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Bruton's Tyrosine Kinase Associates With the Actin-Based Cytoskeleton in Activated Platelets

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Abstract Bruton's tyrosine kinase (Btk) plays a crucial role in the maturation and differentiation of B-lymphocytes and immunoglobulin synthesis. Recently Btk has been described to be present in significant amount in human platelets. To investigate the regulation of this kinase in the platelets we studied its subcellular redistribution in the resting and activated cells. In the resting platelets Btk was almost absent from the actin-based cytoskeleton. Upon challenge of the platelet thrombin receptor upto 30% of total Btk appeared in the cytoskeleton and the protein underwent phosphorylation on tyrosine. Translocation of Btk to the cytoskeleton but not aggregation was prevented by cytochalasin B, which inhibits actin polymerization. Wortmannin and genistein (inhibitors of phosphoinositide 3-kinase and protein tyrosine kinase, respectively) decreased while phenylarsine oxide (a tyrosine phosphatase inhibitor) increased the cytoskeletal content of Btk. The association of Btk with the cytoskeleton was regulated by integrin $\alpha_{IIb}\beta_3$ and partly reversible. Taken together, these data suggest that Btk might be a component of a signaling complex containing specific cytoskeletal proteins in the activated platelets. J. Cell. Biochem. 81:659–665, 2001. © 2001 Wiley-Liss, Inc.

Key words: Platelet; cytoskeleton; Bruton's tyrosine kinase; integrin; thrombin receptor; phosphoinositide 3-kinase; protein tyrosine kinase

Bruton's tyrosine kinase is a 77 kD cytoplasmic tyrosine kinase belonging to the Tec family. Present particularly in B-lymphocytes, it has been implicated in the causation of X-linked agammaglobulinemia in humans and X-linked immunodeficiency in mice [Rawlings et al., 1993; Vihinen et al., 1995; Hyvonen and Saraste, 1997]. Although it shares a number of structural characteristics with the Src family kinases, it possesses several unique domains such as pleckstrin homology (PH) domain, a proline-rich region (PRR) and a Ras GTPase-activating protein (RasGAP) motif.

Btk has recently been reported to be present in the platelets [Quek et al., 1998; Laffargue et al., 1999]. It is activated downstream of the receptors for thrombin and collagen, as well as the FcγRIIA receptor in platelets. Members of the Src family of protein tyrosine kinases, Syk and focal adhesion kinase (FAK) are already described in significant amounts in the platelets and their regulation and physiology studied in detail. While Btk plays a central role in the development and differentiation of B- lymphocytes [de-Weers et al., 1994], degranulation and cytokine production in mast cells [Kawakami et al., 1994] and also as a dual regulator of apoptosis [Uckun, 1998], platelets are not immunocompetent and have relatively distinct functions related to haemostasis and thrombus formation. Thus the function of Btk in the platelets remains relatively unknown. In the present study we provide evidence that Btk specifically associates with the actin-based cytoskeleton upon challenge of the platelet thrombin and FcγRIIA receptors.

MATERIALS AND METHODS

Reagents

Polyclonal antibodies against Btk, monoclonal antibody against phosphotyrosine (clone

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PY99), horseradish peroxidase-labeled secondary antibodies and Protein G-agarose were purchased from Santa Cruz. The mAb IV.3 specific for FcγRIIA receptor was from Medarex. Goat IgG was procured from Bangalore Genei, India. The Super Signal West Pico chemiluminescent substrate was from Pierce. Cross linking goat anti murine $F(ab')_2$ fragment, human thrombin, apyrase, EGTA, sodium orthovanadate, acetylsalicylic acid, bovine serum albumin fraction V, Triton X-100, leupeptin, pepstatin A, aprotinin, phenylmethylsulfonyl fluoride, dimethylsulfoxide (DMSO), phenylarsineoxide (PAO), Arg-Gly-Asp-Ser (RGDS), HEPES, wortmannin and cytochalasin B were the products of Sigma. Tween 20 and genistein were from Bio-Rad and Biomol (Germany) respectively. Protease inhibitor tablets were from Boehringer Maanheim, Germany. The reagents for electrophoresis were procured from SRL, India. All other reagents were of analytical grade.

Platelet Preparation

Blood from volunteers was collected in 0.1 vol of 3.8% trisodium citrate and centrifuged at 180 *g* for 20 min. Platelet rich plasma was incubated with 1 mM acetylsalicylic acid and 0.15 ADPase units of apyrase/ml for 15 min at 37°C. After addition of citric acid (9 mM) and EDTA (5 mM), platelets were pelleted by centrifugation at 800 *g* for 15 min. They were resuspended in washing buffer (20 mM HEPES, 138 mM NaCl, 2.9 mM KCl, 1 mM $MgCl_2$, 0.36 mM NaH_2PO_4, 1 mM EGTA, supplemented with 5 mM glucose and 0.6 ADPase units/ml of apyrase, pH 6.2). Platelets were finally resuspended in resuspension buffer (pH 7.4) which was same as washing buffer but without EGTA and apyrase. The final cell count was adjusted to $0.8–1.2 \times 10^9$/ml.

Platelet Activation and Aggregation

Platelets were stirred at 37°C in a Chrono-log platelet ionized calcium aggregometer (model 600) for 2 min prior to addition of thrombin receptor-activating peptide (TRAP, SFLLRN) (10 μM) or thrombin (1.6 U/ml). Aggregation was measured by change in light transmission (%). In some experiments, aggregation was prevented by incubating the platelets at 37°C in the absence of stirring or by preincubation with EGTA (2 mM) for 20 min or RGDS (0.5 mM) for 3 minutes. In other experiments, platelets were pretreated at 37°C with genistein (150 μM), PAO (2 μM) for 5 min or wortmannin (100 nM) for 20 min or cytochalasin B (20 μM) for 15 min prior to addition of agonist. In the control samples platelets were incubated with DMSO (0.2%). For stimulation of platelet FcγRIIA receptors platelets were incubated with 0.5 μg of mAb IV.3 for 1 min with stirring, followed by 15–30 μg of the cross-linking anti-mouse $F(ab')_2$ fragment.

Isolation of Detergent Lysates of Platelets

Platelets were lysed by adding equal volumes of ice-cold 2 × Triton lysis buffer (pH 7.5) containing 2% Triton X-100, 100 mM Tris-HCl, 10 mM EGTA, 10 mM EDTA, 2 mM sodium orthovanadate, 21 μM leupeptin, 2 mM phenylmethylsulfonyl fluoride, 20 μM pepstatin A and 0.56 trypsin inhibitor unit/ml aprotinin. After keeping on ice for 10 min, samples were briefly vortexed and spun at 12,000 *g* for 20 min at 4°C in a microcentrifuge. The pellets (Triton-insoluble cytoskeleton) were washed without resuspension in 1 × Triton lysis buffer and finally solublized in 1 × sample buffer by heating at 95°C for 10 minutes.

Immunoblotting Studies

Platelet proteins were separated on 10% SDS-PAGE gels and electrophoretically transferred to Immobilon-P PVDF membranes (Millipore Corp.) by using Mini Trans-Blot Cell (Bio-Rad) or Nova Blot semidry system (Pharmacia) as per manufacturer's instructions. The membranes were blocked with 10% bovine serum albumin in 10 mM Tris-HCl, 150 mM NaCl, pH 8.0 (TBS) containing 0.05% Tween 20 for 2 h at room temperature. The blots were then incubated for 2 h with different dilutions of primary antibodies as follows: goat anti-Btk, 1:750 or anti-phosphotyrosine, 1:2000. Following washing, the blots were incubated for 1 h with horseradish peroxidase-labeled anti-goat IgG (1:15000) for Btk or anti-mouse IgG (1:15000) for phosphotyrosine. The antibody binding was detected using enhanced chemiluminescence and bands were quantified by a Shimadzu CS-9000 dual wavelength flying spot scanner. Whenever necessary, blots were stripped of primary and secondary antibodies by incubation for 10 min at 80°C in the stripping buffer (TBS containing 0.05% Tween 20, 2% SDS and 1% 2-mercaptoethanol) and reprobed. To quantify actin in platelet cytoskeletal preparations, platelet proteins were separated by SDS-PAGE,

stained with Coomassie Brilliant Blue and the bands corresponding to actin were measured by densitometry. In some experiments, the actin bands were quantified densitometrically in the reflectance mode after the proteins were transferred to PVDF membranes and stained with Coomassie Brilliant Blue.

Immunoprecipitation Experiments

Platelet suspensions (0.5 ml) were lysed in equal volume of 2 × radioimmunoprecipitation assay (RIPA) buffer (pH 7.4, 2% Triton X-100, 2 mM EGTA, 2 mM EDTA, 100 mM HEPES, 150 mM NaCl, 2 mM Na_3VO_4, one protease inhibitor cocktail tablet/5 ml) for 30 min on ice. The lysates were clarified by centrifugation at 12000 *g* for 15 min at 4°C. The supernatant was precleared by goat IgG and protein G-agarose, and then incubated with 7 μg of anti-Btk antibody for 1 h followed by 20 μl of Protein G-agarose. The immunoprecipitates were pelleted by centrifugation for 10 min at 12000 *g*, washed twice in 1 × RIPA buffer and once each with high salt buffer (100 mM HEPES, 0.5 M NaCl, pH 8) and 0.5 M HEPES (pH 8) and solublized in 50 μl of 2 × sample buffer.

Presentation of Data

Data are presented as mean ± SEM of individual experiments from different blood donors. Immunoblots shown are representative of at least three different experiments.

RESULTS

The Translocation of Btk to the Platelet Cytoskeleton is Induced by Thrombin Receptor Activation and FcγRIIA Cross Linking

In the present study, we used a polyclonal antibody raised against the carboxyl terminus of Btk to detect the protein in human platelets. The corresponding peptide fragment completely blocked the appearance of the 77 kD band, thus confirming its identification. We next examined the redistribution of Btk to the platelet cytoskeletal compartment following stimulation of the cells with either human thrombin or TRAP (which corresponds to the amino terminus of cleaved thrombin receptor and acts as a tethered ligand) [Vu et al., 1991]. In the unstimulated platelets, more than 95% of Btk was recovered in the Triton-soluble fraction. Upon platelet stimulation the protein appeared in the cytoskeleton. The cytoskeletal content of Btk positively correlated with the extent of platelet aggregation. TRAP (10 μM) induced cytoskeletal association of about 15% of total cellular Btk at 95% aggregation (Fig. 1a) while thrombin (1.6 U/ml) was twice as potent in inducing the cytoskeletal translocation of this kinase (Fig. 1c). Appearance of Btk in the cytoskeleton was associated with disappear-

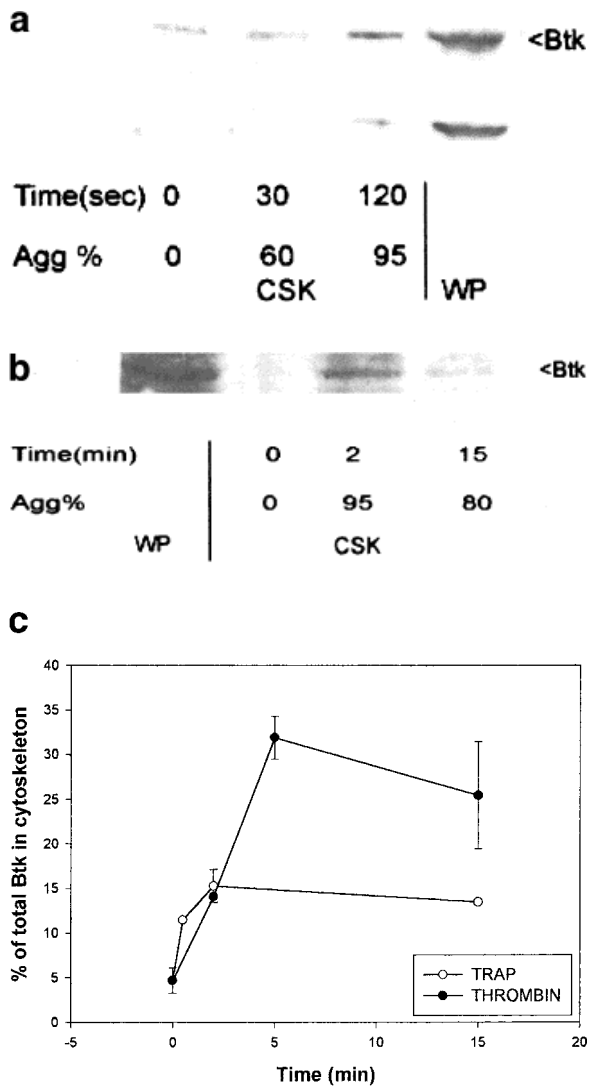

Fig. 1. Translocation of Btk to the cytoskeleton of the aggregating platelets. **(A)** and **(B)** Aliquots of washed platelets were aggregated with TRAP (10 μM) for the times indicated. Platelets were lysed with Triton lysis buffer. The cytoskeletal (CSK) fractions were isolated, proteins were separated by SDS-PAGE and immunoblotted using a polyclonal anti-Btk antibody. WP, whole platelet lysate. **(C)** Platelets were aggregated with 10 μM TRAP (open circle) or 1.6 U/ml thrombin (closed circle) and the content of Btk in cytoskeleton was quantified densitometrically. Results are the mean ± SEM of at least three experiments.

ance from the Triton-soluble fractions. Following 15 min stimulation with either TRAP or thrombin about 20% of Btk dissociated from the cytoskeleton (Fig. 1b,c) and reappeared in the Triton-soluble supernatant. Cytoskeletal association of Btk was also induced by cross-linking of FcγRIIA receptors on the platelet surface (data not shown).

Platelet aggregation induced by activation of the thrombin receptor is associated with a rapid increase in tyrosine phosphorylation of multiple proteins [Clark et al., 1994]. Btk was not tyrosine phosphorylated in the resting platelets. However, in the platelets aggregated either with thrombin or TRAP, Btk was found to be phosphorylated on tyrosine under conditions in which it translocated to the cytoskeleton. This is consistent with previous reports [Quek et al., 1998; Laffargue et al., 1999]. The extent of tyrosine phosphorylation of Btk remained the same when platelets were stimulated for a longer period (15 min) (Fig. 2).

The Redistribution of Btk is Regulated by Activities of Protein Tyrosine Kinases and Phosphatases, PI 3-Kinase, Actin Polymerization and Activation of the $\alpha_{IIb}\beta_3$ Integrin

We sought the role of protein tyrosine phosphorylation in the process of Btk translocation by using genistein [Dhar et al., 1990] and PAO [Greenwalt and Tandon, 1994; Yanaga et al., 1995], the inhibitors of protein tyrosine kinases and phosphatases, respectively. Genistein (150 μM) and PAO (2 μM), respectively, caused global decrease and increase in the tyrosine phosphorylation of platelet proteins stimulated with TRAP. In genistein-treated platelets cytoskeletal association of Btk was 77.4 ± 1.4% (n=3) of control, while aggregation was reduced by about 68%. On the other hand PAO, which also reduced aggregation by about 46%, increased the cytoskeletal association of Btk by 55.3 ± 15.9% (n=3) (Fig. 3). These data implicate the role of protein tyrosine phosphorylation in the cytoskeletal association of Btk.

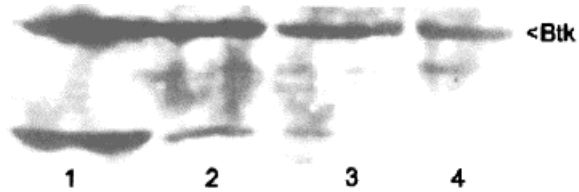

Fig. 3. Effect of genistein and PAO on the cytoskeletal association of Btk. Suspensions of washed platelets were pretreated with DMSO (0.2%, control) **(lane 3)** or genistein (150 μM) **(lane 4)** or PAO (2 μM) **(lane 2)** for 5 min at 37°C, followed by aggregation with TRAP (10 μM) for 2 min. Platelets were lysed with the Triton lysis buffer, cytoskeletal (CSK) fractions were isolated and immunoblotted using the polyclonal antibody against Btk. **Lane 1**, whole platelet lysate. Immunoblot shown is representative of three experiments.

We asked next whether increase of actin polymerization is necessary for the association of Btk with the cytoskeleton. Preincubation of platelets with cytochalasin B (20 μM) prevented thrombin-induced actin polymerization but had no effect on platelet aggregation, as earlier reported [Kirkpatrick et al., 1980]. Cytochalasin B treatment completely inhibited the cytoskeletal association of Btk, indicating that actin polymerization is a prerequisite for this process (Fig. 4).

The products of Phosphoinositide 3-kinase (PI 3-kinase) activity have been shown to bind

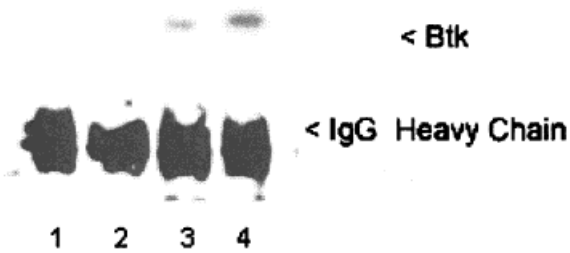

Fig. 2. Tyrosine phosphorylation of Btk in the thrombin-stimulated platelets. Aliquots (0.5 ml) of washed platelets were stimulated with thrombin for 2 min (lane 3) or 15 min (lane 4). Platelet lysates were precleared and incubated either with goat IgG **(lane 1)** or anti-Btk antibody **(lanes 2–4)**. The immunoprecipitates were analysed by anti-phosphotyrosine immunoblotting. Lanes 1 and 2, unstimulated platelets.

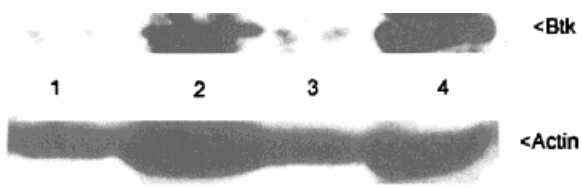

Fig. 4. Effect of cytochalasin B on the cytoskeletal association of Btk. Washed platelets were preincubated with either DMSO (0.2%) **(lane 2)** or cytochalasin B (20 μM) **(lane 1)** for 15 min at 37°C, followed by aggregation with thrombin (1.6 U/ml) for 5 min. Cytoskeletal (CSK) fractions were isolated and immunoblotted against Btk. **Lane 3**, CSK from resting platelets; **lane 4**, whole platelet lysate. The corresponding actin contents following Coomassie staining are shown in the lower panel.

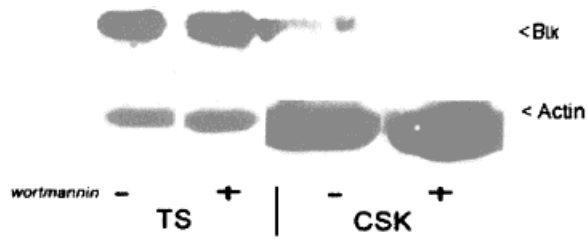

Fig. 5. Effect of wortmannin on the cytoskeletal association of Btk. Washed platelets were preincubated with either wortmannin (100 nM) or with DMSO (0.2%) for 20 min at 37°C, followed by aggregation with thrombin (1.6 U/ml) for 5 min. Triton soluble (TS) and cytoskeletal (CSK) fractions were isolated and immunoblotted against Btk. The corresponding actin contents following Coomassie staining are shown in the lower panel. Figures shown are representative of three experiments.

with high affinity to the PH domain of Btk [August et al., 1997] and regulate Btk activation in the platelets [Laffargue et al., 1999]. In order to find out the role of this kinase in the cytoskeletal association of Btk, platelets were preincubated with wortmannin at 100 nM, which is sufficient to prevent PI 3-kinase activation [Kovascsovics et al., 1995]. Consistent with earlier reports, wortmannin caused inhibition of thrombin-induced aggregation by about 70%, while actin content of cytoskeleton was not altered. Interestingly, in wortmannin-treated platelets cytoskeletal association of Btk was reduced to 44.4 ± 10.2% (n = 3) of control (Fig. 5).

The major platelet integrin $\alpha_{IIb}\beta_3$ acts as a receptor for fibrinogen in the activated platelets and triggers post-ligand binding changes in the cells. To study the role of aggregation, platelets were stimulated with TRAP in the absence of stirring or following preincubation with EGTA (2 mM) or RGDS (0.5 mM). Aggregation was almost completely inhibited under these conditions. However, the cytoskeletal association of Btk was reduced by 30–50% as compared to the normal TRAP-aggregated platelets (Table I).

DISCUSSION

In the present study we show that Btk associates with the cytoskeleton of platelets in an integrin-regulated manner. Btk is known to be activated downstream of G protein-coupled thrombin receptor, collagen receptor and FcγRIIA receptor in the platelets. As it lacks a myristylation site like in Src, phosphatidylinositol 3,4,5-trisphosphate (a product of PI 3-kinase activity) interacts with the PH domain of Btk and recruits it from the cytoplasm to the membrane [Li et al., 1997; Varnai et al., 1999], thus bringing it in close proximity to the Src family kinases [Rawlings et al., 1996]. Transphosphorylation by Src kinases at Y551 of Btk leads to its activation and autophosphorylation at Y223 [Wahl et al., 1997]. PI 3-kinase activity has been reported to be a prerequisite for the activation of Btk in the thrombin-stimulated platelets. Interestingly, the p85α regulatory subunit of PI 3-kinase is also known to translocate to the cytoskeleton of the thrombin-activated platelets with a significant increase in its activity in this subcellular fraction [Guinebault et al., 1995]. Here we show that translocation of Btk to the cytoskeleton is dependent on PI 3-kinase activation. Therefore, it is possible that the cytoskeletally associated PI 3-kinase in active form may facilitate the cytoskeletal recruitment and activation of Btk, thus promoting the downstream Btk signaling.

Btk is tyrosine phosphorylated under conditions in which it translocates to the platelet cytoskeleton. Hence, the active enzyme could interact with its substrates/effectors in the cytoskeleton. WASP (Wiskott Aldrich Syndrome Protein) has been shown to be a substrate of Btk in a pre B cell line and in rat tumor mast cells [Guinamard et al., 1998; Baba et al., 1999]. WASP is also the downstream effector of CDC42Hs [Symons et al., 1996], which regulates specific cytoskeletal reorganization

TABLE I. Role of Platelet Integrin $\alpha_{IIb}\beta_3$ in the Cytoskeletal Association of Btk

	% Cytoskeletal Btk	% Aggregation
Control	100	100
Non-stirred platelets	66.6 ± 6.4 (n = 2)	12.4 ± 3.6 (n = 4)
EGTA-preincubated platelets	52.6 ± 19.3 (n = 2)	35.6 ± 3.3 (n = 7)
RGDS-preincubated platelets	69.2 ± 7.4 (n = 2)	26.5 ± 4.5 (n = 3)

Washed platelets were stimulated with TRAP (10 μM) for 2 min under the conditions: (a) without stirring, (b) preincubated with EGTA (2 mM) at 37°C for 20 min and (c) preincubated with RGDS (0.5 mM) at 37°C for 3 minutes. Platelets were lysed with Triton lysis buffer. Cytoskeletal fractions were isolated and immunoblotted with an antibody against Btk. Values are expressed as percentage (mean ± SEM) of Btk present in the cytoskeleton of the TRAP-aggregated control platelets.

[Adams et al., 1990]. Thus, WASP provides a novel link between CDC42Hs, Btk, and the actin cytoskeleton. As CDC42Hs has been shown to translocate to the cytoskeleton of activated platelets in an integrin $\alpha_{IIb}\beta_3$-dependent manner [Dash et al., 1995a], it would be interesting to speculate that all these three proteins form a multimeric signaling complex on the cytoskeletal scaffold of activated platelets and play an active role in its regulation. Phosphopholipase C (PLCγ2) has been shown to be a substrate of Btk in the B cells [Takata and Kurosaki, 1996] and it, too, translocates to the reorganized cytoskeleton of thrombin-stimulated platelets [Banno et al., 1996]. Thus, Btk could also interact with PLCγ2 in the cytoskeleton of activated platelets.

The cytoskeletal association of Btk was only partially reversed upon continued stimulation of platelets for 15 min whereas tyrosine phosphorylation of Btk was not affected. In contrast, the cytoskeletal distribution of other signaling proteins like FAK, Src, CDC42Hs, and Rap IB, has been shown to be completely reversed upon prolonged stimulation of platelets [Dash et al., 1995b], associated with complete dephosphorylation of FAK on tyrosine [Dash, 1997]. These data point to differential regulation of cytoskeletal association of Btk and other proteins and a possible role of Btk in the long-term post-ligand binding changes in the platelets.

Btk associates with the cytoskeleton in the agonist-activated platelets whereas aggregation further augments the cytoskeletal redistribution of Btk by about 30–50%. Fibrinogen binding to the integrin $\alpha_{IIb}\beta_3$ and subsequent platelet aggregation induces outside-in signaling responses in the platelets. These include activation of the specific protein tyrosine kinases and extensive cytoskeletal reorganizations [Shattil et al., 1997, 1998]. Our data show that, the cytoskeletal association of Btk is decreased by genistein, a protein tyrosine kinase inhibitor and increased by PAO, an inhibitor of protein tyrosine phosphatases. Further, cytochalasin B inhibited actin polymerization and cytoskeletal association of Btk but not platelet aggregation in the thrombin-stimulated platelets. Thus, signals emanating downstream of the thrombin receptor supplement those triggered by integrin clustering and aggregation in inducing the cytoskeletal translocation of Btk. Although at this stage, a role for Btk in the platelet physiology is speculative, possible involvement of this kinase in the post-ligand binding events cannot be ruled out.

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