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# Integrin-Dependent Tyrosine Phoshorylation and Cytoskeletal Translocation of Tec in Thrombin-Activated Platelets

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**Using a specific polyclonal anti-Tec antibody, we have shown that Tec is expressed in human platelets. In addition, Tec was found to undergo tyrosine phosphorylation during platelet activation. The phosphorylation increased after 1 min and remained stable after 3 min of thrombin treatment. The tetrapeptide RGDS inhibited more than 90% of thrombin-induced tyrosine phosphorylation of Tec and blocked its translocation to the cytoskeleton. These results suggest that Tec participates in platelet signaling downstream of integrin activation.** © 1997 Academic Press

The stimulation of platelets by thrombin results in a dramatic increase in tyrosine phosphorylation of multiple proteins, and this process is essential to the appropriate onset and progression of platelet function. However, thrombin receptors belong to the family of protease-activated receptors coupled to heterotrimeric G proteins, without intrinsic protein tyrosine kinase (PTK) activity (1,2). Various nonreceptor PTKs, such as c-Src and Syk, that are activated downstream of the thrombin receptors have been shown to play a crucial role in platelet activation (3-7), but the mechanisms of stimulation of these kinases following G protein activation remain obscure.

Recently, a new group of PTKs has been described, called the Tec family, that contain a pleckstrin homology (PH) domain. The most prominent member of this family is Bruton's tyrosine kinase (Btk), which is deficient in X-linked agammaglobulinemia (8,9). So far, the role of Tec family kinases has been considered only during cell activation by cytokines and other agonists specific of hematopoietic cells, where they are preferentially expressed. For example, Btk, that plays a critical role in B-cell differentiation, is activated downstream of B-cell receptor (10,11). Similarly, the T-cell specific Tec family kinase Itk participates in the activation of T cells (12,13), and Tec itself is involved in the signaling pathways used by various cytokines (14-16). However, the presence of a PH domain in the N-terminal region of the Tec family kinases suggests that they could participate in other signaling pathways, *via* associations with conventional PH-domain-binding molecules including phosphoinositides and the G protein $\beta\gamma$ subunits. Supporting this hypothesis, Btk and Itk have been described as $\beta\gamma$-activated kinases using cotransfection assays (17). In addition, the PH domain of Btk has been recently shown to bind specifically phosphatidylinositol 3,4,5-trisphosphate, one product of phosphoinositide 3-kinase formed during cell activation (18). Since various megakaryoblastic cell lines were reported to express Tec (14,19,20), we have checked whether Tec would also be expressed in platelets, and studied its involvement in thrombin-induced platelet activation.

## MATERIALS AND METHODS

*Cloning of Tec, expression in COS, and antibody preparation.* The full-length cDNA of human Tec was cloned by RT-PCR from the megakaryoblastic cell line Dami using the sequence published by Sato *et al.* (20) (sense primer: GCGGAATTCG CAGGAGGAGT AATCAGAAGA CGGAGATG; antisense primer: GCTTGGGAAG CTTGGAGCCA CTGG). The PCR product was cloned into the Eco RI and Hind III sites of pGEX-KG (21), then sequenced. For eukaryotic expression, Tec was initially subcloned into pET21d (Novagen), to incorporate the T7 epitope tag sequence in fusion with Tec cDNA. Thereafter, T7-Tec was subcloned into pCIneo (Promega), allowing transient expression in COS cells transfected using the Lipofectamine procedure (Gibco BRL). For preparation of a polyclonal antibody, the sequence coding for the amino-acids 1-359 of Tec was subcloned into pGEX-KG, and the GST fusion protein thereby obtained was used to immunize a rabbit. The anti-Tec antibody was purified from immune serum onto GST-Tec(1-359) immobilized on glutathione-

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Abbreviations: GST, glutathione *S*-transferase; PH, pleckstrin homology; PTK, protein tyrosine kinase; pY, phosphotyrosine; RT-PCR, reverse transcription-polymerase chain reaction.



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Sepharose beads (Sigma), followed by a negative purification step versus immobilized GST alone.

*Incubation of platelet suspensions and Tec immunoprecipitation.* Platelet suspensions ($1\times10^9$ cells/ml) in Tyrode's albumin buffer lacking calcium were prepared from human platelet concentrates as previously described (22). Platelets (500 $\mu$l) were equilibrated for approximately 5 min at 37°C. $CaCl_2$ (1mM) was added just before the addition of 1 unit/ml of thrombin (Sigma). In some experiments, platelet suspensions were preincubated for 10 min with 500 $\mu$M RGDS (Sigma). Incubations were terminated upon addition of 125 $\mu$l of five-fold concentrated lysis buffer, thus achieving the following final concentrations: 20 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% (v/v) Triton X-100, 0.2% (w/v) sodium dodecyl sulfate, 5 mM sodium pyrophosphate, 1 mM $Na_3VO_4$, 10 $\mu$g/ml aprotinin, 10 $\mu$g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride. The lysate was then sonicated twice for 20 s. Insoluble material was removed by centrifugation (12,000 g, 10 min, 4°C) and the supernatant was incubated overnight at 4°C with purified anti-Tec antibody. Then, the material precipitated overnight was removed by centrifugation, and 50 $\mu$l of a 10% (w/v) suspension of protein A-Sepharose (Sigma) was added to the supernatant for 2 h at 4°C. The immunoprecipitate was then collected by brief centrifugation, washed four times in lysis buffer and boiled in SDS-PAGE sample buffer.

*Isolation of platelet cytoskeletons.* Incubations of platelets with thrombin were terminated by addition of a buffer containing 1% Triton X-100. The platelets cytoskeletons were allowed to precipitate and separated from the Triton-soluble fraction as previously described (4).

*Immunoblotting.* Proteins were analysed by immunoblotting using standard procedures. The phosphotyrosine content of Tec was determined with 1/1000 dilution of 4G10 antibody (Upstate Biotechnology Inc.). Immunoblots were developed using the enhanced chemiluminescence system (ECL, Amersham). Densitometric analysis was performed using a Gel Doc 1000 system (BioRad).

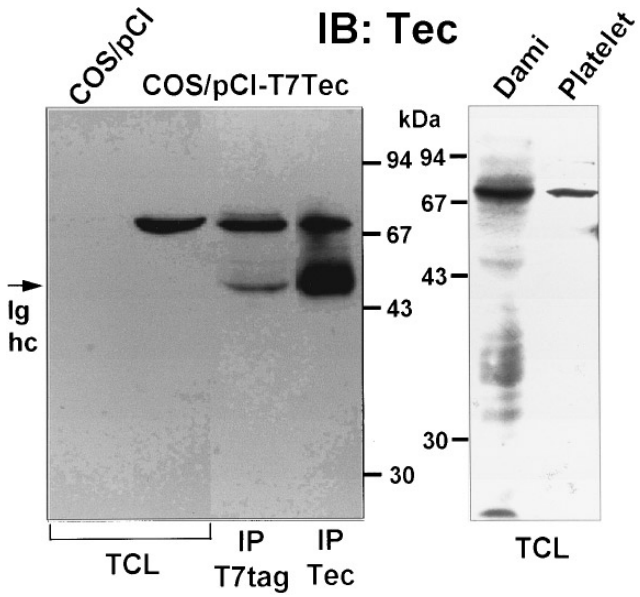


**FIG. 1.** Characterization of the polyclonal antibody raised against Tec. The cDNA of human Tec was cloned using RT-PCR, and the sequence coding for amino-acids 1–359 was subcloned in pGEX-KG, to obtain a GST fusion protein that was used to immunize a rabbit. Left panel: Following affinity purification, the specificity of the anti-Tec antibody was determined using immunoblotting of total cell lysates (TCL, 2 left lanes) of COS cells transfected with pCIneo, either wild type (COS/pCI), or containing the full-length cDNA of Tec in fusion with the T7 epitope tag (COS/pCI-T7Tec). In the right two lanes, Tec was immunoprecipitated (IP) from COS/pCI-T7Tec using either T7tag or Tec antibodies as indicated. Right panel: Immunoblotting of total cell lysates from Dami and platelets with the purified anti-Tec antibody. Ig hc: immunoglobulin heavy chain.

## RESULTS

We have first designed the production of an antibody that could immunoprecipate and reveal Tec on immunoblots. The cDNA of human Tec was cloned by RT-PCR from the megakaryoblastic cell line Dami, and the N-terminal half of the molecule was expressed as a fusion protein with GST, then injected to a rabbit. The immune serum was affinity purified versus GST-Tec(1-359), and the specificity of the purified antibody was determined in transfected COS cells. As shown in Fig. 1 (left panel), anti-Tec immunoblotting revealed a single band around 72-75 kDa in lysates from COS transfected with T7-tagged full-length Tec cDNA, that fits the theoretical 76.4 kDa of the tagged protein. In COS cells transfected with the vector alone, we did not observe any comparable signal, although longer exposure revealed a faint band close to 75 kDa, along with other non specific signals (not shown), suggesting that COS cells express Tec endogenously at a low level, at best. In addition, following immunoprecipitation from Tec-transfected COS cells using either an anti T7-tag antibody or our anti Tec antibody, immunoblotting with the anti-Tec revealed the 75-kDa protein, indicating that our antibody had the ability to immunoprecipitate Tec (Fig. 1, IP T7tag and IP Tec, respectively). The specificity of our antibody was further validated by performing anti-Tec immunoblotting of total lysate from Dami cells, where a single band around 75 kDa was labeled. Interestingly, in platelet lysates, a protein of identical size was revealed, indicating that Tec was also expressed in these cells (Fig. 1, right panel). Finally, we have verified that our antibody did not cross-react with Btk, another member of the Tec family (not shown).

Tyrosine phosphorylation is a pivotal process to regulate the function of signaling proteins in platelets. To determine whether Tec is involved in platelet signaling, we have studied its tyrosine phosphorylation during platelet activation. Tec was immunoprecipitated from lysates of thrombin-treated platelets and analysed by anti-phosphotyrosine (pY) immunoblotting. As shown in Fig. 2, an increased tyrosine phosphorylation of Tec was observed after 1 min of incubation with thrombin, and remained stable until 3 min at which time irreversible aggregation was achieved. At 10s, the pY content of Tec was similar to that of control platelets. In addition, anti-pY immunoblotting of Tec immunoprecipitates invariably labeled a 62-65 kDa protein that was not detected by the anti-Tec antibody, and its

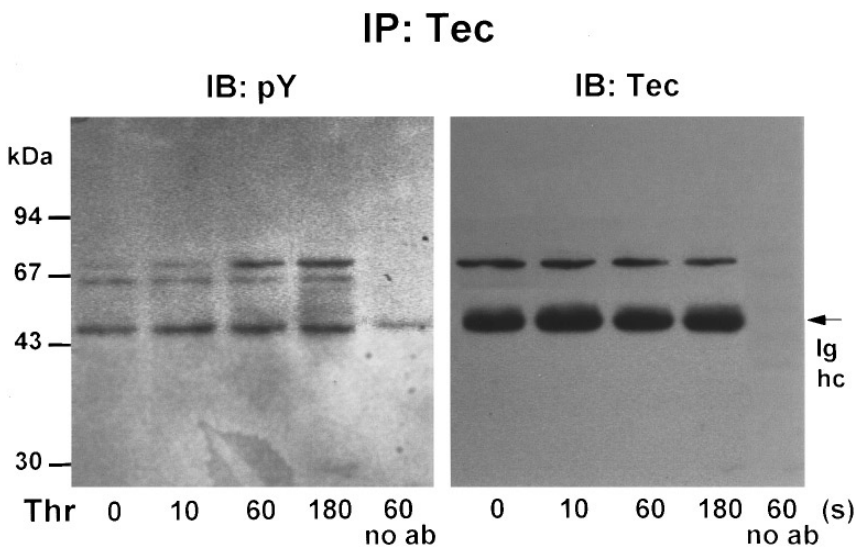


**FIG. 2.** Tyrosine phosphorylation of Tec during activation of platelets with thrombin. Washed platelets were incubated for various times with 1 IU/ml of thrombin, then the reactions were blocked in lysis buffer. Tec was immunoprecipitated using our polyclonal purified antibody and its phosphotyrosine content was analysed by antiphosphotyrosine immunoblotting using the 4G10 antibody (left panel). Thereafter, the blotting membrane was stripped off and reprobed using the anti-Tec antibody, to control the amount of protein immunoprecipitated in each lane (right panel). The lane "no ab" is a mock immunoprecipitate performed without adding anti-Tec antibody. Ig hc: immunoglobulin heavy chain. This figure is representative of four different experiments.

tyrosine phosphorylation was not significantly modified during thrombin stimulation.

The delayed time-course of Tec phosphorylation suggested that this process resulted from an event occurring in the late phase of platelet activation, such as aggregation. Since the role of the integrin $\alpha_{IIb}/\beta_3$ is critical to platelet aggregation, we have studied the effect on Tec phosphorylation of the tetrapeptide RGDS that inhibits fibrinogen binding to $\alpha_{IIb}/\beta_3$. Fig. 3 shows that the pY content of Tec after 1 and 3 min of thrombin was strongly reduced when platelets were preincubated with RGDS. Densitometric analysis of anti-pY immunoblots from 3 different experiments showed that RGDS blocks more than 90% of Tec tyrosine phosphorylation observed after 3 min of thrombin treatment (Fig. 3, graph).

During platelet activation, the cytoskeleton has been shown to be a major site of redistribution of PTKs essential to platelet function, such as Src (4,5) and Syk (23). We have studied the intracellular localization of Tec during platelet activation using immunoblotting of the cytoskeletal fraction isolated from thrombin-stimulated platelets. Thrombin was shown to induce translocation of Tec to the cytoskeleton (Fig. 4, upper panel). This process was maximal in the late phase of platelet activation, suggesting that it is an aggregation-dependent event, involving integrin activation. Indeed, Fig. 4 shows that RGDS and EDTA, that both inhibit activation of the integrin $\alpha_{IIb}/\beta_3$ by different mechanisms, strongly suppressed the translocation of Tec. In the presence of cytochalasin B, the association of Tec to the Triton-insoluble fraction was also blocked.

## DISCUSSION

Although the Tec family has been described as a group of nonreceptor PTKs activated by agonists specific of hematopoietic lineages, we report here that Tec kinase participates in thrombin-induced signaling in platelets, thus locating for the first time one of these kinases downstream of a G protein-coupled receptor. However, both tyrosine phosphorylation and cytoskeletal translocation of Tec appear as late events in terms of platelet activation, being maximal after at least 1 min of stimulation. In addition, the phosphorylation and translocation of Tec were nearly abolished by the tetrapeptide RGDS, which impairs integrin engagement. Therefore, the mobilization of Tec does not seem to be directly linked to thrombin receptor signaling, but rather occurs downstream of integrin signaling. Furthermore, although the cytokine-induced tyrosine phosphorylation of Tec has been reported to increase its catalytic activity (14,15), we failed to observe a higher PTK activity of Tec in immunoprecipitates from thrombin-treated platelets using an autokinase assay (not shown). This suggests that the tyrosine phosphorylation of Tec during platelet activation may play a role in controling its intracellular distribution or its association with signaling proteins rather than stimulating its catalytic activity. Interestingly, Park and coworkers have recently identified on Btk, a member of Tec family, a tyrosine residue that is phosphorylated during cell activation but does not increase the catalytic activity of Btk (24). Therefore, it will be important to determine which tyrosine residue is phosphorylated on the

Tec molecule during platelet activation. In addition, although the role of Tec in platelet is still unknown, our results suggest that it may participate in the last wave of tyrosine phophorylation occuring downstream of integrin engagement (25). Therefore, the identification of the targets of Tec, i.e. its substrates or associated proteins, may help elucidating Tec function in platelets.

The mechanisms that govern the involvement of Tec in platelet activation are not known, but there are various candidates that could recruit Tec into the integrin signaling pathways. For example, Src family PTKs are important regulators of Tec family kinases during lymphocyte activation (13,26-28). Therefore, one can speculate that the c-Src kinase, an essential component of platelet activation, interacts with Tec in platelets. We observed that Tec immunoprecipitated from platelets was constitutively associated with a 62-65 kDa tyrosine-phosphorylated protein (see Fig. 2 and 3), but this protein did not react with anti-c-Src antibodies (not shown). However, we cannot exclude that a putative association between Tec and c-Src did not resist to the immunoprecipitation procedure that requires 0.2% SDS to partly disaggregate the platelet cytoskeleton. Similarly, Tang *et al.* (14) observed in a megakaryocytic cell line a 62-kDa tyrosine-phosphorylated protein associated with Tec that did not react with antibodies specific for various proteins including c-Src and Lyn. In our hands, the 62-65 kDa Tec-associated protein did not react with anti SHP-1 antibodies either, and we are currently trying to identify this protein using microsequencing. Interestingly, Tec has been recently reported to associate *in vitro* with the 65-kDa Wiskott-Aldrich syndrome protein (WASP) (29).

Other interesting candidates will have to be considered as potential regulators of Tec during platelet activation. Tec family members have been shown to associate with G protein $\beta\gamma$ subunits (17,30) and protein kinase C (31,32), through interactions with the PH domain. Moreover, a role of the phosphoinositide 3-kinase

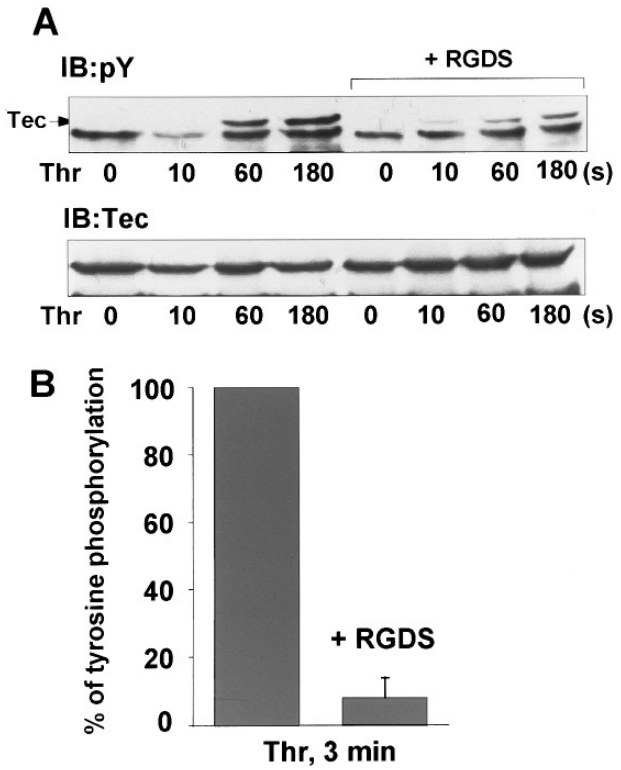


**FIG. 3.** The tyrosine phosphorylation of Tec occurs downstream of integrin signaling in thrombin-stimulated platelets. (A) Anti-Tec immunoprecipitates were performed from thrombin-treated platelets, preincubated, or not, with RGDS. Immunoprecipitates were analyzed by immunoblotting with 4G10 antiphosphotyrosine antibody (upper panel), then stripped off and reprobed with anti-Tec antibody (lower panel). (B) Tyrosine phosphorylation of Tec in platelets treated for 3 min with thrombin, alone and with RGDS, was measured by computer densitometry. Data represent mean ± s.e.m. of three different experiments.

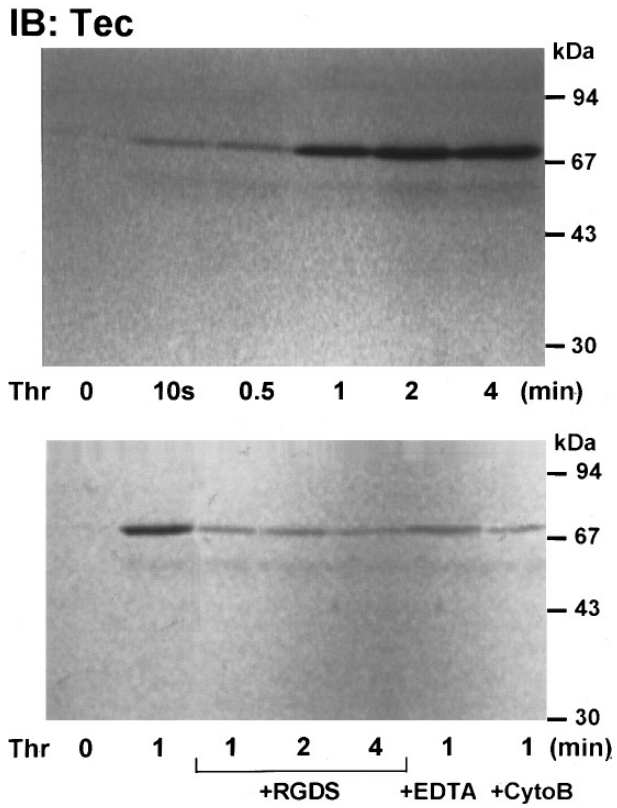


**FIG. 4.** Integrin-dependent translocation of Tec to the cytoskeleton of thrombin-stimulated platelets. Washed human platelets were treated with thrombin (1 IU/ml), and cytoskeletons were then immediately extracted as indicated in Materials and Methods, and resuspended in SDS-PAGE sample buffer for anti-Tec immunoblotting analysis. Upper panel: Time course of Tec translocation to the cytoskeleton of thrombin-stimulated platelets. Lower panel: Translocation of Tec is blocked by integrin inhibitors. Platelets were stimulated with thrombin, alone, or in the presence of the RGDS peptide, EDTA, or cytochalasin B, as indicated below each lane. This figure is representative of two different experiments with similar results.

in Tec regulation has to be examined, in light of recent data showing that the PH domain of Btk, that is highly homologous to that of Tec, binds specifically one product of the phosphoinositide 3-kinase (18). Quite interestingly, this lipid kinase is strongly stimulated during platelet activation (33). Therefore, the role of the phosphoinositide 3-kinase products in the recruitment of Tec during platelet activation will be an important issue to address. It could both unveil the mechanism of activation of Tec and reveal a new target of phosphoinositide 3-kinase products.

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