BCR activation of PI3K is Vav-independent in murine B cells

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Abstract
BCR (B-cell antigen receptor)-induced Ca\(^{2+}\) signalling is initiated by activation of tyrosine kinases, which in concert with adaptor proteins and lipid kinases regulate PLC (phospholipase C) \(\gamma\)2 activation. Vav and PI3K (phosphoinositide 3-kinase) are required for optimal Ca\(^{2+}\) responses, although it has not been established, in primary B-cells, if both proteins are components of the same pathway. \textit{In vitro} evidence suggests that binding of the PI3K lipid product PIP3 to Vav pleckstrin homology domain contributes to Vav activation. However, pharmacological inhibition of PI3K by wortmannin or deletion of the p110\(\delta\) catalytic subunit has no effect on Vav activation in response to BCR engagement, suggesting that this mechanism does not operate \textit{in vivo}. We also show that PI3K recruitment to phosphorylated-tyrosine-containing complexes is Vav-independent. Taken together with our previous observation that protein kinase B phosphorylation is normal in Vav-deficient B-cells, we suggest that PI3K activation is Vav-independent in response to strong signals delivered by multivalent cross-linking.

Introduction
Stimulation of the BCR (B-cell antigen receptor) results in the activation of protein tyrosine kinases and the recruitment of scaffold proteins leading to the assembly of a multiprotein complex, termed a signalosome. This complex regulates major signalling events such as intracellular calcium flux and activation of mitogen-activated protein kinase, which control a variety of transcription factors and induction of gene expression [1–3]. Increases in intracellular calcium levels are controlled by the IP3 (inositol-1,4,5-trisphosphate)-mediated release of calcium from stores in the endoplasmic reticulum and by the influx of calcium through plasma membrane channels, termed as capacitative calcium entry. Evidence from gene-targeted mice [4,5] suggests that BCR-induced IP3 production is principally mediated by PLC (phospholipase C) \(\gamma\)2, which catalyses the hydrolysis of PIP2 (phosphatidyinositol-4,5-bisphosphate) to produce IP3 and diacylglycerol. Moreover, mutation of other components of the signalosome, such as Vav, or PI3K (phosphoinositide 3-kinase), profoundly impairs the Ca\(^{2+}\) response [6–10]. However, the molecular mechanisms by which Vav proteins and PI3K regulate calcium flux downstream of the BCR are not completely understood [11,12].

Activation of PI3K in response to BCR cross-linking results in the accumulation of PIP3, which recruits certain PH (pleckstrin homology) domain-containing proteins to the plasma membrane. Class Ia PI3K form heterodimers composed of a regulatory and a catalytic subunit. In mammals, three genes, p85\(\alpha\), p85\(\beta\) and p55\(\gamma\), encode the regulatory subunits. The p85\(\alpha\) gene encodes an 85 kDa isoform as well as variants of 55 and 50 kDa that arise from alternative splicing. There are also three catalytic subunits, p110\(\alpha\), p110\(\beta\) and p110\(\delta\). Studies of mice deficient in PI3K components have demonstrated roles for p85\(\alpha\) and p110\(\delta\) in B-cell development and function [12].

Vav is a family of three highly homologous genes, termed Vav1, Vav2 and Vav3 to reflect the order of their discovery. Each member possesses a GEF (guanine nucleotide exchange factor) domain for small GTPases of the Rho/Rac family, a PH domain and an array of two SH3 and one SH2 domains. In response to BCR stimulation, Vav proteins are phosphorylated at tyrosine residues, which gives rise to activation of the GEF activity. For Vav1, the regulatory residue is Tyr-174. \textit{In vitro} studies have shown that, in addition, Vav1 GEF activity is regulated by inositol lipids through binding...
to the PH domain [13]. However, studies performed in Vav3-deficient avian DT40 cells do not support this idea, as pharmacological inhibition of PI3K activity did not affect Vav3 phosphorylation in response to antigen receptor stimulation [14]. Instead, recent studies [14–16] support the view that Vav proteins may, at least in part, regulate calcium signalling by activating PI3K downstream of antigen receptors. However, the inter-relationship between Vav and PI3K has not been established in B-cells. In the present paper, we provide evidence to demonstrate that PI3K is downstream of Vav in primary murine B-cells.

**Experimental methods**

**Mice and cells**

Mutant mice harbouring null mutations in Vav1, Vav2, p110δ and p85α have all been described previously [6,9,17,18] and were maintained according to UK Home Office guidelines. B-cells were purified following complement lysis of T-cells as described previously [19]. Bal-17 B-cells were grown in RPMI 1640 culture media supplemented with 5% (v/v) fetal calf serum.

**Antibodies and immunoprecipitation**

Goat anti-mouse IgM F(ab)2 (Jackson Immunoresearch Laboratories, West Grove, PA, U.S.A.), rabbit antisera recognizing the p85 subunit of PI3K, monoclonal anti-phosphotyrosine (4G10) and Vav1 antibodies were purchased from Upstate Biotechnology and anti-473 Phospho-Ser PKB (protein kinase B) and anti-pan PKB were from Cell Signalling Technology. The antibody against Vav1 pTyr 174 was a gift from X. Bustelo (University of Salamanca, Spain). Immunoprecipitations and Western blots were performed as described previously [19].

**In vitro PI3K assay**

This assay was performed by the methods described by Whitman et al. [20,21] with some modifications. In brief, 5 × 10^7 purified B-cells were stimulated with 10 ng/ml anti-IgM F(ab)2 for 2 min and lysed in 2× lysis buffer (20 mM Tris/HCl, pH 7.4, 137 mM NaCl, 1 mM CaCl2, 1 mM MgCl2 and 0.1 mM Na3VO4). p-Tyr proteins were immunoprecipitated using 4G10 antibody, washed sequentially twice in lysis buffer, twice in LiCl wash buffer (10 mM Tris/HCl, pH 7.4, 5 mM LiCl and 0.1 mM Na3VO4), then in TNE (10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA and 0.1 mM Na3VO4). To ensure equal recovery of precipitated proteins, an aliquot corresponding to 3 × 10^7 cell equivalents was resolved by SDS/PAGE for blotting. Kinase assays were performed using 20 μg of sonicated, detergent-free PI (Sigma) and 20 μCi of [γ-32P]ATP for 10 min at 37°C. After organic extraction, samples were spotted onto TLC plates and developed in chloroform/methanol/water/NH4OH (60:47:11.3:2). Migration of PI-3P was related to a standard generated using recombinant PI3Ky [22] generously provided by Dr P. Hawkins and Dr L. Stephens (The Babraham Institute, Cambridge, U.K.).

![Figure 1](image.png)

**Figure 1** | PI3K activity is not required for Vav phosphorylation

(A) BAL-17 B-cells were pretreated for 10 min with 100 nM wortmannin (Wort) or equivalent volume of DMSO vehicle and stimulated with F(ab)2 α-IgM (10 μg/ml) for 2 min. Immediately, cells were lysed followed by precipitation with anti-Vav1 antibodies and blotting with anti-phosphotyrosine-174 Vav1 (pY174). Wortmannin inhibition was controlled by blotting with anti-phosphoserine-473 PKB (p-PKB). The blots were subsequently stripped and re-probed with antibodies to Vav1 or PKB respectively. (B) Wild-type or p110δ-deficient B-cells were stimulated with F(ab)2 α-IgM (10 μg/ml) for the indicated time points, lysed and blotted as indicated in (A).

The incorporated radioactivity was determined using a Fuji phosphorimaging system.

**Results and discussion**

The *in vitro* binding of PI3P analogues to the Vav1 PH domain promotes Vav phosphorylation on Tyr-174 by Lck [13,23]. If activation of Vav proteins by lipid binding operated *in vivo*, pharmacological blockade of PI3K should inhibit Vav1 phosphorylation at Tyr-174. We first tested this hypothesis using BAL-17 B-cells. Stimulation of BAL-17 B-cells through the BCR led to an increase in Vav1 phosphorylation at Tyr-174 revealed by using a phosphospecific antibody (Figure 1A). When BAL-17 B-cells were stimulated in the presence of wortmannin, phosphorylation of Vav1 at Tyr-174 was not affected (Figure 1A). Under the same stimulation conditions, antigen-receptor-induced PKB phosphorylation was abrogated in the presence of wortmannin, confirming that PI3K activity was inhibited (Figure 1A). Similar results were obtained using the A20 B-cell line stimulated with anti-IgG antibodies (results not shown). Thus, in cultured B-cell lines, the phosphorylation of Vav1 triggered by BCR ligation does not require PI3K activity. We extended this observation to primary cells by using B-cells deficient in p110δ. Recent studies from our laboratory and others have implicated the p110δ catalytic subunit of PI3K in BCR signalling [9,10,24]. PI3K activity induced by the BCR is...
severely impaired in the absence of p110δ as judged by PIP3 production or PKB phosphorylation ([9] and Figure 1B). However, analysis of Vav1 phosphorylation in response to anti-IgM cross-linking yielded similar results in wild-type and p110δ-deficient B-cells (Figure 1B). Previous experiments using avian DT40 B-cells found that Vav3 phosphorylation, in response to antigen receptor stimulation, was insensitive to PI3K inhibitors [14]. We conclude that in B-cells the BCR-induced activation of Vav is PI3K-independent.

Recent studies support the view that Vav proteins when activated by antigen receptors regulate PI3K activity, which in turn regulates PLCγ and calcium flux in thymocytes, mast cells and avian B-cells [14–16]. In these studies, PI3K activity was monitored by measuring PIP3 production or PKB phosphorylation. We have observed that PKB phosphorylation elicited by BCR stimulation is not significantly affected in Vav1/2 or Vav1/2/3-deficient B-cells (E. Clayton, E. Vigorito and M. Turner, unpublished work), implying that, in primary B-cells, PI3K activation is Vav-independent. This finding was somewhat unexpected but could reflect differences between cell lineages, species, nature of the stimulus or transformed versus primary cells. To extend the results we obtained on PKB phosphorylation directly to PI3K activation, we assessed PI3K activity in response to α-IgM in B-cells. Owing to limitations in the number of B-cells recoverable from Vav1/2/3-deficient mice, we performed this analysis on Vav1/2 double-deficient B-cells. Defective PI3K activation should be evident in Vav1 and Vav2 double-deficient B-cells as they have profound defects in calcium responses elicited by the BCR [6,7]. Thus we measured IgM-dependent PI3K activity that inducibly associates with phosphotyrosine protein complexes by an in vitro kinase assay. In this assay, α-IgM stimulation of wild-type B-cells gave rise to an 8–12-fold increase in PI3K activity. Similar results were observed in Vav1, Vav2 and Vav1/2 double-deficient B-cells (Figure 2). We also observed that recruitment of p85 was not affected in the absence of Vav proteins (Figure 2A). Our results suggest that BCR stimulation leads to both Vav and PI3K activation, which independently regulate Ca2+ flux (Figure 3). The mechanism by which Vav proteins regulate calcium flux under these conditions remains to be elucidated fully; we present some possibilities in Figure 3. Vav proteins do regulate PLCγ function in α-IgM-stimulated B-cells since we have observed reduced production of IP3 in Vav-deficient mouse B-cells (E. Vigorito and M. Turner, unpublished work). Although we have not yet clarified the mechanism for this effect on IP3 levels several non-exclusive mechanisms may operate. Vav proteins may contribute to increasing the efficiency of PLCγ2, although PLCγ2 phosphorylation is not affected in Vav1/2-deficient B-cells [7]. Alternatively, Vav proteins could regulate lipid kinases such as PIP5K responsible for the provision of PIP2, which may become rate-limiting for inositol lipid hydrolysis [25]. Other PI3K-independent mechanisms by which Vav proteins regulate calcium may be through the adapter function of Vav [15] or through

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**Figure 2** | PI3K activity associated with tyrosine-phosphorylated proteins in activated B-cells

(A) Purified splenic B-cells were stimulated with 10 µg/ml F(ab)′2, α-IgM for 2 min and PI3K activity was determined by incubation of phosphotyrosine-containing immunocomplexes with PI in the presence of [32P]ATP. Immunoprecipitation of p85 was used as loading control.

(B) Graph plot showing the relative PI3K activity expressed as fold induction relative to resting wild-type B-cells. The open bars correspond to unstimulated cells and the filled bars to activated B-cells.

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**Figure 3** | Schematic representation of Vav and PI3K pathways leading to Ca2+ activation

BCR cross-linking activates the PI3K pathway, which requires the p85α and p110δ subunits for elicitation of Ca2+ fluxes. Studies on p110δ-deficient cells have implicated activation of Btk and PLCγ2 as mediators of that response. In addition, BCR ligation results in Vav activation, which is also required for Ca2+ responses. Vav proteins may contribute to enhance PLCγ2 activity or to the synthesis of PIP2. In contrast, co-ligation of the BCR and CD19 results in activation of Vav, which in turn activates PI3K resulting in elicitation of Ca2+ flux by mechanisms still not understood. The p85α and p110δ PI3K subunits are implicated in this response. DAG, diacylglycerol.
the function of the calponin homology domain, which is essential for sustained calcium flux independent of GEF activity [19,26]. Regarding PI3K, we observed that both p85α and p110δ subunits substantially contributed to the overall PI3K activity. As the mice deficient in p85α still express p55α and p50α regulatory subunits of PI3K, it is possible that these subunits contributed to the residual PI3P production. For p110δ, both p110α and p110β are expressed in B-cells. The mechanism by which PI3K contributes to Ca2+ fluxes may involve regulation of Btk (Bruton’s tyrosine kinase) and PLCγ2 activity [9].

Co-receptors on B-cells regulate the threshold for BCR responses. Antigens bound by complement co-ligate the CD21/35 + CD19 complex with the BCR enhancing B-cell responses such as germinal centre formation [27]. Co-cross linking of BCR with CD19 gave rise to a synergistic Ca2+ response of similar magnitude to that elicited by ligation of IgM at high avidity, but qualitatively different. We have observed that after co-ligation of BCR with CD19, the Ca2+ response was entirely dependent on PI3K activity as well as on Vav1 and Vav2 proteins (E. Vigorito and M. Turner, unpublished work). This observation is consistent with the demonstration that calcium flux elicited by simultaneous ligation of IgM and CD19 requires Tyr-482 and Tyr-513 of Vav [28]. Furthermore, we found that PKB was synergistically phosphorylated after BCR and CD19 co-cross-linking, in agreement with the observation that mlg and CD19 co-ligation synergistically activates PI3K [29]. 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 co-ligation of mlg and CD19 (E. Vigorito and M. Turner, unpublished work). These results indicate that, in primary murine B-cells, CD19 regulation of PI3K activity is primarily dependent on Vav proteins, whereas that elicited by BCR is not (represented in Figure 3). We have also observed strong dependence on the p85α and p110δ PI3K subunits for the synergistic response (E. Vigorito, G. Bardi, E. Lam and M. Turner, unpublished work).

The regulation of Ca2+ flux by Vav and PI3K in response to co-ligation of the BCR and CD19 is poorly understood. Vav1 has been implicated in the activation of a synthetic pathway leading to PI2P2 production [25]. Although this mechanism accounts for defective sustained responses, it may not explain the loss of the initial response found in Vav1/2 double-deficient B-cells. Ca2+ fluxes elicited by synergistic responses are strongly dependent on PLCγ2 [30]. However, co-ligation of the BCR with CD19 does not lead to PLCγ2 or Btk phosphorylation ([25] and results not shown), suggesting that other mechanisms may regulate PLCγ2 activity.

In conclusion, our results demonstrate that, in murine B-cells, Vav activation after BCR ligation is PI3K-independent. We also show that PI3K activation downstream of the BCR is Vav-independent. This contrasts with murine B-cells activated through CD19 or antigen receptor-triggered chicken DT40 B-cells, murine thymocytes or mast cells, where Vav appears to be essential for activation of PI3K.

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References

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