Association of thrombospondin-1 with the actin cytoskeleton of human thrombin-activated platelets through an α IIB β 3- or CD36-independent mechanism

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Thrombospondin-1 (TSP-1) is an adhesive glycoprotein which, when secreted from α -granules of activated platelets, can bind to the cell surface and participate in platelet aggregate formation. In this study, we show that thrombin activation leads to the rapid and specific association of a large amount of secreted α -granular TSP-1 with the actin cytoskeleton. This cytoskeletal association of TSP-1 was correlated with platelet secretion, but not aggregation, and was inhibited by cytochalasin D, an inhibitor of actin polymerization. Association of TSP-1 with the actin cytoskeleton was mediated by membrane receptors, as shown by using MAII, a TSP-1-specific monoclonal antibody that inhibited both TSP-1 surface binding to activated platelets and cytoskeletal association. TSP-1 and its potential membrane receptors, e.g. α IIB β 3 integrin, CD36 and CD47, concomitantly associated with the actin cytoskeleton. However, studies on platelets from a patient with type I Glanzmann's thrombasthenia lacking α IIB β 3 and another with barely detectable CD36 showed normal TSP-1 surface expression and association with the actin cytoskeleton.

Likewise, no involvement of CD47 in TSP-1 association with the actin cytoskeleton could be inferred from experiments with control platelets using the function-blocking anti-CD47 antibody B6H12. Finally, assembly of signalling complexes, as observed through translocation of tyrosine-phosphorylated proteins and kinases to the actin cytoskeleton, was found to occur in concert with cytoskeletal association of TSP-1, in control platelets as well as in thrombasthenic and CD36-deficient platelets. Our results imply a role for the actin cytoskeleton in the membrane-surface expression process of TSP-1 molecules and suggest a possible coupling of TSP-1 receptors to signalling events occurring independently of α IIB β 3 or CD36. These results provide new insights into the link between surface-bound TSP-1 and the contractile actin microfilament system which may promote platelet aggregate cohesion.

Key words: adhesive protein, membrane-surface expression, platelet activation.

INTRODUCTION

Thrombospondin-1 (TSP-1) is a ubiquitous 420 kDa trimeric molecule that belongs to a family of multidomain glycoproteins implicated in diverse cellular functions and biological processes, such as cell adhesion and migration, tissue differentiation, angiogenesis and tumour metastasis [1–3]. TSP-1 participates in cell-to-cell recognition and cell adhesion to extracellular matrix. Its ability to form multiprotein complexes with cytokines at the cell surface could contribute to regulating extracellular matrix composition and cell behaviour [1,4]. TSP-1 was first identified as being secreted from α -granules upon platelet activation [5], together with other adhesive glycoproteins (e.g. fibrinogen and von Willebrand factor) [6,7], and it was shown to promote the formation of platelet aggregates [5,8–10]. TSP-1 is able to bind to different membrane receptors expressed on cells, thereby explaining its multifunctional biological properties. TSP-1 receptors include proteoglycans, sulphated glycolipids, integrins of the β_1 and β_3 families, CD36 and CD47 (integrin-associated protein) [1,4]. CD36 is associated with protein kinases of the Src family [11] and CD47, through activation of a heterotrimeric G_i-protein,

associates with and modulates the functions of integrins of the β_1 , β_2 and β_3 families [12]. In platelets, the α IIB β 3 and $\alpha_v\beta_3$ integrins, CD36 and CD47 are potential membrane receptors for TSP-1 [13–15]. Upon its ligation to a peptide from the C-terminus of the TSP-1 sequence, CD47 was shown to activate the major platelet integrin α IIB β 3, allowing its binding to fibrinogen [13]. TSP-1 also binds to activated platelet-bound fibrinogen [5,8,14]. This protein complex was demonstrated to increase fibrinogen affinity for α IIB β 3 [8], but might also participate in outside-in signalling induced upon α IIB β 3–fibrinogen complexation. Fibrinogen, α IIB β 3 and CD36 are present in platelet α -granules [16,17] along with TSP-1, and like TSP-1 become membrane-expressed upon platelet activation. However, the identity of the platelet receptors for TSP-1 remains uncertain; thus the molecular mechanism of TSP-1 recruitment from the α -granules to the platelet surface during exocytosis and the signals coupled to TSP-1 receptors are not known. They probably require the actin cytoskeleton, which besides its mechanical properties also provides a framework that locally brings various signalling components together with transmembrane receptors to facilitate activation of the signalling pathways [18–25]. In the

Abbreviations used: DTT, dithiothreitol; FAK, focal adhesion kinase; 5-HT, 5-hydroxytryptamine creatinine sulphate; PE, phycoerythrin; PI-3K, phosphoinositide 3-kinase; TRAP-6, thrombin-receptor-activator hexapeptide 6; TSP-1, thrombospondin-1.

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present study, we have investigated whether the actin cytoskeleton could play a role in the membrane-surface expression process of the endogenous α -granular adhesive protein TSP-1, promoting platelet recognition and aggregation.

Using purified ligands, we and others have shown previously that exogenous fibrinogen and TSP-1 that are bound to the surface of thrombin-activated platelets undergo late incorporation into the actin cytoskeleton [22,26,27]. Herein, we provide the first evidence for a rapid and specific association of endogenous α -granular TSP-1 with the actin cytoskeleton of thrombin-activated platelets, occurring concurrently with its surface binding to membrane receptors. We demonstrate that TSP-1 cytoskeletal association is not mediated by α IIB β 3 or CD36 and is accompanied by the redistribution of signalling components to the actin cytoskeleton independently of α IIB β 3 or CD36 activation. Results from experiments showing no effect of a function-blocking CD47-specific antibody on both TSP-1 membrane-surface expression and cytoskeletal association are discussed.

EXPERIMENTAL

Materials

Prostaglandin E_1 , BSA (fraction V), human thrombin (2600 NIH units/mg of protein), hirudin (2200 units/mg), aprolinin, leupeptin, benzamidine, sodium orthovanadate, NaF, Triton X-100, Tween 20, cytochalasin D, DMSO and imipramine were obtained from Sigma (St Louis, MO, U.S.A.). The thrombin-receptor-activator hexapeptide 6 (TRAP-6), Ser-Phe-Leu-Leu-Arg-Asn, was from Bachem Biochimie Sarl (Voisins-le-Bretonneux, France). 5-Hydroxy[14 C]tryptamine creatinine sulphate ([14 C]5-HT) was purchased from Amersham Bioscience (Little Chalfont, Bucks, U.K.). The phycoerythrin (PE)-conjugated mouse IgG1 antibody to TSP-1, P10, was from Immunotech (Marseille, France), and the PE-labelled mouse IgG1 antibody to CD62 and the PE-labelled isotype control mouse antibody were from Becton Dickinson (San Jose, CA, U.S.A.). The bicinchoninic acid kit for protein determination was obtained from Pierce (Rockford, IL, U.S.A.) and all reagents for electrophoresis were from Bio-Rad (Richmond, CA, U.S.A.).

TSP-1 was purified from the supernatant of thrombin-activated human platelets, as described previously [26]. The mouse anti-TSP-1 ascitic fluids, MAI and MAII, and the rabbit anti-TSP-1 serum R1 were a gift from Professor Jack Lawler (Harvard Medical School, Boston, MA, U.S.A.). Characterization and purification of these antibodies have been reported previously [9,28]. Rabbit polyclonal antibody directed against human fibrinogen was from Dako (Glostrup, Denmark). Purified CD36 and rabbit polyclonal antibody to CD36 were a gift from Dr George Jamieson (American Red Cross, Rockville, MD, U.S.A.). Rabbit antisera to α IIB and β 3 were a gift from Dr Dominique Pidard (Institut Pasteur, Paris, France), and the mouse monoclonal antibody to CD47, B6H12, was a gift from Dr Eric Brown (Washington University, St Louis, MO, U.S.A.). Peroxidase-conjugated anti-phosphotyrosine monoclonal antibody PY20 was obtained from Zymed Laboratories (San Francisco, CA, U.S.A.), the monoclonal antibody to the focal adhesion kinase (FAK) was from Transduction Laboratories (Lexington, KY, U.S.A.), the monoclonal antibody to the non-receptor tyrosine kinase Syk was from Chemicon International (Temecula, CA, U.S.A.) and the monoclonal antibody to the non-receptor tyrosine kinase Src and polyclonal antibody to phosphoinositide 3-kinase (PI-3K) were from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Peroxidase-conjugated secondary antibodies were purchased from Dako.

Patients

A patient with severe type I Glanzmann's thrombasthenia, whose case history has already been reported, was studied with the courtesy of Professor Jacques Caen (Hôpital Lariboisière, Paris, France). This patient's platelets have no detectable α IIB β 3 complexes and their fibrinogen level is only 5% of the normal values [29,30].

Platelets from another patient, who has a mild bleeding disorder associated with prolonged, > 15 min, bleeding time, a coagulation factor VIII deficiency (48% of the normal value), low von Willebrand cofactor activity (46% of the normal value) and subnormal platelet aggregation induced in plasma by collagen, ADP or adrenaline, were studied by courtesy of Dr Marie-Dominique Dautzemberg (Hôpital Necker, Paris, France). These platelets were CD36-deficient with undetectable surface expression, as assessed by flow-cytometry analysis using different monoclonal antibodies specific to CD36.

Platelet preparation and activation

Platelets were isolated from freshly drawn human blood collected from aspirin-free healthy adult volunteers and washed by centrifugation in the presence of prostaglandin E_1 , as described previously [31]. In some studies, platelets in plasma were incubated for 30 min at room temperature with 0.6 μ M [14 C]5-HT (0.033 μ Ci/ml) to measure platelet dense-granule secretion. Isolated platelets were resuspended to a final concentration of 3×10^8 /ml in BSA-free 5 mM Hepes-buffered Tyrode's solution, pH 7.4, containing 2 mM $CaCl_2$ and 1 mM $MgCl_2$. When platelets were labelled with [14 C]5-HT, imipramine (final concentration, 3 μ M) was added to the final suspension to block [14 C]5-HT reuptake. Platelets (600 μ l) were activated at 37 °C with thrombin, either without stirring or in an aggregometer (Chrono-Log; Beckman Coulter France, Paris, France) with constant stirring (1000 rev./min). At the end of platelet activation, thrombin was inactivated by adding a unit-for-unit 10-fold excess of hirudin to the platelet suspension, aliquots (100 μ l) were mixed with 25 μ l of ice-cold 0.1 M EDTA, centrifuged for 1 min at 12000 g and [14 C]5-HT was measured in the supernatant by liquid scintillation counting. Results were expressed as a percentage of the total amount of [14 C]5-HT incorporated into 100 μ l of the initial platelet suspension. In some experiments, platelets were preincubated for 5 min at 37 °C with 10 μ M cytochalasin D in DMSO (0.1% final concentration) or with 0.1% DMSO alone before the addition of thrombin.

In experiments using the mouse monoclonal antibody to CD47, B6H12 a 'non-responsive' donor as well as a 'responsive' donor to this antibody were selected for the study. Indeed, like some other mouse monoclonal antibodies to platelet glycoproteins, B6H12 has been shown to directly induce aggregation of platelets from some normal donors, but not others [32]. This effect involves the human Fc receptor, Fc γ RII, for mouse monoclonal antibodies and, because of a polymorphism of Fc γ RII, only platelets from some donors can bind the Fc portion of mouse monoclonal antibodies. Donors were selected based on their platelet phenotyping for Fc γ RII, which was performed by Dr Christilla Bachelot-Loza (Faculté de Pharmacie, Paris, France).

Actin cytoskeleton extraction

Platelets (3×10^8 /ml, 600 μ l) were solubilized by adding 60 μ l of cold $10 \times$ Triton lysis buffer, containing 10% Triton X-100 and

the following protease inhibitors: 20 mM leupeptin, 10 mM benzamide, 1.5 trypsin inhibitor units/ml aprotinin, 20 mM sodium orthovanadate and 100 mM NaF in BSA-free 5 mM Hepes-buffered Tyrode's solution (pH 7.4). After stirring for 15 min at 4 °C, platelet lysates were centrifuged for 5 min at 16000 *g*. Supernatants containing Triton X-100-soluble proteins were saved. Pellets containing Triton X-100-insoluble actin cytoskeleton and associated proteins were washed once without resuspension with 1 × Triton lysis buffer and again centrifuged for 5 min at 16000 *g*. Final pellets of insoluble cytoskeletal proteins were resuspended in Tyrode's solution containing 1 % Triton X-100, 3 % SDS and 100 mM dithiothreitol (DTT) and solubilized by heating for 5 min at 100 °C. Corresponding Triton X-100-soluble proteins were similarly solubilized in the presence of 3 % SDS and 100 mM DTT.

SDS/PAGE and Western-blot analysis

Proteins were separated on SDS/polyacrylamide (7.5%) gels and either stained with Coomassie Brilliant Blue or electrotransferred on to nitrocellulose membranes (0.2 µm pore size; Bio-Rad). Nitrocellulose membranes were blocked overnight at 4 °C with 5 % low-fat milk in Tris-buffered saline (20 mM Tris/HCl, 150 mM NaCl and 0.05 % Tween 20, pH 7.4). Membranes were washed and incubated for 1 h at room temperature with the appropriate primary specific antibodies diluted in Tris-buffered saline. After washing, nitrocellulose membranes were incubated with horseradish peroxidase-coupled secondary antibodies for 1 h at room temperature. Immunoreactive bands were revealed by a chemiluminescence reaction (DuPont NEN, Boston, MA, U.S.A.).

Quantification of TSP-1 associated with the actin cytoskeleton

To quantify the amount of membrane-surface-expressed TSP-1 on activated platelets that associated with the actin cytoskeleton upon activation with thrombin, we determined the amount of TSP-1 present in the Triton X-100-soluble fraction that contained both the extracellularly secreted TSP-1 and membrane-surface-bound TSP-1 not associated with the actin cytoskeleton and/or solubilized from the membrane during the actin cytoskeleton-extraction procedure. To determine extracellularly secreted TSP-1, an aliquot of thrombin-activated platelets was taken before Triton X-100 solubilization and centrifuged for 2 min at 12000 *g*. The supernatant was centrifuged for a further 1 h at 100000 *g* and solubilized by heating for 5 min at 100 °C in the presence of 1 % Triton X-100, 3 % SDS and 100 mM DTT. Purified TSP-1 (0.25–2 µg), used to establish the standard curve, Triton X-100-insoluble and -soluble fractions and supernatant from thrombin-activated platelets were subjected to SDS/PAGE on the same gel. Intensities of Coomassie Brilliant Blue-stained bands of TSP-1 were quantified by scanning gels with a laser densitometer connected to a computerized image analyser (Biocom, Les Ulis, France), and TSP-1 in the different platelet fractions was quantified within the linear range of the standard curve.

Flow-cytometry analysis

Platelets (3×10^8 /ml, 100–200 µl), either non-activated or activated for 3 min at 37 °C with 0.1 unit/ml thrombin under non-stirring conditions, were diluted to 1.5×10^8 /ml and incubated with PE-labelled antibodies to either TSP-1 (diluted 1:5) or CD62 (diluted 1:8) or PE-labelled mouse control antibody (diluted 1:8) for 30 min at room temperature. Platelet suspensions were analysed using a FACScan (Becton Dickinson).

RESULTS

Specific association of TSP-1 with the actin cytoskeleton of thrombin-activated platelets

To investigate whether TSP-1 associated with the actin cytoskeleton during its membrane-surface expression, platelets were activated with thrombin, either for various times with a fixed concentration of thrombin (0.1 unit/ml) or for 5 min with various concentrations of thrombin (0.025–0.2 unit/ml), under aggregating (stirred) or non-aggregating (unstirred) conditions. Actin cytoskeleton was recovered from solubilized platelets as Triton X-100-insoluble material and analysed by SDS/PAGE. As shown on the Coomassie Brilliant Blue-stained gels for the kinetics of platelet activation with 0.1 unit/ml thrombin under stirring conditions (Figure 1A, left-hand panel), the composition of the actin cytoskeleton was modified rapidly following the addition of thrombin, as expected [18,19]. In particular, the amounts of filamin (actin-binding protein), talin, myosin, α -actinin, actin and a 180 kDa protein increased rapidly and time-dependently within 10 s of platelet activation under stirring conditions. This coincided with induction of platelet secretion, as measured by dense-granule [14 C]5-HT secretion, which preceded detectable aggregation. The 180 kDa protein was identified as TSP-1 by Western blotting (Figure 1B). TSP-1 incorporation into the actin cytoskeleton was shown to occur independently of platelet aggregation, as it occurred within 30 s of platelet activation under non-stirring conditions, correlating with modifications of the actin cytoskeleton composition (Figure 1A, right-hand panel). In addition, the extent of TSP-1 incorporation was thrombin dose-dependent, as it was detected at 0.025 unit/ml and reached its maximum at 0.1 unit/ml. Comparison of TSP-1 distribution between the Triton X-100-insoluble actin cytoskeleton and the corresponding Triton X-100-soluble fraction by Western blots, as shown in Figure 1(B), indicated that large amounts of TSP-1 associated progressively with the actin cytoskeleton. We therefore performed quantification experiments, which showed that approx. 50 % and 70 % of the platelet-bound TSP-1 associated with the actin cytoskeleton of non-aggregated and aggregated platelets, respectively, at 5 min of stimulation with 0.1 unit/ml thrombin (results not shown).

The role of actin polymerization in the actin-cytoskeleton association of TSP-1 was investigated by treating platelets with cytochalasin D, an inhibitor of actin polymerization. Exposing platelets to cytochalasin D (10 µM) before stimulation by thrombin inhibited both the reorganization of the actin cytoskeleton and the cytoskeletal association of TSP-1 (Figure 2).

We next examined whether fibrin, detected as a γ - γ dimer in the actin cytoskeleton preparation, could be responsible for the recovery of TSP-1 in the actin cytoskeleton. Whereas fibrin binds to activated platelets and associates with the actin cytoskeleton through membrane receptors [33], polymerizing fibrin to which TSP-1 was shown to bind [34] non-specifically sediments during isolation of the actin cytoskeleton from Triton X-100-solubilized platelets. Therefore, platelets were stimulated with 20 µM TRAP-6, which induced platelet aggregation and [14 C]5-HT secretion, similar to that induced by 0.1 unit/ml thrombin, but prevented fibrin formation. When analysed on a Coomassie Brilliant Blue-stained gel, actin cytoskeleton from TRAP-6-activated platelets was reorganized to a lesser extent than that from thrombin-activated platelets and, as a result, it had incorporated less TSP-1. Nonetheless, it was demonstrated clearly that TSP-1 associated with the actin cytoskeleton in the absence of fibrin (Figure 3).

Finally, we determined whether TSP-1 association with the actin cytoskeleton of thrombin-activated platelets occurred

Figure 1 Time- and thrombin-dose-dependent association of TSP-1 with the actin cytoskeleton of thrombin-activated platelets

Washed platelets (3×10^9 /ml) were activated with 0.1 unit/ml thrombin for the indicated times under stirred (aggregating) or unstirred (non-aggregating) conditions or with increasing doses of thrombin for 5 min under unstirred conditions. Aggregation rates (Aggr.) and [¹⁴C]5-HT secretion, measured at the end of incubation with thrombin, are indicated. Platelets were lysed by adding Triton X-100 lysis buffer and the Triton X-100-insoluble actin cytoskeleton was recovered by centrifugation of the platelet lysate for 5 min at 16 000 *g*. Triton X-100-soluble and -insoluble fractions were solubilized in an SDS- and DTT-containing buffer and analysed by SDS/PAGE, as described in the Experimental section. (A) Electrophoresed cytoskeletal proteins from 150 μ g of the initial platelet suspension [approx. $(6-7) \times 10^7$ platelets] were stained with Coomassie Brilliant Blue. (B) Triton X-100-insoluble and -soluble proteins from 30 μ g of the initial platelet suspension were analysed by Western blotting, using a purified polyclonal antibody to TSP-1, R1 (10 μ g/ml).

specifically through its membrane-surface receptors. Platelets were stimulated under non-stirring conditions in the presence of MAII, a monoclonal antibody to TSP-1 known to inhibit TSP-1 membrane-surface expression on activated platelets [9], or with MAI, a non-blocking monoclonal antibody to TSP-1, used as a control. Although cytoskeleton reorganization was fully achieved in platelets activated in the presence of either antibody, TSP-1 association with the actin cytoskeleton was selectively inhibited by MAII (Figure 4), which inhibited approx. 50% of TSP-1 membrane-surface expression, as assessed by flow cytometry (results not shown), whereas MAI had no effect.

Role of α IIB β 3, CD36, CD47 and fibrinogen in mediating association of TSP-1 with the actin cytoskeleton of thrombin-activated platelets

As a first step to identifying the transmembrane receptors mediating TSP-1 association with the actin cytoskeleton of thrombin-activated platelets, we investigated whether the potential receptors for TSP-1, α IIB β 3, CD36 and CD47, as well as fibrinogen, the main ligand for α IIB β 3 and a well-known TSP-1-binding molecule [5,8,14], were recovered together with TSP-1 in the Triton X-100-insoluble actin cytoskeleton. As illustrated in Figure 5(A, panel 2), for control platelets, α IIB β 3 and CD36 were found to associate with the actin cytoskeleton simultaneously with TSP-1. Comparison with the Triton X-100-soluble

fractions indicated that large amounts of these potential receptors for TSP-1 were recovered in the actin cytoskeleton (results not shown). CD47 was also incorporated into the actin cytoskeleton, but its analysis was complicated by the presence of artifactual bands at 100 and 200 kDa, which correspond to CD47 aggregates formed during electrophoresis, as reported previously [35]. However, it seemed that relatively little CD47 was incorporated into the actin cytoskeleton because of the very large amount of CD47 remaining in the corresponding Triton X-100-soluble fraction (results not shown). On the other hand, fibrinogen and fibrin were mostly associated with the actin cytoskeleton upon thrombin activation of platelets.

The role of α IIB β 3 in mediating association of platelet surface-bound TSP-1 with the actin cytoskeleton was investigated by analysing the reorganization of the actin cytoskeleton in platelets from a patient with type I Glanzmann's thrombasthenia with no detectable α IIB β 3 complexes [30]. Compared with control platelets under stirred activation conditions, thrombasthenic platelets exhibited normal rapid thrombin-activation-induced cytoskeletal incorporation of filamin, myosin, α -actinin and actin, as reported previously by Fox et al. [36] (see Figure 5A, panel 1). Some other proteins were present in lower or barely detectable amounts, particularly those corresponding to the electrophoretic migration of the α , β and γ chains of fibrinogen and the fibrin γ - γ dimer. Nevertheless, time-dependent incorporation of TSP-1 into the actin cytoskeleton was normal in thrombasthenic

Figure 2 Cytoskeletal association of TSP-1 is linked to actin cytoskeleton reorganization

Washed platelets (3×10^8 /ml), untreated or treated for 5 min at 37 °C with 10 μ M cytochalasin D in 1% DMSO (+Cyto. D) or 1% DMSO alone (+DMSO), were activated (Thrombin) or not (0) for 3 min with 0.1 unit/ml thrombin under aggregating conditions. Aggregation rates (Aggr.) and [¹⁴C]5-HT secretion are indicated. The actin cytoskeleton was extracted as described in the legend to Figure 1 and cytoskeletal proteins from 150 μ g of the initial platelet suspension were analysed by SDS/PAGE followed by Coomassie Brilliant Blue staining.

Figure 4 TSP-1 association with the actin cytoskeleton is linked to its membrane-surface expression

Washed platelets (3×10^8 /ml) were activated for 5 min at 37 °C with 0.1 unit/ml thrombin under non-aggregating (unstirred) conditions, in the absence or presence of 1 μ M of MAI or MAII antibodies to TSP-1. Platelet secretion of [¹⁴C]5-HT, measured at the end of platelet incubation with thrombin, is indicated. The actin cytoskeleton was extracted as described in the legend to Figure 1 and cytoskeletal proteins from 150 μ g of the initial platelet suspension were analysed by SDS/PAGE followed by Coomassie Brilliant Blue staining.

Figure 3 TSP-1 association with the actin cytoskeleton is not dependent on fibrin formation

Washed platelets (3×10^8 /ml) were activated for 3 min at 37 °C with 0.1 unit/ml thrombin or 20 μ M TRAP-6 under aggregating (stirred) conditions. Aggregation rates (Aggr.) and [¹⁴C]5-HT secretion are indicated. The actin cytoskeleton was prepared as described in the legend to Figure 1 and cytoskeletal proteins from 150 μ g of the initial platelet suspension were analysed by SDS/PAGE followed by Coomassie Brilliant Blue staining.

platelets and was further confirmed by Western-blot analysis (Figure 5A, panel 2). TSP-1 membrane-surface expression, as measured by flow cytometry (results not shown), as well as the

kinetics of dense-granule [¹⁴C]5-HT secretion by these platelets, were also normal (Figure 5A). Fibrinogen, present in very low amounts in the platelets of this thrombasthenic patient (5% of the normal value) [29], was found to associate with the actin cytoskeleton despite the absence of α IIb β 3, but no fibrin could be detected. This observation demonstrated that neither membrane-bound fibrinogen nor membrane-bound fibrin was essential for the mechanism of cytoskeletal association of TSP-1. Note that the presence of the β 3 subunit in the cytoskeleton of this patient's platelets was most likely to be due to cytoskeletal association of the α v β 3 receptor, which is detected in normal amounts in type I thrombasthenic platelets containing trace amounts of β 3 [15,26,36a]. Interesting was the finding that CD36 did not associate with the actin cytoskeleton of thrombin-activated thrombasthenic platelets lacking α IIb β 3, as it remained entirely in the Triton X-100-soluble fraction (results not shown). In addition to the fact that this result demonstrated the selective α IIb β 3-dependent cytoskeletal association of CD36, it indicated that CD36 did not mediate the association of TSP-1 with the actin cytoskeleton. Finally, in contrast to CD36, CD47 was incorporated rapidly into the actin cytoskeleton of thrombasthenic platelets upon thrombin activation, as in control platelets, but apparently to a lesser extent.

Studies on thrombasthenic platelets showed that CD36 could not be responsible for primary linking of TSP-1 to the actin cytoskeleton. This issue was addressed more directly by analysing cytoskeleton reorganization in platelets from a patient with no detectable membrane-surface CD36. Western blotting with a polyclonal antibody specific to CD36 showed that CD36 in the total platelet lysate of this patient was hardly detectable (Figure 5B). However, aggregation and [¹⁴C]5-HT secretion of these CD36-deficient platelets were normal, and normal time-dependent actin cytoskeleton reorganization and TSP-1 redistribution to the actin cytoskeleton were observed (Figure 5A).

Figure 5 TSP-1 association with the actin cytoskeleton of thrombin-activated platelets is not mediated by α IIb β 3 or CD36

(A) Washed platelets (3×10^8 /ml) from a normal control donor, a patient with type I Glanzmann's thrombasthenia and a patient with platelet CD36 deficiency were activated with 0.1 unit/ml thrombin for the indicated times under stirred conditions. Aggregation rates (Aggr.) and [¹⁴C]5-HT secretion, measured at the end of incubation with thrombin, are indicated. The actin cytoskeleton was prepared as described for Figure 1. Electrophoresed Triton X-100-insoluble cytoskeletal proteins from 150 μ g (panel 1) or 50 μ g (panel 2) of the initial platelet suspension were analysed either by Coomassie Brilliant Blue staining (panel 1) or Western blotting (panel 2) using polyclonal antibodies specific to TSP-1 (R1, 10 μ g/ml), CD36 (20 μ g/ml) and fibrinogen (10 μ g/ml), polyclonal antisera raised against α IIb (1:1000 dilution) and β 3 (1:1000), and a monoclonal antibody specific to CD47 (B6H12, 10 μ g/ml). Artifactual aggregate forms of CD47 are indicated by asterisks. (B) Platelet lysates prepared from the whole platelet suspension (50 μ g of proteins) from a control donor and the patient with platelet CD36 deficiency were analysed by Western blotting for the total content of CD36, using a polyclonal antibody to CD36 (20 μ g/ml). Purified CD36 (4.5 μ g) was used as the control.

Thrombin also induced normal membrane-surface expression of TSP-1 and CD62, an α -granule membrane component, on these platelets (results not shown). Finally, it was noted that the absence of CD36 from the platelet membrane did not impair the association of α IIb β 3 with the actin cytoskeleton, which was apparently normal in these CD36-deficient platelets (Figure 5A, panel 2). From these results with α IIb β 3- and CD36-deficient platelets, we concluded that neither α IIb β 3 nor CD36 was essential for the mechanism of TSP-1 membrane-surface ex-

pression and its subsequent association with the actin cytoskeleton.

The role of CD47 was explored by analysing the effect of the function-blocking anti-CD47 antibody, B6H12, on TSP-1 membrane-surface expression and cytoskeletal association in normal platelets. This antibody was demonstrated previously to inhibit ligation of platelet CD47 to a synthetic peptide derived from the C-terminal sequence of TSP-1 [13,37]. B6H12, used at 20 and 40 μ g/ml, did not exhibit any effect on thrombin-induced

Figure 6 TSP-1 association with the actin cytoskeleton of thrombin-activated platelets is not affected by the presence of the function-blocking monoclonal antibody to CD47, B6H12

Washed platelets (3×10^8 /ml) from a non-responsive donor (see the Experimental section) were preincubated for 1 min with or without $20 \mu\text{g/ml}$ B6H12, then activated with 0.05 unit/ml thrombin for the indicated times under stirred conditions. Aggregation rates (Aggr.) and [^{14}C]5-HT secretion, measured at the end of incubation with thrombin, are indicated. The actin cytoskeleton was prepared as described for Figure 1. Cytoskeletal proteins from $150 \mu\text{g}$ (panel 1) or $50 \mu\text{g}$ (panel 2) of the initial platelet suspension were subjected to SDS/PAGE and analysed by either Coomassie Brilliant Blue staining (panel 1) or Western blotting (panel 2) using antibodies specific to TSP-1 (R1, $10 \mu\text{g/ml}$) and CD47 (B6H12, $10 \mu\text{g/ml}$). Artifacts of aggregate forms of CD47 are indicated by asterisks.

platelet aggregation of a non-responsive donor (see the Experimental section), whereas it spontaneously aggregated the platelets from a responsive donor in the absence of thrombin, as expected (results not shown). Normal time-dependent actin cytoskeleton reorganization (Figure 6, panel 1) as well as TSP-1 and CD47 redistribution to the actin cytoskeleton were observed in platelets from the non-responsive donor in the presence of B6H12 ($20 \mu\text{g/ml}$; Figure 6, panel 2). Thrombin was also found to induce normal membrane-surface expression of TSP-1 and CD62 on these platelets activated in the presence of B6H12, as measured by flow cytometry (results not shown). In conclusion, we could not provide evidence that CD47 constituted a membrane receptor for the α -granular TSP-1 on thrombin-activated platelets that could mediate TSP-1 association with the actin cytoskeleton.

Translocation of signalling proteins into the TSP-1-enriched actin cytoskeleton of thrombin-activated $\alpha\text{IIb}\beta 3$ - or CD36-deficient platelets

We have provided evidence for specific association of TSP-1 with the actin cytoskeleton of thrombin-activated platelets occurring

in the absence of either of two major signalling receptors, i.e. $\alpha\text{IIb}\beta 3$ integrin or CD36. As shown for $\alpha\text{IIb}\beta 3$, engagement of signalling receptors induces their connection with the actin cytoskeleton, which subsequently undergoes major modifications and triggers the cytoskeletal association of numerous components important for intracellular signalling [18,20,25]. We therefore examined whether the association of TSP-1 with the actin cytoskeleton in $\alpha\text{IIb}\beta 3$ - or CD36-deficient platelets was accompanied by significant cytoskeletal translocation of signalling components as tyrosine-phosphorylated proteins and tyrosine kinases.

As shown in Figure 7(A), many of the thrombin-induced tyrosine-phosphorylated proteins in control platelets selectively translocated to the cytoskeleton, as already reported [36,38], but this was shown here to occur with a time-dependent rate of incorporation similar to that of TSP-1 (see Figure 5A). A similar time-dependent pattern of tyrosine-phosphorylated proteins was found in the cytoskeleton of CD36-deficient platelets. In thrombasthenic platelets, the time-dependent translocation of tyrosine-phosphorylated proteins to the cytoskeleton was also observed upon platelet activation. However, lower amounts of most of the proteins were detected as less tyrosine phosphorylation of the proteins occurred in these platelets, as shown by the analysis of the corresponding Triton X-100-soluble protein fractions and as also reported previously by others [36,39]. The presence of the tyrosine kinases Src, Syk and FAK and the lipid and tyrosine kinase PI-3K along with tyrosine-phosphorylated proteins was detected in the actin cytoskeleton from $\alpha\text{IIb}\beta 3$ - and CD36-deficient platelets (Figure 7B). In control and CD36-deficient platelets, the kinases were observed to translocate extensively to the actin cytoskeleton, as compared with the Triton X-100-soluble fraction, whereas in thrombasthenic platelets they appeared to translocate to a lesser extent. These results indicated that signalling complexes associated with the actin cytoskeleton of thrombin-activated platelets simultaneously with TSP-1 and independently of $\alpha\text{IIb}\beta 3$ or CD36.

DISCUSSION

Platelet activation by thrombin is shown herein to induce a rapid association of TSP-1 with the actin cytoskeleton that coincides with remodelling of the actin cytoskeleton, induction of platelet secretion and TSP-1 membrane-surface expression, but which does not require platelet aggregation. Studies addressing the identity of the membrane receptors responsible for TSP-1 cytoskeletal association demonstrated that the absence of neither $\alpha\text{IIb}\beta 3$ nor CD36 affected TSP-1 association with the actin cytoskeleton. Although these receptors were identified previously as potential membrane receptors for TSP-1 (see [14]), other studies have shown that thrombasthenic and platelet-specific alloantigen Nak^a-negative platelets, lacking $\alpha\text{IIb}\beta 3$ and CD36, respectively, displayed normal amounts of membrane-expressed TSP-1 upon activation [14,40,41]. These contradictory results could be related to the ability of TSP-1 to bind several receptors simultaneously; thus the absence of a single receptor would not be sufficient to prevent its binding to the platelet membrane. Interestingly in this study, we observed that CD36 in thrombasthenic platelets did not associate with the actin cytoskeleton in the absence of the integrin $\alpha\text{IIb}\beta 3$ (see Figure 5A). This result indicated that the normal association of TSP-1 with the actin cytoskeleton of thrombasthenic platelets required the role of receptor(s) for TSP-1 other than $\alpha\text{IIb}\beta 3$ and CD36. A likely candidate was CD47, which was shown to be present in high levels in platelets [35]. Previous studies have identified CD47 as being a potential platelet receptor for the C-terminal cell-binding

Figure 7 Translocation of tyrosine-phosphorylated proteins and protein kinases into the TSP-1-enriched actin cytoskeleton of thrombin-activated α IIb β 3- or CD36-deficient platelets

Washed control, type I Glanzmann's thrombasthenic and CD36-deficient platelets (3×10^8 /ml) were activated with 0.1 unit/ml thrombin for the indicated times under stirred conditions. **(A)** Triton X-100-insoluble actin cytoskeleton and Triton X-100-soluble fractions, from 50 µg of the initial platelet suspension, were analysed by Western blotting using a monoclonal antibody specific to tyrosine-phosphorylated proteins (PY20, 0.5 µg/ml). Molecular masses of the main tyrosine-phosphorylated proteins are indicated. The protein band corresponding to the tyrosine kinase Src is shown. **(B)** Western-blot analysis of the Triton X-100-insoluble actin cytoskeleton and the Triton X-100-soluble fraction from control platelets using monoclonal antibodies specific to the tyrosine kinases FAK (1 µg/ml), Syk (2 µg/ml) and Src (1.6 µg/ml) and a polyclonal antiserum raised against the lipid kinase PI-3K (1:500 dilution).

domain of TSP-1, using a synthetic peptide from this domain of the TSP-1 molecule and the function-blocking anti-CD47 antibody, B6H12 [13,37]. However, the role of CD47 as a membrane receptor for the native α -granular TSP-1 has not been addressed so far. In the present study, we failed to detect any inhibitory effect of B6H12 on both membrane expression and cytoskeletal association of the α -granular TSP-1 in thrombin-activated platelets. This result seems to indicate that CD47 does not behave as a receptor for the native α -granular TSP-1 and raises the question of the existence of another as-yet unidentified physiological TSP-1 receptor on platelets. Alternatively, this result could be relevant to the mechanism of TSP-1 secretion from the α -granules with direct expression to the plasma membrane, as discussed below. Indeed, it is not known whether an endogenous pool of CD47, with high affinity for TSP-1, exists in the membrane of the α -granules to which epitopes for the blocking anti-CD47 antibody would not be accessible due to TSP-1 occupancy on platelet secretion. Further studies are required to define the localization and possible contribution of CD47 to the molecular process of TSP-1 membrane expression and association with the actin cytoskeleton. Other receptors, like the integrins $\alpha_v\beta_3$ and $\alpha_2\beta_1$, reported to associate with CD47 [12], are themselves potential receptors for TSP-1 [15,42] and may participate in linking TSP-1 to the actin cytoskeleton.

The association of TSP-1 with the actin cytoskeleton through as-yet unidentified receptor(s) may have important implications for TSP-1 functions. First, it could be relevant to the mechanism of TSP-1 recruitment from the α -granules to the platelet surface.

Studies by us and others have indicated that during α -granule exocytosis induced by a strong agonist like thrombin, adhesive proteins, e.g. fibrinogen and TSP-1, seemed to reach the platelet surface already bound to intracellular membrane receptors [7,17,31,43,44]. By immunoelectron microscopic analysis, we showed that, within the first minute of platelet activation by thrombin, TSP-1 molecules concentrated along the membrane of the open canalicular system, originating from plasma-membrane invaginations, and with which α -granule membranes fused, indicating that secreted TSP-1 could indeed bind to receptors present in the open canalicular system and/or α -granules [31], as shown for fibrinogen- α IIb β 3 [17]. During this process, subsequent association of the occupied receptors with the actin cytoskeleton would provide mechanical forces for the rapid outward transport of α -granular proteins and their platelet-surface redistribution. In connection with this, we found that two other α -granular proteins, fibrinogen (illustrated in Figure 5) and von Willebrand factor (N. De Jesus and V. Dubernard, unpublished work), became associated with the actin cytoskeleton concomitantly with TSP-1, sharing the same pattern of cytoskeletal association as TSP-1. Pertinently, immunofluorescence studies on the distribution of membrane-bound TSP-1 in thrombin-activated platelets showed large clusters in the periphery of control platelets, whereas TSP-1 molecules were organized into smaller clusters and/or ring-like patterns in thrombasthenic platelets in the absence of fibrinogen (A. Saumet and V. Dubernard, unpublished work). This could indicate that connection of membrane-bound adhesive proteins with the actin

cytoskeleton favours complexation between adhesive proteins, leading to their redistribution in clusters and the delineation of highly adhesive areas in the plasma membrane.

The association of TSP-1 with the actin cytoskeleton of thrombin-activated platelets, as described herein, may also couple TSP-1 receptors to intracellular effectors needed to initiate and/or amplify platelet responses, such as granule secretion, which proceeds normally in thrombasthenic platelets despite their severe aggregation deficiency (see Figure 5A). In relation to this, we have shown previously that TSP-1 was involved in the secretion-dependent phase of platelet aggregation [9,10]. In the present study, assembly of signalling complexes, as observed through translocation of tyrosine-phosphorylated proteins and kinases to the actin cytoskeleton, was found to occur in concert with cytoskeletal association of TSP-1, and independently of either one of two major signalling receptors, α IIb β 3 or CD36. Whether TSP-1 receptors may be coupled directly to these signalling complexes remains to be determined. This concomitant association of TSP-1 and signalling complexes with the actin cytoskeleton in thrombin-activated platelets could also influence actin cytoskeleton remodelling itself, which was not impaired significantly in the absence of α IIb β 3 or CD36. Indeed, we found significant translocation of the signalling enzymes Src, Syk, FAK and PI-3K kinases to the actin cytoskeleton of thrombasthenic platelets, although translocation of these kinases was shown previously to be dependent on α IIb β 3 ligation [36,38,45,46]. These apparently contradictory findings probably reflect the different experimental procedures used, as we intentionally extracted the actin cytoskeleton in Triton X-100 lysis buffer without EDTA or EGTA, to avoid dissociation of membrane- and actin cytoskeleton-bound TSP-1 (N. De Jesus and V. Dubernard, unpublished work). Under these conditions, our Coomassie Brilliant Blue-stained electrophoretic patterns of extracted cytoskeletal proteins appear very similar to those of others [19,36,46]. Nevertheless, the presence of the tyrosine kinases Src, Syk and FAK along with tyrosine-phosphorylated proteins in the actin cytoskeleton is consistent with the idea that the actin cytoskeleton brings enzymes together in close proximity to their respective substrates, and thus generates the tyrosine phosphorylation events that we and others [36,38] observed in thrombasthenic platelets.

This is the first report demonstrating a specific association between membrane-surface-bound α -granular TSP-1 and the reorganizing actin cytoskeleton in platelets upon thrombin activation. This association could have critical implications for platelet adhesive behaviour. Identification of the transmembrane receptors linking TSP-1 to the actin cytoskeleton and the signals directly coupled to TSP-1 receptors, currently under investigation, will improve our understanding of the functions of membrane-expressed TSP-1 on activated platelets.

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