Cytoskeletal Protein Binding Kinetics at Planar Phospholipid Membranes

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ABSTRACT It has been hypothesized that nonspecific reversible binding of cytoskeletal proteins to lipids in cells may guide their binding to integral membrane anchor proteins. In a model system, we measured desorption rates k_{off} (off-rates) of the erythrocyte cytoskeletal proteins spectrin and protein 4.1 labeled with carboxyfluorescein (CF), at two different compositions of planar phospholipid membranes (supported on glass), using the total internal reflection/fluorescence recovery after photobleaching (TIR/FRAP) technique. The lipid membranes consisted of either pure phosphatidylcholine (PC) or a 3:1 mixture of PC with phosphatidylserine (PS). In general, the off-rates were not single exponentials and were fit to a combination of fast, slow, and irreversible fractions, reported both separately and as a weighted average. By a variation of TIR/FRAP, we also measured equilibrium affinities (the ratio of surface-bound to bulk protein concentration) and thereby calculated on-rates, k_{on}. The average off-rate of CF-4.1 from PC/PS (~0.008/s) is much slower than that from pure PC (~1.7/s). Despite the consequent increase in equilibrium affinity at PC/PS, the on-rate at PC/PS is also substantially decreased (by a factor of 40) relative to that at pure PC. The simultaneous presence of (unlabeled) spectrin tends to substantially decrease the on-rate (and the affinity) of CF-4.1 at both membrane types. Similar experiments for CF-spectrin alone showed much less sensitivity to membrane type and generally faster off-rates than those exhibited by CF-4.1. However, when mixed with (unlabeled) 4.1, both the on-rate and off-rate of CF-spectrin decreased drastically at PC/PS (but not PC), leading to a somewhat increased affinity. Clearly, changes in affinity often involve countervailing changes in both on-rates and off-rates. In many of these studies, the effect of varying ionic strength and bulk concentrations was examined; it appears that the binding is an electrostatic attraction and is far from saturation at the concentrations employed. These results and the techniques implemented carry general implications for understanding the functional role of nonspecific protein binding to cellular lipid membranes.

INTRODUCTION

Much attention has been paid in the literature to the specific binding of cytoplasmic proteins to membrane anchor proteins (Bennett, 1990; Gilligan and Bennett, 1993; Nakao, 1990; Nelson, 1996). Cytoplasmic protein binding directly to lipids may also be biologically significant, both in its total amount and in its reversibility. Direct binding to phospholipids, even if weak, might be significant enough to increase the local concentration of cytoplasmic proteins at the membrane surface and/or induce lipid clustering. Direct binding to lipid may enhance the cytoskeletal network's ability to assemble, rearrange, and disassemble, thereby affecting the membrane's (and cell's) ability to deform (Zot et al., 1992). Lipid-protein interactions may even act as a driving force for such processes (Burn, 1988).

Binding of erythrocyte cytoskeletal proteins spectrin and protein 4.1 (henceforth 4.1) to biological membranes has been shown to be affected by their lipid content (Bazzi and Nelsestuen, 1991; Gascard et al., 1993; Sato and Ohnishi, 1983; Middelkoop et al., 1988; and Rybicki et al., 1988). Spectrin and 4.1 also bind to liposomes and monolayers of various compositions (Bitbol et al., 1989; Bonnet and Begard, 1984; Cohen et al., 1986, 1988; MacDonald, 1993; Maksymiw et al., 1987; Michalak et al., 1990, 1993, 1994; Mombers et al., 1980; Sato and Ohnishi, 1983; Shiffer et al., 1988; Sikorski et al., 1987). Moreover, 4.1 has been shown to enhance spectrin binding to phosphatidylserine vesicles (Takeshita et al., 1993).

Most of the literature cited above has been concerned with parameters of binding equilibria. However, little is known about the kinetics of spectrin and 4.1 binding to lipids. The individual kinetic rates rather than equilibrium concentrations might directly affect cell motility, possible enhancement of specific reaction rates by reduction of dimensionality at the membrane (Adam and Delbruck, 1968; Berg and Purcell, 1977; Wang et al., 1992; Axelrod and Wang, 1994), and mechanisms of two-dimensional signaling (Stossel, 1993). Some recent studies indicate that 4.1 binds reversibly to the cytoplasmic side of the erythrocyte membrane with an average desorption rate equal to 0.09 s^{-1} (Stout and Axelrod, 1994). The present work measures the equilibrium binding kinetics of spectrin and 4.1 to planar phospholipid bilayer membranes supported on glass. We find that both spectrin and 4.1 bind to PC and mixed (3:1) PC/PS planar membranes with a combination of reversible and irreversible (out to 800 s) fractions.

Most of the work here is based on the technique of total internal reflection/fluorescence recovery after photobleaching (TIR/FRAP) (Thompson et al., 1981, 1993), which directly measures off-rates (desorption) of protein/lipid membrane binding. The technique is further developed here in the calculation of on-rates. On-rates are a measure of the probability that a collision will lead to successful binding.

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THEORY

TIR/FRAP, kinetic rates, and equilibrium constants

In TIR/FRAP, the liquid within ~100 nm of a solid/liquid interface is selectively illuminated by an evanescent field of totally internal reflected laser light. A fluorescent solute at concentration A in the liquid is in equilibrium with its surface-bound form at concentration C. A brief intense TIR pulse photobleaches much of the surface-bound material, and subsequent exchange with unbleached fluorophore in the bulk is monitored in real time by the same but much dimmer probe TIR beam. The adsorption/desorption system can be modeled most simply by the chemical equation

$$A_U(t) + B \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} C_U(t) \quad (1)$$

where A_U is the equilibrium concentration (mol/cm^3) of unbleached free bulk molecules, here consisting of either spectrin or 4.1; B is the equilibrium surface concentration (mol/cm^2) of unoccupied surface binding sites on the lipid (which remains constant during the course of the photobleaching experiment); C_U is the equilibrium surface concentration of unbleached surface-bound spectrin or 4.1 (mol/cm^2); k_{on} is the on-rate (M^{-1} s^{-1}, also known as the forward, association, or adsorption rate constant); and k_{off} is the off-rate (s^{-1}, also known as the backward, dissociation, or desorption rate constant). By not including any spatial dependence in the concentrations, we are assuming that the reaction is slow compared to bulk diffusion (the "reaction limit"; see Thompson et al., 1981), and the bleaching area is large enough that surface diffusion plays no role. (These assumptions were tested experimentally.) Under these conditions, the recovery rate should be an exponential with a single rate k_{off} for a homogeneous sample with a one-step desorption process, and a multiexponential sum for a heterogeneous sample or for any sample with a multistep desorption process.

Although TIR/FRAP measures a kinetic rate k_{off}, chemical equilibrium holds for the total (bleached + unbleached) concentrations A and C of protein in the free and surface-bound states, respectively. The on-rate is

$$k_{on} = k_{off}(C/A)(1/B) \quad (2)$$

This on-rate represents the number of successful (i.e., leading to adsorption) collisions of the solute protein at the surface per second, per unit concentration of molecules in the bulk, per unit *concentration* of unoccupied surface sites, B. To calculate k_{on} from TIR/FRAP experimental results for k_{off} and C/A, we need to know B. Unfortunately, B is often difficult to determine accurately (as discussed in the Results) and is not really relevant for a lipid surface with its somewhat delocalized binding "sites." We therefore define a new representation of the on-rate,

$$k^*_{on} \equiv k_{on}B = k_{off}(C/A) \quad (3)$$

Rate k^*_{on} (in units of cm/s) gives the number of successful collisions per second, per unit concentration of molecules in the bulk, per unit *area* of the surface. Rate k^*_{on} is essentially the on-rate in a "unimolecular" model of the adsorption reaction:

$$A_U(t) \underset{k_{off}}{\overset{k^*_{on}}{\rightleftarrows}} C_U(t) \quad (4)$$

The ratio of surface protein concentration to bulk protein concentration (C/A) is called the "binding affinity" here. C/A has units of length (cm) and indicates the depth of bulk solution containing the same number of molecules as on the surface (per unit area). The binding affinity is a key parameter that can be measured directly by TIR/FRAP. Aside from enabling calculation of k^*_{on} from k_{off} as above, C/A is also used to 1) determine if the system is in the reaction limit (Thompson et al., 1981); 2) determine whether there is depletion of the bulk solute concentration (Mc Kiernan, 1995); and 3) calculate how much of the fluorescence measured is due to bulk fluorescence.

In principle, kinetic rates can be measured with TIR but without FRAP by stepping the bulk concentration A either up or down and monitoring the subsequent fluorescence relaxation toward a new equilibrium. In practice, this technique is slower than TIR/FRAP because of the time required for the solution changeover. Concentration jumping was not employed here, but conceivably it could confirm the values of the lowest kinetic rates measured. In the reaction limit, concentration jump experiments report the rate sum ($k_{on}A + k_{off}$), where A is the adsorbate bulk concentration after the jump. However, for the nonzero k_{off} values directly observed here by TIR/FRAP, for their corresponding k_{on} values deduced from C/A, and for the actual A concentrations used and a reasonable estimate of B, one can show that $k_{on}A \ll k_{off}$ here. Concentration jump methods would thereby provide direct information only about k_{off}, as does TIR/FRAP.

MATERIALS AND METHODS

Protein purification and labeling

Unless otherwise noted, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Human red cells (450 ml) were obtained from the University of Michigan Hospital Blood Bank in Ann Arbor. Red cells were immediately passed through a leukocyte filter (Pall Biomedical Products Corp., East Hills, NY) to remove white cells, and placed into four or five sterile 50-ml polystyrene centrifuge tubes and stored at 4°C. The proteins spectrin and 4.1 were extracted from human red blood cells (RBCs) with 1 M Tris and separated on a CL Sepharose 6B column (Ohanian and Gratzer, 1984). 4.1 was purified by salt gradient elution from a DEAE cellulose column. Spectrin fractions were then precipitated with saturated ammonium sulfate, $(NH_4)_2SO_4$, adjusted to pH 7.3 with tetrasodium EDTA. The spectrin solution was pelleted, resuspended, and dialyzed against buffer A (100 mM NaCl; 25 mM Tris, pH 7.6; 0.2 mM dithiothreitol; 0.1 mM EDTA). Spectrin was further separated into fractions of oligomers, tetramers, and dimers on a column of Sepharose 4B preequilibrated at 4°C with buffer A, and the pooled fractions were concentrated by ammonium sulfate precipitation. Spectrin dimers were used to obtain the results reported here. Each pellet was resuspended with 1.5 ml of buffer B

for dye labeling (100 mM NaCl; 5 mM NaP_i, pH 8.0) and dialyzed against this buffer overnight. 4.1 purification followed the protocol given by Stout and Axelrod (1994), with some modifications (Mc Kiernan, 1995).

Before fluorescence labeling, all proteins were centrifuged for 30 min at 27,200 × *g* at 4°C to remove any protein aggregates from solution. Protein concentrations were determined by measuring A_{280}, using extinction coefficients ϵ_{280} = 1.07 $(mg/ml)^{-1}$ $(cm)^{-1}$ for spectrin and ϵ_{280} = 0.8 $(mg/ml)^{-1}$ $(cm)^{-1}$ for 4.1 (Tyler et al., 1980). A 1.0 mg/ml solution of 5,6-carboxyfluorescein (CF) succinimidyl ester (Molecular Probes, Eugene, OR) in DMSO (dimethylsulfoxide) was added in a quantity sufficient for 2:1 molar ratio of dye:protein, and the mixture was incubated in the dark at 4°C for 1.5 h. After this time the mixtures were dialyzed for 2 days in the dark at 4°C against buffer C (100 mM KCl; 20 mM NaCl; 25 mM Tris, pH 7.6; 0.2 mM dithiothreitol; 0.1 mM EDTA), a buffer at physiological ionic strength. The final protein concentrations of CF-spectrin and CF-4.1 were determined by measuring the absorbance of the protein solutions at 280 nm and correcting for the contribution of the dye to the absorbance at 280 nm (Stout and Axelrod, 1994). Preparations with a final dye:protein ratio between 1.0 and 2.0 were used in the experiments reported here.

Supported lipid membrane preparation

Supported lipid membranes were made by exposing glass coverslips to a sonicated dispersion of phospholipid. Egg phosphatidylcholine (EPC) was purchased from Sigma (L-a-lecithin, 99% pure, Sigma no. P-2772) and stored at −20°C. Phosphatidylserine (PS) was purchased from Avanti Polar Lipids (Alabaster, AL) (L-a-phosphatidylserine, bovine brain extract, disodium salt, purity >99%, Avanti no. 840032) and stored at −20°C. NBD-PC was used as the fluorescent lipid in the focused spot photobleaching experiments for membrane characterization, and was purchased from Avanti Polar Lipids (Avanti no. 810121) (NBD = 1-oleoyl-2-[12-{(7-nitro-2–1,3-benzoxadiazol-4-yl)amino}dodecanoyl]).

Lipids (as commercially provided in chloroform solutions) were first dried onto the walls of a glass vial under a stream of nitrogen gas for several minutes. The dried lipids were then resuspended in 1 ml phosphate-buffered saline (PBS) buffer (2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 8.1 mM $Na_2HPO_47H_2O$, pH 7.6), gently vortexed, and transferred to a 5-ml plastic tube for 60–90 s tip sonication (30-s bursts) (Branson Sonifier, model 185; 20 kHz ultrasonic generator) on ice. Just before use, a final spin on a tabletop centrifuge removed any precipitates. The quantity of lipid used in the initial aliquots before resuspension was such that 1 ml of sonicated dispersion contained 1.5 mM EPC + 0.5 mM PS for the PC/PS dispersion and 2 mM EPC for the all-PC dispersion. For dye-labeled vesicle solutions, 2 mol% NBD-PC (0.4 mM) was added to the initial aliquots.

All glass coverslips (no. 1 thickness, both 25- and 33-mm diameters) were first cleaned by boiling in 5% Linbro 7× cleaning solution (Flow Laboratories, McLean, VA) for at least 45 min, rinsed in running deionized water, and finally rinsed three times with acetone and placed in a small laboratory oven at 110–150°C for 3 h to overnight to dry. Coverslips were allowed to cool for 45 min and then plasma-cleaned (model PDG-3XG; Harrick Scientific Corp.) for 1 min under argon gas just before immediate use. A 35-μl aliquot of lipid dispersion was placed on a 33-mm-diameter coverslip in the middle of a 60-μm-thick teflon ring (Nicholson Precision Instruments, Gaithersburg, MD), which was cut into two halves. A 25-mm-diameter coverslip was then gently placed on top of the lipid vesicle aliquot such that the teflon ring halves served as a spacer, with the gaps between the halves acting as an in-port and an out-port for washing the membrane that forms on each coverslip face (Fig. 1).

The coverslip assembly was placed in a humid environment for at least 45 min to allow lipid vesicles to fuse with the plasma-cleaned glass surfaces. The membrane assembly was then rinsed with 10 35-μl aliquots of PBS buffer to completely wash out any remaining vesicles by delivering PBS aliquots to the in-port and adsorbing the excess with a folded paper tissue at the out-port. The assembly was always washed with the final buffer to be used in particular TIR/FRAP protein/membrane experiments at least 10 times with 35-μl aliquots. The 11th 35-μl aliquot contained the labeled protein (spectrin or 4.1) at the bulk concentration specified for each experiment (depletion of bulk protein by adsorption to the surface was calculated to be negligible; Mc Kiernan, 1995). The coverslip assembly containing sample was then securely clamped to the flat floor of a custom-machined aluminum sample chamber with fiberglass tweezer tips. This chamber featured a hole in the bottom for inverted microscope viewing and a removable glass plate top that could be sealed "airtight" with vacuum grease to form a locally humid environment. Each sample was allowed to equilibrate in the humid environment for at least 15 min before data aquisition and as long as necessary for the measured surface fluorescence to reach a steady level (up to 35–50 min for the lower bulk protein concentrations).

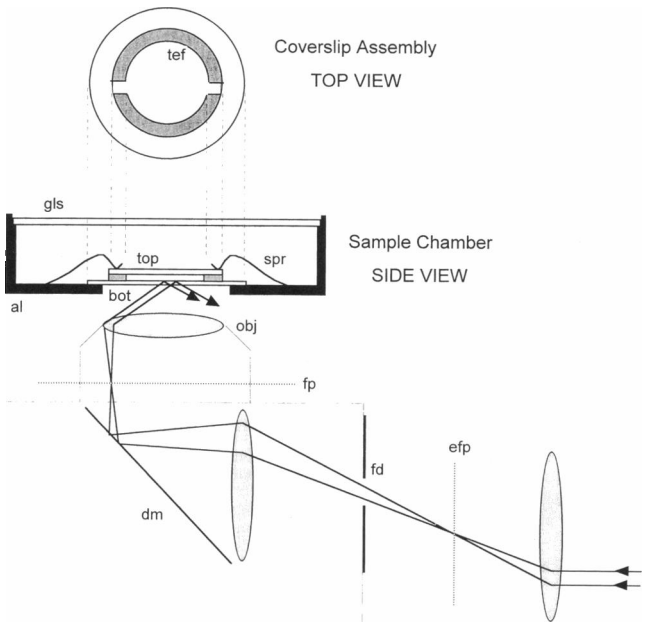

FIGURE 1 Optical configuration used here for TIR/FRAP on a supported planar lipid membrane (not drawn to scale). The membrane is formed on the walls of a glass coverslip sandwich with a 60-μm-thick slotted teflon spacer (tef), spring-clamped (spr) in an aluminum chamber (al). The top (top) and bottom (bot) coverslips are 25- and 33-mm-diameter circles. The top glass slide (gls) is removable but sealed in place with vacuum grease to prevent water evaporation. Part of the optical path used to produce prismless TIR is also shown. A standard epiillumination module with a dichroic mirror (dm) is used, but the objective (obj; Nikon 60×, oil immersion) must have a numerical aperture of 1.4 to produce TIR. The beam must be focused off-axis at an equivalent focal plane (efp) of the objective, so that the beam focuses off-axis at the actual focal plane (fp) and is thereby collimated at a fixed supercritical angle as it emerges from the objective. At the same time, the width of the beam must be fairly small at the center of the field diaphragm (fd), so that the illuminated region on the sample is intense enough to produce significant bleaching with a short-duration pulse. The beam itself is the first-order diffraction from an acoustooptical modulator (not shown).

Microscopy and data acquisition

TIR was achieved in an inverted fluorescence microscope (Leitz Diavert) by epiillumination (or "prismless" TIR) through the periphery of a high-aperture (Nikon 60×, NA = 1.4) objective (see Fig. 1), as previously described (Stout and Axelrod, 1989; Mc Kiernan, 1995). Other features of the photon detection and PC-based counting electronics have also previously been described (Stout and Axelrod, 1994). All FRAP experiments

were carried out with an argon laser (model 95 ion laser; Lexel) at 488-nm excitation. Typically, 200 prebleach and 800 postbleach observation points were collected per FRAP run. Data were collected on a new area of the membrane for each FRAP run, and a variable number of these (from 1 to more than 60) were signal-averaged into PC data files. The illuminated area was approximately a circle with a diameter equal to 58 μm (area $\approx$ 2600 μm^2), and at longer time scales the laser intensity was adjusted downward with the appropriate neutral density filters (NDFs) to avoid significant photobleaching during postbleach probe excitation. At the shortest time scale (1 ms), the approximate laser power incident upon the sample was 85 mW.

Desorption rates from TIR/FRAP

A TIR/FRAP data set consisted of all data files taken with a particular bulk concentration of labeled protein at a particular membrane composition, regardless of the sample time. Because the range of characteristic times for recoveries could extend over several orders of magnitude, it was necessary to combine data files with different bleach depths and sample times into one curve. To accomplish this, data files with different sampling times belonging to one data set were merged into a single large data file covering a wide range of time scales. The resultant merged file was then fitted to obtain protein desorption rates. We discuss here the merging and fitting procedures.

In merging two files at different time scales, the amplitude scale and offsets of inverted recovery curves (i.e., $1 - f(t)$, where $f(t)$ is the postbleach recovery curve normalized to the prebleach fluorescence) are adjusted so that the slopes and heights of the TIR/FRAP fitted functions are continuous at a time point that the two files share in common (see Stout and Axelrod, 1994; Mc Kiernan, 1995; for more details); here that time point is the midway point for the shorter time file. In the merged file, adjusted data points before that midway time come from the shorter time file and adjusted points after come from the longer time file.

The resulting inverted and merged data file was then fit to a double-exponential $g(t)$:

$$g(t) = g_1 \exp(-k_{1\text{off}}t) + g_2 \exp(-k_{2\text{off}}t) + g_3 \qquad (5)$$

After $g(t)$ is normalized such that $g(0)$ is set equal to unity, coefficients g_1 and g_2 give the population fraction of off-rates $k_{1\text{off}}$ and $k_{2\text{off}}$, respectively, and g_3 gives the irreversibly bound fraction ($k_{3\text{off}} = 0$). (This straightforward physical interpretation of $g_{1,2,3}$ is the main advantage for presenting results in their inverted form.) For simplicity in reporting results, a weighted average $\langle k_{\text{off}} \rangle$ is also calculated as $(g_1k_{1\text{off}} + g_2k_{2\text{off}})$. This $\langle k_{\text{off}} \rangle$ thereby implicitly includes the zero-rate contribution of the irreversibly bound fraction. The Windows-based graphics software program Origin (MicroCal) was used for all of the nonlinear least-squares fitting procedures.

Ideally, one might like to calculate from Eq. 3 the separate on-rates $k^*_{1\text{on}}$, $k^*_{2\text{on}}$, and $k^*_{3\text{on}}$ corresponding to each off-rate and then perform a weighted average of these rates to obtain $\langle k^*_{\text{on}} \rangle$. However, the numerical values and physical meaning of such separate rate can be ambiguous, depending on how many components are specified in the curve fitting (three here) and on whether the different components compete for the same free surface sites. An alternative is to calculate an average on-rate simply by plugging the average off-rate $\langle k_{\text{off}} \rangle$ into Eq. 3. The resulting $\langle k^*_{\text{on}} \rangle$ serves as a relatively robust semiquantitative figure for comparing one sample with another.

Binding affinity *C/A* from TIR/FRAP

TIR/FRAP can be used to measure not only kinetic rates but also equilibrium constants (Zimmermann et al., 1990) by relying on measurements of bleaching depth (i.e., the ratio of the difference between the prebleach and the immediate postbleach fluorescent count rate to the prebleach fluorescent count rate). Before the bleach pulse (time $t < 0$) in a TIR/FRAP experiment, the total prebleach fluorescence arises from both bulk and surface fluorescence. The fluorescence from the bulk originates from those molecules in the bulk that happen to be within the finite depth of the evanescent field. Immediately after a bleach pulse, both contributions become partially photobleached. But within a short time thereafter (time $t = 0+$), the bulk fluorescence recovers completely because the time it takes the labeled protein to diffuse in the bulk solution out of the evanescent field is much shorter than the bleach and sampling times. The bulk fluorescence thereby appears to be "unbleachable." By examining the apparent bleach depth as a function of the number of bleaching photons used (intensity times duration), we can derive an estimate of the surface to bulk concentration *C/A*.

In the extreme case of high photobleaching, all of the surface fluorophores will be bleached and the remaining fluorescence is all bulk fluorescence. The ratio of surface to bulk fluorescence before the bleach pulse can then easily be calculated. However, in actual experiments, we use a more moderate bleaching pulse that does not completely bleach all of the surface fluorescence. The measured bleaching depth observed on a protein/lipid sample containing a reversible part must then be compared to the bleaching depth on a sample in which the protein binding is entirely irreversible, with essentially no free bulk fluorescence. Such samples are made by adsorbing the CF-labeled protein onto bare cleaned glass (rather than planar lipid-coated glass) and washing extensively with buffer C. In this comparison, we assume that 1) the bleachability of the fluorophore attached to protein is the same whether the protein is immobilized on glass or bound to the lipid membrane; and 2) the number of bleach photons (proportional to some parameter K) is set to be equal in the two sets of experiments by adjusting the product of bleach durations and illumination intensities (set with NDFs). As discussed by Stout and Axelrod (1994), the fraction f_B of the reversible sample fluorescence originating from the bulk is

$$f_B = \frac{f(K) - f_S(K)}{1 - f_S(K)} \qquad (6)$$

where $f_S(K)$ is the ratio of the (surface-only) early postbleach fluorescence observed at time $t = 0+$ to the prebleach fluorescence at time $t < 0$, as measured on the irreversibly bound sample (protein on glass); and $f(K)$ is the corresponding ratio as measured on the reversible protein/lipid sample.

The bulk fraction f_B can be converted to the *C/A* ratio (to a good approximation) by integrating over the exponentially decaying depth of the evanescent field d (which is known from the incidence angle, the refractive indices, and the wavelength), giving the result

$$\frac{C}{A} = d\frac{(1 - f_B)}{f_B} \qquad (7)$$

RESULTS

We first present results of "control" experiments designed to 1) verify the presence of a supported lipid membrane; 2) check whether free dye adsorbs to the membranes; 3) check for bleaching flash-induced cross-linking artifacts; and 4) investigate the kinetics of CF-protein adsorption to bare glass. After that, we present the results for binding affinity (*C/A*) to the membranes, and finally, the main TIR/FRAP results for kinetic rates of CF-4.1 and CF-spectrin adsorption to the membranes. Unless otherwise noted, the bulk concentration of CF-4.1 and CF-spectrin in the results presented here was in the range of 0.9–1.1 μM in physiological ionic strength (buffer C).

Controls: characterization of the surface

To confirm the presence of a supported lipid membrane on the glass surface, we examined the lateral mobility of a

fluorescent lipid probe (NBD-PC) mixed with the PC or PC/PS using standard (non-TIR) spot FRAP. In principle, if lipid does not adsorb to the glass except for some adsorbed dye, or if the lipid does adsorb but not as a contiguous planar layer, then any fluorescence arising from surface-bound NBD-PC would show no FRAP recovery after photobleaching. However, the actual recovery curves showed that NBD-PC was mobile within both (PC and PC/PS) model membranes. The mobile fraction of NBD-PC for the PC membrane was ~100%, and for the PC/PS membrane it ranged from 70% to 90% (data not shown). In addition, the NBD-PC fluorescence on the surface appeared uniform, with no appearance of dark "holes," gaps, or visible irregularities.

To determine whether CF dye alone bound reversibly and significantly to phospholipid membranes, 3.2 μM CF dye (the same concentration used in the dye-protein complexes) was equilibrated with a PC/PS membrane in the sample chamber. TIR fluorescence intensity was only ~1/40 of that of CF-4.1 binding to the same type of membrane. Furthermore, TIR/FRAP experiments produced no bleach depth for any sample times or bleach durations on this sample.

As to be discussed, all samples showed some fraction of irreversible binding of protein to planar lipid membranes (as detected by an incompletely recovered FRAP curve in the time scale employed). This irreversibility appears not to be a bleaching flash-induced cross-linking artifact. First, the amount of irreversibility depends greatly on the protein/lipid combination, even with the same bleaching depth. Second, repeated bleaching in the same spot does not increase the apparent irreversible fraction, as would be expected from a photochemical artifact, but rather decreases it substantially, as irreversibly bound proteins are bleached and removed from the fluorescent pool early on. Third, increasing either the bleach intensity or the bleach duration by a factor of 2 shows at most a minor increase (less than 0.1) in the irreversible fraction in any case investigated.

A standard concentration of protein was equilibrated with a bare glass coverslip to determine if the behavior of the protein is sensitive to the presence of surface lipid. TIR/FRAP showed that the protein bound irreversibly to bare glass: there was no recovery after the photobleaching pulse and the TIR/FRAP "curves" were flat out to 800 s. In the presence of a planar lipid coating, however, a significant recovery is seen (discussed below).

Off-rates for 4.1

Fig. 2 shows two typical merged data sets for the postbleach recovery of CF-4.1 at PC/PS and PC membranes, plotted as the normalized inverted fluorescence, $g(t)$, on a logarithmic time scale. It is qualitatively clear from these "raw" data that 4.1 desorbs from the PC/PS membrane more slowly than from the PC membrane.

Fig. 3 shows the results of quantitative fits (to two exponentials + constant) of such merged data sets, giving the

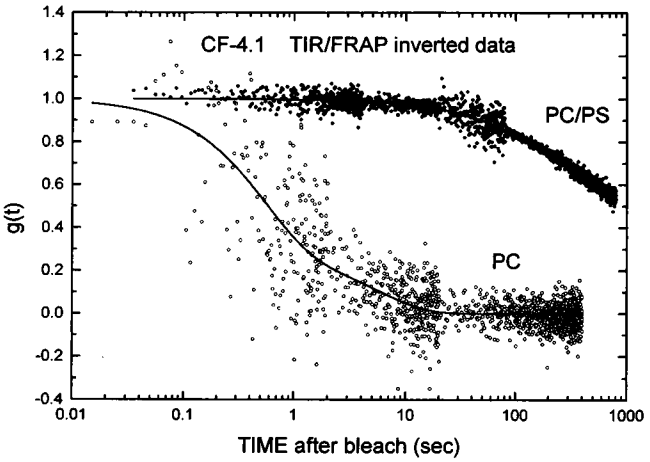

FIGURE 2 CF-4.1 (1.0 μM) merged data, shown as normalized inverted fluorescence $g(t)$ for both PC/PS (●) and pure PC (○) membranes supported on fused silica coverslips. In both, actual data are represented by circles, and nonlinear least-squares fitted curves are represented by solid lines that consist of two decaying exponentials plus a constant. Note that the binding at PC/PS is only slowly reversible with a significant irreversible fraction (out to 800 s), whereas the binding at PC has a much larger off-rate and complete reversibility. The merged data are derived from a total of 99 and 74 runs for the PC/PS and PC membranes, respectively. For the weaker binding at pure PC and at faster time scales, photon shot noise was significant. For the purpose of this figure only, the pure PC data points at times less than 2 s have been smoothed by a Savitzky-Golay sliding fit over five adjacent points. In the case of PC/PS, the original (premerged) data were taken (over N runs each) with sample times of 0.01 s (N = 4), 0.05 s (N = 12), 0.2 s (N = 4), 0.5 s (N = 64), and 1.0 s (N = 13). In the case of pure PC, the original data were taken with sample times of 0.01 s (N = 23), 0.05 s (N = 50), and 0.5 s (N = 1).

fraction of zero, slow, and fast off-rates for 4.1 binding to PC/PS (*filled circle*) and PC (*empty circle*) membranes. This figure shows that a substantial fraction of 4.1 binds irreversibly to PC/PS membrane out to 800 s, and a larger fraction binds with a slow off-rate rather than a fast off-rate. By contrast, very little 4.1 is irreversibly bound to the PC membrane, and the largest fraction consists of the fast off-rate component.

Increasing ionic strength significantly speeds the desorption kinetics. For PC/PS membranes at low ionic strength (buffer D; 10 mM Tris, pH 7.0, 0.2 mM dithiothreitol, 0.1 mM EDTA), the average off-rate (including a 0.9 irreversible fraction) was 0.0034/s (± 0.0006/s), whereas at physiological ionic strength (buffer C), the average off-rate (including a 0.32 irreversible fraction) was an order of magnitude faster at 0.040/s (± 0.002/s). The increase in off-rate with increasing ionic strength also holds for pure PC membranes, although all of the rates are much faster than for PC/PS: at low ionic strength, the average off-rate (including a 0.47 irreversible fraction) was 0.99/s (± 0.19/s), whereas for physiological ionic strength, the off-rate was doubled to 1.94/s (± 0.12/s).

Changing the CF-4.1 concentration in the bulk in several steps from 0.3 μM to 2.2 μM had no significant effect on the off-rates for either membrane type.

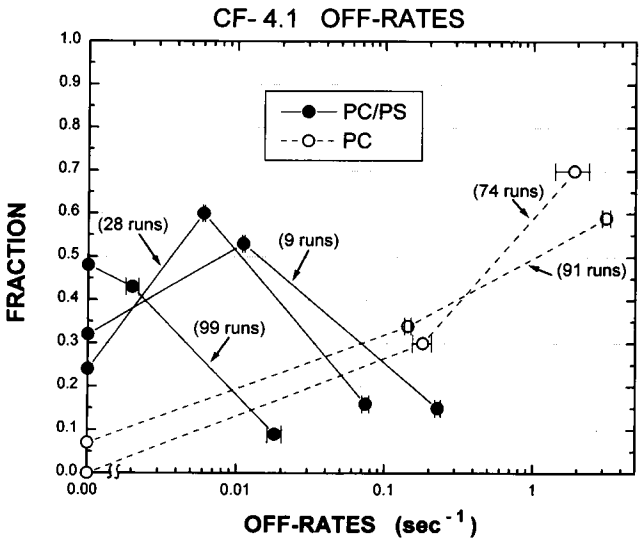

FIGURE 3 Parameters of the two-exponential plus a constant (Eq. 5) fits of merged data for CF-4.1 are shown here as points (k_{1off}, g_1), (k_{2off}, g_2), and (0, g_3). The lines connecting these points are intended only to pictorially link parameters arising from the same merged data set. The extreme left points on the ordinate axis correspond to the irreversible fraction with an off-rate of zero (note break in abscissa axis). Connected points/lines generally skewed to the left (as are the PC/PS runs; ●) arise from slower off-rates and larger irreversible fractions than do those skewed to the right (as are the PC runs; ○). The three separate merged data sets for PC/PS represent data taken at slightly different concentrations (all ranging between 0.9 and 1.1 μM) and different substrates (glass versus fused silica), but these differences are inconsequential. Likewise, the difference (also inconsequential) between the two PC merged data sets is in concentration (0.9 and 1.1 μM). The error bars in off-rates are standard errors calculated in the fitting procedure and originate from photon shot noise. The standard errors in amplitudes are generally smaller than the size of the points and are not shown.

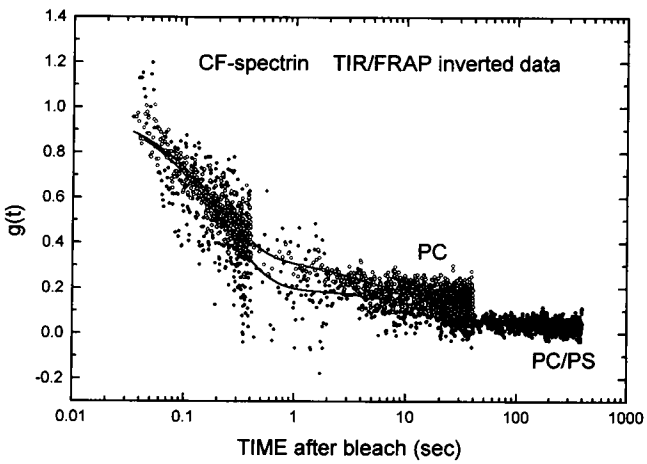

FIGURE 4 CF-spectrin (1.1 μM) TIR/FRAP merged data are shown here as normalized inverted fluorescence $g(t)$ for both PC/PS (●) and pure PC (○) membranes supported on fused silica coverslips. Actual data are represented by circles, and nonlinear least-squares fitted curves are represented by solid lines that consist of two decaying exponentials plus a constant. Note that the recoveries are relatively fast, and almost all of the binding is reversible (out to 400 s). The merged data are derived from a total of 338 and 369 runs for the PC/PS and PC membranes, respectively. To facilitate distinguishing the two closely overlapped data curves for this figure only, all of the data points have been smoothed by a Savitzky-Golay sliding fit over five adjacent points. In the case of PC/PS, the original premerged data were taken (over N runs each) with sample times of 0.001 s (N = 300), 0.02 s (N = 14), 0.05 s (N = 10), 0.1 s (N = 12), and 0.5 s (N = 2). In the case of pure PC, the original data were taken with sample times of 0.001 s (N = 300) and 0.05 s (N = 69).

Off-rates for spectrin

Fig. 4 shows two typical merged data sets for the postbleach recovery of CF-spectrin at PC/PS and PC membranes, plotted as the normalized inverted fluorescence, $g(t)$, on a logarithmic time scale. It is qualitatively clear from these "raw" data that CF-spectrin behaves somewhat similarly at both membrane types, unlike CF-4.1. A comparison with the corresponding curves for CF-4.1 (Fig. 2) shows that spectrin's off-rate from either PC/PS or PC is much higher than for CF-4.1 on PC/PS.

Fig. 5 shows the results of quantitative fits (to two exponentials + constant) of such merged data sets, giving the fraction of zero, slow, and fast off-rates for spectrin binding to PC/PS (*filled circle*) and PC (*empty circle*) membranes. It is clear from the plots that the fastest off-rates predominate, and very little spectrin is irreversibly bound to either membrane (data taken out to 400 s for PC/PS and 40 s for PC cases).

Increasing ionic strength had a much different effect on the desorption kinetics of CF-spectrin than it had for CF-4.1. For PC/PS membranes at both low or physiological ionic strength, the average CF-spectrin off-rate (including irreversible fractions) was either 3.7/s (± 0.5/s) or 3.6/s (± 0.8/s), respectively. But this average was achieved in very different ways: for low ionic strength, most of the binding was irreversible (0.74 fraction), with the remainder at a rather high off-rate (14/s ± 2/s), whereas for physiological ionic strength, only a small fraction was irreversible (0.04), but the majority showed a more moderate off-rate (4.0/s ± 1.0/s). On pure PC membranes, ionic strength had only a slight effect on CF-spectrin off-rates, but in the direction opposite that seen for CF-4.1. At low ionic strength, the average off-rate (including a 0.25 irreversible fraction) was 6.2/s (± 0.9/s) and at physiological ionic strength, and the average off-rate (including a 0.17 irreversible fraction) was 3.9/s (± 0.4/s).

Changing the CF-spectrin concentration in the bulk in several steps from 0.3 μM to 2.2 μM had no significant effect on the off-rates for either membrane type.

Equilibrium binding affinities and on-rates for 4.1, spectrin, and mixtures

From the bleaching depth analysis of TIR/FRAP (see Theory section), we measured the surface/bulk concentration (C/A) ratio at the bulk concentration A = 1.1 μM in physiological ionic strength (buffer C). The results are shown in Table 1 for both pure 4.1 and spectrin, and for mixtures, on both PC and PC/PS membranes. Similar experiments for

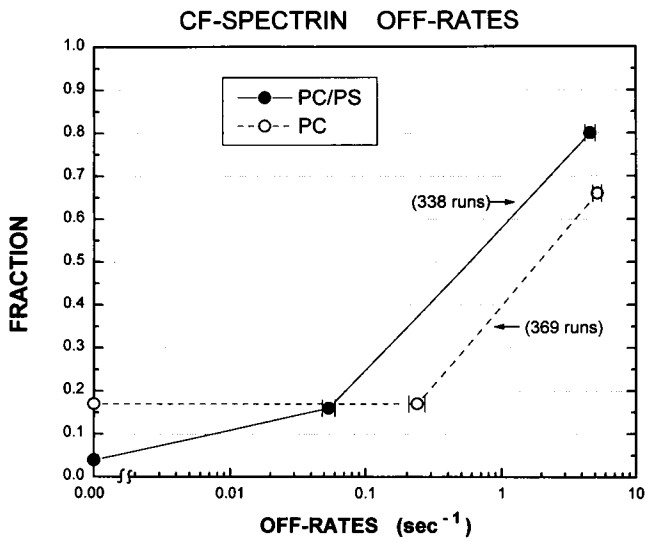

FIGURE 5 Parameters of the two-exponential plus a constant (Eq. 5) fits of merged data for 1.1 μM CF-spectrin are shown here as points (k_{1off}, g_1), (k_{2off}, g_2), and (0, g_3). The lines connecting these points are intended only to pictorially link parameters arising from the same merged data set. The extreme left points on the ordinate axis correspond to the irreversible fraction with an off-rate of zero (note break in abscissa axis). Both the PC/PS (●) and the PC (○) data are similarly skewed to the right, indicating a large proportion of faster off-rates. The error bars in off-rates are standard errors calculated in the fitting procedure and originate from photon shot noise. The standard errors in amplitudes are generally smaller than the size of the points and are not shown.

other concentrations in the A = 0.3–2.2 μM range, together with those reported in Table 1, showed no clear evidence of the binding approaching saturation, even at the higher concentrations; C/A increased linearly with A (although the results are noisy for spectrin, particularly at low A). As a consequence, it was not possible to obtain an estimate of the maximum number of surface binding sites for each protein from the binding isotherms. Note that C/A represents the combined effect of fast, slow, and irreversible components.

The separate fast, slow, and irreversible results for k_{off} shown in Fig. 3 (for 4.1) and Fig. 5 (for spectrin) can be combined into a single average $\langle k_{off} \rangle$ for each protein/membrane type. Together with the binding affinity C/A, this average $\langle k_{off} \rangle$ permits a simple calculation of an average on-rate $\langle k^*_{on} \rangle = \langle k_{off} \rangle (C/A)$, as discussed in the Theory section (see Eq. 3). Figs. 6 and 7 are bar graphs showing the results for the average on-rates $\langle k^*_{on} \rangle$ and off-rates $\langle k_{off} \rangle$ for CF-4.1 and CF-spectrin, respectively. Included are the results for mixtures of labeled 4.1 with unlabeled spectrin and vice versa. The magnitudes of $\langle k_{off} \rangle$ and $\langle k^*_{on} \rangle$ are given by the left and right ordinate axes, respectively.

TABLE 1 Equilibrium concentrations and binding affinities

Protein:membrane	C (10^{-15}) (mol/cm^2)	C/A (10^{-4} cm)
CF-4.1:PC/PS	97.8 (6.7)	0.89 (.06)
CF-4.1:PC	17.1 (2.0)	0.16 (.02)
CF-Spectrin:PC/PS	6.4 (1.9)	.058 (.017)
CF-Spectrin:PC	5.8 (0.9)	.053 (.008)
CF-4.1 + Spectrin:PC/PS	23.3 (1.6)	0.21 (.01)
CF-4.1 + Spectrin:PC	4.9 (0.6)	.044 (.005)
CF-Spectrin + 4.1:PC/PS	15.4 (4.6)	0.14 (.04)
CF-Spectrin + 4.1:PC	3.2 (0.5)	.029 (.004)

Numbers in parentheses are standard errors.

Fig. 6 shows that CF-4.1 has slow off-rates and on-rates to PC/PS membrane, but in the presence of unlabeled spectrin its on-rate decreases even further. For the PC membrane case, both the 4.1 off-rate and on-rate are higher than for the PC/PS case. However, in the presence of unlabeled spectrin, the on-rate of 4.1 again decreases. Fig. 7 shows that CF-spectrin on-rates and off-rates are similar for all cases except the PC/PS membrane in the presence of unlabeled 4.1. For this case both the on-rate and off-rate drop by one to two orders of magnitude.

Reaction limit, surface fluorescence dominance, bulk depletion, and surface diffusion

The C/A values have a significance not only for calculating on-rates but also for determining if the adsorption/desorption process is reaction-limited (Thompson et al.,

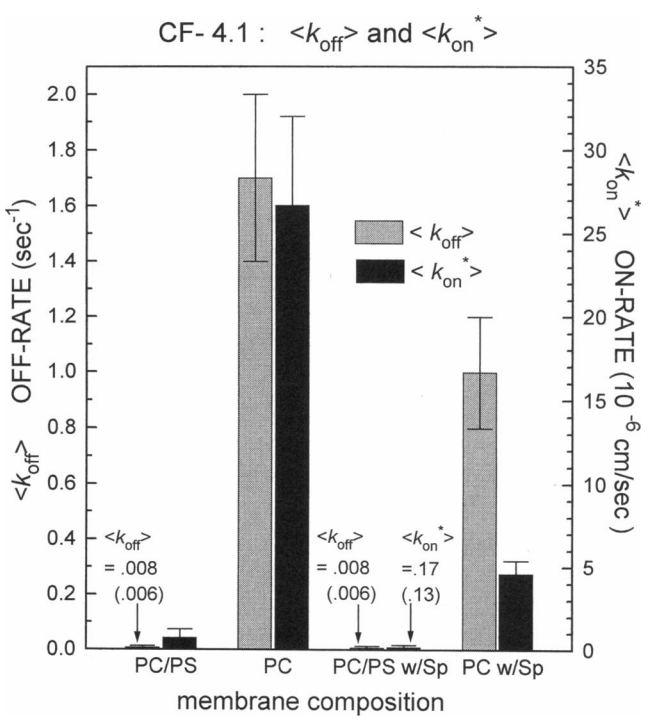

FIGURE 6 Average off-rates, $\langle k_{off} \rangle$, (including irreversible fractions) and on-rates, $\langle k^*_{on} \rangle$, for 1.1 μM CF-4.1 are shown for the indicated membrane compositions. For the two pairs of bars on the right, unlabeled spectrin (1.1 μM) was added to the CF-4.1 solution. The standard error uncertainties arise from a combination of photon shot noise and variations over different data sets. Note the differences in behavior of rates for PC/PS and PC membrane types.

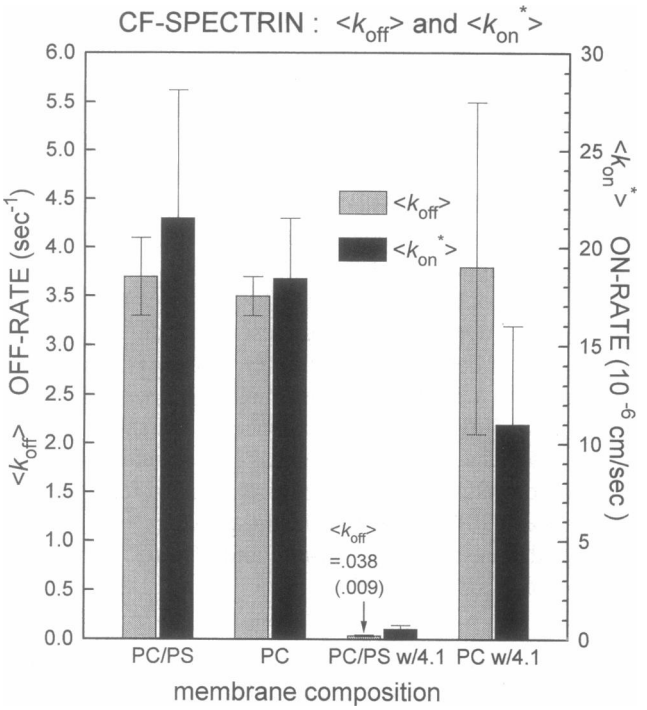

FIGURE 7 Average off-rates, $\langle k_{off} \rangle$, (including irreversible fractions) and on-rates, $\langle k^*_{on} \rangle$, for 1.1 μM CF-spectrin are shown for the indicated membrane compositions. For the two pairs of bars on the right, unlabeled 4.1 (1.1 μM) was added to the CF-spectrin solution. The standard error uncertainties arise from a combination of photon shot noise and variations over different data sets. Note the drop in kinetic rates at the PC/PS membrane in the presence of 4.1.

1981), so that the TIR/FRAP results indeed reflect off-rates (as assumed thus far) rather than bulk diffusion rates. *C/A* is the distance into the bulk that contains the same number of solutes as are surface-adsorbed in any given area. This is the distance through which unbleached solutes must diffuse rapidly enough to replace bleached molecules as they desorb. If the bulk diffusion time t_B through distance *C/A* is short compared to the shortest characteristic time of the TIR/FRAP recovery, then the process is indeed reaction-limited. Given the experimental *C/A* values in Table 1, it can easily be shown that all of the experiments were within the reaction limit by at least two orders of magnitude (Mc Kiernan, 1995).

C/A can also be used to verify whether the total TIR fluorescence originates primarily from the surface rather than the nearby bulk. The ratio *R* of bulk to surface fluorescence is $d/(C/A)$, where d is the evanescent field depth (estimated here to be 94 nm). From the *C/A* data of Table 1, *R* varies from ~0.1 (for CF-4.1 on PC/PS) to ~3.2 (for CF-spectrin + unlabeled 4.1 on PC). A dominance of bulk over surface fluorescence in some of the weaker binding cases does not invalidate the k_{off} results, because recoveries on the slow time scale seen can only arise from desorption kinetics rather than bulk diffusion. However, a high *R* does tend to bury the surface signal in the photon shot noise of the bulk fluorescence, thereby reducing statistical accuracy.

Given *C/A* values, it is also of interest to calculate whether the surface adsorption of protein to lipid membrane might have depleted the protein concentration in the bulk to a level significantly below what was introduced. This depletion can be shown to be negligible in these experiments, even for the strongest binding cases (Mc Kiernan, 1995).

Surface diffusion of adsorbed protein, even if it exists, is unlikely to have affected the fluorescence recoveries on the time scales observed here (maximum of 125 s) because of the large TIR illuminated spot size. With a spot radius of ~29 μm, a surface diffusion rate of 1×10^{-8} cm^2/s would give a recovery time no shorter than ~210 s.

DISCUSSION

Protein 4.1

We find that protein 4.1 kinetic behavior is greatly affected by the presence of PS lipid in the membrane: it binds for at least 125 s to membranes containing 25 mol% PS before desorbing, and binds for only 0.6 s (a decrease of a factor of 200) to the PS-free membranes. In addition, the irreversibly bound fraction of 4.1 is higher for PS-containing (42%) than for PS-free (4%) membranes. Because PS has a net negative charge, whereas PC is neutral at physiological pH, 4.1 binding to PS may be electrostatic, as other researchers have suggested (Cohen et al., 1988; Shiffer et al., 1988; Ben-Tal et al., 1996). This conclusion is supported by the reduced off-rates in low-ionic-strength buffer. It is possible that 4.1 binds to several PS molecules and might even gather them via lipid lateral diffusion, making the off-rate even slower than it otherwise might be (but in principle thereby reducing the maximum amount of 4.1 binding at saturation).

The large decrease in CF-4.1 on-rates associated with the presence of PS is surprising. The on-rate k^*_{on} is proportional to the probability p_{on} that a Brownian collision of the protein solute with the surface will lead to a binding event. A low on-rate reflects a low probability of successful binding per Brownian collision. This low probability could result from an electrostatic repulsion between the overall negatively charged 4.1 and the negatively charged PS. However, once a 4.1 overcomes this barrier, it may reorient so that its more positively charged region (30-kDa region) faces the membrane and binds specifically and strongly to PS, remaining bound for a relatively long time (125 s). At PC membranes, there may be less of a general repulsive barrier, but once that lower barrier is overcome, only weak attachments to PC sites form, with 4.1 remaining bound for only a short time (0.6 s).

The largest on-rate $\langle k^*_{on} \rangle$ among all of the sample combinations is for CF-4.1 binding to PC (~2.7×10^{-5} cm/s). Given an estimate of the number of those collisions per second per area (Axelrod and Wang, 1994), we can deduce that p_{on} is only ~1.8×10^{-8}. Therefore, the probability of successful protein adsorption per collision for any of our protein/lipid systems is very low.

When CF-4.1 is mixed with an equimolar amount of unlabeled spectrin, there is no significant change in the 4.1 off-rate, as directly measured by TIR/FRAP (Fig. 6). However, there is a clear decrease in the 4.1 on-rate for both PC/PS (by a factor of 4) and PC membranes (by a factor of 6), as inferred from corresponding changes in the equilibrium *C/A* ratio. Spectrin might reduce the 4.1 on-rate by blocking either potential 4.1 target sites on the membrane surface or a lipid binding site on 4.1 itself. The first alternative seems unlikely, because *C* versus *A* TIR/FRAP measurements with CF-spectrin on the membranes showed no evidence of saturation in the concentration range used here (Mc Kiernan, 1995). The second alternative seems more likely, particularly because it could explain the decreased on-rate of 4.1 at PC as well as PC/PS membranes, and because spectrin has been shown to bind directly to 4.1 (Tyler et al., 1980; Podgorski and Elbaum, 1985; Becker et al., 1990). The PS binding site on 4.1 is located on the 30-kDa peptide fragment (near the N-terminal end; Cohen et al., 1988), whereas its spectrin-binding site is located on the 10-kDa peptide fragment (near the C-terminal end) (Correas et al., 1986a; Discher et al., 1993). Nevertheless, the 10-kDa fragment contains many (almost 50%) charged amino acid residues, and most of those are basic (Correas et al., 1986b). Therefore, this spectrin-binding end could also bind PS in the absence of spectrin.

Another, more elaborate explanation might account for the observed decrease in the 4.1 on-rate in the presence of spectrin. 4.1 is thought to be a somewhat globular but elongated molecule with multiple flexible lobes (Shahrokh et al., 1991), and it is possible that 4.1 undergoes a conformational change upon binding spectrin, which renders its 30-kDa lipid-binding site less exposed for binding. Although there is no direct experimental evidence reported in the literature for such a conformational change upon spectrin binding, it has been suggested by several researchers (Chao and Tao, 1991; Horne et al., 1990) to explain the inhibitory effect of cAMP-dependent phosphorylation on the association of 4.1 with spectrin and actin. (The actual phosphorylation sites on 4.1 are not at the spectrin/actin-binding site but nonetheless inhibit the association with spectrin.) The similar drop in on-rate for the PC membrane may likewise indicate that spectrin may affect 4.1 conformation in such a manner that the PC binding "mode" or region becomes inaccessible.

Spectrin

In physiological salt buffer, the presence of PS in the membrane has only minor effects on spectrin kinetic rates, reversible fractions, or equilibrium concentrations and binding affinities. In contrast to 4.1 behavior, spectrin binding is relatively insensitive to PS. This result is surprising in view of other accounts of spectrin's binding preference for PS-containing lipid monolayers (Mombers et al., 1980).

Although average off-rates of spectrin were not much affected by the presence of PS or by physiological versus low-salt buffer in our TIR/FRAP experiments, the irreversibly bound fraction was strongly enhanced by PS in low-salt buffer only. A reasonable inference is that the irreversible binding of spectrin to PS is electrostatic, as previously suggested by Maksymiw et al. (1987). Those investigators claim that although spectrin has an overall net negative charge of 200 at physiological pH (Elgsaeter et al., 1976), spectrin is a very flexible molecule that could be highly folded along its length in such a manner as to expose its basic residues, and these basic residues would enable spectrin to electrostatically bind the negatively charged lipids. Their findings indicate that spectrin does not interact with lipid layers via the hydrophobic effect. In contrast, earlier studies had suggested that hydrophobic interactions do play a role (Mombers et al., 1977; Sweet and Zull, 1970).

Independent evidence of PS/spectrin binding comes from electron micrograph studies (Cohen et al., 1986), which showed that spectrin bound to PS vesicles at multiple sites along its length, including at the spectrin dimer tail end region. At higher spectrin dimer concentrations the micrographs showed that spectrin was able to cross-link PS vesicles (500-Å-diameter vesicles), suggesting that both ends of the spectrin dimer molecules have binding sites for PS (Cohen et al., 1986).

Although there is an abundance of literature describing the spectrin-PS interaction as being greater than the spectrin-PC interaction (Bonnet and Begard, 1984), there are other reports indicating no difference between spectrin-PS and spectrin-PC specificity. Bitbol et al. (1989) found that spectrin bound to both DMPC (dimyristoyl phosphatidylcholine) and DMPS:DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphoserine:dimyristoylphosphatidylcholine, 1:1, w/w), but spectrin had no effect on the conformation of the lipid headgroups, as studied by deuterium NMR spectroscopy. They conclude that spectrin does not penetrate into the membrane surface and probably associates with the lipids superficially. Sikorski et al. (1987) have also found no difference in spectrin's specificity for PS over PC. Their results are based on the amount of spectrin intrinsic fluorescence quenched by phospholipid suspensions.

When CF-spectrin is mixed with an equal molar amount of unlabeled 4.1, there is a dramatic 100-fold drop in the spectrin off-rate at the PC/PS membrane, but not at the PC membrane. However, this is accompanied by only a twofold increase in the amount of spectrin bound, as indicated by the measured *C/A* ratio. Therefore we conclude that the presence of 4.1 also reduces the on-rate of spectrin at the PC/PS membrane (by a factor of 50).

The binding enhancement of spectrin to the PC/PS membrane in the presence of 4.1 is in agreement with the results of Takeshita et al. (1993). These authors report a 10-fold increase in CF-spectrin binding to PS vesicles in the presence of 2 μM 4.1. However, only the amount of spectrin binding increased, whereas the affinity of spectrin binding did not increase.

Spectrin may be held on or near the membrane by 4.1. Michalak et al. (1990) noted that spectrin (from pig erythrocytes) may experience conformational changes upon binding to PS-containing lipid vesicles. Therefore, the ability of 4.1 to hold one end of a spectrin molecule near the membrane may also serve to shift more spectrins toward a conformation that allows stronger binding to PS lipids.

Comparison with 4.1 kinetics on erythrocyte membranes

Stout and Axelrod (1994) recently studied 4.1 binding kinetics to the cytoplasmic surface of flattened erythrocyte ghost membranes by TIR/FRAP. They found that a substantial portion of the 4.1 binding (41%) was reversible. Treating the erythrocyte membranes with trypsin or with anti-glycophorin C antibodies reduced the total amount of irreversibly bound 4.1. The authors therefore suggest that the reversible binding of 4.1 to erythrocytes represents direct binding to phospholipids. Indeed, the present study of 4.1 binding to model phospholipid membranes shows that most of the phospholipid binding is reversible. However, in light of the present results that 42% of CF-4.1 binding to PC/PS membrane is irreversible, it is possible that some of the irreversibly bound 4.1 at the erythrocyte membrane might be associated directly with phospholipids.

The measured 4.1 off-rate (reported below as reversible off-rates, not as total) at the erythrocyte membrane is faster than our measured off-rates at model PC/PS membranes. The reversible CF-4.1 off-rates at trypsin-treated erythrocytes averaged $0.09 \pm 0.03\ s^{-1}$, with a reversible fraction of 0.56 ± 0.10, whereas our results for PC/PS show an average reversible off-rate of $0.012 \pm .009\ s^{-1}$ (the error indicates variability among three data sets), with a reversible fraction of 0.58 ± 0.10. The faster off-rate at the erythrocyte submembrane may indicate that the binding to phospholipids in erythrocytes may be weaker (perhaps because of other peripheral proteins binding to lipids at the submembrane or because of restricted lipid lateral mobility, thereby making lipids less accessible to 4.1). The similarity in the reversible fraction of 4.1 binding supports the suggestion that much of the reversible binding of 4.1 to the erythrocyte membrane is directly to lipids.

Biological relevance

On the basis of the present results on model membranes, we consider here whether direct spectrin and 4.1 binding to phospholipids might be significant and functionally important at biological membranes. What is the amount of these proteins in the erythrocyte submembrane that might be bound to phospholipids? The answer depends on the fraction of the protein molecules that is actually available for binding to the phospholipids, a fraction that is unknown. Considering spectrin first, assume that only 1% of the 2.4×10^5 spectrins per cell (Liu and Derick, 1992; Lux and Palek, 1995) are available for phospholipid binding and that they are confined to a 10-nm-deep membrane-proximal layer. Then using our *C/A* value for spectrin at a model PC/PS membrane, one can estimate a phospholipid-bound concentration of 150 spectrins/μm^2 erythrocyte membrane. If the 1% estimate is too low, or if the available spectrins are highly concentrated in a thinner membrane-proximal region, then the surface density of phospholipid-bound spectrins will be even higher. A similar rough estimate for 4.1 (at 2.0×10^5/cell; Nakao, 1990) gives 1900 molecules bound/μm^2 erythrocyte membrane. It is therefore reasonable to suggest that a significant number of spectrin and 4.1 molecules may be bound directly to lipids in the erythrocyte membrane.

Significant spectrin and 4.1 reversible binding to lipids may have physiological functions. For elongated molecules like spectrin, "nonspecifically" tying down one end to lipid increases the effective concentration of the rest of the molecule proximal to the membrane, which may thereby increase reaction rates of the middle of the dimer with "specific" membrane target sites such as ankyrin. Alternatively, nonspecific binding to lipids coupled with surface diffusion along the lipids may also increase the reaction rate with specific sites, a mechanism usually referred to as "reduction of dimensionality" (RD) rate enhancement (Adam and Delbruck, 1968; Berg and Purcell, 1977; Wang et al., 1992; Axelrod and Wang, 1994). Calculations based on these models using the kinetic results presented here show that such surface diffusion along the lipids might enhance reaction rates if the concentration of available, unbound specific targets (ankyrin, band 3, or glycophorin C) is on the order of a few percent or less of their total respective cellular concentrations in the erythrocyte membrane (Mc Kiernan, 1995).

The presumption of three distinct components (fast, slow, and irreversible) for both 4.1 and spectrin binding at phospholipids is largely a matter of mathematical convenience; the actual binding may display more rates or even a continuum of rates. A range of rates rather than a single rate perhaps is to be expected, considering that 1) proteins can adsorb in different orientations; 2) they can partially unfold upon adsorption, exposing a variable number of attractive sites; 3) the adsorption or desorption process may consist of several serial steps; and 4) the target surface is quite fluid and variegated, with certain protein-binding lipid molecules (e.g., PS), possibly vacating some areas to form clusters of variable size under adsorbed proteins. In any case, multirate binding appears to be a ubiquitous feature of protein adsorption to planar lipid membranes (Thompson et al., 1993).

Apart from the specific question about reaction rate enhancement, the cytoskeleton and its interaction with the lipid membrane should be viewed as a continually changing dynamic system rather than a static structure. A growing class of proteins appears to interact directly, reversibly, and functionally with lipid membranes as well with specific protein targets (Burn, 1988; Zot et al., 1992; Lemmon et al., 1996). The equilibrium binding affinity of such proteins for

lipids obviously matters. The present work 1) shows experimentally that changes in the equilibrium binding affinity under a variety of conditions can arise from either alterations in the off-rate or in the on-rate (or both) and 2) provides a generally applicable experimental protocol for determining the contribution of each rate. Further detailed knowledge of the kinetics in both model lipid and cellular systems is likely to further our understanding of cytoskeletal dynamics in cells.

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