Persistence of Cooperatively Stabilized Signaling Clusters Drives T-Cell Activation†

Stephen C. Bunnell, 1‡* Andrew L. Singer, 2‡§ David I. Hong, 1 Berri H. Jaque, 3 Martha S. Jordan, 2 Maria-Cristina Seminario, 3 Valarie A. Barr, 1 Gary A. Koretzky, 2 and Lawrence E. Samelson 1

Laboratory of Cellular and Molecular Biology, Center for Cancer Research, NCI, National Institutes of Health, Bethesda, Maryland 1; Abramson Family Cancer Research Institute, Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 2; Laboratory of Cellular and Molecular Biology, NIA, National Institutes of Health, Baltimore, Maryland 3; and Program in Immunology, Tufts University School of Medicine, Boston, Massachusetts 4

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Antigen recognition triggers the recruitment of the critical adaptor protein SLP-76 to small macromolecular clusters nucleated by the T-cell receptor (TCR). These structures develop rapidly, in parallel with TCR-induced increases in tyrosine phosphorylation and cytosolic calcium, and are likely to contribute to TCR-proximal signaling. Previously, we demonstrated that these SLP-76-containing clusters segregate from the TCR and move toward the center of the contact interface. Neither the function of these clusters nor the structural requirements governing their persistence have been examined extensively. Here we demonstrate that defects in cluster assembly and persistence are associated with defects in T-cell activation in the absence of Lck, ZAP-70, or LAT. Clusters persist normally in the absence of phospholipase C-γ1, indicating that in the absence of a critical effector, these structures are insufficient to drive T-cell activation. Furthermore, we show that the critical adaptors LAT and Gads localize with SLP-76 in persistent clusters. Mutation analyses of LAT, Gads, and SLP-76 indicated that multiple domains within each of these proteins contribute to cluster persistence. These data indicate that multivalent cooperative interactions stabilize these persistent signaling clusters, which may correspond to the functional complexes predicted by kinetic proofreading models of T-cell activation.

Adaptive immune responses depend on the ability of the T-cell receptor (TCR) to distinguish foreign antigens from self-antigens. These peptide antigens are displayed by major histocompatibility complexes (MHC) on antigen-presenting cells (APCs). Peptide-MHC complexes (pMHC) that engage the TCR trigger the sequential recruitment of kinases, scaffolds, and effectors to signaling complexes (5, 46). However, effective T-cell activation is linked to the half-life of the TCR-mediated signaling events. Antigen recognition triggers the recruitment of the critical adaptor protein SLP-76 to small macromolecular clusters nucleated by the T-cell receptor (TCR). These structures develop rapidly, in parallel with TCR-induced increases in tyrosine phosphorylation and cytosolic calcium, and are likely to contribute to TCR-proximal signaling.

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‡ S.C.B. and A.L.S. contributed equally to this work.
§ Present address: Department of Surgery, The Johns Hopkins Hospital, Baltimore, MD 21287.

* Corresponding author. Present address: Department of Pathology, Tufts University Medical School, Jaharis Bldg., Rm. 512, 150 Harrison Ave., Boston, MA 02111. Phone: (617) 636-2174. Fax: (617) 636-2990. E-mail: Stephen.Bunnell@tufts.edu.

Modern imaging techniques have begun to reveal macromolecular signaling complexes induced by TCR ligation. These antigen-induced structures bridge the gap between the protein-protein interactions probed by classical biochemical analyses and higher-order structures, such as the cSMAC. TCR-rich microclusters 200 to 500 nm in diameter assemble within seconds of T-cell–APC contact (10, 12, 21, 26, 59). Our own dynamic imaging studies employing immobilized stimulatory antibodies have shown that these microclusters are the predominant sites of TCR-induced tyrosine phosphorylation and rapidly induce the coaccumulation of tyrosine kinases and critical adaptor proteins, such as SLP-76 (2, 6, 10). We refer to these macromolecular assemblies of signaling molecules as
signaling clusters to distinguish them from the signaling complexes probed by traditional biochemical methods. Similar antigen receptor-based signaling clusters arise in T-cell lines, primary human T cells, and mast cells (2, 10, 50, 54, 55, 59). T cells responding to antigen-presenting lipid bilayers or to peptide-bearing APCs develop similar signaling clusters, confirming the relevance of these structures in vivo (12, 59). Critically, a single TCR microcluster is sufficient to induce intracellular calcium elevations, suggesting that individual signaling clusters are competent to initiate critical T-cell activation pathways (10).

The relationship between signaling clusters and the cSMAC is not well understood. In pMHC-induced synapses, TCR microclusters gradually coalesce to form a cSMAC (19, 27, 61). In contrast, signaling clusters containing the critical adaptor SLP-76 are observed in freshly initiated synapses but disappear at later times and fail to colocalize with the cSMAC (59). In the lipid bilayer model, TCR microclusters form in the periphery and move towards the center of the stimulatory interface. Microclusters containing the critical adaptor SLP-76 initially colocalize with the TCR and move towards the center of the contact with similar kinetics. However, the TCR accumulates in a central cluster that is largely devoid of phosphotyrosine, whereas SLP-76 disappears from these structures prior to its centralization (59). In our model system, TCR microclusters remain immobilized by the stimulating antibody but give rise to SLP-76-containing signaling clusters that segregate from the TCR and move towards the center of the stimulatory interface (10). This data indicate that the fates of TCR microclusters and SLP-76-containing signaling clusters diverge during T-cell activation.

Although the adaptor proteins LAT, Grb2, Gads, and SLP-76 cocluster with the TCR early in T-cell activation, relatively little is known about the formation and composition of SLP-76-containing signaling clusters (10, 15, 21). Lipid rafts may play a role in the initiation of signaling; nevertheless, protein-protein interactions appear to play the dominant role in the stabilization of these structures. Given the complexity of the potential interactions among the expected components of these clusters, complexes with diverse stoichiometries are theoretically possible. However, cooperative interactions among the components of these clusters may contribute to the avidities of specific complexes, ensuring that certain protein-protein interactions are favored in vivo and in vitro (6, 13, 22, 31, 62). In this manner, cooperative protein-protein interactions may contribute to the formation of persistent and functionally competent signaling clusters similar to those postulated by kinetic proofreading models.

In our current studies we demonstrate that persistent SLP-76-containing clusters move laterally within the immunological synapses induced by staphylocoocal enterotoxin E (SEE), and we examine the properties of analogous SLP-76-containing structures in cells stimulated by immobilized antibodies. In particular, we (i) establish the genetic requirements for the assembly of persistent and mobile SLP-76-containing clusters, (ii) more fully characterize the compositions of these structures, and (iii) demonstrate that multivalent protein-protein interactions play unexpected and critical roles in the assembly and persistence of these structures. Finally, our observations indicate that the persistence of the SLP-76-containing signaling clusters is a decisive factor regulating T-cell activation.

MATERIALS AND METHODS

Plasmid constructs. All SLP-76 expression constructs used in this study have been described previously (10, 49). All murine cDNA (mC)-Gads chimera were derived by subcloning mutant fragments from murine Gads constructs provided by Jane McGlade (Hospital for Sick Children) into existing yellow fluorescent protein (YFP)–Gads chimeras and replacing YFP with monoclonal Gads bearing an A206K mutation that disrupts the weak dimer interface found in all green fluorescent protein (GFP)–derived proteins (10, 32, 60). The dominant-negative Gads construct was generated by cutting the parental mC-Gads construct with SacI and XmnI, trimming the 3' overhang with T4 polymerase, and ligating the construct to delete the entire amino terminus of the Gads. The LAT-CFP and LAT-DrRed chimeras were generated by subcloning the EcoRI/BamHI fragment encoding human LAT from the LAT-enhanced GFP (EGFP) chimera (described previously) into the pECFP-N1 and pDsRed-N1 expression vectors from Clontech (10). All constructs were validated by sequencing.

Cell lines, transfections, and cell culture. Parental E6.1 Jurkat T cells and LAT-deficient J.CaM2.5 Jurkat T cells were obtained from ATCC; LAT-deficient J.CaM2.5 Jurkat T cells and SLP-76-deficient J14 Jurkat T cells were gifts from Robert Abraham, and David Strauss (Medical College of Georgia). The LAT chimera were kindly provided by John Wood (University of Colorado) and Mark Uhr (University of Miami). J14 cell lines stably expressing wild-type and mutant EGFP-SLP-76 chimeras were either described previously or derived by identical means (49). The J14-derived stable line stably overexpressing SLP-76-YP was also described previously (10). All cells were maintained as described previously (49). Cells used for transient transfections were maintained in RPMI 1640 supplemented with 10% fetal calf serum, glutamine (20 mM), and ciprofloxacin (10 μg/ml). Immediately prior to transfection, cells were rinsed briefly in serum-free medium and resuspended at 4 × 10^6 cells/ml in complete ciprofloxacin-containing medium. Aliquots of cells (300 μl) were briefly preincubated with no more than 30 μg of experimental and vector DNAs and were transfected in 4-mm-gap cuvettes, using a BTX ECM 830 electroporator set to deliver a single 300-V, 10-ms pulse. After 10 min at room temperature, cells were transferred to prewarmed ciprofloxacin-containing complete medium and allowed to recover for 12 to 16 h prior to analysis.

Antibodies and Western blotting. Three equivalent antibodies against the human CD3ε chain were used interchangeably to trigger T-cell activation and complex formation. Purified OKT3 was obtained from Bio-Express Cell Culture Services (West Lebanon, NH), and purified HIT-3a and UCHT1 were obtained from BD Pharmingen. Each antibody performed similarly in our complex formation assays. Western blotting was performed as described previously to confirm the presence or absence of protein expression in the mutant cell lines (48) (see Fig. S1 in the supplemental material). The following antibodies were used: anti-Lck (sc-433) and secondary detection reagents from Santa Cruz Biotechnology, Inc., anti-SLP-76 (AS5-P) from Antibody Solutions, Inc., anti-LAT (06-807) and anti-PLC-γ1 (05-163) from Upstate Biotechnology, Inc., and anti-ZAP-70 (610240) from BD Transduction Laboratories, Inc.

Cellular imaging and image analysis. As described previously, complex formation was monitored in Jurkat T-cell lines by using a modified T-cell spreading assay (8). Live images were collected as vertical Z stacks and then subsampled over the plane of the coverslip. Alternately, single z sections were captured over time to improve the rate of image acquisition; in these cases, proper focus was maintained using guide cells with distinguishable fluorescence properties to mark the plane of the coverslip. In studies employing transiently transfected cells, expression levels are heterogeneous. To restrict our analyses to cells expressing physiological levels of protein, we imaged them under fixed conditions, varying only the exposure time. Cells expressing moderate levels of the SLP-76-YFP chimera were identified by comparing the optimal exposure times required to image the experimental cells and control cells stably expressing known levels of the SLP-76 chimera. Only these cells were employed in subsequent analyses. All images were collected using a Perkin-Elmer Ultraview spinning-wheel confocal system mounted on an Axiovert 200 microscope. EGFP and DrRed were detected sequentially using 488-nm and 568-nm laser lines in conjunction with a Perkin-Elmer RGA dichroic filter and the supplied fluorophore-matched emission filters. CFP and YFP were detected similarly, using 442-nm and 514-nm laser lines in conjunction with a Perkin-Elmer CFP/YPF dichroic filter. Samples
were maintained at 37°C. All subsequent image manipulation and analysis were performed using IPLab (Scanalytics).

**Functional assays.** For CD69 upregulation experiments, 5 × 10^5 cells/ml were plated in wells containing medium alone, wells containing medium with 20 ng/ml phorbol myristate acetate (PMA), or wells previously coated with C305 antibody, as described previously (49). After overnight culture, cells were stained with APC-conjugated anti-CD69 (BD Pharmingen), and surface expression was analyzed on a FACScalibur cytometer (BD Biosciences). NF-AT assays were set up in glass-bottomed 96-well plates (Whatman) coated with poly-l-lysine, incubated overnight at 4°C with the indicated dilutions of OKT3 in phosphate-buffered saline, and blocked for 1 h at 37°C with 1% bovine serum albumin in phosphate-buffered saline (8). Cells to be assayed for NF-AT activation were transfected with 15 μg of a composite NF-AT/AP-1 luciferase reporter plasmid and 3 μg/ml of a control Renilla luciferase expression vector, pRL-TK (16). After recovering overnight, transfected cells were plated at 10^5 cells/well in a total volume of 100 μl. Positive controls were treated with 50 ng/ml PMA and 1 μM ionomycin. After 6 h at 37°C, plates were assayed for luciferase activity by adding 100 μl of SteadyLite reagent (Perkin-Elmer) directly to the wells of the plate. Renilla luciferase activity was assayed in the same wells by adding 25 μl RenLite reagent to each well, as described previously (45). For both assays, plates were read on a TriLux counter (Perkin-Elmer) approximately 15 min after the addition of the relevant reagent. Controls confirmed that there is no cross-reactivity between AP-1 and Renilla luciferase activities assayed in this fashion. All values were normalized to internal Renilla controls and then to the maximal responses observed in the presence of PMA and ionomycin.

**RESULTS**

**Lateral movement of persistent SLP-76-containing clusters in SEE-induced synapses.** SLP-76-containing signaling clusters have been observed in response to immobilized antibodies and stimulatory lipid bilayers and within the synapses formed by T cells and APCs (10, 12, 59). To examine the dynamics of cluster movement within synapses, we generated conjugates by using SLP-76-deficient Jurkat T cells (J14 cells) stably reconstituted with YFP-tagged SLP-76 (SLP-76.YFP), Nalm-6 B cells, and the bacterial superantigen SEE. To track the lateral movement of SLP-76-containing clusters, we continuously collected three-dimensional image stacks spanning the synapse. In Fig. 1, reconstructed views of the synapse surface (x-z face) reveal that SLP-76-containing clusters formed at the periphery of the synapse, moved laterally within the plane of the synapse, and eventually merged with a larger central cluster of SLP-76 (see Video S1 in the supplemental material). This central cluster faded periodically and appeared to be refreshed by newly arriving clusters. Thus, the lateral movement of persistent SLP-76-containing clusters within the plane of the synapse is a common feature observed with all three imaging systems discussed above (10, 12, 59).

**Upstream requirements for cluster formation, persistence, and translocation.** We postulated that the persistent and mobile SLP-76-containing signaling clusters observed with the three imaging systems correspond to the minimal effective complexes predicted by kinetic proofreading models of T-cell activation. To test the correspondence between cluster formation and T-cell activation, we employed antibody-coated glass surfaces as described previously. Given that persistent and laterally mobile SLP-76-containing clusters formed in each model system, the two planar imaging systems offered several advantages. Both systems immobilize the stimulated cells, allow the prediction of the plane of stimulation, and permit the capture of the entire contact surface in a single image. In particular, our plate-bound assay immobilizes engaged TCRs and therefore enables the visualization of SLP-76 apart from the initiating receptor. In addition, our spinning-disc confocal assay is more amenable to dynamic multicolor studies than are standard total internal reflection/fluorescence-based systems.

Lck, ZAP-70, LAT, and PLC-γ1 have been predicted to play roles in cluster assembly or stabilization, and they play critical roles in T-cell activation. To test whether cluster formation, persistence, or movement correlated with normal activation, we examined Jurkat-derived T-cell lines lacking these proteins (J.CaM1, P116, J.CaM2, and Jy1, respectively) (31). The loss and reconstitution of these signaling proteins are confirmed in Fig. S1 in the supplemental material. To quantitate differences in cluster formation, persistence, and movement in these cell lines, we identified moving complexes in maximum-over-time projections, traced individual cluster trajectories in kymographs, and presented the average behavior of these clusters, using composite kymographs and mean translocation plots (see Fig. S2 in the supplemental material). To limit our analyses to cells expressing approximately physiological levels of protein, we identified and analyzed only those cells that expressed moderate levels of SLP-76.YFP, as determined by comparison with a control cell line stably overexpressing known amounts of SLP-76 (see Materials and Methods). Representative maximum-over-time projections (Fig. 2A) reveal that all aspects of

![FIG. 1. SLP-76-containing complexes in SEE-induced synapses.](http://mcb.asm.org/figure/1)

J14 Jurkat T cells stably expressing SLP-76.YFP were allowed to form synapses in the presence of Nalm-6 B cells and 5 μg/ml SEE. Conjugates were detected 10 to 20 min after the initiation of the assay. SLP-76-containing complexes were observed in the synapses of all conjugates. SLP-76 was imaged within synapses by continuously collecting three-dimensional image sets spanning the synapse. Images were collected with a vertical spacing of 0.3 μm, and full image sets were collected every 16 seconds. The images shown here were collected at 32-second intervals. At each time point, two views of the synapse were created, including a maximum projection of the entire image set, revealing all of the clusters in the synapse in a single image, and a reconstructed head-on view of the synapse. Asterisks mark the position of the Nalm-6 B cell, and arrows identify sites of SLP-76 nucleation (white) and consolidation (gray).
Cluster formation are compromised in the absence of Lck, ZAP-70, or LAT. Overall, fewer cells formed clusters, and those that did not persisted nor translocated (Fig. 2B). These defects are specific to the deficiencies in question, as reconstitution restored normal cluster behavior in these cell lines. Thus, cluster persistence and movement are correlated with effective T-cell activation.

The quantitation of cluster persistence and movement revealed that clusters that formed in the parental Jurkat line persisted for 5 to 6 min, moved 4 to 5 μm from their sites of origin, and initially traveled at speeds of 2 μm/minute, with individual clusters achieving speeds as high as 4 μm/minute (see Fig. S2 in the supplemental material). On average, the few clusters that formed in the mutant cell lines persisted for no more than 2 min, moved no more than 1 μm, and moved no faster than 0.5 μm/minute (Fig. 2C and D). In contrast, the SLP-76-containing clusters observed in the Lck-, ZAP-70-, and LAT-reconstituted cell lines were grossly normal. These clusters persisted for 4 to 6 min and traveled 2 to 4 μm at average speeds between 0.5 and 1.5 μm/minute. Individual clusters achieved speeds of 2 to 4 μm/minute. These data further indicate that normal cluster persistence and movement are associated with effective T-cell activation.

Since abortive cluster formation was observed in the Lck- 
ZAP-70-, and LAT-deficient cell lines, we hypothesized that a distinct Fyn-dependent pathway might account for residual cluster-nucleating activity. PP2, a drug that inhibits both Lck and Fyn, blocked the formation of SLP-76-containing complexes at doses that prevented the recruitment of ZAP-70 to the TCR (Fig. 2E). This is consistent with a role for Fyn in the

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**FIG. 2.** Genetic requirements for complex assembly. Jurkat variants and their stably reconstituted partners were transiently transfected with SLP-76.YFP. (A) Maximum-over-time projections are shown for representative mutant (top row) and reconstituted (bottom row) cells. (B) Summary of the observed microcluster phenotypes in transiently transfected cells. For each condition, at least three independent transfections were performed, and three to five imaging runs were performed per transfection. All cells meeting the expression criteria described in Materials and Methods were sorted into the following three categories: cells displaying no clustering, cells displaying transient clusters that failed to move, and cells displaying persistent, mobile clusters. Overexpression of the SLP-76 chimera was estimated on a per-cell basis by comparing the experimental exposure time to the time required to image a cell expressing a known amount of an identical chimera (not shown). (C) Movement traces of the most persistent and mobile complexes observed in each cell were extracted and compiled into composite kymographs. This approach understates the defects observed in nonreconstituted cell lines. (D) Mean translocation plots were compiled from the individual movement traces shown in panel C. The numbers of traces compiled are indicated. (E) Jurkat T cells stably expressing either SLP-76.YFP or ZAP-70.YFP were assayed for complex formation after a 30-min pretreatment with 10 μM PP2 or with carrier alone (dimethyl sulfoxide [DMSO]). Maximum-over-time projections are shown for representative SLP-76.YFP-expressing cells (top row), whereas still frames are shown for the ZAP-70.YFP-expressing cells (bottom row).

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formation of the labile SLP-76-containing clusters observed in the absence of Lck, ZAP-70, or LAT. Although PLC-γ1 interacts with multiple components of LAT-nucleated signaling complexes and may participate in cooperative interactions within the complex, SLP-76-containing clusters behaved normally in the PLC-γ1-deficient Jy1 cell line (Fig. 2A to D) (6, 31). Since these cells are incapable of normal activation, these data indicate that complex formation is insufficient for maximal activation in the absence of an essential downstream effector. Although this formally indicates that PLC-γ1 does not play a significant role in cluster stabilization in this model system, the Jy1 cell line expresses the highly related protein PLC-γ2. This PLC isomorph is phosphorylated in response to TCR ligation and may compensate to some degree for the absence of PLC-γ1 (24).

Multiple domains of SLP-76 contribute to cluster formation, persistence, and translocation. To identify the molecular mechanisms regulating the formation, persistence, and translocation of SLP-76-containing clusters, we monitored the behavior of these clusters in SLP-76-deficient Jurkat cells stably reconstituted with EGFP-tagged variants of SLP-76 (Fig. 3A). The primary role in the recruitment of SLP-76 to LAT has been attributed to the Gads-binding motif of SLP-76. Additional protein-protein interaction domains include (i) a triply tyrosine-phosphorylated domain that interacts with the SH2 domains of Vav, Nck, and Itk; (ii) the P1 domain, a 67-amino-acid proline-rich region initially identified as a PLC-γ1-binding site; and (iii) the SH2 domain, which binds the adaptor ADAP and the serine/threonine kinase HPK1 (7, 9, 14, 32, 36, 43, 47, 49, 58). To minimize the risk of clonal artifacts, our stable lines were derived as polyclonal populations by high-speed cell sorting and matched for EGFP.SLP-76 expression by flow cytometry and Western blotting (49). These amino-terminally tagged SLP-76 chimeras (EGFP.SLP-76) translocate to the center of the contact surface (Fig. 3B) (49), as originally shown for SLP-76 chimeras tagged at the carboxy terminus (SLP-76.YFP) (10). These clusters traveled approximately 2 μm inward, persisted for approximately 4 min, and achieved speeds of approximately 1 μm/minute (Fig. 3C). In contrast, the assembly, persistence, and movement of clusters were severely defective in all mutant lines (Fig. 3B and C; see Video S2 in the supplemental material). In particular, the P1-deleted (P1) and tyrosine mutant (Y3F) chimeras assembled into clusters that dissipated within 90 seconds, without undergoing significant translocation, whereas the Gads-binding mutant (G2) and SH2 domain mutant (RK) chimeras were not recruited into clusters at all. Although the tyrosine-phosphorylated domain, the P1 domain, and the SH2 domain are commonly viewed as effector docking sites, our results indicate that these domains play unexpectedly important roles in the assembly, persistence, and movement of SLP-76-containing clusters. We predict that each of the domains tested here contributes molecular interactions that cooperate to stabilize SLP-76-containing clusters.

Cluster formation, persistence, and translocation are associated with effective T-cell activation. Previous studies have shown that all of the SLP-76 domains tested above contribute to T-cell activation (17, 37, 49, 58). However, some experiments suggest a limited role for the SLP-76 SH2 domain in T-cell activation (4). To determine whether differences in the mode of stimulation account for these apparent differences in the function of the SH2 domain, we subjected several of the SLP-76-expressing cell lines to NF-AT/AP-1 reporter assays in conjunction with our previous studies, these data indicate that every SLP-76 mutation that results in altered cluster formation or persistence impairs T-cell activation, as summarized in Table 1 (38, 49). Thus, the cooperative stabilization of SLP-76-containing clusters appears to be required for effective T-cell activation.

SLP-76 and Gads cooperate to facilitate cluster formation. To further address the cooperative interactions regulating cluster formation, we examined the role of Gads in greater detail. Although we initially reported that Gads is only transiently recruited to signaling clusters, the Gads-binding G2 domain of SLP-76 is absolutely required for cluster nucleation (10). To clarify the role of Gads in cluster formation, we coexpressed a monomeric CFP-tagged wild-type Gads chimera (mCFP.Gads) with SLP-76.YFP (Fig. 4A; see Fig. S3 in the supplemental material). Under these conditions, Gads entered persistent clusters in response to TCR ligation, colocalizing precisely with SLP-76 (Fig. 4B; see Video S3 in the supplemental material).
Unexpectedly, Gads did not enter clusters in the absence of SLP-76 (Fig. 4C, left panel). The physiological levels of SLP-76 in reconstituted J14 cells enabled the recruitment of Gads into clusters that dissipated rapidly (Fig. 4C, center panel), whereas higher levels of SLP-76 permitted the recruitment of Gads into persistent, mobile clusters (Fig. 4C, right panel).

These observations clearly indicate that SLP-76 controls the localization of Gads. However, this observation contradicts existing biochemical data indicating that Gads controls the recruitment of SLP-76 into complexes (32). To confirm that Gads can regulate the localization of SLP-76, we created a dominant-negative version of Gads by fusing the carboxy-terminal SH3 domain of Gads to an amino-terminal mCFP tag (Fig. 4A). This dominant-negative construct blocked the recruitment of SLP-76 into clusters, whereas a control construct with a single SH3 domain-inactivating
point mutation had no effect on cluster formation (Fig. 4D; see Fig. S3 in the supplemental material). As anticipated, this dominant-negative effect was strongly dose dependent (see Fig. S4 in the supplemental material). Jointly, these observations demonstrate that the recruitment of Gads to TCR requires Gads. These observations are incompatible with linear models of cluster formation but are easily reconciled in models where Gads and SLP-76 cooperate to influence cluster nucleation and persistence. 

Multiple domains of Gads contribute to cluster formation and translocation. The isolated carboxy-terminal SH3 domain of Gads potently inhibited the recruitment of SLP-76 to LAT clusters and did not perturb the localization of SLP-76 (Fig. 5C). In contrast, mutation of the SH2 domain resulted in the generation of a dominant-negative Gads chimera that was not recruited into complexes and inhibited the recruitment of SLP-76 into clusters (Fig. 5C; see Video S6 in the supplemental material). This result confirms that the SH2 domain plays an essential but partially redundant role in cluster formation. The mutation of both SH3 domains created a less potent dominant-negative Gads chimera that was not recruited into clusters but only partially inhibited the recruitment of SLP-76 into clusters (Fig. 5C). In contrast, mutation of the SH2 domain and the carboxy-terminal SH3 domain created an inert Gads chimera that was not recruited into clusters and did not perturb the localization of SLP-76 (Fig. 5C; see Video S6 in the supplemental material). These data reveal an unexpected complexity of Gads interactions. There are at least two functional regions within Gads, namely, the carboxy-terminal SH3 domain, which is sufficient to direct the interaction between Gads and SLP-76, and an amino-terminal region comprising the first SH3 domain and the SH2 domain, which each appear to promote the interaction of Gads with LAT.

Protein-protein interactions govern the recruitment of LAT into signaling clusters. Phosphotyrosine-dependent protein-protein interactions play an important role in the recruitment of LAT into signaling clusters (10, 15, 21). Since our data indicate that the entry of Gads into clusters is influenced by its interaction with SLP-76, we hypothesized that the recruitment of LAT into signaling clusters is regulated by Gads and SLP-76. In the absence of exogenous SLP-76, LAT chimeras entered clusters that dissipated rapidly (10). In this study, we expressed a CFP-tagged wild-type LAT chimera (LAT.CFP.WT) in SLP-76-YFP-reconstituted J14 cells. This LAT chimera was recruited into persistent SLP-76-containing clusters that were transported medially (Fig. 6A, top row; see Video S7 in the supplemental material). Four critical carboxy-terminal tyrosines in LAT have been implicated in binding Gads, Grb2,
A mutant LAT chimera lacking these tyrosines (LAT.CFP.4YF) remained uniformly distributed in the plasma membrane following TCR ligation, even though the coexpressed SLP-76 chimera, presumably interacting with endogenous LAT, clustered normally (Fig. 6A, bottom row; see Video S8 in the supplemental material). These data indicate that phosphotyrosine-mediated protein-protein interactions drive the clustering of LAT, as we and others have argued previously (10, 15, 21).

To test whether the impaired association of the LAT-4YF mutant with Gads, SLP-76, and PLC-γ1 is responsible for the failed assembly of LAT into signaling clusters, we tagged wild-type LAT with a red fluorescent protein. We expressed the resulting chimera, LAT.DsRed, in J14 cell lines stably reconstituted with either the wild-type EGFP.SLP-76 chimera or matched levels of the P1/G2 double-mutant chimera, which cannot bind Gads or PLC-γ1. Although the DsRed fluorescent tag induced some nonspecific aggregation of LAT, the LAT.DsRed chimera clearly assembled into clusters with wild-type EGFP.SLP-76 (Fig. 6B, top row). LAT and SLP-76 persisted in these clusters and coordinately moved to a central compartment. Although the LAT.DsRed chimera entered signaling clusters in the P1/G2 mutant line, these clusters were less persistent (not shown) and did not display directed movement (Fig. 6B, bottom row), indicating that the translocation of LAT required its interaction with Gads and SLP-76. Since the LAT chimera behaved in the same manner in the absence of SLP-76 (compare the left and right cells in the bottom row of Fig. 6B), the residual LAT clustering must be independent of SLP-76. These observations indicate that the assembly of LAT into clusters capable of translocation requires SLP-76 but that alternate tyrosine-dependent mechanisms recruit LAT into transient, static clusters.

## DISCUSSION

SLP-76-containing signaling clusters participate in T-cell activation. SLP-76-containing clusters have been reported for T cells stimulated by three distinct mechanisms, i.e., in response to immobilized antibodies, in response to lipid bilayers bearing stimulatory pMHC, and in response to antigen (or superantigen)-presenting B cells (10, 12, 59). In all three systems, micrometer-scale clusters rich in SLP-76 form at sites of TCR ligation at the periphery of the contact surface and persist...
while moving towards the center of the contact surface. These structures arise in regions of tight contact in the F-actin-rich cell periphery, recruit critical adaptors and effector proteins within seconds of TCR ligation, and suffice to initiate cytoplasmic calcium increases (2, 10–12, 59). In addition, every cell line displaying impaired cluster assembly or persistence fails to respond effectively to TCR ligation. These observations indicate that SLP-76-containing clusters are functional. Furthermore, these structures assemble within the kinetic window dictated by the half-lives of effective TCR-pMHC interactions (10, 19). Thus, these SLP-76-containing clusters may correspond to the essential signaling clusters predicted by the kinetic proofreading model.

**Fates of SLP-76-containing signaling clusters.** In all three imaging systems discussed above, SLP-76-containing clusters are seen to persist, move towards the center of the contact interface, and ultimately diverge from their initiating TCR microclusters. In our study, the SLP-76-containing clusters induced by superantigen move laterally within the contact interface and can be detected within this interface for 20 to 30 min. Although lateral cluster movement and central accumulation were not observed in the superantigen-induced contacts studied by Yokosuka et al., their studies examined SLP-76-containing clusters 30 min after contact formation (59). By this time, the clusters observed in our studies had typically dissipated. Antigen-presenting lipid bilayers induce signaling clusters that colocalize with the TCR in mobile clusters until SLP-76 disappears during its transit towards the center of the contact surface (59). The failure to observe centralized SLP-76-containing clusters on lipid bilayers may result from the failure to detect internalized structures, as the illumination employed in total internal reflection/fluorescence-based studies only penetrates to depths of 50 to 100 nm. Alternately, centralized SLP-76-containing structures may not be observed because of differences in the nature of the responding cell (primary cell versus cell line), the quality of the TCR ligand (pMHC versus superantigen), or the costimulatory molecules presented by the stimulatory surface (lipid bilayer versus intact B cell). Finally, immobilized antibodies induce SLP-76-containing clusters that segregate from initiating TCR microclusters and move towards...

**FIG. 5.** Gads domains contribute to complex assembly. (A) Schematic of mCFP.Gads mutants. (B) mCFP.Gads constructs containing mutations in individual domains were transiently transfected into J14 cells stably reconstituted with SLP-76.YFP. Complex formation assays were performed, and maximum-over-time projections for both SLP-76 (left panels, green overlay) and Gads (middle panels, red overlay) are presented for representative cells. (C) mCFP.Gads constructs containing mutations in multiple domains were analyzed as described for panel B.
The center of the contact surface (10). Although this last system, which was employed in these studies, is the only system to give rise to SLP-76-containing clusters that demonstrably persist apart from the TCR, this difference may be due to the artificial immobilization of the TCR by stimulatory antibodies. Although further studies will be required to determine how differences in the methods of stimulation and visualization influence cluster formation and persistence, our observations indicate that SLP-76-containing clusters can persist apart from the TCR and that these clusters are capable of temporarily maintaining their integrity in the absence of continuous interactions with TCR microclusters.

Multiple protein-protein interactions regulate cluster nucleation and persistence. The SLP-76-containing clusters described above are poised to regulate T-cell activation. However, the precise composition of these clusters remains undefined. The studies presented here confirm that the anticipated protein-protein interactions play critical roles within these clusters (i.e., the Gads SH2 domain, the Gads C-SH3 domain, and the Gads-binding G2 site in SLP-76). However, our studies have demonstrated that several protein interaction domains previously thought to function as effector recruitment sites play important roles in the assembly and stabilization of signaling clusters.

Dramatically, the inactivation of the SLP-76 SH2 domain abolishes the recruitment of SLP-76 into clusters, blocks CD69 upregulation, and prevents the activation of NF-KB. Although some studies have indicated a limited role for the SH2 domain, a partially redundant role in cluster nucleation may have been obscured by the constitutive membrane localization of the SLP-76 chimeras used in these studies (4). Additional studies performed with SLP-76-deficient cells stably reconstituted with SH2 domain mutants or Gads-binding-site mutants confirmed that the roles played by these two sites are comparable and essential (38, 58). The critical SH2 domain ligand supporting complex nucleation has not yet been defined. The adaptor ADAP and the serine-threonine kinase HPK1 are likely candidates, as these proteins bind the SLP-76 SH2 domain and are capable of engaging components of the TCR-proximal signaling complex independently of SLP-76 (14, 33, 47). Therefore, multivalent interactions involving SLP-76 and ADAP or HPK1 could stabilize the observed signaling clusters.

The proline-rich P1 domain and the amino-terminal tyrosines of SLP-76 are best known for their effector-binding properties. Nevertheless, both domains are required for persistent cluster formation (38, 49). Given the important role of the P1 domain and the biochemical evidence in favor of a cooperative bridging model in which PLC-γ1 links SLP-76 to LAT, we were surprised to find that PLC-γ1 is not required for the formation, persistence, or movement of SLP-76-containing clusters (31). Thus, the cooperative interactions reported to recruit PLC-γ1 into signaling clusters may not contribute to the overall stability of individual SLP-76-containing clusters. Although PLC-γ2 is present in the Jy1 line and may compensate for any structural role associated with PLC-γ1, our mutational analyses also indicated that the P1 domain contributes to cluster stability and effective T-cell activation (24). Thus, as yet uncharacterized ligand of the P1 domain is likely to play an important role in cluster stabilization (58). Similarly, we do not yet understand how the amino-terminal tyrosines of SLP-76 contribute to cluster stability, i.e., whether they work through cooperative protein-protein interactions or through the catalytic functions of associated proteins (9, 56, 57). Regardless of how these domains contribute to complex stability, our observations emphasize the utility of imaging approaches that directly evaluate cluster formation as a complement to traditional biochemical and functional approaches.

Cooperative interactions stabilize SLP-76-containing clusters. Cooperative models of complex formation have been invoked to explain the requirement that several distinct phosphorylation sites be present on each LAT molecule (31). Our observations are inconsistent with common linear models of complex formation. First, four distinct regions within SLP-76 contribute to cluster formation and persistence, indicating that these regions cooperate to stabilize signaling complexes. Second, SLP-76 controls the recruitment of Gads into clusters. Finally, Gads does not act as a simple bifunctional bridge between LAT and SLP-76. Instead, the amino-terminal SH3 domain and the SH2 domain of Gads are partially redundant.
with respect to cluster formation and cooperate to recruit Gads into LAT-nucleated clusters. These data support a model in which LAT, Gads, and SLP-76 act as a core signaling cluster stabilized by multiple cooperative interactions among these proteins and additional cluster constituents (6).

Defective signaling clusters are rapidly terminated. While certain alterations abolish cluster formation altogether, diverse perturbations of the SLP-76-containing clusters result in the dissociation of these structures within 90 to 120 seconds, prior to the initiation of medial cluster movement. This effect has been observed in cells lacking Lck, ZAP-70, or LAT, in cells expressing SLP-76 mutants affected in either the amino-terminal tyrosines or the P1 domain, and in cells expressing dominant-negative variants of Gads. The similarity of these behaviors suggests that the failure to persist is regulated by a conserved inhibitory mechanism triggered in response to partial signaling, as predicted by modified versions of the kinetic proofreading model (51). We speculate that this inhibitory process is dampened in cells that assemble mature SLP-76-containing clusters.

Implications of cooperative cluster formation. We have demonstrated a strong relationship between the persistence of cooperatively stabilized SLP-76-containing signaling clusters and optimal T-cell activation (Table 1). We predict that this relationship arises because cooperative cluster stabilization serves several critical needs. First, cooperative interactions may support cluster formation and antagonize cluster dissociation by enabling the assembly of high-avidity macromolecular complexes from multiple low-affinity protein-protein interactions. In this manner, cooperative interactions may increase antigen sensitivity and sharpen the thresholds regulating the discrimination of antigen from self. Second, the diverse interactions within a cooperatively assembled cluster may facilitate the integration of multiple costimulatory or suppressive inputs by providing multiple targets for the modulation of T-cell responses. Finally, highly favorable cooperative interactions may constrain the topology of mature signaling clusters, enabling the selective recruitment of adaptors to LAT in vivo (13, 22, 31, 62). A better understanding of these complex intermolecular interactions will enable the modulation of immune responses by endogenous signaling pathways and by targeted pharmacologic agents.

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