

## **SLP-65: A New Signaling Component in B Lymphocytes which Requires Expression of the Antigen Receptor for Phosphorylation**

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### **Summary**

The B cell antigen receptor (BCR) consists of the membrane-bound immunoglobulin (Ig) molecule as antigen-binding subunit and the Ig- $\alpha$ /Ig- $\beta$  heterodimer as signaling subunit. BCR signal transduction involves activation of protein tyrosine kinases (PTKs) and phosphorylation of several proteins, only some of which have been identified. The phosphorylation of these proteins can be induced by exposure of B cells either to antigen or to the tyrosine phosphatase inhibitor pervanadate/H<sub>2</sub>O<sub>2</sub>. One of the earliest substrates in B cells is a 65-kD protein, which we identify here as a B cell adaptor protein. This protein, named SLP-65, is part of a signaling complex involving Grb-2 and Vav and shows homology to SLP-76, a signaling element of the T cell receptor. In pervanadate/H<sub>2</sub>O<sub>2</sub>-stimulated cells, SLP-65 becomes phosphorylated only upon expression of the BCR. These data suggest that SLP-65 is part of a BCR transducer complex.

**Key words:** antigen receptor • tyrosine phosphorylation • Src homology 2 domain • SLP-65

Engagement of the B cell antigen receptor (BCR)<sup>1</sup> triggers the phosphorylation of proteins on tyrosine and the concomitant activation of a variety of signaling pathways. The tyrosine phosphorylation of phospholipase C- $\gamma$  leads to hydrolysis of phosphoinositides which produces second messengers able to elevate intracellular [Ca<sup>2+</sup>] and activate protein kinase C (1–3). The BCR-mediated activation of the ras/Raf/Map kinase pathway is less well understood but is likely to involve p120rasGAP and the Grb-2–Sos complex (1–3). Tyrosine-phosphorylated Vav exhibits GDP/GTP exchange activity for Rac-1, a small G protein implicated in cell proliferation and cytoskeletal organization (4). The activation of these and other signaling cascades is not restricted to BCR-stimulated B cells, and some signaling elements are used by many receptors. The stimulated BCR must both maintain signal specificity and avoid interference with other types of receptor that are not stimulated. A pre-

formed BCR transducer complex would make this possible by allowing the rapid and selective activation of BCR-specific signaling pathways, even in those cases where some signaling elements are common to the BCR and another receptor. The analysis of substrate phosphorylation in J558L B cells treated with pervanadate/H<sub>2</sub>O<sub>2</sub> suggests that as soon as it is expressed, the BCR indeed recruits and organizes intracellular signaling elements into a signaling complex (5). The existence of preformed signaling complexes has been directly demonstrated for the photoreceptor system in drosophila (6) and for the osmoadaptation–pheromone response system in yeast (7). In both cases, specific adaptor proteins were found to organize the physical and functional coupling of intracellular signaling proteins to receptors in the plasma membrane. We have tried to identify protein tyrosine kinase (PTK) substrate proteins that become tyrosine phosphorylated early after BCR stimulation. Here we report on the identification and cloning of such a protein, which we named SLP-65 (Src homology [SH2] domain-containing leukocyte protein of 65 kD). This B cell-specific adaptor protein contains a number of protein–protein interaction domains and is constitutively associated with Grb-2 and Vav. In the absence of a BCR, treatment of the cells with pervanadate/H<sub>2</sub>O<sub>2</sub> does not induce phos-

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<sup>1</sup>Abbreviations used in this paper: BCR, B cell antigen receptor; GST, glutathione S-transferase; HA, hemagglutinin; ORF, open reading frame; PTK, protein tyrosine kinase; RACE, rapid amplification of cDNA ends; SH2 and SH3 domain, Src homology 2 and 3 domain, respectively; SLP-65, SH2 domain-containing leukocyte protein of 65 kD.

phorylation of SLP-65. Our results suggest a scaffolding function for SLP-65 which would couple BCR activation to intracellular signaling pathways.

## Materials and Methods

**Materials.** Immobilized PT66 antiphosphotyrosine-agarose beads (Sigma-Aldrich, Deisenhofen, Germany) and soluble 4G10 antiphosphotyrosine antibodies (Upstate Biotechnology Inc., Lake Placid, NY) were used for precipitation and immunoblot analysis of phosphotyrosine-containing proteins, respectively. Anti-Vav and anti-Grb-2 rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Polyclonal anti-SLP-65 antibodies were produced by immunizing rabbits with KLH-coupled SLP-65 peptides encompassing amino acids 148–161 (KARLA peptide; see Fig. 1). The glutathione *S*-transferase (GST)-Grb-2 fusion proteins contain amino acids 1–168 (SH3-SH2) and 49–168 (SH2) of the human Grb-2 protein, respectively. Primer sequences used for amplifications of the respective Grb-2 cDNA fragments are available upon request. The plasmid encoding the GST-Grb-2 (SH3) fusion protein was a gift of Dr. William Wishart, Novartis (Basel, Switzerland). The GST fusion protein encompassing the complete murine Grb-2 was obtained from Upstate Biotechnology Inc.

**Protein Analysis.** J558L cell lines, pervanadate/H<sub>2</sub>O<sub>2</sub> stimulations, immunoprecipitations, affinity purifications with GST fusion proteins, and Western blot analysis were described previously (5, 8).

**Large-scale Preparation and Amino Acid Sequencing of PTK Substrate Proteins.** In total, 10<sup>10</sup> J558L $\delta$ m7.1 cells were stimulated in aliquots of 3  $\times$  10<sup>7</sup> cells per ml RPMI 1640 medium for 3 min with 50  $\mu$ M pervanadate/H<sub>2</sub>O<sub>2</sub> at 37°C (5). After lysis of the cells in 600  $\mu$ l of 1% NP-40 lysis buffer (5), postnuclear supernatants were collected and precleared for 3 h with 7.5 ml of mouse IgG agarose (Sigma-Aldrich). Subsequently, the lysate was subjected to antiphosphotyrosine immunoprecipitation with 6 ml of agarose-conjugated PT66 mAb (Sigma-Aldrich). Settled beads were extensively washed with 1% NP-40 lysis buffer, and bound proteins were eluted eight times with 7.5 ml 50 mM phenyl phosphate/200 mM NaCl. Proteins were concentrated with centrifugal filter devices (microsep 10K; Pall Filtron Corp., East Hills, NY), separated by 10% SDS-PAGE, and stained with Coomassie blue. The 65-kD protein band was excised, subjected to in-gel digestion with lysyl endopeptidase C (Lys-C), and the resulting HPLC-purified peptides were sequenced (TOPLAB, Munich, Germany; see Fig. 1).

**cDNA Isolation.** Several independent clones of a 2-kb cDNA (GenBank accession no. Y17159) were isolated using a combination of standard PCR with primers derived from a partial cDNA sequence in GenBank (accession no. AJ222814) and 5' rapid amplification of cDNA ends (5'RACE; GIBCO BRL, Gaithersburg, MD) and completely sequenced.

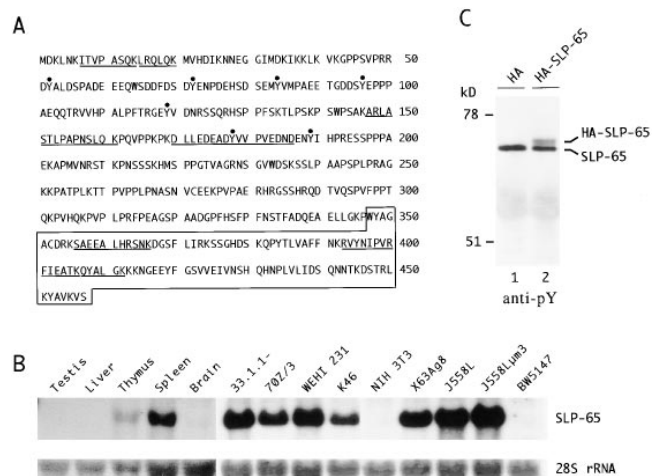
**Northern Blot Analysis.** As described previously (9), total cellular RNA was isolated, and 15  $\mu$ g per sample was separated in a denaturing agarose gel. After blotting, hybridization was performed with a randomly primed 795-bp PstI fragment from nucleotide 650 to 1445 (the open reading frame [ORF] extends from 478 to 1851).

**Transient Protein Expression.** For transient expression of SLP-65 in J558L $\mu$ m3 cells, a 1490-bp PCR fragment (amplified with PfuI [Stratagene Inc., La Jolla, CA]) containing the entire ORF was cloned downstream of the CMV promoter in pCGN (10).

The resulting expression vector, pCGN-p65, encodes a SLP-65 fusion protein containing an NH<sub>2</sub>-terminal, 20 amino acid peptide tag which is derived from the hemagglutinin protein of human influenza virus (HA tag [11]). The conditions for electroporation have been described previously (9).

## Results and Discussion

To identify PTK substrate proteins in B cells, we affinity purified these proteins from the lysate of pervanadate/H<sub>2</sub>O<sub>2</sub>-stimulated J558L $\delta$ m7.1 cells via antiphosphotyrosine antibody columns and determined their peptide sequence. We focused on a 65-kD protein which is the most rapidly phosphorylated substrate after exposure of J558L $\delta$ m7.1 cells to antigen or to pervanadate. Several of the sequenced peptides matched with a partial sequence in GenBank (accession no. AJ222814). Using mouse splenic cDNA as template, we used a combination of conventional and 5'RACE PCR to isolate  $\sim$ 2 kb of the corresponding cDNA (accession no. Y17159). The cDNA we obtained has an ORF encoding a protein of 457 amino acids (Fig. 1 A). Seven of the peptides sequenced from the purified protein were found in the predicted sequence (Fig. 1 A, *underline*). The NH<sub>2</sub>-terminal portion of the protein contains seven tyrosine residues which are putative targets for phosphorylation (Fig. 1 A, *dots*). Five of these are found in a YxxP context otherwise found in the PTK substrates Cas, p62dok, Cbl, and SLP-76 (12–15). The central part of the protein is

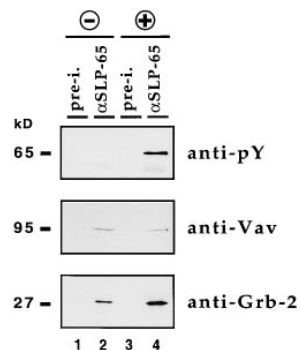


**Figure 1.** The SLP-65 gene encodes a protein with several regions of similarity to SLP-76 and is transcribed in lymphoid tissues. (A) Deduced amino acid sequence of murine SLP-65. *Dots*, Potential tyrosine-phosphorylation sites are marked. *Box*, The COOH-terminal SH2 domain. *Underline*, Peptide sequences obtained from affinity-purified SLP-65. (B) SLP-65 transcripts of  $\sim$ 2 kb were detected by Northern blot analysis of RNA from the indicated tissues and cell lines. The SLP-65 probe used was derived from the ORF, and a reference signal for ribosomal 28S RNA was used as a loading control. (C) Anti-SLP-65 precipitates were prepared from postnuclear supernatants of pervanadate/H<sub>2</sub>O<sub>2</sub>-stimulated J558L $\mu$ m3 cells, transiently transfected either with the parental vector (lane 1) or an expression vector encoding SLP-65 with an NH<sub>2</sub>-terminal HA tag (lane 2). Precipitated proteins were analyzed by antiphosphotyrosine (*anti-pY*) immunoblotting.

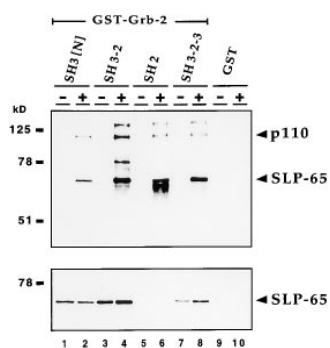
rich in prolines and contains several SH3 domain-binding motifs. The COOH-terminal part contains an SH2 domain which, when compared with the database, is most similar to the SH2 domain of SLP-76, a recently identified PTK substrate in T lymphocytes (15). Both proteins share the same overall structure. A comparison with human and mouse SLP-76 also reveals a modest similarity in the NH<sub>2</sub> terminus (not shown). We conclude that the 65-kD protein substrate is the B cell analogue of SLP-76, and call it SLP-65. In a Northern blot analysis, the 2-kb SLP-65 transcript is found only in spleen and weakly in thymus, but not in liver, testis, or brain (Fig. 1 B). The transcript is also found in B cell lines representing different developmental stages from the pre-B to the plasma cell stage, but not in a T cell or a fibroblast line (Fig. 1 B). These results suggest that SLP-65 is primarily expressed in B lymphocytes but perhaps to some extent also in thymocytes.

SLP-65 has a predicted pI of 8.2, in good agreement with our experimental data showing a pI of 8.5 (data not shown). The predicted molecular mass of 50.7 kD is considerably smaller than the apparent size of 65 kD in SDS-PAGE. To test whether this could be due to aberrant migration, and to obtain further evidence that we had indeed isolated the cDNA encoding the tyrosine-phosphorylated 65-kD substrate, we transiently expressed SLP-65 in J558L $\mu$ m3 cells as a fusion protein with an NH<sub>2</sub>-terminal HA tag (see Materials and Methods). Anti-SLP-65 peptide antibodies (see Materials and Methods) precipitated the HA-tagged fusion protein from pervanadate/H<sub>2</sub>O<sub>2</sub>-stimulated J558L $\mu$ m3 transfectants but not from control cells. The HA-tagged SLP-65 migrates slightly above the endogenous SLP-65 as detected by antiphosphotyrosine antibodies (Fig. 1 C) and anti-HA antibodies (data not shown). We conclude that the cDNA we isolated encodes the 65-kD substrate for activated PTKs.

In T cells, the SLP-76 adaptor protein is bound by Grb-2 and Vav (16–18). Anti-SLP-65 antibodies, but not the preimmune serum, coprecipitate Grb-2 and Vav from lysates of unstimulated and pervanadate/H<sub>2</sub>O<sub>2</sub>-stimulated J558L $\mu$ m3 cells (Fig. 2, lanes 2 and 4). The phosphorylated form of SLP-65 is only found in the lysates of pervanadate/H<sub>2</sub>O<sub>2</sub>-stimulated cells (Fig. 2, *top*, lane 4). These data show



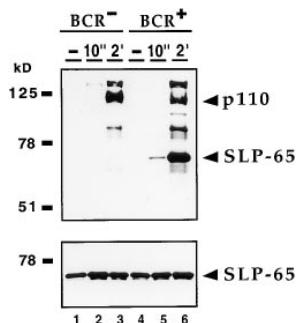
**Figure 2.** SLP-65 is constitutively associated with Vav and Grb-2. J558L $\mu$ m3 cells were left unstimulated (lanes 1 and 2) or stimulated for 2 min with 50  $\mu$ M pervanadate/H<sub>2</sub>O<sub>2</sub> (lanes 3 and 4). Cleared cellular lysates were subjected to immunoprecipitation with preimmune serum (*pre-i.*, lanes 1 and 3) or anti-SLP-65 antibodies (lanes 2 and 4). Tyrosine-phosphorylated SLP-65 was detected by antiphosphotyrosine immunoblotting (*anti-pY*, *top*). The Vav and Grb-2 proteins were detected by probing the same filter with anti-Vav (*middle*) and anti-Grb-2 antibodies (*bottom*).



**Figure 3.** The NH<sub>2</sub>-terminal SH3 domain of Grb-2 is sufficient to bind SLP-65. Grb2-binding proteins were purified from lysates of unstimulated (lanes 1, 3, 5, and 7) and pervanadate/H<sub>2</sub>O<sub>2</sub>-stimulated J558L $\mu$ m3 cells (lanes 2, 4, 6, and 8) using GST fusion proteins containing different domains of Grb-2: NH<sub>2</sub>-terminal SH3 domain (lanes 1 and 2), NH<sub>2</sub>-terminal SH3 plus SH2 domain (lanes 3 and 4), SH2 domain (lanes 5 and 6), and complete Grb-2 (lanes 7 and 8). GST was used as a control (lanes 9 and 10). Proteins were detected by antiphosphotyrosine and anti-SLP-65 immunoblotting (*top* and *bottom*, respectively).

that SLP-65 is constitutively associated with Grb-2 and Vav, supporting the notion that SLP-65 is the B cell analogue of SLP-76. Using different GST-Grb-2 fusion proteins, SLP-65 could be purified from lysates of unstimulated and pervanadate/H<sub>2</sub>O<sub>2</sub>-stimulated J558L $\mu$ m3 cells with the NH<sub>2</sub>-terminal SH3 domain of Grb-2 (Fig. 3, *bottom*, lanes 1 and 2) and to an even stronger extent with the combined SH3 and SH2 domains (Fig. 3, lanes 3 and 4). SLP-65 bound only weakly to the SH2 domain of Grb-2 and not at all to GST alone (Fig. 3, lanes 5, 6, 9, and 10). Probing the same blot with antiphosphotyrosine antibodies showed that, in addition to SLP-65, the Grb-2 SH3 domain coprecipitates a phosphoprotein of 110 kD. This protein may be identical or similar to the SLP-76-associated protein SLAP130/FYB (19, 20). These proteins, as well as other phosphoproteins, were more efficiently purified by the combination of the SH3 and SH2 domains of Grb-2 but not by GST alone (lanes 4 and 10). Two proteins with characteristics similar to SLP-65 have been also found in a human B cell line (21).

We have previously shown that after exposure to pervanadate, a 65-kD protein is rapidly phosphorylated only in BCR-positive cells (5). To verify that SLP-65 is identical to this early PTK substrate, we stimulated the BCR-negative J558L line and its BCR-positive transfectant J558L $\mu$ m3 for different times with pervanadate/H<sub>2</sub>O<sub>2</sub>. Similar amounts of SLP-65 were purified from these cells with the GST-Grb-2[SH3-SH2] fusion protein (Fig. 4, *bot-*



**Figure 4.** Tyrosine phosphorylation of SLP-65 is dependent on BCR expression. BCR-negative J558L cells (lanes 1–3) and BCR-positive J558L $\mu$ m3 cells (lanes 4–6) were unstimulated (lanes 1 and 4) or stimulated with 25  $\mu$ M pervanadate/H<sub>2</sub>O<sub>2</sub> either for 10 s (lanes 2 and 5) or 2 min (lanes 3 and 6). Proteins complexed with GST-Grb-2[SH3-SH2] fusion proteins were detected by antiphosphotyrosine and anti-SLP-65 immunoblotting (*top* and *bottom*, respectively).

tom, lanes 1–6). However, the SLP-65 protein remained completely unphosphorylated in the BCR-negative line, even after 2 min of stimulation (Fig. 4, top, lane 3). In the BCR-positive transfectant, SLP-65 is rapidly phosphorylated, 10 s after stimulation (lane 5). Other PTK substrate proteins in the BCR-negative and -positive lines require longer times of stimulation for detection with antiphosphotyrosine antibodies (Fig. 4, top, lanes 3 and 6). Thus, the rapid phosphorylation of SLP-65 in J558L $\mu$ m3 B cells is dependent on the BCR surface expression.

Our data suggest that SLP-65 is part of a BCR transducer complex comprising not only kinases, phosphatases, and their substrates, but also adaptor proteins which are necessary to physically and functionally connect these elements. This may be a common theme in signal transduction (6, 7). The early and BCR-dependent phosphoryla-

tion of SLP-65, together with its restricted expression pattern, suggest that SLP-65 is critically involved in coupling intracellular signaling elements specifically to the BCR. This may contribute to a basal level of signaling in the absence of antigen, a mechanism that may be necessary for B cell maintenance (22, 23). Such a central role in signal transduction is also suggested for SLP-76. Overexpression of SLP-76 in T cells can augment signal transduction from the TCR, leading to increased nuclear factor of activated T cells (NFAT) activation and IL-2 secretion (24, 25). Furthermore, mice deficient for SLP-76 contained no peripheral T cells as a result of an early block in T cell development, whereas B cells develop normally (26). To better understand the function of the SLP protein family, it will be necessary to reveal exactly how they are connected to the antigen receptors.

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