Platelet activation, we examined the possibility that a TP antagonist, indicating the specificity of the signal. Occurrence of shape change. These biochemical and morphological events are both inhibited by SQ 29548, a cytoskeletal protein cortactin. Tyrosine phosphorylation of cortactin is kinetically correlated with the level of phosphorylation. These data suggest a novel pathway for a G protein-coupled TxA\(_2\) receptor to the protein-tyrosine kinase Syk, which is associated with cortactin in the very early steps of platelet activation.

Tyrosine Phosphorylation of Cortactin Associated with Syk Accompanies Thromboxane Analogue-induced Platelet Shape Change*

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Thromboxane A\(_2\) (TxA\(_2\)) is a potent vasoconstrictor and platelet agonist. Pharmacological studies have defined two classes of thromboxane receptors (TPs) in human platelets; sites that bind the agonist 1S-(1,2(5Z),3-(1E,3S),4)-7–3-(3-hydroxy-4(4’-iodophenoxy)-1-butanyl)-7-oxabicyclo-2.2.1-heptan-2-yl-5-heptenoic acid (I-BOP) with high affinity support platelet shape change, whereas low affinity sites that bind irreversibly the antagonist GR 32191 transduce platelet aggregation. As the mechanisms of signal transduction involved in platelet aggregation begin to be elucidated, few results concern those involved in platelet shape change, which is independent of the engagement of GPIIb/IIa. To elucidate the respective role of the two classes of pharmacological binding sites of TPs in shape change, platelets were incubated with I-BOP at low concentrations or stimulated by I-BOP at high concentrations after pre-treatment with GR 32191 or activated with low concentrations of 8-epi-prostaglandin F\(_{2\alpha}\). Under these three conditions, there is a rapid stimulation of protein tyrosine phosphorylation of the 80/85-kDa doublet identified as the cytoskeletal protein cortactin. Tyrosine phosphorylation of cortactin is kinetically correlated with the occurrence of shape change. These biochemical and morphological events are both inhibited by SQ 29548, a TP antagonist, indicating the specificity of the signal.

Since tyrosine kinase Syk was activated early during platelet activation, we examined the possibility that cortactin is a potential substrate of Syk in TxA\(_2\)-induced platelet shape change. p72 Syk phosphorylation and kinase activity took place during the period when platelets were changing shape upon low concentrations of I-BOP stimulation. Furthermore, cortactin was associated with Syk, and this association increases along with the level of phosphorylation. These data suggest a novel pathway for a G protein-coupled TxA\(_2\) high affinity receptor to the protein-tyrosine kinase Syk, which is associated with cortactin in the very early steps of platelet activation.

Thromboxane (TxA\(_2\))\(^1\) is the principal autacoid among arachidonic acid metabolites responsible for vasoconstriction and platelet aggregation activities (1). It also stimulates mitogenesis and hypertrophy of vascular smooth muscle cells (2, 3). Its mimetics induce apoptosis of immature thymocytes and may regulate thymocyte differentiation and development (4). These actions are attributed to TxA\(_2\) interacting with its specific receptors, which belong to the heptahelical superfamily of G protein-coupled receptors. Platelet TxA\(_2\) receptors (TPs) have been reported to couple to Go\(_q\), Go\(_{12}\), and Go\(_{13}\) (5–7).

Because of the chemical instability of TxA\(_2\), a number of stable agonists have been synthesized, such as U 46619 and I-BOP. Evidence suggests that two different TPs within the platelets or two different affinity states of the same receptor may exist. Analysis of the binding of \(^{125}\)I-labeled I-BOP by platelet membranes indicates two platelet binding sites of high affinity (K\(_d\) = 270 ± 60 pM) and low affinity (K\(_d\) = 3.9 ± 1–2 nM) (8). The K\(_d\) values correspond to the EC\(_{50}\) values for I-BOP to induce shape change and aggregation. Thus, the binding of I-BOP to low affinity receptors induces secretion and aggregation, whereas its binding to high affinity receptors induces shape change (8). Similarly, a TP antagonist such as GR 32191 shows noncompetitive inhibition of platelet aggregation but competitive inhibition of platelet shape change, consistent with the existence of two subtypes of receptors (9). One possible explanation for the pharmacological evidence for distinct platelet thromboxane binding sites is that the affinity of a single receptor type can be modulated by the type of G proteins that the receptors are coupled to. Allan et al. (10) provide evidence that the affinity state of the TP can be altered by Go\(_{13}\) and Go\(_{q}\) when cotransfected with Go\(_{13}\) and Go\(_{q}\) into COS-7 cells.

Stimulation of TPs leading to platelet aggregation involves (i) an activation of phospholipase C\(_{\beta}\) and subsequent generation of inositol triphosphate and diacylglycerol (5); (ii) an increase in intracellular free Ca\(^{2+}\), phosphorylation of the myosin light chain of 20 kDa and of plecstrin of 47 kDa through activation of myosin light chain kinase and protein kinase C, respectively (9, 11); and (iii) exposure of glycoprotein IIb/IIIa binding sites for fibrinogen and subsequent induced pathways such as tyrosine kinases (11).

As the mechanisms of signal transduction involved in platelet aggregation begin to be elucidated, few results concern those involved in platelet shape change (12). We have previ-
ously reported that cortactin was the only tyrosine-phosphorylated substrate that occurred in thrombin-induced Glanzmann thrombasthenic platelets, that is to say in the absence of integrin engagement and aggregation (13). To enhance understanding of the signal transduction that occurs in the absence of integrin engagement and that leads to morphological changes, we used thromboxane analogues that only induce the first steps of platelet activation. To elucidate the respective roles of the two classes of pharmacological binding sites of TP in thromboxane-mediated platelet shape change, platelets were (i) incubated with I-BOP at low concentrations; (ii) stimulated by I-BOP at high concentrations after pretreatment with GR 32191, a TP antagonist that dissociates very slowly, if at all, from the low affinity binding sites (9); or (iii) activated with low concentrations of 8-epi-PGF_2α. This isoprostane is synthesized mostly independently of the activity of cyclooxygenase (in vivo) through the free radical-catalyzed peroxidation of arachidonic acid and may be active as a partial platelet activator at high concentrations (14–16).

Therefore, by using appropriate concentrations of thromboxane analogues that only induce shape change, we found that a 80/85-kDa protein, cortactin, a substrate involved in the cytoskeletal reorganization, is tyrosine-phosphorylated in platelets, and cortactin associates with the tyrosine kinase Syk. We demonstrated that both Syk kinase activation and tyrosine phosphorylation of cortactin are kinetically correlated with platelet shape change, which is the earliest platelet response.

**EXPERIMENTAL PROCEDURES**

**Materials**—The mouse anti-phosphotyrosine 4G10 and the anti-cortactin (p80/85) monoclonal antibodies (mAbs) were from Upstate Biotechnology Inc. (Lake Placid, NY). Rabbit anti-Syk polyclonal antibody (pAb) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Donkey anti-rabbit peroxidase-conjugated IgG was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Triton X-100, sodium orthovanadate, Nonidet P-40, cytochalasin D, and ATP were from Sigma; diethiothreitol and EDTA were from ProLabo (Paris, France); Pefabloc was from Pentapharm (Basel, Switzerland); sodium fluoride was from Aldrich; sodium pyrophosphate was from Merck (Darmstadt, Germany); and I-BOP, SQ 29548, and prostaglandin E_1 were from Cayman Chemical (Ann Arbor, MI). GR 32191 was a kind gift from Dr. B. Bain (Glaxo Laboratories). Sheep antimaus horseradish peroxidase-conjugated IgG, protein A-Sepharose, ECL reagents, and Hyperfilm-ECL (Glaxo Laboratories). Sheep anti-mouse horseradish peroxidase-conjugated IgG for 60 min at room temperature. After washing with TBS, bound primary antibodies were detected after incubation with sheep anti-mouse horseradish peroxidase-conjugated IgG for 60 min. Membranes were further washed in TBS and treated with ECL reagents. Immunoreactive bands were visualized by chemiluminescence according to the manufacturer’s instructions.

Tyrosine-phosphorylated protein bands were measured by laser densitometry (Ultrorcan XL; Amersham Pharmacia Biotech). Phosphorylation intensity was expressed in arbitrary units in fold over basal level at time 0.

**Immunoprecipitation Studies**—Platelet suspensions (0.4 ml, 6 × 10^8 platelets/ml), obtained as described above, were lysed in an equal volume of radioimmune precipitation buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.5% deoxycholic acid, 1 mM EDTA, 0.1% SDS, 1 mM dithiothreitol, 50 mM NaF). Insoluble cell fractions were centrifuged at 10,000 × g for 4 min at 10 °C. Cortactin (p80/85) was immunoprecipitated using 5 μg of mouse-anticortactin mAb and 5 μg of rabbit anti-mouse IgG pAb. After a 1-h incubation at 4 °C, protein A-Sepharose was added for 3 h at 4 °C. Syk kinase activation was assessed using 3 μg of rabbit anti-Syk kinase pAb under similar conditions. After three washings in modified radio-immune precipitation buffer, immunoprecipitates were recovered in Laemmli sample buffer under reducing conditions, subjected to SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membrane, and immunoblotted with (i) anti-phosphotyrosine mAb (1: 5000), (ii) anti-cortactin mAb (1:1000), or (iii) anti-Syk pAb (1:1000).

**Preparation of Human Platelets**—Human blood was taken from healthy volunteers after informed consent in accordance with the French Etablissement de Transfusion Sanguine. Blood was obtained by venipuncture on ACD-C (100 mM citric acid, 124 mM NaCl, 110 mM glucose; 1 volume for 9 volumes of blood) in the presence of 1 mM acetylsalicylic acid to prevent TxA_2 endogenous synthesis. Platelet-rich plasma was obtained by centrifugation at 120 × g for 15 min at room temperature. For some experiments, part of the platelet-rich plasma was separated by ultracentrifugation at high speeds (120,000 × g) for 1 h at 4 °C to obtain platelet-poor plasma. Platelet lysates were heated for 30 min at 60 °C in the presence of β-mercaptoethanol (5% w/v). Proteins were separated by SDS-polyacrylamide gel electrophoresis on a 7% polyacrylamide gel and transferred onto a nitrocellulose membrane using a semidy transfer system ( Hoefer, Amersham Pharmacia Biotech.). Nonspecific binding was blocked by incubating membranes with TBS (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.05% Tween 20) containing 5% (w/v) low fat powder milk before the incubation with anti-phosphotyrosine mAb (1:5000) for 60 min at room temperature. After washing with TBS, bound primary antibodies were detected after incubation with sheep anti-mouse horseradish peroxidase-conjugated IgG for 60 min. Membranes were further washed in TBS and treated with ECL reagents. Immunoreactive bands were visualized by chemiluminescence according to the manufacturer’s instructions.

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**In Vitro Syk Kinase Assay**—Syk kinase activation was calculated as autophosphorylation activity as described previously (17). Following stimulation with I-BOP, platelets (1.2 ml; 6 × 10^8 platelets/ml) were lysed in a buffer containing 10 mM Tris-HCl, pH 7.4, 1.3 × 10^10 X-Triton, 50 mM NaCl, 1 mM sodium orthovanadate, 1 mM Pefabloc, 30 mM sodium pyrophosphate, 50 mM NaF). Clarified platelet lysates were immunoprecipitated with 3 μg of anti-Syk pAb for 1 h at 4 °C. To examine the autophosphorylating activity of Syk kinase, the immunoprecipitates were washed in the same buffer twice; washed once in 10 mM Tris-HCl pH 7.4, 100 mM NaCl, 5 mM MnCl_2, and then incubated in 25 μL of kinase assay buffer (10 mM Tris-HCl, pH 7.4, 5 mM MnCl_2, 1 mM ATP) containing 10 μCi of [γ-32P]ATP for 15 min at 25 °C. The reaction was stopped by washing with 1 ml of ice-cold buffer B. Immunoprecipitates were recovered in Laemmli sample buffer before being subjected to 7% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane. Labeled proteins were visualized by autoradiography.

**RESULTS**

**I-BOP-induced Platelet Activation**—The addition of 10 nM of I-BOP to washed platelets for 2 min with constant stirring caused the platelets to aggregate. Fig. 1 shows that the light transmission after 2-min stimulation was about 40%. The increase in light transmission was preceded by a decrease in light transmission in the 10 first seconds, which corresponded to a shape change (Fig. 1A). In contrast, the addition of 1 nM of
I-BOP only induced the shape change in the absence of any aggregation (Fig. 1A).

Fig. 1B shows the shape change at high and low concentrations of I-BOP. The addition of the TP antagonist SQ 29548 (Fig. 1C) inhibited the I-BOP-induced platelet aggregation and the shape change, which was observed whether the I-BOP concentration was high or low.

Effect of I-BOP and 8-epi-PGF$_2$α on Tyrosine Phosphorylation—In the absence of I-BOP (Fig. 2), only a few proteins were significantly phosphorylated at tyrosine residues, including the 60-kDa protein previously identified as the Src kinase and the 64- and the 130-kDa bands. After 20- and 90-s stimulation with 10 nM I-BOP, phosphorylation of these substrates increased and additional tyrosine-phosphorylated proteins appeared with molecular masses of 80/85, 100/105, and 130 kDa. The increase in tyrosine phosphorylation correlated with the increase in platelet aggregation, which reached about 40% at 90 s (Fig. 2).

In the absence of platelet aggregation, 20–90-s stimulation with 1 nM of I-BOP resulted in a shape change of 16 mm. Under these conditions, we only observed the additional tyrosine-phosphorylated band of 80 kDa, which slightly decreases at 90 s (Fig. 2). The treatment of platelets with SQ 29548 inhibited the increase in tyrosine phosphorylation induced by low or high concentrations of I-BOP. This inhibition was accompanied by the inhibition of shape change and aggregation.

Two other conditions induced platelet shape change in the absence of aggregation. Platelets were stimulated either with high concentrations of I-BOP after pretreatment with GR 32191 or with 5 μM of 8-epi-PGF$_2$α (Fig. 3B). Fig. 3A shows that when platelets were pretreated with GR 32191 before the addition of 10 nM I-BOP (middle), or when they are stimulated with 8-epi-PGF$_2$α for 30 s (bottom), the tyrosine phosphorylation of 80-kDa substrate was the major substrate obtained. As in the presence of 1 nM I-BOP, the addition of SQ 29548 inhibited both the tyrosine phosphorylation of the 80-kDa substrate (Fig. 3A) and the shape change observed under these conditions (Fig. 3B).

Quantification by densitometry demonstrates that the level of phosphorylation of 80 kDa is increased by about 10-fold upon stimulation with low concentrations of I-BOP, 8-fold with 8-epi-PGF$_2$α, or 5-fold upon high concentrations of I-BOP after pretreatment with GR 32191. However, this represents a much lower level of phosphorylation than that obtained upon stimulation with high concentrations of I-BOP (30-fold over the basal level) (Fig. 3C). The results confirm that tyrosine phosphorylation of the 80-kDa band is subjected to two different waves of phosphorylation, the second one due to the integrin engagement (13).

Immunoprecipitation of Cortactin—The 80-kDa band appeared as the main phosphorylated substrate obtained during the early steps of platelet activation. Cortactin is a cytoskeletal tyrosine-phosphorylated protein that migrates as a doublet within the 80-kDa range and was reported previously to be tyrosine-phosphorylated in activated platelets (13). We intended to analyze further whether the 80 kDa corresponded to cortactin. We thus immunoprecipitated I-BOP- (1 nM) and 8-epi-PGF$_2$α-activated platelet lysates with a mAb specific for cortactin, followed by Western blot analysis with an anti-phosphotyrosine Ab (Anti-PY).

Fig. 4 (top) shows that an 80/85-kDa phosphorylated doublet corresponding to cortactin was obtained in platelets stimulated by I-BOP or 8-epi-PGF$_2$α, whereas it was strongly abolished when platelets were pretreated with SQ 29548. No phosphorylated doublet was observed either in resting platelets (I-BOP and 8-epi, Fig. 4, top) or in the presence of SQ 29548 alone (not shown). Blotting of the immunoprecipitates with the anti-cortactin mAb showed constant distribution of cortactin protein under the different conditions (Fig. 4, bottom).

Effect of Protein-tyrosine Kinase Inhibitor A23 and Cytochalasin D on Tyrosine Phosphorylation of Cortactin and Shape Change—Shape change was induced either by low concentrations of 8-epi-PGF$_2$α or I-BOP. Preincubation of platelets with the protein-tyrosine kinase inhibitor, tyrphostin A23 at 1 μM inhibited both platelet shape change and tyrosine phosphorylation of the cortactin (Fig. 5A). Scanner densitometry indicates that upon stimulation by 8-epi-PGF$_2$α or I-BOP, the level of phosphorylation of cortactin returns to the basal level in the presence of A23, whereas the structurally inactive analogue tyrphostin A1 slightly decreases the level by 25% (Fig. 5C). By preincubation of platelets with 10 μM cytochalasin D, which induces depolymerization of cytoplasmic actin, cortactin phosphorylation and shape change induced by I-BOP were also inhibited (Fig. 5B).

I-BOP-induced Syk Kinase Phosphorylation and Activity: Association of Cortactin with Syk—The kinase activities responsible for the phosphorylation of cortactin are not well defined in platelets activated with thromboxane analogues. We wondered whether the 72-kDa Syk tyrosine kinase, which exhibits early activation during platelet activation (18), would be a potential candidate for cortactin phosphorylation throughout I-BOP-induced shape change.

We first examined whether I-BOP activated Syk in platelets. After I-BOP stimulation, platelets were solubilized as de-
scribed under “Experimental Procedures” at the indicated times, and Syk was immunoprecipitated by anti-human Syk pAb. Fig. 6A shows that Syk is tyrosine-phosphorylated in response to low concentrations of I-BOP (1 nM) at early times (10 and 20 s) followed by a slight dephosphorylation (line 1). Then an in vitro kinase assay was performed as described under “Experimental Procedures.” Syk kinase activity was increased at 10 and 20 s after I-BOP stimulation and then
slightly decreased at 90 s (line 2). To address the relation between Syk activation and phosphorylation of cortactin, the same blots were immunblotted with anti-cortactin antibody. Line 3 shows that there was an increased association of cortactin at early times (10 and 20 s) after stimulation, whereas the association decreased at 90 s. It is noteworthy that cortactin seems to be associated with Syk even in the absence of stimulation by I-BOP (Fig. 6A, line 3). The amount of precipitated Syk did not change throughout the time course judging from the immunoblot analysis with anti-human Syk mAb (Anti-Syk) (line 4).

In our experiments, Syk could not be detected in the immunoprecipitates with anti-cortactin mAb. One possibility is that the anti-cortactin mAb destabilized cortactin-Syk complexes. A similar observation was described in K 562 cells (29). Finally, SQ 29548 inhibited Syk phosphorylation at all times (Fig. 6B), indicating the specificity of the signal sent by the activated TP receptor.

We have shown previously that A23 inhibited cortactin phosphorylation upon stimulation by low concentrations of I-BOP. We wondered if the tyrosine kinase inhibitor would affect the association of cortactin with Syk. Fig. 6C shows that A23 inhibits not only the tyrosine phosphorylation of Syk in response to low concentrations of I-BOP at early times (20 s) (line 1) but also the association of cortactin, which was previously detected (line 2). The amount of precipitated Syk did not change throughout the different conditions (line 3).

DISCUSSION

In this study, we demonstrate that upon stimulation of platelets by low concentrations of I-BOP or by high concentrations in platelets pretreated with GR 32191 (which blocks the low affinity sites involved in aggregation) or 8-epi-PGF$_2$$\alpha$, there is a rapid stimulation of protein tyrosine phosphorylation of the
80/85-kDa doublet identified by immunoprecipitation as the cytoskeletal protein cortactin. Tyrosine phosphorylation of cortactin is kinetically correlated with the occurrence of shape change. These effects therefore appear to be mediated by receptors with high affinity for I-BOP.

The molecular basis for this functional segregation of pharmacological TP receptors subtypes is unclear. A single gene encodes the human TP receptor (19), of which there are two identified splice variants, TPα and TPβ (20). Although mRNA for these isoforms has been described in human platelets (21), there was no information that related either TPα or TPβ to the subtypes of TPs that have been defined pharmacologically as high or low affinity receptors. However, it has recently been shown that TPα was the major isoform expressed in platelets and that rapidly induced phosphorylation of the TPα by high I-BOP concentrations appeared to involve low affinity binding sites. However, neither low I-BOP concentrations (which induce platelet shape change) nor high agonist concentrations on platelets pretreated with GR 32191 (which blocks the low affinity sites) cause TPα phosphorylation (22).

The inhibition of both tyrosine phosphorylation of cortactin as well as platelet shape change by SQ 29548 (23) demonstrates the role of the TP receptor inducing these biochemical and morphological events.

Cortactin was initially described as a 80/85-kDa p60 c-Src substrate. It was phosphorylated on tyrosine residues in v-Src-transformed chicken embryo cells (24). In normal cells, cortactin is known to be phosphorylated in response to various stimulants including growth factors and thrombin (25–27). In platelets, tyrosine phosphorylation of cortactin and activation of Syk were induced by thrombin treatment both in the presence or absence of integrin engagement (13, 28). Moreover, a direct association between Syk and cortactin has been demonstrated at an early stage of megakaryocytic differentiation in K 562 cells upon TPA stimulation (29).

The following evidence suggests here that cortactin could be a substrate for Syk at low I-BOP concentrations. (i) p72 Syk phosphorylation took place during the period when platelets were changing shape (10–30 s). This phosphorylation is accompanied by a kinase activity. (ii) Tyrosine phosphorylation of cortactin is kinetically correlated with the activation of Syk kinase. (iii) Cortactin is associated with Syk, and this association increases along with the level of phosphorylation. A slight association of cortactin with Syk is already detected before I-BOP stimulation. The same results were observed when K 562 cells were stimulated with 12-O-tetradecanoylphorbol-13-acetate (29).

Thus, these data indicate that the kinase activity and tyrosine phosphorylation of p72 Syk can be induced through a G protein coupled to TP receptors of high affinity. TP receptors of human platelets are known to couple endogenously to at least four G proteins (Gαq, Gα12, Gα13, and Gαq). The low affinity platelet TP receptor that mediates platelet aggregation seems to be regulated via Gαq (6), but no data were available for possible G proteins mediating platelet shape change via the apparent high affinity TP receptor. Allan et al. (10) present the first pharmacological evidence that at least two of the Gα proteins (Gαq and Gα13) thought to interact with the human platelet TP receptors are capable of regulating the affinity state of the megakaryocyte-like HEL cell TP receptor. Gα13 appears to increase the affinity of the HEL TP receptor for 125I-labeled I-BOP, although Gαq does not increase the affinity to the same extent. Moreover, platelets from mice deficient in Gαq can still change shape but do not aggregate after the addition of U 46619, a thromboxane analogue, therefore emphasizing the coupling of the low affinity TP receptors to Gαq (30). Taking into account these two observations, it is suggested that high affinity TP receptors could be regulated via Gαq. This novel pathway would lead to Syk activation necessary for the phosphorylation of cortactin, a specific substrate that accompanies platelet shape change. It is noteworthy that Syk activation, cortactin phosphorylation, and shape change are kinetically correlated.

What would be the role of cortactin phosphorylation within...
fibrinogen binding to GPIIb/IIIa and aggregation remains to be elucidated.

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