Thrombopoietin stimulates cortactin translocation to the cytoskeleton independently of tyrosine phosphorylation

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Cortactin is an F-actin-binding protein expressed in platelets. During aggregation by thrombin, cortactin associates with Src, is tyrosine phosphorylated, and then translocates to the cytoskeleton. It is also found to associate with Syk during platelet shape change. Since cortactin undergoes tyrosine phosphorylation in platelets activated by thrombopoietin (TPO) that exhibit neither shape change nor aggregation, we investigated whether it could also relocate to the detergent-insoluble fraction. We demonstrate that cortactin was present as a tyrosine-phosphorylated protein and co-localized with Syk in the Triton X-100-insoluble fraction of TPO-activated platelets. TPO stimulated Syk activation and association with cortactin. Conversely,

cortactin associated with the kinases, Syk and Src. Cortactin tyrosine phosphorylation was blocked by Syk kinase inhibitor, piceatannol or Src family kinase inhibitor, PP2, suggesting that it depends on these two kinases. However, piceatannol or PP2 did not prevent cortactin translocation to the detergent-insoluble fraction. These data suggest that tyrosine phosphorylation is not required for cortactin translocation to the detergent-insoluble compartment. Furthermore, TPO activates, through its receptor c-Mpl, a signalling pathway to the cytoskeleton.

Key words: blood platelets, cortical actin-binding protein, tyrosine kinases.

INTRODUCTION

Cortactin is a ubiquitously expressed F-actin cross-linking protein of 550 amino acids, which was initially described as a major phosphotyrosine-containing protein in *v*-Src-transformed chicken embryo fibroblasts [1]. The human homologue, the product of the *EMS1* gene, was discovered as an oncogene which is frequently amplified in human colon carcinomas and tumour cell lines [2,3]. It contains distinct functional domains: an N-terminal domain with several repeats of 37 amino acids, the fourth of which binds F-actin; a proline-rich domain; numerous tyrosine residues; and an SH3 domain at the C-terminus [4].

Cortactin strongly binds F-actin *in vitro* [5] and *in vivo* [4]. In fibroblasts transformed by *v*-Src or *c*-Src, or activated by epidermal growth factor, and in endothelial cells cultured on extracellular matrix, cortactin relocates within peripheral structures, such as lamellipodia, pseudopodia and membrane ruffles, where actin undergoes dynamic reorganization [2,4,6–8]. This relocation may depend on its state of tyrosine phosphorylation, since cortactin is known to be tyrosine phosphorylated in these cells [6–8]. In platelets aggregated with thrombin, cortactin is tyrosine phosphorylated [9], and translocates to the detergent-insoluble fraction sedimented at low speed [10]. Tyrosine-phosphorylated cortactin is also detectable in thrombasthenic platelets unable to aggregate in response to thrombin [9] and in normal platelets stimulated by thrombopoietin (TPO) [11], which activates platelets without shape change or aggregation [12,13]. This indicates that cortactin tyrosine phosphorylation can occur independently of integrin engagement during the early phase of platelet activation. However, it is not established whether cortactin is able to translocate to the detergent-insoluble fraction in the absence of shape change and aggregation.

Cortactin has been described as a prominent substrate for Src tyrosine kinase [1,14,15], and associates with Src in fibroblast growth factor-1-activated fibroblasts [16]. However, cortactin is found constitutively associated with Syk in the erythroid megakaryocytic cell line K562, and is tyrosine phosphorylated by Syk in response to phorbol ester, as demonstrated by overexpression of a dominant negative form of Syk in these cells [17]. In platelets, during early shape change induced by the thromboxane A2 analogue I-BOP, cortactin was found associated with Syk [18], whereas, in platelets aggregated with thrombin, it associated with autophosphorylated Src [19]. The implication of Syk or Src in the tyrosine phosphorylation of cortactin after TPO stimulation is unknown and requires investigation.

In the present study, we demonstrate that tyrosine-phosphorylated cortactin relocates to the detergent-insoluble fraction after shape change-independent platelet activation by TPO. By using tyrosine kinase inhibitors, we further investigated whether tyrosine phosphorylation is required for cortactin translocation.

MATERIALS AND METHODS

Materials

Recombinant human TPO was obtained from R & D Systems (Abingdon, Oxfordshire, U.K.). Human thrombin was purchased from Diagnostica Stago (Asnières, France). The anti-p80/85 cortactin monoclonal 4F11 antibodies were from Upstate Biotechnologies (Lake Placid, NY, U.S.A.). The anti-phosphotyrosine monoclonal antibody PY72 was a gift from Dr P. Mayeux (Institut Cochin de Génétique Moléculaire, Hôpital

Abbreviations used: TPO, thrombopoietin; LSP, low-speed pellet; HSP, high-speed pellet.

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Cochin, Paris, France). The rabbit polyclonal and the mouse monoclonal 4D10 anti-Syk antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The monoclonal anti-Src Ab-1 antibody was from Oncogene Science (Uniondale, NY, U.S.A.). Glutathione S-transferase-HS1 peptide for Syk kinase assay was a gift from Dr U. Blank (Pasteur Institute, Paris, France). The tyrosine kinase inhibitors piceatannol (*trans*-3,3',4,5'-tetrahydroxystilbene) and PP2 {4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine} were from Calbiochem (La Jolla, CA, U.S.A.).

Platelet isolation and stimulation

Human platelets were isolated from aspirin-free volunteer donors. Briefly, blood was diluted 9/1 (v/v) with ACD-C anticoagulant (12 mM trisodium citrate, 13 mM citric acid and 11 mM glucose) containing 1 μ M prostaglandin E1. Platelet-rich plasma was obtained by centrifugation at 160 *g* for 15 min at room temperature. Platelet isolation was carried out using metrizamide (Sigma) gradients and platelet concentration was adjusted to $3\text{--}4 \times 10^8$ platelets/ml in suspension buffer [10 mM Hepes (pH 7.4), 140 mM NaCl, 3 mM KCl, 0.5 mM MgCl₂, 5 mM NaHCO₃ and 10 mM glucose]. Stimulation was performed using 0.4 ml aliquots of metrizamide gradient-platelets in the cuvette of a Chrono-log lumi-aggregometer, and the aggregation was followed at 37 °C with constant stirring (1000 rpm) after addition of the agonist. In some experiments, platelets were preincubated with tyrosine kinase inhibitors piceatannol or PP2 (5–50 μ M), or the corresponding dilutions of the vehicle (DMSO) as control, for 30 min at 37 °C without stirring. The variation of light transmission was expressed as the percentage of the attenuation of platelets in suspension buffer.

Subcellular fractionation

After stimulation either by TPO or thrombin, platelets were solubilized in 50 mM Tris/HCl, pH 7.4, 5 mM EGTA, 1% (v/v) Triton X-100, 1 mM Na₃VO₄, 1 mM PMSF and 10 μ g/ml leupeptin. Cell lysates were sonicated and centrifuged at 10000 *g* for 15 min at 4 °C to isolate the detergent-insoluble fraction sedimented at low speed [low-speed pellet (LSP)]. Supernatants were centrifuged at 100000 *g* for 1 h at 4 °C in a Beckman TL-100 rotor to separate the detergent-insoluble fraction sedimented at high speed [high-speed pellet (HSP)] from the detergent-soluble cytosol, as previously described [20]. LSP and HSP were resuspended in Laemmli buffer and samples were separated on SDS/PAGE (8% gels).

Immunoprecipitations and immunoblots

Immunoprecipitation experiments were carried out under non-denaturing conditions as follows. For anti-cortactin immunoprecipitations, platelets were lysed with an equal volume of 2 $\times$ extraction buffer A: 20 mM Tris/HCl, pH 7.4, 300 mM NaCl, 2% (v/v) Triton X-100, 4 mM EDTA, 2 mM Na₃VO₄, 20 mM sodium pyrophosphate, 20 mM sodium fluoride, 2 mM PMSF, 20 mM benzamidine, and 20 μ g/ml aprotinin, leupeptin and soybean trypsin inhibitor. Triton X-100-insoluble fractions sedimented at low speed (10000 *g*) for 5 min at 4 °C, were re-extracted with RIPA buffer [1 $\times$ extraction buffer A containing 1% (w/v) sodium deoxycholate and 0.1% (w/v) SDS]. For anti-Syk or anti-Src immunoprecipitation, stimulated platelets were

lysed for 30 min on ice in 2 $\times$ extraction buffer B: 100 mM Tris/HCl, pH 7.4, 300 mM NaCl, 2% (w/v) digitonin, 10 mM EDTA, 2 mM Na₃VO₄, 2 mM PMSF, 20 mM benzamidine, and 20 μ g/ml aprotinin, leupeptin and soybean trypsin inhibitor. Lysates were centrifuged at 10000 *g* for 10 min at 4 °C and supernatants were incubated with the immunoprecipitating antibodies for 2 h on ice. Immune complexes were collected on Protein G-Sepharose beads, washed three times in 1 $\times$ extraction buffer, and twice in 1 $\times$ extraction buffer containing 0.1% Triton X-100 or digitonin. Proteins were solubilized in Laemmli buffer at 100 °C for 5 min and separated by SDS/PAGE (8% gels). In some experiments, Triton X-100-insoluble fractions were washed twice in 1 $\times$ extraction buffer containing 0.1% Triton X-100 before solubilization in Laemmli buffer. Immunoblots were performed after protein electrotransfer on to a nitrocellulose filter membrane. Membranes were saturated in Tris-buffered saline Tween [20 mM Tris/HCl, pH 7.4, 137 mM NaCl and 0.1% (v/v) Tween 20] containing 5% low-fat powder milk (w/v) and incubated with the primary monoclonal antibody diluted in the same buffer overnight at 4 °C. Blots were washed four times prior to incubation with a horseradish peroxidase-coupled sheep anti-mouse antibody as secondary antibody (Roche Diagnostics, Mannheim, Germany). After further washing, proteins were visualized by chemiluminescence. Densitometric analysis was performed with a Visio-Mic system (Genomic, Collonges-sous-Saleve, France).

In vitro kinase assays

Syk or Src was immunoprecipitated as described above from digitonin-soluble fractions of platelets. Anti-Syk immunoprecipitates collected on Protein G-Sepharose were washed three times in 1 $\times$ extraction buffer and twice in 1 $\times$ extraction buffer containing 0.1% digitonin, and an additional time in kinase buffer 1 (25 mM Hepes, pH 7.4, 2 mM MnCl₂, 10 mM MgCl₂ and 1 mM Na₃VO₄). Syk kinase activity was assayed by incubating immunoprecipitates with 10 μ Ci of [γ -³²P]ATP (3000 Ci/mmol; NEN Life Science Products, Boston, MA, U.S.A.), 10 μ M ATP and 10 μ g glutathione-S-transferase-HS1 peptide (EQEDE-PEGDYEEVLE) as a substrate, for 5 min at 30 °C in a final volume of 60 μ l of kinase buffer 1 [21]. Src kinase activity was assayed as an autophosphorylation assay as previously described [22]. Anti-Src immunoprecipitates were washed once in kinase buffer 2 (20 mM Tris/HCl, pH 7.4, 5 mM MgCl₂, 5 mM MnCl₂, 10 mM NaF and 100 μ M Na₃VO₄) and phosphorylated for 5 min at 37 °C with 2 μ Ci [γ -³²P]ATP in a final volume of 50 μ l of kinase buffer 2. Reactions were stopped by the addition of 20 μ l of 4 $\times$ Laemmli buffer containing 40 mM EDTA. Samples were electrophoresed on SDS/PAGE (8% gels) under reducing conditions. Dried gels were subjected to autoradiography overnight at –80 °C.

RESULTS

TPO induces cortactin translocation to the Triton X-100-insoluble fraction

TPO addition to platelet suspension is known to activate platelets, despite the absence of shape change and aggregation. We have previously shown [11] that TPO stimulated cortactin tyrosine phosphorylation, and that this tyrosine phosphorylation was not inhibited when $\alpha_{\text{IIb}}\beta_3$ engagement was prevented by RGDS peptide. Thus we wondered whether TPO also stimulated

Figure 1 Translocation of tyrosine-phosphorylated cortactin to the Triton X-100-insoluble fraction of TPO-stimulated platelets

(A) Subcellular immunolocalization of cortactin. Triton X-100-insoluble fraction sedimented at 10000 *g*, LSP and Triton X-100-insoluble fraction sedimented at 100000 *g*, or HSP from 6×10^7 unstimulated (0), TPO-(100 ng/ml) or thrombin (THR; 1 unit/ml)-treated platelets, were analysed by immunoblotting using the anti-cortactin 4F11 monoclonal antibody. (B) Triton X-100-insoluble fractions sedimented at 10000 *g* and re-extracted in RIPA buffer, and Triton X-100-soluble fractions corresponding to 1×10^9 unstimulated platelets (0), TPO- or THR-stimulated platelets for 3 min at 37 °C, were immunoprecipitated with anti-cortactin antibody and sequentially analysed by immunoblotting using the anti-phosphotyrosine (pTyr) PY72 and the 4F11 monoclonal antibodies. Lane C represents controls without immunoprecipitating antibody. (C) Densitometric analysis is expressed as a ratio of tyrosine phosphorylated cortactin to total cortactin, normalized to thrombin effect (100%) in the soluble fraction (means $\pm$ S.E.M.). Results are representative of six (A) or three (B, C) separate experiments.

cortactin translocation to cytoskeleton. Subcellular localization of cortactin was studied by immunoblotting of HSP and LSP fractions (Figure 1A). After 3 min of TPO stimulation (and 0.2 min of stimulation by thrombin), cortactin translocated from the HSP to the LSP. However, cortactin translocation was slower and less effective after TPO stimulation than after platelet aggregation with thrombin. As shown in Figure 1(B), we demonstrated that cortactin was detected as a tyrosine-phosphorylated protein in the Triton X-100-insoluble fraction re-extracted in RIPA buffer of platelets stimulated for 3 min by TPO or thrombin. Anti-cortactin immunoblotting in the same experiment confirmed that cortactin translocation to the Triton X-100-insoluble fraction only occurred after stimulation. Densitometric analysis showed that tyrosine-phosphorylated cortactin represented 25% and 100% of the Triton X-100-soluble cortactin, whereas it represented 12% and 30% of the

Figure 2 TPO affects Syk subcellular localization and stimulates Syk kinase activity

(A) Immunodetection of Syk and Src in the LSP and HSP fractions. Actin and $\alpha_{11b}\beta_3$ expression were used as controls of protein loading for the LSP and HSP respectively. Anti-Syk (B) and anti-Src (C) immunoprecipitates were prepared from the digitonin-soluble fraction of unstimulated (0), TPO- or THR-treated platelets. Syk kinase activity was assayed in a HS1 peptide phosphorylation assay and Src kinase activity was assayed in an autophosphorylation assay. Following SDS/PAGE (13% gels), phosphorylated HS1 peptide of 27 kDa and autophosphorylated p60^{c-src} were revealed by autoradiography. Controls without immunoprecipitating antibody are shown in lane C. Results are representative of six (A) or three (B, C) experiments.

pool of cortactin translocated to the Triton X-100-insoluble fraction after TPO and thrombin stimulation respectively (Figure 1C). Thus cortactin did translocate to the Triton X-100-insoluble fraction in the absence of platelet shape change and aggregation, but tyrosine phosphorylated cortactin was not enriched in this fraction.

TPO stimulates Syk translocation to the LSP and Syk activation

Because Syk and Src have been shown to translocate to the cytoskeleton of thrombin-aggregated platelets [22–24], they are supposed to co-localize with tyrosine-phosphorylated cortactin in this fraction. After 3 min of TPO stimulation, we found that Syk translocated from the HSP to the LSP, with a rate similar to the rate of cortactin redistribution. Src massively relocated to the LSP after 0.2 min of thrombin stimulation, but not after TPO stimulation (Figure 2A). The absence of Src translocation in TPO-activated platelets correlated with the lack of platelet aggregation. Thus Syk and cortactin co-localized in the LSP after TPO stimulation.

We then investigated the ability of TPO to activate these kinases. In low-stringency conditions of platelet solubilization with digitonin-containing buffer, Syk kinase activity was analysed by an *in vitro* phosphorylation assay of the HS1 peptide, a highly specific substrate for this kinase [21]. TPO incubated with platelets for 3 min, as well as thrombin used as control, induced a strong phosphorylation of the HS1 peptide (Figure 2B). After TPO stimulation Src autophosphorylation did not increase above the basal level, whereas it decreased after thrombin stimulation (Figure 2C). The decrease of Src activity in response to thrombin

Figure 3 Cortactin associates with Syk and Src after TPO stimulation

(A) Co-immunoprecipitation of Syk with cortactin after TPO or thrombin (THR) stimulation. Anti-cortactin immunoprecipitates (IP) prepared from 1.6×10^8 unstimulated (0), TPO- or THR-treated platelets were analysed for the presence of Syk by immunoblotting using the 4D10 anti-Syk monoclonal antibody. (B) Association of tyrosine-phosphorylated cortactin with Syk. Anti-Syk immunoprecipitates were sequentially analysed by immunoblotting with anti-phosphotyrosine (pTyr) PY72, anti-cortactin 4F11 and anti-Syk 4D10 monoclonal antibodies. (C) Detection of cortactin in anti-Src immunoprecipitates analysed by immunoblotting with anti-cortactin or with anti-Src 327 monoclonal antibody. Controls without immunoprecipitating antibody are shown in lane C. Each of these results are representative of two or three separate experiments.

was more likely due to the massive translocation of Src to the LSP (Figure 2A). These data show that TPO stimulated Syk kinase activity and translocation to the LSP.

Association of cortactin with Syk and Src in TPO-stimulated platelets

Taking into account that TPO activated Syk, we wondered whether TPO could induce the association of cortactin with Syk. As shown in Figure 3(A), we found that Syk co-immunoprecipitated with cortactin in the Triton X-100-soluble fraction of platelets stimulated for 3 min by TPO or thrombin. The Syk level associated with cortactin began to decrease after 10 min of stimulation by both agonists. Conversely, we investigated whether cortactin associated with Syk, and if the cortactin

Figure 4 Pharmacological evidence for the involvement of Syk and a Src family kinase in the tyrosine phosphorylation of cortactin

Platelets were preincubated for 30 min at 37 °C with increasing concentrations of piceatannol (PIC; A) and Src family kinase inhibitor PP2 (B) before stimulation by TPO or thrombin (THR). Tyrosine phosphorylation of cortactin was studied as described in Figure 2(B). (C) Densitometric analysis is expressed as a ratio of tyrosine-phosphorylated cortactin/total cortactin normalized to thrombin effect (100%) with DMSO (means $\pm$ S.E.M.). Results are representative of three separate experiments. ND, not determined; pTyr, phosphotyrosine.

associated with Syk was tyrosine phosphorylated. In order to preserve protein–protein interactions, platelets were solubilized in digitonin-containing buffer. Tyrosine-phosphorylated proteins of 80/85 kDa co-precipitated with Syk after TPO or thrombin stimulation (Figure 3B). A tyrosine phosphorylated protein of 72 kDa, presumed to be Syk, was also revealed. By re-probing the membrane with anti-cortactin antibodies, we found that cortactin co-migrated with the previously identified phosphoproteins of 80/85 kDa. The interaction of tyrosine-phosphorylated cortactin with Syk was observed at 3 min and lost after 10 min of stimulation by TPO. In contrast, when platelets underwent full aggregation in response to thrombin, the pool of cortactin co-precipitating with Syk was higher after 10 min than after 3 min of stimulation. These results demonstrated that Syk interaction with cortactin is inducible in the absence of platelet shape change and aggregation. Whether cortactin also interacts with Src in these latter conditions is unknown. As shown in Figure 3(C), cortactin co-immunoprecipitated with Src after 3 min of stimulation by TPO. The pool of cortactin associated with Src was lower after thrombin than after TPO stimulation because of the translocation of Src to the LSP (Figure 2A).

Taken together, these data demonstrate that cortactin associates with the two intracellular kinases Syk and Src in the absence of shape change as well as in aggregating platelets.

Effects of piceatannol and PP2 on TPO-induced cortactin tyrosine phosphorylation

We examined the ability of the Syk inhibitor piceatannol and Src family kinases inhibitor PP2 to block TPO-stimulated cortactin tyrosine phosphorylation. Piceatannol and PP2 were used in a range of concentrations that did not affect thrombin-induced aggregation (results not shown). We found that piceatannol inhibited TPO-induced cortactin tyrosine phosphorylation in a

Figure 5 Tyrosine phosphorylation is not required for cortactin translocation to the Triton X-100-insoluble fraction

Triton X-100-insoluble fractions of platelets, preincubated with increasing concentrations of piceatannol (PIC) or PP2 before TPO or thrombin (THR) addition for 3 min at 37 °C, were solubilized in Laemmli buffer. Cortactin was detected by specific immunoblotting. Results are representative of three separate experiments.

dose-dependent manner. An 80% decrease was observed with 5 μM piceatannol (Figure 4A). Piceatannol also effectively reduced thrombin-induced cortactin tyrosine phosphorylation. In addition, PP2 (10 μM) reduced the TPO-induced tyrosine phosphorylation of cortactin by 65%. Thrombin-induced cortactin tyrosine phosphorylation was not affected by 5 μM PP2, and was reduced by 40% by 25 μM PP2 (Figure 4B). This was indicated by the densitometric analysis reported in Figure 4(C). These experiments show that piceatannol and PP2 can inhibit TPO-induced cortactin tyrosine phosphorylation, supporting an important role for Syk and Src family kinases in shape change-independent cortactin tyrosine phosphorylation.

Piceatannol and PP2 did not prevent cortactin translocation to the Triton X-100-insoluble fraction

Whether the translocation of cortactin to the Triton X-100-insoluble fraction was dependent on tyrosine phosphorylation was further investigated. We found that increasing concentrations of piceatannol had no influence on cortactin relocalization to the Triton X-100-insoluble fraction after TPO stimulation (Figure 5A). Piceatannol (50 μM) only slightly diminished cortactin translocation induced by thrombin. In addition, the translocation induced by both agonists was equivalent in the presence or in the absence of PP2 (Figure 5B). These data suggest that cortactin association with the detergent-insoluble fraction, occurring either in the absence of shape change or in aggregating conditions, was independent of tyrosine phosphorylation.

DISCUSSION

In the present study, we demonstrated that cortactin is present as a tyrosine-phosphorylated protein and that it is co-localized with Syk in the detergent-insoluble fraction of platelets activated by TPO or thrombin. We showed that TPO stimulates Syk kinase activity. Moreover, cortactin associated with the kinases Syk and

Src after stimulation by TPO and thrombin. Syk and Src family kinase inhibitors blocked the tyrosine phosphorylation of cortactin induced by TPO, but did not modify its relocalization to the detergent-insoluble fraction.

Despite the absence of platelet shape change and aggregation, cortactin was able to translocate to the LSP during platelet activation by TPO. Ozawa et al. [10] have shown that, within the first few seconds of thrombin stimulation, a minor pool of cortactin translocated to the LSP. This translocation was not inhibited by the RGDS peptide. In contrast, additional translocation of a major pool of cortactin was inhibited by the RGDS peptide and thus required $\alpha_{IIb}\beta_3$ engagement. These results [10] and the present data suggest that cortactin redistribution to the LSP occurs in two waves, the first during early platelet activation and subsequently during platelet aggregation. In activated endothelial cells or fibroblasts, and also in thrombin-stimulated platelets, the redistribution of cortactin to cytoskeleton correlates with its tyrosine phosphorylation [6–8,19]. In the present study, we present evidence that tyrosine phosphorylation is not required for cortactin translocation. Tyrosine-phosphorylated cortactin did not accumulate in the detergent-insoluble fraction during the early phase of platelet activation by TPO, and during the late phase of platelet aggregation in response to thrombin. Moreover, in the presence of tyrosine kinase inhibitors, PP2 or piceatannol, cortactin translocated to the detergent-insoluble fraction as well as in the absence of inhibitors. In agreement with these data, it has been recently demonstrated [25] that cortactin could accumulate under the adherence site of *Escherichia coli* to HeLa epithelial cells without tyrosine phosphorylation. However, it is possible that the modulation of actin cytoskeleton reorganization by cortactin may depend on its state of tyrosine phosphorylation. Indeed, it has been demonstrated that *in vitro* tyrosine phosphorylation of recombinant cortactin by Src down-regulates its F-actin cross-linking activity [5]. The two forms of cortactin, p80 and p85, exhibit a distinct number of N-terminal repeats and are both tyrosine phosphorylated in stimulated cells. We report here a disparity in the degree of tyrosine phosphorylation of p80 and p85 after TPO or thrombin stimulation. Despite an equivalent expression of p80 and p85 in platelets, p80 was always the main tyrosine phosphorylated form, either in the absence of platelet aggregation in RGDS-treated platelets stimulated by thrombin and TPO-stimulated platelets [11] or in thrombin-aggregated platelets. Because the level of cortactin tyrosine phosphorylation was low in the absence of platelet aggregation, we hypothesize that tyrosine-phosphorylated p85 was under the threshold of detection in TPO-stimulated platelets. Alternatively, tyrosine phosphorylation of p80 and p85 may result from distinct integrin-independent and integrin-dependent signalling pathways. However, PP2 or piceatannol greatly inhibited p80 tyrosine phosphorylation induced by TPO, and also more efficiently reduced p85 than p80 tyrosine phosphorylation after thrombin stimulation (Figure 4). This suggests that Syk and Src account for the tyrosine phosphorylation of p80 and of p85. In epidermal growth factor-treated human carcinoma cells with *EMSI* gene amplification, p85 is more heavily charged in phosphoserine and phosphothreonine residues than p80, and is also the major form translocated at the cell-matrix contact sites [26]. In agreement with these data, we have observed that the ratio between p85 and p80 clearly increased in the detergent-insoluble fraction of thrombin-stimulated platelets, suggesting that the translocation of p85 is facilitated (Figure 5). Thus the requisite state to cortactin relocalization to F-actin-containing structures might be a serine/threonine, rather than tyrosine phosphorylation.

We have established that the TPO receptor, c-Mpl, mediated Syk activation and Syk interaction with the detergent-insoluble

fraction in the absence of shape change. Other receptors of the cytokine superfamily such as granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor and erythropoietin receptors also activate Syk [27–29]. In agreement with our data, Syk is also rapidly stimulated during platelet shape change induced by thromboxane analogue [18] or oxidized low-density lipoprotein [30], and in the absence of platelet activation when integrin $\alpha_{IIb}\beta_3$ dissociates under EGTA treatment [31]. We have shown that piceatannol or PP2 inhibited the tyrosine phosphorylation of cortactin. At the low concentration used (5 μ M), piceatannol is known to block Syk and the focal adhesion kinase, but neither platelet aggregation nor Src family kinase activity [32,33]. Considering that TPO leads the integrin $\alpha_{IIb}\beta_3$ to an activated state [34], but is unable to activate focal adhesion kinase in the absence of integrin ligand, Syk is the main target for piceatannol inhibitory effect. In addition, the Src family inhibitor PP1, which is closely related to PP2 [35], has recently been reported [36] as a potent inhibitor of Src in platelets. Thus we suggest from our data that cortactin tyrosine phosphorylation requires both Syk and a Src family kinase. What would be the role of each kinase? The interaction of cortactin with Src is mediated by the binding of a phosphorylated tyrosine residue of cortactin to the Src SH2 domain [15]. Src SH2 domain specifically binds to a YE E/D peptide [37], which is the optimal motif for specific phosphorylation by Syk [21]. Therefore Syk could phosphorylate cortactin on tyrosine residues required for the SH2-dependent binding of Src.

In conclusion, evidence for a TPO-dependent translocation of cortactin to the detergent-insoluble fraction of platelets suggests the existence of a signalling pathway from c-Mpl to the cytoskeleton.

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