Involvement of the N-terminal part of cyclophilin B in the interaction with specific Jurkat T-cell binding sites

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Cyclophilin B (CyPB) is secreted in biological fluids such as blood or milk and binds to a specific receptor present on the human lymphoblastic cell line Jurkat and on human peripheral blood lymphocytes. This study was intended to specify the areas of CyPB that are involved in the interaction with the receptor. A synthetic peptide corresponding to the first 24 N-terminal amino acid residues of CyPB was shown to specifically recognize the receptor. Moreover, modification of Arg¹⁸ of CyPB by *p*-hydroxyphenylglyoxal led to a dramatic loss of affinity for the

receptor. However, when this residue was replaced by an alanine residue using site-directed mutagenesis, no modification of the binding properties was found, suggesting that Arg¹⁸ is not directly involved but is sufficiently close to the interaction site to interfere with the binding when modified. Competitive binding experiments using a chimaeric protein made up of the 24 N-terminal amino acid residues of CyPB fused to the cyclophilin A core sequence confirmed the involvement of this region of CyPB in receptor binding.

INTRODUCTION

Cyclophilins were first identified as the high-affinity binding proteins for the immunosuppressive drug cyclosporin A (CsA). CsA is a cyclic undecapeptide which was isolated over 20 years ago based on its ability to inhibit a mixed lymphocyte reaction [1]. Its clinical introduction greatly improved the outcome of solid organ and bone-marrow transplantation and, despite toxic side-effects, it remains a widely used immunosuppressive agent [2]. Cyclophilins are members of the peptidyl prolyl *cis-trans* isomerase family, which includes also the FK506-binding proteins (FKBPs) [3] and the parvulins [4]. The FKBPs are able to bind the macrolide FK506, which is also used to prevent graft rejection but is structurally unrelated to CsA [5]. All these proteins catalyse the prolyl bond isomerization rate of synthetic peptides and the refolding of a few denatured proteins [3,6–8]. CsA inhibits cyclophilin prolyl isomerase activity [8], while FK506 inhibits FKBP prolyl isomerase activity [3].

Several cyclophilin isoforms with distinct subcellular localizations have been described in various prokaryotic and eukaryotic organisms. The first form characterized is cyclophilin A (CyPA), an abundant cytosolic protein which is considered to be the main target of CsA *in vivo* [9]. Indeed, the complex formed by the immunosuppressive drug and CyPA binds and modulates the activity of calcineurin [10], a critical intermediate in the signal transduction pathway recruited upon T-cell activation [11]. Cyclophilin B (CyPB) [12–14] and cyclophilin C (CyPC) [15] are related to CyPA but contain an N-terminal signal sequence thought to mediate translocation into the endoplasmic reticulum. Mitochondrial cyclophilin (CyPD) is the fourth member of the human cyclophilin family [16,17].

CyPB has been reported as being retained in specialized portions of the endoplasmic reticulum [18] but also as a protein which follows the secretory pathway until its secretion [19]. Its

purification from human milk has provided the evidence that CyPB is secreted into this medium [14]. Specific antibodies against peptides corresponding to the most divergent parts between CyPA and CyPB were used to show that CyPB, but not CyPA, is also present in human blood [20]. The binding capacity of CyPB to cells of the immune system was furthermore investigated and indeed surface binding sites characterized on both Jurkat T-cells and human peripheral blood T-lymphocytes. A dissociation constant (K_d) of 12 nM was calculated and the number of binding sites estimated to be 35000/cell [21]. The CyPB–CsA complex binds to the T-lymphocytes with a similar K_d value and to approximately the same number of sites. At 37 °C, both the CyPB–CsA complex and free CyPB are internalized after binding. However, while CyPB is subsequently degraded in acidic compartments, CsA is accumulated within the cell. Thus, over a defined range of CyPB concentrations, the CyPB–CsA complex may target the drug to lymphocytes and increase the cellular concentration of CsA (see the accompanying paper [21a]). The presence of these surface binding sites is consistent with the hypothesis that CyPB is secreted into biological fluids such as milk or blood [14,20] and then interacts with specific receptors on target cells.

In an attempt to specify the areas of CyPB that interact with the surface binding sites of Jurkat cells, several competitive binding assays have been carried out. By comparing the amino acid sequence of CyPA, which does not interact with the Jurkat T-cell surface binding sites [21], and CyPB, it appears that the N- and C-terminal parts of CyPB are specific to this protein and may therefore contain the interaction site. We have therefore investigated the possible involvement of the N- and C-terminal extremities of CyPB in the binding to the Jurkat receptor by competitive experiments using synthetic peptides corresponding to these regions. CyPB mutants modified at specific locations, either chemically or by site-directed mutagenesis, and a CyPB/

Abbreviations used: AMCA-HPDP, *N*-[6-(7-amino-4-methylcoumarin)-3-acetamidohexyl]-3'-(2'-pyridyldithio)propionamide; CyPA, CyPB and CyPC, cyclophilins A, B and C; CsA, cyclosporin A; DPBS, Dulbecco's PBS; FKBP, FK506-binding protein; IL-8, interleukin 8.

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CyPA chimaera were furthermore used to gain information on the sites involved in the recognition of the specific receptor.

EXPERIMENTAL

Materials

CyPA, CyPC and the PCGF-72 plasmid coding for human CyPB were generous gifts from Sandoz (Basel, Switzerland). Synthetic peptides were kindly provided by Professor A. Tartar, Laboratoire de Chimie des Biomolécules, Unité de Recherche Associée no. 1319 du Centre National de la Recherche Scientifique, Lille, France.

N-[6-(7-Amino-4-methylcoumarin)-3-acetamidohexyl]-3'-(2'-pyridyldithio)propionamide (AMCA-HPDP) and *p*-hydroxyphenylglyoxal were purchased from Pierce (Rockford, U.S.A.).

Construction of CyPB mutants

The CyPB/A chimaera was constructed in two steps. First, the region coding for the N-terminal part of mature CyPB up to Arg²⁶ was amplified by PCR while adding a flanking *Nco*I site, including the ATG initiator codon, at the 5' side, and flanking *Mlu*I and *Hind*III restriction sites at the 3' end. The *Mlu*I site was selected because it was compatible with the desired final amino acid sequence and not present in the original cyclophilin coding sequences. The *Hind*III site was used, together with *Nco*I, for subcloning into the pKK233-2 vector (Pharmacia, Uppsala, Sweden), after purification on a low-melting-point agarose (FMC) gel. Secondly, the CyPA coding region from Gly¹⁸ to the stop codon was amplified by PCR while introducing flanking *Mlu*I and *Hind*III restriction sites. After gel purification and digestion with the appropriate enzymes this fragment was subcloned downstream of the CyPB coding portion in pKK233-2. The resulting plasmid codes for a protein composed of the CyPB-specific N-terminal region (up to Val²⁴) fused to amino acids Gly¹⁸ to end residue Glu of CyPA.

For the CyPB^{L171;R18A} construct, the region coding for the N-terminal region of CyPB up to Gly²⁵ was amplified with PCR using the same 5' primer as above and a 3' primer designed so as to introduce the desired mutations while adding flanking *Aat*II and *Hind*III restriction sites. The *Aat*II site was selected because it was compatible with the desired final amino acid sequence and not present in the original cyclophilin coding sequence. This fragment was subcloned between the *Nco*I and *Hind*III sites of pKK233-2. The region coding for the remainder of CyPB was amplified by PCR while introducing flanking *Aat*II and *Hind*III sites. It was subcloned downstream of the above-mentioned fragment in pKK233-2 to generate a plasmid coding for a mutant form of CyPB where Leu¹⁷ and Arg¹⁸ have been changed to Ile and Ala, respectively.

All the constructs were verified by complete DNA sequencing on the cyclophilin-coding part and the flanking regions, and then used for transformation of competent *Escherichia coli*. A single colony was picked in each case for the expression studies. Cultures (1.5–3 l) of transformed *E. coli* were grown at 37 °C to an absorption value at 550 nm of 2. Induction was with 1 mM isopropyl-D-thiogalactopyranoside for 4 h before harvesting.

Preparation of recombinant wild-type and mutant CyPB

Purifications of recombinant wild-type CyPB and CyPB^{L171;R18A} were performed using the procedure described in [14]. Purification of the CyPB/A chimaera was performed using the same procedure but the ion-exchange chromatography step on a Mono S column was performed at pH 6.0 instead of pH 6.5 due to the lower pI of the protein.

Peptide synthesis

Peptides were synthesized using the conventional solid-phase 'Boc-benzyl strategy' [22] on 'PAM' resins [23] in an automated Applied BioSystem 470A peptide synthesizer, using an *N,N*-dicyclohexylcarbodi-imide/hydroxybenzotriazole-based coupling procedure. After fluorohydric acid cleavage from the resin in the presence of scavengers, crude deprotected peptides were purified by trifluoroacetic acid/diethyl ether precipitation followed by gel permeation.

Derivatization of recombinant CyPB with *p*-hydroxyphenylglyoxal and AMCA-HPDP

Labelling of CyPB with AMCA-HPDP

A sample (0.536 mg) of AMCA-HPDP was dissolved in 1 ml of DMSO (1.55 mM final concentration). CyPB was diluted in PBS/EDTA (1 mM) to a final concentration of 0.5 mg/ml. CyPB was conjugated with AMCA-HPDP at a molar ratio of reagent to CyPB of 10:1. Incubation was performed at room temperature for 90 min. The reaction of AMCA-HPDP with the sulphhydryl group of CyPB was followed with spectrophotometry since the reaction leaving group, pyridine-2-thione, has a characteristic absorption at 343 nm. Free AMCA-HPDP was removed by gel filtration on a Sephadex G-25 PD10 column (Pharmacia). The ratio of AMCA-HPDP to CyPB was estimated by measuring the absorbance values at 280 nm and 345 nm according to the instructions of the manufacturer.

Modification of arginine residues with *p*-hydroxyphenylglyoxal

An aliquot (0.5 mg) of CyPB (or CyPB^{L171;R18A} or *D¹EKKKG-PKVTVKVYFDLRIGDEDV²⁴ peptide) was reacted with 0.5 mg of *p*-hydroxyphenylglyoxal in 0.5 ml of 0.1 M sodium borate buffer, pH 9.0. The reaction mixture was incubated for 1 h at 25 °C in the dark. The excess of *p*-hydroxyphenylglyoxal was eliminated by gel filtration on a Sephadex G-25 PD10 column (Pharmacia) for CyPB and on a Sephadex G-10 column (1.5 cm × 10 cm) for the D¹EKKKGPKVTVKVYFDLRIGDEDV²⁴ peptide. The content of modified arginine residues was estimated based on the molar absorption coefficient for the product. Modified arginine residues exhibit a maximum absorbance at 335 nm with a molar absorption coefficient of $1.85 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at pH 9.0 [24].

Cells

The human lymphoblastic T-cell line Jurkat was routinely cultured in RPMI 1640 medium (Life Technologies, Inc.) containing 2 mM L-glutamine, 20 mM Hepes, 25 mM NaHCO₃ and 5 µg/ml gentamicin (pH 7.3), supplemented with 10% heat-inactivated fetal calf serum. The culture was diluted every 2 days in order to maintain a cell concentration ranging from 0.5×10^6 to 1×10^6 cells/ml.

Competition binding assays

¹²⁵I-CyPB was obtained by incubating 100 µg of protein with 0.3 mCi of carrier-free Na¹²⁵I (ICN Biochemicals, Costa Mesa, U.S.A.) in the presence of 100 µg of Iodo-Gen (Pierce, Rockford, U.S.A.) for 15 min at room temperature. Free iodine was removed by gel filtration on a Sephadex G-25 PD10 column. The specific radioactivity was approx. $(2-3) \times 10^6$ c.p.m./µg.

The cells were washed twice and diluted in Dulbecco's PBS (DPBS)–0.5% BSA to a final concentration of 4×10^6 cells/ml. The specificity of CyPB binding was studied by incubating

Jurkat T-cells (2×10^6 cells/sample) with 25 nM ^{125}I -CyPB and a molar excess of competitors for 1 h at 4 °C. After incubation, the cells were centrifuged (400 g, 5 min) and washed three times with 3 ml of cold DPBS. Cell-associated radioactivity was measured using a model 1282 Compugamma LKB–Wallac counter.

RESULTS

Competitive binding assays with cyclophilin isoforms

The specificity of CyPB binding to Jurkat T-cells was examined by competitive binding experiments in the presence of two other cyclophilins, CyPA and CyPC. Unlabelled CyPA, CyPB or CyPC were tested for their ability to compete with ^{125}I -CyPB for binding to the receptor. To this end, cells were incubated with recombinant ^{125}I -CyPB in the presence of increasing concentrations of unlabelled cyclophilins (Figure 1). As expected, ^{125}I -CyPB binding was inhibited by 80% in the presence of a 500 molar excess of unlabelled CyPB. Conversely, neither CyPA nor CyPC had a competitive effect on the binding of ^{125}I -CyPB to Jurkat T-cells, showing that these two proteins have no affinity for the CyPB receptor present on these cells.

Competitive binding assays with the synthetic peptides corresponding to CyPB extremities

The interactions of the synthetic peptides corresponding to the first 24 N-terminal amino acid residues (D¹EKKKGPKVT-VKVFYDLRIGDEDV²⁴) and to the last 15 C-terminal amino acid residues (C¹⁷⁰GKIEVEKPFIAIAKE¹⁸⁴) of CyPB (Figure 2) were characterized by competitive binding experiments using Jurkat T-cells. The cells were incubated with recombinant ^{125}I -CyPB in the presence of various concentrations of unlabelled peptides (Figure 3). The binding of ^{125}I -CyPB to Jurkat cells was specifically inhibited by addition of the N-terminal peptide but not by the C-terminal peptide derived from CyPB. Increased concentrations of the N-terminal peptide (from 10 to 500 molar excess) inhibited the binding of ^{125}I -CyPB up to 38%. The

Figure 1 Competitive binding assays of ^{125}I -CyPB to Jurkat T-cells with other cyclophilins

Jurkat T-cells were incubated with ^{125}I -CyPB for 1 h at 4 °C together with increasing concentrations of CyPA (○), CyPB (■) or CyPC (●). The amount of bound ^{125}I -CyPB, as a percentage of ^{125}I -CyPB bound in the absence of competitor, is plotted against the molar ratio of ^{125}I -CyPB to the competitors. Data are expressed as mean values from three separate representative experiments.

Figure 2 Amino acid sequence alignment of CyPB with CyPA and CyPC

The D¹EKKKGPKVT-VKVFYDLRIGDEDV²⁴ and C¹⁷⁰GKIEVEKPFIAIAKE¹⁸⁴ peptides are boxed and respectively termed N-terminal (Nt) peptide and C-terminal (Ct) peptide. The L¹⁷¹R¹⁸⁴ sequence in CyPB which is replaced by I¹⁷¹A¹⁸⁴ in CyPA is typed in white on black. Arg¹⁸ and Cys¹⁷⁰, which are modified by *p*-hydroxyphenylglyoxal and AMCA-HPDP respectively, are indicated by arrows.

concentration of unlabelled N-terminal peptide required for half-maximal inhibition (IC₅₀) of ^{125}I -CyPB binding was 2.2 μM.

Competitive binding assays with modified CyPB and modified N-terminal peptide

CyPB was modified either by AMCA-HPDP for targeting the single cysteine residue, or by *p*-hydroxyphenylglyoxal for the arginine residues. The modified proteins were then used as competitors in competitive experiments carried out as described above.

Modification of cyclophilins with *p*-hydroxyphenylglyoxal

To investigate the possible involvement of arginine residues in the binding of CyPB to Jurkat T-cells, CyPB was treated with *p*-hydroxyphenylglyoxal under mild conditions. This compound modifies proteins by reacting highly selectively with the guanidyl group of the arginine residues to form a stable complex [24]. The *p*-hydroxyphenylglyoxal reaction with arginine residues in proteins is accompanied by a marked spectral change. Figure 4 shows the UV absorption spectra of *p*-hydroxyphenylglyoxal-treated CyPB and CyPB^{L171;R184}. The CyPB spectrum (Figure 4, curve a) has a maximum absorption at 335 nm as expected for a *p*-hydroxyphenylglyoxal-modified protein. A molar absorption coefficient of $1.85 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at pH 9.0 was used to determine the amount of modified arginine residues [24]. The extent of reaction of arginine residues with *p*-hydroxyphenylglyoxal calculated using this value and the absorption at 335 nm, was 1.15 residues per molecule. In contrast, the modified CyPB^{L171;R184} spectrum (Figure 4, curve b), which lacks the

Figure 3 Competitive binding assays of ^{125}I -CyPB to Jurkat T-cells with synthetic peptides corresponding to CyPB extremities

Jurkat T-cells were incubated with ^{125}I -CyPB for 1 h at 4 °C together with increasing concentrations of CyPB (■), D¹EKKKGPKVTVKVF²⁴ peptide (○) (Nt peptide) and C¹⁷⁰GKIEVEKPF¹⁸⁴ (●) (Ct peptide). The amount of ^{125}I -CyPB, as a percentage of ^{125}I -CyPB bound in the absence of competitor, is plotted against the molar ratio of ^{125}I -CyPB to the competitors. Data are expressed as mean values from three separate representative experiments.

Figure 4 UV absorption spectra of *p*-hydroxyphenylglyoxal-treated cyclophilins

Curve (a): CyPB treated with *p*-hydroxyphenylglyoxal at pH 9.0 for 1 h at 25 °C. Curve (b): CyPB^{L171:R18A} treated with *p*-hydroxyphenylglyoxal under the same conditions. The spectra were recorded at pH 9.0 and at room temperature.

Arg¹⁸, showed only a weak absorption at 335 nm, corresponding to 0.2 modified arginine residue per molecule. These values confirm that only a limited derivatization has taken place under

Figure 5 Competitive binding assays of ^{125}I -CyPB to Jurkat T-cells with modified CyPB or N-terminal (Nt) peptide

Jurkat T-cells were incubated with ^{125}I -CyPB for 1 h at 4 °C together with increasing concentrations of CyPB (■), AMCA-HPDP-labelled CyPB (○) (Cys-modified CyPB), *p*-hydroxyphenylglyoxal-modified CyPB (●) (Arg-modified CyPB) and *p*-hydroxyphenylglyoxal-modified D¹EKKKGPKVTVKVF²⁴ peptide (□) (Arg-modified Nt peptide). The amount of ^{125}I -CyPB, as a percentage of ^{125}I -CyPB bound in the absence of competitor, is plotted against the molar ratio of ^{125}I -CyPB to the unlabelled cyclophilins. Data are expressed as mean values from three separate representative experiments.

the conditions used since a total of seven and six arginine residues are present in CyPB and CyPB^{L171:R18A} respectively.

Competitive binding assays with modified CyPB, CyPB^{L171:R18A} or the N-terminal peptide

The effect of the modification of CyPB with *p*-hydroxyphenylglyoxal on the association to the Jurkat T-cells is shown in Figure 5. The data demonstrate that the modification of one arginine residue of CyPB is accompanied by a loss of the binding to the Jurkat T-cells. A confirmation was obtained by modifying the only arginine residue of the D¹EKKKGPKVTVKVF²⁴ peptide. The modified peptide also failed to compete with ^{125}I -CyPB for binding to its receptor (Figure 5).

The efficiency of the CyPB labelling by AMCA-HPDP was controlled by the measurement of absorbances at 280 nm and 345 nm. A fluorochrome/protein ratio of 0.82 was obtained, consistent with the fact that CyPB has only one cysteine residue, localized at position 170. As shown in Figure 5, derivatization of CyPB with AMCA-HPDP did not significantly interfere with its binding to Jurkat T-cells. Less than 20 % of deviation of the IC₅₀ value was observed using AMCA-HPDP-labelled CyPB as a competitor, even in 500 molar excess.

Competitive binding assays with CyPB mutants

In order to further confirm the importance of the N-terminus of CyPB in the interaction with the receptor, two mutant forms were engineered and tested. In one mutant the N-terminal region specific to CyPA was replaced by the N-terminal CyPB specific region to give a CyPB/A chimera. In the second mutant, the L¹⁷R¹⁸ residues of CyPB were exchanged for the IA residues located at equivalent positions in the N-terminal region of CyPA, to give the CyPB^{L171:R18A} mutant. Competitive binding experiments were carried out to test the affinity of these CyPB

Figure 6 Competitive binding assays of ^{125}I -CyPB to Jurkat T-cells with CyPB mutants

Jurkat T-cells were incubated with ^{125}I -CyPB for 1 h at 4 °C together with increasing concentrations of CyPB (■), CyPB^{L171; R18A} (□) and CyPB/A (●). The amount of labelled CyPB, as a percentage of ^{125}I -CyPB bound in the absence of ^{125}I -CyPB, is plotted against the molar ratio of competitor to the unlabelled cyclophilins. Data are expressed as mean values from three separate representative experiments.

mutants for the Jurkat T-cell receptor (Figure 6). The CyPB/A chimera was able to inhibit the binding of ^{125}I -CyPB but only to a certain extent. The IC_{50} of CyPB/A chimera was estimated to be 21 μM and corresponds to a 70-fold lower affinity for the receptor than CyPB. In contrast, the CyPB^{L171; R18A} mutant inhibited ^{125}I -CyPB binding as well as CyPB did. The IC_{50} values of CyPB and CyPB^{L171; R18A} binding to the specific receptor differ by less than 20%.

DISCUSSION

Although CyPA and CyPC are closely related to CyPB, they do not bind to the Jurkat T-cell receptor [21]. This is accountable for CyPA which is exclusively cytosolic and exhibits several marked structural divergences from CyPB. It is more surprising for CyPC since it shares more than 70% sequence identity with CyPB. It has so far not been demonstrated that CyPC is secreted despite the presence of a signal sequence which directs it to the secretory pathway [9,15]. The only known ligand for CyPC, the CyPC-associated protein (CyCAP), is also a protein which follows the secretory pathway [25]. Our results demonstrate the fine specificity of the interaction between CyPB and its receptor on Jurkat T-cells.

Major sequence differences between CyPA and CyPB are located in the N- and C-terminal regions, which were therefore obvious choices for use in competitive binding experiments. Antibodies raised against the corresponding peptides D¹EKKKGPKVTVKVYFDLRIGDEDV²⁴ and C¹⁷⁰GKIEVEKPFAIAKE¹⁸⁴ do not recognize CyPA [20], testifying to the specificity of these peptides. While the C¹⁷⁰GKIEVEKPFAIAKE¹⁸⁴ peptide had no inhibitory effect on the binding of ^{125}I -CyPB to Jurkat T-cells, the D¹EKKKGPKVTVKVYFDLRIGDEDV²⁴ peptide inhibited the binding down to 38% of control levels. The incomplete inhibition observed for this peptide (only a 5-fold increase of IC_{50}) may be explained by secondary

structure differences and/or by the fact that all the residues involved in the interaction with the receptor are not included in this peptide.

The comparison of the X-ray structures of CyPA and CyPB shows that the proteins share the same overall conformation except for two loops, one of them located between the residues 19 to 24 of CyPB [26]. The area covered by the D¹EKKKGPKVTVKVYFDLRIGDEDV²⁴ peptide therefore encompasses one of the two main structural differences between CyPA and CyPB, consistent with the fact that, unlike CyPB, CyPA does not bind to Jurkat T-cells [21].

Chemical modification of arginine residues has been proved valuable in understanding structure-function relationships in proteins [27]. In our study, we modified the arginine residues of CyPB with *p*-hydroxyphenylglyoxal. This compound specifically neutralizes the positive charge of arginine by complexation with the guanidyl group but leaves the other amino acids unchanged. Our results clearly demonstrate that the derivatization of one arginine residue in CyPB abolishes its capacity to inhibit the binding of ^{125}I -CyPB to Jurkat T-cells. The arginine residue modified by *p*-hydroxyphenylglyoxal in CyPB might be Arg¹⁸, since only one arginine residue is modified in the protein under mild conditions and modified D¹EKKKGPKVTVKVYFDLRIGDEDV²⁴ peptide is also unable to inhibit the binding of ^{125}I -CyPB to Jurkat T-cells. Another piece of evidence that Arg¹⁸ is the modified arginine residue is provided by the treated CyPB^{L171; R18A}, which shows only 0.2 modified arginine residue. We surmise that this residue is particularly exposed at the surface of the molecule and easily modified by chemical agents. There is no corresponding arginine residue in CyPA. Taking all this together, it is likely that Arg¹⁸ is at least indirectly involved in the interaction with the receptor. It is close enough to the CyPB-interacting site to prevent binding by steric hindrance when modified. Actually, it has been shown that two molecules of *p*-hydroxyphenylglyoxal react with one arginine residue to form a high size complex [24].

Labelling a protein in the vicinity of the receptor-binding site by a fluorophore is a good tool with which to inhibit protein-protein interaction by masking the interaction site. CyPB has a single cysteine residue located at position 170 just before the C-terminal extension. Furthermore, the N- and C-terminus ends of CyPB are aggregated together and point away from the molecular bulk to form a β -strand [25]. To hide this part of the protein, AMCA-HPDP was used because of its high specificity for sulphhydryl groups and the presence of a six-carbon arm creating a high steric hindrance. But, despite a fluorophore/protein ratio of 0.82, the labelling of CyPB by AMCA-HPDP had only a minor effect on the binding to the Jurkat T-cell receptor, indicating that the receptor binding site is not in the area close to the modified cysteine residue. This cysteine is also found in the C¹⁷⁰GKIEVEKPFAIAKE¹⁸⁴ peptide, and the absence of inhibitory effect of this peptide is in agreement with this finding.

Two CyPB mutants were designed to complete the results described above. In the CyPB/A chimera, the N-terminus of CyPB up to Val²⁴ was exchanged for the corresponding region of CyPA. The chimaeric protein binds to the Jurkat receptor, however, with a 70-fold lower affinity than CyPB. There are at least two possible explanations for this. First, the lower affinity might be due to a different conformation of D¹EKKKGPKVTVKVYFDLRIGDEDV²⁴, when placed in CyPA instead of CyPB. This is supported by the fact that the IC_{50} of the free D¹EKKKGPKVTVKVYFDLRIGDEDV²⁴ peptide is 10-fold higher than the one measured for the CyPB/A chimera. Secondly, it is possible that another part of CyPB is involved in the interaction with the receptor. This is observed for instance

with interleukin-8 (IL-8) binding to both its A and B type receptors. While the E⁴LR⁶ sequence is necessary for the binding, the Tyr¹³ and Lys¹⁵ residues are required for a high-affinity binding [28]. There are some similarities between the cyclophilins and IL-8. In fact, like the cyclophilins, IL-8 is able to specifically bind to CsA. IL-8 was described as a dimeric molecule with a three-dimensional structure resembling that of the cyclophilins [29]. Moreover, the E⁴LR⁶ consensus sequence of IL-8 is quite similar to the D¹⁶LR¹⁸ sequence of CyPB, suggesting that these residues may be important for the CyPB interaction with the receptor. In contrast to the results obtained when using *p*-hydroxyphenylglyoxal, a CyPB mutant where the L¹⁷R¹⁸ sequence was modified to the corresponding I¹¹A¹² sequence of CyPA binds to the Jurkat receptor as well as CyPB does, suggesting that neither Leu¹⁷ nor Arg¹⁸ directly interact with the receptor. Consequently, the best explanation for the loss of competitive binding of Arg-derivatized CyPB is probably that the modification forms a complex sufficiently large and close to the binding site to prevent the interaction with the receptor.

In conclusion, our results demonstrate that the first 24 amino acid residues of CyPB are involved in the binding to Jurkat T-cells. This region contains a single arginine residue, preventing the interaction with the receptor when derivatized but not when mutated to Ala. The use of molecular modelling to visualize the effects of the chemical modifications of arginine and cysteine residues will be another step towards a better characterization of the CyPB-binding site. Site-directed mutagenesis will allow a more precise characterization of the amino acid residues directly implicated in the interaction with the receptor. We also confirm the specificity of the Jurkat T-cell binding sites for CyPB, as opposed to CyPA, in keeping with the fact that CyPB is secreted in biological fluids such as blood and milk and that CyPA is exclusively cytosolic. CyPC does not bind to Jurkat T-cells either despite the presence in its N-terminal region of a stretch highly similar to the 24 N-terminal amino acids of CyPB. Interestingly, the amino acid corresponding to Arg¹⁸ of CyPB is conserved in CyPC. A fine analysis of the differences between the different cyclophilins should provide further clues as to the residues involved in receptor binding by CyPB.

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