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The Adaptor Protein Bam32 Regulates Rac1 Activation and Actin Remodeling through a Phosphorylation-dependent Mechanism*[S]

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The B cell adaptor molecule of 32 kDa (Bam32) is an adaptor that links the B cell antigen receptor (BCR) to ERK and JNK activation and ultimately to mitogenesis. After BCR cross-linking, Bam32 is recruited to the plasma membrane and accumulates within F-actin-rich membrane ruffles. Bam32 contains one Src homology 2 and one pleckstrin homology domain and is phosphorylated at a single site, tyrosine 139. To define the function of Bam32 in membrane-proximal signaling events, we established human B cell lines overexpressing wild-type or mutant Bam32 proteins. The basal level of F-actin increased in cells expressing wild-type or myristoylated Bam32 but decreased in cells expressing either an Src homology-2 or Tyr-139 Bam32 mutant. Overexpression of wild-type Bam32 also affected BCR-induced actin remodeling, which was visualized as increases in F-actin-rich membrane ruffles. In contrast, Bam32 mutants largely blocked the BCR-induced increase in cellular F-actin. The positive and negative effects of Bam32 variants on F-actin levels were closely mirrored by their effects on the activation of the GTPase Rac1, which is known to regulate actin remodeling in lymphocytes. Bam32-deficient DT40 B cells showed decreased Rac1 activation and a failure of Rac1 to co-localize with the BCR, whereas cells overexpressing Bam32 had increased constitutive Rac1 activation. These results suggest that Bam32 regulates the cytoskeleton through Rac1. Bam32 variants also affected downstream signaling to JNK in a manner similar to that of Rac1, suggesting that the effect of Bam32 on JNK activation may be at least partially mediated through Rac1. Our results demonstrate a novel phosphorylation-dependent function of Bam32 in regulating Rac1 activation and actin remodeling.

Signaling through antigen receptors expressed by T and B lymphocytes is critical for the induction and regulation of immune responses. These multi-subunit protein tyrosine kinase-linked receptors share many common signaling mechanisms with other protein tyrosine kinase-linked receptors, including the recruitment of the activated receptor to lipid rafts, the activation of several protein tyrosine kinases, and the use of adaptor proteins to link the kinase cascade to downstream amplification and effector pathways. The B cell antigen receptor (BCR)[1] complex activates a variety of signaling pathways including small GTPases such as Ras, Rac, and Rap, phosphatidylinositol 3-kinases (PI3Ks), phospholipase C-γ, calcium, protein kinase C, and the mitogen-activated protein kinases ERK, JNK, and p38 MAPK (1–5). These interrelated signaling pathways initiate cellular responses by directly altering the cellular cytoskeleton and by altering gene transcription through inducible transcription complexes such as NF-κB and NF-AT (nuclear factor of activated T cells).

PI3K signaling plays a critical and indispensable role in B cell activation and differentiation (6–9). A major mechanism of action for PI3Ks is to recruit cytoplasmic signaling molecules to activated membranes through the interaction of their pleckstrin homology (PH) domains with the lipid products of PI3K (10). The PH domain-containing targets of PI3K expressed in B cells include the kinases Btk and Akt, which play critical roles in B cell maturation, survival, and activation (11–15). PH domain-dependent membrane recruitment of these kinases is thought to regulate their accessibility to phosphorylation by upstream activating kinases such as the Src family kinases in the case of Btk (16) or phosphoinositide-dependent kinase-1 in the case of Akt (17, 18). PI3K-dependent membrane-targeting of Btk may also regulate its ability to participate in membrane-associated signaling complexes, leading to the activation of phospholipase C-γ2 (12, 19).

We and others have identified a family of PH domain-containing adaptor proteins expressed in B cells that include members designated Bam32/DAPP1 (dual adaptor for phosphotyrosine and 3-phosphoinositide-1), TAPP1, and TAPP2 (tandem PH domain-containing protein) (20–23). These adaptor proteins bind PI3K lipid products *in vitro* (22–24) and are recruited to the plasma membrane *in vivo* in a PI3K-dependent manner (20, 25). Intriguingly, we found that, after BCR stimulation, these adaptor molecules are recruited with delayed and sustained kinetics as compared with Btk (21), and, unlike Btk, their recruitment is not inhibited by the lipid phosphatase SHIP (SH2-containing inositol phosphatase) (26). This obser-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental material in the form of a movie featuring time-lapse imaging of Bam32-EGFP with labeling of highly motile structures.

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[1] The abbreviations used are: BCR, B cell antigen receptor; Bam32, B cell adaptor molecule of 32 kDa; EGFP, enhanced green fluorescence protein; GEF, guanine nucleotide exchange factor; JNK, c-Jun N-terminal kinase; MemBam32, plasma membrane-targeted Bam32; PBS, phosphate-buffered saline; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEKK, MAPK/ERK kinase kinase; PI3K, phosphatidylinositol 3-kinase; PH, pleckstrin homology; SH2, Src homology-2; WT, wild-type.

vation suggests a differential regulation of these PI3K-regulated effector molecules at the level of membrane recruitment, which is likely due to differences in their precise lipid binding specificities.

Whereas the function of TAPP1 and TAPP2 in B cell activation is not yet well established, Bam32 clearly plays a role in BCR signaling. Bam32 is phosphorylated upon BCR ligation and can regulate BCR-induced NF-AT activation (20). Bam32-deficient DT40 B cells show a set of defects in BCR signaling, including defective activation of ERK and JNK (27). Bam32-deficient mice have a unique phenotype, showing normal differentiation of follicular B cells but a specific defect in proliferation triggered by BCR engagement (28, 29). Strikingly, these mice also have a specific defect in the generation of antibody responses to type 2 T-independent antigens. Bam32-deficient mouse B cells also displayed markedly reduced ERK and JNK activation in response to BCR ligation. However, the mechanisms by which Bam32 links PI3K activation to these downstream signaling responses have not been fully defined.

In addition to its C-terminal PH domain, Bam32 contains an N-terminal SH2 domain that can associate with phospholipase C-γ2 (20). Bam32 also has a single centrally located tyrosine phosphorylation site. Tyr-139 is phosphorylated in response to BCR or platelet-derived growth factor stimulation, and a blockade of Src family protein tyrosine kinases or a mutation of Tyr-139 abolishes Bam32 phosphorylation (30, 31). Here, we have determined the effect of an SH2 mutant and a Y139F Bam32 mutant on BCR signaling and show that these mutants exert a dominant negative effect on Rac1 activation and actin remodeling. Our results demonstrate a functional link between Bam32 and Rac1-dependent cytoskeletal rearrangement and indicate that Bam32 phosphorylation is critical for this function.

EXPERIMENTAL PROCEDURES

Expression Constructs and Transfection—Constructs encoding Myc-tagged Bam32 and the Bam32-enhanced green fluorescent protein (EGFP) fusion protein were described previously (20). R61K and Y139F point mutants were generated by PCR mutagenesis of these constructs (QuikChange; Stratagene, La Jolla, CA). For generation of membrane-targeted Bam32, a PCR fragment encoding the Lyn myristoylation/palmitoylation sequence (amino acids 1–16) was amplified from human B cell cDNA and cloned into pcDNA3.1 Myc/His (Invitrogen) and pEGFP-N1 (Clontech). We confirmed that this Lyn-derived sequence can target EGFP to the plasma membrane.[2] The Bam32 cDNA sequence was then inserted into the Lyn-pcDNA3.1 vector in frame with the Lyn sequence. Prior to use, all constructs were verified by DNA sequencing. Cells of the sporadic human Burkitt's lymphoma line BJAB were cultured and transfected as described previously (26). Stably transfected BJAB clones were screened for the expression of exogenous Bam32 by Western blotting with anti-Myc monoclonal antibody 9E10 (Santa Cruz Biotechnology, Santa Cruz, CA). Clones were checked for matched BCR expression level relative to parental cells.[2] The relative expression of endogenous and exogenous Bam32 in the selected clones was subsequently determined by immunoprecipitation with the anti-Bam32 monoclonal antibody UW32 and Western blotting with rabbit anti-Bam32 serum.[2]

Confocal Imaging Analyses—BJAB cells were stimulated for the indicated times using 10 μg/ml goat anti-human IgM F(ab′)$_2$ fragments (Jackson ImmunoResearch, West Grove, PA). Confocal imaging of EGFP fusion proteins and image analysis were carried out essentially as described (26). For analysis of membrane ruffling, live unstimulated BJAB transfectant or control BJAB cells were plated in glass-bottomed chambered cover glass slides (NalgeNunc, Rochester, NY) and imaged under transmitted light on a microscope equipped with differential interference contrast optics. For BJAB immunofluorescence staining, cells were transiently transfected with Bam32-EGFP vector or empty EGFP vector and then washed, fixed with 3.7% paraformaldehyde, permeabilized with PBS containing 1% bovine serum albumin and 0.05% saponin (wash buffer), intracellularly stained with mouse anti-Rac1 or isotype control antibodies (BD Biosciences), and then washed in wash buffer. Cells were then stained with rhodamine-conjugated F(ab′)$_2$ goat anti-mouse Ig (BIOSOURCE) on ice before washing twice with washing buffer. Stained cells were plated in chambered cover glass slides and imaged on a confocal microscope. The correlation coefficients for paired green/red images of single cells were calculated using ImagePro Plus software (Media Cybernetics, Silver Spring, MD). For DT40 immunofluorescence staining, cells were incubated with fluorescein isothiocyanate-conjugated goat anti-chicken IgM at 4 °C for 30 min, washed, and warmed to 37 °C for 5 min. Cells were washed and fixed with 3.7% paraformaldehyde for 10 min, washed and permeabilized with 0.1% Triton X-100 in PBS, and stained with mouse anti-Rac1. Cells were then transferred to slides and analyzed on the confocal microscope.

Determination of Filamentous Actin Levels—Cells were stimulated with unlabeled goat anti-human IgM F(ab′)$_2$ (10 μg/ml) for the indicated times, washed twice with ice-cold PBS containing 2% fetal calf serum, fixed with 3.7% paraformaldehyde, and permeabilized by washing twice in PBS containing 1% fetal calf serum and 0.1% Triton X. F-actin was then stained using 0.1 μg/ml rhodamine-phalloidin or Alexa 488-phalloidin (Molecular Probes, Eugene, OR), followed by washing twice with PBS and 1% fetal calf serum. For confocal microscopy analysis, cells were loaded on poly-L-lysine slides, covered with the anti-fading agent Mowiol, and finally imaged using an Olympus Fluoview instrument. For flow cytometric analysis, the mean fluorescence was evaluated using a FACSCalibur™ flow cytometer (BD Biosciences).

Determination of Active Rac1—To assess Rac1 activation, 1–2× 10^7 cells were lysed in lysis buffer (25 mM Hepes pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10 mM $MgCl_2$, 1 mM EDTA, 2% glycerol, 2 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin) and incubated with PAK1-PBD (p21-binding domain from human p21-activated kinase-1) bound to glutathione-agarose beads (Upstate Biotechnology, Lake Placid, NY) for 30 min at 4 °C. After a wash in the lysis buffer, bound proteins were eluted from the beads by boiling in SDS-PAGE sample buffer. Bead eluates and aliquots of each unfractionated cell lysate were then blotted with mouse anti-Rac1 (Upstate Biotechnology). The resulting chemiluminescence signals were quantified using a FluorChem 8800 instrument (Alpha Innotech, San Leanardo, CA). The degree of Rac1 activation was expressed as the ratio of the optical density of immunoblots of PAK1-PBD precipitates to the optical density of the total Rac1 immunoblots. The data are normalized to the unstimulated control cells.

JNK Phosphorylation Analysis—JNK phosphorylation was detected essentially as described (27). Chemiluminescence signals were quantitated using a FluorChem 8800 instrument. For the subsequent detection of total cellular JNK, bound antibodies were removed by 30 min of exposure to a stripping solution (0.25 M glycine, pH 2.5) and re-probing with 1 μg/ml anti-JNK (BD Biosciences). JNK phosphorylation is expressed in arbitrary units relative to the phosphorylated JNK/total JNK ratio of unstimulated BJAB cells.

RESULTS

BCR-induced Membrane Recruitment of Bam32 Variants—To determine the role of the SH2 domain and tyrosine phosphorylation site of Bam32, we generated Bam32 constructs carrying inactivating point mutations in either the SH2 domain (R61K Bam32) or the tyrosine phosphorylation site (Y139F Bam32) and expressed these constructs in the human B cell line BJAB (Fig. 1*A*). Because PI3K-dependent membrane recruitment of Bam32 is required for its phosphorylation and function (20, 31), we also generated and expressed constructs encoding Bam32 targeted to the plasma membrane (Mem-Bam32) by the addition of the Lyn myristoylation/palmitoylation sequence at its N terminus. We first examined whether the R61K or Y139F mutations affected the membrane recruitment of Bam32 by using EGFP-tagged proteins (Fig. 1*B*). Images taken between 15–45 min post-BCR stimulation showed that wild-type Bam32 is concentrated in membrane ruffles and lamellipodia. As expected, the myristoylated Bam32 (Mem-Bam32) showed strong constitutive membrane recruitment (Fig. 1*B*). No significant difference in the degree of membrane recruitment was observed in the R61K or Y139F mutants *versus* the wild-type (WT) Bam32, as assessed by visual scoring or

[2] A. Allam and A. J. Marshall, unpublished observations.

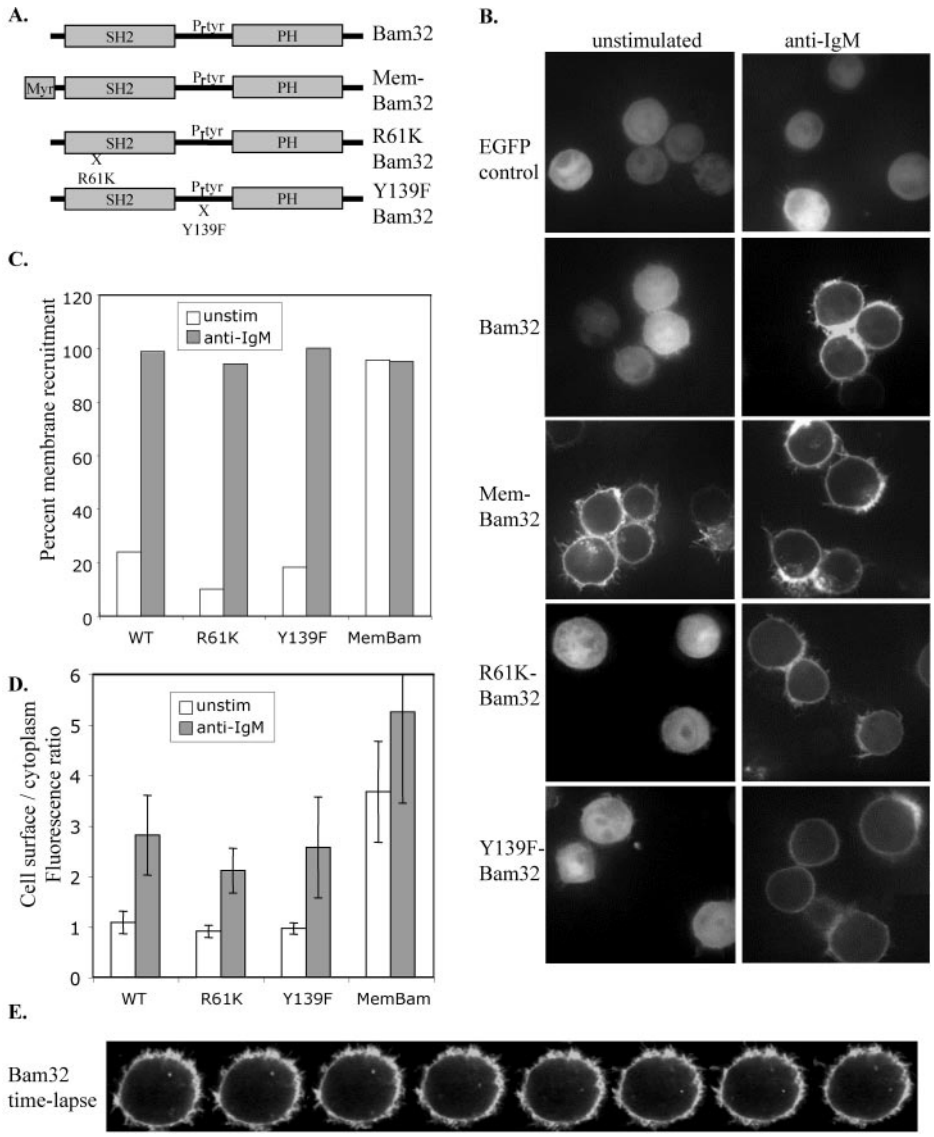

FIG. 1. **BCR-induced membrane recruitment of Bam32 variants.** *A*, schematic diagram illustrating the Bam32 variants used in this study. Note that constructs are tagged with EGFP at their N terminus or Myc/His epitope tags at their C terminus, with the exception of Mem-Bam32, which is tagged with EGFP at its C terminus. *Myr*, myristoylation sequence; *P-tyr*, phosphorylated tyrosine. *B*, BJAB cells were transfected with the indicated EGFP fusion proteins, stimulated with anti-BCR antibodies for 30 min, and imaged by confocal microscopy. Note that the R61K and Y139F mutants are still recruited to the plasma membrane but that the association with membrane ruffles and intracellular structures is less pronounced. *C*, visual scoring of membrane recruitment of Bam32 variants. 30–50 cells per condition were scored for visible accumulation of fluorescence at the cell perimeter. *unstim*, unstimulated. *D*, quantitation of membrane recruitment. Average fluorescence intensity within defined regions of the cell perimeter or interior was used to approximate the membrane/cytoplasmic ratio. Data represent the mean ± S.E. for 10–15 cells per condition. *E*, time-lapse imaging of Bam32-EGFP, showing labeling of highly motile structures. The time series shown represents images taken at 3-s intervals beginning 30 min after BCR stimulation. The full image sequence can be seen as a movie available in the supplementary on-line material.

image analysis (Fig. 1, *C* and *D*). These results are consistent with previous findings suggesting that short term membrane localization of Bam32 is determined primarily by the PH domain (20) and further suggest that long term docking at the membrane is relatively independent of the SH2 domain and tyrosine phosphorylation site.

Bam32 was also frequently observed in discrete accumulations inside the cells that are very motile and difficult to image clearly (Fig. 1, *B* and *E*). In time-lapse experiments, these structures were observed to undergo rapid linear movements consistent with cytoskeletally directed motion (Fig. 1*E* and supplemental material available in the on-line version of this article). These structures were less apparent in cells expressing the R61K or Y139F mutants (Fig. 1*B*). Visual inspection of the images also indicated that cells expressing R61K or Y139F Bam32 generally have a less ruffled appearance. These results suggest that Y139F and R61K mutant Bam32 transfectants are recruited to the plasma membrane normally but may be defective in post-recruitment trafficking events.

Bam32 Regulates F-actin Levels—The association of Bam32 with motile structures suggests that Bam32 may play a functional role in regulating BCR-induced cytoskeletal rearrangements. Because we found previously that Bam32 is concentrated within filamentous actin-rich membrane ruffles within the first few minutes after BCR cross-linking (21), we hypothesized that Bam32 may regulate F-actin assembly. We compared filamentous actin formation in BJAB transfectants expressing WT, membrane-targeted, or mutant Bam32 using flow cytometry and confocal microscopy assays (Fig. 2). The flow cytometric analyses showed that the basal F-actin content is ~35% higher in cells transfected with WT Bam32 than in parental BJAB cells (Fig. 2*A*). Basal F-actin was elevated to a similar extent in cells expressing membrane-targeted Bam32 but was unaffected or slightly reduced in the cells expressing Y139F or R61K Bam32 transfectants. After anti-BCR stimulation, the level of F-actin was slightly elevated in the parental BJAB cells, and this response was markedly elevated in WT Bam32 transfectants (Fig. 2*B*). The distribution of F-actin in BCR-stimulated control or Bam32-tranfected cells was examined by confocal microscopy, and WT Bam32 transfectants had observable increases in F-actin-rich membrane ruffles (Fig. 2*C*). In contrast to WT Bam32 transfectants, BCR-stimulated F-actin formation was reduced in cells expressing Y139F or R61K Bam32 transfectants (Fig. 2*B*), suggesting that these mutants have a dominant negative function. These results provide evidence for a functional role of Bam32 in regulating F-actin formation and suggest that tyrosine phosphorylation of Bam32 and the SH2 domain of Bam32 is required for this function.

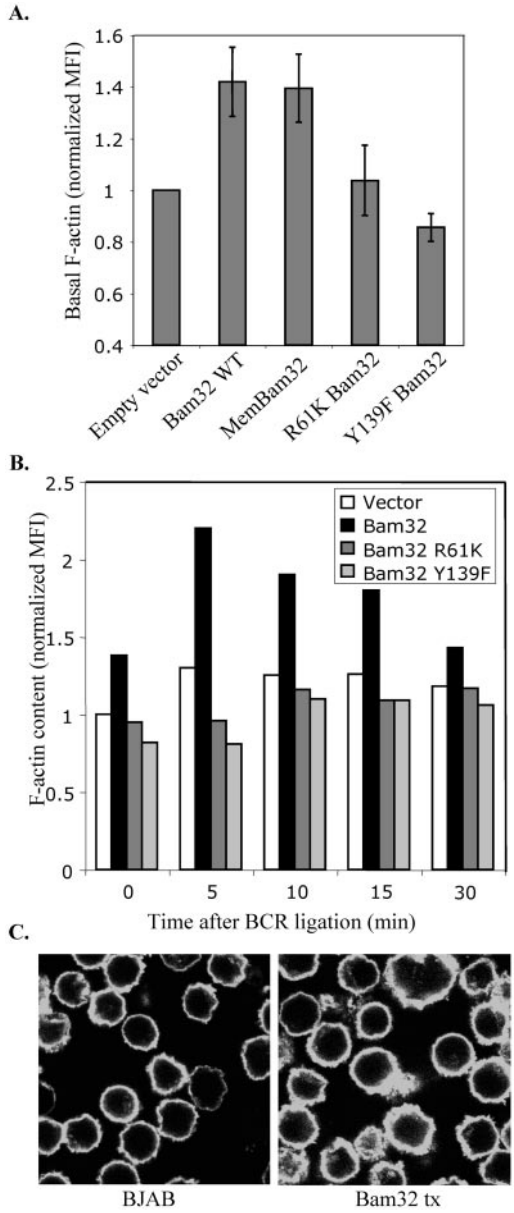

FIG. 2. **Differential effect of wild-type and mutant Bam32 on basal and BCR-induced actin polymerization.** *A*, basal F-actin levels in the indicated transfectants were determined by Alexa 488-phalloidin staining. Mean fluorescence intensity (*MFI*) values were normalized to those from cells transfected with the empty vector. The data shown represents the average and standard error of 3–4 determinations for each transfectant. *B*, the indicated transfectants were stimulated with anti-BCR antibodies for the indicated times and then fixed, permeabilized, and assayed for F-actin content as for *panel A*. Note that the BCR-induced increase in F-actin is enhanced by wild-type Bam32 but inhibited by Bam32 mutants. Data are representative of eight independent experiments. *C*, control or Bam32-expressing cells were stimulated with anti-BCR antibodies for 10 min and then fixed, permeabilized, and stained with rhodamine-phalloidin. Representative panels of cells are shown.

Bam32 Co-localizes with Rac and Regulates Rac Activation after BCR Cross-linking—Reorganization of the actin cytoskeleton in response to extracellular stimuli is orchestrated by Rho family GTPases (32), and the Rho-family members Rac1 and Cdc42 have been shown to play critical roles in the cytoskeletal reorganization responses of lymphocytes (33–36). We found that Bam32 and Rac co-localize in membrane ruffles within a few minutes after stimulation and on endocytic structures formed after longer time periods (Fig. 3, *A* and *B*). To determine whether Bam32 regulates Rac activity, we assessed the basal and BCR-stimulated levels of the active form of Rac in our panel of BJAB transfectants. Rac1-GTP was pulled down using PAK1-PBD beads and detected by anti-Rac Western blot (Fig. 3, *C* and *D*). Total Rac proteins from the same cell extracts were run as a control, and equivalent levels of Rac1 were observed in all cells (Fig. 3*C*). We observed a low basal Rac1 activity in parental BJAB cells and a substantial increase in Rac1-GTP levels after BCR activation. Transfectants expressing WT Bam32 or membrane-targeted Bam32 showed significantly increased Rac activation both before and after BCR stimulation. Strikingly, BCR-induced Rac activation was virtually abrogated in transfectants expressing Y139F or R61K Bam32. We also examined the effect of Bam32 deficiency on Rac activation using the DT40 chicken B cell model (Fig. 4*A*). Bam32-deficient DT40 cells have reduced levels of BCR-stimulated Rac activity compared with those of the parental cells. Re-expression of high levels of Bam32 in the Bam32-deficient cells led to increased basal Rac activity (Fig. 4*A*), consistent with our observations in BJAB cells. We also found that co-capping of Rac1 with the BCR after BCR cross-linking was reduced in the Bam32-deficient cells (Fig. 4*B*). Together, these results demonstrate that Bam32 regulates Rac1 activation through a tyrosine phosphorylation-dependent mechanism. These findings clearly implicate Rac1 as an effector molecule linking Bam32 to cytoskeletal rearrangement.

Role of Bam32 in Membrane Ruffling—Rac1 plays a critical role in membrane ruffling and lamellipodia formation (37, 38). To investigate whether membrane ruffling is affected by Bam32, we assessed our Bam32 transfectants morphologically using differential interference contrast imaging. Rac1 dominant negative (N17) or constitutively active (V12) transfectants were generated as negative and positive controls, respectively. Consistent with the relatively high basal Rac activity in untransfected BJAB cells, the unstimulated control cells had a significant amount of membrane ruffling. As expected, ruffling is less apparent in Rac N17 transfectants (Fig. 5). Y139F Bam32 transfectants have reduced membrane ruffling compared with the control cells, whereas membrane-targeted Bam32 transfectants have visibly increased membrane ruffling that is comparable with that of the Rac V12 transfectants. These results suggest that Bam32 regulates membrane ruffling by affecting Rac1 activity.

Bam32 Affects JNK Phosphorylation following BCR Stimulation—We next examined whether Bam32 affects Rac1 in specific manner to induce cytoskeletal reorganization or whether it generally affects pathways regulated by Rac1. Activation of Rac1 is required for BCR-induced JNK activation (39–41), and Bam32-deficent cells have defective BCR-induced JNK phosphorylation (27, 28). Therefore, we examined JNK expression and phosphorylation in our human B lymphoma transfectants by Western blotting for total or phospho-JNK before and after BCR cross-linking (Fig. 6). BCR-stimulated phosphorylation of JNK1 was virtually abolished in Y139F and R61K Bam32 transfectants, whereas phosphorylation was increased in transfectants expressing WT Bam32 or membrane-targeted Bam32, mirroring the Rac activation results. Thus, in this model Bam32 regulation of JNK phosphorylation correlates with Rac1 activation, suggesting that Bam32 may link to JNK activation through Rac1.

DISCUSSION

Our previous studies and two recent studies on Bam32-deficient mice have provided definitive evidence that Bam32 plays an indispensable role in BCR signaling (20, 27–29). Here, we provide the first evidence that Bam32 functions by regulating cytoskeletal rearrangement pathways through Rac1. We found that Bam32 can influence Rac1 activation in several

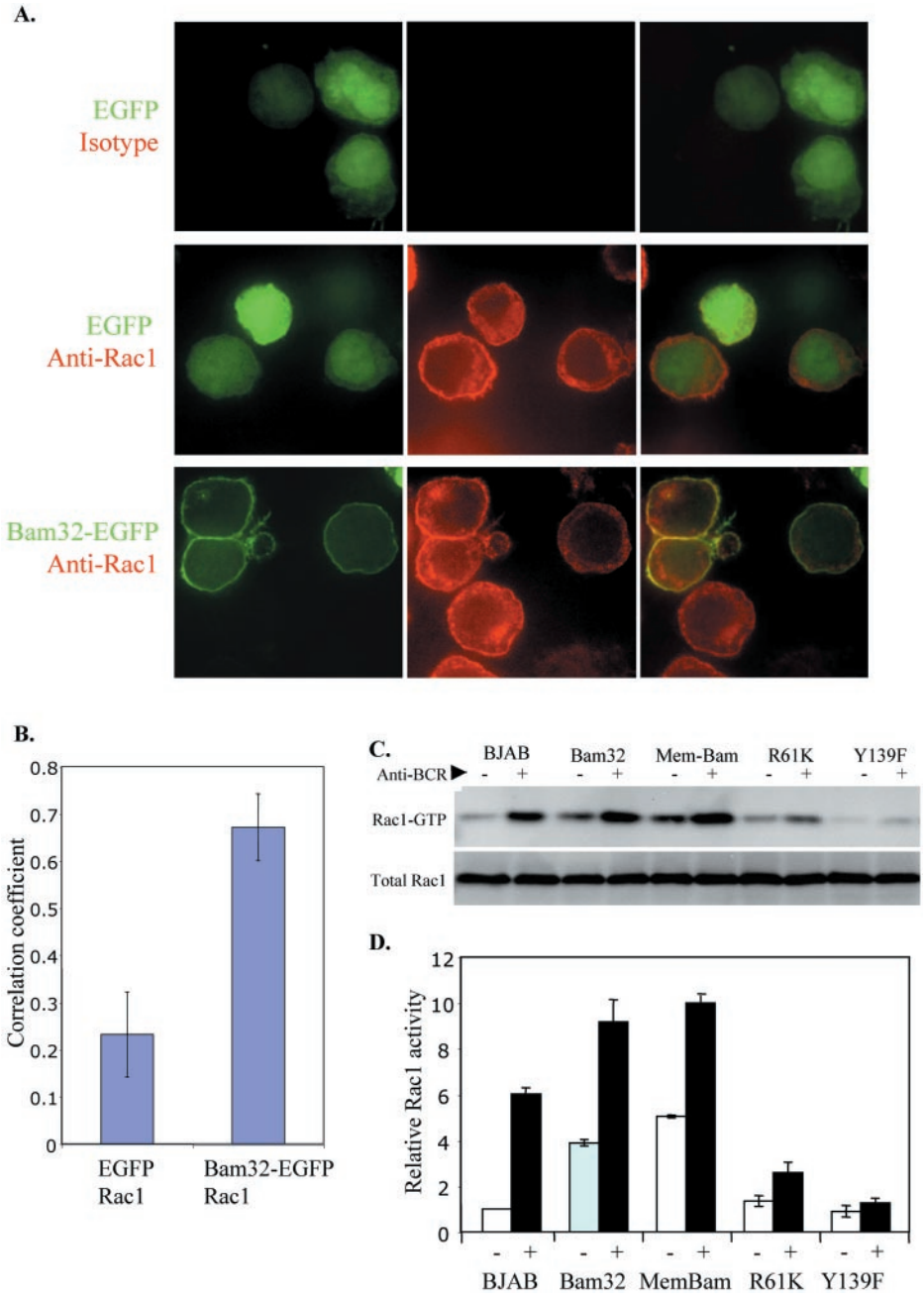

FIG. 3. **Differential effect of wild-type and mutant Bam32 on basal and BCR-induced Rac activation.** *A*, BJAB cells were transfected with EGFP or Bam32-EGFP, stimulated for 30 min with anti-BCR antibodies and then fixed, permeabilized, and stained with anti-Rac1 or isotype control antibodies. *B*, quantitative analysis of Bam32-EGFP and Rac1 co-localization. Correlation coefficients of green and red fluorescence were calculated for 10 EGFP-transfected and 10 Bam32-EGFP-transfected cells. *C*, the indicated cells were stimulated with anti-BCR antibodies for 3 min, lysed and then Rac1-GTP pull-downs were performed using the PAK1-PBD protein. Rac1 was detected in the total cell lysates or the pull-downs by Western blotting with anti-Rac1. *D*, relative Rac1 activity was determined using a chemiluminescence imager. Rac1-GTP signals were normalized in reference to the intensity of the respective total Rac1 bands and expressed as a fold increase compared with unstimulated BJAB cells. Results are expressed relative to unstimulated BJAB cells and represent the average and S.E. of three determinations.

contexts, including gain-of-function and dominant negative experiments in human B lymphoma cells and loss-of-function in DT40 cells. Because the effects Bam32 on F-actin levels closely paralleled Rac1 activation, it is likely that these effects on the cytoskeleton are mediated by Rac1. Consistent with this interpretation, we have found that BJAB cells expressing dominant negative (N17) or constitutively active (V12) Rac1 show reduced and increased basal F-actin levels, respectively.[2] The finding that Bam32 can also regulate membrane ruffling, a response requiring new actin polymerization and known to be regulated by Rac1, also argues in favor of a model where Bam32 regulates actin remodeling at least partially through Rac1 (Fig. 7).

Although the function of the Bam32 PH domain in membrane-targeting was quite well established, the functional roles of the SH2 domain and single tyrosine-phosphorylation site remained obscure. Our present findings provide strong evidence that these motifs play important roles in the regulation by Bam32 of Rac1 and F-actin. Previous studies, as well our own unpublished data, indicate that Bam32 phosphorylation requires PI3K activation and PH domain-mediated membrane recruitment (30, 31).[3] Phosphorylation of Bam32 in response to EGF or BCR stimulation is blocked by inhibitors of Src family kinases (31),[4] and SH2 mutant Bam32 fails to be phosphorylated (43), suggesting that this domain plays a role in bringing Bam32 together with the Src family kinases that act upon Tyr-139. Together, these results suggest a multi-step model whereby PH domain-mediated membrane recruitment of Bam32 facilitates SH2 domain-mediated interactions with other membrane-proximal proteins, allowing access to Src family kinases that phosphorylate Tyr-139. Our present data indicate that Tyr-139 phosphorylation is required for Bam32 function in promoting Rac1 activation and actin remodeling; however, the direct target(s) of phosphorylated Bam32 are not

[3] H. Niiro, and E. A. Clark, unpublished observations.
[4] Niiro, H., Allam, A., Stoddart, A., Brodsky, F., Marshall, A. J., and Clark, E. A. (2004) *J. Immunol.*, in press.

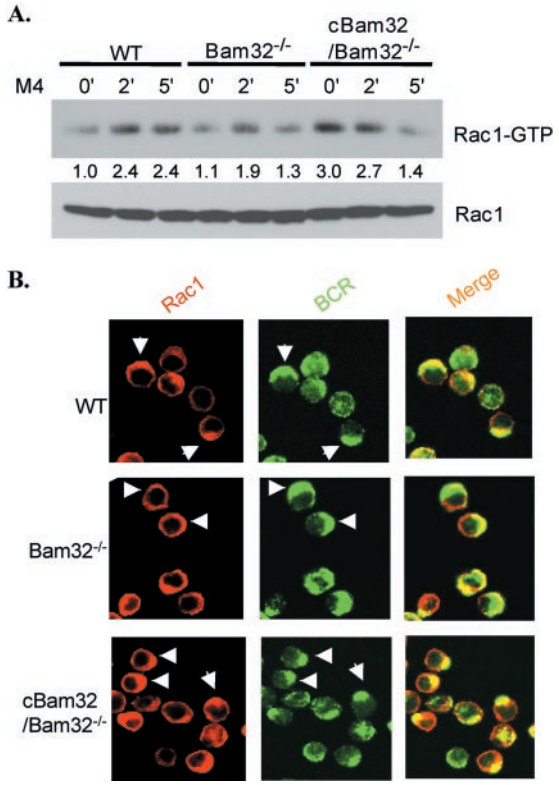

FIG. 4. **Bam32-deficient B cells show reduced BCR-induced activation of Rac1 activation and reduced recruitment of Rac1 to BCR clusters.** *A*, DT40 cells (wild-type, Bam32$^{-/-}$, and Bam32$^{-/-}$ stably transfected with chicken Bam32 (*cBam32*)) were stimulated with anti-BCR antibody for the indicated time periods, and Rac1 activation was determined. The results are representative of three independent experiments. *B*, DT40 cells (wild-type, Bam32$^{-/-}$, and Bam32$^{-/-}$ stably transfected with chicken Bam32 (*cBam32*)) were incubated at 4 °C with fluorescein isothiocyanate-conjugated goat anti-chicken IgM for 30 min. The cells were washed and warmed to 37 °C for 5 min. Cells were fixed, permeabilized, stained with anti-Rac1 monoclonal antibody, and analyzed by confocal microscopy. The images shown are representative of three independent experiments.

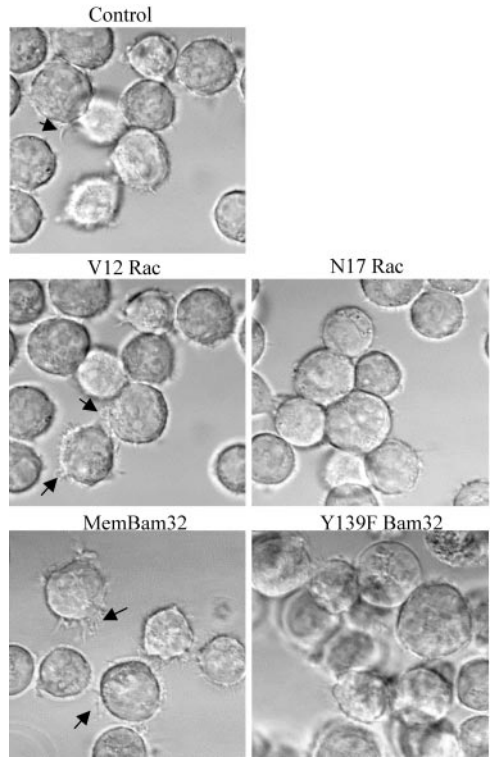

FIG. 5. **Effect of Bam32 variants on membrane ruffling.** The indicated transfectants were plated in glass-bottomed dishes and imaged on a confocal microscope equipped with differential interference contrast optics to visualize membrane ruffles. Representative panels of cells are shown, and *arrows* indicate areas of pronounced membrane ruffling. Note that control BJAB cells have some degree of membrane ruffling as is characteristic of these cells, but the degree of ruffling is increased in V12 Rac or MemBam32 transfectants and decreased in Rac N17 Rac or Y139F Bam32 transfectants.

known. It is intriguing that in all the studies the single phosphorylation of Bam32 correlates with a pronounced (~3–4-kDa) shift in its mobility on SDS-PAGE gels. We speculate that Bam32 phosphorylation may cause a stable conformational change that alters its intermolecular interactions or perhaps even promotes intramolecular interaction.

Our confocal imaging data suggest that, after initial translocation of Bam32 to the plasma membrane via binding to 3-phosphoinositides, the SH2 domain and tyrosine phosphorylation of Bam32 may regulate its further trafficking. The association of Bam32 with motile membrane ruffles and intracellular vesicles appears to be impaired by mutations in the SH2 and Tyr-139 domains. This observation could be explained by a failure of these mutants to target to these structures or by a failure of these structures to form in the presence of the mutant EGFP fusion proteins. The present data suggest that membrane ruffling is reduced in Y139F-expressing cells, supporting the latter possibility. We have also found recently that Bam32 colocalizes with endocytosed BCR and that Y139F mutant Bam32 inhibits BCR endocytosis,[3] indicating that the role of Bam32 in regulating the cytoskeleton extends to receptor endocytosis. Consistent with our findings in B cells, a study using ectopically expressed Bam32 in porcine aortic endothelial cells found that Bam32 is recruited to the plasma membrane, phosphorylated in response to platelet-derived growth factor stimulation, and subsequently localized to motile intracellular vesicles containing internalized platelet-derived growth factor receptors (43). This study also observed that Tyr-139 mutant Bam32 failed to be recruited to endosomes, but the effect of this mutant on platelet-derived growth factor receptor internalization as a whole was not indicated. Interestingly, the present data indicate that a portion of myristoylated Bam32 associates with an intracellular compartment in unstimulated or BCR-stimulated cells (Fig. 1*B*). It is tempting to speculate that this represents specific targeting to an endocytic compartment via the SH2 domain and/or Tyr-139; however, further work will be required to test this idea.

In addition to membrane ruffling and receptor endocytosis, the regulation of F-actin formation by Bam32 may also be important for other cell functions such as cellular motility and adhesion responses, and it will be important to assess the potential role of Bam32 in these responses. The actin cytoskeleton is also increasingly implicated in the assembly of signaling complexes leading to lymphocyte proliferation and differentiation (33, 35), and it is likely that regulation of the cytoskeleton by Bam32 may explain some of its effects on signaling pathways. Actin reorganization in response to extracellular stimuli requires temporally and spatially controlled assembly, disassembly, and architectural rearrangement of actin filaments. The Rho family GTPases, including Rac1, are major players in actin remodeling responses. Active Rac1 can promote *de novo* nucleation of actin filaments by the Arp (actin-related protein) 2/3 complex through its association with a protein complex containing the WASP (Wiskott-Aldrich syndrome protein) family protein WAVE (37, 44). Thus, the promotion of Rac activation by Bam32 can explain the increases in F-actin-rich membrane ruffles; however, we cannot rule out the possibility that Bam32 has additional Rac1-independent effects that contrib-

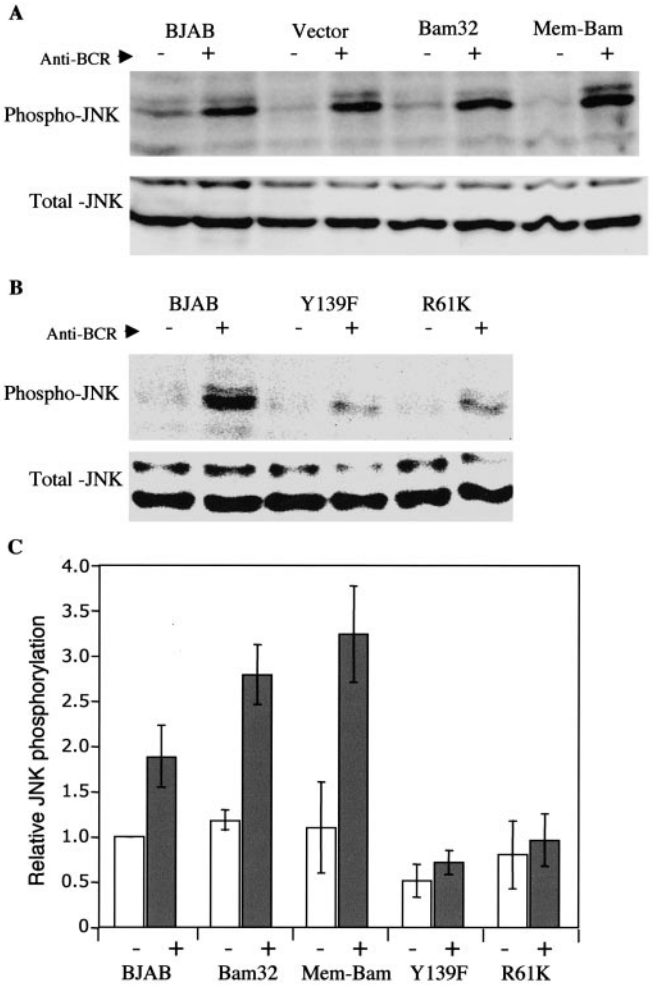

FIG. 6. **Disrupted JNK activation in Bam32 stable transfectants.** *A*, effect of Bam32 or MemBam32 (*Mem-Bam*) on JNK phosphorylation. Cells were stimulated with anti-BCR antibodies for 5 min and lysed, and JNK phosphorylation was determined by blotting sequentially with anti-phospho-JNK or anti-total JNK antibodies. *B*, effect of mutant Bam32 molecules on JNK phosphorylation. *C*, relative JNK phosphorylation was determined using a chemiluminescence imager. Results are expressed relative to unstimulated BJAB cells and represent the average and S.E. of 3–5 determinations per cell line.

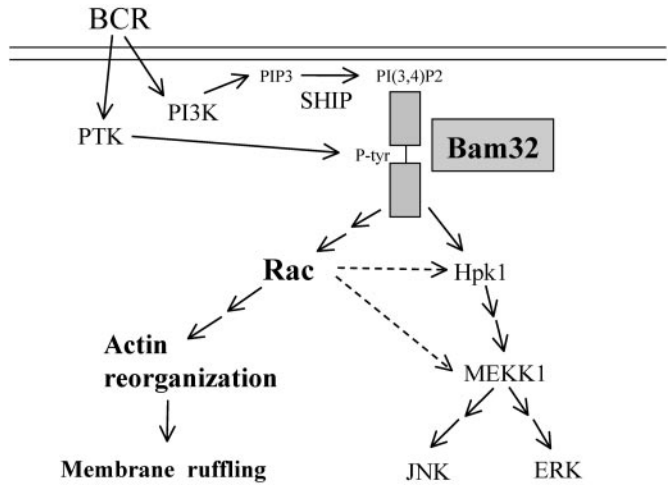

FIG. 7. **Model for Bam32 function integrating the results of this and other studies.** Bam32 is recruited to the plasma membrane through binding to PI(3,4)P2 and then phosphorylated by a Src family kinase (most likely Lyn). Membrane-bound, phosphorylated Bam32 enhances the activation of Rac1, stimulating actin reorganization and membrane ruffling. Active Rac1 also enhances JNK activation. Bam32 may also enhance activation of JNK and ERK acting through Hpk1 and MEKK. *Single arrows* indicate potentially direct interactions, whereas *double arrows* represent interactions that likely involve intermediate steps. *Dotted arrows* indicate that Bam32-enhanced activation of Rac1 may also feed into the MAPK pathway at the level of Hpk1 or MEKKs as discussed. *Boldfaced* text indicate aspects of Bam32 function discovered in this study. *P-tyr*, phosphorylated tyrosine.

ute to increased F-actin levels. The mechanisms by which antigen receptor signaling regulates the actin cytoskeleton are just beginning to be understood, but they clearly involve some lymphocyte-specific regulatory mechanisms. Our results suggest that Bam32 provides a specific mechanism linking BCR signaling with the cytoskeleton.

How does Bam32 regulate BCR-induced Rac1 activation? Several possibilities can be envisaged. First, Bam32 may physically interact with and regulate Rac1 subcellular targeting and activation in B cells. In a number of experiments, we have been unable to detect a direct physical association of Bam32 with Rac1 by co-immunoprecipitation.[2,3] However, any interaction of Bam32 and Rac1 would likely be facilitated by their close association with the plasma membrane, so it is possible that the interaction may be very sensitive to disruption by detergents. There is also an issue concerning the recovery of proteins associated with the cytoskeleton; we have found that a relatively large proportion of Bam32 pellets with the insoluble fraction in commonly used detergents.[2] Also, in one study the co-expression of Bam32 with Src-family kinases, which leads to its constitutive phosphorylation, caused a specific reduction in Bam32 recovery from cell lysates (30). Thus, we cannot rule out the possibility that Bam32 directly or indirectly associates with Rac1/WAVE complexes anchored to the cytoskeleton. Membrane association of Rac1 is thought to be driven primarily by its C-terminal prenylation (45, 46); however, our data suggest that Bam32 may regulate the targeting of Rac1 to BCR signaling complexes. Also consistent with the hypothesis that Bam32 regulates the sub-membrane targeting of Rac1, we have found that Bam32 influences the partitioning of Rac1 to lipid raft fractions.[3]

Another possibility is that Bam32 regulates molecules upstream of Rac1 activation in B cells. The switching of inactive (Rac-GDP) to active forms of Rac (Rac-GTP) is mediated by Dbl family Rac-guanine nucleotide exchange factors (GEFs) including Vav, Sos, Tiam1, and SWAP-70 (47). In addition to the Dbl-family of Rac-GEFs, some members of the CDM family adaptor proteins such as Dock180 and Dock2 appear to bind to and promote activation of Rac proteins (48). Intriguingly, a recent study suggests that the association of Dock2 and a PH domain-containing molecule called Elmo1 is critical for Dock2-induced Rac activation (49). It is thus possible that Bam32, like Elmo1, cooperates with specific Rac-GEFs to trigger BCR-induced Rac activation. Importantly, PI3K signaling is known to regulate Rac activation (50, 51), perhaps partially through influencing GEFs such as Vav that have PH domains (52). However, the mechanisms of PI3K-dependent amplification of Rac1 responses are not well understood, and it is tempting to speculate that Bam32 may play a role in linking PI3K signaling to Rac1 activation. Further work is needed to substantiate such possibilities.

Consistent with findings in chicken and mouse cells (27, 28), our data show that Bam32 can regulate BCR-induced activation of JNK in human lymphoma cells. It is well established that Rac1 plays an important role in the activation of JNK in response to activation by growth factor and antigen receptors (39, 53, 54); thus, it is likely that Bam32 regulates JNK activation at least partially through Rac1 (Fig. 7). Bam32 was recently reported to associate with the Hpk1 and regulate its activation (28), suggesting another possible mechanism by

which Bam32 may influence JNK activation. Hpk1 is related to the p21(Cdc42/Rac1)-activated kinase and may function as a MAP4K (55, 56). BCR-induced activation of the MAP3K MEKK1 is also reduced in Bam32-deficient B cells (28), consistent with the interpretation that Bam32 acts proximally to influence MAP4K activity. Although it was originally reported that Hpk1 does not bind directly to Rac1 (56), Rac1 is known to functionally cooperate with Hpk1 and MEKKs in activating JNK (42, 57, 58); thus, it is entirely possible that Bam32 may coordinate Rac1 with MAP4Ks and/or MAP3Ks during BCR signaling, resulting in enhanced JNK activation. In summary, our results identify a functional linkage between Bam32 and Rac1 that can explain the effects of Bam32 on several downstream signaling events including actin remodeling, membrane ruffling, and JNK activation.

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Mechanisms of Signal Transduction:

The Adaptor Protein Bam32 Regulates Rac1 Activation and Actin Remodeling through a Phosphorylation-dependent Mechanism

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