further separated in the second dimension by SDS-PAGE before silver staining.

Plate Inversion Assays

LFA-3 isoforms reconstituted in planar membranes or coated on plastic were prepared in 96-well microtiter plates. Jurkat cells were labeled by 2',7'-bis-(2-carboxyethyl)-5(and-6)carboxyfluorescein (acetoxymethyl ester) (Molecular Probes, Eugene, OR) in binding medium (RPMI, 10% FCS, 25 mM Hepes, pH 7.4) at 22°C for 20 min and washed. Labeled cells (10^5/well) were allowed to settle onto substrates at 22°C for 1 h. The plates were then inverted for 1 h in a tank filled with PBS (pH 7.4) containing 0.1% BSA to detach unbound cells. Bound cells were measured by a fluorescence plate reader (Pandex, Baxter Healthcare Corp., Mundelein, IL). Percentages of cells bound were determined by dividing the fluorescence of bound cells by that of total input cells.

Laminar Flow Adhesion Assays

Planar membranes containing either of the LFA-3 isoforms were formed in a demarcated area on a glass slide (45 × 60 mm). The glass slide was assembled in a parallel plate laminar flow chamber (300 μm gap thickness) in which uniform wall shear stress is produced (Lawrence et al., 1987). Jurkat cells (3 × 10^6 cells/ml) were introduced into the chamber through an injection port and allowed to settle. All cells came in contact with the membrane within 40 s, as indicated by their entry into the same focal plane. Controlled flow was applied following incubation periods ranging from 3 to 60 min. The initial shear force was 0.5 dyne/cm^2; this force was increased every 20 s to a maximum of 16 dyne/cm^2. Plots of detachment with time showed two rates; with 95% of the cells that ultimately detached being removed within 20 s and the remaining 5% over the next 140 s. When cell contact times were limited to 5 min or less, shear stresses were not sequentially incremented. Instead, each determination of binding at a given shear stress was an independent measurement. Each membrane was used for no more than three cell injections to minimize the effect of possible loss of LFA-3 or disruption of the membrane. A minimum of 10 microscopic fields were scanned before and after starting flow to obtain the percentage of Jurkat cells bound. All experiments were recorded on videotapes.

*Table I. Lateral Mobility of LFA-3 Isoforms and a Phospholipid Analogue in Planar Membranes**

Probe	Target molecules‡	Diffusion coefficient	Fractional recovery	Measurements
		(10^{-9} cm^2/s)	(%)	*n*
FITC-TS2/9	TM LFA-3	§	<10	76
FITC-TS2/9	GPI LFA-3	2.3 ± 1.0‖	73 ± 13‖	55
NBD-PE	NBD-PE	14 ± 5	94 ± 7	56

* Data were taken from planar membranes containing either LFA-3 isoform at surface densities of 50–250 sites/μm^2.
‡ Although the higher site densities of GPI-anchored LFA-3 used in the cell binding experiments were not subjected to FRAP measurements, the effect of high site densities (such as 1,500 sites/μm^2) on lateral diffusion was probably negligible since <1% of the membrane surface area was occupied by LFA-3 molecules (Jacobson et al., 1987).
§ Diffusion coefficient could not be determined because fractional recovery was <20%.
‖ The lateral diffusion of GPI-anchored LFA-3 was completely abolished by cross-linking this isoform with unconjugated TS2/9 mAb followed by FITC-conjugated goat anti-mouse Ig (data not shown).

Results

The GPI Isoform of LFA-3 Is Mobile and the TM Isoform Is Immobile in Planar Membranes

FITC-conjugated TS2/9 mAb was used to label the GPI and TM isoforms of LFA-3 incorporated in the upper leaflet of the egg PC planar membranes. The lateral mobilities of these isoforms were determined by FRAP at 22°C (Table I). The TM isoform was essentially immobile, with <10% fractional recovery. Immobilization of the TM isoform in the membrane bilayer was probably due to interaction of the cytoplasmic domain (12 amino acid residues) with the glass surface, as previously postulated for other transmembrane proteins (Brian and McConnell, 1984; Watts et al., 1984; McConnell et al., 1986). The lack of mobility of the TM-anchored LFA-3 also indicated that the planar membranes were uniformly composed of a single lipid bilayer since exposed TM-anchored proteins would not be able to interact with the glass substrate in multilamellar bilayers and would thereby be mobile.

The lateral diffusion coefficient of the GPI isoform was 2.3 ± 1.0 × 10^{-9} cm^2/s with 73 ± 13% fractional recovery. NBD-PE, a fluorescent phospholipid analog incorporated into the egg PC planar membrane, had a diffusion coefficient of 1.4 ± 0.5 × 10^{-8} cm^2/s with 94 ± 7% fractional recovery. The mobility parameters of GPI-anchored LFA-3 and NBD-PE are similar to those reported for other GPI-anchored proteins on cell surfaces and phospholipid analogs in liposomes, glass-supported lipid membranes, and cell membranes, respectively (Watts et al., 1984; Tamm and McConnell, 1985; Ishihara et al., 1987; Noda et al., 1987; Tamm, 1988). The data also suggest that the diffusion of GPI-anchored LFA-3 in the upper leaflet of membrane bilayers was not affected by the LFA-3 molecules incorporated in the lower leaflet which were likely immobilized due to interaction with the glass substrate via the extracellular domains.

The Two LFA-3 Isoforms Have Similar Levels of Negative Charge

Obtaining homogeneous GPI and TM isoforms of LFA-3 re-

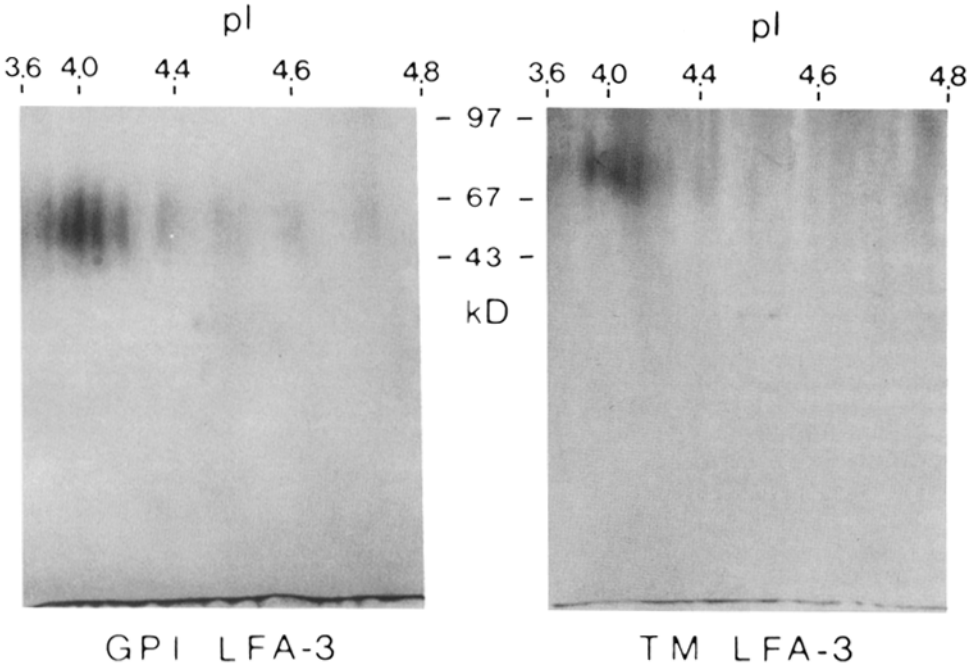

Figure 1. Determination of isoelectric points of the GPI and TM isoforms of LFA-3. Purified LFA-3 isoforms were analyzed in the first dimension by isoelectric focusing and in the second dimension by SDS-10% PAGE under nonreducing conditions. Protein separation was visualized by silver staining. The streaking pattern of silver staining from the top of the gel analyzing TM-anchored LFA-3 is due to reaction with a component of the agarose used to secure the tube gel to the slab gel.

quired purification from different cell types. The GPI isoform was isolated from erythrocytes and is 50–55 kD in molecular weight. The TM isoform was isolated from a mutant B lymphoblastoid line, JY clone 33, that is deficient in GPI-anchored proteins and is 60–70 kD in molecular weight (Dustin et al., 1987*a*; Hollander et al., 1988). The variation in molecular weight of each isoform is due to glycosylation differences (Dustin et al., 1987*b*). Although the carbohydrate portion of LFA-3 is not required for CD2 binding (Chan and Springer, unpublished observation), sialic acids on glycoproteins contribute negative charge which may result in significant repulsive forces between molecules. To determine whether the difference in glycosylation of the two isoforms results in different levels of negative charge, the isoelectric points (pI) of the two LFA-3 isoforms were measured (Fig. 1). Both isoforms resolved around pI 4.0 at the acidic end. They should therefore exert similar repulsive effects upon interaction with CD2.

The Two LFA-3 Isoforms Present Equivalent Binding Affinities for an mAb

To further compare the binding properties of the two isoforms of LFA-3 and validate the site density determination of the isoforms (see discussion below), the affinities of the isoforms for the TS2/9 mAb were determined. mAb binding to either the GPI or TM isoform reached saturation between 2 and 3 μg/ml (Fig. 2 *a*). Scatchard plots showed similar dissociation constants (K_d), 1.8×10^{-9} M for the GPI isoform and 2.2×10^{-9} M for the TM isoform (Fig. 2 *b*).

Jurkat Cells Bind More Efficiently to Lipid Bilayers Containing Mobile than to Immobile LFA-3

GPI and TM isoforms of LFA-3 were reconstituted in planar membranes at site densities of 10–1,500 molecules/μm^2 and used to compare the efficiency of cell binding to mobile and immobile adhesion receptors. Most of the experiments were performed at 22°C, a temperature where cytotoxic T lymphocytes conjugate with target cells (Balk and Mescher, 1981). The CD2/LFA-3 interaction is one of the two major adhesion pathways in conjugation between antigen-specific T lymphocytes and target cells (Springer, 1990). Jurkat T lymphoma cells were allowed to bind to the planar membranes at 22°C for 1 h and unbound cells were removed by gravity in plate inversion assays. Jurkat cells adhered to planar bilayers containing LFA-3 (Fig. 3 *a*) but not to planar membranes lacking LFA-3 (1.1 ± 0.6%) or to glass coverslips alone. Jurkat cells bound to the GPI isoform at site densities one order of magnitude lower than those required for binding to the TM isoform.

Jurkat Cells Bind Similarly to GPI and TM Isoforms of LFA-3 Immobilized on Plastic

To test whether the differences in Jurkat cell binding efficiency were due to differences in the lateral mobility of the GPI and TM isoforms in planar membranes, we measured cell binding to both isoforms immobilized on plastic (Fig. 3, *b* and *c*). When the LFA-3 proteins were coated on plastic in buffer containing 0.1% octyl-β-D-glucopyranoside (OG), below the critical micelle concentration of this detergent (0.7%), Jurkat cell binding to the GPI isoform (Fig. 3 *b*) decreased to a level comparable to that for binding to the TM isoform in planar membranes (Fig. 3 *a*). The site density at 50% cell binding was twofold lower for the GPI isoform than the TM isoform (Fig. 3 *b*). To eliminate the potential effects of different cluster sizes of the immobilized LFA-3 isoforms (Dustin et al., 1989), LFA-3 was coated in monomeric form on plastic in buffer containing 0.8% OG, above the critical micelle concentration of OG. Under these conditions, Jurkat cell binding to the GPI isoform decreased further, and became twofold less efficient than binding to the TM isoform (Fig. 3 *c*). Cell binding to LFA-3 at higher site densities could not be tested because the adsorption of LFA-3 to plastic was less efficient in OG concentrations above the critical micelle concentration. Dispersion of LFA-3 mono-

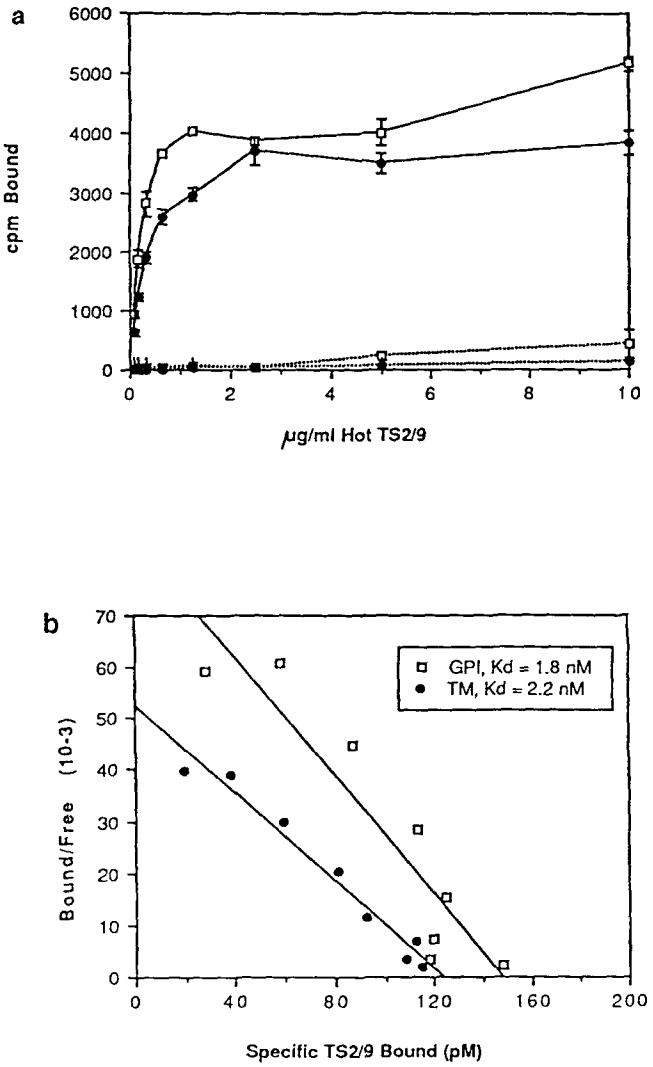

Figure 2. Determination of the affinity of TS2/9 mAb binding to both LFA-3 isoforms. (*a*) The GPI (*open squares*) and TM (*solid circles*) isoforms of LFA-3 were coated on plastic in buffer containing 0.1% OG and were subjected to saturation binding of ^{125}I-labeled TS2/9 mAb in the presence (*broken lines*) or absence (*solid lines*) of 100-fold excess unlabeled mAb. Standard deviations were derived from triplicate samples and experiments were performed twice with similar results. (*b*) Specific binding of ^{125}I-labeled TS2/9 mAb to each of the GPI and TM isoforms was used in Scatchard analysis to determine their dissociation constants (K_d).

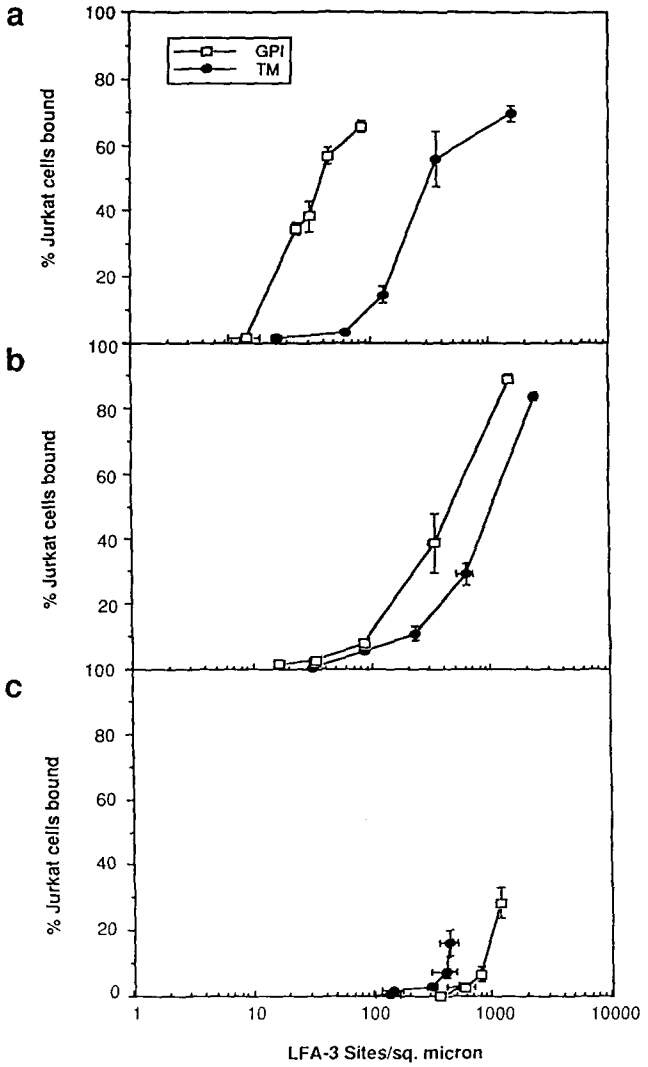

Figure 3. Binding of Jurkat cells to LFA-3 isoforms reconstituted in (*a*) planar membranes or coated on plastic in (*b*) 0.1% OG or (*c*) 0.8% OG, below and above the critical micelle concentration, respectively. Jurkat cells were allowed to bind to LFA-3 for 1 h before unbound cells were removed by plate inversion. Cells did not adhere to blank planar membranes or plastic. Standard deviations were derived from triplicate samples. Experiments were performed thrice with similar results.

mers on plastic resulted in low cell binding levels for both isoforms. These data together showed that immobilization of the two LFA-3 isoforms led to significant decreases in both cell adhesion efficiency and the differences in binding between the isoforms.

Cell Adhesion to the GPI Isoform of LFA-3 Increases with Site Density and Time

A laminar flow system was used to generate controlled shear force capable of detaching cells bound to the LFA-3 isoforms in planar membranes. In these laminar flow assays, Jurkat cells were allowed to settle for 20 min onto planar membranes containing the GPI isoform at 25–1,500 sites/μm^2. A shear force of 0.5 to 16 dyne/cm^2 was then applied for 20 s. The increase in the Jurkat cell adhesion strength with increasing LFA-3 site density was illustrated by the higher percentage of cells bound at a given shear stress (Fig. 4 *a*) as well as the increase in shear stress required to detach 50% of bound cells (Fig. 4 *c*). Jurkat cell binding to the TM isoform increased similarly with site density (Fig. 4 *b*), consistent with results from the plate inversion assays described above (Fig. 3 *a*). However, half-maximal shear stress resistance was developed at a fivefold lower site density for the GPI isoform (Fig. 4 *c*). Jurkat cell binding was more efficient to the GPI isoform than to the TM isoform at lower site densities (<350 sites/μm^2), whereas the strength of binding to both isoforms was equivalent at higher site densities (1,000–1,500 sites/μm^2).

To study the effects of incubation time on the development of cell adhesion strength, laminar flow assays were per-

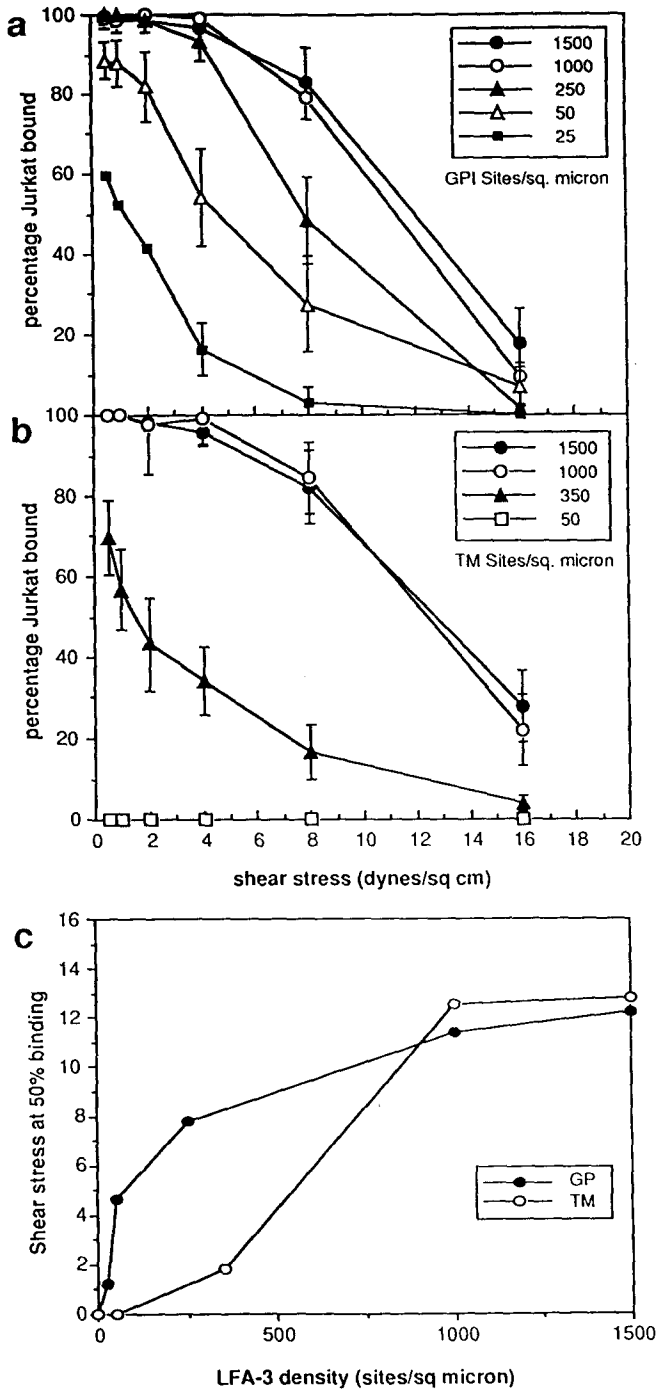

Figure 4. Shear stress resistance of Jurkat cells bound to the (*a*) GPI and (*b*)TM isoforms of LFA-3 in planar membranes at different site densities. Jurkat cells were allowed to bind to LFA-3 at 22°C for 20 min before fixed levels of shear force were applied. Cells did not adhere to blank planar membranes. Percentages of cell binding at different shear forces were mean values of two to five experiments; standard errors are indicated in bars. (*c*) The shear stress at the 50% level of Jurkat cell binding to both isoforms was plotted against the site densities of the GPI and TM isoforms.

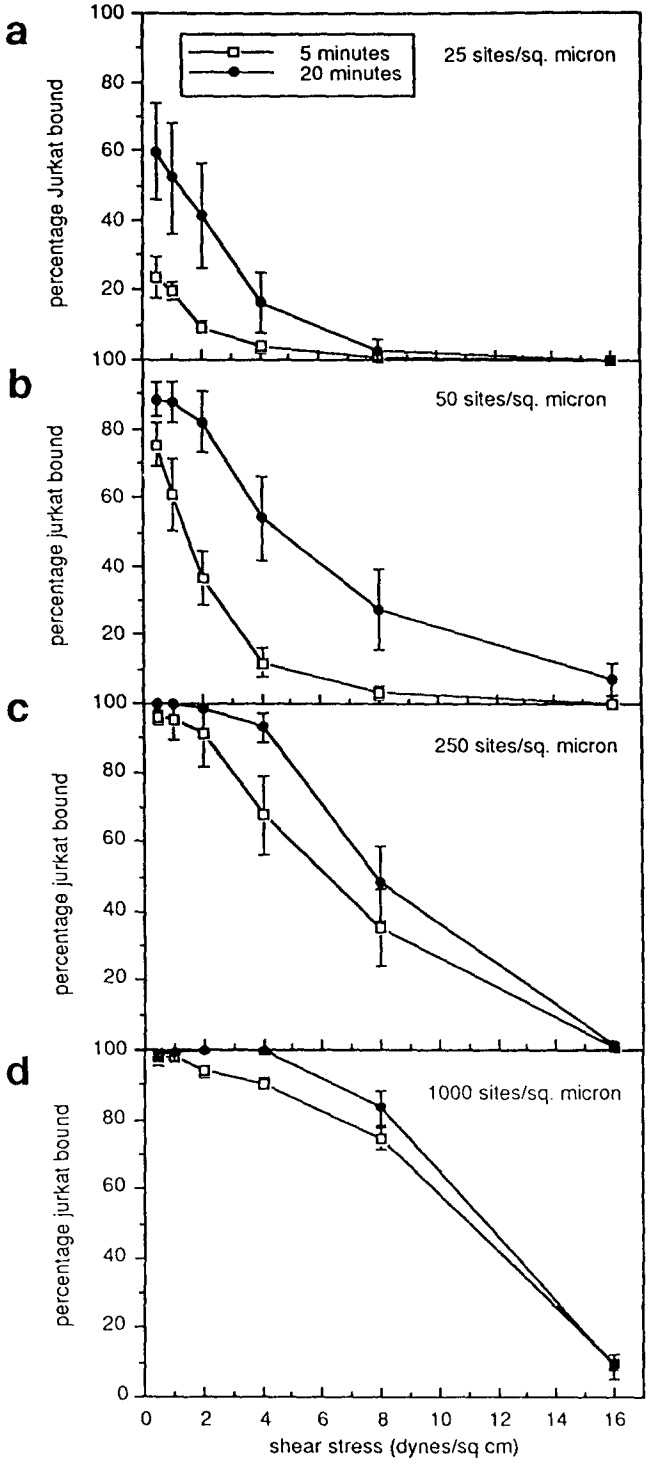

Figure 5. Shear stress resistance of Jurkat cells bound to the GPI isoform of LFA-3 in planar membranes after different contact times. Planar membranes containing GPI-anchored LFA-3 at (*a*) 25, (*b*) 50, (*c*) 250, and (*d*) 1,000 sites/μm^2 were subjected to Jurkat cell binding experiments as described in Fig. 4, except that the shear force was applied after either 5 or 20 min of contact. Percentages of cell binding were mean values of two to five experiments; standard errors are shown in bars.

formed using the GPI isoform of LFA-3 in planar membranes and Jurkat cell contact times of 5 and 20 min (Fig. 5). Cell binding increased with time at site densities of 25 and 50 sites/μm^2 (Fig. 5, *a* and *b*), whereas the increase at the site density of 250 sites/μm^2 was small (Fig. 5 *c*). At 1,000

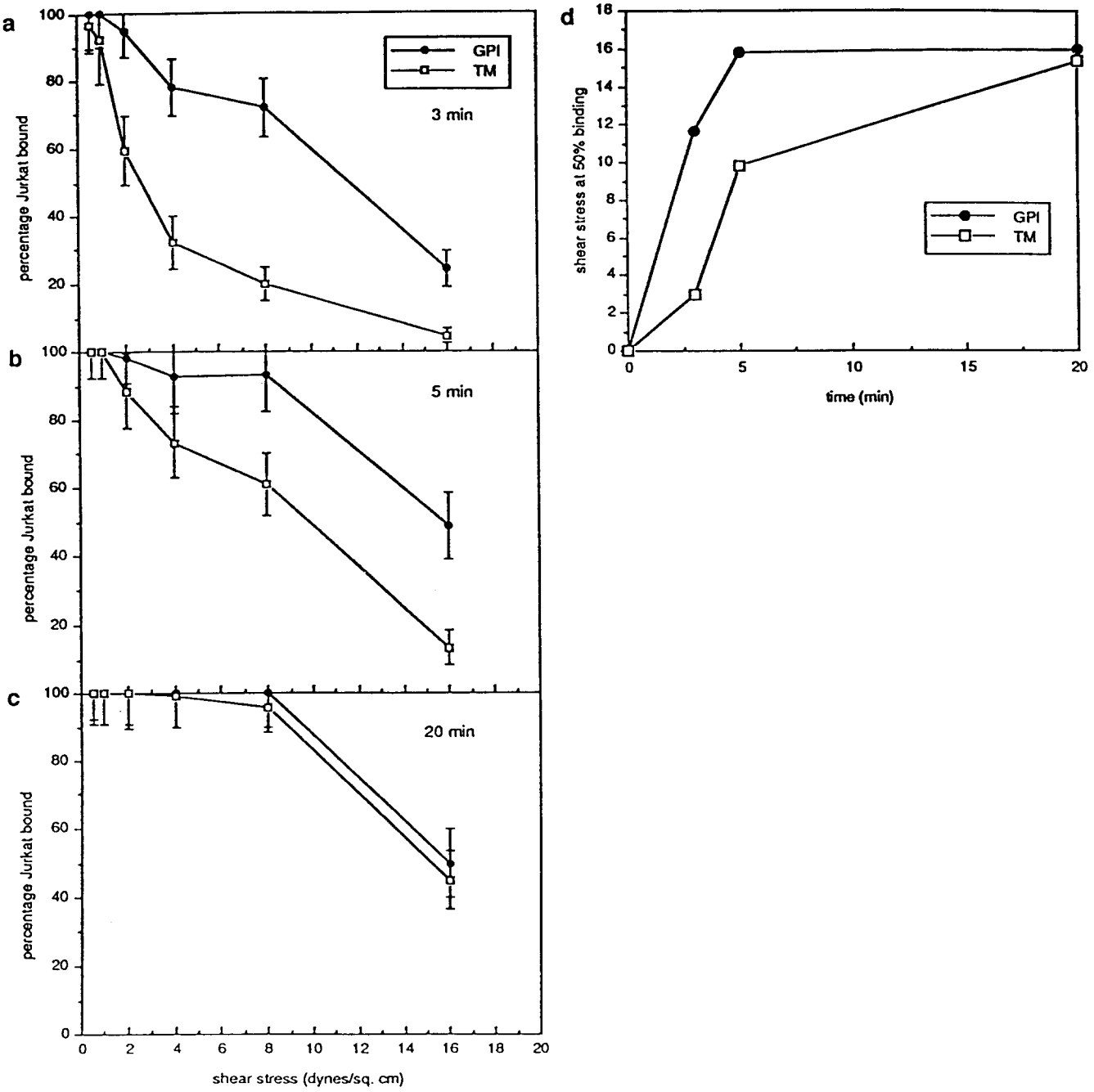

Figure 6. Shear stress resistance of Jurkat cells bound to LFA-3 isoforms at high site densities in planar membranes after different contact times. Jurkat cells were allowed to bind at 22°C to membranes containing the GPI or TM isoforms at 1,500 sites/μm^2 for (*a*) 3, (*b*) 5, or (*c*) 20 min before detachment by shear force as described in Fig. 4. Percentages of cell binding were mean values of two experiments; standard errors are shown in bars. (*d*) The shear stress at the 50% level of Jurkat cell binding to both isoforms was plotted against contact time.

sites/μm^2 the increase in cell binding with time was insignificant, indicating that cell adhesion strength had reached a plateau by 5 min (Fig. 5 *d*).

The dependence of cell adhesion strength on the density of the GPI isoform of LFA-3 and the length of contact time was significant in the range of 25–50 sites/μm^2 and 5–20 min of cell contact, respectively. Since an average Jurkat cell is 14 μm in diameter (Ferguson et al., unpublished observation) and has a mean CD2 cell surface expression of 8×10^4 molecules per cell (Plunkett and Springer, 1986; based on monovalent binding of mAb at a saturating concentration), the CD2 density on an average Jurkat cell is estimated to be ~125 sites/μm^2. The effect of LFA-3 site density and contact time on cell binding suggests that, at LFA-3 site densities less than the surface density of CD2 on Jurkat cells, the GPI isoform accumulates gradually within 20 min into the contact area.

At High Site Densities of LFA-3 Jurkat Cell Binding to the GPI and TM Isoforms Is Equivalent at Equilibrium but has Different Kinetics of Adherence Strengthening

The observations that Jurkat cell binding to the GPI isoform

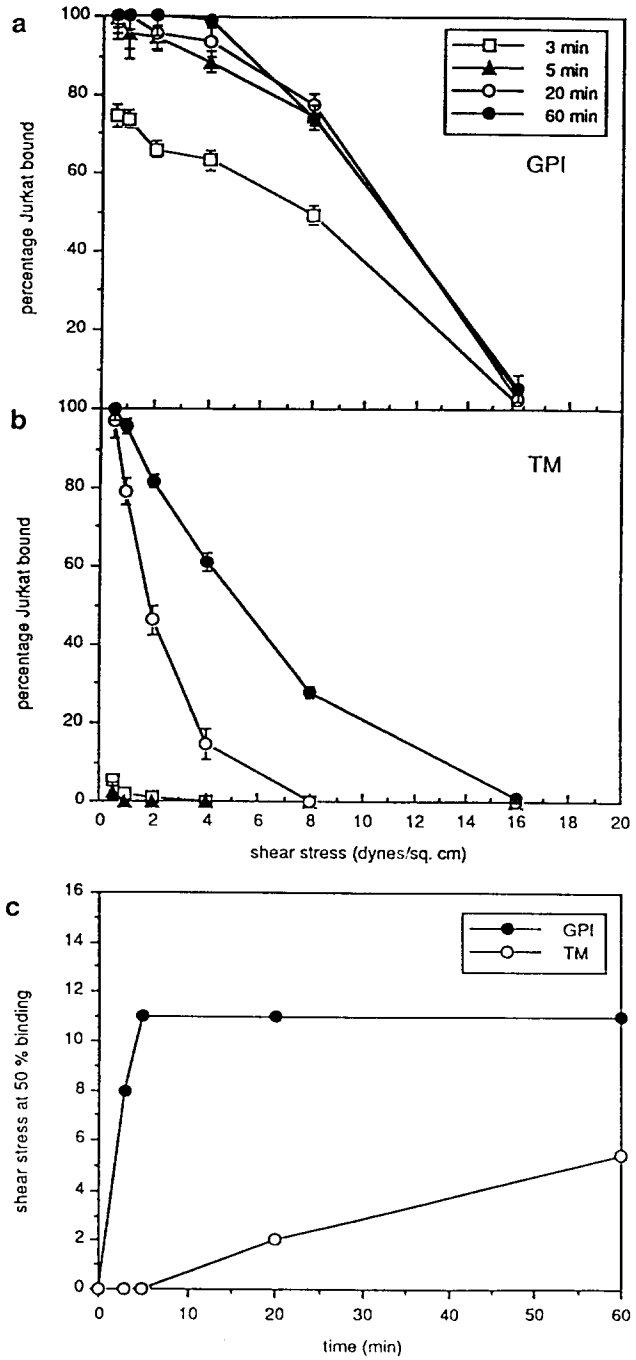

Figure 7. Effects of lower temperature on Jurkat cell binding to LFA-3 isoforms at high densities in planar membranes. Jurkat cells were allowed to bind to the (*a*) GPI or (*b*) TM isoforms at 1,500 sites/μm^2 at 5°C for 3, 5, 20, or 60 min before detachment by shear force as described in Figs. 4 and 5. Error bars represent standard deviations of cell binding in 10–20 video fields. (*c*) The shear stress at the 50% level of Jurkat cell binding to both isoforms was plotted against contact time.

of LFA-3 at a density of 1,000 sites/μm^2 reached maximal adhesion strength within 5 min, and that Jurkat cells bound equally well to the GPI and TM isoforms at densities <1,000 sites/μm^2 and contact time of 20 min, suggested that the LFA-3 on the substrate saturated the CD2 on Jurkat cells and the accumulation of GPI-anchored LFA-3 into the cell contact area did not occur or least was not be critical at such high site densities. To test whether the kinetics of adhesion strengthening differed, we used laminar flow assays to measure development of shear resistance at a LFA-3 density of 1,500 sites/μm^2 after 3, 5, and 20 min of contact (Fig. 6, *a–c*). As shown by the shear stress required to detach 50% of bound cells, the development of strong cell binding to the GPI isoform was faster than that to the TM isoform (Fig. 6 *d*). Cell binding to the GPI isoform reached maximum strength between 3 and 5 min, whereas the binding to the TM isoform reached maximum between 5 and 20 min. The time required to reach half-maximal shear stress resistance was 2 and 4.5 min for the GPI and TM isoform, respectively. As found in the experiments described above, the cell binding strength was equivalent for both isoforms after 20 min of contact time (Fig. 6, *c* and *d*).

We further assessed the importance of the mobility of CD2 in the cell contact area for the kinetics of adhesion strengthening. To focus on the effects of CD2 mobility and redistribution, laminar flow assays were carried out after 3, 5, 20, and 60 min of cell contact at 5°C (Fig. 7, *a* and *b*). The lateral diffusion of lipids in the egg PC planar membranes is not reduced at this temperature (Tamm and McConnell, 1985), whereas the diffusion of glycoproteins in plasma membranes is significantly diminished (~50-fold for the major histocompatibility complex (MHC) molecules) (Petit and Edidin, 1974). The rate of Jurkat cell adhesion strengthening was reduced 16-fold for the TM isoform of LFA-3 under these conditions; development of a half-maximal shear stress resistance of 5.5 dyne/cm^2 took 60 min at 5°C (Fig. 7 *c* compared with Fig. 6 *d*). Maximum Jurkat cell binding to the GPI isoform was reached in 5 min at both temperatures, and half-maximal shear stress resistance of 5.5 dyne/cm^2 was reached in 1.9 min at 5°C and 1.4 min at 22°C. The adhesive strength at equilibrium for GPI-anchored LFA-3 was lower at 5°C, 11 dyne/cm^2 compared with 16 dynes/cm^2 at 22°C.

Discussion

We have used a glass-supported planar membrane system reconstituted with mobile GPI or immobile TM isoforms of the adhesion molecule LFA-3 to examine the influence of receptor lateral mobility on the strength of cell adhesion. Both equilibrium and kinetic measurements of cell adhesion showed that the ability of membrane-bound receptors to diffuse laterally enhanced adhesion strength markedly. To quantify the rate and extent of adhesive bond formation, the adhesive strength of CD2-bearing Jurkat cells was measured at defined levels of detachment force. At low densities of the GPI isoform of LFA-3 (25–50 sites/μm^2), the strength of cell adhesion was likely determined by the accumulation of mobile GPI-anchored LFA-3 into the cell contact area, thus increasing the number of bonds formed. Low densities of the immobile TM isoform of LFA-3, in contrast, mediated only weak adhesion. At the highest site density (1,500 sites/μm^2), both the GPI and TM isoforms of LFA-3 mediated strong adhesion after long periods of cell contact. Measurements at shorter cell contact times showed a greatly enhanced rate of adhesion strengthening for the GPI isoform, however, implying a higher rate of CD2/LFA-3 bond formation for the GPI isoform than for the TM isoform. Experiments at 5°C demonstrated the importance of CD2 mobility in strengthening

cell adhesion to lipid bilayers containing the immobile TM isoform.

In both plate inversion and laminar flow assays, Jurkat cells bound to the mobile GPI isoform of LFA-3 at densities lower by an order of magnitude than the densities of immobile TM isoform required for binding. Comparison between the isoelectric points of the two LFA-3 isoforms suggests no gross charge differences that could account for a 10-fold difference in the affinity of the isoforms for CD2. Previous estimates of the avidity of purified CD2 for either the GPI isoform on erythrocytes or a mixture of GPI and TM isoforms on JY cells were similar (Selvaraj et al., 1987*a*,*b*; Sayre et al., 1989). Furthermore, the GPI and TM isoforms have equivalent affinities for TS2/9 mAb, resulting in equivalent estimation of site densities. Finally, immobilization of the GPI isoform on plastic reduced its binding efficiency to a level comparable to that of the TM isoform. Together, these observations strongly suggest that the differences in Jurkat cell binding efficiency to the two LFA-3 isoforms in planar membranes were due to differences in LFA-3 mobility and not to differences in the affinity of the isoforms for CD2 or the determination of site density.

Development of adhesive strength between Jurkat cells and planar membranes containing the GPI isoform of LFA-3 showed significant time dependence at LFA-3 site densities lower than the surface density of CD2 on Jurkat cells (125 sites/μm^2). This suggests that when Jurkat cells make contact with the planar membranes and LFA-3 becomes ligated to CD2 in the contact area, net diffusion of free LFA-3 into the contact area occurs, allowing further bond formation and increased concentration of total LFA-3 in the contact area. Based on the measured diffusion coefficient, the root mean square displacement of freely diffusive GPI-anchored LFA-3 is 16.6 μm in 5 min (Sheetz et al., 1990). Since the average diameter of a Jurkat cell is 14 μm, the rapid mobility of GPI-anchored LFA-3 makes a mechanism in which the LFA-3 concentration increases in the contact area due to accumulation of mobile LFA-3 physically plausible. Indeed, we found that the adhesive strength of Jurkat cell binding to the GPI isoform of LFA-3 increases significantly between 5 and 20 min. In this model of adhesion strengthening then, at low densities of GPI-anchored LFA-3 the reaction between CD2 and LFA-3 is initially limited by the density of LFA-3. To bind a substantial percentage of CD2 molecules, accumulation of LFA-3 into the contact area is required. Diffusion-dependent entrapment of mobile ligands would be predicted to ultimately equilibrate at a level that depends on the CD2/LFA-3 affinity, the CD2 density, and the initial LFA-3 density.

Consistent with this model of receptor accumulation, strong Jurkat cell binding required less cell contact time at densities of GPI-anchored LFA-3 that were significantly greater than the density of CD2 on Jurkat cells. Furthermore, at the highest LFA-3 density of 1,500 sites/μm^2, the strength of Jurkat cell binding after 20 min of cell contact was equivalent for the TM and the GPI isoforms. These observations suggest that, at such high LFA-3 densities, most of the available CD2 molecules in the cell contact area were ligated and little accumulation of the GPI-anchored LFA-3 might take place. Therefore, the number of adhesive bonds formed with the TM and GPI isoforms is approximately the same at equilibrium. Jurkat cell binding to the GPI isoform reached maximum strength in significantly less time (5 min) than binding to the TM isoform (20 min). It is likely that, even at high site densities, the mobility of GPI-anchored LFA-3 results in a higher rate of molecule encounters with CD2 and thus a higher initial rate of bond formation compared to the immobile TM isoform.

The rate of CD2 ligation to the TM isoform of LFA-3 appears to be limited by the lateral mobility of CD2 on Jurkat cells. The diffusion coefficient of CD2 on Jurkat cells is not known, but its large cytoplasmic domain rich in basic amino acid residues (Sewell et al., 1986; Sayre et al., 1987; Seed and Aruffo, 1987) suggests that it may be associated with cytoskeletal elements and may therefore by less mobile than the GPI isoform of LFA-3 in planar membranes. Disruption of actin filaments with cytochalasin B enhanced Jurkat cell binding to planar membranes containing the GPI isoform of LFA-3, possibly because the mobility of CD2 was increased (Chan, P.-Y., and T. Springer, unpublished observation). Data from laminar flow assays performed at 5°C further support this hypothesis. Under low temperature conditions, TM-anchored receptors on nucleated cells have been shown to diffuse more slowly than at ambient temperatures (Petit and Edidin, 1974; Jacobson et al., 1984). The mobility of the MHC molecules on lymphocytes was reduced by 50-fold from 22°C to 5°C and that of CD44 on fibroblasts was decreased by 2-fold from 24°C to 9°C. The reduction in receptor mobility may be partly attributed to the reduction in membrane fluidity as well as partly to the decrease in random and transient cytoskeletal-directed translocation of membrane proteins, which is energy dependent (Kucik et al., 1989). On the other hand, the lateral mobility of lipids in planar membranes was not reduced (Tamm and McConnell, 1985), so the mobility predicted for the GPI-anchored LFA-3 at 4°C is comparable to 22°C. Binding of CD2 on Jurkat cells to the immobile TM isoform of LFA-3 in planar membranes was drastically diminished compared with the binding at 22°C. Binding to the mobile GPI isoform showed little temperature dependence, presumably because the high diffusivity of this species in planar membranes ($2.3 \pm 1.0 \times 10^{-9}$ cm^2/s compared with CD2 in the order of 10^{-10}–10^{-11} cm^2/s) dominated the cell binding kinetics. It thus appears that the ability of CD2 to diffuse laterally regulates the formation of adhesive bonds with the immobile TM isoform of LFA-3, but has little effect on bond formation with the mobile GPI isoform. Since <1% of the planar membrane surface is occupied by LFA-3 at 1,500 sites/μm^2, lateral diffusion of CD2 or LFA-3 is required for efficient bond formation. Whereas the GPI isoform can readily diffuse and encounter CD2 molecules to form bonds, CD2 must be able to diffuse to form bonds with the immobile TM isoform.

Assuming that bond formation between CD2 and LFA-3 can be represented as a bimolecular reaction between species A (CD2) and B (LFA-3) to form C (CD2/LFA-3), one can estimate the effect of lateral diffusivity on the bond formation rate (Bell, 1978). The reaction is modeled as taking place in two consecutive and reversible steps:

$$A + B \underset{d_-}{\overset{d_+}{\rightleftharpoons}} (AB) \underset{r_-}{\overset{r_+}{\rightleftharpoons}} C$$

Encounter complex formation — Intrinsic reaction

First, the two receptors must diffuse into sufficiently close proximity to permit formation of a bond, i.e., they need to form an encounter complex (AB). The equilibrium constant for encounter complex formation in this model is directly proportional to the ratio of the forward and reverse rate constants d_+ and d_-, which in turn depend on the diffusivities of A and B. At this stage, the encounter complex can either dissociate or form a bond. The equilibrium constant for the second step of the bond formation is not diffusion-dependent and is determined by the ratio of forward and reverse rate constants (r_+/r_-) of the intrinsic reaction. Since the extracellular domains of the two isoforms are identical, the rate constants of the intrinsic reaction should be the same for the two isoforms of LFA-3 once an encounter complex has formed. Assuming that the diffusion coefficient of CD2 is $\sim 10^{-10}$–10^{-11} cm^2/s (Jacobson et al., 1987), the initial rate of formation of an encounter complex with the GPI isoform of LFA-3 is estimated to be two to three orders of magnitude higher than that with the TM isoform (2×10^{13} collisions cm^{-2}s^{-1} for the GPI isoform versus 1×10^{10}–1×10^{11} collisions cm^{-2}s^{-1} for the TM isoform, assuming a LFA-3 density of 1,500 sites/μm^2). The effect of collision rate on the overall bond formation rate depends on the magnitudes of the intrinsic reaction rate constants (r_+ and r_-). Under circumstances where the forward rate of the intrinsic reaction is very high (i.e., not a limiting factor) compared with the reverse rate of the encounter complex formation, the rate of the overall bond formation or the kinetics of approaching equilibrium is controlled by the diffusivities of A and B (Bell, 1978). The ability of the GPI-anchored LFA-3 to diffuse laterally will result in a higher initial bond formation rate and a more rapid approach to equilibrium for this isoform than for the immobile TM isoform. In addition, the model predicts that Jurkat cell adhesion to the TM isoform should display a stronger dependence on the diffusivity of CD2 than adhesion to the GPI isoform, consistent with the temperature effect observed with binding.

We have studied the CD2/LFA-3 interaction, one of two major adhesion pathways involved in the conjugation between antigen specific T lymphocytes and target cells (Springer, 1990). We used an in vitro planar membrane system to compare cell binding to mobile and immobile isoforms of LFA-3 of known diffusion coefficients. Koyasu et al. (1990) have earlier demonstrated redistribution of CD2 to the site of adhesion with LFA-3-bearing target cells, suggesting that the ability of these adhesion receptors to diffuse laterally is physiologically relevant to T cell interactions. A distinct mechanism for adhesion strengthening has been illustrated by the increased avidity of LFA-1 for ICAM-1 that is triggered by the T cell antigen receptor complex (Dustin and Springer, 1989). In this study of LFA-3 and CD2, we have demonstrated another mechanism of cell adhesion strengthening, which does not depend on intracellular signalling. The lateral mobility of membrane receptors significantly affects cell adhesion. High diffusivity increases the adhesive strength at equilibrium as well as the rate of adherence stabilization, probably by allowing receptor accumulation into the cell contact area and more frequent encounters between the receptor and counter-receptor, respectively. It is predicted that the effect of receptor accumulation on adherence stabilization would dominate when the initial receptor density is low. Such accumulation requires diffusion over large distances comparable to the size of the cell contact area. In circumstances where receptor density is high, the effect of enhanced rate of bond formation would dominate instead. This enhanced rate of bond formation involves diffusion over much smaller distances, the distance required before collision with the complementary receptor. The latter process is predicted to occur over a much shorter time scale. An overall enhanced rate of adherence stabilization is particularly important when cell–cell encounter time is limited, such as in the circulation and in migration of lymphocytes through tissues.

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