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Ig-α Cytoplasmic Truncation Renders Immature B Cells More Sensitive to Antigen Contact

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Summary

To study the function of Ig-α in the selection of autoreactive B cells, we have analyzed *mb-1* cytoplasmic truncation mutant mice (*mb-1*$^{\Delta c/\Delta c}$), which coexpress transgenes encoding hen egg lysozyme (HEL) and HEL-specific immunoglobulin. We demonstrate that in the presence of soluble HEL (sHEL) and dependent on the *mb-1*$^{\Delta c}$ mutation, most immature B cells bearing the HEL-specific Ig transgene undergo rearrangements of endogenous κ light chains, resulting in loss of HEL specificity. Moreover, immature B cells from Ig-α mutant mice respond to BCR cross-linking with an exaggerated and prolonged calcium response and induction of protein tyrosine phosphorylation. Our data imply a negative signaling role for Ig-α in immature B cells.

Introduction

The B cell antigen receptor complex (BCR) transmits signals that regulate the survival, differentiation, proliferation, and migration of B lymphocytes (Rajewsky, 1996; Reth and Wienands, 1997; Healy and Goodnow, 1998). The signaling function of the BCR has been ascribed to the mIg associated Ig-α and Ig-β (CD79a/CD79b) heterodimers that are encoded by the genes *mb-1* and *B29*, respectively. Both Ig-α and Ig-β contain a single immunoreceptor tyrosine-based activation motif (ITAM), a common motif found also in the receptor complexes of other cells in the immune system including T, NK, and mast cells (Reth, 1989; Cambier 1995a). BCR cross-linking leads to the activation of protein tyrosine kinases (PTKs) and subsequently to the phosphorylation of the two ITAM tyrosines (reviewed in Cambier, 1995b; Reth and Wienands, 1997). These phosphorylated tyrosine residues represent binding sites for Src homology 2 (SH2) domain–containing proteins including Src family protein tyrosine kinases and Syk (Clark et al., 1992; Johnson et al., 1995). In several studies with Ig-α and Ig-β chimeric molecules expressed in B cell lines, the requirement of Ig-α and Ig-β ITAMs in signal transduction has been well documented (Kim et al., 1993; Sanchez et al., 1993; Flaswinkel and Reth, 1994; Taddie et al., 1994; Williams et al., 1994; Pao et al., 1998). The consensus of these experiments is that tyrosine phosphorylation of ITAMs is required for the induction of protein tyrosine phosphorylation and calcium mobilization. In one study (Flaswinkel and Reth, 1994) using the J558L myeloma cell line, mutant Ig-α molecules were tested in the context of a complete BCR. The ability to activate PTKs and induce tyrosine phosphorylation was severely reduced in cells expressing Ig-α molecules with a truncated cytoplasmic tail or an ITAM in which both tyrosines were changed to phenylalanine, even in the presence of endogenous Ig-β.

Transgenic and gene targeting experiments in mice have demonstrated the importance of Ig-α and Ig-β for B cell development in vivo. Either Ig-α or Ig-β chimeric molecules expressed as transgenes have been sufficient for supporting steps in B cell maturation and inducing allelic exclusion (Papavasiliou et al., 1995a, 1995b; Teh and Neuberger, 1997). In Ig-β knockout mice, a complete block in B cell development at the pro-B cell stage was observed, probably because the BCR cannot reach the cell surface in the absence of Ig-α/Ig-β heterodimer formation (Gong and Nussenzweig, 1996). *mb-1*$^{\Delta c/\Delta c}$ mice express a truncated Ig-α protein that lacks the ITAM and has a cytoplasmic tail that consists of 20 instead of 61 amino acids (Torres et al., 1996). B lymphocytes in *mb-1*$^{\Delta c/\Delta c}$ mice are compromised in their development resulting in a 2- to 4-fold reduction in the number of pre- and immature B cells in the bone marrow and a drastic 100-fold decrease of mature B lymphocytes in the spleen. Moreover, it was demonstrated that mature B cells are dependent on Ig-α-mediated signals to mount an immune response to a T-independent antigen (Torres et al., 1996). In the present study, we further evaluate the signaling function of Ig-α in the hen egg lysozyme (HEL) transgenic mouse model (Goodnow et al., 1988) to determine the impact of Ig-α on positive versus negative signaling. *mb-1*$^{\Delta c/\Delta c}$ mice were generated expressing HEL-specific Ig (Ig^{HEL}) alone or coexpressing either soluble (sHEL; Goodnow et al., 1988) or membrane bound HEL (mHEL; Hartley et al., 1991), to study whether tolerance induction either by "anergy" or clonal deletion was still operational. One might also envision that the expression of the transgenic anti-HEL receptor on its own or in combination with HEL might overcome the developmental block observed in *mb-1*$^{\Delta c/\Delta c}$ mice and lead to antigen-driven maturation of B lymphocytes. Our data demonstrate that autoreactive B cells or their receptors are more efficiently counterselected in *mb-1*$^{\Delta c/\Delta c}$ than in wild-type mice and suggest that this is due to hyperresponsiveness of mutant cells to BCR cross-linking.

Results

In order to examine how an Ig-α cytoplasmic truncation affects BCR-mediated signaling in relation to positive

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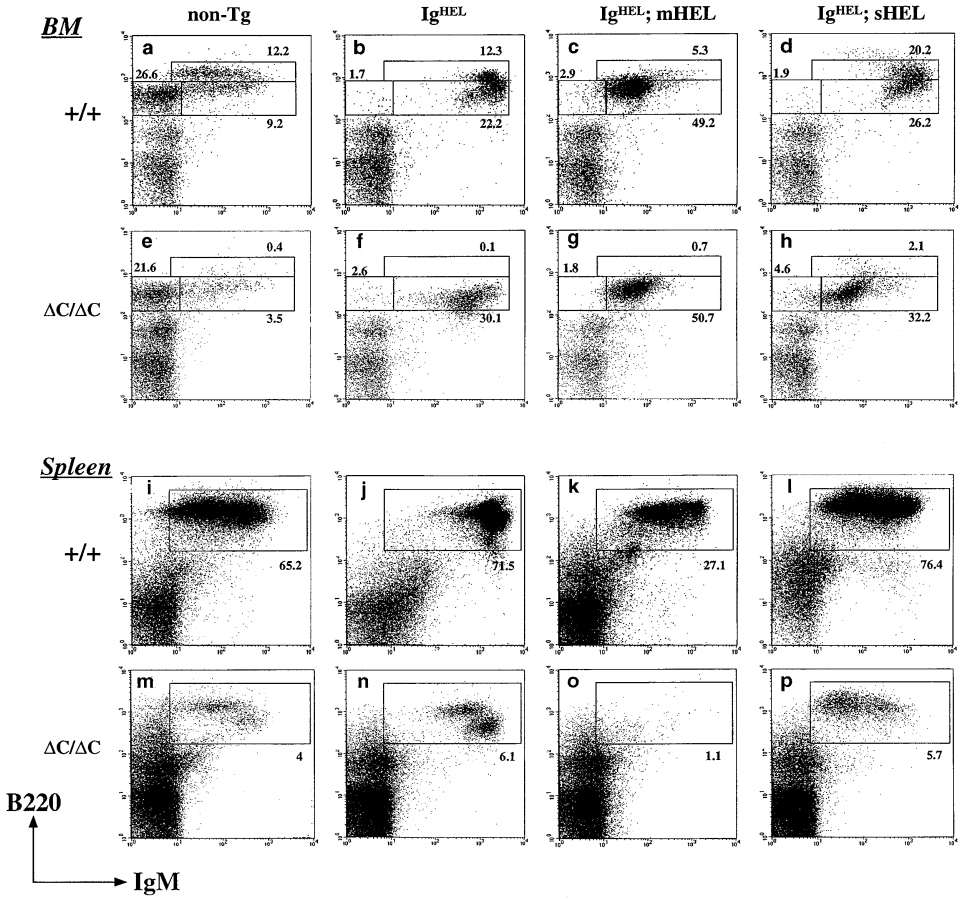

Figure 1. Enhanced Counterselection of Autoreactive B Cells in *mb-1*$^{\Delta c/\Delta c}$ Mice

B cell development in *mb-1*$^{\Delta c/\Delta c}$ and *mb-1*$^{+/+}$ mice, either nontransgenic or carrying a combination of transgenes for a HEL-specific immunoglobulin receptor and a soluble or membrane bound form of HEL, was analyzed by flow cytometry. B lymphocytes in the bone marrow (A–H) and spleen (I–P) were revealed by staining with anti-B220 and anti-IgM mAbs. The frequency of cells in the windows is shown as percentage of cells within the lymphocyte gate. Due to the low number of peripheral B cells in *mb-1*$^{\Delta c/\Delta c}$ mice, dot plots showing splenic cells of mice with the *mb-1*$^{\Delta c}$ mutation display ten times more events compared to wild-type controls.

and negative selection of B lymphocytes, *mb-1*$^{\Delta c/\Delta c}$ mice (Torres et al., 1996) carrying Ig^{HEL} transgenes alone or coexpressing either mHEL or sHEL (Goodnow et al., 1988; Hartley et al., 1991) were analyzed. Flow cytometric analysis showed that in *mb-1*$^{\Delta c/\Delta c}$Ig^{HEL} transgenic mice almost all B cells express the transgenic receptor on their surface and bind fluorochrome-coupled lysozyme, as in *mb-1*$^{+/+}$Ig^{HEL} controls (Figures 1B, 1F, 1J, 1N, 2Bb, and 2Be). These *mb-1*$^{\Delta c/\Delta c}$Ig^{HEL} bone marrow B cells are phenotypically similar to the immature B cells found in *mb-1*$^{+/+}$Ig^{HEL} transgenic mice (Figures 1B and 1F). However, they express about two times less surface IgM and have 2- to 4-fold reduced B220 surface levels compared to *mb-1*$^{+/+}$Ig^{HEL} B cells. Note that in all Ig^{HEL} transgenic mice, probably due to the early expression of the immunoglobulin transgenes, the pro- and pre-B cell compartments are almost absent. Therefore, the population of IgM^{+}B220^{low} cells in the bone marrow of Ig^{HEL} transgenic mice might be of an earlier developmental stage than immature B cells in nontransgenic mice.

Deletion of Autoreactive *mb-1*$^{\Delta c/\Delta c}$ B Cells Recognizing mHEL

Whether tolerance induction in B cells by clonal deletion depends on the Ig-α cytoplasmic tail was tested in *mb-1*$^{\Delta c/\Delta c}$Ig^{HEL} mHEL transgenic mice. Expression of mHEL, which causes clonal deletion of *mb-1*$^{+/+}$ HEL-specific B cells (Hartley et al., 1991), led to a marked decrease of surface IgM within the bone marrow B cell population of *mb-1*$^{\Delta c/\Delta c}$Ig^{HEL} mHEL mice, similar to that in *mb-1*$^{+/+}$Ig^{HEL} mHEL controls (Figures 1B, 1C, 1F, and 1G). In comparison to *mb-1*$^{\Delta c/\Delta c}$Ig^{HEL} mice, the number of splenic B cells was further decreased 10- to 20-fold (Figures 1N and 1O; 1%–3% versus 0.1%–0.2% of spleen cells). These results show that the cytoplasmic part of Ig-α containing the ITAM is not essential for clonal deletion of autoreactive B cells in response to a multivalent, membrane-bound autoantigen.

Soluble HEL Causes Loss of Receptor Specificity in Ig-α-Truncated B Cells

In *mb-1*$^{\Delta c/\Delta c}$ mice, B cells lack most of the cytoplasmic tail of Ig-α, including the ITAM. The absence of this signal regulator might lead to a lowered constant signaling via the BCR and cause the dramatic decrease in peripheral B cell numbers (Flaswinkel and Reth, 1994; Torres et al., 1996; Lam et al., 1997; Figures 1I and 1M). We speculated that in *mb-1*$^{\Delta c/\Delta c}$Ig^{HEL} mice, the expression of a weak self-antigen might promote the maturation and/or persistence of B cells in the periphery. To address this

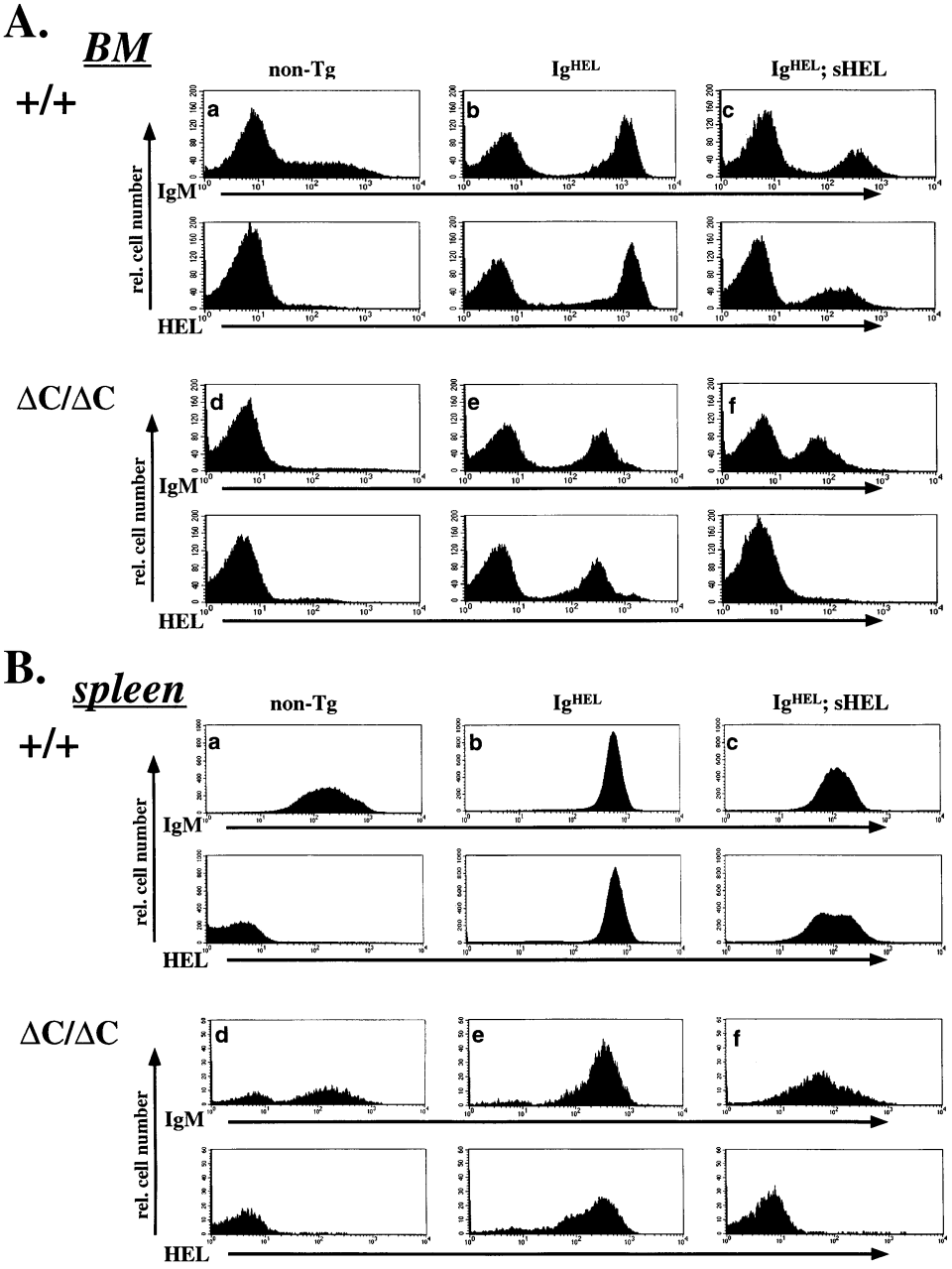

Figure 2. Ig-α Truncation Causes Loss of HEL Receptor Specificity in Ig^{HEL} Transgenic B Lymphocytes in the Presence of sHEL

Bone marrow (A) and splenic (B) cells were stained with anti-IgM and anti-B220 mAbs to define B lymphocytes and with fluorochrome-coupled HEL to detect receptor specificity for HEL. Histograms show IgM surface expression levels and the corresponding HEL binding specificity of cells within the lymphocyte gate (A) or of $B220^+$ cells (B).

issue, mice were generated that express Ig^{HEL} and soluble HEL, in addition to the *mb-1*$^{\Delta c}$ mutation. In such animals, the soluble autoantigen did not cause a significant accumulation of splenic B cells (Figures 1M, 1N, and 1P). On average, they had similar numbers of splenic B cells as mice that do not express the autoantigen, i.e., 0.3–1.3 $\times$ 10^6 cells/spleen. This compares to 28.2 $\pm$ 5.3 $\times$ 10^6 cells/spleen in Ig^{HEL} and 13.9 $\pm$ 0.5 $\times$ 10^6 cells/spleen in Ig^{HEL} sHEL mice.

FACS analysis to define the surface IgM levels and HEL binding specificity was performed in order to determine whether autoreactive *mb-1*$^{\Delta c/\Delta c}$$Ig^{HEL}$ sHEL B cells retain specificity for HEL and modulate their BCR expression levels. A comparison of IgM surface expression levels with the corresponding HEL surface binding intensity is shown in Figures 2A and 2B.

In control mice, sHEL is sufficient to trigger anergy, which is indicated by reduced IgM surface levels, but not the loss of HEL specificity (Goodnow et al., 1988, 1989; Figures 2Bb and 2Bc). By contrast, the *mb-1*$^{\Delta c/\Delta c}$ mutation causes a drastic decrease in receptor specificity for HEL. HEL-binding splenic B cells were not detectable by flow cytometric analysis in *mb-1*$^{\Delta c/\Delta c}$$Ig^{HEL}$ sHEL mice. In four independent experiments, the degree of

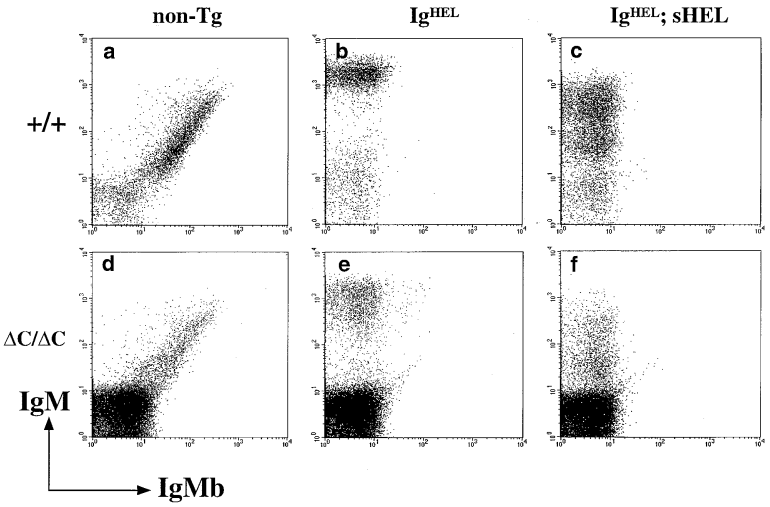

Figure 3. B Lymphocytes of *mb-1*$^{\Delta c/\Delta c}$Ig^{HEL} sHEL Mice Express the Transgenic Heavy Chain

Flow cytometric analysis of splenic cells from nontransgenic, Ig^{HEL}, and Ig^{HEL} sHEL transgenic mice expressing a truncated Ig-α, and the corresponding wild-type controls. Cells were stained with anti-IgM and b allotype–specific anti-IgM. Due to the low number of peripheral B cells in *mb-1*$^{\Delta c/\Delta c}$ mice, dot plots showing splenic cells of mice with the *mb-1*$^{\Delta c}$ mutation display ten times more events compared to wild-type controls.

HEL specificity loss ranged from 35- to 90-fold (Figures 2Be and 2Bf), while the range of IgM surface expression levels was broader and in total 2- to 6-fold reduced compared to *mb-1*$^{\Delta c/\Delta c}$Ig^{HEL} mice (Figures 2Be and 2Bf). Further characterization of splenic B cells in the *mb-1*$^{\Delta c/\Delta c}$Ig^{HEL} sHEL mice showed that they were B220^{high} (Figure 1P) CD43^{-} and had normal expression levels of CD19 (data not shown). More than 80% were IgD^{+} and did not stain for the immature B cell marker 493 (Rolink et al., 1998; data not shown). Thus, the majority of these cells have a mature phenotype.

Rearrangements of Endogenous Light Chains in *mb-1*$^{\Delta c/\Delta c}$Ig^{HEL} B Cells in the Presence of sHEL

The unexpected loss of HEL receptor specificity in the *mb-1*$^{\Delta c/\Delta c}$Ig^{HEL} sHEL B cells may be due to either of two processes. Soluble HEL in the *mb-1*$^{\Delta c/\Delta c}$ mice might trigger a signal that causes deletion of HEL-specific B cells, comparable to the situation in wild-type Ig^{HEL} mice expressing mHEL. Alternatively, the B cells might undergo receptor editing and express endogenous immunoglobulins. Deletion of autoreactive B cells in the presence of membrane-bound HEL occurs in the bone marrow (Hartley et al., 1991). As shown in Figure 1, under such conditions B cells with or without an Ig-α cytoplasmic truncation express low levels of surface IgM. The soluble form of HEL causes a similar downmodulation of IgM levels (Figures 1D and 1H) and induces a loss of detectable receptor specificity for HEL in the *mb-1*$^{\Delta c/\Delta c}$ mutant but not in control mice (Figures 2Ac and 2Af). Thus, the change of receptor specificity occurs in immature B cells in the bone marrow.

To investigate whether the non-HEL-specific B cells had indeed rearranged and expressed endogenous immunoglobulin chains, we looked first for the surface expression of endogenous heavy chains. Since the *mb-1*$^{\Delta c/\Delta c}$ mutant and the HEL transgenic mice are on the C57BL/6 background with allotype *Igh*^{b}, whereas the transgene-encoded heavy chains are *Igh*^{a}, it is possible to detect endogenous heavy chains by an anti-allotypic antibody. As shown in Figure 3, IgM^{b} is only expressed in splenic B cells of nontransgenic mice. Therefore, *mb-1*$^{\Delta c/\Delta c}$Ig^{HEL} sHEL B cells still express the transgene-encoded heavy chains.

With respect to light chain rearrangements, we first determined the fraction of B cells in *mb-1*$^{\Delta c/\Delta c}$Ig^{HEL} sHEL mice that express endogenous λ light chains in comparison to *mb-1*$^{\Delta c/\Delta c}$Ig^{HEL} mice. Flow cytometric analysis revealed no significant difference between these mice, with less than 1.5% of B cells showing surface expression of λ1 light chains (data not shown). For the analysis of κ light chain rearrangements, we employed a single-cell polymerase chain reaction (PCR) approach (Novobrantseva et al., 1999). IgM^{+}B220^{+} cells were sorted from bone marrow preparations of *mb-1*$^{\Delta c/\Delta c}$Ig^{HEL} sHEL, *mb-1*$^{\Delta c/\Delta c}$Ig^{HEL}, and Ig^{HEL} sHEL mice and analyzed for the presence of germline or rearranged κ alleles. Since B cells from Ig^{HEL} transgenic mice have a transgene-encoded VκJκ2 rearrangement, PCR products always included such a joint. As a consequence, it was possible to detect endogenous Igκ rearrangements with the exception of VκJκ2 (Figure 4).

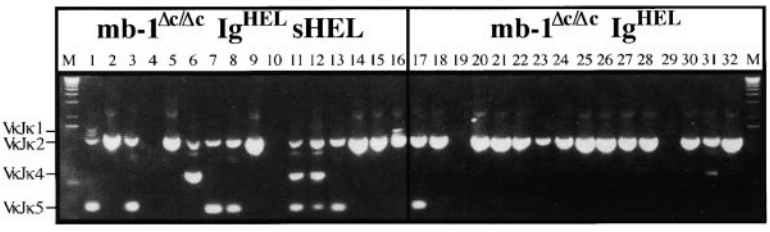

Figure 4. Representative Single-Cell PCR Analysis of VκJκ Joints in Immature B Cells of *mb-1*$^{\Delta c/\Delta c}$Ig^{HEL} sHEL and *mb-1*$^{\Delta c/\Delta c}$Ig^{HEL} Mice

PCR products were fractionated by agarose gel electrophoresis to determine the presence of a specific Jκ element within a VκJκ joint, which corresponds to a defined product size (Novobrantseva et al., 1999). Numbers represent single cells used for VκJκ joint analysis. The presence of a nonrearranged Igκ locus was tested in a separate PCR (data not shown).

Table 1. Single-Cell Analysis of Igκ Rearrangements in Immature B Lymphocytes

Genotype	Number of Cells Analyzed	Cells with PCR Products		Cells with Igκ Joints Other than VκJκ2		Cells with a κ Germline Locus	
mb-1$^{\Delta c/\Delta c}$Ig^{HEL}	40	36	90%	4	11%	22	61%
mb-1$^{\Delta c/\Delta c}$Ig^{HEL} sHEL	45	34	76%	20	59%	13	38%
mb-1$^{+/+}$Ig^{HEL} sHEL	53	45	85%	2	4.4%	37	82%

Single IgM$^+$B220$^+$ bone marrow cells of *mb-1*$^{\Delta c/\Delta c}$Ig^{HEL} sHEL, *mb-1*$^{+/+}$Ig^{HEL} sHEL, and *mb-1*$^{\Delta c/\Delta c}$Ig^{HEL} mice were analyzed by PCR for VκJκ rearrangements. The total number of samples is shown in column 2. Since single-cell sort and PCR efficiencies are below 100%, VκJκ joint amplification products were obtained in a fraction of all analyzed samples as indicated by the numbers and percentages in column 3. Column 4 shows the numbers and percentages of cells with an Igκ light chain rearrangement other than VκJκ2. In column 5, numbers and percentages indicate the cells in which an Igκ locus in germline configuration could be detected.

As shown in Table 1, 20 out of 34 cells (59%) from the *mb-1*$^{\Delta c/\Delta c}$Ig^{HEL} sHEL mice contained additional Igκ rearrangements to VκJκ2. In the absence of sHEL, such rearrangements were found in only 4 out of 36 cells (11%). Likewise, only 2 out of 45 B cells (4.4%) from *mb-1*$^{+/+}$Ig^{HEL} sHEL mice bear additional VκJκ joints. In line with these data, we found less *mb-1*$^{\Delta c/\Delta c}$Ig^{HEL} sHEL cells with an Igκ locus in germline configuration (38%) compared to *mb-1*$^{\Delta c/\Delta c}$Ig^{HEL} (61%) or *mb-1*$^{+/+}$Ig^{HEL} sHEL cells (82%; Table 1). Taking these results together, we conclude that in Ig^{HEL} sHEL mice, the Ig-α cytoplasmic truncation favors the appearance of nonautoreactive B cells expressing endogenous κ light chains.

Strong and Extended Calcium Response in Ig-α-Truncated B Cells

The response of Ig-α-truncated, Ig^{HEL} transgenic, immature B cells toward sHEL, including downregulation of IgM and secondary rearrangements, suggested that these cells are not hypo- but rather hyperresponsive. To examine their BCR signaling capacity, we analyzed IgM$^+$B220^{low} B cells from the bone marrow of *mb-1*$^{\Delta c/\Delta c}$Ig^{HEL} and *mb-1*$^{+/+}$Ig^{HEL} mice. Indo-1 loaded B cells were stimulated with F(ab)′$_2$ goat anti-mouse IgM fragment, anti-IgM, or anti-Ig-β monoclonal antibody and changes in intracellular calcium were monitored by flow cytometry. In *mb-1*$^{\Delta c/\Delta c}$Ig^{HEL} B cells, stimulation induced an elevated and more sustained response compared to the control B cells (Figure 5). However, the calcium response was slightly delayed in immature B cells with an Ig-α truncation. Collectively, these data demonstrate a role for Ig-α in both the initiation and the containment of calcium mobilization.

Ig-α-Truncated Immature B Cells Show Strong Protein Tyrosine Phosphorylation upon BCR Cross-Linking

To further assess the ability of Ig-α-truncated BCRs to transduce signals, we analyzed the effect of the truncation on protein tyrosine kinase activation in immature B cells. Purified Ig^{HEL} transgenic bone marrow B cells were stimulated with F(ab)′$_2$ goat anti-mouse IgM fragment, and protein tyrosine phosphorylation in whole-cell lysates was revealed by anti-phosphotyrosine immunoblotting. As shown in Figure 6, stimulation for 1–30 min showed that the mutant BCRs were capable of inducing protein tyrosine phosphorylation. Moreover, in several experiments the loss of the Ig-α ITAM did not cause an overall decrease of protein tyrosine phosphorylation levels. Total protein tyrosine phosphorylation levels in *mb-1*$^{\Delta c}$ mutant cells were slightly increased and the stimulation was more persistent in time course experiments (Figure 6), or both were comparable to controls (data not shown). However, protein tyrosine phosphorylation levels in B220$^+$ bone marrow B cells from *mb-1*$^{\Delta c/\Delta c}$Ig^{HEL} and *mb-1*$^{+/+}$Ig^{HEL} mice cannot be directly compared, since the cells analyzed comprise different fractions of mature and immature B cells (Figures 1B and 1F; data not shown).

Discussion

In this study we describe a novel aspect of Ig-α function in BCR-mediated signaling. Previous reports have ascribed a positive signaling role to Ig-α (reviewed in Kurosaki, 1999). Consistent with this idea, truncation of the Ig-α cytoplasmic domain in the *mb-1*$^{\Delta c/\Delta c}$ mice leads to a perturbed early B cell development, resulting in a severe reduction in the number of mature B cells (Torres et al., 1996). We now present evidence that Ig-α can also play a negative regulatory role, at least at an early stage of B cell differentiation.

In various transgenic systems, multivalent and widely distributed self-antigens such as double-stranded DNA, blood cell surface antigens, or membrane-bound HEL cause either the deletion of self-reactive B lymphocytes or receptor editing of endogenous immunoglobulin chains (Nemazee and Burki, 1989; Hartley et al., 1991; Okamoto et al., 1992; Gay et al., 1993; Radic et al., 1993; Tiegs et al., 1993; Pelanda et al., 1997). It has been shown by Hartley et al. (1993) that immature self-reactive B cells are eliminated by apoptosis. The role of Ig-α in apoptosis has been investigated with chimeric molecules in vitro. For instance, Tseng et al. (1997) have shown that only heterodimers but not homodimers of Ig-α and Ig-β could induce apoptosis in the immature B cell line WEHI-231. In contrast, Yao et al. (1995) have demonstrated in another B cell lymphoma, CH31, that either Ig-α or Ig-β was capable of inducing apoptosis, and this function was dependent on the presence of a functional ITAM. Consistent with the latter results, our data show that in vivo deletion of immature autoreactive B cells in the presence of mHEL can occur in the absence of Ig-α ITAM, suggesting that Ig-β alone is sufficient for induction of apoptosis.

We have further examined the influence of Ig-α on selection of B cells in the Ig^{HEL} transgenic mice coexpressing sHEL. Interestingly, the Ig-α truncation led to

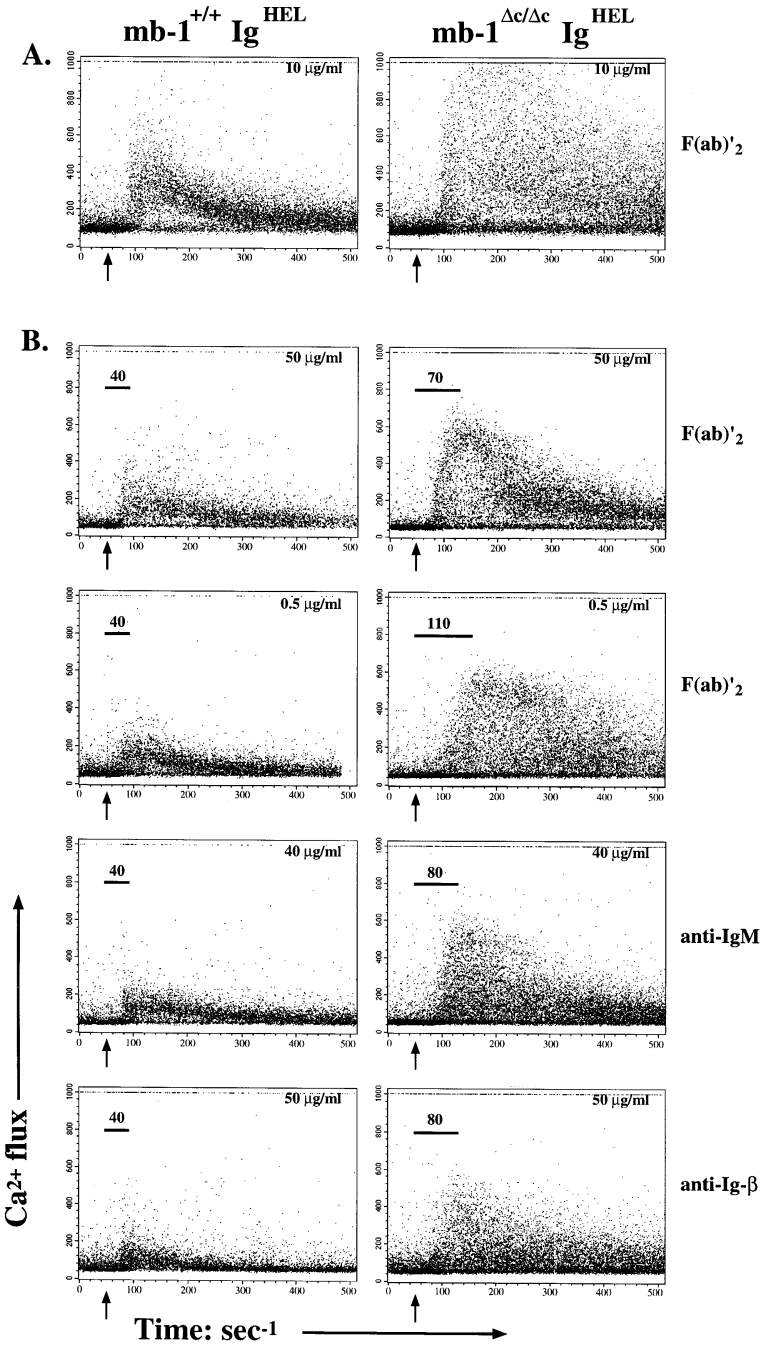

Figure 5. Ig-α-Truncated Immature B Cells Respond to B Cell Receptor Cross-Linking with an Exaggerated Calcium Flux

Changes in intracellular calcium levels were assessed in immature bone marrow cells from *mb-1*$^{\Delta c/\Delta c}$Ig^{HEL} and *mb-1*$^{+/+}$Ig^{HEL} mice by flow cytometry. The dot plots present the measurement of intracellular calcium, gated on B220^{low} cells, as the fluorescence 395/510 nm ratio of Indo-1 emission recorded over time. Baseline fluorescence ratios were aquired for 50 s before F(ab)′$_2$ goat anti-mouse IgM fragments, anti-IgM or anti-Ig-β antibodies were added (indicated by an arrow). Final antibody concentrations are indicated in each plot. (A) Representative measurement of calcium flux induced by stimulation with 10 mg/ml F(ab)′$_2$ goat anti-mouse IgM fragments. Note that these acquisition conditions fit the measurement of calcium flux in wild-type cells, but many Ig-α mutant fluxing cells went off-scale. (B) Representative calcium fluxes induced by stimulation at various concentrations of F(ab)′$_2$ goat anti-mouse IgM fragments, anti-IgM, or anti-Ig-β antibodies. Here the signal amplification of the flow cytometer was lowered to allow presentation of fluxing cells. Results are representative of four independent experiments. The bars and numbers indicate the approximate time in seconds between stimulation and peak of the response.

a strong counterselection of autoreactive B lymphocytes or of their receptors, in contrast to the anergy seen in mice bearing a wild-type Ig-α gene. Similar results have been obtained in the analysis of *motheaten viable* and *lyn* knockout mutants in the HEL system (Cyster and Goodnow, 1995; Cornall et al., 1998). In particular, lack of SHP-1 or Lyn results in the absence of HEL-specific B cells in the periphery. Moreover, these mutant B cells exhibit reduced surface IgM expression and exaggerated calcium mobilization. In contrast to these observations, B cells deficient in CD45 are positively selected into the periphery in the presence of sHEL. Cross-linking of these CD45-deficient, HEL-specific B cells with HEL also elicits a relatively reduced intracellular calcium mobilization; however, this could be overcome by anti-IgM stimulation (Cyster et al., 1996). Overall, these data indicate that Ig-α truncation exerts a similar effect on selection of B cells and B cell signaling strength in the compartment of immature B cells as the absence of SHP-1 or Lyn. These latter molecules are thought to be negative regulators, suggesting a similar role for Ig-α.

We provide evidence that B cells found in the *mb-1*$^{\Delta c/\Delta c}$ mice coexpressing both Ig^{HEL} and sHEL have lost HEL receptor specificity due to endogenous VκJκ rearrangements. In single-cell PCR analysis, 59% of bone marrow B cells showed additional endogenous VκJκ

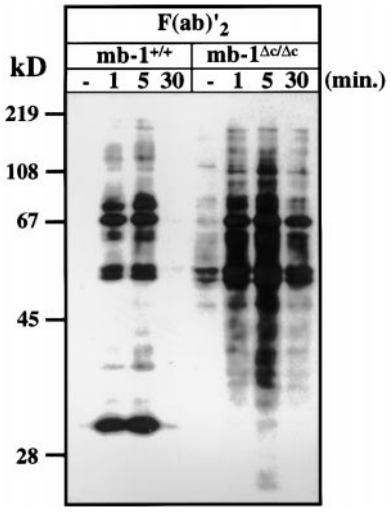

Figure 6. Tyrosine Phoshorylation in Immature Ig-α-Truncated B Cells following BCR Cross-Linking

Cell lysates were prepared from bone marrow B220$^+$ cells of *mb-1*$^{\Delta c/\Delta c}$Ig^{HEL} and *mb-1*$^{+/+}$Ig^{HEL} mice of unstimulated cells ("-") and after stimulation with F(ab)′$_2$ goat anti-mouse IgM fragments for different time periods. Proteins were fractionated by SDS-PAGE and transferred to a PVDF membrane. Phosphotyrosine containing proteins were detected by anti-phosphotyrosine immunoblotting.

rearrangements besides VκJκ2. The percentage of bone marrow B cells undergoing endogenous rearrangement may still be higher, since the PCR strategy could not distinguish between the endogenous and transgenic VκJκ2 joints. Evidence for receptor editing in the HEL system has recently been reported for mice harboring the bcl-x$_L$, Ig^{HEL}, and mHEL transgenes (Fang et al., 1998). In these mice, a subpopulation of transgenic B cells can escape deletion by losing HEL specificity, and this appears to be accomplished by endogenous κ light chain rearrangement as evidenced by endogenous VκJκ1 joints found within this population. Similar to our observations, Fang et al. found no increase in λ chain usage.

It is unclear at present whether receptor editing in *mb-1*$^{\Delta c/\Delta c}$Ig^{HEL} sHEL mice is actively induced by the self-antigen. With defective Ig-α signaling, sHEL might also be sufficient to trigger deletion of immature B cells rather than inducing receptor editing. In this picture, only cells that have acquired a functional rearrangement prior to contact with the self-antigen would persist and go to the periphery. In the single-cell analysis, we found that 11% of B lymphocytes in the bone marrow of *mb-1*$^{\Delta c/\Delta c}$Ig^{HEL} mice had rearrangements of endogenous κ light chains. Therefore, considering the high turnover and proliferation of cells in the bone marrow, we cannot distinguish between a random acquisition or (self-) antigen-dependent induction of secondary rearrangements. The observation of higher numbers of splenic B cells in *mb-1*$^{\Delta c/\Delta c}$Ig^{HEL} sHEL and *mb-1*$^{\Delta c/\Delta c}$Ig^{HEL} mice than in (deleting) *mb-1*$^{\Delta c/\Delta c}$Ig^{HEL} mHEL mice might favor the view of inducibility of receptor editing. However, in the presence of mHEL but not sHEL, cross-reactivity toward HEL of the transgenic heavy chain in combination with most endogenous light chains could be sufficient to induce apoptosis and account for the reduction in cell number.

In Figure 5 we present data indicating that protein tyrosine kinases can be activated such that high protein tyrosine phosphorylation levels can be induced in *mb-1*$^{\Delta c/\Delta c}$ immature B cells. This is in contrast to results obtained in the J558L myeloma cell line (Flaswinkel and Reth, 1994). Expression of either a truncated form of Ig-α or an ITAM mutant, in which the tyrosines were exchanged for phenylalanine, led to a drastic decrease of total tyrosine phosphorylation compared to a wild-type control Ig-α. This difference might be due to the different status of maturation of the transformed cell line compared to the immature B cells taken ex vivo. This is manifested, for example, by the absence of B220, CD19 expression and lack of calcium mobilization following BCR cross-linking in J558L cells (Justement et al., 1990, 1991; Buhl et al., 1997). In addition, several groups have reported the importance of Ig-α ITAM tyrosines for protein tyrosine kinase activation and calcium mobilization in the context of chimeric molecules in transformed B cell lines (Kim et al., 1993; Sanchez et al., 1993; Choquet et al., 1994; Flaswinkel and Reth, 1994; Taddie et al., 1994; Williams et al., 1994; Pao et al., 1998). Furthermore, Ig-α was implicated to be the major signaling component of the BCR (Kim et al., 1993; Sanchez et al., 1993; Flaswinkel and Reth, 1994). However, our data suggest that immature B cells expressing BCR with truncated Ig-α can signal through Ig-β sufficiently to activate protein tyrosine kinases. Calcium flux analysis indicates that immature *mb-1*$^{\Delta c/\Delta c}$Ig^{HEL} B cells reach the peak of the response later compared to *mb-1*$^{+/+}$Ig^{HEL} controls upon BCR cross-linking. This is consistent with previous data demonstrating a role for Ig-α and the importance of its ITAM in calcium mobilization (Choquet et al., 1994; Flaswinkel and Reth, 1994; Pao et al., 1998), although the delayed response in our analysis might also be due to lowered IgM surface expression levels in Ig-α-truncated cells. However, the calcium response of *mb-1*$^{\Delta c/\Delta c}$Ig^{HEL} immature B cells was higher and more persistent than in control cells. We speculate that signals through Ig-α may lower the threshold for calcium mobilization but are also involved in the negative regulation and termination of the response. In *mb-1*$^{\Delta c/\Delta c}$ mice, the developmental blocks and depletion of peripheral B cells could be due to an elevated threshold for calcium mobilization and thus reduced signaling through the BCR at low signal strength. In addition, hyperresponsiveness to BCR cross-linking leading to enhanced negative selection may contribute to the reduction in the number of immature B cells in *mb-1*$^{\Delta c/\Delta c}$ mice. In conclusion, we provide evidence in this study that in additon to its activating function, Ig-α is also involved in the negative regulation of signaling cascades.

Experimental Procedures

Mice

mb-1$^{\Delta c/\Delta c}$ mice (Torres et al., 1996) were bred with C57BL/6 MD4 immunoglobulin transgenic mice (Ig^{HEL}) (Goodnow et al., 1988) and two different transgenic strains expresssing HEL. C57BL/6 ML5 HEL transgenic mice encode soluble HEL (sHEL) at a concentration of around 17.3 ng/ml in serum (Goodnow et al., 1988), whereas KLK3 transgenic mice express a membrane bound form of HEL (mHEL) (Hartley et al., 1991). The offspring were intercrossed, i.e., an Ig^{HEL} transgenic mouse was bred with a mouse carrying either a sHEL

or mHEL transgene. Mice were kept under SPF conditions. The genotype of the mice was determined by polymerase chain reaction (PCR) as follows: screening for the *mb-1*$^{\Delta c}$ allele used a forward primer (5′-TGCAGCAGGCGTCGAC-3′) with the last two 3′ nucleotides complementary to two base substitutions in intron IV of the *mb-1*$^{\Delta c}$ sequence. The corresponding primer, fully complementary to the wild-type allele, was used to amplify the *mb-1*$^{+}$ allele (5′-GCAGCAGGCGTCGGG-3′). A common reverse primer was included in both reactions (5′-AGGCAGGGATGCTGGAGT-3′). PCR was performed under the following conditions: denaturation for 3 min at 94°C followed by 30 cycles of 94°C for 1 min, 60.5°C for 1 min, and 72°C for 1 min. A final extension step was performed at 72°C for 10 min. The amplified product was 283 bp in size. PCR conditions and primers for Ig^{HEL}, mHEL, and sHEL transgenes were as described previously (Hartley et al., 1991).

Flow Cytometry

Single-cell suspensions from bone marrow and spleen were stained directly with fluorochrome (fluorescein isothiocyanate [FITC] and phycoerythrin [PE])-conjugated monoclonal antibodies (mAbs) or biotinylated mAbs followed by Streptavidin-Cy-Chrome (Pharmingen). Receptor specificity for HEL was determined by staining with PE- or FITC-conjugated HEL. Samples were acquired on a FACScan and analyzed using CellQuest software (Becton Dickinson). Dead cells were excluded using propidium iodide. Monoclonal antibodies that were prepared and conjugated in our laboratory include R33–24.12 (anti-IgM), MB86 (anti-IgM^{b}), and RA3–6B2 (anti-B220). Monoclonal antibodies to CD19 were purchased from Pharmingen; anti-493 antibodies were a gift from A. Rolink.

Single Cell PCR Analysis of VκJκ Rearrangements

Bone marrow cells were stained with HEL-PE, anti-IgM-FITC (R33–24.12), and anti-B220-biotin (RA3–6B2) mAbs. Biotin-conjugated anti-B220 was revealed with Streptavidin-Cy-Chrome (Pharmingen). IgM^{+}B220^{+} cells were sorted by a dual laser flow cytometer (FACStar, Becton Dickinson) as described by Ehlich et al. (1994). Single cells from the mouse embryonic stem cell line E14.1 were sorted and used as negative controls. PCR reactions for the analysis of VκJκ rearrangements were performed as described by Novobrantseva et al. (1999).

Measurement of Ca^{2+} Mobilization

Bone marrow cells (5×10^6/ml) were loaded with Indo-1 AM (Molecular Probes) at a final concentration of 2 μM in RPMI containing 2% FCS. After incubation for 30 min at 37°C, the cells were washed and surface staining was performed with anti-B220-FITC (RA3–6B2) and anti-Mac-1-PE (M1/70.15.11). Fluorescence ratios of Indo-1 emission at 395/510 nm were measured by flow cytometry on a dual laser FACStar (Becton Dickinson) after gating on the B220^{low} population. Aquisition was first conducted for 50 s without stimulus followed by the addition of either F(ab)′$_2$ goat anti-mouse IgM fragments (Jackson Immunochemicals; final concentrations of 0.005–50 μg/ml) or goat anti-mouse IgM antibodies (Jackson Immunochemicals; final concentrations of 0.1–20 μg/ml). For anti Ig-β stimulation, cells were first incubated with biotinylated anti-Ig-β monoclonal antibody HM79 (Koyama et al., 1997; final concentrations of 0.1–40 μg/ml) for 5 min. Further cross-linking was achieved by addition of streptavidin (Boehringer Mannheim; final concentration 50 μg/ml). Data were collected for 512 s and analyzed using CellQuest (Becton Dickinson) and FlowJo (TriStar) software.

Phosphotyrosine Analysis

B cells from bone marrow preparations of *mb-1*$^{\Delta c/\Delta c}$*Ig*^{HEL} *and mb-1*$^{+/+}$Ig^{HEL} mice were purified by magnetic cell sorting for B220^{+} cells following manufacturer's instructions (Miltenyi Biotec). B lymphocytes (3×10^5) were stimulated with 20 μg/ml F(ab)′$_2$ goat anti-mouse IgM fragments (Jackson Immunochemicals) for various time periods at 37°C, followed by the addition of ice-cold inhibition buffer (150 mM NaCl, 4 mM EDTA, 10% glycerol, 10 mM Tris-HCl [pH 7.4], 10 ng/ml leupeptin, 10 ng/ml aprotinin, 1 mM PMSF, 1 mM Na_3VO_4, 10 mM NaF). Cells were collected by centrifugation and lysed with inhibition buffer containing 1% NP-40. The cleared lysates were resolved by 10% SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membrane, and immunoblotted with anti-phosphotyrosine antibody Py99 (Santa Cruz). The proteins recognized by Py99 were detected by goat anti-mouse IgG-HRP (Amersham) followed by enhanced chemiluminescence (Super Signal, Pierce).

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