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The B Lymphocyte Adaptor Molecule of 32 Kilodaltons (Bam32) Regulates B Cell Antigen Receptor Internalization

Hiroaki Niiro,* Atef Allam,‡ Angela Stoddart,§ Frances M. Brodsky,§ Aaron J. Marshall,‡ and Edward A. Clark2§†

The B lymphocyte adaptor molecule of 32 kDa (Bam32) is an adaptor that plays an indispensable role in BCR signaling. In this study, we found that upon BCR ligation, Bam32 is recruited to the plasma membrane where it associates with BCR complexes and redistributes and internalizes with BCRs. BCR ligation induced colocalization of Bam32 with lipid rafts, clathrin, and actin filaments. An inhibitor of Src family protein tyrosine kinases (PTKs) blocked both BCR-induced tyrosine phosphorylation of Bam32 and BCR internalization. Moreover, BCR internalization is impaired in Bam32−/− and Lyn−/− cells, and expression of Bam32 with a mutation of its tyrosine phosphorylation site (Y139F) inhibited BCR internalization. These data suggest that Bam32 functions downstream of Src family PTKs to regulate BCR internalization. Bam32 deficiency does not affect tyrosine phosphorylation of clathrin or the association of clathrin with lipid rafts upon BCR cross-linking. However, BCR-induced actin polymerization is impaired in Bam32−/− cells. Collectively, these findings indicate a novel role of Bam32 in connecting Src family PTKs to BCR internalization by an actin-dependent mechanism. The Journal of Immunology, 2004, 173: 5601–5609.

Adaptor proteins play pivotal roles in the integration of BCR signals (1, 10, 11). The B lymphocyte adaptor molecule of 32 kDa, Bam32, also termed dual adaptor for phosphotyrosine and 3-phosphoinositides or 3-phosphoinositide-interacting Src homology-containing protein (12, 13), is an adaptor protein containing an N-terminal Src-homology 2 domain, one tyrosine phosphorylation site, and a C-terminal pleckstrin homology domain (14). Upon BCR cross-linking, Bam32 is tyrosine-phosphorylated and inducibly associated with phospholipase C (PLC)γ2 in human B cell lines (14). Moreover, activation of the PLCγ2-related signaling pathways is partially defective in Bam32−/− DT40 cells (15). However, because the BCR signaling profiles of Bam32−/− and PLCγ2−/− cells are not identical (16), we hypothesized that Bam32 also regulates the PLCγ2-independent pathways in B cells. Recently, two groups generated Bam32−/− mice and showed that Bam32 is required for B cell proliferation mediated through the BCR, but not through CD40 or TLRs (17, 18). Moreover, Bam32 regulates the activation of upstream kinases, hemopoietic progenitor kinase 1 and MEK kinase 1, to control BCR-induced activation of ERK and JNK (18). Thus, Bam32 channels upstream signals into a unique downstream branch of the BCR signaling pathway (19).

A previous study showed that Bam32 redistributes into unidentified vesicles upon BCR cross-linking (20). However, it remained unclear whether these vesicles were related to BCR internalization or whether Bam32 might regulate BCR trafficking pathways. In this study, we show that upon BCR cross-linking, Bam32 colocalizes with the BCR and components of endocytic machinery. In addition, we show that BCR internalization is impaired in Bam32−/− as well as Lyn−/− cells. Our results suggest that Bam32 works downstream of Src family PTKs and functions to link BCR signaling with Ag processing in B cells.

Materials and Methods

Reagents

Goat anti-human IgM F(ab′)2, rhodamine-conjugated anti-human IgM, biotinylated anti-human IgM, FITC-conjugated anti-mouse IgM, and FITC-conjugated anti-goat Ig were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Mouse anti-chicken IgM (M4) mAb was...
described previously (21). Unconjugated or FITC-conjugated goat polyclonal antigen serum specific for chicken IgM was from Bethyl Laboratories (Montgomery, TX). Mouse anti-clathrin H chain (CHC) mAbs for confocal microscopy and PE-conjugated anti-mouse IgM were from BD Biosciences (San Jose, CA). Rhodamine-conjugated F(ab′)2 goat anti-mouse Ig was from BioSource International (Camarillo, CA). Alexa 590-conjugated transferrin (TI), Alexa 594-conjugated cholera toxin subunit B (CT-B), MitoTracker Red and rhodamine-phalloidin were purchased from Molecular Probes (Eugene, OR). Anti-CHC mAbs (X22 and TD.1) were previously described (9). Mouse anti-phosphotyrosine (4G10) mAb was purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit anti-human phospho-ERK, phospho-JNK, phospho-p38 MAPK, phospho-protein kinase C (PKC)ζ, and anti-mouse Akt sera were purchased from Cell Signaling Technology (Beverly, MA). Rabbit anti-chicken Lyn sera was a gift from Dr. T. Kurosaki at Osaka University, Japan. Cytochalasin B (Cyt B), latrunculin A (Lat A), and monodansylcadaverine (MDC) were obtained from Sigma-Aldrich (St. Louis, MO). PP1 was purchased from BIOMOL (Plymouth Meeting, PA). An anti-Bam32 serum and mAb (UW32) were described elsewhere (14).

Flow cytometric analysis

DT40 cells (1 × 10⁶) were incubated with M4 (5 μg/ml) at 4°C for 30 min, then washed and warmed to 37°C for indicated time periods. Where indicated, cells were pretreated with PPI (50 μM), Cyt B (40 μM), or Lat A (2.5 μM) at 37°C for 30 min before the ligand binding, and these inhibitors were included in the ligand binding and chase medium. At the end of each time point, internalization was terminated by placing cells at 4°C and adding ice-cold PBS. Cells were then stained at 4°C for 30 min with FITC-conjugated anti-mouse IgM Ab. Mean fluorescence intensities of IgM remaining on the cell surface were obtained at each time point using flow cytometry; data are presented as the percentage of surface BCR remaining.

For BJAB cells, the cells were incubated on ice for 15 min with 10 μg/ml goat-unlabeled anti-IgM before washing with ice-cold medium and warming at 37°C for the indicated time points. Cells were washed with ice-cold PBS containing 2% FBS and 0.2% sodium azide (Fisher Scientific, Fairlawn, NJ) to stop the internalization at the assigned time point and to remove the unbound Ab. The remaining surface BCR was stained with FITC-labeled rabbit anti-goat Ig and quantified by flow cytometry. Similar results were obtained by using biotinylated anti-IgM cross-linking and streptavidin-FITC detection (data not shown).

Subcellular fractionation

Cells (2 × 10⁶) were stimulated with M4 (5 μg/ml) for 2 min in regular chicken medium at 37°C. Cells were washed with PBS containing 4 mM EDTA, and suspended in 1 ml of TNEV buffer (25 mM Tris, pH 7.5, 0.5% Triton X-100, 150 mM NaCl, 5 mM EDTA, and 1 mM Na2VO4) supplemented with protease and phosphatase inhibitors, and were homogenized with 20 strokes in a loose-fitting Dounce homogenizer (Wheaton, Millville, NJ). The lysates were gently mixed with 1 ml of 85% (w/v) sucrose in TNEV buffer and placed in the bottom of a centrifuge tube. The samples were then overlaid with 6 ml of 35% sucrose and 3.5 ml of 5% sucrose in TNEV buffer, and supercentrifuged for 16 h at 200,000 × g in a SW41 rotor (Beckman Coulter, Palo Alto, CA) at 4°C. Among 12 fractions collected from the top of the gradient, fraction 4, at the interface between 5 and 35% sucrose, was confirmed as lipid rafts by Western blotting with anti-Lyn sera.

BCR-induced actin polymerization

The amount of polymerized F-actin was analyzed by flow cytometry as previously described (23). Cells (1 × 10⁶) were stimulated with M4 (10 μg/ml) for various time periods at 37°C. Reactions were terminated by adding PBS-containing formaldehyde, and FITC-phalloidin (Sigma-Aldrich). The fixed cells were subjected to fluorescence flow cytometry, and the mean relative fluorescence of each sample was determined. All of the data are presented as a fold-increase in F-actin levels compared with those of the sample before BCR stimulation.

Results

Bam32 is recruited to the plasma membrane and redistributes with the BCR complex after BCR ligation

To visualize Bam32 in intact cells, we used a mammalian plasmid construct encoding Bam32 fused to the fluorescent marker protein, EGFP (14). This construct was transiently expressed in human BJAB cells, and the cells were stimulated with rhodamine-labeled F(ab′)2 anti-IgM sera to trigger BCR signaling and internalization (Fig. 1A). BCR cross-linking caused a translocation of Bam32 from the cytosol to the plasma membrane forming a ring at the membrane paralleling the BCR complex. Within a few minutes, the BCR complexes and Bam32 reorganized together at the cell surface forming large patches and caps. Then, BCR complexes were internalized, and, interestingly, Bam32 remained colocalized with BCRs (Fig. 1A). Scoring of Bam32 and BCR distribution in samples fixed after 5, 10, or 30 min indicated that visible accumulations of Bam32 and BCR in membrane patches/caps or internalized structures occur with similar kinetics (Fig. 1B). Two-color

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Bam32 REGULATES BCR INTERNALIZATION

Cells were lysed as described (9). Subsequently, protein G-Sepharose (Amersham Biosciences, Piscataway, NJ) precleared lysates or solubilized fractions were incubated with anti-Bam32 or X22 mAb for 1 h at 4°C then immunoprecipitated with protein G for 1 h at 4°C. Precipitated proteins were resolved on a 12.5 or 8% SDS-PAGE gel, transferred onto Millipore Immobilon (Bedford, MA) polyvinylidene difluoride membrane, and blotted with 4G10, anti-Bam32 sera, or CHC (TD.1) mAb, followed by incubation with secondary HRP-conjugated Abs specific for primary Ab. Blots were visualized with ECL (Amersham Biosciences).

Western blot analysis

Unstimulated or stimulated cells (5 × 10⁵) were lysed as described (15). Lysates were then denatured in an equal volume of 2 × SDS sample buffer, resolved by a 10% SDS-PAGE gel, and electrotransferred 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 anti-phospho-ERK (1/2,000), anti-phospho-JNK (1/1,000), anti-phospho-p38 MAPK (1/1,000), anti-phospho-PKCζ (1/1,000), and anti-Akt (1/1,000), followed by 1/15,000 anti-rabbit or anti-mouse HRP-conjugated IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Blots were developed with ECL.
FIGURE 1. Bam32 traffics with BCR complex after BCR ligation. A, BJAB cells were transiently transfected with full-length EGFP-Bam32 protein and stimulated with rhodamine-conjugated anti-human IgM. At various time points after stimulation, the cells were fixed and examined by confocal microscopy. Superimposed Bam32 and anti-IgM images are shown in the right panels, with areas of yellow representing colocalization of Bam32 (green) and IgM (red). Representative images are shown for the BCR ring (3 min), patching (6 min), capping (12 min), and internalization (30 min). B, Visual scoring results showing the percentage of cells with accumulations of fluorescent label in membrane patches or caps (top panel) or in internal structures (bottom panel). Cells were fixed at the indicated time after stimulation with rhodamine-labeled anti-IgM. Approximately 100 cells per time point were scored for presence of patching/capping or internal structures in either the green channel (Bam32) or the red channel (BCR). Note that the categories used are not mutually exclusive, and the majority of cells at the later time points show both patching/capping and internal distributions. C, A representative Z plane series through an unfixed cell at an advanced stage of internalization (30-min poststimulation), showing colocalization with clusters of internalized BCR. D, A representative time-lapse series showing the colocalization of Bam32 with the BCR during capping and initial internalization. Rhodamine-labeled anti-IgM is added to Bam32-EGFP-transfected cells while under microscopic observation on a temperature-controlled stage set to 37°C. The first frame was taken before stimulation, and then at 2-, 4-, 6-, and 8-min poststimulation.
Z-series imaging of cells at 30–60 min post-cross-linking showed that Bam32 was colocalized with the majority of internalized BCR clusters within cells (Fig. 1C). Control transfectants expressing EGFP show no discernable changes in localization of green fluorescence after stimulation (data not shown). Analysis of fluorescence correlation coefficients showed that Bam32-EGFP and BCR colocalize to significantly higher degree than EGFP and BCR (0.75 ± 0.21 vs 0.28 ± 0.12 at 30-min poststimulation). Time-lapse imaging of BCR uptake indicated that internalized Bam32-BCR clusters could be visualized as early as 5 min after BCR cross-linking (Fig. 1D). These early clusters were often highly mobile and difficult to image as they rapidly moved through different focal planes with the cell (data not shown). In summary, Bam32 colocalizes with the BCR throughout the processes of patching/capping, internalization, and trafficking within the cell.
**Bam32 colocalizes with lipid rafts, clathrin, Tf, and F-actin after BCR cross-linking**

We next tested whether Bam32 localizes in lipid rafts after BCR cross-linking. We examined the localization of Bam32 with GM1, a marker of lipid rafts (Fig. 2A). BJAB cells were stained with CT-B, which binds to GM1 (24). Before stimulation, CT-B staining appears in a relatively homogeneous distribution at the plasma membrane. Upon BCR cross-linking, Bam32 was relatively concentrated in CT-B patches and caps, implying that Bam32 is also a signaling component of the raft-associated BCR signalosomes. Because actin polymerization is crucial for BCR internalization and trafficking to endosomes (25–28), we determined whether Bam32 associates with F-actin during internalization. After BCR cross-linking, we observed colocalization of Bam32 with F-actin caps as well as with F-actin clusters inside cells (Fig. 2B).

Previous studies showed that BCR internalization involves clathrin-mediated processes (9, 25). We found that Bam32 partially colocalized with CHC upon BCR cross-linking (Fig. 2C). In addition, Bam32 partially localized with Tf, a well-established marker for early and recycling endosomes normally internalized through a clathrin-dependent pathway (29). Internalization of Tf resulted in a characteristic punctate pattern of staining which partially colocalized with Bam32-BCR complexes in the cytoplasm (Fig. 2D). In contrast, control stainings showed no colocalization between Bam32 and mitochondria (Fig. 2E), and isotype control stainings of Bam32-EGFP transfectants were negative, indicating no bleedthrough of the green signal into the red channel (data not shown). Together, these data suggest that Bam32 can associate with some components of the endocytic machinery driving BCR internalization.

**BCR internalization is impaired in Bam32−/− cells**

We next tested whether or not Bam32 affects BCR internalization using Bam32−/− DT40 cells. First, we monitored the level of surface BCR expression in DT40 cells after BCR cross-linking (Fig. 3A). BCR cross-linking led to a rapid decrease in the level of surface BCR detected in wild-type cells. A significant decrease in BCR levels was already evident within 5–10 min after stimulation, and further decreases were detectable at 30–60 min. This decrease in BCR levels was less pronounced in two independent Bam32−/− cells (nos. 1 and 2), suggesting that BCR internalization is impaired in the absence of Bam32. Because BCR internalization was blocked to a similar extent in both Bam32−/− cells, we mainly used Bam32−/− cells (no. 1) for additional experiments. In contrast to the absence of Bam32, overexpression of Bam32 in Bam32−/− (no. 1) cells resulted in faster kinetics of BCR internalization (Fig. 3A, bottom). Collectively, these findings suggest that Bam32 regulates BCR internalization.

We next directly visualized BCRs in DT40 B cells using confocal microscopy. To clearly distinguish between surface and internalized BCRs, we stimulated DT40 cells with anti-chicken IgM for various times and then stained the cells with a FITC-conjugated anti-mouse Ig under nonpermeabilized conditions (Fig. 3B). In wild-type cells, BCR complexes exhibited an homogenous or slightly patchy distribution before stimulation, and within 5 min after stimulation, BCRs formed into tight caps on the surface. However, after this time point, fluorescence levels were greatly reduced because the BCR complexes were already internalized. When BCRs were stained under permeabilized conditions, a punctate pattern of internalized BCR was clearly visualized. In Bam32−/− cells, the distribution of BCR complexes at 5 min was...
similar to that in wild-type cells. However, a high surface expression of BCRs was still evident on Bam32−/− cells even 30 min after stimulation, again suggesting that BCR internalization is impaired in the absence of Bam32. Moreover, consistent with the findings using the flow cytometry, overexpression of Bam32 in Bam32−/− cells not only completely abolished the internalization defect, it also accelerated BCR internalization. BCR surface fluorescence was reduced even 5 min after stimulation. Collectively, these findings demonstrate that Bam32 regulates BCR internalization after BCR cross-linking.

**Phosphorylation of Bam32 by Src family PTK is critical for its function in BCR internalization**

Consistent with previous studies (7–9), BCR internalization is also inhibited either in Lyn−/− cells or in wild-type cells treated with PP1, an inhibitor of Src family PTKs (Fig. 4A). We and others have shown that Bam32 is phosphorylated upon BCR cross-linking (14, 30). Consistent with a previous study using overexpression of Bam32 in endothelial cells (31), PP1 blocked BCR-induced tyrosine phosphorylation of Bam32 in a dose range that blocks BCR internalization (Fig. 4B). This confirms that Src family PTKs are required for tyrosine phosphorylation of Bam32. To further determine whether phosphorylation of Bam32 on its single site (Y139) (31) influences its function, we generated BJAB cells stably expressing a point mutant (Y139F) of Bam32. As shown in Fig. 4C, BCR internalization was impaired in these cells, presumably by exerting a dominant-negative effect over the endogenous Bam32 in these cells. Collectively, these data suggest that Bam32 needs to be tyrosine phosphorylated probably by Src family PTKs to regulate BCR internalization. Because activation of the PLCγ2-related signaling pathways is partially crippled in Bam32−/− DT40 cells (15), we also tested whether PLCγ2 activation is required for BCR internalization. BCR internalization was normal in PLCγ2−/− DT40 cells (data not shown). This is consistent with previous findings that activation of PKC or Ca2+ flux, both of which are generated upon PLCγ2 activation, is not required for BCR internalization (26). Thus, BCR internalization is Src family PTK and Bam32 dependent, but does not require PLCγ2.

**Bam32 does not regulate clathrin phosphorylation and its recruitment into lipid rafts**

Both tyrosine phosphorylation of CHC and CHC association with lipid rafts are critical events for BCR internalization (9). In addition, BCR-induced tyrosine phosphorylation of CHC is blocked in either Lyn−/− DT40 cells or B cells treated with an inhibitor of Src family PTKs (9), suggesting that clathrin is also, like Bam32, a downstream target for Src family PTKs to regulate BCR internalization. Because Bam32 colocalized with CHC upon BCR cross-linking (Fig. 2C), we determined whether Bam32 directly regulates the function of clathrin. CHC was only minimally phosphorylated before stimulation. BCR cross-linking induced a strong phosphorylation of CHC within 5 min in wild-type, Bam32−/−, and Bam32-overexpressing Bam32−/− cells (Fig. 5A). We also tested whether Bam32 influences association of CHC with lipid rafts. The amount of CHC in lipid rafts was slightly increased upon BCR cross-linking, and the absence of Bam32 did not appreciably affect this distribution (Fig. 5B). Together with our previous study (9), these findings suggest that both Bam32 and clathrin are the downstream targets of Src family PTKs to regulate BCR internalization. These data also suggest that Bam32 regulates BCR internalization by a mechanism in conjunction with clathrin rather than by directly regulating the function of clathrin.

**Bam32 regulates BCR-induced actin polymerization**

Consistent with previous findings (25–28), Cyt B and Lat A, both of which prevent actin polymerization by different mechanisms (32), significantly blocked BCR internalization (Fig. 6A). Because Bam32 clearly colocalized with F-actin upon BCR cross-linking (Fig. 2B), we tested whether Bam32 regulates BCR-induced actin polymerization. As shown in Fig. 6B, BCR stimulation caused a small increase in the level of F-actin in wild-type cells, as seen previously (33). In Bam32−/− cells, BCR-induced actin polymerization was partially inhibited and more transient compared with wild-type cells. By contrast, overexpression of Bam32 in Bam32−/− cells resulted in a larger increase in the level of F-actin compared with wild-type cells after BCR stimulation. Put together, these results suggest that Bam32 regulates BCR internalization at the stage where actin polymerization is required.

**BCR internalization regulates BCR-induced JNK activation**

Our results here, together with previous findings (15, 18), suggest that Bam32 regulates both BCR internalization and BCR-induced JNK activation. Thus, we tested whether BCR internalization may be essential for BCR-induced JNK activation. MDC, which prevents clathrin-mediated endocytosis at the receptor invagination step (34), inhibited BCR internalization in DT40 cells as expected (data not shown), and also inhibited BCR-induced JNK activation (Fig. 7A). JNK phosphorylation appeared to be more sensitive to blockade of BCR internalization than other MAPKs. At a higher dose of MDC, phosphorylation of ERK and p38 MAPK was partially inhibited, while PKCφ phosphorylation was normal, again as in Bam32−/− cells. Moreover, Cyt B also inhibited BCR-induced activation of JNK and ERK in DT40 cells (Fig. 7B).
Endocytic vesicles can function as active signaling compartments in other cell types (35, 36). The similar results with Bam32/H11002, MDC- and Cyt B-treated cells are consistent with the idea that BCR-containing vesicles may function in activation of certain BCR signaling pathways such as JNK activation; however, further work is necessary to test this hypothesis. Thus, these results suggest that the BCR signaling defect in Bam32/H11002 cells may be at least partly due to impaired BCR internalization.

Discussion
Upon BCR cross-linking, Bam32 is tyrosine phosphorylated and associates with PLCγ2 (14). Moreover, BCR-induced activation of PLCγ2-related signaling pathways is partially crippled in Bam32−/− DT40 cells (15). However, given that the BCR signaling phenotype of Bam32−/− cells is not exactly the same as that of PLCγ2−/− cells (16), we hypothesized that Bam32 also regulates a PLCγ2-independent signaling pathway in B cells. Consistent with this hypothesis, recent studies of Bam32−/− mice suggest that Bam32 regulates B cell proliferation through the BCR in a PLCγ2-independent fashion (17, 18). In this study we show that Bam32 regulates BCR internalization, which is also a PLCγ2-independent event in B cells, because BCR internalization is normal in PLCγ2−/− cells (data not shown).

Previous studies showed that Src family PTKs such as Lyn play a crucial role in BCR internalization (7–9), suggesting that BCR internalization is controlled by certain substrates phosphorylated by Src family PTKs. Because Bam32 is phosphorylated upon BCR cross-linking (14, 30), we speculated that Src family PTKs play a pivotal role in BCR-induced Bam32 phosphorylation. We found that an inhibitor of the Src family PTKs blocked BCR-induced tyrosine phosphorylation of Bam32 in a dose range that blocks BCR internalization (Fig. 4B). Moreover, because Bam32 has only one potential tyrosine phosphorylation site (Y139; Ref. 31), we were able to show that BCR internalization is impaired in B cell

FIGURE 6. Bam32 regulates BCR-induced actin polymerization. A, Wild-type DT40 cells pretreated with Cyt B (40 μM) or Lat A (2.5 μM) were incubated at 4°C with M4 for 30 min. The cells were washed, warmed to 37°C for indicated time lengths, stained with a FITC-labeled anti-mouse IgM Ab and analyzed by flow cytometry. The results are expressed as the percentage of surface BCR remaining. The data shown are presented by the average and SEM of three independent experiments. B, DT40 cells (wild-type, Bam32−/−, and Bam32−/− stably transfected with chicken Bam32; 1 × 10⁶) were stimulated with M4 (10 μg/ml) for various time periods at 37°C and stained with FITC-phalloidin. The fixed cells were then analyzed by flow cytometry. All of the data are presented as a fold-increase in F-actin levels compared with those of the sample before BCR stimulation. The results shown are representative of three independent experiments.

FIGURE 7. BCR internalization regulates BCR-induced JNK activation. A, Wild-type DT40 cells pretreated with the indicated dose of MDC were stimulated with M4 (5 μg/ml) for the indicated time intervals. Cell lysates were subsequently separated on a 10% SDS-PAGE gel, and were analyzed by Western blotting with anti-phospho-ERK, -JNK, -p38 MAPK, -PKCμ, and anti-Akt sera. The results shown are representative of three independent experiments. B, Wild-type DT40 cells pretreated with Cyt B (40 μM) were stimulated with M4 (5 μg/ml) for indicated time intervals. Cell lysates were subsequently separated on a 10% SDS-PAGE gel, and were analyzed by Western blotting with anti-phospho-ERK, -JNK, and anti-Akt sera. The results shown are representative of two independent experiments.
lines expressing Y139F mutation of Bam32 (Fig. 4C), similar to Bam32 \(^{-/-}\) cells. These results suggest that Bam32 needs to be phosphorylated to exert its function in BCR internalization.

Another downstream target of Src family PTKs for receptor internalization is clathrin, a molecule involved in the process of internalization of many receptors such as epidermal growth factor (Refs. 37–39). We recently showed that tyrosine phosphorylation of clathrin, as well as association of clathrin with lipid rafts, is required for BCR internalization (9). We thus questioned whether Bam32 affects the function of clathrin in BCR internalization. Tyrosine phosphorylation of clathrin as well as association of clathrin with lipid rafts were normal in Bam32 \(^{-/-}\) cells. Our biochemical experiments showed no direct association between Bam32 and CHC (data not shown). It thus appears likely that Bam32 and clathrin are both downstream targets of Src family PTKs for BCR internalization, and that Bam32 regulates BCR internalization without directly regulating the function of clathrin.

A number of the cytosolic proteins involved in clathrin-mediated endocytosis have also been shown to interact with actin (32, 40). Indeed, disruption of actin polymerization affects clathrin-mediated endocytosis in several cell types including B cells, suggesting that actin polymerization contributes to clathrin-mediated endocytosis (25, 40, 41). Consistent with previous findings (25–28), inhibitors of actin polymerization significantly inhibit BCR internalization (Fig. 6A). We found that Bam32 almost completely co-localizes with F-actin upon BCR cross-linking (Fig. 2B). However, our biochemical experiments showed that a small amount of Bam32 is constitutively detected in a detergent-insoluble fraction, and this amount is only slightly increased upon BCR cross-linking (data not shown). Thus, the association of Bam32 with the cytoskeleton may be very sensitive to detergent conditions. Finally, BCR-induced actin polymerization is impaired in Bam32 \(^{-/-}\) cells (Fig. 6B). Based on these findings, we propose that Bam32 regulates BCR internalization at the stage where actin polymerization is required.

Previous studies showed that in addition to internalization, BCR capping involves an actin-dependent process (28, 42). Our findings (Fig. 6B) showed that, in Bam32 \(^{-/-}\) cells, BCR-induced actin polymerization still occurs initially but is not sustained compared with wild-type cells. We thus speculate that this initial spike of actin polymerization in Bam32 \(^{-/-}\) cells is enough to form BCR caps, and a sustained phase of actin polymerization is more critical for BCR internalization. We also biochemically isolated membrane and detergent-insoluble fractions from cells and found that upon BCR cross-linking, Bam32 is translocated into the detergent-insoluble fraction in Bam32 \(^{-/-}\), as well as wild-type cells (data not shown). These findings suggest that initial actin-regulating events such as BCR capping and attachment to the cytoskeleton are less affected in the absence of Bam32.

What actin-regulating molecules are involved in the regulation of BCR internalization by Bam32 remains to be clarified. One possible group is the Rho-family of GTPases such as Rac and RhoA, which regulate a Rac-independent actin remodeling process that is required for BCR internalization. An important clue to defining the molecules involved in this process is the fact that Bam32 needs to be tyrosine phosphorylated to regulate BCR internalization. Indeed, BCR-induced actin polymerization is impaired in BJAB cells expressing a point mutant (Y139F) of Bam32 (44). It is possible that tyrosine phosphorylation of Bam32 causes a conformational change which in turn facilitates the interaction of Bam32 with actin-related molecules. Alternatively, the Y139 site of Bam32 may bind to the molecules containing phosphotyrosine-binding modules such as Src homology 2 domains. We are now testing these possibilities.

Previous studies suggest that Bam32 channels upstream signals into a unique downstream branch of the BCR signaling pathway (19). The present study suggests that Bam32 has an additional function. It bridges BCR signals with another critical role of BCR: the Ag-processing pathways in B cells.

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References


