

Bruton's tyrosine kinase is a substrate of calpain in human platelets

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Abstract Platelet-associated Bruton's tyrosine kinase (Btk) was completely cleaved if treated with calcium ionophore A23187 with appearance of a proteolytic product of 27 kDa size. Aggregation with thrombin also induced about 10% degradation of Btk after 30 min. Calpain inhibitors prevented Btk degradation in both. The proteolytic products of the Wiskott–Aldrich syndrome protein (WASP), a calpain and Btk substrate, and the 27 kDa degradation product of Btk did not redistribute to the Triton-insoluble cytoskeleton in thrombin-aggregated platelets, in contrast to the uncleaved proteins. The degradation of Btk and WASP was independent of their tyrosine phosphorylation status. These results indicate that Btk is an endogenous substrate for calpain, the cleavage of which may have functional consequences in long-term post-aggregation events in platelets. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Platelet aggregation; Calpain; Calcium ionophore; Cytoskeleton; Bruton's tyrosine kinase; Wiskott–Aldrich syndrome protein

1. Introduction

Bruton's tyrosine kinase (Btk) is a 77 kDa cytoplasmic tyrosine kinase belonging to the Tec family. Present particularly in B-lymphocytes, it has been implicated, if mutated, in the causation of X-linked agammaglobulinemia in humans and X-linked immunodeficiency in mice [1,2]. Although it shares a number of structural characteristics with the Src family kinases, it possesses several unique domains such as a pleckstrin homology (PH) domain, a proline-rich region and a Ras GTPase-activating protein motif.

Btk has recently been reported to be present in platelets [3,4] where it is activated downstream of the receptors for thrombin and collagen, as well as the FcγRIIA receptor [3]. Members of the Src family of protein tyrosine kinases, Syk and focal adhesion kinase (FAK) are known to be present in platelets in significant amounts and their regulation and physiologic functions were studied in detail [5]. While Btk plays a central role in the development and differentiation of B-lymphocytes [6], in the degranulation and cytokine production in mast cells [7] and, as a dual regulator in apoptosis [8], platelets are not immunocompetent and have relatively distinct functions related to hemostasis and thrombus formation. The

function of Btk in platelets, thus, remains relatively unknown. Recently, we have shown that Btk specifically associates with the actin-based cytoskeleton in an integrin $\alpha_{IIb}\beta_3$ -regulated manner upon challenge of the platelet thrombin and FcγRIIA receptors [9], suggesting that Btk is a component of a signaling complex containing specific cytoskeletal proteins in activated platelets. The calcium-dependent thiol proteases calpains are highly expressed in human platelets [10,11] and are important regulators of signaling events in platelets, including clot retraction [12] and microvesicle shedding [13]. As the active form of calpain has also been reported to associate with the cytoskeleton of activated platelets [14], we asked whether calpains could regulate Btk function. Interestingly, both Btk and its substrate, the Wiskott–Aldrich syndrome protein (WASP), were found to be degraded by calpain in a similar fashion in either thrombin- or calcium ionophore-stimulated platelets. Furthermore, the degradation products of both were distributed to the Triton-soluble supernatant.

2. Materials and methods

2.1. Materials

Polyclonal antibodies against Btk (sc-1107), FAK (sc-557), SHP-1 (sc-287) and horseradish peroxidase-labeled secondary antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA). The anti-WASP rabbit polyclonal antibody (503) was described previously [15]. The Super Signal West Pico chemiluminescent substrate was from Pierce (Rockford, IL, USA). Human thrombin, apyrase, EGTA, sodium orthovanadate, acetylsalicylic acid, bovine serum albumin fraction V, Triton X-100, leupeptin, pepstatin A, aprotinin, phenylmethylsulfonyl fluoride, dimethylsulfoxide (DMSO) and HEPES were purchased from Sigma (St. Louis, MO, USA). A23187 and calpeptin, a cell permeable inhibitor of calpain, were from Biomol (Plymouth Meeting, PA, USA). Tween 20 was obtained from Bio-Rad (Richmond, CA, USA). Calpain inhibitor Z-Leu-Abu-CONH-(CH₂)₃-4-morphonyl was a gift from Dr. James Powers, Georgia Institute of Technology. The reagents for electrophoresis were procured from Sisco Research Laboratory and Spectrochem, India. All other reagents were of analytical grade.

2.2. Platelet preparation

Blood from volunteers was collected in citrate-phosphate-dextrose 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 buffer A (20 mM HEPES, 138 mM NaCl, 2.9 mM KCl, 1 mM MgCl₂, 0.36 mM NaH₂PO₄, 1 mM EGTA, supplemented with 5 mM glucose and 0.6 ADPase units of apyrase/ml, pH 6.2). Platelets were finally resuspended in buffer B (pH 7.4), which was the same as buffer A, but without EGTA and apyrase. The final cell count was adjusted to 0.8–1.2×10⁹/ml.

2.3. Platelet activation and aggregation

Platelets were stirred at 37°C in a Chrono-log platelet ionized cal-

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cium aggregometer (model 600) for 2 min prior to the addition of agonists. Platelets were aggregated with thrombin (1 U/ml) or A23187 (1 μ M) in the presence or absence of extracellular calcium (2 mM) under constant stirring (1000 rpm). In some experiments, calcium was chelated with EGTA (5 mM) 1 min before the addition of the agonist. In other experiments, platelets were preincubated at 37°C with either calpeptin (30 μ M) or Z-Leu-Abu-CONH-(CH₂)₃-4-morphoniyl (50 μ M) for 30 or 15 min, respectively, prior to the addition of the agonist in the absence of stirring. Aggregation was measured from the change in light transmission. Control platelets were incubated with DMSO at concentrations similar to A23187, calpeptin or Z-Leu-Abu-CONH-(CH₂)₃-4-morphoniyl. The cells were lysed in the sample buffer supplemented with 5 mM EGTA and 5 mM EDTA in order to inhibit calpain activity during cell lysis.

2.4. Isolation of detergent lysates of platelets

Platelets were lysed by one-seventh volume of 8× Triton lysis buffer (1× lysis buffer consisting of 1% Triton X-100, 50 mM Tris-HCl, 5 mM EGTA, 5 mM EDTA, 1 mM sodium orthovanadate, 10 μ M leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 μ M pepstatin A and 0.28 trypsin inhibitor unit/ml aprotinin, pH 7.5). After brief vortexing and keeping on ice for 10 min, samples were spun at 16000×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 solubilized in 1× sample buffer by heating at 95°C for 10 min.

2.5. Immunoblotting of platelet proteins

Platelet proteins were separated on 10% SDS-PAGE gels and electrophoretically transferred to Immobilon-P polyvinylidene fluoride (PVDF) membranes (Millipore Corp., Bedford, MA, USA) by using the Nova Blot semidry system 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, 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 rabbit anti-WASP, 1:1000 or rabbit anti-SHP-1, 1:1000 or rabbit anti-FAK, 1:500. Following washing, the blots were incubated for 1 h with horseradish peroxidase-labeled anti-goat IgG (1:15000) for Btk or anti-rabbit IgG (1:5000 for WASP or 1:12000 for SHP-1 or 1:15000 for FAK). The antibody binding was detected using enhanced chemiluminescence and bands were quantified using an Agfa Duoscan T1200 flatbed scanner and GeneTools software (Syngene, UK). To quantify actin, proteins were separated by SDS-PAGE,

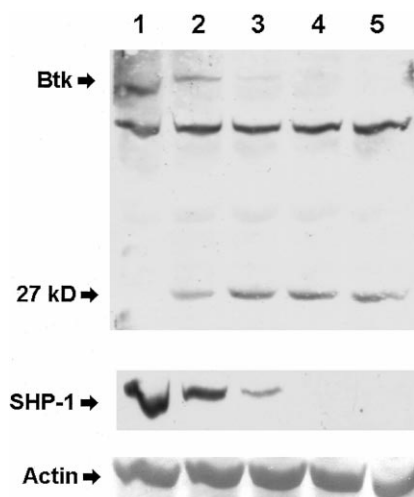


Fig. 1. Btk is degraded in ionophore-treated platelets. Washed platelets were incubated with ionophore A23187 (1 μ M) for 30 s (lane 2), 1 min (lane 3), 2 min (lane 4), and 5 min (lane 5) in presence of 2 mM calcium, followed by lysis with the sample buffer. Platelet proteins were separated by SDS-PAGE, transferred onto the PVDF membranes and immunoblotted using an antibody against Btk (upper panel) or SHP-1 (middle panel). The lower panel shows the corresponding actin bands following Coomassie staining. Lane 1: resting platelets.

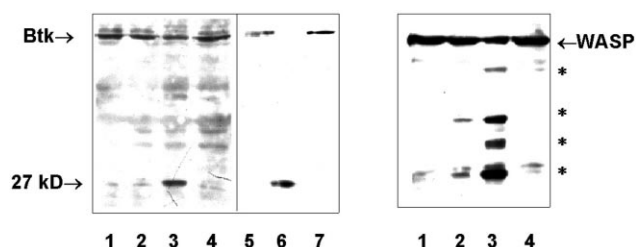


Fig. 2. Ionophore-induced degradation of Btk and WASP is inhibited by calpain inhibitors. Washed platelets were treated with A23187 (lanes 1–3 and 5–6) for 5 min in presence of calcium as described in the legend to Fig. 1. Platelets were preincubated with calpain inhibitors as follows: lanes 1 and 2, calpeptin (60 μ M and 30 μ M, respectively); lane 5, Z-Leu-Abu-CONH-(CH₂)₃-4-morphoniyl (50 μ M). Platelets were lysed in sample buffer and probed for Btk (left panel) and WASP (right panel). Degradation products of WASP are indicated by asterisks. Lanes 4 and 7: resting platelet lysates (without ionophore treatment). Blots corresponding to lanes 1–4 and 5–7 are from different experiments.

transferred onto the PVDF membrane and stained with Coomassie blue. Immunoblots shown are representative of at least three different experiments.

3. Results

3.1. Btk is completely cleaved in a calpain-dependent manner in calcium ionophore-stimulated platelets

Stimulation of platelets with ionophore A23187 (1 μ M) in the presence of extracellular calcium (2 mM) under stirring conditions induced platelet aggregation. This was associated with the disappearance of the 77 kDa Btk band from the cells and concomitant appearance of a new degradation product, a 27 kDa protein, recognized by an antibody against the carboxy terminus of the human Btk. The disappearance of Btk from the blot was completed within 5 min (Fig. 1). Degradation of Btk was also observed in A23187-treated (1 μ M) platelets in the absence of stirring (not shown). However, degradation of Btk and appearance of the proteolytic product was prevented when calcium was excluded from the buffer B or when EGTA (5 mM) was added to chelate extracellular calcium (data not shown). Under similar experimental conditions, we also detected degradation of SHP-1 (Fig. 1), FAK (data not shown) and WASP (Fig. 2, right panel) using specific antibodies, confirming previous findings [16–18]. The kinetics of degradation of these proteins paralleled that of Btk. In order to understand whether calpain was responsible for the calcium-mediated degradation of Btk, we preincubated the platelets with either calpeptin [19,20] or Z-Leu-Abu-CONH-(CH₂)₃-4-morphoniyl [21,22], cell permeable inhibitors of calpains. Calpeptin (30 μ M) or Z-Leu-Abu-CONH-(CH₂)₃-4-morphoniyl (50 μ M), concentrations sufficient to induce inhibition of the calpains, were effective in completely preventing degradation of Btk (Fig. 2, left panel) and WASP (Fig. 2, right panel and data not shown) in ionophore-stimulated platelets. As the cells were lysed in the sample buffer supplemented with 5 mM EGTA and 5 mM EDTA, chances of calpain-mediated proteolysis during cell lysis was ruled out.

3.2. Btk is partially cleaved in a calpain-dependent manner in thrombin-aggregated platelets

As calcium ionophores are not physiological agonists for platelets, we next sought whether thrombin, a natural platelet

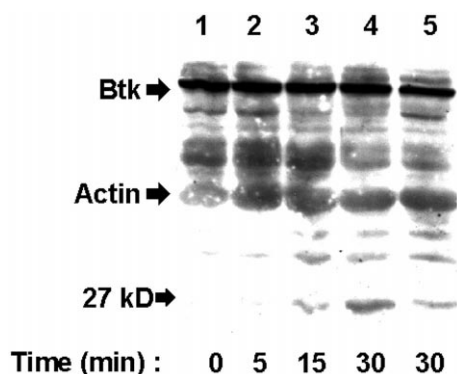


Fig. 3. Degradation of Btk in thrombin-aggregated platelets. Washed platelets were stimulated with thrombin (1 U/ml) in presence of extracellular calcium (2 mM) for the indicated time periods under constant stirring (lanes 2–4) and in absence of stirring (lane 5). Platelets were lysed by sample buffer and probed for Btk as mentioned before.

agonist, was also able to induce Btk proteolysis. When platelets were aggregated with thrombin (1 U/ml) for 5 min in the presence of extracellular calcium (2 mM), no Btk degradation was observed (Fig. 3). This is in striking contrast to another substrate, PTP-1B, which degrades very quickly by calpain in thrombin-aggregated or ionophore-treated platelets [23,24]. Thrombin, however, induced detectable degradation of Btk after 15 min, with about 10% degradation after 30 min of stimulation (Fig. 3). This was associated with appearance of a 27 kDa proteolytic product. Interestingly, similar kinetics of degradation were observed with WASP, which was also about 10% degraded after 30 min of thrombin-induced aggregation (not shown), with the appearance of major degradation products at the 27, 32 and 38 kDa regions [18]. When platelets were stimulated for 30 min in the absence of stirring we did not detect any degradation of Btk, thus implicating the signals elicited by platelet aggregation with Btk proteolysis. Preincubation of platelets with calpeptin (30 μ M) completely prevented proteolysis of Btk and subsequent appearance of the 27 kDa band (Fig. 4). Thus, Btk was also cleaved in a calpain-dependent way in thrombin-aggregated platelets. Preincubation of platelets with another cell permeable calpain inhibitor, Z-Leu-Abu-CONH-(CH₂)₃-4-morphonyl, [21,22] at 50 μ M concentration was equally effective in inhibiting the limited proteolysis of both Btk (Fig. 4) and WASP in thrombin-aggregated platelets (not shown).

We have previously demonstrated that Btk is tyrosine-phos-

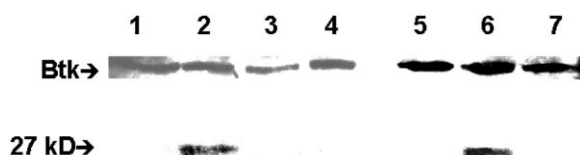


Fig. 4. Degradation of Btk in thrombin-aggregated platelets is inhibited by calpain inhibitors. Washed platelets were aggregated with thrombin (1 U/ml) in presence of calcium (2 mM) for 30 min (lanes 2–4, 6 and 7). Platelets were preincubated with either calpeptin (30 μ M) for 30 min (lane 7) or Z-Leu-Abu-CONH-(CH₂)₃-4-morphonyl (50 μ M, lane 3 and 190 μ M, lane 4) for 15 min or DMSO (lanes 2 and 6) before addition of thrombin. Platelets were lysed in sample buffer and probed for Btk as mentioned before. The 27 kDa degradation product is shown separately in the lower panel. Resting platelet lysates: lanes 1 and 5.

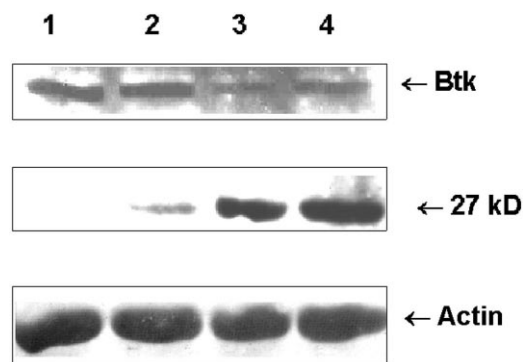


Fig. 5. Calpain-mediated degradation of Btk occurs independent of tyrosine phosphorylation status. Washed platelets in presence of calcium (2 mM) were aggregated with thrombin (1 U/ml) alone for 7 min (lane 2) or with thrombin for 2 min followed by ionophore A23187 (1 μ M) for a further 5 min (lane 3) or with ionophore A23187 (1 μ M) alone for 5 min (lane 4). Platelets were lysed in sample buffer and probed for Btk as mentioned before. The 27 kDa degradation product is shown separately in the middle panel. The lower panel shows the corresponding actin bands following Coomassie staining. Resting platelet lysate: lane 1.

phorylated after 2 min of thrombin receptor activation and the phosphorylated state is maintained for 15 min [9]. As another calpain substrate, SHP-1, is resistant to calpain activity in the tyrosine-phosphorylated state [16], we asked whether tyrosine-phosphorylated Btk could be a substrate of calpain. Platelets were aggregated with thrombin for 2 min in the presence of extracellular calcium and then exposed to A23187 or DMSO for a further 5 min under constant stirring. As expected, thrombin treatment alone for 7 min had negligible effect on the amount of Btk. Interestingly, thrombin followed by A23187 treatment induced degradation of Btk similar in extent to platelets treated with ionophore alone, thus suggesting that tyrosine-phosphorylated Btk was amenable to calpain-mediated proteolysis (Fig. 5). Consistent with an earlier report [16], we did not detect any cleavage of SHP-1 under similar conditions, while WASP, which is also tyrosine-phosphorylated downstream of thrombin activation [18], was degraded similar to Btk (data not shown).

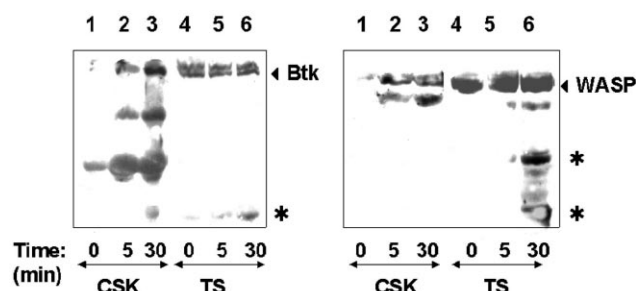


Fig. 6. Degradation products of Btk and WASP are recovered from the Triton-soluble fraction of thrombin-aggregated platelets. Washed platelets were aggregated with thrombin (1 U/ml) in the presence of calcium (2 mM) for the indicated time intervals. Platelets were lysed with Triton lysis buffer. The cytoskeletal (CSK, lanes 1–3) fractions and Triton-soluble (TS, lanes 4–6) fractions were isolated and proteins were separated by SDS-PAGE and immunoblotted using a polyclonal anti-Btk antibody (left panel) or anti-WASP antibody (right panel). The corresponding degradation products are indicated by asterisks.

3.3. Degradation products of Btk and WASP are recovered from the Triton-soluble fraction of thrombin-aggregated platelets

We have recently shown that Btk redistributes to the actin-based cytoskeleton in thrombin-aggregated platelets in an integrin $\alpha_{IIb}\beta_3$ -regulated manner [9]. In order to find out the functional implication of Btk proteolysis, we studied the distribution of its 27 kDa degradation product in activated platelets. When platelets were aggregated with thrombin in the presence of extracellular calcium, Btk associated with the actin-based cytoskeleton and this association was maintained until 30 min. However, the 27 kDa degradation product was detected only in the Triton-soluble fraction upon prolonged stimulation for 30 min (Fig. 6, left panel). WASP, which also translocated to the actin-based cytoskeleton in thrombin-aggregated platelets [18], was found to remain associated with the cytoskeleton at 30 min. Similar to Btk, the degradation products of WASP were also detected in the Triton-soluble supernatant only, and did not translocate to the cytoskeleton (Fig. 6, right panel).

4. Discussion

We present here evidence showing that Btk is a substrate for calpain in platelets. Both calpain [14] and Btk [9] are known to translocate to the cytoskeletal fraction of thrombin-stimulated platelets and undergo activation in an integrin $\alpha_{IIb}\beta_3$ -dependent manner. During activation, the 80 kDa subunit of μ -calpain undergoes autoproteolytic conversion to a 78 kDa 'intermediate' and a 76 kDa fully autolysed form [25,26], which are endowed with distinct substrate specificities and functional roles [24]. The initial conversion to the 78 kDa form has been implicated with the cleavage of PTP-1B, while both the 78 and 76 kDa enzymes are responsible for subsequent degradation of Src in thrombin-aggregated platelets. Interestingly, WASP, a Btk substrate [27], also associates with the cytoskeleton of thrombin-stimulated platelets [18] and is cleaved by calpain [18]. The kinetics of degradation of both Btk and WASP are strikingly similar and suggest them to be possible substrates of the fully cleaved 76 kDa form of μ -calpain.

A 27 kDa degradation product of Btk was recovered from the Triton-soluble supernatant of the thrombin-aggregated platelets. A recent study indicated that Btk interacts with the filamentous actin through a basic 10-residue stretch within the PH domain (at the amino terminal end of Btk) [28]. As the antibody used in this study was directed against the carboxy terminus of Btk, this could explain the absence of the 27 kDa proteolytic fragment from the actin-rich cytoskeleton. The calpain-induced degradation products of Src [29] and WASP also distribute to the supernatant, while the cleaved form of PTP-1B remains associated with the cytoskeleton [30]. Preliminary *in silico* analysis and comparison with the consensus sequence for calpain proteolysis [10] indicates the possible site of degradation to be after Y461 in the Btk sequence.

With the existing data, we cannot formally rule out the possibility of activation of other proteases downstream of calpain-promoting Btk cleavage. A growing number of cytoskeletal signaling enzymes and structural proteins have been identified as calpain substrates [16–18,23,29–34]; the physiological significance of these proteolytic events in platelets has not been well established. Our data suggest that Btk is parti-

ally cleaved by calpain when platelets are aggregated for 30 min with thrombin. In the physiological context, intravascular platelet clots continue to be exposed to the agonists like thrombin in their immediate microenvironment long after aggregation. The ensuing activation of calpain causes proteolysis of molecules like Btk, WASP and Src at a temporally distinct phase, and this could have important implications in the post-aggregation events. The 78 kDa form of μ -calpain is associated with the reduction in platelet-mediated clot retraction [24], while the 76 kDa form appears to regulate the post-ligand binding events like shedding of procoagulant-rich membrane microvesicles [24]. Considering the fact that Btk could play important roles during apoptosis in other cells [8], it is tempting to speculate that calpain-mediated proteolysis of Btk might be functionally related to microvesicle shedding (which simulates membrane blebbing) in platelets as part of platelet destruction.

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