The CD3 ζ Subunit Contains a Phosphoinositide-Binding Motif That Is Required for the Stable Accumulation of TCR –CD3 Complex at the Immunological Synapse

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The CD3 ζ Subunit Contains a Phosphoinositide-Binding Motif That Is Required for the Stable Accumulation of TCR–CD3 Complex at the Immunological Synapse

Laura M. DeFord-Watts,* David S. Dougall,* Serkan Belkaya,* Blake A. Johnson,* Jennifer L. Eitson,* Kole T. Roybal,* Barbara Barylko, † Joseph P. Albanesi, † Christoph Wülling,*‡ and Nicolai S. C. van Oers*§

T cell activation involves a cascade of TCR-mediated signals that are regulated by three distinct intracellular signaling motifs located within the cytoplasmic tails of the CD3 chains. Whereas all the CD3 subunits possess at least one ITAM, the CD3 ε subunit also contains a proline-rich sequence and a basic-rich stretch (BRS). The CD3 ε BRS complexes selected phosphoinositides, interactions that are required for normal cell surface expression of the TCR. The cytoplasmic domain of CD3 ζ also contains several clusters of arginine and lysine residues. In this study, we report that these basic amino acids enable CD3 ζ to complex the phosphoinositides PtdIns(3)P, PtdIns(4)P, PtdIns(5)P, PtdIns(3,5)P₂, and PtdIns(3,4,5)P₃ with high affinity. Early TCR signaling pathways were unaffected by the targeted loss of the phosphoinositide-binding functions of CD3 ζ. Instead, the elimination of the phosphoinositide-binding function of CD3 ζ significantly impaired the ability of this invariant chain to accumulate stably at the immunological synapse during T cell–APC interactions. Without its phosphoinositide-binding functions, CD3 ζ was concentrated in intracellular structures after T cell activation. Such findings demonstrate a novel functional role for CD3 ζ BRS–phosphoinositide interactions in supporting T cell activation. The Journal of Immunology, 2011, 186: 6839–6847.

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containing the cytoplasmic tail of CD3 ε fused to the extracellular and transmembrane domain of the inhibitory NK receptor, KIR2DL3, revealed that interactions between CD3 ε and phospholipids present on the inner leaflet of the plasma membrane sequester the ITAM of CD3 ε, thereby preventing its tyrosine phosphorylation until receptor ligation (17). Yet, subsequent studies, using T cells from mice containing mutations that prevented the BRS of CD3 ε subunits from complexing phosphoinositides, revealed a more defined role for this motif in controlling the cell surface expression of the TCR (15). Given that the phosphorylation state of the CD3 ε ITAM remained similar when comparing T cells expressing the wild-type (WT) versus BRS-modified CD3 ε molecules, the phosphoinositide-binding properties of CD3 ε likely regulate TCR downregulation and/or recycling, rather than the initiation of TCR signaling. This is consistent with the reduced TCR expression after the knockdown of the intraflagellar transport protein (IFT20), which is present in recycling endosomes and associates with CD3 ε (18, 19).

The cytoplasmic tail of the CD3 ε subunit has also been reported to complex phospholipids (20). When a recombinant protein containing just the cytoplasmic domain of CD3 ε was incubated in the presence of liposomes, in vitro kinase reactions revealed that the tyrosine phosphorylation of the ITAMs was significantly inhibited (20). Although such studies establish the ability of CD3 ε to bind phospholipids, the region in CD3 ε responsible for such interactions, the phospholipid-binding specificity of CD3 ε, and the functional role of such interactions within an intact TCR have not been established. In this study, we report that CD3 ε contains a high-affinity phosphoinositide-binding sequence, localized in a BRS signaling motif preceding the second ITAM. This sequence selectively complexed several phosphoinositides, including phosphatidylinositol-3,4,5-trisphosphate [PtdIns(3,4,5)P 3], phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P 2], phosphatidylinositol-4-phosphate [PtdIns(4)P], phosphatidylinositol-5-phosphate [PtdIns(5)P], phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P 2], and phosphatidylinositol-4,5,6-trisphosphate [PtdIns(4,5,6)P 3]. Functionally, mutating the CD3 ε BRS in a manner that eliminated its phosphoinositide-binding ability significantly impaired TCR accumulation at the center of the immunological synapse. This was evident with two distinct TCR transgenic T cells. Despite the dramatic changes in TCR localization, proximal TCR signaling pathways remained intact. Rather, the CD3 ε–phosphoinositide interactions were necessary for the normal redistribution of the TCR after TCR signaling. Taken together, these findings reveal a previously unrecognized role for CD3 ε–lipid interactions in regulating T cell functions.

Materials and Methods

Abs, peptides, and cell lines

Fluorochrome-labeled Abs for flow cytometry were obtained from either Becton-Dickinson (Franklin Lakes, NJ) or eBioscience (San Diego, CA). Fluorochrome-labeled Abs for flow cytometry were obtained from either Becton-Dickinson (Franklin Lakes, NJ) or eBioscience (San Diego, CA). Phospho-specific Abs against protein kinase C θ (PKCθ) (Thr 538; cat. no. 93775), phospholipase C-γ (PLC-γ) (Ser 1248; cat. no. 45018), ZAP-70 (Tyr 319; cat. no. 2701L), Akt (Ser/Thr 473; cat. no. 9271S), and p42/p44/Erk/MAPK (Tyr 202/204; cat. no. 9101S) were obtained from Cell Signaling Technology (Danvers, MA). Anti-actin (cat. no. 4967) and anti–PLC-γ (Tyr 319; cat. no. 2701L), Akt (Ser/Thr 473; cat. no. 9271S), and p42/p44/Erk/MAPK (Tyr 202/204; cat. no. 9101S) were obtained from Cell Signaling Technology (Danvers, MA). Anti-actin (cat. no. 4967) and anti–PLC-γ (cat. no. 2882) were also purchased from Cell Signaling. An anti–phospho–SAP-70 (Ser 736) Ab (cat. no. 2878) was kindly provided by Dr. Bernard Malissen (INSERM, Marseille, France) and has been previously described (23). Biotinylated and cysteine linked peptides were synthesized by The University of Texas Southwestern Medical Center Protein Chemistry Technology Core facility.

Constructs

Selected amino acid substitutions in the cytoplasmic tail of a full-length cDNA of murine CD3 ε were generated using overlapping oligonucleotides combined with PCR-based site-directed mutagenesis procedures, based on the manufacturers’ instructions (Strategene Products, Agilent Technologies, Santa Clara, CA). All nucleotide sequence changes were confirmed by dsDNA sequencing. The various lysine- and arginine-modified constructs were subcloned into pcdf-3E, pEGFP-N1, pGEX, and pET-28a (+) vectors. The pGEX vectors were used to generate GST–fusion proteins according to the manufacturer’s instructions (GE Biosciences, Piscataway, NJ).

Imaging

The CD3 ε sensors were generated by linking full-length WT CD3 ε and the BRS Sub-C construct to the N terminus of GFP (pGC vectors). The sensors were retrovirally transduced into primary, in vitro–primed 3C.C7 TCR transgenic T cells or D011.10 TCR transgenic T cells. The transduced T cells were FACs sorted at defined cell–GFP sensor expression levels selected to be similar to endogenous protein levels, as described previously (24). CD3 ε–GFP expressing 3C.C7 or D011.10 T cells were imaged in three dimensions at various time points before and after T cell–APC interactions with APCs preloaded with moth cytochrome C or OVA peptides, respectively. These images were analyzed as described (15, 24, 25).

Transfections, immunoprecipitations, and T cell activation assays

The transfection of HEK293 cells was performed as described (26). T cell lines were generated with the CD3 ε–deficient BW B T cell hybridoma, transduced with 20 μg plasmid DNA (pcdf-3-murine CD3 ε) premixed with the Transfast transfection reagent, according to the manufacturer’s instructions (Promega Corp., Madison, WI) (23). Stable clones, isolated by G418 drug selection, were selected by the restored expression of the TCR, determined by flow cytometry. For stimulations and phosphoprotein induction, the cells were incubated with biotinylated anti-CD3 ε mAbs, followed by streptavidin cross-linking for various time points as described previously (27). The cells were lysed in a 1% Triton X-100–containing lysis buffer, pH 7.6 (20 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 1 mM NaF, supplemented with protease and phosphatase inhibitors) (27). All other immunoprecipitations and immunoblotting assays were previously described (15, 28, 29). For expression of CD25 and CD69, the cells (106 cells/well) were cultured in the presence of plate-bound anti-CD3 ε for varying time points. In the case of transduced T cells, 1 × 104 T cells were incubated for 20 h with an equivalent number of CH27 B cells that had been preloaded with varying peptide concentrations (0.05–6 μM). IL-2 production was assayed with a mouse IL-2 ELISA assay according to the manufacturer’s instructions (ELISA MAX mouse IL-2; BioLegend).

Phospholipid-binding assays

Phosphatidylinositol phosphates (PIP) strips or PIP arrays were preblocked in 3% fatty acid-free BSA prepared in a Tris-based buffer (TBST; 25 mM Tris•HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20, 0.1% BSA) and has been previously described (23). Biotinylated and cysteine linked peptides were synthesized by The University of Texas Southwestern Medical Center Protein Chemistry Technology Core facility.

The CD3 ε–phosphoinositide complexes regulate T cell activation

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Results

The cytoplasmic tail of CD3ζ contains multiple phospholipid-binding regions

The cytoplasmic domains of both CD3ε and CD3ζ can complex phospholipids, as assayed with liposomes and lipid-embedded membranes (15, 16, 20). While the CD3ε lipid binding domain maps to a basic amino acid-rich stretch (BRS) that follows its transmembrane domain, the corresponding region(s) in CD3ζ and its phospholipid-binding specificity have not been established (15). CD3ζ contains multiple clusters of basic amino acids that are highly conserved in different species (Fig. 1A, Supplemental Fig. 1). To define which, if any, of these clusters were capable of mediating phospholipid interactions, biotinylated peptides spanning distinct subsections of CD3ζ were used to probe membranes embedded with an assortment of lipids (PIP strips). The peptides included three polybasic clusters, termed BRS-1, BRS-2, and BRS-3, as well as the three ITAMs (ITAM-1 to -3) (Fig. 1A). The individual ITAMs were tested because the CD3ε ITAM has been proposed to embed within the inner leaflet of the plasma membrane via lipid binding, thereby modulating its phosphorylation (17). The first BRS (BRS-1) of CD3ζ, located at the juxtamembrane position, did not bind any phospholipids, unlike that reported for CD3ε (Fig. 1B) (15). BRS-2, encompassing a region that preceded the second ITAM, bound to almost every phosphoinositide (Fig. 1B). BRS-2 did not associate with phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine, or sphingosine-1-phosphate, demonstrating a selective inositol binding specificity (Fig. 1B). BRS-3, located between the second and third ITAM, preferentially bound to PtdIns(4)P, PtdIns(5)P, and phosphatidic acid, much like the binding specificity reported for CD3ε (Fig. 1B). The first CD3ζ ITAM (ITAM-1) complexed PtdIns(3,4,5)P₃ weakly, with some binding to PtdIns(3)P noted on longer exposures (Fig. 1B) (data not shown). Peptides encompassing the second and third ITAMs (ITAM-1 and ITAM-3) did not bind to the PIP strips. Substitution of lysine and/or arginine residues with alanines, valines, and glycines in ITAM-1 (ITAM-1 Sub), BRS-2 (BRS-2 Sub), and BRS-3 (BRS-3 Sub) completely eliminated phospholipid binding, confirming the importance of the positively charged amino acids in mediating the CD3ζ–phospholipid interactions (Supplemental Fig. 2A, 2B).

To better define the lipid-binding affinities of ITAM-1, BRS-2, and BRS-3, the corresponding peptides were used to probe PIP arrays containing serial dilutions of the phosphatidylinositol phospholipids (Fig. 1C). BRS-2 had the highest relative affinity for

FIGURE 1. The cytoplasmic tail of CD3ζ contains multiple clusters of arginine and lysine residues that complex phosphoinositides. A. Amino acid sequence of the cytoplasmic tail of murine CD3ζ containing clusters of basic amino acids, as indicated by asterisks. Individual biotinylated peptides containing the three BRS sequences (BRS-1, BRS-2, BRS-3) and the three ITAMs (ITAM-1, ITAM-2, ITAM-3) are shown. The tyrosine residues present in the ITAMs are underlined. B. PIP strips embedded with diverse lipids were probed with the biotinylated peptides. Binding was detected by streptavidin–HRP Western blotting, with the different exposure times indicated below the membrane. Data are representative of four independent experiments. C. Biotinylated peptides containing ITAM-1, BRS-2, and BRS-3 were used to immunoblot lipid arrays containing serial dilutions of the indicated phospholipids ranging from 100 to 1.56 pmol, as shown in the left panel. Peptide binding was assessed as in B. The data are representative of at least four independent binding assays per peptide.
PtdIns(5)P, followed by PtdIns(3,5)P2, binding amounts as low as 3.1 pmol (Fig. 1C). BRS-3 required at least 25 pmol of PtdIns(5)P, PtdIns(3,5)P2, and PtdIns(4)P, suggesting a binding affinity at least 30-fold less than BRS-2. ITAM-1 complexed to PtdIns(3,4,5)P3, but required a minimum of 25 pmol of spotted lipid for detection. These binding affinities differed from each other as well as those observed with the CD3 ε BRS (Supplemental Fig. 2C).

A single BRS within the cytoplasmic tail of CD3 ζ is the predominant phospholipid-binding motif

We next examined the phospholipid-binding specificity of the entire cytoplasmic tail of CD3 ζ using purified GST fusion proteins (Fig. 2A). The full cytoplasmic region of CD3 ζ had an affinity similar to the BRS-2 peptide (Fig. 2B). To determine the contribution of the basic amino acid clusters in this binding, several CD3 ζ-modified constructs were generated in which alanine, glycine, and valine residues were substituted for the indicated arginines and lysines. Because lysine residues in CD3 ζ can serve as ubiquitination sites, the majority of the mutations were introduced at the arginine positions (30). The substitutions were introduced in all three binding subregions (BRS Sub-A), within ITAM-1 and BRS-2 (BRS Sub-B), or just within BRS-2 (BRS Sub-C). These modifications comprised 12, 9, and 4 aa substitutions, respectively (Fig. 2A). The GST–BRS Sub-A, -B, and -C fusion proteins did not bind any phosphoinositides (Fig. 2B). The fact that substitution of only four residues (BRS Sub-C) eliminated lipid associations suggests that the positively charged sequence located within the second BRS (BRS-2) is the principal phospholipid-binding motif in the cytoplasmic tail of CD3 ζ. This was supported by the 30-fold higher affinity of the BRS-2 peptide for phosphoinositides compared with the other phospholipid-binding peptides (ITAM-1 and BRS-3). We next examined whether CD3 ζ could complex phospholipids present in a lipid bilayer. Liposomes containing 80% phosphatidylcholine mixed with 20% of either phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P2] or PtdIns(5)P were incubated with GST, GST–ζ (BRS WT), or GST–BRS Sub-C (Fig. 2C). Greater than 90% of the control GST protein was retained in the supernatant, with little detected in the liposome-containing pellet (Fig. 2C, lanes 1 and 3 versus 2 and 4). In contrast, 78% of GST–ζ (GST–ζ WT) was associated with PtdIns(4,5)P2, and ~37% complexed with PtdIns(5)P-containing liposomes (Fig. 2C, lanes 6 versus 5 and 8 versus 7). GST–BRS Sub-C exhibited much less lipid binding to PtdIns(4,5)P2 (26%), with the majority (74%) detected in the supernatant.

**FIGURE 2.** A single polybasic cluster in the cytoplasmic tail of CD3 ζ complexes selected phosphoinositides with high affinity. A, Amino acid sequence of the cytoplasmic domain of WT CD3 ζ and constructs containing a number of amino acid substitutions at various polybasic cluster positions. These include substitutions of 12 (BRS Sub-A), 9 (BRS Sub-B), and 4 (BRS Sub-C) residues, respectively. The substitutions are boldface and underlined. B, PIP arrays containing serial dilutions of the indicated phosphoinositides were probed with the purified GST–ζ fusion proteins. Binding was detected by anti-GST Western blotting assays. Binding assays with the substituted constructs are shown with 2-min (BRS Wild Type) and 30-min (BRS Sub-A, -B, -C) exposures. The data are representation of three independent experiments. C, Sucrose-loaded liposomes, consisting of the indicated phospholipids, were incubated with GST (lanes 1–4), GST–ζ Wild Type (lanes 5–8), or GST–BRS Sub-C (lanes 9–12). Proteins retained in the supernatant (S) or pellet (P, liposome binding fraction) were detected by SDS-PAGE analysis using Coomassie brilliant blue staining. The percentage of GST fusion protein detected in the supernatant and pellet is listed under the appropriate lanes. These results were obtained in two independent experiments.
HEK cells. This contrasts the increased phosphorylation noted for functions of CD3ζ demonstrated that elimination of the phosphoinositide-binding results in the binding and stabilization of the more heavily anti-CD3 μmunoprecipitation of CD3ζ phosphorylated and migrated in a pattern very similar to WT CD3ζ developed into several phosphorylated intermediates, although, A phosphoproteins ranging from 18 to 23 kDa when analyzed in absence of ZAP-70, WT CD3ζ A (WT, BRS Sub-A, Sub-B, or Sub-C) (Figs. 2,3). Together, these data suggest that modifications to the phospholipid-binding properties of full-length CD3ζ could impact ITAM phosphorylations and/or ZAP-70 associations. To address these possibilities, we characterized the phosphorylation potential of the CD3ζ constructs containing the various BRs modifications. Experimentally, HEK cells were transfected with constructs containing Lck and/or ZAP-70 (tandem SH2 domain) in addition to either vector alone or the indicated CD3ζ constructs (WT, BRs Sub-A, Sub-B, or Sub-C) (Figs. 2A, 3A and 3B). In the absence of ZAP-70, WT CD3ζ appeared as a heterogeneous set of phosphoproteins ranging from 18 to 23 kDa when analyzed in total cell lysates (Fig. 3A, lane 3). The BRs Sub-A and -B also developed into several phosphorylated intermediates, although both exhibited much higher molecular masses of 26–30 kDa (Fig. 3A, lanes 4 and 5). The CD3ζ BRs Sub-C protein was tyrosine phosphorylated and migrated in a pattern very similar to WT CD3ζ, although with a slightly faster mobility (Fig. 3A, lane 6 versus 3). The phosphorylation patterns were confirmed by direct immunoprecipitation of CD3ζ followed by anti-phosphotyrosine and anti-CD3ζ immunoblotting (Fig. 3B, 3C). These experiments demonstrated that elimination of the phosphoinositide-binding functions of CD3ζ did not affect its tyrosine phosphorylation in HEK cells. This contrasts the increased phosphorylation noted for CD3ε when its BRs was mutated, an effect only found in non-T cells (15).

Coexpression of the tandem SH2 domains of ZAP-70 with CD3ζ results in the binding and stabilization of the more heavily phosphorylated CD3ζ intermediates (27). Accordingly, each of the CD3ζ BRs Sub molecules was more heavily phosphorylated when coexpressed with ZAP-70 (Fig. 3D, 3E). This was consistent with the increased percentage of CD3ζ that was tyrosine phosphorylated (Fig. 3F versus 3C). To ascertain whether mutations in CD3ζ affected ZAP-70 association, the CD3ζ precipitates were immunoblotted with anti–ZAP-70 Abs. ZAP-70 complexed the various phoso-CD3ζ intermediates, with the largest amount of ZAP-70 coprecipitating with BRs Sub-C (Fig. 3G). Taken together, these experiments demonstrated that the CD3ζ BRs interactions with phosphoinositides are not required for ITAM accessibility to Src-family kinases or phspho-ITAM binding to ZAP-70.

To examine further the functional role of the CD3ζ BRs–phosphoinositide interactions in regulating signaling pathways, CD3ζ-deficient T cells were reconstituted with WT or BRs Sub-C CD3ζ chains. Clones were selected for identical TCR surface expression and compared for their capacity to initiate TCR-mediated phosphoprotein induction at different time points. The magnitude and kinetics of overall TCR-mediated phosphoprotein induction was comparable in the WT and BRs Sub-C reconstituted T cells (Fig. 4A). These findings were consistent with that observed in HEK cells, suggesting that the phosphoinositide-binding functions of CD3ζ are unconnected from early intracellular signaling events. To address this question more carefully, selected proteins were either immunoprecipitated followed by Western blotting or directly assayed with anti–phospho-specific Abs. These experiments revealed similar levels and kinetics of ZAP-70 (Fig. 4B), CD3ζ (T co-precipitated with ZAP-70 (Fig. 4B), PLC-ζ (Fig. 4D), PKCζ (Fig. 4E), AKT (Fig. 4F), and Erk (Fig. 4G) phosphorylation in WT and BRs Sub-C expressing T cells (Fig. 4B–G, Supplemental Fig. 4). The only protein that showed a statistically significant difference between the two clones was SLP-76 (Fig. 4C, t = 3 min, p = 0.047) (Supplemental Fig. 4). Importantly, both clones expressed similar levels of total actin whereas the levels of CD3ζ were slightly lower in the BRs Sub-C line (Fig. 4H, 4I). TCR signaling processes are required for the expression of activation markers, including CD69 and CD25. To determine if the phosphoinositide-binding function of CD3ζ contributes to these later processes, TCR-induced upregulation of CD69 and IL-2 secretion was compared between the WT and BRs Sub-C reconstituted T cells. There was no significant impairment in CD69 induction or IL-2 production in T cells lacking the lipid-binding functions of CD3ζ (data not shown). These results demonstrated that the phosphoinositide-binding functions of CD3ζ are unconnected from early intracellular signaling events. To address this question more carefully, selected proteins were either immunoprecipitated followed by Western blotting or directly assayed with anti–phospho-specific Abs. These experiments revealed similar levels and kinetics of ZAP-70 (Fig. 4B), CD3ζ (T co-precipitated with ZAP-70 (Fig. 4B), PLC-ζ (Fig. 4D), PKCζ (Fig. 4E), AKT (Fig. 4F), and Erk (Fig. 4G) phosphorylation in WT and BRs Sub-C expressing T cells (Fig. 4B–G, Supplemental Fig. 4). The only protein that showed a statistically significant difference between the two clones was SLP-76 (Fig. 4C, t = 3 min, p = 0.047) (Supplemental Fig. 4). 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CD3 ζ-Phosphoinositide Complexes Regulate T Cell Activation

Z are not required for early TCR-mediated phosphoprotein induction after a strong stimulus. The phospholipid-binding properties of CD3 ζ are required for normal immunological synapse formation. T cell interactions with agonist peptide-loaded APCs causes the TCR to redistribute to a structure at the center of the T cell–APC interface known as the central supramolecular activation cluster or immunological synapse (31). Eliminating the positively charged residues within the BRS of CD3 ε was found to impair significantly the recruitment of the TCR to the immunological synapse (15). To examine whether the CD3 ζ binding to phosphoinositides could have a similar function, 5C.C7 T cells were reconstituted with the various CD3 ζ–GFP constructs, and imaging studies were performed to monitor the distribution of CD3 ζ. Quantitative immunoblotting of the GFP-sorted cells indicated that the levels of the ζ–GFP chimeric molecule were 14.4 ± 7% (n = 3) overexpressed compared with the endogenous protein levels (Supplemental Fig. 5). Upon tight cell couple formation, the localization of BRS Sub-C to two prototypical sites of TCR accumulation (the distal pole and the center of the T cell–APC interface) was dramatically impaired (Fig. 5A). The number of cell couples exhibiting transient accumulation of CD3 ζ–GFP at the distal pole of the T cell was significantly reduced (p < 0.001) from a maximum of 64 ± 7% to 20 ± 4% when comparing WT CD3 ζ–GFP with BRS Sub-C–GFP (Fig. 5B versus 5C). Unlike WT CD3 ζ, BRS Sub-C–GFP was not maintained at the center of the T cell–APC interface for more than 120 s, instead accumulating in internal punctuate structures, most likely vesicles (Fig. 5A, 5C, Supplemental Video 1). Such internal accumulation was seen in 88 ± 4% of the cell couples expressing BRS Sub-C–GFP, and less than half with cell couples with WT CD3 ζ–GFP (p < 0.001). In addition, the percentage of cells exhibiting any accumulation of CD3 ζ at the interface after 7 min of tight cell couples formation dropped significantly from 93 ± 5% in T cells expressing WT CD3 ζ–GFP to 24 ± 5% in T cells expressing BRS Sub-C–GFP (p < 0.001). The experiments were repeated with a distinct TCR transgenic line, D011.10, which expresses an OV A-specific TCR. Consistent with the 5C.C7 results, the TCR in the BRS Sub-C–GFP expressing D011.10 T cells was not maintained at the T cell–APC interface for more than about 2 min and again accumulated in internal punctuate structures (Fig. 5E versus 5D, Supplemental Video 2). This internal accumulation was seen in 88 ± 5% of the cell couples, very distinct from the WT CD3 ζ–GFP (p < 0.001) (Fig. 5D). Unlike the 5C.C7 T cells, the accumulation of the TCR at the distal pole in the D011.01 cells was not affected by the mutation of the BRS. The localization defects noted with CD3 ζ were distinct from those observed when analyzing the redistribution of other CD3 chains containing various signaling mutations. For example, mutation of the CD3 ε BRS motif resulted in delayed and reduced accumulation of the TCR complex at the center of the T cell–APC interface but without the consistent and prominent internal accumulation seen with the CD3 ζ BRS Sub-C–GFP constructs (15). Moreover, substitution of the tyrosine residues in the CD3 ζ ITAMs yielded a slower translocation of CD3 ζ from the...
These data support a unique role for the **CD3**$^\text{z}$BRS signaling motif in the redistribution of the TCR after T cell–APC interactions.

Using these retrovirally reconstituted T cells, we next analyzed late T cell activation events. In a peptide dose-response curve, the levels of IL-2 and the induction of CD69 expression were equivalent between the WT and CD3$^\text{z}$BRS Sub-C reconstituted cells (data not shown). These findings suggest that while CD3$^\text{z}$–phospholipid interactions stabilize synapse formation, this is not critical for induction of CD69 expression and IL-2 production. This could relate to the presence of endogenous CD3$^\text{z}$ molecules in the 5C.C7 cells and the fact that the TCR contains two independent signaling modules, CD3$^\text{zz}$ and CD3$^\text{g}\varepsilon$ (32).

**Discussion**

Polybasic clusters are defined as arginine- and lysine-enriched amino acid sequences that enable diverse transmembrane and cytosolic proteins to bind lipids. In the immune system, the cytoplasmic domains of several ITAM-containing subunits, including CD3$^\varepsilon$ and FcεRγ, have been shown to bind phospholipids due to the presence of such polybasic structures (15, 16, 20). In this study, we report that the CD3$^\text{z}$ subunit contains a key polybasic cluster—which precedes the second ITAM—that complexes select phosphoinositides including PtdIns(3)P, PtdIns(4)P, PtdIns(5)P, PtdIns(3,5)P$^2$, and PtdIns(3,4,5)P$^3$ with a high affinity. CD3$^\text{z}$ does not bind phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine, or sphingosine-1-phosphate. Because the structural basis of polybasic cluster–phosphoinositide interactions is not well understood, we engineered diverse arginine and lysine substitutions in CD3$^\text{z}$ to eliminate any basic amino acid pairs. As lysine residues are polyubiquitination sites, the substitutions were directed at three arginine residues and just one lysine (30, 33). The one lysine substituted (in murine CD3$^\text{z}$BRS Sub-C) does not undergo K-33 linked polyubiquitination and is not conserved in other species (34). Based on the various
amino acid substitutions that were engineered in CD3 ζ, it appears that the phosphoinositide binding requires a tandem pair of basic residues separated by 10 other amino acids.

In prior studies, using just the cytoplasmic region of CD3 ζ, the ability of Src-kinases to phosphorylate the tyrosine residues within its ITAMs was inhibited in the presence of liposomes containing negatively charged phospholipids (20). These observations led to the hypothesis that electrostatic interactions between the cytoplasmic tail of CD3 ζ and charged phospholipids may regulate the phosphorylation state of its ITAM (35). A similar mechanism has been proposed for CD3 ε, where the phosphorylation of its ITAM was hypothesized to be blocked by insertion of the cytoplasmic tail into the plasma membrane (17, 35). However, all these conclusions were based on experiments using recombinant proteins lacking the extracellular and transmembrane regions of the CD3 molecules in the absence of an intact TCR complex. To define the functional contribution of CD3 ζ-phosphoinositide binding in T cells, we expressed a GFP-linked CD3 ζ BRS Sub-C molecule in naïve 5C.C7 and OTII T cells. Remarkably, a functional phospholipid interaction was required for effective recruitment of the TCR to the immunological synapse. Moreover, the inability to bind phosphoinositides resulted in an intracellular accumulation of CD3 ζ in punctate structures. This suggests that efficient redistribution of CD3 ζ after Ag receptor signaling requires CD3 ζ to complex select phosphoinositides. Although the identity of the punctate structure is unknown, recent experiments suggest that it could be recycling endosomes. Imaging studies have revealed an accumulation of phospho-ζ in endosomes (36). In addition, recycling endosomes containing TCR–CD3 polarize to the immunological synapse (18). These distributions could require that CD3 ζ BRS interacts with PtdIns3P, PtdIns(3,5)P2 and low levels of PtdIns(3,4,5)P3, which are enriched in endosomes and the plasma membrane, respectively. A small amount of PtdIns(4)P is also generated at the plasma membrane after T cell activation (19, 37–40). Notably, IFT20 is associated with early and recycling endosomes, as well as the cis- and trans-Golgi network. IFT20 interacts with both CD3 ζ and ε in an activation-dependent manner, implying a functional link between these different organelles and the immunological synapse (19). Furthermore, TCR clustering is impaired when IFT20 is knocked down (19). Further experiments are required to elucidate whether selected phosphoinositides bound by CD3 ζ regulate these patterns of TCR accumulation and redistribution and whether this involves IFT20 interactions.

Our previous work has demonstrated that CD3 ε-phosphoinositide interactions maintained normal cell surface levels of the TCR (15). The phosphoinositide interactions did not affect the TCR-inducible tyrosine phosphorylation of the CD3 ITAMs (15). Our current findings indicate that CD3 ζ–phosphoinositide interactions are also uncoupled from ITAM phosphorylations. Yet, there are clear differences between the CD3 ζ and ε BRS. First, CD3 ζ exhibited a distinct binding preference for particular phosphoinositides. Second, the CD3 ζ BRS was located prior to the second ITAM whereas the CD3 ε BRS was adjacent to the transmembrane region. This could indicate that the CD3 ζ–phosphoinositide interactions are more flexible than that for CD3 ε. Third, transient transfection assays in HEK cells revealed similar levels of tyrosine-phosphorylated WT CD3 ζ and BRS Sub-C, with both capable of complexing ZAP-70. This result was quite distinct from the transient transfection assays performed with CD3 ε, wherein the substitution of the BRS significantly increased the tyrosine phosphorylation of its ITAM (15).

Our results have important implications for a recently proposed model of TCR signal initiation. In this model, interactions between CD3 ε and ζ and negatively charged phospholipids were proposed to be essential for the initiation of TCR signal transmission (17, 35). Although CD3 ζ and ε might contribute to this process, the lipid-binding functions are clearly not required for TCR signaling after TCR interactions with cognate peptide–MHC complexes. As our data establish, CD3 ζ–phosphoinositide interactions regulate TCR clustering, internalization, and/or recycling. The combined ability of CD3 ζ to bind to diverse phosphoinositides is likely to be essential for T cell activation under situations of low peptide–MHC concentrations. In addition to CD3 ζ and ε, the FcεRI γ-chain has also been reported to complex lipids. Our findings suggest that this lipid-binding property could also influence the clustering and aggregation of the high-affinity IgE receptor (16, 41). Other proteins of the innate and adaptive immune system that contain BRS motifs include stomal interaction molecule 1, a calcium regulator, and Trif-related adapter molecule, a TLR adapter protein (42, 43). Ultimately, understanding the role of CD3 ζ-phospholipid interactions in T cell functions will provide greater insight into not only the mechanisms controlling T cell activation but also how other proteins containing such polybasic clusters regulate cellular activity.

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Disclosures

The authors have no financial conflicts of interest.

References


