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Regulated Movement of CD4 In and Out of the Immunological Synapse

Henry Kao,*† Joseph Lin,* Dan R. Littman,† Andrey S. Shaw,*§ and Paul M. Allen†‡

The mechanism underlying the transient accumulation of CD4 at the immunological synapse (IS) and its significance for T cell activation are not understood. To investigate these issues, we mutated a serine phosphorylation site (S408) in the cytoplasmic tail of murine CD4. Preventing phosphorylation of S408 did not block CD4 recruitment to the IS; rather, it blocked the ability of CD4 to leave the IS. Surprisingly, enhanced and prolonged CD4 accumulation at the supramolecular activation cluster in the contact area had no functional consequence for T cell activation, cytokine production, or proliferation. Protein kinase C θ (PKCθ)-deficient T cells also displayed enhanced and prolonged accumulation of wild-type CD4 at the IS, indicating that θ is the critical PKC isoform involved in CD4 movement. These findings suggest a model wherein recruitment of CD4 to the IS allows its phosphorylation by PKCθ and subsequent removal from the IS. Thus, an important role for PKCθ in T cell activation involves its recruitment to the IS, where it phosphorylates specific substrates that help to maintain the dynamism of protein turnover at the IS. The Journal of Immunology, 2008, 181: 8248–8257.

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2 Address correspondence and reprint requests to Dr. Paul M. Allen, Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO 63110. E-mail address: pallen@wustl.edu
3 Abbreviations used in this paper: IS, immunological synapse; SMAC, supramolecular activation cluster; PKCθ, protein kinase C θ; YFP, yellow-fluorescent protein; CFP, cyan-fluorescent protein; WT, wild type; Hb, hemoglobin.

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Materials and Methods

Transgenic mice

Mice expressing the N3.L2 TCR, which recognize hemoglobin 64–76 (Hb64–76/II-E), has been described previously (32). The amino acid sequences for the peptides used in this study are: agonist (Hb64–76), GKKVITAFLTEGLK; and antagonist (T72), GKKVITAFLTEGLK.

Peptides

Altered peptide ligands were synthesized, purified, and analyzed as described previously (34). The amino acid sequences for the peptides used in this study are: agonist (Hb64–76), GKKVITAFTEGLK; and antagonist (T72), GKKVITAFTEGLK.

Constructs

The retroviral construct, GFP-RV, was a gift from Dr. K. Murphy (Washington University, St. Louis, MO). Mouse CD4 cDNA was generated by PCR using the b-a-L3T4-8sl-HII-neo plasmid provided by Dr. C. Benoist (Harvard Medical School, Boston, MA).

Creation of CD4 and S408A nonfusion proteins. Mouse CD4 and S408A cDNA was generated by PCR or site-directed mutagenesis (T72) to generate the serine to alanine mutation) and cloned into the BP/B/IIX/oI site upstream of the internal ribosome entry site.

Creation of CD4-yellow-fluorescent protein (YFP) and S408A-YFP. After removal of the stop codon from CD4 or S408A, enhanced YFP (Clontech) was attached in-frame to the C terminus via a five-amino acid linker (GGAAS) (21) and cloned into the BP/B/IIX/oI site of a retroviral construct in which GFP has been removed.

Creation of CD3ζ-cyan-fluorescing protein (CFP). CD3ζ was excised from the CD3ζ-GFP construct (gift from Dr. M. Davis, Stanford University, Palo Alto, CA) and cloned in-frame with enhanced CFP (Clontech) at the C terminus. Both fusion and nonfusion CD3ζ proteins exhibited identical functional properties in response to PMA when expressed in T cell hybrids (data not shown). All constructs were purified using the Endofree Plasmid Kit (Qiagen) before transfection into packaging cell lines.

Retroviral transduction of primary T cells and T cell hybrids

The Platinum-E packaging cell line (a gift from Dr. T. Kitamura, University of Tokyo, Tokyo, Japan) was transfected with 25–30 μg of retroviral construct DNA with Lipofectamine 2000 (Invitrogen), and viral supernatant was collected 48 h after transfection. To obtain purified CD4+ T cells, splenocytes from CD4KI mice were harvested and depleted of CD4+ D2, B220, MHC class II, CD11b+, and CD11c+ rosetting cells using MACS Microbeads (Miltenyi Biotec). To obtain primary CD4+ N3.L2 T cells, purified cells were cultured using CD4 MACS Beads (Miltenyi Biotec). For T cell activation, 3 × 10^5 T cells were stimulated with 6.5 × 10^5 irradiated B6.K splenocytes loaded with 10 μM Hb64–76. At 20 and 24 h after activation, retroviral supernatant was added to the T cell cultures and spun for 45 min at 1800 rpm at 25°C in the presence of Lipofectamine 2000 (Invitrogen) and 125 U/mL IL-2. At days 6–8 after activation, T cells were sorted for equivalent levels of CD4 either via GFP (for CD4 nonfusion proteins) or YFP (for fusion proteins) using the FACSVantage flow cytometer (BD Biosciences) at the Washington University Department of Pathology and Immunology Cell Sorting Facility. For transduction of T cells with two different retroviral constructs, supernatant from individually transduced Platinum-E cells were collected, mixed together, added to T cells, and treated as described above. CD4+ 3.L2 T cell hybrids were generated as described (35). For stable expression of CD4 or S408A, the T cell hybridomas were retrovirally transduced with either CD4 or S408A and sorted for equivalent expression levels.

CD4 down-regulation

3.L2-CD4 or 3.L2-S408A T cell hybridomas (1 × 10^6) were incubated in each of triplicate wells of a 96-well plate with 10 ng/mL PMA in a total volume of 200 μl and incubated at 37°C for times ranging from 15 min to 8 h. The cells were fixed, stained for CD4 or S408A expression (PK1.5-biotin, streptavidin-CyChrome), and analyzed by FACS. The level of CD4 or S408A expression for each well was determined as a percentage of the mean fluorescence index of the unstimulated T cell hybridomas at time zero.

Confocal microscopy

Confocal and differential interference contrast images were acquired using the Zeiss LSM 510 microscope (Zeiss) fitted with a 1.3-narrow aperture (NA) ×40 Fluor objective. Live cell imaging of primary T cells transduced with either CD4-YFP or S408A-YFP were done in temperature-controlled Delta T dishes (Biotec) using imaging buffer (1% HSA, 1 mM CaCl2, 2 mM MgCl2, in HEPES-buffered saline, pH 7.4). CH27 cells loaded with 10 μM Hb64–76 were attached to surfaces coated with anti-H-2D^d (Biolegend) and used as APCs. No significant signal saturation was observed in any of the images used for analysis. To make three-dimensional reconstructions, 15 differential interference contrast images and YFP images spaced 0.66 μm apart in the z-plane were acquired every 15 s. For quantitation of cell couples and accumulation indices, deconvolved images were scored using Image J software (National Institutes of Health; Ref. 36). The accumulation index is a ratio based on the mean fluorescent intensity at the IS divided by the mean fluorescent intensity from the rest of the membrane minus background (21). For two-color experiments, CFP and YFP emission was collected using CFP and YFP emission filters, respectively.

For fixed conjugates, T cells were added to CH27 cells loaded with 10 μM Hb64–76, allowing for interaction for the indicated times, and fixed with 4% paraformaldehyde for 20 min. CD4 was stained using the Ab, GK1.5, followed by goat anti-rat Ig Alexa 546 Ab (Molecular Probes). For staining of intracellular molecules, conjugates were also permeablized with 0.1% Triton X-100 and stained with either pY394 Lck (15) or Lck (sc-013; Santa Cruz Biotechnologies), followed by donkey anti-rabbit Ig Alexa 555 Ab (Molecular Probes).

Primary T cell proliferation assay, IL-2 bioassay, and T cell hybridoma assay

For proliferation assays, 1.0 × 10^5 purified CD4K1 T cells were incubated with 5 × 10^5 irradiated B6.K splenocytes loaded with increasing amounts of peptide for 48 h, pulsed with 0.4 μCi of [3H]Tdr, and harvested 18–24 h later. Where CD4 dependency was assessed, T cells were also incubated with 2 μg/ml anti-CD4 (OKT4A.1) during the course of the assay. Measurement of IL-2 production, one-fourth of each well was collected 20 h after Ag stimulation, transferred to the IL-2 indicator cell line CTL-L2 for 24 h, pulsed with 0.4 μCi of [3H]Tdr for 18–24 h, and harvested. T cell hybridoma assays were performed as previously described (35). The activation of T cell hybridomas was assessed by measuring IL-2 production by a quantitative ELISA. Briefly, Immulon-2HB 96-well plates were coated with 100 μg of a capture anti-IL-2 mAb (JES6-1A12; Biolegend) per mL overnight at 4°C. The plates were washed and blocked (PBS, 0.5% BSA, 0.1% Tween 20). Dilutions of the samples and IL-2 standards (Peprotech) were added to triplicate wells and incubated at 25°C for 2 h. The plates were washed, and biotin-anti-IL-2 (JES6-5H4; Biolegend) at 0.5 μg/ml was added to each well, incubated at 25°C for 1 h, and washed. 100 μl/well of streptavidin-HRP (Southern Biotech, 1/10,000) were added, incubated at 25°C for 30 min, and washed. To each well, we added 100 μl of 1-Step-Ultra TMB substrate (Thermo Scientific), the reaction was stopped with 2 M sulfuric acid after 15 min, and the product was quantified using a Victor3 plate reader (PerkinElmer). The concentration of IL-2 in each well was calculated from the IL-2 standard curve.

Measurement of calcium mobilization and intracellular cytokine production

For calcium imaging, T cells were loaded for 20 min at 37°C with 1 μM/ml Fura-2-AM (Molecular Probes), rested for 20 min at 37°C, and washed in Ringer’s solution (150 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM glucose, 5 mM HEPES, pH 7.4) before the onset of the experiment (37). The T cells were added onto a monolayer of H-2D^d-adhered CH27 cells loaded with Ag, and the ratio of fura-2 emission at 510 nm was determined by dividing the emission intensities at 340/380 nm excitation. Data acquisition and analysis were performed using Metamorph 6.0 (Universal Imaging). Experiments were done using a Zeiss Axiovert 200 microscope, equipped with a xenon light source, ×20 Fluar objective (NA 0.75), and maintained in a 37°C environmental control chamber.

For intracellular cytokine staining, CD4K1 T cells expressing equivalent levels of either CD4 or S408A were stimulated with irradiated B6.K splenocytes loaded with agonist or altered peptide ligands for 8 h. During the last 2 h of stimulation, 10 μg/ml brefeldin A were added to the culture. Cells were then fixed with 4% paraformaldehyde, permeabilized with 0.5% saponin, and stained for CD25, IFN-γ, and clonotypic Ab for N3.L2 TCR (Cab).
In vitro kinase assays

Lck kinase assays were as previously described (38). For CD4 immunoprecipitation kinase assays, equal numbers of T cell hybrids (2.5 × 10^6) expressing equivalent CD4 levels were lysed in lysis buffer (1% Nonidet P-40, 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.2 mM Na3VO4, 10 mM NaF) and incubated with protein G-Sepharose beads bound with the anti-CD4 Ab, GK1.5, for 2 h at 4°C. The beads were then washed three times in lysis buffer, followed by two washes with kinase buffer (50 mM HEPES (pH 7.5), 10 mM MnCl2, 0.1 mM Na3VO4) and then subjected to in vitro kinase assay reactions with 1.2 mM concentrations of either the substrate peptide (RRLIEDAHYAARG) or the control peptide (RRLIEDAHAAARG) in the presence of 0.2 mM ATP and 10 μCi of [γ-32P]ATP for 10 min at 30°C. Reactions were stopped by the addition of 10% trichloroacetic acid. Reactions were spotted in triplicate onto P81 paper and washed five times with 0.5% phosphoric acid, followed by one wash in acetone. Dry filters were then counted in a scintillation counter. For PKCθ kinase assays, purified PKCθ (Millipore) was added to 10 μg of either CD4 peptide (RRQAAARMSQIKRL) or S408A peptide (RRQQAARMAQIKRL), and the reaction was conducted as described in product literature (Millipore).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 4.0 software. Student’s t test with two-tailed distribution was used to determine statistical significance between any two groups; a p value of <0.05 was considered statistically significant.

Results

Retroviral expression of CD4 and S408A in primary N3.L2 T cells

For this study, we used the 3.L2 TCR transgenic mouse model, specific for Hb/I-Eαβ. To be able to assess the role of CD4 in primary mouse T cells, we took advantage of our previous finding, that we could generate functional CD4<sup>N3.L2</sup> T cells in vivo (termed CD4KI) by expression of an altered peptide ligand in the thymus (33). This system provided us with a unique source of CD4<sup>N3.L2</sup> primary T cells, and bypassed the critical role of CD4 in T cell development.

We generated and used two types of retroviral constructs: 1) for imaging studies, we made fusion proteins consisting of either CD4 or S408A (serine at position 408 mutated to alanine; Fig. 1A) fused in-frame with YFP (designated CD4-YFP or S408A-YFP, respectively); 2) for functional studies, we made nonfusion proteins in which CD4 or S408A was inserted into a GFP-reporter retroviral construct (designated as CD4 or S408A, respectively). Both fusion and nonfusion proteins exhibited identical behavior when expressed in T cell hybrids or primary T cells, displaying similar CD4 down-regulation kinetics, cytokine production, and T cell proliferation (data not shown), suggesting that the addition of a YFP tag did not impact CD4 function in the fusion proteins. To confirm the lack of endocytosis of S408A, we transduced CD4<sup>−</sup> T cell hybridomas expressing the 3.L2 TCR, sorted for equivalent CD4 levels, and measured CD4 cell surface expression after stimulation (Fig. 1B). CD4 surface expression decreased rapidly upon stimulation, to <30% by 2 h. In marked contrast, S408A levels did not decrease during the first 2 h and was maintained for as long as 8 h. These findings confirm the published phenotype of the S408A mutation (22, 30, 31), that it prevents endocytosis of CD4 molecules following stimulation.

We were able to transduce CD4 into CD4KI T cells, and we used these cells for the functional studies in a comparison of CD4 and S408A. Despite repeated transductions and FACS sorting, the expression levels were still ~10-fold less than normal CD4 levels, consistent with other studies involving retroviral expression of proteins in murine T cells (18, 39). However, for the imaging studies, we were concerned that the levels we could achieve may complicate our interpretations of the findings of the movement of CD4, due to the lower expression levels. Therefore, we developed an alternate system whereby we transduced CD4-YFP or S408A-YFP into primary N3.L2 T cells that expressed endogenous CD4, resulting in only a 10% increase in total CD4 levels (data not shown). This was similar to a previous approach using T cell clones (18), and we observed no effects of the slight increase in CD4 levels on T cell proliferation, IL-2 production, or patterns of cytokine secretion such as IFN-γ, IL-4, or IL-5 (data not shown). Thus, we have created a unique system that permitted us to image CD4 dynamics at the IS in a physiological manner and examine its role in T cell signaling and function in normal primary T cells.

**S408A mutation leads to enhanced accumulation at the IS**

We first examined CD4 movement in T cells expressing either CD4-YFP or S408A-YFP in fixed T cell-APC conjugates 30 min after cell contact. We observed very little and transient accumulation of CD4-YFP (Fig. 2A, top), consistent with previous findings (17, 18). In marked contrast, we detected a high level of accumulation of S408A-YFP at the IS, achieving a distinct, centralized accumulation (Fig. 2A, bottom, and C (three-dimensional reconstruction) and supplemental video 1).<sup>4</sup> We quantitated the degree of accumulation at 30 min and found that S408A-YFP accumulation was significantly more than CD4-YFP (accumulation index, 2.45 vs 1.17; p < 0.0001; Fig. 2B, left). The majority of T cells expressing S408A-YFP showed strong accumulation (60.8%), whereas most of the T cells expressing CD4-YFP had weak accumulation (69.2%); Fig. 2B, right). On the basis of the persistence on the cell surface of S408A upon T cell activation (Fig. 1B), we propose that

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<sup>4</sup>The online version of this article contains supplemental material.
the increased accumulation of S408A at the IS is due to its lack of internalization.

To determine the kinetics of the accumulation of CD4-YFP and S408A-YFP during IS formation, we performed live-cell imaging on primary N3.L2 T cells transduced with either construct. We detected modest CD4-YFP accumulation at the interface within 30–60 s after IS formation, which disappeared within 120 s (Fig. 3A and supplemental video 2), consistent with the kinetics observed by Davis and colleagues (18). With S408A-YFP, we observed a much different pattern, with strong accumulation within the first 60 s after IS formation, which increased during the next 60 s, followed by persistence for the duration of the experiment (Fig. 3B and supplemental video 3). This finding reveals that the accumulation we observed at 30 min (Fig. 2A), occurred very rapidly, and was maintained for a long period of time. Quantitation of the kinetics of the accumulation in representative cells for CD4-YFP and S408A-YFP, is shown in Fig. 3C. During the first 2 min after initial contact, we detected a modest and transient accumulation with CD4-YFP (Fig. 3C, left), whereas with S408A-YFP we observed a rapid and strong accumulation (Fig. 3C, right).

Most of the S408A-YFP accumulation exhibited a centralized localization pattern (Fig. 2A and C), consistent with its being in the c-SMAC. To confirm this localization, we examined S408A-YFP accumulation in relationship to CD3ζ-CFP, a known c-SMAC marker (14, 40). As shown in Fig. 3D, we found that S408A-YFP colocalized within the same region as CD3ζ-CFP, indicating that centralized S408A accumulation was occurring in the c-SMAC. These findings show that the S408A mutation did not affect the movement of CD4 into the synapse but significantly altered the movement of CD4 out of the synapse, leading to a large centralized accumulation in the c-SMAC.
S408A accumulation at the IS does not enhance early T cell signaling

From the established role of CD4 as a coreceptor in coordinating Lck accumulation at the IS (20), a prediction would be that enhanced CD4 accumulation at the IS would lead to enhanced T cell signaling and T cell function. We examined the effect of the S408A mutation on calcium mobilization, a well-established early event in T cell activation (41). We reconstituted CD4KI T cells with either CD4 or S408A constructs expressing GFP as a reporter protein, sorted for equivalent GFP levels, and measured calcium levels over time, using the calcium-sensitive dye fura-2, in response to agonist-pulsed APCs. Overall, we found that the calcium levels and patterns were the same between the T cells expressing CD4 or S408 (Fig. 4). We did observe a modest increase in peak calcium levels (Fig. 4A), but the overall pattern was the same. These results show that centralized S408A accumulation had no effect on the early T cell signaling, as measured by calcium mobilization.

S408A accumulation at the IS does not alter early and late T cell functions

We next determined the effect of centralized S408A accumulation at the IS on the later T cell responses of cytokine production and T cell proliferation. When we measured IFN-γ production 8 h after Ag stimulation, we did not detect an increase in IFN-γ production in T cells expressing S408A (Fig. 5A). No significant differences in T cell responses to the weak agonist or antagonist were also seen (Fig. 5A): the differences seen in response to the weak agonist, T72, were within normal variation and not significant (H.K. and P.M.A, unpublished observations). When we extended our analysis to include T cell functional responses 20 or 72 h after Ag stimulation, we detected the same consistent pattern. At 20 h, T cells expressing either CD4 or S408A produced similar levels of IL-2 in response to the agonist or weak agonist in a dose-dependent manner (Fig. 5B). At 72 h, T cells expressing S408A proliferated equivalently well compared with T cells expressing CD4, in a dose-dependent manner to either the agonist or weak agonist (Fig. 5C). Taken together, our results show that centralized CD4 accumulation at the c-SMAC had no effect on early or late T cell signaling or function.

One possibility for the lack of differential T cell functional responses between T cells expressing CD4 or S408A is that the stable expression levels that we were able to achieve by retroviral transduction of primary T cells were not high enough to reveal a difference. To address this issue, we transduced CD4-negative T cell hybridomas expressing the 3.L2 TCR with either CD4 or
S408A, and FACS sorted for high levels of expression. With T cell hybridoma cells, we were able to achieve high expression for both CD4 and S408A (Fig. 6A). These levels were significantly higher than we were able to achieve in the primary T cell blasts, and were approximately 30% of the normal levels. The functional responses of the 3L2 T cells expressing either CD4 or S408A to Hb64–76 were then examined, and their dose-response curves were indistinguishable from each other (Fig. 6B). Thus, despite higher levels of CD4 or S408A expression in the T cell hybridomas, the findings confirm those with the primary T cell blasts (Fig. 5), in that despite the significant accumulation of S408A at the IS, there did not appear to be any significant functional consequence.

**Equivalent Lck activity and localization in T cells expressing CD4 or S408A**

The finding that enhanced CD4 accumulation did not lead to enhanced T cell functional responses led us to examine whether Lck activation or localization was impaired in T cells expressing S408A. We first wanted to establish that the S408A mutation did not affect basal Lck kinase activity (Fig. 7A). We next examined active Lck localization at the IS using an Ab specific for the active form of Lck (42). There were no significant differences between T cells expressing either CD4 or S408A in the localization or levels of active Lck (Fig. 7B). We did observe two patterns of active Lck staining: accumulation either at the p-SMAC (Fig. 7B) or at both the c-SMAC and p-SMAC (data not shown), but they were both found in the CD4 and S408A expressing cells. Total Lck recruitment was also the same between T cells expressing either CD4 or S408A, ruling out the possibility that the S408A mutation affected total Lck recruitment (Fig. 7, C and D). For these experiments, it is important that CD4 and S408A were expressed in primary CD4KI T cells, in which we not able to achieve full wild-type (WT) levels of expression. Because of the low expression levels, we did not visualize any enhanced total Lck accumulation as one might have expected from the expressed S408A. These findings with Lck indicated that the S408A mutation did not affect the activity state or recruitment pattern of Lck.

**PKCθ-deficient T cells exhibit CD4 accumulation at the c-SMAC**

We next investigated what kinase(s) was involved in the phosphorylation of CD4 in the IS. Previous studies had implied that S408 was phosphorylated by a PKC (31, 43). We focused on PKCθ, based on its role in T cell activation and recruitment to the IS (44, 45). The S408 region of CD4 (Fig. 1A) contains potential PKC recognition sites (44). We initially determined whether PKCθ could directly phosphorylate S408 using an in vitro kinase reaction, with rPKCθ and either an S408-containing peptide (WT) CD4) or a control S408A peptide. PKCθ could strongly phosphorylate the WT CD4 peptide but not the control S408A peptide (Fig. 8A). To assess the effect of PKCθ on CD4 in primary T cells, we used two independent approaches. We then treated normal N3.L2 T cells with rottlerin, a PKCθ inhibitor (46), and visualized CD4 movement by staining for CD4 in fixed T cell-APC conjugates. We observed strong centralized CD4 accumulation 30 min after IS formation (Fig. 8B), similar to what we had observed with S408A (Fig. 2, A and C). This finding showed that inhibiting PKCθ substantially affected the movement of CD4 at the IS. Because rottlerin could also inhibit the PKCθ isoform, which has been shown to be involved in T cell activation (47), we then examined CD4 accumulation in PKCθ-deficient N3.L2 T cells. We detected strong centralized CD4 accumulation in N3.L2-PKCθ-deficient T cells, whereas no accumulation was detected with N3.L2 T cells (Fig. 8C). The CD4 accumulation in both the N3.L2-PKCθ-deficient and rottlerin-treated T cells was significantly higher than that of normal N3.L2 T cells (Fig. 8D, left). The distribution of cells exhibiting centralized CD4 accumulation was also greater in rottlerin-treated and N3.L2-PKCθ-deficient compared with WT N3.L2 T cells (Fig. 8D, right). Thus, by either inhibiting or eliminating PKCθ in T cells, we were able to substantially alter the accumulation of CD4 at the IS, recapitulating a pattern generated by the S408A mutation of CD4. These studies reveal a previously unrecognized link between CD4 and PKCθ and strongly support a model in which PKCθ is directly involved in the removal of CD4 from the IS by phosphorylating the S408 residue.
Discussion

In this study, we have established a critical role for the serine phosphorylation of the cytoplasmic tail of the CD4 molecule for its movement at the IS. Mutation of the S408 residue did not prevent CD4 from localizing to the IS upon contact with an APC, indicating that it was not required for the recruitment to the IS. However, phosphorylation of S408 was essential for the removal of CD4 from the IS, in that the S408A mutation resulted in prolonged accumulation of CD4 at the IS. The phosphorylation of S408 was performed exclusively by PKCθ, as evidenced by the accumulation of WT CD4 in T cells treated with a PKCθ inhibitor or in PKCθ−/− deficient cells. PKCθ is the only PKC isoform that is recruited to the IS, and we provide compelling evidence that CD4 is a direct substrate of PKCθ, making it the first PKCθ substrate in the IS to be identified. Thus, our findings support a model in which PKCθ participates in the turnover of proteins in the IS by phosphorylating multiple substrates, including CD4. Our studies also help to clarify the reason for the removal of CD4 from the IS. The S408A mutation and the resulting accumulation of CD4 at the IS did not have any functional consequences on early or late T cell functions. These findings eliminate the possibility that maintenance of CD4 in the IS would be deleterious, and it is removed from the synapse to help coordinate the signaling initiated through the TCR. They do support the concept that coreceptor function of CD4-Lck is acting as a catalyst, being critically important only during the initial TCR signaling events. The reason for the rapid removal, within minutes, of CD4 from the IS is not fully resolved, in that its retention does not appear to be detrimental, and sustained contact of several hours between the T cell and APC is required for full T cell activation.

The IS is now known to be a highly dynamic structure. The turnover of proteins in the IS could be involved in the tuning of a strong stimulus and/or the dissolution of the IS, allowing the relocation and reformation of the IS. Sims et al. (48) have shown,
In CD8+ T cells, PKCθ localizes to both the c-SMAC and p-SMAC (49). CD28 has also been shown to be important for the localization of PKCθ to the c-SMAC (50, 51). These differences most likely reflect the different T cells, antigenic strength, and the coreceptor dependence, among other variables. We observed that CD4 localized to the c-SMAC but did not address the localization of PKCθ and where CD4 and PKCθ interacted. In our studies, we used B cells as APC and primary mouse T cell blasts, and the PKCθ could have localized to c-SMAC, where it encounter CD4. Alternatively, CD4 could encounter PKCθ in the p-SMAC, become phosphorylated, migrate to the c-SMAC, and then leave the p-SMAC. Future studies involving differentially labeled CD4 and PKCθ would help to resolve this issue. The phosphorylation of CD4 initiates several actions, with Lck being dissociated from CD4 and CD4 being endocytosed. The fate of the dissociated Lck in the IS is not currently known. It could remain in the IS, making CD4 an Lck transport molecule, or the dissociated Lck could join the pool of free Lck in the T cell. Lck has been shown to interact directly with PKCθ, which could also provide another mechanism for Lck remaining in the IS (52). In our studies, we did not find any differences in the localization of total Lck or active Lck in T cells expressing either CD4 or S408, but we could not rule out some highly localized Lck effects, that were below our level of detection.

In this study, we found little accumulation of WT CD4 at the IS, similar to results reported by Davis and colleagues (18), who also reported transient accumulation of CD4 at the IS. Our findings were somewhat different from those of Zal et al. (21), who showed strong CD4 accumulation early after IS formation, but not necessarily in an Ag-specific manner. Possibilities for these differences could be the T cells used, T cell hybrids vs primary T cell blasts, or the affinity of the TCR for the pMHC. It is still possible that the little CD4 accumulation seen with WT CD4 in our system is reflective of the passive nature of CD4 movement on the surface of the T cell, with the recruitment of TCR and its proximity to CD4 being the determinant for T cell activation (21). Our studies did not address the important issue of the activation/differentiation state of the T cells on the role and movement of CD4 and PKCθ. The fact that PKCθ has been shown to differentially localize in naive vs activated T cells (48) raised the possibility that its role in the phosphorylation of CD4 may vary depending on the activation state. Th subsets may also have different levels of CD4 involvement, as suggested by the observation of small differences in CD4 expression levels between Th1 and Th2 cells (53), and Ca2+ signaling differences among Th1, Th2, and Th17 cells (37).

PKCθ is an essential component of T cell activation and is involved in many different signaling pathways (44, 54, 55). Here, we have made the novel link between PKCθ and CD4 and provide strong evidence that CD4 is a direct substrate of PKCθ. We cannot rule out the possibility that PKCθ is acting indirectly on the CD4 through the action of another kinase. We favor the simplest explanation that PKCθ is directly phosphorylating CD4 on the S408 residue, as evidenced by the demonstration that purified PKCθ can phosphorylate a S408-containing peptide, and that inhibition of PKCθ or genetic elimination of PKCθ resulted in WT CD4 accumulating at the IS, in a manner similar to that of the S408A mutation. Clearly, the phosphorylation of CD4 by PKCθ is only one of many activities of PKCθ, given that the PKCθ-deficient T cells have a profound defect in signaling (55) and that we found no functional defect in the S408A-expressing T cells.

In this study, we were able to investigate the role of S408 phosphorylation in primary mouse T cells because of our ability in vivo to generate T cells in the absence of CD4, using the CD4KI system (33). Because previous studies have shown a pivotal role for S408...
in controlling CD4 down-regulation in T cell hybrids and non-transformed human cell lines (22, 31), we assumed that this would be similar in primary T cells and that mutating S408 would lead to increased CD4 surface expression. Instead, we detected enhanced S408A accumulation at the c-SMAC, but S408A down-regulation was similar to WT CD4 in mouse primary T cells (data not shown). This implies a different mechanism of control of CD4 down-regulation in primary T cells compared with T cell hybrids, not necessarily dependent on S408. It is also possible that the defect on CD4 down-regulation is more acute and less severe in primary T cells, visible only by imaging, and not by measuring the overall expression of CD4 on the surface.

In conclusion, our findings in mouse primary T cells support the concept that CD4 coreceptor function operates within a spatiotemporal window at the IS, with its primary purpose to first stabilize TCR-pMHC interaction and, second, to deliver Lck to the IS to help initiate T cell signaling. We also uncover a new connection between CD4 and PKCθ, which highlights the importance of phosphorylation by PKCθ in the dynamic nature of protein turnover in the IS.

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Disclosures
The authors have no financial conflict of interest.

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