Journal of Thrombosis and Haemostasis, **1**: 320–329

ORIGINAL ARTICLE

Platelet aggregation induced by the C-terminal peptide of thrombospondin-1 requires the docking protein LAT but is largely independent of αIIb/β3

C. TRUMEL,* M. PLANTAVID,* S. LÉVY-TOLÉDANO,† A. RAGAB,* J. P. CAEN,‡ E. AGUADO, § B. MALISSEN§ and B. PAYRASTRE*

*INSERM, Unité 563, Centre de Physiopathologie Toulouse Purpan, Department of Oncogenesis and Cell Signaling in Hematopoetic Cells, Hôpital Purpan, Toulouse; †INSERM, Unité 348, Hôpital Lariboisière, Paris; ‡IVS, Hôpital Lariboisière, Paris; and §Center d'Immunologie de Marseille-Luminy, INSERM-CNRS-University Medical Case 906, Marseille, France

Summary. Thrombospondin-1 (TSP1) is abundantly secreted during platelet activation and plays a role in irreversible platelet aggregation. A peptide derived from the C-terminal domain of TSP1, RFYVVMWK (RFY) can activate human platelets at least in part *via* its binding to integrin-associated protein. Although integrin-associated protein is known to physically interact with αIIb/β3, we found that this major platelet integrin had only a partial implication in RFY-mediated platelet aggregation. Accordingly, RFY induced a significant Glanzmann type I thrombasthenic platelet aggregation. The αIIb/β3-dependent part of platelet aggregation induced by RFY was mainly due to secreted ADP and thromboxane A2. In the absence of αIIb/β3 and fibrinogen, RFY stimulated a rapid tyrosine phosphorylation of a set of proteins, including Syk, linker for activation of T cells (LAT) and phospholipase Cγ2. This signaling pathway was critical for RFY-mediated platelet activation as revealed by the use of pharmacological inhibitors as well as LAT-deficient mouse platelets. Phosphoinositide 3-kinase activation was also required for RFY-mediated platelet aggregation. Our results unravel a new αIIb/β3 and fibrinogen-independent mechanism for platelet aggregation in response to the active peptide from the C-terminal domain of TSP1.

Keywords: αIIb/β3 integrin, LAT, platelet aggregation, thrombospondin-1.

The critical role of thrombospondin-1 (TSP1) in the irreversible platelet aggregation first described by Leung [1] has now been confirmed by several groups [2–4]. TSP1, a major component of platelet α granules, is secreted upon activation and behaves as a multiadhesive protein, mediating various biological functions. In this respect, at least four cell attachment sites have been documented in TSP1 sequence [5]. A number of recent studies have demonstrated a key functional role of the C-terminal domain of TSP1. For instance, a monoclonal antibody (C6.7) directed against this domain inhibits the secondary phase of ADP-induced platelet aggregation [2]. This antibody also blocks TSP1-dependent effects on cell motility [6–8] and Ca^{2+} influx [9] in other cell types. Two related sequences within this domain, RFYVVM and IRVVM, were described as minimal binding sites able to interact with a receptor at the surface of different cells, including platelets [2,10,11]. One of the receptors for the C-terminal domain of TSP1 has been identified as integrin-associated protein (IAP) or CD47 [12]. This widely expressed receptor has an extracellular immunoglobulin-like domain, five putative membrane-spanning sequences and a short intracellular tail. IAP is abundantly present in platelets and physically associates with αIIb/β3, α2/β1 and αv/β3 integrins [12–16]. α2/β1, quantitatively much less abundant than αIIb/β3 in this model, plays an important role in hemostasis as an adhesive receptor of collagen [17]. Chung *et al.* [18] demonstrated that TSP1 or RFY peptide can induce a strong aggregation of platelets adhered onto immobilized collagen suggesting that TSP1 may be involved in the first steps of platelet adhesion and activation in certain conditions. This effect was no longer observed when platelets from IAP-deficient mice were used, indicating a major role of IAP in this mechanism. Moreover, the same peptide was an important coactivator for the spreading of platelets on fibrinogen and this effect was dependent on intracellular signaling events involving tyrosine kinases and phosphoinositide 3-kinase (PI 3-kinase) [19]. Surprisingly, although TSP1 is not considered as a primary agonist of platelets [5], RFY peptide added to platelets in suspension can induce a rapid tyrosine phosphorylation of several proteins and a strong platelet aggregation [19,20].

The aim of the present study was to investigate the role of αIIb/β3 integrin in RFY-stimulated platelets and to characterize the signaling mechanisms induced by this peptide in the presence or absence of αIIb/β3. Interestingly, we found that platelet aggregation induced by RFY was partially dependent on the binding of fibrinogen to αIIb/β3. The αIIb/β3-dependent part of RFY-mediated platelet aggregation was almost exclusively due to secreted ADP and thromboxane A2 (TXA2), two classical

Correspondence: Dr B. Payrastre, Inserm Unité 563, Hôpital Purpan, 31059 Toulouse Cedex, France.
Tel.: +33 5 61779401; fax: +33 5 61779401; e-mail: payrastr@toulouse.inserm.fr

Received 13 May 2002, revised 13 August 2002, accepted 19 August 2002

© 2003 International Society on Thrombosis and Haemostasis

coactivators of αIIb/β3. The intracellular signaling pathways underlying this new αIIb/β3-independent mechanism of platelet activation and aggregation involved the tyrosine kinase Syk and its substrate, the docking molecule LAT, phospholipase C (PLC) γ2 and PI 3-kinase. Using pharmacological inhibitors and LAT-deficient mice, we found that this pathway was required for RFY-mediated platelet activation. These results will be discussed in light of the potential receptors for the C-terminal sequence of TSP1.

Materials and methods

Reagents

RFY was purchased from Bachem, (Voisin le Bretonneux, France) and RFYGGMWK was synthesized by Sigma-Genosys (Cambridge, UK). Mouse monoclonal antiphosphotyrosine 4G10 and rabbit polyclonal anti-LAT antibody were obtained from Upstate Biotechnology (Lake Placid, NY, USA). Rabbit polyclonal anti-PLCγ2 antibody was from Biotechnology Inc., (Santa Cruz, CA, USA). Rabbit polyclonal anti-Syk antibody was a gift from Dr U. Blank (Departement d'Immuno-Allergie, Institut Pasteur, Paris, France). The anti-α2 integrin antibody P1E6 was from Life Technologies (Cergy Pontoise, France). The enhanced chemiluminescence (ECL) Western blotting reagents and [^{32}P]orthophosphate were from Amersham Pharmacia Biotech (Little Chalfont, Bucks, UK). Piceatannol was obtained from Boehringer Mannheim (Rueil Malmaison, France). C7E3 Fab fragments (abciximab, ReoPro) and SR121566A [21] were kindly provided by Dr P. Savi (Sanofi-Synthelabo, Toulouse, France). All other reagents were purchased from Sigma (St Quentin-Fallavier, France) unless otherwise indicated. To generate LAT-deficient mice, an XhoI-BamHI genomic fragment that contains the complete LAT gene was replaced by a LoxP-flanked neo^r gene. The targeting construct was electroporated into CK35 129/SV embryonic stem (ES) cells. After selection in G418, colonies were screened for homologous recombination by Southern blot analysis. Targeted ES cells were injected into Balb/c blastocysts. The resulting mutant mouse line was first bred to Deleter mice to eliminate the LoxP-flanked neo^r cassette, and intercrossed to produce homozygous LAT−/− mutant mice. Their phenotype is identical to that previously described for an independently derived LAT−/− line [22].

Glanzmann thrombasthenia patients

The two Glanzmann type I thrombasthenic patients (patients 1 and 2) studied have been characterized previously [23]. Their platelets, having less than 5% of αIIb/β3 content, are severely deficient in fibrinogen and characterized by an absence of platelet aggregation with classical agonists and no or minimal clot retraction.

Preparation and activation of human platelets

Control platelets were isolated from concentrates obtained from the local blood bank (Etablissement Français du Sang, Pyrénées-Méditerranée) as previously described [24]. When platelets from Glanzmann thrombasthenia patients were used, blood was anticoagulated with ACD (124 mmol L^{-1} tri-sodium citrate, 130 mmol L^{-1} citric acid, 110 mmol L^{-1} glucose: one volume for nine volumes of blood). Blood was then centrifuged 15 min at 190 *g* to obtain the platelet-rich plasma (PRP). After centrifugation platelets were washed in a buffer (pH 6.5) containing 140 mmol L^{-1} NaCl, 5 mmol L^{-1} KCl, 5 mmol L^{-1} KH_2PO_4, 1 mmol L^{-1} Mg_2SO_4, 10 mmol L^{-1} Hepes, 5 mmol L^{-1} glucose and 0.35% (w/v) bovine serum albumin. The same buffer was added to the final suspension (7.5×10^8 cells mL^{-1}) and the pH adjusted to 7.3 with NaOH. Control platelets were obtained in the same way from healthy volunteer. For inositol-lipid analysis, platelets were incubated with 0.5 mCi mL^{-1} of [^{32}P]orthophosphate during 60 min in a phosphate-free buffer (pH 7.3) at 37 °C. ^{32}P-labeled platelets were then washed in a phosphate containing buffer and finally resuspended in the stimulation buffer, as above. Platelets were stimulated by RFY at 37 °C, under stirring at 900 rev min^{-1} in a dual-channel Payton aggregometer (Payton Associates, Scarborough, Ontario, Canada). In some experiments, platelets were preincubated at 37 °C with 5 μmol L^{-1} creatine phosphate and 40 IU mL^{-1} creatine phosphokinase, 10 μmol L^{-1} indomethacin or 0.5–1 mmol L^{-1} RGDS peptide.

Murine platelet preparation

Blood was collected by cardiac puncture and platelets were isolated from PRP by centrifugation at 2000 *g* for 8 min. The pellet was resuspended in a Tyrode-HEPES buffer [25] in the presence of 500 nmol L^{-1} prostaglandin I2 and platelets were left at 37 °C for 30 min before stimulation. Platelet stimulation was done at 37 °C under stirring at 900 rev min^{-1} in a dual-channel Payton aggregometer.

Lipid extraction and analysis

Reactions were stopped by addition of chloroform/methanol (1 : 1 v/v) containing 0.4 mol L^{-1} HCl and lipids were immediately extracted following a modified Bligh & Dyer procedure as previously described [26]. For PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 quantification, lipids were first resolved by thin-layer chromatography (TLC) using chloroform/acetone/methanol/acetic acid/water (80 : 30 : 26 : 24 : 14 v/v) and the spots corresponding to PtdInsP_2 and PtdInsP_3 were scraped off, deacylated and analyzed by HPLC on a Partisphere SAX column (Whatman International Ltd, UK) as previously described [26]. For phosphatidic acid (PtdOH) quantification, lipids were resolved by TLC using $CHCl_3$/CH_3OH/10 mol L^{-1} HCl (87 : 13 : 0.5 v/v) as described [26]. The radioactivity incorporated in PtdOH was counted by scintillation (TRI-CARB, 1900 TR, Packard).

Immunoprecipitation

Reactions were stopped by addition of one volume of twice-concentrated ice-cold lysis buffer containing 80 mmol L^{-1}

© 2003 International Society on Thrombosis and Haemostasis

Tris-HCl, pH 7.4, 200 mmol L^{-1} NaCl, 200 mmol L^{-1} NaF, 20 mmol L^{-1} EDTA, 80 mmol L^{-1} $Na_4P_2O_7$, 4 mmol L^{-1} Na_3VO_4, 2% (v/v) Triton X-100 and 10 µg mL^{-1} each of aprotinin and leupeptin. After gentle shaking during 20 min at 4 °C and centrifugation (12 000 *g* for 10 min at 4 °C), the soluble fraction was collected and precleared for 30 min with protein A-Sepharose CL4B. The precleared suspensions were then incubated for 2 h at 4 °C with the indicated antibodies and immune complexes were then precipitated by addition of 10% (w/v) protein A-Sepharose CL4B for 1 h at 4 °C and centrifugation (6000 *g* for 5 min at 4 °C). The immunoprecipitates were washed once in lysis buffer and twice in washing buffer containing 10 mmol L^{-1} Tris-HCl, pH 7.4, 100 mmol L^{-1} NaCl, 100 µmol L^{-1} Na_3VO_4, 1 µg mL^{-1} each of aprotinin and leupeptin. Immunoprecipitated proteins were then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting.

Gel electrophoresis and immunoblotting

Proteins were resuspended in the electrophoresis sample buffer containing 100 mmol L^{-1} Tris-HCl, pH 6.8, 15% (v/v) glycerol, 25 mmol L^{-1} dithiothreitol, and 3% SDS, boiled for 5 min, separated on 7.5% SDS–PAGE and transferred onto nitrocellulose membrane (Pall Life Sciences, Ann Arbor, MI, USA). The blots were blocked for 60 min at room temperature with 1% milk powder, 1% bovine serum albumin in a buffer containing 10 mmol L^{-1} Tris-HCl, pH 7.5, 150 mmol L^{-1} NaCl, and 0.1% Tween 20. Immunodetections were performed with the relevant antibodies, and the reactions visualized using peroxidase-conjugated secondary antibodies and the ECL chemiluminescence system.

Results

RFY induces a significant platelet aggregation independently of αIIb/β3

As previously shown [19,20], RFY can induce aggregation of human platelets in a concentration-dependent manner (Fig. 1a). We observed an optimal effect between 50 and 100 µmol L^{-1} RFY depending on the preparations, which might be due to peptide insolubility at high concentrations, and a classically decreased response at 200 µmol L^{-1} (not shown). As a control of specificity, we tested the RFYGGMWK peptide at increasing concentrations from 25 µmol L^{-1} to 200 µmol L^{-1} alone or in combination with RFY. This control peptide was inefficient by itself (Fig. 1a) and did not affect the action of the active RFY peptide (not shown). As shown Fig. 1(b), platelet aggregation induced by RFY was partly affected in the presence of an inhibitor of TXA2 synthesis and an ADP scavenger indicating that these two secreted molecules were required to amplify RFY-mediated platelet aggregation. Therefore, RFY is a secretagogue, but it can also induce a significant aggregation independently of TXA2 and ADP.

We then tested the effect of antagonists of fibrinogen binding to αIIb/β3 on RFY-mediated platelet aggregation. RGDS, the c7E3 antibody and the SR121566A compound [21] were potent inhibitors of platelet aggregation in response to thrombin used at 0.1 IU mL^{-1} (Table 1) and up to 1 IU mL^{-1} (not shown).

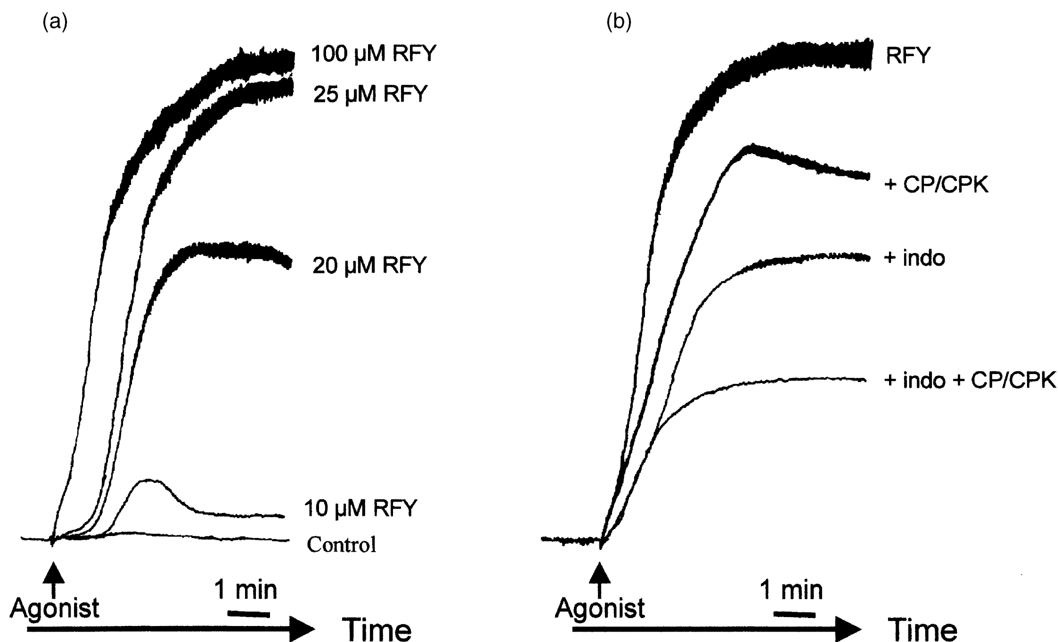

Fig. 1. Role of ADP and TXA2 in human platelets responses induced by RFY Platelets from a healthy volunteer were stimulated by increasing concentrations of RFY (A) or by 100 µmol L^{-1} RFY in the absence or in the presence of indomethacin to block TXA2 synthesis, a mixture of creatine phosphate/creatine phosphokinase (CP/CPK), an ADP scavenger or both together. The aggregation profiles were obtained with a Payton aggregometer with stirring at 900 rev min^{-1} (7.5×10^8 platelets mL^{-1}). The profiles shown are representative of four different experiments with similar results. Control: RFYGGMWK peptide (100 µmol L^{-1})

© 2003 International Society on Thrombosis and Haemostasis

Table 1 Comparative effects of various antiαIIb/β3 on the aggregation of platelets stimulated either by RFY or thrombin

αIIb/β3 inhibitors	RFY (100 μmol L^{-1})	Thrombin (0.1 IU mL^{-1})
None	100	100
RGDS (1 mmol L^{-1})	44 ± 8	7 ± 1
c7E3 (40 μg mL^{-1})	30 ± 2	14 ± 4
SR121566 (3.5 μmol L^{-1})	29 ± 2	7 ± 4

The effect of several unrelated antiαIIb/β3 (3.5 μmol L^{-1} SR121566A, 40 μg mL^{-1} c7E3 or 1 mmol L^{-1} RGDS) on the amplitude of platelet aggregation, stimulated either by 100 μmol L^{-1} RFY or by 0.1 IU mL^{-1} thrombin, was quantified. Data are expressed as percentages of the maximal aggregation obtained without inhibitor and are means $\pm$ SEM of three independent experiments.

Surprisingly, under the same conditions, RFY-induced platelet aggregation was only partially inhibited by these compounds (Table 1, see also Figs 2 and 3) even when used at very high concentrations (not shown). Phase contrast microscopy analysis confirmed the presence of tight aggregates induced by RFY in control platelets similar to those induced by thrombin (Fig. 2). Moreover, as illustrated in Fig. 2, RGDS addition fully abolished platelet aggregation induced by thrombin but did not prevent the formation of platelet aggregates in response to RFY. Interestingly, the ADP- and TXA2-independent aggregation (shown in Fig. 1) was not significantly affected by 1 mmol L^{-1} RGDS ($5\% \pm 3\%$ of inhibition). To further investigate this striking observation, we used platelets from two type I Glanzmann thrombasthenia patients having less than 5% of the normal amount of αIIb/β3 and no intracellular fibrinogen [27]. Figure 3(a) illustrates the aggregation response of normal platelets whereas Fig. 3(b) shows the response of type I Glanzmann thrombasthenic platelets. Even in the absence of αIIb/β3 and fibrinogen (Fig. 3b), RFY was still able to induce a significant change in light transmission (44%) reflecting the presence of small aggregates, clearly visible (not shown), whereas 0.5 IU mL^{-1} of thrombin was almost inefficient (12%). As a control, addition of RGDS did not alter the formation of the small aggregates observed in response to RFY (not shown) and the aggregation curves were comparable.

To further characterize this αIIb/β3-independent aggregation normal human platelets were fixed with 3% paraformaldehyde in order to check whether they would agglutinate in response to RFY. Under these conditions, no aggregation could be detected by the aggregometer (not shown). However, very small aggregates of a few platelets were visible under the light microscope suggesting that RFY can induce a discrete platelet cross-linking mechanism reminiscent of agglutination (not shown).

RFY induces a rapid tyrosine phosphorylation of Syk and LAT independently of αIIb/β3

The intracellular mechanisms induced by RFY, independently of αIIb/β3, were analyzed further. As shown in Fig. 4(a), in normal platelets stimulated by RFY, the tyrosine phosphorylation of several proteins was extremely rapid, reaching a maximum between 10 and 30 s and decreasing thereafter. Two major phosphotyrosyl proteins could be observed, one of 70 kDa, matching Syk, and another one of about 37 kDa matching LAT, a docking protein recently identified in platelets [28]. As shown in Fig. 4(b), immunoprecipitation experiments confirmed the tyrosine phosphorylation of Syk and LAT. Interestingly, these major tyrosine phosphorylation processes were still

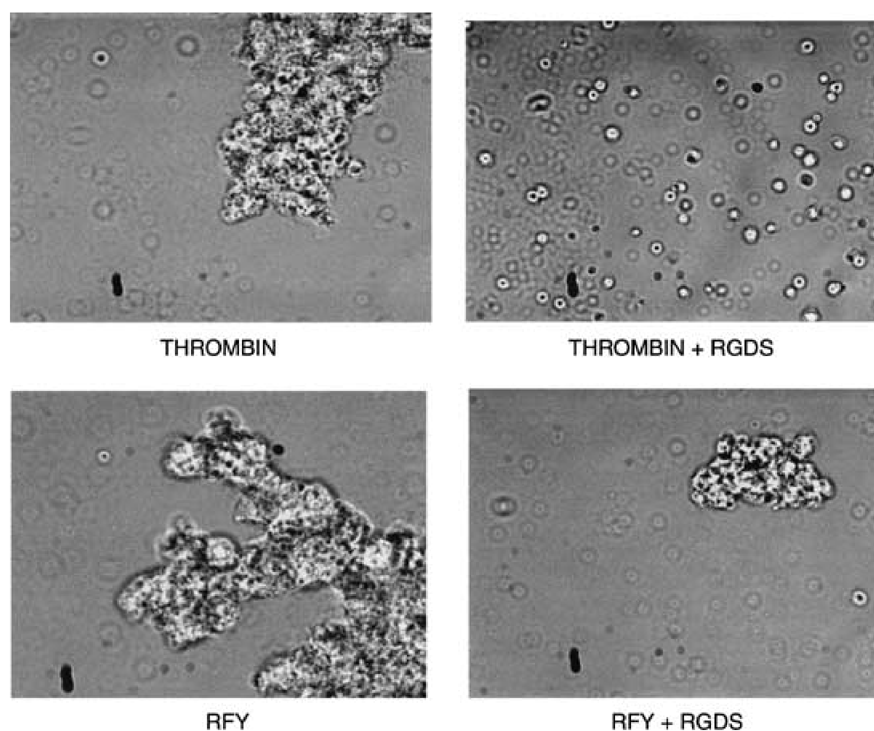

Fig. 2. Visualization of platelet aggregates induced by RFY in the absence of fibrinogen binding. Platelets from a healthy volunteer were stimulated by 0.1 IU mL^{-1} thrombin or by 100 μmol L^{-1} RFY in the presence or in the absence of 1 mmol L^{-1} RGDS under stirring at 900 rev min^{-1}. Platelet stimulation was stopped after 3 min by adding 3% formaldehyde. After 30 min of incubation at room temperature cells were observed by phase contrast microscopy using a Zeiss microscope equipped with a 40 $\times$ objective and a Princeton microMAX camera. Representative data are shown.

© 2003 International Society on Thrombosis and Haemostasis

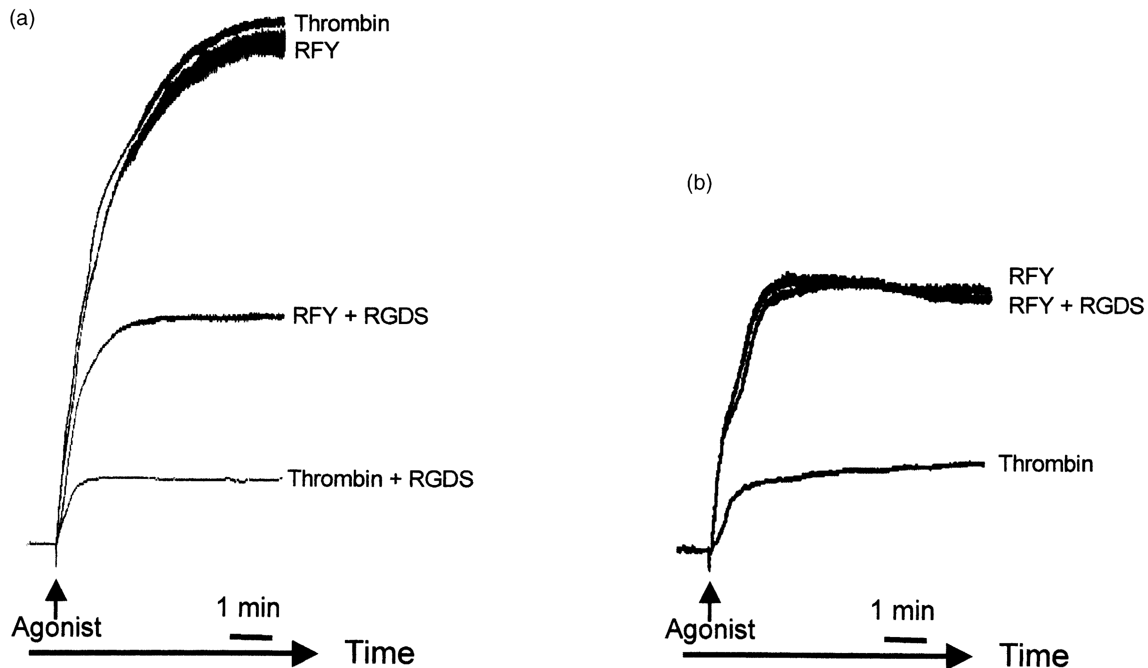

Fig. 3. Platelets from thrombasthenic patients still respond to RFY. Platelets from a healthy volunteer (a) or a type I thrombasthenic patient (b) were stimulated by 100 μmol L^{-1} RFY or by 0.1 IU mL^{-1} thrombin in the presence or in the absence of 1 mmol L^{-1} RGDS. Aggregation profiles shown were obtained with platelets from patient 1 and similar results were obtained with the second patient 2.

observed in RGDS-treated platelets or in thrombasthenic platelets (Fig. 4a) indicating that this mechanism was independent of αIIb/β3 engagement. In agreement, when RFY was added to thrombasthenic or to normal RGDS-treated platelets, similar amounts of Syk were recovered in antiphosphotyrosine immunoprecipitates (Fig. 4c). Conversely, the rapid dephosphorylation of Syk occurring in control platelets at 180 s and 300 s was strongly inhibited or delayed in the absence of αIIb/β3 engagement as shown either in thrombasthenic platelets or in control platelets treated with RGDS (Fig. 4a). These results indicate that αIIb/β3 was not involved in the tyrosine phosphorylation process triggered by RFY but rather in a dephosphorylation mechanism possibly via activation of protein tyrosine phosphatases.

Moreover, as shown Fig. 4(d), PP1, an inhibitor of the Src family tyrosine kinases, was able to strongly inhibit RFY–mediated aggregation amplitude (82% $\pm$ 3% of inhibition by 10 μmol L^{-1} PP1). Addition of inhibitors of ADP and TXA2 did not significantly amplify the effect of PP1 on platelet aggregation (not shown). An other tyrosine kinase inhibitor, piceatannol, was also able to strongly inhibit RFY-mediated aggregation (not shown) suggesting that the rapid activation of tyrosine kinases induced by RFY is required to obtain platelet aggregation.

RFY rapidly stimulates a PLC via a tyrosine kinase-dependent mechanism

In ^{32}P-labeled platelets, the diacylglycerol produced by PLC is mainly converted into [^{32}P]PtdOH by a diacylglycerol kinase [26]. The contribution of phospholipase D to this production of [^{32}P]PtdOH is relatively minor. Thus, the formation of this lipid can be considered as a good reflection of PLC activation in platelets. As shown Fig. 5(a), after a lag time of about 10 s, the synthesis of PtdOH increased rapidly until 1 min, followed by a slow decrease. The αIIb/β3 antagonists (RGDS, SR121566 and c7E3) had no significant effect on this production (Fig. 5a). Furthermore, treatment of thrombasthenic platelets by RFY led to a similar increase in the production of [^{32}P]PtdOH (three- and fourfold increase, in thrombasthenic and in control platelets, respectively). The tyrosine kinase inhibitor piceatannol (50 μmol L^{-1}) strongly inhibited the synthesis of PtdOH (92% $\pm$ 8% of inhibition, $n = 2$). In agreement, RFY induced a rapid tyrosine phosphorylation of PLCγ2 which was not affected by RGDS addition (Fig. 5b). Moreover, PLCγ2 was recovered in antiphosphotyrosine immunoprecipitates performed from thrombasthenic platelets challenged by RFY (Fig. 5c), indicating that αIIb/β3 was not necessary to control this mechanism.

Phosphoinositide 3-kinase is involved in RFY-mediated platelet aggregation

As illustrated in Fig. 6, PtdIns(3,4,5)P$_3$ and PtdIns(3,4)P$_2$ were produced in human platelets stimulated by RFY indicating the activation of a PI 3-kinase. The synthesis of PtdIns(3,4,5)P$_3$ was rapid and sustained whereas PtdIns(3,4)P$_2$ occurred after a lag time of 30 s. It is known that a large part of PdtIns(3,4)P$_2$ production is dependent on αIIb/β3 engagement and platelet aggregation induced by various agonists [29,30]. In agreement,

© 2003 International Society on Thrombosis and Haemostasis

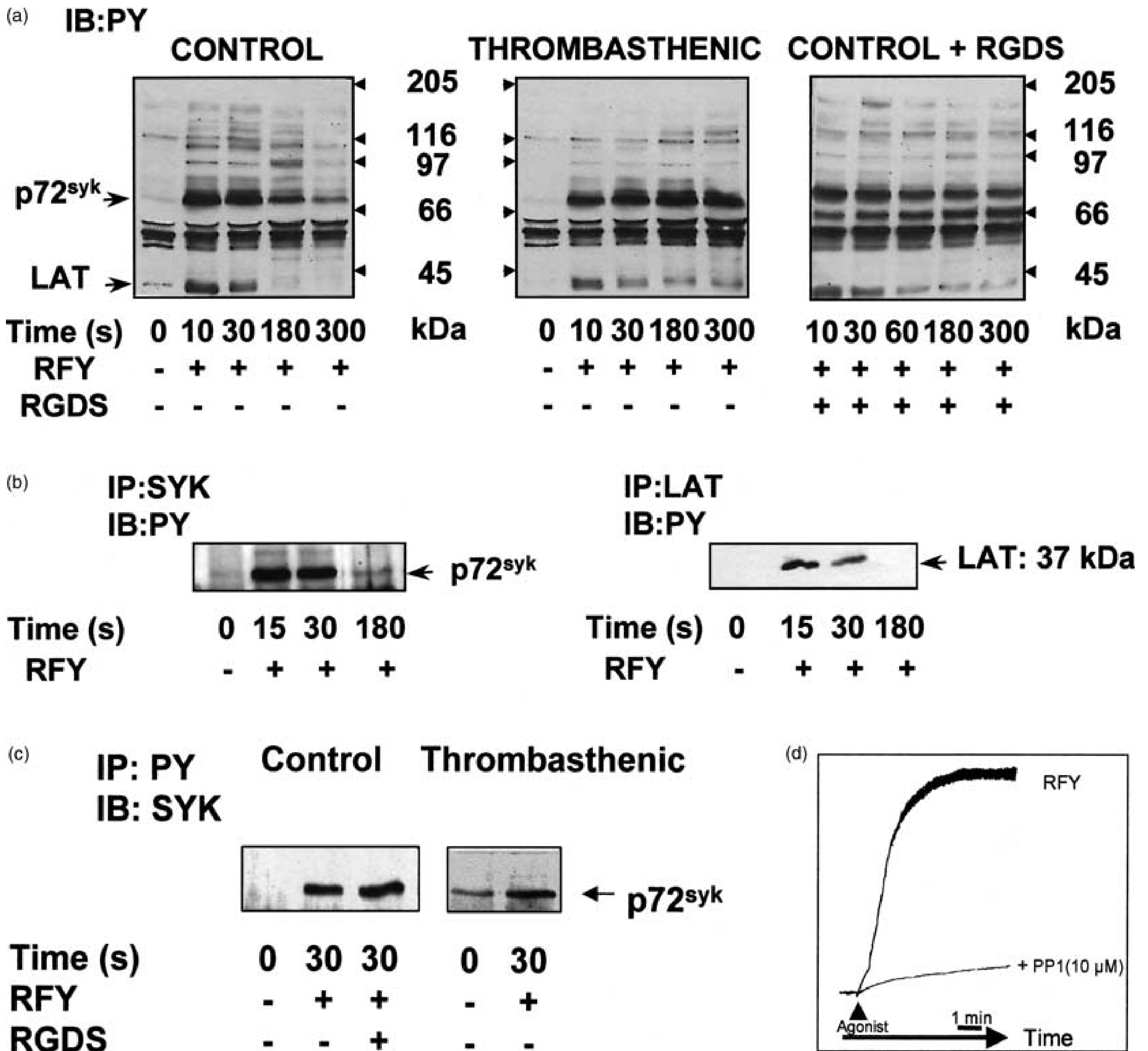

Fig. 4. RFY induces rapid and transient tyrosine phosphorylation of a set of proteins, including p72^{syk} and LAT, in an αIIb/β3-independent manner. Control and thrombasthenic platelets from patient 2 were stimulated with 100 μmol L^{-1} RFY for indicated times, in the presence or in the absence of 1 mmol L^{-1} RGDS. (a) Immunoblotting of whole platelet proteins extract with antiphosphotyrosine antibody 4G10. (b) LAT and p72^{syk} were immunoprecipitated from control platelets with specific antibodies and submitted to immunoblotting with 4G10 antibody. (c) Anti-phosphotyrosine immunoprecipitates were immunoblotted with a polyclonal antip72^{syk} antibody. (d) Platelets from a healthy volunteer were stimulated by 100 μmol L^{-1} RFY in the absence or in the presence of 10 μmol L^{-1} PP1 and aggregation profiles were obtained with a Payton aggregometer with stirring at 900 rev min^{-1} (7.5×10^8 platelets mL^{-1}). Data shown are representative of two to four different experiments.

RGDS treatment decreased the production of PtdIns(3,4)P$_2$ induced by 1 IU mL^{-1} thrombin by 77% $\pm$ 17% ($n = 4$). Conversely, in platelets stimulated by RFY for 3 min, the level of PtdIns(3,4)P$_2$ was only partly reduced (38% $\pm$ 0.1% of inhibition, $n = 3$) by RGDS treatment. These results suggest that the production of this phosphoinositide was largely independent of αIIb/β3 engagement in RFY-stimulated platelets. Moreover, 10 nmol L^{-1} wortmannin treatment [which inhibited PtdIns(3,4,5)P$_3$ and PtdIns(3,4)P$_2$ production by 90% $\pm$ 13 and 95% $\pm$ 6%, respectively] strongly affected platelet aggregation (70% $\pm$ 17% inhibition of aggregation amplitude, $n = 3$) induced by RFY. Similar effects were observed when the unrelated PI 3-kinase inhibitor LY294002 was used (not shown).

LAT is required for RFY -mediated platelet aggregation

The docking protein LAT has been shown to play a critical role downstream of Syk in GpVI-mediated PLCγ2 activation in mouse platelets [31]. Since LAT was a major tyrosine

© 2003 International Society on Thrombosis and Haemostasis

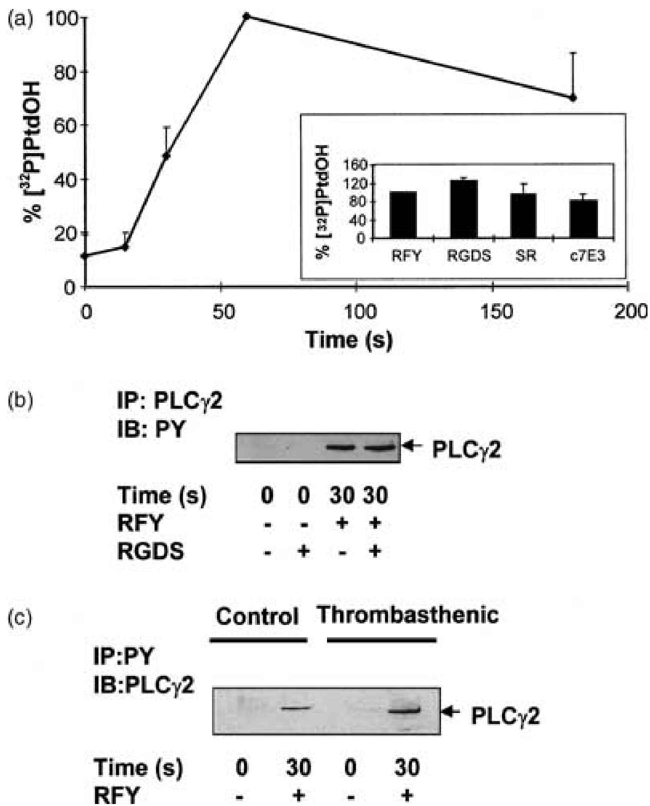

Fig. 5. RFY induces an αIIb/β3-independent production of [^{32}P]PtdOH and a tyrosine phosphorylation of PLCγ2. (a) ^{32}P-labeled platelets were stimulated by 100 μmol L^{-1} RFY for the indicated times and [^{32}P]PtdOH was quantified. The effect of 1 mmol L^{-1} RGDS, 3.5 μmol L^{-1} SR121566, or 40 μg mL^{-1} c7E3 on the synthesis of this lipid was measured at 1 min of stimulation (insert). Results are expressed as percentage of maximal effects and are means ± SEM of at least three independent experiments. Maximal radioactivity measured for [^{32}P]PtdOH: 203 × 10^3 cpm. (b) Immunoprecipitation of PLCγ2 was performed from platelets stimulated or not for 30 s with 100 μmol L^{-1} RFY and preincubated for 1 min before stimulation with 1 mmol L^{-1} RGDS when indicated. Western blotting analysis was carried out with the 4G10 antiphosphotyrosine antibody. Data shown are representative of three independent experiments (c) Immunodetection of PLCγ2 in antiphosphotyrosine immunoprecipitates from control and thrombasthenic platelets (patient 1) activated or not for 30 s by RFY.

phosphorylated protein upon RFY treatment (Fig. 4a,b), we used platelets from LAT-deficient mice in order to test the potential role of this docking protein in this activation process. Interestingly, LAT-deficient platelets aggregated normally to thrombin but were no longer able to aggregate upon addition of RFY at concentrations up to 25 μmol L^{-1} (Fig. 7). At 50 μmol L^{-1} RFY, which is the optimal concentration in wild-type mice platelets, LAT-deficient platelets aggregated weakly. At a higher concentration (100 μmol L^{-1}) the role of LAT was partially by-passed since we observed a 25% increase in the aggregation amplitude compared to LAT-deficient platelets stimulated by 50 μmol L^{-1} RFY (not shown). As observed with human platelets, addition of an inhibitor of TXA2 synthesis and an ADP scavenger strongly reduced RFY-mediated aggregation of control mouse platelets. Interestingly, the ADP and TXA2 –independent platelet aggregation induced by

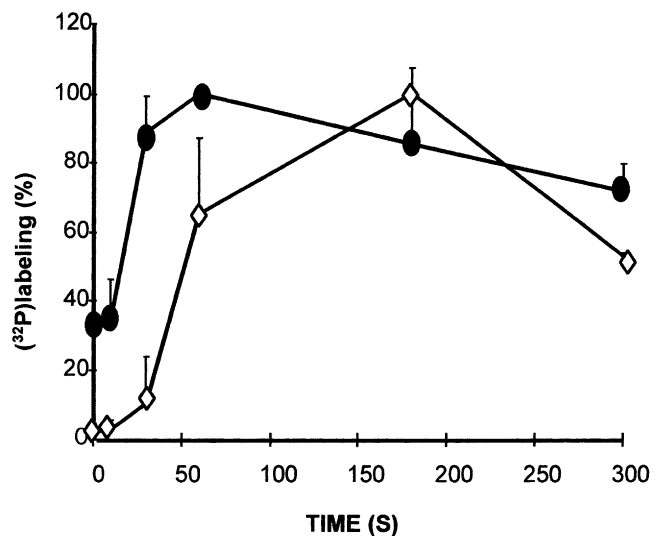

Fig. 6. RFY induces PI 3-kinase activation. Time course of [^{32}P]PtdIns(3,4,5)P_3 (●) and [^{32}P]PtdIns(3,4)P_2 (◇) labeling during platelet stimulation by 100 μmol L^{-1} RFY. [^{32}P]PtdIns(3,4)P_2 and [^{32}P]PtdIns(3,4,5)P_3 were quantified as indicated in Materials and methods. Results are expressed as percentage of maximal effects and are means ± SEM of three independent experiments. Maximal radioactivity measured for each 3D-phosphoinositide was: [^{32}P]PtdIns(3,4,5)P_3, 4210 cpm; [^{32}P]PtdIns(3,4)P_2, 9644 cpm.

50 μmol L^{-1} RFY or 100 μmol L^{-1} (not shown) was strongly inhibited with LAT-deficient platelets.

Discussion

This study was undertaken to investigate the molecular mechanisms underlying platelet activation induced by the active peptide from the C-terminal domain of TSP1, RFY [19,20]. We first observed that platelet aggregation induced by RFY peptide was partly dependent on secreted ADP and TXA2. Interestingly, using either antagonists of fibrinogen binding to αIIb/β3 or type I thrombastenia platelets, we found that the ADP- and TXA2-independent platelet activation and aggregation induced by RFY did not require αIIb/β3 and fibrinogen. This new αIIb/β3-independent platelet activation and aggregation process required a rapid activation of specific signaling pathways. For instance, a very early increase in tyrosine phosphorylation of a set of proteins including the tyrosine kinase Syk and its potential substrates, LAT and PLCγ2 was observed. Moreover, as shown by the production of PtdOH, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, both PLC and PI 3-kinase were rapidly stimulated by RFY. The functional importance of these signaling pathways was assessed by using pharmacological inhibitors of Syk, Src and PI 3-kinases as well as genetically modified mice platelets deficient in the docking protein LAT. Inhibition of tyrosine kinases dramatically decreased RFY-induced platelet aggregation strongly suggesting that this signaling pathway was crucial. Moreover, blockade of PI 3-kinase activity by two unrelated inhibitors also affected platelet aggregation induced by RFY. These results are consistent with previous observations

© 2003 International Society on Thrombosis and Haemostasis

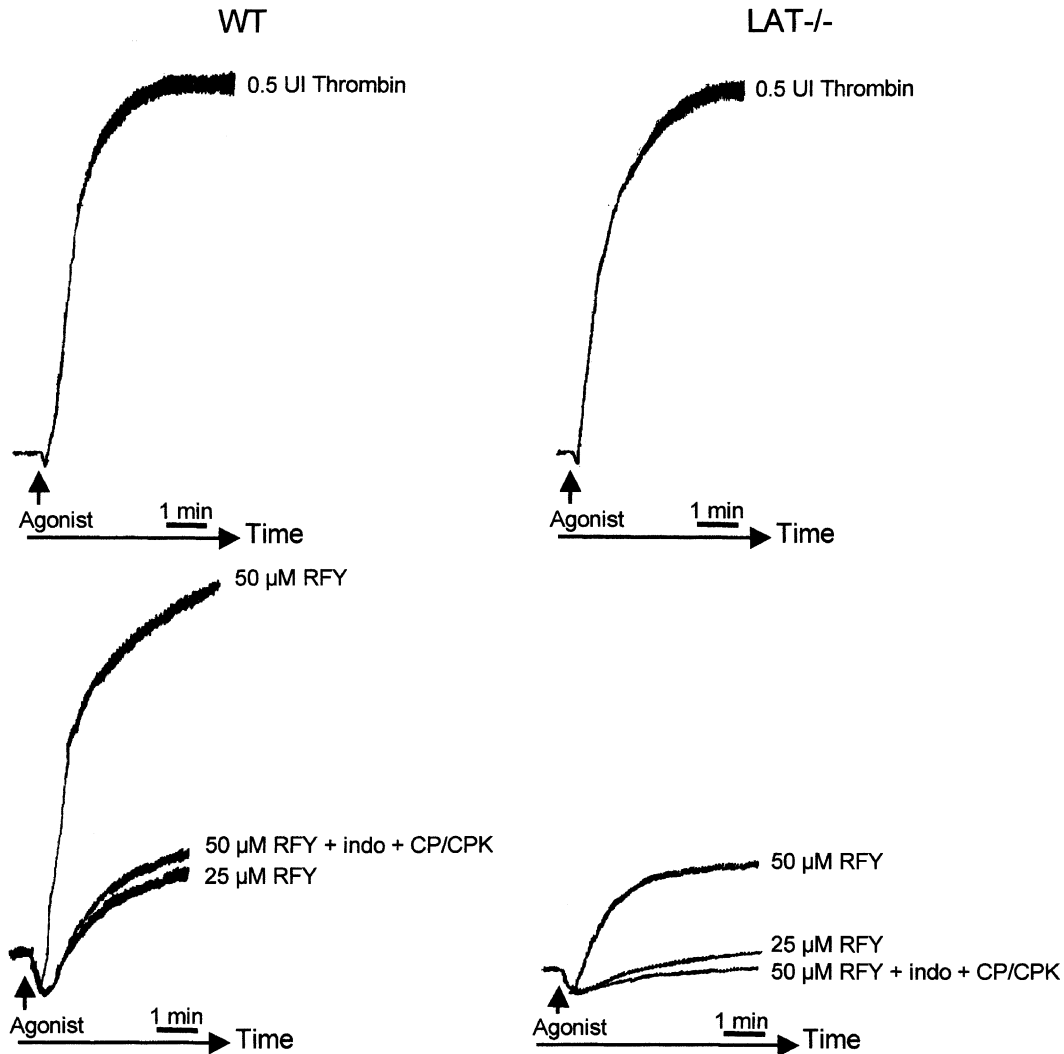

Fig. 7. Platelets from LAT-deficient mice are defective in RFY-induced aggregation. Platelets from control (a) or LAT-deficient (b) mice were stimulated with increasing concentrations of RFY. Aggregation profiles shown are representative of two different platelet preparations with similar results.

indicating that the same peptide was able to play a role as a coactivator of αIIb/β3-mediated spreading of platelets on fibrinogen through a tyrosine kinase- and PI 3-kinase-dependent process [19]. The transmembrane adapter LAT which plays a critical role in immune receptor signaling [32] was clearly tyrosine phosphorylated upon RFY stimulation. In platelets, LAT has an important role in the process of tyrosine phosphorylation and activation of PLCγ2 downstream of GpVI. Accordingly, this adapter is required for normal platelet activation *via* collagen or collagen-related-peptide [33]. Once phosphorylated, LAT can associate with a number of proteins including the p85α subunit of PI 3-kinase, Grb2, Gads, PLCγ2, SLP-76 and Cbl [32]. The impact of LAT in RFY-mediated platelet activation was evaluated by using platelets from LAT-deficient mice. Interestingly, these platelets did not aggregate in response to RFY at concentrations below 25 $\mu mol\,L^{-1}$. At higher concentrations the role of LAT was partially by-passed as also observed for GpVI agonists [34]. At 50 or 100 $\mu mol\,L^{-1}$ (not shown), the weak aggregation observed was fully inhibited by addition of an ADP scavenger and a TXA2 synthesis inhibitor. These results demonstrate the critical role of the adapter molecule LAT in RFY-mediated platelet activation. As shown for GPVI, the signaling cascade Src/Syk/LAT/PLCγ2 seems to be required for RFY-induced platelet stimulation.

In light of these results, an important question is how RFY can mediate this novel mechanism of platelet activation. At first glance, IAP appears to be a good candidate to mediate the rapid signaling pathway initiated by the C-terminal peptide of TSP1 [18,19]. However, in our hands, an antifunctional IAP antibody did not block RFY-mediated platelet aggregation (not shown) suggesting the presence of another functional receptor for the C-terminal sequence of TSP1. Moreover, the two major platelet integrins known to associate with IAP, αIIb/β3 and α2/β1, were not required for RFY–mediated platelet aggregation. Indeed, the P1E6 α2/β1 function blocking antibody [18] did not affect platelet aggregation induced by RFY (not shown) suggesting that this integrin is not involved in this process.

© 2003 International Society on Thrombosis and Haemostasis

During the writing of this manuscript, Tulasne *et al*. [33] published a study showing that the C-terminal peptide of TSP1 can indeed stimulate platelet aggregation independently of IAP. These authors also found that RFY stimulated platelets in an αIIb/β3- and GPIbα-independent manner through an Fc receptor γ-chain associated signaling pathway. This is in agreement with our results, however, these authors propose that agglutination support the αIIb/β3-independent platelet aggregation. Our results suggest that, in addition to agglutination, the αIIb/β3-independent platelet aggregation may involve other mechanisms. Indeed, in the presence of an ADP scavenger and a TXA2 synthesis inhibitor, platelet aggregation induced by RFY was no longer observed in LAT-deficient platelets even at high concentrations of agonist. Moreover, RFY did not induce aggregation of fixed human platelets but rather cross-linking of a few platelets that were only visible by light microscopy (not shown).

Two receptors, GpVI and FcγRIIa, are known to strongly activate the tyrosine kinases of the Src family as well as Syk leading to the phosphorylation of LAT and PLCγ2 [35,36]. We found that FcγRIIa was not tyrosine phosphorylated upon RFY addition (not shown) and Tulasne *et al*. [33] suggest that RFY does not activate GpVI. The present study and the work of Tulasne *et al*. [33] point to a new mechanism of platelet activation and aggregation, however, the initial events involved remain to be explored further.

To consider the functional importance of this new platelet activating mechanism, it is important to note that we could not reproduce the effects of RFY by treating platelets with exogenous full-length TSP1 (not shown). TSP1 stabilizes fibrinogen binding to the activated platelet surface and reinforces the strength of platelet to platelet interactions, supporting the conversion of reversible microaggregates to irreversible macro-aggregates [1,5]. However, one can propose that the RFYVVMWK sequence is masked in full-length TSP1 and that a first interaction of TSP1 with fibrinogen [37] might expose additional binding site present in the C-terminal domain of the molecule. In this case, the use of RFY will help us to better understand the signaling pathways initiated by TSP1 binding to platelet membranes during irreversible aggregation and clot retraction.

To conclude, our results indicate that platelet to platelet contacts can efficiently take place independently of αIIb/β3 and fibrinogen in platelets stimulated by RFY. The first steps of this new mechanism of platelet activation and the molecular events supporting this αIIb/β3-independent aggregation need to be investigated further to evaluate their physiological significance.

Acknowledgements

The authors thank C. Viala for her excellent technical assistance and Drs C. Legrand, M. Jandrot-Perrus and H. Tronchère for helpful discussions. This study was supported by funds from the Institut National de la Santé et de la Recherche Médicale and by grants from the Association pour la Recherche Contre le Cancer (ARECA-Toulouse and ARECA-Marseille).

References

1 Leung LL. Role of thrombospondin in platelet aggregation. *J Clin Invest* 1984; **74**: 1764–72.
2 Dixit VM, Haverstick DM, O'Rourke KM, Hennesy SW, Grant GA, Santoro SA, Frazier WA. A monoclonal antibody against human thrombospondin inhibits platelet aggregation. *Proc Natl Acad Sci USA* 1985; **82**: 3472–6.
3 Tuszynski GP, Rothman VL, Murphy A, Siegler K, Knudsen KA. Thrombospondin promotes platelet aggregation. *Blood* 1988; **72**: 109–15.
4 Tuszynski GP, Rothman VL, Deutch AH, Hamilton BK, Eyal J. Biological activities of peptides and peptide analogues derived from common sequences present in thrombospondin, properdin, and malarial proteins. *J Cell Biol* 1992; **116**: 209–17.
5 Legrand C, Dubernard V, Rabhi-Sabile S, Morandi Da Silva V. Functional and clinical significance of thrombospondin. *Platelets* 1997; **8**: 211–23.
6 Mansfield PJ, Boxer LA, Suchard SJ. Thrombospondin stimulates motility of human neutrophils. *J Cell Biol* 1990; **111**: 3077–86.
7 Taraboletti G, Roberts DD, Liotta LA. Thrombospondin-induced tumor cell migration: haptotaxis and chemotaxis are mediated by different molecular domains. *J Cell Biol* 1987; **105**: 2409–15.
8 Yabkowitz R, Mansfield PJ, Ryan US, Suchard SJ. Thrombospondin mediates migration and potentiates platelet-derived growth factor-dependent migration of calf pulmonary artery smooth muscle cells. *J Cell Physiol* 1993; **157**: 24–32.
9 Tsao PW, Mousa SA. Thrombospondin mediates calcium mobilization in fibroblasts via its Arg-Gly-Asp and carboxyl-terminal domains. *J Biol Chem* 1995; **270**: 23747–53.
10 Kosfeld MD, Frazier WA. Identification of a new cell adhesion motif in two homologous peptides from the COOH-terminal cell binding domain of human thrombospondin. *J Biol Chem* 1993; **268**: 8808–14.
11 Gao AG, Frazier WA. Identification of a receptor candidate for the carboxyl-terminal cell binding domain of thrombospondins. *J Biol Chem* 1994; **269**: 29650–7.
12 Brown E, Hooper L, Ho T, Gresham H. Integrin-associated protein: a 50-kD plasma membrane antigen physically and functionally associated with integrins. *J Cell Biol* 1990; **111**: 2785–94.
13 Lindberg FP, Gresham HD, Schwarz E, Brown EJ. Molecular cloning of integrin-associated protein: an immunoglobulin family member with multiple membrane-spanning domains implicated in alpha v beta 3-dependent ligand binding. *J Cell Biol* 1993; **123**: 485–96.
14 Lindberg FP, Lublin DM, Telen MJ, Veile RA, Miller YE, Donis-Keller H, Brown EJ. Rh-related antigen CD47 is the signal-transducer integrin-associated protein. *J Biol Chem* 1994; **269**: 1567–70.
15 Gao AG, Lindberg FP, Finn MB, Blystone SD, Brown EJ, Frazier WA. Integrin-associated protein is a receptor for the C-terminal domain of thrombospondin. *J Biol Chem* 1996; **271**: 21–4.
16 Wang XQ, Frazier WA. The thrombospondin receptor CD47 (IAP) modulates and associates with alpha2 beta1 integrin in vascular smooth muscle cells. *Mol Biol Cell* 1998; **9**: 865–74.
17 Moroi M, Jung SM. Platelet receptors for collagen. *Thromb Haemost* 1997; **78**: 439–44.
18 Chung J, Wang XQ, Lindberg FP, Frazier WA. Thrombospondin-1 acts via IAP/CD47 to synergize with collagen in alpha2beta1-mediated platelet activation. *Blood* 1999; **94**: 642–8.
19 Chung J, Gao AG, Frazier WA. Thrombospondin acts via integrin-associated protein to activate the platelet integrin alpha (Iib) beta (3). *J Biol Chem* 1997; **272**: 14740–6.
20 Dorahy DJ, Thorne RF, Fecondo JV, Burns GF. Stimulation of platelet activation and aggregation by a carboxyl-terminal peptide from thrombospondin binding to the integrin-associated protein receptor. *J Biol Chem* 1997; **272**: 1323–30.
21 Hérault JP, Lale A, Savi P, Pflieger AM, Herbert JM. *In vitro* inhibition of heparin-induced platelet aggregation in plasma from patients with HIT by SR 121566, a newly developed Gp IIb/IIIa antagonist. *Blood Coagul Fibrinolysis* 1997; **8**: 206–7.

© 2003 International Society on Thrombosis and Haemostasis

22 Zhang W, Sommers CL, Burshtyn DN, Stebbins CC, DeJarnette JB, Trible RP, Grinberg A, Tsay HC, Jacobs HM, Kessler CM, Lkong EO, Love PE, Samelson LE. Essential role of LAT in T cell development. *Immunity* 1999; **10**: 323–32.

23 George JN, Caen JP, Nurden AT. Glanzmann's thrombasthenia: the spectrum of clinical disease. *Blood* 1990; **75**: 1383–95.

24 Cazenave JP, Hemmendinger S, Beretz A, Sutter-Bay A, Launay J. Platelet aggregation: a tool for clinical investigation and pharmacological study. *Methodol Ann Biol Clin* 1983; **41**: 167–79.

25 Guinebault C, Payrastre B, Racaud-Sultan C, Mazarguil D, Breton M, Mauco G, Plantavid M, Chap H. Integrin-dependent translocation of phosphoinositide 3-kinase to the cytoskeleton of thrombin-activated platelets involves specific interactions of p85 alpha with actin filaments and focal adhesion kinase. *J Cell Biol* 1995; **129**: 831–42.

26 Gratacap MP, Payrastre B, Viala C, Mauco G, Plantavid M, Chap H. Phosphatidylinositol 3,4,5-trisphosphate-dependent stimulation of phospholipase C-gamma2 is an early key event in FcgammaRIIA-mediated activation of human platelets. *J Biol Chem* 1998; **273**: 24314–21.

27 Djaffar I, Caen JP, Rosa JP. A large alteration in the human platelet glycoprotein IIIa (integrin beta 3) gene associated with Glanzmann's thrombasthenia. *Hum Mol Genet* 1993; **2**: 2183–5.

28 Gibbins JM, Briddon S, Shutes A,van Vugt MJ, van de Winkel JG, Saito T, Tybulewicz VL, Watson SP. The p85 subunit of phosphatidylinositol 3-kinase associates with the Fc receptor gamma-chain and linker for activitor of T cells (LAT) in platelets stimulated by collagen and convulxin. *J Biol Chem* 1998; **273**: 34437–43.

29 Sultan C, Plantavid M, Bachelot C, Grondin P, Breton M, Mauco G, Plantavid M, Chap H. Involvement of platelet glycoprotein IIb-IIIa (alpha IIb-beta 3 integrin) in thrombin-induced synthesis of phosphatidylinositol 3′,4′-bisphosphate. *J Biol Chem* 1991; **266**: 23554–7.

30 Sorisky A, King WG, Rittenhouse SE. Accumulation of PtdIns (3,4),P2 and PtdIns (3,4,5), P3 in thrombin–stimulated platelets. *Biochem J* 1992; **286**: 581–4.

31 Pasquet JM, Gross B, Quek L, Asazuma N, Zhang W, Sommers CL, Schweighoffer E, Tybulewicz V, Judd B, Lee JR, Koretzky G, Love PE, Samelson LE, Watson SP. LAT is required for tyrosine phosphorylation of phospholipase Cgamma2 and platelet activation by the collagen receptor GPVI. *Mol Cell Biol* 1999; **19**: 8326–34.

32 Wonerow P, Watson SP. The transmembrane adapter LAT plays a central role in immune receptor signalling. *Oncogene* 2001; **20**: 6273–83.

33 Tulasne D, Judd BA, Johansen M, Asazuma N, Best D, Brown EJ, Kahn M, Koretzky GA, Watson GP. C-terminal peptide of thrombospondin-1 induces platelet aggregation through the Fc receptor gamma-chain-associated signaling pathway and by agglutination. *Blood* 2001; **98**: 3346–52.

34 Judd BA, Myung PS, Obergfell A, Myers EE, Cheng AM, Watson SP, Pear WS, Allman D, Shattil SJ, Koretzky GA. Differential requirement for LAT and SLP-76 in GPVI versus T cell receptor signaling. *J Exp Med* 2002; **195**: 705–17.

35 Poole A, Gibbins JM, Turner M, van Vugt MJ, van de Winkel JG, Saito T, Tybulewicz VL, Watson SP. The Fc receptor gamma-chain and the tyrosine kinase Syk are essential for activation of mouse platelets by collagen. *EMBO J* 1997; **16**: 2333–41.

36 Chacko GW, Duchemin AM, Coggeshall KM, Osborne JM, Brandt JT, Anderson CL. Clustering of the platelet Fc gamma receptor induces noncovalent association with the tyrosine kinase p72syk. *J Biol Chem* 1994; **269**: 32435–40.

37 Legrand C, Thibert V, Dubernard V, Begault B, Lawler J. Molecular requirements for the interaction of thrombospondin with thrombin-activated human platelets: modulation of platelet aggregation. *Blood* 1992; **79**: 1995–2003.

© 2003 International Society on Thrombosis and Haemostasis