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SHORT COMMUNICATION

The SRC family kinase LYN redirects B cell receptor signaling in human *SLP65*-deficient B cell lymphoma cells

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SLP65 represents a critical component in (pre-) B cell receptor signal transduction but is compromised in a subset of pre-B cell-derived acute lymphoblastic leukemia. Based on these findings, we investigated (i.) whether SLP65-deficiency also occurs in mature B cell-derived lymphoma and (ii.) whether SLP65-deficient B cell lymphoma cells use an alternative B cell receptor signaling pathway in the absence of SLP65. Indeed, expression of SLP65 protein was also missing in a fraction of B cell lymphoma cases. While SLP65 is essential for B cell receptor-induced Ca^{2+} mobilization in normal B cells, B cell receptor engagement in SLP65-deficient as compared to SLP65-reconstituted B cell lymphoma cells resulted in an accelerated yet shortlived Ca^{2+}-signal. B cell receptor engagement of SLP65-deficient lymphoma cells involves SRC kinase activation, which is critical for B cell receptor-dependent Ca^{2+}-mobilisation in the absence but not in the presence of SLP65. As shown by RNA interference, the SRC kinase LYN is required for B cell receptor-induced Ca^{2+} release in SLP65-deficient B cell lymphoma cells but dispensable after SLP65-reconstitution. B cell receptor engagement in SLP65-deficient B cell lymphoma cells also resulted in tyrosine-phosphorylation of the proliferation- and survival-related MAPK1 and STAT5 molecules, which was sensitive to silencing of the SRC kinase LYN. Inhibition of SRC kinase activity resulted in growth arrest and cell death specifically in SLP65-deficient lymphoma cells. These findings indicate that LYN can short-circuit conventional B cell receptor signaling in SLP65-deficient B cell lymphoma cells and thereby promote activation of survival and proliferation-related molecules.
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Introduction

The linker molecule SLP65 (also known as BLNK (Fu *et al*., 1998), BASH (Hayashi *et al*., 2000) represents a critical component of the (pre-) B cell receptor signaling cascade (Wienands *et al*., 1998). Through a number of phosphorylated tyrosine residues, SLP65 can connect with upstream and downstream signaling molecules including SYK, BTK, NCK, VAV and PLCγ2 (Middendorp *et al*., 2003). These interactions are essential for the assembly of the signaling cascade ultimately leading to the release of Ca^{2+} from the endoplasmic reticulum (Fu *et al*., 1998). In the absence of SLP65, B cell development is arrested at the pro-B to pre-B cell transition (Minegishi *et al*., 1999; Pappu *et al*., 1999), which parallels complete breakdown of (pre-) B cell receptor signaling in SLP65-deficient B cells (Ishiai *et al*., 1999; Pappu *et al*., 1999; Hayashi *et al*., 2003). While one study reported normal expression of SLP65 in childhood acute lymphoblastic leukemia (Imai *et al*., 2004), recent work by us and others (Flemming *et al*., 2003; Hayashi *et al*., 2003; Jumaa *et al*., 2003; Kersseboom *et al*., 2003; Klein *et al*., 2004) indicated a role of SLP65 as a tumor suppressor in human and murine leukemia derived from pre-B cells. Based on these findings, we investigated (i.) whether SLP65-deficiency also occurs in mature B cell lymphoma and (ii.) whether SLP65-deficient B cell lymphoma cells use an alternative B cell receptor signaling pathway in the absence of SLP65.

Results and discussion

SLP65-deficiency in B cell lymphoma cells

Studying SLP65 expression in Non-Hodgkin's B cell lymphomas including small lymphocytic lymphoma/B-CLL, mantle cell lymphoma, follicular lymphoma, Burkitt's lymphoma, diffuse large B cell lymphoma (DLBCL) and plasmablastic B cell lymphoma (PBBCL) by reverse transcription transcription-PCR (RT-PCR) and Western blot, we found normal expression of SLP65 mRNA and protein in the majority of B cell lymphoma cases analysed (Figure 1).

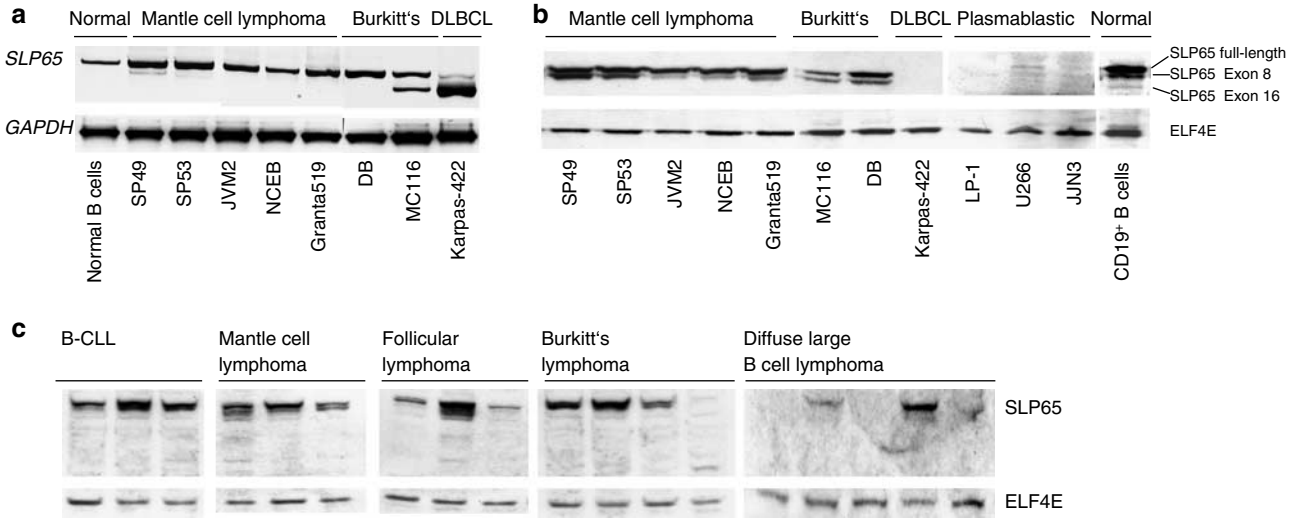

Figure 1 Analysis of SLP65 expression in B cell lymphoma cells. SLP65 mRNA (**a**) and protein (**b**) expression in normal B cells and B cell lymphoma cells were analysed by RT-PCR and Western blot. SLP65 and GAPDH transcripts were amplified as previously described (Klein *et al*., 2004) using the primers listed in Supplementary Table 2. $CD19^+$ B cells were purified from peripheral blood of four healthy donors using immunomagnetic beads against CD19 (Miltenyi Biotech, Bergisch Gladbach, Germany). For Western blot analysis of SLP65 expression, total cell lysates of B cell lymphoma cell lines and normal B cells (**b**) and primary cases of B cell lymphoma (**c**) were used together with antibodies against the N-terminus of human SLP65 and ELF4E (Cell Signaling Technology, Beverly, MA). ELF4E was used as a loading control.

Sequence analysis revealed that truncated SLP65 transcripts amplified do not encode functional SLP65 proteins because of introduction of pre-terminal translation stops or deletion of tyrosine residues that are important for SLP65 function (Supplementary Table 1; sequence data available from EMBL/GenBank under accession numbers AM180327-AM180346). Two other SLP65 mRNAs lacking exons 8 or 16 were amplified from various B cell lymphoma samples. SLP65 mRNA lacking exon 8 was previously described and termed BLNK-S (Fu *et al*., 1998). However, these SLP65 splice variants cannot be attributed to malignant transformation because they were also amplified from $CD19^+$ peripheral blood B cells purified from four healthy donors (Supplementary Table 1, Figure 1).

Protein expression of SLP65 was missing in 3 of 11 cell lines (Figure 1b) and 4 of 18 primary cases of B cell lymphoma (Figure 1c). In conclusion, SLP65-deficiency does not only occur in B cell precursor leukemia but also lymphoma derived from mature B cells.

In one case (DLBCL cell line, Karpas-422), aberrant splicing of SLP65 was the result of a deletion of the 3′ splice site of exon 3 of the *SLP65* gene. Due to a 28-bp deletion of the 3′ part of exon 3 and the 5′ part of intron 3–4, full-length SLP65 can no longer be expressed from this allele (Figure 1b; sequence data available from EMBL/GenBank under accession number AM180347). Of note, the second *SLP65* allele in these DLBCL cells was lost due to a large deletion encompassing 10q24.1 (Stefan Geske and R. S., unpublished FISH data). Chromosomal deletion and loss of heterozygosity by somatic mutation is consistent with a role of *SLP65* as a tumor suppressor gene in these DLBCL cells. In two plasmablastic B cell lymphoma (PBBCL) cell lines (LP-1 and JJN3), SLP65 protein expression was missing (Figure 1b) despite expression of regularly spliced SLP65 transcripts. Studying genomic DNA of these cell lines, no mutations or deletions within the SLP65 coding region and splice sites were found (not shown).

Because SLP65 is essential for B cell receptor signal transduction in normal B cells, we investigated the consequences of SLP65-deficiency on B cell receptor signal transduction in two B cell lymphoma cell lines.

LYN-dependent B cell receptor signaling in SLP65-deficient B cell lymphoma cells

Unexpectedly, B cell receptor-stimulation in two SLP65-deficient B cell lymphoma cell lines excited a very vigorous yet shortlived Ca^{2+}-signal despite SLP65-deficiency of these cells (Figure 2a, d and e). In comparison to normal B cells, Ca^{2+}-release in response to B cell receptor engagement in SLP65-deficient lymphoma cells occurred after a substantially reduced lag-phase. Also the signal amplitude was higher in SLP65-deficient lymphoma cells as compared to normal B cells (Figure 2a, d and e), but the signal was very unstable in the SLP65-deficient lymphoma cells and returned to baseline levels within 90 s whereas elevated cytoplasmic Ca^{2+}-levels were maintained in normal B cells for more than six minutes (Figure 2a). These findings suggest that an alternative SLP65-independent pathway may link the B cell receptor to Ca^{2+} mobilization in diffuse large B cell lymphoma cells. Given that the SLP65-deficient lymphoma cell lines express the SRC kinase LYN, FYN and BLK (not shown) and

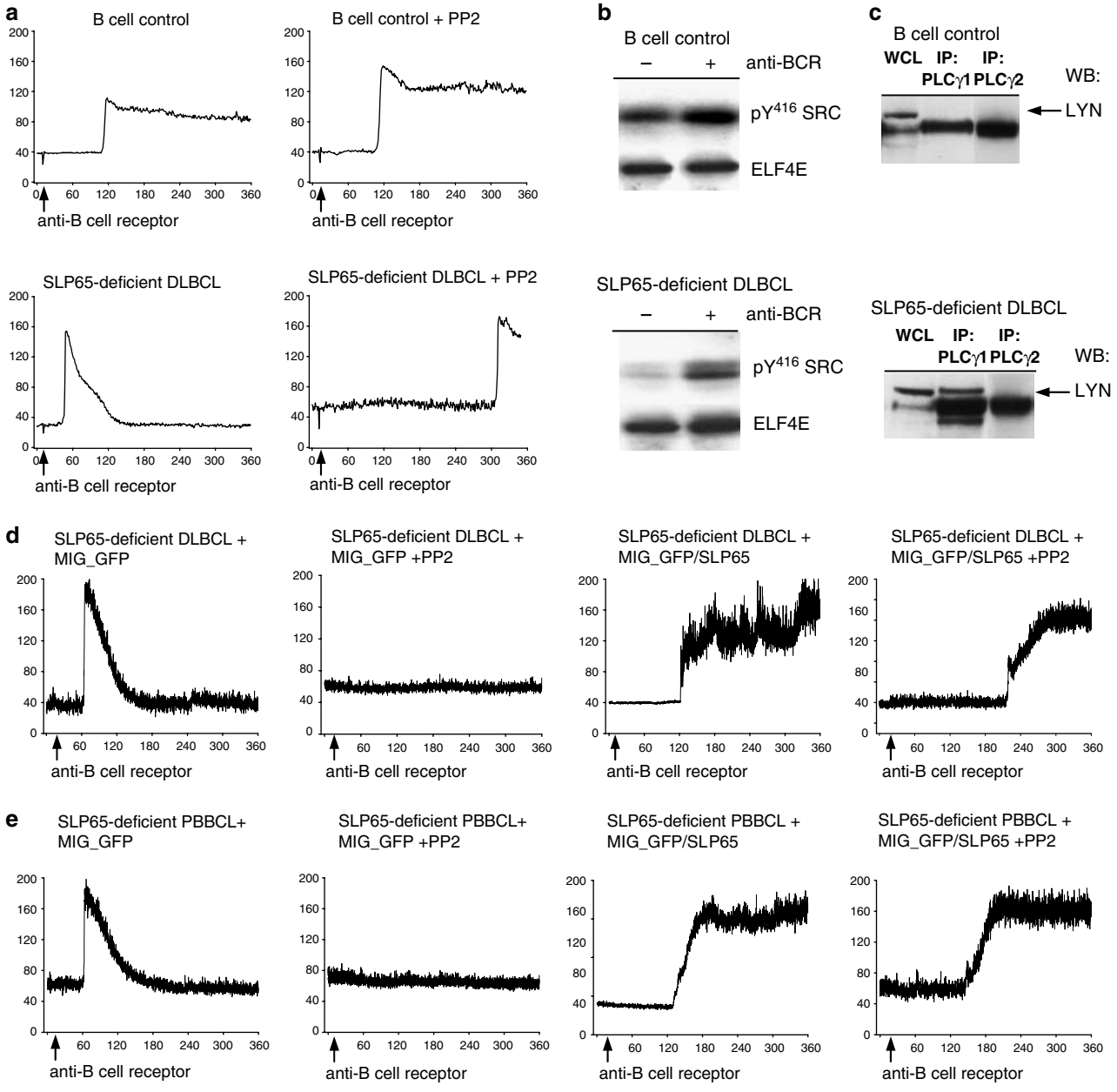

Figure 2 SRC kinase-dependent B cell receptor signaling in SLP65-deficient B cell lymphoma cells. Normal B cells or SLP65-deficient diffuse large B cell lymphoma cells were incubated in the presence or absence of 10 μmol/l of the SRC-kinase inhibitor PP2 (Calbiochem, Bad Soden, Germany) for 24 h prior to Ca^{2+}-measurement (**a**). After pre-incubation, cells were washed and stained with Fluo-3 dye (Calbiochem) for 30 min. Changes of cytosolic Ca^{2+} upon B cell receptor engagement were measured by laser scans using confocal microscopy as previously described (Klein *et al*., 2004). After 30 s of measurement, antibodies against the B cell receptor (Jackson Immunoresearch, West Grove, PA) were added. To measure changes of tyrosine-phosphorylation upon B cell receptor (BCR)-stimulation at a common activation-motif of SRC-kinases (Y416), cell lysates of normal B cells and SLP65-deficient DLBCL cells were analysed by Western blot using phosphotyrosine-specific antibodies against Y416 SRC-kinases (**b**; Cell Signaling Technology). To measure potential interactions between the SRC kinase LYN and tyrosine-phosphorylated PLCγ1 and PLCγ2 in SLP65-deficient and SLP65-expressing B cell lymphoma cells (**c**), whole cell lysates (WCL) were generated and subjected to immunoprecipitation of phosphorylated PLC$\gamma1^{Y783}$ (IP: PLCγ1) or PLC$\gamma2^{Y1217}$ (IP: PLCγ2) as previously described (Feldhahn *et al*., 2005). Thereafter, immunoprecipitated proteins were analysed for the presence of LYN by Western blotting. As a control, normal B cells were used. (**d**–**e**) To analyse the relationship between SRC kinase- and SLP65-dependent signaling pathways, SLP65-deficient DLBCL cells and SLP65-deficient plasmablastic B cell lymphoma cells were transfected with a MIG-GFP or a MIG-GFP/SLP65 vector and incubated in the presence or absence of 10 μmol/l of the SRC-kinase inhibitor PP2. Cells were stimulated with an anti-B cell receptor antibody (arrows) and cytoplasmic Ca^{2+} levels were measured as previously described (Reppel *et al*., 2005).

exhibit activating tyrosine-phosphorylation of SRC-kinases in response to B cell receptor engagement (Figure 2b), we tested whether SRC-kinase activity contributed to B cell receptor-dependent Ca^{2+}-release in these cells. While global inhibition of SRC-kinase activity by PP2 had little effect in normal B cells, B cell receptor-dependent Ca^{2+}-release was completely abolished by PP2 in SLP65-deficient lymphoma cells (Figure 2a, d and e). Only few PP2-treated lymphoma cells responded to B cell receptor engagement at all and with a delay of more than five minutes (Figure 2a).

Consistent with an alternative SRC kinase-dependent pathway of Ca^{2+} mobilization in SLP65-deficient B cell lymphoma cells, we identified the SRC kinase LYN co-immunoprecipitating with tyrosine-phosphorylated PLCγ1, which generates IP3 acting as a ligand for Ca^{2+} channels of the endoplasmic reticulum. Surprisingly, the B cell-specific PLCγ isozyme PLCγ2, which binds to SLP65 in normal B cells, does not interact with LYN (Figure 2c). Conversely, an interaction between LYN and PLCγ1 was not found in normal B cells (Figure 2c), suggesting that this interaction is specific for SLP65-deficient lymphoma cells. However, in a larger panel of SLP65-expressing B cell lymphomas and pre-B cell leukemias, we also identified LYN-PLCγ1 interactions in some SLP65-expressing pre-B cell leukemia cell lines (not shown). These findings indicate that SRC kinase (LYN)- and SLP65-dependent signaling pathways are not mutually exclusive.

Therefore, we tested the relationship between SRC kinase- and SLP65-dependent B cell receptor signaling in SLP65-deficient B cell lymphoma in more detail: Two SLP65-deficient B cell lymphoma cell lines were transfected with a control vector or a vector encoding wildtype SLP65 (Figure 2d and e). Control- and SLP65-transfectants were cultured in the presence or absence of the SRC kinase inhibitor PP2 and B cell receptor responsiveness was measured as Ca^{2+} release after stimulation with anti-B cell receptor antibodies. In the absence of SLP65-expression, both lymphoma cell lines exhibited an atypical Ca^{2+} signal in response to B cell receptor engagement, which was entirely abolished by SRC-kinase inhibition through PP2. Upon reconstitution of SLP65 in the lymphoma cell lines, however, several aspects of normal B cell receptor signaling were restored: as opposed to SLP65-deficient lymphoma cells, the signal was retarded but stable for more than four minutes and no longer sensitive to SRC-kinase inhibition by PP2 (Figure 2d and e).

As PP2 blocks activity of all SRC family kinases, we investigated the specific contribution of the SRC-family kinase LYN, which we found in complex with PLCγ1 (Figure 2c). To this end, normal B cells and SLP65-deficient diffuse large B cell lymphoma cells were transfected with fluorochrome-labeled non-targeting RNAs (siRNAs) and a mixture of three fluorochrome-labeled LYN-targeting siRNA duplices by nucleofection. After repeated nucleofection, the successfully transfected cells were sorted based on uptake of fluorochrome-labeled siRNAs. Efficiency of RNA interference-mediated silencing of LYN was verified by semiquantitative RT-PCR (Figure 3a).

Measurement of Ca^{2+}-release in response to B cell receptor engagement among the transfected cells showed that RNA interference with LYN expression greatly diminished Ca^{2+}-release in SLP65-deficient B cell lymphoma but not in normal B cells (Figure 3b). To test whether SLP65- and LYN-dependent signals are components of mutually exclusive signaling pathways, B cell receptor-dependent Ca^{2+}-release was also measured in SLP65-reconstituted B cell lymphoma cells that were transfected with non-targeting siRNA or siRNA duplices specific for LYN. Reconstitution of SLP65-expression in the B cell lymphoma cells did not only restore the Ca^{2+}-signal kinetics observed in normal B cells (i.e. a sustained signal at a lower amplitude and a longer lag-phase; Figure 3b). Re-expression of SLP65 also rendered B cell receptor-dependent Ca^{2+}-release in the B cell lymphoma insensitive to LYN-inhibition by siRNAs as observed in normal B cells (Figure 3b). These findings suggest that the B cell receptor in SLP65-deficient DLBCL cells signals by default through a pathway dependent on the SRC-kinase LYN.

Taken together, we propose that SLP65-deficient B cell lymphoma cells use an alternative, likely aberrant, B cell receptor signaling pathway that short-circuits SLP65 and potentially other SLP65-binding molecules like BTK and PLCγ2. This alternative B cell receptor signaling pathway is dependent on the SRC kinase LYN and involves tyrosine phosphorylation of PLCγ1. In the absence of SLP65, LYN together with PLCγ1 can maintain the capacity of the B cell receptor to mobilize Ca^{2+} from cytoplasmic stores.

LYN is required for B cell receptor-induced tyrosine phosphorylation of signal transducer and activator of transcription5 and mitogen activated protein kinase1 in SLP65-deficient B cell lymphoma cells

We next investigated, whether LYN-dependent B cell receptor signaling in SLP65-deficient lymphoma cells can promote survival and proliferation. In normal B cells, B cell receptor engagement results in activation of STAT5 (Karras *et al*., 1996) and mitogen activated protein kinases (MAPK; Richards *et al*., 2001), which induce survival and proliferation, respectively. Likewise, B cell receptor engagement induced tyrosine phosphorylation of STAT5 and MAPK1 in two SLP65-deficient and one SLP65-expressing B cell lymphoma cell lines (Figure 4). To determine whether LYN is required for B cell receptor-induced STAT5 and MAPK1 activation in the absence of SLP65, SLP65-deficient and SLP65-expressing B cell lymphoma cells were transfected either with non-targeting fluorochrome-labeled siRNAs or siRNA duplices against LYN.

Comparing transfected and non-transfected cells, non-targeting siRNAs had no effect. However, inhibition of LYN significantly diminished both STAT5- and MAPK1 activation in response to B cell receptor engagement in two SLP65-deficient B cell lymphoma cell lines but had no significant effect on SLP65-expressing B cell

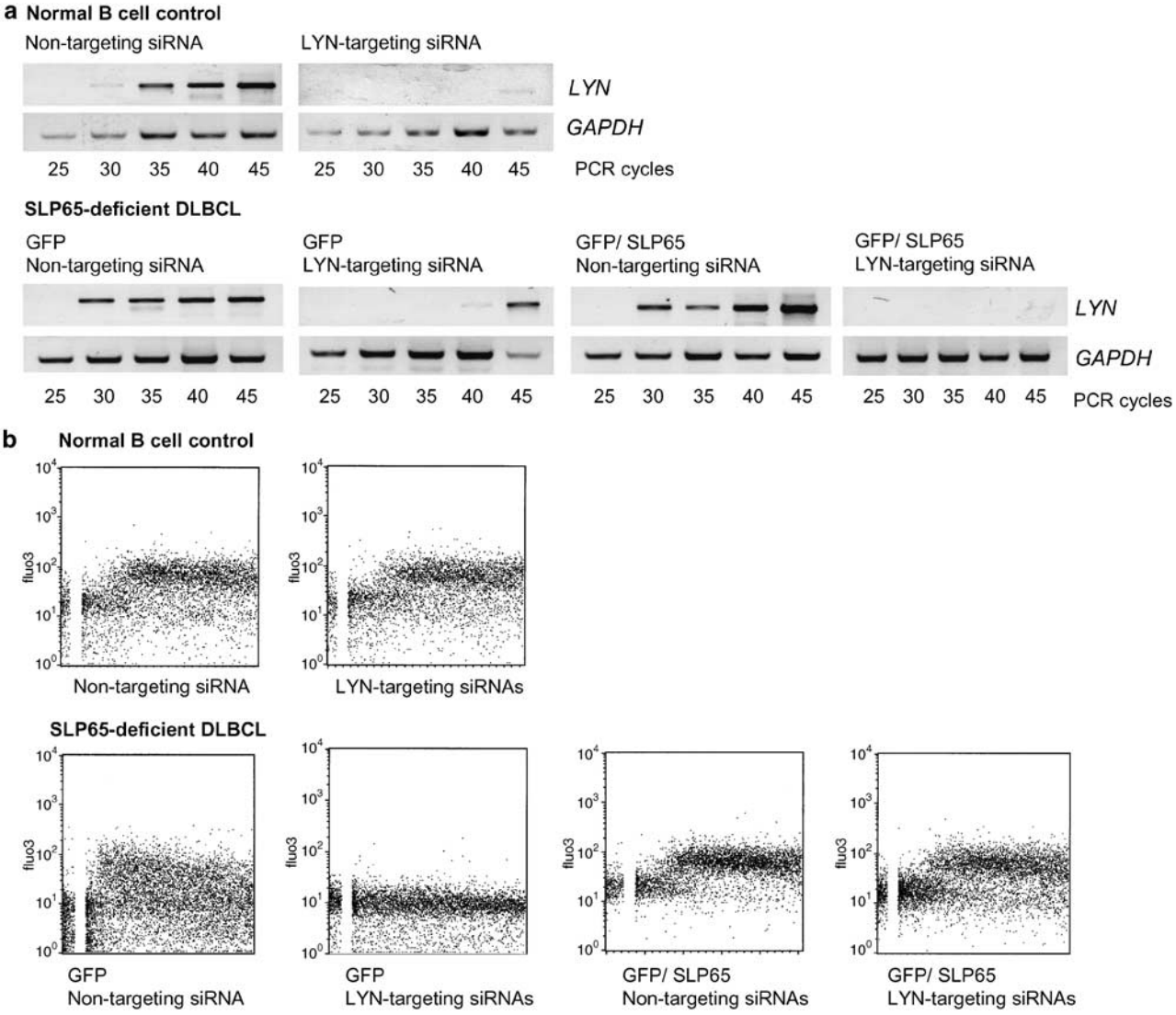

Figure 3 B cell receptor signaling in DLBCL cells in the presence or absence of LYN and SLP65. Normal B cells, SLP65-deficient DLCBL cells and SLP65-reconstituted DLCBL cells were transfected with LYN-targeting siRNAs by nucleofection (Amaxa Biosystems, Cologne, Germany). Three siRNA duplices were synthesized for RNA interference with LYN expression (MWG Biotech, Ebersberg, Germany; Supplementary Table 2). As a control, a non-targeting siRNA duplex was used that does not match a known mRNA sequence (Supplementary Table 2). All siRNA duplices were labeled with CY3 using an siRNA labeling kit (Ambion, Austin, TX) according to the manufacturer's protocol. DLBCL or normal B cells were transfected with a mixture of the three CY3-labeled siRNAs or the non-targeting siRNA at a concentration of 50 nmol/l each by nucleofection (Amaxa Biosystems): Briefly, for each cell line 5×10^6 cells were resuspended in 100 μl nucleofector-solution (Amaxa Biosystems) and 5 μg of plasmid DNA were subsequently added for electroporation. The cells were cultured for 24 h and nucleofection was repeated. After 24 h, CY3-positive cells were sorted under sterile conditions using a FACStar 440 cell sorter and either kept under cell culture conditions or subjected to RNA isolation for RT-PCR analysis. Transfection efficiency was controlled by FACS. The silencing effect of siRNAs for LYN was controlled by RT-PCR analysis of LYN mRNA levels (**a**) using the primers listed in Supplementary Table 1. SLP65-deficient B cell lymphoma cells were transfected with MIG-GFP/SLP65 and a MIG-GFP vector as a control using a nucleofection system according to the manufacturers' protocol (Amaxa Biosystems). GFP-positive cells were sorted under sterile conditions using a FACStar 440 cell sorter. Changes of cytosolic Ca^{2+} were measured by flow-cytometry. After 20 s of measurement, antibodies against the B cell receptor were added (**b**).

lymphoma cells (Figure 4). Of note, these B cell lymphoma cells express also other SRC kinases besides LYN including FYN and BLK (not shown). Therefore, these findings indicate that among SRC kinases, LYN has an important contribution to survival and proliferation signaling in SLP65-deficient B cell lymphoma cells.

SRC kinase activity contributes to survival and proliferation signals in SLP65-deficient B cell lymphoma cells

To directly compare the contribution of SRC-kinases to survival signaling in SLP65-deficient versus SLP65-expressing B cell lymphoma cells, we incubated two SLP65-deficient and three SLP65-expressing B cell

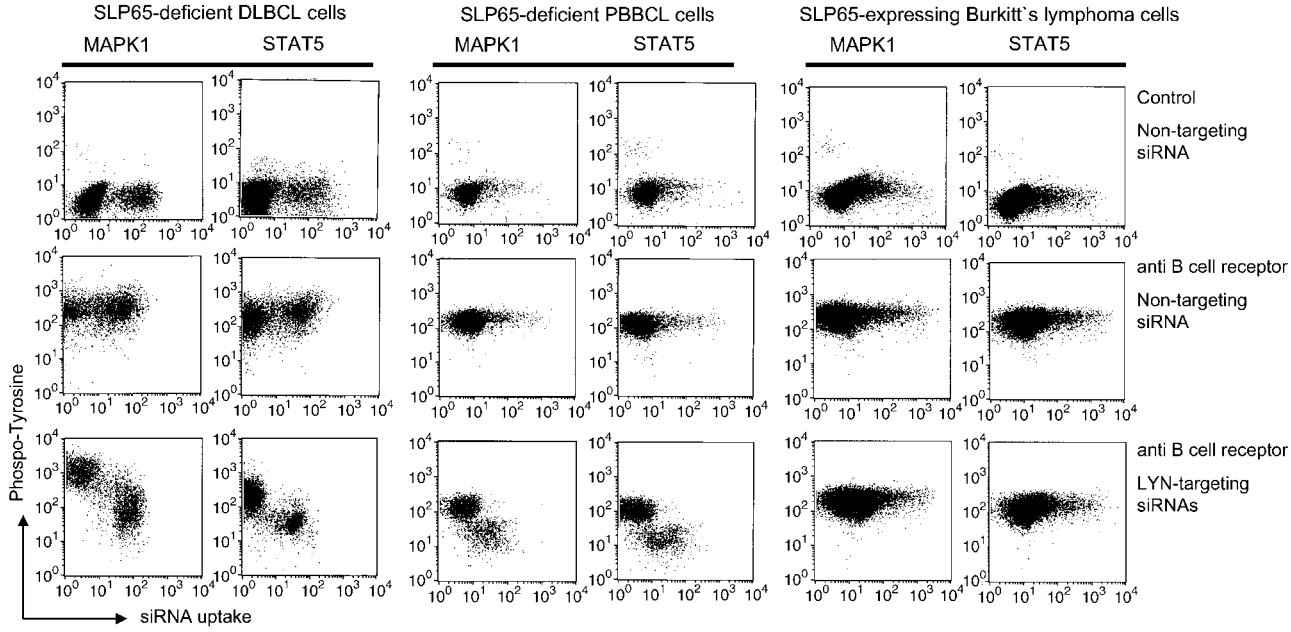

Figure 4 LYN is required for tyrosine-phosphorylation of STAT5 and MAPK1 in SLP65-deficient B cell lymphoma cells. SLP65-deficient diffuse large B cell lymphoma cells, SLP65-deficient plasmablastic lymphoma cells and SLP65-expressing Burkitt's lymphoma cells were transfected either with non-targeting fluorochrome-labeled siRNAs or siRNAs against LYN. The cells were analysed for uptake of CY3-labeled siRNAs and STAT5- and MAPK1-tyrosine phosphorylation in response to B cell receptor engagement. Cytoplasmic staining of phosphorylated $STAT5^{Y694}$ and $MAPK1^{Y204}$ was performed using primary antibodies against these phosphotyrosines (Cell Signaling Technology together with anti-mouse IgG-CY2 and anti-rabbit IgG-CY2 (Jackson Immunoresearch) as secondary antibodies, respectively. Cells were fixed with 0.4% para-formaldehyde and incubated for 10 min in 90% methanol on ice and analyzed by flow-cytometry.

lymphoma cell lines in the presence or absence of the SRC kinase inhibitor PP2 for ten days. In one set of experiments, proliferation of SLP65-deficient and SLP65-expressing lymphoma cells was measured by CSFE-labeling and counting of viable cells in the cell cultures in the presence or absence of PP2. SRC-kinase inhibition had only a mild and transient effect on the number of cell divisions (CSFE labeling (Cooperman *et al*., 2004)) and the growth of viable cells (counting; Supplementary Figure 1A and B) in SLP65-expressing lymphoma cells. In contrast, SRC kinase inhibition resulted in growth arrest in two SLP65-deficient B cell lymphoma cell lines (Supplementary Figure 1A and B).

Likewise, contribution of SRC-kinase activity to viability of SLP65-deficient versus SLP65-expressing lymphoma cells was compared. Viability of the cells (as defined by exclusion of apoptotic or dead cells by staining for annexin V and propidium iodide uptake) was measured as the ratio of the precentage of living cells at specific incubation times and the percentage of living cells at the outset (Supplementary Figure 1C). While SRC-kinase inhibition had no significant effect on SLP65-expressing lymphoma cells, more than 50 percent of cells among the two SLP65-deficient lymphoma cell lines were apoptotic after one week of incubation in the presence of PP2 (Supplementary Figure 1C).

SRC-kinase-dependent activation of survival and proliferation signals in B cell lymphoma is in agreement with previous studies that implicate the SRC-kinase LYN in anti-apoptotic signaling in B cell chronic lymhocytic leukemia (Contri *et al*., 2005) and Philadelphia chromosome-positive acute lymphoblastic leukemia (Hu *et al*., 2004; Ptasznik *et al*., 2004). Also LYN-dependent activation of the proliferation-related MAPK1 in SLP65-deficient lymphoma cells is in accordance with two previous studies showing that LYN is essential for proliferation of lymphoma cells either driven by the Karposi sarcoma-associated herpesvirus K1 protein (Prakash *et al*., 2005) or by interleukin 6 in multiple myeloma (Li *et al*., 2005).

Consistent with an emerging role of LYN in malignant B cell lymphoproliferation (Hu *et al*., 2004; Ptasznik *et al*., 2004; Contri *et al*., 2005; Li *et al*., 2005; Prakash *et al*., 2005), these findings identify the SRC-kinase LYN as an important component of a novel aberrant signaling pathway in SLP65-deficient B cell lymphoma cells.

Abbreviations

B-CLL, B cell chronic lymphocytic leukemia; DLBCL, diffuse large B cell lymphoma; *IGH*, immunoglobulin heavy-chain; PBBCL, plasmablastic B cell lymphoma; SH2, SRC homology domain 2; siRNA, short interfering RNA; SLP65, SH2 domain-containing leukocyte protein of 65 kD.

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Supplementary Information accompanies the paper on Oncogene website (http://www.nature.com/onc)