Roles of the Proline-rich Domain in SLP-76 Subcellular Localization and T Cell Function*§

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The adaptor protein Src homology (SH)2 domain-containing and leukocyte-specific phosphoprotein of 76 kDa (SLP-76) is critical for signal transduction in multiple hematopoietic lineages. It links proximal and distal T cell receptor signaling events through its function as a molecular scaffold in the assembly of multimolecular signaling complexes. Here we studied the functional roles of sub-domains within the SLP-76 proline-rich region, specifically the Gads binding domain and the recently defined P1 domain. To gain a further understanding of the functions mediated by this region, we used three complementary approaches as follows: reconstitution of SLP-76-deficient cells with functional domain deletion mutants, blocking molecular associations through the expression of a dominant negative protein fragment, and directed localization of SLP-76 to assess the role of the domains in SLP-76 recruitment. We find the Gads binding domain and the P1 domain are both necessary for optimal SLP-76 function, and in the absence of these two regions, SLP-76 is functionally inert. Furthermore, we provide direct evidence that SLP-76 localization and, in turn, function are dependent upon association with Gads.

Adaptor molecules, proteins containing modular binding domains and without intrinsic enzymatic activity, serve as essential scaffolds for the construction of multimolecular protein complexes and are indispensable for signal transduction in a variety of cell lineages. The adaptor molecule Src homology (SH)2 domain-containing and leukocyte-specific phosphoprotein of 76 kDa (SLP-76) is expressed in T cells, NK cells, macrophages, mast cells, platelets, and neutrophils (1–5). It is necessary for thymocyte development (6, 7), signaling through the T cell receptor (TCR) in mature T cells (8), FcɛR-dependent mast cell activation (9), platelet activation downstream of the collagen receptor, GPVI (5, 10), and appropriate developmental separation of the vascular and lymphatic circulatory systems (11). SLP-76-deficient mice demonstrate a complete block in thymic development at the CD4/CD8 double negative stage, with an absence of peripheral T cells (6, 7). SLP-76-deficient Jurkat T cells (J14) are grossly impaired in signaling through the TCR with defects in phospholipase C-1 (PLCγ1) activation, calcium flux, mitogen-activated protein kinase (MAPK) pathway activation, and interleukin-2 production (8).

Complementary overexpression and reconstitution studies have identified domains of SLP-76 critical to its function. The amino terminus of SLP-76 contains three tyrosine residues that, upon phosphorylation by the Syk family kinase ZAP-70 (12), bind to molecules containing SH2 domains including Vav (a guanine nucleotide exchange factor) (13–16), Nck (an adaptor molecule) (17), and Itk (a Tec family protein tyrosine kinase) (18, 19). Its central, proline-rich region is constitutively bound to Gads, a homologue of the adaptor molecule Grb2 (20–24). This high affinity interaction occurs through association of the carboxyl-terminal SH3 domain of Gads with a novel SH3 domain binding motif (RXXK) found in SLP-76 (25). Direct association of the PLCγ1 SH3 domain with a portion of the SLP-76 proline-rich region (P1 domain) has also been described recently (26). The carboxyl terminus of SLP-76 contains an SH2 domain that can bind the phosphorylated tyrosine residues of adhesion and degranulation-promoting protein (ADAP) (27, 28) and the serine-threonine kinase HKP-1 (29) after TCR engagement.

A model for the creation of an adaptor-nucleated signaling complex following TCR engagement has emerged. Linker of activated T cells (LAT), a transmembrane adaptor crucial for T cell development and function (30, 31), is phosphorylated by ZAP-70 (32) allowing for the recruitment and assembly of signaling molecules at the cell membrane. Two conserved cysteines near the LAT transmembrane domain are palmitoylated and target LAT and its binding partners to lipid rafts (GEMs), detergent-insoluble membrane fractions enriched in key signaling molecules. Recruitment by these two modified cysteines has been shown to be necessary for LAT function (33). Upon phosphorylation, SLP-76 recruits Vav, Nck, and Itk. This complex, which is constitutively associated with Gads, in turn is translocated from the cytosol to lipid raft microdomains through the interaction of the SH2 domain of Gads with phosphorylated tyrosine residues of LAT (20). PLCγ1, also recruited to lipid rafts after TCR engagement through phosphorylation-dependent association with LAT (33), is activated with resultant cleavage of phosphatidylinositol 4,5-bisphosphate and ac-
tivation of the phosphatidylinositol second messenger pathway (34).

This model of SLP-76 recruitment by LAT and Gads is supported by the observation that signaling via the TCR is rescued in LAT-deficient Jurkat T cells by membrane targeting of SLP-76 (35). There are a number of studies that when taken together suggest our understanding of the role of the SLP-76 proline-rich region is incomplete. First, it has been observed that Gads-deficient mice manifest a less severe T cell phenotype than the mice in either SLP-76 or LAT-deficient mice (36). In addition, transgenic reconstitution of SLP-76-deficient mice with a Gads-binding mutant at least partially rescues T cell development and function (37, 38). Finally, there have been reports of other molecules that can associate with the SLP-76 proline-rich region including, most recently, the direct SLP-76/LAT interaction (26).

In this study we utilize biochemical, genetic, and imaging techniques in a Jurkat T cell model to define further the functional roles of the sub-domains of the SLP-76 proline-rich region. Through reconstitution of SLP-76-deficient Jurkat T cells with wild-type (WT), mutant, membrane-targeted, and dominant negative forms of SLP-76, we show the primary role of the SLP-76/Gads interaction to be SLP-76 recruitment. Association of SLP-76 with PLCγ1 and perhaps other molecules at the P1 domain is also critical for optimal signaling but not solely a result of effects on SLP-76 localization.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—Wild-type SLP-76 cDNA was obtained from murine splenic RNA by reverse transcriptase-PCR and was then subcloned into EGFP-C1 at the XhoI and EcoRI sites (Clontech). The cDNA encoding a-amino-terminally tagged WT SLP-76 was then digested with MfeI and Sall, digested at its 5′ end, and ligated into the MgiR1 vector (a gift from W. Pear, University of Pennsylvania) (39) between HpaI and Sall. The P1, G2, and P1/G2 domain mutations of SLP-76 were then created using Transformer Site-directed Mutagenesis (Clontech). The bases encoding amino acids 157–222 of SLP-76 were deleted in the creation of the P1 mutant. The G2 mutation represents replacement of amino acids 237 and 240 of SLP-76, arginine, and lysine, respectively, with alanine residues. The P1/G2 mutant carries both the domain deletion and amino acid substitutions. For creation of the LAT/SLP-76 fusion, the complete LAT cDNA was obtained from murine splenic RNA by reverse transcriptase-PCR. A partial cDNA encoding the 35 amino acids of LAT, flanked by BamHI and SalI sites, was amplified by PCR and subcloned into the BglII site of MgiR1. The fusion was created by insertion of the full-length SLP-76 cDNA at the BglII and EcoRI sites. The P1 and G2 mutations were then created using Transformer Site-directed Mutagenesis (Clontech). The cDNA encoding the Gads-binding fragment (GBF), amino acids 205–254 of SLP-76, was generated by PCR from a WT SLP-76 plasmid template and then subcloned into DSRed2-C1 at the BglII and HindIII sites (Clontech). All constructs were sequenced for fidelity.

Cell Lines, Transfections, and Cell Culture—E6-1 Jurkat T cells and SLP-76-deficient Jurkat T cells (J14) were the gifts from A. Weiss (University of California, San Francisco). All cells were maintained in RPMI 1640 media with 10% fetal calf serum, penicillin (1,000 units/ml), streptomycin (1,000 units/ml), and glutamine (20 m) in a 5% CO2 humidified atmosphere at 37 °C, as described previously (40). Additionally, stable Jurkat derivatives, expressing WT and mutant forms of SLP-76, were created using the MgiR1 and DSRed2-C1 vectors described above. Cells were electroporated using a Gene Pulser (Bio-Rad) at a setting of 250 V and 975 microfarads, in cuvettes containing 2 × 107 cells in 0.4 ml of serum-free RPMI 1640 and 20 μg of linearized plasmid. After 7 days in culture, cells were sorted for GFP and/or DSRed2 expression by a Cytomation MoFlo (Cytomation, Inc.). After an additional 2 weeks in culture, cells were again sorted to obtain stable transfectants with matched fluorescence. Similar levels of surface TCR expression were demonstrated.

Antibodies—The monoclonal antibody C305 (41) and UCHT1 (Pharmingen) were used for TCR stimulation. For immunoprecipitation and Western biochemistry the following antibodies were used: BD Living Colors A.v. peptide antibody, BD Living Colors DSRed monoclonal antibody (Clontech); anti-SOS, anti-Gab2, and anti-Gads (Upstate Biotechnology, Inc.); and anti-Grb2 (Transduction Laboratories). Sheep polyclonal anti-SLP-76 was prepared as described previously (40).

Immunoprecipitations and Western Blots—Cells were lysed in Nonidet P-40 lysis buffer containing a 1:100 dilution of a protease inhibitor mixture (Sigma), 1 mM phenylmethanesulfonyl fluoride, and the following protein phosphatase inhibitors: 400 μM sodium vanadate, 10 μM sodium fluoride, and 10 μM sodium pyrophosphate. Lysates were resolved by SDS-PAGE for examination of the indicated proteins or were subjected to immunoprecipitation. For either immunoprecipitation, 4 μg of antibody were conjugated to GammaBind Plus-Sepharose Beads (Amersham Biosciences) for 12 h; lysates were tumbled with the antibody-conjugated beads for 4 h at 4 °C; and the immune complexes were washed three times with Nonidet P-40 lysis buffer, subjected to SDS-PAGE, and transferred to nitrocellulose for immunoblot analysis using the indicated antibodies.

Isolation of GEM Fractions—1 × 106 cells were resuspended in 1 ml of phosphate-buffered saline and rested at 37 °C, 5% CO2, for 30 min. Cells were lysed at 4 °C for 10 min and resuspended after 5 min in 1 ml of MES-buffered saline (25 mM MES, pH 6.5, 150 mM NaCl) containing 1% Triton X-100 and protease and phosphatase inhibitors as described above. The lysates were then mixed with 1 ml of 80% sucrose in MES-buffered saline and transferred to ultracentrifuge tubes. The supernatant was overlaid with 2 ml of 30% sucrose in MES-buffered saline, followed by 1 ml of 5% sucrose in MES-buffered saline, all at 4 °C. The Tritron-insoluble fractions were separated from the cell lysates by ultracentrifugation for 18 h at 44,000 rpm in a Beckman SW55Ti rotor at 4 °C (no break). Fractions (350–μl) were removed sequentially starting from the top of the gradient.

Measurement of T Cell Activation Markers—Cells at 5 × 106/ml were cultured for 18 h in wells containing media alone, media containing 20 ng/ml PMA, or in media containing wells that had earlier been coated with C305 antibody. To prepare antibody-coated wells, C305 ascites was diluted in phosphate-buffered saline at 1:500, allowed to incubate at 37 °C for 4 h in 48-well dishes, and aspirated prior to transfer of cells for stimulation. After overnight culture, cells were stained with APC-anti-CD69 (Pharmingen), and surface expression of activation markers was analyzed on a FACSCalibur cytomter (BD Biosciences).

Calcium Flux—Intracellular free Ca2+ measurement was performed as described previously (42). Briefly, cells were loaded with 2 μg/ml of the ratiometric dye Indo-1 at 37 °C for 30 min. Base-line Ca2+ levels were measured for 30 s after which time cells were stimulated with C305 antibody. Calcium mobilization was detected by flow cytometry on a LSR Benchtop Flow Cytometer (BD Biosciences). Data were analyzed using FlowJo software (Tree Star Inc.), and histograms represent the average ratio of FL1/FL2 fluorescence over time. Roughly 500–1000 events were collected per sample.

Luciferase Assay—2 × 106 cells were transfected with 25 μg of the NF-AT reporter construct (a gift from G. Crabtree, Stanford University), using a Gene Pulser (Bio-Rad) as described above. After 24 h, 5 × 105 cells/well were placed into 96-well dishes and cultured for 6 h in media containing C305 antibody (1:10,000), C305 antibody (1:10,000) plus 20 ng/ml PMA, 20 ng/ml PMA plus 1 μM ionomycin, or were left unstimulated. Luciferase activity was assessed as per the manufacturer’s instructions (Promega). To standardize for transfection efficiency, luciferase light units were normalized to values obtained upon treatment with PMA plus ionomycin.

Cellular Imaging—As described previously, the formation and translocation of signaling complexes in Jurkat variant T cells were monitored using a modified T cell spreading assay (43). Briefly, four-chambered cover glasses (Lab-Tek II, Nunc/Nalgene) were coated with UCHT1, C305, and co-loaded with sensitized and B2a-deficient Jurkat T cells and then incubated with 25 μM Hesper. To initiate an assay, 2 μl of cells at 2 × 106/ml were injected into the chambers immediately above the apera- ture of the objective. Jurkat variants were visualized using a PerkinElmer Life Sciences Ultraview spinning wheel confocal system equipped with an Orca-ER II CCD camera (Hamamatsu) standard fluorescein isothiocyanate and TRITC filter sets suitable for the detection of GFP and DSRed2, respectively. These live culture movies were illuminated with the 488- and 568-nm laser lines from a krypton/argon laser. Live images were collected as vertical Z-stacks and then sub-sampled for the plane of the coverslip. Images were collected using a 63× Plan-Apochromat objective (Carl Zeiss). The temperature of the sample was maintained at 37 °C by using a hot air blower (Nertek) and an objective heater (Bioptechs).
The three functional domains of SLP-76 are shown with their binding partners. Phosphorylatable tyrosines are present at amino acids 112, 128, and 145.

Fig. 2. SLP-76 expression constructs. A, schematic of WT and mutated GFP-tagged SLP-76. For the G2 mutation, asterisks represent replacement of amino acids 237 and 240 of SLP-76, argueine and lysine, respectively, with alanine residues. P1 mutation represents deletion of SLP-76 amino acids 157–222. B, expression of the SLP-76 fusion constructs in stably transfected J14 cells was determined by fluorescence-activated cell sorter analysis. C, expression of the SLP-76 fusion constructs was determined by examining lysates from 0.5 × 10⁶ cell equivalents per lane by immunoblot (WB) analysis with anti-GFP antibody.

RESULTS

Expression of SLP-76 Proteins with Mutations in the Gads and/or PLCγ1 Binding Domains—Previous studies revealed multiple domains within SLP-76 that mediate protein/protein interactions (Fig. 1). The Gads binding domain was initially mapped to a 20-amino acid stretch within the central region of SLP-76 (Δ20 region) (24). A minimal Gads-binding region of SLP-76 (G2 domain) has now been identified; by using peptide arrays, it was determined that an Arg-X-X-Lys SH3-binding motif confers specificity for the interaction between Gads and SLP-76 in T cell signaling (25). Recently, the P1 domain, a 67-amino acid stretch of the SLP-76 proline-rich region that is distinct from the Gads-binding region, has been shown to associate with PLCγ1 (26). Whereas SLP-76-deficient Jurkat T cells (J14 cells) stably transfected with WT SLP-76 signal normally through the TCR, cells reconstituted with either Δ20 or P1 deletion mutants of SLP-76 demonstrate incomplete functional rescue (26).

To characterize further the roles of the G2 and P1 domains of SLP-76 individually and in combination, in T cell signaling we created stable J14 cell lines expressing WT and mutant forms of SLP-76 (Fig. 2A). All SLP-76 constructs were tagged at their amino terminus with green fluorescent protein (GFP), and stable transfectants, matched for mean fluorescence intensity, were acquired through high speed cell sorting (Fig. 2B). To avoid the possibility of clonal variation artifact, we chose to study these discrete polyclonal populations. Equivalent expression of the SLP-76 constructs was confirmed by anti-GFP immunoblot analysis (Fig. 2C).

Both the P1 and G2 Domains of SLP-76 Are Required for Optimal T Cell Activation—We first examined the capacity of the proline domain mutants to reconstitute signaling in J14 cells. Following TCR engagement, cleavage of phosphatidylinositol 4,5-bisphosphate yields inositol 1,4,5-trisphosphate and diacylglycerol (34). The liberation of intracellular calcium through the activity of inositol 1,4,5-trisphosphate, necessary for activation of the calcineurin-dependent signaling pathway, is absent in J14 cells (8). Thus, we assessed the capacity of the J14 stable transfectants to flux intracellular calcium following TCR stimulation. To ensure fusion of GFP onto the amino terminus of SLP-76 does not alter SLP-76 function, we compared TCR-induced Ca²⁺ mobilization in J14 cells reconstituted with SLP-76 fused or not fused to GFP. As shown in Fig. 3A, the responses were indistinguishable, indicating that GFP/SLP-76 functions as wild type in the Jurkat-based assay. Moreover, GFP/SLP-76 appears to function similarly to the unfused molecule in vivo as reconstitution of SLP-76-deficient mice with GFP/SLP-76 leads to normal T cell development (data not shown). Analysis of the mutant constructs revealed that whereas the WT-reconstituted cells demonstrate robust Ca²⁺ mobilization after TCR ligation, this response is partially diminished in the G2 and P1 mutants and nearly absent in the P1/G2 double mutant (Fig. 3B). It should be noted that the degree of Ca²⁺ mobilization in WT-reconstituted J14 cells is similar to that detected in the parental Jurkat line and that all cells respond equivalently to treatment with ionomycin (data not shown).

Whereas CD69 is expressed normally by J14 cells following treatment with PMA, reflecting intact signaling through the Ras/MAPK pathway, they do not demonstrate TCR-inducible CD69 expression (8). As another measure of T cell function, we examined the reconstituted J14 cell lines for their capacity to up-regulate CD69 following overnight stimulation (Fig. 3C). The pattern of response mirrors that seen in the calcium flux studies described above; normal CD69 up-regulation following TCR engagement in the WT transfectants, impaired expression
in the G2 and P1 mutants, and no response by the P1/G2 double transfectants. All cells responded equally to pharmacologic treatment with PMA.

TCR-inducible activation of the NF-AT transcription factor, dependent upon integration of signals through both calcium and Ras/MAPK pathways (44), is also grossly impaired in J14 cells. Thus, as an additional means by which to determine functional rescue by the SLP-76 mutants, we transiently trans-
fected the stable cell lines with an NF-AT-luciferase reporter construct and assessed NF-AT activation following TCR stimulation (Fig. 3D). Here we found restoration of T cell activation in the WT-reconstituted cells and minimal responses by the cells expressing G2, P1, or P1/G2 domain mutants of SLP-76.

Disruption of the SLP-76/Gads Association Blocks SLP-76 Recruitment Following TCR Stimulation—One critical step in transducing signals from cell surface proteins is the subcellular redistribution of signaling molecules following receptor engagement (45). Biochemical data suggest that an important role of the association of Gads with SLP-76 is to mediate TCR-induced recruitment of SLP-76 and its binding partners to the immunologic synapse, the interface between the T cell and antigen-presenting cell (24, 35). These studies, however, have relied upon structural mutation of either SLP-76 or Gads. In such studies, there is always the possibility that the experimental observations are reflections of nonspecific changes in secondary or tertiary molecular structure. Therefore, as a complementary, alternative approach, we sought to interrupt the association of SLP-76 and Gads without genetic manipulation of either molecule. To this end, we created a construct encoding a 50-amino acid fragment containing the Gads binding domain of SLP-76, amino-terminally tagged with the fluorescent molecule DSRed2 (Fig. 4A). We hypothesized that high level expression of this protein would interfere with SLP-76/Gads binding through a competitive mechanism.

To assess the efficiency and selectivity of this strategy, we first stably transfected Jurkat T cells with the DSRed2-tagged GBF or with DSRed2 alone. Populations matched for expression of the constructs were obtained by cell sorting and then were subjected to biochemical analysis (Fig. 4B). Cellular lysates were immunoprecipitated with an anti-Gads antibody and were blotted for SLP-76 and DSRed2. The GBF fusion protein, but not DSRed2 alone, bound Gads. In the presence of the GBF but not in cells expressing only DSRed2, the SLP-76/Gads association was wholly eliminated. Given the recent report (46) of the SH3-mediated association of Gads with Gab2, another hematopoietic cell adaptor, we also probed the Gads immunoprecipitates for Gab2. We found that expression of the GBF partially but not completely reduced Gab2/Gads binding. Finally, to evaluate the possibility that the GBF might interrupt other SH3 associations, we immunoprecipitated SOS, a guanine nucleotide exchange factor, from cellular lysates of these stable Jurkat transfectants and blotted for the consti-
tutive SOS-binding partner, Grb2 (47). GBF expression did not measurably interfere with the Grb2/SOS association.

As real time imaging techniques have advanced, it has become possible to visualize molecular recruitment and assembly of adaptor-centered signaling complexes (48–51). By utilizing spinning disk confocal microscopy, we have demonstrated the unique movement of fluorescently tagged SLP-76 in Jurkat T cells following TCR engagement (52). To test the hypothesis that interruption of the SLP-76/Gads association through expression of the GBF would interfere with SLP-76 recruitment, we first stably transfected the J14 cells that had been reconstituted previously with GFP-tagged, WT SLP-76 with either DsRed2 or the DsRed2/GBF fusion protein (Fig. 4C). We then imaged these GFP/SLP-76-expressing cells as they were dropped on anti-TCR-coated coverslips (Fig. 4D). Those cells expressing GFP/SLP-76 and DsRed2 demonstrated initial clustering of SLP-76 at the periphery of the T cell as it contacted the stimulatory surface with subsequent movement to the central point of contact (Video 1 of the Supplemental Material), a pattern identical to that described previously (52) for SLP-76. The DsRed2 protein was diffusely distributed throughout the cytoplasm. In those cells expressing the GFP/SLP-76 and DsRed2/GBF fusion proteins, no clustering of GFP/SLP-76 was observed following TCR engagement (Video 2 of the Supplemental Material). Also, no aggregation of the DsRed2/GBF was seen.

Disruption of the SLP-76/Gads Association Significantly Impairs TCR-mediated Signaling—By having demonstrated the impact of GBF expression on the SLP-76/Gads association and SLP-76 trafficking, we next sought to determine its functional consequence. We assessed the capacity of the GFP-tagged WT SLP-76-reconstituted J14 cells, in the presence of either DsRed2 or the DsRed2/GBF fusion protein, to flux calcium and up-regulate CD69. J14 cells were stably transfected with DsRed2 alone for use as negative controls. We found that cells expressing GFP/SLP-76 and DsRed2 retained the capacity to flux calcium, whereas those coexpressing GFP/SLP-76 and the DsRed2/GBF showed a marked decrease in Ca\(^{2+}\) mobilization (Fig. 5A). The GBF also blocked up-regulation of CD69 following sustained TCR stimulation (Fig. 5B).

The G2 but Not the P1 Domain of SLP-76 Is Dispensable for Jurkat T Cell Function in Cells in Which SLP-76 Is Targeted to Lipid Rafts—We have shown previously (35) that SLP-76 is efficiently targeted to membrane lipid raft microdomains, when fused with the transmembrane domain of LAT including its two juxtamembrane cysteine residues but not the remainder of its cytoplasmic domain. To determine whether the primary function of the P1 and G2 domains of SLP-76 is to mediate SLP-76 recruitment, we generated three additional J14 stable transfectants in which the WT SLP-76 and proline domain mutants were raft-localized through fusion with the LAT transmembrane domain (Fig. 6A). The three cell lines were matched...
for GFP expression through cell sorting (data not shown). To ensure proper raft localization of the SLP-76 fusion proteins, membrane fractions were isolated following sucrose gradient centrifugation, and raft fractions were identified by anti-LAT Western blot. Western blot analysis using an anti-SLP-76 antibody verified the presence of all three SLP-76 fusion proteins in the raft fractions (Fig. 6B).

We measured calcium flux and CD69 up-regulation to gauge function of the reconstituted J14 cells. Cells expressing LAT/SLP-76 fusions, both WT and G2 domain mutated, had an
equivalent capacity to flux calcium in response to C305 stimulation (Fig. 6C). Calcium liberation was equal to that seen in J14 cells stably expressing GFP-tagged WT SLP-76. J14 cells expressing the LAT/P1 fusion showed only minimal calcium mobilization. Following overnight stimulation through the TCR, the LAT/WT- and LAT/G2-reconstituted J14 cells displayed similar CD69 up-regulation with lower expression seen in cells expressing the LAT/P1 fusion (Fig. 6D).

Disruption of the SLP-76/Gads Association Does Not Block Function of Lipid Raft-localized SLP-76—If the principal role of the SLP-76/Gads interaction is to mediate SLP-76 recruitment, we reasoned that interruption of their association through expression of the GBF should have little functional impact in cells in which SLP-76 was constitutively lipid raft-targeted through fusion with the LAT transmembrane domain. To test this premise, we stably transfected the J14 cells that had been reconstituted previously with LAT/SLP-76 with either DSRed2 alone or the DSRed2-tagged GBF as in Fig. 3. A, Ca²⁺ flux. B, expression of CD69. Data are representative of at least three separate experiments.

**Fig. 7.** Interruption of the SLP-76/Gads association by the GBF does not block function of lipid raft-localized SLP-76. TCR signaling was assessed in J14 cells expressing the LAT/SLP-76 and either DSRed2 alone or the DSRed2-tagged GBF as in Fig. 3. A, Ca²⁺ flux. B, expression of CD69. Data are representative of at least three separate experiments.

DISCUSSION

The adaptor SLP-76 links proximal and distal TCR signaling events through its function as a molecular scaffold in the assembly of multiprotein signaling complexes. Accumulated biochemical evidence has suggested that SLP-76 is recruited by the Grb2 family adaptor Gads to LAT (24, 35), a transmembrane adaptor that is constitutively localized to lipid rafts by virtue of palmitoylation of tandem cysteine residues within its proximal cytoplasmic region (33). These data support a model in which phosphorylation-dependent association of Gads with LAT following TCR engagement shuttles SLP-76 and its inducible binding partners to the immune synapse, allowing for signal transduction complex assembly with subsequent activation of PLCγ1 and the production of phosphoinositide-derived second messengers.

We employed the following three complementary approaches to gain a further understanding of the functions mediated by the SLP-76 proline-rich region: 1) reconstitution of SLP-76-deficient cells with functional domain deletion mutants; 2) blocking molecular associations through the expression of a dominant negative protein fragment; and 3) directed localization of SLP-76 to assess the role of the domains in SLP-76 recruitment. By using a Jurkat T cell model, we demonstrate the requirement for multiple, cooperative molecular associations at sub-domains within the SLP-76 proline-rich region for optimal SLP-76 function. Specifically, we show the P1 and G2 domains of SLP-76 are both necessary for SLP-76 function, and in the absence of these two regions, SLP-76 is functionally...
inert. Furthermore, we provide direct evidence that SLP-76 localization and, in turn, function are dependent upon association with Gads.

Earlier in vitro and in vivo reports have described the suboptimal capacity of the P20 form of SLP-76, a deletion mutant lacking 20 amino acids within the SLP-76 proline-rich region necessary for Gads association, to functionally rescue signaling through the TCR in SLP-76-deficient cells (26, 37, 38). Our results obtained using J14 cells reconstituted with G2-mutated SLP-76, the minimal Gads-binding mutant, are concordant with these previous studies because we found that these cells demonstrated much reduced capacity to transduce signals through the TCR relative to J14 cells that had been stably transfected with WT SLP-76. Measures of calcium flux, expression of the activation marker CD69, and activation of NF-AT transfected with WT SLP-76. This finding may reflect suboptimal SLP-76 association with the signaling complex when constrained by the membrane tether.

Although the signaling defect resulting from mutation of the G2 domain of SLP-76 is restored by raft targeting through fusion with the LAT transmembrane region, there is no rescue of TCR function observed in cells expressing the LAT/P1 fusion. Thus, the P1 domain clearly mediates some process other than recruitment. Given the large 66-amino acid span of this domain, it will be essential for future studies to identify the “minimal” P1 domain. Despite the fact that protein expression is unaffected by the deletion, it remains possible that the phenotype seen in the P1-reconstituted J14 cells merely reflects a failure of SLP-76 to fold properly thus interfering with a known, or yet unknown, function.

Alternatively, the P1 domain may be important because of the two domains might serve similar roles with respect to SLP-76 function. For example, phosphorylation-dependent recruitment of Gads and PLCγ1, both constitutively bound to SLP-76 through their SH3 domains, to LAT following TCR engagement might provide necessary and overlapping mechanisms for SLP-76 localization. However, another possible explanation was that the two domains served different roles. The SLP-76/Gads association might mediate SLP-76 recruitment, whereas molecular associations at the P1 domain function primarily to recruit PLCγ1 to LAT or to stabilize the PLCγ1/LAT interaction after signaling complex formation.

To begin to assess these possibilities, we first focused on the requirement of the Gads association for SLP-76 recruitment. We found that expression of the GBF, a fragment containing the G2 domain of SLP-76, blocked SLP-76/Gads binding while sparing other related SH3-mediated associations. Hence, although both SLP-76 and Gab2 contain similar Gads-binding motifs (RXXK) (46), the specificity of the GBF suggests that the sequence flanking this motif likely plays an important role in binding specificity. Our current study confirms that the SLP-76/Gads association is required for SLP-76 recruitment. Through live confocal imaging, we show that recruitment of GFP-tagged SLP-76 molecules following TCR engagement is absent in cells expressing the GBF.

In the absence of SLP-76 recruitment, we find concomitant inhibition of TCR function. We expected the J14 cells stably transfected with G2-mutated SLP-76 to be functionally equivalent to those cells expressing GFP-tagged WT SLP-76 plus the GBF, and we found them comparable in their capacity to flux calcium following TCR stimulation. Most interesting, compared with the G2 transfectants, CD69 expression after overnight stimulation through the TCR was slightly higher in those cells in which the SLP-76/Gads association was blocked through expression of the GBF. Although our biochemical and imaging data suggest complete interruption of SLP-76/Gads binding by the GBF, it is possible that there is minimal and transient SLP-76/Gads association that is below the threshold of detection for these assays. Only in the setting in which the impact of this nominal SLP-76/Gads interaction is summed over a long period of time, as in the CD69 activation assays, does it become apparent.

Further evidence for the role of Gads in the recruitment of SLP-76 is our observation that targeting SLP-76 to lipid rafts obviates the need for the G2 domain. We have shown previously (35) that SLP-76, fused with the transmembrane domain of LAT, is targeted to lipid rafts of the cell membrane. In this report we show that expression of LAT/SLP-76 fusions, both WT and G2 domain mutated, are matched with respect to their capacity to restore TCR-induced calcium mobilization and CD69 expression to J14 cells. As would be predicted, we found that blocking the SLP-76/Gads interaction through expression of the GBF had no impact on the raft-localized LAT/WT SLP-76 fusion function. Somewhat surprisingly, we noted that CD69 expression after overnight anti-TCR stimulation was lower in J14 cells stably transfected with the LAT/WT SLP-76 fusion than in those reconstituted with GFP-tagged WT SLP-76. This finding may reflect suboptimal SLP-76 association with the signaling complex when constrained by the membrane tether.

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