Brief Definitive Report

The B Lymphocyte Adaptor Molecule of 32 kD (Bam32) Regulates B Cell Antigen Receptor Signaling and Cell Survival

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Abstract

The B lymphocyte–associated adaptor protein 32 kD in size (Bam32) is expressed at high levels in germinal center (GC) B cells. It has an NH_2-terminal src homology 2 (SH2) domain which binds phospholipase C (PLC)γ2, and a COOH-terminal pleckstrin homology (PH) domain. Thus, Bam32 may function to integrate protein tyrosine kinase (PTK) and phosphatidylinositol 3-kinase (PI3K) signaling pathways in B cells. To further define the role Bam32 plays in B cells, we generated Bam32-deficient DT40 cells. These $Bam32^{-/-}$ cells exhibited lower levels of B cell antigen receptor (BCR)-induced calcium mobilization with modest decreases in tyrosine phosphorylation of phospholipase C (PLC)γ2. Moreover, BCR-induced activation of extracellular signal-regulated kinase (ERK), c-jun NH2-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK) pathways was impaired in $Bam32^{-/-}$ cells but not the activation of Akt-related pathways. Activation of downstream transcription factors such as nuclear factor of activated T cells (NF-AT) and nuclear factor of κ binding (NF-κB) was also impaired in $Bam32^{-/-}$ cells. Furthermore, $Bam32^{-/-}$ cells were more susceptible to BCR-induced death. Taken together, these findings suggest that Bam32 functions to regulate BCR-induced signaling and cell survival most likely in germinal centers.

Key words: adaptor protein • cell death • germinal center • PH domain • signal transduction

Introduction

Signal transduction pathways initiated through the B cell antigen receptor (BCR) complex determine the fate of B cells depending on the context of Ag, expression levels of coreceptors, and the differentiation stage of the B cell (1). Upon BCR cross-linking, immature B cells undergo apoptosis or become anergized, while mature B cells enter the cell cycle and differentiate into memory or antibody-secreting plasma cells (2). BCR engagement induces the activation of three distinct protein tyrosine kinases (PTKs): src-family PTKs (such as Lyn), Syk, and Btk, which in turn regulate several downstream signaling pathways. The significance of these PTKs in B cell function has been underscored by studies with genetically deficient animals or humans (1, 3). In addition to PTKs, a growing family of immune cell–restricted adaptors fine tune BCR signaling cascades (1). As the expression levels of these adaptors change during B cell differentiation, we postulated that B cell adaptors play pivotal roles in decisions affecting B cell fate (1).

Bam32, a B cell adaptor molecule 32 kD in size, was originally identified during screens for genes highly expressed in human germinal center (GC) cells (4). Bam32 contains an NH2-terminal src homology 2 (SH2) domain, one potential tyrosine phosphorylation site, and a COOH-terminal pleckstrin homology (PH) domain but no apparent catalytic domain. Bam32 was also identified by others as a novel PH-domain containing adaptor protein designated dual adaptor for phosphotyrosine and 3-phosphoinositides (DAPP1) or 3′-phosphoinositide-interacting src homology–containing protein (PHISH; references 5 and 6). Based on its structure, Bam32 (DAPP1/PHISH) may function to integrate PTK and phosphatidylinositol 3-kinase

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(PI3K) signaling pathways. Indeed, Bam32 is efficiently targeted to the plasma membrane upon BCR cross-linking in a PI3K-dependent manner (4). Given its preference and high affinity for binding to PI(3,4,5)P3 (Bam32 >> Btk/Akt; reference 5), Bam32 may be one of the first proteins recruited to the plasma membrane after PI3K activation in B cells. Bam32 also associates with several tyrosine-phosphorylated proteins after BCR cross-linking, including phospholipase C (PLC)γ2 (4).

To further define the role of this adaptor in B cells, we prepared Bam32-deficient DT40 cells. We found that the absence of Bam32 attenuates a number of BCR-induced processes including activation of c-jun NH2-terminal kinase (JNK) and nuclear factor of activated T cells (NF-AT) and also increases BCR-induced cell death.

Materials and Methods

Reagents. Mouse anti–chicken IgM (M4) mAb and rabbit anti–chicken PLCγ2 Ab were described previously (7). Rabbit anti–human phospho-extracellular signal-regulated kinase (ERK), rabbit anti–rat ERK, rabbit anti–human phospho-JNK, rabbit anti–human phospho-p38 mitogen-activated protein kinase (MAPK), rabbit anti–mouse Akt phospho-serine 473-specific, rabbit anti–human phospho-Glycogen synthase kinase (GSK)-3α/β, rabbit anti–mouse Akt, and rabbit anti–human phospho-Mek1/2 sera were purchased from Cell Signaling Technology. Mouse anti–human JNK1 mAb was purchased from BD PharMingen. Mouse anti–human c-Myc mAb (9E10) and rabbit anti–mouse p38 MAPK sera were purchased from Santa Cruz Biotechnology, Inc. Mouse anti–human Raf-1 phospho-serine 338-specific mAb and biotinylated anti-phosphotyrosine (4G10) mAb were obtained from Upstate Biotechnology, Inc.; Phorbol myristate acetate (PMA), ionomycin, and indo-1/AM, from Calbiochem; and FITC-conjugated anti-chicken IgM sera, from Bethyl Laboratories. Propidium iodide and EGTA were purchased from Sigma-Aldrich.

Cells. Parental chicken DT40 cells and Bam32-deficient DT40 cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% heat-inactivated chicken serum, 10 U/ml penicillin, 10 μg/ml streptomycin, 2 mM L-glutamine, 1 mM pyruvate, and 50 mM 2-mercaptoethanol at 37°C and 5% CO_2.

Generation of Bam32-deficient DT40 Cells. A lamda Zap DT40 cDNA library was screened by human Bam32 cDNA. The chicken *Bam32* gene exhibited ~80% identity to human counterpart at the amino acid level. Using the isolated cDNA of chicken Bam32, a chicken DNA library was screened to obtain genomic clones. After subcloning, the targeting vectors pBam32-Neo and pBam32-HisD were made by replacing the genomic fragment containing exons corresponding to amino acid residues 106–148 with neo- and hisD-cassette (Fig. 1 A). These cassettes were flanked by 2.5 and 1.8 kb of chicken Bam32 genomic sequence on the 5′ and 3′ sides, respectively. Targeting constructs were sequentially transfected into wild-type DT40 cells by electroporation to obtain null mutants. Selection for drug-resistant clones was performed using G418 (2 mg/ml) and histidinol (1 mg/ml), and clones were screened by Southern analysis. Evidence for null DT40 mutants was demonstrated by Northern analysis using the probe containing an entire coding region (Fig. 1 C). No truncated transcripts were observed. Wild-type chicken Bam32 cDNA with a COOH-terminal flanking myc epitopic tag was cloned into the pApuro vector (8). Bam32-deficient DT40 cells were transfected with this myc-tagged cDNA construct and selected in the presence of 0.5 μg/ml puromycin (Calbiochem). Transfection was performed by electroporation at 350V, 960 μF.

Measurement of Intracellular Free Calcium. Cells were collected and resuspended to 10^7 cells/ml in RPMI 1640 containing 10% FBS. After incubation at 37°C for 15 min, Indo-1/AM (7 μg/ml final concentration) was added, and the cells were incubated for a further 45 min with resuspension every 15 min. The cells were centrifuged and resuspended in RPMI 1640 at 2×10^6 cells/ml. The cells were stimulated with anti-μ (M4, 2 μg/ml) in the presence or absence of EGTA (8 mM) and the fluorescence intensity of intracellular Indo-1 was monitored and analyzed using a BD LSR system (Becton Dickinson).

Western Blot Analysis. Unstimulated or stimulated cells (5×10^6) were lysed in 100 μl RIPA lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 10 mM $Na_4P_2O_7$, 25 mM sodium β-glycerophosphate, 1 mM EDTA, 1% [wt/vol] NP-40, 0.5% [wt/vol] sodium deoxycholate, 0.1% [wt/vol] SDS, 1 mM PMSF, 1 mM Na_3VO_4, 10 μM E-64, 1 μg/ml pepstatin, 10 μg/ml aprotinin, 10 μg/ml leupeptin). After incubation on ice for 30 min, cell lysates were centrifuged at 16,000 *g* for 15 min at 4°C. Lysates were then denatured in an equal volume of 2× SDS sample buffer, resolved by 10% or 12.5% SDS-PAGE and electrotransfered to nitrocellulose membranes in non-SDS–containing transfer buffer (25 mM Tris, 0.2 M glycine, 20% methanol, pH 8.5). Western blotting was performed with the following primary Abs and indicated dilutions: anti-p-ERK (1:1,000), anti-ERK (1:1,000), anti-p-JNK (1:1,000), anti-JNK1 (1 μg/ml), anti-p-p38 MAPK (1:1,000), anti-p38 MAPK (0.25 μg/ml), anti-p-Akt (1:1,000), anti-p-GSK-3α/β (1:1,000), anti-p-Mek1/2 (1:1,000), and anti-Akt (1:1,000) sera, and anti-p-Raf-1 mAb (1 μg/ml) followed by 1:15,000 anti–rabbit horseradish peroxidase (HRP)-conjugated IgG (Jackson ImmunoResearch Laboratories) and 1:2,000 anti–mouse HRP-conjugated IgG (Santa Cruz Biotechnology, Inc.). Blots were developed with a Renaissance® Western blot chemiluminescence reagent (NEN Life Science Products).

Luciferase Assays. Cells (1×10^7) were transfected by electroporation with either NF-AT- (gift from Dr. Gary Koretzky, University of Pennsylvania, Philadelphia, PA) or NF-κB-luciferase reporter constructs (pGL2–4 × NF-κB/tk) as described (9). After 18–20 h, cells were harvested and plated in 96-well plates at 2×10^5/well. Triplicate cultures were incubated in media alone, with graded dose of M4 mAb or with 50 nM PMA and 2.5 μM ionomycin. After 6 h, cells were lysed in 50 μl reporter lysis buffer (Promega) for 15 min at room temperature. Luciferase activity was assayed by adding 20 μl luciferase substrate (Promega) to 50 μl lysate and immediately measuring with a TD-20/20 Luminometer (Turner Designs). To control for transfection efficiency, the relative luciferase activity of medium and BCR stimulation was calculated relative to PMA/ionomycin stimulation.

Flow Cytometric Analysis. For DNA content analysis, cells (5×10^5) were cultured with or without anti-μ (M4, 10 μg/ml) in RPMI 1640 supplemented with 10% heat-inactivated FBS, 1% heat-inactivated chicken serum, 10 U/ml penicillin, 10 μg/ml streptomycin, 2 mM L-glutamine, 1 mM pyruvate, and 50 mM 2-mercaptoethanol at 37°C and 5% CO_2. After 24 h, cells were pelleted and resuspended in hypotonic DNA staining solution (50 μg/ml propidium iodide, 0.1% sodium citrate, 0.1% Triton X-100). Samples were kept at 4°C overnight, and then analyzed with a FACScan™ (Becton Dickinson). For cell surface expression of BCR, DT40 cells were washed, incubated with FITC-conju-

gated anti–chicken IgM sera, and analyzed using a FACScan™. The x and y axes for the histograms indicate fluorescence intensity (four-decade log scales) and relative cell number, respectively.

Results and Discussion

Generation of Bam32-deficient DT40 Cells. To disrupt the *Bam32* locus in DT40 cells, we transfected a targeting construct into parental DT40 cells (Fig. 1 A). Screening by Southern analysis identified two independent clones (M80–11 and M82–5) that successfully underwent homologous recombination at both *Bam32* loci (Fig. 1 B). Lack of Bam32 expression was confirmed by Northern analysis (Fig. 1 C). We could not detect endogenous Bam32 protein in either wild-type or mutant DT40 cells, perhaps because the levels of endogenous Bam32 protein are relatively low. Flow cytometric analysis showed that surface IgM expression on both Bam32$^{-/-}$ cells was almost similar to that of wild-type cells (Fig. 1 D). BCR cross-linking rapidly induces tyrosine phosphorylation on a number of cellular proteins. However, after anti–chicken IgM stimulation there was no obvious difference between wild-type and Bam32$^{-/-}$ cells in the overall pattern of new protein tyrosine phosphorylation (data not shown).

Role of Bam32 in BCR-induced Ca^{2+} Mobilization and Tyrosine Phosphorylation of PLCγ2. As BCR-induced calcium mobilization is dramatically reduced in both PLCγ2-deficient DT40 cells and B cells from PLCγ2-deficient mice (10, 11), we evaluated BCR-induced calcium mobilization in Bam32$^{-/-}$ cells. In both Bam32$^{-/-}$ cells, this response was partially impaired compared with wild-type cells (Fig. 2 A). BCR-induced calcium response comprises an initial release phase and a subsequent sustained plateau phase due to release of Ca^{2+} from intracellular stores and Ca^{2+} influx across the plasma membrane, respectively (12). Pretreatment of cells with EGTA resulted in almost complete inhibition of the sustained phase of the calcium response. Under these conditions, the partial inhibition of BCR-induced calcium response in Bam32$^{-/-}$ cells was still observed, suggesting that Ca^{2+} release from intracellular stores is defective in Bam32$^{-/-}$ cells (Fig. 2 B). We also confirmed that thapsigargin-sensitive calcium stores are normal in Bam32$^{-/-}$ cells (data not shown). Tyrosine phosphorylation of PLCγ2 is required for an increase in its catalytic activity (13). Tyrosine phosphorylation of PLCγ2 after anti-IgM stimulation was modestly attenuated in Bam32$^{-/-}$ cells compared with wild-type cells (Fig. 2, C and D). These results suggest that Bam32 regulates the activation of PLCγ2, but is not essential for PLCγ2 activation upon BCR ligation in DT40 cells.

Bam32 Regulates BCR-induced Activation of MAPK Pathways, but Not Akt-related Pathways. Three structurally related MAPK subfamilies have been described in mammalian cells: the p42 and p44 kinases (ERKs), the JNKs, and the p38 MAPK family (14). Different patterns of activation of MAPKs may well direct B cells to different cell fates such as proliferation, differentiation, and apoptosis. In addi-

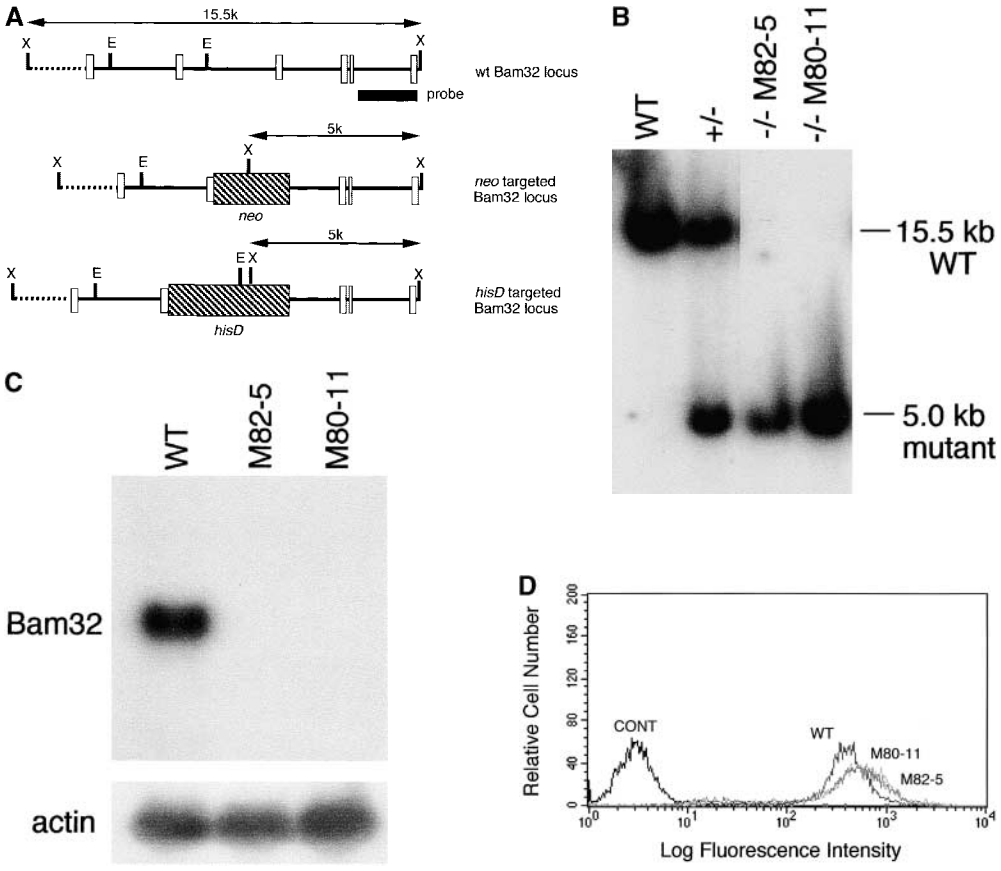

Figure 1. Disruption of the *Bam32* gene in chicken DT40 B cells. (A) Partial restriction map of chicken *Bam32* locus and expected structure of the targeted *Bam32* alleles. The restriction endonuclease cleavage sites are abbreviated: X, XbaI; E, EcoRI. (B) Southern analysis of genomic DNA from wild-type DT40 cells (lane 1), neo-targeted cells (+/−, lane 2), and neo- and hisD-targeted cells (−/−, lane 3 and 4). Xba I-digested genomic DNA separated on an agarose gel was blotted and hybridized with a chicken Bam32 DNA probe shown in A. Wild-type (WT) and targeted alleles show 15.5- or 5.0-kb fragments, respectively. (C) Northern analysis using a chicken cDNA probe for Bam32 (top) and β-actin (bottom). Wild-type (WT) DT40 cells contain a hybridizable RNA species of ~3,000 nucleotides. (D) Cell surface expression of BCR. BCR expression on the surface of wild-type (WT) and two independent Bam32-deficient (M80–11, M82–5) DT40 cells was analyzed by flow cytometry. Unstained cells were used as negative controls.

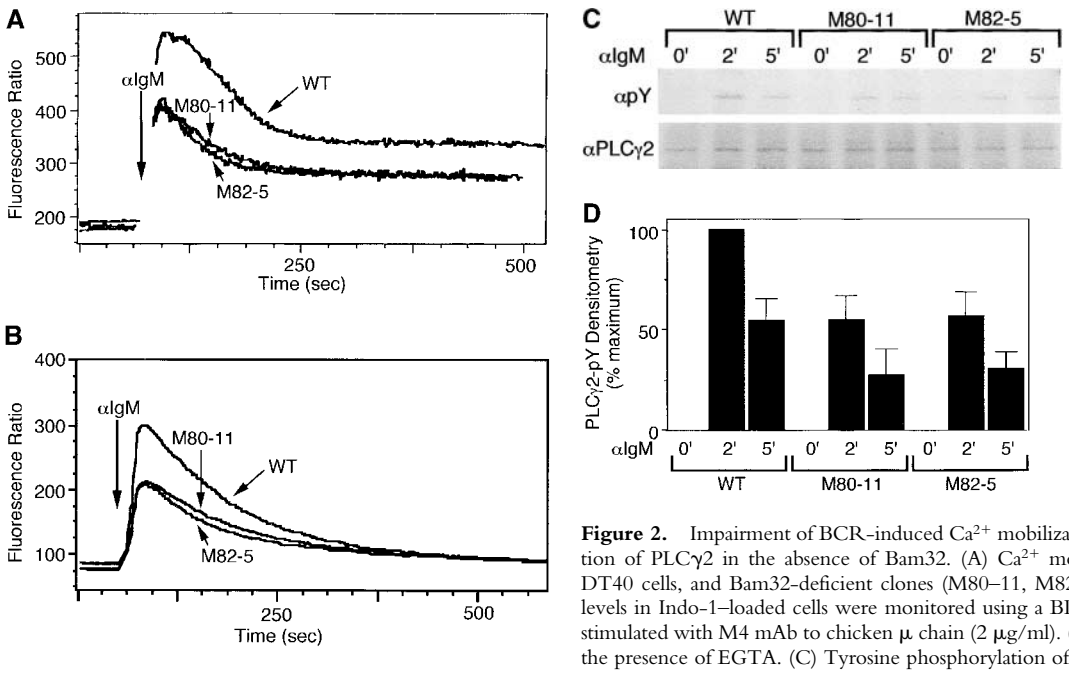

Figure 2. Impairment of BCR-induced Ca^{2+} mobilization and tyrosine phosphorylation of PLCγ2 in the absence of Bam32. (A) Ca^{2+} mobilization in wild-type (WT) DT40 cells, and Bam32-deficient clones (M80–11, M82–5). Intracellular free calcium levels in Indo-1–loaded cells were monitored using a BD LSR system, after cells were stimulated with M4 mAb to chicken μ chain (2 μg/ml). (B) Intracellular Ca^{2+} release in the presence of EGTA. (C) Tyrosine phosphorylation of PLCγ2. At the indicated time points after BCR stimulation (M4, 5 μg/ml), immunoprecipitates with anti-PLCγ2 Ab were separated on a 10% SDS-PAGE gel, and were analyzed by Western blotting with biotinated anti-phosphotyrosine mAb (top, αpY) or anti-PLCγ2 Ab (bottom, αPLCγ2). (D) Densitometrical analyses of tyrosine phosphorylation of PLCγ2. The resulting values were expressed as the percentage in reference to that of maximal response in BCR-stimulated wild-type DT40 cells. The results were shown by average and SEM of three independent experiments.

tion, PLCγ2 is required for full activation of all three MAPKs in DT40 B cells (15). Thus, we tested whether the phosphorylation of these MAPKs could be affected by the absence of Bam32 (Fig. 3 A). The kinetic profile of activation of the three MAPKs after anti-IgM stimulation of wild-type DT40 cells was consistent with our previous findings (15, 16). In $Bam32^{-/-}$ cells, the phosphorylation of JNK1 was consistently reduced by >85% compared with wild-type cells. In addition, the phosphorylation of both ERK2 and p38 MAPK was partially impaired in $Bam32^{-/-}$ cells. These findings are consistent with studies showing that JNK activation is drastically attenuated either

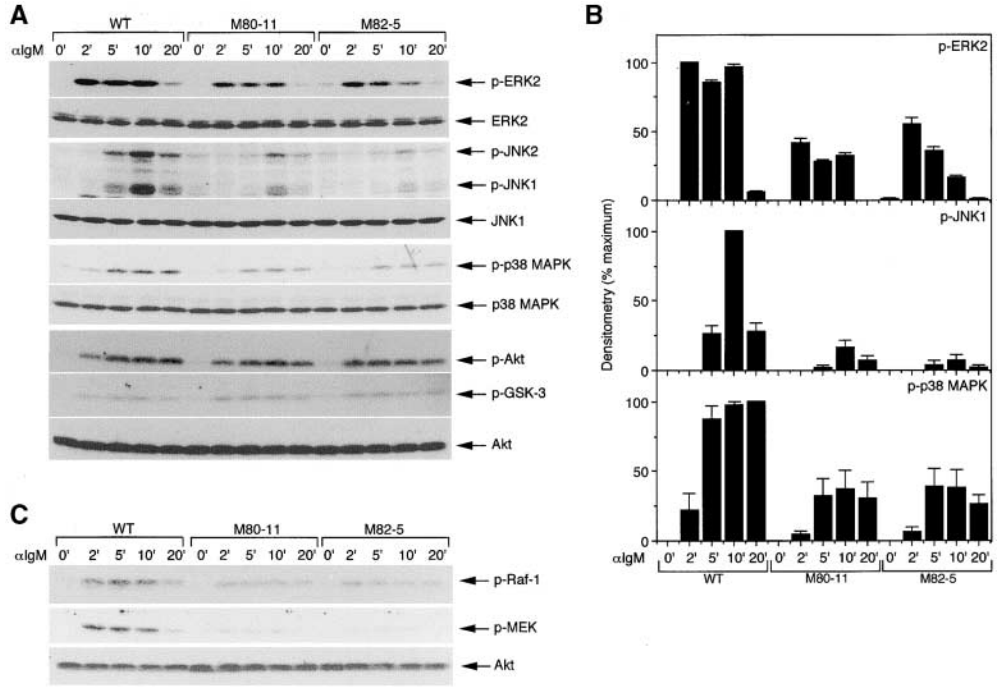

Figure 3. BCR-induced activation of MAPK and Akt pathways in wild-type and Bam32-deficient DT40 cells. (A) Cells were stimulated with anti-μ (M4, 5 μg/ml) for indicated time periods. Cell lysates were subsequently separated on a 10% SDS-PAGE gel, and were analyzed by Western blotting with anti-phospho-ERK, anti-phospho-JNK, anti-phospho-p38 MAPK, anti-phospho-Akt, anti-phospho-GSK-3, and anti-Akt sera. (B) Densitometrical analyses of BCR-induced activation of MAPK. The resulting values were expressed as the percentage in reference to that of maximal response in BCR-stimulated wild-type DT40 cells. The results were shown by average and SEM of three independent experiments. (C) Cells were stimulated as described in A. Cell lysates were subsequently separated on a 10% SDS-PAGE gel, and were analyzed by Western blotting with anti-phospho-Raf-1 mAb, and anti-phospho-Mek sera.

by blocking intracellular calcium release of wild-type B cells treated with BAPTA-AM or in inositol 1,4,5-triphosphate receptor (IP_3R)-deficient B cells (15, 16). The fact that the activation of all three MAPKs was affected suggests again a role for Bam32 in BCR-induced PLCγ2 activation.

While the activation of MAPKs and calcium mobilization have been well studied in DT40 cells, PI3K activation has only been recently examined. Mice deficient in the gene encoding the regulatory p85 subunit of PI3K have profound defects in B cell proliferative responses and B cell survival (see references 1 and 3). PI3K plays a role in BCR signaling via generation of a key second messenger PI(3,4,5)P3 which in turn recruits a number of PH domain-containing signaling molecules to the vicinity of BCR signaling complex. Bam32 is recruited to the plasma membrane upon BCR activation in a PI3K-dependent manner (4). Thus, we tested if the absence of Bam32 affected PI3K-regulated molecules, and in particular Akt, as Akt activation is crucial for DT40 cell survival (17). However, the phosphorylation of Akt after anti-IgM stimulation was not altered in $Bam32^{-/-}$ cells (Fig. 3 A). This is consistent with the fact that decreasing level of Ca^{2+} release does not affect Akt activation in DT40 cells (18). GSK-3 is a critical downstream element of Akt pathway, and can be regulated by Akt-mediated phosphorylation (19). Like Akt, the phosphorylation of GSK-3 was not affected in $Bam32^{-/-}$ cells (Fig. 3 A). In summary, Bam32 appears to regulate MAPK-family kinases and especially JNK but not the PI3K-regulated kinases Akt and GSK-3.

Role of Bam32 in BCR-induced Activation of NF-AT and NF-κB. BCR cross-linking leads to the activation of two pivotal transcription factors NF-AT and NF-κB in B cells. The absence of either of these transcription factors leads to significant defects in B cell functions (20, 21). BCR-induced NF-AT activation is almost completely blocked in both PLCγ2- and IP_3R-deficient DT40 cells (22), consistent with the crucial role of calcium in the activation of NF-AT. Anti-IgM stimulation caused a dose-dependent induction of NF-AT activation in wild-type cells (Fig. 4, top panel). This activation was significantly attenuated in both $Bam32^{-/-}$ cell lines. BCR-induced NF-κB activation is almost completely eliminated either in PLCγ2-deficient DT40 cells or by blocking BCR-induced calcium mobilization in wild-type DT40 cells with EGTA/BAPTA-AM (23). Anti-IgM–induced activation of NF-κB was only partially blocked in $Bam32^{-/-}$ cells (Fig. 4, bottom panel). Notably, the level of NF-AT inhibition was consistently more pronounced than that of NF-κB inhibition in $Bam32^{-/-}$ cells.

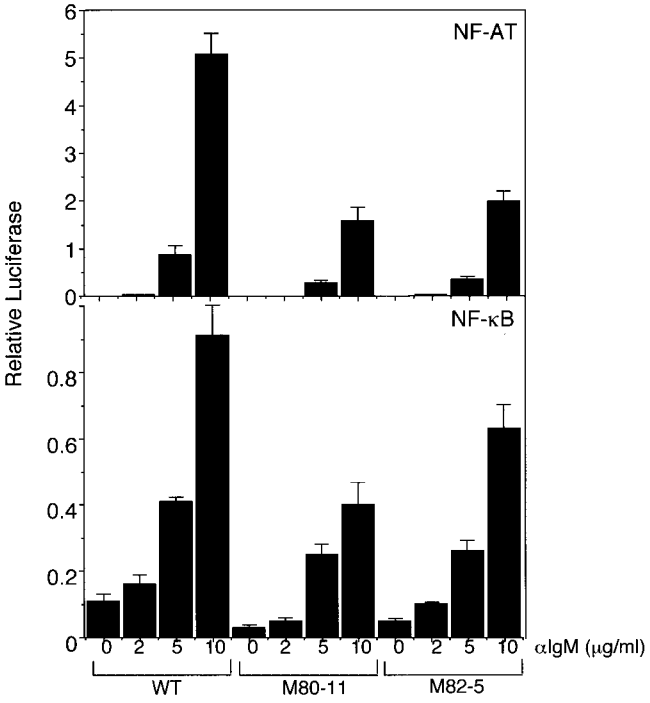

Figure 4. Impairment of BCR-induced NF-AT and NF-κB activation in the absence of Bam32. Cells transfected with NF-AT (top) or NF-κB (bottom) luciferase reporter construct were stimulated with graded dose of anti-μ (M4) for 6 h, lysed, and luciferase activity was assayed using a luminometer. The relative luciferase activity of medium and BCR stimulation was expressed in reference to that of PMA/ionomycin stimulation. The results were shown by average and SEM of triplicate cultures. One experiment representative of four independent experiments is shown.

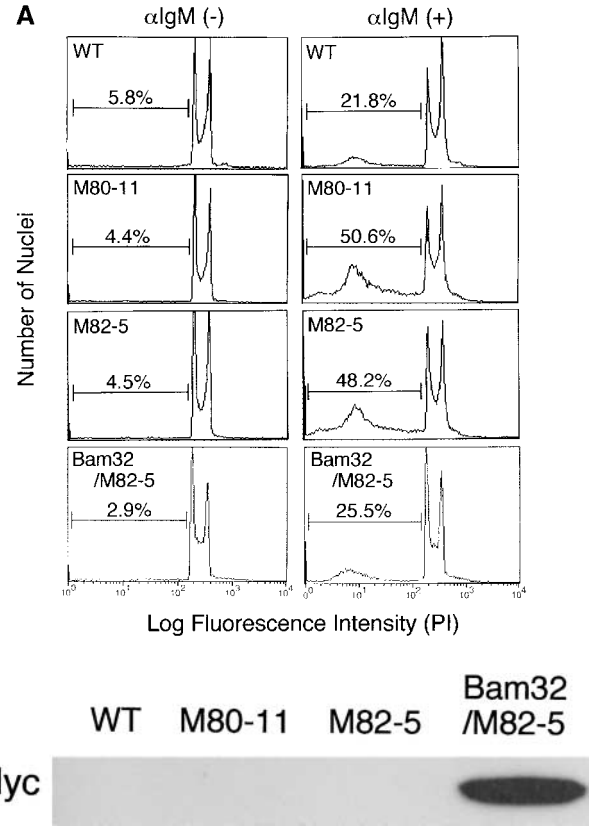

Figure 5. Induction of apoptosis in wild-type and Bam32-deficient DT40 cells. (A) Wild-type cells (WT), Bam32-deficient clones (M80–11, M82–5), and Bam32-deficient clones expressing wild-type Bam32 were cultured with or without anti-μ (M4, 10 μg/ml) for 24 h, treated in hypotonic DNA staining solution containing 50 μg/ml propiduim iodide, and subjected to analysis by FACScan™. The percentage of fragmented nuclei is indicated. (B) The expression of chicken Bam32 transgene. Cell lysates were separated on a 12.5% SDS-PAGE gel, and analyzed by Western blotting with anti-Myc mAb (0.5 μg/ml).

Bam32 Regulates BCR-induced Cell Death. BCR cross-linking leads to growth inhibition and apoptosis in immature B cell lines including DT40 cells. Treatment of wild-type DT40 cells for 24 h after anti-IgM stimulation results in a significant increase in apoptotic cells (Fig. 5). In both PLCγ2- and IP_3R-deficient DT40 cells, this BCR-induced apoptotic response is significantly decreased (22). In contrast, both Bam32$^{-/-}$ cell lines were much more susceptible to BCR-induced cell death (Fig. 5). As expected, transfection of chicken Bam32 cDNA into Bam32-deficient DT40 cells restored cells to a wild-type phenotype.

How might Bam32 promote B cell survival? In considering this question it is useful to compare the phenotypes of Bam32$^{-/-}$ cells, where BCR-induced death is increased, to that of PLCγ2$^{-/-}$ and $IP_3R^{-/-}$ cells, where BCR-induced cell death is greatly attenuated (10, 22). Either a change in a BCR-induced death pathway or a BCR-induced survival pathway could account for this difference. As JNK is not activated in Bam32$^{-/-}$ cells (Fig. 3 A) or in PLCγ2$^{-/-}$ and $IP_3R^{-/-}$ cells (15), JNK is an unlikely candidate for regulating BCR-induced death. However, BAPTA-AM, which chelates intracellular free calcium ([Ca^{2+}]i), blocks BCR-induced apoptosis in DT40 cells (16), and after BCR ligation PLCγ2$^{-/-}$ and $IP_3R^{-/-}$ cells neither release [Ca^{2+}]i nor die (10, 22). As [Ca^{2+}]i is essential for BCR-induced death, it is likely that the partial release of calcium in Bam32$^{-/-}$ cells (Fig. 2 A) is sufficient to induce a cell death pathway not activated in PLCγ2$^{-/-}$ and $IP_3R^{-/-}$ cells.

The release of [Ca^{2+}]i alone does not explain why Bam32$^{-/-}$ cells are more susceptible to BCR-induced death than wild-type cells (Fig. 5). Here the difference may be due to a deficiency in Bam32$^{-/-}$ cells of a BCR-induced survival pathway. Three candidate pathways come to mind.

The first candidate is the Raf/Mek/ERK pathway. Several lines of evidence suggest that this pathway has an important role in modulating cell survival (24). In addition to partial inhibition of ERK2 activation in Bam32$^{-/-}$ cells, Mek activation was significantly inhibited (Fig. 3 C). However, specific Mek inhibitors do not block BCR-induced apoptosis in the WEHI-231 line (25). And the same is true in DT40 cells (data not shown). Recent papers also showed that disruption of the *Raf-1* gene results in a hyperapoptotic phenotype without affecting Mek-ERK activation (26, 27). Thus, Raf-1 might provide a survival signal independent of Mek-ERK activation. Raf-1 requires phosphorylation of the serine at position 338 (S338) for activation (28). Interestingly, BCR-induced Raf-1 phosphorylation at S338 is more significantly inhibited in Bam32$^{-/-}$ cells than ERK activation (Fig. 3 C). Thus, reduced Raf-1 activation may contribute to elevating death in Bam32$^{-/-}$ cells and Raf-1 may play a role in B cell survival.

A second candidate for regulating cell survival is the NF-κB pathway. A number of anti-apoptotic proteins including Bcl-2 are upregulated by the transcription factor NF-κB (29). And Bam32$^{-/-}$ cells do have a partial reduction in BCR-induced NF-κB activation (Fig. 4).

A third candidate for regulating cell survival is the PI3K pathway, known to play a key role in B cell survival (1). Disruption of the gene encoding a novel B cell adaptor for PI3K activation (BCAP) results in inhibition of BCR-induced PI3K activation and also a hyperapoptotic phenotype (30). Likewise, treatment of DT40 cells with a PI3K inhibitor, LY294002, drastically accelerates BCR-induced cell death (data not shown). Therefore, molecules downstream of PI3K clearly play a role in B cell survival. The Akt kinase is an obvious candidate because inactivation of both *Akt* alleles leads to death of DT40 cells (17). Our data suggest that Bam32 does not regulate an Akt-dependent pathway. However, it remains possible that Bam32 regulates other PI3K-regulated molecules affecting B cell survival. In this context, Vav-Rac pathway is a compelling possibility, because this pathway not only regulates [Ca^{2+}]i and JNK activation but also cell death independent of PLCγ2 activation (31). Whether Bam32 regulates the Vav-Rac pathway remains to be elucidated.

In summary, our results suggest that Bam32 may normally function to regulate the fate of B cells triggered by antigen through the BCR complex. BCR ligation promotes the survival of GC B cells (32). Bam32 is expressed at high levels in GC B cells and upregulated after CD40 ligation (4). The increased levels of Bam32 in CD40-triggered GC B cells could facilitate the survival of B cells selected by antigen in the GCs during T cell–dependent immune responses.

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