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Phosphoinositide 3-kinase and integrin signalling are involved in activation of Bruton tyrosine kinase in thrombin-stimulated platelets

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Abstract Bruton tyrosine kinase (Btk) plays a crucial role in the differentiation of B lymphocytes and belongs to the group of Tec kinases, which are characterised by the presence of a pleckstrin homology domain. Here we show that Btk is activated and undergoes tyrosine phosphorylation upon challenge of platelet thrombin receptor, these responses requiring engagement of α_{IIb}/β_3 integrin and phosphoinositide 3-kinase activity. These data unravel a novel signalling pathway involving Btk downstream of an adhesive receptor via a complex regulation implicating the products of phosphoinositide 3-kinase, which might act to anchor Btk at the membrane.

Key words: Bruton tyrosine kinase; Platelet; Phosphoinositide 3-kinase; α_{IIb}/β_3 integrin; Thrombin

1. Introduction

Platelets play a key role in haemostatic reactions and are activated by numerous agonists, including thrombin. Upon cleavage of its seven-transmembrane domain receptor, thrombin induces three successive waves of tyrosine phosphorylation involving several non-receptor protein tyrosine kinases (PTK) [1]. Recently, a new subfamily of cytoplasmic PTK that share sequence homology with Src has been identified. They include Bruton tyrosine kinase (Btk), Itk, Tec and Bmx [2]. In addition to the catalytic, Src homology 2 (SH2) and SH3 domains, Btk has a characteristic N-terminal region consisting of a pleckstrin homology (PH) domain and a proline-rich region referred to as the tec homology (TH) domain [3]. These kinases are predominantly expressed in the haematopoietic lineage, but Tec and Bmx seem to be more ubiquitous. Btk, which is particularly present in B lymphocytes, is activated by stimulation of interleukin receptors [4], IgE receptor (FcεRI) [5], and B cell receptor [6]. Btk deficiency results in human and murine genetic disorders such as X-linked agammaglobulinaemia, indicating that this kinase plays an important role in B-cell differentiation [3,7].

Btk can also be activated by G_q protein α subunit [8], while a direct activation of both Btk and Itk by G protein βγ subunits has been observed in vitro [9]. In addition, the N-terminus of the PH domain of Btk has been shown to bind with high affinity to inositol 1,3,4,5-tetrakisphosphate and (or) phosphatidylinositol 3,4,5-trisphosphate [10–13], this kind of interaction promoting association of Btk with the plasma membrane [13]. These data suggest that the PH domain of Tec kinases might be critical for their activation. Moreover, the PH domain of Btk is able to interact with several isoforms of protein kinase C (PKC), causing a decrease of its tyrosine kinase activity [14]. This observation contrasts with the positive effects of PKC described for another PTK from the Tec family (Itk) specific to T lymphocytes [15]. If the role of PKC in the modulation of PH domain-containing PTK seems to be yet unclear, the implication of the Src kinase family in stimulating the enzymatic activity of Btk is now well documented and involves complex interactions between the proline-rich sequence of Btk TH domain and the SH3 domain of Src kinases [16,17].

In this study, we provide evidence that this PH domain-containing PTK is present in platelets, and participates in a signalling pathway occurring downstream of integrin α_{IIb}/β_3. We also show that phosphoinositide (PI) 3-kinase is involved in both the activation of Btk and its translocation to the membrane of activated platelets.

2. Materials and methods

2.1. Materials

Human thrombin, thrombin-receptor activating peptide (TRAP), tetrapeptide RGDS, wortmannin, protein A-Sepharose, glutathione-Sepharose, anti-mouse and anti-rabbit IgG horseradish peroxidase conjugate were purchased from Sigma. A polyclonal antibody directed against the PH+TH domain of Btk was obtained by injecting a rabbit with the fusion protein GST-Btk (1–216). Anti-phosphotyrosine monoclonal antibody 4G10 was from Upstate Biotechnology Inc. (Euromedex, Strasbourg, France). [γ-^{32}P]ATP (3000 Ci/mmol) was from Amersham (Les Ulis, France). The enhanced chemiluminescence SuperSignal Western blotting substrate was obtained from Pierce (Interchim, Montluçon, France).

2.2. Incubation of platelet suspensions and Btk immunoprecipitation

Platelet suspensions (1×10^9 cells/ml) in Tyrode's buffer containing albumin but lacking calcium were prepared from human platelet concentrates (Etablissement de Transfusion Sanguine Pyrénées-Garonne, France) as previously described [18,19]. In some experiments, platelet suspensions (500 μl) were incubated with either RGDS (500 μM), RGES (500 μM), or wortmannin (10 or 100 nM) for 20 min before addition of TRAP (10 μM). Incubations were terminated by addition of 250 μl of lysis buffer thus achieving the following final concentrations: Tris-HCl, pH 8.0, 20 mM; NaCl, 150 mM; EDTA, 2 mM; Triton X-100, 1% (v/v); sodium dodecyl sulphate (SDS), 0.2% (w/v); Na_3VO_4, 1 mM; aprotinin, 10 μg/ml; leupeptin, 10 μg/ml; phenylmethylsulphonyl fluoride, 1 mM; sodium pyrophosphate, 5 mM. After standing on ice for 15 min, the lysates were sonicated (twice for 20 s) and centrifuged ($12\,000\times g$ for 10 min at 4°C) to remove

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insoluble material. The clear lysates (700 μl) were incubated for 30 min with 40 μl of a 10% suspension of protein A-Sepharose and centrifuged as above. The supernatants were then incubated for 2 h at 4°C with rabbit polyclonal anti-Btk antibody, followed by addition of 80 μl of a 10% suspension of protein A-Sepharose for 1 h at 4°C. The immunoprecipitates were then collected by centrifugation ($6000 \times g$ for 5 min at 4°C), washed five times in lysis buffer and solubilised in Laemmli's sample buffer for polyacrylamide gel electrophoresis [20].

2.3. In vitro kinase assay of Btk

Clarified whole lysate from 500 μl platelet suspension was used for immunoprecipitation as described above, except that SDS was omitted. The immune complex was washed twice in lysis buffer and twice again in 50 mM Tris-HCl (pH 7.4) containing 0.5 mM Na_3VO_4. The kinase reaction was carried out for 20 min at 30°C under shaking in 40 μl of kinase buffer (50 mM Tris-HCl, pH 7.4, 2.5 mM $MgCl_2$ and 5 mM $MnCl_2$) containing 10 μM ATP, 10 μCi [γ-^{32}P]ATP and 10 μg of GST-PLCγ1. The latter fusion protein contained amino acids 538–780 of PLCγ1, including the two SH2 domains as well as phosphorylatable tyrosine residues 771 and 775. In some experiments a truncated GST-PLCγ1 (amino acids 538–757) was also used. To better determine the substrate specificity of Btk, another fusion protein (GST-HS1) containing HS1 peptide (amino acids 388–403), which is specifically phosphorylated by Syk tyrosine kinases, was used in some experiments.

2.4. Detection of Btk translocation to the platelet membrane

Washed platelets (7.5×10^8/ml) were activated by TRAP (10 μM) after a previous incubation (20 min at 37°C) in the absence or in the presence of wortmannin (10 or 100 nM). Reactions were stopped by addition of one volume of twice concentrated ice-cold buffer I, which contained 50 mM Tris (pH 7.4), 200 mM KCl, 6 mM ATP, 6 mM $MgCl_2$, 10 mM benzamidine, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 2 mM phenylmethylsulphonyl fluoride and 0.2 mM Na_3VO_4. All subsequent steps were performed at 4°C. Cells were sedimented immediately by centrifugation at $12\,000 \times g$ for 20 s, resuspended in 600 μl of buffer I, and sonicated four times for 10 s. The membrane fraction was isolated by centrifugation at $120\,000 \times g$ for 40 min. Membranes were washed once with 100 μl of buffer I, then suspended in 500 μl of buffer I for protein determination using a Bio-Rad assay system, and 15 μg of protein from each sample were analysed by Western blotting using anti-Btk polyclonal antibody.

2.5. Protein electrophoresis and immunoblotting

Immunoprecipitates were boiled with 20 μl of Laemmli's sample buffer, proteins were separated on an 8% polyacrylamide gel in the presence of SDS under reducing conditions [20], transferred to nitrocellulose membranes and checked by immunoblotting essentially as described in [18,21], using the enhanced chemiluminescence system.

3. Results

3.1. Platelet Btk is tyrosine phosphorylated and activated upon thrombin stimulation

In order to examine whether Btk is implicated in thrombin signalling, human platelets were stimulated for various periods of time with TRAP (10 μM) and the tyrosine kinase was immunoprecipitated. Western blotting with anti-phosphotyrosine antibody showed that Btk was slightly phosphorylated at 10 s, tyrosine phosphorylation reaching a maximum at 3 min of stimulation (Fig. 1A). It was verified that equal amounts of Btk were recovered in the immunoprecipitates (Fig. 1B), and that no signal was detected with immunoprecipitates obtained with non-immune serum (not shown).

We also tested whether TRAP stimulation would increase Btk activity using an immune complex kinase assay. Tyrosine phosphorylation of Btk was correlated with a significant increase in its activity, detected using GST-PLCγ1 (538–780) as an exogenous substrate, maximal activity (2.05 ± 0.26-fold increase) being observed after 3 min of thrombin stimulation (Fig. 1C). As a control, GST-PLCγ1 (538–757), which lacks tyrosine residues 771 and 775, was not phosphorylated by Btk (Fig. 1D). Moreover, this Btk activity appeared to be sub-

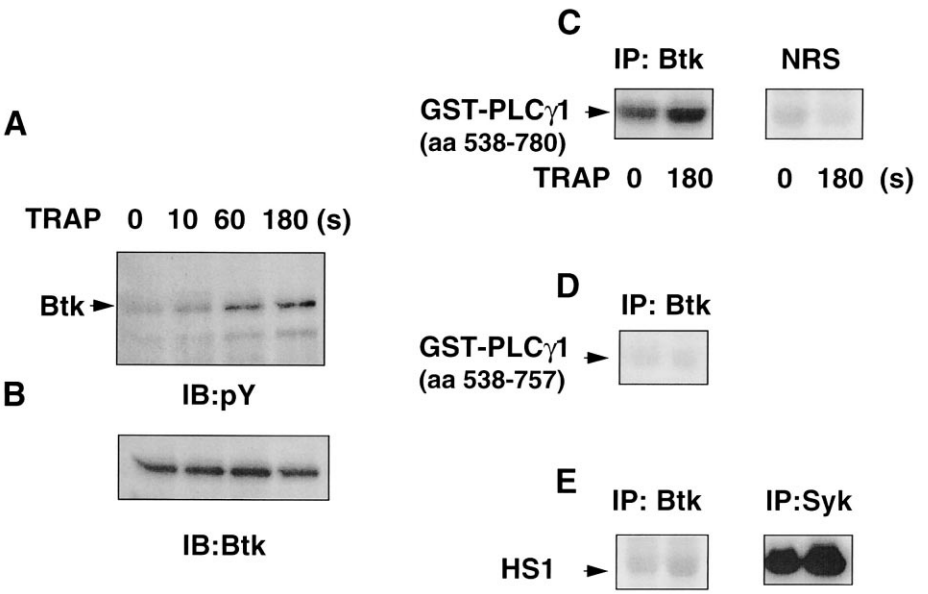

Fig. 1. Time course of Btk tyrosine phosphorylation and activation in thrombin-stimulated platelets. Platelets (5×10^8) were stimulated with TRAP (10 μM) for the indicated times and subjected to immunoprecipitation (IP) as described in Section 2. Immunoprecipitates were analysed by anti-phosphotyrosine immunoblotting (A). The level of immunoprecipitated Btk was revealed by immunoblotting (IB) using anti-Btk antibody (B). Immunoprecipitates were subjected to an immune complex kinase assay using as a substrate GST-PLCγ1 (538–780), which contains the two SH2 domains as well as tyrosine residues 771 and 775 (C). In some experiments, this substrate was replaced by a fusion protein (GST-PLCγ1 538–757) lacking the two phosphorylatable tyrosine residues (D) or by GST-HS1 (E). Radiolabelled proteins were separated by SDS-PAGE and visualised by PhosphorImager scanning. Data are representative of three experiments. NRS, normal rabbit serum.

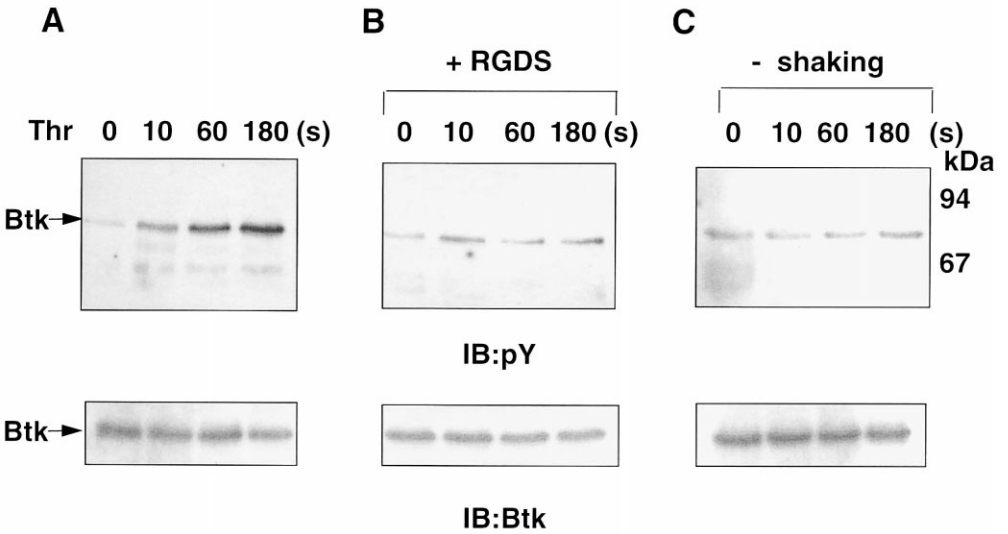

Fig. 2. Btk tyrosine phosphorylation is dependent on integrin α_{IIb}/β_3 engagement and on platelet aggregation. Platelets (5×10^8) were stimulated for the indicated times with thrombin (1 NIH unit/ml) with (A and B) or without (C) shaking. In B, platelets were preincubated with RGDS (500 μM). Lysates were subjected to immunoprecipitation with anti-Btk antibody and analysed by antiphosphotyrosine immunoblotting (IB: pY, upper panel). After stripping, the level of Btk protein was revealed by immunoblotting (IB: Btk, lower panel). Three experiments were performed with the same conclusions.

strate-specific since GST-HS1, which was shown to be a good substrate for Syk at tyrosine 397 [22], was not phosphorylated by Btk (Fig. 1E).

3.2. Btk tyrosine phosphorylation and activation are coupled to integrin α_{IIb}/β_3 engagement

Addition of RGDS to thrombin-stimulated platelets abolished aggregation by preventing fibrinogen binding to integrin. This treatment inhibited by 76 ± 5% Btk tyrosine phosphorylation occurring at 3 min of thrombin stimulation (Fig. 2A,B). Moreover, when platelet aggregation was prevented by the absence of shaking during stimulation, Btk tyrosine phosphorylation was also strongly reduced (Fig. 2C). In addition, TRAP-induced activation of Btk was inhibited by 74 ± 9% by RGDS, whereas RGES was without significant effect (Fig. 3). These data clearly indicate that Btk phosphorylation is dependent on platelet aggregation via integrin α_{IIb}/β_3 engagement.

3.3. PI 3-kinase is involved in thrombin-induced activation and membrane association of Btk

The stimulation of Btk activity promoted by TRAP (10 μM) was reduced by 57 ± 8% when platelets were preincubated with 10 nM wortmannin (Fig. 3). As shown in Fig. 4, TRAP (10 μM) promoted the translocation of Btk to the platelet membrane, which reached around 4 times the basal level after 1 min of stimulation. Membrane association of Btk promoted by TRAP was decreased by 66 ± 12% or 94 ± 8% in the presence of 10 or 100 nM wortmannin, respectively (Fig. 4). These results indicate that PI 3-kinase products are actually involved in the translocation of Btk to the membrane of activated platelets.

4. Discussion

From our investigation, it is obvious that Btk is present in significant amounts in platelets and appears to participate in the signalling pathways evoked upon activation of thrombin receptor. Btk tyrosine phosphorylation and activation observed herein appear mainly as secondary events related to integrin α_{IIb}/β_3 engagement, since they are inhibited when aggregation is suppressed by competing for fibrinogen binding to its receptor. These results indicate that Btk is not restricted to immunocompetent cells [3–7] and might also be involved in cell signalling evoked by adhesion receptors, the data reported herein representing the first example of such a possible link between Btk and integrins. We and others have also previously described the same relationship between another PH domain-containing tyrosine kinase (Tec) and platelet α_{IIb}/β_3 integrin [23,24].

Platelets constitute a very useful model to delineate a number of signalling events evoked by integrin engagement and (or) its immediate functional consequence, i.e. platelet aggregation. These include Ca^{2+} influx [25] and various tyrosine phosphorylations possibly involving Src kinases, Syk or the focal adhesion kinase $pp125^{FAK}$ [26,27]. It is thus tempting to speculate upon which of the signalling events occurring downstream of integrin α_{IIb}/β_3 could be related to the tyrosine phosphorylation and activation of Btk. Two of them deserve some discussion and include Src kinases and PI 3-kinase.

Several reports have shown that the activation loop for tyrosine phosphorylation of Btk is generated by a combination of the activities of both Src kinases and Btk itself [16,17]. Indeed, the SH3 domain of Src kinases is able to bind to the TH domain of Btk [28]. This binding is followed by tyrosine phosphorylation of Y551 in the Btk catalytic domain. This transphosphorylation by Src kinases leads to an increase in the catalytic activity of Btk and to autophosphorylation at the second site present in its SH3 domain [29,30]. In platelets, $pp60^{src}$ is activated early after thrombin stimulation, possibly leading to Btk tyrosine phosphorylation. This would be analogous to the process already described for Btk activation in B cells upon antigen stimulation, since Lyn and Blk are stimulated within seconds after B cell receptor activation, Btk phosphorylation occurring later on at 5 min [31]. In the same way, Li et al. [32] have shown a correlation between membrane

targeting and an increase of Btk tyrosine phosphorylation, suggesting that Btk may have to be placed in the vicinity of a Src kinase to become activated.

In this context, the fact that thrombin stimulation promotes translocation of Btk to the membrane is particularly interesting to consider. This phenomenon has been already reported by Kawakami et al. [5] in mast cells stimulated by IgE. Some membrane phospholipids, essentially the products of PI 3-kinase, might be involved, as suggested by the effect of wortmannin. Since a specific interaction between the PH domain of Btk and phosphatidylinositol 3,4,5-trisphosphate has been reported, it is tempting to suggest that the products of PI 3-kinase might play a crucial role in the behaviour of Btk observed in thrombin-stimulated platelets. Our observations are in agreement with previous studies describing similar specific interactions between PI 3-kinase products and Btk or Itk in immunocompetent cells [12,13,33,34]. Similar interactions also occur between a serine/threonine kinase bearing a PH domain (Akt) and PI 3-kinase products [35]. However, it is still difficult to conclude whether the inhibitory effect of wortmannin on Btk activation is due to the lack of Btk translocation or to the fact that wortmannin inhibits α_{IIb}/β_3 integrin and platelet aggregation [36].

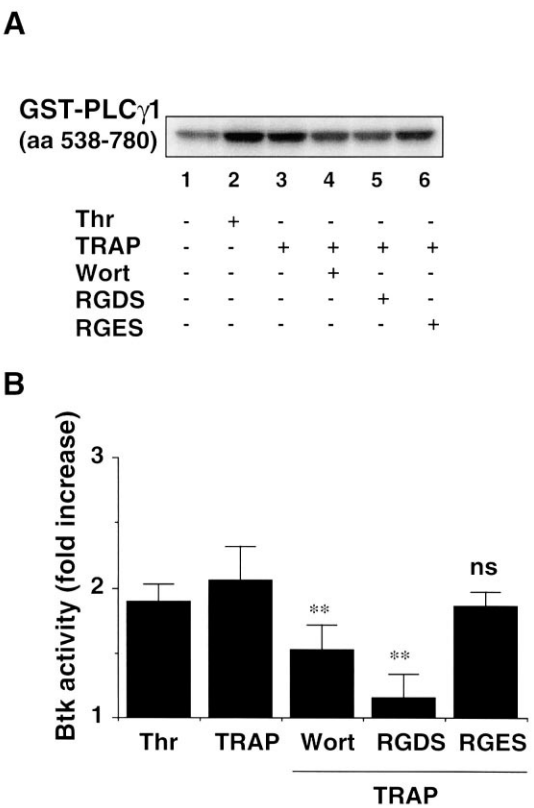

Fig. 3. PI 3-kinase and α_{IIb}/β_3 integrin are involved in Btk activation by TRAP. A: Platelets (5×10^8) were preincubated for 20 min in the absence of inhibitors (lanes 1–3), with 10 nM wortmannin (Wort, lane 4), 500 μM RGDS (lane 5) or 500 μM RGES (lane 6) before addition of 10 μM TRAP (lanes 3–6) or 1 NIH unit/ml of thrombin (Thr, lane 2) and further incubation for 3 min at 37°C. In vitro kinase assay of Btk was performed using GST-PLCγ1 (538–780) as a substrate. Proteins were separated on a 10% polyacrylamide gel and radioactivity was detected with PhosphorImager. The data shown are representative of four different experiments with identical results. B: The radioactivity of the substrate was quantified using Imagequant program and data (means ± S.E.M., $n=4$ experiments) are expressed as fold increase of Btk activity. $^{**}P<0.01$, probability of significance according to Student's t-test; ns, non-significant.

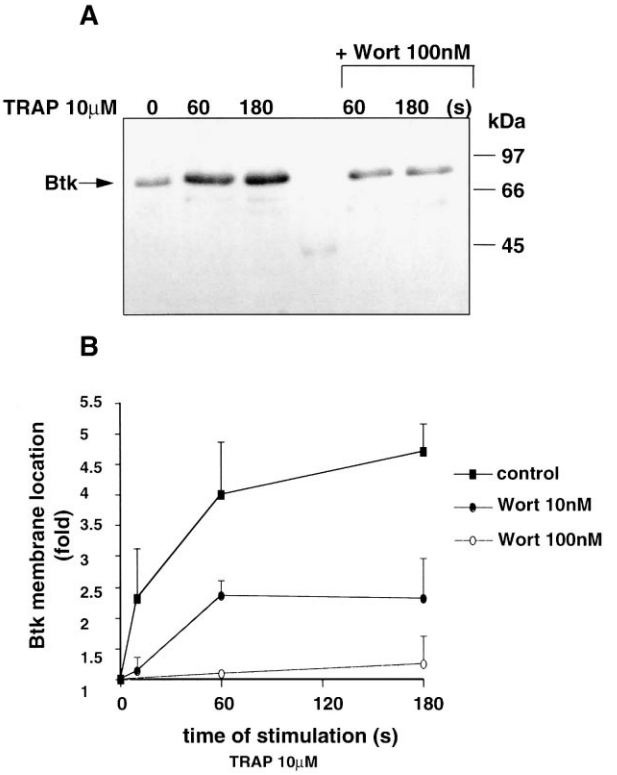

Fig. 4. Effect of wortmannin on Btk translocation to the platelet membrane. Platelet suspensions were preincubated or not for 10 min at 37°C with 10 nM or 100 nM wortmannin (Wort), followed by addition of TRAP (10 μM) and further incubation for the indicated times. At the end of the incubations, the platelet membrane was isolated as described in Section 2 and probed for the presence of Btk by immunoblotting. A typical immunoblot is shown in A, whereas B presents results of immunoblot quantification. Data (stimulation fold compared to non-activated control platelets) are means ± S.E.M. of three different experiments.

In most examples described so far, Btk tyrosine phosphorylation is accompanied by activation of its kinase activity [16,17], which we also observed in platelets. However, it still remains to identify specific substrates of Btk. To date, only PLCγ2 was found to be tyrosine phosphorylated and activated by Btk in B lymphocytes [37]. This is in agreement with the fact that PLCγ1 appeared as a selective substrate of Btk in our in vitro kinase assay.

During the preparation of this article, it was reported that Btk is tyrosine phosphorylated in collagen- and thrombin-treated platelets, only collagen response being altered in patients lacking Btk [38]. This led the authors to suggest that Btk is not essential in the mechanism of platelet activation by thrombin. If this is true for the primary events evoked downstream of platelet thrombin receptor, the present study brings evidence that Btk is activated in the signalling pathway evoked by an integrin. This should stimulate further studies dealing with other PTK of the Tec family and in other cells than platelets, where adhesive events are known to play an important role in signalling pathways regulating cell proliferation.

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