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Vav-Dependent and Vav-Independent Phosphatidylinositol 3-Kinase Activation in Murine B Cells Determined by the Nature of the Stimulus¹

Elena Vigorito,^{2*} Giuseppe Bardi,* Janet Glassford,[†] Eric W.-F. Lam,[†] Elizabeth Clayton,* and Martin Turner*

We show in this study that B cell activation following high avidity ligation of IgM or coligation of membrane Ig with CD19 elicits similar levels of Ca²⁺ flux using different mechanisms. Each form of activation requires the function of Vav and PI3K. However, Vav regulates Ca²⁺ flux independently of PI3K following anti-IgM cross-linking. By contrast, Vav function is essential for PI3K activation following membrane Ig (mIg)/CD19 coligation. Inhibition of PI3K revealed anti-IgM-stimulated Ca²⁺ flux has a PI3K-independent component, while Ca²⁺ flux following mIg/CD19 coligation is totally PI3K dependent. The p85 α and p110 δ subunits of PI3K both participate in anti-IgM and mIg/CD19 coligation-induced Ca²⁺ flux, although the defects are not as severe as observed after pharmacological inhibition. This may reflect the recruitment of additional PI3K subunits, as we found that p110 α becomes associated with CD19 upon B cell activation. These data show that the nature of the Ag encountered by B cells determines the contribution of Vav proteins to PI3K activation. Our results indicate that the strong signals delivered by multivalent cross-linking agents activate B cells in a qualitatively different manner from those triggered by coreceptor recruitment. *The Journal of Immunology*, 2004, 173: 3209–3214.

The development and responses of B lymphocytes are controlled by signal transduction pathways initiated by pre-BCRs as well as the mature BCR, membrane Ig (mIg)³ (1). Surface expression and signaling capacity of mIg are mediated by the associated nonpolymorphic subunits CD79a and CD79b (Ig α and Ig β). CD79a and CD79b contain ITAMs within their cytoplasmic domains that, when phosphorylated, recruit tyrosine kinases, including Syk and Lyn. CD79a may also bind the adapter protein B cell linker protein (BLNK; also named Src homology 2 domain-containing leukocyte protein of 65 kDa/B cell adaptor containing an Src homology 2 domain) through additional tyrosines in its cytoplasmic domain (2). The recruitment of further signaling proteins is facilitated by BLNK, which serves as a scaffold protein for the assembly of a multiprotein complex that includes phospholipase C γ 2 (PLC γ 2), Bruton's tyrosine kinase

(Btk), Vav, and PI3K. This complex has been termed the signalosome and regulates the activity of PLC γ 2 in B cells (3–5). A number of biochemical and genetic studies support this idea. Thus, B cells from mice deficient in both Vav1 and Vav2 display a phenotype similar to that of B cells from mice lacking BLNK, the p85 subunit of PI3K, Btk, or PLC γ 2 (3, 6, 7). The signalosome is thought to act as a pacemaker, which determines the initial peak of intracellular calcium and additionally controls the accumulation of phosphatidylinositol 3, 4, 5 trisphosphate and the activation of kinases such as those of the mitogen-activated protein kinase family (ERK, JNK, p38).

Elevation of the level of intracellular calcium is a major signaling event in response to mIg engagement. Increases in intracellular calcium levels are controlled by inositol 1,4,5-trisphosphate (IP₃)-mediated release of calcium from stores in the endoplasmic reticulum and, subsequently, by the influx of calcium through plasma membrane channels. Evidence from gene-targeted mice (8, 9) suggests that mIg-induced IP₃ production is principally mediated by PLC γ 2, which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) to produce IP₃ and diacylglycerol. The phosphorylation and activation of the Vav family of guanine nucleotide exchange factors contribute to the regulation of calcium flux through mechanisms that are incompletely understood (10). PI3Ks also regulate Ca²⁺ flux, possibly by activating Btk, which in turn phosphorylates PLC γ 2. A recent study using Vav3 mutant avian DT40 cells has indicated that Vav3 regulates PI3K activity downstream of surface IgM (11). Moreover, in immature T cells and mast cells, Vav1 regulates PI3K downstream of the Ag receptor (12, 13). These studies suggest that Vav may, at least in part, regulate calcium signaling by activating PI3K. However, it is not known whether, or which, Vav proteins regulate PI3K in mammalian B cells. Stimulation through mIg initiates signal transduction events that lead to proliferation, differentiation, or apoptosis. However, B cell responses to foreign Ags typically result from the integration of signals from mIg and other receptors. Ags bound by

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³ Abbreviations used in this paper: mIg, membrane Ig; BCAP, B cell adaptor for phosphoinositide 3-kinase; BLNK, B cell linker protein; Btk, Bruton's tyrosine kinase; IP₃, inositol 1,4,5-trisphosphate; PIP₂, phosphatidylinositol 4,5-bisphosphate; PIP₃, phosphatidylinositol 3,4,5-trisphosphate; PKB, protein kinase B; PLC, phospholipase C.

complement coligate the CD21/35⁺CD19 complex with mIg, enhancing B cell responses such as germinal center formation and Ab secretion by several orders of magnitude (14). In this context, the CD21/35⁺CD19 receptor complex links innate immune responses to the acquired immune system. At the cellular level, signal transduction by CD19 leads to increased intracellular Ca²⁺, activation of MAPK, and PI3K. CD19 is rapidly phosphorylated as a consequence of mIg engagement, and the cytoplasmic domain of CD19 contains nine tyrosine residues with the potential to recruit Src homology 2 domain-containing proteins. PI3K is recruited to CD19 through binding of the Src homology 2 domain of the regulatory subunit to tyrosines 482 and 513 (15), while Vav1 and Vav2 have been reported to bind to tyrosines 391 (16, 17) and 421 (18). Vav1 (16) and PI3K (19) have been implicated in CD19 signaling, but the contribution of Vav2 to CD19 signal transduction has not been determined. Additionally, it is not known which catalytic or regulatory subunits of PI3K are crucial for CD19 function. Recent work from our laboratory and others has highlighted a role for the p110 δ catalytic subunit of PI3K in B cell function (20–22). The phenotype of B cells from these mice closely matched that of CD19-deficient mice; however, it was not tested in these studies whether p110 δ was involved in signal transduction by CD19. Moreover, it remains unclear whether p110 δ is the only class Ia PI3K catalytic subunit activated by mIg.

In this study, we have investigated the contribution of Vav proteins and PI3K subunits to signal transduction triggered by polyclonal anti-IgM Abs or by coligation of CD19 to mIg, which mimics Ag bound to complement. These stimuli elicit quantitatively similar increases in Ca²⁺ flux and PI3K activation, but display differential requirements for Vav proteins. Unexpectedly, in primary B cells, anti-IgM activated PI3K independently of Vav, indicating that, in this context, Vav regulates Ca²⁺ flux independently of PI3K. By contrast, when mIg was coligated with CD19, Ca²⁺ flux was entirely PI3K and Vav dependent. Following mIg:CD19 coligation, PI3K activation was critically dependent upon Vav function. We also show that PI3K activity stimulated by strong mIg cross-linking or by mIg:CD19 coligation was reduced, but not abrogated in p110 δ - and p85 α -deficient B cells, and provide evidence to implicate p110 α in B cell activation.

Materials and Methods

Mice and cells

Mutant mice harboring null mutations in Vav1, Vav2, Vav3, p110 δ , and p85 α have all been described previously (6, 21, 23–25). Vav1, Vav2, and Vav3 mice were intercrossed to generate Vav double- and triple-deficient mice. All mice were maintained according to United Kingdom Home Office guidelines. B cells were purified following complement lysis of T cells, as previously described (17). Bal-17 B cells were grown in RPMI 1640 culture medium supplemented with 5% FCS.

Abs and immunoprecipitation

The following Abs were used: goat anti-mouse IgM F(ab')₂ (Jackson ImmunoResearch Laboratories, West Grove, PA), 1D3 Ab to mouse CD19, rabbit Ab to CD19 (26), rabbit antisera recognizing the p85 subunit of PI3K, monoclonal anti-phosphotyrosine (4G10) and Vav1 from Upstate Biotechnology (Lake Placid, NY), Abs p110 α and p110 β from Santa Cruz Biotechnology (Santa Cruz, CA), rabbit p110 δ Ab (21), anti-473 phospho-Ser protein kinase B (PKB) and anti-pan PKB from Cell Signaling Technology (Beverly, MA), and LO-MK-1 rat Ab to mouse κ (Zymed Laboratories, San Francisco, CA). Fab' fragments were produced by papain digestion and were subsequently biotinylated. Immunoprecipitations and Western blots were performed, as previously described (17). For densitometric analysis, films were scanned, bands of interest were quantitated, and in-lane background was subtracted. To determine specific phosphorylation level, the signal from phosphorylated band was divided by the signal from the appropriate loading control, and all values were normalized to the level of unstimulated wild type.

Calcium flux analysis

Purified splenic B cells were loaded for 30 min at room temperature in the dark with 3 mM Fluo-4 AM (Molecular Probes, Eugene, OR) at a density of 6×10^6 /ml in 0.5% BSA/PBS. The cells were washed in indicator-free medium and then resuspended at 3×10^6 /ml in 0.5% BSA/PBS containing 1 mM CaCl₂. After a further incubation of 30 min to allow complete de-esterification of intracellular Fluo-4 AM ester, the variations in absorbance were measured using a PerkinElmer (Wellesley, MA) LS55 luminescence spectrometer. Intracellular Ca²⁺ was calculated, as previously described (27).

Results

Ca²⁺ mobilization following IgM cross-linking at high avidity

Pharmacological blockade of PI3K or mutation of p110 δ or Vav1 and Vav2 in murine B cells variably inhibit calcium mobilization following IgM cross-linking at high avidity (anti-IgM) (6, 7, 20–22). In Vav1- or Vav2-deficient B cells, anti-IgM-stimulated Ca²⁺ flux was similar to that of wild-type B cells (Fig. 1). Combined loss of Vav1 and Vav2 or mutation of p110 δ leads to a greatly reduced Ca²⁺ flux following anti-IgM stimulation. The effect of loss of the p85 α regulatory PI3K subunit on Ca²⁺ flux following anti-IgM has not previously been reported. Deficiency in p85 α resulted in impaired calcium flux, indicating an important contribution for this subunit in the regulation of Ca²⁺ signaling by the BCR. To establish the contribution of anti-IgM-stimulated phosphatidylinositol 3,4,5-triphosphate (PIP₃) to calcium flux in each of the mutants, we stimulated B cells from wild-type, Vav1^{-/-}, Vav2^{-/-}, Vav1^{-/-}/Vav2^{-/-}, p85 α , or p110 δ mice in the presence or absence of PI3K inhibitors. PI3K inhibition using either wortmannin or LY294002 dramatically reduced Ca²⁺ flux in anti-IgM-stimulated wild-type B cells and B cells lacking either Vav1 or Vav2 (Fig. 1, and data not shown). Anti-IgM-stimulated Ca²⁺ flux in Vav1- and Vav2-deficient B cells was reduced further following PI3K inhibition (Fig. 1). These findings are consistent with PI3K activation being independent of Vav proteins when the BCR is cross-linked by anti-IgM. In the absence of p85 α or p110 δ , further inhibition of Ca²⁺ flux was observed when PI3K was inhibited (Fig. 1). This reduction, although modest in magnitude, was consistent in all experiments performed, suggesting that additional PI3K regulatory and catalytic subunits contribute to calcium flux following BCR cross-linking by anti-IgM. The residual

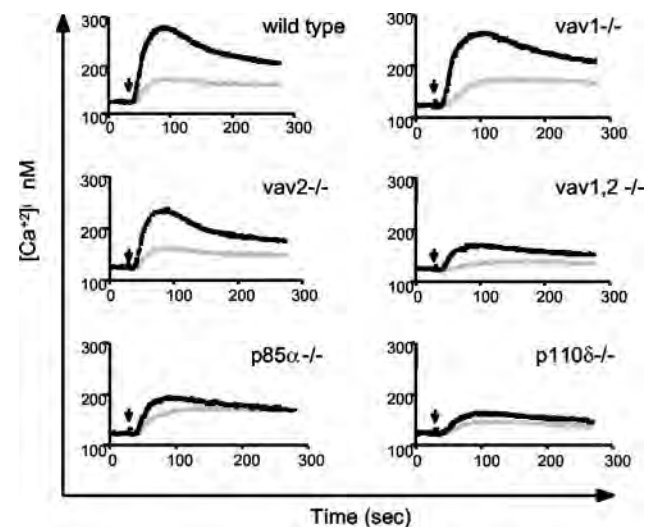


FIGURE 1. Defective IgM-stimulated Ca²⁺ responses in primary B cells from mutant mice. Intracellular calcium concentration in B cells of the indicated genotypes after stimulation with F(ab')₂ anti-IgM (10 μ g/ml) in presence (gray trace) or absence (black trace) of 100 nM wortmannin. The arrows indicate the time of addition of the stimulating Ab.

intracellular Ca^{2+} flux observed in wild-type and mutant B cells following PI3K inhibition indicates the existence of a PI3K-independent component of the Ca^{2+} flux triggered following anti-IgM.

Anti-IgM stimulated PKB phosphorylation

The effects of PI3K inhibition upon Ca^{2+} flux in the mutant B cells provided indirect evidence that PI3K was activated to some extent in these mutant cells. However, we wished to know whether PI3K was activated in the absence of Vav1 and Vav2, and whether PI3K subunits other than p85 α or p110 δ had any role in B cell activation. As a more sensitive alternative to measurement of PIP₃ levels in primary resting B cells, we assessed PI3K activation through measurement of PKB serine 473 phosphorylation. This phosphorylation is critically dependent on PI3K and has been widely used to monitor PI3K activity (28). As expected, PKB phosphorylation induced by anti-IgM treatment of primary mouse B cells was inhibited by wortmannin and LY294002 (Fig. 2A, and data not shown). The extent of PKB phosphorylation following anti-IgM stimulation was quantitated by densitometry and found not to be

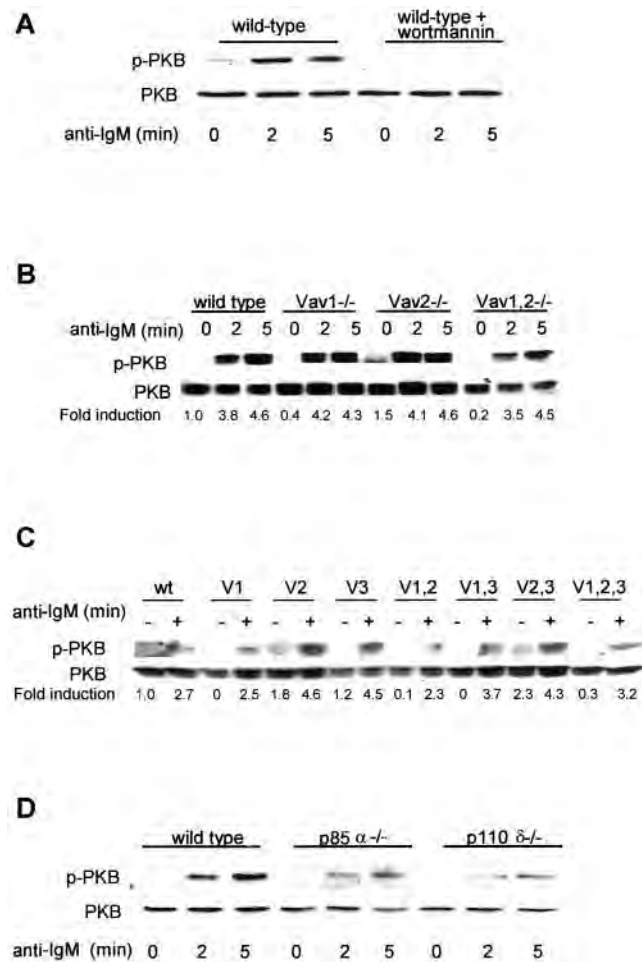


FIGURE 2. PI3K, but not Vav1,2, required for PKB phosphorylation following anti-IgM. **A**, Purified splenic B cells from wild-type mice were pretreated for 10 min with 100 nM wortmannin or equivalent volume of DMSO and stimulated with F(ab')₂ anti-IgM (10 mg/ml) for the indicated times. Cells were lysed and analyzed by Western blotting with anti-phospho PKB (p-PKB) Abs. **B–D**, Splenic B cells of the indicated genotypes (Vav abbreviated as “v” in **C**) were stimulated with anti-IgM, as in **A**. In each case, the blots were subsequently stripped and reprobed with Abs to PKB. Densitometry was performed, as described in *Materials and Methods*, and all signals were normalized to the levels of wild-type unstimulated cells.

significantly affected in B cells lacking any combination of Vav proteins (Fig. 2, *B* and *C*). Thus, Vav proteins are dispensable for PI3K activation when B cells are stimulated by anti-IgM. Anti-IgM-stimulated PKB phosphorylation was absolutely dependent upon PI3K (Fig. 2A); PKB phosphorylation was not totally abolished following anti-IgM stimulation of B cells deficient in p85 α or p110 δ (Fig. 2C). We note our findings on PKB phosphorylation in p85 α mutant B cells contrast with those by Koyasu and colleagues (29), who failed to detect significant PKB phosphorylation following anti-IgM stimulation. Because we are using the same mice as this previous study, our results most likely reflect the higher relative sensitivity of the PKB phosphorylation assay in our hands. Collectively, our results suggest that catalytic and adapter subunits other than p85 α or p110 δ can contribute to anti-IgM-stimulated PI3K activation.

The calcium flux elicited through mIg and CD19 coligation is critically dependent upon Vav1, Vav2, and PI3K

CD19 is a B cell coreceptor that acts as an adapter protein providing docking sites for a variety of signal-transducing proteins, including the Vav proteins and PI3K (14, 30). Coligation of mIg together with CD19, at levels that by themselves are suboptimal, leads to a synergistic activation of PI3K and Ca^{2+} flux (14). We established conditions under which coligation of mIg and CD19, initiated by avidin cross-linking of biotinylated monovalent Fab' derived from mAbs, gave rise to a synergistic calcium response (Fig. 3A). In wild-type B cells, this synergistic response was of similar magnitude to that induced by optimal stimulation by anti-IgM (compare Figs. 1 and 3A). However, unlike anti-IgM stimulation, mIg/CD19 coligation was totally dependent on PI3K activity, as it was completely abolished in cells pretreated with wortmannin (Fig. 3B). Synergistic Ca^{2+} responses were observed in p85 α - and p110 δ -deficient B cells, but at much reduced levels (Fig. 3B). This result cannot be attributed to defects on receptor expression because the levels of κ L chain and CD19 on splenic B cells were not different in the absence of p85 α or p110 δ (data not shown). Thus, while p85 α and p110 δ are important, other PI3K

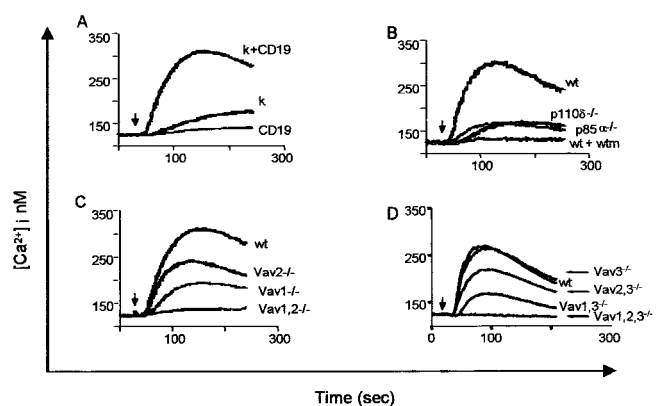


FIGURE 3. Ca^{2+} flux induced by synergistic coligation of Ig κ and CD19. **A**, Splenic B cells from wild-type mice were incubated with biotinylated Fab' anti-Ig κ (anti- κ) fragments (0.1 μ g/ml), anti-CD19 (1 μ g/ml), or a combination of anti- κ and anti-CD19 for 1 min. Ca^{2+} flux was recorded for 30 s before addition of avidin (indicated by the arrow). **B**, Synergistic coligation of Ig κ and CD19 in wild-type, p85 α ^{-/-}, and p110 δ ^{-/-} B cells and wild-type B cells in the presence of 100 nM wortmannin (wtm), as described in **A**. **C**, Synergistic coligation of Ig κ and CD19 in wild-type, Vav1^{-/-}, Vav2^{-/-}, and Vav1,2^{-/-} splenic B cells, as described in **A**. **D**, Synergistic coligation of Ig κ and CD19 in wild-type, Vav3^{-/-}, Vav1,3^{-/-}, Vav2,3^{-/-}, and Vav1,2,3^{-/-} splenic B cells, as described in **A**.

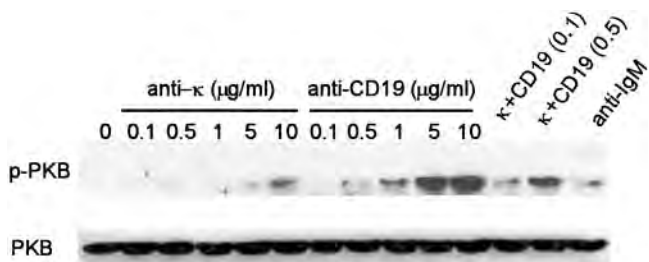


FIGURE 4. Synergistic phosphorylation of PKB following coligation of Ig κ and CD19. Purified splenic B cells were incubated with the indicated concentrations of anti- κ , anti-CD19, or anti- κ (0.1 μ g/ml) + anti-CD19 (0.1 or 0.5 μ g/ml) biotinylated F(ab')₂ for 10 min at room temperature. Cells were washed and stimulated by the addition of avidin (20 μ g/ml) for 2 min. Alternatively, cells were stimulated with F(ab')₂ anti-IgM (10 μ g/ml) for 2 min. Cells were lysed and analyzed by Western blotting with anti-phospho PKB (p-PKB) Abs and subsequently stripped and reprobed with Abs to PKB.

subunits also participate in this response. The Ca²⁺ flux following synergistic coligation of mIg and CD19 has been previously shown to display a partial requirement for Vav1 (16). We reproduced this finding and additionally demonstrated reduced Ca²⁺ flux in Vav2-deficient B cells (Fig. 3, C and D). Our analysis further revealed that in the absence of both Vav1 and Vav2, the synergy response was profoundly impaired (Fig. 3C). Again, the defects did not reflect altered receptor expression levels (data not shown). These data show that Vav1 and Vav2 are partially redundant in the synergy response. We also observed that Vav3 cannot compensate for the lack of Vav1 or Vav2 (Fig. 3D). In addition, we observed similar responses comparing Vav1 with Vav1,Vav3, Vav2 with Vav2,Vav3, and Vav1,Vav2 with Vav triple-deficient B cells, indicating that Vav3 is not required for the mIg/CD19 synergistic response.

PI3K activation elicited through mIg and CD19 synergy requires Vav proteins

We tested the levels of PKB phosphorylation following ligation of mIg alone, CD19 alone, or mIg in combination with CD19. Stim-

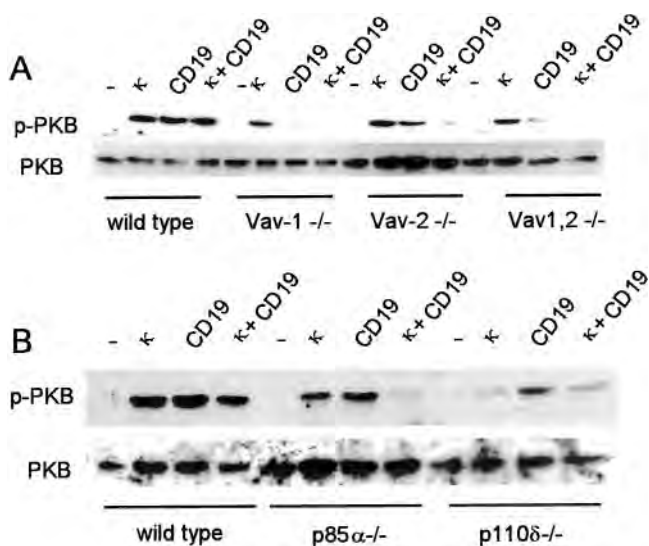


FIGURE 5. PKB phosphorylation stimulated by coligation of Ig κ and CD19 requires Vav1. A and B, Cells of the indicated genotypes were stimulated with optimal concentrations of κ (anti- κ)- or CD19 (anti-CD19)-specific Fab' (10 μ g/ml each; see Fig. 4). Alternatively, B cells were stimulated under synergistic conditions using doses of κ (0.1 μ g/ml)- and CD19 (0.5 μ g/ml)-specific F(ab')₂ that were by themselves suboptimal (anti- κ + anti-CD19).

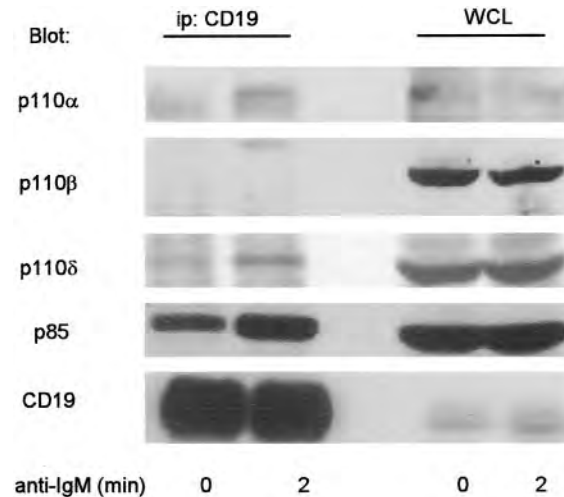


FIGURE 6. PI3K subunits associated with CD19 upon Ag receptor stimulation. Bal-17 B cells were either unstimulated (–) or stimulated with anti-IgM for 2 min (+), after which CD19 was immunoprecipitated (IP CD19). Association of PI3K subunits was detected by Western blot using the indicated Abs. Whole cell lysates (WCL) of Bal-17 were also analyzed to assess the level of expression of the individual proteins.

ulation with anti-IgM was included as a control for comparative purposes. Cross-linking of mIg alone or CD19 alone promoted a dose-dependent phosphorylation of PKB (Fig. 4). Phosphorylation of PKB was synergistically amplified by coligation of mIg with CD19 at levels that were by themselves suboptimal (Fig. 4). Moreover, the levels of PKB phosphorylation obtained under synergy conditions equaled or exceeded that achieved by ligation of IgM with polyclonal Ab (Fig. 4). We next analyzed the phosphorylation status of PKB following optimal ligation of mIg alone, CD19 alone, or the synergistic response of mIg and CD19 in Vav-deficient cells (Fig. 5A). When mIg was cross-linked by optimal concentrations of biotin Fab' anti- κ Vav-deficient B cells showed similar levels of PKB phosphorylation to that found in wild-type B cells. By contrast, CD19-stimulated PKB phosphorylation was profoundly reduced in Vav1-deficient and in Vav1- and Vav2-double-deficient cells. B cells deficient in Vav1 also showed impaired PKB phosphorylation in response to the CD19:mIg synergy conditions. This effect was recapitulated in Vav1- and Vav2-double-deficient cells (Fig. 5A). In Vav2-deficient cells, defects in PKB phosphorylation were evident, but less pronounced following synergy stimulation (Fig. 5A). Following mIg cross-linking using optimal levels of anti- κ B cells lacking p110 δ showed reduced PKB phosphorylation (Fig. 5B). By contrast, PKB phosphorylation elicited by optimal CD19 was less severely affected by p110 δ deficiency. Synergistic responses to mIg and CD19 coligation were much reduced in p110 δ mutant cells. We found p85 α -deficient B cells behaved similarly, although the extent of reduction of mIg-stimulated PKB phosphorylation was less than seen in p110 δ -deficient B cells following optimal mIg cross-linking (Fig. 5B). Taken together, these data show that Vav proteins are critical for PKB activation in response to mIg:CD19 coligation. Moreover, p110 δ and p85 α contribute significantly to PKB activation following mIg:CD19 coligation.

Recruitment of PI3K catalytic subunits to CD19 upon IgM cross-linking

Stimulation of B cells leads to phosphorylation of CD19 on multiple tyrosines; this, in turn, recruits PI3K activity through binding of the PI3K regulatory subunit to CD19 tyrosines 485 and 513

(14). Because several of the results described above suggested a role for catalytic subunits other than p110 δ in B cell activation, we attempted to identify which PI3K components became associated with CD19. Coligating mIg and CD19 in primary B cells resulted in the recruitment of both proteins into a detergent-insoluble complex that precluded recovery of PI3K associated with the complex (data not shown). Thus, to gain insights into which PI3K catalytic subunits have the potential to be involved in CD19 signal transduction, Bal-17 B cells were stimulated with F(ab')₂ anti-IgM, and CD19 was immunoprecipitated. Western blot analysis showed that both p110 α and p110 δ , but not p110 β , were inducibly associated with CD19 (Fig. 6). These results suggest p110 α may have a role in B cell activation.

Discussion

Previous studies using murine thymocytes, cultured mast cell lines, or DT40 avian B cells have suggested the existence of a pathway from the Ag receptor via Vav proteins to the activation of PI3K and Ca²⁺ flux (11–13). In this study, we have extended these findings to demonstrate that the functional relationships between Vav proteins and PI3K depend on the nature of the stimulus used to activate B cells. Both Vav1 and Vav2 proteins were required for Ca²⁺ flux following cross-linking of IgM at high avidity, and this calcium response was, to a large extent, PI3K dependent. However, the normal PKB phosphorylation following IgM cross-linking of Vav1- and Vav2-deficient B cells or of Vav triple knockout cells indicated PI3K activation was Vav independent. Our results therefore suggest that Vav proteins regulate Ca²⁺ flux following IgM cross-linking in a PI3K-independent manner. The impairment of Ca²⁺ responses observed in Vav1,2-deficient B cells is unlikely to reflect altered ratios of splenic B cell subsets, as the defect was specific and spared other signaling responses. The mechanism by which Vav proteins regulate calcium flux under these conditions remains to be fully elucidated; however, Vav proteins do regulate PLC γ function in anti-IgM-stimulated B cells because we have observed reduced production of IP₃ in Vav-deficient mouse B cells (E. Vigorito and M. Turner, unpublished observations). Although we have not yet clarified the mechanism for this effect on IP₃ levels, several nonexclusive mechanisms may operate. Vav proteins may contribute to the activation of tyrosine kinases such as Btk, which phosphorylate PLC γ 2 (31), or of lipid kinases such as type I phosphatidylinositol 4-phosphate 5-kinase that may be responsible for the provision of PIP₂ substrate within signaling microdomains (16). Presently, this latter possibility is impossible to test using biochemical methods, as global measurements of PIP₂ levels are unlikely to reflect events in signaling microdomains. Other PI3K-independent mechanisms by which Vav proteins regulate calcium may be through the adapter function of Vav (12) or through the function of the calponin homology domain that is essential for sustained calcium flux independent of guanine nucleotide exchange factor activity (17, 32).

Ca²⁺ flux and PKB phosphorylation were reduced following IgM cross-linking on p85 α - and p110 δ -deficient B cells. These defects do not mirror general signaling deficiencies, as protein tyrosine phosphorylation or ERK phosphorylation is normal in p85 α - or p110 δ -deficient B cells stimulated by anti-IgM (data not shown) (20). Both of these responses were further reduced by wortmannin or LY294002 (data not shown). These findings suggest involvement of other PI3K catalytic and regulatory subunits in the activation of B cells. Because B cells from mice deficient in p85 α overexpress the alternatively spliced p55 α and p50 α isoforms of the p85 α regulatory subunit (E. Vigorito and M. Turner, unpublished data), it is possible that the p55 α and p50 α subunits

or p85 β permit some anti-IgM-stimulated PIP₃ production. These regulatory subunits may also play a role in B cell activation in normal cells. Our data also suggest other PI3K catalytic subunits besides p110 δ function in mIg signal transduction. Both p110 α and p110 β are expressed in Bal-17 B cells, and p110 α is additionally recruited to CD19 and activated upon IgM cross-linking. In the absence of p110 δ , p110 α may provide sufficient PIP₃ to permit residual PKB activation.

Co-cross-linking of mIg with CD19 gave rise to a synergistic Ca²⁺ response that was of similar magnitude, but qualitatively different, from that elicited by ligation of IgM at high avidity. Following coligation of mIg with CD19, the Ca²⁺ response was entirely dependent upon PI3K activity as well as on Vav1 and Vav2 proteins. This observation is consistent with the demonstration that calcium flux elicited by simultaneous ligation of IgM and CD19 requires tyrosines 482 and 513 of CD19 (33). Furthermore, our finding that PKB was synergistically phosphorylated following mIg and CD19 co-cross-linking is consistent with the observation that mIg and CD19 coligation synergistically activates PI3K (15). Importantly, both Vav1- and Vav1,2-deficient B cells showed severe defects in PKB phosphorylation under optimal stimulation conditions for CD19 alone as well as following the synergistic coligation of mIg and CD19. In the case of the Vav2-deficient B cells, the defects were evident, but less pronounced. Thus, our results show that in primary murine B cells, CD19 regulation of PI3K activity is primarily dependent upon Vav1, while that elicited by mIg is not. This result distinguishes primary murine B cells from chicken DT40 B cells and from murine thymocytes or mast cells, in which Vav appears to be essential for Ag receptor activation of PI3K. Other adapter molecules such as B cell adaptor for phosphoinositide 3-kinase (BCAP) and Gab may participate in PI3K recruitment and activation upon mIg ligation (30). Thus, it is possible that following optimal mIg cross-linking, PI3K is recruited to signaling complexes and activated by CD19-dependent and -independent mechanisms. While under the synergy conditions binding to CD19 may be the major route for PI3K recruitment and Vav proteins are required for PI3K activation. It is not yet clear how Vav proteins influence PI3K activity upon CD19 engagement. One possibility is through activation of Rac proteins, as Rac-2-deficient B cells show some defects in the flux of intracellular Ca²⁺ in cells stimulated by cross-linking the mIg and CD19 (34). There is evidence suggesting that Rac binds and activates PI3K (11, 35, 36). Another possibility is that BCAP could act in concert with Vav proteins. Mice deficient in Vav1 and Vav2 proteins share some phenotypic characteristics with BCAP-deficient mice. BCAP-deficient B cells have reduced Ca²⁺ flux and normal PKB activity in response to anti-IgM (37). It is possible BCAP may be required for normal activation of Vav proteins. However, BCAP is also phosphorylated by cross-linking of CD19 and contributes to PI3K activation in this context (38).

The p85 α and p110 δ PI3K subunits were important for stimulation by mIg/CD19. However, the defective Ca²⁺ flux was less severe than that observed in wild-type cells treated with wortmannin, indicating other PI3K subunits can participate in the synergy response. One candidate PI3K is p110 α , as we found it can be recruited to CD19 in response to IgM stimulation.

In conclusion, our results suggest that PI3K activation and function as well as Ca²⁺ flux are differentially regulated in B cells following high avidity ligation of mIg compared with ligation by low avidity complement-modified Ags. Defining the roles of additional PI3Ks in B lymphocytes should provide further insights into these processes.

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