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The Non-Ig Parts of the VpreB and λ5 Proteins of the Surrogate Light Chain Play Opposite Roles in the Surface Representation of the Precursor B Cell Receptor

Marko Knoll,* Yuki Yanagisawa,† Szandor Simmons,*+ Niklas Engels,§ Jürgen Wienands,§ Fritz Melchers,* and Kazuo Ohnishi†

The VpreB and λ5 proteins, together with Igκ-μ H chains, form precursor BCRs (preBCRs). We established λ5−/−/VpreB1−/−/VpreB2−/− Abelson virus-transformed cell lines and reconstituted these cells with λ5 and VpreB in wild-type form or with a deleted non-Ig part. Whenever preBCRs had the non-Ig part of λ5 deleted, surface deposition was increased, whereas deletion of VpreB non-Ig part decreased it. The levels of phosphorylation of Syk, SLP65, or PLC-γ2 were dependent on the levels of surface-bound preBCRs. It appears that VpreB probes the fitness of newly generated VH domains of IgH chains for later pairing with IgL chains, and its non-Ig part fixes the preBCRs on the surface. By contrast, the non-Ig part of λ5 crosslinks preBCRs for downregulation and stimulation. The Journal of Immunology, 2012, 188: 6010–6017.

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The precursor BCR (preBCR) is composed of two identical Igκ-μ H chains and two surrogate L chains (SLC) (1, 2). PreBCRs are selectively expressed during preB cell development on large preB II cells that have undergone productive V6q to D3μq rearrangements on the IgH chain locus and express a μH chain capable of pairing with SLC (3, 4). More than half of the in-frame V6qD3μq rearrangements do not pair with the SLC. Therefore, cells expressing such μH chains do not form a preBCR on their surface (5).

The SLC is composed of VpreB (either VpreB1 or VpreB2) and λ5 proteins that associate noncovalently into an Ig L chain-like structure, because both VpreB and λ5 have Ig domain-like structures (6). VpreB lacks the seventh β sheet in its Ig domain, whereas λ5 has an additional β sheet within the aminoterminal portion extending beyond its Ig domain. Deletion of this additional β sheet in λ5 abolishes the capacity of VpreB and λ5 to form a noncovalently bound L chain-like structure, suggesting that the extra β sheet of λ5 occupies the structural features of the seventh β sheet of a classical Ig-V domain in VpreB (7). Both VpreB and λ5 also have non-Ig-like structures, VpreB at its C-terminal end and λ5 at its N-terminal end. The experiments of Minegishi et al. (7) have suggested that the non-Ig part of λ5 acts as a chaperone in the assembly of the preBCR. Without VpreB, the non-Ig part-containing wild-type form of λ5 cannot form disulphide bonds between the first constant domain of the H chain and the Ig domain of λ5. Binding of VpreB to λ5 induces a conformational change in λ5, so that SLC can now covalently be bound by disulphide bonds to H chain (7). In an IgL chain-like structure, the non-Ig parts of VpreB and λ5 protrude, close to each other, at the location where the third CDR is found in classical IgL chains (8, 9). Hence, the non-Ig parts of the SLC might be accessible for binding to ligands. Potential ligands, such as galectin-1 (10, 11) or heparan sulfate (12), might be provided in the bone marrow, possibly by stromal cells (13, 14). Deletion of the non-Ig parts of VpreB and λ5 does not interfere with the capacity of the two proteins to associate with each other to form an IgL chain-like structure (7) and to link covalently by disulphide bridge to Igκ-μ H chain to form a preBCR-like structure (15, 16). However, deletion of the non-Ig parts of VpreB, λ5, or both could alter the ways in which SL chain is assembled, deposited in the surface membrane, and shed or internalized from the surface of the SLC-expressing cells.

The preBCR is thought to signal large preB II cells to proliferate for several rounds of divisions (17, 18). The non-Ig part of λ5 and specifically the seven arginine residues in it have been shown to change the surface deposition and internalization of the preBCR measured by aggregation and internalization of the preBCR in Abelson virus-transformed, μH chain-expressing preBCR cell lines established from λ5-deficient mice (15). With the development of a triple VpreB1-, VpreB2-, and λ5-deficient strain (19), we are now able to generate μH chain-expressing, triple SL-deficient preBCR cell lines to study the roles of the non-Ig parts of both VpreB and λ5 in assembly and turnover of preBCRs on preB cells. In this study, we have transduced triple-deficient preBCR cell lines with all possible combinations of wild-type and mutant (i.e., non-Ig part-deleted) forms of VpreB1 and λ5. We describe in this study the surface deposition and turnover as well as anti-μH chain-induced preBCR signaling of wild-type and mutant forms of preBCRs. The results of our experiments show that the non-Ig...
transformed preB cell lines from these clone was transduced with vectors containing conditioned media (equivalent to 10 ng rIL-7/ml) at 37˚C. Abelson virus-transformed preB cell lines from these X5−/−, VpreB1−/−, and VpreB2−/− mice were established with a temperature-sensitive Abelson murine leukemia virus (20). After virus transformation, these cell lines grew independently of OP9 stromal cells and IL-7–conditioned RPMI 1640 media (Life Technologies) supplemented with 10% FCS at 34˚C. The resulting SLC-deficient cell lines were then cloned by limiting dilution, and resulting clones were further selected for expression of a cytoplasmic μH-chain. These clones were then tested for their pairing capacities with SLC in an assay described by ten Boekel et al. (3). The clone 7538 expressed a good pairing μ-chain and was chosen for further experiments. The 7538 clone was transduced with vectors containing 5 and/or VpreB in wild-type form or with deleted non-Ig portions (designated as mutant). The cells transduced with both vectors were enriched by sorting for GFP and YFP double-positive cells expressing the preBCR on their surface identified with the mAb SL156 (for an example, see Fig. 1).

**Materials and Methods**

**Cells**

PreB cell lines and clones were established from fetal liver at day 18 of gestation from SLC triple-deficient (Δ5−/−, VpreB1−/−, VpreB2−/−) mice and propagated on a semiconfluent layer of gamma-irradiated (30 Gy) OP9 stromal cells in RPMI 1640 medium (Life Technologies) supplemented with 10% FCS (Sigma-Aldrich, Seelze, Germany) and 1% mouse IL-7–conditioned media (equivalent to 10 ng rIL-7/ml) at 37˚C. Abelson virus-transformed preB cell lines from these X5−/−, VpreB1−/−, and VpreB2−/− mice were established with a temperature-sensitive Abelson murine leukemia virus (20). After virus transformation, these cell lines grew inde-

**Abs**

The Abs used in flow cytometry were: allophyocyanin-conjugated anti-mouse IgM (M41; our own production) (21), anti-mouse X5 (LM34; our own production) (22), anti-rat IgE L chain (MAR18.5; our own production) (23), biotinylated anti-mouse preB CR (SL156; our own production) (24), anti-VpreB (Vp245; our own production) (22), and Vp181 (K. Onishi). Note that Vp245 and Vp181 recognize only the wild-type form of VpreB. Anti-mouse x-k L chain (187.1; Southern Biotechnology Associates) was Pacific Blue conjugated. For stimulation experiments, a polyclonal rabbit F(ab′)2 Ab to mouse IgM (unlabeled; Southern Biotechnology Associates) and an anti-human (hu)CD8 mAb (MEM-31, unlabeled; kindly provided by V. Horejsi, Czech Republic) were used.

**Genes and vectors**

Genes, vectors, and site-directed mutagenesis were performed as described previously (18). Briefly, mouse X5, VpreB1, and SP6-κ IgL cDNA were cloned into pBluescript II. To delete the N-terminal non-Ig region of X5-ELVC vector was used to introduce the leader sequence of SP6 IgL as described previously. This procedure gives rise to the N-terminal 3 aa, QVD, in place of original sequence, and WHY, after splicing and translation. For the VpreB1 WT and VpreB1 mut, PCR using the sense primer 5′-CTTIGAGCTTGAAGCTGATGGGCTCTAGATGATCAAAATTCTGATA-3′ (VpreB1 WT) or 5′-GATGGATCCTCCTAAGATCCCAAATCTGTATA-3′ (VpreB1 mut) and the reverse primer 5′-GATTGAGCTTCTCTAGAGCCACGAGCACATGTA-3′ (VpreB1 mut) was conducted, and the PCR products were cloned into pcR2.1-TOPO (Invitrogen). This procedure gave rise to the full-length VpreB1 protein (VpreB1 WT) and the truncated form of VpreB1 in which the C-terminal 23 aa were deleted (VpreB1 mut) (Supplemental Fig. 1A, 1B). The chimeric CD8-IgG construct was generated by fusing the 5′ region of the cd8α-1 gene to μ heavy chain sequence provided by B. Schraven, Magdeburg, Germany) encoding extracellular and transmembrane regions to the coding sequence of the cytoplasmic domain of murine IgG (codons 163–220). The construct was then ligated into pMVCpuro (Clontech).

**Production of retroviral particles and transduction of cells**

For the production of retroviral particles, the retroviral packaging cell line Phoenix ec0 was transiently transfected using Lipofectamine reagent (Invitrogen, Karlsruhe, Germany). The Phoenix cells were seeded with 5 × 105 cells/2 ml/well of a six-well-plate (Costar CellBIND; Corning Life Sciences). After 24 h, the cells were transfected with 2 μg endotoxin-free retroviral vector plasmid mixed with 20 μg Lipofectamine for 5 h 30 min. For transduction of transformed preB cells, supernatant containing virus particles was harvested 48 h after transfection, and 1 × 106 Abelson-transformed preB cells were spin-infected with 1 ml virus supernatant (1150 × g, 210 min, 30˚C). After transduction, the supernatant was removed, and cells were resuspended and cultured as described before.

**Cytoplasmic staining**

To detect cytoplasmic proteins, cells were fixed with Fix Buffer I (BD Biosciences, Heidelberg, Germany) for 10 min at 37˚C and permeabilized in Phosflow Perm/Wash Buffer I (BD Biosciences) for 10 min at room temperature followed by 10 min blocking with Fcγ-R blocking Ab (clone 2.4G2; our own production) and then stained with the specific Abs.

**Internalization assay**

For steady-state internalization, 5 × 106 cells were incubated with an SL156 F(ab′)2 fragment for 30 min on ice. After thorough washing, the cells were incubated in cell culture media at 37˚C, and aliquots were taken at the indicated time points. After a short centrifugation (1150 × g, 1 min), the cells were immediately fixed in Fix Buffer I (BD Biosciences) for 10 min at 37˚C and then kept in FACS buffer (1× PBS, 2% heattreated FCS) until further processing. The fixed cells were stained with an allopbyocyanin-labeled Ab to rat IgE (mAb MAR 18.5), and the mean fluorescence intensities in GFP/YFP double-positive were measured using a FACS LSR II (BD Biosciences). The internalization rate was calculated as follows: internalized (%) = 100 − [(A0 − A1)/T − T0 − A0] × 100, where T0 is total bound SL156 on the surface at time 0 and A0 is residual SL156 at the indicated time point. A1 is the background fluorescence at the indicated points on P-l−/−/ knockout cells where no preBCR is expressed.

**Ca2+ flux measurement**

Approximately 2 × 106 preB cells were harvested and loaded with Indo-1 in RPMI 1640 medium containing 0.01% Pluronic F-127 and 1 μM Indo-1-AM under mild agitation at 30˚C for 25 min. After two wash steps with Krebs–Ringer solution containing 1 mM CaCl2, Ca2+ mobilization was assessed following stimulation of cells with polyclonal rabbit F(ab′)2 fragments to mouse IgM. Unless stated otherwise, the measurements were done in the presence of Ca2+. To selectively mobilize Ca2+ from intracellular stores, the cells were shortly centrifuged and resuspended in Krebs–Ringer solution containing 0.5 mM EGTA instead of CaCl2. The instrument setup and cellular loading with Indo-1 was controlled by treating with 1 × 106 cells with ionomycin (1 μg/ml). After the baseline was established for 30 s, cells were stimulated with polyclonal rabbit F(ab′)2 fragments to mouse IgM (10 μg/ml) or anti-huCD8 Ab (MEM-31, 10 μg/ml), and Ca2+ mobilization was measured for 6 or 12 min as indicated. Measurements were done with a BD LSR II flow cytometer (BD Biosciences). Raw data files were exported as FCS2.0 files and analyzed using FlowJo software (Tree Star).

**Phosflow analysis**

For analysis of the phosphorylation status of the components of the preBCR signaling complex, 5 × 106 preB cells were harvested and resuspended in RPMI 1640 without FCS. Thereafter, they were preheated to 37˚C for 5 min before stimulation or without stimulation, respectively. The cells were incubated with polyclonal rabbit F(ab′)2 Ab to mouse IgM (10 μg/ml) for exactly 2 min. Cells were then immediately fixed with preheated Phosflow Fix Buffer I (BD Biosciences) for 10 min at 37˚C. After the fixation step, cells were permeabilized in Phosflow Perm/Wash Buffer I (BD Biosciences) for 10 min at room temperature followed by 10 min blocking with Fcγ-R blocking Ab. After blocking, the cells were stained with Alexa Fluor 647-conjugated anti-phosphorylated Syk (PY352), SL65 (pY84), PLC-γ2 (pY759), p38 MAPK (pT180/pY182), and ERK1/2 (pT202/pY204). As control for the same levels of SL65 and PLC-γ2, Alexa 647-conjugated anti-mouse SL65 (clone 2B11) and PLC-γ2 (clone K86-1161) were used. All components in this assay were purchased from BD Biosciences. Stained cells were analyzed using a BD LSR II flow cytometer (BD Biosciences).

**Immunoblotting**

A total of 5 × 105 cells was harvested and washed twice in 1× PBS. Cells were either unstimulated or stimulated with polyclonal rabbit F(ab′)2. Ab to mouse IgM (10 μg/ml) for 2 min at 37˚C, and cells were shortly centrifuged and immediately lysed with digitonin lysis buffer (1% w/v) as described before (15). Reducing protein loading buffer (Fermentas) was added to the cell lysates and then incubated at 95˚C for 5 min. Cell lysates were separated by electrophoresis and blotted onto a nitrocellulose membrane (1 mA/cm²). The nitrocellulose membrane was blocked in 1× PBS with 0.1% Tween 20 (PBS-T) with 5% BSA for 30 min, shortly washed, and then incubated with a phospho-Syk–specific Ab in 1× PBS-T with 2% BSA (Y525/526 clone C87/cel Cell Signaling) overnight at 4˚C. After being washed three times for 10 min in 1× PBS-T, the membrane was incubated with an HRP-labeled polyclonal secondary anti-rabbit specific Ab (Pierce).
Detection was done using ECL kit (PerkinElmer), and chemiluminescence was measured using an LAS3000 imaging system (Fujifilm).

**STI517**
Cells were incubated with 10 μM STI517 (imatinib mesylate; Cayman Chemicals) for 24 h [i.e., under conditions used by Muljo and Schlissel (25)]. A fresh 1:1000 stock solution was prepared before each experiment by dissolving 5.8 mg STI517 (imatinib mesylate) in 1 ml 1× PBS (sterile filtered). We isolated total protein after 12 and 24 h, separated the lysates of 3 × 10⁷ cells per lane by electrophoresis on 8% AA gel, and blotted onto a nitrocellulose membrane for 45 min (1 mA/cm²). Blocking and incubation with phospho-specific Abs was done as described in the Immunoblotting section above. For detection of phosphorylated tyrosines, the mAb 4G10, HRP labeled (Millipore), was used.

**Results**

**Generation of preB cell lines expressing different combinations of wild-type and mutant λ5 and VpreB1**

To probe the influence of the non-Ig parts of λ5 and VpreB on surface deposition of SLC in association with μH-chains (i.e., preBCR formation as well as their influence on preBCR-dependent Ca²⁺ mobilization and on the preBCR-dependent phosphorylation status of downstream signaling components in preB cell lines), we first established a fetal liver-derived preB cell line of VpreB1-, VpreB2-, and λ5-deficient mice (SLC-knockout). Then we transformed the cells with a temperature-sensitive Abelson murine leukemia virus to freeze the cells in an IL-7–independent, proliferating preB cell-like state (26, 27). We selected a clone based on the capacity of the endogenous μH chain to pair with wild-type forms of λ5 and VpreB to form preBCRs on the surface. Coexpression of both SLC genes in one preB cell was detected by gating on GFP and YFP double-positive cells. Flow cytometry analysis of preBCR expression on GFP- and/or YFP-positive cells showed that preBCRs were formed on the cell surface only when both λ5 (enhanced GFP [EGFP]) and VpreB1 (enhanced YFP [EYFP]) were coexpressed (Fig. 1A, 1B, gate 2). Thereafter, the SLC⁺/⁻ preB cell line was transduced with retroviruses carrying the four possible combinations of either wild-type or the non-Ig part-deleted (i.e., mutant) forms of λ5 and VpreB1, generating the transduced cell lines L5WT/VpreBWT, L5mut/VpreBWT, L5WT/VpreBmut, and L5mut/VpreBmut. As control, we also transduced the Igκ L chain gene from the SP6 hybridoma cell line (28), resulting in preB cells expressing mature BCRs on their surface. In the transducing retroviral constructs, λ5 was linked to EGFP via an internal ribosome entry site cassette, whereas VpreB1 was linked to EYFP.

**Intracellular assembly and surface deposition of wild-type and non-Ig mutant preBCRs**

We further tested for surface deposition of preBCR molecules in SLC⁺/⁻ cell lines reconstituted with λ5 and VpreB1 in wild-type or mutant forms with a series of mAbs recognizing different parts of preBCRs. Whereas mAb LM34 recognizes λ5 protein in free as well as in μH chain-bound forms, mAb SL156 binds λ5 protein only when bound to μH chains, mAb Vp181 detects only the wild-type but not the mutant form of VpreB, mAb M41 binds μH chains, and mAb 187.1 detects λL chains. The results of these analyses showed that cells transduced with the wild-type forms of λ5 and VpreB displayed normal surface deposition of the preBCR (SL156), λ5 (LM34), and VpreB (Vp181) (Fig. 2, second panel from the top). When the non-Ig part of VpreB was deleted, surface deposition decreased (Fig. 2, third panel from top). By contrast, and in agreement with previous analyses with λ5-reconstituted cells (15), the surface deposition of the preBCR was strongly increased when the non-Ig part of λ5 was deleted, even more so when VpreB was present in wild-type form than when its non-Ig part was deleted (Fig. 2, fourth panel from top). Interestingly, the surface deposition was lowered (as on λ5–wild-type–/VpreB-mutant form-expressing cells) when the non-Ig parts of both VpreB and λ5 were deleted (Fig. 2, fifth panel from top) but were still higher than in λ5WT transfectants. As expected, the preB cell lines expressed mature BCRs on the surface when transduced with λL chains (Fig. 2, bottom panel). In conclusion, surface deposition of the preBCR in different forms was dependent on the non-Ig parts of SL chain.

When we transduced Rag1⁻/⁻ SLC⁻/⁻ fetal liver-derived preB-I cells that are μH-chain negative with the different combinations of λ5 and VpreB, λ5WT/VpreBWT showed the highest deposition of λ5 and VpreB (LM34), gradually decreasing when the non-Ig parts were deleted (Supplemental Fig. 2A). In the absence of a pairing μH chain, surface expression of λ5 did not increase when its non-Ig part was deleted. This was not influenced by different amounts of intracellular pools, as all cells produced equal amounts of total λ5 (Supplemental Fig. 2B). We conclude that deposition of SLC in the surface membrane of μH-chain–negative pre- and preB cells, possibly in association with a collection of proteins [one of them BILL-cadherin (29)], possibly with (so far unknown) functions in preBCRs, differ from those of SLC in preBCRs.

To investigate whether the surface deposition of preBCRs in the reconstituted preB cell lines was effected from similarly large intracellular pools, the transfectants were stained for intracellular expression of total λ5-protein (LM34). Data in Fig. 3B (red lines) show that similar quantities of total λ5-protein were detected in all of the different cell lines. However, on the cell surface, these pools were different (Fig. 3B, black lines). Cells transduced with the wild-type form of λ5 did not display as many molecules on the surface as those transduced with the mutant λ5. Deletion of the non-Ig part of VpreB had an opposite effect on surface deposition of preBCRs; it led to decreased surface pools of preBCR molecules both in association with wild-type and with mutant λ5. We conclude from these experiments that the non-Ig portion of VpreB appears to be required for optimal surface expression of preBCRs, whereas that of λ5 appears to interfere with that process.

So far, our results indicated that the quantities of preBCRs on the surface of preB cells appear to be controlled by the presence or absence of the non-Ig portions of λ5 and VpreB. This might be the result of an influence of the non-Ig domains of SLC components on preBCR stability (i.e., on its turnover by internalization or shedding from the surface) or from differences in the assembly of the
preBCRs from intracellular pools. To test for possible differences in the internalization or shedding of surface-bound preBCRs, we compared the quantities of surface-bound preBCRs in an internalization assay (Fig. 3C). The results show that the surface expression of wild-type preBCRs, and even more so of mutant VpreB-containing preBCRs, decreased by internalization on live cells by 56 and 72%, respectively. By contrast, preB cells expressing mutant lambda5-containing preBCRs retained surface expression of preBCR molecules, and the internalization rate was lowered (mut/WT 30%, mut/mut 38%). We conclude from these results that preBCRs with non-Ig part-deleted lambda5 turn over more slowly, a turnover that is increased by the deletion of the non-Ig-part of VpreB. These results showed that the functions of the non-Ig parts of lambda5 and VpreB in preBCR turnover are different. It suggests that the non-Ig part of VpreB slows down preBCR turnover.

Phosphorylation of Syk, SLP65, and PLC-γ2 in wild-type and mutant preBCR-expressing cell lines

To investigate possible connections between Ca2+ mobilization and phosphorylation states of critical components of the signaling pathways of preBCRs, we analyzed the phosphorylation status of the downstream signaling components Syk, SLP65, and PLC-γ2 that are known to be involved in the transmission of preBCR-mediated signals. The cells were either left unstimulated or stimulated with polyclonal rabbit F(ab)2 Ab to mouse IgM for 2 min. Subsequently, the cells were fixed and stained with Abs specific for phosphorylated Syk (p-Y352), SLP65 (p-Y84), and p-PLC-γ2 (p-Y759) mAbs.

Neither of the preBCR-expressing cell lines showed detectable levels of Syk, SLP65, or PLC-γ2 phosphorylation in the absence of anti-IgM stimulation (Fig. 4A–C, top panels). After preBCR stimulation with anti-IgM F(ab)2 fragments, phosphorylation of Syk, SLP65, and PLC-γ2 became detectable (Fig. 4A–C, bottom panels). Whenever the non-Ig part of lambda5 was deleted, regardless of the type of VpreB coexpressed, the highest phosphorylation signals could be detected (Fig. 4, orange and blue lines) in intensities comparable to the control cell line expressing the mH chain together with a κL chain as a BCR (Fig. 4, black dashed line). In the presence of wild-type lambda5, Syk, SLP65, and PLC-γ2 phosphorylations were low (Fig. 4, red and green lines). To determine the degree of phosphorylation of Syk by an alternative method, Western blots of total cellular lysates from preBCR-stimulated cells were done (Fig. 4D). The results were comparable with the flow cytometric analyses, again showing that the highest signals of phosphorylated Syk were detected when the non-Ig part of lambda5 was deleted, whereas only very low levels of phosphorylated Syk were detectable when lambda5 was in wild-type form (Fig. 4D).

Ca2+ signaling in wild-type and mutant-preBCR-expressing cell lines

The influence of preBCRs composed of the four possible combinations of wild-type and mutant VpreB and lambda5 proteins, expressed in and on the μH chain-producing, Abelson virus-transformed preB cell lines on Ca2+ mobilization, was measured before and after crosslinking with polyclonal rabbit F(ab)2 Ab to mouse IgM. The preB cells, either not transduced with SLC constructs or
transfected with κ chain to form, with the μH chain, a BCR on
the surface, served as controls.

In the absence of external ligands. Before the addition of cross-
linking polyclonal rabbit F(ab')2 Ab to mouse IgM, the baseline of
the Ca2+ mobilization measurements was only marginally differ-
ent from background in all of the different cell lines tested.

FIGURE 3. Intracellular expression and internalization rates of preBCRs. Comparison of cytoplasmic (red line) versus surface (black line) expression of μH-chains (M41), λ5 (LM34), and preBCR (SL156) in SLC-/- preB cells reconstituted with all possible combinations of λ5, VpreB, and κ L chain (A, B). Internalization of surface-expressed preBCRs (C). Transfectants were labeled with mAb SL156 F(ab')2 fragment, and internalization rates were determined by staining with allophycocyanin-labeled anti-rat Igκ mAb (MAR18.5). The dotted vertical line was adjusted to λ5 WT/VpreB WT transfectants to show that equal amounts of μHC, λ5, and preBCR are expressed intracellularly. Experiments were repeated at least five times, and representative staining patterns are shown.

in the absence of external ligands. Before the addition of cross-
linking polyclonal rabbit F(ab')2 Ab to mouse IgM, the baseline of

FIGURE 4. Phosphorylation status of preBCR proximal signaling molecules. Phosphorylation of Syk (A), SLP65 (B), and PLC-γ2 (C) in resting (top panels) or anti-IgM–stimulated (bottom panels) cells was analyzed by intracellular staining with phospho-specific Abs followed by detection with Alexa 647-labeled Abs. Syk phosphorylation was validated by Western blot analysis with a p-Syk (Y525/526 clone C87C1)-specific Ab of whole cellular lysates after 2 min stimulation with 10 μg/ml polyclonal anti-IgM F(ab')2 fragments. β-actin Ab was used as loading control (D). Data are representative of three independent experiments.
different forms of preBCRs. As expected, addition of polyclonal rabbit F(ab')2 Ab to mouse IgM mobilized Ca\(^{2+}\) in the control cells expressing κL chain/μH chain BCR (Fig. 5A, black dashed line). It did not mobilize Ca\(^{2+}\) in SLC-deficient, μH chain-expressing cells (Fig. 5A, black line). These results suggest that the μH chain-expressing preB cells can mobilize Ca\(^{2+}\) after μH chain-directed crosslinking only when the μH chains form a BCR with κL chains.

Cells expressing mutant λ5 showed a strong and sustained Ca\(^{2+}\) flux that was comparable to cells expressing a mature IgM/κ-containing BCR (Fig. 5A, blue, orange, and dashed lines). By contrast, cells expressing wild-type λ5 responded only very poorly to preBCR stimulation (Fig. 5A, red and green lines). These results suggest that expression of wild-type λ5 containing preBCRs rendered the cells refractory to anti-IgM-induced Ca\(^{2+}\) mobilization. VpreB, in wild-type or mutant form, did not influence the Ca\(^{2+}\) mobilizations in any of the different preBCR-expressing preB cell lines. Thus, λ5 wild-type form-expressing preB cells remained refractory to external, μH chain-mediated Ca\(^{2+}\) mobilization, regardless of whether VpreB was in its wild-type or mutant form (Fig. 5A, red and green lines). We interpret these results to indicate that the functions of the non-Ig parts of λ5 and VpreB are different and that the non-Ig part of VpreB does not influence an anti-IgM-induced, preBCR-mediated signaling and Ca\(^{2+}\) mobilization in the different Abelson-transformed preB cell lines.

**Mediated by CD8-specific-Ab on CD8-Igα transected cells.** To test the preB cell lines expressing the different forms of preBCRs for their capacities to mobilize their intracellular Ca\(^{2+}\) stores by a preBCR (μH-IC)-unrelated stimulus, we stably transduced these cell lines with a retroviral construct that encoded a chimeric transmembrane protein consisting of the extracellular part of huCD8α and the cytoplasmic domain of Igα in the different preB cell lines. Stimulation of these transfectants with anti-CD8 Abs showed that all of them were capable of eliciting a Ca\(^{2+}\) response irrespective of the structure of the preBCR expressed (Fig. 5B). We conclude from these experiments that all preB cell lines have Ca\(^{2+}\) stores that can be mobilized by appropriate signaling.

Next, we first stimulated the different cell lines with IgM-specific Ab and subsequently exposed the stimulated cells to a second stimulation by anti-CD8 Abs (Supplemental Fig. 3A, 3B). The results of these experiments show that cells exposed to IgM-specific Abs that had mobilized Ca\(^{2+}\) (i.e., in cells expressing preBCRs with non-Ig deleted λ5) could no longer mobilize Ca\(^{2+}\). In contrast, in those cells refractory to Ca\(^{2+}\) mobilization by IgM-specific Abs (i.e., in cells expressing preBCRs with wild-type non-Ig λ5), CD8-specific Abs mobilized Ca\(^{2+}\). We conclude from these results that the inability of preB cell lines expressing wild-type λ5 forms of preBCRs to mobilize Ca\(^{2+}\) is most likely determined by the quantity of the preBCRs on the surface.

The phosphorylation of Syk, SLP65, and PLC-γ2 seen after stimulation with Abs to mouse IgM in the different preB cell lines complement the results seen in the Ca\(^{2+}\) mobilization experiments after the same stimulation (Fig. 5), showing that preBCR signaling in preB cell lines is inducible to high levels by ligation of μH chains on cells expressing mutant λ5, but inducible to only low levels in cells expressing the wild-type form of λ5. Both functional tests show that the role of the non-Ig parts of λ5 and VpreB are different. In fact, the extent of Ca\(^{2+}\) mobilization and induced phosphorylation of Syk appears to be correlated with the amount of preBCR expressed on the surface of the different preB cell lines (Table I).

**Discussion**

The experimental results presented in this paper show that the quantities of preBCRs deposited on the surface of Abelson virus-transformed preB cell lines are regulated by the non-Ig parts of the two proteins forming the SLC of the preBCR, VpreB and λ5. Deletion of the non-Ig part of λ5 increases this surface representation, whereas deletion of the non-Ig part of VpreB decreases it. Median fluorescence intensities (MFI) measured by flow cytometry (Fig. 2, Table I) show that surface representation is lowest with VpreB–non-Ig–mutated preBCRs, higher with normal, wild-type preBCRs, still higher with VpreB–non-Ig–/λ5–non-Ig–mutated preBCRs, and highest with VpreB–non-Ig–/wild-type/λ5–non-Ig–mutated preBCRs.

The two different non-Ig parts might have different, structurally complementary binding partners, most probably in the surface membrane. It is also conceivable that such structurally complementary binding partners are provided by the stromal cell microenvironment of preB cells. For the non-Ig part of λ5, galectin-1 (10, 11) and heparansulfate (12) have been proposed as chemically complementary molecules expressed on stromal cells in contact with preB cells.

The partners of VpreB should retain preBCRs in the surface membrane, whereas those of λ5 should lower the preBCR surface representation. The quantities of surface preBCRs are a reflection of a steady state of transport from intracellular pools of synthesis and transport to the surface, measured in this study to be equally large in the four different preB cell types, and of turnover by shedding or internalization from the surface. Although our experiments are able to show that deletion of the non-Ig part of VpreB...
increases preBCR turnover (Fig. 3C), the turnover of non-Ig-deleted λ5 was much higher. Turnover of preBCRs on normal preB cells has been measured as half-lives of internally labeled molecules to be in the range of several hours (30) (i.e., much longer than the 1 h in the present internalization assay) (Fig. 3C), and this needs to be done with the preB cell lines used in this study. Our results confirm and extend previous results obtained with λ5-only-deficient preB cells, which had suggested that any partner molecule interacting with the non-Ig part of λ5 should carry oppositely charged residues.

We have probed the sensitivity of preBCRs to stimulation by external ligands in experiments in which we induced, by μH-chain–specific Ab the phosphorylation of Syk, SLP-65, and PLC-γ2 as three signal-transducing molecules connected to preBCR signaling for Ca2+ mobilization and differentiation (31). In Table I, we quantitated these results by MFI of the total Ca2+ mobilizations and, as one representative signal transducer, of the total phosphorylation intensities of Syk as a measure of the extent of anti-μH chain-Ab stimulations in the four different preB cell lines. Comparing the quantities of surface-bound preBCRs, it becomes evident that they determine the quantities of mobilized Ca2+ and of phosphorylated Syk stimulated by μH-chain-specific Ab.

The quantities of preBCRs deposited on the surface membrane of preB cells are also dependent on the pairing (i.e., on qualities of interactions of different IgH chains with the SLC) (3, 32), possibly by interactions of the VpreB-part of SL chain with the VH domain of the IgH chain (33). In our preB cell lines used in this study, one and the same pairing IgH chain was used, with the intention to keep the influence of pairing on preBCR deposition and function constant. In a polyclonal collection of preBCR-expressing preBII cells, the quality of pairing of SLC with IgH chain is expected to control the quantity of preBCRs deposited on the surface and, thereby, the extent of clonal expansion of different preB cells, whereas the non-Ig parts of the SL chain proteins control the quality of surface deposition and turnover of these preBCRs.

The non-Ig part of λ5 has also been found to influence another signaling reaction that results in a basal, constitutive phosphorylation of preBCR-associated Igα (15). These findings suggested that the non-Ig part of λ5 might interact with the non-Ig part of VpreB on the neighboring preBCR molecule, with a yet-unknown ligand in or on the preB cell, or on the environmental stromal cells to effect crosslinking, hence preBCR-mediated signaling for proliferation of large preBII cells (1). However, it has become clear from our results presented in this study that the four different forms of VpreB– or λ5–non-Ig part-containing or deleted preBCR do not phosphorylate Syk, SLP-65, or PLC-γ2 or mobilize Ca2+ to high or at least reproducibly measurable levels prior to the stimulation induced by Abs to mouse IgM. This indicates that the levels of constitutive stimulation in the absence of Abs to mouse IgM that were detected as phosphorylations of preBCR-associated Igα in these previous studies (15) must be very low. These previous analyses did not measure Ca2+ mobilizations, and the measured levels of Igα phosphorylation were not determined as quantities relative to the total available Igα in the preB cells.

The previous studies (15), as well as those presented in this study, have been conducted with preB cells transformed with the same, temperature-sensitive form of Abelson virus (20). Hence, the cells may not be optimal for such studies because they are already activated by viral action to proliferate without the stimulatory activities of IL-7, stromal cells, or the preBCR. Abelson virus transformation strongly activates pathways that stimulate preB cell proliferation and survival (including Ras, PI3K, Raf, Myc, Bcl-2, and Akt-mediated pathways (34) and JAK1, JAK3, STAT5, and STAT6 signal transductions (35)), but do not activate the Syk/SLP-65/PLC-γ2/Ca2+ mobilization pathway implied in preB cell differentiation (31). Thus, in the current study, we were able to stimulate this Syk/SLP-65/PLC-γ2/Ca2+ mobilization pathway so well either with μH-chain-specific Abs or CD8-specific Abs because of the unstimulated state of these cells (Figs. 4, 5, Supplemental Fig. 3). It might well be that the strong virus-induced activation of proliferation, in fact, inhibits the preBCR-mediated stimulation of signals that start with the phosphorylation of Igα and Syk (36, 37). To study the potential role of the four different forms of preBCRs, it will be necessary to establish experimental conditions that lead to an inactivation of the preB cell transformation by Abelson virus (25, 38, 39), thereby providing the opportunity to activate the Syk/SLP-65/PLC-γ2/Ca2+ mobilization pathway that might include a potential activation of high levels of constitutive phosphorylation of preBCR-associated Igα. STI571 has been found to inhibit the activity of Abelson virus (40). Mult and Schlissel (25) have shown that STI571-treated Abelson virus-transduced preB cell lines activate RNA expression of a series of genes involved in preBCR-mediated signaling, such as Igα, Syk, and SLP-65. In contrast, Syk and SLP-65 proteins are already present in such Abelson virus-transformed preB cell lines before the STI571 treatment, shown, for example, by our experiments presented in Fig. 4 and Table I, in which exposure to anti-μH chain-specific Ab stimulated, within 2 min, the phosphorylation of Syk, SLP-65, and PLC-γ2. Unfortunately, our preB cell lines, treated with STI571 for 12 or 24 h with STI571, did not activate the phosphorylation of Syk, SLP-65, or PLC-γ2 when treated with STI571 for either 12 or 24 h (Supplemental Fig. 4, showing the inhibitory effect of STI571 on total protein phosphorylation in our different cell lines, as well as the very low Syk phosphorylation in untreated as well as STI571-treated cells, in comparison with the high levels of anti-μH chain-specific Ab-stimulated phosphorylation of Syk). Thus, it appears that any constitutive signaling activity of the preBCR in the phosphorylation of the Syk/SLP-65/PLC-γ2 pathway is not activated by STI571-mediated inhibition of Abelson virus activity.

Our experimental results suggest different roles of the VpreB and λ5 proteins and their non-Ig parts for the function of the preBCR. VpreB, which was shown to pair with VH domains of IgH chains in the absence of λ5 (33), pairs with fitting VH domains

<table>
<thead>
<tr>
<th>MFI of SL156 Staining</th>
<th>MFI of pSyk Staining</th>
<th>MFI of Calcium Signaling</th>
</tr>
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<tbody>
<tr>
<td>λ5 WT / VpreB mut</td>
<td>1034.9</td>
<td>47</td>
</tr>
<tr>
<td>λ5 WT / VpreB WT</td>
<td>2952.9</td>
<td>109</td>
</tr>
<tr>
<td>λ5 mut / VpreB mut</td>
<td>7161.9</td>
<td>176</td>
</tr>
<tr>
<td>λ5 mut / VpreB WT</td>
<td>14234.9</td>
<td>354</td>
</tr>
</tbody>
</table>

The values of the MFI are shown from staining with SL156 (preBCR) in the first column (see Fig. 2), from staining with phospho-Syk Ab after stimulation with anti-μH-chain–specific Ab in the second column (see Fig. 5), and from Ca2+ mobilization in the third column (see Fig. 4). The background intensities in untransfected SLC–/– were subtracted from all values. The table is sorted for ascending values.
to form a preBCR-like complex, in which the non-Ig part of VpreB anchors the complex in the surface membrane. The better the fit between VH and VpreB, the more complexes will be deposited (41). In this way, the preBCR screens the repertoire of VH domains on newly generated IgH chains for later structural fitness in the pairing with IgL chains to form fitting BCRs and Abs. The A5 protein, in contrast, covalently attaches via the classical S-S bond of the first constant domain of the IgH chain to the C region-like domain of A5 (6) to form the full preBCR structure. In it, the non-Ig part of A5 crosslinks preBCR molecules and downregulates their surface expression by internalization. It will be interesting to see whether non-Ig part deletions, as we have introduced them in the SLC, can be mimicked in a preB cell by molecular interactions with antagonists that inhibit the binding of the non-Ig parts to their partners on preB cells and whether this offers further opportunities to modulate the strength of preBCR signaling.

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Disclosures

The authors have no financial conflicts of interest.

References

30. Rolink, A. G., H. M. Ja¨ck, and Jana Winckler for professional help with experiments.
Supplementary figure 1
Supplementary figure 2
Supplementary figure 3
Supplementary figure 4
**Supplementary Figure 1: Coding capacity of λ5 and VpreB**

Coding capacity of λ5 with the non-Ig part at its N-terminus and VpreB with the non-Ig part at its C-terminus is compared to a conventional light chain (A). An array of arginine residues is evolutionary conserved in the non-Ig part of λ5. In the non-Ig part of VpreB only 4 arginine residues are more or less conserved. L, leader sequence; V, variable domain; C, constant domain; J, joining region; CDR, complementarity determining region; R, arginine; E, glutamic acid; K, Lysine; C, cystein. Deletion of the non-Ig domains of VpreB1 and λ5 are outlined in (B) compared to a conventional light chain.

**Supplementary Figure 2: Surface deposition of surrogate light chain components in the absence of μH-chains**

Fetal liver derived preB cells from Rag1<sup>−/−</sup> SLC<sup>−/−</sup> reconstituted with the different forms of λ5 and VpreB (A). Cytoplasmic staining for λ5 (LM34) revealed that equal amounts of λ5 are produced but differ in their surface deposition (B). Note that anti-VpreB (Vp181) antibody detects only the wild type form of VpreB.
**Supplementary Figure 3: Anti-IgM stimulation prior to anti-CD8 stimulation**

PreB cell lines expressing the wild type \( \lambda_5 \) preBCRs (A) or the mutated \( \lambda_5 \) preBCRs (B) were additionally transduced to express a chimeric CD8/Ig\( \alpha \) fusion protein. Cells were first stimulated with anti-IgM F(ab)\( _2 \) and monitored for 5.5 minutes. Subsequently the cells were stimulated with 10\( \mu \)g/ml anti-CD8 antibodies and monitored for additional 6 minutes. Results are representative of three independent experiments. Surface staining with anti-huCD8 antibody revealed that all cell lines transduced had comparable densities of surface-bound huCD8 (C).

**Supplementary Figure 4: Phosphorylation after treatment with STI517**

Phosphorylation patterns of proteins from the different cell lines before, and 24 hours after STI571 treatment. Total protein in cell lysates either before or 24 hours after STI571 treatment were applied to PAGE (see Materials and Methods). Phosphorylated proteins (from 3\times10^5 cells per lane) were developed by Western blotting from untreated (left gels) or from cells treated with STI571 for 24 hours (right gels) with ant total P-Tyr-specific mAb 4G10 (A), or with P-Syk-specific mAb (B). Note in (A) the shorter exposure times for gels before, and longer exposure times for gels after STI571 treatment. Note that in (B) the signals were so low that the exposure time had to be increased to 10 minutes, and a tonal correction had to be made to gain signals.