Reversible Binding Kinetics of a Cytoskeletal Protein at the Erythrocyte Submembrane

Andrea L. Stout* and Daniel Axelrod*‡
*Biophysics Research Division and ‡Department of Physics, University of Michigan, Ann Arbor, Michigan USA

ABSTRACT Reversible binding among components of the cellular submembrane cytoskeleton and reversible binding of some of these components with the plasma membrane likely play a role in nonelastic morphological changes and mechanoplastic properties of cells. However, relatively few studies have been devoted to investigating directly the kinetic aspects of the interactions of individual components of the membrane skeleton with the membrane. The experiments described here investigated whether one component of the erythrocyte membrane cytoskeleton, protein 4.1, binds to its sites on the membrane reversibly and if so, whether the different 4.1-binding sites display distinct kinetic behavior. Protein 4.1 is known to stabilize the membrane and to mediate the attachment of spectrin filaments to the membrane. Protein 4.1 previously has been shown to bind to integral membrane proteins band 3, glycophorin C, and to negatively charged phospholipids. To examine the kinetic rates of dissociation of carboxymethyl fluorescein-labeled 4.1 (CF-4.1) to the cytofacial surface of erythrocyte membrane, a special preparation of hemolyzed erythrocyte ghosts was used, in which the ghosts became flattened on a glass surface and exposed their cytofacial surfaces to the solution through a membrane rip in a distinctive characteristic pattern. This preparation was examined by the microscopy technique of total internal reflection/fluorescence recovery after photobleaching (TIR/FRAP). Four different treatments were employed to help identify which membrane binding sites gave rise to the multiplicity of observed kinetic rates. The first treatment, the control, stripped off the native spectrin, actin, 4.1, and ankyrin. About 60% of the CF-4.1 bound to this control binded irreversibly (dissociation time >20 min), but the remaining ~40% binded reversibly with a range of residency times averaging ~3 s. The second treatment subjected these stripped membranes to trypsin, which presumably removed most of the band 3. CF-4.1 binded significantly less to these trypsinized membranes and most of the decrease was a loss of the irreversibly binding sites. The third treatment simply preserved the native 4.1 and ankyrin. CF-4.1 binded less to this sample too, and the loss involved both the irreversible and reversible sites. The fourth treatment blocked the gycophorin C sites on the native 4.1-stripped membranes with an antibody. CF-4.1 again binded less to this sample than to a nonimmune serum control, and almost all of the decrease is a loss of irreversible sites. These results suggest that 1) protein 4.1 binds to membrane or submembrane sites at least in part reversibly; 2) the most reversible sites are probably not proteinaceous and not glycophorin C, but possibly are phospholipids (especially phosphatidylserine); and 3) TIR/FRAP can successfully examine the fast reversible dynamics of cytoskeletal components binding to biological membranes.

INTRODUCTION

Erythrocytes can undergo a variety of shape changes attributed to rearrangements of skeleton-membrane contacts (Lerche et al., 1991; Whatmore et al., 1992). Spectrin, a filamentous molecule, is the main component of the meshlike membrane skeleton; attachments of spectrin to the membrane itself are mediated by two independent proteins, ankyrin and protein 4.1. One connection is between spectrin and the cytoplasmic portion of the anion exchanger, band 3, made via ankyrin. The other connection is provided by protein 4.1 (also called band 4.1), which binds to band 3, glycophorin C (another integral membrane protein), and to negatively charged phospholipids (for a review, see Bennett (1990)). If these connections do indeed play a role in cell shape change and maintenance, it would imply that the linkages between ankyrin or 4.1 and their binding sites on the membrane are reversible. Protein 4.1 (see Leto and Marchesi (1984) for a structural study) has been shown to be essential for membrane stability (Takakuwa et al., 1986). Protein 4.1 also appears to increase the maximal extent of binding of spectrin to phosphatidylserine vesicles (Takeshita et al., 1993). The ability of protein 4.1 to interact with its binding sites can be altered profoundly by factors such as phosphorylation state and the phosphatidylinositol composition of the membrane (Danilov et al., 1990; Gascard et al., 1992).

Although the dynamic nature of skeleton-membrane attachments has been postulated, relatively few studies have been devoted to investigating the kinetic aspects of the interactions of individual components of the membrane skeleton with the membrane. In the experiments described below, we investigated whether protein 4.1 binds to its sites on the membrane reversibly and if so, whether the different 4.1-binding sites display distinct kinetic behavior. Such information could be relevant to understanding both the dynamics of erythrocyte morphology and possibly also motility of living cells.

This study used total internal reflection-fluorescence recovery after photobleaching (TIR/FRAP) (Axelrod et al., 1992; Tamm, 1993; Thompson et al., 1981) which allows

Received for publication 7 January 1994 and in final form 23 June 1994.
Address reprint requests to Dr. Daniel Axelrod, Biophysics Research Division, University of Michigan, 930 N. University St., Ann Arbor, MI 48109-1055. Tel.: 313-764-5280; Fax: 313-764-3323; E-mail: daniel axelrod@umich.edu.
Andrea L. Stout's present address is School of Applied and Engineering Physics, Cornell University, Ithaca, NY 14853.

0006-3495/94/09/1324/11 $2.00

both selective visualization of molecules reversibly bound to a surface in equilibrium with a solution of fluorescent molecules and quantitative measurements of binding kinetics, surface diffusion, equilibrium constants, and concentration. This technique has been previously used to study the surface-binding dynamics of bovine serum albumin (BSA) at glass or plastic (Burghardt and Axelrod, 1981; Tilton et al., 1990a, b), the kinetics of protein binding to lipid bilayers supported on glass (Pisarchick et al., 1992; Pearce et al., 1992; McKiernan et al., 1993), and the dynamics of protein binding at biological membranes and receptors (Hellen and Axelrod, 1991; Fulbright and Axelrod, 1993).

MATERIALS AND METHODS

TIR/FRAP

Light that is incident upon an interface (e.g., glass/water) from the high refractive index side (e.g., glass) with a sufficiently high incidence angle will suffer total internal reflection (TIR). However, an exponentially decaying field (the "evanescent field") exists on the low refractive index side, which can be used to selectively excite fluorescent molecules within its range (about 100 nm; see Axelrod et al., (1992) and Tamm (1993) for detailed reviews). If the incident light is briefly pulsed at a bright intensity, the fluorophores in the evanescent field (which are mainly surface-bound ones for sufficiently strong surface binding) will be subject to photobleaching. Subsequent exchange of bleached surface-bound fluorescent molecules with unbleached ones farther out in the bulk solution will lead to a fluorescence recovery as excited by the same, but much dimmer evanescent field. This scheme can easily be adapted to microscope optics.

The surface association/dissociation reaction can be symbolically written as $A + B \leftrightarrow C$, where A is the concentration of free fluorescent (unbleached) molecules in the bulk, B is the concentration of free (available) binding sites on the surface, and C is the concentration of binding sites on the surface occupied by unbleached fluorescent molecules in chemical equilibrium. The forward (association) rate is denoted as k_a, and the backward (dissociation rate) is denoted as k_d. The TIR optical system senses fluorescence proportional to concentration C (apart from a small amount of fluorescence from the nearby bulk; see Appendix). After the bleaching pulse, the fluorescence recovers according to a complicated function of k_a, k_d, the bulk diffusion coefficient, and the surface diffusion coefficient (Thompson et al., 1981). However, in the special but common case where 1) surface diffusion is negligible, and 2) the average residency time of a surface-bound molecule is long enough to give ample time for dissociating bleached molecules to diffuse far from the surface (the "reaction rate limit"), then the fluorescence recovery becomes a very simple function. In this special case, A near the surface is essentially a constant in space and time, equal to the distant unbleached bulk concentration. Also, in all TIR/FRAP experiments, B is a constant in time, because the bleaching pulse affects only the ratio of bleached to unbleached surface-bound fluorophores, but not the overall amount of surface binding. Therefore, in the kinetic equation appropriate to our symbolic reaction, $\partial C/\partial t = k_a AB - k_d C$, the first term on the right is a constant in time, and the relaxation of C becomes a simple exponential, dependent only upon the dissociation rate k_d, not k_a at all. Based on the time scale of the TIR/FRAP results here, it can be shown that the reaction limit approximation is entirely appropriate (see Appendix).

Protein purification and labeling

Unless stated otherwise, all chemicals were reagent grade and purchased from Sigma Chemical Corp (St. Louis, MO). Protein 4.1 was purified from erythrocyte membrane skeletons following the procedure of Ohanian and Gratzer (1984) with modifications made by Drs. Robert and Ruby MacDonald of Northwestern University. Human red cells were obtained from the University of Michigan Hospital blood bank. In most cases the blood was not yet outdated; the collection date varied from several days to 2 weeks before its availability. Immediately after the red cells were acquired, they were passed through a leukocyte filter (Pall Biomedical Products, East Hills, NY) into sterile 50-ml polystyrene centrifuge tubes and stored at 4°C until use.

Briefly, 100 ml of filtered red cells were washed three times in an equal volume of buffer A (150 mM NaCl, 5 mM NaP_i, pH 8.0, 0.1 mM ethylenediaminetetraacetic acid (EDTA)) at 4°C for 10 min at 3000 × g. After washing, the packed red cells were lysed for 3–5 min in 5 volumes of ice-cold buffer B (5 mM NaP_i, pH 8.0, 0.1 mM EDTA) with 0.4 mM diisopropylfluorophosphate (DFP) and 2.08 μg/ml pepstatin A. This mixture was then centrifuged at 4°C for 40 min at 31,000 × g. The supernatant was aspirated off, leaving a loose pellet of red cell ghosts and a button rich in protease activity adhered to the bottom of the centrifuge tube. The ghosts were carefully poured into clean tubes and washed twice more in buffer B with 0.4 mM DFP resulting in removal of most of the hemoglobin from the red cell membranes. The ghost membranes were detergent-solubilized by mixing with an equal volume of the following ice-cold solution: 25 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethane sulfonic acid) (HEPES), pH 7.0, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 2 mM DFP, 4% Triton X-100. This mixture was layered over a cushion of 30% sucrose containing 1.5 M NaCl, 25 mM HEPES, pH 7.0, 0.5 mM adenosine triphosphate (ATP), 1.25 mM DTT, 0.5 mM EGTA, and 1 mM DFP; it was then spun in a Beckman ultracentrifuge (Beckman Instruments, Inc., Porterville, CA) using an SW28 swinging bucket rotor at 4°C and 112,000 × g for 1 h.

After the 1-h spin, the Triton and sucrose solutions were removed, leaving pellets of detergent-insoluble material consisting of the membrane skeletons. These pellets were dissolved in 2 M tris(hydroxymethyl)-aminomethane hydrochloride (Tris), pH 7.0, with 0.5 mM EGTA at room temperature. After about 10 min the solutions were diluted to 1 M Tris and centrifuged for 1 h at 4°C, 130,000 × g in a Beckman SW40Ti swinging bucket rotor (Beckman Instruments, Inc., Porterville, CA) to pellet any undissolved material. The three major components of the membrane skeleton (spectrin, actin, and protein 4.1) were separated by gel filtration on a column of Sepharose CL-6B (2.5 × 75 cm) equilibrated at 4°C with 1 M Tris, pH 7.2, 1 mM EGTA, 0.2 mM DTT. The fractions containing protein 4.1 were pooled and further purified by anion exchange chromatography (after dialysis into buffer C (100 mM KCl, 25 mM Tris, pH 7.8)) on a 5-ml DEAE-Sephacel column eluting with a linear KCl gradient from 0.01 to 0.2 M KCl. The purified protein 4.1 was then dialyzed into 0.1 M NaCl, 5 mM NaP_i, pH 8.0. This method of purification resulted in protein 4.1 that was intact and free of any major contaminants, as determined by SDS-PAGE (data not shown).

Fluorescence labeling of 4.1

In preparation for fluorescent labeling, a 1 mg/ml solution of (5-and-6)-carboxyfluorescein succinimidyl ester (Molecular Probes, Eugene, OR) in dimethylsulfoxide (DMSO) was prepared. In the pH range used here, this dye is most likely to react with lysine residues on the protein (Brinkley, 1992). The dialyzed protein 4.1 solution was centrifuged for 30 min at 4°C at 31,000 × g to remove any protein aggregates. The protein concentration was determined by measuring A_{280} using $\epsilon = 0.8\ (mg/ml)^{-1}(cm)^{-1}$ for 4.1 (Tyler et al., 1980). The portion of protein solution to be labeled (usually 4–5 ml) was allowed to warm up to room temperature. Then, while vortexing, a 4–5-mol excess of carboxyfluorescein in DMSO was slowly added to the 4.1. The mixture was incubated in the dark at 4°C for 2 h or less; it was then dialyzed in the dark at 4°C for 2 days against two 1-l changes of buffer D (100 mM KCl, 20 mM NaCl, 25 mM Tris pH 7.6, 0.2 mM DTT, 0.1 mM EDTA). Unlabeled 4.1 was also dialyzed in this buffer. The final protein concentration of the carboxymethyl fluorescein-labeled 4.1 (CF-4.1) solution was determined by the BCA protein assay (Pierce, Rockford, IL) or by measuring absorbance at 280 nm and correcting for the contribution of the dye to absorbance at this wavelength. The extinction coefficients at 280 and 491 nm of free carboxyfluorescein in buffer D were estimated experimentally by measuring the absorbance of solutions with known concentrations of CF at these wavelengths; these were then used to determine

both the concentration of dye in the CF-4.1 solution and the contribution of the dye to A_{280}. At 280 nm, the extinction coefficient for free CF in buffer D ranged from 10,000 to 22,000 $M^{-1}cm^{-1}$, whereas that for free CF in buffer D at 491 nm usually ranged from 27,000 to 57,000 $M^{-1}cm^{-1}$. This procedure for labeling the 4.1 resulted in dye:protein ratios that were always between 1.0 and 3.0.

Preparation of polylysinated coverslips

For early experiments a method of covalently attaching poly-L-lysine to glass or quartz based on a procedure of Jacobson et al. (1978) and modified by Fulbright and Axelrod (1993) was used. For later experiments a much quicker but equally effective method of polylysination was employed. #1 glass coverslips were cleaned by boiling in a 20% (v/v) solution of Linbro detergent (Flow Laboratories, McLean, VA) for 1–2 h. They were then rinsed repeatedly in tap water, then several times in distilled water, then twice in 95% ethanol and stored in a 110°C oven until use. When needed for an experiment, coverslips were cleaned in a plasma cleaner (Harrick Scientific Corp., Ossining, NY) under argon gas for 5 min. Immediately after plasma cleaning, 150 μl of a 40 mg/ml aqueous solution of poly-L-lysine (230 kDa) was placed on each coverslip and allowed to sit for 40–50 min. After repeated rinsing with distilled water, the coverslips were ready for use.

Preparation of red cell membranes adhered to coverslips

The procedure for preparing surface-adhered flattened erythrocyte membranes (referred to here as "flattened ghosts") is based on the Fulbright and Axelrod (1993) procedure with slight modifications. Four to five drops of freshly-drawn human blood were placed in 3 ml Hank's Balanced Salt Solution (HBSS) without Ca^{2+} or Mg^{2+} (GIBCO BRL, Grand Island, NY) and centrifuged at low speed in a clinical centrifuge for 10 min at room temperature. The supernatant was aspirated and the red cells were washed again in the same buffer. A more dilute suspension of red cells was made by placing 0.5 ml of the resuspended red cells in a clean centrifuge tube with 3.5 ml HBSS. 200 μl of the dilute red cell suspension was allowed to sit on a glass coverslip prepared as above for 40 min at room temperature. The coverslip was rinsed with HBSS and then placed under 3 ml of hemolysis buffer (5 mM NaP_i, 0.1 mM EDTA, pH 8.0) at room temperature for 4.5 min. The coverslip was rinsed with HBSS, and then either used as it was or subjected to further treatments to remove membrane skeleton proteins.

During hemolysis a rip occurs in the upper-facing surface of the membrane (the portion not directly adhered to the glass). The upper membrane pulls away from this rip, attracted by and becoming adhered to the charged glass surface. This leaves part of the lower membrane covered by a flap of upper membrane (the "crescent") and part of it exposed to the surrounding solution (the "bite"). The terms "crescent" and "bite" refer to the appearance of flattened ghosts either pre- or postlabeled with fluorescent probe-conjugated lectins, which bind exclusively to the extracellular sides of the membranes (Fulbright and Axelrod, 1993).

To remove just the native spectrin and actin from the glass-adhered red cell membranes, the coverslip with flattened ghosts was placed under 3 ml of a low-salt buffer (0.3 mM NaP_i, 0.2 mM EDTA, pH 8.0) and incubated for 40 min at 37°C. It was then either rinsed with HBSS and kept refrigerated until use or subjected to further depletion of membrane skeleton proteins. To remove the native protein 4.1 and ankyrin from the membranes, the coverslip was then placed under 3 ml of a high-salt buffer (1 M KI, 7.5 mM NaP_i, 0.1 mM EDTA, pH 8.0) and incubated for 40 min at 37°C. It was then rinsed with HBSS and refrigerated until use.

For some experiments, membranes that had been depleted of all native membrane skeleton proteins (spectrin, actin, ankyrin, and 4.1) were subjected to a mild trypsin treatment to cleave off the cytoplasmic portion of the integral membrane protein band 3, one of the proteins thought to serve as a membrane binding site for 4.1. The coverslip was placed under 3 ml of 0.5 μg/ml trypsin in ice-cold hemolysis buffer for 20 min. Digestion was terminated by repeated rinsing with 0.115 mM phenylmethylsulfonyl fluoride in hemolysis buffer.

Sample mounting for TIR/FRAP

For a typical TIR/FRAP experiment, the sample coverslip was mounted cell-side down as the top coverslip in a Dvorak-Stotler perfusion chamber (Nicholson Precision Instruments, Gaithersburg, MD). This chamber consists primarily of a circular aluminum frame and a metal spacer ring with inlet and outlet ports. One coverslip is placed on either side of the spacer ring; a C-spring on top of a rubber gasket holds the coverslips in place. Once assembled, a 1-cc syringe was used to inject the chamber with the desired labeling solution and the inlet and outlet ports sealed to minimize diffusion of oxygen into the chamber. To prepare the labeling solution for an experiment, the stock solution of CF-4.1 in buffer D was diluted in the same buffer with 0.5% β-D-glucose to CF-4.1 concentrations ranging from 40 to 300 nM. Before adding the CF-4.1, N_2 gas was bubbled through the buffer D for 2–3 min. Just before filling the sample chamber, 12 μl of a stock solution of 10 mg/ml glucose oxidase and 3 mg/ml catalase was added to the diluted CF-4.1 (total volume = 612 μl) as a means of deoxygenation (Englander et al., 1987). Deoxygenation was necessary to avoid bleach-induced cross-linking (Fulbright and Axelrod, 1993) on the surface, a phenomenon characterized by a rapid postbleach increase in fluorescence emission from the membrane surface. However, this low concentration of oxygen in solution was linked to another artifact, slowly reversible photobleaching (Stout and Axelrod, 1994), correctable as described later.

Microscopy and data collection

A Leitz Diavert inverted epifluorescence microscope with dichroic mirror and barrier filter appropriate for fluorescein was used for TIR/FRAP experiments. The optical setup was very similar to that depicted in Fig. 3 of Axelrod (1990). Illumination was provided by the 488-nm line of an argon-ion laser (Lexel, Palo Alto, CA). The intensity of the excitation light was controlled by computer (a Zenith 8 MHz 286-based PC) via an acousto-optic modulator (NEC Corporation, Mountain View, CA); in a typical experiment the intensity of the photobleaching pulse was 9,000 to 10,000 times that of the probe illumination, which was ~0.12 μW at the sample. To achieve total internal reflection of the incoming laser light, mirrors directed the beam to a cubical glass prism coupled to the top sample coverslip by immersion oil. The beam, focused to a small spot by a lens located just upbeam from the prism, was incident on a vertical face of the prism at an angle of ~74°. The resulting illumination spot had an intensity profile characterized by an elliptical Gaussian with diameters of ~50–60 μm (major axis) and 15–20 μm (minor axis). Fluorescence from the sample was collected by a 40 × 0.75 numerical aperture water immersion objective (Zeiss, Thornwood, NY). A photomultiplier tube (Hamamatsu R943–02) mounted atop the microscope's trinocular head and cooled to −22°C by a Peltier device (Pacific Instruments, Concord, CA) with an internal amplifier-discriminator detected fluorescence emitted from the sample.

Photon pulses from the amplifier-discriminator were collected by the PC equipped with a counter/timer board (Keithley/Metrabyte CTM-05, Taunton, MA). A custom program allowed the user to input experimental parameters such as sample times, duration of bleaching pulses, and duration of the postbleach recording time. The counter/timer board also provided a transient pulse that electrically shorted (through an external transistor) the photomultiplier's photocathode and first dynode to protect the photomultiplier during the bleaching flash. In these experiments, sample times of 15–500 ms were used. Only one photobleaching run was done on any particular cell. To maximize the signal/noise ratio, runs on individual cells were averaged together by the data acquisition program and stored for later analysis.

Analysis of TIR/FRAP results

A typical individual "set" of TIR/FRAP data consisted of 600–800 postbleach points representing an average of 20–40 TIR/FRAP bleach/probe sequences (called a "run"). Each run was done on a different red cell so that no cell was ever subjected to more than one bleach pulse. Depending on the sample time used, the length of a data set in these experiments could be as

short as 12 s or as long as 500 s. Data from different time scales on the same sample preparation were then merged into one composite data set (Fulbright and Axelrod, 1993). Therefore, each merged set represented data gathered from 30 to 50 separate ghost membranes.

Before the composite data files could be curve-fit for determination of kinetic parameters it was necessary to correct them for a component of the fluorescence recovery, arising not from on/off kinetics but instead from slowly reversible photobleaching (Stout and Axelrod, 1994), which occurs only under deoxygenated conditions. We assume here a model in which bleached fluorophores bound to the surface can follow two possible courses: they can dissociate, leaving a concentration of originally bleached fluorophores that decays according to some function $g_d(t)$, a function that contains information about adsorption/desorption kinetics; or they can spontaneously recover their fluorescence, leading to a decay of bleached concentration according to some function $g_r(t)$. The overall bleached concentration on the surface will therefore decay according to the function $g_t(t) = g_d(t)g_r(t)$. If one could determine $g_r(t)$ empirically, one could then extract the function of interest $g_d(t)$ from the measured $g_t(t) = 1 - f(t)$, where $f(t)$ is the post-bleach fluorescence recovery curve, normalized to the prebleach fluorescence. Estimates of $g_r(t)$ were made by performing TIR/FRAP experiments on glass coverslips coated with immobilized CF-4.1 at surface concentrations that yielded fluorescence values as close as possible to those measured for CF-4.1 on red cell membranes. Use of samples with similar surface fluorescence for the determination of $g_r(t)$ is important because the extent of reversible photobleaching appears to be somewhat dependent on surface concentration of fluorophores (Stout and Axelrod, 1994). The resulting rate constants and corresponding weights obtained from corrected data taken under deoxygenated conditions were similar to those from data sets of experiments done in the presence of oxygen where no correction was necessary (data not shown). All data reported here are on deoxygenated samples.

After dividing out the contribution of $g_r(t)$ to the total signal, the remaining decay, presumably due to adsorption/desorption kinetics alone, was subjected to a least-squares fitting routine to either a one- or two-exponential decay (representing the reversible component) plus a constant (representing the irreversible component) using commercially available software. (An F-test was used to determine whether or not the improvement of fit given by a double exponential was statistically significant). Each component is associated with an amplitude fraction. The "irreversible fraction" essentially represents CF-4.1, which does not dissociate during the maximum duration of a single experiment, about 2000 s. To combine information on average rates and fractions from data subjected to the two types of fits, the two reversible desorption rates from each data set fit to a double exponential were combined in a weighted average according to their amplitude fractions and are thereby reported as one average rate except as noted. Despite the use of one or two discrete exponentials to characterize the recoveries quantitatively, the actual kinetic behavior may be a continuum of rates.

RESULTS

Characterization of flattened ghosts

Fig. 1 schematically depicts the "crescent" as a flap of the upper membrane that has been pulled back to reveal a portion of the lower membrane, the "bite." To demonstrate that the flattened ghost preparation does indeed present this configuration, and in particular, contains areas of exposed cytoplasmic membrane, flattened ghosts were exposed to several fluorescent probes, each specific for a particular region of the bilayer.

Fig. 2 shows flattened ghosts that were depleted of their native membrane skeletons and then labeled with the amphiphilic lipid probe, diI-C_{18}-(3) (diI, Molecular Probes). Note that most cells appear as a bright rim (the crescent) around a somewhat dimmer area (the bite). When red cells are labeled with diI, the crescent is brighter than the bite because it consists of two layers of membrane, whereas the bite is only one membrane thick. The bite, although dimmer than the crescent, nonetheless is brighter than the bare glass background because it does consist of a labeled membrane.

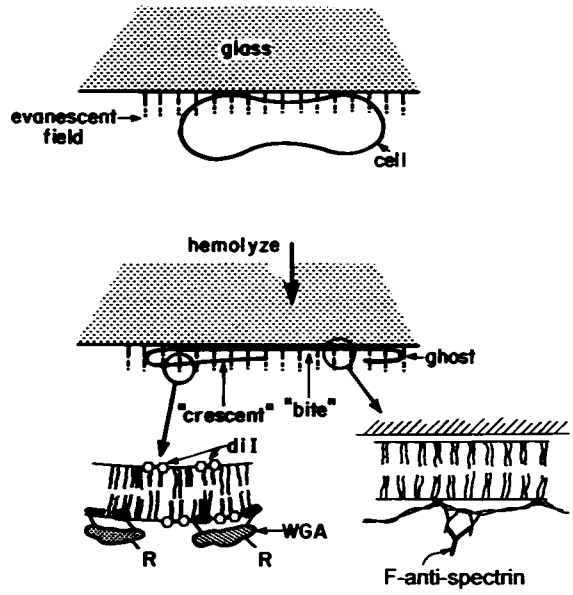

FIGURE 1 Schematic sectional view of a polylysine glass-adhered erythrocyte as it hemolyzes and flattens itself. A rip develops in the upper membrane that exposes the cytofacial surface of the lower membrane (the "bite" region). The exofacial surface is exposed where a flap of upper membrane covers the lower membrane (the "crescent" region). The putative locations of the fluorescent components used to confirm this membrane configuration are indicated: R-WGA, tetramethylrhodamine wheat germ agglutinin; diI, 3,3'-dioctadecylindocarbocyanine; F-anti-spectrin, fluorescein second antibody to anti-spectrin.

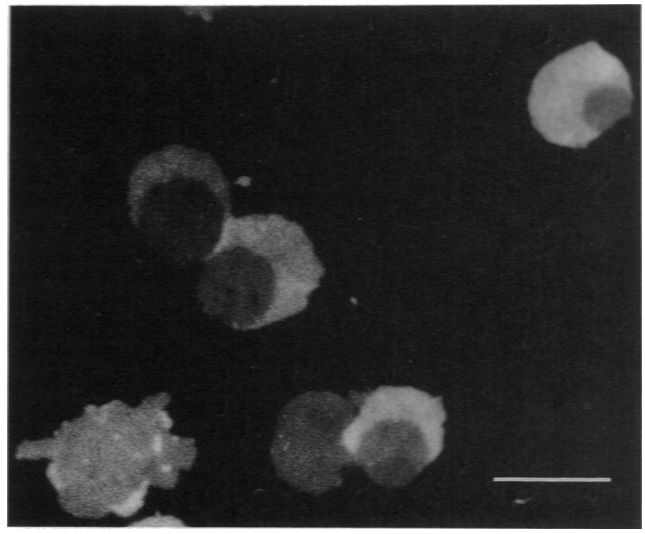

FIGURE 2 Flattened erythrocytes labeled with the amphiphilic lipid probe diI-(C-18) after adhesion and hemolysis by incubating the coverslip in a 0.5% (v/v) dilution into HBSS of a 1-mg/ml solution of diI in 95% ethanol at 37°C for 5 min. The pattern shows the characteristic "crescent-bite" configuration. The excitation here is by standard epiillumination at λ = 514.5 nm, although the pattern appears the same under TIR illumination. The objective used was a 40×, 0.75 N.A. water immersion and the space bar = 10 μm.

Fig. 3 shows flattened ghosts exposed to two different probes. In Fig. 3 *a*, the lectin tetramethylrhodamine-wheat germ agglutinin (R-WGA, Molecular Probes) binds to carbohydrate groups that are present only on the extracellular side of the membrane; labeling the opened red cells results in bright crescents at which the outside surface is accessible, but the bite regions where the cytoplasmic surfaces of the cells are exposed are dark. In Fig. 3 *b*, the cells are labeled with fluorescent second antibodies to rabbit anti-spectrin bound to the membrane skeletons. Under these conditions, the bites are very brightly labeled, for it is in these regions that the antibodies have access to the spectrin located on the inside surfaces of the membranes. The crescents are quite dark, indicating that the flap of upper membrane is in such close apposition to the lower membrane that entry of the two antibodies into the space between the two membranes is obstructed. In Fig. 3, *c* and *d*, the flattened ghosts were depleted of spectrin and actin as described in Materials and Methods before labeling with (*c*) R-WGA and (*d*) anti-spectrin. The R-WGA labeling is still bright and specific for the crescents. The F-anti-spectrin labeling, however, is much dimmer and more diffuse, indicating that most of the native spectrin is indeed gone. After undergoing membrane skeleton depletion, a cell often loses most of its crescent flap as it pulls away from the tightly attached lower membrane and becomes more fragmented in appearance, consistent with previous observations that removal of spectrin from red cells causes the cells to form small, inverted vesicles (Bennett and Branton, 1977).

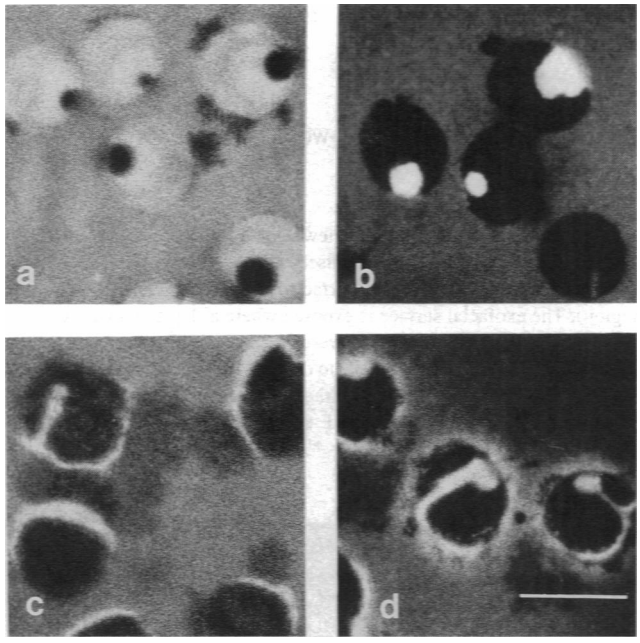

FIGURE 3 Comparison of flattened erythrocytes with membrane skeletons left intact (*a* and *b*) and with membrane skeletons depleted ("stripped") of native spectrin and actin by low ionic strength buffer. (*a*) Intact membranes postlabeled with R-WGA. Note the bright crescents and dark bites. The slide supporting the membranes was incubated in 200 μl of a 0.25 mg/ml solution of R-WGA in 0.1 M NaCl, 5 mM NaP_i, pH 7.9 for 15 min, then washed extensively with HBSS. Excitation was by epiillumination at λ = 514.5 nm. (*b*) Intact membranes postlabeled with anti-spectrin and a fluorescein second antibody. Note the dark crescents and bright bites. The slide supporting the membranes was incubated in 200 μl of a 1:4 dilution of polyclonal anti-spectrin (rabbit) (ICN Immunobiologicals, Irvine, CA) for 15 min, then washed extensively with HBSS, incubated in 200 μl of a 1:4 dilution of fluorescein isothiocyanate-anti-rabbit IgG (goat) (Organon-Teknika-Cappel, West Chester, PA) for 15 min, and again washed extensively with HBSS. Excitation is by TIR illumination at λ = 488.2 nm light. The sample is deoxygenated with the enzyme system discussed in the text. (*c*) Stripped membranes postlabeled with R-WGA, with the same labeling and illumination protocol as in (a). The bites are still dark but the bright crescents are much thinner than in (a) and frequently appear as strands overlying the bite region. (*d*) Stripped membranes postlabeled with a fluorescein second antibody to anti-spectrin, with the same labeling and illumination protocol as in (b). The bite regions here appear dark, unlike in (b), and they comprise almost the entire membrane area. The crescents are almost entirely retracted except for a thin circumference and some strands connected to the circumference overlaying the bites. These mutilated crescents perhaps retract into these shapes before all of their spectrin is removed; evidently, they still contain some spectrin sufficiently exposed to be labeled. For all photographs, the objective used was a 40×, 0.75 N.A. water immersion; space bar = 10 μm.

Maximal CF-4.1 binding at equilibrium

To maximally tag the 4.1 binding sites with CF-4.1, the native 4.1 was removed by the low-salt treatment (which removes spectrin and actin) followed by high-salt treatment (which removes 4.1 and ankyrin) as described in Materials and Methods. As shown in Fig. 4, the resulting flattened erythrocytes, stripped of all native membrane skeletal proteins, can bind CF-4.1 in distinctly bright bite regions (visible at CF-4.1 concentrations as low as 20 nM), leaving the crescents dark. The fact that the crescents are considerably darker than the bites indicates that 1) the CF-4.1 had only minimal access to the space between the upper and lower membranes, and 2) the CF-4.1 did not penetrate into the narrow region between the ghosts and the substrate. The bites are bright but the off-cell background is even brighter because of CF-4.1 adsorbed directly to the polylysine coated glass. The amount of CF-4.1 bound to the glass could be reduced by incubating the sample coverslip with its flattened erythrocytes with a 2-mg/ml solution of BSA for at least 20 min before assembling it into the sample chamber and injecting the CF-4.1 solution.

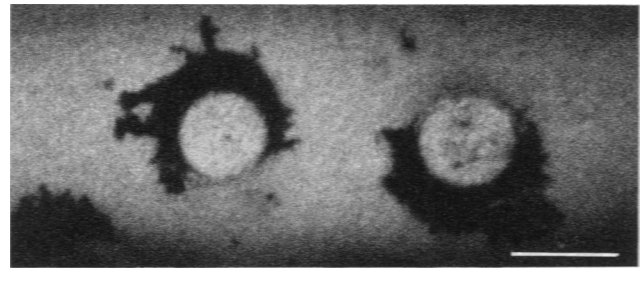

FIGURE 4 Flattened erythrocytes stripped of native spectrin, actin, and 4.1, and then exposed continuously to CF-4.1. Note the cytofacial surface (the bites) is brighter than the exofacial surface (the crescents). The sample chamber is filled with 70 nM CF-4.1 in deoxygenated buffer D as described in the text. The objective used was a 40×, 0.75 N.A. water immersion and excitation is by TIR illumination at λ = 488.2 nm. Space bar = 10 μm.

Fig. 5 shows a binding isotherm of CF-4.1 to the bite regions of stripped flattened erythrocytes. Because the evanescent field resulting from TIR did penetrate a short distance beyond the glass/solution interface, it was necessary to subtract the contribution of bulk fluorescence from the total fluorescence observed at each CF-4.1 concentration. The experimental method for the simultaneous determination of bulk and surface fluorescence and the corresponding concentrations was based entirely on a variation of TIR/FRAP and is described in the Appendix.

The binding isotherm in Fig. 5 demonstrates that the CF-4.1 binds to the cytoplasmic surface of stripped flattened erythrocytes in a saturable manner, with a K_D of 150 ± 50 nM. Because the TIR/FRAP results presented below clearly show a multiplicity of binding types, this K_D must be considered to be an average value. For each data point in Fig. 5, the system was allowed to equilibrate for 30 min or longer, somewhat longer than the duration of most TIR/FRAP recoveries, so binding that appears irreversible in TIR/FRAP may still show a bulk concentration-dependent binding in the equilibrium isotherm.

The range of reported K_D values for 4.1 binding to stripped inside-out red cell membrane vesicles is quite large, extending from 20–50 nM (Shiffer and Goodman, 1984; Pasternak et al., 1985) up to 600 nM (Gascard et al., 1992). The range of measured binding capacities is equally broad. Our number falls well within the range of reported dissociation constants, so it appears that CF-4.1 as prepared here behaved in a manner similar to the ^{125}I-4.1 most often used by others. However, 4.1 is a rather unstable protein, easily subject to degradation. The apparent binding affinities of any particular fluorescent 4.1 preparation may be strongly affected by the storage duration and conditions and the labeling protocol and degree. In one set of experiments in another lab (R. MacDonald, personal communication), CF-4.1 suffered a 15-fold decrease of binding affinity to phosphatidylserine relative to unlabeled 4.1 while maintaining the same maximal binding level. Therefore, quantitative equilibria and kinetics measurements on labeled 4.1 should be treated with some caution.

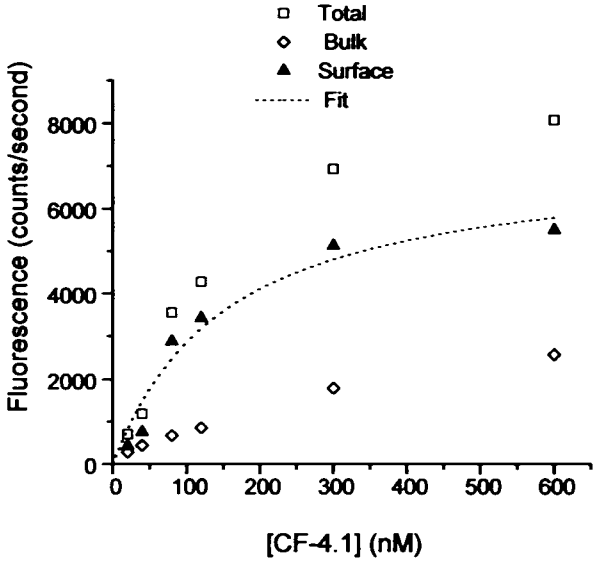

FIGURE 5 Equilibrium binding isotherm for CF-4.1 interacting with stripped, flattened erythrocytes. The contribution of bulk fluorescence to the total fluorescence was measured using the method described in the Appendix. To determine K_D, data were fit to the monocomponent function form $F(C) = F(\infty)C/(K_D + C)$, where C denotes the CF-4.1 surface concentration and $F(C)$ is the surface fluorescence (total minus bulk). This procedure yields K_D = 150 ± 50 nM, which must be considered to be an average value because TIR/FRAP curves clearly show a multicomponent nature to the binding.

Reduced CF-4.1 binding at equilibrium

To help identify the targets of 4.1 binding, flattened erythrocytes were treated to affect some or all of the maximal 4.1 binding, as summarized in Table 1. Mild trypsin digestion before labeling led to a 42% reduction in CF-4.1 fluorescence. Trypsin has been shown to efficiently cleave the cytoplasmic portion of band 3, one of the known binding sites for 4.1, but with minimal cutting of the cytoplasmic part of glycophorin C (glyC), the high-affinity 4.1 binding site (Danilov et al., 1990). It has been observed, however, that a substantial portion of the glycophorin population can be cleaved by trypsin under some conditions (C. Cohen, personal communication).

If flattened erythrocytes are left with their native 4.1 and ankyrin intact (i.e., no high-salt treatment), then a 49% reduction in CF-4.1 binding is observed. Such a treatment should block any high-affinity binding sites occupied by 4.1 in the intact cell; these sites could be the glyC sites as well as some band 3 sites.

In an attempt to block the glycophorin binding sites selectively, fully-stripped flattened erythrocytes were incubated with polyclonal antibodies to a peptide from the cytoplasmic portion of glyC (a gift from P. Low, Purdue University). This treatment (at antibody dilutions of 1:50 and 1:250) led to an average (46 ± 13)% decrease in bound CF-4.1 from the level obtained after exposure of the flattened ghosts to a control (nonimmune rabbit serum) at the same dilutions.

TABLE 1 Loss of total binding capacity (reversible + irreversible) of CF-4.1 at equilibrium with flattened erythrocyte ghosts upon various treatments that reduce binding

Treatment	n	Fraction lost ± SEM	p-value (1-sample t-test)
(a) Trypsin	7	0.42 ± 0.26	0.155
(b) Spectrin-deplete only	5	0.49 ± 0.22	0.096
(c) Anti-glyC	2	0.46 ± 0.13	0.092

Loss fractions are based on steady-state (i.e., no bleaching) TIR surface fluorescence measurements averaged over CF-4.1 bulk concentration ranging from 40 to 600 nM. Surface fluorescence (total minus bulk fluorescence) was calculated (according to the method described in the Appendix) using a conservative estimate of the bulk fluorescence fraction as 0.19. For treatments (a) and (b), the "fraction lost" is relative to high-salt (KI) stripped flattened erythrocytes with no further treatment. For treatment (c), the fraction lost is relative to treatment with non-immune serum. The anti-glyC dilutions were 1:50 and 1:250 in the two trials.

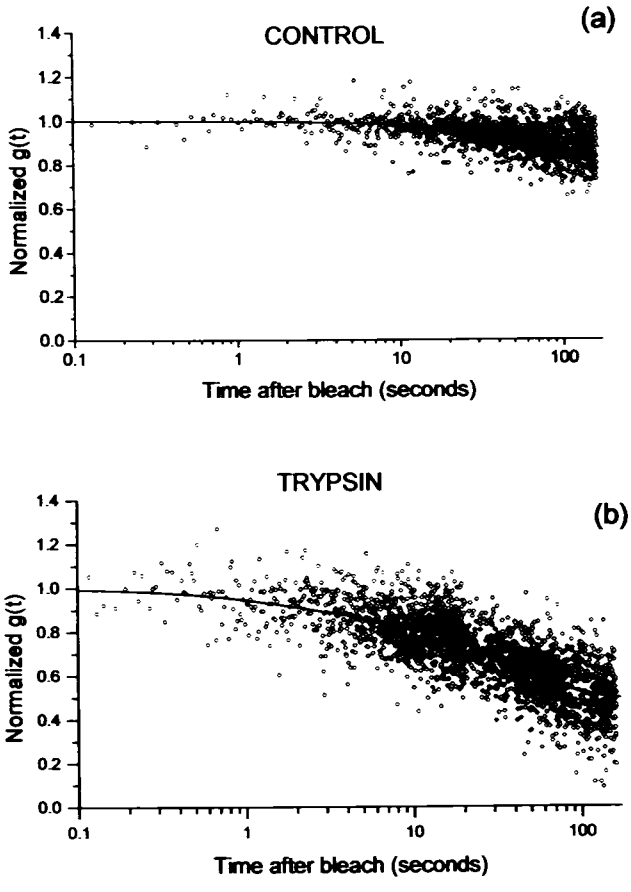

FIGURE 6 Typical inverted TIR/FRAP recovery curves for CF-4.1 binding to stripped erythrocytes, with data from several time scales merged together. The actual fluorescence recovery curve is normalized to its average prebleach value, forming $f(t)$; the ordinate here is the complimentary function $1 - g(t)$, showing the loss of bleached fluorophore on the surface. Note the logarithmic scale of the abscissa. The solid line is a two-exponential best fit. (*a*) "Control" membranes, with no treatment after low and high salt stripping of native proteins, and in equilibrium with 200 nM CF-4.1 in the bulk; (*b*) membranes prepared similarly on the same day, then exposed to trypsin as discussed in the text, and then placed in equilibrium with 200 nM CF-4.1 in the bulk. The reversible fractions can be seen as the amount of decline in these curves; this was somewhat variable from day to day. The control shown here displays a reversible fraction somewhat less than the 41% obtained as an average over many data sets.

Kinetics of CF-4.1 binding

TIR/FRAP experiments for measuring CF-4.1 dissociation rates were performed with bulk CF-4.1 concentrations ranging from 40 to 600 nM. Typical curves are shown in Fig. 6. There was no discernible effect of concentration on the measured irreversibly bound fraction between 40 and 400 nM on the "stripped" membrane controls, so results for all these bulk concentrations are combined. The fraction of 4.1 bound irreversibly to the "stripped" (control) membranes in these experiments was quite variable but the average was around 0.59 ± .05 (SEM), as shown in Table 2.

Concerning the reversibly bound CF-4.1, the weighted average dissociation rates observed by TIR/FRAP also are summarized in Table 2. For the stripped membrane controls, the average dissociation rate was 0.34 ± .07 s^{-1}, giving a characteristic time of 2.9 s spent by CF-4.1 on the membrane before dissociation. Individual merged data runs yielded rate parameters that were quite variable. Some merged recovery curves were well fit by single exponentials, and others by two exponentials. The individual reversible dissociation rates all fell in the range between 0.01 s^{-1} and 1.0 s^{-1}, although the TIR/FRAP technique could easily have detected much faster or slower rates if they were present.

TABLE 2 Parameters of reversible binding of CF-4.1 to flattened erythrocytes with various treatments

Treatment	N	k_d ± SEM	f_{rev} ± SEM
		(s^{-1})	
(a) Control	21	0.34 ± 0.07	0.41 ± 0.05
(b) Trypsin	6	0.09 ± 0.03	0.56 ± 0.10
(c) No KI extraction	4	0.20 ± 0.1	0.39 ± 0.05
(d) Anti-glyC	2	0.10 ± 0.02	0.94 ± 0.04

The dissociation rate k_d (obtained by averaging rates from single-exponential fits and weighted-average rates from double-exponential fits) and the fraction of the binding f_{rev} that is reversible on the time scale of the experiments (several hundred seconds) for each treatment. N is the number of merged data sets. Each data set merged was generated from signal averaged TIR/FRAP results on 30 to 50 separate flattened erythrocytes. Treatments are the same as in Table 1.

In an effort to associate a subset of dissociation rates with a particular type of site, TIR/FRAP experiments were performed on flattened erythrocytes that had been treated in various ways to remove classes of potential 4.1 binding sites. Stripped membranes that underwent the mild trypsin digestion described in Materials and Methods exhibited irreversibly adsorbed CF-41 fractions 0.44 ± .03, somewhat lower than the 0.59 ± .05 for the nontrypsinized cases. The post-trypsinization dissociation rates ranged between 0.01 s^{-1} and 0.3 s^{-1} with a weighted average of 0.09 ± .03 s^{-1}.

Allowing most of the native 4.1 and ankyrin to remain associated with the flattened erythrocytes by omitting the 1 M KI extraction did not appear to affect the measured dissociation rates or the relative amounts of irreversibly and reversibly bound 4.1 on the membranes (although the total binding decreased as mentioned earlier). Exposure of stripped flattened erythrocytes to polyclonal antibodies against a peptide from the cytoplasmic side of glyC (anti-glyC) resulted in a substantial decrease in the fraction of irreversibly bound CF-4.1 and a threefold decrease in the off-rate for desorption of reversibly bound CF-4.1.

DISCUSSION

Most previous studies of protein 4.1-erythrocyte membrane interactions have focused on the affinity as quantified by equilibrium constants. This is the first TIR/FRAP study that allows direct measurement of fast reversible binding kinetic rates in addition to equilibrium parameters. The most important result is that the binding residence times are multicomponent, ranging from almost irreversible (>2000 s) down to as short as 1 s. Furthermore, these components change both their relative weights and their absolute amplitudes (i.e., amount of bound CF-4.1 corresponding to a particular rate)

when the membrane or 4.1 is altered in a defined way. Based on these alterations, we will discuss whether a particular component of kinetic behavior can be attributed to one or more of the known 4.1 binding sites (band 3, glyC or other glycophorins, or phospholipids).

Trypsin

Mild trypsinization of erythrocytes is an established means of removing the cytoplasmic domains of band 3 molecules in red cell membranes with relatively little effect on the cytoplasmic domains of glyC (Pasternak et al., 1985; Danilov et al., 1990; Gascard et al., 1992). This treatment led to a net 42% decrease in surface fluorescence after the contribution from bulk fluorescence is taken into account. This implies that, on the average, trypsinization removes 42% of the potential 4.1 binding sites from the stripped flattened erythrocytes, a figure somewhat smaller than the 60–70% usually reported. (Danilov et al., 1990; Pasternak et al., 1985). The effect from trial to trial was somewhat variable, perhaps because overcleavage of extracellular proteins might reduce the membrane-substrate separation, thereby exposing the membranes to a more intense region of the evanescent field and exciting more fluorescence from fewer fluorophores.

Of the CF-4.1 binding sites that remained after trypsin treatment, an average of (56 ± 10)% of them bound CF-4.1 reversibly. This reversible fraction corresponds to (32 ± 21)% of the total number of sites that were present on the membrane before trypsinization. The average dissociation rate of these posttrypsinization sites was somewhat slower than that of 4.1 bound to reversible sites before trypsinization. It is possible that some previously irreversible sites are rendered slowly reversible by trypsin. The posttrypsinization irreversible sites account for (26 ± 16)% of the total number of sites present before trypsinization.

These fractions are depicted in the pie charts of Fig. 7. A whole circle represents all the sites originally present. Fig. 6 *a* shows those sites divided into reversible and irreversible sectors of the nontrypsinized control. Fig. 7 *b* shows a large slice that becomes unavailable after trypsin, and the division of the remainder into reversible and irreversible fractions. It is clear that trypsinization has the greatest impact on the irreversible sites of the nontrypsinized controls.

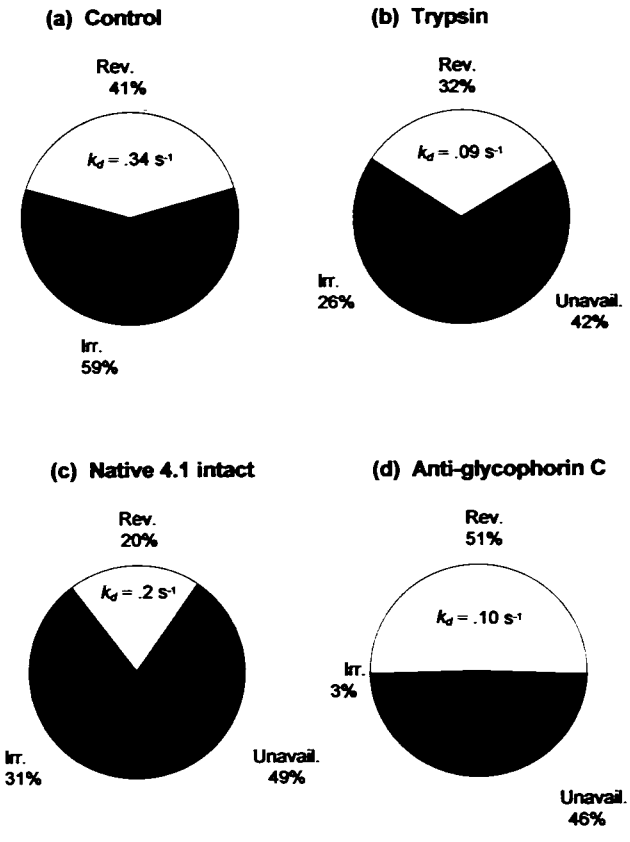

FIGURE 7 Effect of various treatments that reduce the equilibrium binding of CF-4.1 to flattened erythrocytes, with amounts of the residual reversible and irreversible binding shown in pie charts. In each case (*a–c*), the full circle represents the total number of binding sites present in the controls (stripped membranes treated to both low and high salt, otherwise untreated). (*a*) Controls, divided into reversible and irreversible fractions. (*b*) After trypsin treatment, in which 42% of the original control sites are eliminated ("unavailable"). (*c*) Membranes with spectrin and actin removed by low-salt treatment, but native ankyrin and 4.1 left intact by deleting the high-salt (KI) extraction. This reduces the binding by 49% from the control level. (*d*) Stripped membranes after treatment with anti-glyC. Here the full circle represents the total binding to stripped membranes after treatment with nonimmune serum. In that sector of each circle corresponding to the reversible binding fraction, the dissociation rate k_d of that fraction is shown. (See Table 2 for complete statistical information).

Blocking by native 4.1 and ankyrin

Some of the binding sites for CF-4.1 can be blocked simply by not stripping off the native 4.1 and ankyrin with a high-salt (KI) treatment. Overall, the effect of not performing the KI extraction is to decrease the number of available sites by 49%, again somewhat variable from trial to trial. However, one can roughly estimate from published binding data (Bennett and Stenbuck, 1980; Lombardo et al., 1992) that native 4.1 and ankyrin occupy only about 25% of the total number of glyC-plus-band 3 sites on the cytofacial surface of the membrane. It is possible that: 1) some of these "unoccupied" band 3 molecules actually may be occupied by other membrane-associated proteins not released during hemolysis or spectrin-actin removal; or 2) some band 3 molecules are not in the oligomeric state preferred by protein 4.1. As with trypsin treatment, blockage by native 4.1 and ankyrin reduces the irreversible binding population more than the reversible binding population: reversibly binding sites still account for (20 ± 9) % of the total number of sites despite blockage (see Fig. 7 *c*).

Anti-glyC

Exposure of the flattened erythrocytes to polyclonal antibodies to the cytoplasmic domain of glyC (anti-glyC) removed roughly 75% of the original binding sites, and almost all of the remainder bound CF-4.1 only reversibly (see Fig. 7 *d* pie chart). This 75% reduction is much higher than the

15–25% generally accepted as the fraction of total binding capacity because of glyC binding sites (Gascard et al., 1992; Lombardo et al., 1992). This large discrepancy could be due to: 1) partial blocking of many band 3 sites by the anti-glyC antibodies bound to glyC because of some association of these two integral membrane proteins; or 2) anti-glyC was able to bind nonspecifically to the cytoplasmic side of band 3, thereby blocking more potential 4.1 binding sites.

Nature of reversible binding

Regardless of how the membranes have been prepared as discussed above, 20–40% of the total number of CF-4.1 binding sites originally present on the stripped controls are reversibly binding. In general, the treatments affect the number of irreversible sites more than the number of reversible ones. Given that none of the treatments used are purported to affect the phospholipids of the membrane, this observation suggests that the "irreversible" interactions of 4.1 involve its protein binding sites, and the reversible interactions involve mainly phospholipids. The phospholipid sites probably include PS. The reported affinity of 4.1 for PS-containing vesicles is similar in magnitude to that of 4.1 for the protein band 3, with a K_D of 330 nM (Cohen et al., 1988). In addition, TIR/FRAP experiments in our lab performed on supported planar membranes consisting of 25% PS and 75% phosphatidylcholine (PC) in equilibrium with CF-4.1 yield similar dissociation rates to those measured here, ranging from 0.008 s to 0.08 s^{-1}. The fraction of CF-4.1 bound "irreversibly" (on the time scales measurable in those experiments) to PS/PC membranes is only around 17% (McKiernan and Axelrod, 1993).

It seems unlikely that the reversible recovery could be due to the lateral diffusion of "fresh" 4.1 protein binding sites into the observation region from the crescent region where these sites had been evidently sheltered from binding to CF-4.1. Lateral diffusion of band 3 and glycophorin in intact erythrocyte membrane is rather slow, no larger than about 4×10^{-11} cm^2/s (Peters et al., 1974; Corbett et al., 1994). At that rate, a characteristic time to diffuse from the crescent to the center of the bite regions (estimated at about 3 μm) would be at least 500 s, longer than the characteristic times for fluorescence recovery measured here. In addition, both proteins have extracellular sugars and are probably stuck tightly to the polylysine-coated substrate. Lateral diffusion of "fresh" lipid binding sites from the crescent, which is probably fast enough to account for the observed recovery, could contribute to the recovery if the exposed lipids in the bite were virtually saturated with adsorbed 4.1 so that the arrival of fresh lipid sites would be significant. But the potentially huge number of lipid binding sites and the rather low surface concentration of 4.1 (see Appendix) makes this theoretical possibility unlikely.

If 20–40% of exogenous 4.1 binds to reversible sites on phospholipids, even at low concentrations, it is of interest to speculate what biological function such an interaction might have in vivo. One possible role is the facilitation of reactions with protein targets such as band 3 of glyC on the surface: if ligands can adsorb "nonspecifically" to a surface and diffuse in two dimensions before desorbing, the rate of reaction with specific targets may be enhanced ("reduction of dimensionality" enhancement: see Adam and Delbruck, 1968; Berg and Purcell, 1977; Wang et al., 1992; Axelrod and Wang, 1994). The quantitative models that predict the degree of reaction rate enhancement by surface diffusion (see Wang et al. (1992) for diffusion-limited targets; and Axelrod and Wang (1994) for reaction-limited targets) use exactly those parameters measured experimentally here by TIR/FRAP: the dissociation rate and the equilibrium constant for the reversible binding.

Our calculations based on these theories indicate that the parameters of reversible binding of 4.1 to flattened erythrocytes is not in the regime in which reaction rate enhancement by 2D diffusion along the phospholipids might occur, even if the fastest physically reasonable surface diffusion rates are assumed. For the equilibrium constant determined for this system, there are just too many targets in the red cell membrane for surface diffusion to contribute appreciably to the total flow of 4.1 toward irreversible targets. However, in the intact red cell, it is possible that only a small percentage of the total number of potential binding sites is available to the protein 4.1 molecules that are not already incorporated into the membrane skeleton. If this were the case, there may still be a possibility of reaction rate enhancement by surface diffusion along phospholipids.

Even in the absence of true surface diffusion as a reaction rate-enhancing mechanism, nonspecific binding to phospholipids by 4.1 might still enhance its binding rates to specific protein sites. Because 4.1 molecules are effectively tethered to each other by binding at opposite ends of a spectrin tetramer, nonspecific association of one 4.1 to the phospholipids in the membrane restricts the range of exploration of the 4.1 at the other end to the immediate submembrane bulk volume, thereby increasing the local concentration there. Even when the spectrin end that is reversibly bound to phospholipids through 4.1 becomes dissociated from the membrane, the 4.1 at the other end might still be attached to the nearby membrane cytoskeleton, thereby increasing the likelihood of nonspecific rebinding.

Previous kinetics and mechanoplasticity data

Because of the novelty of the techniques used here, little data on the dynamics of 4.1-membrane interactions are available for direct comparison with these TIR/FRAP results. However, observations of the time-dependent Ca^{2+}-induced proteolysis of membrane skeletal constituents revealed that loss of the ability to reverse the change of red cell shape from discocyte to echinocyte coincided with the degradation of 80% of the protein 4.1 in the cell (Whatmore et al., 1992). The half-time for the loss of 4.1 to proteolysis in these experiments was about 17 min; if susceptibility to Ca^{2+}-dependent proteases occurs only upon release of protein 4.1 from its sites on the membrane, this result indirectly implies

an off-rate of 0.0007 s^{-1}, which is slow enough to appear "irreversible" in the TIR/FRAP experiments described here.

Measurements of the dissociation kinetics of another cytoskeletal protein, ankyrin bound to inside-out red cell vesicles, showed that ankyrin remains bound to band 3 sites for a characteristic time of 300 s, corresponding to a dissociation rate of 0.0033 s^{-1} (Thevenin and Low, 1990). In that study, two classes of kinetic behavior were detected and assigned to two distinct oligomeric states of the band 3 binding sites.

Summary and future studies

Although a large portion of the 4.1-erythrocyte membrane interaction appears to be very stable on the time scale examined here (up to 2000 s), it is still possible that modulation of the 4.1-membrane interaction is involved in slow red cell shape changes. The significant portion of binding that is rapid may represent binding to phospholipids but even that portion may act as a buffer reservoir or a means to enhance reaction rates. It will be very interesting to discover whether alteration of the 4.1 by factors present in vivo (such as phosphorylation by protein kinase C) changes its kinetic behavior so that reversible interactions are favored. Further experiments along these lines may lead to insights as to how more complex cells use regulation of cytoskeletal protein-membrane interactions to maintain or change shape in response to external stimuli.

APPENDIX

Bulk fluorescence, equilibrium binding, and the reaction limit

The TIR evanescent field penetrates into the liquid medium for a short distance (about 100 nm here). It is desirable to measure quantitatively the proportion of fluorescence excited in the bulk solution relative to that from surface bound fluorophores, for three purposes: 1) to subtract out the contribution from the bulk; 2) to evaluate whether or not the system of interest is in the "reaction limit", as defined in Thompson et al. (1981); 3) to obtain an equilibrium binding isotherm; and 4) to estimate quantitatively the concentration of surface binding sites. The method for determining the bulk fluorescence contribution used here is entirely internal in that it uses the same TIR/FRAP system as the kinetics experiments.

The method is a modification of the approach described by Zimmerman et al. (1990). If the time for diffusional replacement of bleached fluorophores in the bulk portion of the evanescent field is much shorter than the residency time of surface-adsorbed fluorophores, then the bulk portion will appear essentially "unbleachable" for intermediate bleach duration and sample times. This is because bleached fluorophores in the bulk will almost complete exchange with unbleached ones even before the first reading of postbleach fluorescence is made, whereas bleached fluorophores on the surface will still be in residence at the time of the first reading. By using a very strong bleaching pulse (of either sufficient duration or intensity), the surface fluorescence can be completely extinguished, leaving only bulk fluorescence. By measuring the prebleach total (surface + bulk) fluorescence and the immediately ($t = 0+$) postbleach (bulk only) fluorescence, one can calculate the ratio of fluorophore concentration in the two regions.

In practice, a complete bleach of the surface fluorescence is not always possible or advisable (e.g., because of laser power limitations or heating), and a partial bleach (with lower intensity or duration) can be used as follows. Let the prebleach surface and total fluorescence (normalized to the total prebleach fluorescence) be denoted by $f_S(-)$ and $f_T(-)$, respectively, and the immediately postbleach surface and total fluorescence (at $t = 0+$) be denoted by $f_S(K)$ and $f_T(K)$. Here K is the bleaching strength parameter proportional to the product of the bleaching intensity, duration, and intrinsic probability of bleaching per absorbed photon (the "bleachability"). Given that the normalized bulk fluorescence f_B (the parameter we wish to calculate) is effectively unbleachable, it has no argument. It can be easily shown that:

$$f_B = \frac{f_T(K) - f_S(K)}{1 - f_S(K)}$$

In principle, $f_S(K)$ at a particular K can be found on a special control sample consisting of only irreversibly adsorbed labeled protein, by using the same particular combination of bleach duration and intensity as on the sample of interest. In our case, this control sample was prepared by exposing a clean glass coverslip to CF-4.1 and washing extensively. Ideally, fluorophores in the control sample should reside in a similar local environment (concentration, hydrophilicity, etc.) as that on the sample of interest. If the local environments are different, then the bleachabilities of the fluorophore may be different. In that case, $f_T(K)$ on the sample of interest should be tested at least two different K values and the control sample should be used to measure $f_S(K)$ over a range of K values. Further details of this procedure can be found in Stout (1993).

The relative bulk fluorescence f_B can be converted into an equilibrium concentration ratio C/A where C is the surface-adsorbed CF-4.1 concentration and A is the bulk CF-4.1 concentration. The bulk fluorescence is simply an integral over distance from the substrate of the bulk concentration weighted by a product of the exponentially decaying intensity of the evanescent field and the (slightly) distance-dependent collection efficiency of the fluorescence (see Hellen and Axelrod, 1987).

Aside from allowing the subtraction of the bulk concentration from the total isotherm points of Fig. 5 to obtain the true surface binding isotherm, ratio C/A is also needed to determine whether the TIR/FRAP results were in the reaction limit. C/A has units of distance and represents the depth of the bulk that contains the same number of fluorophores as the surface, per unit cross-sectional area. If the experimental TIR/FRAP recovery times are much longer than the time required for a fluorophore to traverse that depth by diffusion, then the process is in the reaction limit and the recovery times do indeed represent the surface dissociation time (k_d^{-1}) (Thompson et al., 1981). This calculation need be performed only for the binding components with the shortest residency time, i.e., the most rapidly reversible components. For example, in Fig. 5 the surface fluorescence is about 70% of the total fluorescence at a bulk 4.1 concentration of 400 nM. Assuming 41% of this is rapidly reversible (i.e., on the time scale of TIR/FRAP experiments), we can calculate the ratio C_{rev}/A where C_{rev} is the reversibly bound surface concentration of 4.1. We find that this ratio is about 170 nm. The time for diffusional transport across that distance is around 4×10^{-4} s, much shorter than any characteristic time spent on the surface by reversibly bound 4.1 measured in these experiments. The system is therefore well within the reaction limit.

The ratio C/A, measurable directly by TIR/FRAP, can be related to the equilibrium dissociation constant K_D. This procedure provides a bonus: an estimate of the absolute number of surface binding sites per μm^2. Note that for the reaction $A + B = C$ we have $K_D = B/(C/A)$ where B is the concentration of free surface binding sites. Given a TIR/FRAP measurement of $C/A = 415$ nm for the total rather than just reversible C and an equilibrium dissociation constant of 150 nM from the isotherm of Fig. 5, we obtain $B = 37/\mu m^2$. Given that C/A was measured at the bulk concentration of 400 nM, at which point Fig. 5 shows the surface sites to be about 75% occupied, we can then calculate the total concentration of 4.1 surface binding sites to be $\sim 150/\mu m^2$.

We are grateful to Drs. Robert and Ruby MacDonald of Northwestern University for their generous and invaluable help with the purification protocols for protein 4.1 and for their advice and comments on all aspects of this project. We also thank Dr. Robert M. Fulbright for help with data analysis and custom software, Dr. Carl Cohen of St. Elizabeth's Hospital (Boston, MA) for useful discussions, Dr. P. Low of Purdue University for the gift of anti-glyC, and Dr. Ariane McKiernan for useful discussions and help with

the biochemistry. This project was supported by National Science Foundation grants DMB8805298 and National Institutes of Health grant R01-NS14565 (to D.A.), and NIH grant P01-HLA45168 (to R. C. MacDonald).

REFERENCES

Adam, G., and M. Delbruck. 1968. Reduction of dimensionality in biological diffusion processes. *In* Structural Chemistry and Molecular Biology. A. Rich and N. Davidson, editors. W. H. Freeman and Company, San Francisco. 198–215.

Axelrod, D. 1990. Total internal reflection fluorescence at biological surfaces. *In* Noninvasive Techniques in Cell Biology, J. K. Foskett and S. Grinstein, editors. Modern Cell Biology 9, Wiley-Liss, New York. 93–127.

Axelrod, D., E. H. Hellen, and R. M. Fulbright. 1992. Total internal reflection fluorescence. *In* Fluorescence spectroscopy: principles and applications, Vol. 3: Biochemical applications. J. Lakowicz, editor. Plenum Publishing Corp., New York. 289–343.

Axelrod, D. and M. D. Wang. 1994. Reduction-of-dimensionality kinetics at reaction-limited cell surface receptors. *Biophys. J.* 66:588–600.

Bennett, V. 1990. Spectrin-based membrane skeleton: a multipotential adaptor between plasma membrane and cytoplasm. *Physiol. Rev.* 70:1029–1065.

Bennett, V., and D. Branton. 1977. Selective association of spectrin with the cytoplasmic surface of human erythrocyte plasma membranes. Quantitative determination with ^{32}P spectrin. *J. Biol. Chem.* 252:2753–2763.

Bennett, V. and P. J. Stenbuck. 1980. Association between ankyrin and the cytoplasmic domain of band 3 isolated from the human erythrocyte membrane. *J. Biol. Chem.* 255:6424–6432.

Berg, H. and E. M. Purcell. 1977. Physics of chemoreception. *Biophys. J.* 20:193–239.

Brinkley, M. 1992. A brief survey of methods for preparing protein conjugates with dyes, haptens, and cross-linking reagents. *Bioconj. Chem.* 3, 2–13.

Burghardt, T. P., and D. Axelrod. 1981. Total internal reflection/fluorescence photobleaching recovery study of serum-albumin adsorption dynamics. *Biophys. J.* 33:455–467.

Cohen, A. M., S. C. Liu, J. Lawler, L. Derick, and J. Palek. 1988. Identification of the protein 4.1 binding site to phosphatidylserine vesicles. *Biochemistry.* 27:614–619.

Corbett, J. D., M. R. Cho, and D. E. Golan. 1994. Deoxygenation affects fluorescence photobleaching recovery measurements of red cell membrane protein lateral mobility. *Biophys. J.* 66:25–30.

Danilov, Y. N., R. F. Fennell, E. Ling, and C. M. Cohen. 1990. Selective modulation of band 4.1 binding to erythrocyte membranes by protein kinase C. *J. Biol. Chem.* 265:2556–2562.

Englander, S. W., D. B. Calhoun, and J. J. Englander. 1987. Biochemistry without oxygen. *Anal. Biochem.* 161:300–306.

Fulbright, R. M., and D. Axelrod. 1993. Dynamics of nonspecific adsorption of insulin to erythrocyte membranes. *J. Fluor.* 3:1–16.

Gascard, P., T. Pawelczyk, J. M. Lowenstein, and C. M. Cohen. 1992. The role of inositol phospholipids in the association of band 4.1 with the human erythrocyte membrane. *Eur. J. Biochem.* 211:671–681.

Hellen, E. H., and D. Axelrod. 1987. Fluorescence emission at dielectric and metal-film interfaces. *J. Opt. Soc. Am. B.* 4:337–350.

Hellen, E. H., and D. Axelrod. 1991. Kinetics of epidermal growth factor/receptor binding on cells measured by total internal reflection/fluorescence recovery after photobleaching. *Biophys. J.* 1:113–128.

Jacobson, B. S., J. Cronin, and D. Branton. 1978. Coupling polylysine to glass beads for plasma membrane isolation. *Biochim. Biophys. Acta.* 506: 81–96.

Lerche, D., M. M. Kozlov, and W. Meier. 1991. Time-dependent elastic extensional RBC deformation by micropipette aspiration: redistribution of the spectrin network? *Eur. Biophys. J.* 19:301–309.

Leto, T. L., and V. T. Marchesi. 1984. A structural model of human erythrocyte protein 4.1. *J. Biol. Chem.* 259:4603–4608.

Lombardo, C. R., B. M. Willardson, and P. S. Low. 1992. Localization of the protein 4.1-binding site on the cytoplasmic domain of erythrocyte membrane band 3. *J. Biol. Chem.* 267:9540–9546.

McKiernan, A. M., R. C. MacDonald, R. I. MacDonald, and D. Axelrod. 1993. Kinetics of spectrin and band 4.1 binding to phospholipid model membranes. *Biophys. J.* 64:A75, manuscript in preparation.

Ohanian, V., and W. Gratzer. 1984. Preparation of red-cell-membrane cytoskeletal constituents and characterization of protein 4.1. *Eur. J. Biochem.* 144:375–379.

Pasternak, G. R., R. A. Anderson, T. L. Leto, and V. T. Marchesi. 1985. Interactions between protein 4.1 and band 3. *J. Biol. Chem.* 260: 3676–3683.

Pearce, K. H., R. G. Hisket, and N. L. Thompson. 1992. Surface binding kinetics of prothrombin fragment 1 on planar membranes measured by total internal reflection fluorescence microscopy. *Biochemistry.* 31: 5983–5995.

Peters, R., J. Peters, K. H. Tews, and W. Bahr. 1974. Microfluorimetric studies on translational diffusion in erythrocyte membranes. *Biochim. Biophys. Acta.* 367:282–294.

Pisarchick, M. L., D. Gesty, and N. L. Thompson. 1992. Binding kinetics of an antidinitrophenyl monoclonal Fab on supported phospholipid monolayers measured by total internal reflection with fluorescence photobleaching recovery. *Biophys. J.* 63:215–233.

Shiffer, K. A., and S. R. Goodman. 1984. Protein 4.1: its association with the human erythrocyte membrane. *Proc. Natl. Acad. Sci. USA.* 81: 4404–4408.

Stout, A. L., and D. Axelrod. 1994. Spontaneous recovery of fluorescence by photobleached surface-adsorbed proteins. *Photochem. Photobiol.* In press.

Takakuwa, Y., G. Tchernia, M. Rossi, M. Benabadji, and N. Mohandas. 1986. Restoration of normal membrane stability to unstable protein 4.1-deficient erythrocyte membranes by incorporation of purified protein 4.1. *J. Clin. Invest.* 78:80–85.

Takeshita, K., R. I. MacDonald, and R. C. MacDonald. 1993. Band 4.1 enhances spectrin binding to phospha'idylserine vesicles. *Biochem. Biophys. Res. Commun.* 191:165–171.

Tamm, L. K. 1993. Total internal reflectance fluorescence microscopy. *In* Optical Microscopy: Emerging Methods and Applications. B. Herman and J. J. Lemasters, editors. Academic Press, San Diego. 295–337.

Thevenin, B. J.-M., and P. S. Low. 1990. Kinetics and regulation of the ankyrin-band 3 interaction of the human red blood cell membrane. *J. Biol. Chem.* 265:16166–16172.

Thompson, N. L., T. P. Burghardt, and D. Axelrod. 1981. Measuring surface dynamics of biomolecules by total internal reflection fluorescence with photobleaching recover or correlation spectroscopy. *Biophys. J.* 33: 435–454.

Tilton, R. D., A. P. Gast, and C. R. Robertson. 1990a. Surface diffusion of interacting proteins. Effect of concentration on the lateral mobility of adsorbed bovine serum albumin. *Biophys. J.* 58:1321–1326.

Tilton, R. D., C. R. Robertson, and A. P. Gast. 1990b. Lateral diffusion of bovine serum albumin adsorbed at the solid-liquid interface. *J. Colloid Interface Sci.* 137:192–203.

Tyler, J. M., B. N. Reinhardt, and D. Branton. 1980. Associations of erythrocyte membrane proteins. Binding of purified bands 2.1 and 4.1 to spectrin. *J. Biol. Chem.* 255:7034–7039.

Wang, D., S.-Y. Gou, and D. Axelrod. 1992. Reaction rate enhancement by surface diffusion of adsorbates. *Biophys. Chem.* 43:117–137.

Whatmore, J. L., E. K. Y. Tang, and J. A. Hickman. 1992. Cytoskeletal proteolysis during calcium-induced morphological transitions of human erythrocytes. *Exp. Cell Res.* 200:316–325.

Zimmerman, R. M., C. F. Schmidt, and H. E. Gaub. 1990. Absolute quantities and equilibrium kinetics of macromolecular adsorption measured by fluorescence photobleaching in total internal reflection. *J. Colloid Interface Sci.* 139:268–280.