Igβ tyrosine residues contribute to the control of B cell receptor signaling by regulating receptor internalization

Anna Gazumyan,[1,2] Amy Reichlin,[3] and Michel C. Nussenzweig[1,2]

[1]Laboratory of Molecular Immunology and [2]Howard Hughes Medical Institute, The Rockefeller University, New York, NY 10021
[3]Department of Pediatrics, Division of Neonatology, New York University Medical School, New York, NY 10016

Immunoglobulin (Ig)α and Igβ initiate B cell receptor (BCR) signaling through immune receptor tyrosine activation motifs (ITAMs) that are targets of SH2 domain–containing kinases. To examine the function of Igβ ITAM tyrosine resides in mature B cells in vivo, we exchanged these residues for alanine by gene targeting ($Ig\beta_{AA}$). Mutant mice showed normal development of all B cell subtypes with the exception of B1 cells that were reduced by fivefold. However, primary B cells purified from $Ig\beta_{AA}$ mice showed significantly decreased steady-state and ligand-mediated BCR internalization and higher levels of cell surface IgM and IgD. BCR cross-linking resulted in decreased Src and Syk activation but paradoxically enhanced and prolonged BCR signaling, as measured by cellular tyrosine phosphorylation, Ca^{++} flux, AKT, and ERK activation. In addition, B cells with the ITAM mutant receptor showed an enhanced response to a T-independent antigen. Thus, Igβ ITAM tyrosines help set BCR signaling threshold by regulating receptor internalization.

CORRESPONDENCE
Anna Gazumyan:
gazumya@mail.rockefeller.edu

Abbreviations used: BCR, B cell receptor; CGG, chicken γ globulin; ITAM, immune receptor tyrosine activation motif; MAPK, mitogen-activated protein kinase; NP, 4-hydroxy-3-nitrophenylacetyl; PLC-γ2, phospholipase C-γ2; SHIP, SH2-containing inositol polyphosphate 5-phosphatase; SHP-1, SH2-containing tyrosine phosphatase 1.

B lymphocyte survival, development, and function are dependent on signals produced by the B cell receptor (BCR), which comprises membrane-bound Ig and a dimer of Ig superfamily signal transducers Igα and Igβ (CD79a and CD79b) (1–6). Although neither Igα nor Igβ has enzymatic function, transfection and transgenic mouse experiments showed that the cytoplasmic domain of either Igα or Igβ was sufficient to initiate BCR signaling (7–13). Gene targeting revealed that the cytoplasmic domains of either Igα or Igβ were not absolutely required for early stages of B cell development, but a fully intact BCR was essential for complete B cell maturation (14–16) and survival in vivo (17).

The Igα-Igβ dimer is noncovalently associated with membrane Ig through polar residues in the transmembrane domain of Ig (10, 11, 18–21), and it initiates BCR signaling through immune receptor tyrosine activation motifs (ITAMs) (22). Tyrosine residues imbedded in the ITAMs serve as substrates for Src and Syk kinases and as a platform for recruiting and organizing other activated SH2 domain–containing tyrosine kinases (3–6). Syk has two SH2 domains, both of which must be engaged by the BCR for efficient activation (23). Once activated, Syk binds cooperatively to the ITAMs of Igα and Igβ and phosphorylates downstream adaptors and kinases triggering a cascade that leads to nuclear effectors (3–6). Syk is an essential kinase in the BCR pathway. In the absence of Syk, there is no BCR signaling in DT40 cells, and $Syk^{-/-}$ mouse B cells fail to develop beyond the pro–B cell stage (24–26).

Despite the importance of the Igα and Igβ cytoplasmic domains in initiating BCR signaling and Syk recruitment, loss of either produced hyperresponsive Ig^{HEL} transgenic B cells, suggesting an unexpected negative regulatory function for Igα and Igβ (16, 27, 28). Experiments with Igα ITAM mutant B cells ($Ig\alpha_{FF}$) demonstrated that this unexpected phenomenon was mediated by Igα ITAM tyrosines, but the mechanism of negative regulation by Igα was not established (15).

Here we show that Igβ ITAM tyrosines modulate ligand-induced signaling by regulating BCR internalization.

RESULTS

$Ig\beta_{AA}$ mice

To determine the function of Igβ ITAM tyrosines in mature B cells, we replaced these residues with alanine residues by gene targeting

The online version of this article contains supplemental material.

(Fig. 1 A). Expression of the mutant protein was confirmed by immunoprecipitation and Western blotting on B cell lysates using antibodies specific for the cytoplasmic domains of Igα and Igβ (Fig. 1 B) (29, 30). Mutant Igβ was coimmunoprecipitated with Igα and vice versa (Fig. 1 B and Fig. S1 A, which is available at http://www.jem.org/cgi/content/full/jem.20060221/DC1). After BCR cross-linking, Igα was tyrosine phosphorylated in both wild-type and mutant B cells (Fig. 1 B). In the wild-type, small amounts of phosphotyrosine were also found on Igβ in response to receptor cross-linking, but we found no phosphorylation of mutant Igβ in $Igβ_{AA}$ B cells (Fig. 1 B and Fig. S1 B). We conclude that $Igβ_{AA}$ B cells produce the mutant protein and that it is associated with Igα.

B cell development in $Igβ_{AA}$ mice

We used flow cytometry to examine the effect of the $Igβ_{AA}$ mutation on B cell development and to compare it with $Igα_{FF}$, in which the ITAM tyrosine residues of Igα were mutated to phenylalanine (15). We found normal numbers of pro–B ($IgM^-B220^{low}CD25$), pre–B ($IgM^-B220^{low}CD25^+$), immature B ($IgM^+B220^{low}IgD^{-/low}$), and recirculating B cells ($IgM^+B220^{hi}IgD^{hi}$) in the bone marrow of $Igβ_{AA}$ mice (Fig. 1 C). The only reproducible difference between developing B cells in $Igβ_{AA}$ and wild-type mice was in the higher levels of surface IgM and IgD on immature and recirculating B cells (Fig. 1 C).

In the spleen, the number of $Igβ_{AA}$ B cells was similar to wild-type, as was the proportion of marginal zone ($CD21^{hi}CD23^{lo}$) and follicular B cells ($CD21^{hi}CD23^{hi}$) (Fig. 1 C). This is in contrast to $Igα_{FF}$ mice that appear to have fewer marginal zone B cells (Fig. 1 C) (15). In the spleen, as in the bone marrow, $Igβ_{AA}$ B cells showed higher levels of surface IgM and IgD expression than wild-type B cells, but normal CD19 levels (Fig. 1, C and D, and not depicted). Finally, in the peritoneal cavity, $Igβ_{AA}$ mice showed a fivefold reduction in the number of B1a B cells similar to that found in $Igα_{FF}$ mice (Fig. 1 C) (31–36). Thus, Igβ ITAM tyrosines are essential for normal levels of cell surface BCR expression but not required for most other aspects of B cell development.

To determine whether the combined ITAMs in Igα and Igβ were required for B cell development, we crossed $Igα_{FF}$ and $Igβ_{AA}$ to obtain double mutant mice. We found that

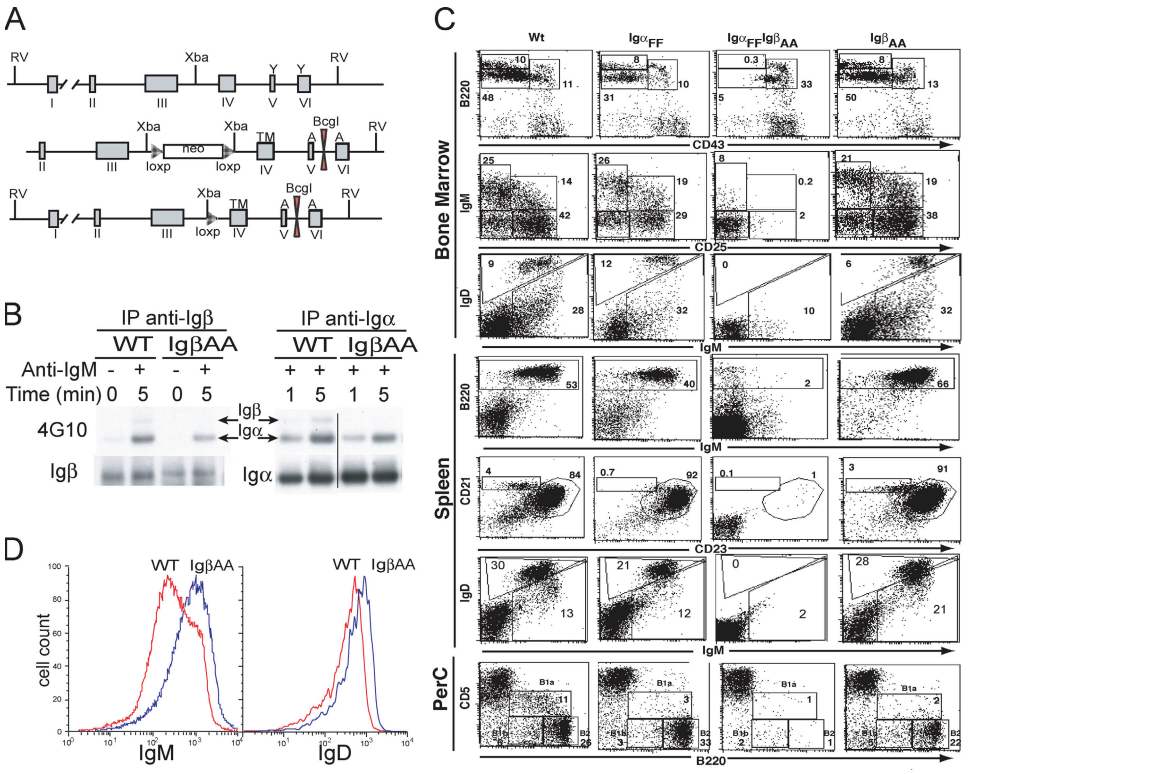

Figure 1. Targeting the Igβ locus. (A) Diagram shows the endogenous Igβ locus (top), targeting construct (middle), and the targeted locus (bottom). Boxes labeled with roman numerals indicate exons, and the transmembrane domain (TM), diagnostic BclI site, and position of alanine (A) substitutions are shown. (B) Purified splenic B cells from wild-type and $Igβ_{AA}$ mice were stimulated with anti-IgM, and extracts were immunoprecipitated with anti-Igα or anti-Igβ and blotted with anti-phosphotyrosine 4G10 antibodies or anti-Igα or anti-Igβ antibodies. (C) Flow cytometry analysis of bone marrow, spleen, and peritoneal cavity B cells from wild-type, $Igα_{FF}$, $Igα_{FF}/Igβ_{AA}$, and $Igβ_{AA}$ mice. Staining antibodies are indicated. Numbers show relative percentages of cells within indicated gates. (D) Histogram plots show expression of surface IgM and IgD by splenic $B220^+$ B cells in wild-type (red) and $Igβ_{AA}$ (blue) mice.

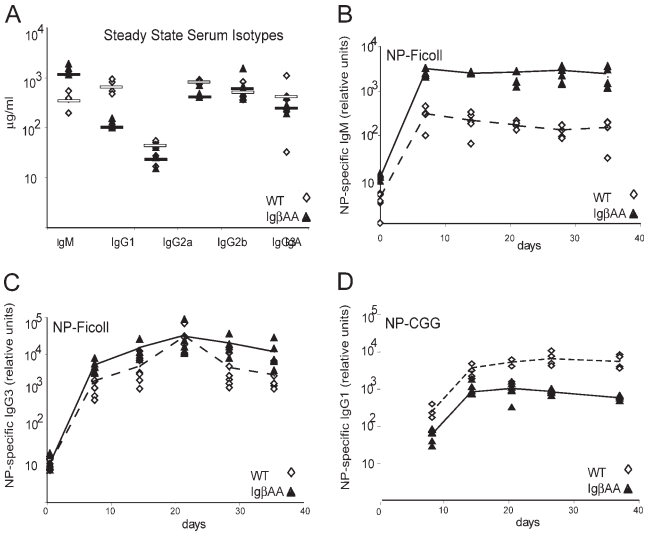

Figure 2. Steady-state antibody levels and antibody responses. Individual wild-type and $Ig\beta_{AA}$ mice are represented by open circles and filled triangles, respectively. Bold lines indicate mean values for each group. Plots show total serum Ig or relative binding to NP2-BSA. (A) Steady-state serum Ig concentrations (µg/ml) in age- and sex-matched wild-type and $Ig\beta_{AA}$ mice. (B) IgM response to immunization with NP-Ficoll. (C) IgG3 response to immunization with NP-Ficoll. (D) IgG1 response to immunization with NP-CGG.

B cells that express a BCR with no cytoplasmic ITAM tyrosine residues fail to develop beyond the pro–B cell stage (Fig. 1 C; $IgM^{-}B220^{low}CD25^{-}$). We conclude that a single intact ITAM in either Igα or Igβ is both necessary and sufficient for normal B cell development with the exception of B1a cells.

Enhanced B cell responses in vivo

$Ig\beta_{AA}$ mutant mice showed a threefold increase in serum IgM levels, a fivefold decrease in IgG1, normal levels of IgG2a, IgG2b, IgG3, and IgA, and no increase in anti-DNA antibodies (Fig. 2 A and not depicted). To determine whether $Ig\beta_{AA}$ B cells respond normally to antigen in vivo, we immunized mice with a T-independent antigen 4-hydroxy-3-nitrophenylacetyl (NP)-Ficoll or a T-dependent antigen NP coupled to chicken γ globulin (NP-CGG) and measured anti-NP–specific antibody responses by ELISA (Fig. 2, B–D). $Ig\beta_{AA}$ mice showed a 10-fold increase in anti-NP–specific IgM, a small increase in IgG3 responses to T-independent antigen, no increase in proliferative responses to anti-IgM in vitro, and slightly decreased T-dependent antibody responses (Fig. 2, B–D, and not depicted). In contrast, there was no apparent difference in antibody responses to the same antigens in $Ig\alpha_{FF}$ mice (15). We conclude that $Ig\beta_{AA}$ B cells express increased levels of surface IgM and are hyperresponsive to stimulation with T-independent antigen in vivo.

Decreased BCR internalization

Increased BCR surface expression in $Ig\beta_{AA}$ B cells was not due to increased mRNA expression (not depicted). To determine whether the increased surface levels of BCR found on $Ig\beta_{AA}$ B cells was associated with altered BCR internalization, we measured spontaneous internalization (Fig. 3 A). Cell surface BCRs were labeled with monovalent biotinylated Fab′ anti-IgM to avoid receptor cross-linking, incubated at 37°C, and visualized with streptavidin. Wild-type B cells internalized 30% of their receptors in 20 min, but $Ig\alpha_{FF}$ and $Ig\beta_{AA}$ internalized only 5–7% of their receptors during the same time (Fig. 3 A). Thus, both $Ig\alpha_{FF}$ and $Ig\beta_{AA}$ B cells were severely impaired in constitutive BCR internalization

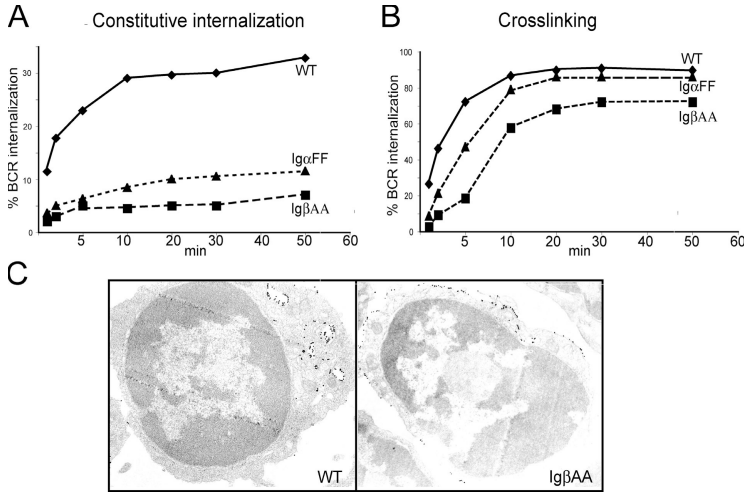

Figure 3. BCR internalization. (A) Endocytosis. Plots show relative rates of BCR internalization by purified $Ig\alpha_{FF}$, $Ig\beta_{AA}$, and wild-type B cells at 37°C as measured with a biotinylated Fab′ anti-IgM antibody. (B) Ligand-mediated internalization. Internalization of a biotinylated $F(ab')_2$ anti-IgM antibody by $Ig\alpha_{FF}$, $Ig\beta_{AA}$, and wild-type B cells at 37°C. Experiments in A and B were repeated three times with similar results. (C) Electron micrographs show BCR labeling on $Ig\beta_{AA}$ and wild-type B cells with 10 nm of gold-labeled anti–rabbit antibodies 10 min after cross-linking by Fab'_2 anti-IgM at 37°C.

compared with wild-type. $Ig\beta_{AA}$ B cells were more impaired than $Ig\alpha_{FF}$ B cells (Fig. 3 A).

To examine BCR internalization in response to BCR cross-linking, we incubated B cells with biotinylated $F(ab')_2$ anti-IgM. BCR internalization in response to receptor cross-linking was both decreased and delayed in $Ig\beta_{AA}$ B cells (Fig. 3, B and C). Although wild-type cells internalized 50% of the cell surface BCR in 5 min, $Ig\beta_{AA}$ B cells reached this level of BCR internalization only after 20 min. In contrast, $Ig\alpha_{FF}$ B cells displayed a more modest decrease in receptor internalization in response to cross-linking. They internalized 50% of their receptors in 9 min (Fig. 3 B). By ultrastructural analysis, wild-type cells ($n = 65$ cells) internalized 65% of the gold-labeled surface-bound $F(ab')_2$ in 10 min, whereas only 20% of the same tracer was internalized by $Ig\beta_{AA}$ B cells ($n = 64$ cells) at this time point (Fig. 3 C). Thus, the ITAM tyrosine residues in both Igα and Igβ are required for constitutive and ligand-mediated BCR internalization. However, the $Ig\beta_{AA}$ mutation results in a more profound block in BCR internalization than the $Ig\alpha_{FF}$ mutation.

Enhanced Ca^{2+} responses

To determine whether signaling is affected by the $Ig\beta_{AA}$ mutation, we initially measured Ca^{2+} flux responses to BCR cross-linking with $F(ab')_2$ anti-IgM antibodies. We found that $Ig\beta_{AA}$ B cells showed increased Ca^{2+} flux responses to anti-IgM when compared with wild-type or $Ig\alpha_{FF}$ B cells at all doses of anti-IgM tested (Fig. 4 A).

To determine whether the enhanced Ca^{2+} signaling is simply the result of increased surface IgM expression by $Ig\beta_{AA}$ B cells, we compared Ca^{2+} responses in wild-type and mutant B cells with low (L), medium (M), and high (H) levels of IgM expression (Fig. 4 B). B cells were stained with Fab′ anti-IgM and gated based on expression of similar levels of surface IgM. Although Ca^{2+} flux was lower in cells expressing lower levels of surface IgM (Fig. 4 B, L), direct comparison of $Ig\beta_{AA}$ and wild-type B cells expressing equivalent levels of surface IgM showed that Ca^{2+} flux responses were always higher in $Ig\beta_{AA}$ B cells (Fig. 4 B). We conclude that $Ig\beta_{AA}$ are hyperresponsive to BCR cross-linking and that this effect is not simply due to higher levels of surface BCR expression.

Tyrosine phosphorylation

BCR cross-linking leads to phosphorylation of several cellular substrates. To determine whether tyrosine phosphorylation responses were altered in $Ig\beta_{AA}$ B cells, we measured total cellular tyrosine phosphorylation (Fig. 5 A). Consistent with increased Ca^{2+} flux responses, we found increased and prolonged general cellular tyrosine phosphorylation in $Ig\beta_{AA}$ B cells compared with wild-type control (Fig. 5 A).

BCR phosphorylation is mediated by Src and Syk family kinases. To determine whether Src and Syk activation was altered in $Ig\beta_{AA}$ B cells, we measured their phosphorylation in responses to BCR cross-linking by Western blotting with phospho-Src–specific antibodies (Fig. 5 B) and by immunoprecipitation with anti-Syk (Fig. 5 C). In contrast to increased Ca^{2+} flux and general tyrosine phosphorylation responses, we found a decrease in Src and Syk activation in $Ig\beta_{AA}$ B cells (Fig. 5, B and C). In addition, Syk phosphorylation was delayed (Fig. 5 C).

Syk becomes associated with phosphorylated Igα-Igβ ITAM tyrosines after BCR cross-linking by a mechanism that requires cooperative binding of both of its SH2 domains (23, 37). To determine whether this association was altered in $Ig\beta_{AA}$ B cells, we immunoprecipitated BCR with anti-Igα and immunoblotted it with 4G10 and anti-Syk antibodies. Although Iga phosphorylation was marginally reduced (Fig. 1 B

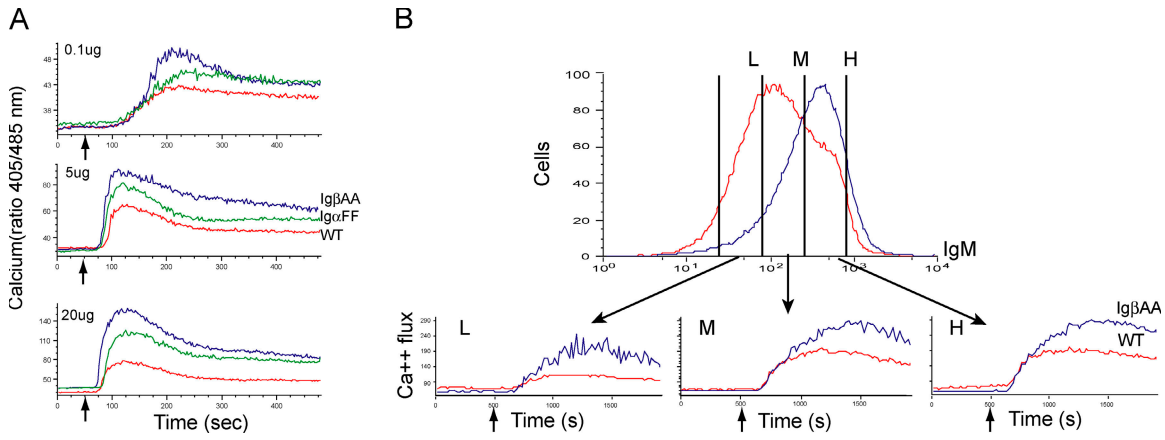

Figure 4. Ca^{2+} flux responses. (A) Ca^{2+} flux response to BCR cross-linking with 0.1, 5, and 20 μg/ml anti-IgM in splenic B cells from $Ig\beta_{AA}$ (blue), $Ig\alpha_{FF}$ (green), and wild-type (red) B cells. Histograms show fluorescence 405/485 nm ratio of Indo-1-AM emission (y axis) in response to BCR cross-linking plotted as a function of time in seconds (x axis). (B) Ca^{2+} flux analysis in response to BCR stimulation in $Ig\beta_{AA}$ (blue) and wild-type (red) B cells gated based on similar levels of surface IgM. Top panel shows IgM expression by B cells stained with FITC-labeled Fab′ and gating based on the level of IgM expression. Bottom panel shows histogram plots of Ca^{2+} flux response to BCR cross-linking with 5 μg/ml Fab′2 anti-IgM on the gated populations as indicated by arrows. The time points of anti-IgM addition are indicated by small arrows.

and Fig. S1 B), we found decreased association of phosphorylated Syk with the BCR in $Ig\beta_{AA}$ B cells after receptor cross-linking when compared with wild-type controls despite equivalent levels of cellular Syk expression in wild-type and $Ig\beta_{AA}$ B cells (Fig. 5, D and E). BLNK and c-Cbl are central adaptor proteins that are directly downstream of Syk. Consistent with decreased Syk activation, we found decreased BLNK and c-Cbl phosphorylation in $Ig\beta_{AA}$ B cells after BCR cross-linking but normal levels of BLNK and c-Cbl expression (Fig. 5 F and not depicted). We conclude that phosphorylation of BCR proximal kinases is impaired but overall cellular tyrosine phosphorylation is enhanced and prolonged in $Ig\beta_{AA}$ B cells.

Phospholipase C-γ2 (PLC-γ2) amplifies BCR signals by producing second messengers, diacylglycerol and inositoltriphosphate. To determine whether PLC-γ2 activation was altered in $Ig\beta_{AA}$ B cells, we measured its phosphorylation by immunoblotting with phospho-specific antibodies (Fig. 5 G). Despite the reduced Syk activity and increased Ca^{2+} flux responses, we found no difference in the level of PLC-γ2

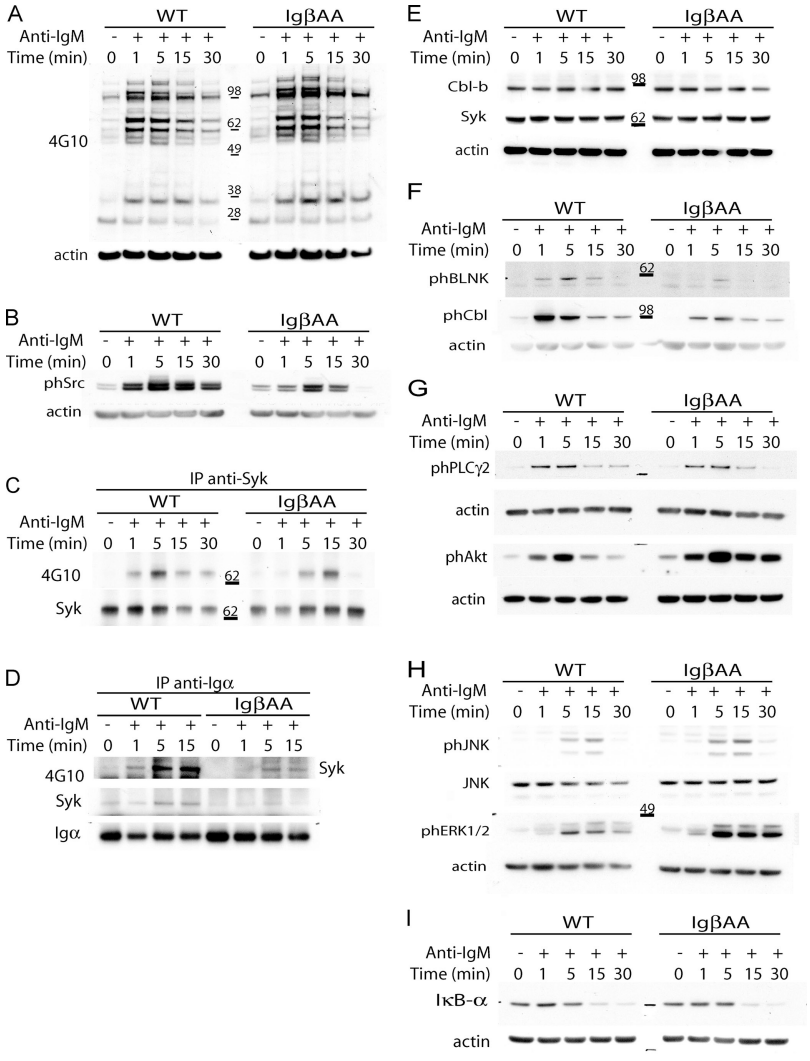

Figure 5. Signaling responses. $Ig\beta_{AA}$ or wild-type splenic B cells were stimulated with 20 μg/ml $F(ab')_2$ anti-IgM at 37°C for the indicated times. Anti-actin, anti-Iga, anti-Syk, and anti-JNK antibodies were used for loading controls as indicated. (A) Tyrosine phosphorylation of total cellular proteins analyzed by immunoblotting with 4G10 antibody. (B) Src kinase phosphorylation in total cellular lysates measured by immunoblotting with anti–phospho-src–specific antibodies. (C) Syk tyrosine phosphorylation analyzed by immunoblotting of anti-Syk immunoprecipitates with 4G10. (D) Association of Syk with the BCR and its phosphorylation. Anti-Igα immunoprecipitates were immunoblotted with anti-Syk or 4G10 antibody. (E) Cbl-b and Syk levels in total cellular lysates were measured by immunoblotting with anti–Cbl-b and anti-Syk antibodies. (F) Phosphorylation of BLNK and c-Cbl in total cellular lysates measured by immunoblotting with anti–phospho-Cbl or anti–phospho-BLNK antibodies. (G) PLC-γ2 and Akt phosphorylation in total cellular lysates measured by immunoblotting with anti–phospho-Akt or anti–phospho-PLC-γ2 antibodies. (H) JNK and ERK phosphorylation in total cellular lysates measured by immunoblotting with anti–phospho-JNK and anti–phospho-ERK. (I) NF-κB activation was measured by degradation of the IkB-α subunit in total cell extracts using anti–IkB-α antibodies. All experiments were repeated three times with similar results.

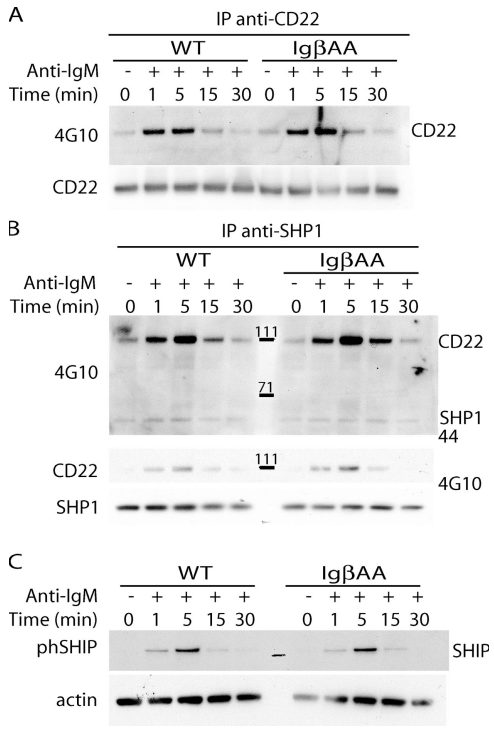

Figure 6. Negative regulators. Igβ$_{AA}$ or wild-type splenic B cells were stimulated with 20 μg/ml F(ab′)$_2$ anti-IgM at 37°C for the indicated times. (A) CD22 tyrosine phosphorylation analyzed by immunoblotting of anti-CD22 immunoprecipitates with 4G10. Anti-CD22 is shown as a loading control. (B) Recruitment of SHP-1 to CD22. Anti–SHP-1 immunoprecipitates were immunoblotted with 4G10 or anti-CD22 antibodies. The positions of SHP-1 and CD22 are indicated. Anti–SHP-1 immunoblot is shown as a loading control. (C) SHIP tyrosine phosphorylation analyzed in cellular lysates by immunoblotting with anti–phospho-SHIP antibodies. Anti-actin is shown as a loading control.

phosphorylation in the mutant B cells. PI3K is another essential kinase that amplifies BCR signaling by producing phosphatidylinositol-(3,4,5)-triphosphate. Phosphatidylinositol-(3,4,5)-triphosphate amplifies signaling by recruiting PH domain–containing proteins to the plasma membrane, including serine/threonine kinase Akt. Although we were unable to detect significant changes in p85 phosphorylation by Western blotting, we found that activation of Akt was dramatically increased and prolonged in Igβ$_{AA}$ B cells in response to BCR cross-linking (Fig. 5 G and Fig. S2, which is available at http://www.jem.org/cgi/content/full/jem.20060221/DC1). Therefore, Igβ ITAM mutation enhances Akt activation in response to BCR signaling.

Mitogen-activated protein kinases (MAPKs) and NF-κB are nuclear effectors of BCR signaling. To determine whether MAPK and activation NF-κB were altered in Igβ$_{AA}$ B cells, we measured phosphorylation of MAPK and NF-κB as well as degradation of NF-κB by Western blotting. We found that ERK phosphorylation was enhanced and prolonged, but p38, JNK, and IκBα phosphorylation as well as NF-κB degradation were not altered (Fig. 5, H and I, and not depicted). We conclude that there is a discrepancy between proximal and distal signaling responses in Igβ$_{AA}$ B cells. Proximal signaling, as exemplified by Syk activation, is impaired, whereas phosphorylation of distal effectors, such as Akt and ERK, is enhanced by the same mutation.

Negative regulators of BCR signaling

In addition to activators, BCR signaling is also controlled by several negative regulators, including CD72, CD45, CD5, FcγRIIb, and CD22 (38). We found no differences in the expression levels of the constitutive negative regulators CD72, CD45, and CD5 by flow cytometry (not depicted). FcγRIIb was ruled out because all of the signaling experiments were performed using F(ab′)$_2$ antibodies and because BCR cross-linking with intact and Fab′2 antibody had similar effects on Igβ$_{AA}$ B cells. CD22 becomes phosphorylated by the Lyn kinase in response to BCR cross-linking (39–42) and terminates BCR signaling by recruiting SH2-containing tyrosine phosphatase 1 (SHP-1) and SH2-containing inositol polyphosphate 5-phosphatase (SHIP) (43–45). We found normal levels of CD22 and SHP-1 phosphorylation after BCR cross-linking in Igβ$_{AA}$ B cells (Fig. 6, A and B). In addition, association between SHP-1 and phosphorylated CD22 was unchanged in Igβ$_{AA}$ B cells as measured by coimmunoprecipitation (Fig. 6 B). Finally, SHIP phosphorylation was normal in response to BCR cross-linking in Igβ$_{AA}$ B cells (Fig. 6 C). Thus, neither CD22 nor SHIP phosphorylation nor absence of SHP-1 recruitment can account for prolonged and enhanced BCR signaling in Igβ$_{AA}$ B cells.

BCR internalization decreases signaling

Others have shown that BCR internalization can regulate the magnitude and duration of signaling as measured by total tyrosine phosphorylation and ERK activation in B cell lines (46, 47). To determine whether blocking BCR internalization enhances signaling in primary B cells, we partially blocked BCR internalization by inhibiting actin polymerization with cytochalasin D (Fig. S3 A, available at http://www.jem.org/cgi/content/full/jem.20060221/DC1). Signaling was measured by immunoblotting with anti-phospho ERK antibodies after receptor cross-linking. We found that 10 μM cytochalasin D inhibited ligand-mediated BCR internalization and enhanced ERK phoshorylation, though to a lesser extent than Igβ$_{AA}$ mutation (Fig. S3 B). Thus, our findings are consistent with the idea that normal termination of BCR signal transduction appears to require BCR internalization (46, 47).

Activation-induced ubiquitylation

Ubiquitylation regulates the internalization of many cell surface receptors, including the TCR (for review see references 48 and 49). To determine whether BCR ligation results in its ubiquitylation and whether Igβ regulates ubiquitylation, we performed Western blotting experiments with anti-ubiquitin antibodies. Wild-type and Igβ$_{AA}$ B cells were stimulated with F(ab′)$_2$ anti-IgM antibodies, and ubiquitylation was measured

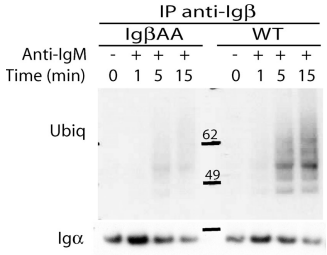

Figure 7. BCR ubiquitylation upon receptor ligation. Wild-type and $Ig\beta_{AA}$ splenic B cells were stimulated with $F(ab')_2$ anti-IgM at 37°C for the indicated times, and extracts were immunoprecipiated with anti-Igβ antibodies. Ubiquitylation was analyzed by immunoblotting with anti-ubiquitin antibodies. Igα immunoblotting was a loading control.

on BCRs purified by immunoprecipitation with anti-Igβ or anti-Igα. We found inducible BCR ubiquitylation, and consistent with decreased c-Cbl phosphorylation and reduced $Ig\beta_{AA}$ BCR internalization, we found decreased ubiquitylation of Igα or Igβ precipitates from $Ig\beta_{AA}$ B cells (Fig. 7 and not depicted). We conclude that BCR-associated proteins are ubiquitilated upon receptor ligation and that the Igβ ITAM tyrosines are required for normal BCR ubiquitylation.

DISCUSSION

To evaluate how Igβ might regulate BCR signaling, we mutated Igβ ITAM tyrosine residues to alanine. We selected the alanine substitution because it destroys phosphorylation and internalization motifs, whereas phenylalanine substitution might only interfere with phosphorylation (50). In contrast to Igα or Igβ cytoplasmic domain deletion, which altered B cell maturation in the periphery (16, 27), B cell development in $Ig\alpha_{FF}$ and $Ig\beta_{AA}$ mice was nearly indistinguishable from wild-type (15). Non-ITAM residues must account for these phenotypic differences and may do so by stabilizing or facilitating BCR signaling (51–54).

Although $Ig\beta_{AA}$ B cells showed normal distribution of most cell surface antigens, they expressed higher levels of surface BCRs that were defective in steady-state and ligand-mediated endocytosis. The role of Igα and Igβ ITAM tyrosine residues in endocytosis was previously studied in cell lines using chimeric FcγRIIB receptors (55). In those experiments, Igα was able to mediate endocytosis by a mechanism that required an intact ITAM. Consistent with those observations, we found decreased ligand-mediated receptor internalization in $Ig\alpha_{FF}$ mice (Fig. 3) and enhanced internalization in Igαα mice whose BCRs carry two Igα cytoplasmic domains (56). In contrast to Igα, Igβ was inactive in endocytosis in transfection experiments (55). Thus, the observation that the $Ig\beta_{AA}$ mutant BCR was profoundly defective in endocytosis in B cells in vivo reveals a novel and essential function for the Igβ ITAM. In addition, the finding that $Ig\alpha_{FF}$ is nearly as defective in steady-state endocytosis as $Ig\beta_{AA}$ suggests that constitutive ITAM phosphorylation is an important regulator of BCR internalization.

The role of Igα and Igβ ITAM tyrosine residues in ligand-mediated BCR internalization was originally studied by measuring antigen presentation by B cell lines expressing chimeric Ig, Fc, and PDGF receptors by several groups (11, 55, 57–59). In all cases, both Igα and Igβ were active in ligand-mediated internalization (11, 55, 57–59). The role of the ITAM phosphorylation varied with the experimental system and was required in some (59) but not in others (56, 60). Our results with $Ig\alpha_{FF}$ and $Ig\beta_{AA}$ mice are consistent with a requirement for phosphorylation in ligand-mediated receptor internalization in vivo. The difference in the internalization phenotype between the two mutants could be due to non-ITAM residues (51, 52) or to partial preservation of the internalization signal by the phenylalanine substitution in $Ig\alpha_{FF}$ (50). In addition, we show that Igβ regulates activation-induced ubiquitylation, which may control receptor levels by enhancing endocytosis or by targeting surface BCR to the degradative lysosomal compartment (48, 49).

$Ig\beta_{AA}$ B cells resembled $Ig\alpha_{FF}$ B cells in several important ways, including the unexpected finding that both mutants showed enhanced Ca^{2+} and tyrosine phosphorylation responses to BCR cross-linking (15). The observation that $Ig\alpha_{FF}$ BCRs were hyperresponsive led to the suggestion that a major function of the Igα ITAM was to recruit negative regulators (15, 27, 28). This model predicts that Igβ ITAM mutation should produce an inactive BCR due to unopposed negative regulation by Igα. On the contrary, we found that $Ig\beta_{AA}$ BCRs were also hyperactive in vitro and in vivo. $Ig\beta_{AA}$ B cells produced enhanced T-independent antibody responses to NP-Ficoll. Thus, increased BCR signaling in $Ig\alpha_{FF}$ cannot be due to inability to recruit an Igα-specific negative regulator, but must be due to an abnormality in a pathway downstream of both Igα and Igβ ITAMs. The enhanced responses of $Ig\beta_{AA}$ B cells to BCR cross-linking may account for the enhanced T-independent immune responses to NP-Ficoll and increased steady-state levels of serum Ig in these mice despite the decrease in B1 cells, which together with MZ B cells normally produce the majority of circulating antibodies in mice. Finally, decreased BCR internalization is also consistent with decreased T-dependent immune responses, which would be dependent on antigen internalization and processing for presentation to cognate T cells.

The BCR initiates signal transduction by activation of nonreceptor Src and Syk tyrosine kinases. Therefore, it was surprising to find that $Ig\beta_{AA}$ B cells were hyperresponsive to BCR stimulation as measured by cellular tyrosine phosphorylation and Ca^{2+} flux, Akt, and ERK activation despite impaired Src and Syk family kinase activation. These kinases phosphorylate Igα and Igβ, which in turn recruit activated Syk by providing docking sites for its two SH2 domains (2–6). Our observation of decreased Syk activation and association with the $Ig\beta_{AA}$ mutant BCR and decreased BLNK phosphorylation was consistent with the requirement for two functional ITAMs for maximal Syk activation (23). Thus, the $Ig\beta_{AA}$ BCR is less functional than its wild-type counterpart in activating and recruiting proximal nonreceptor tyrosine kinases, but

paradoxically, this leads to increased Ca^{2+} flux, Akt, and ERK activation, as well as total cellular tyrosine phosphorylation.

Enhanced signaling in the face of decreased Src and Syk activation could be due to decreased activation of negative regulators, such as CD45, CD72, CD5, FcR, and CD22 (38, 61). However, we found no abnormalities in any of these pathways in $Ig\beta_{AA}$ B cells. CD45, CD72, and CD5 levels were all normal, and the involvement of FcγRIIb was ruled out by comparing $F(ab')_2$ and intact antibodies. Negative regulation by CD22 is initiated by BCR-mediated activation of lyn kinase, which phosphorylates CD22 leading to recruitment of the SHP-1 tyrosine phosphotase to the activated BCR (39–45). Although B cells deficient in any of the elements of the CD22 pathway show enhanced BCR signaling responses (38, 61), CD22 and SHP-1 phosphorylation and SHP-1 recruitment to CD22 were all normal in $Ig\beta_{AA}$ B cells. In addition, activation of SHIP, which negatively regulates the BCR by interfering with accumulation of inositol phosphate second messengers, was also unaltered in $Ig\beta_{AA}$ B cells (62–64). Finally, Cbl-b and c-Cbl are ubiquitin ligases that have been implicated as negative regulators of the BCR by targeting Syk for degradation by ubiquitinylation (65–67). Absence of Cbl-b in mice and overexpression of dominant-negative c-Cbl in B cell lines lead to enhanced Syk activation and signal transduction (66, 68). However, the role of Cbl-b and c-Cbl as negative regulators in B cells is debated, and they have also been implicated as positive regulators of BCR signaling in gene-targeting experiments (68, 69). Although there was decreased c-Cbl phosphorylation and a corresponding decrease in BCR-associated ubiquitylation in $Ig\beta_{AA}$ B cells, we found no corresponding increase in Syk activation. Indeed, Syk phosphorylation was decreased in response to BCR ligation. Thus, we cannot attribute the increased BCR signaling in $Ig\beta_{AA}$ mutant B cells to alterations in known negative regulators, but we cannot rule out effects of yet to be defined negative components.

Experiments with inhibitors of tyrosine kinases and phosphotases have implicated ligand-induced tyrosine phosphorylation in regulating BCR internalization (70, 71). How phosphorylation activates endocytosis of the BCR has not been determined, but several nonmutually exclusive endocytic pathways, including coated pits, rafts, actin, and ubiquitinylation, have been implicated. Blocking BCR endocytosis with pharmacologic agents or by deletion of the clathrin heavy chain in chicken B cell lines leads to enhanced signaling, suggesting that receptor internalization is an important mechanism for attenuating BCR signaling (46, 47). However, the mechanism by which BCR regulates its internalization was not determined. Our experiments establish a molecular link between Igβ, BCR internalization, and termination of signaling responses in vivo.

MATERIALS AND METHODS

Mice. Igβ cytoplasmic tyrosines 195 and 206 were replaced with alanine by gene targeting. The long arm of the targeting vector was 9-kb long with a 3′ end at the SpeI site located in the third intron of Igβ (Fig. 1 A). The short arm was a 2.5-kb fragment from the SpeI site in the third intron of Igβ to the NotI site located 1.8 kb downstream of the termination codon. TAT codons at positions 195 and 206 in the cytoplasmic tail were replaced with alanines by substitution with GCT. A LoxP-flanked neomycin-resistance gene was used for positive selection, and a diphtheria toxin gene was used for negative selection (72). The targeting construct was linearized with PacI and transfected into 129/Sv embryonic stem cells. 100 embryonic stem cell clones were screened, three positive clones were injected into C57B/6 blastocysts, and two produced chimeric mice that transmitted the mutation. The mutation was confirmed by sequencing mRNAs cloned from mutant B cells (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20060221/DC1). Heterozygous $Ig\beta_{AA}$ mice were backcrossed to C57BL/6 for two generations after Cre deletion in the germline before intercrossing. All mice were maintained under specific pathogen-free conditions, and experiments were performed under Rockefeller University Institutional Animal Care and Use Committee–approved protocols.

Flow cytometry. Single cell suspensions from bone marrow, spleen, and peritoneal cavity were stained with FITC, PE, APC, and biotin-conjugated monoclonal antibodies for 20 min on ice. Biotinylated antibodies were visualized with streptavidin PerCp. The following monoclonal antibodies were used: anti-CD43, anti-IgM, anti-B220, anti-CD25, anti-IgD, anti-CD19, anti-CD5, anti-CD21, anti-HSA, and anti-CD23 (BD Biosciences). Data were collected with a FACSCalibur and analyzed using CellQuest software (Becton Dickinson).

Immunizations and ELISA. Age- and sex-matched 8–12-wk-old mice were injected intraperitoneally with either 12.5 g NP190-Ficoll or 50 g of alum-precipitated NP21-CGG (both from Biosearch Technologies) in 300 μl PBS. To measure serum antibody levels, goat anti–mouse Ig (H+L) was used for capture and horseradish peroxidase–conjugated goat anti–mouse isotype-specific antibodies (SouthernBiotech) was used for detection. Values were calculated by comparison with mouse Ig standards (SouthernBiotech). To measure NP-specific IgM and IgG antibody levels, we used 5 μg/ml NP_2BSA (Biosearch Technologies) for capture and horseradish peroxidase–conjugated goat anti–mouse IgM or anti–mouse IgG for detection (Jackson ImmunoResearch Laboratories). Serial dilutions were performed for each sample, and readings were taken within the linear range for each sample and adjusted for dilution. Results reflect relative absorbance for each sample compared with the standard control. All plates were developed using a peroxidase substrate kit (Bio-Rad Laboratories), and absorbance was measured at 415 nm.

BCR internalization. To measure endocytosis, splenocytes were preequilibrated at 4°C in RPMI 0.5% FBS and incubated with either biotin goat anti–mouse IgM Fab′ or goat anti–mouse IgM $(Fab')_2$. Cells were then either fixed in 0.5% paraformaldehyde in PBS (T_o) or incubated at 37°C for the indicated times (T_n) before termination with 0.5% paraformaldehyde in PBS. Fixed cells were stained with PE-B220 and streptavidin Red 670 (Invitrogen), and surface BCR expression was determined by flow cytometry. Percent internalization was calculated by the formula $[\%\ sIgM(T_0) - \%\ sIgM(T_n)]/\%\ IgM(T_0) \times 100$ (Fig. 3 A) or $[MFI\ sIgM(T_0) - MFI\ sIgM(T_n)]/MFI\ IgM(T_0) \times 100$ (Fig. S3 A), and the results were comparable. For electron microscopy, B cells were incubated with 10 μg/ml rabbit anti–mouse $F(ab')_2$ (Jackson ImmunoResearch Laboratories) for 20 min on ice, washed twice with PBS 0.5% FBS, and incubated for an additional 30 min on ice with a 1:5 dilution of 10 nm gold-labeled goat anti–rabbit IgG (GE Healthcare). Excess reagent was removed by washing cells twice with PBS 0.5% FBS. Cells were then either fixed with 2.5% glutaraldehyde for time zero or incubated for 2, 5, or 10 min at 37°C before fixation and processing for electron microscopy. 20–30 grids were examined for each time point and labeled cells were photographed.

Ca^{2+} flux. 5×10^6 spleen cells were incubated with 2 μM Indo-1 AM (Invitrogen) in RPMI 2% FBS for 30 min at 37°C. The cells were washed and stained with anti–B220-FITC or PE–anti-B220 and Fab′ FITC goat

anti–mouse IgM at room temperature (Jackson ImmunoResearch Laboratories) to determine gating. Fluorescence ratios of Indo-1 emission at 405/485 nm were measured on cells gated based on B220 and IgM expression by flow cytometry on a FACSVantage SE (Becton Dickinson). Data were acquired for 30 s without stimulation, and Ca^{2+} flux was induced by the addition of μ chain-specific $F(ab')_2$ goat anti–mouse IgM (Jackson ImmunoResearch Laboratories). Data were collected for 500 s and analyzed using FlowJo (TriStar) software.

Western blotting and immunoprecipitation. B cells were purified from the spleen by negative selection with anti-CD43 magnetic beads (MACS; Miltenyi Biotec). Cells were stimulated with 20 μg/ml of $F(ab')_2$ fragment goat anti–mouse IgM (Jackson ImmunoResearch Laboratories) at 37°C and lysed in 50 mM Tris HCl, pH 7.7, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM EGTA, 0.1% DOC, 10% glycerol, 1 mM PMSF, 0.2 mM Na_3VO_4, and protease inhibitors for Western, or 50 mM Tris HCl, pH 7.7, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.1% DOC, 1 mM PMSF, and 0.2 mM Na_3VO_4 for immunoprecipitation. Lysates were incubated for 15 min on ice, and cellular debris was sedimented at 14,000 rpm for 15 min at 4°C. Lysates or immunoprecipitates were resolved on a 4–12% NuPage gel (Invitrogen) and transferred onto Immobilon-P membranes (Millipore). The following antibodies were used in these experiments: anti-JNK1/2, anti-p38, and phosphorylation site-specific antibodies to ERK1/2, JNK1/2, p38, BLNK, c-Cbl, Akt, PLC-γ2, Src, IkB-α (112B2), and anti–phospho-IkB-α (Cell Signaling Technology); anti-ERK1/2 antibodies (Promega); anti-Syk (N-19), anti-phBLNK, anti-ubiquitin (P4D1), anti-Cbl-b (H-121), anti-Cbl (C-15), and anti-phospho pPI 3-kinase p85α (Tyr 508; Santa Cruz Biotechnology, Inc.); anti-phophotyrosine antibody 4G10 and anti-SHIP (Upstate Biotechnology); anti-phospho SHIP (StemCell Technologies Inc.); anti-actin (Sigma-Aldrich); SHP-1 and anti-CD22 for IP (BD Biosciences); and Igα, Igβ (10), and anti-CD22 (provided by H. Wortis, Tufts University, Boston, MA).

Online supplemented material. Fig. S1 shows Igβ expression and phosphorylation in wild-type and mutant B cells. Fig. S2 shows PI3 kinase p85 and AKT phosphorylation in wild-type and mutant B cells. Fig. S3 shows internalization and ERK phosphorylation in cytochalasin D–treated cells. Fig. S4 shows Igβ cDNA sequence for wild-type and mutant B cells. Figs. S1–S4 are available at http://www.jem.org/cgi/content/full/jem.20060221/DC1.

We thank Dr. Henry Wortis for anti-CD22 antibody, Dr. Eva Besmer for her help with the manuscript, Thomas Eisenreich for his help with mice, and Klara Velinzon for flow cytometry.

This work was supported by National Institutes of Health grant AI051573 (to M.C. Nussenzweig) and the Howard Hughes Medical Institute (to M.C. Nussenzweig).

The authors have no conflicting financial interests.

Submitted: 27 January 2006
Accepted: 2 June 2006

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