SLP-65 Signal Transduction Requires Src Homology 2 domain-mediated Membrane Anchoring and a Kinase-independent Adaptor Function of Syk*

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The family of SLPs (Src homology 2 domain-containing leukocyte adaptor proteins) are cytoplasmic signal effectors of lymphocyte antigen receptors. A main function of SLP is to orchestrate the assembly of Ca2+-mobilizing enzymes at the inner leaflet of the plasma membrane. For this purpose, SLP-76 in T cells utilizes the transmembrane adaptor LAT, but the mechanism of SLP-65 membrane anchoring in B cells remains an enigma. We now employed two genetic reconstitution systems to unravel structural requirements of SLP-65 for the initiation of Ca2+ mobilization and subsequent activation of gene transcription. First, mutational analysis of SLP-65 in DT40 B cells revealed that its C-terminal Src homology 2 domain controls efficient tyrosine phosphorylation by the kinase Syk, plasma membrane recruitment, as well as downstream signaling to NFAT activation. Second, we dissected these processes by expressing SLP-65 in SLP-76-deficient DT40 cells and found that a kinase-independent adaptor function of Syk is required to link phosphorylated SLP-65 to Ca2+ mobilization. These approaches unmask a mechanistic complexity of SLP-65 activation and coupling to signaling cascades in that Syk is upstream as well as downstream of SLP-65. Moreover, membrane anchoring of the SLP-65-assembled Ca2+ initiation complex, which appears to be fundamentally different from that of closely related SLP-76, does not necessarily involve a B cell-specific component.

The SH2 domain-containing leukocyte adaptor proteins, SLP-65 (1) (BLNK (2), BASH (3)), and SLP-76 (4), are instrumental to integrate and collect signals from antigen receptors on B and T lymphocytes, respectively. In their N-terminal half, the two effector proteins encompass a variety of similar consensus motifs for either inducible tyrosine phosphorylation by Syk/ZAP-70 family kinases or constitutive association to proteins with Src homology (SH)3 domains. Both SLP adaptors possess a C-terminal SH2 domain with a very high degree of sequence similarity and reported binding specificity for phosphorylated tyrosine residues in the consensus motif YXXDV (in single letter code for amino acids, with X being any amino acid). The similar overall structure of the two SLP family members matches their closely related signaling roles. Most prominently, their scaffold functions are mandatory for the correct subcellular localization and activation of phospholipase C-γ (PLC-γ) isoforms to induce mobilization of the second messenger Ca2+.

Surprisingly and despite the common protein architecture, the molecular mechanisms through which SLP-65 and SLP-76 assemble and target the Ca2+ initiation complexes in B and T cells, respectively, appear to be different (5). Downstream of the B cell antigen receptor (BCR), phosphorylated SLP-65 provides docking sites for the SH2 domains of Bruton’s tyrosine kinase (Btk) and PLC-γ2 (2, 6–9). Simultaneous recruitment of both enzymes must occur in cis, i.e. to a given SLP-65 molecule because only tri-molecular complex formation enables Btk to phosphorylate and thereby activate its target PLC-γ2 (9). To provide PLC-γ2 with access to its lipid substrate phosphatidylinositol 4,5-bisphosphate, the assembled Ca2+ initiation complex needs to be tethered at the plasma membrane. As shown more recently, membrane anchoring requires an N-terminal leucine zipper motif in SLP-65, but the exact mechanism remains elusive (10). However, the N terminus of SLP-76 does not contain such a leucine zipper. In fact, nucleation of the Ca2+ initiation complex upon engagement of the T cell antigen receptor (TCR) involves not only the intracellular adaptor SLP-76 but also the transmembrane linker of activated T cells) LAT (5, 11, 12). Tyrosine-phosphorylated LAT binds PLC-γ1 directly and associates simultaneously with a SLP-76/Itk complex through the Grb2 family member Gads. Itk is the T cell parologue of Btk. LAT- and Gads-related molecules in B cells are NTAL (13) (alternatively named Lab (14)) and Grb2 itself. We recently showed that the NTAL/Grb2 module is indeed critically involved in BCR-triggered Ca2+ elevation but through a SLP-65-independent mechanism (15, 16).

One mode of stimulation-dependent membrane recruitment of SLP-65 involves its SH2 domain, which directly binds the

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The abbreviations used are: SH, Src homology; BCR, B cell antigen receptor; Btk, Bruton’s tyrosine kinase; PLC, phospholipase C; TCR, T cell antigen receptor; ITAM, immunoreceptor tyrosine-based activation motif; HA, hemagglutinin; EGFP, enhanced green fluorescent protein.
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phosphorylated BCR signaling subunit Ig-α (17). The corresponding phosphotyrosine residue in Ig-α is located outside of the immunoreceptor tyrosine-based activation motif (ITAM) (17, 18). The non-ITAM phospho-acceptor site of Ig-α is conserved within and across species but absent in other ITAM-containing immunoreceptor signaling subunits (19), suggesting an important and B cell-specific signaling function. Indeed, B cells expressing an Ig-α mutant lacking the SLP-65-binding site mount impaired responses to T cell-independent antigens in vivo (20). Hence, SH2-mediated SLP-65 recruitment to the BCR appears to amplify signaling rather than to provide indispensable membrane linkage for the SLP-65-assembled Ca²⁺ initiation complex. A second known ligand for the SLP-65 SH2 domain and also for that of SLP-76 is the serine/threonine kinase HPK1 (21, 22), which has, however, not been implicated in the onset of Ca²⁺ mobilization. Interestingly, the SH2 domain of SLP-76 functions in another completely different pathway. It regulates TCR-induced integrin activation and T cell adhesion by binding tyrosine-phosphorylated ADAP, which couples to Rac1 activation (23–26). Hence, although similar in many aspects, the structural entities of SLP-65 and SLP-76 execute cell-type-specific functions by binding distinct interaction partners. We have explored this topic further and show herein that the SH2 domain of SLP-65 controls the subcellular localization of the adaptor, which is critical for BCR-proximal and -distal activation signals in a Syk-dependent manner.

**EXPERIMENTAL PROCEDURES**

**Cells, Antibodies, and Reagents**—Jurkat T cells, their derivative J14 (slp-76−/−, syk−/−, kindly provided by D. Yablonski, Haifa, Israel) and DT40 B cells deficient for either SLP-65 or PLC-γ2 (kindly provided by T. Kurosaki, Yokohama, Japan) were cultured as described (27) and stimulated through their antigen receptors with 20 μg/ml anti-human CD3 (C305, kindly provided by B. Schraven, Magdeburg, Germany) and 10 μg/ml anti-chicken IgM (M4, Southern Biotechnology, BioMol, Hamburg, Germany), respectively. T cells were solubilized in Nonidet P-40 lysis buffer I (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 10 mM NaF, 1 mM Na₃VO₄, 10 mM Na₂MoO₄, 1% Nonidet P-40, and protease inhibitors P2714; Sigma-Aldrich) and B cells in Nonidet P-40 lysis buffer II (10 mM Tris/HCl, pH 7.8, 137 mM NaCl, 0.5 mM EDTA, 2 mM Na₂VO₄, 1 mM NaF, 1% Nonidet P-40, 10% glycerol, and protease inhibitors P2714). Lysates of 3 × 10⁶ cells were subjected to immunoprecipitation with either anti-PLC-γ2 (Q20; Santa Cruz Biotechnology, Heidelberg, Germany), anti-Syk (4D10; Santa Cruz Biotechnology, Heidelberg, Germany), or anti-HA antibodies (12CA5; Roche Applied Science). In the latter case, antibodies were immobilized on NHS-activated Sepharose beads (Amersham Biosciences) according to manufacturer’s instruction. Antibodies for immunoblotting are specific for HA epitope (3F10; Roche Applied Science), chicken SLP-65 (kindly provided by T. Kurosaki, Yokohama, Japan), human actin (Sigma-Aldrich), phosphotyrosine (4G10; BioMol, Hamburg, Germany), human SLP-76 (BioMol, Hamburg, Germany), human Syk (4D10), and PLC-γ2 (Cell Signaling Technology, Beverly, MA).

**Expression Constructs**—The cDNA encoding N-terminally HA-tagged chicken SLP-65 was ligated into pENTR/SD/D-TOPO (Invitrogen). Site-directed mutagenesis was employed to generate expression constructs for the SH2 domain deletion mutant of chicken SLP-65 (ΔSH2; amino acids 1–441) and to inactivate the SH2 domain by a single amino acid exchange, i.e., R468L. The resulting cDNAs were ligated into the expression vector pAbes-puro (28) using Gateway cloning technology (Invitrogen) and introduced into SLP-65-negative DT40 B cells by electroporation (250 V, 960 microfarad). The cDNA encoding C-terminally EGFP–tagged chicken SLP-65 was ligated via BglII/NotI into pMSCV vector (Becton Dickinson-Clontech, Heidelberg, Germany), and SLP-65-deficient B cells were retrovirally transduced as described (15). Wild-type murine slp-65 cDNA was cloned by reverse transcription-PCR from EL-4 T cells and ligated via BamHI and EcoRV into pAbes-puro. Using a PCR-generated expression cassette encoding N-terminal HA-tagged murine SLP-65 inserted into pCRII-TOPO vector (Invitrogen), the leucine zipper deletion mutant (ΔLZ; amino acids 46–442) and the SH2 domain deletion mutant (ΔSH2; amino acids 1–339) of SLP-65 were generated by site-directed mutagenesis and ligated via EcoRI into pApuro. Wild-type human syk cDNA was isolated from pDSyk vector (a kind gift from M. Reth, Freiburg, Germany) and ligated into pcDNA3 (Invitrogen) via EcoRI. A kinase-inactive mutant of Syk (Syk*) and truncated Syk constructs were created by amino acid substitution (K402G; see also Ref. 29) or deletion using site-directed mutagenesis, respectively. The tandem SH2 domains of Syk were inactivated by replacement of R42G/Q43G/S44I in the N-terminal and R195G/R197L in the C-terminal SH2 domain of Syk*(SH2*)2. To delete the N-terminal, C-terminal, or tandem SH2 domains or the kinase domain of Syk, cDNA fragments encoding amino acids 1–168 (Syk*(SH2*)N), 167–260 (Syk*ΔSH2N), 167–260 (Syk*ΔSH2C), 257–355 (Syk*ΔKD) of Syk* were ligated into pcDNA3, respectively. J14 cells were transfected by electroporation and selected in the presence of G418 (2 mg/ml) and/or puromycin (0.5 μg/ml).

**Ca²⁺ Analysis and Luciferase Reporter Gene Assay**—The concentration of intracellular free Ca²⁺ ions was measured by flow cytometry using the ratiometric Ca²⁺ chelator dye Indo1-AM (Molecular Probes, BioMol, Hamburg, Germany) as described (27). For monitoring NFAT/AP1-driven gene transcription, 1 × 10⁶ DT40 cells were transiently cotransfected with 10 μg of the β-galactosidase expression plasmid pCMV-β and 20 μg of the pNFAT-luc construct (kindly provided by T. Brummer, Sydney, Australia), which harbors three tandem copies of an NFAT/AP1 composite element of the human interleukin-2 promoter (position −286 to −257 (30)) to drive transcription of the luciferase cassette. Induction of luciferase protein expression upon BCR activation was measured as described (27).

**Confocal Microscopy**—A total of 1 × 10⁶ DT40 cells were resuspended in Krebs-Ringer solution composed of 10 mM HEPES (pH 7.0), 140 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, and 10 mM glucose. After 30 min of seeding onto chambered coverslips (Labtek, Stadthofern, Germany), the cells were examined on a Leica TCS SP2 confocal laser scanning microscope (Leica, Wetzlar, Germany) in the absence or presence of...
BCR stimulation via anti-IgM antibodies as described in Ref. 16. EGFP was excited at a wavelength of 488 nm, and emission was recorded at 510 nm.

RESULTS

Tyrosine Phosphorylation and Membrane Recruitment of SLP-65 Requires a Functional SH2 Domain—To investigate the functional relevance of the SLP-65 SH2 domain, we reconstituted SLP-65-deficient DT40 B cells (31) with HA-tagged wild-type SLP-65 and SH2 domain mutants, which harbor an inactivated SH2 domain (single amino acid exchange of R468L) or lack the SH2 domain at all (ΔSH2). Anti-HA immunoblotting revealed that all SLP-65 proteins were expressed by the transfectants in similar amounts (Fig. 1A). Anti-phosphotyrosine immunoblotting of anti-HA precipitates obtained from resting or anti-IgM-stimulated transfectants revealed robust phosphorylation of wild-type SLP-65 in activated cells (Fig. 1B, lanes 1–4). In contrast, the SH2 domain mutants of SLP-65 were slightly but significantly less phosphorylated (Fig. 1B, lanes 5–8). Hence, efficient communication between the activated BCR and its proximal effector SLP-65 is dependent on a functional SLP-65 SH2 domain.

SLP-65 is a specific substrate of the protein tyrosine kinase Syk (1, 2), which upon cellular activation translocates from the cytosol to the phospho-ITAMs of Ig-α/β at the plasma membrane (32–35). Therefore, we next analyzed the subcellular localization of wild-type and mutant SLP-65 proteins. For this purpose, we transfected slp-65−/− DT40 cells with expression constructs encoding fusion proteins between the EGFP and either wild-type SLP-65 or SLP-65ΔSH2. Untreated and BCR-activated transfectants were examined by laser scanning microscopy (Fig. 1C). In unstimulated cells, the majority of SLP-65 was randomly distributed in the cytosol (upper left image). Following BCR activation, wild-type SLP-65 became almost quantitatively recruited to the plasma membrane (upper right image). The dotted staining pattern of these cells indicates that SLP-65 is concentrated and clustered at specific sites at the plasma membrane where signaling occurs. This stimulation-dependent translocation of wild-type SLP-65 was completely lost for the ΔSH2 mutant of SLP-65 (lower left and right images). The result identifies the SH2 domain as an indispensable structural element for membrane anchoring of SLP-65 in activated B cells.

The SH2 Domain of SLP-65 Controls the Activation of Downstream Signaling Cascades—To assess whether impaired phosphorylation and lack of membrane recruitment of SLP-65 SH2 domain mutants also affects SLP-65-regulated downstream signaling, we analyzed BCR-induced activation of PLC-γ2 and concomitant Ca²⁺ mobilization as well as the transcriptional activity of NFAT in the cell lines described above (Fig. 2). Consistent with previous reports (31), BCR-induced PLC-γ2 phosphorylation required SLP-65 expression (Fig. 2A, lanes 1–4). Similar to SLP-65-negative DT40 B cells, almost no PLC-γ2 phosphorylation was observed in transfectants expressing SH2-defective SLP-65 proteins (Fig. 2B, lanes 5–8). Block of PLC-γ2 activation was associated with drastically diminished assembly of the Ca²⁺ initiation complex (data not shown) and directly correlated with defective Ca²⁺ mobilization (Fig. 2B). Only a very weak and transient Ca²⁺ flux was observed in cells expressing the R468L mutant of SLP-65 (red line). BCR-induced Ca²⁺ mobilization was completely abrogated in cells expressing SLP-65ΔSH2 (magenta line) or no SLP-65 at all (black line).

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FIGURE 1. BCR-induced tyrosine-phosphorylation and subcellular translocation of SLP-65 requires a functional SH2 domain. A, SLP-65-deficient DT40 B cells (slp−/−, lane 1) were reconstituted with either wild-type chicken SLP-65 (wt, lane 2) or SH2 domain mutants, R468L and ΔSH2 (lanes 3 and 4). Expression of SLP-65 proteins was tested by immunoblotting of cleared cellular lysates with antibodies to the N-terminal HA peptide tag. (upper panel). Protein loading was controlled by anti-actin immunoblotting (lower panel). B, cells described in A were left untreated (lanes 1, 3, 5, and 7) or stimulated through their BCR for 2 min (lanes 2, 4, 6, and 8) and from the cleared cellular lysates, SLP-65 proteins were purified by anti-HA immunoprecipitation. Obtained proteins were analyzed by anti-phosphotyrosine (α-pTyr, upper panel) and anti-chicken SLP-65 (α-chSLP-65, lower panel) SLP-65 immunoblotting. The relative molecular masses of marker proteins are indicated on the left in kDa. C, SLP-65-deficient DT40 cells were reconstituted with EGF-tagged wild-type SLP-65 (wt, upper left and right panels) or SLP-65ΔSH2 (ΔSH2, lower left and right panels) and analyzed by confocal laser scanning microscopy in the absence (left panels) or presence (right panels) of BCR stimulation via anti-IgM treatment for 3 min.
expression plasmid allowed for normalization of the reporter gene activity according to the transfection efficiencies. As previously published (27, 36, 37), anti-BCR stimulation of cells expressing wild-type SLP-65 resulted in strong activation of NFAT-mediated gene transcription, which was almost undetectable in SLP-65-deficient cells (Fig. 2C, column pairs 1 and 2). Reconstitution with SH2 domain mutants of SLP-65 did not restore this response (column pairs 3 and 4). Collectively, this set of experiments reveals that the SLP-65 SH2 domain is critically involved in proper formation of the Ca^{2+} initiation complex to activate PLC-γ2 and to raise intracellular Ca^{2+} concentrations above a threshold level that allows activation of gene transcription.

A Concerted Action of Phosphorylated SLP-65 and Syk Compensates for Loss of SLP-76 in T Cells—The indispensable role of the SLP-65 SH2 domain for efficient coupling to upstream as well as downstream signaling partners of Ca^{2+} mobilization suggested another fundamental difference to the T cell parologue SLP-76, whose SH2 domain is involved neither in SLP-76 membrane anchoring nor TCR-mediated Ca^{2+} mobilization (38, 39). To further confirm this notion and to directly assess whether the SLP-65 SH2 domain needs a B cell–specific ligand, we expressed HA-tagged wild-type SLP-65 in the SLP-76-deficient Jurkat T cell mutant J14 (40) (Fig. 3A, upper two panels, lanes 2 and 4). As control, J14 cells were reconstituted with wild-type SLP-76 (lanes 2 and 3). Because the J14 variant lacks not only SLP-76 but also Syk (third panel, lanes 1–4), we generated additional J14 transfectants expressing either Syk alone or in combination with HA-tagged wild-type SLP-65 (lanes 5 and 6).

After having established these cell lines, we first tested for TCR-induced tyrosine phosphorylation of SLP-65. Anti-HA precipitates were prepared from resting and stimulated J14 parental cells as well as SLP-65 single transfectants and SLP-65/Syk double transfectants. Subsequently, purified proteins were analyzed by anti-phosphotyrosine or anti-HA immunoblotting (Fig. 3B, upper and lower panel, respectively). SLP-65 became inducibly tyrosine-phosphorylated in the single and double transfectants (lanes 3–6). The presence of Syk in the latter cells caused a low level of constitutive SLP-65 phosphorylation (lane 5). No specific signal was obtained in the analysis of J14 parental cells (lanes 1 and 2). These data show that SLP-65 can be coupled to the TCR signaling machinery independent of Syk. Most likely, SLP-65 phosphorylation is accomplished by the Syk-related kinase ZAP-70. However, flow cytometric analysis of TCR-induced Ca^{2+} mobilization in the various T cell lines described above showed that expression and tyrosine phosphorylation of SLP-65 alone is not sufficient to restore the Ca^{2+} response in SLP-76-deficient J14 cells (Fig. 3C, red and black lines). In marked contrast to the SLP-65 single transfectants, the SLP-65/Syk double transfectants of J14 mounted robust Ca^{2+} signaling (orange line) similar to that observed in wild-type Jurkat T cells or SLP-76-reconstituted J14 cells (dark and light blue lines, respectively). Expression of Syk alone did not confer TCR responsiveness to J14 cells (magenta line), even though the single transfectants produced Syk in large amounts (Fig. 3A, lane 5). Indeed, the analysis of a panel of independent SLP-65/Syk double transfectants revealed

To test whether the residual Ca^{2+} flux observed in cells with mutant SLP-65 is still sufficient to trigger nuclear responses, we monitored Ca^{2+}–sensitive activation of the transcription factor NFAT. For this purpose, the slp-65^{-/-} parental cells and the different SLP-65 transfectants were equipped with a luciferase reporter gene construct under the transcriptional control of a NFAT-responsive promoter. Empty vector-transfected cells served as control, and cotransfection of a β-galactosidase

FIGURE 2. The SLP-65 SH2 domain controls Ca^{2+} mobilization and subsequent nuclear signaling events. A, SLP-65-negative DT40 cells and their various SLP-65 transfectants described in Fig. 1A as well as PLC-γ2-deficient DT40 cells were left untreated or stimulated through their BCR for 3 min. PLC-γ2 was immunoprecipitated from the cleared cellular lysates and analyzed by immunoblotting with antibodies to phosphotyrosine (upper panel) and PLC-γ2 (lower panel). The relative molecular mass of marker protein is indicated on the left in kDa. B, cells described in Fig. 1A were loaded with the Ca^{2+}-sensitive dye Indo-1 AM and stimulated through their BCR, and elevation of intracellular Ca^{2+} concentrations (Ca^{2+}) was measured by flow cytometry (for details see “Experimental Procedures” and for color code see the right side of the panel). C, upon transient cotransfection of the various SLP-65-negative and -positive DT40 cells with an NFAT reporter gene construct and a β-galactosidase expression vector (for determination of transfection efficiency), enzymatic activities of luciferase and β-galactosidase were determined for resting cells (open bars) and cells stimulated through their BCR for 6 h (black bars). The data were normalized according to transfection efficiency, and the standard deviations were calculated from three independent experiments. wt, wild type.
Cell-specific Signaling Elements

that it is the coexpression of Syk and SLP-65 rather than the level of expression of each protein that allows the cells to mobilize intracellular Ca\(^{2+}\) (only one representative SLP-65/Syk-positive clone is shown in Fig. 3A, lane 6). Hence, SLP-65 and Syk appear to form a functional unit that is required for coupling phosphorylated SLP-65 to downstream Ca\(^{2+}\) signaling. It is important to note that this function of Syk cannot be studied in B cells because here Syk is already upstream of SLP-65 activation in that it is required for SLP-65 phosphorylation.

The C-terminal Half of Syk Couples Phospho-SLP-65 to Ca\(^{2+}\) Signaling in a Kinase-independent Manner—Given the lack of Ca\(^{2+}\) mobilization in the presence of phosphorylated SLP-65, we next asked the question of whether the enzymatic activity of Syk is at all required to restore the Ca\(^{2+}\) response in SLP-65 expressing J14 cells. An expression vector for a kinase-negative mutant of Syk (Syk\(^{*}\)) was constructed (Fig. 4A) and expressed in SLP-65-positive J14 cells (Fig. 4B, upper panel, lanes 1–4). We additionally generated transfectants expressing a Syk\(^{*}\) variant in which both SH2 domains were inactivated by amino acid exchanges (Syk\(^{(SH2)*}\)) (Fig. 4, A and B, upper panel, lanes 5 and 6). Following TCR activation of the cells, the Syk\(^{*}\) variant became tyrosine-phosphorylated (presumably by ZAP-70), whereas the Syk\(^{(SH2)*}\), mutant did not (Fig. 4B, lower panel) because it lacks functional SH2 domains required for Syk recruitment to the ITAMs of the activated TCR. Yet both Syk mutants were equally capable of fully reconstituting a TCR-induced Ca\(^{2+}\) response (Fig. 4C). These results were unexpected because they show that neither the function of the kinase domain nor that of the tandem SH2 domains of Syk is required for coupling phosphorylated SLP-65 to Ca\(^{2+}\) elevation. The data indicated the existence of an as yet unknown functional element in Syk that operates downstream of SLP-65 phosphorylation and critically supports SLP-65-mediated Ca\(^{2+}\) elevation.

To further dissect the structural requirements of Syk, a series of Syk deletion mutants were constructed (Fig. 4D, see also schematic representation in Fig. 4A) and tested for their Ca\(^{2+}\) signaling capacity in SLP-65-positive J14 cells (Fig. 4E). Ablation of single or tandem SH2 domains in the Syk\(^{*}\) variant did not significantly alter the ability of this kinase-inactive mutant to support Ca\(^{2+}\) mobilization. In marked contrast but identical to J14 control cells, no Ca\(^{2+}\) response was detected in SLP-65-positive J14 transfectants expressing a kinase deletion mutant of Syk (Syk\(^{ΔKD}\)), i.e. only the N-terminal tandem SH2 domains of Syk. Collectively, these reconstitution experiments show that the C terminus of Syk possesses a kinase-independent adaptor function that links phospho-SLP-65 to Ca\(^{2+}\) mobilization. Apparently ZAP-70 cannot provide this accessory function, although it is able to substitute Syk for SLP-65 phosphorylation. We consequently conclude that in B cells, Syk possesses a dual role for SLP-65 function. As an upstream activator, Syk phosphorylates SLP-65, allowing the recruitment of Btk and PLC-γ2. Subsequently, a kinase-independent adaptor function in the C-terminal half of Syk orchestrates the signaling efficiency of the Ca\(^{2+}\) initiation complex.

Both SLP-65 Membrane Anchors Function in the Absence of B Cell-specific Signaling Elements—So far our J14 reconstitution experiments suggested that the SLP-65/Syk module can trigger Ca\(^{2+}\) flux independently of additional B cell signaling elements. However, data published by Kohler et al. (10) and our own mutational analysis in DT40 B cells (Figs. 1 and 2) revealed that SLP adapters in B and T cells utilize distinct mechanisms for anchoring the Ca\(^{2+}\) initiation complex at the plasma membrane. This prompted us to investigate the importance of the
two membrane anchors of SLP-65 for Ca\(^{2+}\) signaling in J14 cells. We reasoned that if in contrast to B cells, the leucine zipper and/or the SH2 domain are dispensable to restore Ca\(^{2+}\) signaling in J14 cells, SLP-65 must be artificially recruited to the T cell membrane, revealing a specialty of the experimental system. In case both targeting devices are also required in J14 cells, ligands for these domains must exist in T cells, indicating that membrane recruitment of SLP-65 does not necessarily involve B cell-specific molecules.

Two HA-tagged SLP-65 deletion mutants lacking either the leucine zipper (ΔLZ) or the SH2 domain (ΔSH2) were expressed together with the Ca\(^{2+}\) signaling-competent Syk\(^*\) variant in J14 cells (Fig. 5A, lanes 3 and 4). J14 parental cells and J14 transfectants expressing wild-type SLP-65 in addition to Syk\(^*\) served as negative and positive control, respectively (lanes 1 and 2). Immunoblot analysis of anti-HA precipitates confirmed the TCR-induced tyrosine phosphorylation of wild-type SLP-65 in J14 cells (Fig. 5B, lanes 3 and 4; see also Fig. 3B), which was reduced for the SLP-65 mutants (Fig. 5B, lanes 5–8) as already observed in DT40 cells (see Fig. 1 and data not shown). Importantly, a robust Ca\(^{2+}\) response was detected only in the positive control cells coexpressing wild-type SLP-65 and Syk\(^*\) but not in the transfectants that express the ΔLZ or ΔSH2 mutant of SLP-65 showing no or weak Ca\(^{2+}\) flux, respectively (Fig. 5C). Hence, the presence of both the N- and C-terminal membrane anchors is necessary to permit a functional cooperation between SLP-65 and kinase-inactive Syk for Ca\(^{2+}\) mobilization in T cells. We conclude that the Syk/SLP-65 module does not require a B cell-specific membrane recruiter.

**DISCUSSION**

With this report we have elucidated structural and mechanistic details about the signaling function of the central BCR transducer SLP-65. We first identified the SH2 domain of SLP-65 as a mandatory component for early and late BCR signaling events. In the absence of a functional SH2 domain,
SLP-65 phosphorylation by Syk is diminished but not completely abolished. The SH2 domain is indispensable for BCR-triggered membrane relocalization, which identifies the C terminus as a second and critical membrane targeting device in addition to the leucine zipper in the N terminus (10). The functional significance of our finding is demonstrated by the inability of SH2 mutants of SLP-65 to efficiently support PLC-γ2 activation and their failure to elevate intracellular Ca^{2+} level above a threshold that suffices for activation of NFAT-induced gene transcription. Hence, the signaling function of SLP-65 relies on bidental membrane anchoring in a sequential manner. First, the leucine zipper confers constitutive membrane association to SLP-65 (10), which in stimulated cells may prepare for initial phosphorylation by Syk that is recruited to and activated by the BCR. How the leucine zipper fulfills this function is not known, but the mechanism operates also in non-B cells (10) and may account for the residual phosphorylation of the SLP-65 SH2 mutants observed in our DT40 transfectants. Second, the SH2 domain provides stimulation-dependent membrane targeting that facilitates or amplifies SLP-65 phosphorylation and is necessary for full B cell activation. A likely candidate transmembrane resident that could accomplish this function was Ig-α because its phosphorylated non-ITAM tyrosine provides a specific docking site for the SLP-65 SH2 domain (17, 18). Indeed, genetic engineering in mice supports an important role for the Ig-α/SLP-65 complex for B cell activation (20). Our reconstitution experiments in J14 T cells show, however, that the SLP-65 SH2 domain, although functionally indispensable, does not necessarily need a B cell-specific ligand for Ca^{2+} signaling. Nonetheless, we cannot formally rule out the existence of such a phosphoprotein, and experiments are underway to isolate and characterize the responsible linker molecules that confer membrane association to SLP-65 in resting and stimulated B cells.

A second key finding of our studies is the kinase-independent adaptor function in the C-terminal part of Syk, which connects tyrosine-phosphorylated SLP-65 to Ca^{2+} elevation. This discovery was greatly facilitated by the J14 T cell reconstitution system because in B cells, Syk is already required for SLP-65 phosphorylation and thus upstream of SLP-65 activation for Ca^{2+} mobilization. It is therefore difficult to unmask a downstream role of Syk subsequent to SLP-65 phosphorylation in B cells. In T cells, SLP-65 phosphorylation can be achieved by other kinases, most likely ZAP-70, which, however, cannot provide the final link to SLP-65-regulated Ca^{2+} mobilization. Interestingly, Syk but not ZAP-70 accommodates in its C-terminal end a conserved tyrosine phosphorylation motif YXDV, which perfectly matches the consensus binding site of the SLP-65 SH2 domain found in Ig-α (17, 18) and HPK1 (21, 22).

Indeed, a stimulation-dependent coimmunoprecipitation of SLP-65 and Syk has been reported by Fu et al. (2) and by our group (17). It is thus tempting to speculate that only in conjunction with phospho-Syk, SLP-65 becomes efficiently translocated to and/or maintained at the plasma membrane. In this case, Syk itself provides stimulation-dependent membrane association for SLP-65 in B cells. However, in our J14 T cell system, SLP-65-mediated Ca^{2+} flux is accomplished by coexpression of a kinase-inactive Syk variant (Syk^{−/−}(SH2^{−/−})) that is not phosphorylated (Fig. 4, B and C). This suggests that Syk and SLP-65 may also bind each other in an SH2 domain-independent, perhaps constitutive manner, which prepares for SLP-65 phosphorylation and subsequently keeps phospho-SLP-65 in a conformational status that allows efficient membrane targeting or recruitment of Btk and PLC-γ2. This possibility is directly supported by the recent finding that in resting B cells, SLP-65 exists in a 180-kDa protein complex, which is not formed by...
SLP-65 oligomerization but mandatory for BCR-induced SLP-65 phosphorylation (41). Whether or not this complex also contains Syk was not addressed. In any case, the preformed and stimulation-dependent BCR signaling complexes described earlier (42, 43) and in this report show that the structural and functional linkage between Syk and SLP-65 appears to be more than just a simple enzyme-substrate interaction. Both proteins may have coevolved as a functional transducer unit of the BCR. This makes it plausible why the SLP adaptors in B and T cells on their own cannot functionally substitute each other despite their similar structural composition. SLP-76 expression alone fails to restore Ca\(^{2+}\) signaling in SLP-65-deficient DT40 cells but additionally requires LAT and Gads for membrane targeting (44). The SH2 domain of SLP-76 appears to be involved in TCR-induced T cell adhesion (38) but is dispensable for Ca\(^{2+}\) flux (39). We have shown here that ectopic expression of SLP-65 together with kinase-inactive Syk is required to reconstitute Ca\(^{2+}\) signaling in SLP-76-deficient T cells. In future experiments, a combination of biochemical and genetic approaches with B and non-B cells will be required to elucidate the exact mechanism of constitutive and stimulation-dependent SLP-65 membrane targeting by the N-terminal leucine zipper and the C-terminal SH2 domain, respectively.

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