HEMOSTASIS, THROMBOSIS, AND VASCULAR BIOLOGY

Cyclophilin B Binding to Platelets Supports Calcium-Dependent Adhesion to Collagen

By Fabrice Allain, Sandrine Durieux, Agnès Denys, Mathieu Carpentier, and Geneviève Spik

We have recently reported that cyclophilin B (CyPB), a secreted cyclosporine-binding protein, could bind to T lymphocytes through interactions with two types of binding sites. The first ones, referred to as type I, involve interactions with the conserved domain of CyPB and promote the endocytosis of surface-bound ligand, while the second type of binding sites, termed type II, are represented by glycosaminoglycans (GAG). Here, we further investigated the interactions of CyPB with blood cell populations. In addition to lymphocytes, CyPB was found to interact mainly with platelets. The binding is specific, with a dissociation constant (kd) of 9 ± 3 nmol/L and the number of sites estimated at 960 ± 60 per cell. Platelet glycosaminoglycans are not required for the interactions, but the binding is dramatically reduced by active cyclosporine derivatives. We then analyzed the biologic effects of CyPB and found a significant increase in platelet adhesion to collagen. Concurrently, CyPB initiates a transmembranous influx of Ca2+ and induces the phosphorylation of the P-20 light chains of myosin. Taken together, the present results demonstrate for the first time that extracellular CyPB specifically interacts with platelets through a functional receptor related to the lymphocyte type I binding sites and might act by regulating the activity of a receptor-operated membrane Ca2+ channel.

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Materials and Methods

Materials. Human citrated venous blood samples from healthy donors were obtained from the local blood transfusion center (Etablissement de Transfusion Sanguine, Lille, France). Recombinant human CyPA and CyPB were produced and purified as previously described.1,6 Recombinant human CyPC and cyclosporine derivatives (CsA, CsG, CsH)19 were a generous gift from Novartis (Basel, Switzerland).

CYCLOPHILINS are known to be the main binding proteins for the immunosuppressive drug cyclosporine A (CsA).1,2 The first characterized isoform was cyclophilin A (CyPA), an abundant cytosolic protein that is considered to be the major target for CsA into the cell.3,4 Cyclophilin B (CyPB)5-6 and cyclophilin C (CyPC)7 are two other isoforms structurally related to CyPA, but their mRNA encodes a signal sequence thought to mediate translocation into the endoplasmic reticulum. Both the cyclophilins and the structurally unrelated FK506-binding proteins (FKBP) exhibit peptidyl-prolyl cis-trans isomerase activity (PPlase)8-10 and inhibit the phosphatase activity of calcineurin in the presence of their respective ligand.11,12 The latter property is thought to be relevant to the immunosuppressive activity of both drugs. Indeed, the inhibition of calcineurin activity has been shown to be a crucial step that effectively blocks the early T-cell activation cascade and constitutes the basis of the prevention of graft rejection.13

The presence of a released form of CyPB in human milk and plasma14 has led us to investigate the properties of this protein. We first characterized specific surface binding sites on T lymphocytes,15 mainly associated with the helper/inducer T-cell subset.16 Most recently, we provided evidence that interactions of CyPB with sulfated glycosaminoglycans (GAG) may occur on the T-cell surface. In addition, we identified a second type of CyPB binding sites, referred as type I sites versus type II for GAG interactions.17 The binding of CyPB to type I and type II sites requires interactions with two distinct areas of the protein, the catalytic/CsA-binding domain and the N-terminal extremity of CyPB, respectively. Moreover, we demonstrated that the type I binding sites are involved in an endocytosis process of the protein, which supports the hypothesis that they may correspond to a functional receptor of CyPB.17 The presence of surface binding sites on T lymphocytes was consistent with the hypothesis that secreted CyPB may interact with specific membrane receptors. However, the presence of CyPB binding sites on the other human blood cells has not yet been investigated. In the present report, we analyzed the distribution of CyPB binding sites in blood cell populations. In addition to the interactions with T lymphocytes, we found a significant binding of the protein to platelets. CyPB interacts with platelets in a specific manner, with a dissociation constant (kd) value similar to that of T cells. However, CyPB binding to platelets did not involve interactions with GAG, while it was strongly reduced in the presence of active cyclosporine derivatives. Platelets are largely represented in human blood and their activation is critically important in blood coagulation and inflammatory events.18 Here, we demonstrated that incubation of platelets together with CyPB enhances adhesion to collagen, but is ineffective in terms of degranulation or aggregation. In addition, CyPB binding initiates an influx of extracellular Ca2+ and some kinase activation, which demonstrates that the platelet receptor is coupled to a transduction pathway. The present results suggest that CyPB interacts with platelets through a functional receptor related to the lymphocyte type I binding sites and is probably involved in the regulation of a receptor-operated membrane channel.

From the Laboratoire de Chimie Biologique, Unité Mixte de Recherche no. 8576 du CNRS, Université des Sciences et Technologies de Lille, Villeneuve d’Ascq, France.

Supported by the Université des Sciences et Technologies de Lille, CNRS (Unité Mixte de Recherche no. 111; Director: Professor A. Verbert) and by a grant from the Conseil Régional du Nord/Pas-de-Calais (contract: “Maladies neurodégénératives et Vieillissement”).

Address reprint requests to Prof Geneviève Spik, Laboratoire de Chimie Biologique, Unité Mixte du CNRS no. 111, Université des Sciences et Technologies de Lille, 8576 Villeneuve d’Ascq Cedex, France; e-mail: genevieve.spik@univ-lille1.fr.

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Human citrated venous blood samples from healthy donors were obtained from the local blood transfusion center (Etablissement de Transfusion Sanguine, Lille, France). Recombinant human CyPA and CyPB were produced and purified as previously described.1,6 Recombinant human CyPC and cyclosporine derivatives (CsA, CsG, CsH)19 were a generous gift from Novartis (Basel, Switzerland).

Blood, Vol 94, No 3 (August 1), 1999; pp 976-983
Peptides that corresponded to the N- and C-terminal extensions of CyPB were synthesized as described and were provided by the tetrapeptide RGDS by Professor A. Tartar (Institut Pasteur de Lille, France). Collagen mixture (essentially of type I and II) was purified from rat tail. \(^{14}\) CyPB was prepared as described. \(^{15}\) The specific radioactivity ranged from 4 to \(6 \times 10^3\) cpm/μg.

**Preparation of platelets.** Platelet-rich plasma (PRP) was obtained by centrifugation of whole blood at 200g for 30 minutes. Washed platelets were prepared by filtration on a Sepharose 4-B column (Pharmacia, Uppsala, Sweden) equilibrated in Tyrode’s buffer (NaCl, 137 mM; KCl, 2.7 mM; CaCl\(_2\), 2 mM; MgCl\(_2\), 1 mM; NaHPO\(_4\), 0.2 mM; NaHCO\(_3\), 12 mM; HEPES, 5 mM; 0.1% glucose), pH 7.4, and resuspended in Tyrode’s buffer containing 0.5% bovine serum albumin (BSA). Concentrated platelet suspension was obtained by centrifugation of PRP (1,600g, 10 minutes) and resuspension of the resulting pellet with PRP to a final concentration of \(2 \times 10^9/\text{mL}\).

**Surface binding experiments with [\(^{125}\text{I}\)]CyPB.** Surface binding of [\(^{125}\text{I}\)]CyPB to blood cell populations was performed by incubating blood samples (1 mL) in the presence of radiolabeled ligand for 1 hour at room temperature. After washing off the plasma, the surface-bound CyPB was counted in the blood cell pellet, to obtain the total binding capacity, and in each isolated cell population, after separation of blood cells on Ficoll separation medium (Nycocem, Oslo, Norway). For platelet binding experiments, PRP was previously incubated in the presence of 1 μg/mL prostaglandin E\(_1\) for 30 minutes at 37°C. Platelets (2 \(\times 10^9\) per sample) were then allowed to bind [\(^{125}\text{I}\)]CyPB at various concentrations. After 1 hour at 22°C, the platelet suspension was filtered over a Whatman glass fiber filter (Maidstone, UK) under mild vacuum and washed twice. Filter-associated (bound ligand) and incubation medium (free ligand) radioactivities were then measured. To analyze interactions with GAG, washed platelets were treated with either 1 U/mL heparinase I, 2 U/mL chondroitinase ABC, or both (Sigma Chemical, St Louis, MO) for 2 hours at room temperature and directly used for binding experiments as described. Control untreated platelets were prepared under the same protocol. For all binding experiments with [\(^{125}\text{I}\)]CyPB, nonspecific interactions were determined in the presence of a 200-fold molar excess of unlabeled ligand and radioactivity was measured using a model 1282 Compugamma LKB-Wallac counter (Gaithersburg, MD).

**Platelet function analysis.** Platelet aggregation was typically performed at 37°C for 3 minutes using a turbidimetric method. Aggregation was induced by the addition of fibrinogen (1 mg/mL) or autologous plasma to washed platelet mixture (2 \(\times 10^9/\text{mL}\) followed by the addition, 30 seconds later, of CyPB or agonist. To measure platelet degranulation, PRP was incubated with 5-\[^3\text{H}\]hydroxytryptamine (5-HT) (0.05 mCi/mL) (ICN Biochemicals, Costa Mesa, CA) for 30 minutes at 37°C. After gel filtration, platelets were incubated with CyPB or agonist and processed as described.\(^{21}\) Released 5-\[^3\text{H}\]HT was analyzed using a model LS 6100-TA Beckman counter (Allendale, NJ). For platelet adhesion assays, 96-well microtiter plates were coated with 1 μg/well of collagen in a sodium carbonate buffer, 10 mM, pH 9.6, overnight at 4°C. Nonspecific adhesion sites were blocked by addition of 2% BSA. Platelets (1 \(\times 10^7\) per well) were incubated in the presence of various concentrations of CyPB and added to collagen-coated wells for a 30-minute incubation at 37°C. After washing, the adherent platelets were quantified using the BCA reagent kit for protein assay (Pierce Chemicals, Rockford, IL).

**Calcium measurements.** Platelets were loaded with 3 μM of Fluo 3-acetoxyxymethylster (Fluo 3-AM) (Molecular Probes, Leiden, Netherlands)\(^{22}\) for 30 minutes at 37°C. After gel filtration to remove extracellular Fluo 3, the final platelet concentration was adjusted to 1 \(\times 10^9/\text{mL}\) in Tyrode’s buffer. Stimulation was induced by the addition of either various concentrations of CyPB or thrombin at 37°C. Changes in fluorescence were recorded by flow cytometry using a Becton Dickinson FACScan cytofluorimeter (Mountain View, CA), with excitation and emission wavelengths set on 488 and 515 nm, respectively. This method allowed the analysis of 2,000 fluorescent particles every 30 seconds. The Ca\(^{2+}\)-Fluo 3 fluorescence was calibrated with a maximum response induced by the addition of ionomycin to the suspension. The levels of cytosolic Ca\(^{2+}\) were calculated for each fluorescence mean value, with a kd of 400 nM/mL for Fluo 3.\(^{21}\)

**Measurement of inositol phosphate formation.** Concentrated platelet suspensions were labeled with myo-[\(^{3}\text{H}\)]inositol (50 μCi/mL) (ICN Biochemistry) for 3 hours at 37°C. Following washing and resuspension in a myo-inositol–free buffer, platelets were stimulated with either CyPB or thrombin and processed as described.\(^{23}\)

**Protein phosphorylation analysis.** Platelets (2 \(\times 10^9/\text{per sample}\) were incubated in the presence of either CyPB or thrombin at 37°C. At various times, platelets were transferred into Tyrode’s buffer containing 10 mM EDTA, 1 mM o-vanadate and 100 mM NaF, to inhibit protein phosphatases and rapidly washed by centrifugation (2,500g, 30 seconds). Proteins from platelet lysates were separated on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE) and transblotted onto nitrocellulose paper.\(^{22}\) After blocking in Tris-buffered saline (TBS), pH 8.2, which contained 3% gelatin, blots were incubated with mouse monoclonal antibodies to phosphoserine or phosphotyrosine residues (Sigma) in TBS-gelatin 0.5% for 2 hours and exposed to horseradish peroxidase–labeled antismouse IgG antibodies (1/2,500) (BioSys, Compiègne, France) for another 2-hour incubation. Development was performed with \(o\)-phenylene diamine kit (Sigma).

Because platelet controls from different individuals showed varying degrees of protein phosphorylation, the effects of CyPB were evaluated in terms of variations of phosphorylation intensity using Quantiscan software (Biosoft, Cambridge, UK).

**Statistical analysis.** Results are expressed as mean values \(\pm\) SEM for at least three independently performed experiments conducted with separate donors. Statistical significance between the different values was analyzed by Student’s \(t\)-test for unpaired data with a threshold of \(P < .05\).

**RESULTS**

Characterization of CyPB binding sites on human blood cells. On the assumption that only T lymphocytes were involved in the interactions with CyPB, the total binding capacity of [\(^{125}\text{I}\)]CyPB was estimated at 50 fmol/mL in whole blood. Indeed, we previously reported that approximately 40% of the peripheral blood lymphocyte population showed significant binding of CyPB, with a capacity ranging from 30,000 to 120,000 sites per cell.\(^{15,17}\) Surprisingly, the surface-bound ligand was found to be \(415 \pm 80\) fmol/mL, much higher than the calculated value. The distribution of [\(^{125}\text{I}\)]CyPB was then analyzed after separation of the blood cell populations (Table 1). Ten percent to 15% of the total binding capacity of [\(^{125}\text{I}\)]CyPB was found associated with the lymphocyte fraction, which corresponds to the expected value we had calculated. No significant amounts of radioactivity were measurable in the

<table>
<thead>
<tr>
<th>Table 1. Surface Binding of CyPB to Human Blood Cell Populations</th>
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<tbody>
<tr>
<td><strong>Surface-Bound CyPB (fmol/mL) % of Cellular Binding</strong></td>
</tr>
<tr>
<td>Whole blood cells</td>
</tr>
<tr>
<td>Lymphocytes</td>
</tr>
<tr>
<td>Monocytes</td>
</tr>
<tr>
<td>Granulocytes</td>
</tr>
<tr>
<td>Platelets</td>
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<td>Erythrocytes</td>
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Abbreviation: NS, not significant (below the limit of detection).
monocyte population. A weak but significantly measurable proportion of CyPB was associated with granulocytes. This value might reflect a poor expression of CyPB binding sites on the membrane of this whole cell population, or a restricted CyPB binding on a specific subpopulation of polymorphonuclear cells. More than 80% of the bound protein was found associated with the platelet fraction. Owing to the large amount of platelets in human blood, this value implies that the number of surface-bound CyPB would range from 500 to 1,200 on platelets. Finally, the radioactivity found associated with the erythrocyte population is probably due to [125I]CyPB binding to contaminating platelets or lymphocytes; otherwise it would correspond to less than one binding site per erythrocyte, according to the number of red blood cells.

Surface binding of CyPB to platelets. The binding parameters of CyPB were determined by incubating platelets with increasing concentrations of [125I]CyPB. Figure 1 illustrates one representative experiment performed with washed platelets. The binding was specific since a 200-fold molar excess of unlabeled ligand inhibited [125I]CyPB binding by 60% to 80%. After subtraction of nonspecific interactions, the binding was found to be concentration-dependent and saturable (Fig 1A). Scatchard analysis resulted in a linear plot compatible with a single affinity binding site. The apparent kd was 9 ± 3 nmol/L and the number of binding sites was estimated at 960 ± 60 per platelet (Fig 1B). Similar data were obtained when Ca2+ and Mg2+ were omitted from the incubation medium, which indicates that the presence of the divalent cations is not required for CyPB binding to platelets. In additional experiments, platelets were incubated in the presence of 50 nmol/L [125I]CyPB for 1 hour at 22°C, then extensively washed to remove unbound ligand and resuspended in the same buffer containing a 10-fold molar excess of unlabeled CyPB. A rapid removal of surface bound radiolabeled ligand was observed, which indicates that the binding of CyPB to platelets is reversible (data not shown). To ensure that CyPB binding may occur under physiologic conditions, PRP was adjusted to 2 × 108 platelets/mL with citrated buffer and directly used for binding experiments (n = 3). In this case, the kd value and number of sites were estimated at 10 ± 3 nmol/L and 900 ± 120 per platelet, which indicates that the presence of plasma does not significantly modify the binding parameters. In addition, endogenous plasma CyPB levels were measured by enzyme-linked immunosorbent assay (ELISA)14 and found to be less than 5 nmol/L, which is too low to account for a large part in the binding site occupancy.

Specificity of CyPB binding to platelets. The specificity of CyPB binding to platelets was first analyzed by competitive experiments. For these studies, platelets were incubated with 50 nmol/L of [125I]CyPB in the presence of various concentrations of unlabeled CyPA, CyPB or CyPC (Fig 2A). As expected, [125I]CyPB binding was inhibited by greater than 80% from a 200-fold molar excess of unlabeled CyPB. The concentration of CyPB required for half-maximal inhibition (IC50) of the total [125I]CyPB binding was estimated at 250 nmol/L. CyPA was unable to displace the radio-iodinated ligand from the platelet membrane, which shows that this isoform has no affinity for CyPB binding sites. In contrast, increasing concentrations of CyPC inhibited [125I]CyPB binding to platelets, but to a lesser extent than CyPB. The IC50 was estimated at 2,950 nmol/L, which shows that CyPC has a lower affinity for platelet binding sites. We then examined the involvement of nonconserved regions of CyPB in the ligand binding by using synthetic peptides that copy the most divergent parts of the protein. As previously reported, increasing concentrations of the C-terminal peptide were unable to reduce [125I]CyPB binding. Surprisingly, the N-terminal peptide was also inefficient at competing with the ligand for binding to the platelet receptor, although it was previously shown to strongly reduce interactions of CyPB with the lymphocyte type II sites.27 Contrary to CyPA and CyPC, only CyPB possesses a specific RGD motif.17 Nevertheless, the involvement of this specific tripeptide in the interactions of CyPB with platelets is unlikely, since the addition of increasing concentrations of the tetrapeptide RGDS, which binds to glycoprotein (GP)IIb/IIIa and inhibits its interaction with RGD-containing ligands such as fibrogin25 was also ineffective at reducing [125I]CyPB binding (Fig 2A). We then investigated the role of the CsA-binding/catalytic domain of CyPB in the interactions with the platelet receptor, by using cyclosporine derivatives as competitive inhibitors (Fig 2B). Both CsA and the less active CsG reduced [125I]CyPB binding to the platelet membrane. A significant decrease was only obtained from a 10-fold molar excess of both drugs, which indicates that the inhibition requires that CyPB was maintained in a complexed form. By contrast, CsH, which is unable to interact with CyPB, failed to prevent the ligand binding, which confirms that occupancy of CsA-binding/catalytic domain by active cyclosporine derivatives accounts for the loss of binding activity. On the other hand, treatment of platelets with GAG-degrading enzymes did not significantly modify the binding of [125I]CyPB to platelets. Moreover, incubation of CyPB together with protamine, a polypeptide that inhibits interactions with heparin-like molecules, had no more effect on CyPB binding to platelets, which confirms that CyPB does not interact with platelet GAG (Fig 2B).

Analysis of CyPB activity on platelet functions. In an attempt to understand the biological relevance of CyPB binding, we analyzed its effects on platelet functions. The addition of CyPB at different concentrations did not induce any signifi-
The cyclophilin B receptor on platelets was examined. Competitors, are plotted against the molar ratios of [125I]CyPB to the ligand. Washing, the amounts of remaining surface-bound [125I]CyPB, expressed as a percentage of the ligand bound in the absence of any treatment. Data are mean values from 3 separate experiments conducted with platelets from different donors.

Fig 2. Specificity of [125I]CyPB binding to platelets. (A) Competitive binding assays with cyclophilin isofoms and synthetic peptides copying specific sequences of CyPB. Platelets were incubated in the presence of 50 nmol/L [125I]CyPB and increasing concentrations of unlabeled CyPA (●), CyPB (○), CyPC (△), N-terminal peptide of CyPB (□), C-terminal peptide of CyPB (△), or RGDS peptide (○). After washing, the amounts of remaining surface-bound [125I]CyPB, expressed as a percentage of the ligand bound in the absence of competitors, are plotted against the molar ratios of [125I]CyPB to the competitors. Data are expressed as mean values from 3 separate experiments conducted with platelets from different donors. (B) Sensitivity of CyPB binding to cyclosporine derivatives, protamine, and GAG-degrading enzymes. Platelets were incubated with 50 nmol/L [125I]CyPB in the absence (1) or presence of CsA 500 nmol/L (2); CsA 5 μmol/L (3); CsG 500 nmol/L (4); CsG 5 μmol/L (5); CsH 500 nmol/L (6); CsH 5 μmol/L (7); protamine 500 nmol/L (8); or protamine 5 μmol/L (9). In the last cases, platelets were first pretreated with heparinase type I (10), chondroitinase ABC (11), or both (12), and directly used for binding experiments. After washing, the amounts of remaining surface-bound [125I]CyPB were expressed as a percentage of the ligand bound in the absence of any treatment. Data are mean values ± SEM from 3 separate experiments conducted with platelets from different donors.

Table 2. Effects of CyPB on Platelet Aggregation and Degranulation

<table>
<thead>
<tr>
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<th>Platelet Aggregation (%)</th>
<th>5-HT Release (%)</th>
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<tbody>
<tr>
<td>No ligand</td>
<td>6 ± 5</td>
<td>8.4 ± 4.5</td>
</tr>
<tr>
<td>CyPB</td>
<td></td>
<td></td>
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<tr>
<td>10 nmol/L</td>
<td>5 ± 4</td>
<td>11.1 ± 3.5</td>
</tr>
<tr>
<td>50 nmol/L</td>
<td>6 ± 4</td>
<td>10.0 ± 2.5</td>
</tr>
<tr>
<td>500 nmol/L</td>
<td>7 ± 3</td>
<td>10.1 ± 3.4</td>
</tr>
<tr>
<td>Thrombin: 0.05 U/mL</td>
<td>26 ± 6</td>
<td>12 ± 5</td>
</tr>
<tr>
<td>+ CyPB 50 nmol/L</td>
<td>28 ± 5</td>
<td>16 ± 3.5</td>
</tr>
<tr>
<td>+ CyPB 500 nmol/L</td>
<td>25 ± 8</td>
<td>14 ± 4</td>
</tr>
<tr>
<td>Thrombin: 0.5 U/mL</td>
<td>84 ± 5</td>
<td>89 ± 6</td>
</tr>
</tbody>
</table>
Results are expressed as mean values conducted with platelets from different donors.

collagen in complete Tyrode’s buffer supplemented with 0.5% BSA.

coated well. Percentages of initially added platelets (1 × 10^7 platelets per well) for a 30-minute incubation at 37°C. After washing, adherent platelets were quantified using a BCA protein assay and expressed as percentages of initially added platelets (1 × 10^7 platelets per well).

Results are expressed as mean values ± SEM from quadruplicates and are representative from at least 3 separate experiments conducted with platelets from different donors.

between the platelet membrane and the collagen fiber surface. To check this hypothesis, collagen-coated plates were pretreated with CyPB and extensively washed before the addition of platelets. In these conditions, a CyPB-mediated increase in platelet adhesion was not observed, which demonstrates that CyPB does not act by forming a link between collagen and the platelet receptor. In contrast, when platelets were pretreated with CyPB and extensively washed to remove unbound ligand, the enhancing effect of the protein on platelet adhesion was preserved, which suggests it may be related to the binding of CyPB to a platelet signalling receptor (Table 3). We then analyzed the role of divalent cations, by incubating platelets in a Ca^{2+}/Mg^{2+}-depleted medium supplemented with 2 mmol/L EGTA. In this case, platelet adhesion was significantly reduced, from 30% to 12%. Nevertheless, no significant increase occurred in the presence of CyPB, which implies that the presence of divalent cations is required for the protein to enhance platelet adhesion. When citrated plasma was used in place of buffer that contained EGTA, platelet adhesion was approximately 20%, which indicates the participation of plasma factors in promoting cation-independent adhesion. Nevertheless, this mechanism was unmodified when CyPB was added to citrated plasma. Finally, adhesion experiments were reproduced with recalcified plasma. To prevent spontaneous aggregation, platelets were pretreated with aspirin (100 µmol/L) and the drug was conserved all along the experiment. In this case, the enhancing effect of CyPB on platelet adhesion to collagen was partially restored, with an almost 1.5-fold increase in adhesion by comparison to control (Table 3). Taken together, these results demonstrate that the action of CyPB is only dependent on the presence of divalent cations and may occur under physiologic conditions.

Effects of CyPB on Ca^{2+} movements and protein phosphorylation. In the following experiments, we investigated whether the enhancing effect of CyPB on platelet adhesion may be related to the transduction of intracellular signals. In this way, we analyzed possible pathways of CyPB-induced platelet response by measuring the dose- and time-responses of intraplatelet Ca^{2+} signal generation and protein kinase activation following CyPB addition.

To analyze Ca^{2+} responses, a series of spectrofluorimetric experiments was performed on platelets loaded with the Ca^{2+} fluorophore Fluo-3. Addition of CyPB (10 to 500 nmol/L) induced an increase in cytosolic free Ca^{2+} and the concentration of ligand required for a maximum response was estimated at 100 nmol/L. These values are consistent with the CyPB concentrations required for surface binding site occupancy. Stimulation with CyPB resulted in a low and durable Ca^{2+} flux, with an increase from 90 ± 35 nmol/L to 225 ± 45 nmol/L in the first minute (Fig 4A). This elevation in cytosolic free Ca^{2+} concentration was nevertheless not comparable to that induced by thrombin, which was estimated at 1,220 ± 205 nmol/L. To inhibit extracellular Ca^{2+} entry, platelets were diluted 1 minute before analysis in a CaCl_{2}-depleted buffer containing 2 mmol/L EGTA. In this case, the effect of CyPB was similar to that observed in the absence of any activator (Fig 4B), which suggests that the elevation in cytosolic free Ca^{2+} initiated by CyPB is likely to be generated by a transmembranous influx of extracellular Ca^{2+} through a membrane channel. We then examined the influence of CyPB binding on the formation of inositol phosphate (InsPs) derivatives, after platelet labeling with myo-[3H]inositol. The exposure of platelets to CyPB for times varying from 1 to 10 minutes did not increase the levels of these second messengers (1,260 ± 290 cpm within 5 minutes) in comparison to basal levels (930 ± 240 cpm), while thrombin induced a large and rapid increase in InsPs concentration (14,400 ± 1,080 cpm within 5 minutes). These results further demonstrate that CyPB-induced Ca^{2+} flux is quite different from that initiated by thrombin and not dependent on the activation of PLC.

To analyze the effect of CyPB on protein kinase activation, platelets were incubated in the presence of various concentrations of CyPB and the patterns of phosphorylation were compared with those obtained in the absence of agonist, or in the presence of thrombin (0.5 U/mL) taken as a positive control.

Table 3. Effects of the Modification of Either Incubation Medium or Treatment Procedure on CyPB-Mediated Adhesion of Platelets to Collagen

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Absence of CyPB</th>
<th>Presence of CyPB (50 nmol/L)</th>
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</thead>
<tbody>
<tr>
<td>Control*</td>
<td>29 ± 3</td>
<td>49 ± 5</td>
</tr>
<tr>
<td>Modified incubation medium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca^{2+}/Mg^{2+}-depleted medium</td>
<td>12 ± 4</td>
<td>12 ± 3</td>
</tr>
<tr>
<td>Citrated plasma</td>
<td>19 ± 2</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>Recalcified plasma</td>
<td>33 ± 5</td>
<td>53 ± 4</td>
</tr>
<tr>
<td>Modified incubation procedure:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pretreatment of platelets</td>
<td>28 ± 4</td>
<td>48 ± 6</td>
</tr>
<tr>
<td>Pretreatment of coated collagen</td>
<td>29 ± 1</td>
<td>28 ± 2</td>
</tr>
</tbody>
</table>

Results are expressed as percentages of initially added platelets (1 × 10^7 platelets per well) remaining associated to the collagen-coated well.

*Control corresponds to direct incubation of platelets with coated collagen in complete Tyrode’s buffer supplemented with 0.5% BSA.
CyPB did not induce any significant changes in tyrosine phosphorylation (data not shown). We then compared the profiles of serine phosphorylation of P-47 pleckstrin, substrate for protein kinase C (PKC) and P-20 myosin light chains, substrate for myosin light chain kinase (MLCK), as expected, the addition of thrombin to platelets resulted in the rapid phosphorylation of P-47 and P-20. After CyPB stimulation, an increase in serine phosphorylation was essentially observed for the 20-kD proteins, while P-47 was not significantly modified (Fig 4C). Phosphorylation of P-20 rapidly increased and was maximum within 5 to 10 minutes, which shows that it occurred after the generation of Ca$^{2+}$ influx. Moreover, the optimal concentration of CyPB was estimated at 100 nM/L, with a threefold increase in intensity, at a level similar to that obtained with platelets challenged with thrombin (290% ± 10% and 250% ± 15% for CyPB and thrombin, respectively). In contrast, CyPB did not induce any increase in P-20 phosphorylation when similar experiments were reproduced in the absence of extracellular Ca$^{2+}$. Taken together, these results indicate that the dose- and time-dependent responses to CyPB for increasing P-20 phosphorylation paralleled those observed for elevation of cytosolic free Ca$^{2+}$ concentration, which suggests that both events are related.

**DISCUSSION**

Our previous work showed that CyPB specifically binds to the surface of human T cells. Here, we present new data demonstrating that specific binding sites are also expressed at the surface of human platelets and exhibit similar affinity for CyPB. No binding was observed to erythrocytes and monocytes, and most probably the methods used were not sensitive enough to conclude to the presence or absence of surface CyPB binding sites on granulocytes. We reported that CyPB interacts with two types of binding sites present on the membrane of T lymphocytes. The first ones, termed type I binding sites, involve interactions with the CsA-binding domain of CyPB, while the type II sites are mainly represented by GAG present on the T-cell membrane and involve interactions with the N-terminal extension of the protein. The present data demonstrate that the interactions of CyPB with the platelet receptor and the lymphocyte type II binding sites are quite different, which excludes a role for platelet GAG in CyPB binding. Actually, the lymphocyte type II sites were found to mainly correspond to molecules of the heparin/heparan sulfate family, which interacts with the platelet receptor. However, the area of the RGDS peptide in the interactions of a large family of ligands, such as fibronectin and von Willebrand factor, with platelet membrane receptors has been largely documented. Nevertheless, the RGDS peptide was unable to compete with CyPB binding, which rules out the role of integrins of the GPIIb/IIIa family in the binding of CyPB. We then demonstrated that both CsA and CsG, but not CsH, reduced CyPB binding to the platelet membrane. Actually, active drugs overlay the binding domain of CyPB when complexed to the protein and probably lead to a loss of accessibility for the platelet receptor. In addition, CyPC, but not CyPA, was found here to compete with CyPB for binding to platelet receptor. However, the area of the cyclophilin isoforms that interacts with cyclosporine derivatives is strongly conserved, which disagrees with the role of this conserved catalytic domain in receptor recognition. Most probably, divergent regions differentially influence the spatial conformation of these proteins and therefore explain variations in the interactions with specific receptors. Such properties were also observed for the binding of CyPB to lymphocyte type I sites, which strongly suggests that both CyPB receptors are related.

The events initiated by CyPB binding to the platelet receptor were unexpected and appeared to differ in important ways to those induced by agonists like thrombin. CyPB was found to increase platelet adhesion to collagen, but was unable to promote degranulation or aggregation. The mechanism by which CyPB increases platelet adhesion to collagen is dependent on the presence of extracellular Ca$^{2+}$ and is accompanied...
by the elevation of cytosolic free Ca\(^{2+}\) concentration and P-20 phosphorylation. However, it does not require the generation of InsPs, which suggests that stimulation of CyPB membrane sites mediates activation of effectors other than PLC. Moreover, the absence of the PKC-dependent phosphorylation of P-47 demonstrates that there is no generation of diacylglycerol and further confirms the absence of any activation of PLC. In contrast, the effect of CyPB on phosphorylation of P-20 indicates that the increase in cytosolic free Ca\(^{2+}\) concentration probably led to the activation of MLCK. Indeed, phosphorylation of the P-light chains of myosin may be induced by direct activation of this kinase by the low elevation of intracellular Ca\(^{2+}\) concentration.\(^{18}\) Similar events are observed when platelets are exposed to cold temperatures. Chilling platelets was reported to promote by the elevation of cytosolic free Ca\(^{2+}\) and adhesion.\(^{21,28,29,35}\) It is therefore conceivable to postulate in the contractile events associated with platelet shape changes that CyPB-mediated activation of MLCK and Ca\(^{2+}\) operated channel and initiate a transmembraneous influx of Ca\(^{2+}\). In this way, CyPB might interact with a receptor-chains of myosin may be induced by direct activation of this kinase by the low elevation of intracellular Ca\(^{2+}\) concentration.\(^{18}\) This is consistent with our hypothesis that CyPB-mediated Ca\(^{2+}\) influx is related to the phosphorylation of P-20 in promoting the activation of MLCK. On the other hand, a CyPB-associated protein, termed calcium-signal–modulating cyclophilin ligand, was already reported to participate in the transmission of Ca\(^{2+}\) influx signals in T cells.\(^{34}\) This protein was postulated to regulate intracellular Ca\(^{2+}\) release or generate a signal responsible for opening plasma membrane channels.\(^{34}\) Such a CyPB-binding protein might be expressed at the surface of platelets and be involved in the control of Ca\(^{2+}\) influx in association with extracellular CyPB. In this way, CyPB might interact with a receptor-operated channel and initiate a transmembrane influx of Ca\(^{2+}\), leading to the activation of the Ca\(^{2+}\)-dependent MLCK.

The phosphorylation of P-light chains of myosin and increase in cytosolic free Ca\(^{2+}\) concentration are thought to play a key role in the contractile events associated with platelet shape changes and adhesion.\(^{21,28,29,35}\) It is therefore conceivable to postulate that CyPB-mediated activation of MLCK and Ca\(^{2+}\) entry is related to the enhancing effect of the protein on platelet adhesion.

The addition of CsA significantly reduced the enhanced platelet adhesion. These results indicate that the surface binding and activity of CyPB are related and can be abolished by occupancy of the CsA-binding domain of the protein. This inhibitory effect is likely to be dependent on the concentration of the drug. Most probably, CsA divided between CyPB and other binding sites, eg, platelet proteins and lipids, and large molar excesses of the drug are necessary to form a stable and inactive complexed form of CyPB. Elevated concentrations of CsA are currently measured in blood from transplant recipients, which suggests that the drug could interfere with the biologic activity of CyPB and hemostatic parameters. Many in vivo observations report that CsA increases the risk of thromboembolism in transplant patients, which is mainly related to endothelial damage and abnormalities in platelets and the coagulolytic system. However, data concerning the effect of CsA on hemostasis and platelet functions are often confusing. A prothrombotic effect of the drug was generally reported and attributed to an increased activation of PKC after stimulation by agonists.\(^{36,37}\) In contrast, CsA therapy was reported to increase the levels of antithrombin and protein C, two proteins known to protect against venous thromboembolism.\(^{38}\) Most recently, CsA was demonstrated to have both proaggregatory but anticoagulant effects, which are largely related to platelet reactivity and drug concentration.\(^{39}\) Further studies will therefore be necessary to determine the mechanisms by which CyPB in combination with hemostatic agents could modulate platelet reactivity and coagulation in the presence of CsA.

We reported in a previous work that CyPB levels in plasma from healthy donors were in the range of 5 nmol/L.\(^{14}\) However, we demonstrated here that CyPB-mediated Ca\(^{2+}\) flux generation and increase in platelet adhesion to collagen required higher concentrations. Such concentrations have been measured in the plasma from patients with sepsis, which indicates that CyPB may be secreted as an inflammatory response and exert a cytokine-like activity.\(^{40}\) On the other hand, CyPB was demonstrated to escort and stabilize procollagen chains all along the secretory pathway.\(^{41}\) Thus, CyPB could be secreted together with collagen and accumulate in the subendothelial matrix. As a response to blood vessel offense, platelets adhere to the site of injury and become activated. These events are mainly mediated by the contact of collagen with platelets.\(^{18}\) Therefore, the occurrence of collagen might be associated with the liberation of CyPB from the subendothelial matrix at the site of the injured vessel, which would allow the protein to exert its enhancing effect on platelet adhesion.

In nonexcitable cells such as platelets, extracellular Ca\(^{2+}\) entry is thought to be controlled in part by agonists that act directly on plasma membrane Ca\(^{2+}\) channel.\(^{42}\) Studies with permeabilized platelets demonstrated that guanine nucleotide regulatory proteins were involved in the adhesive process to collagen.\(^{53}\) In this way, the platelet CyPB receptor might be associated with such regulatory proteins and control the activity of a membrane channel. Our objective is now to characterize the platelet receptor as a possible Ca\(^{2+}\) channel-associated protein and to ascertain whether it is related to the lymphocyte type I binding sites. This should allow further understanding on the biologic functions of released CyPB.

**ACKNOWLEDGMENT**

We are grateful to Dr J.J. Huart, Director of the Etablissement de Transfusion Sanguine, Lille, for providing us with blood samples, and to Prof A. Tartar for the synthesis of the peptides used in this work. We also thank Drs J.F. Borel and M. Zurini for generous gifts of cyclosporine derivatives and human recombinant CyPC.

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Cyclophilin B Binding to Platelets Supports Calcium-Dependent Adhesion to Collagen

Fabrice Allain, Sandrine Durieux, Agnès Denys, Mathieu Carpentier and Geneviève Spik