Conventional Light Chains Inhibit the Autonomous Signaling Capacity of the B Cell Receptor

Sonja Meixlsperger,1 Fabian Köhler,1 Thomas Wossning,1 Michael Reppel,2 Markus Müschen,3 and Hassan Jumaa1,*

1 Institute for Biology III, Albert-Ludwigs University of Freiburg and Max Planck Institute for Immunobiology, Stuebeweg 51, 79108 Freiburg, Germany
2 Institute for Neurophysiology, Universität zu Köln, 50931 Cologne, Germany
3 Laboratory for Molecular Stem Cell Biology, Heinrich-Heine-Universität, 40225 Düsseldorf, Germany
*Correspondence: jumaa@immunbio.mpg.de
DOI 10.1016/j.immuni.2007.01.012

SUMMARY

Signals from the B cell antigen receptor (BCR), consisting of μ heavy chain (μHC) and conventional light chain (LC), and its precursor the pre-BCR, consisting of μHC and surrogate light chain (SLC), via the adaptor protein SLP-65 regulate the development and function of B cells. Here, we compare the effect of SLC and conventional LC expression on receptor-induced Ca2+ flux in B cells expressing an inducible form of SLP-65. We found that SLC expression strongly enhanced an autonomous ability of μHC to induce Ca2+ flux irrespective of additional receptor crosslinking. In contrast, LC expression reduced this autonomous μHC ability and resulted in antigen-dependent Ca2+ flux. These data indicate that autonomous ligand-independent signaling can be induced by receptor forms other than the pre-BCR. In addition, our data suggest that conventional LCs play an important role in the inhibition of autonomous receptor signaling, thereby allowing further B cell differentiation.

INTRODUCTION

B cell development is characterized by a series of developmental stages defined by the rearrangement status of the immunoglobulin genes and the expression of surface markers (Rollink et al., 1999; Schlissel, 2003). At the pro-B cell stage, the μ heavy chain (μHC) gene is rearranged. For this process, the recombination activating genes 1 and 2 (RAG1 and 2) are essential (Bassing et al., 2002). Productive rearrangement leads to μHC expression on the cell surface in the context of the pre-BCR, in which μHC is associated with the Ig-α, Ig-β heterodimer and the surrogate light chain (SLC) components β5 and VpreB (Karasuyama et al., 1994; Tsubata and Reth, 1990). The pre-BCR serves as an important checkpoint in B cell development, and loss of any of the components of the pre-BCR results in developmental arrest at the pro-B cell stage (Kitamura et al., 1992; Mundt et al., 2001). Signaling from the pre-BCR induces proliferation, followed by the downregulation of the pre-BCR and light chain (LC) recombination (Burnows et al., 2002). Prior to LC recombination, the kappa locus is transcribed in its germine configuration, indicating the activation of the kappa gene locus for RAG-mediated recombination (Schlissel and Baltimore, 1989). Transcription factors such as Spi-B and Irf4 are involved in this process (Muljo and Schlissel, 2003). Upon productive rearrangement, κLC is expressed with the μHC and the Ig-α, Ig-β complex as the BCR on immature B cells (Matthias and Rolink, 2005). Although it is clear that binding of antigen activates the BCR, it remains controversial whether the pre-BCR requires a ligand (Bradi et al., 2003; Gauthier et al., 2002) or can signal autonomously (Ohrishi and Melchers, 2003).

SLP-65 is a central molecule in signaling downstream of the pre-BCR and BCR. SLP-65 is phosphorylated by the tyrosine kinase Syk, thereby providing docking sites for Bruton’s tyrosine kinase (Btk) and phospholipase Cγ2 (PLCγ2) (Kurosaki and Tsukada, 2000). This close contact allows the activation of PLCγ2 by Syk and Btk and results in the generation of Ca2+ signals (Hashimoto et al., 1999; Ishiai et al., 1999; Su et al., 1999). These Ca2+ signals are involved in the regulation of proliferation, differentiation, and function of B cells. The central role of SLP-65 is demonstrated by SLP-65-deficient mice that show severe defects in B cell signaling and partial blocks in B cell development (Hayashi et al., 2000; Jumaa et al., 1999; Pappu et al., 1999; Xu et al., 2000). Pre-B cells isolated from the bone marrow of SLP-65-deficient mice express high amounts of the pre-BCR on their surface and can be cultured in IL-7-supplemented medium. Reconstitution with SLP-65 allows the cells to resume their differentiation program and to become κLC-expressing immature B cells (Flemming et al., 2003).

The ligand-binding domain of the estrogen receptor (ER) provides a powerful tool for inducible activation of protein function. Fusion of the protein of interest to the ER renders protein activity dependent on the presence
of estradiol. ERT2 is a variant of ER, which was mutated to make it hardly responsive to estradiol but very sensitive to the synthetic ligand 4-hydroxytamoxifen (OHT) (Feil et al., 1997). In the absence of OHT, heatshock proteins (Hsps) bind to ERT2 and keep the ERT2 fusion protein in an inactive state. Addition of OHT releases the Hsps and allows relocalization and activation of the fusion protein.

For this study, we generated an inducible form of SLP-65 by fusing it to ERT2 at the N terminus. With this system, we showed that expression of SLC greatly enhanced the autonomous, SLP-65-dependent signaling capacity of µHC. In contrast, expression of conventional LC abolished this signaling capacity, pointing to a new role for LCs in the regulation of B cell development.

RESULTS

Inducible Activation of SLP-65

To study the kinetics of SLP-65 function in B cells, we generated an inducible form of SLP-65 by fusing it to ERT2 at the N terminus. With this system, we showed that expression of SLC greatly enhanced the autonomous, SLP-65-dependent signaling capacity of µHC. In contrast, expression of conventional LC abolished this signaling capacity, pointing to a new role for LCs in the regulation of B cell development.
Activation of the Kappa Locus by SLP-65

To address the molecular mechanisms of SLP-65-controlled differentiation processes, we performed RT-PCR analysis with RNA isolated from sorted ERT2-SLP-65-expressing Oct cells that were incubated with OHT for different periods of time. We analyzed the expression of \( Irf4 \), which encodes a transcription factor that binds to the kappa LC locus enhancer and promotes transcription of the unrecombined kappa locus. The time course showed that SLP-65 promotes \( Irf4 \) transcription, which was already maximally upregulated after 4 hr of induction of SLP-65 function (Figure 2). Subsequently, kappa transcripts were upregulated within 8 hr and reached full activation after 24 hr of induction. In contrast, SLP-65 activation resulted in downregulation of \( \lambda 5 \) expression. These data indicate that SLP-65 transmits pre-BCR signals that terminate pre-BCR expression and activate the kappa gene locus, thus making it accessible to the recombination machinery. Moreover, they showed that the kappa gene locus is tightly regulated in the absence of OHT, providing further evidence for the efficiency of this inducible system.

SLP-65 Provokes Ligand-Independent Ca\(^{2+} \) Flux

Kappa transcription required only SLP-65 activation and no additional receptor crosslinking, but SLP-65 is required for Ca\(^{2+} \) mobilization in pre-B cells (Köhler et al., 2005; Su and Jumaa, 2003), so we tested whether induction of SLP-65 function in the ERT2-SLP-65-positive Oct cells was sufficient to induce Ca\(^{2+} \) flux. Indeed, the experiments showed that robust and sustained Ca\(^{2+} \) flux was generated about 2 min after addition of OHT (Figure 3A). These data suggest that activating SLP-65 function in pre-B cells is sufficient to induce Ca\(^{2+} \) flux in the absence of additional receptor stimulation via crosslinking antibodies or ligands. Importantly, adding OHT to SLP-65-deficient pre-B cells expressing only ERT2 did not induce Ca\(^{2+} \) flux (Figure S2A), ruling out the possibility that the robust Ca\(^{2+} \) flux is a side effect of the OHT treatment. Moreover, incubating the ERT2-SLP-65-positive Oct cells with wortmannin (a PI3-K inhibitor) drastically reduced their ability to mobilize Ca\(^{2+} \), suggesting that physiological signaling pathways initiated by PI3-K are required for Ca\(^{2+} \) flux in these cells (Figure S2B).

To further confirm the hypothesis of autonomous ligand-independent pre-BCR activity, we performed Ca\(^{2+} \) flux experiments with ERT2-SLP-65-expressing pre-B cells in medium lacking fetal calf serum (FCS). These experiments showed that addition of OHT was sufficient to induce Ca\(^{2+} \) flux in these cells (Figure S2C), ruling out the possibility that some unknown factors in FCS might serve as pre-BCR ligands that triggered the receptor. Furthermore, Ca\(^{2+} \) flux was measured in single cells (data not shown), suggesting that cell-cell contact is not required for pre-BCR signaling and supporting the model of autonomous pre-BCR function.

To test whether preactivation of SLP-65 affects the signaling capacity of the pre-BCR, we incubated the ERT2-SLP-65-positive cells with OHT overnight to mimic pre-BCR signaling in pre-B cells expressing wt SLP-65. Compared to ERT2-SLP-65-positive cells that were incubated with the solvent alone, the OHT-pretreated cells showed elevated Ca\(^{2+} \) baseline amounts (Figure 3B). An additional OHT dose showed no effect on the Ca\(^{2+} \) signal of these OHT-pretreated cells. Crosslinking the pre-BCR with anti-IgM antibodies (right) showed a clear response, indicating that the pre-BCR is functional in these cells.
anti-μHC, however, led to a slight increase in Ca\(^{2+}\) flux (Figure 3B). These data suggest that constitutive pre-BCR signaling leads to adaptation or exhaustion of the involved signaling pathways, thereby reducing pre-BCR-signaling capacity.

**Pre-BCR Is Required for SLP-65-Induced Ca\(^{2+}\) Flux**

With this inducible system for SLP-65 function, we asked whether or not the pre-BCR itself is required for the robust Ca\(^{2+}\) flux induced by SLP-65 activation. To study this, we used a pre-B cell line derived from the bone marrow of a SLP-65, RAG2, λ5 triple-deficient mouse. Because of RAG2 deficiency, triple-deficient cells cannot express the pre-BCR or the BCR. To test SLP-65 function in the absence of the pre-BCR, we expressed the inducible ERT2-SLP-65 fusion in triple-deficient cells and sorted them for GFP expression. Activation of SLP-65 by OHT treatment did not result in Ca\(^{2+}\) flux in these cells, showing that the expression of SLP-65 by itself is not sufficient for the generation of Ca\(^{2+}\) signals (Figure 4A, first row). Subsequently, we transduced the ERT2-SLP-65-positive triple-deficient cells with an expression vector for a murine μHC (B1-8). As reported previously, the μHC can come to the surface with the Ig-α, Ig-β heterodimer and form a signaling-competent receptor in the absence of SLC (Galler et al., 2004; Schuh et al., 2003; Su et al., 2003). Therefore, we tested whether μHC expression is sufficient to form functional receptors in pre-B cells. In fact, OHT treatment of cells sorted for μHC and ERT2-SLP-65 expression induced a delayed Ca\(^{2+}\) signal with intermediate intensity, suggesting that the SLC-deficient pre-BCR form is capable of inducing autonomous ligand-independent signaling (Figure 4A, second row).

**Associated LCs Modulate Ca\(^{2+}\) Signal Intensity**

Because triple-deficient cells cannot express the pre-BCR or the BCR, they can be used to reconstitute these receptors and compare their function in cells with the same genetic background. Hence, we addressed the question of whether SLP-65 activation downstream of the BCR is also sufficient to induce Ca\(^{2+}\) flux in the absence of additional ligands.

Therefore, we first expressed λ5 in the ERT2-SLP-65, μHC-positive triple-deficient cells (shown in Figure 4B, first row) to reconstitute pre-BCR expression. After sorting the cells for pre-BCR expression, we analyzed Ca\(^{2+}\) flux upon SLP-65 activation. In agreement with the results obtained from the Oct pre-B cell line, activation of SLP-65 in pre-BCR-expressing cells led to a massive Ca\(^{2+}\) signal (Figure 4B, first row). Because a recent report proposed a model in which the non-immunoglobulin (non-Ig) portion of λ5 mediates the crosslinking of pre-BCR complexes on single cells and allows ligand-independent signal transduction (Ohnishi and Melchers, 2003), we generated a mutant λ5, in which the non-Ig region was deleted (non-Ig\(^{-}\) λ5). We expressed this mutant in the μHC, ERT2-SLP-65-positive triple-deficient cells and sorted the cells. Although high pre-BCR expression was detected (Figure 4B, second row), activation of SLP-65 in these cells resulted in a greatly reduced Ca\(^{2+}\) signal as compared to the Ca\(^{2+}\) signal in cells with wild-type pre-BCR (Figure 4B, compare first and second row). However, stimulation of the non-Ig\(^{-}\) λ5 containing pre-BCR with μ chain-specific antibody induced a strong Ca\(^{2+}\) signal (Figure S2D). This indicates that the pre-BCR can signal in the absence of a ligand in vitro and that, in addition to SLP-65, the non-Ig region of λ5 is essential for pre-BCR signaling. To investigate BCR function in our system, we next expressed λLC (Wossning and Reth, 2004) in ERT2-SLP-65, μHC-positive triple-deficient cells (shown in Figure 4B, first row) to reconstitute BCR expression. In contrast to the massive Ca\(^{2+}\) signal in pre-BCR-positive cells, activation of SLP-65 in BCR-positive cells failed to generate a Ca\(^{2+}\) signal (Figure 4C, first row). We obtained the same result when expressing the λLC in the ERT2-SLP-65, μHC-positive triple-deficient cells to generate the BCR (data not shown).

In addition, we confirmed that the BCR was functional and capable of inducing Ca\(^{2+}\) signal. Therefore, we stimulated the BCR-expressing cells with nitro-iodo-phenol (NIP) as the μHC and λLC used were derived from a hybrid-oma cell line generating antibodies specific for NIP (Reth et al., 1978). These experiments showed that addition of NIP resulted in a rapid Ca\(^{2+}\) signal without additional ERT2-SLP-65 activation (Figure 4C, second row). This is in agreement with our recent data showing that BCR engagement on SLP-65-deficient human B cells induces Ca\(^{2+}\) flux, which is dependent on the activity of src-family kinases (Sprangers et al., 2006). However, activation of SLP-65 by OHT resulted in a slightly increased and more sustained BCR-induced Ca\(^{2+}\) signal (Figure 4C, second row, compare left and right panels). Similar results were obtained via crosslinking antibodies recognizing μHC (data not shown). These data show that the BCR is functional but requires a ligand to induce a Ca\(^{2+}\) response.

In summary, the data support the model that the pre-BCR is an active complex that can signal autonomously, unlike the BCR that requires crosslinking antigen for Ca\(^{2+}\) signaling. Moreover, the fact that the parental μHC-positive cells were able to flux Ca\(^{2+}\) in contrast to the BCR-positive cells argues for a negative influence of the LC on the aggregation and signaling competence of the μHC (compare Figure 4A, second row and Figure 4C, first row).

**Activation-Induced SLP-65 Phosphorylation**

The activation of SLP-65 was sufficient to induce robust Ca\(^{2+}\) flux with the pre-BCR but not the BCR, so we investigated ERT2-SLP-65 expression in the corresponding cell lines. Immunoblot analysis revealed similar amounts of ERT2-SLP-65 in pre-BCR- and BCR-expressing triple-deficient cells. Moreover, ERT2-SLP-65 expression in these cells was comparable to wild-type SLP-65 expression in other B cell lines such as WEHI231 (Figure 5A and data not shown). This shows that the observed effects are not caused by overexpression of ERT2-SLP-65 in the pre-BCR-expressing cells. μHC expression, however, was higher in BCR-positive compared to pre-BCR-positive cells, which is in agreement with the FACS analysis (Figures 5A, 4B, first row, and 4C).
Figure 4. Distinct Requirements for Ca²⁺ Flux in Pre-BCR- and BCR-Positive Cells

(A) FACS analysis for µHC expression and Ca²⁺ measurement of ERT2-SLP-65-positive SLP-65, RAG2, λ5 triple-deficient cells without and with addition of 1 µM OHT (first row). FACS analysis for µHC expression of these ERT2-SLP-65-positive triple-deficient cells that were transduced and sorted for µHC expression (gray) as compared to the untransduced cells (black), and Ca²⁺ measurement of the µHC-positive cells either without or with addition of 1 µM OHT (second row).

(B) FACS analysis and Ca²⁺ measurement of ERT2-SLP-65, µHC-positive triple-deficient cells (shown in [A], second row) that were transduced with λ5 and sorted for receptor expression. FACS staining for pre-BCR expression (µHC versus SLC) and Ca²⁺ measurement without or with addition of 1 µM OHT is shown (first row). The same is shown for ERT2-SLP-65, µHC-positive triple-deficient cells transduced with λ5 with a deleted non-Ig portion (second row).

(C) As in [B], but FACS staining (µHC versus λLC) and Ca²⁺ measurement for cells expressing BCR (first row). Ca²⁺ measurement without or with addition of 1 µM OHT in combination with stimulation with 10 µg/ml NIP-BSA is shown for the same cells (second row).
We next tested whether inducing ERT2-SLP-65 by OHT leads to any alterations in tyrosine phosphorylation of SLP-65 or other signaling proteins. In addition, we stimulated pre-BCR-positive and BCR-positive triple-deficient cells by receptor crosslinking with anti-μHC in the presence of OHT. Immunoblot analysis of total cellular lysates (TCL) suggested that addition of OHT may lead to increased ERT2-SLP-65 tyrosine phosphorylation, particularly in pre-BCR-positive cells (Figure 5B, lanes 2 and 5). In contrast, anti-μHC stimulation induced stronger overall tyrosine phosphorylation in BCR-positive as compared to pre-BCR-positive cells (Figure 5B, lanes 3 and 6). To confirm the increased tyrosine phosphorylation after OHT addition, we performed immunoprecipitations (IPs) for ERT2-SLP-65. These IPs showed that basal ERT2-SLP-65 phosphorylation is already detectable without stimulation in pre-BCR-positive cells as compared to BCR-positive cells (Figure 5B, lanes 8 and 11). In addition, they showed that OHT treatment resulted in a clear increase of ERT2-SLP-65 phosphorylation in pre-BCR-positive cells, which was only minimally augmented when the cells were also stimulated by receptor crosslinking (Figure 5B, lanes 8 to 10). OHT treatment of BCR-positive cells resulted in low, but apparent, induction of ERT2-SLP-65 phosphorylation, which was drastically increased upon receptor engagement (Figure 5B, lanes 11 to 13). Importantly, co-IP of other phosphorylated proteins correlates closely with the phosphorylation status of ERT2-SLP-65.

In summary, OHT treatment seems to induce higher tyrosine phosphorylation of SLP-65 in pre-BCR-positive cells as compared to BCR-positive cells. This may explain the differences seen in the requirements for the generation of Ca²⁺ flux in these cell lines.

Human Pre-BCR and BCR Behave Like Their Murine Counterparts

To confirm our finding that the pre-BCR acts autonomously but is dependent on SLP-65 function whereas the BCR requires a specific ligand, we used SLP-65-deficient human cell lines expressing either pre-BCR or BCR. The inducible ERT2-SLP-65 system was not established for human cells, so we transiently expressed normal human SLP-65 (hSLP-65) in the SLP-65-deficient Oct pre-B cells (lane 4), and of WEHI231 B cells as control (lane 5) on the same blot. μHC, SLP-65, and Ig-α expression were tested with the respective antibodies. Immunoblotting with anti-actin served as loading control.

In summary, OHT treatment seems to induce higher tyrosine phosphorylation of SLP-65 in pre-BCR-positive cells as compared to BCR-positive cells. This may explain the differences seen in the requirements for the generation of Ca²⁺ flux in these cell lines.

DISCUSSION

In this study, we define a new property of conventional LCs by showing that they inhibit the autonomous signaling
capacity of the BCR. We propose that this property plays an important role in the selection of developing B cells. In contrast to conventional LCs, we show that SLC induces constitutive autonomous receptor signaling. We obtained these results by using ERT2-SLP-65 fusions, which allowed us to efficiently induce the function of the essential signaling protein SLP-65 in BCR- or pre-BCR-expressing cells. Interestingly, our data showed that efficient induction of SLP-65 activity requires N-terminal fusion of SLP-65 to ERT2, as indicated by the fact that SLP-65 activity was leaky in the C-terminal fusion. A possible explanation for the discrepancy between N-terminally and C-terminally fused ERT2 is that the N terminus of SLP-65, which we have shown to be essential for membrane recruitment and activity of SLP-65 (Köhler et al., 2005), might be accessible, leading to membrane association of SLP-65 in the C-terminal fusion. 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With this SLP-65-inducible system, we found that, in contrast to the BCR, pre-BCR-induced Ca\textsuperscript{2+} signaling was independent of ligand and cell-cell interactions. Moreover, our data indicate a unique mode of Ca\textsuperscript{2+} signaling in pre-BCR-expressing cells that show autonomous repetitive Ca\textsuperscript{2+} flux. An obvious question is how SLP-65 activates autonomous pre-BCR-dependent Ca\textsuperscript{2+} flux. It is conceivable that induction of SLP-65 phosphorylation leads to the assembly and translocation of preactivated signaling proteins to the pre-BCR. In BCR-expressing cells, however, SLP-65 phosphorylation does not reach the required threshold, probably resulting from inefficient preactivation of the responsible kinases, which seem to be preactivated in pre-B cells. This is in line with data showing increased Btk, Blk, and Fyn activation in pre-BCR-expressing cells as compared to BCR-expressing cells (Aoki et al., 1994a, 1994b; and our unpublished results).

In addition to SLP-65, the non-Ig portion of λ5 is required for efficient pre-BCR signal transduction, as demonstrated by recent data (Ohnishi and Melchers, 2003). Our results are in full agreement with these data and, by showing that the autonomous capacity of the pre-BCR to induce Ca\textsuperscript{2+} flux could be detected in isolated single cells, they provide additional evidence for ligand-independent pre-BCR signaling. We can not, however, exclude the existence of potential cis-acting ligands located on the membrane that allow interaction between pre-BCRs of the same cell. On the other hand, it has been suggested that molecules produced by stromal cells in the bone marrow can serve as ligands for the pre-BCR. For instance, murine pre-B cells can bind heparan sulfate (Bradl et al., 2003), and human pre-B cells attach to galectin-1 (Gauthier et al., 2002). However, neither stromal cells nor FCS were required for Ca\textsuperscript{2+} mobilization in ERT2-SLP-65-positive pre-B cells, arguing against the existence of specific pre-BCR ligands in our experimental system.

The constitutive and autonomous signaling capacity of the pre-BCR suggests that critical pre-BCR signals have already been delivered in commonly used pre-B cell lines that express SLP-65 (Aoki et al., 1994a, 1994b;
Karasuyama et al., 1997). In fact, when we mimicked constitutive pre-BCR signaling by inducing SLP-65 activity in pre-B cells overnight, only minor Ca\(^{2+}\) signals were generated after pre-BCR crosslinking. This suggests that constitutive signaling hampers the analysis of pre-BCR signaling in commonly used pre-B cells, whereas inducible activation of SLP-65 provides a potent system to trigger pre-BCR signaling in a timely regulated manner. Our results showed that few hours after triggering pre-BCR signaling, the LC gene locus was accessible for V(D)J recombination and transcription of the SLC component \(\lambda\)5 was downregulated. Thus, inducible SLP-65 activation not only allows the characterization of immediate pre-BCR signals, such as Ca\(^{2+}\) flux, but also provides a powerful system for analyzing the molecular requirements for connecting pre-BCR signaling with LC gene activation and pre-B cell differentiation.

We measured Ca\(^{2+}\) in triple-deficient cells expressing different receptor forms (\(\mu\)HC, conventional, or mutant pre-BCR and BCR) after activation of SLP-65. This confirmed the importance of SLC as an internal ligand for pre-BCR signal transduction. Introduction of a \(\lambda\)LC to express BCR, however, prevented the intermediate Ca\(^{2+}\) signals, which were observed with triple-deficient cells expressing only \(\mu\)HC. This may indicate that conventional LCs are selected for inhibiting autonomous BCR aggregation. From these data, we propose a model in which the SLC and LC play opposite roles in regulating receptor activation (Figure 7). Without a SLC or conventional LC, we assume that \(\mu\)HC possesses an intrinsic capacity to aggregate and form multimeric complexes that can signal if the intracellular signaling machinery is intact. Binding of the SLC to the \(\mu\)HC enhances the aggregation capacity and results in higher autonomous signaling, which is mediated by the non-Ig portion of \(\lambda\)5 (Ohnishi and Melchers, 2003). In contrast, the negative effect of conventional LC may prevent the aggregation capacity of \(\mu\)HC on the cell surface. Only binding of a ligand allows aggregation with subsequent signal transduction. In accordance with the current view of the role of the pre-BCR (Melchers, 2005), this concept of receptor activation would allow proliferation of all pre-B cells, which express a \(\mu\)HC capable of pairing with the SLC. On the other hand, a negative role of conventional LCs in BCR aggregation may provide an important model for the selection of immature B cells. Naturally arising autoantibodies are often polyreactive (Wardemann et al., 2003, 2004), so it is well likely that they possess increased ability to interact and aggregate on one single cell. This aggregation might lead to autonomous signaling comparable to that induced by the pre-BCR, suggesting that polyreactive receptors may induce proliferation. However, conventional LCs that prevent autonomous receptor aggregation on immature B cells enable further differentiation (Figure 7). The ability of some \(\mu\)HCs to form pre-BCR like complexes with conventional LCs is supported by data showing that a prematurely expressed LC can rescue the deficient B cell development in mice lacking pre-BCR expression (Pelanda et al., 1996). In addition, it provides a cell-autonomous mechanism for removing polyreactive antibodies from developing B cells and is in full agreement with the data showing that LCs are involved in silencing autoreactive antibodies (Wardemann et al., 2004).

Of note, the inability of the BCR to induce Ca\(^{2+}\) mobilization after SLP-65 activation is not in conflict with the importance of BCR expression for B cell survival indicating
Role of Light Chains in B Cell Signaling

**EXPERIMENTAL PROCEDURES**

**Culture Conditions**

Oct (Su and Jumaa, 2003) and SPL-65, RAG2, λ5 triple-deficient cells were cultured in complemented Iscove’s medium (Storch et al., 2007). Supernatant of J558L cells stably transfected with a vector for murine IL-7 was added. Differentiation experiments were done by culturing the cells for 4 days in complemented Iscove’s medium lacking IL-7. HPB null (Taguchi et al., 2004) and KARPAS-422 (Sprangers et al., 2006) cells were cultured in complemented RPMI medium containing 10% FCS. Cells were incubated and stimulated with 1 µM 4-Hydroxytamoxifen (OHT, Sigma-Aldrich). As negative control, cells were treated with the same volume of EtOH, the solvent of OHT.

**Retroviral Constructs and Transductions**

The ERT2 cassette (Indra et al., 1999) was fused to the N terminus or the C terminus of SLP-65 in pMIG or pMOWS, respectively. λ5 was cloned by PCR with primers covering the start and stop codon and inserted into the pMOWS vector. Deletion of the non-ig portion of λ5 (JC mutant) was done as described (Ohnishi and Melchers, 2003). The λLC and the μHC were amplified from μ3-11 cDNA (Wossing and Reth, 2004) and inserted into respective retroviral vectors. Retroviral transductions of murine cells were done as described (Storch et al., 2007). Human SLP-65 deficient B cell leukemia and lymphoma cells were transfected with pMIG-GFP-SLP-65 or pMIG-GFP vector as control by a nucleofection system according to the manufacturer’s instructions (Amaxa Biosystems). Cells were sorted under sterile conditions with a FACStar 440 or a MoFlo (Dako Cytomation) cell sorter.

**Ca²⁺ Measurements**

Ca²⁺ measurements were done as described (Storch et al., 2007). Cells were stimulated with 1 µM OHT. As negative control, cells were treated with the same volume of EtOH, the solvent of OHT. NIP-BSA (Biosearch Technologies) and anti-IgM (μHC-specific, Southern Biotechnology/Biozol) at a final concentration of 10 µg/ml for 3 min at 37°C. SLP-65 was activated by incubating the cells in 1 µM OHT for 3 min at 37°C. Antibodies recognizing phospho-tyrosine (4G10, Upstate Biotech), SLP-65 (generated in our department by B. Wollscheid), μHC (Pierce), actin (Santa Cruz), Ig-α (kind gift of H.M. Jäck), HA (Roche), eIF-4A (kind gift of P. Nielsen), or P-Btk (New England Biolabs) were used.

**RT-PCR Analysis**

Cells were cultured with or without 1 µM OHT for the indicated time. RNA was purified with Trizol according to the manufacturer’s instructions (Invitrogen Life Technologies). 1 µg RNA was used to perform cDNA synthesis as described (Jumaa et al., 1999), with 0.1 µg random hexamer oligonucleotides. The resulting cDNA was used in PCR assays to amplify kappa transcripts of the unrecombined kappa locus (also referred to as germine transcripts), Ir4, λ5, and HPRT gene transcripts. The PCR fragments were separated on agarose gels and detected by ethidium bromide staining. The following primers were used: κGL, λ5, and HPRT (Grawunder et al., 1995) and Ir4 (Muljo and Schlissel, 2003).

**Supplemental Data**

Two Supplemental Figures can be found with this article online at http://www.immunity.com/cgi/content/full/26/3/323/DC1/.

**ACKNOWLEDGMENTS**

We would like to thank C. Eschbach and I. Fiedler for excellent technical assistance, A. Wuerch for cell sorting, and M. Reth for critical discussions. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 620 and SFB 746). S.M. is supported by a Boehringer Ingelheim Fonds fellowship.

Received: February 28, 2006
Revised: December 14, 2006
Accepted: January 26, 2007
Published online: March 1, 2007

**REFERENCES**


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Tsubata, T., and Reth, M. (1990). The products of pre-B cell-specific genes (lambda 5 and VpreB) and the immunoglobulin mu chain form a complex that is transported onto the cell surface. J. Exp. Med. 172, 973–976.


