Involvement of two classes of binding sites in the interactions of cyclophilin B with peripheral blood T-lymphocytes

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Cyclophilin B (CyPB) is a cyclosporin A (CsA)-binding protein, mainly associated with the secretory pathway, and is released in biological fluids. We recently reported that CyPB specifically binds to T-lymphocytes and promotes enhanced incorporation of CsA. The interactions with cellular binding sites involved, at least in part, the specific N-terminal extension of the protein. In this study, we intended to specify further the nature of the CyPB-binding sites on peripheral blood T-lymphocytes. We first provide evidence that the CyPB binding to heparin-Sepharose is prevented by soluble sulphated glycosaminoglycans (GAG), raising the interesting possibility that such interactions may occur on the T-cell surface. We then characterized CyPB binding to T-cell surface GAG and found that these interactions involved the N-terminal extension of CyPB, but not its conserved CsA-binding domain. In addition, we determined the presence of a second CyPB binding site, which we termed a type I site, in contrast with type II for GAG interactions. The two binding sites exhibit a similar affinity but the expression of the type I site was 3-fold lower. The conclusion that CyPB binding to the type I site is distinct from the interactions with GAG was based on the findings that it was (1) resistant to NaCl wash and GAG-degrading enzyme treatments, (2) reduced in the presence of CsA or cyclophilin C, and (3) unmodified in the presence of either the N-terminal peptide of CyPB or protamine. Finally, we showed that the type I binding sites were involved in an endocytosis process, supporting the hypothesis that they may correspond to a functional receptor for CyPB.

INTRODUCTION

Cyclophilins are ubiquitous, highly conserved proteins known to be the main binding proteins for the immunosuppressive drug cyclosporin A (CsA) [1,2]. They are also termed peptidylprolyl *cis-trans* isomerases, as they are able to accelerate the *cis-trans* isomerization of artificial substrates and the refolding of denatured proteins. Cyclophilins are represented in human tissues by several highly conserved isoforms. The proteins of this family contain a conserved core domain which supports CsA binding and the catalytic activity, but they differ from each other by variable flanking N- and C-termini that presumably encode distinct subcellular-targeting information [3,4]. The first characterized form, cyclophilin A (CyPA), is an abundant cytosolic protein which is considered to be the main target of CsA *in vivo* [5]. Indeed the complex CyPA–CsA binds to and modulates the activity of calcineurin [6], a critical intermediate in the signal transduction pathway recruited upon T-cell activation. Cyclophilin B (CyPB) [7–9] and cyclophilin C (CyPC) [10] are structurally related to CyPA but contain an N-terminal signal sequence thought to mediate translocation into the endoplasmic reticulum. Both proteins have been reported to be mainly localized within the intracellular vesicles, but they were found secreted in biological fluids such as human milk and plasma [9,11], and in the culture medium supernatants of fibroblasts [12] and leptomeningeal cells [13]. The secretion process is still unknown, but CyPB secreted in human milk was reported to lack the last five C-terminal amino acid residues [14], which are part of a sequence described previously as a retention signal within the endoplasmic reticulum [15]. Moreover, CyPB levels were shown to be increased in plasma from CsA-treated graft recipients [16] and patients suffering from HIV-infection [17] or sepsis [18], suggesting that the protein may act as an inflammatory mediator.

We reported previously the specific binding of CyPB to human peripheral T-lymphocytes and lymphoblastic T-cells [19]. The surface-bound ligand is internalized into T-cells and subsequently degraded within acidic vesicles. CsA-complexed and free CyPB exhibit similar binding activity, suggesting that the drug does not affect the interactions of the protein with cell binding sites [20]. Moreover, the specific binding of the CyPB–CsA complex to the T-cell surface was found to enhance the incorporation of the drug, even in the presence of other blood cells, implying targeting of the CyPB-complexed drug. Finally, we demonstrated that CyPB-complexed CsA retains its immunosuppressive activity and that CyPB enhances the ability of CsA to suppress CD3-induced T-cell proliferation. Therefore, these results led us to postulate that CyPB binding sites, mainly associated with the CD4 helper/inducer T-cell subset, may enhance the immunosuppressive activity of CsA through the specific docking of CyPB-complexed drug on the cell membrane [16,21]. The areas of the protein which interact with the lymphocyte receptor were specified by competitive experiments. Using either recombinant cyclophilin isoforms, CyPB mutants and synthetic peptides corresponding to the specific N- and C-terminal parts of CyPB, we demonstrated that neither CyPA nor CyPC were able to displace CyPB from its lymphocyte binding sites and that the first 24 amino acid residues of CyPB were required for receptor recognition [22]. Nevertheless, the lower inhibitory effect observed with CyPB mutants and the N-terminal peptide has suggested that another part of CyPB may be involved in the interaction with T-cell binding sites. Moreover, CyPB endocytosis involved only 20% of the total ligand binding, indicating that

Abbreviations used: CyPA, CyPB and CyPC, cyclophilin A, B and C; CsA, CsG and CsH, cyclosporin A, G and H; DPBS, Dulbecco's PBS; GAG, glycosaminoglycan(s); HBP, heparin-binding protein(s); CyCAP, CyPC-associated protein.

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other binding sites might exist that do not participate in this process.

CyPB is a highly basic protein and its purification by affinity chromatography on heparin- and DNA-cellulose has already been reported [23]. In particular, the N-terminal extremity, which was shown to be involved in cellular CyPB binding, is highly cationic as it includes many lysine residues and may interact with negatively charged molecules on T-cells through electrostatic interactions. Glycosaminoglycans (GAG), mainly of chondroitin- and heparan-sulphate families, are the major sources of all surface negative charges. Within the lymphoid population these molecules are, however, poorly represented [24], but some reports have described their role in the interactions with heparin-binding proteins (HBP) which exhibit inflammatory [25] or immunomodulatory properties [26,27]. We then looked for the potential involvement of surface GAG in cellular CyPB binding. We report that CyPB interacts, via its N-terminal extremity, with heparin-like molecules present on the T-cell membrane. In addition, we characterized the presence of a second set of CyPB binding sites, which probably involve a CsA-binding domain. These results indicate that CyPB may be considered as a new member of the expanding family of HBP and raises the interesting possibility that this protein may have selective activity on T-lymphocytes through interactions with membrane binding sites.

MATERIALS AND METHODS

Materials

Recombinant human CyPA and CyPB were purified as described previously [9,28]. Recombinant human CyPC [29] and cyclosporin derivatives (CsA, CsG and CsH) [30] were a generous gift from Novartis (Basel, Switzerland), and [^{3}H]CsA (10.1 Ci/mmol) was from Sandoz-France (Ruel-Malmaison, France). N- and C-terminal peptides of CyPB were synthesized as described in [11]. Heparan sulphate was purchased from Fluka (Buchs, Switzerland) and heparin was from Roche Pharma AG (Reinach BL, Switzerland). Chondroitin sulphate A/B mixture was isolated from ovine cartilage and kindly provided by Dr. Michalski (Unité Mixte de Recherche du Centre National de Recherche Scientifique no. 111, Université des Sciences et Technologies de Lille, Villeneuve d'Ascq, France). Heparinase type I (EC 4.2.2.7), chondroitinase ABC (EC 4.2.2.4), neuraminidase (EC 3.2.1.18), trypsin (EC 3.4.21.4), protamine, hyaluronate and chondroitin sulphate C were from Sigma Chemical Co. (St Louis, MO, U.S.A.). Both GAG-degrading enzyme preparations were provided free of any contaminating enzymes such as β-galactosidase, β-hexosaminidase, α- and β-mannosidases and proteases, by the manufacturer. ^{125}I-CyPB and the CyPB–[^{3}H]CsA complex were prepared as described previously [19,20]; the specific radioactivities were estimated at $(4–6) \times 10^6$ c.p.m./μg of CyPB and $(0.8–1) \times 10^6$ c.p.m./μg of CyPB respectively. Fluorescein-S-CyPB was prepared as described previously [21].

Cells

Human citrated venous blood was obtained from the Etablissement de Transfusion Sanguine (Lille, France). Peripheral blood mononuclear cells were isolated by density centrifugation on Lymphoprep separation medium (Nycomed, Oslo, Norway) and T-lymphocytes were collected as described in [20]. The purity of the population and the cell viability were estimated by flow cytofluorimetry analysis. Cells were then resuspended in Dulbecco's PBS (DPBS; Sigma), supplemented with 0.5 % (w/v) BSA, to a final density of 4×10^6 cells/ml.

Enzyme treatments

Peripheral blood T-lymphocytes were washed twice with DPBS and incubated for 3 h at room temperature in RPMI-BSA containing either heparinase type I (0.5 unit/ml) or chondroitinase ABC (10 units/ml), or both. At the end of the incubation period, cells were washed twice with cold DPBS and directly used for binding experiments. Contamination of GAG-degrading enzyme preparations by proteases was controlled by measuring the degradation of FITC-labelled casein [31] under the same conditions used for treating the cells. The absence of residual protease activity was further ensured by comparing the ligand binding to treated cells either in the absence or presence of protease inhibitors (0.5 mM PMSF, 10 μM pepstatin A, 10 μM leupeptin, 1 μM aprotinin). Lymphocytes treated with neuraminidase (0.1 unit/ml) for 30 min at 37 °C were also used to assess the susceptibility of CyPB binding sites to digestion by this enzyme. Trypsin treatment was performed by incubating cells for 30 min at 4 °C in the presence of trypsin (1 mg/ml). Enzymic treatment was stopped by washing the cells with RPMI supplemented with 10 % (v/v) heat-inactivated fetal-calf serum, and CyPB binding was assessed in the same medium to avoid potential degradation of the ligand by residual protease. In all cases, the conditions of treatment were optimized to obtain maximal enzymic activities without affecting cell integrity.

Ligand binding to heparin-Sepharose column

Cyclophilins were applied to a 1 ml heparin-Sepharose column (Pharmacia, Uppsala, Sweden) equilibrated with 20 mM phosphate buffer, pH 7.2, and eluted with the same buffer in the presence of a gradient of NaCl from 100 nM to 1 M. The profile of elution was monitored by measuring the absorbance at 260 nm, and the presence of cyclophilins in the different fractions was assessed by Coomassie Blue staining after SDS/PAGE. The relative affinity of each isoform was estimated based on the concentration of salt required to eluate the ligand from the column. Specificity of CyPB binding to heparin was studied by simultaneously incubating 100 μl of heparin-Sepharose with 50 nM ^{125}I-CyPB and various concentrations of competitors. After a 1 h incubation at room temperature, the gel was extensively washed with DPBS, pH 7.4, and the remaining heparin-associated radioactivity was measured using a model 1282 Compugamma LKB-Wallac counter.

Surface binding assay of ^{125}I-CyPB

CyPB binding to peripheral blood T-lymphocytes was investigated as reported previously [19,20], with the following modifications. The specificity of CyPB binding was studied by incubating peripheral blood T-lymphocytes in the presence of 50 nM ^{125}I-CyPB and increasing concentrations of competitors for 1 h at 4 °C. After washing off the excess unbound ligand with cold DPBS, cells were treated with a buffer containing a high NaCl concentration, and the radioactivity was measured in the cell pellets (NaCl-resistant fraction) and eluates (NaCl-sensitive fraction). The surface binding assay was performed by incubating cells with increasing concentrations of ^{125}I-CyPB. Non-specific binding was determined in parallel experiments in the presence of a 200-fold molar excess of unlabelled CyPB. After a 1 h incubation, the supernatant was removed and the cells were treated as above with a high NaCl concentration to discriminate between the binding of the ligand to NaCl-sensitive and NaCl-resistant binding sites.

Flow cytometry analysis

Cells were washed in cold DPBS and resuspended in DPBS-BSA to obtain a final cell count of 2×10^5 cells/sample. Fluorescein-S-CyPB was added to a final concentration of 100 nM for 1 h at 4 °C. Non-specific binding was determined in parallel experiments in the presence of a 100-fold molar excess of unlabelled CyPB. After incubation at 4 °C, cells were either washed with cold DPBS, or treated with a high NaCl concentration buffer, and then resuspended in DPBS for analysis. Data were monitored on a Becton Dickinson FACScan cytofluorimeter equipped with an argon ion laser at 488 nm. The light-scatter channels were set on a linear gain, and the fluorescence channels were set on a logarithmic scale. Cells were gated for forward- and side-angle light scatters, and 10000 fluorescent particles of the gated population were analysed. The data collected with logarithmic amplification were analysed by the Becton-Dickinson computer.

Internalization kinetics of ^{125}I-CyPB

Two different methods were applied to measure the kinetics of the internalization of ^{125}I-CyPB into peripheral blood T-lymphocytes. In both experiments, cell suspensions (4×10^6 cells/ml) were incubated in RPMI 1640 medium containing 20 mM HEPES, pH 7.4, supplemented with 0.5 % (w/v) BSA at 37 °C. ^{125}I-CyPB was added to a final concentration of 50 nM. In the first method, cells were allowed to bind ^{125}I-CyPB for various times at 37 °C in the absence or presence of a 250-fold molar excess of protamine. At various times, aliquots of the cell suspension were removed, washed and treated for 10 min at 4 °C with a buffer containing 0.5 M NaCl/0.2 M glycine, pH 4. Finally, cell-associated and cell-released radioactivities were counted in the cell-pellet and acidic washes respectively. In the second method, cells were first allowed to bind ^{125}I-CyPB at a final concentration of 50 nM for 1 h at 4 °C, until the binding site capacity reached a steady state. After washing off ligand bound to NaCl-sensitive binding sites, cells were resuspended in pre-warmed binding medium at 37 °C. Cell-free supernatants were collected and the cells were treated with a buffer containing 0.5 M NaCl/0.2 M glycine, pH 4, as described above. The surface-bound, internalized and released ^{125}I-CyPB were counted in acid-sensitive eluate, cell extract and incubation medium respectively. To determine whether internalized CyPB is released in a degraded form, supernatants were precipitated with 10 % (v/v) trichloroacetic acid for 20 min at 4 °C, and then centrifuged for 30 min at 10000 ***g***; the radioactivity in intact and degraded ^{125}I-CyPB was counted in precipitated and soluble extracts respectively. For all experiments, non-specific binding was determined as described above.

Statistical analysis

Values are means ± S.D. for the indicated number of independent experiments. Statistical significance was determined using the Student's *t* test for unpaired data, and values of $P < 0.05$ were considered to be significant.

RESULTS

Binding of CyPB to heparin-Sepharose column

The relative affinity of cyclophilins for heparin was analysed based on the concentration of salt required to elute proteins from heparin affinity column. CyPB was eluted as a single peak from a heparin-Sepharose column with 0.6 M NaCl, while CyPA and CyPC were rapidly eluted with 0.2 M and 0.25 M NaCl respectively. These results demonstrate that CyPB, but not other isoforms, strongly interacts with heparin, suggesting that these interactions may occur under physiological conditions. The specificity of CyPB binding was examined by competitive binding experiments in the presence of various GAG (Figure 1A). As expected, ^{125}I-CyPB binding to heparin-Sepharose was highly reduced with increasing concentrations of free heparin. For instance, when 1 μg of ^{125}I-CyPB was loaded onto 300 μg of Sepharose-linked heparin, the amount of free heparin required for 50 % inhibition of ^{125}I-CyPB binding was 250 μg. Heparan sulphate, chondroitin sulphate A/B mixture and chondroitin sulphate C were less active competitors, since the amount of these compounds required for 50 % inhibition of binding was 3–4-fold higher than that of free heparin. Moreover, the non-sulphated GAG, hyaluronate, failed to compete with ^{125}I-CyPB for binding to heparin-Sepharose. These results demonstrated that CyPB potently interacts with heparin, and to a lower extent with other sulphated GAG, implying that it may be considered as a HBP.

To examine the areas of CyPB involved in these interactions, ^{125}I-CyPB was incubated with heparin-Sepharose in the presence

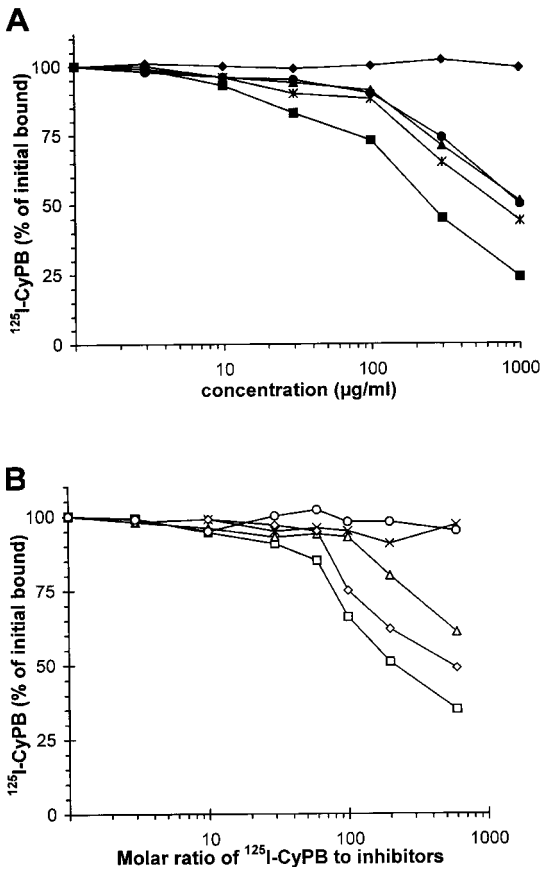

Figure 1 Specificity of CyPB binding to Heparin-Sepharose

^{125}I-CyPB (1 μg) was applied to a heparin-Sepharose column (300 μg of conjugated heparin) in the presence of increasing concentrations of competitors. After washing with DPBS, remaining radiolabelled ligand was eluted with 0.6 M NaCl buffer (3 ml) as described in the Materials and methods section, and the radioactivity was counted in aliquots (500 μl) and compared with the control obtained in the absence of competitors. 100% corresponds to 512700 ± 23600 c.p.m. Three separate experiments were performed in triplicate for each competitor. (**A**) Effects of heparin (■), heparan sulphate (●), chondroitin sulphate C (✱), chondroitin sulphate A/B mixture (▲) and hyaluronate (◆) on ^{125}I-CyPB binding to heparin-Sepharose. (**B**) Effects of unlabelled CyPB (□), N-terminal peptide (△) and C-terminal peptide (×) of CyPB, CsA (○) and protamine (◇) on ^{125}I-CyPB binding to heparin-Sepharose.

of increasing concentrations of various putative competitors (Figure 1B). Protamine, a well known polypeptide interacting with cationic molecules such as DNA and heparin, was as efficient as unlabelled CyPB in inhibiting the binding of ^{125}I-CyPB. Indeed, 50 % inhibition of binding was obtained with a 200- and 600-fold molar excess of CyPB and protamine respectively, demonstrating that both proteins shared similar binding properties to heparin. On the other hand, the peptide corresponding to the N-terminal extension of CyPB also reduced the binding of ^{125}I-CyPB, but with a 5-fold lower efficiency by comparison with CyPB. In contrast, the C-terminal peptide did not exhibit any inhibitory effect. CsA, which interacts with the conserved catalytic domain of CyPB, had no competitive effect on the binding of ^{125}I-CyPB to heparin. Moreover, the CyPB–[^{3}H]CsA complex was eluted from heparin-Sepharose in a single peak at the same NaCl concentration as that required for uncomplexed CyPB. The free radiolabelled drug was not retained, implying that the CsA-binding domain of CyPB was not involved in these interactions. Finally, we checked that the elution profile of the CyPB–[^{3}H]CsA complex on a hydrophobic LH-20 column was not modified by the addition of free heparin, further supporting the hypothesis that the ternary complex, heparin–CyPB–CsA, may be formed (results not shown). Taken together, these results indicate that CyPB probably interacts with heparin through its N-terminal specific extension, allowing the CyPB–CsA complex to exhibit similar binding activity to free CyPB.

Characterization of two CyPB binding sites on the T-lymphocyte surface

To ascertain whether the interactions of CyPB with heparin are related to the cellular binding properties of this protein, we analysed the sensitivity of CyPB binding sites present on T-cells to the NaCl washing procedure. To determine whether CyPB could be removed from the cell surface by this procedure, peripheral blood T-lymphocytes were allowed to bind ^{125}I-CyPB and were then washed with DPBS containing increasing concentrations of NaCl. Most of the surface-bound ^{125}I-CyPB could be released by 0.6 M NaCl. However, no more than 70 % of the ligand was recovered in the eluates, even in the presence of 1 M NaCl. As CyPB bound to the heparin-Sepharose column was eluted with a 0.6 M NaCl wash, the remaining binding of ^{125}I-CyPB to the cell surface could correspond to a NaCl-resistant binding site. We then analysed the sensitivity of CyPB binding to acidic pH. It has been shown in the literature that the binding of proteins to their receptors can be disrupted at low pH, without affecting interactions with GAG [32]. One-step mild-acid treatment with cold PBS/citrate, pH 4, led to approx. 34 % release of surface-bound ^{125}I-CyPB from peripheral blood T-lymphocytes, which might correspond to the ligand bound to NaCl-resistant sites. However, a two-step washing procedure with 0.6 M NaCl followed by PBS/citrate, pH 4, did not allow the release of the whole surface-bound ligand. Less than 15 % was recovered in the acidic wash eluate, which might be due to the rebinding of released ligand to NaCl-sensitive binding sites. Indeed, when peripheral blood T-lymphocytes were washed with 0.5 M NaCl/0.2 M glycine buffer, pH 4, allowing the addition of both high salt concentration and low pH actions, all bound ^{125}I-CyPB was released from the cell membrane. Therefore, these results suggest that the binding of CyPB to peripheral blood T-lymphocytes involves two classes of binding sites, which can be discriminated by their sensitivity to high NaCl concentrations and acidic pH.

Using flow cytometry, we demonstrated recently that CyPB binding sites were heterogeneously expressed on the surface of a sub-population of T-cells [21]. Similar binding experiments were reproduced here with cells either untreated (Figure 2A), or treated with 0.6 M NaCl buffer (Figure 2B). Addition of fluorescein-S-CyPB to untreated cells resulted in an asymmetrical fluorescence flow profile. The first peak of fluorescence corresponded to cells which did not bind CyPB, as it overlaid that of the control cells. As expected, the second peak displayed a broad distribution, that has been related to the highly variable expression of CyPB binding sites on these cells. In contrast, after an NaCl wash, the fluorescence flow profile was quite different. The binding of fluorescein-S-CyPB resulted in a single peak of fluorescence, distinct from the non-specific binding peak and without a broad distribution. Such a profile suggests that NaCl-resistant binding sites are poorly expressed on T-cells, and that the broad heterogeneity of CyPB binding may be related to the highly variable expression of NaCl-sensitive sites on T-cells.

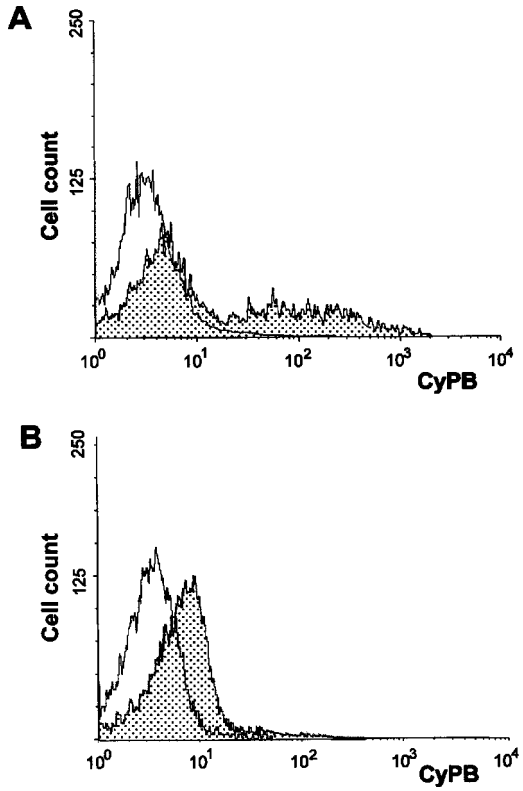

Figure 2 Sensitivity of the surface binding of CyPB to a 0.6 M NaCl wash

T-cells were incubated in the presence of 100 nM fluorescein-S-CyPB. After washing, with either DPBS (**A**) or 0.6 M NaCl buffer (**B**), surface binding of CyPB (filled peak) was analysed by flow cytometry, as described in the Materials and methods section. Non-specific binding (open peak) was determined in the presence of a 100-fold molar excess of unlabelled CyPB. Data are representative of separate experiments with similar results.

To support this hypothesis, the binding capacity of CyPB to both binding sites was investigated using ^{125}I-CyPB (Table 1). Cells were allowed to bind various concentrations of ^{125}I-CyPB. As above, the two binding sites were separated by washing the cells with 0.6 M NaCl at pH 7.4; the radioactivity counted in the cell pellets and eluates related to the binding of CyPB to NaCl-resistant and NaCl-sensitive sites respectively. In both fractions, CyPB binding was specific since a 200-fold molar excess of unlabelled ligand inhibited ^{125}I-CyPB binding by 75–85 %. After subtraction of the non-specific interactions from total counts, the binding on both sites was found to be concentration-dependent

Table 1 Binding constants for CyPB interactions with type I and type II binding sites on T-lymphocytes

Peripheral blood T-cells were incubated in the presence of increasing concentrations of ^{125}I-CyPB for 1 h at 4 °C. After removal of unbound ligand, cell-associated radioactivity was either counted directly (total) or separated between 0.6 M NaCl resistant (type I sites) and sensitive (type II sites) fractions. Binding parameters were analysed according to the method of Scatchard. Values are means ± S.D. from five separate experiments conducted with T-cells from separate individuals.

Binding sites	K_d (nM)	Number of sites per cell
Total binding sites	12 ± 3	94200 ± 33000
Type I binding sites	14 ± 4	25400 ± 10000
Type II binding sites	10.5 ± 1.5	68700 ± 26800

and saturable. Analysis of the ^{125}I-CyPB remaining bound to cell surface, by the Scatchard procedure, showed the presence of only one binding site, referred to as type I, with a K_d of 14 ± 4 nM, and an estimated capacity varying from 10000–36000 sites per cell. In the same way, Scatchard analysis of the ^{125}I-CyPB released in the NaCl washes indicated the presence of one binding site, referred to as type II, with a K_d of 10.5 ± 1.5 nM. The number of type II binding sites varied from 31200–106000 per cell, reflecting a large inter-individual heterogeneity ($n = 5$) (Table 1). In both cases, the K_d values were close to the mean K_d value (12 ± 3 nM), which explains why these sites were not discriminated by Scatchard analysis previously [19,20]. In contrast, the binding capacity of CyPB on these sites is quite different. Type II binding sites represented from 65–80 % of the total binding sites, confirming that most of the binding capacity of CyPB to T-cells may be related to its expression.

Interactions of CyPB with GAG

After demonstrating that protamine blocked CyPB binding to heparin, we then investigated whether this polypeptide could inhibit the interactions of CyPB with cell-surface binding sites. As expected, addition of protamine to the binding mixture, containing ^{125}I-CyPB, partially competed with CyPB binding on the cell surface. When both binding sites were separated by 0.6 M NaCl, only the type II binding sites were found to be sensitive to protamine. Indeed, the interactions of ^{125}I-CyPB with NaCl-sensitive sites were inhibited by 75 % with 12.5 μM protamine, while the binding to NaCl-resistant sites remained unchanged (Table 2). These results indicated that CyPB binding to type II sites could be mediated by interactions with a polyanionic domain from GAG chains present on the T-cell membrane. To further investigate whether CyPB binding was dependent on the interactions with GAG, cells were treated with heparinase I, chondroitinase ABC, or both (Table 2). The binding of ^{125}I-CyPB was decreased by more than 50 % on peripheral blood T-lymphocytes treated with heparinase, in comparison with untreated cells, while chondroitinase treatment removed 25 % of the CyPB binding capacity. Moreover, the combination of the two enzymes reduced CyPB binding to a similar extent to the 0.6 M NaCl treatment, suggesting that the type II binding sites may correspond to heparin/heparan sulphate and chondroitin sulphate molecules. To ensure that the loss in the cellular binding capacity of CyPB was related to the effective removal of membrane GAG, we examined the possibility of the presence of adventitious protease activity under the same conditions used for treating the cells. As expected, protease activity was undetectable in our GAG-degrading preparations. Moreover, treatment of cells with heparinase and/or chondroitinase in either the presence or absence of protease inhibitors resulted in a similar decrease in the binding of ^{125}I-CyPB (results not shown). It is therefore highly improbable that the observed effect produced on the binding capacity of CyPB by GAG-degrading enzymes could be due to contaminating proteases. In contrast, treatment with neuraminidase did not modify CyPB binding, ruling out the involvement of sialic acid in these interactions. When cells were treated with trypsin, the specific binding of ^{125}I-CyPB was completely abolished. These results indicate that the type II binding sites may correspond to membrane GAG present on peripheral blood T-lymphocytes. These GAG structures would be mainly represented by heparin-like molecules, probably associated to cell membrane proteoglycans. On the other hand, the type I binding sites correspond to distinct proteins, since the binding to both sites was abolished after trypsin treatment.

Table 2 Discrimination of CyPB binding sites on T-lymphocytes

Peripheral blood T-lymphocytes (2×10^6 cells per sample), pretreated with these agents, as described in the Materials and methods section, were incubated in the presence of 50 nM ^{125}I-CyPB for 1 h at 4 °C. After washing, the surface-bound radioactivity was measured and compared with controls, obtained in the absence of any agents, as the percentage remaining cellular binding of CyPB. 100 % corresponds to 22400 ± 3400 c.p.m. Data are expressed as mean values from three separate representative experiments performed in triplicate.

Treatment of lymphocytes	Cellular CyPB binding
Control	100 %
0.6 M NaCl wash	25 ± 5 %
Protamine (12.5 μM)	30 ± 4 %
Enzymes	
Heparinase I	46 ± 5 %
Chondroitinase ABC	75 ± 10 %
Heparinase I + Chondroitinase ABC	32 ± 9 %
Neuraminidase	100 ± 10 %
Trypsin	5 ± 1 %

Discrimination of binding domains within CyPB

In order to determine the areas of CyPB involved in the interactions with both binding sites, we first examined the competitive properties of CyPA, CyPC and peptides corresponding to the N- and C-terminal extensions of CyPB. To this end, cells were incubated with ^{125}I-CyPB in the presence of increasing concentrations of each competitor. Since both binding sites can be effectively separated by a 0.6 M NaCl wash, this procedure was used to study the ability of the competitors to reduce the binding of ^{125}I-CyPB to type I or II sites. As shown in Figure 3(A), ^{125}I-CyPB binding to type II sites was inhibited by 75 and 85 % in the presence of a 25- and 250-fold molar excess of unlabelled CyPB respectively, and by 55 and 80 % in the presence of the same molar excess of N-terminal peptide. The concentrations for 50 % inhibition of binding were estimated to be 0.35 and 1 μM respectively, indicating that the N-terminal peptide has a 3-fold lower affinity for type II binding sites than CyPB. In contrast, neither the C-terminal peptide, nor CyPA and CyPC reduced the binding of ^{125}I-CyPB to these sites, indicating that the specific N-terminal extension of CyPB, which is different from those of CyPA and CyPC, is required for the interaction with the type II binding sites. In contrast, the same competitors showed quite different inhibitory effects on CyPB binding to type I sites (Figure 3B). None of the peptides corresponding to the C- and N-terminal extensions of CyPB exhibited any competitive activity. ^{125}I-CyPB binding was inhibited by 70 and 85 % in the

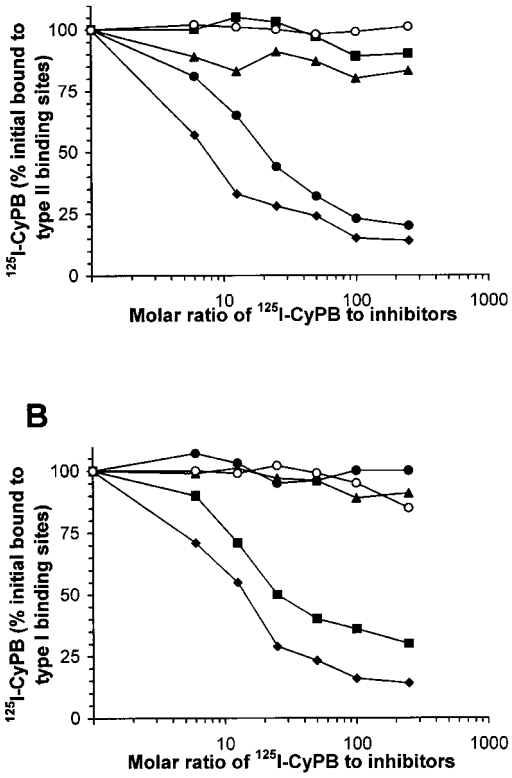

Figure 3 Competitive experiments for CyPB binding to T-lymphocytes

Competitive binding experiments were performed by incubating T-cells (2×10^6 cells per sample) in the presence of 50 nM ^{125}I-CyPB and either unlabelled CyPB (◆), CyPA (▲), CyPC (■) or N-terminal peptide (●) and C-terminal peptide (○) of CyPB at the indicated molar excess. After 1 h at 4 °C, cells were washed twice with DPBS and once with 0.6 M NaCl. Radiolabelled CyPB present in released (**A**) and remaining cell-associated (**B**) fractions was measured and compared with a control obtained in the absence of any competitor. 100% of released and remaining cell-associated fractions corresponds to 19200 ± 4600 c.p.m. and 5900 ± 800 c.p.m. respectively. Data represent the mean values of triplicates at each ligand concentration.

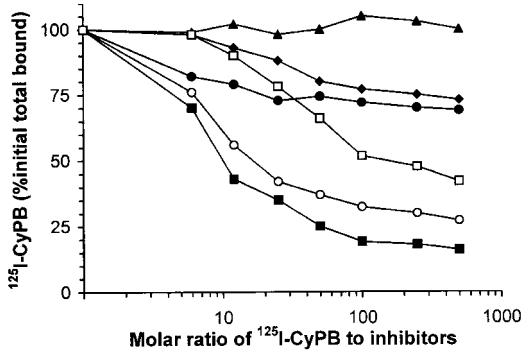

Figure 4 Inhibition of CyPB binding to T-lymphocytes by cyclosporin derivatives

Lymphocytes were incubated for 1 h at 4 °C in the presence of 50 nM ^{125}I-CyPB and either unlabelled CyPB (■), CsA (●), CsG (◆), CsH (▲), protamine (□) or protamine/CsA combination (○) at the indicated molar excess. Data represent the percentages of initial surface-bound ^{125}I-CyPB and are expressed as the mean value of triplicates at each ligand concentration. 100% corresponds to 23100 ± 4900 c.p.m.

presence of a 25- and 250-fold molar excess of unlabelled CyPB respectively, and by 50 and 70% in the presence of the same concentrations of CyPC. The concentrations for 50% inhibition of binding were estimated to be 0.65 and 1.25 μM respectively, showing that CyPC interacts with type I binding sites with a 2-fold lower affinity in comparison with CyPB. In contrast, increasing concentrations of CyPA were ineffective in inhibiting ^{125}I-CyPB binding to these sites. These results suggest that the type I binding sites are shared by CyPB and CyPC, and that these interactions probably involve a conserved domain in both proteins.

To further confirm the involvement of a conserved domain of CyPB in the binding to type I binding sites, competitive binding experiments were carried out with CsA to test whether the occupancy of the conserved binding domain could reduce the interactions. Thus, the total binding capacity of ^{125}I-CyPB was inhibited by 25% by a 10-fold molar excess of CsA, but higher concentrations of the drug did not modify this level of inhibition (Figure 4). CsG, a less cyclosporin-active derivative [30], required higher concentrations to reduce the binding of ^{125}I-CyPB to the same extent as CsA. In contrast, the non-active CsH derivative [30], which does not bind to CyPB, failed to prevent the ligand binding, indicating that the occupancy of the conserved domain of this protein was responsible for the partial inhibition of CyPB binding (Figure 4). However, association of the 0.6 M NaCl wash and high levels of CsA affected cell integrity, ruling out the use of this procedure to discriminate between the inhibitory effects of the drug on the two binding sites. To bypass this problem, cells were incubated together with protamine, since we demonstrated above that this polypeptide strongly inhibited the binding of CyPB to type II binding sites. As expected, the combination of increasing levels of CsA and protamine was much more effective at reducing ^{125}I-CyPB binding than CsA alone, and the resulting inhibition was close to that observed in the presence of unlabelled CyPB (Figure 4). Therefore, these results indicate that CsA has an additive inhibitory effect with protamine, supporting the hypothesis that CyPB interacts with T-cells through two different domains of the protein involved in the selective recognition of both types of binding sites.

Endocytosis of CyPB

In a previous study, we demonstrated that surface bound CyPB was internalized into T-cells and subsequently released into medium in a degraded form [19]. These experiments were reproduced here in order to determine the role of each binding site in the endocytosis process of CyPB (Figure 5A). When incubated at 37 °C in the presence of 50 nM ^{125}I-CyPB, cell-associated radioactivity reached a plateau after 20 min of incubation, that corresponded to 200 fmol/10^6 cells. In parallel, the fraction of internalized ligand slowly increased but did not exceed 25% of the cell-associated radioactivity, demonstrating that most of the surface-bound ^{125}I-CyPB did not enter into the cells. We then reproduced these experiments in the presence of protamine (12.5 μM), in order to block the interactions with heparin-like molecules present on the membrane of T-cells. In these conditions, cell-associated radioactivity was strongly reduced, while the fraction of internalized ligand was unchanged. Interestingly, the two fractions corresponded to 50 and 35 fmol/10^6 cells respectively after 20 min of incubation, indicating that most of the ^{125}I-CyPB bound to the type I sites was rapidly internalized within the cells (Figure 5A). To further support this result, peripheral blood T-lymphocytes were incubated at 4 °C for 1 h in the presence of 50 nM ^{125}I-CyPB, washed with 0.6 M NaCl and rapidly exposed at 37 °C to determine the fate of the remaining ligand bound to the type I sites (Figure 5B).

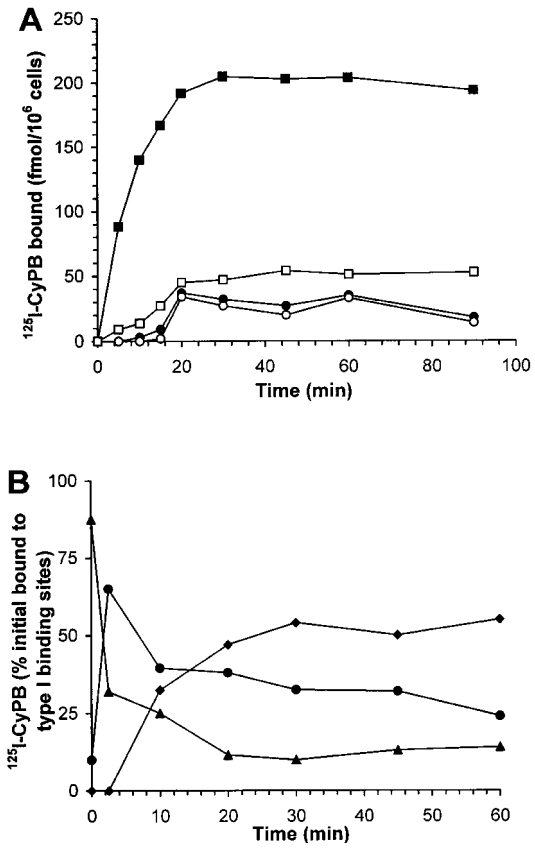

Figure 5 Endocytosis of CyPB into T-lymphocytes

(**A**) Time course of internalization of ^{125}I-CyPB into T-lymphocytes. Cells were incubated with 50 nM ^{125}I-CyPB at 37 °C in the absence (■,●) or presence of protamine (12.5 μM) (□,○). At indicated times, cells were washed either with DPBS (□,■) or 0.5 M NaCl/0.2 M glycine, pH 4 (○,●) as described in the Materials and methods section and cell-associated radioactivities were counted. (**B**) Time course of the fate of ^{125}I-CyPB bound to type I binding sites. After a 1 h incubation at 4 °C, lymphocytes (4×10^6 cells per sample) were washed with 0.6 M NaCl and were allowed to internalize ^{125}I-CyPB at 37 °C. At indicated times, aliquots were removed and incubation supernatants, eluates from acid-treated cells and cell-pellet radioactivities were counted. Ordinates represent the percentages of the total radioactivity initially bound to type I binding sites at 4 °C which was segregated within the cells (●), surface bound (▲) and released in the incubation medium in a degraded form (◆). 100% corresponds to 15500 + 950 c.p.m. Data are representative of separate experiments performed in triplicate.

During the first minutes, the surface bound fraction decreased rapidly, as the internalized fraction increased simultaneously to reach almost 60% of the initially surface-bound ligand after 3 min of incubation. Thereafter, the internalized fraction decreased slowly, as degraded ^{125}I-CyPB started to appear in the incubation medium. These results strongly support the hypothesis that only type I binding sites are involved in the endocytosis process of the ligand, explaining why we previously reported that CyPB was only partially internalized within T-cells [19].

DISCUSSION

In previous work, we showed that CyPB specifically binds to the surface of human T-cells, even in the presence of CsA [19,20], and that these interactions involve, at least in part, the N-terminal extension of the ligand [22]. In an attempt to further specify the binding sites for CyPB on peripheral blood T-lymphocytes, we have first investigated the ability of this protein to interact with heparin. Indeed, Galat and Bouet [23] have reported that bovine CyPB has a strong tendency to bind to cation exchangers including DNA and heparin, raising the interesting possibility that this interaction might be involved in CyPB cell-surface binding. Thus, we demonstrated that, in spite of the high sequence similarity between cyclophilin isoforms, only CyPB interacts with heparin with high avidity. The interactions could be inhibited by a synthetic peptide corresponding to the N-terminal extension of CyPB, but not by CsA, confirming that the conserved CsA-binding domain is not involved in the binding of CyPB to heparin. The N-terminal extension is highly specific for CyPB and contains the sequence ^{1}DEKKKGPK^{8}, which resembles the consensus sequences BBXB and BXXBBXB (where B is a basic residue) involved in GAG recognition and found in many other HBP [33]. Moreover, the fact that other sulphated GAG exhibit a lower avidity for CyPB by comparison with heparin, is also a common feature to most of the known HBP. These results strongly suggest that the cellular binding properties of CyPB might be related to interactions with heparin-like molecules present on T-cell membrane.

We then analysed the sensitivity of cellular CyPB binding to a NaCl wash and demonstrated that most of the surface-bound ligand was also removed at 0.6 M NaCl, confirming that CyPB probably interacts with GAG present on the T-cell membrane. In addition, the presence of a resistant fraction led to the hypothesis of a second, less represented, type of binding site. This finding is, however, not surprising since the existence of two types of binding sites has been already demonstrated for many HBP [34,35]. The most abundant binding represents interactions with GAG expressed on the cell membrane, while the second corresponds in most cases to a signalling receptor for the HBP.

The less represented binding sites of CyPB, which we named type I sites, are characterized by a K_d value of 14 ± 4 nM, and there are less than 30000 sites per cell. These type I receptors interact with the conserved CsA-binding domain of CyPB, since we showed that the interactions can be inhibited in the presence of the drug and can compete with CyPC. Indeed, CyPC reduces the binding of CyPB to the type I sites, which are poorly represented, without affecting interactions with GAG, explaining why its competitive properties on the total binding capacity of CyPB could not be discriminated previously [22]. On the other hand, CyPA was confirmed to be unable to compete with CyPB binding. However, Sherry et al. [36] have recently characterized the presence of a signalling receptor for CyPA on T-lymphocytes that probably interacts through its CsA-binding domain. Thus, a possibility remains that CyPA binds to the CyPB receptor with a lower affinity than CyPB and CyPC, explaining the inability of CyPA to compete with CyPB. Therefore, in agreement with our results, the receptor for CyPA described by Sherry et al. [36] might correspond to the type I binding site of CyPB, and cyclophilins may be classified as CyPB, CyPC and CyPA in accordance with the relative binding to this lymphocyte receptor. Such a discrimination between cyclophilin isoforms in the recognition of a membrane protein has also been reported for CyPC [37]. A 77 kDa protein, termed CyCAP, for CyPC-associated protein, was found to specifically interact with CyPC. CsA was shown to inhibit these interactions while CyPA was unable to interact with this receptor. The areas of CyPC which interacted with CyCAP were thought to be localized in a loop without any sequence similarity with the other cyclophilin isoforms and localized close to the enzymic site, explaining the specificity of recognition and the inhibition by CsA through steric hindrance. However, this restricted binding might also be related to large differences in the affinity of cyclophilin isoforms for CyCAP, raising the interesting possibility of the existence of a family of cyclophilin receptors with redundancy in their binding specificity.

The other CyPB binding sites, which we named type II sites, correspond to GAG chains of proteoglycans present at the surface of T-cells. This conclusion rests on the observations that cellular CyPB binding is inhibited by high concentrations of NaCl and protamine, is eliminated by treatment with GAG-degrading enzymes and trypsin, and involves the N-terminal extension of CyPB, which was demonstrated to be required for the interactions with heparin. This basic extremity is localized on the opposite side of the CsA binding pocket [38], explaining the absence of an inhibitory effect of CsA on CyPB binding to the type II binding sites, and the ability of the CyPB–CsA complex to conserve its binding efficiency. The type II binding sites are characterized by a K_a value of 10.5 ± 1.5 nM, and number around 69000 sites per cell. However, this binding capacity showed broad intra- and inter-individual variabilities. Recently, we reported that the distribution of CyPB binding sites showed a broad heterogeneity in the different subsets of peripheral blood T-lymphocytes, with a more pronounced expression on the CD4-positive cells [21]. Therefore, it is conceivable to postulate that this heterogeneity may be correlated chiefly with the variable expression of the type II binding sites.

In order to approach the biological significance of these two types of binding sites, we analysed their involvement in the ligand endocytosis. We have shown previously that CyPB was internalized into T-cells and subsequently degraded within acidic compartments, such as lysosomes [19]. However, this endocytosis was partial and never involved more than 25% of the surface-bound ligand. Here, we reproduced similar experiments and demonstrated that only the type I binding sites were required for CyPB internalization. Indeed, an endocytosis process occurred even in the presence of protamine or after removal of the ligand bound to type II sites. Moreover, the internalized fraction corresponds to the binding capacity of type I sites, explaining why the endocytosis process was found previously to involve less than 25% of the total binding capacity [19]. While type II binding sites are not involved in CyPB internalization, they may participate in CyPB-mediated incorporation of CsA within T-cells. Indeed, we reported previously that the CyPB–CsA complex interacts with the T-cell membrane to an extent similar to CyPB, allowing drug targeting to sensitive lymphocytes and an enhanced immunosuppressive activity [16,20,21]. We determined here that the CyPB–CsA complex could interact with heparin to the same extent as CyPB, suggesting that interactions between CsA-complexed CyPB and lymphocyte GAG may occur. Therefore, the binding of the CyPB–CsA complex and the resulting enhanced activity of the drug are probably due to the interactions with type II sites, allowing a specific docking of the complexed drug onto T-lymphocytes. This phenomenon is likely to increase the local concentration of the drug around T-lymphocytes and the elevation of the intracellular CsA level probably results from the dissociation of the membrane-bound complex and the crossing of the free drug through the lipid bilayer [39].

The interactions of CyPB with heparin are of particular interest in view of the prominent role of this GAG in physiological processes, raising the possibility that it may regulate some of the actions of CyPB *in vivo* [40]. Moreover, the role of cellular GAG in HBP function was suggested to be related, for ensuring localization or local presentation. In this way, interactions of many growth factors and cytokines with GAG present on the cell membrane were shown to be a pre-requisite for binding to specific receptors and enhancing cellular responses. On the other hand, it has been suggested that vascular endothelial cell surface GAG of the heparin/heparan sulphate class mediate the presentation of some HBP to leucocytes [34,35]. Therefore, the type II binding sites might also contribute to the binding of CyPB to its receptor and regulate the activity of this protein. However, it remains to be determined whether the type II sites correspond to any of the previously described cell-surface proteoglycans. The effects of heparinase and chondroitinase on lymphocyte CyPB binding suggest that both families of GAG are involved. It is thus possible that tight binding of CyPB to type II sites requires interactions with both types of GAG chains. The existence of proteoglycans containing heparan sulphate and chondroitin sulphate may indicate the presence of either multiple proteoglycans, each bearing one class of GAG, or hybrid proteoglycans, in which both GAG are attached to the same core protein. We have recently characterized extensive binding of CyPB to cultured human endothelial cells from various origins, e.g. capillary, aorta and umbilical vein. The binding capacity was effectively reduced after treatment of the cells with heparinase or chlorate, indicating the involvement of GAG chains in the interactions. However, the affinity of CyPB binding sites was about 30-fold lower on endothelial cells compared with T-lymphocytes, suggesting that differences in the structure of GAG chains could result in variable interactions with CyPB (M. Carpentier, F. Allain, A. Denys and G. Spik, unpublished work). In contrast with those in endothelial cells, lymphocyte proteoglycans are of low molecular mass, with relatively small GAG chains, and are likely to have few different GAG chains on each core protein [24]. This has already been found to be the case for the invariant chain of class II major histocompatibility antigen and adhesion molecule CD44 [41,42].

In conclusion, our results show that CyPB binds specifically to T-lymphocytes through two types of binding sites, termed type I and type II. The binding domains are localized on opposite sides of the CyPB molecule, the catalytic/CsA-binding site and the N-terminal extremity of CyPB respectively. The knowledge of the relevance of both T-lymphocyte binding sites for CyPB function is therefore important as an aid to our understanding of the role of released CyPB. Studies are now in progress to investigate the functional importance of these interactions, and the mapping of binding domains in CyPB by mutagenesis may offer the opportunity to gain further insight into the biological role of different binding sites expressed on the T-cell surface.

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