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Membrane binding mode of intrinsically disordered cytoplasmic domains of T cell receptor signaling subunits depends on lipid composition

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ABSTRACT

Intrinsically disordered cytoplasmic domains of T cell receptor (TCR) signaling subunits including ζ_{cyt} and CD3ε_{cyt} all contain one or more copies of an immunoreceptor tyrosine-based activation motif (ITAM), tyrosine residues of which are phosphorylated upon receptor triggering. Membrane binding-induced helical folding of ζ_{cyt} and CD3ε_{cyt} ITAMs is thought to control TCR activation. However, the question whether or not lipid binding of ζ_{cyt} and CD3ε_{cyt} is necessarily accompanied by a folding transition of ITAMs remains open. In this study, we investigate whether the membrane binding mechanisms of ζ_{cyt} and CD3ε_{cyt} depend on the membrane model used. Circular dichroic and fluorescence data indicate that binding of ζ_{cyt} and CD3ε_{cyt} to detergent micelles and unstable vesicles is accompanied by a disorder-to-order transition, whereas upon binding to stable vesicles these proteins remain unfolded. Using electron microscopy and dynamic light scattering, we show that upon protein binding, unstable vesicles fuse and rupture. In contrast, stable vesicles remain intact under these conditions. This suggests different membrane binding modes for ζ_{cyt} and CD3ε_{cyt} depending on the bilayer stability: (1) coupled binding and folding, and (2) binding without folding. These findings explain the long-standing puzzle in the literature and highlight the importance of the choice of an appropriate membrane model for protein–lipid interactions studies.

Introduction

Intrinsically disordered proteins (IDPs) are proteins that lack a well-defined three-dimensional structure under physiological conditions [1]. In this context, cytoplasmic regions of signaling subunits of immune receptors, including those of ζ and CD3ε signaling subunits (ζ_{cyt} and CD3ε_{cyt}, respectively) of T cell receptor (TCR), represent a novel class of IDPs [2–5]. These regions all have one or more copies of an immunoreceptor tyrosine-based activation motif (ITAM) [6], tyrosine residues of which are phosphorylated upon receptor engagement in an early and obligatory event in the signaling cascade. IDPs are thought to undergo coupled binding and folding upon interaction with their partners [7]. In contrast, random coil ζ_{cyt} remains unfolded upon homodimerization [3,4,8] or interaction with the well-structured core domain of simian immunodeficiency virus Nef [9], as shown by circular dichroic (CD) and nuclear magnetic resonance (NMR) spectroscopy. Perhaps even more intriguing is the fact that no chemical shift changes and significant changes in peak intensities are observed in the ^{1}H–^{15}N heteronuclear single quantum coherence spectra of ^{15}N-labeled ζ_{cyt} in ζ_{cyt} dimer [8] or ζ_{cyt}–Nef complex [9], thus highlighting unusual biophysical features of this and, possibly, other ITAM-containing proteins. Considering a crucial role of ζ_{cyt} and CD3ε_{cyt} in TCR signaling and their close proximity to the cell membrane, the question whether or not lipid binding of these IDPs promotes folding of ζ_{cyt} and CD3ε_{cyt} ITAMs and thus leads to inaccessibility of the ITAM tyrosines for phosphorylation is of fundamental importance. However, little is known about lipid-binding activity of the ITAM-containing cytoplasmic domains and the existing data are contradictory [2,4,10].

In 2000 [2], it has been shown that α-helical folding transition of ζ_{cyt} upon binding to acidic phospholipids prevents ITAM phosphorylation. The authors concluded that this folding transition can represent a conformational switch to regulate TCR triggering [2]. Later, the other group of investigators extended these findings to CD3ε_{cyt} and showed that binding of this protein to acidic phospholipids is accompanied by folding of ITAM, leading to inaccessibility of the ITAM tyrosines for phosphorylation *in vitro* [10]. This led the authors [10,11] to the conclusion that the conformational model of TCR activation previously suggested for ζ_{cyt} [2] can be extended to CD3ε_{cyt}. In contrast, we have previously shown that binding of ζ_{cyt} and CD3ε_{cyt} as well as ITAM-containing cytoplasmic domain of Fc receptor, FcRγ_{cyt}, to acidic phospholipids is not accompanied by a disorder-to-order structural transition [4]. This

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questions the possibility of ITAM folding upon membrane binding *in vivo* and challenges the suggested model of TCR activation [2,10,11].

Lipid bilayers are self-assembled structures, the mechanical properties of which are derived from noncovalent forces such as the hydrophobic effect, steric forces, and electrostatic interactions. In this context, the electrostatic force is of special interest because biological membranes are rich in anionic lipids [12] and are therefore charged in aqueous solution. Importantly, electrostatic interactions play the critical role in membrane stability [13]. Thus, considering that net charges of ζ_{cyt}, CD3ε_{cyt}, and FcRγ_{cyt} are + 5, + 11, and + 3, respectively, binding of these proteins to acidic phospholipids can potentially destabilize and disrupt lipid bilayers.

In this study, using CD and fluorescence spectroscopy, we demonstrated that α-helical folding of ζ_{cyt}, CD3ε_{cyt}, and FcRγ_{cyt} upon binding to acidic lipids depends on the membrane model structures used (lipid-mimetic detergent micelles vs. lipid vesicles) and the lipid composition of vesicles. For ζ_{cyt}, we used synthetic peptides and a lipid-binding assay employing sucrose-loaded large unilamellar vesicles (LUVs) of palmitoyloleoylphosphatidylglycerol (POPG) [14] to map the lipid-binding region(s) of ζ_{cyt}. The assay revealed that not ITAMs but clusters of basic residues outside ITAMs are involved in protein binding to stable acidic phospholipid vesicles. As shown by electron microscopy (EM) and dynamic light scattering (DLS), binding of ζ_{cyt} to small unilamellar vesicles (SUVs) and LUVs of dimyristoylphosphatidylglycerol (DMPG) but not POPG promotes vesicle fusion and rupture, highlighting the importance of ensuring the integrity of model membranes upon protein binding.

Materials and methods

Reagents and proteins. Peptides A–G (Table 1) were from Sigma. Lysomyristoylphosphatidylglycerol (LMPG), DMPG, and POPG were from Avanti Polar Lipids. Cytoplasmic domains of CD3ε, ζ, and FcRγ were expressed and purified as described previously [4].

Vesicle binding assay. Sucrose-loaded LUVs of POPG were prepared and the membrane binding assay was performed as described [4,14].

Vesicle preparation. LUVs of DMPG and POPG were made after five freeze–thaw cycles of the hydrated lipids by extruding multilamellar vesicles 10 times through a stack of two polycarbonate filters (100 nm pore diameter) in an Avanti Mini-Extruder (Avanti Polar Lipids). Filters with 30 nm pore diameter were used to prepare extruded SUVs of DMPG and POPG. Alternatively, SUVs were prepared by sonication of the hydrated lipids to clarity in a high intensity bath sonicator (Laboratory Supplies).

CD and fluorescence spectroscopy. Far-UV CD spectra were recorded on an Aviv 202 spectropolarimeter (AVIV Instruments) as described previously [4]. The intrinsic tyrosine fluorescence spectra were taken at 25 °C using a Spex Fluoromax-2 spectrofluorimeter as reported [4].

Table 1
Binding of T cell receptor ζ cytoplasmic domain peptides to POPG LUVs.

Peptide/protein	Net charge	% of bound peptide/protein
A LRVKFSRSADAPAYQQGQNQL	+2	50
B LYNELNLGRREEYDVL	−2	3
C DKRRGRDPEMGGKPRRKNPQEL	+4	42
D LYNELQKDKMAEAYSEI	−2	0
E GMKGERRRGKGHDGL	+3	54
F LYQGLSTATKDTYDAL	−1	2
G LYQGLSTATKDTYDALHMQALPPR	0	8
ζ_{cyt}	+5	90

Dynamic light scattering. Scattering data were collected at 20 °C with a DynaPro-MS800 instrument (Protein Solutions) and hydrodynamic radius values were calculated as described previously [4].

Electron microscopy. Samples of 3 mM extruded LUVs of POPG and DMPG as well as sonicated SUVs of DMPG alone or in the presence of 10 μM ζ_{cyt} were prepared in 20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), 0.1 M NaCl, pH 7.0. Negatively stained grids were prepared by applying 8-μl drops to carbon-coated Formvar 300-mesh gold grids, blotting away excess sample after 1 min, then soaking the grid on six successive drops of 1% (w/v) uranyl acetate, blotting, and allowing to dry. Micrographs were taken at an initial magnification of 28,500 with a Philips CM10 transmission electron microscope (Philips) operating at 80 kV.

Results

Mapping of ζ_{cyt} domains involved in lipid binding

To map the lipid-binding ζ_{cyt} sites, we used a sucrose-loaded vesicle-binding assay [14] and synthetic ζ_{cyt} peptides of varying length that cover the entire protein sequence (Fig. 1). The binding data clearly show that the peptides A, C, and E that correspond to the regions outside ITAMs and contain clusters of basic amino acids bind to POPG LUVs while the ITAM peptides (B, D, and F)—do not (Table 1).

These findings experimentally prove our early hypothesis that binding of ζ_{cyt}, CD3ε_{cyt}, and FcRγ_{cyt} to acidic phospholipid LUVs is driven by the clusters of basic amino acid residues rather than the overall net charge [4]. In addition, we specifically show that ITAMs do not contribute to membrane binding by ζ_{cyt}. As recently reported [10], CD3ε_{cyt} ITAM is not involved in binding to DMPG LUVs. Thus, taken together, these findings suggest that binding of ITAM-containing ζ_{cyt}, CD3ε_{cyt}, and highly possibly, FcRγ_{cyt}, to acidic phospholipid bilayers involves the protein regions outside ITAMs. This raises an important question: if ITAMs do not contribute substantially in binding, then what determines lipid binding-mediated folding of these functionally important domains and therefore accessibility (or inaccessibility) of the ITAM tyrosines for phosphorylation? To answer this question, we further investigated the induction of secondary structure upon binding of ζ_{cyt}, CD3ε_{cyt}, and FcRγ_{cyt} to acidic micelles and vesicles of different size and composition.

Structure of ζ_{cyt}, CD3ε_{cyt}, and FcRγ_{cyt} in the presence of micelles and vesicles

Considering current discrepancies in the literature on whether or not lipid binding of intrinsically disordered ζ_{cyt}, CD3ε_{cyt}, and FcRγ_{cyt} is accompanied by a disorder-to-order transition [2,4,10], we hypothesized that membrane binding mode of these proteins can depend on the membrane model used. To test this hypothesis, we examined binding of ζ_{cyt}, CD3ε_{cyt}, and FcRγ_{cyt} to micelles and different vesicles: LMPG micelles, extruded or sonicated DMPG and POPG SUVs as well as LUVs of DMPG and POPG. Among vesicles, POPG LUVs likely represent the best model to mimic the cell membrane because of higher vesicle stability (POPG vs. DMPG) and lower degree of membrane curvature (LUVs vs SUVs).

The CD data clearly show that ζ_{cyt} remains unfolded upon binding to POPG SUVs obtained by sonication (Fig. 2a) or extruding (data not shown) and POPG LUVs (Fig. S1a). Similarly, CD studies of CD3ε_{cyt} and FcRγ_{cyt} do not reveal any detectable secondary structure induction upon binding to POPG SUVs (Figs. S2a and S3a) and LUVs (data not shown). In contrast, the CD spectra of ζ_{cyt} taken in the presence of LMPG micelles (Fig. 2a), DMPG SUVs obtained by sonication (Fig. 2a) and extruding (data not shown) as