Ionic CD3–Lck interaction regulates the initiation of T-cell receptor signaling

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Antigen-triggered T-cell receptor (TCR) phosphorylation is the first signaling event in T cells to elicit adaptive immunity against invading pathogens and tumor cells. Despite its physiological importance, the underlying mechanism of TCR phosphorylation remains elusive. Here, we report a key mechanism regulating the initiation of TCR phosphorylation. The major TCR kinase Lck shows high selectivity on the four CD3 signaling proteins of TCR. CD3 ϵ is the only CD3 chain that can efficiently interact with Lck, mainly through the ionic interactions between CD3 ϵ basic residue-rich sequence (BRS) and acidic residues in the Unique domain of Lck. We applied a TCR reconstitution system to explicitly study the initiation of TCR phosphorylation. The ionic CD3 ϵ –Lck interaction controls the phosphorylation level of the whole TCR upon antigen stimulation. CD3 ϵ BRS is sequestered in the membrane, and antigen stimulation can unlock this motif. Dynamic opening of CD3 ϵ BRS and its subsequent recruitment of Lck thus can serve as an important switch of the initiation of TCR phosphorylation.

T-cell receptor | Lck | initial phosphorylation | substrate selectivity | ionic interaction

T cells play key roles in adaptive immunity. Appropriate T-cell response is required for efficient cleaning of invading pathogens and transformed cells based on the recognition of non-self-antigens (1). Abnormal activity of T cells to self-antigens leads to undesirable inflammation and tissue damage that causes autoimmune diseases (2). Harnessing T-cell activity and T-cell-based cell therapies have been successfully applied in clinic to treat cancers and other diseases (3–6). However, current T-cell therapies have limited efficacy. Understanding the molecular basis of T-cell activation therefore is desired to pave the way for new concepts of immunotherapies.

A key feature of T-cell immune response is the specificity. Different antigens can elicit diverse T-cell responses via different T-cell signaling programs (7). The first step of T-cell signaling is the antigen-induced T-cell receptor phosphorylation (8). A T-cell regulator (TCR) protein complex is composed of an antigen recognition subunit TCR $\alpha\beta$ and three signaling subunits CD3 $\epsilon\delta$, CD3 $\epsilon\gamma$, and CD3 $\zeta\zeta$ (9). The cytoplasmic domains of CD3 ϵ , CD3 δ , CD3 γ , and CD3 ζ all contain the conserved Immunoreceptor Tyrosine-based Activation Motif (ITAM). CD3 ζ has three ITAMs whereas other CD3 chains each contain one ITAM. Different antigens are able to induce distinct phosphorylation programs of the 10 ITAMs of a TCR to trigger different T-cell signaling pathways that lead to antigen-specific T-cell immune response (1).

Lck and Fyn, both belonging to the Src kinase family, are the major kinases to phosphorylate TCR in T cells. Genetic experiments show that Lck plays a more important role than Fyn in TCR signaling (10). Lck can either be loaded on coreceptor CD4/CD8 or remain free (11–13). It is suggested that Lck molecules are all loaded on coreceptors in thymocytes (12). However, super-resolution imaging shows that CD4 and Lck localize in different

nanodomains in mature T cells (13). Functionally, the coreceptor contributes to antigen sensitivity of T cells but is not essential for T-cell activation in mature T cells (14). A recent study shows that free Lck molecules in mature T cells are responsible for initial TCR phosphorylation (15).

In addition to a C-terminal tyrosine kinase domain, Lck contains four regulatory domains, i.e., an SH4 domain, a Unique domain (UD), an SH3 domain, and an SH2 domain. The N-terminal SH4 domain contains a myristoylation site and a palmitoylation site. The lipid modifications are responsible for targeting Lck to cellular membranes, particularly the inner leaflet of the plasma membrane (16–18). Following the SH4 domain is the UD, which is less conserved among Src family members. The N-terminal part of the Lck UD domain forms a zinc clasp with coreceptor CD4 or CD8 using its highly conserved CxxC motif (11). However, whether the UD domain has other functions is unknown. The SH3 and SH2 domains of all Src kinases are highly conserved. The SH3 domain binds the PxxP motif as well as the proline-independent tyrosine-based RKxxYxxY motif (19, 20), thus regulating the substrate specificity. The SH2 domain binds to phosphotyrosine, and its interaction with an inhibitory pTyr site at the Lck C terminus leads to an auto-inhibitory folding (21, 22). Earlier experiments show that ~40% of Lck molecules in resting T cells are constitutively active (23) and antigen stimulation leads to local activation of Lck molecules (24, 25). However, how Lck recognizes CD3 substrates and initiates ITAM phosphorylation remains elusive. This earliest event of TCR signaling is crucial because it determines the downstream signaling programs. More recently, residues surrounding ITAM tyrosines have been found to be important for interaction with the Lck kinase domain and affect substrate selectivity of Lck (26). Whether other Lck domains contribute to CD3 selectivity is still unclear.

Significance

Antigen-induced phosphorylation of T-cell receptor (TCR) is the first signaling event in T cells that determines the functional outcome of antigen stimulation. Here, we report a key regulatory mechanism of the initiation of TCR phosphorylation. Using biochemical and biophysical tools, we find that a basic residue-rich sequence in CD3 ϵ subunit of TCR can efficiently recruit Lck via ionic interaction to initiate TCR phosphorylation. The mechanism reported here can aid in the design of better T-cell therapies such as the Chimeric Antigen Receptor T-cell therapies.

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Here we first used biochemical and biophysical experiments to show that Lck has strong selectivity on four CD3 chains and that CD3 ϵ is the most favorable substrate because of the ionic interactions between CD3 ϵ basic residue-rich sequence (BRS) and acidic residues in Lck UD. Functional experiments showed that the recruitment of Lck by CD3 ϵ directly controls the initiation of TCR phosphorylation.

Results

CD3 Signaling Proteins Have Different Membrane Binding Properties.

The cytoplasmic domains of four CD3 chains have low sequence similarity except the conserved ITAM. Previous studies strongly suggest that they should have different functional roles in TCR signaling (27–29). The CD3 ϵ cytoplasmic domain (CD3 ϵ _{CD}) has a BRS at the juxtamembrane region, followed by a nonredundant proline residue-rich sequence (PRS) and an ITAM (30). CD3 ζ _{CD}

contains three ITAMs and BRS in between ITAMs. CD3 δ _{CD} and CD3 γ _{CD} each have one ITAM but no enrichment of basic residues (Fig. 1A). Under physiological pH, human CD3 ϵ _{CD} and CD3 ζ _{CD} carry +11 and +5 charges, respectively, whereas CD3 δ _{CD} and CD3 γ _{CD} carry 0 charges. In resting T cells, both CD3 ϵ _{CD} and CD3 ζ _{CD} can bind to acidic phospholipids in the plasma membrane via ionic interactions, which can sequester key tyrosines within the membrane bilayer to prevent spontaneous phosphorylation by the constitutively active Lck (31–37). Such a safety control can be removed by Ca²⁺ and other factors in activated T cells to make CD3 ϵ _{CD} and CD3 ζ _{CD} available for tyrosine phosphorylation (36, 38, 39). The reversible lipid binding of CD3 ϵ _{CD} and CD3 ζ _{CD} thus enables a precise regulation of ITAM phosphorylation. However, whether this mechanism also applies to CD3 δ and CD3 γ is unknown. We used a live-cell FRET experiment to compare the membrane binding properties of the cytoplasmic domains of four

Fig. 1. Different membrane binding properties of CD3 signaling proteins. (A) The protein sequences of human CD3 ϵ , CD3 ζ , CD3 δ , and CD3 γ cytoplasmic domains. BRS, PRS, and ITAM were highlighted by cyan, yellow, and green, respectively. (B–E) The membrane binding of cytoplasmic domain of each CD3 chain was measured by dequenching FRET in the context of KIR2DL3-CD3_{CD}-mTFP constructs. The FRET donor was the mTFP fused to the C terminus of the CD3 cytoplasmic domain, and the FRET acceptor was R18 dye inserted into the plasma membrane. The dileucine motif in CD3 δ or CD3 γ was mutated to alanine to avoid protein down-regulation. The short linker construct, 3 amino acid (a.a.)-mTFP, served as the positive control. (B) The FRET efficiency. Jurkat cells with different constructs were sorted to have matched mTFP level. The (C) surface R18 staining level and (D) photobleaching efficiency for Jurkat cells with different CD3 constructs were comparable. Each dot represents the value from one cell; $n = 24, 22, 27, 20$, and 19 for 3a.a.-mTFP, hCD3 ϵ _{CD}-mTFP, hCD3 ζ _{CD}-mTFP, hCD3 δ _{CD}(LL-AA)-mTFP, and hCD3 γ _{CD}(LL-AA)-mTFP, respectively. The result was a representative of two independent experiments. Error bars represent mean $\pm$ SD. P values were determined by the one-way ANOVA with Tukey's multiple comparisons test; n.s., not significant; **** $P < 0.0001$; ** $P < 0.01$. (E) Representative pictures of each construct. (Scale bar, 5 μ m.)

CD3 signaling proteins in the context of HA-KIR2DL3-CD3_{CD}-mTFP chimeric construct. The data clearly showed that CD3_ε_{CD} and CD3_ζ_{CD} bound to the membrane and thus generated high FRET values, whereas CD3_δ_{CD} and CD3_γ_{CD} were exposed in the cytosol and generated low FRET values (Fig. 1 *B–E*). Consistently, CD3_δ_{CD} and CD3_γ_{CD} within the native TCR–CD3 complex also showed cytosol-exposed conformation (Fig. S1). Given that constitutively active Lck molecules are largely present (23), it is difficult to understand why the well-exposed ITAMs in CD3_δ and CD3_γ are not highly phosphorylated by Lck before antigen stimulation.

Lck Is Highly Selective on Different CD3 Signaling Proteins. We next set up an *in vitro* phosphorylation (IVP) assay to test the phosphorylation efficiency of Lck on individual GST-CD3_{CD}-His fusion protein. Under the same condition, CD3_ε was strongly phosphorylated and CD3_ζ was modestly phosphorylated. However, CD3_δ and CD3_γ were barely phosphorylated by Lck (Fig. 2*A*). The phosphorylation efficiency of different CD3 chains is thus highly correlated with CD3 charge property. Intriguingly, Fyn didn't show the same selectivity as Lck. CD3_ζ appeared to be a better substrate for Fyn than CD3_ε (Fig. 2*B*). CD3_δ and CD3_γ again were barely phosphorylated by Fyn.

To study why CD3_ε was the best substrate of Lck, we generated two CD3_ε mutants, CD3_ε^{BRSmut} and CD3_ε^{PRSmut}. In CD3_ε^{BRSmut}, the first six basic residues in BRS were mutated to serines. In CD3_ε^{PRSmut}, proline residues in PRS were mutated to alanines. The IVP result showed that BRS mutation significantly impaired CD3_ε phosphorylation, whereas PRS mutant only had a minor effect (Fig. 2*C*). To further test the importance of BRS, we used the CD3_ε BRS (SKNRKAKAK) to replace the nine N-terminal residues in

CD3_δ_{CD} and CD3_γ_{CD} to generate CD3_δ-_ε^{BRS} and CD3_γ-_ε^{BRS} mutants. The phosphorylation efficiency of both mutants was largely increased (Fig. 2*D*). These data together highlighted the importance of BRS in the Lck-mediated CD3 phosphorylation.

Ionic Interactions Determine the Substrate Selectivity of Lck. We analyzed the protein sequence of Lck and found that its UD domain is enriched of acidic residues, carrying −8 charges at neutral pH (Fig. S2). These acidic residues are highly conserved across different species. Because Lck favored the substrate with positive charge, we proposed that the ionic interaction between acidic Lck UD and basic CD3_ε BRS might play an important role in the substrate selectivity of Lck.

To test this proposal, we set up a biolayer interferometry (BLI) experiment using the Octet Red 96 system. Human Lck protein containing the UD and SH3 domains (Lck_{UD+SH3}) was purified, biotinylated, and immobilized onto streptavidin biosensors. Then, the Lck_{UD+SH3}-coated biosensor was immersed into solutions that contained only one kind of CD3 peptide, and then back to blank solution to study the reaction kinetics and thermodynamics. The association and dissociation curves indicated that Lck_{UD+SH3} showed a stronger binding with CD3_ε than with CD3_ζ. Nearly no Lck binding was observed for CD3_δ or CD3_γ (Fig. 3*A*). This result agreed well with the IVP data in Fig. 2*A*. Mutating CD3_ε BRS not only impaired the binding strength but also slowed down the binding kinetics (Fig. 3*B*), whereas PRS mutation only affected the binding strength (Fig. 3*C*). Moreover, introducing CD3_ε BRS to CD3_δ or CD3_γ chain led to evident binding to Lck_{UD+SH3} (Fig. 3*D* and *E*), which further demonstrated the importance of BRS in the interaction between Lck kinase and CD3 substrate. We noticed that

Fig. 2. Lck was highly selective on different CD3 chains. (*A* and *B*). Purified GST-CD3_{CD}-His protein was phosphorylated by (*A*) Lck or (*B*) Fyn in an IVP assay. (*C*) BRS-mutated CD3_ε^{BRSmut} mutant and PRS-mutated CD3_ε^{PRSmut} mutant were generated and subjected for Lck phosphorylation. (*D*) The nine N-terminal residues in CD3_δ or CD3_γ were replaced by CD3_ε BRS, and the mutants were compared with WT proteins in the IVP assay. The bands were quantitated by ImageJ. The pTyr signal of CD3 peptide was divided by the corresponding GST-CD3 signal. The pTyr/GST-CD3 value of different CD3 peptides was further normalized to (*A–C*) that of CD3_ε or to (*D*) that of CD3_δ/CD3_γ WT. Average represents the average value of three independent experiments.

CD3 δ - ϵ BRS and CD3 γ - ϵ BRS mutants did not show binding kinetics that were as fast as those of CD3 ϵ and CD3 ϵ PRSmut, suggesting that there might be another motif in CD3 ϵ that contributes to fast Lck binding. An earlier study shows that the Lck SH3 domain can bind to an RKxxYxxY motif in a proline-independent manner (20). The RKxxxxY motif in CD3 ϵ ITAM region thus might be another motif contributing to the fast binding with Lck.

The above molecular interaction data (Fig. 3) and the phosphorylation data (Fig. 2) together showed that CD3 ϵ BRS played a more important role than PRS in interacting with Lck to result in efficient phosphorylation.

Determination of the Binding Sites for CD3 ϵ -Lck Interaction. To define the CD3 ϵ -Lck interface at atomic resolution, we performed a series of NMR experiments. We first made backbone chemical shift assignments for CD3 ϵ CD residues and Lck_{UD+SH3} residues by standard triple-resonance experiments, and then performed titration experiments to map the key residues involved in CD3 ϵ -Lck interaction.

The ^{15}N -labeled CD3 ϵ CD WT was titrated by unlabeled Lck_{UD+SH3} with molar ratios ranging from 0 to 10, and the resonances of CD3 ϵ CD residues were traced in ^{15}N - ^1H heteronuclear

single-quantum coherence (HSQC) spectra (Fig. S3A). Substantial changes in chemical shifts were observed for most of the regions in CD3 ϵ CD except the C-terminal region (Fig. S3B and C). Furthermore, some residues showed a large decrease of intensity after titration. These changing residues were likely to be involved in CD3 ϵ -Lck interaction. It has been reported that the Lck SH3 domain directly interacts with PRS and the RKxxYxxY motif (20); therefore, the chemical shift and intensity changes in the PRS and the first half of the ITAM of CD3 ϵ might be caused by their direct interaction with the Lck SH3 domain. Interestingly, we found that the interaction between CD3 ϵ PRS and Lck SH3 was also dependent on CD3 ϵ BRS. Mutating BRS residues made PRS residues less vulnerable to Lck_{UD+SH3} titration (Fig. S4).

To further map the CD3 ϵ -Lck interface, we titrated ^{15}N -labeled Lck_{UD+SH3} by unlabeled CD3 ϵ CD WT or mutants. As expected, both the Lck UD and SH3 domains showed systematic changes in chemical shift signals and peak intensities under CD3 ϵ CD WT titration (Fig. 4A, D, and E). We identified E10 to E15 in the UD domain (distal to the coreceptor binding site), and S71 to L80, Q93 to Q101, and I111 to A117 in the SH3 domain as the most affected regions under CD3 ϵ CD WT titration (Fig. 4E). We further

Fig. 3. CD3 interaction with the Lck UD-SH3 domain. The molecular interaction between different CD3 chains and Lck was measured by BLI. (A) Binding of Lck_{UD+SH3} to CD3 ϵ CD, CD3 δ CD, CD3 γ CD, or CD3 ζ CD. (B) Binding of Lck_{UD+SH3} to CD3 ϵ WT or varying concentrations of CD3 ϵ BRSmut. (C) Binding of Lck_{UD+SH3} to CD3 ϵ WT or varying concentrations of CD3 ϵ PRSmut. (D) Binding of Lck_{UD+SH3} to CD3 δ WT or varying concentrations of CD3 δ - ϵ BRS. (E) Binding of Lck_{UD+SH3} to CD3 γ WT or varying concentrations of CD3 γ - ϵ BRS. These results are representative of three independent experiments.

Fig. 4. The ^{15}N -labeled Lck_{UD+SH3} NMR signals in response to CD3 ϵ titration. (A–C) HSQC spectra of ^{15}N -labeled Lck_{UD+SH3} titrated by (A) CD3 ϵ_{WT} , (B) CD3 ϵ_{BRSmut} , or (C) CD3 ϵ_{PRSmut} . The molar ratio of CD3 ϵ /Lck was 0 to 10. (D) Representative residues from Lck UD and SH3. (E) A bar graph showing the resonance changes of Lck residues caused by CD3 ϵ titration. Some residues were left blank because we were unable to detect them (such as prolines), or they were unassigned (such as S6, H24, K33, and K84), or due to peak overlapping. The residues with peak overlapping are indicated by "O" symbols. Asterisks indicate peak broadening caused by the CD3 ϵ titration.

used unlabeled CD3 ϵ_{BRSmut} or CD3 ϵ_{PRSmut} peptide to titrate ^{15}N -labeled Lck $_{\text{UD-SH3}}$ (Fig. 4 *B* and *C*). Clearly, PRS mutation strongly abolished the chemical shift changes of residues in the SH3 domain but did not affect the changes of residues in UD, which confirmed the interaction between the CD3 ϵ PRS and Lck SH3 domains (Fig. 4 *B* and *D*). Differently, BRS mutation not only strongly abrogated the chemical shift changes of residues in UD but also largely influenced the changes of residues in the SH3 domain (Fig. 4 *C* and *D*). This result strongly suggests that CD3 ϵ BRS directly interacts with Lck UD and promotes the interaction between CD3 ϵ PRS and Lck SH3.

The above NMR experiments further confirmed the importance of CD3 ϵ BRS in interacting with Lck.

CD3 ϵ Helps the Phosphorylation of Other CD3 Chains. In the TCR complex, the four subunits are assembled together by strong ionic

interactions in the transmembrane domains and other interactions in the extracellular domains (40–42). The cytoplasmic domains of CD3 chains are therefore proximal to each other within the complex. The specific interaction between CD3 ϵ and Lck should recruit the kinase to the TCR complex to facilitate the phosphorylation of other CD3 chains. To test this proposal, we set up an on-bead phosphorylation assay to test the help effect of CD3 ϵ .

In this assay, we coated GST-tagged CD3 chain on glutathione bead and incubated the bead with Lck solution to mimic the phosphorylation process of membrane-anchored CD3 chain by free Lck (Fig. 5*A*). The selectivity of Lck on anchored CD3 chains was the same as the result observed in the IVP assay in which both CD3 and Lck were in solution (Fig. 5*B*). We further performed the cocoating experiment. CD3 ϵ WT or CD3 ϵ_{BRSmut} was coated together with another GST-tagged CD3 chain, i.e., CD3 δ , CD3 γ , or CD3 ζ , and then subjected to Lck phosphorylation. Immunostaining showed

Fig. 5. Recruitment of Lck by CD3 ϵ helped the phosphorylation of other CD3 subunits. (A) Schematic illustration of the on-bead kinase assay. GST-CD3 ϵ_{CD} WT-His or GST-CD3 ϵ_{CD} BRSmut-His was coated together with another GST-tagged CD3 chain onto GST beads at the molar ratio of 1:1. (B) Comparison of phosphorylation level of CD3 ϵ , CD3 δ , CD3 γ , and CD3 ζ on beads. (C) The α -CD3 and α -GST staining to show the well mixture of different CD3 proteins on the bead. (D) Comparison of phosphorylation level of CD3 δ , CD3 γ , and CD3 ζ in the presence of CD3 ϵ_{WT} or CD3 ϵ_{BRSmut} . The bands were quantitated by ImageJ. The pTyr signal of CD3 peptide was divided by the corresponding GST-CD3 signal. In *B*, the pTyr/GST-CD3 value of different CD3 peptides was further normalized to that of CD3 ϵ . In *D*, the pTyr/GST-CD3 value under the CD3 ϵ_{BRS} mutant condition was normalized to that under the CD3 ϵ_{WT} condition. Average represents the average value of three independent experiments.

Fig. 6. Mutating CD3 ϵ -Lck interface residue significantly attenuated TCR phosphorylation upon antigen stimulation. (A) The 1G4 TCR-CD3 complex and Lck WT or Mutant (D12N), as well as other essential proteins for initial TCR phosphorylation (CD45, CSK, CBP, CD2, and ICAM-1) into HEK293FT cells as previously described (43). Raji cells expressing MHC I loaded with the cognate ESO9V antigen served as antigen-presenting cells (APCs) (43). (B) The α -CD3 (UCHT1) staining and confocal imaging showed the successful surface expression of TCR in HEK293FT cells. (Scale bar, 50 μ m.) (C) Flow cytometry (FACS) analysis showed that the TCR surface level of the reconstituted HEK293FT cells was similar to that of Jurkat T cells. (D) HEK293FT-1G4TCR cells and Raji-pMHC cells were mixed at 1:1 ratio. TCR stimulation was stopped at indicated time points, and cells were lysed for Western blot analysis. The bands were quantitated by ImageJ. The pCD3 $\epsilon/\delta/\gamma$, pCD3 ζ Y142/CD3 ζ , and pCD3 ζ Y83/CD3 ζ ratios were obtained and further normalized to the value of 0 min of Lck WT condition. Average represents the average value of four independent experiments.

that CD3 ϵ was evenly mixed with the other CD3 chain on the bead (Fig. 5C). The phosphorylation level of CD3 δ , CD3 γ , or CD3 ζ was much higher under the condition of CD3 ϵ WT cocating (Fig. 5D). It is thus evident that the ionic interaction between CD3 ϵ BRS and the Lck UD domain could significantly increase the phosphorylation level of other CD3 chains.

Ionic CD3 ϵ -Lck Interaction Controls TCR Phosphorylation. We further studied the role of ionic CD3 ϵ -Lck interaction in the intact TCR complex. In T cells, TCR phosphorylation is not only regulated by Lck but also affected by feedback regulations from downstream signaling molecules, thus making it difficult to explicitly address the contribution of CD3 ϵ -Lck interaction in the initiation of TCR

phosphorylation. To preserve the essential elements of initial TCR phosphorylation but leave out other downstream molecules, we take the advantage of previously reported TCR reconstitution system (43) (Fig. 6A).

The 1G4 TCR complex was stably reconstituted into HEK293FT cells and stably expressed on the surface at the physiological level (Fig. 6B and C). Lck and its direct regulators were then transiently transfected. This system enabled the investigation of Lck-mediated TCR phosphorylation without the influences of downstream feedback regulations. Mutation of CD3 ϵ BRS significantly impaired the TCR surface level, which is consistent with previous reports (32, 34). Therefore, we generated an Lck mutant with the acidic residue D12 mutated to asparagine that can be normally expressed and had normal membrane localization similar to that of the WT Lck (Fig. S5). Moreover, NMR titration experiments show that Lck D12N had impaired binding with CD3 ϵ , especially with the BRS region of CD3 ϵ (Fig. S6). We then used either peptide–major histocompatibility complex (pMHC) presented by B cells or stimulating antibody OKT3 to trigger the reconstituted TCR in HEK293FT cells. The phosphorylation levels of tyrosine 394 and 505 in Lck WT or Lck D12N mutant were comparable at 0 min, indicating that the kinase activity of Lck is not affected by the mutation (Fig. 6D). However, compared with Lck WT, Lck D12N mutant showed impaired ability to phosphorylate TCR (Fig. 6D and Fig. S7), which further supports the notion that the ionic CD3 ϵ –Lck interaction plays a crucial role in initiating TCR phosphorylation. Consistently, the recruitment of Lck D12N to the TCR–pMHC contact area was also impaired (Fig. S8).

Discussion

Antigen-induced TCR phosphorylation is the first signaling event in T cells to trigger T-cell immune responses. The 10 ITAMs, i.e., 20 tyrosine residues, in a single TCR–CD3 complex enables a wide variety of TCR phosphorylation programs to induce distinct downstream signaling pathways in response to different antigen stimulations. How the 10 ITAMs of TCR are phosphorylated by Lck kinase is largely unknown. Moreover, why TCR can have distinct phosphorylation programs also remains elusive. In this study, we find a key mechanism that regulates the initiation of TCR phosphorylation. The basic BRS in CD3 ϵ can ionically interact with the acidic UD in Lck to recruit the kinase and initiate the phosphorylation of all CD3 chains. The exposure level of CD3 ϵ BRS upon antigen engagement might determine the final phosphorylation program of TCR to induce specific downstream signaling.

The BRS motif is present in both CD3 ϵ and CD3 ζ . CD3 ϵ BRS is directly after the transmembrane domain, whereas CD3 ζ BRS motifs are in between ITAMs and distal to the transmembrane domain. In addition to BRS, CD3 ϵ also has PRS that is not present in other CD3 chains. Lck has four regulatory domains: SH4, UD, SH3, and SH2. Compared with CD3 ζ , it is more spatially favorable for CD3 ϵ to interact with Lck on multiple sites. The N-terminal CD3 ϵ BRS can ionically interact with the N-terminal Lck UD, and, simultaneously, CD3 ϵ PRS and the first half of ITAM can interact with the Lck SH3 domain (Fig. S9). Our BLI and NMR experiments showed that BRS–UD interaction was more dominant than PRS–SH3 interaction (Figs. 3 and 4 and Figs. S3 and S4). Fig. 2 also supported the notion that CD3 ϵ BRS is functionally more important than PRS in mediating CD3 phosphorylation. Note that the interaction between ITAM and Lck kinase is also important for determining CD3 phosphorylation (26).

The physiological importance of CD3 ϵ BRS has been fully demonstrated by transgenic and retrogenic mouse models (32, 34, 44). Truncation of CD3 ϵ_{CD} led to the block of early thymocyte maturation at double-negative-3 stage, whereas truncation of both CD3 δ_{CD} and CD3 γ_{CD} did not cause such a defect. CD3 ϵ mutant that has intact BRS but truncated PRS and ITAM is sufficient to support successful double-negative to double-positive thymocyte

differentiation and single-positive thymocyte production (44). Furthermore, mutating CD3 ϵ BRS significantly impaired the thymocyte differentiation, TCR signaling, and TCR surface expression level (32, 34). Our finding of the key role of CD3 ϵ BRS in initiating TCR phosphorylation can explain these strong phenotypes observed in previous animal studies.

Another role of CD3 ϵ BRS is to bind acidic phospholipids (35, 45). Previous studies show that the addition of acidic phospholipids can fully abolish CD3 ϵ phosphorylation mediated by Lck (35). In resting T cells, despite the presence of active Lck molecules, CD3 ϵ BRS mainly binds to the plasma membrane, which leads to the sequestration of the whole CD3 ϵ cytoplasmic domain within the membrane (35). These findings strongly suggest that acidic phospholipids should have stronger binding with CD3 ϵ BRS than does Lck. BRS–lipid interaction not only sequesters the ITAM of CD3 ϵ chain within the membrane but more importantly also prevents CD3 ϵ –Lck interaction, which therefore prevents the spontaneous phosphorylation of all CD3 chains, especially the ITAMs of CD3 δ and CD3 γ that are fully exposed in the cytosol. Such a safety control mechanism can keep TCR molecules at low basal activity before antigen engagement. In activated T cells, several factors can trigger the dissociation of the CD3 ϵ cytoplasmic domain from the membrane at different signaling stages (36, 38, 39). At the initial antigen stimulation stage, it is likely that antigen-induced allosteric regulation and/or mechanical force could dislodge the CD3 ϵ cytoplasmic domain from the membrane, and antigens with different TCR affinities could cause different exposure levels of the CD3 ϵ cytoplasmic domain. Indeed, it has been shown that strong antigen but not weak antigen can induce the exposure of CD3 ϵ PRS to recruit Nck (46, 47). At the later signal amplification and sustainment stages, Ca²⁺ can directly bind to the phosphate groups of acidic phospholipids to neutralize their negative charges, which therefore disrupts CD3 ϵ –lipid interaction. Strong antigen can elicit a higher level of Ca²⁺ influx than weak antigen (48, 49) and therefore should cause a higher exposure level of the CD3 ϵ cytoplasmic domain. Therefore, either at the initial or later TCR signaling stage, the exposure level of the CD3 ϵ cytoplasmic domain, including the key BRS, should be different in response to different antigen stimulations. Given the central role of CD3 ϵ BRS in interacting with Lck, different exposure levels of CD3 ϵ BRS can directly control the recruitment level of Lck. The final TCR phosphorylation program of 20 tyrosines is then determined by Lck, CD45, Zap70, and other regulatory factors (43, 50–56).

In summary, here we report a key mechanism of the initiation of TCR phosphorylation. The BRS motif of CD3 ϵ can recruit Lck via ionic interactions to initiate TCR phosphorylation. Given the central role of CD3 ϵ BRS in TCR signaling, its exposure level in response to different antigen stimulations may serve as a key regulator of antigen-specific immune responses. Membrane-proximal BRS has been also found in many other immunoreceptors, such as CD28, CD19, and IL-10R. Indeed, CD28-BRS is required for efficient Lck interaction (57). We anticipate that the mechanism identified here might have a general application in immunoreceptor signaling.

Materials and Methods

Cells and Reagents. The cells and reagents are described in *SI Materials and Methods*.

Live-Cell FRET Measurement. Live-cell FRET was performed as previously described (35). Details of the experimental procedure are described in *SI Materials and Methods*.

IVP Assay. Soluble or bead-coated CD3 peptides were incubated with Lck or Fyn in a 50- μ L reaction volume at 30 °C for 30 min; the tyrosine-phosphorylated

and total protein levels were detected by Western Blot. Details of the experimental procedure are described in *SI Materials and Methods*.

NMR Experiments. All NMR spectra were acquired at 25 °C on Bruker Ascend 600 MHz with cryoprobe. Details of the experimental procedure are described in *SI Materials and Methods*.

TCR Stimulation in Reconstituted TCR–CD3–Lck Cells. HEK-1G4 cells and APCs (Raji cells) were generated as previously described (43). For APC triggering, HEK-1G4 cells and APCs were mixed together and incubated in a 37 °C water bath to trigger TCR phosphorylation. For OKT3 triggering, HEK-1G4 cells were stimulated by 5 µg/mL of OKT3 in a 37 °C water bath to trigger TCR phosphorylation.

Details of the experimental procedure are described in *SI Materials and Methods*.

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