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Bam32 Links the B Cell Receptor to ERK and JNK and Mediates B Cell Proliferation but Not Survival

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Summary

Bam32 is an adaptor protein recruited to the plasma membrane upon B cell receptor (BCR) crosslinking in a phosphoinositol 3-kinase (PI3K)-dependent manner; however, its physiologic function is unclear. To determine its physiologic function, we produced Bam32-deficient mice. Bam32$^{-/-}$ B cells develop normally but have impaired T-independent antibody responses in vivo and diminished responses to BCR crosslinking in vitro. Biochemical analysis revealed that Bam32 acts in a novel pathway leading from the BCR to MAPK/ERK Kinases (MEK1/2), MAPK/ERK Kinase Kinase-1 (MEKK1), extracellular signal-regulated kinase (ERK), and c-*jun* NH2-terminal kinase (JNK), but not p38 mitogen-activated protein kinase (p38). This pathway appears to be initiated by hematopoietic progenitor kinase-1 (HPK1), which interacts directly with Bam32, and differs from all previously characterized BCR signaling pathways in that it is required for normal BCR-mediated proliferation but not for B cell survival.

Introduction

The B cell receptor (BCR) is essential for B cell development, specific response to antigen, and survival (Benschop and Cambier, 1999; King and Monroe, 2000; Kurosaki, 2000; Niiro and Clark, 2002; Rajewsky, 1996; Reth et al., 2000). These diverse functions are mediated by a receptor that is composed of membrane bound immunoglobulin (mIg) and an associated signal transducer composed of the Igα-Igβ dimer (Hermanson et al., 1988; Hombach et al., 1988; Sakaguchi et al., 1988). The Igα-Igβ dimer initiates BCR signaling by recruiting and activating Src family, Syk, and Btk tyrosine kinases (Kurosaki, 2000; Niiro and Clark, 2002). These BCR proximal protein tyrosine kinases (PTKs) phosphorylate a number of cellular substrates, leading to activation of phosphoinositol 3-kinase (PI3K), AKT/Protein Kinase B (AKT), phospholipase Cgamma2 (PLCγ2), intracellular Ca^{2+} mobilization, and nuclear mediators of survival and proliferation including NFκB and mitogen-activated protein kinases (MAPKs) (Chang and Karin, 2001; Karin and Lin, 2002; Kurosaki, 2000; Niiro and Clark, 2002).

Adaptor proteins diversify BCR signaling by linking together different enzymatic cascades and by recruiting signaling molecules to the membrane (Jordan et al., 2003; Kelly and Chan, 2000; Kurosaki, 2002). For example, BLNK/SLP65 is an adaptor composed of a tyrosine-rich target domain for protein tyrosine kinases, a proline-rich domain that interacts with src-homology 3 (SH3) domains, and a src-homology 2 (SH2) domain that interacts with phosphotyrosine (Fu et al., 1998; Goitsuka et al., 1998; Wienands et al., 1998). When phosphorylated by Syk, BLNK provides docking sites for Btk and PLCγ2, which results in the activation of PLCγ2 and diversification of the response by initiating Ca^{2+} flux and activating protein kinase C β (PKCβ) and thereby NFκB (Fu et al., 1998; Ishiai et al., 1999; Jumaa et al., 2001; Kurosaki and Tsukada, 2000; Saijo et al., 2002). BLNK and its associated proteins are essential for B cell proliferation, development, and survival (Cheng et al., 1995; Fruman et al., 2000; Ishiai et al., 1999; Jumaa et al., 1999; Kaisho et al., 2001; Kerner et al., 1995; Khan, 2001; Kurosaki and Tsukada, 2000; Pasparakis et al., 2002; Saijo et al., 2002; Wang et al., 2000; Xu et al., 2000).

Bam32, also known as Dapp1 or PHISH, is a recently described adaptor consisting of an N-terminal SH2 domain and a C-terminal pleckstrin homology (PH) domain and is recruited to the cell membrane in a PI3K-dependent manner following BCR crosslinking (Anderson et al., 2000; Dowler et al., 1999; Marshall et al., 2000a; Niiro et al., 2002; Rao et al., 1999). Bam32 is expressed in B cells and appears to function as a dual adaptor for phosphotyrosine and 3-phosphoinosides, but its physiologic function has not been determined. To examine the role of Bam32 in B cell development and function, we used gene targeting to produce Bam32 null mutant mice. Here, we report that Bam32 links the BCR to ERK and JNK through a novel pathway. This pathway is required for normal B cell proliferative responses but not for B cell survival.

Results

Bam32 Expression in Mice

In humans, Bam32 is largely restricted to hematopoietic cells with the exception of T cells, and its expression is thought to be enhanced in germinal center B cells (Marshall et al., 2000a). In mice, we found the expression of Bam32 to be similar to what was reported in humans, with some notable differences. We detected no increase in Bam32 mRNA in isolated germinal center B cells or activated splenic B cells, including those stimulated with anti-CD40, anti-CD40 + anti-IgM, or anti-CD40 + IL-4 (Figure 1). Rather, in comparison with naive splenic B cells, B cells that were stimulated with LPS, anti-CD40, anti-IgM, or isolated germinal center B cells expressed reduced levels of Bam32 mRNA. We also found murine Bam32 to be expressed in thymus and in isolated thymic and splenic T cells, albeit at lower levels than were observed in B cells (Figure 1). We conclude that in mice, Bam32 is largely restricted to hematopoietic cells with particularly high expression in B cells, and that expression is not enhanced with B cell activation.

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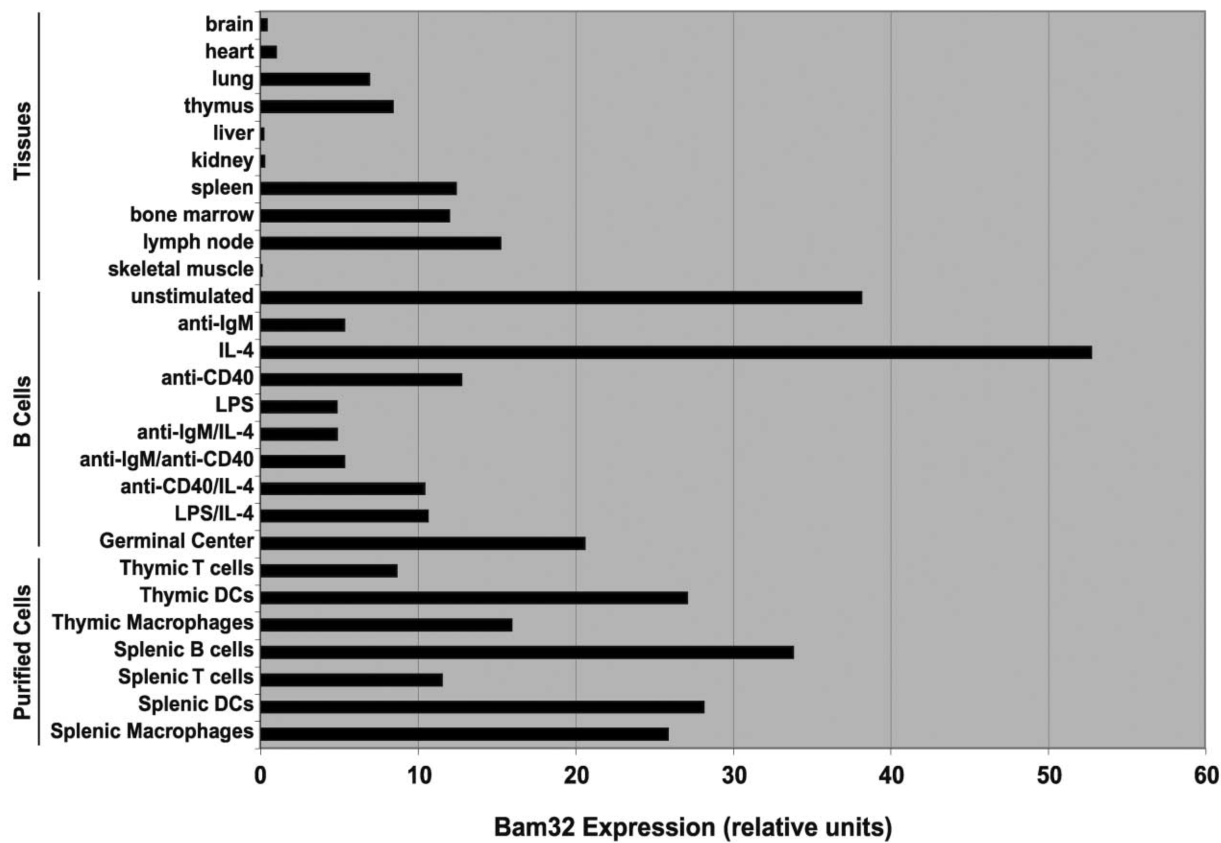

Figure 1. Expression of Bam32 in Mice

Quantitative RT-PCR analysis on tissues (top), B cells (middle), and isolated cells from spleen and thymus (bottom). B cells were purified from resting spleen and cultured in the presence various stimuli, or isolated from splenic germinal centers as indicated. Results are expressed as relative units of Bam32 mRNA normalized using GAPDH mRNA. Data shown are representative of two independent experiments.

Bam32-Deficient Mice

To examine the function of Bam32 in vivo, we targeted Bam32 by replacing exon 1 with a neomycin resistance cassette (Figures 2A and 2B). Stop codons are present in all reading frames 3′ to the neomycin resistance cassette. Absence of Bam32 protein expression was confirmed by Western blotting on B lymphocyte extracts with a polyclonal antibody raised against full-length Bam32 (Figure 2C). No additional bands that might represent aberrant forms of Bam32 were detectable in Bam32$^{-/-}$ lysates. Flow cytometry revealed normal proportions of pro-B, pre-B, immature, and recirculating B cells in the bone marrow, and normal proportions of type 1 and type 2 transitional (T1 and T2, respectively), marginal zone, and follicular B cells in spleen (Figure 3A). The only B cell subset affected by Bam32 deficiency was peritoneal B-1a B cells, which were 3- to 5-fold reduced (Figure 3A, lower left). We also found normal numbers of total cells and B cells in the bone marrow and spleen (Supplemental Table S1 [http://www.immunity.com/cgi/content/full/19/4/621/DC1]). Consistent with the normal numbers of B cells, Bam32$^{-/-}$ mice had normal serum antibody levels (Figure 3B). We conclude that Bam32 is not required for most aspects of B cell development but is required for normal development or maintenance of the peritoneal B-1a compartment.

Bam32 Is Essential for Normal T-Independent Immune Responses

To determine whether Bam32 is essential for B cell responses to antigen in vivo, we immunized mice with 4-hydroxy-3-nitrophenylacetyl (NP) coupled to chicken γ globulin (NP-CGG) or Ficoll (NP-Ficoll) to elicit T cell-dependent or T cell-independent immune responses, respectively. We found no deficiency in T cell-dependent immune responses as measured by specific IgM and IgG1 responses (Figure 4A). In addition, sequencing of the V_H186.2 gene cloned from germinal center B cells isolated from NP-CGG immunized mice revealed that somatic hypermutation was normal (Bothwell et al., 1981; data not shown). In contrast, T cell-independent immune responses were disabled in Bam32$^{-/-}$ mice. The NP-specific IgM response was reduced 2- to 3-fold, and, more impressive, the IgG3 response was reduced 14-fold (Figure 4B). Thus, Bam32$^{-/-}$ B cells are defective in T cell-independent but not in T cell-dependent responses to antigen in vivo.

Bam32 Mediates BCR-Induced B Cell Proliferation

To determine whether Bam32$^{-/-}$ B cells show a cell autonomous defect in response to BCR stimulation, we purified B cells and stimulated them with anti-IgM or various B cell mitogens in vitro. We found that Bam32$^{-/-}$

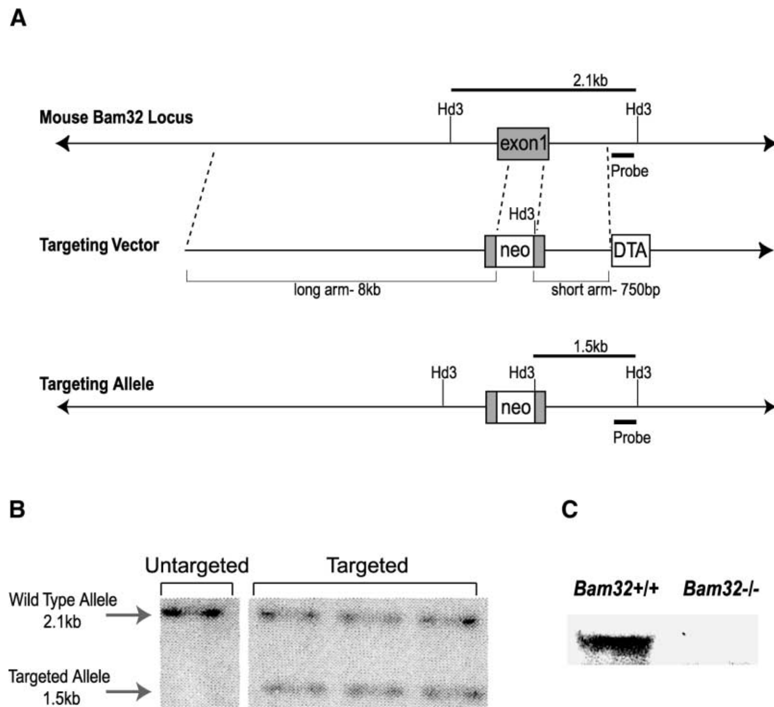

Figure 2. Targeting the Bam32 Locus

(A) The endogenous Bam32 locus (top), the targeting construct (middle), and the targeted locus (bottom). Relevant HindIII sites, probe region, and size of the bands expected in Southern blotting are indicated.

(B) Southern blot analysis of HindIII-digested ES cell DNA using the probe indicated in (A). The wild-type allele is 2.1 kb while the mutant allele is 1.5 kb. An untargeted clone is included as a control.

(C) Western blot analysis for Bam32 expression in whole-cell lysates of purified splenic B cells from wild-type and Bam32$^{-/-}$ mice using anti-Dapp1/Bam32 antibody (Babraham).

B cells responded normally to anti-IgM by upregulating MHCII and CD86 expression (Figure 5A), but proliferative response to anti-IgM stimulation was severely impaired (Figure 5B). Bam32$^{-/-}$ B cells essentially failed to divide at doses of 0.6 μg/ml and 1.2 μg/ml of anti-IgM, while wild-type B cells underwent multiple divisions (Figure 5B). At saturating doses of anti-IgM (10 μg/ml), Bam32$^{-/-}$ B cells did divide but to a lesser extent than wild-type B cells (Figure 5B). The decreased proliferative response to anti-IgM was not the result of an intrinsic defect in B cell response to mitogens, as we found normal cell division after stimulation of knockout B cells with LPS, anti-CD40, anti-CD40 + IL-4, anti-RP105, anti-CD38, and CpG (Figure 5B and data not shown). No defects in proliferation were observed at suboptimal doses of LPS or anti-CD40 in Bam32$^{-/-}$ B cells (Supplemental Figure S2 [http://www.immunity.com/cgi/content/full/19/4/621/DC1]). Finally, the defective response to anti-IgM was complemented by costimulation with either IL-4 or anti-CD40 (Figure 5B). Thus, signaling pathways induced by LPS, IL-4, anti-CD40, anti-CD38, anti-RP105, and CpG are unaffected by Bam32 deletion. In addition, Bam32 is not required for BCR-mediated upregulation of MHCII and CD86. Bam32 specifically affects BCR-mediated proliferation.

Mutations in all previously characterized BCR signaling components that impede proliferation also affect B cell survival (Clayton et al., 2002; Fruman et al., 2000, 1999; Kaisho et al., 2001; Khan, 2001; Kurosaki, 2000; Maas and Hendriks, 2001; Niiro and Clark, 2002; Okkenhaug et al., 2002; Pasparakis et al., 2002; Pogue et al., 2000; Saijo et al., 2002). To determine whether the Bam32 mutation is also associated with altered B cell survival, we stained cultured cells with a combination of 7-amino-actinomycin D (7-AAD) and Annexin V (Figure 5C). We found no differences in survival between Bam32$^{-/-}$ and wild-type B cells cultured in the absence of mitogens or with anti-IgM, LPS, IL-4, anti-CD40, anti-IgM + IL-4, or anti-IgM + anti-CD40, after 24, 48, or 72 hr of culture, while PKCβ$^{-/-}$ cells included as a control were clearly impaired in survival (Figure 5C and data not shown). We conclude that Bam32$^{-/-}$ B cells differ from previously characterized BCR signaling mutants in that they show a defect in proliferative responses to BCR crosslinking without an associated increase in cell death.

Bam32 Links the BCR to ERK and JNK

To determine which signaling pathways are affected by Bam32, we performed biochemical analysis of mutant B cells. BCR proximal signaling was initially evaluated by measuring protein tyrosine phosphorylation and Ca^{2+} flux responses. Consistent with the normal upregulation of MHCII and CD86 in response to anti-IgM, we found no significant differences in either tyrosine phosphorylation or Ca^{2+} flux (Figures 6A and 6B). This suggests Bam32 is not essential for activation of proximal elements of the BCR signaling cascade including src family and Syk kinases, PI3K, Btk, and PLCγ2. AKT and NFκB are downstream of PI3K and have been implicated in regulating B cell survival (Kaisho et al., 2001; Pasparakis et al., 2002; Pogue et al., 2000). In agreement with the observation that Bam32$^{-/-}$ B cells survive normally in culture, in response to BCR stimulation, we found normal phosphorylation of AKT and an intact NFκB pathway as assessed by phosphorylation of IKKα/β and IκB and degradation of IκB (Figure 6C).

In chicken DT40 cells, Bam32 was found to regulate MAPKs, and these enzymes are implicated in regulating BCR-mediated proliferative responses (Kurosaki, 2000;

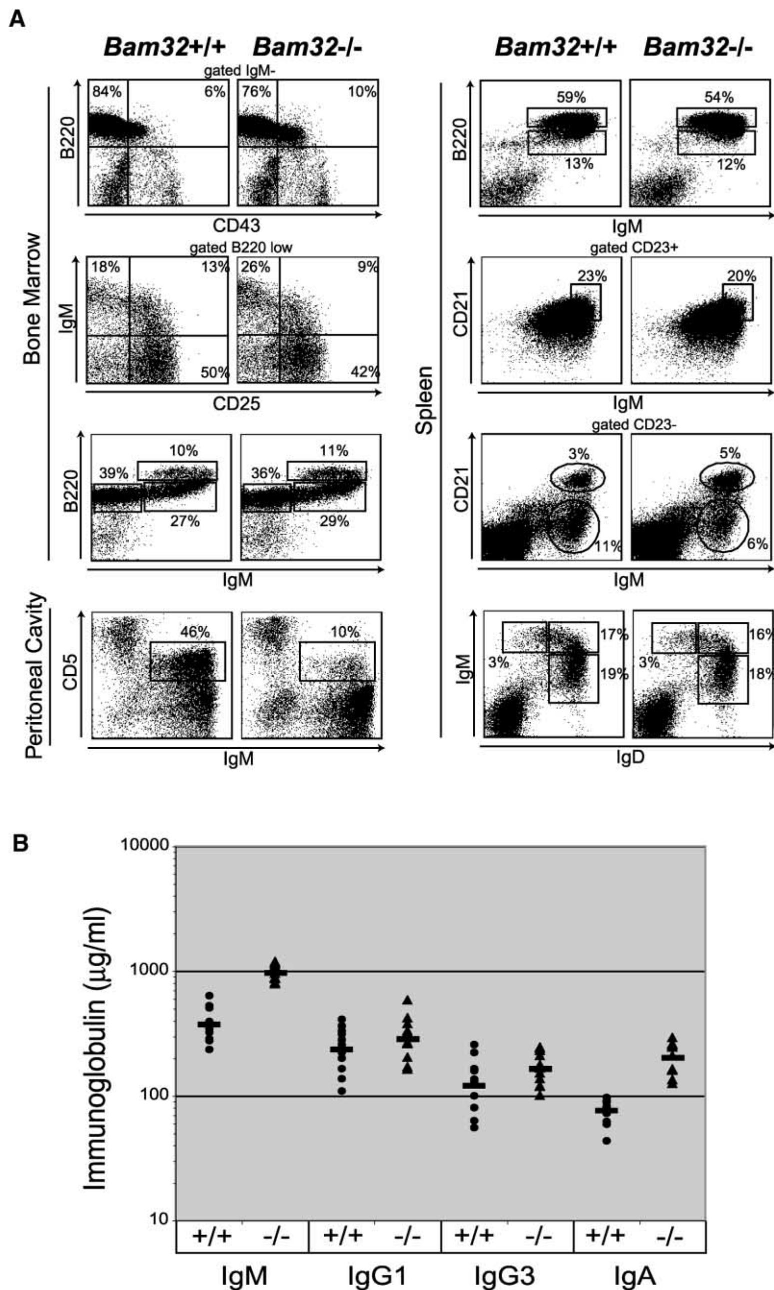

Figure 3. B Cell Development and Serum Antibody Titers in Bam32$^{-/-}$ Mice

(A) Flow cytometric analysis of cells from the bone marrow (upper left), spleen (right), and peritoneal cavity (lower left). Staining antibodies are indicated adjacent to the axis. Cells within the lymphocyte gate are shown, and further gating is indicated directly above the dot plots where applicable. Numbers show relative percentages of cells within indicated gates. In the bone marrow: top row, staining for pro-B cells (IgM$^-$, CD43$^+$, B220$^+$) and pre-B cells (IgM$^-$, CD43$^-$, B220$^+$); middle row, staining for pre-B (B220^{lo}, CD25$^+$, IgM$^+$) and immature B cells (B220^{lo}, CD25$^-$, IgM$^+$); bottom row, staining for pro/pre-B (B220^{lo}, IgM$^-$), immature (B220^{lo}, IgM$^+$), and mature recirculating (B220^{hi}, IgM$^+$) B cells. In the spleen: top row, staining for immature (B220^{lo}, IgM$^+$) and mature (B220^{hi}, IgM$^+$) B cells; second row, staining for transitional type-2 B cells (CD23$^+$, CD21$^+$, IgM$^+$); third row, staining for marginal zone (CD23$^-$, CD21$^+$, IgM$^+$) and transitional type-1 (CD23$^-$, CD21$^-$, IgM$^+$) B cells; bottom row, staining for immature (IgM^{hi}, IgD^{lo}) and mature (IgM^{lo}, IgD^{hi}) B cells. In the peritoneal cavity: staining for B-1a cells (CD5$^+$, IgM$^+$). Each dot plot shown is representative of at least three independent experiments.

(B) Serum Ig concentrations (μg/ml) in age- and sex-matched Bam32 mutant and wild-type mice. Individual wild-type mice are represented by circles, and individual knockout mice are represented by triangles. Mean values are indicated by bold lines.

Niiro and Clark, 2002; Niiro et al., 2002; Richards et al., 2001). To determine whether Bam32 regulates MAPKs in mouse B cells, we measured activation of extracellular signal-regulated kinase (ERK), c-*jun* NH2-terminal kinase (JNK), and p38 mitogen-activated protein kinase (p38) in response to anti-IgM stimulation in Bam32$^{-/-}$ B cells and controls. Western blot analysis showed that ERK and JNK phosphorylation, and therefore activation, was reduced for all doses of anti-IgM tested, but p38 was unaffected in the absence of Bam32 (Figure 7A, Supplemental Figure S2 [http://www.immunity.com/cgi/content/full/19/4/621/DC1]).

To determine how Bam32 might be linked to ERK and JNK, we examined MAPK kinases upstream of ERK and JNK. MAPK/ERK kinases (MEK1/2) are members of the MAPK kinase (MAP2K) superfamily and are known to activate ERK. We found that MEK1/2 phosphorylation, and therefore activation, in response to anti-IgM stimulation was severely impaired in Bam32-deficient B cells (Figure 7B). We conclude that BCR signals leading to MEK1/2 and kinases downstream of MEK1/2 are impaired in the absence of Bam32.

MEK1/2 are activated by members of the MAPKK kinase (MAP3K) superfamily including Raf and MEKK1 (Brummer et al., 2002; Lange-Carter et al., 1993; Yujiri et al., 1998). To determine which of these is linked to the Bam32 pathway, we measured their activation in response to BCR stimulation in Bam32$^{-/-}$ B cells and controls. We found no defect in Raf activation in Bam32$^{-/-}$ B cells in response to receptor stimulation as

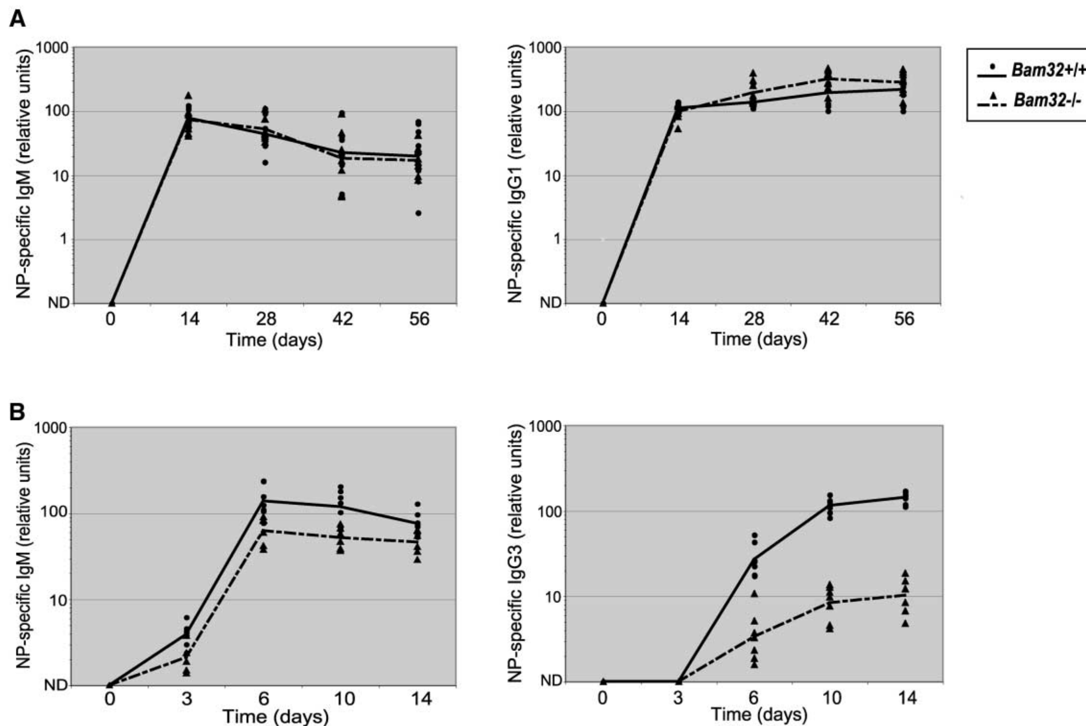

Figure 4. Responses to Immunization in Bam32 Knockout Mice

(A) Serum NP-specific response to immunization with TD antigen NP-CGG as determined by ELISA. Individual wild-type mice (circles) and Bam32 knockout mice (triangles) were immunized, and serum antibodies were assessed at various time points, as indicated on the *x* axis. The mean antibody levels are indicated for wild-type (solid line) and knockout mice (dashed line). Levels reflect relative binding of serum IgM (left) or IgG1 (right) to NP_2-BSA, within the linear range when compared to a standard. Samples with antibody levels below detection threshold are represented as ND (none detected).

(B) Serum NP-specific response to immunization with TI antigen NP-Ficoll. Levels reflect relative binding of serum IgM (left) or IgG3 (right) to NP_2-BSA. Samples with antibody levels below detection threshold are represented as ND (none detected).

measured by Western blotting for phosphorylated Raf (Figure 7B). In contrast, MEKK1 kinase activation in Bam32$^{-/-}$ B cells was diminished when compared to wild-type controls (Figure 7B). We conclude that signaling pathways leading to MEKK1 activation are impaired in the absence of Bam32.

MEKK1 and other MAP3Ks are activated by Ste20 family kinases that act as MAP3K kinases (MAP4K). Hematopoietic progenitor kinase 1 (HPK1) is one such MAP4K which is expressed in B cells (Hu et al., 1996; Kiefer et al., 1996; Liou et al., 2000). To determine whether HPK1 activation is impaired in the absence of Bam32, we performed kinase assays on Bam32$^{-/-}$ and control B cells after BCR stimulation. We found that HPK1 kinase was less active in response to BCR crosslinking in the absence of Bam32 (Figure 7B). To determine whether Bam32 interacts with HPK1, we performed immunoprecipitation experiments using the human B cell line BJAB. We found that Bam32 was coimmunoprecipitated with HPK1, and conversely that HPK1 was coimmunoprecipitated with Bam32; this association was independent of BCR crosslinking (Figure 7C). This association was confirmed in mouse splenic B cells where HPK1 was also coimmunoprecipitated with Bam32 (Figure 7D). As a further control, we performed parallel coimmunoprecipitation experiments in Bam32 knockout mice and found that HPK1 was not detectable in anti-Bam32 immunoprecipitates of Bam32$^{-/-}$ B cell lysates (Supplemental Figure S3 [http://www.immunity.com/cgi/content/full/19/4/621/DC1]). We conclude that Bam32 associates with HPK1, and Bam32$^{-/-}$ B cells are impaired in HPK1 kinase activation.

Discussion

In the absence of Bam32, B cells develop normally in the bone marrow and spleen, but there is a deficiency in the peritoneal B-1a cell compartment. The selective B-1a deficiency is in agreement with the idea that BCR crosslinking by self-antigen is required for maintenance of this self-renewing B cell compartment (Hardy and Hayakawa, 2001). Normal B cell development in the absence of Bam32 is consistent with the finding that the Bam32 mutation has no effect on B cell survival and the idea that the role of BCR signaling in development is to maintain B cell survival through inhibition of proapoptotic signals (Lam et al., 1997; Meffre and Nussenzweig, 2002; Rajewsky, 1996).

Adaptor proteins diversify cellular signaling by linking different groups of signaling proteins (Jordan et al., 2003; Kelly and Chan, 2000; Kurosaki, 2002). Bam32, an adaptor implicated in BCR signaling, is composed of an SH2 and a PH domain and is recruited to the membrane by PI3K activation (Anderson et al., 2000; Marshall et

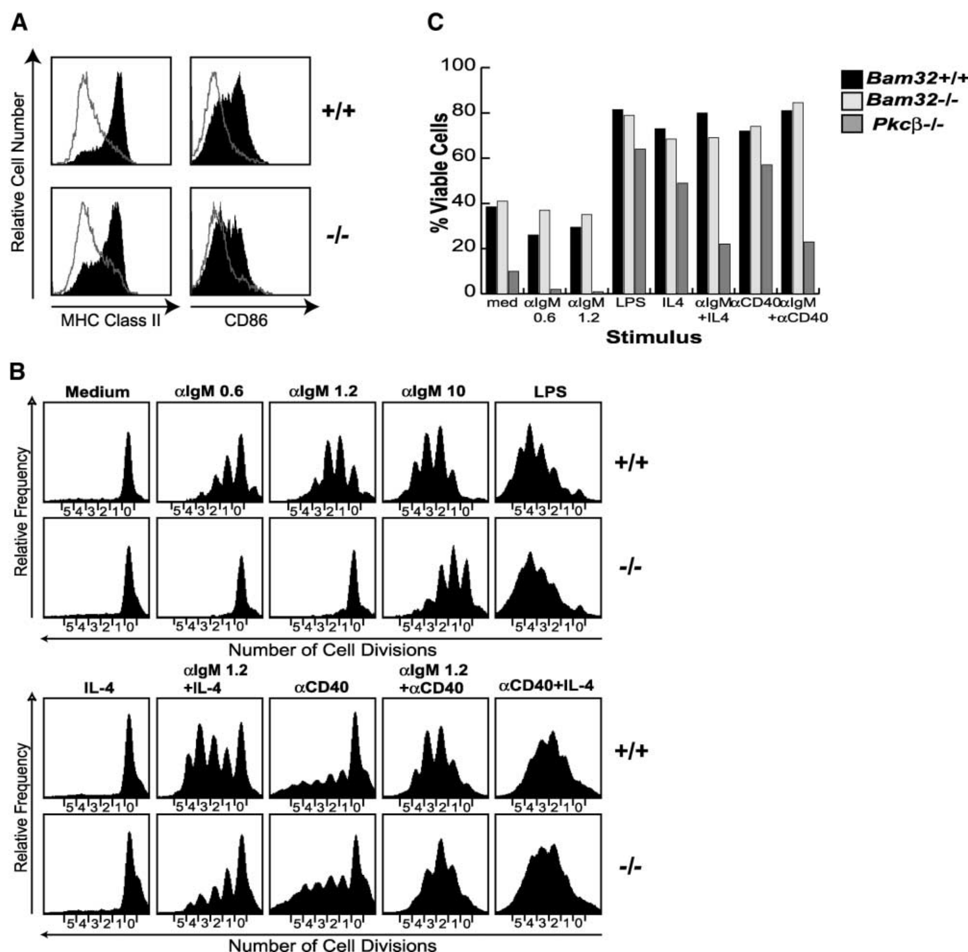

Figure 5. Responses of Bam32 Knockout B Cells to Stimulation In Vitro

(A) Activation of wild-type and knockout B cells in response to 1.2 μg/ml anti-IgM as assessed by MHCII upregulation (left) and CD86 upregulation (right). Histograms represent Class II or CD86 expression with stimulation (filled plot) and without (unfilled plot). Data shown are representative of three independent experiments.

(B) Proliferative responses of CFSE-labeled B cells from wild-type and knockout mice incubated for 72 hr in the presence of indicated mitogenic stimuli. Number of cell divisions as assessed by CFSE dye dilution is indicated on the *x* axis. Data shown are representative of four independent experiments.

(C) Cell survival as determined by flow cytometric analysis on isolated splenic B cells cultured in the presence of indicated mitogens for 48 hr. Cells negative for 7-AAD and Annexin V staining were scored as viable. Numbers show percentage of viable cells. Black bars show wild-type, light gray bars show Bam32$^{-/-}$, and dark gray bars show PKCβ$^{-/-}$ B cells, included as a control. Results represent the mean of two experiments, and the variation between experiments was less than 10%.

al., 2000a). Based on studies in DT40 cells, it was initially proposed that Bam32 mediates Ca^{2+} flux through PLCγ2 and consequently, NFκB and MAPK activation and cell survival (Marshall et al., 2000a; Niiro et al., 2002). However, the function of Bam32 and its relation to PLCγ2 was unclear in DT40 cells. Bam32-deficient DT40 cells exhibit only mild impairment in PLCγ2 phosphorylation and Ca^{2+} flux in response to BCR stimulation (Niiro et al., 2002). Furthermore, in response to anti-IgM stimulation, apoptosis is impaired in PLCγ2-deficient DT40 cells, while apoptosis is enhanced in Bam32-deficient DT40 cells (Niiro et al., 2002; Takata et al., 1995). In mice, we found no defects in Ca^{2+} flux or NFκB activation in Bam32$^{-/-}$ B cells and no direct link between Bam32 and PLCγ2. The observed differences between the data presented here and previous data from DT40 cells may reflect redundancy of signaling pathways in mammalian as opposed to chicken B cells. Others have demonstrated such redundancy in mutant mouse models, including Syk/Zap70 and Btk/Tec double mutants and Src family kinase triple mutants (Ellmeier et al., 2000; Saijo et al., 2003; Schweighoffer et al., 2003). The differences may also reflect aberrant responses in transformed DT40 cells, or intrinsic differences between chicken DT40 and mouse B cells in response to BCR stimulation. For example, it is not possible to measure proliferation in response to BCR stimulation in DT40 cells. In contrast to splenic mouse B cells which undergo proliferation, DT40 cells undergo apoptosis in response to BCR stimulation, mimicking elimination of self-reactive B cells (Takata et al., 1995). Our findings in mice that Bam32 is not linked to PLCγ2, Ca^{2+} flux, or NFκB activation are consistent with our observations that Bam32$^{-/-}$ B cells show no evidence of increased cell death in vivo or in

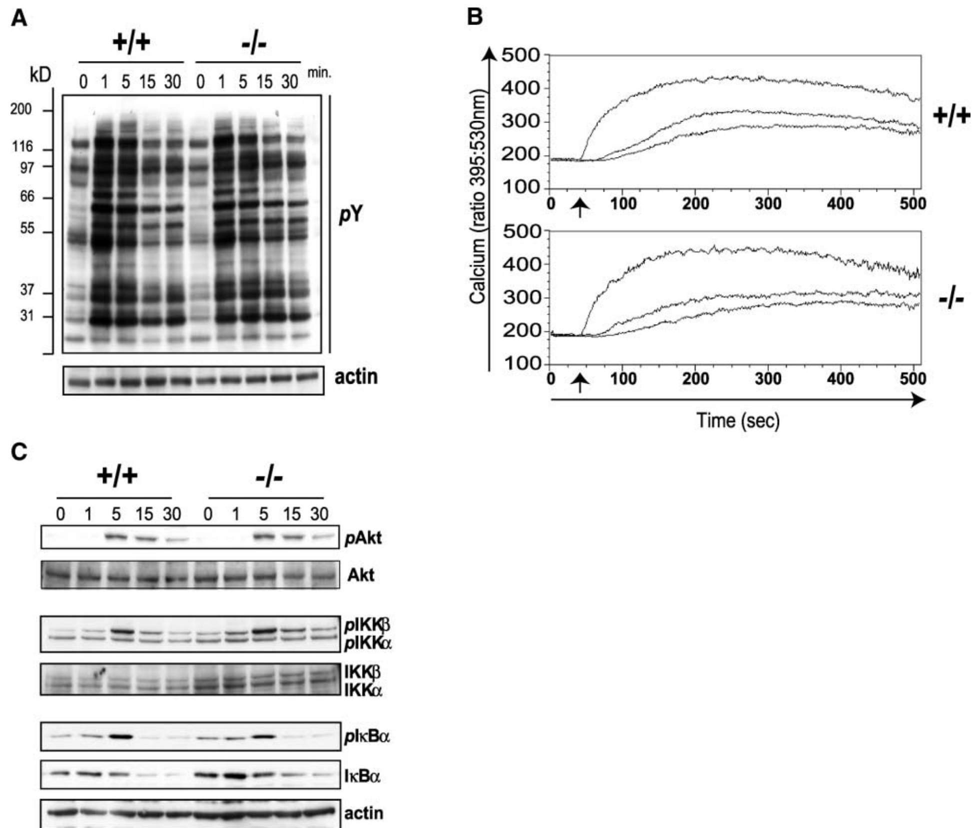

Figure 6. BCR-Mediated Signaling in Bam32$^{-/-}$ B Cells

(A) Western blot analysis for tyrosine phosphorylated proteins in isolated splenic B cells from knockout and wild-type mice when stimulated with anti-IgM. Cell lysates were subject to Western blotting with anti-phophotyrosine. Blot was probed for actin for loading control. Data are representative of two experiments.

(B) Calcium Flux analysis in response to BCR stimulation is wild-type (top) and knockout (bottom) B cells. Flow cytometry analysis depicting calcium flux (*y* axis) as a function of time (*x* axis) in cells stimulated with either 0.6 μg/ml (lower lines within plot), 1.2 μg/ml (middle line), or 10 μg/ml (upper line) of anti-IgM. Arrows indicate time point of simulation. Data shown are representative of three independent experiments.

(C) Western blot analysis to detect BCR-induced phosphorylation of Akt (top), IKKα/β (middle), and IκBα (bottom), and degradation of IκBα (bottom) in response to BCR stimulation. For loading control, blots were probed to detect total AKT, IKKα/β, and actin. Data shown are representative of two (NF-κB pathway) or three (AKT) independent experiments.

vitro. We propose that Bam32 affects MAPK activation through means independent of PLCγ2.

PI3K is a central effector of BCR signaling that amplifies cellular signaling responses by producing phophoinositol 3,4,5-triphosphate ($PtdInsP_3$) (Kurosaki, 2000; Niiro and Clark, 2002; Okkenhaug and Vanhaesebroeck, 2003). This second messenger in turn mediates several parallel signaling cascades including those of AKT and PLCγ2 (Kurosaki, 2000; Niiro and Clark, 2002; Okkenhaug and Vanhaesebroeck, 2003). PLCγ2 further diversifies signals by producing the additional second messengers inositol 1,4,5-triphosphate and diacylglycerol, which induce Ca^{2+} flux and activate PKCs, and therefore NFκB (Marshall et al., 2000b; Saijo et al., 2002). Given the importance of AKT and NFκB in cell survival (Kaisho et al., 2001; Pasparakis et al., 2002; Pogue et al., 2000), it is not surprising that numerous mouse mutations in PI3K-related signaling proteins such as Btk, PKCβ, BLNK/SLP-65, PLC-γ2, Vav1/Vav2, BCAP, and PI3K subunits p85α and p110δ are characterized by X-linked immunodeficiency-like (xid-like) defects (Fruman et al., 2000; Khan, 2001; Maas and Hendriks, 2001) in B cell development, proliferation, and survival (Clayton et al., 2002; Doody et al., 2001; Fruman et al., 1999; Jumaa et al., 1999; Khan et al., 1995; Maas and Hendriks, 2001; Okkenhaug et al., 2002; Saijo et al., 2002; Tedford et al., 2001; Wang et al., 2000; Xu et al., 2000; Yamazaki et al., 2002). In contrast, the isolated defects in proliferation and B-1a cell maintenance which we observe in Bam32 knockout mice are unlike those of the aforementioned xid-like mutants, suggesting that Bam32 activates a previously unappreciated pathway from PI3K to the nucleus which clearly differs from pathways affected by xid-like mutations.

A number of signaling pathways triggered by the BCR are believed to converge on activation of MAPKs including a PLCγ-2-dependent pathway that is essential for activation of JNK and p38, and a PLCγ-2-independent pathway to ERK that is poorly understood but is essential for BCR-induced B cell proliferation in mature B cells (Hashimoto et al., 1998; Ishiai et al., 1999; Richards et al., 2001). We propose that PI3K initiates a novel PLCγ2-independent pathway to ERK and JNK activation that functions through Bam32 and provide evidence that this

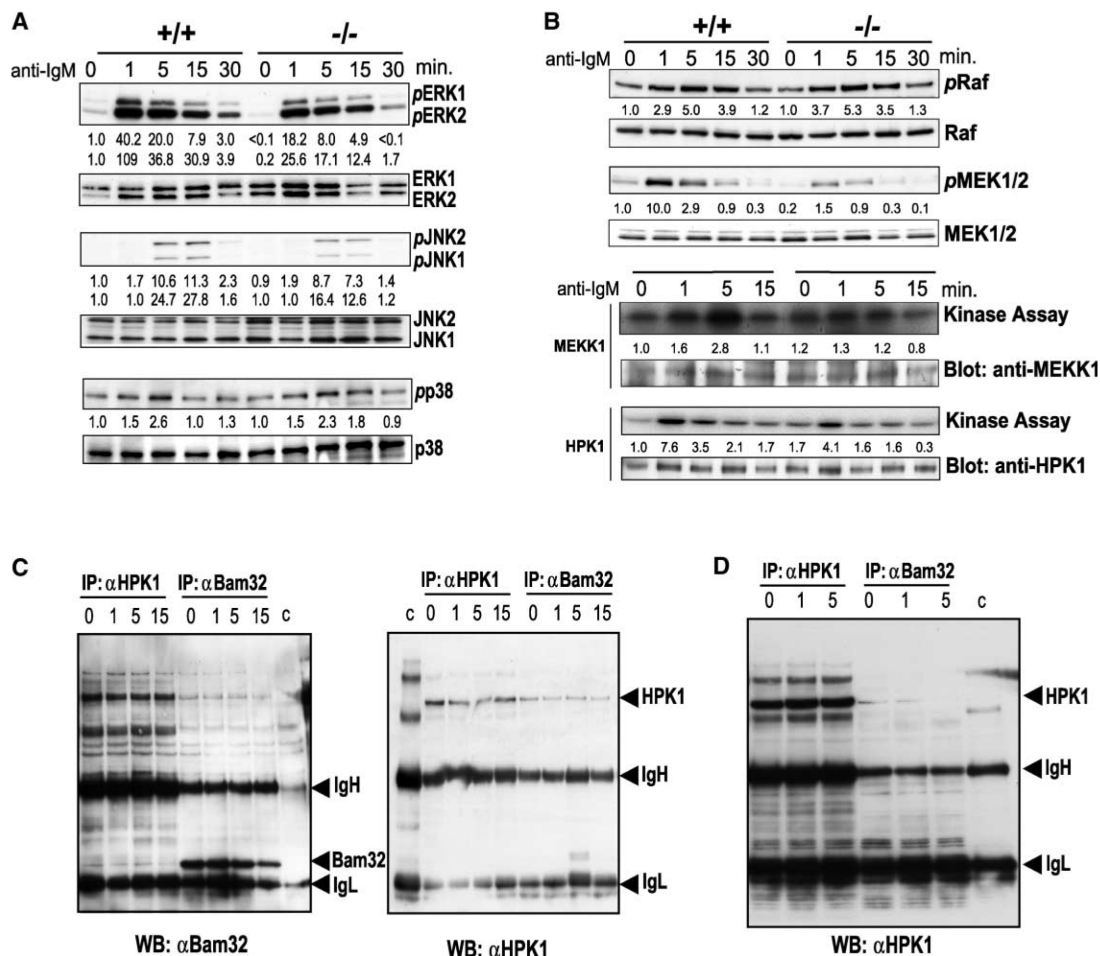

Figure 7. MAP Kinase Pathways in Bam32$^{-/-}$ B Cells

(A) Western blot analysis for phophorylated ERK, JNK, and p38 MAPK in isolated splenic B cells. B cells from knockout and wild-type control mice were stimulated with anti-IgM and lysed at different time points. Blots were reprobed to detect total ERK, JNK, and p38. Numbers reflect the relative intensity of bands representing phosphorylated active protein in relation to total protein. Data shown are representative of three (ERK, p38) or four (JNK) independent experiments.

(B) Raf, MEK1/2, MEKK1, and HPK1 activation. Western blot analysis for phosphorylated forms of Raf and MEK1/2 (upper) and in vitro kinase assay performed on immunoprecipitated MEKK1 and HPK1 using MBP substrate (lower). For Raf and MEK1/2, numbers reflect relative intensity of bands representing phorphorylated active protein in relation to total protein. For MEKK1 and HPK1, numbers reflect the relative kinase activity in relation to protein levels. Data shown are representative of three independent experiments.

(C) Bam32 and HPK1 coimmunoprecipitation in BJAB. Immunoprecipitation on BJAB lysates using anti-Bam32 (left half of blots) or anti-HPK1 (right half of blots), probed with anti-Bam32 (left blot) or anti-HPK1 (right blot). Bands representing Bam32 or HPK1 and control immunoprecipitations performed with species-matched control antibodies are indicated. Data shown are representative of four independent experiments.

(D) Bam32 and HPK1 coimmunoprecipitation in mouse splenic B cells. Lysates were immunoprecipitated using anti-HPK1 (left half) or anti-Bam32 (right half) and probed with anti-HPK1. Data shown are representative of two independent experiments.

pathway functions through intermediates MEK1/2 and MEKK1. MEKK1 is selective for ERK and JNK but not p38, and activates ERK through MEK1/2 (Schlesinger et al., 1998; Yujiri et al., 1998). MEKK1 is not the only enzyme that can activate MEK1/2: Raf is an additional MAP3K implicated in BCR-mediated proliferation, but we find that Raf activation does not require Bam32 (Brummer et al., 2002; Lange-Carter et al., 1993).

The means through which MEKK1 is activated is poorly understood. HPK1 is member of the Ste20 group kinases, which may function as a MAP4K (Hu et al., 1996; Kiefer et al., 1996). HPK1 has been implicated in MEKK1 activation, and, like Bam32, its expression is largely restricted to hematopoietic cells. Here, we provide evidence that Bam32 physically interacts with HPK1 and that HPK1 kinase activity is impaired in Bam32 mutant B cells. These data suggest a means through which Bam32 may affect MEKK1 activation.

Numerous groups have reported HPK1 as a positive regulator of JNK (Hu et al., 1996; Kiefer et al., 1996; Liou et al., 2000) However, with regard to ERK, it was previously reported that HPK1 does not affect ERK in B cell lines, but negatively regulates ERK and AP-1 in T cell lines (Liou et al., 2000; Tsuji et al., 2001). HPK1 has also been implicated in NFκB signaling both as a positive and negative regulator (Arnold et al., 2001; Hu

et al., 1999; Tsuji et al., 2001). These differences may be due to caspase-mediated cleavage of HPK1, which can modulate its activity (Arnold et al., 2001; Chen et al., 1999). HPK1 has also been shown by others to associate with numerous other adaptors in different cell types, including BLNK (Ensenat et al., 1999; Ling et al., 2001, 1999; Liou et al., 2000; Sauer et al., 2001; Tsuji et al., 2001). Bam32 may direct localization of HPK1 to the plasma membrane, thereby allowing activation of MEKK1 without significantly altering the function of HPK1 in other parallel pathways. Consistent with this idea, our data show a comparatively mild impairment in HPK1 activity in Bam32$^{-/-}$ B cells. It is also possible that Bam32 affects MEKK1 through other MAP4Ks, independently of HPK1. Thus, the precise relationship between Bam32 and HPK1 as it pertains to MAPK activation remains to be determined.

The Bam32 pathway to MAPKs is not directly linked to NFκB, AKT, or Ca^{2+} flux, and therefore disruption of this arm of BCR signaling does not affect cell survival. In addition, PI3K-independent pathways to MAPK activation should not be affected by Bam32 deficiency. Indeed, complementation of the Bam32 deficiency by anti-CD40 or IL-4 in vitro is likely to result from ERK and JNK activation through such alternative means. This may also explain why Bam32$^{-/-}$ mice show normal T-dependent antibody responses in vivo.

Experimental Procedures

Bam32-Deficient Mice

Bam32 knockout mice carry a Bam32 gene in which exon 1 is replaced by a neomycin resistance cassette. The genomic DNA used for targeting was derived from a BAC genomic clone (Research Genetics). The long arm of the targeting vector was 8 kb of DNA 5′ of the first codon, and the short arm was 750 bp of DNA 3′ to exon 1. A neomycin resistance cassette inserted between the long and short arms was used for positive selection. Exon 1 encodes a conserved amino acid sequence and the beginning of the SH2 domain. Stop codons are present in all reading frames 3′ to the neo cassette, and no in-frame methionines are present until the end of the consensus SH2 domain. No aberrant splice products were detectable in RT-PCR reactions using primers derived from Bam32 5′ untranslated sequences upstream of the Neo cassette or using primers derived from within the Neo cassette. A diphtheria toxin gene, inserted distal to the short arm, was used for negative selection (Yagi et al., 1990). A HindIII site, engineered within the neomycin resistance cassette, was used for screening purposes. The targeting construct was transfected into E14 (129/Ola) embryonic stem (ES) cells; G418 resistant clones were screened for homologous recombination by PCR and confirmed with Southern blot analysis on HindIII-digested DNA. The genomic fragment used as a probe for Southern blotting was generated by PCR with the primers 5′-TACTGTCCCTTGCATAGGATGGC-3′ and 5′-GTCTGACATATCATCTGTGTGGAG-3′. Following digestion with HindIII, the wild-type allele gives a 2.1 kb fragment while the knockout allele gives a 1.5 kb fragment. Mice were genotyped by PCR with the following primers: 5′-CCTCTTACTCACAGCCGTGTAGT-3′ (common), 5′-CGAGCACGTACTCGGATGGAAG-3′ (targeted) and 5′-ATGGGCAGAGCAGAACTTCTAGGA-3′ (wild-type). A PCR product of approximately 500 bp represents the wild-type allele, while a product of approximately 850 bp represents the knockout allele. Positive ES cell clones were injected into C57BL/6 blastocysts. Mice were backcrossed to C57BL/6 for 10 generations during time of analysis. Data presented here reflect experiments performed on either C57BL/6 congenic Bam32 knockout mice with age- and sex-matched controls, or on littermates. All mice were bred and maintained under specific pathogen-free conditions, and all mouse protocols were approved by the Rockefeller University IACUC.

Flow Cytometry

Single-cell suspensions from indicated organs were depleted of erythrocytes by lysis in ACK buffer (0.155 M ammonium chloride, 0.1 mM disodium EDTA, and 0.01 M potassium bicarbonate). 1×10^6 cells per condition were stained with antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), or biotin. Biotin conjugates were visualized with either Red^{670}-streptavidin (GIBCO-BRL, Rockville, MD) or PE-Cy7-streptavidin (Caltag). The following anti-mouse mAbs (BD Pharmingen) were used: anti-B220 (RA3-6B2), anti-CD43 (S7), anti-IgM (R6-60.2), anti-IgD (11-26c.2a), anti-CD21 (7G6), anti-CD23 (B3B4), and anti-CD25 (PC61). Data were collected on a FACS Calibur cytometer and analyzed with Cellquest software (Becton Dickinson). For Calcium flux analysis, cells were loaded in 2 μM Indo 1 (Molecular Probes). B cells were gated with anti-B220 and cells were stimulated with indicated concentrations of $F(ab')_2$ anti-mouse IgM (Jackson Immunoresearch). Data were acquired on a BD LSR cytometer (Becton Dickinson) and analyzed with FloJo software (Treestar).

In Vitro Survival, Proliferation, and Activation

B cells were purified from spleen by depletion of CD43-expressing cells with anti-CD43 magnetic beads (MACS; Miltenyi Biotec). For proliferation analysis, 1×10^7 cells/ml in RPMI were labeled with 5 μM CFSE for 10 min at 37°C. Cells were cultured in RPMI containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-mercaptoethanol. Cells were stimulated with 0.6, 1.2, or 10 μg/ml (as indicated) of $F(ab')_2$ fragment goat anti-mouse IgM (Jackson Immunoresearch), 0.6 μg/ml anti-CD40 antibody (HM40-3; BD Pharmingen), 5 μg/ml lipopolysaccharide (LPS; Sigma), 25 U/ml recombinant mouse IL-4 (Genzyme), or with combinations as indicated. For survival analysis, cells were washed in Annexin V binding buffer (10 mM HEPES [pH 7.5], 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$) and stained with Annexin V biotin (Roche) and 7-aminoactinomycin D (7-AAD, Sigma). Cells were analyzed by flow cytometry as previously indicated. For activation, cells were stained 24 hr after culture with anti-CD86 (BD Pharmingen) or anti-MHCII (M5/114).

Immunizations

Age- and sex-matched 8- to 12-week-old C57BL/6 control mice (Jackson Labs) and C57BL/6 congenic Bam32$^{-/-}$ mice were injected intraperitoneally with either 12.5 μg of NP_{190}-Ficoll or 50 μg of alum precipitated NP_{21}-CGG (both from Biosearch Technologies) in 300 μl of PBS. Doses within the linear range by ELISA (see below) were determined by titration of antigen and immunizations in wild-type mice. Mice were bled from the tail vein at various time points as indicated.

ELISA

Unimmunized age- and sex-matched 8- to 12-week-old C57BL/6 control mice and C57BL/6 congenic Bam32$^{-/-}$ mice were bled and ELISA was carried out using unlabeled goat anti-mouse Ig (H+L) for capture and HRP-conjugated goat anti-mouse isotype-specific antibodies (Southern Biotechnology) for detection. Plates were developed using a Peroxidase Substrate Kit (Bio-Rad) and absorbance measured at 415 nm. Values were compared to mouse immunoglobulin standards (Southern Biotechnology). The relative amount of NP-specific immunoglobulin was measured using NP_2-BSA (Biosearch Technologies) as the coating antigen. Plates were coated at 4°C for 15 hr with 100 μl of PBS containing 5 μg/ml NP_2-BSA. Serial dilutions were performed for each sample and a standard control. Plates were developed as described above. Results reflect relative absorbance for each sample compared with the standard control. Readings were taken within the linear range for each sample, and adjusted for dilution.

Western Blotting, Immunoprecipitation, and In Vitro Kinase Assay

To confirm the absence of Bam32 protein in knockout mice, 1×10^7 splenic B cells were prepared in 1% NP-40 lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 10% Glycerol) supplemented with a protease inhibitor cocktail (Sigma). Lysates were resolved on a 4%–12% NuPage gel (Invitrogen) and transferred onto Immobilon-P membranes (Millipore). Western blot-

ting was performed with anti-Dapp1 antibody (Babraham). For activation of signaling pathways, 1×10^6 B cells were stimulated with 20 μg/ml goat-anti-mouse IgM $F(ab')_2$ fragment (Jackson Immunoresearch) for the indicated times. Lysates were prepared in 1% NP-40 lysis buffer supplemented with a protease inhibitor cocktail (Sigma), 2 mM Na_3VO_4, and 5 nM CalyculinA (Calbiochem). Whole-cell lysates were resolved and transferred as described. Total tyrosine phophorylation was determined by Western blot with anti-phophotyrosine antibody (Upstate Biotech) and, following stripping, anti-actin (Oncogene) for loading control. For MAPK, MEK1/2, Raf, and AKT activation, membranes were developed with phosphorylation site-specific antibodies to ERK1/2, JNK1/2, p38, MEK1/2, Raf (Ser338), and AKT (all purchased from Cell Signaling Technology). For loading controls, the membranes were stripped and incubated with antibodies to nonphosphorylated forms of ERK1/2 (Promega), JNK1/2, p38, MEK1/2, Raf, and AKT (Cell Signaling Technology). For in vitro kinase assays, 2×10^7 purified B cells were lysed in 1% NP-40 lysis buffer plus protease and phosphatase inhibitors as described above and containing 0.1% SDS and 0.5% Sodium deoxycolate. Lysates were precleared with rabbit IgG for MEKK1 and goat IgG for HPK1 plus Protein-G-sepharose (Amersham Biosciences) for 1 hr at 4°C. Immunoprecipitation was done with anti-MEKK1 or anti-HPK1 antibodies (Santa Cruz Biotechnology) for 1 hr followed by incubation with Protein-G-sepharose at 4°C for 30 min. Beads were washed twice with lysis buffer and twice with kinase buffer. Kinase reactions were performed with half of the immune-complex on the beads in kinase buffer (50 mM HEPES [pH 7.5], 10 mM $MnCl_2$, 10 mM $MgCl_2$, 1 mM DTT, and 10 μCi γ-ATP) for 15 min at 30°C using 10 μg myelin basic protein (MBP) peptide (Upstate Biotech) as substrate. The rest of the immune-complex on beads was resolved on a 4%–12% NuPAGE gel to check the equal loading by Western blotting. Coimmunoprecipitation of Bam32 and HPK1 was examined with 1×10^7 BJAB cells lysed in 0.5% NP-40 lysis buffer as described above. After preclearing, lysates were immunoprecipitated with anti-HPK1 or anti-Dapp1 (Babraham) as described above. For controls, immunoprecipitation was performed with species-matched antibodies: goat IgG for HPK1 and sheep IgG for Bam32 (both from Sigma). For coimmunoprecipitation in mouse splenic cells, 9×10^7 splenic B cells (isolated and stimulated as previously described) were lysed in 1% CHAPS buffer. All quantifications were done with NIH image software.

Quantitative RT-PCR

Quantitative RT-PCR was performed using the SYBR green system and master mix (Applied Biosystems) with an ABI Prism 7700 Sequence Biodetector (PE Applied Biosystems). Total RNA was extracted from sorted cells and tissues with Trizol (Life Technologies) and reverse transcribed using Superscript II and random hexamers (Life Technologies). Parallel quantitative RT-PCR reactions were performed using primers for Bam32 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Bam32 mRNA levels were normalized to GAPDH to account for differences in total mRNA present in each sample. Data are represented as relative expression of Bam32 mRNA in each sample. Bam32 mRNA level in the heart was arbitrarily set at 1, and all samples were adjusted accordingly. The following primers were used: Bam32, 5′-GGCACCAAAGAAGGCTATCTCA-3′, 5′-CCATCTCAGTATTTTGATCCATT-3′; GAPDH, 5′-TGAAGCAGG CATCTGAGGG, 5′-CGAAGGTGGAAGAGTGGGAG-3′. $CD11c^+$ cells (representing predominantly dendritic cells) were isolated through positive selection for CD11c by MACS (Miltenyi Biotec). $CD11b^+$, $CD11c^-$ cells (representing predominantly macrophages) were isolated by positive selection of $CD11b^+$ cells from CD11c depleted samples. T and B cells were isolated by positive selection using MACS for CD90 or CD19, respectively. For culture, splenic B cells were isolated and stimulated as described above for proliferation studies. The dose for anti-IgM stimulation was 1.2 μg/ml. Germinal center B cells were purified from mice immunized with NP-CGG. Fourteen days postimmunization, spleen cells were prepared and depleted of CD43-expressing cells. Cells were then stained with anti-Fas, anti-GL7 (both from BD Pharmingen), and Fas^{hi}; $GL7^+$ germinal center B cells were purified by cell sorting using a FACS Vantage (Becton Dickinson).

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