Phosphorylation of T cell receptor ζ is regulated by a lipid dependent folding transition

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The cytoplasmic domain of the T cell receptor ζ subunit (ζ_{cyt}) is sufficient to couple receptor ligation to intracellular signaling cascades, but little is known about its structure or mechanism of signaling. In aqueous solution, ζ_{cyt} is unstructured. Here we report that in the presence of lipid vesicles ζ_{cyt} assumes a folded structure. The folding transition is reversible and dependent on the presence of acidic phospholipids. In the lipid-bound conformation, ζ_{cyt} is refractory to phosphorylation by src family tyrosine kinases, which are believed to play a key role in signal initiation *in vivo*. In the lipid-free, unstructured form, ζ_{cyt} is readily phosphorylated, and phospho-ζ_{cyt} exhibits neither membrane association nor structure induction. The conformational change may provide a mechanism for coupling receptor clustering to cytoplasmic signaling events.

T cells are activated by multivalent engagement of their antigen receptors with major histocompatibility complex (MHC)–peptide complexes carried on the surface of antigen presenting cells. Clustering of the antigen-specific αβ subunits of the T cell antigen receptor (TCR) activates phosphorylation of the nonvariable ζ_2 and CD3 γδε TCR subunits, in an early, obligatory step in the T cell activation pathway[1]. The ζ_2 and CD3 subunits are phosphorylated by the src family kinases lck and/or fyn, on Tyr residues found in characteristic Yxx(L/I)x_{6-8}Yxx(L/I) (x represents any amino acid type) sequences called immunoreceptor tyrosine-based activation motifs (ITAMs)[2] that are found also in other signaling systems[3]. Doubly phosphorylated ITAMs engage SH2 domains of kinases and adapter proteins, and activate downstream signaling pathways[2,4]. The TCR ζ subunit is a monotopic type I transmembrane protein, found as a disulfide-linked dimer, with the bulk of the protein exposed to the cytoplasm (112 of 142 amino acid residues), and with three ITAMs spaced along its cytoplasmic domain. TCR ζ is sufficient to carry oligomerization signals into the cell in the absence of other subunits[5–7]. As for many receptors, the molecular events responsible for communicating the ligand binding signal across the membrane are not known. Here, we show that the cytoplasmic domain of the TCR ζ subunit exhibits a lipid-dependent folding transition that controls its accessibility to src family protein kinases, suggesting a novel mechanism for transmembrane signal transduction regulated by reversible membrane association.

Lipid-dependent folding of ζ_{cyt}

We studied the structural properties of recombinant TCR ζ cytoplasmic domain (ζ_{cyt}) in the presence of lipid vesicles. We used a variety of lipid species characteristic of T cell membranes, reasoning that these might simulate the *in vivo* environment where the TCR ζ cytoplasmic domain is found, and that this interaction could affect ζ_{cyt} structure. As reported[8], the far UV circular dichroism (CD) spectrum of ζ_{cyt} in aqueous solution is characteristic of an unstructured protein (Fig. 1*a*, no lipid). However, in the presence of vesicles containing the acidic lipid dimyristoylphosphatidylglycerol (DMPG), ζ_{cyt} exhibited a large increase in secondary structure, with a CD spectrum characteristic of a partly helical protein (Fig. 1*a*, DMPG). By contrast, the presence of the zwitterionic lipid dimyristoylphosphatidylcholine (DMPC) did not cause any change in ζ_{cyt} secondary structure (Fig. 1*a*). A disulfide-linked homodimeric form (ζ_{cyt}–ζ_{cyt}), designed to mimic the naturally occurring homodimer, exhibited similar behavior to the ζ_{cyt} monomer (Fig. 1*b*). In the presence of other acidic lipids, including dimyristoylphosphatidylserine (DMPS), phosphatidylinositol (PI, mixed acyl chains), or mixtures of PC with either phosphatidylinositol-4-phosphate (PIP, not shown), or phosphatidylinositol-4,5-bisphosphate (PIP_2), ζ_{cyt} exhibited a

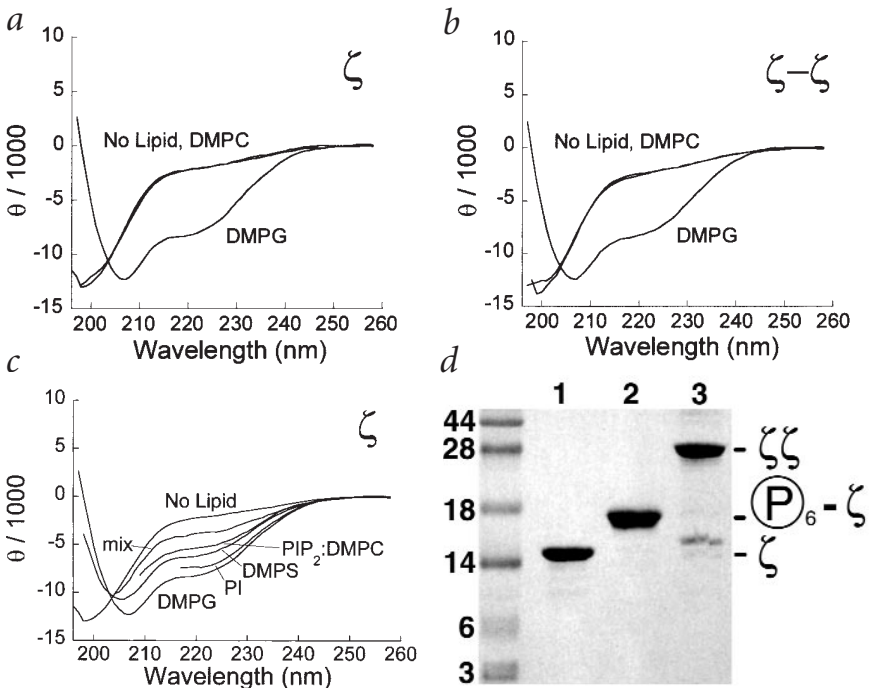

Fig. 1 Acidic phospholipids induce folding of the cytoplasmic domain of TCRζ. ***a***,***b***, Far UV CD spectra of ζ_{cyt} (*a*) or ζ_{cyt}–ζ_{cyt} (*b*) in the absence or presence of vesicles of the zwitterionic lipid DMPC (dimyristoylphosphatidylcholine) or the acidic lipid DMPG (dimyristoylphosphatidylglycerol). ***c***, CD spectra of ζ_{cyt} in the absence or presence of vesicles of DMPG, DMPS (dimyristoylphosphatidyl serine), PI (bovine liver phosphatidylinositol), PIP_2:PC (10% phosphatidylinositol-4,5-bisphosphate, 90% DMPC), or a mixture of synthetic lipids approximating the composition of the T cell plasma membrane[14] ('mix', 55% dipalmitoylphosphatidylcholine, 25% palmitoyloleoylphosphatidylethanolamine, 10% PI, 10% DMPS). The single chain lipid analog detergents lysomyristoylphosphatidylglycerol (LMPG) and lysomyristoylphosphatidylcholine (LMPC) behaved identically to their parent lipids DMPG and DMPC (not shown). θ is the molar ellipticity in units of deg cm^2 $dmol^{-1}$. ***d***, SDS-PAGE of purified ζ_{cyt} (1), phospho-ζ_{cyt} (2) and ζ_{cyt}–ζ_{cyt} (3). Phospho-ζ_{cyt} shows a decrease in gel mobility relative to ζ_{cyt}, as reported[8].

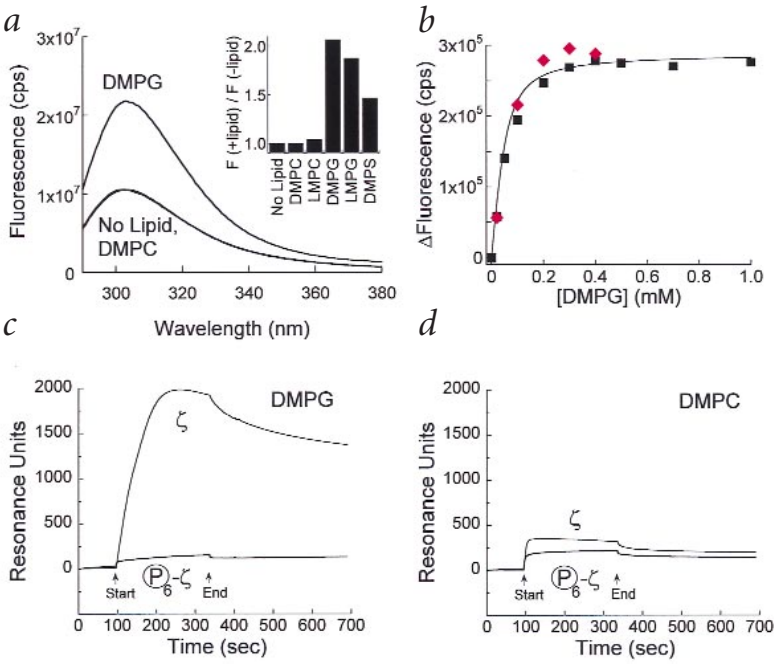

Fig. 2 Lipid binding activity of the cytoplasmic domain of TCR ζ. ***a***, Tyrosine fluorescence emission spectra of ζ_{cyt} in the presence or absence of DMPG or DMPC vesicles. Inset shows relative fluorescence increase in the presence of various other lipids and lysolipids. ***b***, Concentration dependence of ζ_{cyt} fluorescence increase induced by DMPG vesicles. Squares, addition of vesicles to ζ_{cyt} solution (binding); diamonds, dilution of preformed ζ_{cyt}–vesicle complexes (release). The curve describes a model in which 25 lipid molecules bind to each ζ_{cyt}, with a K_d of 1 μM (see Methods). ***c***,***d***, Surface plasmon resonance assay of binding of ζ_{cyt} to DMPG (*c*) or DMPC (*d*) hybrid bilayers on alkylthiolated gold chip. In both assays, lipids with acidic but not zwitterionic head groups bind to ζ_{cyt}. Phosphorylated phospho-ζ_{cyt} does not bind to either surface.

similar structure to that in the presence of DMPG (Fig. 1*c*). A mixture of zwitterionic and acidic lipids designed to mimic the bulk composition of T cell plasma membranes (see Methods) induced similar but smaller changes in the CD spectrum (Fig. 1*c*). Lipid-dependent folding behavior induced by acidic but not zwitterionic phospholipids has been seen previously with several other proteins that interact with membranes, including α-synuclein[9], apocytochrome *c*[10], herpes virus infected cell protein 47 (ICP47)[11], and cytolytic peptides including the magainins and mellitins[12].

The cytoplasmic face of the plasma membrane should provide an environment suitable for ζ folding; acidic lipids such as phosphatidylserine and phosphatidylinositol are predominantly found on the inner leaflet[13], each representing ~20% of the total plasma membrane phospholipid composition[14], and therefore up to 40% of the inner leaflet. Acidic lipids are also enriched in microdomains[15] having distinct lipid and protein compositions that may play a role in T cell signaling[16]. Thus, ζ_{cyt} in its natural environment, tethered to the cytoplasmic face of the plasma membrane may adopt a folded structure similar to that observed here.

Reversible membrane association

The lipid-dependent folding of ζ was accompanied by membrane association, as shown by changes in intrinsic fluorescence and by a direct binding assay. An increase in tyrosine fluorescence, consistent with interaction with a lipid surface, was observed for ζ_{cyt} in the presence of DMPG or other acidic phospholipids, but not their zwitterionic counterparts (Fig. 2*a*). The binding was saturable with increasing lipid concentration (Fig. 2*b*). Similar fluorescence intensities were observed in experiments in which lipid vesicles were added to soluble ζ_{cyt} (Fig. 2*b*, squares) or in which bound complexes were diluted with buffer (Fig. 2*b*, diamonds), indicating that the binding was readily reversible since the same state could be reached by starting with either quantitatively lipid-bound or lipid-free ζ_{cyt}. Specific binding of ζ_{cyt} to an acidic lipid surface was confirmed by surface plasmon resonance, using lipid monolayers assembled on an alkylthiol-coated gold surface (Fig. 2*c*,*d*). As observed for structure induction, ζ_{cyt} binding activity was specific for acidic lipids. DMPG monolayers exhibited much greater levels of ζ_{cyt} binding compared to those of DMPC (Fig. 2*c*,*d*). DMPS and PI monolayers also bound ζ_{cyt} (data not shown). ζ_{cyt} is expected to be positively charged in the physiological pH range (pI ~9), indicating that the acidic lipid binding activity may include a substantial electrostatic component. Membrane binding activity has been observed for other lipid binding proteins such as the protein kinase C substrate myristoylated alanine-rich C-kinase substrate (MARCKS)[17] and the

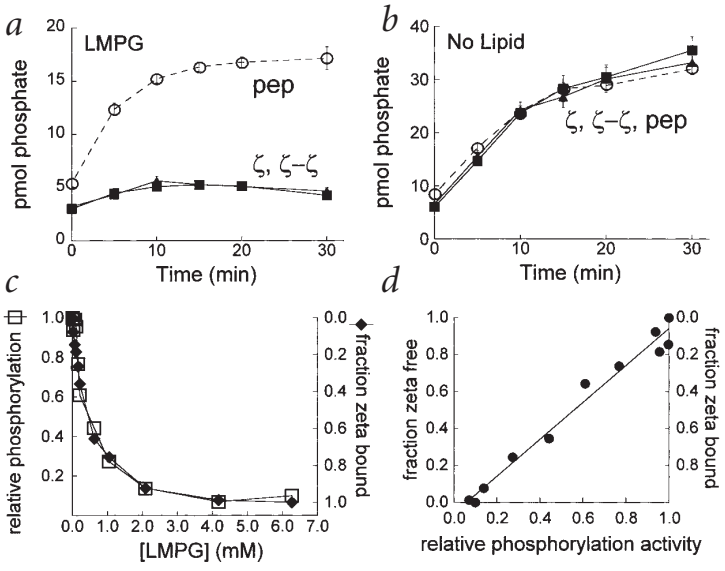

Fig. 3 Lipid association blocks phosphorylation of ζ_{cyt} by src(86–536). ***a***,***b***, Time course of phosphorylation of ζ_{cyt} (squares), ζ_{cyt}–ζ_{cyt} (triangles) or control cdc2 peptide (circles) by src(86–536) in the presence (*a*) or absence (*b*) of LMPG . Phosphorylation of ζ_{cyt} and ζ_{cyt}–ζ_{cyt} is strongly inhibited in the presence of LMPG but not LMPC, while the control peptide is phosphorylated efficiently under both conditions. ***c***, Comparison of fractional ζ_{cyt} phosphorylation and lipid binding activities, as a function of LMPG concentration. Fractional phosphorylation activity relative to that of the test peptide substrate is shown. ***d***, Correlation plot for binding and phosphorylation activities (correlation coefficient = 0.99). The best-fit line (shown) has a slope of 1.0.

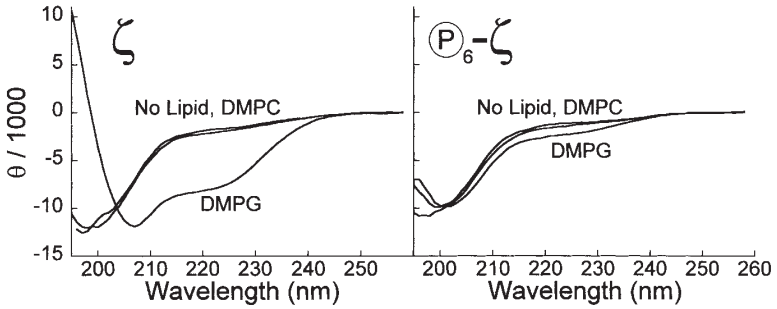

Fig. 4 Multiply phosphorylated ζ_{cyt} does not exhibit lipid dependent folding and binding activities. CD spectra of nonphosphorylated ζ_{cyt} (left) and multiply phosphorylated phospho-ζ_{cyt} (right).

C-terminal lipid binding tail of ras[18], and characteristically includes a strong electrostatic component in addition to hydrophobic effects[18,19].

Lipid binding regulates ζ_{cyt} phosphorylation

To investigate the role of the lipid-dependent folding of ζ_{cyt} in TCR-mediated signaling, we tested the accessibility of lipid-bound ζ_{cyt} to phosphorylation by recombinant src tyrosine kinase (Fig. 3). LMPG completely blocked the phosphorylation of ITAM Tyr residues on both ζ_{cyt} (Fig. 3*a*, squares) and ζ_{cyt}–ζ_{cyt} (triangles), whereas a control peptide substrate was phosphorylated efficiently (open circles). In the absence of lipid (Fig. 3*b*) or the presence of the zwitterionic lipid LMPC (data not shown), ζ_{cyt}, ζ_{cyt}–ζ_{cyt} and control substrates were phosphorylated equivalently. The degree of lipid binding of ζ_{cyt} in various concentrations of LMPG, as measured by the fluorescence assay, correlated closely with the degree of resistance to src-mediated tyrosine phosphorylation (Fig. 3*c*,*d*). After phosphorylation, ζ_{cyt} exhibited neither the acidic lipid induced helical structure (Fig. 4) nor the binding activity (Fig. 2*c*) associated with the resting, nonphosphorylated form.

T cell receptor activation model

An intriguing aspect of signaling in T cells is that clustering of receptors, rather than simply ligand engagement, is the proximal activation stimulus[20–22]. The mechanism that triggers tyrosine phosphorylation of clustered TCR is not known. Could the ζ lipid dependent conformational change and its effect on phosphorylation play a role in this process? One possibility is that TCR clustering could increase the local concentration of ζ_{cyt}, causing a competition for membrane surface and release of ζ_{cyt}, allowing the ITAMs to be accessed by protein kinases (Fig. 5). Once released from the membrane, ζ_{cyt} would become accessible to protein kinases such as lck or fyn, initiating the cytoplasmic signaling cascade. Phosphorylated ζ would not reassociate with the membrane, and could engage ZAP-70 SH2 domains[23] and other downstream components of the activation complex[2].

A structural model for clustered TCR extracellular domains[24] indicates they will have a membrane footprint of 30–40 nm^2. MHC oligomers with ~30 Å crosslinkers activate T cells[21], suggesting that the activating cluster has a footprint on the membrane similar to that of the TCR structural model. These values can be compared to an estimated 30 nm^2 for the intracellular domains of a ζ dimer, as arranged in a helical conformation parallel to the membrane plane. TCR CD3 cytoplasmic domains may also bind to the membrane, as described for the related cytoplasmic domain of the FCγRIIb1 subunit[25] of a receptor for the Fc region of IgG, further increasing the footprint on the cytoplasmic face of the membrane. This analysis suggests that receptor clustering could be in sufficiently close apposition that there would not be sufficient membrane space to accommodate the cytoplasmic domains on the inner face of the membrane. Alternately, TCR crosslinking is known to induce changes in the local lipid environment[16], and this might be sufficient to release ζ_{cyt} and allow phosphorylation and signal initiation.

Reversible membrane association, mediated by acidic lipids, has been proposed to regulate the activities of numerous cytoplasmic proteins[26], including vinculin[27], CTP:phosphocholine cytidyldyl transferase[28], and MARCKS[17]. In this last case, the reversible membrane binding of the myristoylated cytoplasmic protein MARCKS is regulated by protein kinase C mediated serine phosphorylation[17]. We provide here the first evidence for such regulation of the cytoplasmic domain of a transmembrane receptor. Reversible membrane binding may thus play a general role in both transmembrane and cytoplasmic signaling mechanisms.

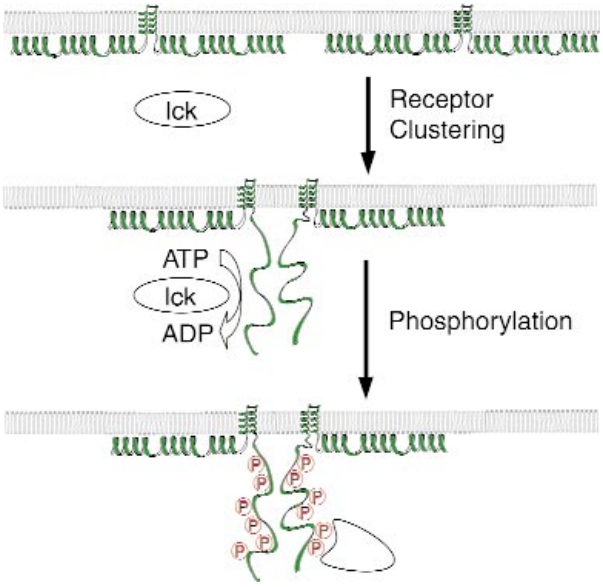

Fig. 5 Model for T cell signal transduction through the ζ subunit. In the pre-activation state, ζ_{cyt} is bound to the inner leaflet of the membrane. Receptor engagement and crosslinking of the TCR forces the cytoplasmic domain to be released from the membrane, either through a competition for membrane space or an alteration in the local lipid environment. Protein tyrosine kinases can phosphorylate the newly accessible ζ ITAMs. After phosphorylation, phospho-ζ_{cyt} remains free of the membrane, and recruits phospho-ITAM binding proteins such as ZAP-70 and Grb2. For simplicity, the other components of the T cell receptor complex are not shown.

Methods

ζ_{cyt}, ζ_{cyt}–ζ_{cyt} and phosphorylated ζ_{cyt}. The complete cytoplasmic domain of human TCR ζ (Swiss Prot ID P20963) (54–163) with a two-residue linker (sequence: MKKFSR…LPPR), or of ζ (51–163) with a five-residue linker carrying a unique Cys residue used to form the ζ_{cyt}–ζ_{cyt} dimer (MKKCGLRVKFSR…LPPR), were expressed in *Escherichia coli*. Plasmids for expression of ζ_{cyt} and ζ_{cyt}–ζ_{cyt} were provided by W. Weissenhorn[8]. Disulfide-linked ζ_{cyt}–ζ_{cyt} (1.2 mg ml^{-1}) was

formed by air oxidation in the presence of 1 mM $CuSO_4$ and 3.9 mM *o*-phenanthroline. ζ_{cyt} (0.75 mg ml^{-1}) was phosphorylated *in vitro* using protein tyrosine kinase lck (4 μg ml^{-1}) at 37 °C for 4 h as described[8]. Protein purifications were performed as described[8] with an additional reverse phase chromatography step. Recombinant baculovirus for expression of lck protein tyrosine kinase (lck53–509, EVRD…QPQP) was a gift of S. Harrison (Harvard). Complete phosphorylation in phospho-ζ_{cyt} (six moles of phosphate per mole protein) was confirmed by matrix-assisted laser desorption mass spectrometry.

Lipid vesicles. Synthetic lipids (DMPC, DMPG, DMPS, DMPA), purified natural lipids (PI, PIP, PIP_2) from bovine liver, or lipid mixtures, in chloroform or chloroform:methanol solution, were dried to films under vacuum to remove residual solvent, and then resuspended in buffer and sonicated to clarity in a high intensity bath sonicator (Laboratory Supplies Company Inc., Hicksville, New York). All lipids were purchased from Avanti Polar Lipids.

Circular dichroism. Far UV CD measurements were made using an Aviv 62DS spectropolarimeter in a 0.1 cm path length quartz cuvette at 37 °C. Spectra were recorded from 190–260 nm for solutions of ζ_{cyt} (23 μM) or ζ_{cyt}-ζ_{cyt} (11 μM) containing various lipids or lysolipids (5 mM) in 20 mM Tris, pH 7.5 (Fig. 1), or of ζ_{cyt} or phospho-ζ_{cyt} (23 μM) in 10 mM Tris, pH 7.5, 5 mM NaCl with 6 mM DMPG or DMPC vesicles (Fig. 4). Lipid absorbance limited measurements to wavelengths >215 nm for samples containing natural lipids.

Lipid binding. Fluorescence spectra of ζ_{cyt} were obtained using a Spex Fluoromax-W spectrofluorimeter, with an excitation wavelength of 275 or 280 nm and emission wavelengths from 290–450 nm in a 1 cm cell at 37 °C, using 8 μM ζ_{cyt} and 1.5 mM lipid in 20 mM Hepes, pH 7.7, 150 mM NaCl (Fig. 2*a*) or else 2 μM ζ_{cyt} in 10 mM Tris, pH 7.5, 5 mM NaCl, with various concentrations of lipid vesicles prepared as described above (Fig. 2*b*). The plot of lipid induced fluorescence change (304 nm) *versus* DMPG concentration (Fig. 2*b*) was described by a three parameter (n, K_d, ΔF_{max}) quadratic binding equation for two-state binding of one molecule of ζ_{cyt} to an aggregate of n lipid molecules:

$$[\zeta_{bound}] / [\zeta_{tot}] = \Delta F / \Delta F_{max} = ([L_{tot}] / n + K_d + [\zeta_{tot}]) - (([L_{tot}] / n + K_d + [\zeta_{tot}])^2 - 4([L_{tot}][\zeta_{tot}]) / n)^{1/2}) / (2[\zeta_{tot}])$$

where K_d is the dissociation constant, ΔF_{max} is the tyrosine fluorescence for fully bound ζ_{cyt}, and $[\zeta_{tot}]$ and $[L]_{tot}$ are the total ζ_{cyt} and lipid concentrations, respectively. The experimental data are consistent with values of K_d from 0.3 μM (n = 40) to 50 μM (n = 1), and more definitive values for the binding affinity and stoichiometry remain to be determined. Plasmon resonance measurements were made using a BIACORE 2000 instrument. Thioalkylated gold HPA chips (BIACORE, Piscataway, New Jersey) were coated with lipid monolayers by fusion of DMPG and DMPC vesicles (1 mM) in 20 mM Hepes pH 7.7, 25 mM NaCl, as recommended by the manufacturer. ζ_{cyt} and phospho-ζ_{cyt} (0.8 μM) were passed over the chip at 10 μl min^{-1} and 25 °C in 20 mM Hepes, pH 7.7, 25 mM NaCl. The signal (resonance units) at time 0 for each profile was subtracted from the remaining data.

Phosphorylation assay. ζ_{cyt} (2 μM), ζ_{cyt}–ζ_{cyt} (1 μM) or control peptide kinase substrate cdc2(6–20) (KVEKIGEGTYGVVYK; 5 μM; ref. 29) were phosphorylated using recombinant src(86–536) (0.2 μM) in 20 mM Hepes, pH 7.7, 150 mM NaCl, 1 mM MgATP (0.2 μCi μl^{-1} [γ-^{32}P]ATP), 6.5 mM $MgCl_2$, 50 μM Na_3VO_4 at 37 °C, in the presence or absence of LMPG or LMPC (2 mM unless otherwise noted). Aliquots (10 μl) were removed, spotted onto P81 phosphocellulose squares (Whatman), washed extensively with 0.5% phosphoric acid and dried, and bound ^{32}P was determined by scintillation counting. Protein tyrosine kinase src 86–536 (VTTF…GENL), carrying SH2, SH3, and kinase domains, was produced in baculovirus infected Sf9 insect cells as described[30].

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