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Btk/Tec kinases regulate sustained increases in intracellular Ca^{2+} following B-cell receptor activation

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Bruton's tyrosine kinase (Btk) is essential for B-lineage development and represents an emerging family of non-receptor tyrosine kinases implicated in signal transduction events initiated by a range of cell surface receptors. Increased dosage of Btk in normal B cells resulted in a striking enhancement of extracellular calcium influx following B-cell antigen receptor (BCR) cross-linking. Ectopic expression of Btk, or related Btk/Tec family kinases, restored deficient extracellular Ca^{2+} influx in a series of novel Btk-deficient human B-cell lines. Btk and phospholipase Cγ (PLCγ) co-expression resulted in tyrosine phosphorylation of PLCγ and required the same Btk domains as those for Btk-dependent calcium influx. Receptor-dependent Btk activation led to enhanced peak inositol trisphosphate (IP_3) generation and depletion of thapsigargin (Tg)-sensitive intracellular calcium stores. These results suggest that Btk maintains increased intracellular calcium levels by controlling a Tg-sensitive, IP_3-gated calcium store(s) that regulates store-operated calcium entry. Overexpression of dominant-negative Syk dramatically reduced the initial phase calcium response, demonstrating that Btk/Tec and Syk family kinases may exert distinct effects on calcium signaling. Finally, co-cross-linking of the BCR and the inhibitory receptor, FcγRIIb1, completely abrogated Btk-dependent IP_3 production and calcium store depletion. Together, these data demonstrate that Btk functions at a critical crossroads in the events controlling calcium signaling by regulating peak IP_3 levels and calcium store depletion.

Keywords: calcium stores/FcγRIIb1/phospholipase Cγ/Syk/thapsigargin

Introduction

In electrically non-excitable cells, activation of cell surface receptors leads to oscillatory changes in intracellular calcium concentrations $[Ca^{2+}]_i$ which provide cell-specific information essential for cell division, gene transcription and/or cell death (reviewed in Clapham, 1995). The signaling events which integrate the release of Ca^{2+} from intracellular stores and the entry of extracellular calcium across the plasma membrane have not been fully defined. Release of Ca^{2+} from intracellular stores occurs, at least in part, via activation of phospholipase C (PLC) isoforms, which results in increased production of inositol 1,4,5-trisphosphate (IP_3), and emptying of IP_3 receptor-gated stores (reviewed in Gardner, 1989; Berridge, 1993; Bootman and Berridge, 1995; Noh *et al.*, 1995). Regulation of extracellular Ca^{2+} influx may involve several alternative (or potentially overlapping) mechanisms including: IP_3-dependent plasma membrane Ca^{2+} channels; Na^+-dependent Ca^{2+} channels; L-type Ca^{2+} channels (Kahn *et al.*, 1992; Wacholtz *et al.*, 1992, 1993; Akha *et al.*, 1996); and plasma membrane calcium channels directly responsive to depletion of intracellular Ca^{2+} stores. This latter mechanism, referred to as a store-operated calcium entry (SOC), leads to sustained increases in $[Ca^{2+}]_i$ throughout a broad range of cell types, including hematopoietic and other cell lineages (Hoth and Penner, 1992; Putney and Bird, 1993; Zweifach and Lewis, 1993; Fanger *et al.*, 1995; Serafini *et al.*, 1995).

Activation of B cells in response to B-cell antigen receptor (BCR) cross-linking is an example of a cell lineage-specific signal which controls cell growth decisions in part by regulating the balance of Ca^{2+} store depletion, calcium entry and store refilling (Braun *et al.*, 1979; Ransom *et al.*, 1986; Wilson *et al.*, 1987; Brent *et al.*, 1993; Yamada *et al.*, 1993; Takata *et al.*, 1995; Sugawara *et al.*, 1997; reviewed in Perlmutter *et al.*, 1993; Weiss and Littman, 1994). Sustained increases in $[Ca^{2+}]_i$ are required for the proliferation and differentiation of naive B cells. Failure to reach a threshold intracellular Ca^{2+} level, as seen in anergic B cells, leads to altered cell migration and ultimately to cell death (Dolmetsch *et al.*, 1997; Healy *et al.*, 1997). Finally, modulation of BCR cross-linking-dependent calcium signaling is also achieved through the engagement of several key inhibitory co-receptors on B cells (reviewed in Scharenberg and Kinet, 1997). Identification of the signaling molecules regulating this balance of calcium signals in B-lineage cells therefore has important implications for the understanding of both normal and abnormal immune responses.

B cells from mice with the X-linked immunodeficiency (XID) exhibit a blunted increase in $[Ca^{2+}]_i$ and fail to proliferate following BCR cross-linking (Rigley *et al.*, 1989; reviewed in Wicker and Scher, 1986). Normal Ca^{2+}

flux and cell proliferation, however, can be restored using Ca^{2+} ionophores and protein kinase C activation, suggesting that the signaling deficit in XID B cells is upstream of the elevation in $[Ca^{2+}]_i$. Both XID and the human B-cell immunodeficiency, X-linked agammaglobulinemia (XLA), result from mutations in the cytoplasmic Bruton's tyrosine kinase (Btk) (Rawlings *et al.*, 1993; Thomas *et al.*, 1993; Tsukada *et al.*, 1993; Vetrie *et al.*, 1993). Btk is a member of an expanding family of non-receptor tyrosine kinases which includes Btk, Tec, Itk, Txk, Bmx and *Drosophila* Src28C (reviewed in Rawlings and Witte, 1995). These proteins contain a catalytic domain, SH2 and SH3 protein interaction domains, and a unique N-terminal pleckstrin homology (PH) domain capable of directing protein and phospholipid interactions (Tsukada *et al.*, 1994; Langhans-Rajasekaran *et al.*, 1995; Fukuda *et al.*, 1996; Salim *et al.*, 1996; Franke *et al.*, 1997; Rameh *et al.*, 1997; Snyder *et al.*, 1997; reviewed in Gibson *et al.*, 1994; Lemmon *et al.*, 1996). XID B cells also fail to respond to a variety of additional activating signals including thymus-independent type I antigens, interleukin-5 (IL-5), IL-10, CD38 and possibly CD40 surface receptor cross-linking (reviewed in Wicker and Scher, 1986; Rawlings and Witte, 1995). These abnormalities in receptor signaling and B-lineage development suggest that activated Btk uniquely regulates signaling events essential for normal B-lineage development and proliferation.

Studies demonstrating altered calcium flux in both XID B cells and Btk-deficient chicken B-lymphoma cells (Takata and Kurosaki, 1996) indicate that Btk/Tec kinases play a critical role in the regulation of calcium signaling. In the studies presented here, we have used ectopic expression of Btk and Btk mutant proteins in both normal and Btk-deficient XLA B cells to determine the specific role(s) of Btk in receptor-dependent calcium signaling. Expression of Btk significantly enhanced the sustained increase in $[Ca^{2+}]_i$ following receptor activation in normal B cells and fully restored deficient Ca^{2+} signaling in XLA B cells. Most notably, Btk activation led to a marked increase in peak IP_3 levels, depletion of intracellular calcium stores and enhancement of extracellular calcium influx. These Btk-dependent effects on calcium influx could be blocked completely by co-cross-linking of the FcγRIIb1 inhibitory receptor. Taken together, our data suggest that Btk family kinases uniquely modulate BCR cross-linking-dependent increases in $[Ca^{2+}]_i$ by controlling IP_3-dependent intracellular Ca^{2+} store depletion and store-operated Ca^{2+} influx.

Results

Increased dosage of Btk enhances extracellular Ca^{2+} influx in B cells following BCR cross-linking

Vaccinia virus-driven expression of Btk was used to evaluate the effect of increased Btk dosage on BCR cross-linking-dependent Ca^{2+} signaling. Ramos B cells were infected with equivalent titers of recombinant vaccinia viruses (Figure 1A). While infection with wild-type vaccinia or kinase-inactive Btk resulted in no change, overexpression of wild-type Btk enhanced the sustained increase in $[Ca^{2+}]_i$ following BCR receptor cross-linking. This effect was enhanced further by expression of the

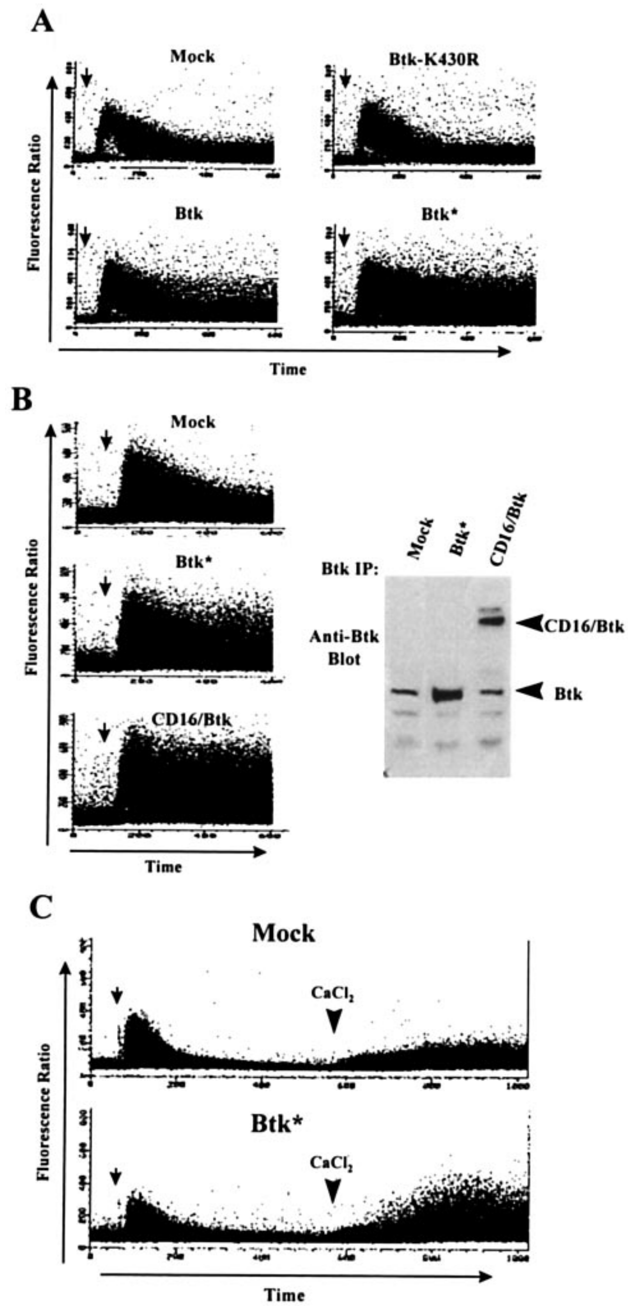

Fig. 1. Increased dosage of Btk increases intracellular Ca^{2+} in B cells following BCR cross-linking by enhancing extracellular Ca^{2+} influx. (**A**) Ramos B cells were infected with the indicated viruses [mock, kinase-inactive Btk (BtkK430R), wild-type Btk (Btk) or the activated Btk mutant, Btk*] and then activated by anti-IgM cross-linking (arrow), and Ca^{2+} mobilization of indo-1-loaded cells was monitored continuously using flow cytometry. (**B**) Left panel: Ramos B cells were infected with viruses including the membrane-targeted chimeric construct CD16/Btk and monitored for Ca^{2+} flux following addition of anti-IgM (arrow). Right panel: the relative expression level of endogenous Btk, Btk* and CD16/Btk (arrowhead). (**C**) Mock- (top) or Btk*-infected (bottom) Ramos cells were resuspended in Ca^{2+}-free media plus 1.8 mM EGTA, activated by anti-IgM cross-linking (arrow) and Ca^{2+} mobilization was monitored. Calcium was restored to the media (arrowhead) to evaluate extracellular Ca^{2+} influx. Representative data from one of $>$10 similar experiments are shown.

activated Btk mutant, Btk* (Figure 1A). Expression of a constitutively membrane-associated Btk chimeric construct, CD16/Btk, resulted in an even greater enhancement

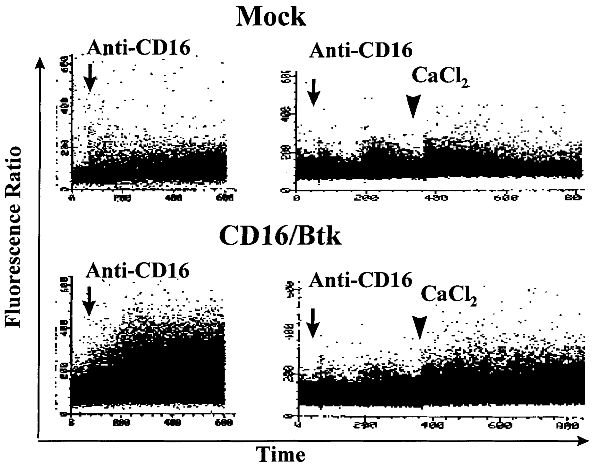

Fig. 2. Btk dimerization enhances extracellular Ca^{2+} influx. Mock- (top) or CD16/Btk-infected (bottom) Ramos cells were activated by anti-CD16 cross-linking (arrow) and monitored for total Ca^{2+} flux in the presence (left panels) or absence of extracellular Ca^{2+} (right panels), demonstrating specific enhancement of extracellular Ca^{2+} influx following CD16/Btk cross-linking.

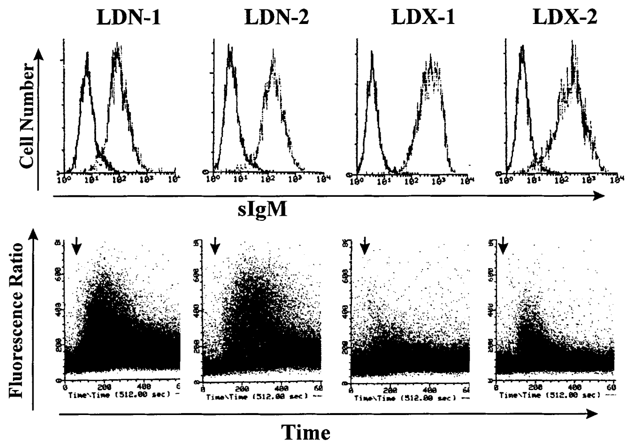

Fig. 3. XLA B cells exhibit reduced Ca^{2+} flux in response to BCR cross-linking. Top: LMP2-deficient B-cell lines were derived from normal individuals (LDN-1 and LDN-2) and patients with XLA (LDX-1 and LDX-2). Cells were evaluated by FACS analysis for surface IgM expression (sIgM). Results of isotype control (left histogram) and anti-IgM (right histogram) staining are shown. Bottom: cell lines were activated by anti-IgM cross-linking (arrow) and monitored for Ca^{2+} flux.

of BCR cross-linking-dependent Ca^{2+} flux (Figure 1B). The increase in $[Ca^{2+}]_i$ correlated directly with the dosage of Btk. Notably, increased dosage of Btk, Btk* or CD16/Btk each led to increased sustained levels of $[Ca^{2+}]_i$ following receptor cross-linking but resulted in only minimal effects on the overall peak of the Ca^{2+} response. These results and our previous data (Afar *et al.*, 1996; Park *et al.*, 1996; Rawlings *et al.*, 1996; Li *et al.*, 1997) suggest that the strength of the calcium signals from Btk, Btk* and CD16/Btk is dependent upon their relative degree of membrane association which facilitates receptor cross-linking-dependent transphosphorylation by Src family kinases.

To define further the potential signaling mechanisms by which Btk activation led to the sustained elevation in $[Ca^{2+}]_i$, we evaluated the phases of calcium signaling in infected B cells. Cells were activated in medium containing 1.8 mM EGTA to evaluate the release of calcium from internal stores. This was followed by addition of calcium in molar excess of EGTA to quantitate extracellular calcium influx (Figure 1C). Compared with wild-type vaccinia infection, the peak and morphology of the fluorescence ratio reflecting the initial phase of the calcium response was unaltered or slightly reduced following infection with wild-type Btk or activated Btk (Figure 1C and data not shown). In contrast, the slope, peak and duration of the secondary phase of the Ca^{2+} response were significantly enhanced by Btk, Btk* or CD16/Btk expression (Figure 1C and data not shown). This increase directly paralleled the results in total calcium flux and was dependent upon the dosage of Btk and the Btk construct expressed.

In order to evaluate the effects of Btk activation in the absence of BCR cross-linking, we also expressed CD16/Btk in Ramos B cells and activated Btk by dimerization using anti-CD16 antibody cross-linking (Kolanus *et al.*, 1993; Rivera and Brugge, 1995). Btk dimerization resulted in a gradual increase in $[Ca^{2+}]_i$ that was dependent upon the level of CD16/Btk protein expression (Figure 2 and data not shown). The Ca^{2+} flux following CD16/Btk dimerization was delayed in comparison with that observed following BCR receptor cross-linking, and lacked the initial, rapid peak in $[Ca^{2+}]_i$. While there was little effect on intracellular calcium release, CD16/Btk dimerization resulted in a significant increase in extracellular Ca^{2+} influx (Figure 2, right panels). Taken together, these results demonstrate that activation of Btk either via BCR cross-linking or by dimerization results in increased $[Ca^{2+}]_i$ that occurs predominantly through enhancement of extracellular calcium influx.

XLA B cells exhibit deficient extracellular Ca^{2+} influx following BCR cross-linking which is restored specifically by Btk/Tec family kinases

Our data indicated either that Btk was essential for Ca^{2+} signaling or that Btk activation augmented BCR-dependent Ca^{2+} signaling. To distinguish between these alternatives and to evaluate potential differences between human B cells and those of other species, we derived a panel of novel BCR signaling-competent, human B-cell lines from normal individuals and from XLA patients. In order to establish these cell lines, B cells were transformed with a mutant Epstein–Barr virus (EBV) lacking EBV latent membrane protein 2 (LMP2; Miller *et al.*, 1995; Fruehling *et al.*, 1996). This was required because immune receptor tyrosine-based activation motifs (ITAMs) in LMP2, also present in the cytoplasmic tail of the BCR, act in a dominant-negative manner to block surface receptor-generated signals. Cell lines matched for surface IgM expression were derived from unrelated normal individuals (LMP2-deficient normal, LDN) and from XLA patients with previously identified mutations in Btk (LMP2-deficient XLA, LDX; Figure 3, top panel). Both normal and XLA LMP2-deficient B-cell lines responded to BCR cross-linking as evidenced by increased tyrosine phosphorylation of multiple cellular substrates, while, as previously suggested, wild-type EBV-transformed B-cell lines derived from the same individuals failed to respond (Miller *et al.*, 1995; and data not shown).

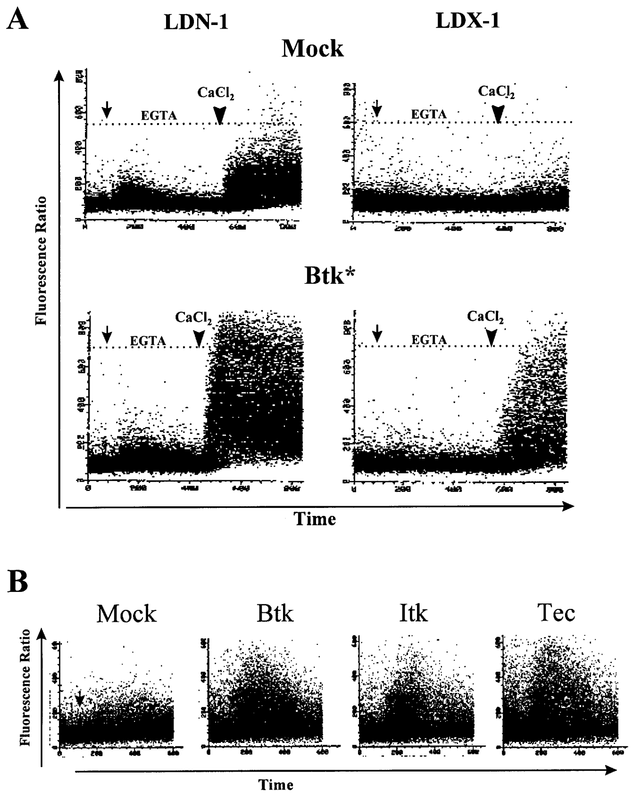

Fig. 4. Deficient extracellular Ca^{2+} influx in XLA B cells is restored by expression of Btk/Tec family kinases. (**A**) Extracellular Ca^{2+} influx in XLA B cells is restored by Btk* activation. LDN-1 and LDX-1 cell lines were infected with wild-type vaccinia virus (mock; top) or Btk* (bottom), and resuspended in Ca^{2+}-free medium plus 1.8 mM EGTA. Cells were activated by anti-IgM cross-linking (arrow) and calcium was restored to the media at 3 min (arrowhead). Similar but less pronounced influx was obtained following expression of Btk. (**B**) Expression of Tec or Itk restores BCR cross-linking-dependent calcium signaling in XLA B cells. LDX-1 cells were infected for 14 h with wild-type vaccinia virus (mock) or vaccinia viruses expressing wild-type Btk, Itk or Tec, and Ca^{2+} mobilization was monitored in indo-1-labeled cells following BCR cross-linking (arrow). Expression of Btk, Tec and Itk was confirmed by Western blot analysis of infected cells (data not shown).

Following BCR cross-linking, LDN cell lines exhibited a Ca^{2+} flux similar to EBV-negative IgM^{+} B cell lines (Figure 3, bottom). In contrast, LDX cell lines had detectable but markedly blunted Ca^{2+} responses. These results indicated that Btk activity is essential for normal BCR-dependent Ca^{2+} signaling in human B cells. Expression of wild-type Btk fully restored Ca^{2+} flux in LDX cells, while expression of kinase-inactive Btk had no effect. Btk* expression restored and further enhanced Ca^{2+} flux to levels greater than those in LDN lines (Figure 4A and B; and data not shown). Ectopic expression of Btk or Btk* markedly enhanced the peak and duration of secondary phase in both LDN-1 and LDX-1 cells (Figure 4A, bottom). The increase in extracellular calcium influx paralleled the restoration of total calcium flux and the dosage of Btk and Btk*.

While Btk is the major Btk family kinase expressed in B cells, it shares significant homology with additional non-receptor tyrosine kinases expressed in both hematopoietic and non-hematopoietic cells (reviewed in Rawlings and Witte, 1995). Examples include Itk, with expression restricted to T and natural killer (NK) cells, and Tec, present in multiple hematopoietic lineages including myeloid cells and some B-cell lines, as well as in liver, kidney, ovary and heart (Siliciano *et al.*, 1992; Heyeck and Berg, 1993; Mano *et al.*, 1993; Sato *et al.*, 1994). Equivalent titers of vaccinia viruses expressing either Itk or Tec were used to infect LDX-1 cells. Strikingly, both Itk and Tec expression restored BCR cross-linking-dependent calcium signaling in Btk-deficient cells to levels equivalent to that following wild-type Btk infection (Figure 4B). Similarly to Btk, Itk and Tec each enhanced the peak and duration of the secondary phase of the calcium response and specifically enhanced extracellular calcium influx (Figure 4B, and data not shown). While these cell lines express both Btk and Tec, differences in relative expression and/or other targeting events are likely to explain the inability of endogenous Tec to rescue Btk signaling events in LDX-1 cells. Finally, in contrast to these results using Btk/Tec kinase proteins, overexpression of Src (Lyn or Fyn) and Syk kinases each failed to restore calcium signaling in XLA cells (data not shown), further supporting the specificity of the Btk in these events. Together, these results suggest that regulation of receptor cross-linking-dependent extracellular calcium influx is a general and unique property of activated Btk/Tec family kinases.

Btk activation leads to depletion of intracellular Ca^{2+} stores

In most cell types, including B cells, emptying of intracellular calcium stores leads directly via SOC and/or via alternative mechanisms to sustained extracellular calcium influx (Takemura *et al.*, 1989; Gouy *et al.*, 1990; reviewed in Gardner, 1989; Premack and Gardner, 1992; Lewis and Cahalan, 1995). The dramatic effect of Btk overexpression on sustained calcium influx suggested that Btk might act by controlling the emptying of intracellular calcium stores in B cells. In order to test this hypothesis, we utilized the drug thapsigargin (Tg), a sesquiterpene lactone which selectively inhibits Ca^{2+} reuptake via sarco/endoplasmic reticulum Ca^{2+}-ATPases (SERCAs; Thastrup *et al.*, 1989; Lytton *et al.*, 1991). Eukaryotic cells sequester Ca^{2+} within a group of biochemically and/or physically distinct intracellular calcium stores, including a dynamic Ca^{2+} store capable of both rapid Ca^{2+} release in response to IP_3 receptor engagement and rapid Ca^{2+} reuptake via SERCA pumps. In the absence of extracellular calcium, Tg can be used to block the refilling of these intracellular calcium stores and to measure their relative size without initiation of calcium influx. We evaluated the potential effects of Btk on the relative size of this dynamic calcium store in cells overexpressing Btk both before and after BCR cross-linking.

Prior to BCR cross-linking, Tg-sensitive stores were identical in normal B cells infected with either wild-type vaccinia or vaccinia expressing Btk (Figure 5A). Following BCR cross-linking, however, Tg-sensitive stores consistently were more depleted in Btk-overexpressing cells. In repeated experiments, infection with Btk led to 80–90% depletion of initial Tg stores versus 35–50% depletion in mock-infected cells (Figure 5A, compare lower right panels in mock- versus Btk-infected cells). Store depletion paralleled the relative level of increase in $[Ca^{2+}]_i$ after BCR cross-linking, and correlated with both

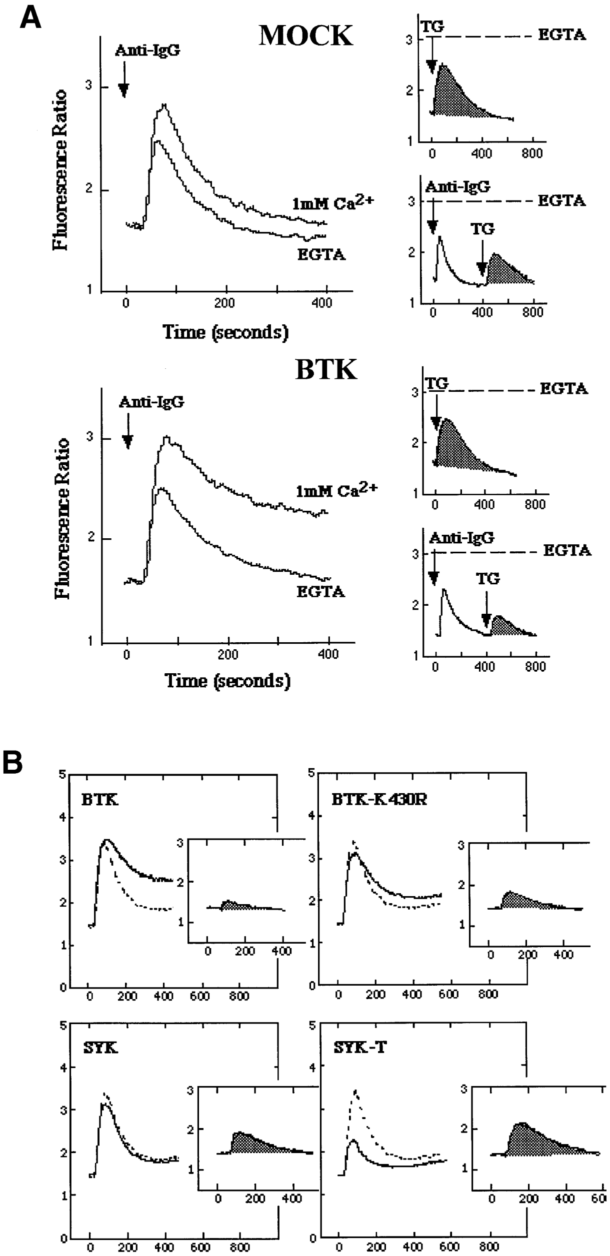

Fig. 5. Btk activation leads to depletion of thapsigargin-sensitive intracellular Ca^{2+} stores. (**A**) A20 murine B cells were infected for 15 h with wild-type vaccinia (mock; top) or vaccinia expressing wild-type Btk (bottom). Cells were activated by BCR cross-linking (anti-IgG, arrow) in the presence or absence of extracellular calcium (left panels), and Ca^{2+} mobilization of fura-2-loaded cells was monitored by spectrofluorimetry. ER calcium stores were evaluated by addition of 1 μM thapsigargin (Tg; right) both before (0 s) and after BCR cross-linking (425 s). Shaded regions demonstrate the relative size of calcium stores under each of the experimental conditions. Representative data from one of >5 similar experiments are shown. (**B**) Increased dosage of Syk has no effect on Tg-sensitive Ca^{2+} stores. A20 cells were infected (as above) with the indicated vaccinia viruses including wild-type Syk and a dominant-negative Syk mutant lacking the catalytic domains (Syk-T). Infected cells were activated by BCR cross-linking in the presence of extracellular calcium, and monitored for Ca^{2+} mobilization. The hatched tracing indicates calcium mobilization in wild-type vaccinia-infected cells. ER calcium stores were evaluated by addition of 1 μM Tg after BCR cross-linking (insets), and the relative store size is depicted by shaded regions. Eqivalent expression of wild-type and kinase-inactive Btk and of Syk and dominant-negative Syk was confirmed by Western blot analysis (data not shown).

the dosage and the specific Btk construct expressed (Btk, Btk* or CD16/Btk). Notably, essentially identical measurements of the relative size of calcium stores were also obtained in experiments comparing ionomycin and Tg, supporting the premise that Tg treatment provides an accurate correlate of the content of the calcium stores in A20 cells (data not shown). The requirement for BCR cross-linking for initiation of store depletion additionally supports the specificity of Btk activation for these events. Finally, expression of either Tec or Itk also led to similar enhancement of BCR cross-linking-dependent, Tg-sensitive store depletion in normal B cells (data not shown). Together, these results support a model in which Btk/Tec kinases maintain sustained calcium influx through depletion of Tg-sensitive calcium stores and initiation of plasma membrane calcium influx.

Previous studies have demonstrated a role for both Src and Syk/Zap70 family kinases in calcium signaling in hematopoietic cells (Kolanus *et al.*, 1993; Takata *et al.*, 1994; Kong *et al.*, 1995; Rivera and Brugge, 1995; Qin *et al.*, 1996; reviewed in Weiss and Littman, 1994). In contrast to the effect of Btk, however, overexpression of Syk (Figure 5B) or of the Src kinases Lyn and Fyn (data not shown) in either normal or Btk-deficient B cells resulted in no enhancement in the sustained phase of the calcium response. Expression of these proteins also resulted in no further enhancement in Tg-sensitive calcium store depletion compared with uninfected or mock vaccinia-infected cells (Figure 5B). However, while wild-type and kinase-inactive Btk each had a minimal detectable effect on intracellular calcium release, inhibition of Syk function using a dominant-negative Syk (Scharenberg *et al.*, 1995) specifically blocked the initial phase release of Ca^{2+} from internal stores, resulting in a greatly reduced overall peak calcium response (Figure 5B; and data not shown). Because the tandem SH2 domains in dominant-negative Syk are predicted to function by binding to phosphorylated ITAMs, this interaction may interfere with the recruitment of additional signaling molecules to the receptor, thereby enhancing its inhibitory effect on the calcium signal. Taken together, however, the lack of effect of wild-type Syk and the early phase inhibitory effect of dominant-negative Syk strongly support the conclusion that Btk and Syk family kinases regulate distinct events in calcium signaling.

Btk activation promotes phosphorylation and downstream signaling of PLCγ isoforms resulting in enhancement of peak and sustained IP_3 levels

Tg-sensitive calcium stores at least partially overlap IP_3-gated Ca^{2+} stores in the vast majority of cell types (Verma *et al.*, 1990; Takemura *et al.*, 1991; Zacchetti *et al.*, 1991; Gamberucci *et al.*, 1995). Tyrosine phosphorylation of PLCγ activates its enzymatic activity leading to increased production of IP_3 and release of Ca^{2+} from IP_3 receptor-gated calcium stores (reviewed in Gardner, 1989; Berridge, 1993). We therefore evaluated the effects of Btk activation on IP_3 production and the activation of PLCγ isoforms. Expression of Btk in A20 B cells led to marked enhancement of IP_3 production (Figure 6A). Peak IP_3 levels increased nearly 6-fold in cells infected with vaccinia-expressing Btk compared with only an ~1.5-fold increase in mock-infected cells. Btk overexpression also led to a

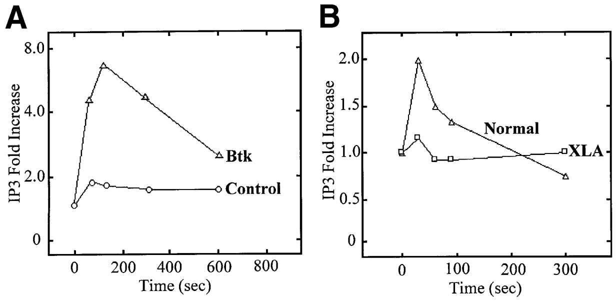

Fig. 6. Btk activation controls peak and sustained IP_3 levels. (**A**) A20 murine B cells were infected for 15 h with equivalent titers of wild-type vaccinia (control; ○) or vaccinia expressing wild-type Btk (Btk; △). Cells were activated by BCR cross-linking (time 0), and kinetic analysis of IP_3 production was performed by radioreceptor assay and displayed as IP_3 fold increase versus time. (**B**) LMP2-deficient EBV B-cell lines derived from a normal individual (LDN-1; △) and a patient with XLA (LDX-1; □) were activated by anti-IgM cross-linking (time 0), and IP_3 production was measured. Representative results of >3 independent experiments are shown.

clear increase in sustained IP_3 levels at all time points for >5 min. Similar results were also observed using Btk overexpression in Ramos B cells (data not shown). Conversely, peak IP_3 levels were significantly lower (1.5- to 2-fold reduction) in Btk-deficient XLA B cells in comparison with LMP2-deficient EBV-transformed B cells from normal individuals (Figure 6B). Together, these data demonstrate that Btk activation leads to enhancement of both peak and sustained IP_3 levels, and that Btk activity is required for normal BCR cross-linking-induced IP_3 production in human B cells.

As predicted by the effect of Btk on IP_3 generation, PLCγ served as a direct substrate of co-expressed Btk (Figure 7A). Co-expression of either Btk or Lyn alone with PLCγ2 resulted in a slight increase (~2-fold) in the overall level of tyrosine phosphorylation of PLCγ2 (Figure 6A). As previously described, co-expression of Btk and Lyn results in a significant enhancement of Btk kinase activity (Rawlings *et al.*, 1996). We therefore evaluated the effect of Btk activated by Lyn on the phosphorylation state of co-expressed PLCγ. Under these conditions, PLCγ2 tyrosine phosphorylation increased ~10-fold (Figure 6A, lane 4; similar results were obtained using PLCγ1, data not shown), strongly supporting the conclusion that PLCγ is a direct substrate of Btk. Phosphorylation of PLCγ required the Btk ATP-binding site (lane 5), the activation loop tyrosine (Y551; lane 6) and the SH2 domain (lane 7), but not the Btk SH3 (lane 8), proline-rich (lane 9) or PH domains (lanes 10–11).

As an alternative assay system, we also evaluated the transformation capacity of Btk and/or PLCγ isoforms in rat fibroblasts expressing a non-transforming Src mutant capable of activating Btk. This assay system previously has permitted receptor cross-linking-independent analysis of Btk activation and signaling (Li *et al.*, 1995, 1997; Afar *et al.*, 1996; Park *et al.*, 1996). Retroviral vectors expressing Btk and/or PLCγ isoforms were used to infect rat fibroblasts stably expressing Src-E378G. Following acute infection with vectors expressing one or both proteins, cells were plated in soft agar and evaluated for the number and size of colonies after 2–3 weeks of cell growth. Expression of Btk or PLCγ2 alone resulted in formation of <5 transformed colonies per plate. In contrast, co-infection with viruses expressing both Btk and PLCγ2 resulted in a 30- to 40-fold increase in the number of transformed colonies (Figure 7B). Together, these series of *in vitro* and *in vivo* data directly support the conclusion that Btk activation leads to enhancement of PLCγ-dependent signaling.

We also compared the tyrosine phosphorylation of PLCγ before and after BCR cross-linking in normal and XLA B-cell lines. PLCγ2 was minimally phosphorylated in both unstimulated normal and XLA B cells (Figure 7C). Surprisingly, phosphorylation of PLCγ2 increased equivalently in both normal and Btk-deficient B cells and remained consistent throughout an extended time course following receptor activation. A small but equivalent increase in PLCγ1 phosphorylation was also observed in both cells lines (data not shown). In contrast to our data, Btk-deficient chicken B-lymphoma cells exhibit reduced tyrosine phosphorylation of PLCγ2 following BCR cross-linking (Takata and Kurosaki, 1996). There are several possible reasons for this discrepancy. First, global PLCγ tyrosine phosphorylation, in contrast to measurement of IP_3 levels, is a relatively insensitive measure of PLCγ activity which is dependent upon a number of factors including localization of activated enzyme with its substrate and intracellular calcium levels. Consistent with this, we also observed only modest enhancement in PLCγ phosphorylation in normal B-cell lines despite marked overexpression of Btk and significantly increased IP_3 levels (data not shown; A.M.Scharenberg *et al.*, 1998). The differences between these human and chicken cell lines may also reflect: the use of alternative sites of PLCγ tyrosine phosphorylation (in normal and Btk-deficient B cells) not distinguishable by measurement of global tyrosine phosphorylation; partial retention of PLCγ phosphorylation via activation of other Tec family kinases (including Tec, present in the XLA cell lines and capable of phosphorylating co-expressed PLCγ; data not shown); the possibility that Btk may activate additional PLC isoforms leading to enhanced peak IP_3 production; and species or genetic differences in these transformed cell lines. Finally, there may also be a selective advantage in those rare peripheral B cells present in XLA patients (and successfully transformed using EBV) for up-regulation of signals capable of partially rescuing Btk-dependent signals.

Co-cross-linking of the FcγRIIb1 inhibitory receptor blocks Btk-dependent calcium influx and calcium store depletion

BCR cross-linking-dependent calcium responses are modulated by several key inhibitory co-receptors. The low affinity receptor for IgG, FcγRIIb1, plays a critical role in the negative feedback regulation of B-cell activation and immunoglobulin production by immune complexes (Muta *et al.*, 1994; Daeron *et al.*, 1995). Because the inhibitory response initiated by FcγRIIb1 results primarily in a blockade of extracellular calcium influx (Wilson *et al.*, 1987; Choquet *et al.*, 1993; Diegel *et al.*, 1994; Muta *et al.*, 1994), we evaluated the effect of FcγRIIb1 co-cross-linking on Btk-dependent calcium influx and calcium store depletion (Figure 8A). A20 B cells were infected with equivalent titers of wild-type or Btk-expressing vaccinia viruses. The relative responses to BCR versus BCR and FcγRIIb1 co-cross-linking were compared fol-

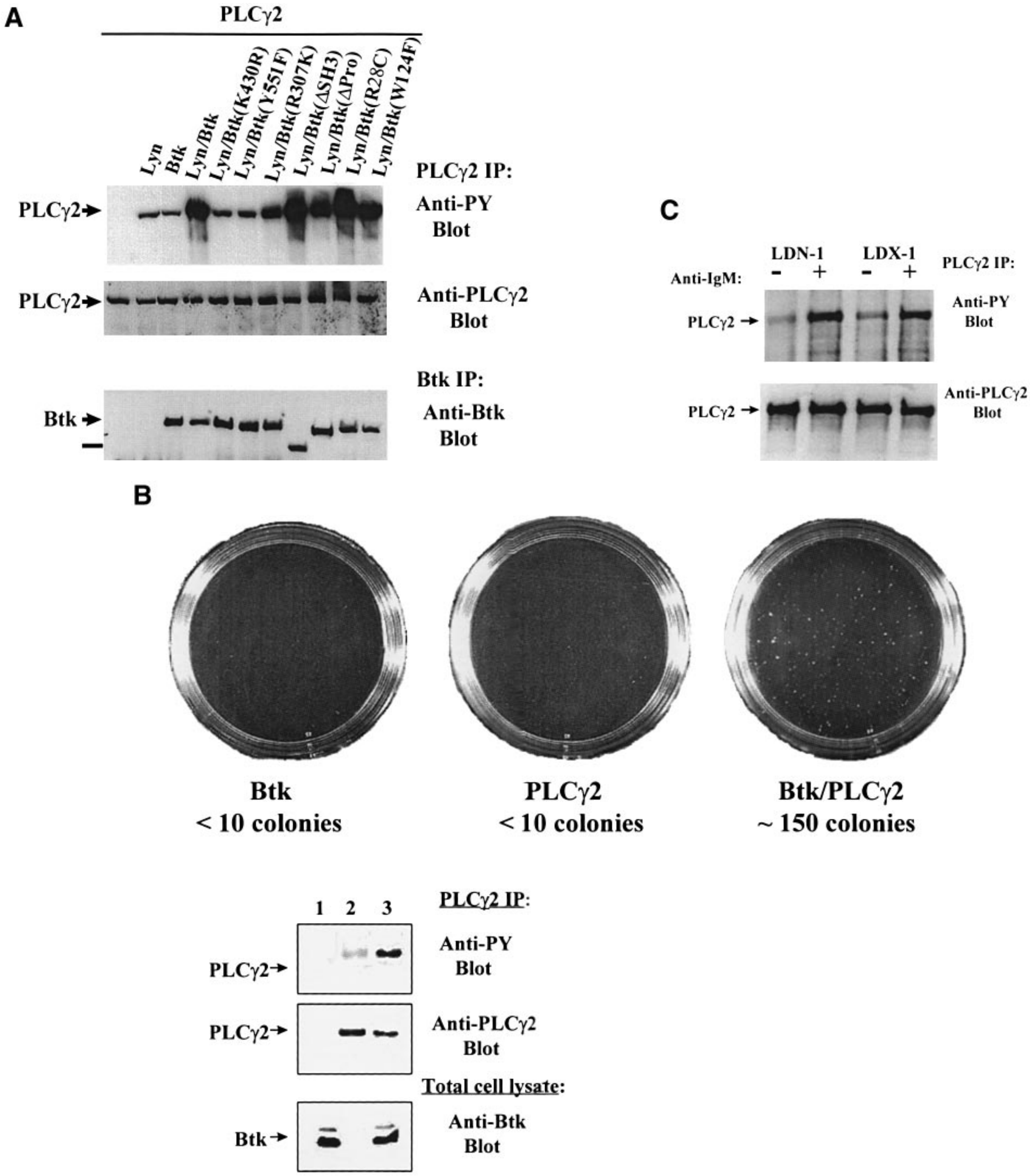

Fig. 7. Btk activation results in enhanced phosphorylation and downstream signaling of PLCγ isoforms. (**A**) PLCγ2 and/or Lyn and wild-type and mutant Btk constructs were co-expressed in NIH 3T3 fibroblasts. Cells were lysed and PLCγ2 was immunoprecipitated and subjected to anti-phosphotyrosine (anti-PY) immunoblotting (top); the blot was then stripped and reblotted with anti-PLCγ2 antibody, demonstrating equivalent recovery of PLCγ2 (middle). Lysates were also subjected to anti-Btk immunoprecipitation and blotted with anti-Btk antibody (bottom). Similar results were obtained following co-expression of PLCγ1 and/or Lyn and Btk (data not shown). (**B**) PLCγ2 synergizes with Btk for transformation of fibroblasts. Top: rat fibroblasts stably expressing an activated but non-transforming Src mutant were infected with retroviruses expressing wild-type Btk, PLCγ2 or both proteins and plated in soft agar. Plates were photographed and colonies $\geqslant$0.5 mm were counted and photographed 3 weeks after plating. Similar data were obtained using infection with PLCγ1 and Btk (data not shown). Bottom: anti-phosphotyrosine Western blot of PLCγ2 immunoprecipitates in cells infected with Btk (lane 1), PLCγ2 (lane 2) or both Btk and PLCγ2 (lane 3). Consistent with the vaccinia co-expression studies, the level of tyrosine phosphorylation of PLCγ2 was also increased 2- to 5-fold in cells co-infected with Btk and PLCγ2. Expression of both Btk and PLCγ2 was confirmed by Western blot analysis in 11/12 transformed colonies derived from co-infected cells (data not shown). (**C**) Normal and XLA B cells exhibit equivalent phosphorylation of PLCγ2 in response to BCR cross-linking. LDN-1 and LDX-1 cells were lysed before and 5 min after activation by anti-IgM cross-linking and subjected to anti-PLCγ2 immunoprecipitation followed by anti-phosphotyrosine (anti-PY) immunoblotting (top panel); the blot was then stripped and reblotted with anti-PLCγ2 antibody, demonstrating equivalent recovery of PLCγ2 (bottom panel). The position of PLCγ2 is indicated (arrows). These cell lines also exhibited indistinguishable patterns of tyrosine phosphorylation of PLCγ1 following IgM cross-linking (data not shown).

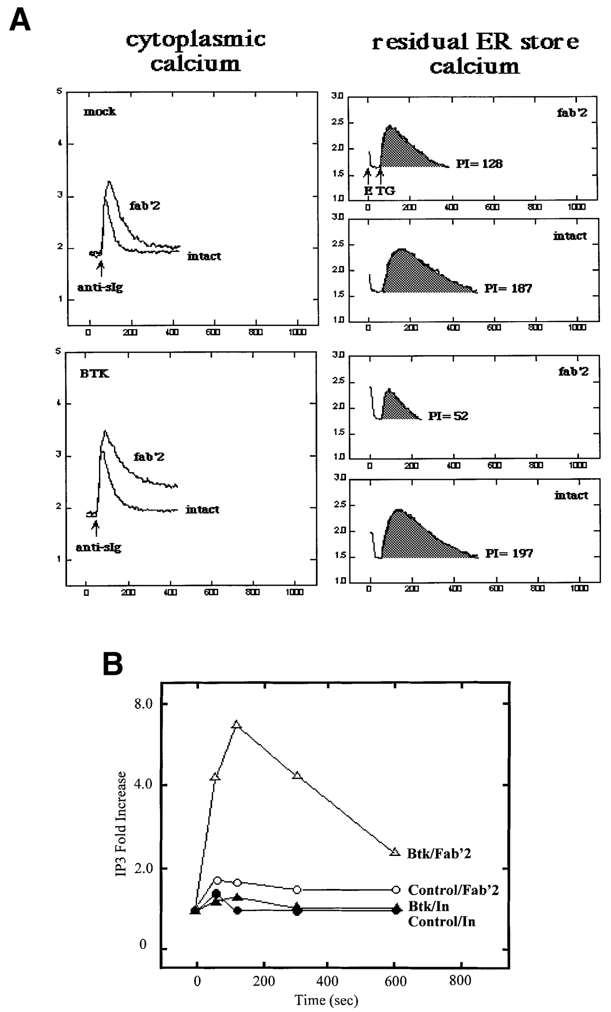

Fig. 8. Co-cross-linking of FcγRIIb1 and the BCR inhibits Btk-dependent calcium influx and calcium store depletion by controlling peak IP_3 production. (**A**) A20 B cells were infected for 15 h with wild-type vaccinia (mock; top panels) or vaccinia expressing wild-type Btk (Btk; bottom panels). Right panels: cells were activated by BCR cross-linking (anti-sIg, arrow) using either a Fab'_2 anti-IgG fragment (Fab'_2 trace) or by co-cross-linking of the BCR and the low affinity receptor for IgG, FcγRIIb1 ('intact' antibody trace). Left panels: Ca^{2+} mobilization of fura-2-loaded cells was monitored by spectrofluorimetry in the presence of extracellular Ca^{2+}. In the right panels, ER calcium stores after BCR cross-linking or co-cross-linking were evaluated by addition of EGTA (E) at 500 s, followed 30 s later by addition of 1 μM thapsigargin (Tg). Shaded regions demonstrate the relative size of calcium stores (quantitated as relative peak integral; PI) under each of the experimental conditions. Representative data from 1–3 similar experiments are shown. (**B**) A20 B cells were infected with control (circles) or wild-type Btk (triangles) vaccinia viruses as in Figure 6 and then activated with either a Fab'_2 anti-IgG fragment (Fab'_2) or intact antibody (In). Kinetic analysis of IP_3 production was performed and is displayed as IP_3 fold increase versus time.

lowing the addition of either an anti-mouse IgG Fab'_2 fragment or an identical intact anti-mouse IgG antibody. BCR activation with the Fab'_2 fragment resulted in higher sustained levels of $[Ca^{2+}]_i$ in Btk-overexpressing cells. Strikingly, this Btk-dependent enhancement in $[Ca^{2+}]_i$ was completely abrogated by FcγRIIb1 co-cross-linking (Figure 8A, left panels, compare Fab'_2 versus 'intact' tracings). As described above, Tg-sensitive stores were significantly more depleted in Btk-overexpressing cells following activation with the Fab'_2 fragment. In contrast, Tg-sensitive stores in Btk-overexpressing cells and mock-infected cells were essentially identical following FcγRIIb1 co-cross-linking (Figure 8A, right panels, compare peak integral store size following 'intact' activation of mock-versus Btk-infected cells). Thus, FcγRIIb1 co-cross-linking completely abrogated the Btk-dependent depletion of Tg-sensitive calcium stores.

We also evaluated the effect of FcγRIIb1 co-cross-linking on the Btk-dependent enhancement of IP_3 production. As previously noted, Btk overexpression resulted in a marked increase in peak IP_3 levels following BCR cross-linking using the anti-mouse IgG Fab'_2 fragment (Figure 8B; compare Fab'_2 tracings of Btk- and control-infected cells). Consistent with its effect on calcium influx and store depletion, FcγRIIb1 co-cross-linking completely abrogated the Btk-dependent increase in IP_3 (Figure 8B; compare 'intact' tracings of Btk- and control-infected cells). These observations demonstrate that Btk-dependent calcium influx is down-modulated specifically by this key inhibitory receptor, suggesting that Btk functions at a critical crossroads in the events regulating both positive and negative calcium signaling in B-lineage cells.

Discussion

Btk kinases are likely to regulate extracellular calcium influx by modulating the Tg-sensitive, IP_3-gated calcium store controlling SOC

Our data complement and significantly extend previous studies identifying a role for Btk in BCR-dependent calcium signaling. Consistent with previous studies in mice and chicken B cells, our results demonstrate that Btk is also essential for BCR cross-linking-dependent calcium flux in human B cells. Ectopic expression of Btk or other members of the Btk/Tec kinase family fully restored calcium signaling in Btk-deficient XLA B cells. Consistent with our previous data regarding Btk activation (Rawlings *et al.*, 1996), Ca^{2+} mobilization required both the kinase activity of Btk and the presence of the Btk activation loop tyrosine, Y551. Restoration of Ca^{2+} flux also required the Btk SH2 and PH domains, but was independent of the Btk SH3 and proline-rich domains, or the major Btk tyrosine autophosphorylation site (Y223; data not shown). Most notably, our data extend earlier studies in Btk-deficient models by identifying a unique role for Btk/Tec kinases in regulation of extracellular Ca^{2+} influx. Btk activation led to a dramatic, dose-dependent enhancement of BCR cross-linking-dependent extracellular Ca^{2+} influx in both normal and XLA B cells. A role for Btk in these events additionally was supported by the calcium signal induced by direct Btk dimerization in the absence of BCR cross-linking, and by the lack of a similar effect on extracellular Ca^{2+} influx following overexpression of either Src or Syk family kinases.

Previous work suggested that altered PLCγ activation may be responsible for the blunted or absent calcium signaling in XID B cells and Btk-deficient chicken B lymphoma cells (Rigley *et al.*, 1989; Takata and Kurosaki, 1996). Our studies demonstrate directly that activation of Btk leads to tyrosine phosphorylation of co-expressed PLCγ isoforms. Consistent with our data identifying the Btk subdomains required for sustained Ca^{2+} influx, PLCγ phosphorylation was also abrogated by mutations of the Src kinase transphosphorylation site, the ATP-binding site and the Btk SH2 domain. The requirement for the Btk SH2 domain suggests that a PLCγ phosphotyrosine–Btk SH2 binding interaction may stabilize the interaction of these proteins and/or permit additional transphosphorylation of PLCγ. Most importantly, using three independent assay systems, our data also demonstrate that Btk activation leads to enhancement of PLCγ-dependent signaling *in vivo*: PLCγ synergized with activated Btk for transformation of rat fibroblasts; Btk activation in normal B cells led to enhanced peak and sustained production of IP_3; and deficient Btk function in XLA B cell lines was associated with significantly reduced BCR cross-linking-dependent IP_3 production.

A striking observation in these studies was that Btk activation led to marked depletion of Tg-sensitive calcium stores. Release of Tg-sensitive calcium stores triggers SOC in nearly all eukaryotic cell types. SOC regulates one or more electrophysiologically defined ion channels, including the calcium release-activated calcium channel (I_{CRAC}) present in both T cells and mast cells (Hoth and Penner, 1992; Zweifach and Lewis, 1993; Fanger *et al.*, 1995; Serafini *et al.*, 1995; reviewed in Putney and Bird, 1993; Birnbaumer *et al.*, 1996). Recent electrophysiological data indicate that the opening of I_{CRAC} channels is non-linear and occurs only upon generation of a high peak level of IP_3 (Parekh *et al.*, 1997). In mast cells, low levels of IP_3 result in intracellular calcium release but no activation of I_{CRAC}. In contrast, a significantly higher (micromolar) threshold level of IP_3 is required to activate calcium influx. Thus, while ~90% of calcium stores are sensitive to low IP_3 levels, a critical, apparently biochemically distinct subset of these stores requires generation of peak IP_3 levels for calcium release initiating SOC. Together, our data demonstrating Btk-dependent enhancement of peak IP_3 levels, calcium store depletion and sustained calcium influx suggest that Btk activation leads to release of an analogous, functionally distinct, Tg-sensitive Ca^{2+} store controlling calcium influx in B cells. Depletion of this calcium store following Btk activation most likely leads to sustained increases in $[Ca^{2+}]_i$ through enhancement of SOC. This conclusion is supported additionally by the observation that Btk activation does not appear to alter the electrochemical gradient driving calcium influx (as demonstrated by the lack of a direct effect of Btk on Tg-induced calcium influx) and by the lack of Ca^{2+} influx in B cells with targeted disruption of the IP_3 receptor (Sugawara *et al.*, 1997).

Our results help to define further the relative roles of Syk and Btk kinases in calcium signaling. Disruption of either Btk or Syk expression in chicken B cells leads to a complete loss of calcium signaling, suggesting that these kinases exert non-redundant effects in the calcium response (Takata *et al.*, 1994; Takata and Kurosaki, 1996). Our results indicate that Btk and Syk kinases primarily may control distinct phases in BCR-dependent calcium signaling. In multiple experiments using wild-type or activated Btk, Btk overexpression led to marked enhancement in extracellular calcium influx. Expression of both kinase-active and kinase-inactive Btk, however, had only a very limited effect on the initial calcium release. In contrast, overexpression of wild-type Syk had no effect on sustained calcium influx, while overexpression of dominant-negative Syk dramatically inhibited the initiation of calcium signaling. In addition, while Syk and Btk activation each clearly led to enhanced PLCγ tyrosine phosphorylation *in vitro*, we failed to identify synergy between these kinases for PLCγ activation or calcium signaling (unpublished data). The initial phase calcium response results from the rapid release of the majority of the IP_3-gated calcium stores in response to low level increases in IP_3 (Parekh *et al.*, 1997). Because Btk activation is delayed relative to activation of Src and Syk kinases following receptor cross-linking (Saouaf *et al.*, 1995), the rate and overall peak of the initial phase response is most likely to be dependent primarily upon activation of these alternative tyrosine kinases. The Btk-dependent increase in peak IP_3 levels, therefore, may result in only a slight additional increase in this initial phase store release, but appears to be essential to activate the critical subset of stores that are required for store-operated influx. Finally, the modest, long-term increase in IP_3 levels following receptor activation is likely to keep intracellular calcium stores in an empty state, thereby maintaining calcium influx. Thus, both peak and sustained IP_3 levels in Btk-overexpressing cells are likely to have only a limited effect on the release phase but a major effect on calcium influx. Additional studies evaluating the interaction of Btk with PLCγ, its substrate and/or other regulatory proteins, the role of membrane phospholipids in these events and the Btk- and Syk-dependent PLCγ phosphorylation sites utilized *in vivo* will be critical for a more complete understanding of how Btk/Tec kinases control receptor-dependent calcium signals.

Btk-dependent Ca^{2+} influx is modulated by B-cell surface co-receptors

Engagement of FcγRIIb1 inhibits BCR cross-linking-dependent phosphoinositide generation and extracellular Ca^{2+} influx (Bijsterbosch and Klaus, 1985; Wilson *et al.*, 1987; Choquet *et al.*, 1993; Muta *et al.*, 1994; Diegel *et al.*, 1994). Our work demonstrates that co-cross-linking of FcγRIIb1 and the BCR leads to near-complete inhibition of Btk-dependent IP_3 production and thereby blocks the effect of Btk overexpression on calcium store depletion. This allows calcium store refilling and blocks SOC influx responsible for sustained calcium signaling. FcγRIIb1 cross-linking results in recruitment of the inositol polyphosphate 5′-phosphatase, SHIP, which is necessary and sufficient for its inhibitory effect (Ono *et al.* 1997). The effects of FcγRIIb1 engagement are nearly identical to the inhibition of BCR cross-linking-dependent calcium influx observed following treatment with the phosphatidylinositol 3-kinase (PI3-K) inhibitor, wortmannin (Hippen *et al.*, 1997; Kiener *et al.*, 1997). Because the Btk PH domain binds avidly to several phosphoinositides including the SHIP substrate, phosphatidylinositol-(3,4,5)-trisphosphate (PtdIns-3,4,5-P_3; Fukuda *et al.*, 1996; Rameh *et al.*, 1996; Salim *et al.*, 1996), we also evaluated the effect of inhibitory signaling on PtdIns-3,4,5-P_3 production and Btk activation. These studies demonstrate that the FcγRIIb1 inhibitory signal is accompanied by a loss of detectable PtdIns-3,4,5-P_3, and a block in Btk/PtdIns-3,4,5-P_3-dependent PLCγ phosphorylation and IP_3 production via activation of SHIP (Scharenberg *et al.*, 1998).

Together, our studies provide an important insight into the previously observed selectivity of FcγRIIb1 for inhibition of calcium influx. Earlier work has suggested that recruitment of SHIP to the membrane may act on inositol-(1,3,4,5)-P_4 and/or PtdIns-3,4,5-P_3 to directly inhibit the opening of I_{CRAC} channels and thereby block calcium influx (Ono *et al.*, 1996). If this were the case, however, then co-cross-linking of FcγRIIb1 and the BCR would be expected to result in even greater calcium store depletion (since both calcium influx and store refilling would be inhibited) than that observed following BCR engagement alone. Our data demonstrate the opposite result, with significantly greater calcium stores present in cells following FcγRIIb1 inhibitory signaling. These data, together with the work of Parekh *et al.* (1997), support an alternative model for the apparent influx selectivity of the inhibitory signal. In such a model, FcγRIIb1 engagement would permit sufficient IP_3 production to allow emptying of the stores responsible for the initial phase calcium signal, but not for release of those stores essential for SOC influx. Termination of Btk-dependent peak IP_3 production by the inhibitory signal would block the release of these critical calcium stores, inhibit SOC influx and lead to store refilling.

Several other B-cell co-receptors also interact in BCR signal transduction to positively or negatively modulate extracellular Ca^{2+} influx and may exert their regulatory effects, in part, by controlling Btk activation. Splenic B cells from mice with targeted disruption of CD45 fail to proliferate, and they exhibit reduced extracellular calcium influx in response to BCR cross-linking (Benatar *et al.*, 1996). Because CD45 can interact specifically with Lyn and regulate the activity of Src kinases, the reduced Ca^{2+} influx in these cells may result from deficient Src kinase-mediated Btk activation (Brown *et al.*, 1994; reviewed in Justement *et al.*, 1994). Finally, cross-linking of the B-cell co-receptor CD22 results in recruitment of the tyrosine phosphatase, SHP-1, and a block in calcium release from intracellular calcium stores (D'Ambrosio *et al.*, 1995; O'Keefe *et al.*, 1996; Ono *et al.*, 1996). It will be important to determine if CD22 cross-linking and SHP-1 activation can modulate Btk-dependent calcium influx by controlling the activation of Btk and/or the generation of Btk-dependent tyrosine-phosphorylated substrates.

The capacity to regulate sustained increases in [Ca^{2+}] may explain the critical role of Btk in B-lineage development

Genetic data support a role for Btk at two key transition points during B-lineage development regulated by signaling through the pre-B- and B-cell antigen receptors, respectively (reviewed in Wicker and Scher, 1986; Conley *et al.*, 1994; Rawlings and Witte, 1994; Sideras and Smith, 1995). Dysregulated signaling at each of these transition points can result in failure of these cell populations to proliferate, and subsequent cell death. Following BCR-dependent activation of naive B cells, both peak threshold and sustained increases in $[Ca^{2+}]_i$ are required for induction of the mitogen-activated protein kinase (MAPK), C-Jun N-terminal kinase (JNK) and p38 kinase cascades, and the calcium-dependent transcription factors NF-AT and NF-κB (Dolmetsch *et al.*, 1997; Healy *et al.*, 1997). These and possibly other signals lead to cell proliferation and differentiation. In contrast, intermittent or sub-threshold oscillations in $[Ca^{2+}]_i$ result in activation of only a subset of these downstream signals and lead to anergy and cell death via apoptosis. Btk/Tec kinase-dependent calcium signals are likely to regulate a subset of transcriptional events essential for B-lineage growth or survival. Identification of these signals will be important in understanding cell activation and/or cell death in hematopoietic and other cell lineages, and may lead to therapies specifically targeting these events in disease states such as B-cell autoimmunity and malignancy.

Materials and methods

Cell culture and vaccinia virus infection of cell lines

Ramos B cells were maintained in RPMI 1640 supplemented with 10–20% fetal calf serum, 2 mM L-glutamine at 37°C in 5% CO_2 at 0.5–2$\times10^6$ cell/ml. Ramos B cells and LMP2A-deficient EBV-transformed B cell lines from normal and XLA patients were infected in RPMI plus 10% calf serum with 5 p.f.u./cell of the indicated recombinant vaccinia viruses for 14–16 h prior to analysis for calcium flux and protein expression.

NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% calf serum. Vaccinia virus infections of NIH 3T3 fibroblasts were performed by seeding cells into 10 cm^2 dishes 18 h prior to infection. The monolayer was washed with DMEM containing 0.1% bovine serum albumin (BSA) and 25 mM HEPES, and then overlaid with the same media containing 5 p.f.u./cell of the indicated viruses. Single infections contained an additional 5 p.f.u./cell of control virus. Cells were placed at 4°C for 30 min to synchronize the infection, and then grown for 6 h at 37°C.

Cells were lysed in 150 mM NaCl, 200 mM Na borate (pH 8.0), 5 mM EDTA, 5 mM NaF, 5 μM leupeptin, 10 μM pepstatin, 10 μM aprotinin and 1 mM Na vanadate. Lysates were spun at 100 000 r.p.m. for 30 min in an Eppendorf microfuge and the supernatant was subjected to the indicated immunoprecipitations or analysis of total cell lysates. Protein expression was confirmed by Western blotting, and enzymatic activity was measured by *in vitro* kinase assay as described below.

Generation of LMP2A-deficient EBV B-cell lines

Peripheral blood or bone marrow were obtained with informed consent from XLA patients and normal blood donors. Mononuclear cells were infected with LMP2-deficient EBV viral supernatant (Longnecker *et al.*, 1993) and then cultured on fibroblasts stably expressing the human CD40 ligand (gift from Dr J.Banchereau, Schering-Plough, Dardilly, France) in media supplemented with 100 U/ml IL-10 (gift from Dr S.Narula, Schering-Plough, Kenilworth, NJ). This protocol significantly increased the efficiency of B-cell transformation and was essential for the establishment of cell lines from XLA patients with limited numbers of peripheral blood B cells (A.-C.Fluckiger, R.Longnecker, O.N.Witte and D.J.Rawlings, in preparation). LMP2-deficient B-cell lines were matched by the expression level of surface immunoglobulin and additional B-lineage differentiation markers including CD10, CD19, CD20, CD22 and CD38. The nearly identically matched $sIgM^+$ cell lines, LDN-1 and -2 from normal individuals, and LDX-1 and -2 from unrelated XLA patients, containing missense mutations in the Btk kinase domain (Arg525Gln and Gly613Asp, respectively), were used in the studies described. Consistent with the clinical diagnosis and Btk sequence analysis, LDX-1 and -2 cells lacked detectable Btk *in vitro* kinase activity although both lines expressed normal levels of Btk protein (data not shown).

Generation of recombinant vaccinia viruses and retroviruses

Recombinant vaccinia viruses expressing murine Btk and Btk mutant proteins, wild-type porcine Syk, truncated Syk (Syk-T) and murine Lyn were constructed as described (Scharenberg *et al.*, 1995; Rawlings *et al.*, 1996). The Btk mutant constructs have been described previously (Li *et al.*, 1995; Park *et al.*, 1996; Rawlings *et al.*, 1996) and included: Btk XID PH domain mutant (Btk-R28C); Btk*, containing a point mutation in the PH domain (E41K) which is associated with increased Btk tyrosine phosphorylation and transforming activity; Btk-ΔPRR, eliminating the conserved prolines in each of the two predicted SH3-binding proline motifs of Btk; Btk SH2 mutant, containing a mutation in the phosphotyrosine-binding pocket (R307K); BtkΔSH3, with a complete deletion of the SH3 domain; the Btk autophosphorylation site mutant (Y223F); kinase-inactive Btk, containing a point mutation at the ATP-binding site (K430R); and the Btk activation loop tyrosine mutant (Y551F). CD16/Btk was constructed as described (Kolanus *et al*., 1993; Li *et al*., 1997).

Murine Tec cDNA (kindly provided by James Ihle), human Itk cDNAs (obtained from ATCC), and bovine PLCγ1 and PLCγ2 cDNAs (kindly provided by Genetics Institute) were subcloned into the pSC-65 vaccinia recombination plasmid (gift of S.Chakrabarti and B.Moss, unpublished), and recombinant vaccinia viruses were selected, amplified and titered using standard techniques (Earl *et al*., 1987).

Immunoprecipitation and Western blot analysis

Btk immunoprecipitations and Western blotting were performed as described (Rawlings *et al*., 1996) using an affinity-purified anti-Btk antiserum. The monoclonal anti-phosphotyrosine antibody 4G10 (1.0 μg/ml, Upstate) and a horseradish peroxidase (HRP)-conjugated sheep anti-mouse secondary antibody (Amersham) were used, respectively, for anti-phosphotyrosine immunoblots as recommended by the manufacturer. The anti-Lyn and anti-Syk antibodies and immunoprecipitation, *in vitro* kinase assay and blotting protocols were as described (Scharenberg *et al*., 1995). Expression of Tec and Itk was confirmed by Western blotting using antibodies provided by James Ihle or purchased from Santa Cruz Biotechnology, respectively. Anti-PLCγ1 and PLCγ2 (Santa Cruz Biotechnology) immunoprecipitations and blotting protocols were performed as recommended by the manufacturer. Primary incubation using rabbit antiserum was followed by incubation in HRP-conjugated goat anti-rabbit immunoglobulin (Amersham). Membranes were developed using the enhanced chemiluminescence system (ECL; Amersham) performed as recommended by the manufacturer. Western blots were stripped using 100 mM 2-mercaptoethanol, 2% SDS and 67.5 mM Tris pH 6.8 for 30 min at 55°C and then washed four times in TBS (10 mM Tris pH 7.5, 150 mM NaCl) and reblotted with the indicated second antibody as described above.

BCR cross-linking and intracellular Ca^{2+} assays

After 14–16 h of vaccinia infection, B cells (5×10^6 cells/ml) were loaded with 10 μM indo-1 acetoxylmethylester (indo-1 AM; Molecular probes) for 30 min at 37°C in RPMI without sera, washed twice and resuspended at 2×10^6 cells/ml in calcium-free Hank's buffered saline (HBSS; pH 7.4). Aliquots of cells were incubated at 37°C for 2 min at final concentration of 2 mM $CaCl_2$ and maintained at this temperature during analysis of $[Ca^{2+}]_i$. The indo-1 fluorescence ratio of individual cells was measured using a FACS Vantage flow cytometer (Becton Dickinson) before and after the addition of activators. Analysis was performed using an ion laser (Inova Enterprises) optimized for UV argon ions, set for 351–364 nm range excitation at a power setting of 50 mW. The 400:530 nM fluorescence ratio was acquired as a function of time. For analysis of BCR cross-linking, cells were activated at 30 s with 1–10 μg/ml goat anti-human IgM Fab'_2 fragment (Southern Biotechnology) and analysis continued for a total of 5 min. For analysis of CD16/Btk cross-linking, cells were resuspended at 2×10^6 cells/ml in HBSS, in the presence and absence of extracellular Ca^{2+}, with 0.5 μg/ml of biotin-labeled anti-CD16 antibody (3G8-Biotin; Medarex) for 2 min at 37°C prior to analysis. After baseline analysis, strepavidin–phycoerythrin was added (20 μl/10^6 cells; Southern Biotechnology) and analysis was continued for a total of 5 min. The more prominent enhancement of sustained $[Ca^{2+}]_i$ levels in CD16/Btk-overexpressing cells induced by BCR cross-linking versus CD16 cross-linking suggests that Src kinase transphosphorylation represents a more efficient mechanism for Btk activation. Western blot analysis was also used to quantitate the level of exogenous protein(s) expression in all experiments using vaccinia-infected cells.

For experiments evaluating Tg-sensitive calcium stores and co-cross-linking of the low affinity receptor for IgG, FcγRIIb1, semi-confluent monolayers of A20 murine B cells were grown overnight in RPMI supplemented with 8% fetal calf serum and 2 mM L-glutamine. Cells were infected with 7 p.f.u./cell of the indicated vaccinia viruses for 15 h and loaded with the calcium-sensitive dye fura-2. Intracellular calcium measurements were obtained in buffer containing 1mM calcium (with or without addition of 1.5 mM EGTA immediately prior to BCR cross-linking) and monitoring of 510 nm fluorescence emission after excitation at either 340 or 380 nm using a Deltascan bulk spectrofluorimeter (Photon Technologies International). Cells were activated after 20 s of data acquisition by the addition of 15 μg/ml of rabbit anti-mouse IgG Fab'_2 fragment or using 30 μg/ml of an identical intact rabbit anti-mouse IgG antibody (Jackson Immunoresearch) followed by evaluation of endoplasmic reticulum (ER) calcium stores by addition of EGTA at 500 s followed, 20 s later, by addition of 1 μM Tg.

Measurement of IP_3

Ramos or LDX-1 B cells were infected with wild-type vaccinia virus or viruses expressing wild-type, kinase-inactive Btk or Btk* for 16 h, washed in HBSS, and activated with 1 μg/ml goat anti-human IgM in the presence of 1.5 mM EGTA. Kinetic analysis of IP_3 production was performed by radioreceptor assay as recommended by the manufacturer (DuPont NEN). Western blot analysis of aliquots of infected cells demonstrated equivalent expression levels of Btk, Btk* and kinase-inactive Btk. Results are representative of more than three independent experiments.

Soft agar transformation assays

Recombinant Btk, PLCγ1 and PLCγ2 were also cloned individually into the retroviral vector, pSRαMSVtk-neo, as described (Li *et al*., 1995). Helper-free Btk and PLCγ retroviral stocks were prepared by transient transfection of 293T cells and used to infect rat-2 fibroblasts expressing an activated mutant of Src for soft agar transformation assays performed as described (Src-E378G; Afar *et al*., 1996). Fibroblasts were infected with retroviruses encoding the neomycin resistance gene, wild-type Btk, PLCγ1 and PLCγ2, grown for 48–72 h, and then plated in duplicate in soft agar at different cell densities (1×10^4 cells/6 cm dish) in media containing 10% fetal calf serum. Colonies ⩾0.5 mm were counted at 2–3 weeks after plating, and the color of the media was recorded. Expression of both Btk and PLCγ2 was confirmed by Western blot analysis in 11/12 transformed colonies derived from co-infected cells.

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