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Bruton's tyrosine kinase regulates B cell antigen receptor-mediated JNK1 response through Rac1 and phospholipase C-γ2 activation

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Abstract Bruton's tyrosine kinase (Btk) is essential for B cell development and B cell antigen receptor (BCR) function. Recent studies have shown that Btk plays an important role in BCR-mediated c-Jun NH_2-terminal kinase (JNK) 1 activation; however, the mechanism by which Btk participates in the JNK1 response remains elusive. Here we show that the BCR-mediated Rac1 activation is significantly inhibited by loss of Btk, while this Rac1 activation is not affected by loss of phospholipase C-γ2 (PLC-γ2). Since PLC-γ2 is also required for BCR-mediated JNK1 response, our results suggest that Btk regulates Rac1 pathway as well as PLC-γ2 pathway, both of which contribute to the BCR-mediated JNK1 response.

Key words: B cell antigen receptor; Bruton's tyrosine kinase; c-Jun NH_2-terminal kinase; Rac1; Phospholipase C-γ2

1. Introduction

Antigen-mediated clustering of B cell antigen receptor (BCR) induces complex cascades of biochemical events that culminate in proliferation, differentiation, or apoptosis. The BCR utilizes sequential activation of at least three types of cytoplasmic protein tyrosine kinases (PTKs), Src-PTK, Syk, and Bruton's tyrosine kinase (Btk), to regulate downstream effectors [1–3]. Critical downstream signaling events include the activation of mitogen-activated protein kinase (MAPK) family members, comprising extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK), and p38 MAPK. Different patterns of activation of these MAPKs may lead to differential expression of genes, and thereby contrasting cellular phenotypes such as growth or apoptosis in B cells [4–6].

As in mast cells in which Btk is essential for FcεRI-mediated JNK1 response [7], we have recently provided evidence that Btk regulates the JNK1 response in BCR signaling context [8]. Since this JNK1 response requires both Rac1 and phospholipase C-γ2 (PLC-γ2) pathways [9], two models could be put forward to account for the requirement of Btk in the JNK1 response. In the first model, given the requirement for Btk in the PLC-γ2 pathway, Btk acts on only PLC-γ2 pathway, which in turn regulates the JNK1 response. In the second model, both PLC-γ2 and Rac1 pathways lie downstream of Btk, and thereby leading to activation of JNK1. Here we show the decrease of BCR-induced Rac1 activation in Btk-deficient B cells, suggesting that Btk utilizes both pathways for JNK1 activation.

2. Materials and methods

2.1. Cells and antibodies

Chicken B cell line DT40 wild-type and its derived mutant cells (Btk-, PLC-γ2-deficient cells, and Btk-deficient cells expressing Btk) were previously described [10,11]. Human B cell lines, C1LCL from the neonatal cord blood and NH-1 from the peripheral blood of an X-linked agammaglobulinemia (XLA) patient in whom the Btk mutation leading to Btk protein deficiency had been identified [12], were established by infection with a mutant Epstein–Barr virus (EBV) lacking EBV latent membrane protein 2 (LMP2) as described [13,14]. These cell lines were maintained in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, penicillin and streptomycin. The following antibodies were used: anti-chicken IgM mAb, M4 (μ, κ) [15], $F(ab')_2$ fragments of a goat anti-human IgM (μ) Ab (Biosource International, Camarillo, CA, USA), anti-human JNK1 mAb (Pharmingen, San Diego, CA, USA), and anti-human Rac1 mAb (Upstate Biotechnology, Lake Placid, NY, USA).

2.2. Biochemical analyses

In vitro kinase assay of JNK1 was performed as described previously [9]. For affinity precipitation assay using GST–CRIB (Cdc42/Rac interactive binding domain [CRIB] of rat PAK1 [amino acid 1–125] fused to glutathione *S*-transferase), bacterially expressed GST–CRIB prebound glutathione-Sepharose beads (20 μl packed beads, 40 μg of protein) were prepared as described [16]. Cell lysates in lysis buffer (25 mM HEPES [pH 7.3], 150 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 20 mM β-glycerophosphate, 10 mM NaF, 2 mM sodium vanadate, 0.5% Triton X-100, 4% glycerol, 5 mM DTT, 0.5 mM PMSF and 5 μg/ml each leupeptin and pepstatin) [16] were incubated with the beads for 10 min at 4°C. After one wash in the lysis buffer, bound proteins were eluted with SDS–PAGE sample buffer and resolved on 12.5% SDS–PAGE and subjected to Western blotting using anti-Rac1 mAb.

3. Results and discussion

Using DT40 chicken B cells, we have previously shown that

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Abbreviations: BCR, B cell antigen receptor; Btk, Bruton's tyrosine kinase; JNK, c-Jun NH_2-terminal kinase; PLC-γ2, phospholipase C-γ2; PTK, protein tyrosine kinase; XLA, X-linked agammaglobulinemia; CRIB, Cdc42/Rac interactive binding domain

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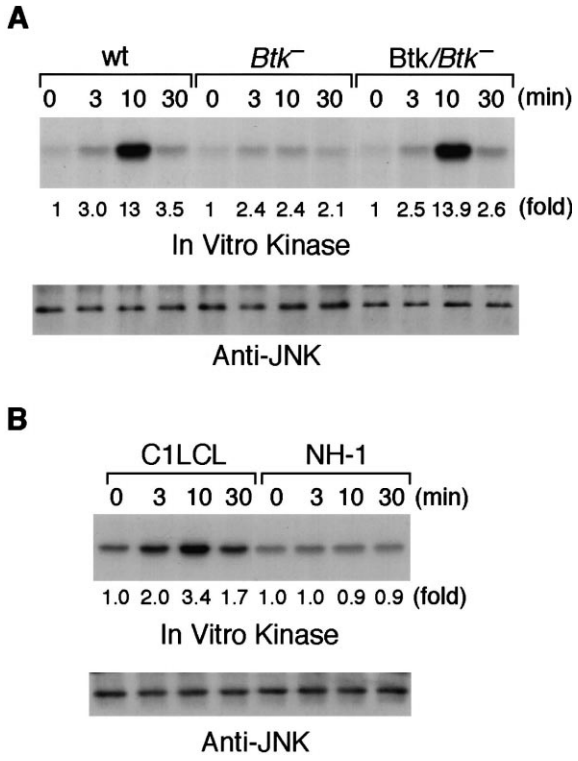

Fig. 1. Inhibition of BCR-induced JNK1 activation in Btk-deficient cells. A: Various DT40 cells (5×10^6/sample) were stimulated with M4 (4 μg/ml) for indicated times. JNK1 was immunoprecipitated and the precipitates were divided. The half of them were used for Western blot analysis using anti-JNK1 mAb (lower panel). The remaining half were used for kinase activity using GST–c-Jun as a substrate. The kinase reaction products were resolved by 12.5% SDS–PAGE and their phosphorylation was quantified by autoradiography (upper panel). B: Human B cell lines, C1LCL and NH-1 (2×10^6/sample), were stimulated for indicated times with $F(ab')_2$ fragments of a goat anti-human IgM (μ) Ab (14 μg/ml), lysed and immunoprecipitated with anti-JNK1 mAb. JNK1 activity was assessed as described in (A).

BCR-mediated JNK1 activation is a downstream event of Btk [8]. To evaluate whether this conclusion is applicable to other species, we examined human B-cell lines from normal individuals (C1LCL) and from XLA patients (NH-1). In order to establish these cell lines, B cells were transformed with a mutant EBV lacking LMP2 [13,14]. As shown in Fig. 1B, normal B cells evoked JNK1 activation upon BCR ligation, while XLA B cells failed to induce the JNK1 response despite the same expression level of BCR to that of normal B cells (data not shown).

We and others have demonstrated that Btk regulates PLC-γ2 activation in BCR signaling [11,17,18]. Given the evidence that BCR-mediated JNK1 response requires both PLC-γ2 and Rac1 activation [9], these observations indicate that Btk regulates only PLC-γ2, leading to the JNK1 response, or that both of these two pathways lie downstream of Btk. To distinguish between these possibilities, we studied directly the amount of GTP-bound Rac1 in wild-type and Btk-deficient DT40 cells. To measure Rac1 activity, we used an affinity assay to precipitate Rac1-GTP from cell lysates [16,19,20]. In this assay, a fragment of p65/p21-activated kinase ($p65^{PAK}$) including the CRIB, a Rac1 effector that binds with high affinity to Rac1-GTP but not to Rac1-GDP, was used to construct an affinity matrix. Stimulation by BCR led to a rapid and transient activation of Rac1 in wild-type DT40 cells, whereas this activation was significantly inhibited in Btk-deficient DT40 cells (Fig. 2A,B). Transfection of Btk into Btk-deficient DT40 cells restored Rac1 activation as well as JNK1 response upon BCR ligation (Fig. 1A and 2A,B), confirming that these defects are due to Btk. Although the stimulation-fold in normal human B cells was lower than that in wild-type DT40 B cells, the BCR-mediated Rac1 activation was reproducibly observed. In contrast to normal B cells, XLA B cells failed to activate Rac1 (Fig. 2C). Together, these results indicate that Rac1 activation is regulated by Btk in BCR signaling context.

To determine whether Rac1 activation lies downstream or in parallel to PLC-γ2 activation in BCR signaling context, we determined Rac1 activation in PLC-γ2-deficient DT40 cells [10]. As shown in Fig. 2A,B, Rac1 activation occurred normally even in the absence of PLC-γ2, indicating that Rac1 is activated independently of PLC-γ2 activation.

Using wild-type and Btk-deficient B cells, we have found that BCR cross-linking induces the activation of Rac1, which is regulated by Btk. Although the involvement of Btk in Rac1 activation is clear, the residual Rac1 activation in Btk-deficient DT40 B cells implicates that other PTKs such as Syk, in addition to Btk, may play a role in Rac1 activation; this additional PTK may be capable of activating Rac1 to some extent even in the absence of Btk in DT40 B cells.

One of the pathways regulated by Rac1 is the JNK1 signal-

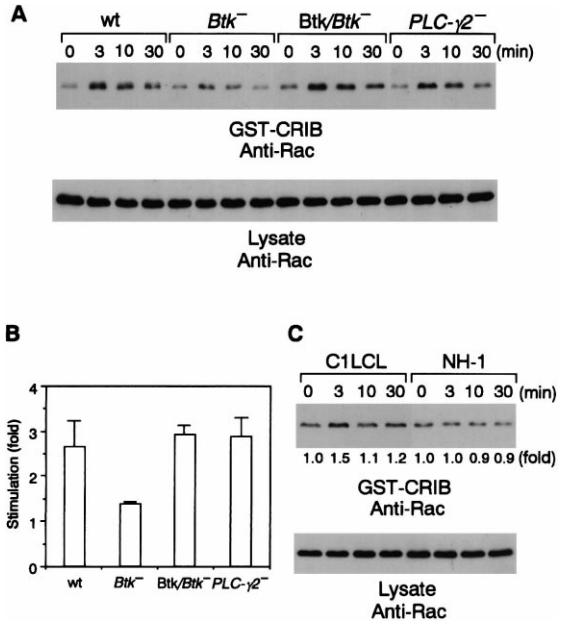

Fig. 2. BCR-induced Rac1 activation requires Btk, but not PLC-γ2. Various DT40 cells and human B cells were stimulated as described in the legend to Fig. 1. After stimulation, cell lysates were used for the affinity precipitation assay in the presence of GST–CRIB as described in Section 2. Proteins bound to GST–CRIB and aliquots of whole-cell lysates were separated on SDS–PAGE (12.5% gel) and were analyzed by Western blotting using anti-Rac1 mAb. A: Representative results of the three independent experiments in DT40 cells are shown. B: Peak activation of Rac1 in DT40 cells (at 3 min) was analyzed by densitometric quantification and expressed as fold increase compared to activity at time 0 (mean ± S.E.M., $n=3$). C: Representative results of the three independent experiments in human B cells were shown. Simulation-fold at 3 min (mean ± S.E.M., $n=3$) is 1.4 ± 0.13 in C1LCL cells and 0.95 ± 0.01 in NH-1 cells.

ing pathway; indeed, a dominant-negative mutant of Rac1 abrogates the BCR-mediated JNK1 activation in DT40 B cells [9]. Thus, it is likely that Btk-regulated Rac1 activation is one critical event for inducing JNK1 response in B cells. The molecular link(s) between Btk and Rac1 still remains elusive. Recent reports demonstrate that Vav has guanine nucleotide exchange activity towards Rho-family G proteins such as Rac1 and Cdc42 [21] and that this activity is dependent on its tyrosine phosphorylation [22]. Moreover, our recent study has revealed that BCR-mediated Rac1 activation is attenuated by $\sim$50% in Vav3-deficient DT40 B cells [23]. Collectively, one potential model is that Btk mediates tyrosine phosphorylation of Vav family proteins, which in turn regulates Rac1 activation. However, BCR-induced tyrosine phosphorylation of Vav3 as well as Vav2 occurred normally in Btk-deficient DT40 B cells, judged by anti-phosphotyrosine mAb blotting (data not shown). These results suggest that Btk may regulate Rac1 independently of Vav families, although we cannot completely exclude the possibility that the small changes of Vav2/Vav3 phosphorylation may have escaped our detection system.

Differential time course between Rac1 and JNK1 activation (stimulation peaks; 3 min versus 10 min) could be explained by the existence of two distinct GTPases which might be involved in the initial phase and the sustained JNK1 activation, respectively. Reminiscence of this type of regulation is nerve growth factor-mediated ERK activation; the early phase of the ERK activation is mediated by Ras, but sustained activation of this pathway is due to Rap1 [24]. Analogous to this mechanism, Rac1 might be involved only in initiation of the JNK1 pathway and an additional GTPase might be required for sustaining this response. Since Rac1 is known to regulate the cytoskeletal organization [25], the alternative possibility is that this Rac1-mediated cytoskeletal organization, in addition to regulating MEKKs, is required for BCR-mediated JNK1 response. This cytoskeletal organization may require a relatively long time, explaining the time lag between Rac1 and JNK1 activation.

Normal BCR-mediated Rac1 activation in the absence of PLC-γ2, together with the evidence that PLC-γ2 is involved in BCR-mediated JNK1 response, suggests that the PLC-γ2-dependent signals, PKC and calcium signals are integrated downstream or in parallel to Rac1, leading to JNK1 activation. Hence, our results strongly support the model that Btk-dependent pathways bifurcate, such that PLC-γ2 and Rac1 signals are generated, leading to convergence on JNK1 response in B cells.

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