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Association of the EF-hand and PH domains of the guanine nucleotide exchange factor SLAT with IP₃ receptor 1 promotes Ca²⁺ signaling in T cells

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Abstract

The guanine nucleotide exchange factor SLAT (SWAP-70-like adaptor of T cells) regulates T cell activation and differentiation by enabling Ca²⁺ release from intracellular stores in response to stimulation of the T cell receptor (TCR). We found a TCR-induced association between SLAT and inositol 1,4,5-trisphosphate (IP₃) receptor type 1 (IP₃R1). The N-terminal region of SLAT, which contains two EF-hand motifs that we determined bound Ca²⁺, and the SLAT pleckstrin homology (PH) domain independently bound to IP₃R1 by associating with a conserved motif within the IP₃R1 ligand-binding domain. Disruption of the SLAT-IP₃R1 interaction with cell-permeable, IP₃R1-based fusion peptides inhibited TCR-stimulated Ca²⁺ signaling, activation of the transcription factor NFAT (nuclear factor of activated T cells), and production of cytokines, suggesting that this interaction is required for optimal T cell activation. The finding that SLAT is an IP₃R1-interacting protein required for T cell activation suggests that this interaction could be a potential target for a selective immunosuppressive drug.

INTRODUCTION

Ca²⁺ signaling plays a crucial role in immune responses by regulating many aspects of lymphocyte biology, including development, activation, and effector functions (1). Impaired Ca²⁺ signaling in T lymphocytes is linked to pathophysiological processes in several autoimmune and inflammatory diseases (2). Antigen recognition through the T cell receptor (TCR) results in the activation and recruitment of several tyrosine kinases and substrates to

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SUPPLEMENTARY MATERIALS

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the TCR-CD3 complex, which eventually results in the activation and recruitment to the plasma membrane of phospholipase- $\text{C}\gamma 1$ (PLC- $\gamma 1$), which in turn leads to the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdInsP₂, also known as PIP₂) to generate the second messengers D-*myo*-inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol.

IP₃ stimulates the release of Ca²⁺ from intracellular stores through binding to IP₃ receptors (IP₃Rs) in the endoplasmic reticulum (ER). Depletion of ER Ca²⁺ stores leads to the activation of calcium release-activated calcium (CRAC) channels at the plasma membrane and results in a sustained influx of Ca²⁺ into the cell. The resulting increased intracellular Ca²⁺ concentration ([Ca²⁺]_i) leads to the activation of Ca²⁺-dependent enzymes and subsequent nuclear translocation of the transcription factor nuclear factor of activated T cells (NFAT), which stimulates the expression of multiple target genes whose products are involved in T cell activation and differentiation.

IP₃Rs are large, tetrameric proteins that reside primarily in the ER. Each monomer has an N-terminal ligand-binding domain (LBD), which is followed by a large coupling domain and a C-terminal domain that contains the channel region (3). Regulation of the IP₃R is complex and involves many mechanisms, including IP₃ and Ca²⁺ binding (3), posttranslational modifications such as tyrosine phosphorylation (4), and protein-protein interactions. Many proteins interact with IP₃Rs and modulate their affinity, conductivity, or localization and cellular distribution (5–8). SWAP-70-like adaptor of T cells (SLAT) (9), also known as Def6 (10) and IBP (11), is a guanine nucleotide exchange factor (GEF) for Rho guanosine triphosphatases (GTPases), and it is predominantly found in thymocytes and peripheral T cells (9, 11, 12). Structurally, SLAT contains, beginning at its N terminus, a putative Ca²⁺-binding, EF-hand domain, which is followed by an imperfect immunoreceptor tyrosine-based activation motif (ITAM)-like sequence, a phosphatidylinositol 3,4,5-trisphosphate (PIP₃)-binding pleckstrin homology (PH) domain (9), and a catalytic Dbl homology (DH) domain, which exhibits GEF activity toward Cdc42 and Rac1 (13–15). SLAT is required for the activation and differentiation of CD4⁺ T cells by controlling Ca²⁺ release from ER stores in response to costimulation of the TCR and the co-receptor CD28 (12). Hence, SLAT-deficient (*Def6*^{−/−}) T cells display a marked defect in Ca²⁺ signaling and NFAT activation, despite having normal PLC- $\gamma 1$ activation and intact IP₃ production (12).

Here, we report a direct, TCR-induced, and Ca²⁺-sensitive physical association between SLAT and type 1 IP₃R (IP₃R1). Both the N-terminal EF-hand domain and the PH domain of SLAT interacted with IP₃R1 independently and were essential for mediating TCR-induced Ca²⁺ signaling. We further demonstrated that a short motif within the LBD of IP₃R1 bound to SLAT. The biological relevance of this association was established by demonstrating that its disruption in CD4⁺ T cells impaired TCR-stimulated Ca²⁺ and NFAT signaling and cytokine production. These findings highlight SLAT as an IP₃R1-interacting protein that critically regulates the TCR-stimulated Ca²⁺ and NFAT signaling pathway.

RESULTS

TCR-stimulated association between SLAT and IP₃R1

Upon TCR stimulation, *Defb*^{-/-} CD4⁺ T cells show a marked defect in Ca²⁺ and NFAT signaling that can be traced to a reduction in the extent of Ca²⁺ release from the ER (12). Given that IP₃ production by these T cells is intact (12), we hypothesized that SLAT is required for proper IP₃R1 function in T lymphocytes, potentially through its physical interaction with the receptor. Through immunoprecipitation experiments with CD4⁺ T cells stimulated with antibodies against CD3 and CD28 (anti-CD3/CD28), we found that endogenous SLAT physically interacted with IP₃R1 within minutes after TCR engagement (Fig. 1A). The extent of the interaction was maximal at 2 min after stimulation and returned to basal level after 15 min (Fig. 1A). Through an in situ Duolink proximity ligation assay (16), we also demonstrated a close proximity between SLAT and IP₃R1 in stimulated CD4⁺ T cells (Fig. 1, B and C). To determine whether SLAT and IP₃R1 were localized within the same cellular compartment in T cells, we performed subcellular fractionation of MCC-T cells, an antigen-specific T cell hybridoma (17). As expected (15), SLAT resided in the cytosolic and plasma membrane fractions (Fig. 1D); however, in addition, it was also detected in the ER fraction, which also contained IP₃R1 (Fig. 1D). Thus, TCR activation stimulated an interaction between SLAT and IP₃R1 in CD4⁺ T cells, and both molecules were found in the ER.

Direct binding of SLAT to the IP₃R1 LBD

To map the SLAT-binding site in IP₃R1, we used a series of glutathione *S*-transferase (GST)-fused IP₃R1 recombinant proteins (Fig. 2A) in pull-down experiments. These proteins correspond to six fragments (f1 to f6) covering the entire length of IP₃R1, with the exception of the transmembrane channel domain. SLAT interacted exclusively with the f2 protein, which corresponds to amino acid residues 346 to 922 of IP₃R1 (Fig. 2B), a region that overlaps the IP₃-binding and the regulatory and coupling domains. To further narrow down the IP₃R1 region responsible for binding to SLAT, we generated shorter (~140-residue) recombinant fragments (f2.1 to f2.4) of f2 (Fig. 2A), and we found that SLAT predominantly bound to fragment f2.1 (residues 346 to 490) and, to a much lesser extent, to fragment f2.2 (residues 491 to 635) (Fig. 2C). Further breakdown of these two fragments into shorter fragments of ~96 residues each (f.A to f.D) revealed that SLAT interacted exclusively with one IP₃R1 fragment, f.A, which corresponds to residues 366 to 441 (Fig. 2D), thereby defining a short SLAT-binding region of IP₃R1 within its LBD.

To determine whether the SLAT-IP₃R1 interaction was direct, we performed pull-down experiments with various recombinant GST-IP₃R1 proteins and purified SLAT. Recombinant SLAT bound to all of the IP₃R1 fusion proteins that contained residues 366 to 441, that is, to fragments f2, f2.1, and f.A, but not f.D (Fig. 2E), which served as a negative control, thus indicating that the association between SLAT and IP₃R1 is direct. Similarly, Far-Western blots with recombinant proteins also showed direct binding of SLAT to IP₃R1 (fig. S1).

Independent interaction of the PH and putative EF-hand domains of SLAT with IP₃R1

To gain further insight into the molecular basis of the SLAT-IP₃R1 interaction, we transfected Jurkat TAg cells with plasmids encoding a series of Myc-tagged SLAT deletion mutants (Fig. 3A) together with plasmid encoding FLAG-tagged full-length IP₃R1, and then analyzed their association by coimmunoprecipitation (Fig. 3B). Both the putative EF-hand domain and the PH domain of SLAT bound to IP₃R1 independently and, consistently, all other SLAT mutants that contained either of these domains (that is, EF-ITAM and PH-DH) also associated with IP₃R1 (Fig. 3B). Conversely, the EF-PH SLAT mutant, which is deficient in both domains, interacted poorly with IP₃R1 compared to full-length SLAT (fig. S2A). Similar results were obtained by performing pull-down experiments with GST-SLAT fusion proteins and lysates from human embryonic kidney (HEK) 293T cells transfected with plasmid encoding FLAG-tagged IP₃R1 (fig. S2, B and C). We additionally performed coimmunoprecipitation experiments with lysates from Jurkat TAg cells cotransfected with plasmid encoding IP₃R1-f2.1 (residues 346 to 490) together with plasmids encoding the putative EF-hand or PH domains of SLAT. As expected, both of these SLAT domains coimmunoprecipitated with f2.1, with the EF-hand domain displaying a greater interaction than the PH domain (Fig. 3C).

To determine whether the EF-hand or PH domains of SLAT independently and directly bound to IP₃R1, we performed GST pull-down experiments with GST-SLAT fusion proteins immobilized on glutathione beads (fig. S2B) and a recombinant His-tagged protein representing IP₃R1 ligand-binding site-1 (Lbs-1; residues 1 to 581). Both the EF-hand and PH domains of SLAT precipitated the His-tagged IP₃R1 Lbs-1 (Fig. 3D), indicating that each of these two domains independently and directly interacted with IP₃R1.

Transfection of Jurkat TAg cells with plasmid encoding SLAT enhances NFAT activation in response to anti-CD3 stimulation (15). To assess the contribution of the EF-hand and PH domains of SLAT to this activity, we tested the ability of SLAT deletion mutants deficient in these two domains to enhance NFAT activation in transfected Jurkat TAg cells. Upon TCR stimulation, the SLAT mutant lacking the EF-hand domain (EF-) and the mutant lacking the PH domain (PH-) stimulated NFAT activity to the same extent as did full-length SLAT (Fig. 3E). Only the simultaneous deletion of both domains (in the EF-PH mutant) reduced NFAT activity (Fig. 3E), demonstrating that each domain alone was sufficient to promote NFAT activation.

Ca²⁺ dependence of the SLAT-IP₃R1 association: Role of the EF-hand domain

At its N terminus, SLAT contains a putative EF-hand domain (residues 1 to 72) (9), consisting of two potential Ca²⁺-binding motifs (residues 19 to 30 and 57 to 68, respectively) (14), whose function has not been formally established. EF-hand domains are conserved, Ca²⁺-binding motifs that are found in a large number of intracellular proteins. Typically, this domain assumes a helix-loop-helix structure that binds to a single Ca²⁺ ion through a 12-residue canonical loop in which conserved residues participate in Ca²⁺ binding through their carboxyl or hydroxyl groups (18). To determine whether the N-terminal region of SLAT contains a functional Ca²⁺-binding domain, we performed a ⁴⁵Ca overlay assay with a recombinant GST fusion protein consisting of the EF-hand and ITAM domains of

SLAT (Fig. 4A); GST protein alone and calmodulin (CaM) served as negative and positive controls, respectively. Autoradiography of the membrane probed with ^{45}Ca showed that the GST-EF-ITAM protein bound to Ca^{2+} (Fig. 4B). Furthermore, deletion of the two Ca^{2+} -binding motifs (EF-ITAM 19–30+ 57–68) substantially, albeit not completely, reduced Ca^{2+} binding, whereas deletion of a single motif (EF-ITAM 19–30) had no noticeable effect (Fig. 4C). Thus, SLAT can bind to Ca^{2+} and, furthermore, Ca^{2+} binding involves the N-terminal EF-hand domain of SLAT.

To determine whether the association between SLAT and $\text{IP}_3\text{R1}$ was Ca^{2+} -dependent, we performed pull-down experiments by incubating cell lysates from unstimulated or anti-CD3-stimulated Jurkat TAg cells with a recombinant GST- $\text{IP}_3\text{R1}$ -f2 protein (Fig. 2A) in the presence of CaCl_2 or EGTA. Chelation of Ca^{2+} by EGTA substantially reduced the binding of cellular SLAT to the recombinant $\text{IP}_3\text{R1}$ protein by 84 and 73%, respectively, in unstimulated or stimulated cells (Fig. 4D). Conversely, inclusion of external Ca^{2+} increased the extent of SLAT- $\text{IP}_3\text{R1}$ binding in unstimulated cells by about two- to threefold (Fig. 4D). This increase was less pronounced in stimulated cells, most likely because anti-CD3 stimulation already induced the maximal increase in binding.

Mapping of a conserved SLAT-binding motif in $\text{IP}_3\text{R1}$

Having defined a 96-amino acid region of $\text{IP}_3\text{R1}$ (residues 346 to 441) that binds to SLAT (Fig. 2D), we next aimed at identifying a minimal $\text{IP}_3\text{R1}$ motif essential for this interaction. We generated five deletion mutants of $\text{IP}_3\text{R1}$ -f.A (Fig. 2A), in which stretches of 18 amino acid residues within this critical region were successively deleted, for bacterial production as GST fusion proteins (Fig. 5A). These recombinant proteins were assayed in pull-down experiments for their ability to precipitate endogenous SLAT from T cell lysates. The results showed that deletion of residues 400 to 417 from $\text{IP}_3\text{R1}$ f.A abrogated its ability to interact with SLAT (Fig. 5B), whereas deletion of other residues had minimal, if any, effect on the interaction, with the possible exception of the A4 mutation, which seemed to reduce the extent of the interaction compared with that of the nondeleted f. A fusion protein. We confirmed the importance of this 18-amino acid region by performing similar pull-down experiments with a larger GST fusion protein of $\text{IP}_3\text{R1}$ f2.1 (residues 346 to 490) from which residues 400 to 417 were deleted (Fig. 5C), and found again that deletion of these 18 residues abolished the interaction with SLAT (Fig. 5D). These results suggest that the short motif consisting of amino acid residues 400 to 417 in $\text{IP}_3\text{R1}$ is critical for the interaction between $\text{IP}_3\text{R1}$ and SLAT. Note that this polar motif, which contains four negatively charged and three positively charged residues, is evolutionarily conserved among IP_3R subtypes (fig. S3).

Inhibition of Ca^{2+} signaling and cytokine production by disruption of the SLAT- $\text{IP}_3\text{R1}$ interaction

To assess the biological relevance of the SLAT- $\text{IP}_3\text{R1}$ interaction, we designed a strategy to disrupt this interaction in T cells, which relied on a cell-permeable recombinant blocking peptide. The independent binding of both the EF-hand and PH domains of SLAT to $\text{IP}_3\text{R1}$ ruled out the feasibility of testing a SLAT-based blocking peptide. Instead, we generated a cell-permeable fusion protein by cloning the complementary DNA (cDNA) encoding $\text{IP}_3\text{R1}$ -

f2.1 (residues 346 to 490), or, as a control, the same region deleted of residues 400 to 417, into a bacterial vector (pTAT-HA) (19) encoding a hemagglutinin (HA)-tagged HIV-1 TAT protein-derived peptide (TAT-HA-2.1 and TAT-HA-2.1 18, respectively) (Fig. 6A). Efficient peptide transduction was assessed and confirmed by performing Western blotting analysis of peptide-treated CD4⁺ T cell lysates with an anti-HA antibody. Through coimmunoprecipitation analysis, we found that preincubation of T cells with the TAT-HA-2.1 peptide reduced the stimulation-induced interaction between endogenous SLAT and IP₃R1 by ~70% compared to the effect of the TAT-HA negative control protein alone. In contrast, the mutated peptide (TAT-HA-2.1 18) caused a mild, ~35% reduction in this interaction (Fig. 6B). This residual inhibitory activity might reflect a more modest contribution of other IP₃R1 residues, distinct from the 18 deleted residues, to the interaction.

We next analyzed the effects of these TAT fusion proteins on different aspects of the TCR-dependent activation of CD4⁺ T cells. Transduction of primary (ex vivo) T cells with the control, mutated TAT-IP₃R1 fusion protein had a minimal, if any, effect on the increase in [Ca²⁺]_i (Fig. 6C); however, treatment with the blocking fusion protein (TAT-HA-2.1) substantially inhibited the TCR-stimulated increase in [Ca²⁺]_i, phenocopying the reduced and delayed response observed in *Defb*^{-/-} CD4⁺ T cells (Fig. 6C), despite the cell having a normal amount of IP₃R1 (fig. S4). Furthermore, upon chelation of extracellular Ca²⁺ by EGTA, only CD4⁺ T cells pretreated with the blocking fusion protein TAT-HA-2.1 showed a defect in Ca²⁺ release from intracellular stores in response to TCR and CD28 cross-linking (fig. S5A). Consistent with our previous study showing that Ca²⁺-independent TCR signaling events are not affected in *Defb*^{-/-} T cells (12), we found that transduction of primary (ex vivo) T cells with either of the two fusion proteins did not alter the TCR-induced phosphorylation of ZAP-70, LAT, PLC-γ1, Akt, glycogen synthase kinase-3β (GSK-3β), or p38 mitogen-activated protein kinase (MAPK) (fig. S5B).

We next evaluated the effect of the IP₃R1 fragment f2.1 on TCR-induced activation of an NFAT-luciferase (Luc) reporter gene. As previously shown (12), full-length SLAT enhanced TCR-stimulated NFAT activity in Jurkat TAg cells (Fig. 6D); however, when the cells were additionally cotransfected with plasmid encoding the IP₃R1-f2.1 protein, SLAT-stimulated NFAT activity was reduced, albeit not entirely abolished (Fig. 6D). In contrast, the TCR-induced stimulation of an NF-κB reporter gene, either in the absence or presence of exogenous SLAT, was not inhibited by the IP₃R1 fragment f2.1 (fig. S5C). Last, we investigated the consequences of disrupting the SLAT-IP₃R1 interaction on interferon-γ (IFN-γ) production. The anti-CD3/CD28-stimulated production of IFN-γ by CD4⁺ T cells transduced with the TAT-HA-2.1 fusion protein was reduced by ~50 to 60% relative to that of cells transduced with the control TAT-HA protein (Fig. 6E). Cells treated with the TAT-HA-2.1 18 fusion protein also showed a weak (~15 to 20%) reduction in cytokine production, which was likely because the TAT-HA-2.1 18 protein retains a weak ability to disrupt the SLAT-IP₃R1 interaction (Fig. 6B).

DISCUSSION

During the course of T lymphocyte activation, the rise in [Ca²⁺]_i is a critical step that regulates the strength and type of immune response by controlling many biological functions

(1, 20, 21). Despite recent breakthroughs made in this area, with the identification of the ER Ca^{2+} sensor STIM1 and of the Orai1 protein as a subunit of the CRAC channel (22–24), many of the processes that regulate Ca^{2+} entry in T lymphocytes remain elusive. Here, we report a direct TCR-stimulated association between SLAT and IP₃R1, and showed that the EF-hand and PH domains of SLAT independently and directly interacted with IP₃R1. In addition, we showed that the N-terminal region of SLAT bound to Ca^{2+} , and we demonstrated that the SLAT-IP₃R1 association was Ca^{2+} -dependent (fig. S6). Last, we identified an 18-residue motif in IP₃R1 that was required for binding to SLAT, and we found that disruption of the SLAT-IP₃R1 association markedly inhibited TCR-dependent Ca^{2+} signaling and reduced cytokine production. Thus, our findings identify SLAT as a previously uncharacterized IP₃R1-interacting protein and reveal a potentially T cell-specific mechanism for IP₃R1 regulation and function. Given the predominant, almost exclusive, presence of SLAT in T lymphocytes, our results implicate SLAT as a potentially promising selective drug target for the treatment of NFAT-dependent, T cell-mediated autoimmune and inflammatory diseases.

The stimulus-induced association between SLAT and IP₃R1 was rapid and declined by ~15 min after TCR stimulation, suggesting that it is required for the early stages of intracellular Ca^{2+} release. The results of the proximity ligation and co-fractionation assays are consistent with this physical association, and demonstrated that a fraction of SLAT is present in the ER-enriched subcellular fraction. SLAT does not contain an ER retention signal motif (25), and it is unlikely that SLAT localizes in the ER lumen. Rather, it most likely interacts with the cytosolic part of IP₃R1, which represents 85% of the protein (8, 26). We also detected IP₃R1 in the membrane fraction, consistent with previous studies that showed the presence of IP₃R1 at the plasma membrane of lymphocytes, where it is present as a potentially functional Ca^{2+} channel (27–29). However, we cannot exclude the possibility that the detection of IP₃R1 in the membrane fraction was due to the coprecipitation of heavy membranes, which include, in addition to the plasma membrane, the rough ER, as well as plasma membrane-associated membranes (PAMs) and mitochondria, in which IP₃R1 has been previously detected (30, 31). Contrary to a previous study (15), we did not detect enrichment of SLAT in the membrane fraction upon TCR stimulation. This discrepancy may be because the subcellular fractionation protocol that we used differs substantially from the one originally used to document the TCR-dependent translocation of SLAT to the plasma membrane (15).

PH domains are best known for their ability to target proteins to the plasma membrane through specific phosphoinositide binding (32), but there is now growing evidence that PH domains can also serve as protein docking sites (33, 34). Indeed, we showed that the SLAT PH domain directly associated with IP₃R1, consistent with our earlier findings that, despite its reported ability to bind to PIP₂ (13), the PH domain of SLAT did not require phosphoinositide binding for its translocation to the plasma membrane or the immunological synapse or for its downstream effector functions (15).

We also identified the N-terminal region of SLAT, which contains a functional Ca^{2+} -binding EF-hand domain, as mediating the SLAT-IP₃R1 interaction. Although we cannot formally exclude the participation of the ITAM-like domain in Ca^{2+} binding, it is highly

likely that Ca^{2+} binding is mediated by the EF-hand domain because loss of both of the putative Ca^{2+} -binding sites greatly reduced the extent of Ca^{2+} binding to SLAT. These Ca^{2+} -binding motifs share 63 and 68% homology with a consensus EF-hand domain sequence (14, 35), and it was previously suggested that because of the mutation of a single residue in these motifs relative to the consensus sequence, the SLAT EF-hand domain may have a low affinity for Ca^{2+} (14). The region of SLAT that contains the EF-hand domain (residues 1 to 72) is the region that is most conserved between SLAT and its SWAP-70 homolog, which suggests that this region performs an important function (9). Together with IP_3 , Ca^{2+} binding to IP_3Rs is a major regulatory feature of IP_3R channel activity, and several Ca^{2+} -binding sites have been identified in the protein (36). One Ca^{2+} -binding site of $\text{IP}_3\text{R1}$ is localized in an N-terminal region encompassing residues 378 to 450 (37), including the 18 residues (400 to 417) that we found to be critical for the interaction with SLAT. It is therefore tempting to speculate that SLAT may function as a Ca^{2+} sensor through its N-terminal Ca^{2+} -binding region and thereby promote $\text{IP}_3\text{R1}$ activation by facilitating the binding of Ca^{2+} to the channel. Alternatively, SLAT may compete with other Ca^{2+} -binding proteins that bind to the IP_3R and thus inhibit its channel activity (5).

Our previous work showed that the isolated PH-DH tandem domains were inactive in T cells and, furthermore, that deletion of the SLAT N-terminal region (containing the EF-hand domain) did not impair its biological activities (15). As an extension of this result, we now demonstrate that only the simultaneous deletion of both the EF-hand and PH domains markedly reduced the ability of SLAT to enhance NFAT activation. On the other hand, the importance of the ITAM-like motif that is localized between the EF-hand and PH domains of SLAT for its proper localization and downstream functions (15) raises the possibility that, upon TCR stimulation, phosphorylation of this ITAM-like motif induces a conformational change that renders the EF-hand and PH domains accessible for interaction with $\text{IP}_3\text{R1}$.

The catalytic DH domain of SLAT was not required for its association with $\text{IP}_3\text{R1}$. Nevertheless, forced membrane targeting of the SLAT DH domain to the plasma membrane was sufficient to restore NFAT activation and T helper 1 (T_H1)– or T_H2 -type differentiation in *Defb^{-/-}* T cells and, furthermore, a constitutively active mutant of Cdc42 (which is a target of the GEF activity of SLAT) restored Ca^{2+} flux in *Defb^{-/-}* T cells (15). These observations imply a role for the GEF activity of SLAT in Ca^{2+} signaling (15). The regulatory interplay between Ca^{2+} signaling and rearrangement of the actin cytoskeleton is essential to sustain and amplify T cell activation and functions (38). Thus, our previous (15) and current findings raise the possibility that SLAT regulates Ca^{2+} signaling in T cells through two independent pathways: one involving the interaction between SLAT and $\text{IP}_3\text{R1}$ through its EF-hand and PH domains, and the other involving its DH domain–mediated GEF activity.

More work is required to gain a full understanding of the mechanism through which SLAT regulates Ca^{2+} signaling in T cells. For example, future elucidation of the crystal structure of the critical $\text{IP}_3\text{R1}$ -binding domains of SLAT, either alone or in combination with the corresponding region of $\text{IP}_3\text{R1}$, might provide critical information that could be used to specifically target this interaction. Such studies will likely enable us to evaluate the effect of disrupting the SLAT- $\text{IP}_3\text{R1}$ interaction in animal models of T cell–mediated autoimmune

and inflammatory diseases. Furthermore, such studies may facilitate the design of new inhibitors, which could act selectively to inhibit Ca^{2+} signaling in T cells without (or only minimally) affecting other TCR signaling pathways.

MATERIALS AND METHODS

Mice

C57BL/6 (B6) mice and *Def6*^{-/-} mice on a B6 background (12) were housed and manipulated according to a protocol approved by the La Jolla Institute for Allergy and Immunology Animal Care Committee and in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care International.

Expression vectors

Plasmids encoding Myc-tagged SLAT or its mutants were generated by polymerase chain reaction (PCR) and subcloned into the pEF-Myc-His A/C mammalian vectors (Life Technologies) to encode an in-frame Myc tag epitope downstream of the insert, or into the pET28a+ bacterial vector (Merck Millipore) to generate an expressed protein with N- and C-terminal His tags. cDNAs encoding the various GST-SLAT fusion proteins were generated by PCR and subcloned into the pGEX-4T1 vector to encode an N-terminal GST fusion protein. The GST EF-ITAM 19–30 and EF-ITAM 19–30+ 57–68 vectors were generated by mutagenesis from the GST EF-ITAM template with the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer's instructions. The plasmid encoding FLAG-IP₃R1 was previously described (39) and was provided by A. R. Marks (Columbia University, New York). cDNAs encoding IP₃R1 fragments f1 to f6 fused to GST at their N termini subcloned into the pGEX-6p2 plasmid, as well as IP₃R1 Lbs-1 His (residues 1 to 581), were described previously (40, 41) and were provided by H. de Smedt (Catholic University, Leuven, Belgium). cDNAs encoding other IP₃R1 fragments were constructed by PCR and subcloned into the pGEX-4T2 plasmid (GE Healthcare Life Sciences). cDNAs encoding IP₃R1 fragments f2.1 or f2.1 18 were generated by PCR, cloned into the pTAT-HA bacterial vector, and produced as previously described (19).

Cell purification, culture, and stimulation

After red blood cells were lysed, splenic CD4⁺ T cells were isolated from peripheral blood by positive selection with anti-mouse CD4 magnetic particles (BD Biosciences) or by negative selection with a CD4⁺ T Cell Isolation Kit II (Miltenyi Biotec). T cells were cultured in RPMI 1640 (Life Technologies) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 1 mM sodium pyruvate, 10 mM Hepes, 1 mM MEM nonessential amino acid solution, and penicillin G and streptomycin (100 U/ml each; Life Technologies). For short stimulations (immunoprecipitation experiments, GST pull-downs, and subcellular fractionations), 5×10^6 to 15×10^6 T cells resuspended in serum-free medium were incubated for 30 min on ice with anti-mouse CD3 ϵ mAb (20 $\mu\text{g/ml}$; 145-2C11, Bio-Legend) and anti-CD28 mAb (20 $\mu\text{g/ml}$; 37.51, BioLegend), followed by cross-linking with goat anti-hamster IgG (Thermo Scientific) for the indicated times at 37°C with gentle shaking. To generate T cell blasts, purified T cells were stimulated with plate-bound anti-CD3 (5 $\mu\text{g/ml}$) and soluble anti-CD28 (2.5 $\mu\text{g/ml}$) mAbs in the presence of human recombinant IL-2 (100

U/ml; PeproTech) for 48 to 72 hours. The mouse MCC-specific T cell hybridoma (MCC-T) (17) was cultured and stimulated as described earlier, and the human Jurkat T cell line derivatives JA16 (42) or Jurkat TAg cells (the latter stably expressing simian virus 40 large T antigen) were cultured and stimulated with an anti-human CD3 mAb (OKT3; BioXCell) followed by cross-linking with goat anti-mouse IgG (Thermo Scientific).

Immunoprecipitations

Cells were stimulated as described earlier and were lysed in 1% NP-40, 150 mM NaCl, 50 mM tris (pH 7.4), 10 mM NaF, 1 mM phenylmethyl-sulfonyl fluoride (PMSF), aprotinin (10 µg/ml), and leupeptin (10 µg/ml). Lysates were collected after centrifugation at 13,000g for 10 min. Immunoprecipitation was performed by adding the antibodies indicated in the figure legends with 30 µl of protein G–Sepharose (GE Healthcare Life Sciences) and incubating the lysates overnight at 4°C. Samples were washed four times with lysis buffer, and the immunoprecipitates were dissolved in 1× Laemmli buffer, subjected to SDS-PAGE, transferred onto a nitro-cellulose membrane, and analyzed by Western blotting with an anti-FLAG M2 mAb (F3165, Sigma), anti-Myc antibody (sc-40, Santa Cruz Bio-technology Inc.), or anti-Xpress antibody (R910-25, Life Technologies). To assess the effect of the TAT fusion proteins on the SLAT-IP₃R1 interaction, cells were prestimulated for 72 hours, rested overnight in medium containing IL-2 (20 U/ml), and then cross-linked with anti-TCR/CD28 mAbs after a 1.5-hour incubation with the indicated TAT fusion proteins in RPMI 1640 containing 0.5% FBS at 37°C.

Proximity ligation assay

Proximity between SLAT and IP₃R1 was analyzed with the Duolink proximity ligation assay according to the manufacturer's instructions (Olink Bioscience). Briefly, B6 CD4⁺ T cell blasts were generated for 3 days, rested overnight, and then seeded for 15 min on coverslips coated with anti-CD3 and anti-CD28 mAbs before being fixed and then permeabilized with saponin. T cells from wild-type mice were stained with mouse anti-IP₃R1 mAb [clone L24/18; UC Davis/National Institutes of Health (NIH) NeuroMab Facility] and rabbit anti-SLAT antibody (9). Negative controls included *Def6*^{-/-} T cells stained with a combination of anti-SLAT and anti-IP₃R1 antibodies or wild-type T cells stained only with the anti-SLAT antibody. Generated fluorescence spots were counted, and the average number of spots per cell was determined.

GST pull-downs

GST fusion proteins were batch-purified as previously described (43) and dialyzed overnight in phosphate-buffered saline containing 10% glycerol. Briefly, GST fusion proteins were immobilized on glutathione–Sepharose 4B beads (GE Healthcare Life Sciences) at 4°C for 1 hour in TEN buffer [20 mM tris (pH 7.4), 0.1 mM EDTA, 100 mM NaCl], washed four times with TEN buffer to remove unbound material, and incubated with pre-cleared whole-cell extracts of stimulated Jurkat TAg cells or MCC-T cells at 4°C for 1 hour. Beads were washed four times with 0.5% NP-40, 20 mM tris (pH 7.4), 0.1 mM EDTA, 300 mM NaCl, and the eluted proteins were resolved by SDS-PAGE and analyzed by Western blotting with anti-GST (sc-138) and anti-His mAbs (sc-8036; both from Santa Cruz Biotechnology Inc.).

To assess the direct interaction between SLAT and IP₃R1, His-SLAT fusion protein (10 µg) was incubated with immobilized GST-IP₃R1 fusion proteins for 1 hour at 4°C in 1% NP-40, 150 mM NaCl, 50 mM tris (pH 7.4), 10 mM NaF, 1 mM PMSF, aprotinin (10 µg/ml), and leupeptin (10 µg/ml), washed four times in the same buffer, and eluted, before being resolved by SDS-PAGE and analyzed by Western blotting.

Subcellular fractionation

MCC-T hybridoma cells (50×10^6) were stimulated as described earlier with anti-CD3 and anti-CD28 mAbs for 2 min at 37°C with gentle shaking, and resuspended in ice-cold buffer (0.25 M sucrose, 1 mM EGTA, 3 mM imidazole). Cells were disrupted by sonication and then homogenized with a 27-gauge needle. Nuclei were pelleted by centrifugation at 800g for 5 min, and supernatants were centrifuged at 1450g for 10 min at 4°C to obtain the membrane fraction (P1). Centrifugation at 17,000g for 10 min at 4°C was performed to obtain the organelle fraction (containing mitochondria, lysosomes, endosomes, and peroxisomes), which we refer to as the P2 fraction. A final centrifugation at 100,000g for 1 hour at 4°C was performed to separate the ER and Golgi (P3) from the cytosolic fraction (SN3). Fractions were resolved on a 4 to 12% gradient gel (Life Technologies) and were analyzed by Western blotting with anti- α 1 sodium-potassium adenosine triphosphatase (ab7671, Abcam) or anti-p38 (#9212, Cell Signaling Technology) antibodies.

Ca²⁺ overlay assay

Purified GST-SLAT recombinant proteins were resolved by 12% SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane. The blot was washed three times for 30 min with binding buffer [60 mM KCl, 5 mM MgCl₂, 10 mM imidazole-HCl (pH 6.8)]. The overlay assay was performed by incubating the blot with ⁴⁵CaCl₂ (5 µCi/ml; Perkin Elmer) for 1 hour at room temperature with gentle shaking, followed by three 5-min washes in distilled water. After autoradiography, the blot was stained with Ponceau S solution to detect proteins.

Intracellular Ca²⁺ measurements

Purified B6 CD4⁺ T cells were incubated with 150 nM of the indicated TAT fusion proteins for 1.5 hours in RPMI 1640, 0.5% FBS at 37°C and then were loaded with indo1-AM (2 µg/ml; I1226, Invitrogen Molecular Probes) in cell loading Hanks' balanced salt solution (HBSS) medium supplemented with CaCl₂ and MgCl₂ (Life Technologies) in the presence of 4 mM probenecid for 45 min at 37°C in the dark. Loaded cells were washed twice with cell loading medium and incubated with anti-CD3 (10 µg/ml) and anti-CD28 (2.5 µg/ml) mAbs for 30 min at room temperature. Unbound antibodies were removed by centrifugation, and cells were then resuspended in cell loading medium to determine baseline [Ca²⁺]_i. A cross-linking goat anti-hamster antibody (10 µg/ml) was then added. To assess Ca²⁺ release from intracellular stores, cells were washed in HBSS (Life Technologies) and resuspended in HBSS containing 1 mM EGTA to chelate extracellular Ca²⁺. Cells were analyzed by reading the emission at 500 nm (FL4 channel) and 460 nm (FL5 channel) on an LSRII instrument (BD) and calculating the FL5/FL4 emission ratio.

Luciferase reporter assays

Jurkat TAg or JA16 cells (20×10^6) were cotransfected with empty pEF plasmid (10 μ g) (empty vector) or with pEF encoding Myc-tagged SLAT mutants, with or without Xpress-tagged IP₃R1-f2.1, together with NFAT-Luc or NF- κ B-Luc (5 μ g each) and β -Gal (5 μ g) reporter genes. Cells were left unstimulated or were stimulated with anti-CD3 (OKT3) mAb for 6 hours at 37°C. Normalized luciferase activity was determined in duplicate samples, and graphs show the means $\pm$ SD. The presence of exogenous protein was determined by Western blotting analysis.

IFN- γ measurement

Purified B6 CD4⁺ T cells were preincubated for 2 hours at 37°C with 150 nM of the appropriate TAT fusion proteins in RPMI 1640, 0.5% FBS. The cells were washed and resuspended in RPMI 1640, 10% FBS containing anti-CD28 mAb (2.5 μ g/ml) and 150 nM fresh TAT fusion protein. Cells (2×10^5 per well) were stimulated at 37°C in 96-well plates coated with anti-CD3 mAb (2.5 μ g/ml) plus soluble anti-CD28 mAb (2.5 μ g/ml) for 48 hours. Fresh TAT proteins (150 nM) in RPMI 1640 (100 μ l) were added to the cells after 12 and 24 hours. Supernatants were collected after 48 hours, and ELISAs were performed to measure IFN- γ concentrations.

Statistics

Statistical significance was analyzed by two-tailed Student's *t* test. Unless otherwise indicated, data represent means $\pm$ SD, with *P* < 0.05 considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. SLAT physically interacts with IP₃R1 in T cells

(A) Murine CD4⁺ T cells were stimulated with anti-CD3 and anti-CD28 antibodies for the indicated times. Samples were subjected to immunoprecipitation (IP) with an anti-SLAT antibody, resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE), and analyzed by Western blotting with antibodies against the indicated proteins. An immunoprecipitation with normal immunoglobulin G (IgG) was used as a negative control. (B) CD4⁺ T cells from wild-type (WT) or *Def6*^{-/-} mice were adhered to coverslips coated with anti-CD3 and anti-CD28 antibodies and were stained with the indicated combinations of anti-SLAT antibody (α -SLAT Ab) and anti-IP₃R1 antibody (α -IP₃R1 Ab). The proximity of the molecules stained with the pair of antibodies was then assessed with the Duolink technology. (C)

Analysis of data from the proximity assays shown in (B). The mean numbers of spots per cell (an indicator of molecular proximity) in different microscopy fields $\pm$ SD are plotted. Each dot represents a single cell. A total of 80 cells for the WT and *Def6*^{-/-} conditions (anti-SLAT and anti-IP₃R1 antibodies) and a total of 49 cells for the WT (anti-SLAT antibody alone) were analyzed. *** $P < 0.0001$ for WT versus *Def6*^{-/-} T cells (anti-SLAT and anti-IP₃R1 antibodies) and for WT versus WT stained with anti-SLAT antibody alone. (D) Subcellular fractionation of MCC-T cells. ER, cytosolic (C), and membrane (M) fractions from nonstimulated MCC-T cells (NS) or MCC-T cells stimulated with anti-CD3 and CD28 monoclonal antibodies (mAbs) were analyzed by Western blotting with antibodies against the indicated proteins. Data are representative of three (A and D) or two (B and C) independent experiments.

Fig. 2. Mapping of the SLAT-binding region of IP₃R1

(A) Schematic representation of the functional domains of IP₃R1 (without the channel pore). The six fragments (f1 to f6) used as initial baits in GST pull-down experiments, as well as the smaller fragments derived from f2, are indicated together with the numbering of the corresponding amino acid residues. (B to D) Lysates from MCC-T cells stimulated with anti-CD3 and anti-CD28 antibodies were precleared and incubated with the indicated glutathione-bound GST-IP₃R1 fusion proteins. GST was used as a negative control. Eluted proteins were resolved by SDS-PAGE and analyzed by Western blotting with antibodies against SLAT and GST. Arrowheads indicate the positions of the GST-IP₃R1 fusion proteins. The positions of molecular mass markers (in kilo-daltons) are indicated to the right of the Western blots. (E) Recombinant His-SLAT protein was incubated with the indicated immobilized GST-IP₃R1 fusion proteins (or with GST protein as a negative control), and samples were analyzed as described for (B) to (D). Eluted proteins were subjected to SDS-PAGE and Western blotting analysis with antibodies against SLAT and GST. Data in (B) to (D) are representative of at least three independent experiments.

Fig. 3. Mapping of the IP₃R1-binding domains of SLAT

(A) Schematic representation of SLAT functional domains, SLAT mutants, and Lbs-1 used in immunoprecipitation experiments. (B) Jurkat TAg cells cotransfected with plasmids encoding the indicated Myc-tagged SLAT mutants or with empty vector (EV) together with plasmid encoding FLAG-tagged IP₃R1 were lysed. Whole-cell lysates (WCL) were subjected to immunoprecipitation (IP) with anti-Myc antibody (left) or were left untreated (right). All samples were then analyzed by Western blotting with antibodies against the indicated tags. Arrowheads indicate the positions of Myc-tagged SLAT proteins. (C) Jurkat TAg cells cotransfected with plasmids encoding the indicated Myc-tagged SLAT domains together with plasmid encoding FLAG-tagged IP₃R1-f2.1 were analyzed as described for (B). Ca²⁺ binding to the EF-ITAM protein was assigned a value of 1 (top) and 100% (bottom). In (B) and (C), Jurkat TAg cells were stimulated for 2 min at 37°C before being lysed. (D) The indicated immobilized recombinant GST-SLAT proteins (or GST alone as a negative control) were incubated with recombinant IP₃R1 Lbs-1 His, and eluted proteins were analyzed as described in Fig. 2E. (E) Jurkat TAg cells were cotransfected with empty vector or with plasmids encoding the indicated SLAT proteins together with NFAT-Luc and β-galactosidase (β-Gal) reporter plasmids. Cells were left unstimulated (–) or were stimulated with an anti-CD3 mAb (CD3) for 6 hours at 37°C. Left: Normalized luciferase activity was determined in duplicates in each experiment, and bars show means ± SD from three independent experiments. Luciferase activity, in relative light units (RLU), for cells expressing WT SLAT was assigned a value of 100%. Right: Expression of exogenous SLAT proteins was detected by Western blotting with an anti-Myc antibody. The Western blot is representative of three independent experiments. **P* < 0.03, SLAT versus EF PH; NS, nonsignificant. Data in (B) to (E) are representative of at least three independent experiments.

Fig. 4. Ca^{2+} binding to SLAT and the Ca^{2+} dependence of the SLAT-IP₃R1 interaction
(A) Schematic representation of the SLAT GST-EF-ITAM recombinant proteins used in the Ca^{2+} overlay assay. **(B and C)** The indicated GST fusion proteins were transferred onto a nitro-cellulose membrane, which was incubated with $^{45}\text{CaCl}_2$, washed, and subjected to autoradiography (top), followed by Ponceau staining (bottom). Numbers above the autoradiogram in (C) indicate values from densitometric analysis of the bands shown. The bar graph in (C) shows pooled densitometry data from two independent experiments. **(D)** Jurkat TAg cells were left unstimulated (NS) or were stimulated with anti-CD3 antibody (α -CD3). Cell lysates were incubated in vitro with GST (negative control) or a GST-IP₃R1-f2 fusion protein in the absence (–) or presence of either EGTA or CaCl_2 (2 mM each). The binding of SLAT in the absence of CaCl_2 or EGTA was assessed in 1% NP-40 lysis buffer [150 mM NaCl, 50 mM tris-HCl (pH 7.4)]. Numbers above the blots indicate the relative SLAT binding to IP₃R1, as determined by densitometry. Bar graphs showing densitometry data pooled from all experiments are shown below the Western blots. Data are representative of at least three (B and D) and two (C) independent experiments.

Fig. 5. Mapping of a minimal SLAT-binding motif in IP₃R1

(A) Schematic representation of the IP₃R1 fragments used as GST fusion proteins. (B) MCC-T cells stimulated with anti-CD3 and anti-CD28 antibodies were lysed, and cell lysates were incubated in vitro with the indicated deletion mutants of GST-IP₃R1-f.A protein. Bound proteins were resolved by SDS-PAGE and analyzed by Western blotting with anti-SLAT (top) and anti-GST (bottom) antibodies. (C) Schematic representation of the IP₃R1 fragments f2.1 and f2.1-18. (D) MCC-T cells were stimulated and lysed as described for (B), and cell lysates were incubated in vitro with the IP₃R1 fragments shown in (C). Data in (B) and (D) are representative of at least three independent experiments.

Fig. 6. Effect of disrupting the SLAT-IP₃R1 interaction on T cell functions

(A) Schematic representation of the TAT-HA fusion proteins. (B) Murine CD4⁺ T cells preincubated with the indicated TAT-HA proteins were lysed and left untreated or were subjected to immuno-precipitation with anti-SLAT antibody. Samples were then analyzed by Western blotting with anti-IP₃R1 and anti-SLAT antibodies. Numbers above the IP₃R1 blot indicate the relative SLAT binding to IP₃R1 as determined by densitometry. Right: Bar graph shows densitometry data pooled from three independent experiments. (C) [Ca²⁺]_i recordings of murine CD4⁺ T cells preincubated with the indicated TAT-HA proteins. (D) Jurkat TAG cells cotransfected with plasmid encoding Myc-tagged SLAT in the absence or presence of plasmid encoding Xpress-tagged IP₃R1-f2.1 together with NFAT-Luc and β-Gal reporter plasmids were left unstimulated or were stimulated with an anti-CD3 mAb. Left: Normalized luciferase activity was determined in duplicates in each experiment, and bars show means ± SD from three independent experiments. Luciferase activity, in relative light units (RLU), in cells transfected with plasmid encoding SLAT and with empty vector (EV) was assigned a value of 100%. Right: Expression of exogenous SLAT and IP₃R1-f2.1 proteins was determined by Western blotting. The Western blot is representative of three independent experiments. ****P* < 0.001, SLAT versus SLAT+TAT-HA 2.1. (E) Murine CD4⁺ T cells preincubated with TAT proteins were stimulated with anti-CD3 and anti-CD28 antibodies. Culture medium was collected after 48 hours, and the amounts of secreted IFN-γ

were determined by enzyme-linked immunosorbent assay (ELISA). $*P < 0.05$, WT+TAT-HA 2.1 versus WT+TAT-HA 18; $**P < 0.002$, WT+TAT-HA versus WT+TAT-HA 2.1; $***P < 0.0001$, WT+TAT-HA versus *Defb*^{-/-}. Data are representative of three independent experiments.