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Identification of a Pre-BCR Lacking Surrogate Light Chain

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Abstract

SLP-65$^{-/-}$ pre-B cells show a high proliferation rate in vitro. We have shown previously that λ5 expression and consequently a conventional pre-B cell receptor (pre-BCR) are essential for this proliferation. Here, we show that pre-B cells express a novel receptor complex that contains a μ heavy chain (μHC) but lacks any surrogate (SL) or conventional light chain (LC). This SL-deficient pre-BCR (SL^{-}pre-BCR) requires Ig-α for expression on the cell surface. Anti-μ treatment of pre-B cells expressing the SL^{-}pre-BCR induces tyrosine phosphorylation of substrate proteins and a strong calcium (Ca^{2+}) release. Further, the expression of the SL^{-}pre-BCR is associated with a high differentiation rate toward κLC-positive cells. Given that B cell development is only partially blocked and allelic exclusion is unaffected in SL-deficient mice, we propose that the SL^{-}pre-BCR is involved in these processes and therefore shares important functions with the conventional pre-BCR.

Key words: B cell development • adaptor • signaling • proliferation • receptor

Introduction

The development of B cells is a highly regulated process that can be divided into distinct stages according to the expression of various surface markers and the recombination status of the heavy chain (HC) and light chain (LC) genes (1, 2). The recombination of the HC locus is initiated in pro-B cells (c-kit^{+}B220^{+}CD43^{+}), which represent the earliest distinguishable B cell population (3). Productive recombination of the HC locus results in μHC expression, which pairs with the SL chain and the signaling components Ig-α/Ig-β to form the pre-BCR on the cell surface (4, 5). The SL chain consists of the noncovalently associated VpreB and λ5 polypeptides that have sequence and presumed structural homology to the Ig V- and C-type domains, respectively (6). Successful LC recombination and the subsequent association between HC and LC proteins lead to the expression of the B cell receptor (BCR) complex on the cell surface and the production of immature B cells that leave the BM and continue differentiation to become mature B cells (7, 8). Binding of the BCR to its specific ligand is an essential requirement for the selection and activation of immature and mature B cells, respectively (9). Although it is still unclear whether ligand binding is required for proper pre-BCR function, recent results indicate that the pre-BCR can react with ligand molecules on stromal cells (10–12). Whereas the biological role of this pre-BCR ligand interaction is not clear at present, it is established that pre-BCR expression is crucial for pre-B cell proliferation and differentiation and therefore represents a key checkpoint in B cell development (13–15). For instance, B cell development is completely blocked at the pro-B cell stage in mice deficient for Ig-α or Ig-β (16, 17). In μMT mice that lack the transmembrane region of the μ chain, a complete block of B cell development and a loss of allelic exclusion were reported (18, 19). Mice deficient for the SL component λ5, VpreB, or both showed an incomplete block of B cell development but intact allelic exclusion (20–22). Since the conventional pre-BCR cannot be expressed in both the μMT mice and the SL-deficient mice, it was suggested that prematurely expressed LCs allow the expression of the μHC on the surface of SL-deficient pre-B cells (23).

Pre-BCR engagement results in activation of protein tyrosine kinases (PTKs) of the Src, Syk, and Tec families that mediate the phosphorylation of substrate proteins (24). One major substrate of Syk is the adaptor protein SLP-65, which is also known as BLNK or BASH (25–27). Phosphorylated SLP-65 is a central modulator of Ca^{2+} response

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Abbreviations used in this paper: BCR, B cell receptor; HC, heavy chain; LAT, linker for activation of T cells; LC, light chain; PLC, phospholipase C; PTK, protein tyrosine kinase; SL, surrogate light chain.

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that couples Syk and the Tec family kinase Btk to phospholipase C (PLC)-γ2 (28–30). After phosphorylation by Syk and Btk, PLC-γ2 is activated and generates the second messenger inositol 1,4,5-trisphosphate which leads to Ca^{2+} release from intracellular stores (31). Mice deficient for SLP-65 show a partial block of B cell development at the pre-B cell stage and, due to a defect of receptor down-regulation pre-B cells from these mice express large amounts of the pre-BCR on their surface (32–34). Here, we exploit the increase of μHC expression on the surface of SLP-65/λ5 double mutant (SLP-65/$\lambda5^{-/-}$) pre-B cells to characterize a novel pre-BCR complex that is expressed without any surrogate or conventional LCs.

Materials and Methods

Mice. Ig-$\alpha^{-/-}$, $\lambda5^{-/-}$, and SLP-$65^{-/-}$ mice were generated as described previously (17, 20, 32). Single deficient mice were crossed to generate the SLP-65/$\lambda5^{-/-}$ and SLP-65/Ig-$\alpha^{-/-}$ mice. All animal experiments were performed in compliance with guidelines of the German law and the MPI for Immunobiology.

Cell Purification, Cell Culture, and Cell Lines. Cell suspension was prepared from the murine BM and cultured in Iscove's medium containing 10% FCS (Vitromex), 100 U/ml penicillin, 100 U/ml streptomycin (GIBCO BRL), 5×10^{-5} M 2-ME, and IL-7 as described previously (35). The SLP-65/$\lambda5^{-/-}$ pre-B cell line mcR2 was established by culturing BM cells in IL-7–supplemented medium for extended times (>6 mo). The pre-B cell lines Dec and Oct were derived from SLP-$65^{-/-}$ mice and the pro-B cell line from SLP-65/Ig$\alpha^{-/-}$ mice.

Flow Cytometry. Aliquots of single cell (10^6) suspensions from cell culture was stained for FACS® analysis (FACSCalibur; Becton Dickinson) using FITC–, cy5– or biotin–anti-IgM (μ chain specific; Southern Biotechnology and Dianova), biotin–anti-IgM^a (Becton Dickinson), FITC– or biotin–anti-κ (Southern Biotechnology), FITC–anti-λ (Southern Biotechnology), and streptavidin-cy5 (Dianova). Anti–mouse surrogate LC (SL-156), anti-VpreB (Vp245), and anti-λ5 (LM34) rat mAbs were gifts from A.G. Rolink (University of Basel, Basel, Switzerland).

Retroviral Constructs and Transduction. Retroviral transductions were performed as described previously (36). pMOWS-λ5 vector encoding murine λ5 was used for transfection performed in the Phoenix™ retroviral producer cell line using GeneJuice™ (Novagen) according to manufacturer's instructions. For transduction, pre-B cells derived from the BM of SLP-65/$\lambda5^{-/-}$ mice, which were cultured for 2–3 d were mixed with viral supernatants and centrifuged at 1,800 rpm at 37°C for 3 h. Transduction efficiency, measured 1 d later, was between 1 and 10%.

Southern Blotting. Genomic DNA was digested with EcoRI at 37°C overnight. 10 μg of DNA was loaded, separated in 0.8% TAE-agarose gel, and blotted to Nylon membrane (PerkinElmer). The membrane was hybridized overnight with a P^{32}-labeled probe corresponding to genomic DNA sequences downstream of JH4 at the μ locus as described previously (34). The results were visualized by the development with Hyperfilm™ MP high performance autoradiography film (Amersham Biosciences).

In Vitro Differentiation Assay. BM-derived cells from SLP-$65^{-/-}$ and SLP-65/$\lambda5^{-/-}$ mice were cultured for 1 wk with IL-7 and then divided into two pools, one cultured continuously with IL-7 and the other without IL-7 for 2 d. The differentiated B cells were analyzed by FACS® using anti-μHC and anti-κLC stain.

Surface Biotinylation, Immune Purification, and Western Blot Analysis. SLP-65/Ig-$\alpha^{-/-}$ pre-B cells, SLP-65/$\lambda5^{-/-}$ pre-B cells (mcR2), SLP-$65^{-/-}$ (Dec) pre-B cells, J558LμM3 (surface μHC and λLC positive), and WEHI-231 (surface μHC and kLC positive) were used in biotinylation experiments of surface cellular proteins. Cells (2×10^7) were washed twice with ice cold PBS. The cell pellets were resuspended in EZ-Link™ Sulfo-NHS-Biotin (Pierce Chemical Co.) at 1 μg/ml in PBS and incubated on ice for 30 min. After PBS washing, the cell pellets were lysed in ice cold digitonin lysis buffer (50 mM Tris-HCl, pH 7.4; 0.5% digitonin [Sigma-Aldrich], 137.5 mM NaCl, 1% glycerol, 1 mM Na-orthovanadate, 0.5 mM EDTA, pH 8, and protease inhibitor cocktail [Sigma-Aldrich]) and kept on ice for 15 min. After 15 min centrifugation at 14,000 rpm and 4°C, the supernatant was collected. μHC and μHC-associated proteins were immune purified from the supernatant by incubation with goat anti–mouse IgM (μ chain specific; Southern Biotechnology) plus protein G sepharose beads (Amersham Biosciences) at 4°C overnight. Purified proteins were washed with PBS or lysis buffer, subjected to 12% SDS-PAGE, and blotted to Immobilon™ PVDF transfer membrane (Millipore). The biotinylated proteins were revealed by streptavidin-HRPO (Pierce Chemical Co.) following ECL developmental system™ (Amersham Biosciences) according to manufacturer's instructions. μHC, κLC, and λLC were detected with the same antibodies used for FACS® analysis. Ig-α was detected with rabbit polyclonal antibody (a gift from J.C. Cambier, Integrated Department of Immunology, Jewish Medical and Research Center, and University of Colorado Health Sciences Center, Denver, CO).

Cell Stimulation. Cell pellets were incubated with 20 μg/ml of goat anti–mouse IgM or goat anti–mouse kappa (Southern Biotechnology) at 37°C for 2 min. Stimulation was stopped by the addition of ice cold PBS, and after centrifugation the cell pellets were lysed in ice cold lysis buffer (50 mM Tris-HCl, pH 7,4); 1% *n*-octyl-β-D-glucopyranosid [Applichem], 137.5 mM NaCl, 1% glycerol, 1 mM Na-orthovanadate, 0.5 mM EDTA, pH 8, supplemented with protease inhibitor cocktail as described above). Tyrosine-phosphorylated cellular proteins were immune purified from the total cellular lysates by incubation with antiphosphotyrosine antibody 4G10 (Upstate Biotechnology) at 4°C overnight. The purified proteins were subjected to 10% SDS-PAGE, revealed by antiphosphotyrosine antibody (4G10, Upstate Biotechnology) followed by HRPO-labeled anti–mouse antibody (Pierce Chemical Co.) and developed as described above.

Ca^{2+} Mobilization. Cells (5×10^6) resuspended in Iscove's medium containing 1% FCS were incubated with 5 μg/ml of Indo-1 a.m. (Molecular Probes) and 0.5 μg/ml of pluronic F-127 (Molecular Probes) at 37°C for 45 min. The cell pellets were then resuspended in Iscove's medium (containing 1% FCS) and kept on ice. Ca^{2+} response was induced by the addition of goat anti–mouse IgM (Southern Biotechnology) at a final concentration of 20 μg/ml or anti–mouse kappa (Southern Biotechnology) at the same concentration as a control.

Results

SLP-65/$\lambda5^{-/-}$ Pre-B Cells Express a Novel μ-Containing Receptor. BM-derived SLP-$65^{-/-}$ pre-B cell cultures repeatedly contained cell populations that were positive for μHC but negative for conventional LCs and the antibody SL-156 recognizing λ5 in association with μ (not depicted). We postulated that μHC might be expressed on

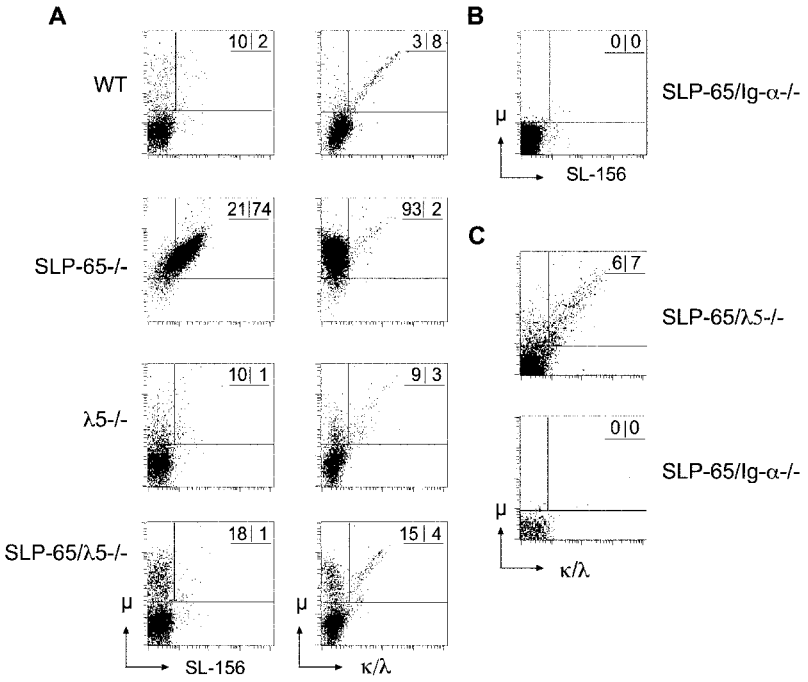

Figure 1. Pre-B cells with a μ-containing, LC-deficient receptor. (A) FACS® analysis of MACS-purified CD19$^+$ BM cells from littermates of the indicated genotypes. The cells were cultured in IL-7 for 4 d and stained for conventional pre-BCR (μ versus SL-156) or BCR (μ versus kappa/lambda). (B) FACS® analysis of SLP-65/Ig-α$^{-/-}$ BM-derived pre-B cells showing that the SL$^-$pre-BCR requires Ig-α for expression on the cell surface. (C) FACS® analysis of gated B cells freshly isolated from the BM of the indicated mice showing that the SL$^-$pre-BCR can be detected in SLP-65/λ5$^{-/-}$ pre-B cells that have not been cultured. Numbers refer to the percentage of cells in each gate.

the surface of pre-B cells in the absence of λ5. To test this hypothesis, we analyzed in parallel BM-derived pre-B cells from WT, SLP-65$^{-/-}$, λ5$^{-/-}$, and SLP-65/λ5$^{-/-}$ littermates by FACS®. At day 4 of in vitro culture, the cells were stained with SL-156, anti-κ/λ, and anti-μHC. No evident pre-BCR expression (μHC$^+$/SL-156$^+$) was detected in the WT pre-B cell culture, and most μHC$^+$ WT cells were also κ/λ$^+$ (Fig. 1 A, top). However, the SLP-65$^{-/-}$ pre-B cell culture contained a clear μHC$^+$/SL-156$^+$ pre-B cell population (74%) in contrast to the WT, λ5$^{-/-}$, and SLP-65/λ5$^{-/-}$ cultures (Fig. 1 A, left). In addition, ~20% of the SLP-65$^{-/-}$ pre-B cells were μHC$^+$/SL-156$^-$/κ$^-$/λ$^-$, indicating that they express the SL$^-$pre-BCR that lacks a conventional or a surrogate LC (Fig. 1 A, second left and right panels). The μHC$^+$/SL-156$^-$ cells in the pre-B cell cultures from λ5$^{-/-}$ and SLP-65/λ5$^{-/-}$ mice consisted of two distinct populations. A minor population that expressed the μHC as a part of the BCR together with a conventional LC (κ or λ) and a major population expressing the μHC as a part of the SL$^-$pre-BCR. The ratio of the SL$^-$pre-BCR–expressing cells and its amount on each cell are increased in SLP-65/λ5$^{-/-}$ compared with λ5$^{-/-}$ pre-B cell cultures (Fig. 1 A, bottom four panels).

To confirm that the μHC of the SL$^-$pre-BCR is expressed on the surface as a subunit of a receptor complex, we generated mice deficient for SLP-65 and the signal transduction subunit Ig-α (SLP-65/Ig-α$^{-/-}$ mice). BM-derived pre-B cells from these mice showed no μHC expression on the cell surface (Fig. 1 B). To show that the SL$^-$pre-BCR can also be detected in cells that have not been cultured in vitro, we analyzed freshly isolated BM cells from SLP-65/λ5$^{-/-}$ and SLP-65/Ig-α$^{-/-}$ mice by FACS®. These experiments showed that a distinct B cell fraction (6%) in the BM of SLP-65/λ5$^{-/-}$ but not of SLP-65/Ig-α$^{-/-}$ mice expressed the SL$^-$pre-BCR (Fig. 1 C).

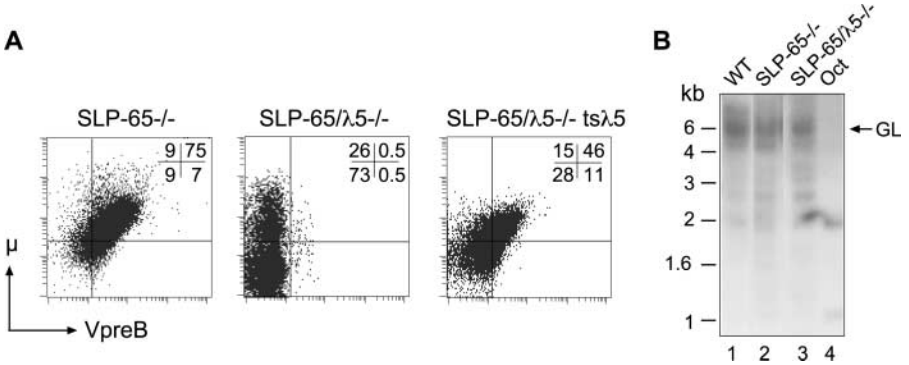

Figure 2. λ5 expression results in pre-B cell proliferation. (A) FACS® analysis of the indicated BM-derived pre-B cells showing that no VpreB expression is detected on SLP-65/λ5$^{-/-}$ cells. Reconstitution of λ5 expression with a retroviral vector (tsλ5) resulted in surface VpreB expression and expansion of the transduced cells. The BM-derived cells were transduced at day 2 of in vitro culture and analyzed 18 d after transduction. Numbers refer to the percentage of cells in each quadrant. (B) Southern blot analysis of genomic DNAs showing the polyclonality of BM-derived SLP-65/λ5$^{-/-}$ pre-B cells. The genomic DNA was extracted from BM-derived pre-B cells of WT, SLP-65$^{-/-}$, and SLP-65/λ5$^{-/-}$ mice cultured for 1 wk (lanes 1–3). Genomic DNA from the clonal SLP-65$^{-/-}$ pre-B cell line (Oct) was used as a control (lane 4). The DNA was digested with EcoRI separated by 0.8% agarose gel. After blotting, the membrane was hybridized with a JH probe (as described in Materials and Methods). GL indicates germ line configuration.

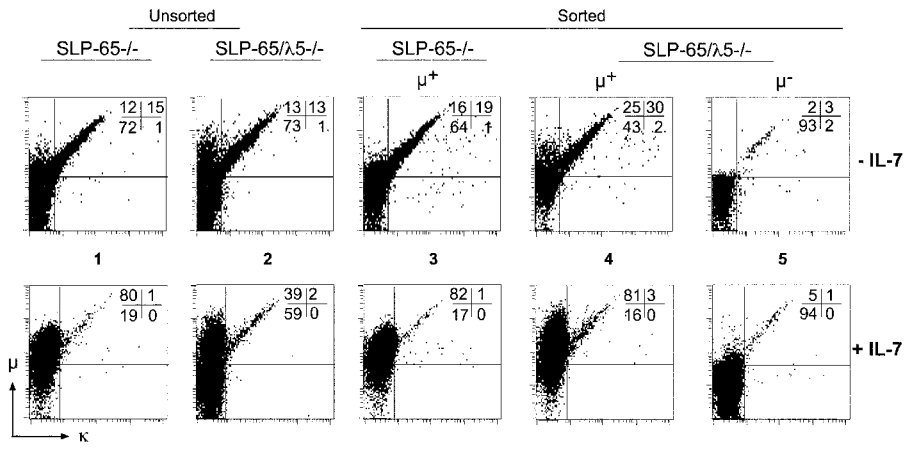

Figure 3. The SL$^-$pre-BCR is associated with enhanced differentiation. BM-derived pre-B cells from a 1-wk culture were incubated for 2 d in the absence or presence of IL-7 (top and bottom, respectively). Unsorted and μHC$^+$ (μ$^+$) and μHC$^-$ (μ$^-$) sorted cells were analyzed in parallel. Sorted μHC$^-$ SLP-65$^{-/-}$ pre-B cells showed poor survival and therefore were not included. μHC versus κLC FACS® profiles of the indicated genotypes are shown. Numbers refer to the percentage of cells in each quadrant.

The SL component VpreB might associate with μHC in the absence of λ5 and allow surface expression of an incomplete pre-BCR (37). To exclude this possibility, we performed FACS® analysis with anti-VpreB and show that VpreB is not associated with μHC in the SL$^-$pre-BCR on the surface of SLP-65/λ5$^{-/-}$ pre-B cells (Fig. 2 A, second panel). However, surface VpreB expression was easily detected when λ5 was retrovirally transduced into SLP-65/λ5$^{-/-}$ pre-B cells, and the ratio of the transduced cells increased from 9% at day 4 to 46% at day 18 posttransduction (Fig. 2 A, third panel and not depicted). Further, we analyzed the Ig HC rearrangement status in genomic DNA from WT, SLP-65$^{-/-}$, and SLP-65/λ5$^{-/-}$ pre-B cell cultures. This analysis showed that similar to WT and SLP-65$^{-/-}$ the DNA from SLP-65/λ5$^{-/-}$ pre-B cells contained several submolar JH-rearranged bands, indicating that the cells were polyclonal and excluding the possibility that few clones with abnormal μH chains gave rise to surface μHC expression (Fig. 2 B). In summary, SLP-65/λ5$^{-/-}$ and λ5$^{-/-}$ pre-B cells express a SL$^-$pre-BCR that cannot provide the cells with a strong selective advantage to dominate the culture, since a considerable proportion of these pre-B cells is μHC$^-$ in contrast to SLP-65$^{-/-}$ pre-B cells that are mostly μHC$^+$ (Fig. 1 A). Further, the reconstitution of pre-BCR (i.e., λ5) expression in SLP-65/λ5$^{-/-}$ pre-B cells resulted in the proliferative selection of μHC$^+$ cells (Fig. 2 A).

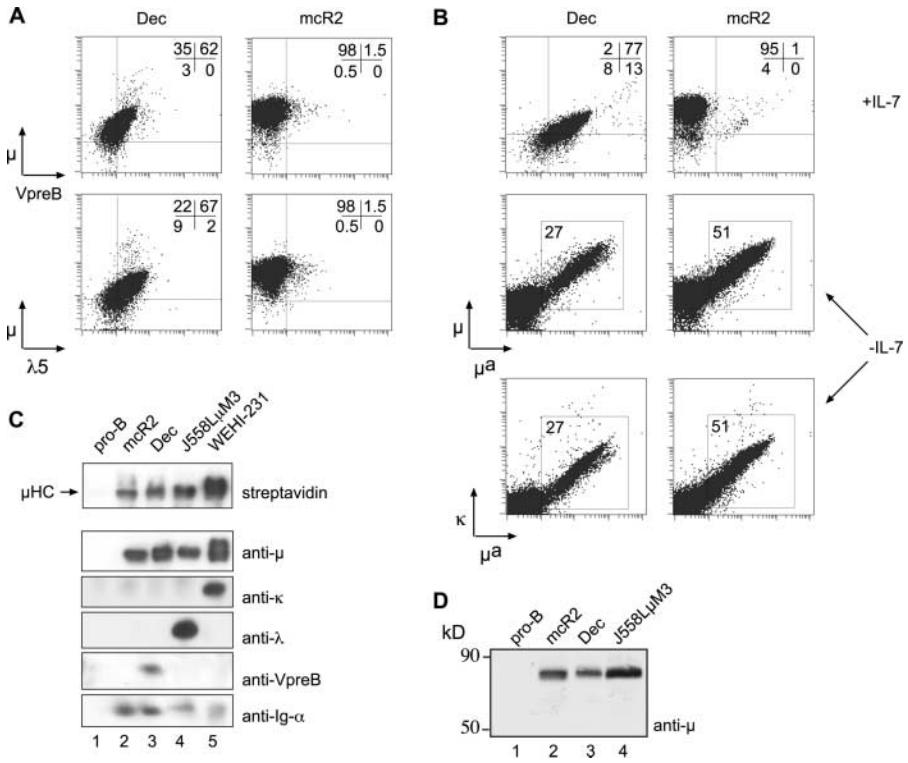

Figure 4. Pre-B cell lines that express the SL$^-$pre-BCR. (A) FACS® analysis of the SLP-65$^{-/-}$ (Dec) or SLP-65/λ5$^{-/-}$ (mcR2) pre-B cell lines stained for conventional pre-BCR (μ versus VpreB, top; μ versus λ5, bottom). (B) FACS® analysis of Dec and mcR2 pre-B cells cultured with IL-7 (top) or without IL-7 for 2 d (middle and bottom). Cells were stained with antibody DS-1 for the μHC^a allotype that detects conventional pre-BCR (Dec) but not the SL$^-$pre-BCR (mcR2) (μ versus μ^a, top). After IL-7 removal, differentiation and κLC expression enable the recognition of the μHC^a allotype in the mcR2 cells (middle and bottom). Numbers refer to the percentage of cells in the gate. (C) Western blot analysis of protein complexes obtained with anti-μ immune purification. The surface cellular proteins of the indicated cell lines were biotinylated before cell lysis. The purified protein complexes were separated by SDS gel electrophoresis. After blotting, the membrane was incubated with streptavidin-HRPO (tol) and subsequently with antibodies recognizing μHC, κLC, λLC, VpreB, or Ig-α. The pro-B cell line was established from BM cells of an SLP-65/Ig-α$^{-/-}$ mouse by extended culture in IL-7. The cell lines J558LμM3 and WEHI-231 were used as controls for cells expressing a BCR with λLC and κLC, respectively. (D) Western blot analysis of total cellular lysates for μHC expression in the indicated cell lines.

The SL⁻pre-BCR Is Associated with Enhanced Differentiation In Vitro. We further tested the ability of SLP-65/λ5$^{-/-}$ pre-B cells to differentiate in vitro. 2 d after withdrawal of IL-7 to induce differentiation, we observed κLC recombination and the appearance of similar amounts of BCR^{+} cells in SLP-65$^{-/-}$ and SLP-65/λ5$^{-/-}$ cultures (Fig. 3, panels 1–2). To show that pre-B cells with a surface SL⁻pre-BCR are not blocked in development, we compared the in vitro differentiation of sorted μHC^{+} pre-B cells from SLP-65/λ5$^{-/-}$ and SLP-65$^{-/-}$ cultures. After 2 d of IL-7 removal, 30 and 19% of the SLP-65/λ5$^{-/-}$ and SLP-65$^{-/-}$ pre-B cells differentiated to BCR^{+} cells, respectively (Fig. 3, panels 3–4). In contrast, the SLP-65/λ5$^{-/-}$ pre-B cells that did not express an SL⁻pre-BCR showed a poor differentiation capacity (Fig. 3, panel 5). This indicates that the SL⁻pre-BCR is capable of inducing differentiation and that the μHC of this receptor can pair with κLC. Sorted μHC^{-} pre-B cells from SLP-65$^{-/-}$ cultures did not grow in culture and therefore were not analyzed.

Pre-B Cell Lines Expressing the SL⁻pre-BCR. Prolonged in vitro culture of BM-derived SLP-65/λ5$^{-/-}$ pre-B cells allowed the generation of cell lines that express the SL⁻pre-BCR. Analysis of five independent lines showed a similar pattern of surface marker expression compared with short-term cultures and were CD19^{+}/CD43^{+}/CD25^{-}/μHC^{+}/VpreB^{-}/λ5^{-} (Fig. 4 A and not depicted). Interestingly, the DS-1 antibody, which is specific for the *IgHC*^{a} allotype present in SLP-65/λ5$^{-/-}$ mice, did not detect the μHC on these cell lines (mcR2) and on cells from short-term culture (Fig. 4 B, top, and not depicted). Given that the DS-1 antibody detects only μHC associated with an SL or conventional LC but not free μHC (38), we conclude that the SL⁻pre-BCR contains a μHC without any light chain. To prove that the μHC of the SLP-65/λ5$^{-/-}$ cells is recognizable by DS-1 after association with a light chain, IL-7 was removed for 2 d from the culture to induce κLC recombination. Indeed, all SLP-65/λ5$^{-/-}$ cells with a surface κLC were readily detected with DS-1 (Fig. 4 B, bottom). Similar to the results of sorted μHC^{+} cells (Fig. 3), the SLP-65/λ5$^{-/-}$ pre-B cells showed a higher differentiation capacity compared with SLP-65$^{-/-}$ pre-B cells. We used the SLP-65/λ5$^{-/-}$ pre-B cell lines to further characterize the SL⁻pre-BCR. Western blot analysis revealed that the SLP-65/λ5$^{-/-}$ pre-B cells mainly contain a complete μHC and not the truncated Dμ protein (Fig. 4 D). Biotinylation of surface proteins followed by anti-μ immune purification and streptavidin Western blot analysis confirmed the FACS® data that μHC is expressed on the surface of SLP-65/λ5$^{-/-}$ pre-B cells (Fig. 4 C, top). Further, the μHC of both the pre-BCR and the SL⁻pre-BCR was associated with Ig-α, whereas VpreB was only detected in the context of the pre-BCR (Fig. 4 C, bottom). In these surface biotinylation experiments, no μHC-associated proteins were detected that might correspond to a light chain or similar molecules (not depicted).

Engagement of the SL⁻pre-BCR Induces a Massive Ca^{2+} Response. To analyze the signaling competence of the SL⁻pre-BCR, we compared the phosphorylation of PTK substrate proteins after anti-μ treatment of SLP-65$^{-/-}$ and SLP-65/λ5$^{-/-}$ short-term cultures and pre-B cell lines. In all cell types, anti-μ treatment induced tyrosine phosphorylation of similar substrate proteins including PLC-γ2, Syk, Ig-α, and the adaptor protein LAT (linker for activation of T cells) (Fig. 5 A). This confirms our recent results showing that LAT is expressed in pre-B cells and phosphorylated upon pre-BCR engagement (35). The induction of substrate tyrosine phosphorylation was lower in the short-term cultures compared with the cell lines (Fig. 5 A, lanes 1–6 and 7–12). Further, the SLP-65/λ5$^{-/-}$ pre-B cells showed the highest induction of tyrosine phosphorylation (Fig. 5 A, lane 11). Compared with the short-term culture of SLP-65$^{-/-}$ pre-B cells and the SLP-65$^{-/-}$ pre-B cell line Dec,

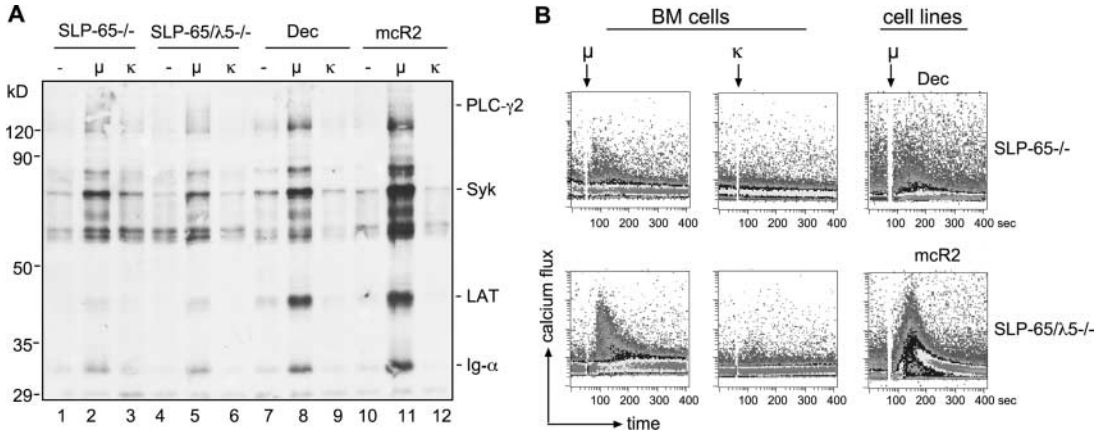

Figure 5. Engagement of the SL⁻pre-BCR induces PTK substrate phosphorylation and a massive Ca^{2+} release. (A) Antiphosphotyrosine Western blot analysis of proteins purified by antiphosphotyrosine precipitation. BM-derived cells from SLP-65$^{-/-}$ and SLP-65/λ5$^{-/-}$ mice cultured for 1 wk (lanes 1–6), pre-B cell lines Dec (lane 7–9) and mcR2 (lanes 10–12) were left unstimulated (lanes 1, 4, 7, and 10), stimulated with 20 μg/ml anti-μ (lanes 2, 5, 8 and 11) or 20 μg/ml anti-kappa (lanes 3, 6, 9 and 12) at 37°C for 2 min. For all lanes, 1 × 10^{7} cell equivalents were loaded. (B) BM-derived pre-B cells and cell lines (described in A) were loaded with Indo-1 and treated with anti-μ (left and right lanes) to induce Ca^{2+} response. Antikappa treatment was used as a negative control (middle lane).

anti-μ treatment of the SLP-65/λ5$^{-/-}$ short-term culture and the pre-B cell line mcR2 induced a strong Ca^{2+} response (Fig. 5 B). Treatment with anti-κ as a control induced neither phosphorylation nor Ca^{2+} release (Fig. 5, A and B, middle). These results demonstrate a profound difference between the SL$^-$pre-BCR and the conventional pre-BCR in the induction of Ca^{2+} release.

Discussion

Our experiments demonstrate that the μHC forms an Ig-α–associated and signaling-competent complex on the surface of pre-B cells that lack any SL or conventional light chains. These cells can be detected in the BM of SLP-65/λ5$^{-/-}$ mice and can be grown in vitro in IL-7–supplemented medium. The SL$^-$pre-BCR on the surface of SLP-65/λ5$^{-/-}$ pre-B cells contains a complete μHC of the same molecular weight as the μHC from control cell lines, so that it is unlikely that deletions within the variable region or the CH1 domain of μHC lead to SL$^-$pre-BCR expression in the absence of SL or conventional light chains. Previous reports showed that some human μH chains with special VH genes could be expressed on the cell surface in the absence of SL or conventional LC (39). These aberrant μH chains induced apoptosis and were only found in pro-B cells, but not in preB or mature B cells, indicating that pro-B cells with this μHC are negatively selected. For several reasons, we assume that a functional rather than an aberrant μHC is expressed in the SLP-65/λ5$^{-/-}$ pre-B cells. First, SLP-65/λ5$^{-/-}$ pre-B cells are polyclonal, arguing against the use of highly selected VH genes able to generate μHC with LC-independent expression on the cell surface. Second, no increased apoptosis was detected when μHC$^+$ SLP-65/λ5$^{-/-}$ pre-B cells were sorted and analyzed for differentiation. Third, the μHC$^+$ SLP-65/λ5$^{-/-}$ pre-B cells differentiated readily to immature B cells, whereas μHC$^-$ SLP-65/λ5$^{-/-}$ cells showed a poor differentiation capacity, demonstrating that the μH chains do not block but rather promote development. It is unlikely that the μH chains of the sorted μHC$^+$ SLP-65/λ5$^{-/-}$ pre-B cells are replaced during differentiation, since this would suggest that replacement in the μHC$^+$ cells is more frequent than de novo recombination in μHC$^-$ cells.

We reported previously that SLP-65$^{-/-}$ pre-B cells show an increased proliferation capacity that can lead to pre-B cells tumors (33, 34). Since the SLP-65/λ5$^{-/-}$ pre-B cells showed a decreased proliferation capacity compared with SLP-65$^{-/-}$ pre-B cells, we concluded that the high proliferation rate of SLP-65$^{-/-}$ pre-B cells in vitro required the expression of the conventional pre-BCR (33). In the present study, we show that reconstitution of λ5 expression in SLP-65/λ5$^{-/-}$ pre-B cells lead to the enrichment of cells with a conventional pre-BCR. Thus, the SL$^-$pre-BCR on SLP-65/λ5$^{-/-}$ pre-B cells does not seem to induce cell proliferation. In full agreement with this, no pre-B cell tumors were observed in SLP-65/λ5$^{-/-}$ mice (>500).

We have shown recently that the adaptor protein LAT is involved in pre-BCR signaling and in the rescue of SLP-65$^{-/-}$ pre-B cells (35). SLP-65/λ5$^{-/-}$ pre-B cells express high amounts of LAT, which explains their capability to mobilize Ca^{2+} in the absence of SLP-65. However, it is currently unclear why receptor engagement induces a strong Ca^{2+} response in SLP-65/λ5$^{-/-}$ pre-B cells compared with SLP-65$^{-/-}$ pre-B cells.

In a recent study, Ohnishi and Melchers (40) demonstrated that mutations in the non-Ig portion of λ5 lead to increased pre-BCR accumulation on the cell surface, suggesting that λ5 controls the internalization of the pre-BCR. Further, they showed that the same λ5 mutations abolished the constitutive, cell autonomous signaling capacity of the pre-BCR. However, the signaling capacity of these mutant pre-BCR molecules was restored after cross-linking with anti-μ, suggesting that λ5 is involved in the cross-linking of the pre-BCR on the surface of pre-B cells (40). These results indicate that, in the uninduced situation, the SL$^-$pre-BCR may be less efficiently cross-linked and internalized than the conventional pre-BCR and that cross-linking of the pre-BCR is required for the proliferation but not the differentiation of pre-B cells.

However, in BM-derived pre-B cells from WT mice we could not detect conventional pre-BCR or SL$^-$pre-BCR–expressing cells, and most μHC$^+$ cells in these cultures were also κLC$^+$, indicating that they were immature B cells. We assume that defective receptor internalization due to a deficiency in SLP-65, λ5, or both simply allows the detection rather than the formation of both the conventional pre-BCR and the SL$^-$pre-BCR.

Although the exact role and significance of the SL$^-$pre-BCR are not fully clear, this receptor may explain previous results. For instance, B cell development is only partially blocked in λ5$^{-/-}$ mice, which is in contrast to the complete developmental block in μMT mice lacking the transmembrane region of μHC (18). In both cases, no expression of a conventional pre-BCR and a block in differentiation are expected. However, the λ5$^{-/-}$ pre-B cells bypass this block, indicating that the μHC forms alternative complexes in the absence of an SL. According to previous reports, prematurely expressed conventional light chains could pair with μHC and compensate the loss of the SL chain in λ5$^{-/-}$ pre-B cells (23). However, this rescue of SL deficiency required an early onset of κLC expression, which might be unlikely because kLC expression is largely restricted to cells at later stages of B cell development (23). We propose that in λ5$^{-/-}$ pre-B cells, an SL$^-$pre-BCR may induce differentiation of these cells and explain the difference between the λ5$^{-/-}$ and μMT mice. In addition, the fact that the μHC forms an SL$^-$pre-BCR on pre-B cells may also explain why allelic exclusion still operates in SL chain deficient but not in μMT mice (19, 22).

Normally, a μHC unpaired with an SL or a conventional LC is retained in the ER by the chaperone BiP (41). BiP binds to the unpaired CH1 domain of μHC and retains it in the ER until an SL or a conventional LC displaces BiP

and allows the transport of the completely assembled receptor to the cell surface. Our experiments with surface biotinylation did not detect additional proteins that might associate with μHC and allow its dissociation from BiP. The staining experiments with the μHC-allotype–specific antibody (DS-1), which recognizes μHC^a only when it is associated with an SL or a conventional LC, suggest that μHC is expressed on the surface without associated proteins. It is currently unclear how the μHC can circumvent the BiP retention control in SLP-65/$\lambda 5^{-/-}$ pre-B cells. A better understanding of the SL^-pre-BCR should help to clarify the requirements for surface μHC expression, allelic exclusion, pre-B cell proliferation, and differentiation.

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