The cytosolic domain of T-cell receptor ζ associates with membranes in a dynamic equilibrium and deeply penetrates the bilayer

Interactions between lipid bilayers and the membrane-proximal regions of membrane-associated proteins play important roles in regulating membrane protein structure and function. The T-cell antigen receptor is an assembly of eight single-pass membrane-spanning subunits on the surface of T lymphocytes that initiates cytosolic signaling cascades upon binding antigens presented by MHC-family proteins on antigen-presenting cells. Its ζ-subunit contains multiple cytosolic immunoreceptor tyrosine-based activation motifs involved in signal transduction, and this subunit by itself is sufficient to couple extracellular stimuli to intracellular signaling events. Interactions of the cytosolic domain of ζ (ζcyt) with acidic lipids have been implicated in the initiation and regulation of transmembrane signaling. ζcyt is unstructured in solution. Interaction with acidic phospholipids induces structure, but its disposition when bound to lipid bilayers is controversial. Here, using surface plasmon resonance and neutron reflection, we characterized the interaction of ζcyt with planar lipid bilayers containing mixtures of acidic and neutral lipids. We observed two binding modes of ζcyt to the bilayers in dynamic equilibrium: one in which ζcyt is peripherally associated with lipid headgroups and one in which it penetrates deeply into the bilayer. Such an equilibrium between the peripherally bound and embedded forms of ζcyt apparently controls accessibility of the immunoreceptor tyrosine-based activation signal transduction pathway. Our results reconcile conflicting findings of the ζ structure reported in previous studies and provide a framework for understanding how lipid interactions regulate motifs to tyrosine kinases and may regulate the T-cell antigen receptor biological activities for this cell-surface receptor system.

The T-cell antigen receptor (TCR) is a multisubunit cell-surface protein complex composed of eight single-pass transmembrane subunits arranged in pairs as αβ-γε-δε-ζζ (Fig. 1). The γε and δε subunits carry the CD3 cell-surface epitope and are referred to by this name. (The homodimeric ζζ subunit carries the CD247 epitope but is not usually referred to by using this designation.) TCR signal transduction is triggered by interaction with MHC-peptide complexes on antigen-presenting cells. Variable and hypervariable regions on the extracellular antigen-binding domains of the TCR αβ subunits contain the binding site for MHC-peptide. CD3γ-δ, and ε possess extracellular immunoglobulin-like domains that form heterodimers and associate with TCRαβ. The ζζ subunits have very small extracellular portions and associate with the receptor complex primarily via transmembrane interactions. The activation mechanism is incompletely understood at the molecular level, but it is well established that extracellular MHC-peptide binding triggers cytosolic signaling pathways (1), with the first step believed to be phosphorylation, by Src-family kinases, of tyrosine residues on intracellular TCR immunoreceptor tyrosine-based activation motifs (ITAMs) of the general sequence, YXX(L/I)X_8YXX(L/I) (2). The cytosolic domains of CD3γ-δ, ε each contain one ITAM, and the cytosolic domain of ζ (TCRζζ) has three. ζcyt, by itself can initiate signal transduction.

The abbreviations used are: TCR, T-cell antigen receptor; ITAM, immunoreceptor tyrosine-based activation motif; POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1′-rac-glycerol); POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPS, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine; stBLM, sparsely tethered lipid bilayer membrane; SPR, surface plasmon resonance; NR, neutron reflectometry; βME, β-mercaptoethanol; EIS, electrical impedance spectroscopy; LUV, large unilamellar vesicle; LMPG, lysomyristoylphosphatidylglycerol; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; SAM, self-assembled monolayer; nSLD, neutron scattering length density; ζcyt, cytosolic domain of ζ.
processes, as shown with chimeric proteins on which ζcyt was fused to an unrelated extracellular domain (3) and used as a basis for chimeric antigen receptor cancer immunotherapy (4).

A schematic representation of the intact TCR(αβ)(γε)(δε) (ζζ) complex is depicted in Fig. 1, but its three-dimensional structure is unknown. However, structures have been determined for several sections (5–13), such as the isolated TCRαβ extracellular antigen-binding domains, both free (5) and MHC-bound (6). Structures of the extracellular domains of CD3γ, δ, ε and -e and TCRζ form substantial cytosolic domains that contain immunoreceptor tyrosine-based activation motifs (open circles).

Figure 1. The T-cell antigen receptor is formed by eight single-pass transmembrane proteins. Subunits TCRα and -β, which interact with extracellular MHC-peptide complexes, form a disulfide-bonded heterodimer. The heterodimers CD3γε and CD3δε associate with TCRαβ through their extracellular and transmembrane domains. A disulfide-bonded homodimer of ζζ-subunits interacts through its transmembrane domains with TCRγ. Structures of the extracellular antigen-binding domains, both free and MHC-present docking sites of the CD3 subunits to TCRbound(6). Structures of the extracellular domains of CD3γ, δ, ε and -e and TCRζ form substantial cytosolic domains that contain immunoreceptor tyrosine-based activation motifs (open circles).

CD3γ, -δ, -ε and TCRζ each have substantial cytosolic domains with 45, 45, 55, and 113 residues, respectively, that are unstructured in solution (18–20). The isolated cytosolic domains of ζ (19, 20) and CD3ε (20, 21), but not those of CD3γ and δ (20), bind to acidic lipids in lipid-detergent micelles (22), bicelles (21), and lipid vesicles (19, 20, 23) even in the absence of their transmembrane anchors. Whereas ζcyt is unstructured in solution, as shown by CD, NMR, and fluorescence assays (18, 19, 22, 24), it interacts with lipid vesicles or lipid-detergent micelles for some (19, 20, 22) but not all (23) lipid compositions, depending on lipid acidity, and such interactions induce helical structure in the peptide and can destabilize or fuse vesicles (23). Cationic clusters on CD3εcyt and ζcyt have been identified that are critical for membrane binding (21, 23, 25–27) and, thereby, the regulation of signaling processes, as binding may sequester key residues in the membrane, where they are inaccessible to cytosolic kinases and other signaling components (19, 21, 27). For example, an NMR structure of CD3εcyt bound to 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1’-rac-glycerol) (POPG)/dihexanoyl-phosphatidylcholine bicelles showed that the protein is peripherally bound with its ITAM tyrosine residues buried within the hydrophobic membrane interior (21).

In vivo, ζcyt binds to the inner plasma membrane as observed with FRET for chimeric protein in which the peptide was fused to the transmembrane and extracellular regions of CD2 or KIR2DL3 (irrelevant proteins in the context of MHC-TCR signaling) (25, 28). The cytosolic domain of TCRζ by itself is thus sufficient to transduce activation signals across the cell membrane (3, 29–31) and has been a focus of studies of the molecular basis of T-cell signaling (25, 32). However, the disposition of ζcyt in the membrane and its implications for kinase accessibility remain unknown. In this study, we use a planar bilayer model, the sparsely tethered lipid bilayer membrane (stBLM) (33), to investigate the membrane association of isolated ζcyt in thermodynamic and structural terms by surface plasmon resonance (SPR) and neutron reflectometry (NR), respectively. Molecular models based on these results visualize the localization of ζcyt relative to the membrane. A phosphorylation assay based upon recombinant Lck tyrosine kinase was used to study the sequestration of ITAM tyrosines within the bilayer. Taken together, these results show conclusively that the interaction of the ζcyt with the bilayer controls accessibility of signaling components and thus regulates an intermediate stage of signal transduction.

Results

Quantification of ζcyt membrane binding by SPR

In the stBLM sample format (Fig. 2), a phospholipid bilayer is tethered to a silicon-supported, atomically flat gold film via synthetic lipid anchors (34) that are spaced out by co-adsorption with β-mercaptoethanol (BME) and thus passivate the solid substrate, provide a hydrated submembrane space, and allow for native-like interaction of membrane proteins (33). Bilayers were of various compositions, either zwitterionic (pure 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC)) or acidic (POPC/POPG = 60:40 or POPC/1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS) = 70:30 or with cholesterol (DOPC/DOPS/cholesterol = 65:35:3), and bathed in HEPES buffer (20 mm, pH 7.4) at high and low ionic strength (25 or 150 mm NaCl). POPC in binary bilayers was investigated for comparison with previous vesicle adsortion studies (19, 20). On the other hand, POPC/POPS = 70:30 provides a better
representation of the inner cell membrane leaflet. Time-dependent SPR responses to \( \zeta_{\text{cyt}} \) incubation of such stBLMs were recorded for protein concentrations between 0.05 and \( \sim 23 \mu M \). SPR measurements were performed at 25.0 °C. While the bilayer membranes are in the fluid phase at 25.0 °C, as they would be at physiological temperature, the entropic barrier to binding is slightly higher at physiological temperatures. Therefore, apparent binding constants observed under our experimental conditions would be expected to be somewhat reduced, but the general behavior of the protein is not expected to be qualitatively different.

Fig. 3 shows representative SPR data. Binding of \( \zeta_{\text{cyt}} \) to pure POPC stBLMs was insignificant, even at 25 mm NaCl, where electrostatic interactions are minimally shielded (Fig. 3A). Binding of \( \zeta_{\text{cyt}} \) to POPG-containing membranes (POPC/POPG, 60:40) leads to a fast increase of the SPR signal after each injection, followed by an approximately exponential decay of the signal to a new baseline above that of the previous, lower concentration (Fig. 3B). This indicates that protein binds to the membrane in two distinct modes: an initial fast association followed by a slow process that does not reach equilibration during the course of the experiment. Extrapolated SPR response values, \( R_{\infty} \), at the end of each incubation are well described by a Langmuir binding model with dissociation constant (\( K_d \)) of 4.0 ± 1.4 \( \mu M \) (\( n = 2 \) measurements). SPR traces for the binding of \( \zeta_{\text{cyt}} \) to POPG-containing stBLMs (POPC/POPG, 70:30) showed a similar response (Fig. 3C), with a dissociation constant of \( K_d = 5.4 ± 2.2 \mu M \) (\( n = 3 \)). Adding cholesterol to a DOPC/DOPS membrane composition (DOPC/DOPS/cholesterol, 65:35:3) does not substantially alter either the observed binding behavior (Fig. 3D) or the apparent binding constant. The DOPC/DOPS/cholesterol data are best fit by a Langmuir binding model with \( K_d = 4.3 ± 0.2 \mu M \) (Fig. 3D), which is comparable with those measured for POPC/POPG and DOPC/DOPS membranes. At 150 mM NaCl, binding of \( \zeta_{\text{cyt}} \) to POPG-containing stBLMs is strongly reduced (Fig. 3E). Here, binding kinetics were considerably slowed down, low concentrations of \( \zeta_{\text{cyt}} \) caused small decreases of the SPR response, and increases in SPR signal were only observed at high \( \zeta_{\text{cyt}} \) concentrations (>6.7 \( \mu M \), \( n = 3 \)), yielding an estimated dissociation constant (\( K_d \)) of >25 \( \mu M \).

**Bilayer integrity**

In light of a previous report that \( \zeta_{\text{cyt}} \) may disrupt vesicles upon incubation (23), we monitored the integrity of bilayers in contact with the protein. Negative-stain transmission EM showed that POPC/POPG (60:40; total lipid 3 \( \mu M \)) vesicles were not affected in their morphology by 10 \( \mu M \) \( \zeta_{\text{cyt}} \) (data not shown). In addition, the hydrodynamic radii of these vesicles without and with 5 \( \mu M \) protein were indistinguishable, as reported by dynamic light scattering. As a more sensitive probe of membrane integrity, we used electrical impedance spectroscopy (EIS), which measures dielectric (insulating) properties and is a sensitive readout of bilayer quality in stBLMs (33). The EIS response has been modeled using an equivalent circuit yielding the capacitance of the intact membrane and the conductance and capacitance of parallel conductive pathways induced by protein interaction (33). Using this approach, we monitored the EIS signatures of POPC/POPS (70:30) bilayers before and after completion of an SPR experiment at which maximum surface coverage has been achieved (Fig. 4). We typically observed an increase in membrane conductance that was <5 \( \mu S \ cm^{-2} \). For comparison, the introduction of a pore-forming toxin like \( \alpha \)-hemolysin would lead to an increase of the membrane conductance of >100 \( \mu S \ cm^{-2} \), well below saturation surface coverage (35). We therefore conclude that our studies of \( \zeta_{\text{cyt}} \) on stBLMs report protein interaction with intact membranes.

**Structural characterization of membrane-bound \( \zeta_{\text{cyt}} \)**

NR is a well-established technique to determine interfacial structure in the normal direction of supported planar bilayers as time and ensemble averages of molecular conformations within the plane of the sample (36). Changes in bilayer structure upon protein incubation and the distribution of proteinaceous material along the bilayer normal are thus obtained. Using NR, we probed the structure of \( \zeta_{\text{cyt}} \) bound to stBLMs (POPC/POPG = 60:40 and POPC/POPS = 70:30) in buffers that contained 25 mm NaCl. Fig. 5 shows an exemplary set of reflectivity curves for a POPC/POPS stBLM before and after the addition of 15.8 \( \mu M \) \( \zeta_{\text{cyt}} \) in isotopically distinct buffers. Modeling established the corresponding neutron scattering length density (nSLD) distributions shown in the inset. Tables 1–3 contain median fit values and 68% confidence limits of the parameterized one-dimensional structural profiles that give rise to these nSLD distributions for three distinct NR experiments.

In 25 mm NaCl, the distribution of \( \zeta_{\text{cyt}} \), as measured with protein in the buffer, occupies the hydrocarbon region and headgroups of the substrate-distal bilayer leaflet (Fig. 6, A and C). Outside the bilayer, the peptide extends away from the membrane surface up to ~60 Å from the bilayer center. After rinsing with protein-free buffer, we observed significantly less bilayer-associated \( \zeta_{\text{cyt}} \), following bimodal distributions of proteinaceous material (Fig. 6, B and D) with one peak within the hydrocarbon chains and the other ~40–50 Å from the membrane center. In 150 mm NaCl, where electrostatic interactions are highly screened, the localization of membrane-bound \( \zeta_{\text{cyt}} \) depended on sample history. Protein incubation of the DOPC/DOPS bilayer

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**Figure 2. Molecular-scale illustration of the stBLMs used in studies of \( \zeta_{\text{cyt}} \) incubation by SPR and NR.** Synthetic lipids tethered to a 10-nm-thick gold film contain an oligo(ethylene oxide) segment that separates a lipid bilayer from the atomically flat gold surface, which is passivated by co-adsorption of \( \beta \)-ME. The hydrated space between the \( \beta \)-ME film and the bilayer avoids direct contacts between the membrane and the solid surface. This configuration is sufficiently resilient to withstand buffer exchanges by flowing solutions across the bilayer.
bilayer that was previously exposed to \( \xi_{\text{cyt}} \) in low-salt buffer and then rinsed (Fig. 6, C and D), resulted in a distribution in which the protein on the membrane surface was essentially removed but membrane-embedded protein was unaffected (Fig. 7A). Subsequent rinsing with protein-free high-salt buffer removed some of the membrane-embedded \( \xi_{\text{cyt}} \) (Fig. 7B). On the other hand, incubation of a pristine POPC/POPS bilayer with 15.8 \( \mu M \) \( \xi_{\text{cyt}} \) resulted in a layer of protein peripherally associated with the membrane (Fig. 7C), and rinsing with protein-free buffer removed most of this protein (Fig. 7D).

The protein distributions measured at low salt concentrations (Fig. 6) can be interpreted as a single homogeneous protein configuration in which one region in the protein intercalates the membrane and another region extends substantially
into the adjacent buffer phase. Alternatively, the same distributions would arise from two distinct protein populations of which one is membrane-inserted and a second one is peripherally associated with the membrane. The observation that rinsing with high-salt buffer removes the membrane-peripheral protein density but leaves the membrane-inserted density intact (Fig. 7, A and B) suggests that latter model is correct. This conclusion is supported by Fig. 8, in which we quantitatively compare the protein distribution profiles on POPS-containing membranes at low and high ionic strength. The difference (red trace) between the profiles in 25 mM NaCl (black) and after subsequent incubation with protein in 150 mM NaCl (green) is identical to the protein distribution profile after incubation of a pristine membrane with $\zeta_{cyt}$ at 150 mM NaCl (blue).

### Table 1

| Parameter | Neat bilayer | 15.8 $\mu M$ $\zeta_{cyt}$, 25 mM NaCl | Rinse, 25 mM NaCl |
|-----------|--------------|----------------------------------------|------------------|
| Substrate |              |                                        |                  |
| Thickness SiO$_2$ layer / Å | 16.1 ± 2.4 |                                  |                  |
| nSLD SiO$_2$ layer / 10$^6$ Å$^{-2}$ | 3.44 ± 0.08 |                                |                  |
| thickness Cr layer / Å | 26.1 ± 2.5 |                                    |                  |
| nSLD Cr layer / 10$^6$ Å$^{-2}$ | 2.96 ± 0.07 |                                |                  |
| Thickness Au layer / Å | 173.0 ± 0.9 |                                |                  |
| nSLD Au layer / 10$^6$ Å$^{-2}$ | 4.50 ± 0.01 |                                |                  |
| Bilayer |              |                                        |                  |
| Thickness tether / Å | 10.9 ± 0.2 |                                    |                  |
| Molar fraction of tether in the inner lipid leaflet | 0.65 ± 0.17 |                  |                  |
| Number of JME molecules per tether molecule | 2.2 ± 0.7 |                                  |                  |
| Hydrocarbon thickness, inner lipid leaflet / Å | 17.1 ± 0.4 | 15.7 ± 1.0 | 12.8 ± 0.4 |
| Hydrocarbon thickness, outer lipid leaflet / Å | 12.0 ± 1.1 | 11.6 ± 1.0 | 10.3 ± 1.0 |
| Bilayer completeness | 1.00 ± 0.01 | 1.00 ± 0.01 | 1.00 ± 0.01 |

### Table 2

| Parameter | Neat bilayer | 15.8 $\mu M$ $\zeta_{cyt}$, 25 mM NaCl | Rinse, 25 mM NaCl | Rinse, 150 mM NaCl |
|-----------|--------------|----------------------------------------|------------------|-------------------|
| Substrate |              |                                        |                  |                   |
| Thickness SiO$_2$ layer / Å | 16.2 ± 2.6 |                                  |                  |                   |
| nSLD SiO$_2$ layer / 10$^6$ Å$^{-2}$ | 3.52 ± 0.09 |                                |                  |                   |
| thickness Cr layer / Å | 34.5 ± 2.6 |                                    |                  |                   |
| nSLD Cr layer / 10$^6$ Å$^{-2}$ | 3.04 ± 0.02 |                                |                  |                   |
| Thickness Au layer / Å | 161.0 ± 0.5 |                                |                  |                   |
| nSLD Au layer / 10$^6$ Å$^{-2}$ | 4.48 ± 0.01 |                                |                  |                   |
| Bilayer |              |                                        |                  |                   |
| Thickness tether / Å | 10.8 ± 0.4 |                                    |                  |                   |
| Molar fraction of tether in the inner lipid leaflet | 0.59 ± 0.22 |                  |                  |                   |
| Number of JME molecules per tether molecule | 1.7 ± 0.8 |                                  |                  |                   |
| Hydrocarbon thickness, inner lipid leaflet / Å | 19.3 ± 1.4 | 18.7 ± 1.3 | 17.9 ± 1.3 |
| Hydrocarbon thickness, outer lipid leaflet / Å | 12.7 ± 1.3 | 12.2 ± 1.2 | 11.7 ± 1.2 |
| Bilayer completeness | 0.99 ± 0.01 | 0.99 ± 0.01 | 1.00 ± 0.01 | 0.99 ± 0.01 | 0.88 ± 0.02 |

### Figure 4

Cole–Cole plots and ECM fits to EIS data of a POPC/POPS (70:30) stBLM before and after SPR $\zeta_{cyt}$ binding measurements at concentrations between 0.05 and 23 $\mu M$ in 25 mM NaCl for 4 h (see Fig. 3C). The bilayer capacity increased insignificantly from 0.93(2) to 0.96(2) microfarads ($\mu F$ cm$^{-2}$), whereas its resistance decreased from 255(21)-kiloohms cm$^2$ exposure to 139(26) kiloohms cm$^2$ during the SPR measurements (standard deviations in parentheses). This indicates minor protein-induced defect formation in the bilayer but rules out large-scale bilayer reorganization. Similar results were obtained for other combined SPR/EIS experiments (not shown).

### Figure 5

NR data and best fits for an stBLM (POPC/POPS = 70:30) without protein and with 15.8 $\mu M$ $\zeta_{cyt}$ in two isotopically distinct bulk solvents (H$_2$O and D$_2$O-based buffer, 25 mM NaCl). The inset shows the nSLD profiles from which fits to the reflectivity were computed. These profiles are an intermediate step in the modeling process and represent the scattering properties of the material along the surface. They derive from a primary model that in turn represents the molecular architecture of the interfacial film in terms of the stBLM and protein, which is chemistry-based and referred to as a composition-space model (see Figs. 6–8).
Accessibility of ITAMs in membrane-bound ξ_cyt, to phosphorylation

To investigate the effects of membrane interaction on phosphorylation of ITAM tyrosine residues, we used recombinant Lck-G2A tyrosine kinase in a phosphorylation assay in which ξ_cyt was exposed to large unilamellar POPC/POPG vesicles (LUVs). These experiments included 6.5 mM Mg^{2+} in the assay buffer, as described previously (19), to allow MgATP-dependent tyrosine kinase activity (37); the increase in ionic strength relative to 150 mM NaCl alone (12%) might weaken but is not likely to substantially alter the nature of the ξ_cyt--membrane interaction. (Ca^{2+}) at high local concentration has been reported to promote release from the membrane of ITAM-containing cytosolic domains (37), but Mg^{2+} interacts with acidic membrane more weakly than Ca^{2+} (38). As shown in Fig. 9, Lck phosphorylation of ξ_cyt, ITAM tyrosines is reduced as the lipid concentration is increased. This decrease cannot be attributed to a lipid-dependent reduction of Lck catalytic activity, because a control experiment with an unrelated Fyn-substrate peptide does not depend on lipid concentration. In contrast to a previous study, which showed that ITAMs on ξ_cyt bound to lysomysterylphosphatidylglycerol (LMPG) micelles were completely protected from phosphorylation (19), we observed only a partial reduction that leveled off near 50% of the phosphorylation in the absence of lipid. Therefore, a fraction of tyrosine residues is still exposed to buffer, or there is a dynamic equilibrium between the membrane-embedded and exposed populations. No significant differences in the time courses of ξ_cyt phosphorylation were observed in the absence or presence of zwitterionic POPC LUVs (Fig. 10A), but substantial differences appeared in the presence of POPC/POPG LUVs, where phosphorylation kinetics slowed significantly at 0.5 mM lipid compared with lipid-free phosphorylation, and only very slow phosphorylation was observed for 7 mM lipid (Fig. 10B). A similar delay in phosphorylation was observed in the presence of pure POPG LUVs, but equivalent levels of phosphorylation were reached at all LUV concentrations after long exposure times to the Lck kinase (Fig. 10C).

Discussion

TCR ξ_cyt (25) and CD3ξ_cyt (21) interact with the inner leaflet of the plasma membrane. Such interactions inhibit ITAM phosphorylation by Src-family kinases (19, 21) and may control the accessibility of ITAM tyrosines in the cellular context (19, 21, 39), providing a regulatory mechanism for signal transmission. However, this hypothesis has been controversial, and mutations designed to disrupt membrane association can in some cases inhibit rather than potentiate tyrosine phosphorylation (25–27, 40, 41). This study was designed to shed light on this long-standing problem by investigating the membrane association of ξ_cyt with well-defined membrane models and to correlate structural and functional aspects of protein--membrane association under well-controlled experimental conditions.

ξ_cyt adopts distinct conformations at the membrane

The ξ_cyt--membrane interaction has often been viewed as a simple equilibrium involving a single bound species (19, 20). ξ_cyt is unstructured in solution but may adopt a helical structure in association with detergent micelles or acidic lipid vesicles, depending on detergent or lipid composition (19, 20, 22, 23). The SPR results in Fig. 3 confirm that ξ_cyt membrane binding is contingent on acidic lipids. Not surprisingly, binding to charged membranes depends on ionic strength of the aqueous buffer because of electrostatic shielding by counterions (42). Accordingly, the dissociation constants (K_d) of ~4–6 μM, measured in 25 mM NaCl increase to ~25 μM in 150 mM NaCl, which is a lower limit because full binding isotherms could not be determined due to limited amounts of protein. This is consistent with a sucrose-loaded vesicle pull-down assay at 100 mM KCl that determined a molar partition coefficient (K) of 2,200 M^{-1} for an equimolar mixture of POPC and POPG and K = 84,000 M^{-1} for 100% POPG (20). Although the interaction of isolated ξ_cyt is relatively weak, because the full-length ξ domain is membrane-associated and the local concentration near the membrane is therefore high, these results indicate a substantial and strong membrane interaction of the soluble domain by itself.

Examination of individual SPR traces in 25 mM NaCl after protein additions shows a slow decay that follows an initial spike (Fig. 3, B and C). Such a decay indicates a reduction of the refractive index of the interfacial layer, which may result from a degradation of the lipid bilayer (i.e. formation of water-filled defects) or bilayer thinning (i.e. rearrangement of the aliphatic chains within the bilayer upon protein adsorption), or the replacement of lipid in the bilayer by protein, which can outweigh the increase in refractive index due to peripherally bound protein. EIS results show that bilayer integrity is maintained (Fig. 4), and therefore membrane degradation is unlikely to contribute to the observed changes in SPR. We observe that bilayer thinning is minimal upon protein incubation, with an upper limit of 5% of the initial hydrocarbon thickness according.
to NR (Tables 1–3). This effect corresponds to an estimated reduction of 3–5 SPR response units at a protein concentration of ~16 μM, where we observe SPR signals of >30 units (Fig. 3). Membrane thinning is therefore a component of the observed reduction in SPR response, but too small to account for much more than 10% of the observed effects. The time courses of the SPR signals therefore most likely result from slow conformational changes of ζcyr following its adsorption to the membrane surface. We propose a process in which ζcyr initially binds peripherally to the membrane and converts to a second, membrane-embedded state thereafter, leading to a reduction in SPR response. NR corroborates this interpretation, as it shows proteinaceous material in two distinct distributions (Fig. 6, B and D): one within the membrane and another that extends from

Figure 6. Composition-space modeling results of NR data for stBLMs exposed to 15.8 μM ζcyr in buffers containing 25 mM NaCl. A and B, component volume occupancy (CVO) profiles of a POPC/POPG = 60:40 stBLM during protein exposure (A) and after rinse with protein-free buffer (B). C and D, CVO profiles of a POPC/POPS = 70:30 stBLM during protein exposure (C) and after rinse with protein-free buffer (D).

Figure 7. Composition-space modeling results of NR data for stBLMs exposed to 15.8 μM ζcyr in buffers containing 150 mM NaCl. A and B, CVO profiles during protein exposure (A) and after rinse with protein-free buffer (B) showing the same POPC/POPS = 70:30 stBLM that was initially exposed to ζcyr at 25 mM NaCl (Fig. 6). C and D, CVO profiles from a pristine POPC/POPS = 70:30 stBLM during protein exposure (C) and after rinse with protein-free buffer (D).
the membrane surface into the buffer, where it is selectively removed by rinsing (Fig. 7, A and B). We conclude that bound \( \zeta_{\text{cyt}} \) coexists in a membrane-embedded and in a membrane-peripheral state at low ionic strengths.

At physiological ionic strength (150 mM NaCl), \( \zeta_{\text{cyt}} \) interaction with the membrane is different; individual SPR traces did not show bimodal time courses (Fig. 3E), and membrane-bound protein does not penetrate the membrane, as shown by NR (Fig. 7, C and D). We conclude that soluble \( \zeta_{\text{cyt}} \) interacts with the membrane too weakly or resides at the membrane too briefly to form a membrane-embedded state. However, this is only true for the truncated cytosolic domain of the protein; hence, the situation may differ for full-length \( \zeta_{\text{cyt}} \) whose transmembrane segment ties the soluble protein region more tightly to the membrane.

Whereas \( \zeta_{\text{cyt}} \) inserts deeply into the bilayer under conditions where it interacts strongly with the membrane, EIS shows that bilayer integrity is not compromised (Fig. 4). In addition to functional relevance of the membrane-inserted state as a regulator of signal strength (see below), membrane insertion may also play a role in sensing or regulating local lipid curvature (43) or in intracellular sorting or trafficking (44, 45).

**TCR\( \zeta_{\text{cyt}} \) membrane association**

Our studies of \( \zeta_{\text{cyt}} \) phosphorylation (Figs. 9 and 10) correlate protein structure on the membrane with function and provide insight into the kinetics of the distinct protein conformations. We considered distinct kinetic models to interpret the data. First, we assumed that ITAM tyrosines of both peripherally bound and membrane-embedded \( \zeta_{\text{cyt}} \) populations were accessible to phosphorylation at different rate constants, such that the time course for \( p_{\zeta_{\text{cyt}}} \) formation is a sum of two exponentials. Alternatively, only the peripherally bound \( \zeta_{\text{cyt}} \) population might be accessible to phosphorylation, such that \( p_{\zeta_{\text{cyt}}} \) follows a single exponential with a rate constant similar to that in the absence of lipids. Here, \( p_{\zeta_{\text{cyt}}} \) is limited by the number of exposed ITAM tyrosines, and due to inaccessibility of membrane-embedded protein, the final phosphorylation level will be lower than in the absence of lipids. Finally, we considered a dynamic equilibrium between membrane-embedded and peripherally bound \( \zeta_{\text{cyt}} \) where only the latter is accessible to phosphorylation. However, because phosphorylation of sur-

Figure 8. Comparison of protein distribution (CVO) profiles from NR experiments on POPC/POPS = 70:30 stBLMs exposed to 15.8 \( \mu \)M \( \zeta_{\text{cyt}} \) in buffers containing 25 mM and 150 mM NaCl. Subsequent incubations with \( \zeta_{\text{cyt}} \) in 25 mM NaCl (black trace; Fig. 6C) and 150 mM NaCl (green trace; Fig. 7A) led to the removal of the membrane-peripheral fraction of protein (red trace; difference plot), whereas the membrane-embedded fraction remained. An independent protein incubation of a pristine stBLM at 150 mM NaCl yields a membrane-peripheral CVO profile (blue trace; Fig. 7C) that is quantitatively consistent with the difference plot (red trace).

Figure 9. Phosphorylation of \( \zeta_{\text{cyt}} \) (2 \( \mu \)M; squares) and Fyn-substrate peptide (500 \( \mu \)M; triangles) as control by Lck as a function of lipid concentration (LUVs; POPC/POPG = 60:40). Circles indicate Lck without substrate.

Figure 10. Phosphorylation of \( \zeta_{\text{cyt}} \) (2 \( \mu \)M) in the absence of lipids (open squares) and, shown with filled symbols, in the presence of LUVs composed of POPC (A), POPC/POPG = 60:40 (B), and POPG (C). Error bars, S.D.
TCRζ<sub>cyst</sub> membrane association

Figure 11. A, primary sequence of ζ<sub>cyst</sub> with two extra N-terminal amino acid residues (Gly and Ser) resulting from the thrombin recognition site. Arginine and lysine residues are highlighted in blue, and key residues of ITAMs (tyrosine, leucine/isoleucine) are shown in magenta. B, hydropathy scores of the Kyte/Doolittle (48) (black line) and Wimley/White (49) (blue line) hydrophobicity scales and secondary structure prediction based on amino acid sequence. Shown are locations of predicted α-helices with 0% (C), 25% (D), and 38% (E) overall helical content used to construct molecular models.

Face-bound ζ<sub>cyst</sub> reduces the interaction with acidic lipids (19, 20), this step is effectively irreversible.

\[
\frac{k_1}{k_{-1}} \xrightarrow{\text{membr}} \frac{k_2}{k_{-2}} \xrightarrow{\text{surf}} p_{\text{surf}}
\]

**Reaction 1**

If the sum of rate constants for interconversion of the surface-associated and membrane-inserted ζ<sub>cyst</sub> conformations \((k_1 + k_{-1})\) is similar to the phosphorylation rate \((k_2)\), a time lag in the formation of \(p_{\text{cyst}}\) is expected. Whereas our experimental phosphorylation data are at odds with the first two models, the observed delay in ζ<sub>cyst</sub> phosphorylation in the presence of acidic vesicles (Fig. 10, B and C) supports the latter, as it is reasonable to assume that the membrane-embedded conformation of ζ<sub>cyst</sub> is less susceptible to tyrosine phosphorylation. Therefore, it is likely that the two conformations are in a dynamic equilibrium, such that interaction of ζ<sub>cyst</sub> with the membrane does not fully abolish phosphorylation but rather controls the reaction rate. The idea of a dynamic equilibrium between a membrane-associated and free state has also been suggested for the cytoplasmic domains of full-length CD3ε (41).

**A molecular model of the membrane-bound conformations of ζ<sub>cyst</sub>**

Molecular models for membrane-associated ζ<sub>cyst</sub> consistent with the NR results took three considerations into account. First, ζ<sub>cyst</sub> is an unstructured protein in aqueous solution, but interaction with lipid vesicles can induce helicity (19, 20, 23); in micellar solutions, ~35% of the protein was α-helical as observed by CD spectroscopy (22). Second, regions of ζ<sub>cyst</sub> that interact with acidic lipid headgroups have been mapped using single amino acid substitutions in the full-length ζ<sub>cyst</sub>, its cytoplasmic domain (27), and peptide fragments of ζ<sub>cyst</sub> (23, 27). These studies revealed basic-rich sequences (Fig. 11A) (25) at residues Lys<sup>40</sup>—Arg<sup>44</sup>, Lys<sup>51</sup>—Lys<sup>55</sup>, and Lys<sup>80</sup>—Lys<sup>87</sup>. Third, phosphorylation of ITAM tyrosines is blocked in LMPG micelles (19), suggesting sequestration of these residues into the hydrophobic cores. Accordingly, we designed molecular models with different levels of helicity in which tyrosine hydroxyls were sequestered by the membrane and most of the Lys and Arg side chains were in contact with phosphates or carboxylates of lipid headgroups. However, we were unable to generate membrane-embedded models consistent with the scattering profiles in which all Lys and Arg side chains of ζ<sub>cyst</sub> were outside the bilayer. Therefore, the final models contain 4–7 buried Arg and 4–6 buried Lys residues. Because of the high energetic cost to immerse an arginine residue into a lipid bilayer, estimated at ~60 kJ/mol (46), such models might seem unfavorable. However, TCR transmembrane domains in close proximity within the intact receptor are replete with buried acid and basic residues, including Lys-Asp, Lys-Glu, and Arg-Asp salt bridges, with an excess of acidic residues (16). Moreover, computer simulations suggest that transferring multiple Arg residues into lipid bilayers is energetically nonadditive (47), so that the energetic penalty of burying multiple Arg sites might be lower than anticipated from single amino acid transfer studies and could be partially compensated by negative transfer energies for neighboring Leu, Ile, and Val residues. We did not explicitly account for any potential temperature- or salt-dependent alterations in bilayer structure or protein stability, but these are not expected to be significant for the conditions investigated.

Potential helical peptide regions were assigned, as shown in Fig. 11, based upon hydropathy scores of the Kyte/Doolittle (48) and Wimley/White (49) hydrophobicity scales as well as secondary structure analysis (50) and guided the design of three models with different helical contents (Fig. 11, C–E) with 0, 25, and 38% helicity. Scaled three-dimensional models of ζ<sub>cyst</sub> with these properties were placed into a mixed lipid bilayer with their N-terminal residues near the membrane surface and basic-rich sequence residues preferentially in contact with lipid headgroups. Depending on whether the models were designed
to conform to the NR-derived protein distribution profiles at 150 mM NaCl within or outside of the bilayer (Fig. 7, A and B), ITAMs and their neighboring residues were either membrane-immersed or membrane-peripheral, as depicted in Figs. 12 and 13, respectively. For the membrane-embedded ζ cyt model, this implies that the central stretch of the peptide chain is located near the membrane surface, whereas overall, the protein may be anchored to the bilayer by its hydrophobic flanks (N terminus to residue 30 and residue 90 to C terminus), which may penetrate the bilayer deeply. Fig. 12 (A–C) shows representative projections of the protein models with different helical contents. NR protein distribution profiles were calculated for these models and compared with the experimental data (black) in 25 mM NaCl.

Figure 12. A–C, ζ cyt models with distinct helical contents (0% (A), 25% (B), and 38% (C)) embedded in a lipid bilayer. α-Helical segments are shown in red, key ITAM residues (Tyr and Leu/Ile) in magenta, and Arg/Lys side chains in blue. D, NR protein distribution profiles calculated for these models are compared with the experimental data (black) in 25 mM NaCl. The color code is as in Fig. 12.

Figure 13. ζ cyt models with distinct helical contents (0% (A), 25% (B), and 38% (C)) peripherally bound to a lipid bilayer. Sections in the ζ cyt-sequences arranged into α-helices are highlighted in red. D, NR protein distribution profiles calculated for these models are compared with the experimental data (black) in 25 mM NaCl. The color code is as in Fig. 12.

Figure 13D shows that models of the membrane-embedded ζ cyt population agree well with the experimental data, independent of their helical content. In the surface-associated ζ cyt configurations (Fig. 13), the protein is also ligated to the bilayer by its hydrophobic flanks, but its hydrophilic central stretch forms loops that stretch out some 40 Å from the membrane surface, thus exposing the ITAM tyrosines to the aqueous compartment, where they are easily accessible to phosphorylation by Src family kinases. Again, reconstructed nSLD profiles of proteins with distinct secondary structures, although indistinguishable, all capture the experimental result quite well. Although these simple models are not unique, and thus not conclusive, they illustrate that peptide configurations that satisfy known constraints in conformational space can rationalize the NR results (Fig. 13D) and phosphorylation data. It will require more elaborate molecular dynamics simulations to quantify the underlying structures of the ζ cyt protein near membranes more stringently, explore the energetic landscapes that govern the distinct association modes, and thus define the conditions that control interconversion between coexisting ζ cyt conformations.

Conclusions

In this work, we presented an unconventional approach to quantify the role of protein–membrane interactions in regulating the accessibility of ITAM tyrosines, and thereby their phosphorylation, on the cytoplasmic ζ domain of the T-cell receptor, a process that is at the root of signal transduction in T-cell signaling. Although the impact of ζ cyt phosphorylation and its variation though membrane interactions have been extensively studied, mostly in the cellular context (25, 27), consensus on these processes has not been established due to contradicting results from distinct experiments (19, 26, 51, 52). In vitro experiments that reconstruct significant aspects of the ζ cyt signaling cascade may help to elucidate this problem (51, 52). Here we took the radical approach to study the soluble ζ cyt domain in the extremely simple but well-controlled experimental environment of tethered synthetic bilayers probed by SPR and NR. This reductionist approach necessarily engenders certain compromises: we used soluble ζ cyt, whereas in the native protein, this domain is covalently attached to a transmembrane span; we modulated protein-lipid interaction by variation of solution ionic strength and lipid bilayer composi-
TCR<sub>c</sub> membrane association

tion, whereas in the physiological cellular environment, the composition of aqueous and membrane phases are complex and dynamic; and we performed SPR and NR at subphysiological temperature because of instrumental constraints. Nonetheless, despite these caveats, we were able to gain insight into basic features of the ζ<sub>c</sub>–membrane interaction.

The results presented here lead to a more complex concept of ζ<sub>c</sub>–membrane interactions than discussed previously. We determined the affinities of (soluble) ζ<sub>c</sub> to acidic membranes to be rather low, in the micromolar range, at low ionic strength and to drop further at physiological salt concentration. Despite this relatively weak interaction, in its native environment, we expect that the ζ<sub>c</sub> component of the polytopic membrane-spanning TCR complex will be membrane-associated, as attested to by the observation of ζ<sub>c</sub>–membrane interaction in living cells (25, 28). The time courses of SPR adsorption traces indicated that the protein undergoes large-scale reconfiguration following adsorption to acidic membranes. The observation that the SPR signal drops slowly after a fast initial rise suggests, after exclusion of more trivial processes, that the protein inserts into the membrane. Indeed, corresponding phosphorylation assays with Lck showed that the ITAM accessibility of soluble ζ<sub>c</sub> is severely compromised in the presence of membranes. Two distinct membrane association states of ζ<sub>c</sub>, one deeply inserted and one peripherally bound, were also detected and characterized with NR, and it is tempting to assume that they are in a dynamic equilibrium at the membrane, as suggested by the phosphorylation assay. Both structures of the ζ<sub>c</sub>–membrane complexes clearly bear the signatures of molecular dimensions: one in which the central part of the protein that holds the ITAMs is membrane-inserted and one in which it is solvent-exposed. Even if we could not discriminate between distinct models with widely varying helicities, these results show clearly that the two association states must be grossly distinct in their functional roles; whereas the ITAM tyrosines are deeply buried within the membrane in the inserted state, they are exposed and thereby accessible to kinase activity in the membrane-peripheral state.

Experimental procedures

Materials

[γ-<sup>32</sup>P]ATP (3,000 Ci/mmole) was from PerkinElmer Life Sciences. Fyn kinase substrate (EGFTYGTLKSKK) was obtained from Enzo Life Science. POPC, POPG, and POPS were from Avanti Polar Lipids.

Protein expression and purification

The cytoplasmic domain (Lys<sup>51</sup>–Arg<sup>164</sup>) of human TCRζ (NCBI accession number NP_000725.1) was cloned in pET32a(+) (Invitrogen) and expressed as a thrombin-cleavable thioredoxin/His<sub>6</sub>-tagged protein in <i>Escherichia coli</i> (strain BL21(DE3)) at 37 °C, as described (20), but with an additional purification step. Bacteria were harvested by centrifugation 4 h after induction with 1 mM isopropyl 1-thio-β-D-galactopyranoside and lysed under denaturing conditions using lysis buffer (10 mM Tris, 100 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 8, 8 M urea). Lysate was stirred for 12–16 h at 4 °C and centrifuged at 10,000 × g. Supernatant was loaded onto Ni<sup>2+</sup>-nitrilotriacetic acid-agarose (Qiagen) preequilibrated with lysis buffer. Protein was eluted by stepwise reduction of the pH (pH 6.3, 5.7, and 4.5). Fractions containing ζ<sub>c</sub> were pooled and dialyzed against 20 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM DTT. Protein was digested with thrombin (MP Biomedicals, LLC) at 22 °C for 1 h in the presence of 5 mM CaCl<sub>2</sub> and 1 mM DTT. Digest was stopped by PMSF (0.5 mM); diluted with 50 mM MES, pH 6, to a final NaCl concentration of 34 mM; and loaded on cation exchange media (POROS HS20). ζ<sub>c</sub> elutes with a linear gradient of 120–360 mM NaCl. Fractions containing ζ<sub>c</sub> were identified by SDS-PAGE (12.5% Tris/Tricine gels) and further purified by reverse-phase HPLC on a C18 Vydac 22 × 250-mm preparative column with a linear gradient of 5–80% acetonitrile containing 0.05% TFA (v/v). Fractions containing ζ<sub>c</sub> were lyophilized, dissolved in 150 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 8.5, and purified by size-exclusion chromatography on a Superdex 200 10/300 (GE Healthcare) column. After final lyophilization, ζ<sub>c</sub> was stored at −20 °C.

Recombinant Lck-G2Δ (N-terminal glycine deleted to prevent myristoylation) containing Src homology 3, Src homology 2, and catalytic kinase domains was expressed using High Five<sup>TM</sup> cells infected with recombinant baculovirus (a gift from M. Eck) at a multiplicity of infection of 0.1 pfu/cell. Cells were harvested by centrifugation 72 h after infection; resuspended in 25 mM HEPES, pH 7.4, 25 mM NaCl, 2 mM DTT, 0.5 mM PMSF, and leupeptin, aprotinin, and pepstatin (2 μg/ml each); and lysed by sonication. Lysate was centrifuged (75,000 × g, 1 h), and the supernatant was loaded on a 5-ml HiLoad DEAE FF-column (GE Healthcare) preequilibrated with 25 mM HEPES, pH 7.4, 25 mM NaCl. Protein eluted with a linear gradient of 25–500 mM NaCl. Fractions containing Lck were pooled and applied to gel filtration chromatography (Superdex 200 16/60) in 25 mM HEPES, pH 7.7, 150 mM NaCl. Further purification was performed after concentrating Lck-containing fractions (Amicon Ultra 4 30 k filter unit) and buffer exchange (25 mM Tris, pH 8.5) by anion-exchange chromatography (POROS HQ20). Protein eluted with a linear gradient of 300–400 mM NaCl. Pooled fractions were stored at −80 °C.

Vesicle preparation

Definite amounts of lipids dissolved in chloroform were well mixed to obtain homogeneity of chosen lipid compositions. Chloroform was evaporated under a continuous N<sub>2</sub> stream and completely removed by lyophilization. Lipids were hydrated in 20 mM HEPES, pH 7.4, 150 mM NaCl and subjected to five freeze-thaw cycles. LUVs were prepared by extrusion (11 times) through a stack of two polycarbonate filters (pore size of 100 nm) using an Avanti Mini-Extruder (Avanti Polar Lipids). Vesicles were characterized by negative-stain electron microscopy and dynamic light scattering. Dynamic light scattering experiments were performed with a DynaPro-MS800 (Protein Solutions) instrument at 20 °C. Samples of LUVs (2 mM) were incubated at 20 °C with or without ζ<sub>c</sub> (45 μM) in 20 mM HEPES, pH 7.5, 25 mM NaCl; diluted (1:10,000); and filtered (Millex filter unit 0.22 μm) before measurements. Scattering data were analyzed using DYNAMICS autocorrelation analysis software (version 5.25.44).

Samples analyzed by negative-stain electron microscopy were prepared by incubating LUVs (3 mM) with or without
centrations, determine a baseline before adding protein in increasing concentrations, stBLMs before and after protein is introduced. In the single-batch setup using a custom-built instrument (SPR Biosystems, Germantown, MD). SAM-covered, gold-coated glass slides (SPR) by vesicle fusion as described previously (34). Briefly, freshly gold-coated substrates using magnetron sputtering (ATC Orion, AJA) were immersed overnight in a 0.2 mM solution of a tether lipid, Z-20-(Z-octadec-9-enoxyloxy)-3,6,9,12,15,18,22-heptaoxatetracont-31-ene-1-thiol (HC18), as described (34) and βME (Sigma-Aldrich) mixed at a molar ratio of 3:7 in ethanol to form mixed self-assembled monolayers (SAMs). The substrates were then rinsed with pure ethanol and dried in an N2 stream, and stBLMs were formed by incubating the SAM with a 10 mg/ml phospholipid vesicle solution in 1 M NaCl, 20 mM HEPES, pH 7.4, for 1 h. The sample was finally rinsed with copious amounts of 0.25 M NaCl, 20 mM HEPES, pH 7.4.

Surface plasmon resonance

SPR measurements were conducted at 25 ± 0.01 °C in a single-batch set-up using a custom-built instrument (SPR Biosystems, Germantown, MD). SAM-covered, gold-coated glass slides were assembled in the Kretschmann configuration by index-matching to a prism. stBLMs were completed by vesicle fusion in situ, as described above. The setup allows for simultaneous SPR and electrochemical impedance spectroscopy (EIS) measurements, which are used to assess the quality of the stBLMs before and after protein is introduced. In the single-batch experiments, the untreated bilayer is measured first to determine a baseline before adding protein in increasing concentrations, c_p, and measuring the equilibrium SPR response, R_eq, R_eq(c_p) was fitted to the Langmuir isotherm to determine the protein affinity in terms of the dissociation constant (K_d) and the surface density (R_p) of bound protein extrapolated to infinite concentration c_p.

\[ R_{eq}(c_p) = \frac{c_p R_p}{c_p + K_d} \]  
(Eq. 1)

Electrochemical impedance spectroscopy

EIS data were taken using a Solartron (Farnborough, UK) system (model 1287A potentiostat and model 1260 frequency response analyzer). Gold-coated glass slides served as the working electrode in a setup that allowed simultaneous SPR and EIS measurements. The cell has a volume (V) of ~250–300 μl and a surface area (A_e) of ~0.33 cm² confined by a Viton O-ring. Copper contrast was used to measure the geometric electrode surface area (53), and raw EIS data were normalized to A_e and a roughness factor (β) of 1.4, estimated from the gold surface oxidation/oxide stripping charge (54). A saturated silver–silver chloride (Ag|AgCl|NaCl(aq,sat)) microelectrode (Microelectrodes, model M-401F) was used as reference. The auxiliary electrode was a 0.25-mm diameter platinum wire (99.99% purity; Sigma-Aldrich) coiled around the barrel of the reference electrode. The distance between the tip of the reference and working gold electrode surface was set to 2–3 mm. Measurements were carried out in aerated solutions with 10-mV alternating current at 0-V bias versus reference electrode. Data analysis was carried out by fitting to an equivalent circuit using ZView (Scribner Associates), as described (33).

Neutron reflectometry

NR measurements were performed at 22 ± 2 °C on the NGD-MAGIK reflectometer at the NIST Center for Neutron Research (55). Reflectivity curves were recorded for momentum transfer values 0.008 Å⁻¹ ≤ q_R ≤ 0.25 Å⁻¹. stBLMs were prepared on 3-inch diameter silicon wafers assembled in a flow cell (56). Samples were measured at room temperature using three isotopically different bulk solvents of distinct H/D contrasts (D₂O, H₂O, and a 2:1 (v/v) mixture of the two), with adequate counting statistics typically obtained after ~6 h. The flow cell allows for in situ buffer exchange; therefore, subsequent measurements were performed on the same sample area. NR data were first collected from an as-prepared stBLM. Thereafter, protein was added to the stBLM, and NR data were collected either during protein incubation or after rinsing the bilayer with buffer.

To characterize the structures of proteins associated with the membrane, one-dimensional nSLD profiles along the lipid bilayer normal were determined by fitting to the experimental NR data. These one-dimensional structural profiles were composed using a hybrid of a stratified slab model for the solid substrate (57), a continuous distribution of lipid components for the stBLM (58), and a monotonic Hermite spline for the model-free envelope of the protein (36). In this compound model, bulk silicon, silicon oxide, a chromium bonding layer (~50 Å), and the substrate-terminal gold layer (~150 Å) were parameterized as subsequent slabs in terms of thickness and nSLD for each layer, except for the bulk silicon, for which the nSLD is known. One global roughness parameter was applied to all substrate interfaces. Individual submolecular groups implemented in the continuous distribution model of the stBLM are as follows: βME, tether PEG chains, tether glycerol groups, substrate-proximal and substrate-distal PC and PS headgroups, and substrate-proximal and substrate-distal methylene and methyl chains of lipid and tether molecules. Fit parameters are the bilayer hydrocarbon thickness for each bilayer leaflet, bilayer completeness, tether surface density, tether thickness, βME surface density, and one roughness parameter applied to all of these distributions.

The Hermite spline that encodes the protein distribution profile is defined by control points that are on average 20 Å apart. The number of control points depends on the spatial extension of the protein along the bilayer normal and is determined iteratively during model optimization. Fit parameters for each control point are the volume occupancy of protein and the deviation from a position defined by equidistant control points throughout the spline. A constant nSLD is associated with the spline functions that thus describe the contributions to the scattering of either completely protonated or partially deuterated, intrinsically homogeneous protein material.
TCRζ<sub>c</sub> membrane association

Data modeling was performed with the ga_refl and Refl1D software packages (56) using a differential evolution Markov chain global optimizer. Reflectivity curves recorded from one sample under different conditions were fitted simultaneously to the same model by sharing fit parameters, for example, for the solid substrate. The recorded Monte Carlo Markov chain was used to determine the fit parameter confidence limits using a statistical analysis. This procedure yields a bias-free and objective estimate of the uncertainties of the resulting nSLD profiles while avoiding overparameterization (36).

Molecular modeling

Two amino acid residues (Gly<sup>3</sup> and Ser<sup>2</sup>) were N-terminally appended to the primary sequence of ζ<sub>c</sub> to account for the thrombin recognition site in the recombinant protein. Residue numbering used here refers to the cytosolic domain of ζ, such that residue Leu<sup>31</sup> in the native full-length protein (with signal sequence) is considered number 3. The hydropathy score and the secondary structure prediction were determined using ExPASy ProtScale (59) and PSIPRED version 3.3 (50), respectively. Molecular models were derived from an initial ideal helix model, which was interspersed with perturbations to maintain helical segments at the desired positions. Three-dimensional conformations of these initial models were adjusted using Lego tools in the O software environment (60) to reflect local conformations found in a library of high resolution protein structures. Final protein models were used to form a protein/membrane system by using the CHARMM-GUI Input Generator (61). Protein structures were first preoriented relative to the membrane normal and then surrounded by lipid-like spheres whose positions were subsequently replaced by lipid molecules (replacement method). A rectangular protein/membrane system was formed choosing DOPC/DOPS molecules in a molar ratio of 70:30.

Tyrosine phosphorylation assay

Phosphorylation of ζ<sub>c</sub> (2 μM), or Fyn kinase substrate (150 μM) by recombinant Lck (0.2 μM) was performed in 20 mM HEPES, pH 7.4, 150 mM NaCl, 6.5 mM MgCl<sub>2</sub>, 50 μM Na<sub>2</sub>VO<sub>4</sub>, 112.5 μM ATP (0.25 μCi/μl [γ<sup>32</sup>]<sub>P</sub>ATP) at 37 °C with or without vesicles. The reaction was stopped by adding 20 μl of 40% trichloroacetic acid to 40 μl of phosphorylation mixture. Aliquots of 30 μl were transferred to P81 phosphocellulose squares (Millipore), washed extensively with 0.75% phosphoric acid, and air-dried. Incorporation of <sup>32</sup>P was quantified by scintillation counting (Wallac MicroBeta<sup>2</sup> 1450, PerkinElmer Life Sciences).

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