THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 52, pp. 36202–36212, December 25, 2009

Vav and Rac Activation in B Cell Antigen Receptor Endocytosis Involves Vav Recruitment to the Adapter Protein LAB

Received for publication, July 2, 2009, and in revised form, September 14, 2009 Published, JBC Papers in Press, October 26, 2009, DOI 10.1074/jbc.M109.040089

Shikha Malhotra[‡], Susan Kovats[§], Weiguo Zhang[¶], and K. Mark Coggeshall[‡1]

From the [‡]Immunobiology and Cancer Program, Oklahoma Medical Research Foundation, and Department of Cell Biology, University of Oklahoma, Oklahoma City, Oklahoma 73104, the [§]Arthritis and Immunology Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104, and the [¶]Department of Immunology, Duke University Medical Center, Durham, North Carolina 27710

The signal transduction events supporting B cell antigen receptor (BCR) endocytosis are not well understood. We have identified a pathway supporting BCR internalization that begins with tyrosine phosphorylation of the adapter protein LAB. Phosphorylated LAB recruits a complex of Grb2-dynamin and the guanine nucleotide exchange factor Vav. Vav is required for activation of the small GTPases Rac1 and Rac2. All these proteins contribute to (and dynamin, Vav, and Rac1/2 are required for) BCR endocytosis and presentation of antigen to T cells. This is the first description of a sequential signal transduction pathway from BCR to internalization and antigen presentation.

All receptors for antigens stimulate a complex series of intracellular biochemical events to stimulate biological changes in the state of the host cell. Antigen receptors have common structural features that include a low molecular weight polypeptide bearing an immunoreceptor tyrosine-based activation motif. The tyrosines of the immunoreceptor tyrosine-based activation motif are phosphorylated to recruit a variety of Src homology 2 (SH2)[2]-containing lipid, tyrosine, and serine/threonine kinases, many adapter proteins, and several enzymes. Together, the enzymes generate second messengers that include increased cytoplasmic Ca^{2+}, inositol trisphosphate, diacylglycerol, and phosphoinositide lipids to cause cell activation (reviewed in Refs. 1–3).

BCR signal transduction causes B cells to increase expression of MHC class II, CD80, and CD86 (4). However, the expression of these proteins by themselves is insufficient to promote B-T interaction and the production of high affinity, class-switched antibodies and long lived memory. Rather, T-B interaction is driven by antigenic peptides derived from BCR-endocytosed antigens (5). Many studies have identified signal transduction pathways that support transcription factor activation leading to up-regulation of surface proteins that support B-T interaction. Very few studies have shown a connection between those signaling events and BCR internalization following antigen binding.

Other receptors in the immune system that are similar to BCR include the T cell antigen receptor and a variety of immunoglobulin receptors, including IgG receptors FcγRI, -II, and -III and IgE receptors FcεRI and -II. The IgG receptors generally do not endocytose their targets upon engagement; instead, these receptors phagocytose large particles having IgG bound to them. The high affinity FceRI is endocytosed in a lipid raft using the GTPase dynamin (6). The low affinity FcεRII uses clathrin and dynamin for endocytosis (7). The T cell antigen receptor is endocytosed after antigen binding along with a protein complex that contains the tyrosine kinase ZAP-70 and two tyrosine-phosphorylated adapter proteins LAT (linker of activated T cells) and SLP-76 (8). T cell antigen receptor internalization is associated with immunoreceptor tyrosine-based activation motif phosphorylation and recruitment of ZAP-70, phosphorylation of ZAP-70 at Tyr-292, and subsequent recruitment of the adapter protein Cbl to phosphorylated ZAP-70 (9). T cell antigen receptor internalization also involves WASp (Wiskott Aldrich syndrome protein), which links actin reorganization and the small GTPase Cdc42 (10). WASp recruits Intersectin 2, a Dbl homology-containing protein that potentially activates Cdc42 to stimulate WASp-catalyzed actin reorganization.

We recently reported that receptor-triggered BCR endocytosis required Vav1 and/or Vav3 isoforms such that BCR endocytosis was completely blocked in B cells of Vav1,3$^{-/-}$ mice. Other BCR-triggered functions in Vav1,3-deficient B cells like up-regulation of MHC class II, CD80, and CD86 and signal transduction events like the influx of Ca^{2+}, the activation of MAPK modules, and of Akt were equivalent to those of wild-type B cells. Thus, Vav1 and/or -3 are uniquely and absolutely required for BCR endocytosis, whereas Vav2 can substitute for all other BCR-triggered functions, including B cell development and maturation.

Vav contains a Dbl homology domain, a pleckstrin homology (PH) domain, and a C-terminal protein interaction domain that includes an Src homology 2 (SH2) domain flanked by two SH3 domains (11). Vav is activated by tyrosine phosphorylation at Tyr-174 (12), which allows the catalytic Dbl homology domain to act on small GTPases (13). Vav phosphorylation is achieved by Vav recruitment to the site of tyrosine kinases associated

[1] To whom correspondence should be addressed. Tel.: 405-271-7209; E-mail: mark-coggeshall@omrf.ouhsc.edu.

[2] The abbreviations used are: SH, Src homology; BCR, B cell antigen receptor; MFI, mean fluorescence intensity; PC, phosphorylcholine; PH, pleckstrin homology; PI3K, phosphatidylinositol 3-kinase; Ova, ovalbumin; siRNA, small interfering RNA; PE, (*R*)-phycoerythrin; GST, glutathione *S*-transferase; GFP, green fluorescent protein; MHC, major histocompatibility complex; IL, interleukin; MAPK, mitogen-activated protein kinase.

with the receptors. Vav is recruited to the scaffolding protein LAT in T cells (14) and to phosphatidylinositol 3-kinase (PI3K)-generated lipids in many cytokine receptors (15–18). Both CD19 tyrosine phosphorylation (19) and PI3K-generated 3-phosphoinositide lipids (20) are induced upon BCR stimulation. It is not clear which of these mechanisms are operative in supporting Vav activation during BCR endocytosis.

The Vav family of proteins (Vav1, -2, and -3) acts as guanine nucleotide exchange factors for many small GTPases, including Rac1/2, RhoA/G, and possibly Cdc42 (11). Vav1 and -3 isoforms prefer Rac1/2 and RhoA/G but do not recognize Cdc42 (21). Vav2 is capable of causing GTP loading of Cdc42 and Rac1/2 but has less activity toward RhoA/G (22, 23). It is not clear which of these small GTPases is the molecular target of Vav in BCR endocytosis.

Here, we have tested the role of these small GTPases using RNA interference to selectively down-regulate their expression and show that Rac1 and Rac2 are required for BCR endocytosis, whereas other Vav targets are not. We also explored the mechanism by which Vav is activated in B cells. We found that PI3K is not required for BCR endocytosis, Vav phosphorylation, or Rac-GTP loading, whereas the protein interaction domain of Vav is involved in these processes. Vav bound to the B cell adapter protein LAB (linker of activated B cells) in a complex that also contains the GTPase dynamin and Grb2. LAB-deficient B cells show an incomplete defect in BCR endocytosis, Vav tyrosine phosphorylation, and Rac activation. We conclude that Vav is activated by recruitment to LAB and another unidentified adapter protein(s), which serves to stimulate Rac-GTP loading to support BCR endocytosis.

MATERIALS AND METHODS

Mice—Animals were obtained from The Jackson Laboratories (Bar Harbor, ME), Duke University Medical Center, or bred at our facility. Wild-type C57BL/6 mice were used as controls for $LAB^{-/-}$. All experiments have been approved by the IACUC.

Cell Culture—The murine B cell lymphoma M12g4Rd (24) and the murine T cell hybridoma DO.11.10 (25) were maintained at 37 °C in RPMI 1640 medium containing 10% fetal bovine serum, 50 μM 2-mercaptoethanol, and penicillin/streptomycin.

Materials—$F(ab')_2$ antibodies to BCR were from Pierce and Zymed Laboratories Inc. Antibodies to Grb2, phosphorylated Akt (pAkt), and Rac were purchased from Cell Signaling (Danvers, MA). Cy5-conjugated rabbit anti-mouse IgG was purchased from Jackson ImmunoResearch (West Grove, PA). Phosphocholine (PC)-conjugated ovalbumin (Ova) or keyhole limpet hemocyanin were obtained from Biosearch Technologies, Inc. (Novato, CA). Ova peptide-(323–339) was purchased from GenScript Corp. (Piscataway, NJ). The Rac activation kit and anti-phosphotyrosine antibodies were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Mouse monoclonal antibody to LAB/NTAL was purchased from Abcam (Cambridge, MA). Antibodies to Vav, phosphorylated Vav (pVav), actin, Rho family GTPases, normal rabbit IgG, siRNA targeting mouse Grb2, LAB, and Rho family GTPases and control (scrambled) siRNA were bought from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). (*R*)-Phycoerythrin (PE)-conjugated goat anti-rabbit IgG was from Southern Biotech (Birmingham, AL). LY294002, latrunculin B, and guinea pig complement was obtained from Sigma or Calbiochem. Recombinant protein A/G-agarose was from Invitrogen. Mouse IL-2 enzyme-linked immunosorbent assay kit and fluorochrome-conjugated antibodies to CD19 were bought from BD Biosciences.

Cell Purification and Stimulations, Lysates—Splenic B cells were prepared from the spleens of 5–8-week-old BALB/c mice as described earlier (26). In brief, erythrocytes were removed by osmotic lysis, and T cells were removed by incubation with anti-Thy-1.2 followed by guinea pig complement. The resulting population was 85–95% pure B cells, based on staining with antibodies to CD19 or B220. For immunoprecipitations, 10×10^6 cells were washed and resuspended in 100 μl of phosphate-buffered saline and stimulated at 37 °C with 15–20 μg/ml $F(ab')_2$ rabbit anti-mouse IgG or IgM. In the indicated experiments, LY294002 was added to temperature-equilibrated cells for 10 min at 37 °C prior to cell stimulation. Cells were then lysed in TN-1 buffer (50 mM Tris-HCl, pH 8.0, 125 mM NaCl, 10 mM EDTA, 1% Triton, 10 mM NaF, 3 mM Na_3VO_4, 10 mM $Na_4P_2O_7$, 10 μg/ml aprotinin, 10 μg/ml leupeptin) and centrifuged at 16,000 $\times$ *g* for 10 min at 4 °C. The cleared lysate was incubated overnight on a rocker at 4 °C with 2–3 μg of immunoprecipitating antibody/ml. Protein A/G-Sepharose was added for 1 h, and immune complexes were washed five times in lysis buffer and eluted with SDS sample buffer. The proteins were separated by SDS-PAGE. The proteins were electrophoretically transferred to nitrocellulose membranes, probed with appropriate antibodies, and visualized by an enhanced chemiluminescence. The bands were quantitated by measuring individual chemiluminescence by using a Lumi-Imager F1 workstation (Roche Applied Science).

BCR Internalization Assay—B cells were incubated with 15 μg/ml unlabeled anti-BCR (primary cells) or 5 μg/ml PC-keyhole limpet hemocyanin (M12g4Rd) for 30 min at 4 °C and washed to remove unbound antibody or antigen. The cells were then incubated at 37 °C for an additional 30 min (for anti-BCR) or 4 h (for antigen). The receptors remaining on the surface were detected by a fluorescently labeled anti-BCR (M12g4Rd) or anti-rabbit Ig (primary cells). Mean fluorescence intensity (MFI) of the samples was determined using a FACS Scan. As controls, samples were either stained with secondary antibody alone or the samples were maintained at 4 °C to prevent BCR internalization. Percent internalized BCR was obtained by the MFI of the sample and the control in the following calculation: percent internalization = ((MFI no internalization, 4 °C sample control) − (MFI sample) ÷ (MFI no internalization, 4 °C sample control)) × 100.

Transfections—For transfections, plasmids containing Δ6AVav, dominant-negative *Rac1* and *Rac2* genes (15), and the other Vav constructs were cloned into the expression vector pLEGFP-C1, which co-expresses green fluorescent protein (GFP) along with the gene of interest. The GST-Grb2 fusion protein has been described elsewhere (27). M12g4Rd cells were transfected by electroporation as follows: 1×10^7 cells were electroporated with the indicated plasmid DNA in a 4-mm gap cuvette containing warm RPMI 1640 medium,

using a Bio-Rad GenePulser at 340 V and 960 microfarads. The cells were resuspended in 8–10 ml of warm growth medium and cultured for 36–72 h before analysis. Following incubation, cells were sorted for equal expression of GFP using MoFlo. For siRNA targeting of LAB, Grb2, or small GTPases, the M12g4Rd cells were electroporated as above with commercial (Santa Cruz Biotechnology) siRNA or scrambled siRNA and kept at 37 °C as above.

Rac Assay—The protocol is based on a Rac-GTP pulldown method using GST-p21-activated kinase as described (28). Briefly, cells were stimulated and lysed as above, and the lysates were incubated with GST-PAK pre-bound to glutathione-agarose beads. After washing, the beads were incubated with Laemmli SDS-PAGE sample buffer, and the eluted proteins were subjected to SDS-PAGE. After the transfer of proteins to a nitrocellulose membrane, Western blotting was performed using a pan monoclonal antibody to Rac isoforms. The amount of GTP-loaded Rac was quantitated by measuring the mass of GTP-Rac in comparison with the mass of total Rac. The value was then normalized to the GTP-Rac/total Rac ratio of unstimulated cells.

Antigen Presentation Assay—M12g4Rd B cells were cultured in RPMI 1640 medium in with PC-Ova (5 μg/ml) to generate class II MHC loaded with Ova peptide on their surface. The cells were washed and co-cultured at 100,000 cells per well with the T cell hybridomas (DO.11.10; (25)) at 100,000/well, and the co-culture was maintained at 37 °C for 24 h. Following incubation, the plates were frozen, thawed, and centrifuged at 2,000 × *g*, and supernatants were harvested. The supernatant was then assayed for IL-2. In the indicated experiments, B cells were co-cultured with DO.11.10 in the presence of 5 μg/ml of an Ova peptide ISQAVHAAHAEINEAGR.

Statistics—All experiments were done in triplicate and repeated multiple times, as indicated in the figure legends. The data are reported as the average and S.E. of the triplicate samples from within each experiment.

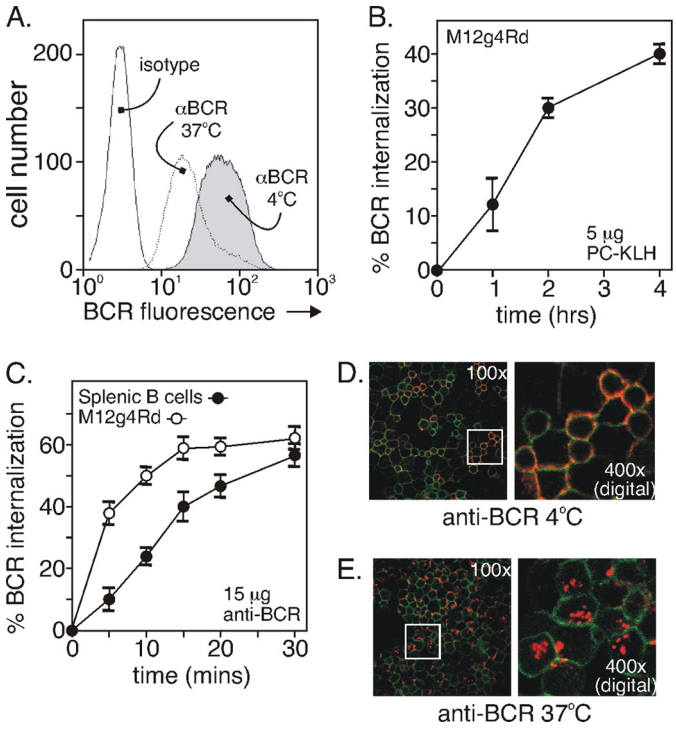

FIGURE 1. **BCR endocytosis assay.** B cells were incubated with goat antibodies to BCR, held at 4 °C or incubated at 37 °C for various times, and then stained with PE-labeled anti-goat Ig or isotype control. *A* shows a typical result of BCR expression level of cells kept at 4 °C (*solid gray*) or incubated for 30 min at 37 °C (*dotted line*); the staining level of the isotype control is shown with a *solid line*. % BCR internalization is determined as the ratio of MFI of the 37 °C sample to the 4 °C sample, minus the MFI or the isotype, as described (29). *B* shows the kinetics of BCR internalization of the M12g4Rd cell line using antigen (5 μg/ml PC-keyhole limpet hemocyanin (*KLH*)), and *C* shows primary splenic B cells (*closed circles*) and M12g4Rd (*open circles*) with 15 μg/ml anti-BCR. All samples were incubated for the indicated time before analysis of remaining surface BCR. The result is the average and S.E. of triplicate samples and representative of many experiments. *D* and *E* are confocal fluorescence photomicrographs of primary splenic B cells stained with Cy5-anti-BCR and fluorescein isothiocyanate-labeled anti-CD45 and kept at 4 °C (*D*) or incubated for 30 min at 37 °C (*E*). The images on the *left* are ×100 magnification; the images on the *right* are a digital magnification (×4) of the area within the *white box*. The results are representative of many experiments.

RESULTS

BCR Is Endocytosed upon Treatment with Antigen or Anti-BCR Antibody—We used two models for BCR endocytosis: primary splenic B cells or a B cell line (M12g4Rd (24)) that has been transfected with cDNA encoding an antigen-specific (PC) BCR. Each model has advantages and disadvantages. Splenic B cells can be derived from various knock-out mice to test the contribution of a known signaling protein to BCR endocytosis. Likewise, splenic B cells form a distinct BCR cap in 2 min following anti-BCR treatment and hence are conducive to fluorescence co-capping studies. However, primary B cells cannot be transfected with dominant-negative constructs or RNA interference reagents and cannot be used for antigen presentation studies because of their polyclonal antigen specificities. The M12g4Rd cell line is easily transfected and can be used for antigen presentation studies with PC-labeled proteins (24), but it does not form a BCR cap.

For endocytosis measurements, B cells are stimulated with unlabeled antigen or goat anti-BCR antibodies at 4 or 37 °C. At various times, the B cells are stained with PE-labeled anti-mouse BCR antibodies (for antigen treatments) or PE-donkey anti-goat (for anti-BCR treatments) and analyzed by flow cytometry. A typical result is shown in Fig. 1*A*. BCR expression is high in cells held at 4 °C (Fig. 1*A*, *solid gray*) and lower in cells incubated at 37 °C with anti-BCR antibodies (*dotted line*). The *solid line* in Fig. 1*A* marks the PE-anti-goat isotype control, showing only background staining. The percent internalization is determined as the ratio of the MFI at 37 and 4 °C, as described in detail previously (29). Using this measurement, we find that both models readily endocytose their BCR upon antigen stimulation (Fig. 1*B*) or anti-BCR stimulation of the M12 cell line or primary B cells (Fig. 1*C*).

To ensure the receptor is contained within the B cell, we stimulated primary splenic B cells with PE-labeled anti-BCR for 30 min at 4 °C (Fig. 1*D*) or at 37 °C (Fig. 1*E*). The B cells were fixed and stained with antibodies to the surface protein, CD45, and visualized by confocal fluorescence microscopy. The images show the red-stained BCR within the cells that are also stained with anti-CD45 to mark the plasma membrane. Our earlier report shows that the receptor is not shed from the sur-

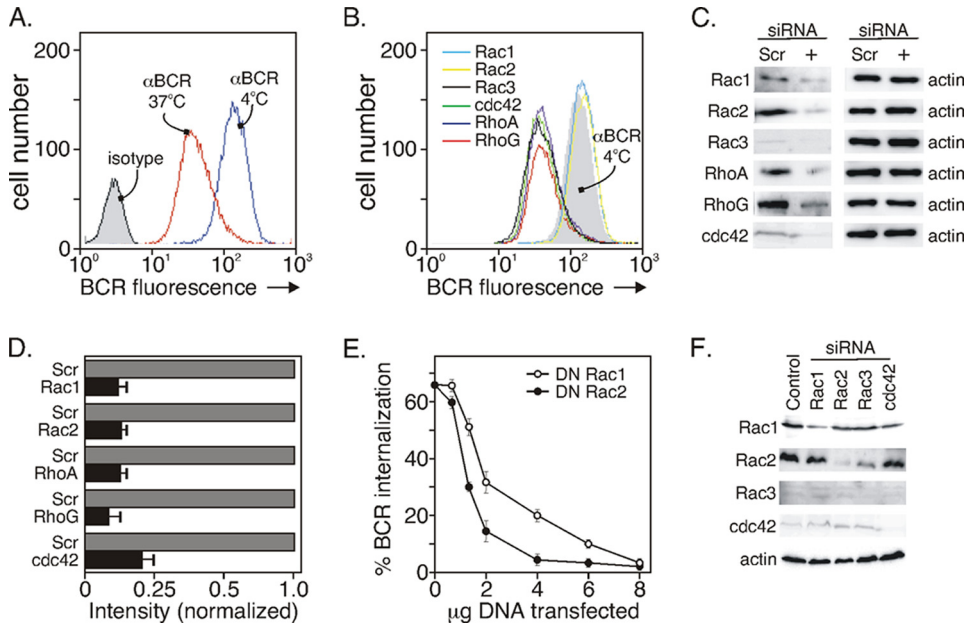

FIGURE 2. **Rac1 and Rac2 but not other Vav targets are required for BCR endocytosis.** *A,* M12g4Rd B cell line was incubated with anti-BCR antibodies for 30 min at 37 °C, when the remaining surface BCR after internalization is detected with fluorochrome-labeled anti-Ig. B cells stained with isotype-matched anti-Ig (*gray peak*) show autofluorescence only; anti-BCR-treated B cells kept at 4 °C (*blue line*) show a high level of BCR fluorescence; anti-BCR-treated B cells transferred to 37 °C (*red line*) show an intermediate fluorescence, indicating loss of surface BCR. These data are representative of many similar measurements. *B,* M12g4Rd B cells were transfected with siRNA targeting the indicated small GTPase. *Gray peak,* cells kept at 4 °C. Note that B cells with siRNA targeting Rac1 (*blue*) or Rac2 (*yellow*) show as much BCR fluorescence as those kept at 4 °C (*gray*). These data are representative of four such experiments. *C,* lysates of M12g4Rd B cells transfected with siRNA targeting the indicated small GTPase (+) or control scrambled (*Scr*) siRNA blotted with antibodies to the indicated small GTPase or actin as a loading control. The data are representative of three similar experiments. *D,* ratio obtained from the immunoblot data in *B* of small GTPase:actin for M12g4Rd B cells transfected with scrambled (*Scr*) or siRNA targeting the indicated small GTPase. Shown is the average and S.E. of three measurements, where the ratio is normalized to 1.0 for the scrambled siRNA. Rac3 was not detected in the lysates and is not quantitated. *E,* anti-BCR-induced BCR endocytosis of M12g4Rd B cells transfected with the indicated amount of cDNA encoding dominant-negative Rac1 or Rac2. The BCR endocytosis is the average and S.E. of three identical dose-response measurements. *F,* immunoblot of Rac1, -2, and -3, Cdc42 and actin after transfection of M12g4Rd B cells with scrambled siRNA (control) or siRNA targeting the indicated small GTPase. The blotting antibody target is shown at *left*. The data are representative of three identical experiments.

face, is not endocytosed at 4 °C, requires actin reorganization, and requires expression of both dynamin and either or both of the Vav1 or -3 isoforms (29). Thus, our assay accurately measures BCR endocytosis with either model.

Rac1 and -2 Are the Molecular Targets of Vav in BCR Endocytosis—To identify which small GTPase acts distal to Vav, we used siRNAs that individually target the small GTPases known to be Vav targets. The siRNAs were transfected into M12g4Rd, and the transfectants were compared with untransfected control cells in the BCR endocytosis assay described in Fig. 1. The controls are shown in Fig. 2*A*, showing the isotype staining control (*gray peak*), the sample kept at 4 °C showing maximal BCR fluorescence (*blue peak*), and the sample incubated for 30 min at 37 °C (*red peak*). The decrease in surface BCR fluorescence indicates BCR endocytosis, and we use the ratio of their MFI as described above.

Upon transfection of siRNA targeting small GTPases, we found (see Fig. 2*B*) that siRNA targeting either Rac1 or Rac2 completely blocked BCR endocytosis such that B cells expressing these RNA interference reagents have as much surface BCR as did the B cells held at 4 °C. In contrast, siRNA targeting Rac3, Cdc42, RhoA, or RhoG or scrambled siRNA to Rac1 or -2 (data not shown) had no effect, and endocytosis was as complete as the untransfected control cells shown in Fig. 2*B* (*red line*). We confirmed (Fig. 2, *C* and *D*) that protein expression of the siRNA target was indeed down-regulated by Western blots of whole cell lysates of the transfectants using antibodies to the individual small GTPases. Rac3 showed low-to-absent expression levels in B cells, so it was not quantitated. The data show indeed that transfection of B cells with all the siRNAs (+), but not the scrambled (Scr) control siRNA, efficiently knocked down expression of the targeted small GTPase to less than 20% of control (scrambled siRNA) levels.

As an additional test for Rac contributions to BCR endocytosis, we transfected M12g4Rd with N17 mutants of Rac1 or Rac2 having high affinity for GDP and hence unable to bind GTP (30). The transfection was done as a dose response, titrating the amount of dominant-negative Rac mutant and measuring the level of BCR endocytosis. We found (Fig. 2*E*) that both dominant-negative mutants of Rac were able to block BCR endocytosis in this model, with the Rac2 mutant consistently acting slightly more efficient than Rac1.

Rac1, Rac2, and Rac3 are over 90% identical and differ primarily in the region near the C-terminal *CAAX* (where *AA* is aliphatic amino acid) prenylation site (31). Because the siRNA reagents are proprietary commercial ones and do not list sequences targeted, we tested whether the siRNAs down-regulate the protein expression of their specific target alone or cross-react with related small GTPases. We therefore transfected M12g4Rd with scrambled siRNA (control) or siRNA targeting Rac1, Rac2, Rac3 or Cdc42 as an additional control, and we measured the expression level of Rac1, Rac2, Rac3, and the less related small GTPase, Cdc42 (32), and used actin as a loading control. We found (Fig. 2*F*) that the siRNAs were indeed specific and only knocked down protein expression of their advertised targets. The siRNA targeting Rac3 showed a slight decrease in Rac2 expression (an off-target effect), but this decrease in Rac2 level was not sufficient to alter BCR endocytosis (Fig. 2*B, purple line*). Together, these findings show that, of all the known small GTPases targeted by Vav isoforms, only Rac is required for BCR endocytosis and surprisingly that both Rac1 and Rac2 isoforms are separately required.

PI3K Is Not Required for BCR Internalization and Does Not Affect Vav or Rac Activation—In colony-stimulating factor-1-stimulated macrophages (15) and a variety of other cytokine receptors, PI3K is necessary for Vav membrane recruitment

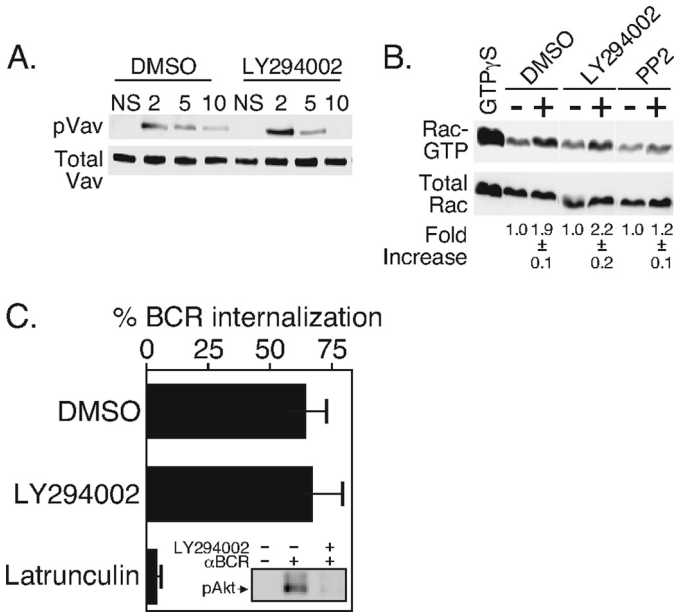

FIGURE 3. **BCR internalization, Vav phosphorylation, and Rac-GTP loading are independent of PI3K.** *A,* splenic B cells treated with 10 μM LY294002 or with an equal volume of DMSO solvent were left unstimulated (*NS*) or stimulated with 15 μg/ml anti-BCR for the indicated time in minutes. Lysates were subjected to Vav immunoprecipitations, and filters were probed with antibodies to phosphotyrosine (labeled *pVav*) or pan-Vav (*total Vav*). The data represent four similar experiments. *B,* splenic B cells were prepared and stimulated as in A (unstimulated, −; 5 min anti-BCR, +). Lysates were precipitated with GST-PAK and probed with antibodies to Rac as described previously (15). The data are representative of four identical experiments. The quantitation of Rac-GTP to total Rac for four experiments is shown below the immunoblot as a fold stimulation, where the unstimulated sample is normalized to 1.0. *GTPγS,* guanosine 5′-3-*O*-(thio)triphosphate. *C,* B cells prepared as in *A* and assessed for BCR internalization after 30 min using anti-BCR. The *inset* shows the level of phosphorylated Akt (*pAkt*) in unstimulated (−) or anti-BCR-stimulated (+) B cells with or without LY294002. The data represent three separate experiments.

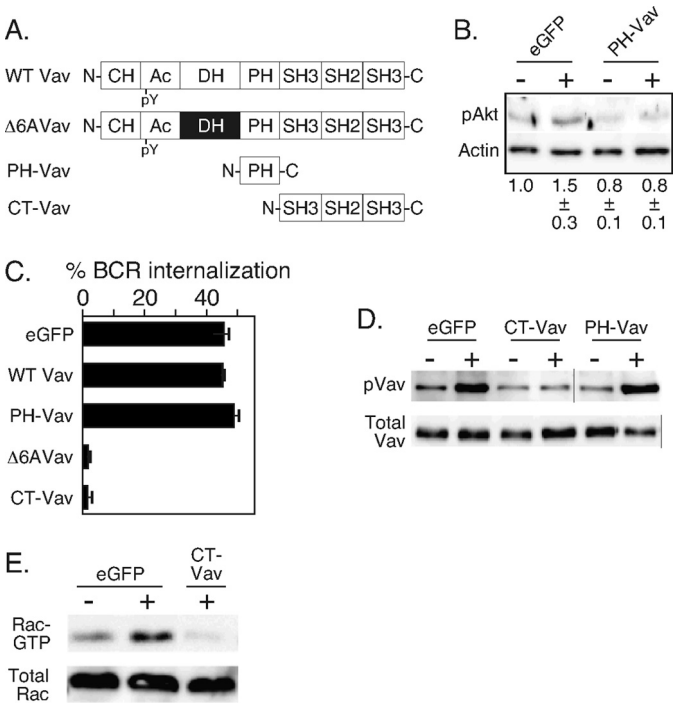

FIGURE 4. **Protein interaction region of Vav is required for Vav-Rac activation.** *A,* Vav constructs used in *B–E*. The Dbl homology (*DH*) domain is mutated in Δ*6A-Vav*, as described previously (15). *CH*, calponin homology; *WT*, wild type. *B,* Akt phosphorylation is blocked in cells overexpressing the Vav PH domain. M12g4Rd B cells were transfected with empty vector (*eGFP*) or PH-Vav (*PH-Vav*) and sorted for GFP expression. Lysates of unstimulated (−) or stimulated for 2 min with anti-BCR (+) were probed with antibodies to pAkt or actin as a loading control. The data are representative of three identical experiments, and the S.E. of those results are *below* the figure. *C,* anti-BCR-induced BCR internalization after 30 min in M12g4Rd B cells transfected and overexpressing the indicated Vav mutants using a vector that co-expresses GFP. The transfectants were sorted for GFP expression to isolate the transfected cells. The data are the average and S.E. of three separate experiments. *D,* Vav phosphorylation measured in unstimulated (−) or anti-BCR stimulated for 2 min (+) M12g4Rd B cells expressing the indicated Vav mutant. The transfectants were sorted on the basis of GFP expression as above. The data are representative of three experiments. *E,* Rac-GTP loading in M12g4Rd B cells transfected with the indicated Vav mutant, sorted for GFP expression as above. Cells were left unstimulated (−) or anti-BCR stimulated for 4 min (+). The data are representative of three separate experiments.

and tyrosine phosphorylation. To test if PI3K acts upstream of Vav tyrosine phosphorylation and Rac activation in mouse B cells, we used a chemical inhibitor of PI3K (LY294002). The level of Vav tyrosine phosphorylation is a marker of Vav activation (13) because phosphorylation relieves auto-inhibition allowing Vav to activate its small GTPase target. We consistently found that treatment of B cells with LY294002 increased rather than inhibited Vav tyrosine phosphorylation (Fig. 3*A*) and had no effect on Rac activation (Fig. 3*B*). Likewise, LY294002 did not alter the rate or extent of BCR internalization induced by anti-BCR (Fig. 3*C*), which requires Vav activity (29). Fig. 3*C, inset*, shows the level of phosphorylated Akt in stimulated or unstimulated B cells, treated or not with LY294002, to establish that PI3K activity was indeed blocked by the drug. The data indicate that PI3K is not needed for BCR-triggered Vav phosphorylation, Rac activation, or BCR endocytosis.

As an additional test for the contribution of PI3K to Vav activation, we overexpressed various Vav mutants shown in Fig. 4*A* in the M12g4Rd B cell line. The Δ*6A Vav* construct has a mutation within the catalytic domain and was used previously (15) to disrupt Vav/Rac-dependent functions. We earlier showed that expression of the Δ*6A Vav* mutant in M12g4Rd B cells completely blocked BCR endocytosis (29). For the experimental conditions, we overexpressed the isolated PH domain of Vav (which blocks PI3K-triggered Vav recruitment by consuming available 3-phosphoinositides (33)) and the isolated protein interaction domains present in the C terminus of Vav (CT-Vav). Expression of either the PH and C-terminal Vav domains interferes with Vav function by virtue of overexpression, above that of endogenous Vav. Accordingly, the overexpressed proteins adsorb mediators (phosphoinositides for the PH domain) or protein-binding sites (phosphotyrosine or proline-rich sequences for the SH2/3 domains).

To establish overexpression of the PH domain, we measured the BCR-induced phosphorylation of Akt, a PI3K-dependent event in B cells (34), and we found that Vav PH domain overexpression completely blocked this event (Fig. 4*B*). Thus, the PH domain was overexpressed sufficiently to block PI3K function. We have established overexpression of Δ6AVav in our previous report (29). It is difficult to establish overexpression (expression greater than endogenous Vav expression) with the CT-Vav mutant because there is no C terminus-specific Vav antibody that recognizes these constructs as well as endogenous Vav. We attempted probes using phosphopeptides con-

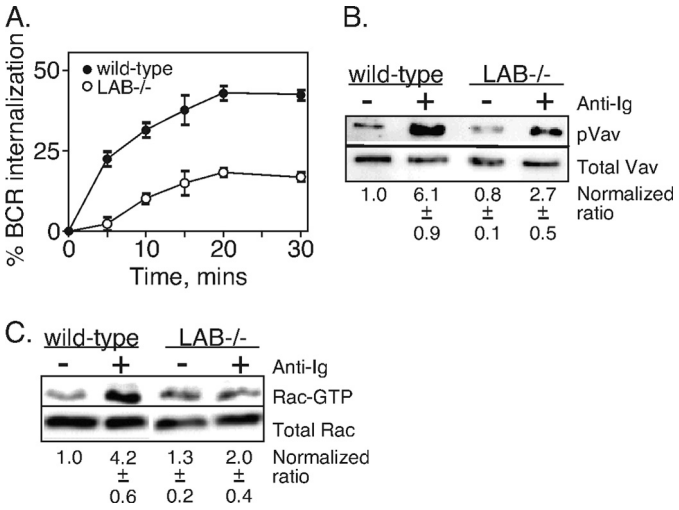

FIGURE 5. **LAB is required for optimal BCR internalization.** *A,* primary B cells were prepared from wild-type or LAB$^{-/-}$ mice splenocytes with anti-Thy-1.2 and guinea pig complement. BCR internalization was measured for the indicated times. The data are representative of five separate experiments and are the average and S.E. for one representative experiment. *B,* splenic B cells of wild-type or LAB$^{-/-}$ mice were left unstimulated (−) or stimulated with anti-BCR for 2 min (+). Lysates were subjected to immunoprecipitation with antibodies to Vav and probed with antibodies to phosphotyrosine (labeled *pVav*) or to Vav (*total Vav*). The ratio of pVav to total Vav for four experiments is shown *below* the figure. *C,* Rac activation using GST-PAK was as described in Fig. 3. Wild-type or LAB$^{-/-}$ splenic B cells were left unstimulated (−) or stimulated with anti-BCR for 5 min (+). The ratio of Rac-GTP to total Rac and normalized to the unstimulated sample is shown *below* the blot for four experiments.

taining a pYESP motif, a sequence found in both ZAP-70 (35) and SLP-76 (36) that directly bind the SH2 domain of Vav in far Western blots. Unfortunately, the peptide failed to bind endogenous Vav or any of the proteins derived from the transfected cDNAs. Nevertheless, and consistent with the result using LY294002 (Fig. 3), we found that overexpression of the Vav PH domain (to block PI3K function) did not alter BCR endocytosis (Fig. 4*C*). In contrast, overexpression of the C-terminal protein interaction domain of Vav or the Δ6AVav mutant completely blocked BCR endocytosis. Likewise, overexpression of the isolated C terminus but not the PH domain of Vav blocked BCR-triggered Vav phosphorylation (Fig. 4*D*) and formation of Rac-GTP (Fig. 4*E*). Thus, although we cannot formally establish overexpression of the C-terminal construct, the fact that we can disrupt Vav function by transfection of the Vav protein interaction domain suggests that Vav protein interactions and not recruitment to 3-phosphoinositides are key in Vav activation for BCR endocytosis.

Linker of Activation in B Cells (LAB/NTAL) Is Required for Optimal Vav Phosphorylation and BCR Internalization—Previous studies indicated that LAB, a scaffolding protein with multiple tyrosine phosphorylation sites, is important in BCR internalization (37). We confirmed the defect in BCR internalization of B cells from LAB$^{-/-}$ mice (Fig. 5*A*) using our endocytosis measurement and applying anti-BCR antibodies. We found that B cells from LAB$^{-/-}$ animals showed an approximate 50% decrease in the extent of BCR internalization compared with wild-type B cells.

The data in Fig. 4 uncovered an important role for the protein interaction C-terminal domain of Vav in regulating its phosphorylation and the formation of Rac-GTP. If LAB is involved in recruitment of Vav as LAT is involved in T cells (14, 38), the LAB-deficient B cells would show a defect in Vav phosphorylation. We tested this possibility by probing Vav immunoprecipitates from resting or stimulated B cells of wild-type or LAB$^{-/-}$ mice with antibodies to phosphorylated Vav (pVav). We found (Fig. 5*B*) that Vav from LAB-deficient B cells showed an approximate 50% decrease in phosphotyrosine level relative to that of wild-type B cells. The decreased Vav tyrosine phosphorylation was associated with a corresponding decrease in Rac-GTP loading, as shown in Fig. 5*C*. These findings suggest that LAB at least in part regulates Vav activation, possibly by recruitment of the C-terminal protein interaction domain to permit Vav tyrosine phosphorylation. Other adapter protein(s) in B cells likely can substitute for LAB in LAB-deficient B cells. The data are remarkably similar to findings in LAT-deficient Jurkat T cells that likewise show suboptimal Vav tyrosine phosphorylation (38).

LAB Forms a Complex with Dynamin and Vav—Our earlier work established an important role for the GTPase dynamin, in addition to Vav (29). Both dynamin and Vav co-capped with BCR upon BCR stimulation. Likewise, in data not shown, we observed that LAB and BCR co-cap upon BCR stimulation, as was shown previously (37). Given these previous findings, it may be that both Vav and dynamin physically interact with the LAB scaffolding protein. To test this possibility, we immunoprecipitated dynamin or Vav from resting or BCR-stimulated B cells and probed the immunoprecipitates with antibodies to LAB. We found (Fig. 6*A*) that LAB associated with both proteins but only in BCR-stimulated samples, indicating that LAB can bind both dynamin and Vav after B cell activation and suggesting the involvement of LAB tyrosine phosphorylation. The finding raises the possibility that LAB can form individual complexes; some that contain only Vav and others that contain only dynamin. Alternatively, LAB might form a single complex that contains both Vav and dynamin. Because LAB contains 10 phosphorylatable tyrosines (39, 40), it is likely that LAB recruits other interacting partners.

We applied an immunodepletion approach to distinguish these possibilities. Here, the strategy is to remove all Vav from the lysate and ask if there are remaining complexes of LAB-dynamin. Similarly, we removed all dynamin from lysates and asked if there is remaining LAB-Vav complexes. We exhaustively immunodepleted Vav or dynamin by three sequential immunoprecipitations from B cell lysates prepared from resting (−) or BCR-stimulated (+) splenic B cells. As a control, we depleted samples with normal rabbit immunoglobulin. The material obtained from the first and third immunoprecipitation was tested by Western blot (Fig. 6*B* for Vav immunodepletion and Fig. 6*D* for dynamin immunodepletion) and showed the presence of the target protein in the first but not the third immunoprecipitate. Thus, we successfully depleted the target protein from the lysate. The supernatant was then subjected to immunoprecipitation with antibodies to dynamin or Vav. We found antibodies to dynamin failed to co-precipitate LAB (Fig. 6*C*), indicating the LAB-dynamin complexes were removed in the immunoprecipitation with anti-Vav. Likewise, we found no LAB-Vav complexes in the samples immunodepleted with anti-

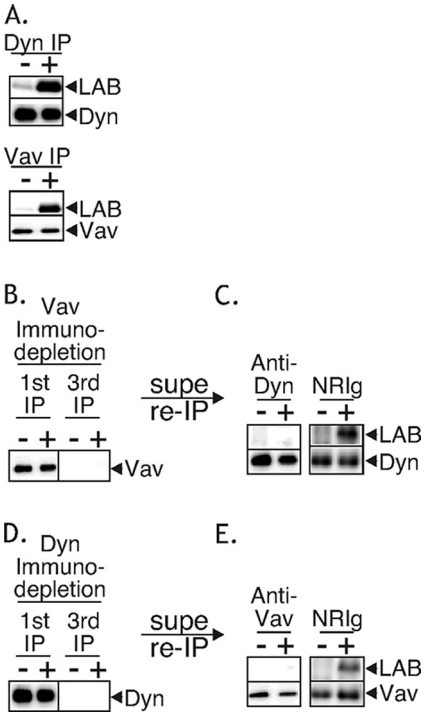

FIGURE 6. **LAB-dynamin-Vav association.** *A,* B cells from spleens of wild-type mice were stimulated (+) for 2 min with anti-BCR or left unstimulated (−). Detergent lysates were immunoprecipitated (*IP*) with dynamin (*Dyn*) or Vav antibodies, and the material was resolved by SDS-PAGE and transferred to nitrocellulose filters. The filters were probed with antibodies to LAB, Vav, or dynamin as indicated. *B* and *C,* anti-Vav (*B*) or anti-dynamin (*C*) or NRIg immunoprecipitates from unstimulated (−) or stimulated (+) samples were probed for the immunoprecipitation target to test for target depletion. The NRIg samples are not shown. *supE re-IP,* supernatant reimmunoprecipitated. *D* and *E,* supernatants of Vav (*B*), dynamin (*C*), or NRIg immunodepletions were subjected to immunoprecipitation with antibodies to dynamin (*D*) or Vav (*E*). The immunoprecipitates were probed with antibodies to LAB. The identical experiment has been done three times with similar results.

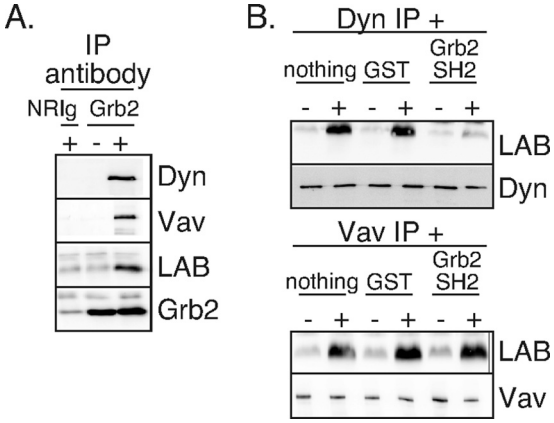

FIGURE 7. **Grb2 is the adapter protein for dynamin to bind LAB.** *A,* splenic B cells of wild-type mice were left unstimulated (−) or stimulated with anti-BCR for 2 min (+). The cells were lysed, and lysates subjected to immunoprecipitation (*IP*) with antibodies to Grb2. The immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, and probed with the antibodies indicated at *right*. The data are representative of three identical experiments. *B,* dynamin (*Dyn*) (*top*) or Vav (*bottom*) immunoprecipitates from unstimulated (−) or anti-BCR-stimulated (+) samples were co-incubated with nothing, recombinant GST alone (0.1 μg/ml), or a GST-Grb2 SH2 domain fusion (0.1 μg/ml). The immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies to LAB. The data are representative of three identical experiments.

bodies to dynamin (Fig. 6*E*). In contrast, when the samples were depleted three times with NRIg, we found complexes of LAB-dynamin (Fig. 6*C*) and LAB-Vav (Fig. 6*E*), indicating that the associated proteins survive the immunodepletion protocol. The data suggest that there are only single complexes of LAB bound to both Vav and dynamin and not individual complexes of LAB-Vav and LAB-dynamin. We cannot exclude single complexes of LAB-Vav or LAB-dynamin that are themselves associated.

Grb2 Adapter Is Needed for BCR Internalization and Bridges Dynamin and LAB—There is no immediately obvious way by which LAB or phosphorylated LAB can associate with dynamin. Unlike Vav, dynamin lacks SH2 and SH3 domains, but dynamin contains a proline-rich region that is known to associate with SH3 domains of various proteins, including that of Grb2 (41). LAB contains five tyrosine residues in a Y*X*N*X* sequence that upon phosphorylation are able to engage the SH2 domain of Grb2 (39, 40). Thus, a distinct possibility is that Grb2 recruits dynamin to tyrosine-phosphorylated LAB. We tested this possibility by immunoprecipitation of Grb2 and probing with antibodies to proteins within the complex: dynamin, Vav, and LAB. We found (Fig. 7*A*) that Grb2 immunoprecipitates did indeed contain all three proteins from activated but not unstimulated splenic B cells, consistent with the notion that dynamin, Vav, and LAB are present within a single complex.

We hypothesize that Grb2 and dynamin are associated with tyrosine-phosphorylated LAB because the SH2 domain of Grb2 is bound to one or more of the five Grb2-binding sites on LAB. To test this hypothesis, we immunoprecipitated the LAB-dynamin complex overnight in the presence or absence of the recombinant SH2 domain of Grb2 as a GST fusion protein as described previously (27). The recombinant SH2 domain of Grb2 maintains its preference for the p*YXNX* sequences as does the full-length protein, as we previously found (42). The expectation in this experiment was that the recombinant SH2 domain, being in excess, would compete with the endogenous Grb2 for binding to LAB and hence disrupt dynamin binding. Indeed, probing the dynamin immunoprecipitates for LAB (Fig. 7*B*) showed that LAB failed to co-immunoprecipitate when the recombinant SH2 domain of Grb2 was present, but not when the GST fusion partner alone was present. These findings are consistent with the hypothesis that a dimer of Grb2-dynamin associates with phosphorylated LAB through the Grb2 SH2 domain. In contrast, including the recombinant SH2 domain of Grb2 did not affect the association of Vav to LAB (Fig. 7*B*), indicating these proteins do not associate through the Grb2 SH2 domain.

Grb2 Is Required for Optimal B-T Interaction—The data above suggest Grb2 is an important protein for dynamin recruitment to permit BCR internalization, antigen processing, and presentation. To more rigorously test a possible connection between Grb2 and antigen-triggered BCR internalization, we applied siRNA targeting Grb2 or a scrambled siRNA to M12g4Rd B cells and tested their ability to internalize their BCR. We found that siRNA targeting Grb2 reduced BCR internalization induced by anti-BCR to about half the level of untreated B cells or B cells exposed to scram-

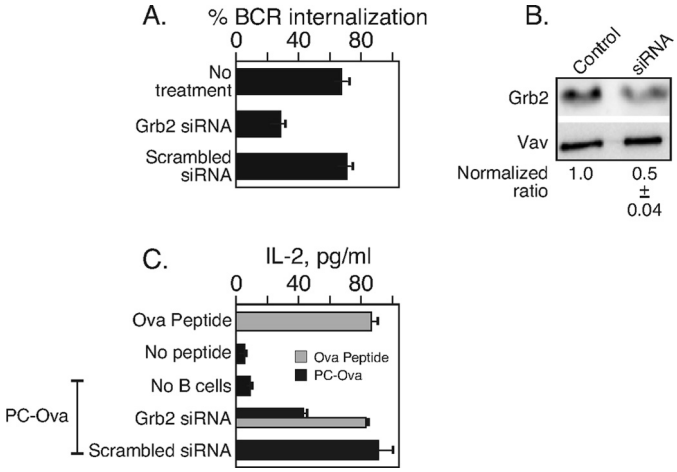

FIGURE 8. **Grb2 is required for optimal BCR endocytosis and antigen presentation.** *A,* M12g4Rd B cells were transfected with the indicated siRNA material, washed, and incubated for 48 h. The cells were then subjected to the BCR internalization assay described in Fig. 1 using anti-BCR. *B,* extent of the Grb2 knockdown was determined by probing total cell lysates with antibodies to Grb2 or Vav as a loading control. The amount of Grb2 was normalized to Vav, and the ratio is shown *below* the blot and are the average and S.E. of four experiments. *C,* M12g4Rd were transfected as in *A* and incubated with PC-Ova protein for 4 h. The cells were washed and mixed with DO.11.10 T cells for 24 h. Samples represented by *gray bars* received ISQAVHAAHAEINEAGR, the Ova peptide corresponding to Ova residues 323–339. The supernatants were assayed for IL-2 production, and the data shown are the average and S.E. of triplicate samples. The data are representative of four separate experiments.

bled siRNA (Fig. 8*A*). Transfection with siRNA targeting Grb2 reduced Grb2 protein expression about 50%, consistent with the reduction in BCR endocytosis (Fig. 7*B*). The data are consistent with a critical role for Grb2 in recruiting dynamin to LAB to support BCR endocytosis.

Once internalized, the BCR and its bound antigen traffic to lysosomes, where the protein antigen is digested into peptides. After lysosome digestion, the peptides are loaded onto MHC class II for presentation to T cells. Thus, conditions that block BCR endocytosis should likewise block presentation of antigenic peptides to T cells. To test this possibility, we applied an antigen presentation system using PC-Ova incubated with M12g4Rd B cells and allowed the Ova-derived peptides to be presented to the Ova-specific T cell line, DO.11.10, as described previously (29). In this model, the responder DO.11.10 T cells are used as an indicator for the presence of antigen (PC-Ova) that was endocytosed via BCR and processed into peptides. The T cells in the presence of the M12g4Rd B cells will also respond by IL-2 production upon addition of the cognate Ova peptide corresponding to residues 323–339 (ISQAVHAAHAEINEAGR), because the peptide will displace existing peptides bound to B cell MHC class II (43).

For these studies, M12g4Rd was pulsed with 5 μg/ml PC-Ova for 4 h to allow endocytosis. The B cells were washed and cocultured with DO.11.10 T cells for an additional 24 h to stimulate IL-2 production. The amount of IL-2 present in the supernatant was measured by enzyme-linked immunosorbent assay, and the data are shown in Fig. 8*C*. Addition of the cognate Ova peptide corresponding to residues 323–339 to a culture containing M12g4Rd and DO.11.10 T cells stimulates the maximum amount of IL-2 in this system (about 80 pg/ml) and served as the positive control. The absence of peptide or protein antigen PC-Ova (no peptide) and the absence of M12g4Rd B cells (no B cells) represent the negative controls. M12g4Rd B cells expressing the same siRNA targeting Grb2 (Grb2 siRNA) but not the scrambled siRNA lowers the DO.11.10 about 50%, consistent with the extent of the Grb2 knockdown shown in Fig. 8*B* and the effect on BCR endocytosis shown in Fig. 8*A* (*black bars*). However, addition of the exogenous 323–339-residue Ova peptide to the B cells expressing Grb2 siRNA completely reconstituted IL-2 production (Fig. 8*A gray bars*). This peptide control indicates there is no defect in the presentation process itself by B cells expressing the Grb2 siRNA and focuses the defect on the BCR endocytic process. Thus, Grb2 is an important part of the signal transduction pathway leading to BCR internalization and B-T interaction.

DISCUSSION

Mature B cells bind soluble protein antigens through a unique and clonally restricted BCR. Antigen binding stimulates a complex signal transduction biochemical cascade that supports two distinct biological events, First, BCR signaling induces transcription factors to cause expression of new genes, particularly those involved in antigen presentation to T cells. Second, BCR signaling stimulates BCR endocytosis that ultimately leads to antigen-dependent B-T interaction to drive the humoral immune response. There have been many studies linking BCR signaling to the activation of transcription factors. Conversely, how BCR and bound antigen is internalized to initiate humoral immunity is relatively unstudied.

We earlier reported (29) that BCR internalization requires the protein dynamin and was completely blocked in B cells lacking Vav1 and -3 isoforms. Remarkably, Vav1,3-deficient B cells were fully competent in other BCR-triggered signaling events, including the influx of extracellular Ca^{2+}, the activation of MAPK modules (p38, c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK)), and the phosphorylation of Akt. Similarly, anti-BCR-stimulated B cells lacking Vav1 and -3 could up-regulate those proteins involved in B-T interaction as follows: MHC class II, CD80, and CD86 (29). These findings suggest that the signal transduction events and proteins that support transcription factor activation and those supporting BCR endocytosis are, at least in some cases, completely distinct.

Here, we have extended those previous findings to identify a molecular target for Vav and a mechanism by which Vav 1/3 proteins are activated. First, we showed that RNA interference-mediated depletion of Rac1 or -2 protein but not other small GTPases known to be targets of Vav (Rac3, Cdc42, RhoA, and RhoG) block BCR endocytosis. Second, we showed that Vav is recruited through its protein interaction C-terminal domain to the adapter protein LAB to form a complex that also contains Grb2-dynamin. The association of dynamin to LAB required the participation of Grb2. We provided evidence that the SH2 domain of Grb2 binds to phosphorylated LAB, and others have shown that the SH3 domain of Grb2 binds to the proline-rich sequence of dynamin (41). We also show that the four proteins, LAB, Grb2, dynamin, and Vav, are all present in a complex. Finally, we show here that Grb2 and earlier that dynamin and Vav (29) are all important for the generation of cognate MHC

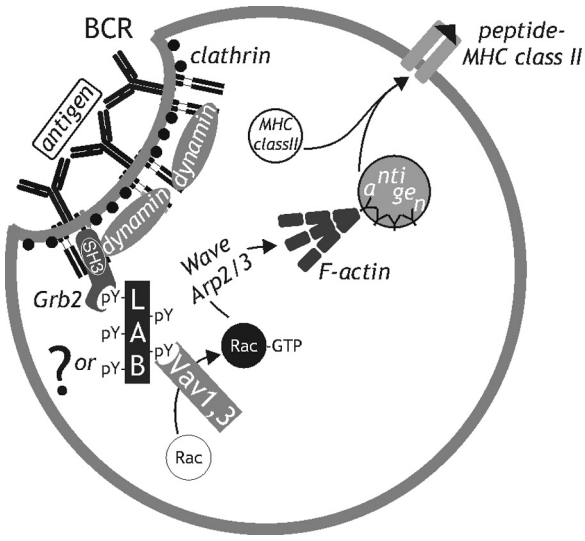

FIGURE 9. **Model for signaling events that support BCR endocytosis and B-T interaction.** BCR clustering by antigen stimulates tyrosine phosphorylation of LAB, which then recruits Grb2-dynamin and Vav1,3. An unknown protein appears to be partially redundant with LAB, indicated by *?* and based on the partial BCR endocytosis effects in LAB-deficient B cells shown in Fig. 4. Grb2 binds to LAB via its SH2 and to dynamin via its SH3 domains. It is not known how Vav interacts with LAB, but the Vav SH2 domain binds to the LAB homolog LAT in T cells (14). Vav binding to LAB and/or to the unknown protein promotes Vav tyrosine phosphorylation and activation. Active Vav serves to stimulate GTP loading on Rac1,2 to promote F-actin reorganization and BCR endocytosis. The endocytosed BCR ultimately traffics to compartments allowing antigen hydrolysis and peptide loading on MHC class II. It is not known what proteins act downstream of Rac in the process, but it may include WAVE and the Arp2/3 complex.

class II-peptide complexes after native antigen internalization, because their reduction or elimination by RNA interference techniques prevents antigen presentation. These data and the previous report (29) are summarized in a model shown in Fig. 9.

It was surprising that both Rac1 and Rac2 are separately required for BCR endocytosis (Fig. 2). These Rac isoforms are difficult to functionally distinguish because the amino acid sequences are highly similar. The main sequence differences are in the C termini; Rac1 has six basic amino acids preceding the C*AAX* site (KKRKRKCLLL) involved in prenylation and in binding to effectors (44). In Rac2, three of the six basic residues are neutral amino acids (RQQKRPCSLL). Isoform-specific knock-out animals have been generated, but Rac1$^{-/-}$ mice are embryonic-lethal (45), whereas Rac2$^{-/-}$ mice show defects in B cell development and generation of antibody-secreting cells (46), consistent with this report. Conditional Rac1,2$^{-/-}$ mice lack all mature peripheral B cells (47) and thus the functional requirements for Rac proteins and potential isoform-specific functions (BCR endocytosis, formation of class II peptide, or humoral immune responses) in mature B cells cannot be addressed in these models. This study establishes that both isoforms are individually required for BCR endocytosis and likely have unique downstream effectors that have not been identified.

There are no data showing any functional consequences toward BCR endocytosis for the formation of the LAB-Grb2-dynamin-Vav protein complex (Figs. 6 and 7). However, an important function is likely because the LAB-deficient B cells show a defect in Vav phosphorylation and Rac1/2 activation (Fig. 5) and because both Vav and Rac are obligatory for BCR endocytosis (29).

In the LAB$^{-/-}$ mice, BCR internalization was decreased as shown earlier (37). Vav and Rac activation were similarly and partially decreased, showing the important scaffolding role LAB plays in these BCR-triggered events. However, these processes were reduced but not absent in LAB-deficient B cells, whereas Rac activation and BCR endocytosis are completely absent in B cells of Vav1,3$^{-/-}$ mice (29). BCR-stimulated Rac activation can only occur through Vav1 or -3 proteins (29), which accounts for the defect in BCR endocytosis in Vav1,3-deficient B cells. The partial effect of LAB deficiency on BCR endocytosis and Vav-Rac activation suggests that Vav activation might have an alternative mechanism besides Vav protein recruitment to LAB or that another scaffolding protein can substitute for LAB. Bam32 is also important for BCR internalization and acts upstream of Rac (48). However, Bam32 requires PI3K for activation (48), and our data suggest BCR internalization does not appear to have such a requirement. It is possible that Bam32 can substitute for LAB in BCR internalization under the unique conditions when LAB is absent. Alternatively, and more likely, there may be an additional scaffolding protein(s) that is at least partially redundant with LAB to support Vav-Rac activation that is essential for BCR endocytosis.

Given the partial effect on BCR endocytosis of the LAB-deficient B cells, the efficiency of humoral immunity in LAB-deficient mice might be decreased but not lost. The efficiency issue has not been addressed, but an earlier report showed that LAB$^{-/-}$ and wild-type mice were not different in IgG responses following a single intraperitoneal injection of 75 μg of trinitrophenyl-Ova and a single measurement of serum IgG levels after 14 days (49).

The interaction of LAB with dynamin and Vav was complex (Figs. 6 and 7). The interaction required that the B cells be stimulated and required Grb2 to recruit dynamin but not Vav. The need for B cell stimulation likely reflects a need for LAB tyrosine phosphorylation to create a docking site for SH2 domain-containing proteins like Grb2 and Vav. Five of the 10 tyrosines in LAB (tyrosines 95, 118, 136, 193, and 233) are present in motifs that are able to bind Grb2 (39, 40). Phosphorylated tyrosine residues 191 (pYVNV) and 226 (pYENL) of LAT, the T cell homolog of LAB, directly bind to the SH2 domain of Vav (14). LAB Tyr-136 is present as YENV (similar to the Vav-binding site YENL of LAT), and Tyr-233 is present as YVNG (similar to the Vav-binding site YVNV of LAT). Studies on Tyr-to-Phe mutants of LAB indicated that Tyr-136, Tyr-193, and Tyr-233 are the primary phosphorylation sites (50). Thus, it is possible that the SH2 domain of Vav directly binds LAB at one or another of these phosphorylated tyrosine residues.

Dynamin appeared to associate with LAB via the adapter protein Grb2 (Fig. 7), although we have no data showing that the interaction is important for any of the processes investigated here. A role for Grb2 in LAB recruitment of dynamin was likely, because Grb2 is known to interact with dynamin (41), and LAB contains numerous Grb2-binding sites (39, 40). Indeed, we found that the recombinant SH2 domain of Grb2 was able to block dynamin association to LAB. However, the recombinant Grb2 SH2 domain did not alter LAB-Vav interac-

tion despite the fact that Grb2 is known to associate with Vav (51), and Vav co-precipitated with Grb2 in stimulated B cells (Fig. 6*A*). It may be that Vav is able to directly bind LAB by its own SH2 domain, as discussed above. The hypothesis that the Vav SH2 domain directly binds to phosphorylated LAB is consistent with the need for the protein interaction C-terminal region of Vav for its own activation (Fig. 4).

It is interesting that we failed to find separate complexes of LAB-Vav or LAB-Grb2-dynamin (Fig. 6), and the reasons for this are not understood. It is possible that separate complexes are present but also associate through LAB-LAB or another protein interaction. We have observed that cells expressing short hairpin RNA targeting dynamin showed a decrease in Vav phosphorylation (data not shown) after BCR stimulation. This latter finding suggests that dynamin recruits a kinase needed for Vav phosphorylation when Vav is present in the LAB protein interaction complex.

We have considered proteins that might act downstream of Rac to reorganize F-actin during BCR endocytosis. WASp is a member of a family of proteins that regulate the actin cytoskeleton organization (52) and include WASp, N-WASp, and WAVE1–3. WASp and N-WASp contain a Cdc42/Rac interaction binding motif that directly binds activated Cdc42 (52). WAVE1–3 lack the Cdc42/Rac interaction binding motif but act downstream of Rac in a variety of situations (53, 54). The members of the WASp family bind actin monomers and Actin-related protein 2 and 3 (Arp2/3 (55)). The Arp2/3 dimer serves as an actin nucleation site by binding to pre-existing actin filaments (56). Arp2/3 thus enhances actin polymerization to produce a branched filament structure. Repeated branching by Arp2/3 and actin monomer binding produces an actin network, and the process is greatly accelerated in the presence of active Rac (57, 58). A recent report showed that WASp was recruited to the BCR cap and that WASp became phosphorylated by Btk upon BCR stimulation (59). Furthermore, BCR-triggered actin reorganization was dependent on Btk, raising the possibility that WASp acts downstream of Btk in actin reorganization. However, this hypothesis contrasts with findings in $WASp^{-/-}$ mice, where WASp appeared not to function in BCR capping or in the humoral immune response to vaccination (60). BCR endocytosis was not measured in either report. There is no information on the role of N-WASp or WAVE in BCR internalization. We are actively pursuing the possible involvement of WAVE and Arp2/3 in BCR endocytosis and in B-T interaction.

Acknowledgments—We are grateful to David Parker and Lou Justement for critical review of the manuscript.

REFERENCES

1. Harwood, N. E., and Batista, F. D. (2008) *Immunity* **28,** 609–619
2. Gupta, N., and DeFranco, A. L. (2007) *Semin. Cell Dev. Biol.* **18,** 616–626
3. Cambier, J. C., Gauld, S. B., Merrell, K. T., and Vilen, B. J. (2007) *Nat. Rev. Immunol.* **7,** 633–643
4. Clark, E. A., and Ledbetter, J. A. (1994) *Nature* **367,** 425–428
5. Kim, Y. M., Pan, J. Y., Korbel, G. A., Peperzak, V., Boes, M., and Ploegh, H. L. (2006) *Proc. Natl. Acad. Sci. U.S.A.* **103,** 3327–3332
6. Fattakhova, G., Masilamani, M., Borrego, F., Gilfillan, A. M., Metcalfe, D. D., and Coligan, J. E. (2006) *Traffic* **7,** 673–685
7. Montagnac, G., Mollà-Herman, A., Bouchet, J., Yu, L. C., Conrad, D. H., Perdue, M. H., and Benmerah, A. (2005) *J. Immunol.* **174,** 5562–5572
8. Barr, V. A., Balagopalan, L., Barda-Saad, M., Polishchuk, R., Boukari, H., Bunnell, S. C., Bernot, K. M., Toda, Y., Nossal, R., and Samelson, L. E. (2006) *Traffic* **7,** 1143–1162
9. Davanture, S., Leignadier, J., Milani, P., Soubeyran, P., Malissen, B., Malissen, M., Schmitt-Verhulst, A. M., and Boyer, C. (2005) *J. Immunol.* **175,** 3140–3149
10. McGavin, M. K., Badour, K., Hardy, L. A., Kubiseski, T. J., Zhang, J., and Siminovitch, K. A. (2001) *J. Exp. Med.* **194,** 1777–1787
11. Turner, M., and Billadeau, D. D. (2002) *Nat. Rev. Immunol.* **2,** 476–486
12. Miletic, A. V., Sakata-Sogawa, K., Hiroshima, M., Hamann, M. J., Gomez, T. S., Ota, N., Kloeppel, T., Kanagawa, O., Tokunaga, M., Billadeau, D. D., and Swat, W. (2006) *J. Biol. Chem.* **281,** 38257–38265
13. Aghazadeh, B., Lowry, W. E., Huang, X. Y., and Rosen, M. K. (2000) *Cell* **102,** 625–633
14. Paz, P. E., Wang, S., Clarke, H., Lu, X., Stokoe, D., and Abo, A. (2001) *Biochem. J.* **356,** 461–471
15. Vedham, V., Phee, H., and Coggeshall, K. M. (2005) *Mol. Cell. Biol.* **25,** 4211–4220
16. Han, J., Luby-Phelps, K., Das, B., Shu, X., Xia, Y., Mosteller, R. D., Krishna, U. M., Falck, J. R., White, M. A., and Broek, D. (1998) *Science* **279,** 558–560
17. Tamás, P., Solti, Z., Bauer, P., Illés, A., Sipeki, S., Bauer, A., Faragó, A., Downward, J., and Buday, L. (2003) *J. Biol. Chem.* **278,** 5163–5171
18. Aoki, K., Nakamura, T., Fujikawa, K., and Matsuda, M. (2005) *Mol. Biol. Cell* **16,** 2207–2217
19. Chalupny, N. J., Kanner, S. B., Schieven, G. L., Wee, S. F., Gilliland, L. K., Aruffo, A., and Ledbetter, J. A. (1993) *EMBO J.* **12,** 2691–2696
20. Brauweiler, A., Tamir, I., Dal Porto, J., Benschop, R. J., Helgason, C. D., Humphries, R. K., Freed, J. H., and Cambier, J. C. (2000) *J. Exp. Med.* **191,** 1545–1554
21. Movilla, N., Dosil, M., Zheng, Y., and Bustelo, X. R. (2001) *Oncogene* **20,** 8057–8065
22. Heo, J., Thapar, R., and Campbell, S. L. (2005) *Biochemistry* **44,** 6573–6585
23. Abe, K., Rossman, K. L., Liu, B., Ritola, K. D., Chiang, D., Campbell, S. L., Burridge, K., and Der, C. J. (2000) *J. Biol. Chem.* **275,** 10141–10149
24. Webb, C. F., Das, C., Coffman, R. L., and Tucker, P. W. (1989) *J. Immunol.* **143,** 3934–3939
25. Kappler, J. W., Skidmore, B., White, J., and Marrack, P. (1981) *J. Exp. Med.* **153,** 1198–1214
26. Phee, H., Rodgers, W., and Coggeshall, K. M. (2001) *Mol. Cell. Biol.* **21,** 8615–8625
27. Tridandapani, S., Pradhan, M., LaDine, J. R., Garber, S., Anderson, C. L., and Coggeshall, K. M. (1999) *J. Immunol.* **162,** 1408–1414
28. Benard, V., Bohl, B. P., and Bokoch, G. M. (1999) *J. Biol. Chem.* **274,** 13198–13204
29. Malhotra, S., Kovats, S., Zhang, W., and Coggeshall, K. M. (2009) *J. Biol. Chem.* **284,** 24088–24097
30. Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D., and Hall, A. (1992) *Cell* **70,** 401–410
31. Didsbury, J., Weber, R. F., Bokoch, G. M., Evans, T., and Snyderman, R. (1989) *J. Biol. Chem.* **264,** 16378–16382
32. Wennerberg, K., and Der, C. J. (2004) *J. Cell Sci.* **117,** 1301–1312
33. Das, B., Shu, X., Day, G. J., Han, J., Krishna, U. M., Falck, J. R., and Broek, D. (2000) *J. Biol. Chem.* **275,** 15074–15081
34. Jacob, A., Cooney, D., Tridandapani, S., Kelley, T., and Coggeshall, K. M. (1999) *J. Biol. Chem.* **274,** 13704–13710
35. Katzav, S., Sutherland, M., Packham, G., Yi, T., and Weiss, A. (1994) *J. Biol. Chem.* **269,** 32579–32585
36. Raab, M., da Silva, A. J., Findell, P. R., and Rudd, C. E. (1997) *Immunity* **6,** 155–164
37. Mutch, C. M., Sanyal, R., Unruh, T. L., Grigoriou, L., Zhu, M., Zhang, W., and Deans, J. P. (2007) *Int. Immunol.* **19,** 19–30
38. Zhang, W., Irvin, B. J., Trible, R. P., Abraham, R. T., and Samelson, L. E. (1999) *Int. Immunol.* **11,** 943–950
39. Brdicka, T., Imrich, M., Angelisová, P., Brdicková, N., Horváth, O., Spicka, J., Hilgert, I., Lusková, P., Dráber, P., Novák, P., Engels, N., Wienands, J.,

Simeoni, L., Osterreicher, J., Aguado, E., Malissen, M., Schraven, B., and Horejsí, V. (2002) *J. Exp. Med.* **196,** 1617–1626
40. Janssen, E., Zhu, M., Zhang, W., Koonpaew, S., and Zhang, W. (2003) *Nat. Immunol.* **4,** 117–123
41. Miki, H., Miura, K., Matuoka, K., Nakata, T., Hirokawa, N., Orita, S., Kaibuchi, K., Takai, Y., and Takenawa, T. (1994) *J. Biol. Chem.* **269,** 5489–5492
42. Yohannan, J., Wienands, J., Coggeshall, K. M., and Justement, L. B. (1999) *J. Biol. Chem.* **274,** 18769–18776
43. Shimonkevitz, R., Colon, S., Kappler, J. W., Marrack, P., and Grey, H. M. (1984) *J. Immunol.* **133,** 2067–2074
44. Knaus, U. G., Wang, Y., Reilly, A. M., Warnock, D., and Jackson, J. H. (1998) *J. Biol. Chem.* **273,** 21512–21518
45. Sugihara, K., Nakatsuji, N., Nakamura, K., Nakao, K., Hashimoto, R., Otani, H., Sakagami, H., Kondo, H., Nozawa, S., Aiba, A., and Katsuki, M. (1998) *Oncogene* **17,** 3427–3433
46. Croker, B. A., Tarlinton, D. M., Cluse, L. A., Tuxen, A. J., Light, A., Yang, F. C., Williams, D. A., and Roberts, A. W. (2002) *J. Immunol.* **168,** 3376–3386
47. Walmsley, M. J., Ooi, S. K., Reynolds, L. F., Smith, S. H., Ruf, S., Mathiot, A., Vanes, L., Williams, D. A., Cancro, M. P., and Tybulewicz, V. L. (2003) *Science* **302,** 459–462
48. Niiro, H., Allam, A., Stoddart, A., Brodsky, F. M., Marshall, A. J., and Clark, E. A. (2004) *J. Immunol.* **173,** 5601–5609
49. Wang, Y., Horvath, O., Hamm-Baarke, A., Richelme, M., Grégoire, C., Guinamard, R., Horejsi, V., Angelisova, P., Spicka, J., Schraven, B., Malissen, B., and Malissen, M. (2005) *Mol. Cell. Biol.* **25,** 4455–4465
50. Koonpaew, S., Janssen, E., Zhu, M., and Zhang, W. (2004) *J. Biol. Chem.* **279,** 11229–11235
51. Smit, L., van der Horst, G., and Borst, J. (1996) *J. Biol. Chem.* **271,** 8564–8569
52. Thrasher, A. J. (2002) *Nat. Rev. Immunol.* **2,** 635–646
53. Innocenti, M., Zucconi, A., Disanza, A., Frittoli, E., Areces, L. B., Steffen, A., Stradal, T. E., Di Fiore, P. P., Carlier, M. F., and Scita, G. (2004) *Nat. Cell Biol.* **6,** 319–327
54. Miki, H., Suetsugu, S., and Takenawa, T. (1998) *EMBO J.* **17,** 6932–6941
55. Paavilainen, V. O., Bertling, E., Falck, S., and Lappalainen, P. (2004) *Trends Cell Biol.* **14,** 386–394
56. Goley, E. D., and Welch, M. D. (2006) *Nat. Rev. Mol. Cell Biol.* **7,** 713–726
57. McGee, K., Zettl, M., Way, M., and Fällman, M. (2001) *FEBS Lett.* **509,** 59–65
58. Eden, S., Rohatgi, R., Podtelejnikov, A. V., Mann, M., and Kirschner, M. W. (2002) *Nature* **418,** 790–793
59. Sharma, S., Orlowski, G., and Song, W. (2009) *J. Immunol.* **182,** 329–339
60. Snapper, S. B., Rosen, F. S., Mizoguchi, E., Cohen, P., Khan, W., Liu, C. H., Hagemann, T. L., Kwan, S. P., Ferrini, R., Davidson, L., Bhan, A. K., and Alt, F. W. (1998) *Immunity* **9,** 81–91