

## Essential Immunoregulatory Role for BCAP in B Cell Development and Function

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### Abstract

BCAP was recently cloned as a binding molecule to phosphoinositide 3-kinase (PI3K). To investigate the role of BCAP, mutant mice deficient in BCAP were generated. While BCAP-deficient mice are viable, they have decreased numbers of mature B cells and B1 B cell deficiency. The mice produce lower titers of serum immunoglobulin (Ig)M and IgG3, and mount attenuated responses to T cell-independent type II antigen. Upon B cell receptor cross-linking, BCAP-deficient B cells exhibit reduced Ca<sup>2+</sup> mobilization and poor proliferative responses. These findings demonstrate that BCAP plays a pivotal immunoregulatory role in B cell development and humoral immune responses.

Key words: mice • knockout • antigen receptor • phospholipase C- $\gamma$  • calcium

### Introduction

B cell development proceeds through sequential stages characterized by stage-specific surface marker expression (1–4). The B lineage cells in the bone marrow first initiate rearrangement of IgH locus and incorporate the resulting protein into the pre-B cell receptor (pre-BCR),\* the expression of which is the hallmark of the development of pro-B cells into pre-B cells. Then, the rearrangement of IgL locus occurs and the BCR is subsequently assembled, marking the progression of pre-B cells to immature B cells. Immature B220<sup>+</sup> B cells highly expressing IgM exit from the bone marrow and immigrate into the spleen. Once in the spleen, immature B cells, identified as IgM<sup>hi</sup>IgD<sup>lo</sup> population, become IgM<sup>hi</sup>IgD<sup>hi</sup> cells, which in turn develop into IgM<sup>lo</sup>IgD<sup>hi</sup> mature B cells (5). These developmental

progressions likely require the signaling ability of the pre-BCR and BCR complex, as exemplified by the block before the pre-B cell stage in mice lacking the tyrosine kinase Syk (6, 7).

The BCR utilizes sequential activation of three distinct families of nonreceptor protein tyrosine kinases (PTKs), such as Src, Syk, and Tec family, as initial activation (8). Indeed, deficiencies in any of these three families of PTKs result in defective or aberrant B cell function and development (8–11). Thus, characterization of the substrates of these activated PTKs is a prerequisite for understanding the mechanism of how such initial activation regulates the biological outcomes of B cell function and development.

The phosphorylation events that these PTKs catalyze both modulate the catalytic activity of effector enzymes and mediate protein–protein interactions that juxtapose critical signal transduction elements. In this context, it is increasingly appreciated that a group of cellular proteins called adaptor proteins regulate the interactions of effector enzymes with the BCR and their targets, thereby amplifying and integrating multiple signaling pathways (12–14). In support of this, the importance of adaptor proteins in normal B lymphocyte development has emerged. For example, mutant mice lacking the adaptor protein B cell linker

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\*Abbreviations used in this paper: BCR, B cell receptor; BLNK, B cell linker protein; ES, embryonic stem; HSA, heat-stable antigen; IP, immunoprecipitation; IP<sub>3</sub>, inositol-1,4,5-trisphosphate; MZ, marginal zone; PI3K, phosphoinositide 3-kinase; PIP<sub>2</sub>, phosphatidylinositol-4,5-bisphosphate; PLC, phospholipase C; PTK, protein tyrosine kinase; TD, T cell-dependent; TI, T cell-independent.

protein (BLNK; alternatively named SLP-65 or BASH) manifest defective maturation of pro-B to pre-B cells as well as impaired responses to T cell-independent (TI) antigens (15–18).

BCAP has recently been isolated as an adaptor molecule that binds to the p85 subunit of phosphoinositide 3-kinase (PI3K; reference 19). It contains several characteristic structures, including an ankyrin repeat, coiled-coil regions, and proline-rich stretches, in addition to several candidate tyrosine phosphorylation sites that could mediate interactions with PI3K, Grb2, and Src homology 2 domain tyrosine phosphatase 2 (SHP-2). Experiments with BCAP-deficient DT40 B cells demonstrated that BCAP regulates BCR-mediated phospholipase C (PLC)- $\gamma$ 2 activation as well as PI3K activation (19).

In an effort to understand the role of BCAP in B cell development and function, we have generated mice deficient in BCAP. Here, we find that BCAP is required for B cell activation and maturation, and suggest that it causes these biological consequences, at least in part, by regulating  $Ca^{2+}$  flux.

## Materials and Methods

**Construction of BCAP Targeting Vector.** Partial BCAP genome in pBleoBAC11 vector was obtained from Genome Systems. A targeting vector was designed to replace a 2.4-kbp genomic fragment with neomycin resistance gene (*neo*) from pMC1-neopoly(A) (Stratagene), deleting the BCAP exon harboring three YxxM motifs. A herpes simplex virus-thymidine kinase cassette (*TK*) mediating negative selection was inserted in the 5' end of the BCAP-*neo* construct.

**Generation of BCAP<sup>-/-</sup> Mice.** The SalI-linearized BCAP targeting construct was electroporated into E14.1 embryonic stem (ES) cells and the selection of ES cells was performed with G418 and gancyclovir as described previously (20). 550 ES cell colonies were screened by PCR. Nine PCR-positive ES clones were obtained, all of which gave the expected bands by Southern blot analysis. Six independent clones were injected into blastocysts and three of them gave rise to chimeric mice that transmitted the mutation to the germline. The mice were maintained under specific pathogen-free conditions. Genotyping of mice was performed by Southern blot analysis.

**Northern Blot Analysis.** RNA was extracted from spleens using RNazol B (Tel-Test Inc.). 20  $\mu$ g of total RNA was separated on a 1% agarose formaldehyde gel, transferred to Hybond-N<sup>+</sup> membrane (Amersham Pharmacia Biotech), and probed with <sup>32</sup>P-labeled mouse BCAP full-length cDNA or  $\beta$ -actin.

**Flow Cytometric Analysis.** The expression of surface antigens was determined by standard flow cytometric methods. Single-cell suspensions from the indicated organs were depleted of erythrocytes by osmotic lysis and aliquots of 10<sup>6</sup> cells were stained with a combination of FITC-, PE-, and CyChrome-conjugated antibodies. The following mAbs used in the flow cytometric analyses were purchased from BD PharMingen: anti-CD4 (GK1.5), anti-CD5 (53–7.3), anti-CD8 (53–6.72), anti-CD11b (M1/70), anti-CD16 (2.4G2), anti-CD21 (7G6), anti-CD23 (B3B4), anti-CD43 (S7), anti-B220 (RA3–6B2), anti-heat stable antigen (HSA; M1/69), and anti-IgM (R6–60.2), and anti-IgD (11–26) was obtained from Southern Biotechnology Associates, Inc. Data were collected on a FACScan<sup>TM</sup> flow cytometer (Bec-

ton Dickinson) and analyzed using CELLQuest<sup>TM</sup> software (Becton Dickinson).

**Intracellular Staining Assays.** Single-cell suspensions from the spleen were stained with a combination of PE- and CyChrome-conjugated antibodies, washed, and fixed in PBS containing 3.7% paraformaldehyde. Subsequently, cells were washed and permeabilized in PBS containing 0.1% Triton X-100. After blocking with PBS with 10% fetal calf serum (staining buffer), cells were stained with an antiserum to BCAP (diluted at 1:1,000 in staining buffer) for 30 min at 4°C. Cells were washed and then stained with goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) at 0.2  $\mu$ g/ml for 30 min at 4°C. Cells were washed and resuspended in ice-cold PBS for FACS<sup>®</sup> analysis.

**ELISA and Immunizations.** Total serum levels of IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA were determined using ELISA quantitation kits (Bethyl Lab.). Bound Ig was detected using TMB microwell peroxidase substrate system (Kirkegaard & Perry Lab.). Concentrations were calculated from curves constructed using mouse Ig standards ELISA quantitation kits contain. To evaluate TI-II responses, wild-type and BCAP<sup>-/-</sup> mice (five per group) were immunized intraperitoneally with 20  $\mu$ g of the thymus-independent antigen TNP-Ficoll (Biosearch Technologies) in PBS. Mice were bled before and 7 d after immunization. To determine responses to a thymus-dependent antigen, wild-type and BCAP<sup>-/-</sup> mice (four per group) were immunized intraperitoneally with 20  $\mu$ g TNP-KLH (Biosearch Technologies) in a 1:1 homogenate with complete Freund's adjuvant (Difco). Mice were bled before and 14 and 21 d after immunization. At day 14, a second immunization (boost) with 20  $\mu$ g TNP-KLH was performed. Serum Abs specific for TNP were measured in Immuron<sup>®</sup> plates (Dynex) coated with TNP-BSA (Biosearch Technologies) using ELISA quantitation kits (Bethyl). Immunoabsorbance was read at 450 nm and titers were calculated relative to control sera from unimmunized mice.

**Proliferative Response of Splenic B Cells.** The spleen cell suspensions were depleted of erythrocytes by osmotic lysis, and their B cells were purified with Dynabead (Dynal) in combination with antibodies against CD4, CD8, CD11b, and Gr-1 (all from BD PharMingen). The resulting preparations (hereafter referred to as splenic B cells) contained 93 and 90% B220<sup>+</sup> cells from wild-type and BCAP<sup>-/-</sup> mice, respectively. Splenic B cells were cultured, either directly or immediately after sorting with a FACS Vantage<sup>TM</sup> (Becton Dickinson) using anti-B220 and anti-HSA antibodies, in 0.2 ml of RPMI 1640 (GIBCO BRL) containing 10% fetal calf serum, 50  $\mu$ M 2-mercaptoethanol, 2 mM L-glutamine, penicillin, and streptomycin at a density of 1.5  $\times$  10<sup>5</sup> cells/well in flat-bottom 96-well plates. The cells were stimulated with 15  $\mu$ g/ml F(ab')<sub>2</sub> goat anti-IgM Ab (Jackson ImmunoResearch Laboratories), 10  $\mu$ g/ml rat agonistic anti-CD40 mAb (clone 3/23; Serotec), or 10  $\mu$ g/ml LPS (Sigma-Aldrich). For the last 8 h of the 48-h culture period the cultures were pulsed with 0.5  $\mu$ Ci/well [<sup>3</sup>H]thymidine. They were then harvested onto glass-fiber filters, and radioactivity was measured using a  $\beta$  counter.

**Ca<sup>2+</sup> Measurement.** Continuous monitoring of fluorescence from the total splenic B cells was performed using Fura-2/AM (Molecular Probes) as an indicator as described previously (21). Ca<sup>2+</sup> mobilization in the subpopulations of splenic B cells was separately measured using a BD LSR (Becton Dickinson) as follows: erythrocyte-depleted splenocytes were loaded with 1.2  $\mu$ M Indo-1AM (Sigma-Aldrich) and then incubated with anti-HSA-FITC and anti-B220-PE antibodies. Cells were washed and stimulated with 15  $\mu$ g/ml F(ab')<sub>2</sub> goat anti-IgM Ab. In-

creases in intracellular  $\text{Ca}^{2+}$  were recorded in real time on live-gated cells.

**Immunoprecipitation and Western Blot Analysis.** Immunoprecipitation (IP) and Western blotting were performed as described previously using the following antibodies: anti-phosphotyrosine (Upstate Biotechnology), anti-Syk (Santa Cruz Biotechnology, Inc.), anti-Btk (43-3B) provided by S. Arai (Hayashibara Biochemical Lab., Okayama, Japan) and S. Tsukada (Osaka University, Osaka, Japan), anti-PLC- $\gamma$ 2 (Santa Cruz Biotechnology, Inc.), and antisera to BCAP and BLNK, which we raised against GST-BCAP and GST-BLNK, respectively. To determine the kinase activity of Akt, *in vitro* kinase assays were done as described previously using histone (H2B) as a substrate (19).

**Inositol-1,4,5-trisphosphate Generation.** Inositol-1,4,5-trisphosphate ( $\text{IP}_3$ ) generation in BCR-stimulated splenic B cells was measured with  $\text{IP}_3$  [ $^3\text{H}$ ] assay system (Amersham Pharmacia Biotech) according to manufacturer's instructions.

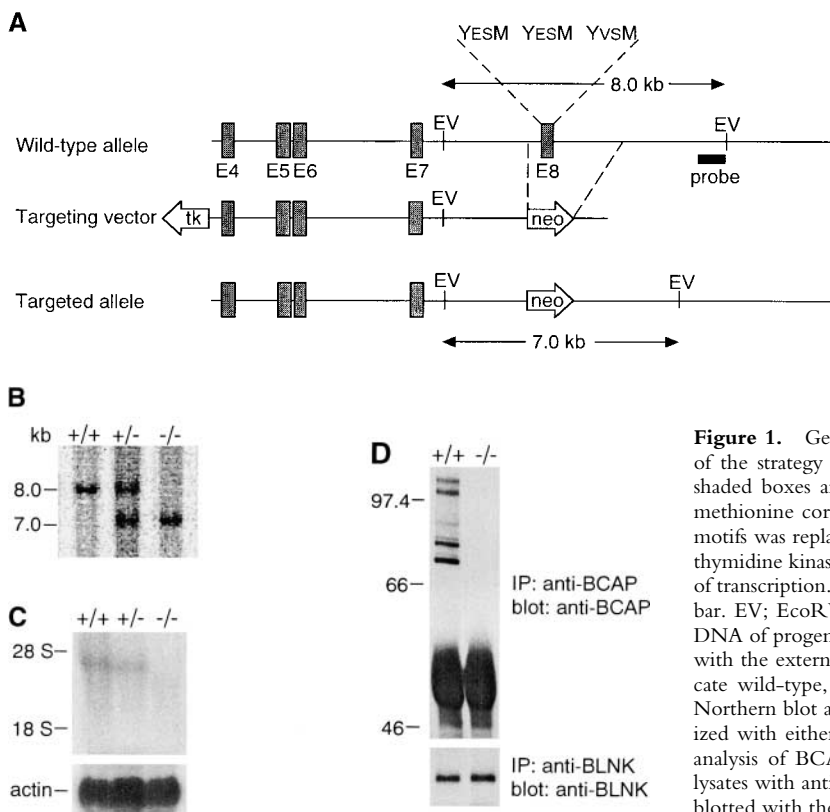
**In Vitro PI3K Assay.** Splenic B cells were stimulated with 15  $\mu\text{g}/\text{ml}$   $\text{F(ab)'}_2$  goat anti-IgM Ab and were lysed in ice-cold lysis buffer (137 mM NaCl, 20 mM Tris-Cl, pH 8.0, 1% NP-40, 10% glycerol, 1 mM  $\text{Na}_3\text{VO}_4$ , 1  $\mu\text{g}/\text{ml}$  leupeptin, 0.1  $\mu\text{g}/\text{ml}$  aprotinin, and 1 mM phenylmethylsulfonyl fluoride). After insoluble materials were removed by centrifugation, the supernatants were incubated with antisera recognizing PI3K p85 subunit (Upstate Biotechnology). Immunoprecipitates were recovered with protein A sepharose, washed sequentially with lysis buffer, wash buffer A (0.1 M Tris-Cl, pH 7.4, 5 mM LiCl, and 0.5 mM  $\text{Na}_3\text{VO}_4$ ) and wash buffer B (10 mM Tris-Cl, pH 7.4, 100 mM NaCl, 1 mM EDTA, and 0.5 mM  $\text{Na}_3\text{VO}_4$ ), and were finally resuspended in 20 mM HEPES (pH 7.4). Kinase reactions were started by addition of 10  $\mu\text{Ci}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP, 15  $\mu\text{g}$  of phosphatidylinositol (Sigma-Aldrich),  $\text{MgCl}_2$  (15 mM final), and ATP (40

$\mu\text{M}$  final). Reactions were terminated with 1 M HCl after incubation at 37°C for 10 min. After organic extraction, aqueous samples were spotted onto TLC plates and phosphorylated products were separated in a  $\text{CHCl}_3/\text{methanol}/4\text{ M NH}_4\text{OH}$  (9:7:2) developing solvent. The incorporated radioactivity was measured using a Fuji FLA2000 bioimaging analyzer (Fuji Photo Film).

## Results

**Generation of  $\text{BCAP}^{-/-}$  Mice.** We constructed a gene-targeting vector such that homologous recombination would replace an exon and its flanking introns with a neomycin resistance gene (*neo*), deleting three YxxM motifs that mediate binding to the p85 subunit of PI3K (Fig. 1 A). Nine ES clones with the appropriately targeted allele were obtained. Six clones were injected into C57BL/6 blastocysts and three of those yielded chimeric mice that transmitted the mutations to their offsprings. Southern blot analysis using an external probe confirmed the presence of the targeted locus (Fig. 1 B). Founder mice showing germline transmission were interbred to produce homozygous  $\text{BCAP}^{-/-}$  mice, which were born at the expected Mendelian ratio.  $\text{BCAP}^{-/-}$  mice appeared healthy and were fertile.

The expression of BCAP in the mutant mice was examined both at the RNA and protein levels. Northern blot analysis using the BCAP full-length cDNA as a probe revealed no messenger RNA in the homozygous mice (Fig. 1 C). We previously showed that mouse splenic B cells express two major BCAP isoforms (100- and 98-kD long iso-

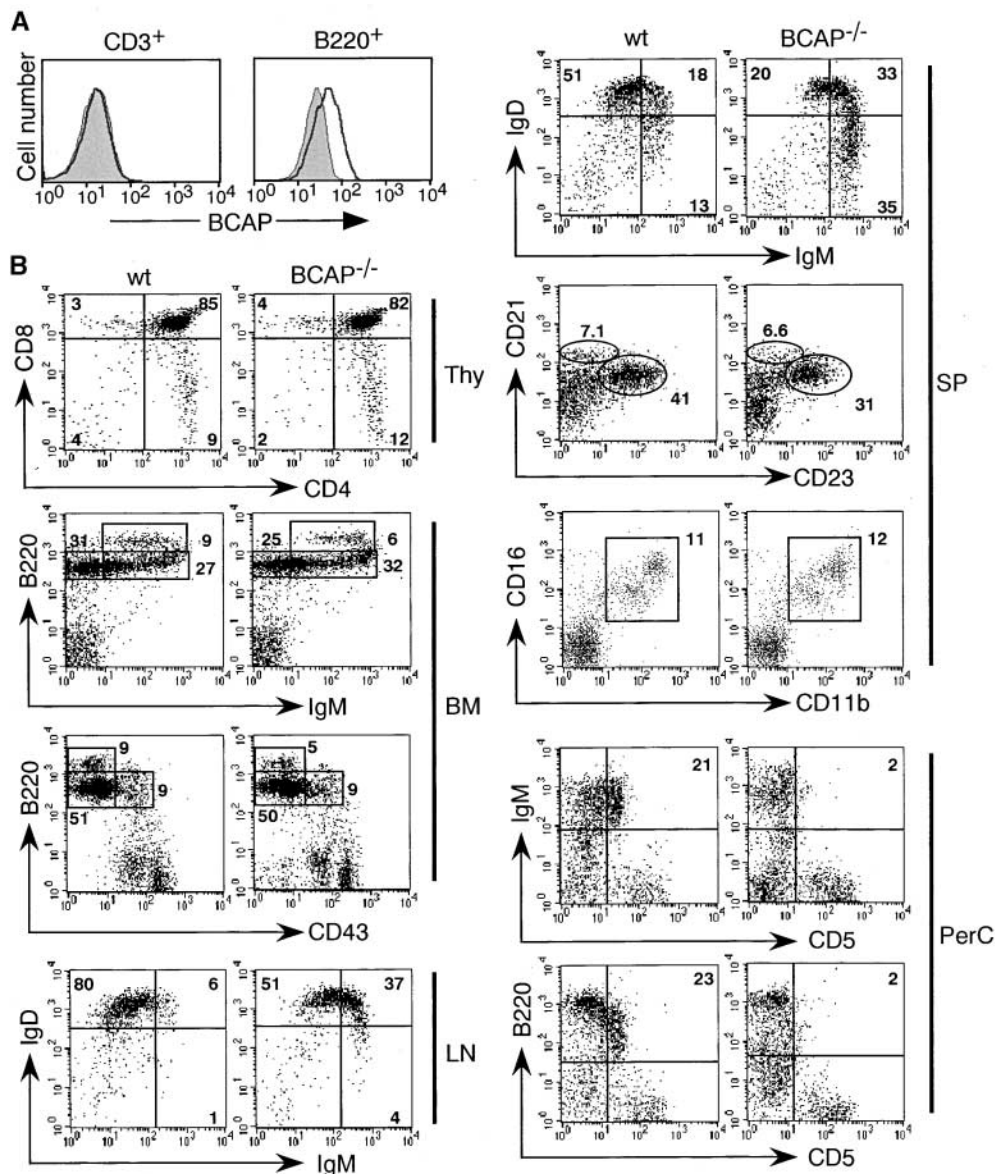


**Figure 1.** Generation of  $\text{BCAP}^{-/-}$  mice. (A) Schematic representation of the strategy used to target the BCAP locus. Exons are presented as shaded boxes and numbered, where the exon containing the initiation methionine corresponds to exon 1. Exon 8 (E8) harboring three YxxM motifs was replaced with the neomycin selection cassette (*neo*). Neo and thymidine kinase (*tk*) are shown as open arrows that indicate the direction of transcription. A probe used for Southern blot analysis is also shown as a bar. EV; EcoRV. (B) Representative Southern blot analysis of genomic DNA of progeny mice. Tail DNA was digested with EcoRV and probed with the external probe shown in panel A. +/+, +/-, and -/- indicate wild-type, heterozygous, and homozygous mice, respectively. (C) Northern blot analysis of total RNA from the spleen. A blot was hybridized with either BCAP full-length cDNA or  $\beta$ -actin. (D) Western blot analysis of BCAP protein from splenocytes. Immunoprecipitates from lysates with antiserum to BCAP or anti-BLNK antibody were immunoblotted with the same antibodies.

forms and 72- and 70-kD short isoforms) presumably by alternative initiation or splicing (19). Western blot analysis of splenic B cells with an antiserum to BCAP (19) displayed no expression of either BCAP isoform in the mutant mice in accord with Northern blot data (Fig. 1 D).

**Defective B Cell Development in BCAP<sup>-/-</sup> Mice.** BCAP expression, as determined by intracellular flow cytometric staining, was detected in B220<sup>+</sup> B cells, but not in CD3<sup>+</sup> T cells (Fig. 2 A). Cellularity in the thymus of BCAP<sup>-/-</sup> mice was equivalent to that in wild-type mice, and thymocyte development was normal, as assessed by expression of CD4 and CD8 (Table I and Fig. 2 B). Then, B cell development in BCAP<sup>-/-</sup> mice was examined by multiparameter flow cytometry. In the bone marrow of BCAP<sup>-/-</sup> mice, no gross developmental arrest was found except for a reduction of pre-B cell subset compared with wild-type mice (Table I and Fig. 2 B). The number of B220<sup>+</sup> cells in

the bone marrow did not differ significantly between wild-type and the mutant mice. In the spleen, the total number of cells was reduced by 20–30% in the mutant mice. Reflecting this decrease, the number of B220<sup>+</sup> cells in the spleen of BCAP<sup>-/-</sup> mice was reduced by 20–30% relative to that in wild-type littermate controls (Table I). Moreover, transition from IgM<sup>hi</sup>IgD<sup>hi</sup> cells to IgM<sup>lo</sup>IgD<sup>hi</sup> mature B cells was considerably blocked, resulting in the lowered ratio of IgM<sup>lo</sup>IgD<sup>hi</sup> to IgM<sup>hi</sup>IgD<sup>hi</sup> cells in the spleen of BCAP<sup>-/-</sup> mice compared with that of wild-type mice ( $0.75 \pm 0.20$  versus  $2.25 \pm 0.20$ ; Fig. 2 B). The percentages of marginal zone (MZ) B cells (CD21<sup>hi</sup>CD23<sup>lo</sup>B220<sup>+</sup>), which are thought to contribute to TI immune responses (22, 23), were comparable in the spleens between BCAP<sup>-/-</sup> and wild-type mice. In contrast, a slight decrease of follicular B cells (CD21<sup>int</sup>CD23<sup>hi</sup>B220<sup>+</sup>) was observed in BCAP<sup>-/-</sup> mice, presumably reflecting a



**Figure 2.** Flow cytometric analysis of lymphocyte populations in BCAP<sup>-/-</sup> mice. (A) Expression of BCAP in splenic B cells. Spleen cells stained for CD3 and B220 were analyzed by flow cytometric intracellular staining with an antiserum to BCAP plus goat anti-rabbit IgG-FITC (thick solid line, wild-type mice; shaded area, BCAP<sup>-/-</sup> mice). Data shown are representative of three independent experiments. (B) Single-cell suspensions from thymus (Thy), bone marrow (BM), lymph node (LN), spleen (SP), and peritoneal cavity (PerC), were stained with the indicated Abs and analyzed using a FACScan<sup>®</sup> (wt, wild-type mice). Numbers indicate the percentages of lymphoid cells in the quadrants or enclosed areas. IgM versus IgD and CD21 versus CD23 profiles are shown for B220<sup>+</sup> cells, and CD11b versus CD16 profile for B220<sup>-</sup> cells. Data shown are representative of six independent experiments.

**Table I.** Lymphocyte Populations in BCAP<sup>-/-</sup> Mice

	Wild-type (n = 6)	BCAP <sup>-/-</sup> (n = 6)
Bone marrow		
Total cells	34.6 ± 8.5	31.3 ± 7.4
Total B cells	8.1 ± 2.9	6.3 ± 1.5
Pro-B cells (fractions A–C)	1.1 ± 0.4	1.4 ± 0.6
Pre-B cells (fraction D)	3.3 ± 0.8	1.6 ± 0.9
Immature B cells (fraction E)	2.6 ± 1.7	2.6 ± 1.4
Recirculating mature B cells (fraction F)	1.1 ± 0.5	0.7 ± 0.2
Spleen		
Total cells	66.5 ± 21.8	48.6 ± 19.8
B cells	38.6 ± 8.0	27.0 ± 12.2
T cells	16.6 ± 3.1	12.4 ± 8.6
Macrophages	11.5 ± 5.0	9.0 ± 7.5
Thymus		
Total cells	295.3 ± 232.1	262.5 ± 93.5

Bone marrow cells were obtained from two femurs of mice 6–8 wk of age. Cell populations were determined on the basis of the total cell count and flow cytometric analysis shown in Fig. 2 B. Data are means ± standard deviation and represent multiples of 10<sup>6</sup> cells. Subsets in the bone marrow are defined as follow: pro-B, B220<sup>+</sup>CD43<sup>+</sup>IgM<sup>-</sup>; pre-B, B220<sup>+</sup>CD43<sup>-</sup>IgM<sup>-</sup>; immature B, B220<sup>lo</sup>IgM<sup>+</sup>; mature B, B220<sup>hi</sup>IgM<sup>+</sup>.

blocked development beyond the immature B cell stage (Fig. 2 B). As in the spleen, the lymph nodes of BCAP<sup>-/-</sup> mice also had a reduced subpopulation of mature IgM<sup>lo</sup>IgD<sup>hi</sup> B cells. In addition, CD5-expressing B1 B cells, usually found in larger amounts in the peritoneal cavity, were virtually absent in BCAP<sup>-/-</sup> mice (Fig. 2 B). Hence, we conclude that BCAP is indispensable for the production of B1 B cells, but not for MZ B cells, and contributes to the development of conventional B cells, particularly to the transition from immature to mature B cells.

BCAP transcript was detected in macrophage cell lines as well as B cell lines (19). We thus determined whether the lack of BCAP affected macrophage development. Although the number of CD11b<sup>+</sup>CD16<sup>+</sup>B220<sup>-</sup> cells containing the macrophage lineage was marginally decreased in the spleens of BCAP<sup>-/-</sup> mice relative to wild-type littermate controls, no difference in the proportion of this subset was found between these mice (Table I and Fig. 2 B). Similarly, the number of CD3<sup>+</sup> T cells in the spleen from BCAP<sup>-/-</sup> mice was marginally decreased (Table I).

**Attenuated Humoral Immune Responses in BCAP<sup>-/-</sup> Mice.** To further assess the consequences of BCAP deficiency, we first measured Ig production in unimmunized mice. Decreased titers of both IgM and IgG3 were seen in BCAP<sup>-/-</sup> mice (Fig. 3 A), corresponding to a lack of CD5-expressing B1 B cells in their peritoneal cavities. A slight reduction in IgG2b was also seen. In contrast, serum

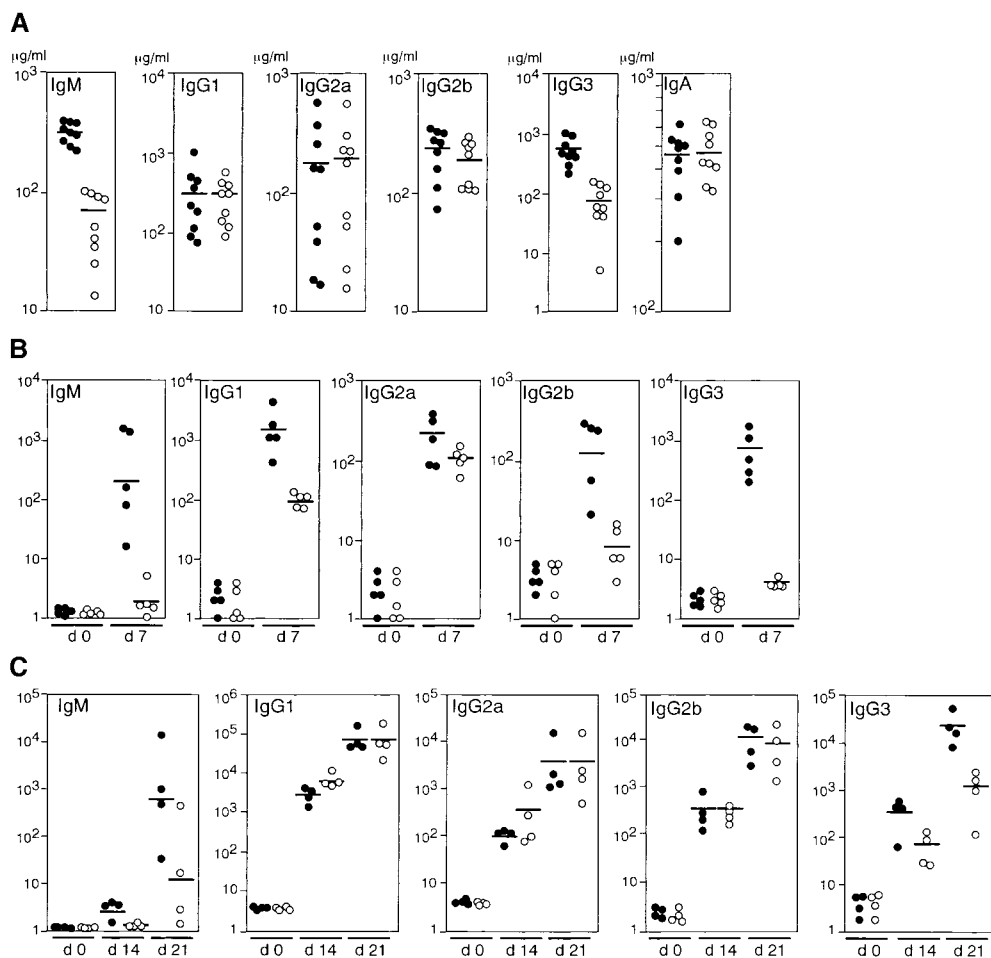
concentrations of IgG1, IgG2a, and IgA were not different from those in littermate controls.

Then, to determine whether BCAP<sup>-/-</sup> mice can respond to TI and T cell-dependent (TD) antigens, we immunized mice intraperitoneally with TI type II (TI-II, TNP-Ficoll) and TD (TNP-KLH) antigens. 7 d after immunization with TNP-Ficoll, we measured serum titers of the hapten-specific immunoglobulins by ELISA. BCAP<sup>-/-</sup> mice produced TNP-specific immunoglobulins of any of the isotypes to a lesser extent than wild-type mice (Fig. 3 B).

14 d after immunization with TNP-KLH, TNP-specific immunoglobulin titers were measured. TNP-specific IgG1, IgG2a, and IgG2b were yielded in BCAP<sup>-/-</sup> mice equivalently to wild-type mice, although the production of both IgM and IgG3 was reduced (Fig. 3 C). Reimmunization of wild-type mice with TNP-KLH 14 d after primary immunization induced a robust secondary TD response. Similarly, BCAP<sup>-/-</sup> mice upon this boosting mounted a remarkable secondary response to TD antigens, nonetheless, with reduced titers of both IgM and IgG3. Therefore, BCAP<sup>-/-</sup> mice are able to respond to TD antigens, in contrast to their attenuated TI-II responses.

**Impaired BCR Signaling in BCAP<sup>-/-</sup> Splenic B Cells.** The basis of humoral response is the ability of individual B cells to respond to an array of antigens, which is tightly regulated by signals through the BCR complex (24, 25). Thus, we directly assessed the growth response of BCAP<sup>-/-</sup> B cells to various stimulations in vitro. As shown in Fig. 4 A, BCAP<sup>-/-</sup> B cells did respond poorly to antigen receptor stimulation with F(ab')<sub>2</sub> anti-IgM Ab compared with wild-type B cells. Interestingly, the ability of BCAP<sup>-/-</sup> B cells to respond to either CD40 ligation or LPS stimulation was reduced to almost 50% of that of wild-type B cells. Given that mature B cells proliferate upon BCR engagement whereas immature B cells are particularly susceptible to BCR-triggered apoptosis (26, 27), the reduced thymidine uptake observed in BCAP<sup>-/-</sup> B cells might reflect the lowered ratio of mature/immature B cells in the spleen of mutant mice (Fig. 2 B). We thus used HSA as a maturation marker to separate splenic B cells into B220<sup>+</sup>HSA<sup>hi</sup> immature B and B220<sup>+</sup>HSA<sup>lo</sup> mature B cells (28, 29). As shown in Fig. 4 B, both fractions of splenic B cells from the mutant mice responded poorly to BCR triggering.

To further evaluate the defects of BCR signals in BCAP<sup>-/-</sup> B cells, we measured the mobilization of intracellular calcium, as Ca<sup>2+</sup> flux is implicated in both B cell maturation and BCR-induced B cell proliferation (21, 30). BCR-elicited Ca<sup>2+</sup> flux in BCAP<sup>-/-</sup> B cells was reduced in amplitude compared with that in wild-type B cells (Fig. 5 A). This overall change cannot be ascribed to developmental differences between wild-type and BCAP<sup>-/-</sup> mice; both immature and mature subsets of BCAP<sup>-/-</sup> B cells exhibited attenuated Ca<sup>2+</sup> flux (Fig. 5 B). As IP<sub>3</sub> is a critical mediator for BCR-induced calcium mobilization, the reduced Ca<sup>2+</sup> flux in BCAP<sup>-/-</sup> B cells could be explained, at least partly, by impaired activation of PLC-γ2. To test this possibility, we directly measured IP<sub>3</sub> generation upon



**Figure 3.** Serum immunoglobulin titers and the humoral responses in  $BCAP^{-/-}$  mice. (A) The concentrations of serum Ig isotypes measured by ELISA. The values for each individual wild-type (●) and  $BCAP^{-/-}$  (○) mouse tested are plotted. Bars indicate mean values. (B) Attenuated TI-II responses in  $BCAP^{-/-}$  mice. Group of five wild-type (●) and  $BCAP^{-/-}$  (○) mice were immunized with the TI-II antigen TNP-Ficoll. Mice were bled before (d 0) and 7 d (d 7) after immunization. TNP-specific immunoglobulins of the indicated isotypes were expressed in relative units compared with a standard titrated serum. Representative data of two independent experiments are shown. (C) Group of four wild-type (●) and  $BCAP^{-/-}$  (○) mice were immunized with the TD antigen TNP-KLH. Mice were reimmunized with the same antigen at day 14, and were bled before (d 0) immunization and at day 14 (d 14) and 21 (d 21). Relative amounts of TNP-specific immunoglobulins were analyzed as described in B. Representative data of two independent experiments are shown.

BCR stimulation. Indeed,  $BCAP^{-/-}$  B cells yielded 20–30% less  $IP_3$  than wild-type controls (Fig. 5 C). Accordingly, we conclude that PLC- $\gamma$ 2 is not sufficiently activated in the absence of BCAP, thereby leading to insufficient calcium flux.

To examine the molecular mechanism underlying the insufficient PLC- $\gamma$ 2 activation in  $BCAP^{-/-}$  B cells, we first compared the BCR-mediated tyrosine phosphorylation status of PLC- $\gamma$ 2 in mutant versus wild-type B cells. PLC- $\gamma$ 2 was normally tyrosine phosphorylated in  $BCAP^{-/-}$  B cells after BCR ligation, as was seen in wild-type B cells (Fig. 6 A). Consistent with this observation, the BCR-mediated tyrosine phosphorylation status of BLNK, Syk, and Btk, molecules responsible for PLC- $\gamma$ 2 phosphorylation (31), did not significantly differ between  $BCAP^{-/-}$  and wild-type B cells (Fig. 6 A).

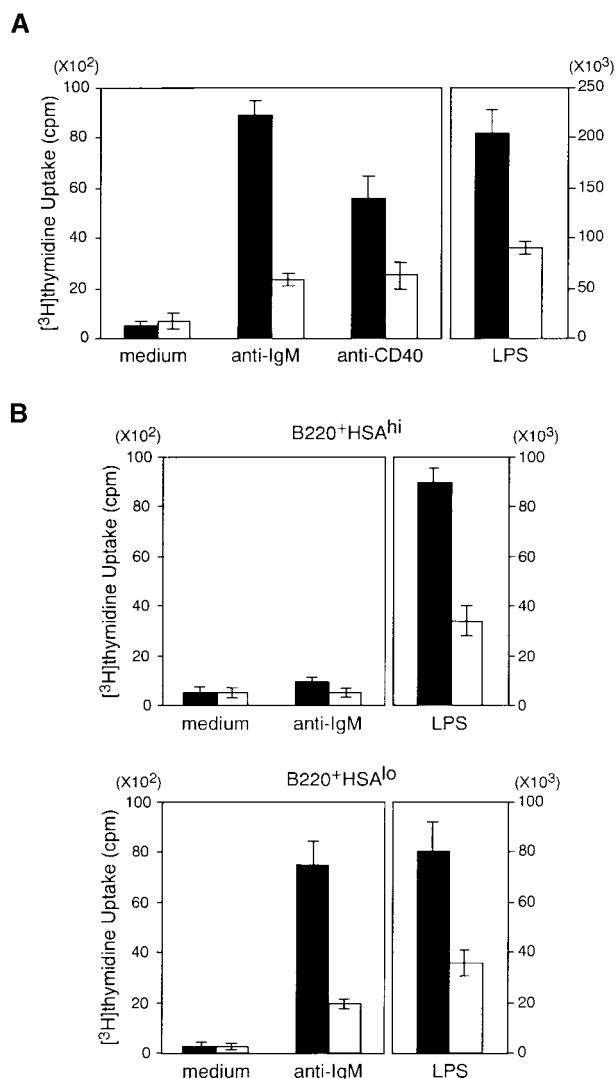
Not only upstream PTKs but PI3K is implicated in PLC- $\gamma$ 2 activation. Phosphatidylinositol-3,4,5-trisphosphate ( $PIP_3$ ) generated by PI3K is thought to mediate membrane targeting of both Btk and PLC- $\gamma$ 2 via its interactions with PH domains, thereby participating in PLC- $\gamma$ 2 activation (32–34). Thus, it is possible that BCAP indirectly facilitates PLC- $\gamma$ 2 activation by activating PI3K. To address this possibility, we directly measured PI3K activity.  $BCAP^{-/-}$  B

cells exhibited PI3K activation similarly to wild-type B cells (Fig. 6 B). In support of this, Akt activity was not significantly affected by the loss of BCAP (Fig. 6 C). As a control, treatment with the PI3K inhibitor LY294002 reduced the Akt kinase activity to the basal levels in both  $BCAP^{-/-}$  and wild-type B cells. Taken together, BCAP is likely involved in PLC- $\gamma$ 2 activation independently of tyrosine phosphorylation and PI3K activation.

## Discussion

We show here that a lack of BCAP, which is a substrate of BCR-activated PTKs, leads to defects in B cell development and humoral immune responses. Although the BCAP protein has been detected in macrophage cell lines (data not shown), BCAP is unlikely to play an essential role in macrophage development in that macrophage numbers are only slightly decreased in spleens of BCAP-deficient mice. Moreover, overall T cell development appears normal in  $BCAP^{-/-}$  mice, which is consistent with no detectable expression of BCAP in  $CD3^+$  T cells (Fig. 2, A and B).

Elimination of BCAP does not result in the severe early B cell developmental arrest. Rather,  $BCAP^{-/-}$  mice manifest mainly defects in peripheral B cell populations. Splenic



**Figure 4.** Impaired proliferative response of BCAP<sup>-/-</sup> splenic B cells. (A) Purified splenic B cells from wild-type and BCAP<sup>-/-</sup> mice were cultured with medium, F(ab')<sub>2</sub> goat anti-IgM Ab (15 μg/ml), rat anti-CD40 Ab (10 μg/ml), or LPS (10 μg/ml). The mean and standard deviations are plotted for wild-type (black bars) and BCAP<sup>-/-</sup> splenic B cells (white bars). Experiments were performed in triplicates. Data shown are representative of three independent experiments. (B) Splenic B cells from the indicated mice were sorted into B220<sup>+</sup>HSA<sup>hi</sup> (immature B) and B220<sup>+</sup>HSA<sup>lo</sup> (mature B) subsets, and experiments were performed in triplicates. Data shown are representative of three independent experiments.

B cells are present, albeit somewhat reduced in number, and are skewed toward an immature phenotype, with an over-representation of IgM<sup>hi</sup>IgD<sup>lo</sup> cells and a decrease of IgM<sup>lo</sup>IgD<sup>hi</sup> fraction (Fig. 2 B). Immature B cells that emigrate from the bone marrow to the periphery are referred to as transitional B cells. Only 10–30% of transitional B cells enter the long-lived mature peripheral B cell compartment (27, 35, 36). This entering mechanism is not entirely clear, but BCR signaling appears to be an important determinant in this transition. Indeed, loss of BCR expression by conditional IgM ablation aborts all further development from the transitional to mature B cell stage (37). Hence, the

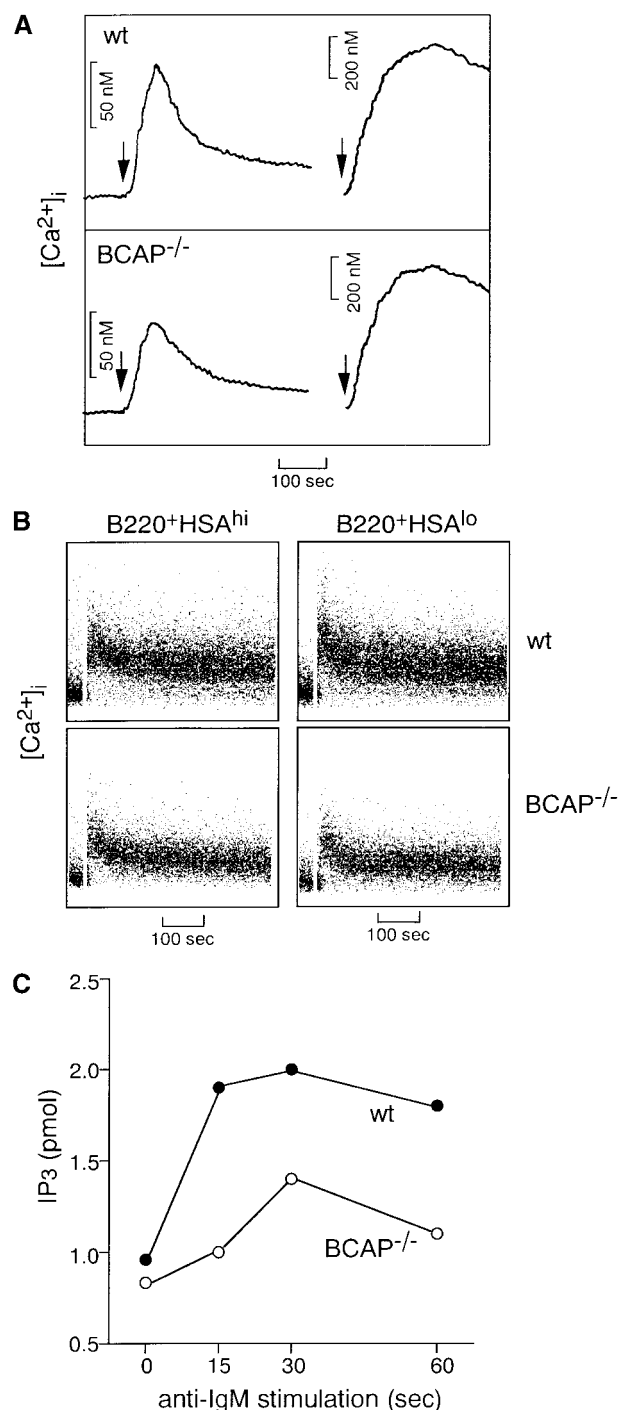
developmental arrest at the immature B cell stage in BCAP<sup>-/-</sup> mice could be explained by two mechanisms; (a) the BCAP mutation affects BCR signaling, thereby interfering with differentiation to mature B cells; (b) although this maturation occurs in BCAP<sup>-/-</sup> mice, albeit at low levels, the survival of mature B cells requires BCAP.

B1 cells often express germline-encoded self-reactive BCRs and favoring the B1 cell development could be a function of BCR signaling and positive selection (38). MZ B cells may be selected similarly to the B1 population, as there are shared characteristics between B1 and MZ B cells (39). For example, B cells harboring a bias for self-reactive BCRs are also enriched in the MZ subset (40). BCAP<sup>-/-</sup> mice display a lack of CD5-expressing B1 B cells (found primarily in the peritoneal cavity), while MZ B cell population, defined as CD21<sup>hi</sup>CD23<sup>lo</sup> B cells, appears normal (Fig. 2 B). Assuming that BCAP functions as a positive regulator in BCR signaling, the straightforward explanation of these data is that the decreased BCR signaling by loss of BCAP is still sufficient to promote the maturation and/or survival of the MZ subset, but not the B1 subset. These results nevertheless allow for the alternative possibility that qualitative differences in BCR signaling determine MZ or B1 cell fate.

TI-II antigens, which are typically derived from polysaccharides, usually consist of complex repeating units that drive B cell responses by extensive cross-linking of specific BCRs. Both B1 and MZ B cells are thought to be responsible for a TI-II response (41). Thus, an impaired TI-II response in BCAP<sup>-/-</sup> mice could be simply explained by the lack of CD5-expressing B1 B cells. However, considering the importance of MZ B cells in the TI-II response (22, 23), dysfunction of MZ B cells in BCAP<sup>-/-</sup> mice, in addition to B1 deficiency, could contribute to the impaired TI-II response. In this regard, we would like to speculate that despite the dispensability of BCAP for MZ B cell development, BCAP could be required for MZ B cells to transduce extensive BCR engagement and thereby generate TI-II responses. In contrast to an impaired response to TI-II antigens, BCAP<sup>-/-</sup> mice mounted a robust response to a TD antigen (Fig. 3 C). These data, together with normal germinal center formation observed in BCAP<sup>-/-</sup> mice (data not shown), suggest that BCAP is not normally involved in the formation and activation of memory B cells.

The phenotype of BCAP<sup>-/-</sup> mice resembles that of mice lacking Btk (*xid*) (42, 43). Both mutant mice manifest reduced levels of mature B cells; an increase in transitional B cells; CD5<sup>+</sup> B1 cell deficiency; low serum IgM and IgG3 levels; defective humoral responses to TI-II antigens; poor proliferative responses after stimulation via the BCR, anti-CD40, and LPS; and reduced Ca<sup>2+</sup> responses upon BCR engagement. A number of signaling molecules whose disruption causes a phenotype similar to *xid* have now been identified, including BLNK (15–18), Vav1/Vav2 (44, 45), PLC-γ2 (21, 30), and PI3K p85α (46, 47). A common property of all these molecules including BCAP is their regulation of calcium flux.

Given the importance of PI3K in calcium flux, we expected that the reduced  $\text{Ca}^{2+}$  response in  $\text{BCAP}^{-/-}$  B cells was attributable to an impairment of BCR-mediated PI3K activation, because we previously showed that BCR-mediated PI3K activation was partly blocked by loss of BCAP in chicken DT40 B cells (19). However, elimination of mouse BCAP does not significantly interfere with PI3K activity upon BCR cross-linking (Fig. 6, B and C). This apparent discrepancy might simply reflect differences between transformed and primary B cells. A recent study has

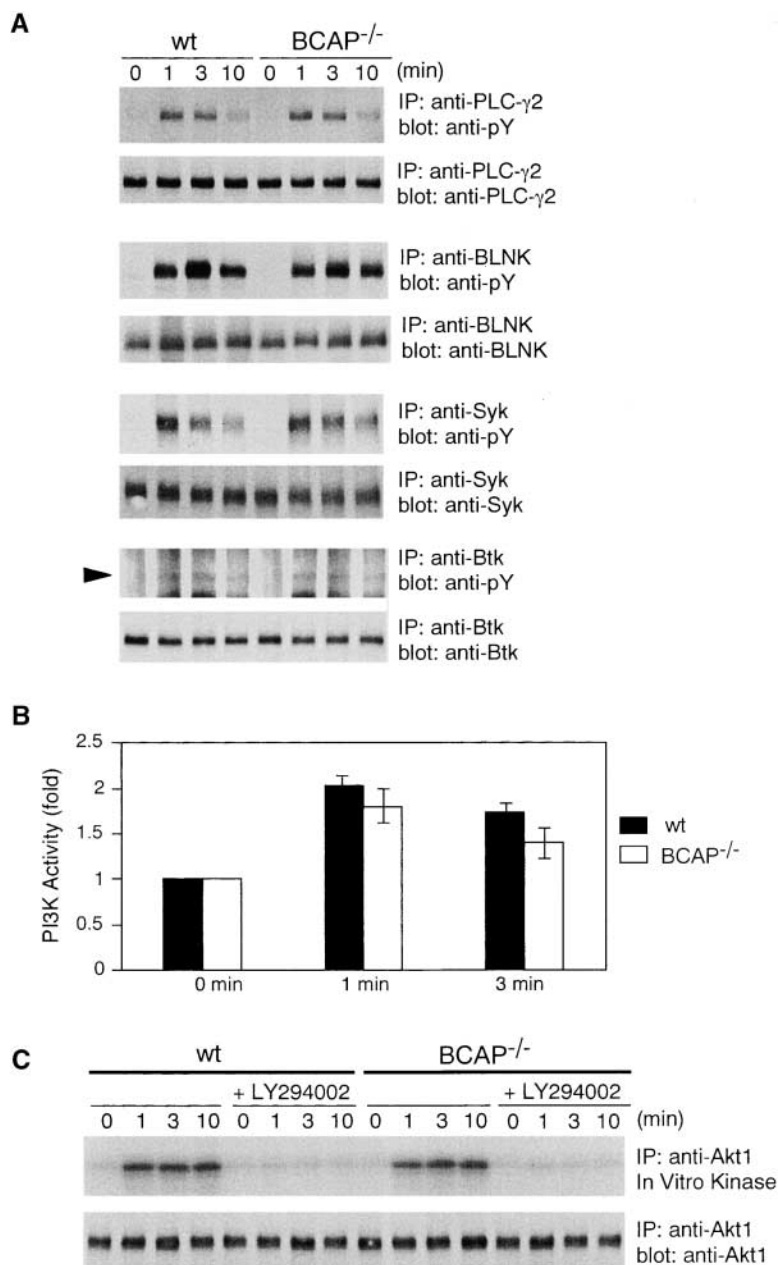


provided the possibility that PI3K pathway is not negatively regulated under a normal fashion in transformed lymphocytes. Indeed, the Jurkat T cell line is shown to be devoid of PTEN, a negative regulator of PI3K signaling (48). Thus, if so in DT40 B cells, the PI3K pathway in this transformed cell line might not reflect the normal regulatory manner in naive B cells. Alternatively, this disparity might reflect species differences between chicken and mouse. In this regard, there exist at least two possibilities. First, mouse B cells might utilize other PI3K binding proteins, such as CD19 (49, 50) and Gab1 (51), rather than BCAP, to activate PI3K pathway upon BCR engagement. Second, chickens have only one isoform of BCAP, whereas mice might have several BCAP isoforms that could function redundantly in BCR-mediated PI3K activation.

Therefore, apparently normal PI3K activation in mouse  $\text{BCAP}^{-/-}$  B cells raises the question of how BCAP is involved in PLC- $\gamma$ 2/calcium pathway. Calcium signals are triggered by the second messenger IP<sub>3</sub>, the product of the hydrolyzing action of PLC- $\gamma$ 2 on phosphatidylinositol-4,5-bisphosphate (PIP<sub>2</sub>). The attenuated IP<sub>3</sub> generation in  $\text{BCAP}^{-/-}$  B cells could be accounted for by the decreased availability of the PLC- $\gamma$ 2 substrate PIP<sub>2</sub> and/or by the decreased hydrolysis of PIP<sub>2</sub> through insufficient PLC- $\gamma$ 2 activation. In this context, if the former mechanism is dominantly operating, we predicted that BCR-mediated PI3K activation was similarly attenuated, as PI3K utilizes the same substrate, PIP<sub>2</sub>, to generate PIP<sub>3</sub>. Thus, normal Akt activation in  $\text{BCAP}^{-/-}$  B cells could place the latter possibility more likely.

BCAP appears to participate in PLC- $\gamma$ 2 activation through phosphorylation-independent mechanisms, because of the normal tyrosine phosphorylation status of PLC- $\gamma$ 2 in  $\text{BCAP}^{-/-}$  B cells. Thus, at least two possibilities might account for the manner by which BCAP regulates PLC- $\gamma$ 2 activation. First, as PLC- $\gamma$ 2 should be targeted to subdomains of the plasma membrane known as rafts for its activation (52), BCAP might contribute to this recruitment. Given that BLNK is required for PLC- $\gamma$ 2 targeting (53), BCAP could act in concert with BLNK,

**Figure 5.** Defective  $\text{Ca}^{2+}$  mobilization in  $\text{BCAP}^{-/-}$  splenic B cells. (A) Splenic B cells were loaded with Fura-2/AM. Cells were washed, stimulated with  $\text{F(ab')}_2$  goat anti-IgM Ab (15  $\mu\text{g}/\text{ml}$ ), and then with ionomycin (1  $\mu\text{M}$ ). Fluorescence from the cell suspension was continuously monitored using a Hitachi F-2000 fluorescence spectrophotometer. In three other experiments, peak values of  $[\text{Ca}^{2+}]_i$  in  $\text{BCAP}^{-/-}$  B cells were 55, 63, and 66% of those in wild-type B cells, respectively. Data shown are representative of four independent experiments. (B) Erythrocyte-depleted splenocytes were loaded with Indo-1AM. Fluorescence from each subpopulation was collected on a BD LSR.  $\text{Ca}^{2+}$  concentrations are represented as the ratio of bound to unbound Indo-1. Data shown are representative of three independent experiments. (C) Reduced IP<sub>3</sub> generation in  $\text{BCAP}^{-/-}$  splenic B cells. Splenic B cells were stimulated with  $\text{F(ab')}_2$  goat anti-IgM Ab (15  $\mu\text{g}/\text{ml}$ ), and lysed. IP<sub>3</sub> generation from  $10^7$  cells at each time point was measured (●, wild-type; ○,  $\text{BCAP}^{-/-}$ ). In three other experiments, amounts of IP<sub>3</sub> in  $\text{BCAP}^{-/-}$  B cells (after 30 s of anti-IgM stimulation) were reduced by 20, 27, and 30%, respectively, compared with those in wild-type B cells. Data shown are representative of four independent experiments.



**Figure 6.** Tyrosine phosphorylation in BCAP<sup>-/-</sup> splenic B cells. (A) BCR-elicited tyrosine phosphorylation status of PLC- $\gamma$ 2, BLNK, Syk, and Btk. Splenic B cells ( $2 \times 10^7$  cells for each time point) were stimulated with F(ab')<sub>2</sub> goat anti-IgM Ab (15  $\mu$ g/ml), lysed, and immunoprecipitated with the indicated antibodies. Immune complexes were resolved by SDS-PAGE and sequentially immunoblotted with anti-phosphotyrosine and the immunoprecipitating antibody. Data shown are representative of three independent experiments. (B) BCR-mediated PI3K activation. The results present the fold activation as a stimulation index between resting and activated cells. The mean and standard deviations of three independent experiments are plotted for wild-type (black bars) and BCAP<sup>-/-</sup> splenic B cells (white bars). (C) BCR-induced activation of Akt. Either with or without 15 min incubation with 10  $\mu$ M LY294002, splenic B cells were stimulated with F(ab')<sub>2</sub> goat anti-IgM Ab (15  $\mu$ g/ml), lysed, and immunoprecipitated with anti-Akt 1 Ab. Half of Akt immunoprecipitates was assayed for in vitro kinase activity using H2B as a substrate. The reactions were separated by SDS-PAGE and autoradiogrammed (top panel). The protein levels of Akt were analyzed using the remaining half of Akt immunoprecipitates (bottom panel). Data shown are representative of three independent experiments.

thereby ensuring sufficient targeting of PLC- $\gamma$ 2. Second, considering the requirement for conformational changes of PLC- $\gamma$ 2 (54), in addition to its targeting, in its full activation, BCAP might be involved in forming the active conformation of PLC- $\gamma$ 2. Indeed, a recent report has demonstrated that binding of PLC- $\gamma$ 1 to multiple adaptors, including linker for activation of T cells (LAT), Gads, and SLP-76, is required for its optimal activation (55, 56). Although it is clear that the insufficient PLC- $\gamma$ 2 activation in BCAP<sup>-/-</sup> B cells contributes to the decreased calcium flux, this mechanism may not entirely account for the effect of BCAP on calcium mobilization. For example, BCAP could directly regulate a calcium influx channel on the plasma membrane. Hence, it might be reasonable to anticipate that BCAP is an important molecule for forming

a spatially compact signaling complex including PLC- $\gamma$ 2 and calcium channels on the plasma membrane ("the signalosome") (57, 58).

The data presented here support the concept that quantitative differences in intracellular Ca<sup>2+</sup> can have clear effects on subsequent activation of downstream signaling events, which in turn affect B cell development. Extracellular signal-regulated kinase (ERK), c-Jun NH<sub>2</sub>-terminal kinase (JNK), p38 cascades, and the Ca<sup>2+</sup>-dependent transcription factors nuclear factor of activated T cells (NFAT) and NF- $\kappa$ B could be potential targets of calcium signaling (59–61). In addition, given the importance of calcium concentration for Rag2 expression and apoptosis in immature B cells (62), BCAP might contribute to B cell tolerance by modulating receptor editing and/or cell deletion.

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