Cyclophilin B is a cyclosporin A-binding protein exhibiting peptidyl-prolyl cis/trans isomerase activity. We have previously shown that it interacts with two types of binding sites on T lymphocytes. The type I sites correspond to specific functional receptors and the type II sites to sulfated glycosaminoglycans. The interactions of cyclophilin B with type I and type II sites are reduced in the presence of cyclosporin A and of a synthetic peptide mimicking the N-terminal part of cyclophilin B, respectively, suggesting that the protein possesses two distinct binding regions. In this study, we intended to characterize the areas of cyclophilin B involved in the interactions with binding sites present on Jurkat cells. The use of cyclophilin B mutants modified in the N-terminal region demonstrated that the Lys-Lys-Lys and Tyr-Phe-Asp clusters are probably solely required for the interactions with the type II sites. We further engineered mutants of the conserved central core of cyclophilin B, which bears the catalytic and the cyclosporin A binding sites as an approach to localize the binding regions for the type I sites. The enzymatic activity of cyclophilin B was dramatically reduced after substitution of the Arg and Phe residues, whereas the cyclosporin A binding activity was destroyed by mutation of the Trp residue and strongly decreased after modification of the Phe residue. Only the substitution of the Trp residue reduced the binding of the resulting cyclophilin B mutant to type I binding sites. The catalytic site of cyclophilin B therefore did not seem to be essential for cellular binding and the cyclophilin A binding site appeared to be partially involved in the binding to type I sites.

Cyclophilins are highly conserved proteins first identified as the main binding proteins for cyclosporin A (CsA), an immunosuppressive drug widely used in the prevention of graft rejection (1, 2). They were later identified as peptidyl-prolyl cis/trans isomerases (PPIase) (3, 4). Such an activity consists of the acceleration of the cis/trans isomerization of Xaa-Pro peptide bonds and has been proposed to be involved in protein folding (5). The enzymatic activity of cyclophilins is strongly inhibited by CsA because of the binding of the drug over the catalytic site of these proteins. Different members of the cyclophilin family have been described. They all contain a conserved core domain, carrying both the CsA binding and isomerase sites, flanked by distinct N and C termini accounting for their specificities (6). The prototype of this family is the abundant cytosolic 18-kDa form now named cyclophilin A (CyPA) (1). Cyclophilin B (CyPB) (7) and cyclophilin C (CyPC) (8) are closely related, but their mRNA encodes a signal peptide that directs them to the secretory pathway. A mitochondrial form called cyclophilin D (9) and a second larger cytosolic form named cyclophilin 40 (10) have also been described. Alignment of amino acid sequences reveals 65% identity between CyPA and CyPB (6, 7) and more than 70% between CyPB and CyPC (8). In the central core of the three forms the conservation in amino acid sequence is over 80%, implying that the regions bearing the CsA binding and isomerase activity are very similar in the different cyclophilins. The three-dimensional structures of CyPA (11), CyPB (12), and CyPC (13) have been solved and as expected the central core is similarly shaped. The structure includes eight antiparallel β strands forming a right-handed β-barrel overlaid by connecting loops and α helices (11). Both active sites are closely localized in a large hydrophobic pocket formed by four β strands and their connecting loops, whereas the N and C termini are located on opposite sides of the molecule.

In the case of CyPB, the C-terminal VEKPFIAKKE sequence has been described as a signal for retention in intracellular vesicles (14). The protein however was reported to follow the secretory pathway (15). Our finding that CyPB is present in human milk and blood plasma provided the first evidence that it is effectively secreted into biological fluids (7, 15, 16). Mariller et al. (17) furthermore demonstrated that CyPB isolated from milk is truncated because of the absence of the C-terminal AIAKE sequence. The presence of CyPB in plasma spurred us on to find out whether specific binding sites for this protein existed on blood cells. Indeed we were able to detect surface binding sites for CyPB on human peripheral blood T lymphocytes (18). The binding parameters were estimated to be 10–20 nM for the dissociation constant (Kd) and 30,000–120,000 for the number of binding sites/cell. We also found that the surface-bound ligand was specifically internalized into T cells and that CsA-complexed CyPB retained its cellular binding properties while promoting increased uptake of the drug.
and enhanced immunosuppressive activity (19–21). More recently, we were able to distinguish two classes of CyPB binding sites on the surface of peripheral blood T lymphocytes (22). Although both types of sites exhibit similar binding affinities, they are easily discriminated by their sensitivity to 0.6 M NaCl. The type I class of binding sites is insensitive to ionic strength and corresponds to functional lymphocyte receptors for CyPB, because endocytosis of the ligand follows the binding (22).

Interaction with type I sites is reduced in the presence of CsA, suggesting that the conserved CsA binding region of CyPB is involved. CyPC also interacts with the lymphocyte receptors, however with a 5-fold lower affinity, whereas CyPA does not, indicating that fine differences in the three-dimensional structure and/or a few specific amino acids may be responsible for variations in binding affinity. The type II class of binding sites represents at least 70% of the total lymphocyte binding capacity and corresponds to sulfated glycosaminoglycans (GAG) (22).

Interestingly, the interaction of CyPB with GAG is strongly inhibited by a peptide corresponding to the 24 N-terminal amino acid residues of CyPB, but remains unchanged in the presence of CsA (22). On the other hand, the binding to lymphocyte type II sites is found exclusively for CyPB, because neither CyPA nor CyPC is able to reduce the ligand binding (22). These results suggest that two distinct regions of CyPB may be involved in the interactions with lymphocyte GAG and specific membrane receptors.

In this study, which aims to understand the functional implications of CyPB association with the two classes of binding sites on T cells, we have tried to identify the GAG binding region and the functional receptor binding region in the CyPB molecule. Using either fragments of CyPB obtained by proteolysis or mutant forms modified by genetic engineering, we localized two amino acid clusters necessary for binding to sulfated GAG. In addition, we provide evidence that the region interacting with the type I binding sites is close to the CsA binding site of CyPB and that the amino acids of the enzymatic site of the protein are not required for the interactions with the receptor.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant human CyPA and CyPB were purified as described previously (7, 23). Recombinant human CyPC was a generous gift from Novartis (Basel, Switzerland). For site-directed mutagenesis, the previously described bacterial expression construct, which contains the coding sequence of mature human CyPB placed between the NcoI and HindIII sites of the pKK233-2 plasmid (Amersham Pharmacica Biotech, Uppsala, Sweden) was used as template. Construction of the CyPB1132RNA mutant has already been outlined (24). For the CyPB1132RNA mutant (CyPB1132RNA), DNA fragments covering the 5’ moiety from the NcoI site up to the mutated residues flanked by a KpnI restriction site (introduced simultaneously as a silent mutation) and the 3’ moiety from the same site to the HindIII site following the stop codon were generated by polymerase chain reaction. After gel purification and appropriate digestion, both fragments were introduced into the CyPB1132RNA plasmid in a three-way ligation. The CyPB1132RNA, (CyPB-BKK), CyPB1146F16 (CyPBΔAF), CyPB1132RA, CyPB1132HA, and CyPB1132RA, mutants were generated using the Quickchange Kit (Stratagene, La Jolla, CA) with minor modifications. Briefly, complementary primers covering the region to be modified were used to generate mutated unmethylated strands. Upon treatment with the DpnI endonuclease twice for 3 h at 37 °C, the methylated template DNA was digested allowing the selective rescue of the mutated strand after bacterial transformation. The DNA sequence of all the mutant plasmids was verified using the dideoxy chain termination method (25) with dATP(dS) and the Sequenase Kit (both from Amersham Pharmacica Biotech, Amersham, UK), and vector- and insert-specific primers. Production and purification of recombinant wild-type and mutated CyPB were performed using the procedure described by Spik et al. (7).

**Purification of Peptides**—Before hydrolysis, clostripain (E.C. 3.4.22.8) (Pharmacia) was activated by incubation in 0.1 M phosphate buffer, pH 7.4, containing 2.5 mM dithiothreitol and 1 mM CaCl2 for 2 h. Hydrolysis was then performed at 37 °C for 24 h with a molar ratio enzyme/substrate equal to 1:25. Clostripain mainly hydrolyzes peptide bonds in the C-terminal of Arg-Ala. At the end of the reaction, the sample was loaded on a QAE-Sephadex A-50 column (Pharmacia). The acidic isoelectric point (pI) of clostripain allowed its retention on the column while peptides were collected in the elution fraction. The peptides obtained were then separated by reverse phase high performance liquid chromatography (HPLC) using an Ultrapore C8 column (Beckman). Elution was achieved by a continuous 0.1% aqueous trifluoroacetic acid in the mobile phase of 0.08% trifluoroacetic acid, and monitored at 215 nm using a SP8450 detector (Spectra-physics, San José, CA). First analysis of peptides of interest was done by mass spectrometry using a matrix-assisted laser desorption ionization technique coupled with an analyzer of time of flight (MALDI-TOF). This preliminary identification was confirmed by partial amino acid sequence determination.

**Enzymatic Activity Assay and CsA Binding Analysis**—The peptidyl-prolyl cis/trans isomerase activity was assessed according to the method of Fisher et al. (26) with the difference that the reaction was allowed to proceed for 90 s at 10 °C. For CsA binding analysis, an automated LH-20 column (Pharmacia) binding assay was used, according to the procedure described by Hanschmacher et al. (1) and Spik et al. (6). The CsA binding capacity of each mutant was calculated by comparing the initially applied amount of radioactivity to that recovered in void volume fractions.

**Interactions with Heparin**—The capacity of wild-type and mutant CyPB to interact with heparin was analyzed on prepacked Hi-trap heparin-Sepharose columns (Pharmacia) previously equilibrated in a 20 mM phosphate buffer, pH 7.4. Purified proteins (1 mg) were loaded onto the column and elution was performed with a NaCl gradient from 0 to 1 M in phosphate buffer at a flow rate of 0.5 ml/min. The elution profile was monitored by following the absorbance at 280 nm.

**Cellular Binding Experiments**—To provide a reference model for the study of CyPB binding to T lymphocytes, the human lymphoblastic Jurkat cell line (E6–1 clone, ATCC TIB-152) was used for binding experiments according to the method described by Allain et al. (18). To discriminate between type I and type II binding sites, cells were washed three times with 3 ml of cold Dulbeco’s phosphate-buffered saline and once with phosphate buffer containing 0.6 M NaCl, as described by Denys et al. (22). The binding curves for each mutant were fitted using the wild-type dissociation constants determined for each type of sites as fixed parameters.

**Molecular Modeling**—Molecular modeling was carried out on a personal computer using the WinMGM software (27). The structures for human CyPA and CyPB were obtained from the Brookhaven National Laboratory protein data bank, as files ICWA and ICYN, respectively. The crystallographic coordinates of N-terminal area 1Asp-Glu-Lys-Lys-Lys (5) of CyPB are not available; therefore the determination of the putative three-dimensional structure of this area was performed on the basis of the partial known coordinates of the molecule by energy minimization on a Silicon Graphics Optane workstation using the SYBYL program.

**Statistical Analysis**—Results are expressed as the mean values ± S.D. from at least three separate experiments performed in triplicates. Statistical analysis was performed using a Student’s t test and a P value less than 0.05 was considered significant.

**RESULTS**

**Discrimination of CyPB Binding Sites on Jurkat cells**—As a prerequisite for our binding experiments, we examined whether, similarly to those found on T lymphocytes, the surface binding sites on Jurkat cells can be divided in two classes. The total surface binding of [125I]-CyPB to Jurkat cells was characterized by a Kd estimated to be 9.2 ± 2.0 nM (Table I) and a number of sites of 80,000/cell. After washing with a buffer containing 0.6 M NaCl, the binding capacity was strongly reduced to less than 20,000/cell, indicating that about 75% of CyPB binding sites were sensitive to NaCl treatment and corresponded to the type II sites previously identified as sulfated GAG. As seen in Table I, the ligand binding affinity for type I sites (Kd = 20 ± 10 nM) was in the same range as that of type II sites (Kd = 9 ± 5 nM). These dissociation constants were used as fixed parameters for fitting the binding curves for each
and close to Arg 18. Importantly, three-dimensional structure derivatized with region contains an Arg residue at position 18 which, when part, the N-terminal extension of the protein (22, 24). This binding sites have earlier been shown to involve, at least in homologous and the main difference occurs in the N-terminal of CyPA and of CyPC (Fig. 1). CyPB and CyPC are highly cells, we compared the amino acid sequence of CyPB with those in the interactions with the type II binding sites on Jurkat cells. Radioactivity was then measured in the supernatant (ligand remaining bound to the type II sites) and in cellular pellets (ligand released from the type II sites). The binding curves for each mutant were fitted using the wild-type dissociation constants ($K_d$) as fixed parameters.

### Table I

**Binding parameters studies of CyPB mutated in its N-terminal part**

The $K_d$ values for the total binding sites were determined by the Scatchard linearization method. Jurkat cells were incubated for 1 h at 4°C with increasing concentrations of radiolabeled ligands. To discriminate between the type I and type II binding sites, cells were washed with Dulbecco’s phosphate-buffered saline containing 0.6 M NaCl and the indicated ligands were used to compete with wild-type [$^{125}$I]-CyPB for binding to Jurkat cells. Radioactivity was then measured in the supernatant (ligand released from the type II sites) and in cellular pellets (ligand remaining bound to the type I sites). The binding curves for each mutant were fitted using the wild-type dissociation constants ($K_d$) as fixed parameters.

| Ligands | Total binding sites | Type I binding sites | Type II binding sites |
|---------|---------------------|---------------------|---------------------|
| CyPB    | 9.2 ± 2.0           | 20 ± 10             | 9 ± 5               |
| CyPBE22K| 10.0 ± 3.0          | 25 ± 11             | N.C.*              |
| CyPBK    | 12.5 ± 1.5          | 30 ± 12             | N.C.               |
| CyPBK22E| 11.0 ± 1.0          | 23 ± 9              | 11 ± 6             |
| CyPBK23P| 12.0 ± 2.0          | 25 ± 14             | 12 ± 7             |

* N.C., no competition.

To verify the specificity of the interactions, we analyzed the binding of CyPA and CyPC to Jurkat cells. CyPA did not interact significantly, whereas CyPC exhibited binding only to the NaCl-resistant sites that correspond to the functional CyPB receptor (not shown). The binding affinity of CyPC to these type I sites was however 6-fold lower ($K_d = 117 ± 65$ nM) than that of CyPB. Taken together, our results indicate that the type I and type II binding sites previously described on peripheral blood T lymphocytes (22) are also expressed on the membrane of Jurkat cells.

### Strategy of Identification of Binding Regions of CyPB to GAG

To delineate specific regions of CyPB possibly involved in the interactions with the type II binding sites on Jurkat cells, we compared the amino acid sequence of CyPB with those of CyPA and of CyPC (Fig. 1). CyPB and CyPC are highly homologous and the main difference occurs in the N-terminal region. Indeed the interactions of CyPB with T lymphocyte binding sites have earlier been shown to involve, at least in part, the N-terminal extension of the protein (22, 24). This region contains an Arg residue at position 18 which, when derivatized with p-hydroxyphenylglyoxal inhibits the binding of CyPB by 70%. Mutation of this residue does not however affect ligand binding, suggesting that other residues located nearby are involved in the interaction with GAG (24). It is known that such interactions are largely electrostatic and require basic amino acids. Indeed, CyPB possesses a highly accessible and basic Lys-Lys-Lys cluster in its N-terminal part, which might establish ionic interactions with the sulfated GAG. However, CyPC, though possessing a basic RKK cluster at a similar position (Fig. 1), does not interact with the lymphocyte GAG. Another region possibly implicated in the establishment of interactions with GAG is the $^{14}$Tyr-Phe-Asp$^{16}$ sequence, which is specific of CyPB and near enough to the Arg$^{18}$ residue to be affected by the chemical modification (22). The residues Glu$^{22}$ and Asp$^{23}$ are other residues specific of CyPB and close to Arg$^{18}$. Importantly, three-dimensional structure analysis (12) has previously revealed these two amino acids to be localized in a loop structure (loop 19–24) not found in other cyclophilins. To study the importance of all these residues of the N-terminal region, which is spatially localized on one side of the molecule (12), far away from the regions involved in PPlase or CsA binding activities, we engineered a series of CyPB mutants. In CyPBK22E, the sequence $^{3}$Lys-Lys-Lys$^{5}$ was replaced by AAA. In the case of CyPBK23P, the $^{14}$Tyr-Phe-Asp$^{16}$ sequence was deleted. CyPBK22E and CyPBK23P were engineered by substituting Glu$^{22}$ and Asp$^{23}$ with the corresponding amino acid residues of CyPC and CyPA. In addition, we used a second method to identify the receptor binding regions of CyPB. It consisted of generating peptides by partial proteolytic cleavage of CyPB and searching which ones inhibited the binding of wild-type CyPB.

### Analysis of CsA Binding and Enzymatic Activities of CyPB Mutated in Its N-terminal Part

Before the analysis of their cellular binding activity, all the CyPB mutants were tested for PPlase activity and efficiency to form a complex with CsA. All mutants, i.e. CyPBK22E, CyPBK23P, CyPBK22E and CyPBK23P retained both activities with values comparable to those found for wild-type CyPB (data not shown). These results are not surprising because the mutations made are confined to the N-terminal region, which is spatially localized on one side of the molecule (12), far away from the regions involved in PPlase or CsA binding activities. Previously, we showed that CyPBYPD and CyPBKXX Mutations with Heparin—To support the hypothesis that $^{14}$Tyr-Phe-Asp$^{16}$ and $^{3}$Lys-Lys-Lys$^{5}$ are required for the interactions of CyPB with GAG, the mutants CyPBYPD and CyPBKXX were analyzed for their ability to interact with heparin (Fig. 2). Wild-type CyPB

![Image](308x358 to 554x729)
and mutants were applied onto a heparin-Sepharose column in a phosphate buffer, pH 7.4. After extensive washing with the same buffer, elution of the proteins was performed by increasing concentrations of NaCl from 0 to 1 M. As previously reported, CyPB was eluted at 0.6 M NaCl only, indicating that it was strongly retained on the column. In contrast, CyPB$_{KKK}$ and CyPB$_{DYFD}$ were more rapidly eluted from the column, at 0.1 and 0.3 M NaCl, respectively. These results indicate that both tripeptides are likely to be involved in the interactions with sulfated GAG. The $^3$Lys-$^4$Lys-$^5$Lys tripeptide seems to be of more crucial importance, because its replacement dramatically reduced the avidity of CyPB for heparin. CyPB$_{KKK}$ was eluted at an ionic strength lower than 0.15 M, indicating that cyclophilin lacking the $^3$Lys-$^4$Lys-$^5$Lys sequence was unable to interact with sulfated GAG under physiological conditions. In contrast, the elution of CyPB$_{DYFD}$ required a 0.3 M NaCl concentration, showing that interactions with heparin may occur in an isotonic medium and supporting our hypothesis for a role of this sequence in stabilizing the interactions of CyPB with sulfated GAG. Molecular modeling was used to examine the relative positions of the two potential GAG binding sites in the folded N-terminal extension of CyPB. The model shown in Fig. 3 clearly illustrates that the two clusters are juxtaposed in the three-dimensional structure and could act synergistically to interact tightly with GAG chains.

**Cellular Binding Properties of CyPB$_{DYFD}$ and CyPB$_{KKK}$ Mutants—** To find out whether the interactions of CyPB with heparin reflect those with the surface binding to the type II sites, we tested the ability of CyPB$_{DYFD}$ and CyPB$_{KKK}$ to compete with wild-type CyPB for binding to Jurkat cells (Table I). Cells were incubated in the presence of 50 nM $^{125}$I-CyPB and increasing concentrations of unlabeled wild-type or mutated proteins. After washing, cells were treated with 0.6 M NaCl, and the distribution of remaining surface-bound (type I sites) and released (type II sites) ligand was analyzed. As expected, CyPB$_{D}$ and CyPB$_{KK}$ were ineffective in reducing the binding of $^{125}$I-CyPB to the type II sites. In contrast, interactions with the type I sites were reduced to an extent similar to what was measured with the wild-type unlabeled CyPB, implying that both mutants could still interact with the type I binding sites but had lost their ability to recognize the type II binding sites. To support the hypothesis that both mutants only interacted with the type I binding sites, we performed direct binding assays. Cells were incubated in the presence of increasing concentrations of either $^{125}$I-CyPB$_{KK}$ or $^{125}$I-CyPB$_{D}$. The nonspecific interactions obtained in the presence of a 200-fold molar excess of the corresponding unlabeled mutant were subtracted from total counts. The binding parameters were determined and compared with those obtained with the wild-type radioiodinated ligand (Table I). The apparent dissociation constants ($K_d$) of $^{125}$I-CyPB$_{KK}$ and $^{125}$I-CyPB$_{D}$ binding were estimated to be 10.0 ± 3.0 nM and 12.5 ± 1.5 nM, respectively. These values are close to the $K_d$ value of the wild-type $^{125}$I-CyPB binding (9.2 ± 2.0 nM). In contrast, the number of binding sites/cell was estimated to be 17,000 ± 2,000 and 15,000 ± 2,000 respectively, less than that found for the wild-type ligand (80,000 ± 5,000). Thus, the substitution of the $^3$Lys-$^4$Lys-$^5$Lys cluster by AAA and the deletion of the $^1$Tyr-$^2$Phe-$^3$Asp cluster led to a similar loss of
about 80% of the whole binding capacity of CyPB, without affecting the affinity of the ligand for its binding sites. Indeed, the 20% remaining binding capacity probably corresponds to the NaCl-resistant fraction referred to as type I sites, which explains why both CyPB\(_{\Delta 22K}\) and CyPB\(_{KKK}\) were only efficient to displace radiolabeled ligand from these sites. Taken together, these results demonstrate the involvement of the 3Lys-Lys-Lys\(_5\) and 14Tyr-Phe-Asp\(_{16}\) clusters in the establishment of tight interactions with the type II binding sites present on Jurkat cells. In addition, the fact that both CyPB\(_{KKK}\) and CyPB\(_{\Delta 22K}\) interact with the type I binding sites similarly to wild-type CyPB implies that a second region, not located in the N-terminal extension of the protein, is required here.

**Cellular Binding Properties of CyPBE\(_{22K}\) and CyPBD\(_{23P}\) Mutants**—To find out whether other CyPB-specific residues of the N-terminal extension are implicated in the interactions with type II sites, we examined the cellular binding properties of CyPBE\(_{22K}\) and CyPBD\(_{23P}\) mutants. The dissociation constants of these modified proteins were estimated to be 23 ± 6 nM and 25 ± 14 nM for the type I sites and 11 ± 6 nM and 12 ± 7 nM for the type II sites, respectively (Table I). These values differed little from those measured for wild-type CyPB, indicating that all three ligands exhibited a similar affinity for the two types of binding sites. These results therefore demonstrate that the Glu\(_{22}\) and Asp\(_{23}\) residues, and by extension the 19–24 loop of CyPB, are not involved in the interactions with GAG sites and further substantiate that the region close to Arg\(_{18}\) and required for cellular binding under physiological conditions is most probably the 14Tyr-Phe-Asp\(_{16}\) cluster.

**Identification of CyPB Binding Regions after Proteolytic Cleavage**—Additionally, a proteolytic approach was used to identify regions of CyPB implicated in the interactions with Jurkat cells. Several proteases were tested but only the results obtained after clostripain treatment are presented here because they were the most conclusive. After clostripain cleavage, the fragments obtained were purified by reverse phase HPLC and the ability of the 16 fractions eluted to compete with \(^{125}\)I-CyPB binding to Jurkat cells was analyzed (Fig. 4). Only two fractions, referred to as fractions A and B, were found to contain fragments able to reduce the binding of \(^{125}\)I-CyPB to Jurkat cells. Fraction A, eluted with 30% of acetonitrile in HPLC, inhibited the binding of radiolabeled CyPB to T cells by 55%, whereas an equivalent amount of unlabeled CyPB inhibited ligand binding by 75%. Thus, fraction A probably contains a large part of the CyPB areas involved in the interactions with T cells. The fraction B, eluted with 50% of acetonitrile, inhibi-
CyPB Regions Involved in Receptor and GAG Recognition

**Table II**

| Ligands | PPI activity | CsA-binding efficiency % wild-type |
|---------|--------------|-----------------------------------|
| CyPB    | 100          | 100                               |
| CyPB<sub>RGD</sub> | 20 ± 5       | ND<sup>a</sup>                    |
| CyPB<sub>F67A</sub> | 8 ± 3        | 100 ± 9                           |
| CyPB<sub>R62A</sub> | 12 ± 5       | 20 ± 5                            |
| CyPB<sub>W128A</sub> | 60 ± 10      | ND                                |

<sup>a</sup> ND, not detectable.

*B<sub>RGD</sub>.*—The 76Arg-Gly-Asp<sup>78</sup> tripeptide is not directly located in the central core of CyPB and the corresponding amino acid residues of CyPA, 69Arg-His-Asn<sup>71</sup>, have not been reported to be essential for activities. Surprisingly the CyPB<sub>RGD</sub> mutant exhibited strongly reduced PPIase activity and no CsA binding (Table II). We assume that the substitution of the 76Arg-Gly-Asp<sup>78</sup> tripeptide led to a wrong folding of the central core of the protein so that the enzymatic activity of the protein was lost. This mutant was retained for our next experiments as an inactive form of CyPB.

**Binding Properties of CyPB<sub>RGD</sub>**.—The binding properties of CyPB<sub>RGD</sub> to Jurkat cells were examined (Table III). Increasing concentrations of CyPB<sub>RGD</sub> reduced the amount of radioactivity released in the 0.6 M NaCl wash comparably with the wild-type CyPB, indicating that the mutant has conserved its binding properties for the type II sites. In contrast, a 5-fold higher concentration of CyPB<sub>RGD</sub> than of CyPB was required to reduce by 50% the amount of [125I]-CyPB associated with the NaCl-resistant fraction. These last results reflect a marked decrease in the affinity of this mutant for the type I sites. When using a synthetic RGDS peptide to compete with the cellular binding of [125I]-CyPB to Jurkat cells no interference was observed, however, which seems to rule out the possibility that the type I sites correspond to proteins of the integrin family (data not shown). We rather surmise that substitution of the 76Arg-Gly-Asp<sup>78</sup> tripeptide most probably led to substantial conformational modifications of CyPB, which perturbed the interactions with type I surface binding sites.

**Strategy of Identification of Binding Regions of CyPB to the Functional Receptor**.—Denys et al. (22) have demonstrated that large excesses of CsA inhibit the binding of CyPB to the type I sites, without affecting the interactions with GAG. These results indicate that the drug probably overlays the domain of CyPB involved in binding to type I and consequently that this domain might be part of the conserved central part of the protein. Zydowski et al. (31) have shown that the areas of CyPA supporting PPIase and CsA binding activities are distinct. In particular, they identified Arg<sup>62</sup> and Phe<sup>69</sup> as being essential for the PPIase catalytic activity, whereas Trp<sup>121</sup> and to a lesser extent Phe<sup>69</sup> are required for the CsA binding activity. Even though similar studies have not been performed yet for CyPB, an analysis of the sequence alignment of CyPA and CyPB and of the three-dimensional structures of both proteins allowed us to pinpoint the three corresponding amino acid residues in CyPB, i.e., Arg<sup>62</sup>, Phe<sup>69</sup> and Trp<sup>121</sup>. Indeed, they are positioned similarly to the Arg<sup>62</sup>, Phe<sup>69</sup> and Trp<sup>121</sup> residues of CyPA, and are located in a comparable spatial environment, suggesting that they could participate in either PPIase or CsA binding activities (Fig. 6). We therefore engineered three mutants, termed CyPB<sub>F67A</sub>, CyPB<sub>R62A</sub>, and CyPB<sub>W128A</sub>, where Arg<sup>62</sup>, Phe<sup>69</sup> and Trp<sup>121</sup> were replaced by an Ala residue.

**Analysis of CsA Binding and Enzymatic Activities of CyPB Active Site Mutants**.—The mutants of the conserved central domain were tested for their enzymatic and CsA binding properties (Table II). As expected, substitution of either Arg<sup>62</sup> or...
The amino acid residues indicated, Arg55, Phe60, and Trp121, are in the constant (curves for each mutant were fitted using the wild-type dissociation constants ($K_d$) as fixed parameters).

| Ligands        | Type I binding sites | Type II binding sites |
|----------------|----------------------|-----------------------|
| CyPB           | 20 ± 10              | 9 ± 5                 |
| CyPB<sub>BGD</sub> | 106 ± 63             | 11 ± 6                |
| CyPB<sub>R62A</sub> | 20 ± 11              | 11 ± 5                |
| CyPB<sub>F67A</sub> | 28 ± 15              | 9 ± 4                 |
| CyPB<sub>W128A</sub> | 135 ± 80             | 11 ± 5                |

Phe<sup>67</sup> residues led to a dramatic loss of PPIase activity, down to 8 and 12% of wild-type CyPB isomerase activity. Moreover, the ability of CyPB<sub>F67A</sub> to interact with CsA was reduced by 80%, whereas CyPB<sub>R62A</sub> was as efficient as wild-type CyPB in complexing the drug. These results are in agreement with the published work of Zydowski <em>et al.</em> (31) concerning CyPA and demonstrate the conservation of the enzymatic site and CsA binding domain between CyPA and CyPB. Moreover, we found the CyPB<sub>W128A</sub> mutant unable to interact with CsA, demonstrating that the requirement of this amino acid residue in the interactions with the drug is also a common feature of CyPA and CyPB. Surprisingly, the CyPB<sub>W128A</sub> mutant retained 60% of the capacity to accelerate the cis-trans isomerization of a Pro-containing substrate, indicating that the Trp<sup>128</sup> residue in CyPB was not essential for PPIase activity. This is not the case for CyPA where mutation of the corresponding Trp<sup>121</sup> residue was reported to strongly reduce the enzymatic activity. Fine differences in the spatial conformation of the central domains of CyPA and CyPB exist, explaining why such variations in the CsA binding capacity might occur. In support of this observation, it was found that the half-inhibitory concentration of CsA required to inhibit PPIase activity is 3-fold higher for CyPA when compared with CyPB, indicating that indeed differences in the three-dimensional structure of the central core may be related to variations in the activities of each cyclophilin (12). In conclusion, we produced three different mutants of the conserved central domain of CyPB, namely CyPB<sub>R62A</sub>, CyPB<sub>F67A</sub>, and CyPB<sub>W128A</sub>—lacking either PPIase activity, CsA binding capacity, or both, respectively.

**Cellular Binding Properties of CyPB<sub>R62A</sub>, CyPB<sub>F67A</sub>, and CyPB<sub>W128A</sub>**—Competitive binding experiments conducted with CyPB<sub>R62A</sub>, CyPB<sub>F67A</sub>, and CyPB<sub>W128A</sub> demonstrated that the three mutants were as efficient as wild-type protein in inhibiting the binding of [125I]-CyPB to the type II binding sites (Table III), in line with our previous results. Moreover, CyPB<sub>R62A</sub> and CyPB<sub>F67A</sub> were as efficient as wild-type CyPB to reduce the amount of radiolabeled ligand associated with the NaCl-resistant fraction. Because both these mutants have lost their PPIase activity, the results strongly suggest that the amino acids of the catalytic site of CyPB are not directly involved in the interactions with the type I sites. In contrast, a 7-fold higher concentration of CyPB<sub>W128A</sub> was required to displace 50% of the radiolabeled ligand bound to the type I sites. Interestingly, this last mutation completely abolished the CsA binding capacity of CyPB but was less critical for enzymatic activity. It may therefore be postulated that the areas of CyPB involved in the interaction with the type I sites are close to the Trp<sup>128</sup> residue and probably overlay the CsA binding site of this protein.

**DISCUSSION**

Before mapping the CyPB regions involved in functional receptor and GAG binding, we checked that the two types of binding sites described by Denys <em>et al.</em> (22) on the membrane of peripheral blood T lymphocytes were also expressed on the membrane of Jurkat cells. Indeed we found that 75% of the total Jurkat cell binding capacity was due to interactions with type II sites and that the remainder corresponded to type I functional receptors previously shown to be involved in ligand endocytosis. Binding to type I receptors was also found for CyPC and was strongly reduced in the presence of CsA, suggesting that part of the conserved CsA binding domains of CyPB and CyPC corresponds to the binding region. The type II binding sites represent about 75% of the total binding capacity and probably involve interactions with sulfated GAG present on cell surface. This binding is highly specific for CyPB and can be effectively reduced in the presence of a synthetic peptide corresponding to the N-terminal part of the protein (22). The role of this N-terminal extension of CyPB in the cellular binding has first been emphasized by Mariller <em>et al.</em> (24) who have also suggested that another region of CyPB may be involved in the interactions.

The results presented here identify two regions of CyPB involved in the interactions with both types of CyPB binding sites present on the T cell membrane. We clearly demonstrate that CyPB possesses two receptor binding regions: one, located in the specific N-terminal extension of the protein, required for the interactions with sulfated GAG and the second one, corre-
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sponding to a part of the central conserved core of CyPB, involved in the recognition of a specific functional receptor. Both binding regions are spatially located on opposite sides of the CyPB molecule (12), raising the possibility that interactions with both types of binding sites could occur simultaneously.

The use of CyPB mutants corresponding to the protein modified within its N-terminal extension provided evidence for the role of the 14Tyr-Phe-Asp16 cluster in the interactions with heparin and the type II binding sites constituted by sulfated GAG. Proteolytic cleavage confirmed the requirement of the 13VYFDLR18 peptide of CyPB in the interactions with these binding sites, indicating that this region covers at least in part the actual binding region present in the N-terminal end of the protein. Most probably the 13VYFDLR18 peptide and more specifically the 14Tyr-Phe-Asp16 sequence are necessary but not sufficient for interactions with the type II binding sites. Conceivably the 14Tyr-Phe-Asp16 sequence is required to stabilize the primary interactions of a highly exposed basic sequence with the type II sites, explaining why a large excess of free 13VYFDLR18 peptide inhibits the binding of CyPB. In this respect, we found that another region which contains the 3Lys-Lys-Lys5 basic cluster was also required for tight interactions, in line with the involvement of two regions from the N-terminal part of CyPB in the binding to GAG chains. CyPBKKK and CyPBYYY were eluted from a heparin-Sepharose column, respectively, with 0.1 and 0.3 M NaCl whereas CyPA, which lacks a KKK cluster, and CyPC, which lacks a YFD cluster, were eluted from 0.2 and 0.25 M NaCl (22). The three-dimensional model of the N-terminal extension of CyPB showed that both the 3Lys-Lys-Lys5 and 14Tyr-Phe-Asp16 clusters can be spatially juxtaposed and may act synergistically to form a cradle-like binding site for the sulfated polysaccharide chain. Unfortunately, the published crystallographic data on CyPB (12) do not give information on the three-dimensional conformation of the 3Asp-Glu-Lys-Lys-Lys5 extremity, which would help confirm this model. This is because of the absence in the crystal structure of the five N-terminal amino acid residues, which were probably cleaved off by proteases during the isolation procedure. Using the molecular modeling, we showed that the 3Lys-Lys-Lys5 and the 14Tyr-Phe-Asp16 clusters form a structural arrangement that could permit the interactions with the GAG. In addition, a YFDLR peptide was described in type IV collagen as a heparin binding domain (32, 33). Indeed, this YFDLR peptide is located in a discontinuity of the triple helix of collagen and was reported to be directly involved in promoting keratinocyte adhesion to heparin sulfated proteoglycans. Taken together, these data clearly document the role of such regions in the interactions with GAG and strongly support our hypothesis that the binding region of CyPB involved in the interactions with type II sites and heparin is probably restricted to the 3Lys-Lys-Lys5 and 14Tyr-Phe-Asp16 clusters.

A role of sulfated GAG was suggested to be related to localization or local presentation of HBP. Because the biological functions of secreted cyclophilins are as yet largely unclear, it is difficult to speculate on the implications of the interaction with GAG. Some cyclophilins were reported to exhibit chemotactic activity for eosinophils, neutrophils, and monocytes (34, 35). Also, CyPB levels measured in blood plasma from CsA-treated graft recipients (21) and from patients suffering from HIV infection (36) or sepsis (37) are increased for reasons not yet understood. Taken together, these observations suggest however that secreted cyclophilins might act as pro-inflammatory mediators. Another family of small chemotaxtractant proteins are the chemokines that are implicated in the attraction and activation of a variety of leukocytes (38). Like CyPB, they bind to functional receptors on target cells and interact with sulfated GAG. The importance of the interactions with sulfated GAG present on cell membrane has also been shown for many growth factors and cytokines. Indeed these primary interactions are a prerequisite for the binding to specific receptors and the enhancement of cellular responses. For instance, soluble heparin was reported to support binding of interleukin-8 to its neutrophil receptor and to increase the intracellular free calcium concentration (39). On the other hand, it has been suggested that interactions with GAG, either at the surface of endothelial cells or in the extracellular matrix, could be responsible for the establishment of an immobilized chemokine gradient and the presentation of these molecules to leukocytes (40, 41). In support of this idea, it was demonstrated that MIP-1β and RANTES (regulated on activation normal T cell expressed) interact with solid phase GAG or activated endothelium and that these immobilized chemokines are then capable of stimulating leukocyte adhesion (42, 43). It is therefore conceivable that the type II sites contribute to the binding of CyPB to its type I lymphocyte receptor and regulate its activity. This possibility may only be further clarified by elucidating the biological role of CyPB and identifying the functional receptor.

Finally, we examined in detail the implication of the CsA binding and PPIase domains of CyPB in type I receptor binding, because the interaction between CyPB and the drug leads to impaired receptor binding. Upon analysis of the homologies between the sequences of CyPA and CyPB, three amino acids residues, namely Arg162, Phe167, Trp128, were identified in CyPB as being possibly required for either PPIase, CsA binding, or both activities. We found that substitution of the Trp128 residue in CyPB prevented the interaction with CsA and markedly reduced the affinity of the protein for the type I sites. These results agree with our previous finding that addition of CsA inhibits the interactions of CyPB with the type I sites present on blood T lymphocytes (22). Surprisingly, PPIase activity was not directly related to the cellular binding properties of CyPB. The substitution of Arg162 and Phe167 effectively resulted in the loss of enzymatic activity but did not affect the interactions with the type I sites. Taken together, our results indicate that the second binding region of CyPB is probably conformational and emphasize the crucial role of the Trp128 residue in the interactions with either CsA or type I sites. The presence of a Trp residue in the CsA binding domain is a common feature of all cyclophilins and may explain in part why CyPC exhibits binding activity for the type I sites. The binding affinity of CyPC was however found to be 6-fold lower than that of CyPB, whereas CyPA was unable to interact with any of the binding sites present on T cells. Such a discrimination of the cyclophilins for the recognition of a receptor has been already reported for CyPC with which a 77-kDa protein, termed CyCAP for CyPC-associated protein, was found to specifically interact (44, 45). CsA inhibits this interaction although neither CyPA nor CyPB is able to bind to this membrane protein. The areas of CyPC that interact with CyCAP are thought to be localized in a loop close to the catalytic site but without any sequence homology with the other cyclophilins, explaining the specificity of recognition. Inhibition of CyCAP binding by CsA is probably because of sterical hindrance (13). Such fine divergences in the spatial conformation of the central domain of the different cyclophilins might therefore well explain the variations seen in the binding affinities of CyPB and CyPC for their specific receptors. In addition, Sherry et al. (35) have very recently characterized a signaling receptor for CyPA on T lymphocytes and suggested that here also the CsA binding domain is involved in the interaction. Possibly, the type I binding sites for CyPB, CyCAP and the lymphocyte CyPA receptor might all be
members of a family of related cyclophilin-binding proteins. The possibility that the different cyclophilins interact to some extent with the various receptor family members cannot be ruled out presently. Resolution of this question will require purification and structure elucidation of the different cyclophilin-binding proteins expressed on the membrane of T cells.

Our studies clearly localize two distinct binding regions in the CyPB molecule that are separately involved in the recognition of GAG and of a functional receptor on T cells. This should allow the design of new experiments to test the importance of the cellular binding of CyPB and to provide new insights into the biological activity of this protein.

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