Delineation of the calcineurin-interacting region of cyclophilin B

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Abstract

The immunosuppressant drug cyclosporin A (CsA) inhibits T-cell function by blocking the phosphatase activity of calcineurin. This effect is mediated by formation of a complex between the drug and cyclophilin (CyP), which creates a composite surface able to make high-affinity contacts with calcineurin. In vitro, the CyPB/CsA complex is more effective in inhibiting calcineurin than the CyPA/CsA and CyPC/CsA complexes, pointing to fine structural differences in the calcineurin-binding region. To delineate the calcineurin-binding region of CyPB, we mutated several amino acids, located in two loops corresponding to CyPA regions known to be involved, as follows: R76A, G77H, D155R, and D158R. Compared to wild-type CyPB, the G77H, D155R, and D158R mutants had intact isomerase and CsA-binding activities, indicating that no major conformational changes had taken place. When complexed to CsA, they all displayed only reduced affinity for calcineurin and much decreased inhibition of calcineurin phosphatase activity. These results strongly suggest that the three amino acids G77, D155, and D158 are directly involved in the interaction of CyPB with calcineurin, in agreement with their exposed position. The G77, D155, and D158 residues are not maintained in CyPA and might therefore account for the higher affinity of the CyPB/CsA complex for calcineurin.

Keywords: calcineurin; cyclophilin A; cyclophilin B; cyclosporin A; phosphatase activity

Cyclophilins (CyPs) are highly conserved proteins first isolated as the main binding proteins for cyclosporin A (CsA), an immunosuppressive drug widely used in the prevention of graft rejection (Handschumacher et al., 1984; Harding et al., 1986). They were later identified as peptidyl-prolyl cis/trans isomerases (PPIases), a family of enzymes catalyzing protein folding (Fischer et al., 1989; Takahashi et al., 1989). The enzymatic activity of CyPs is strongly inhibited by CsA, due to the binding of the drug over the catalytic site. The CyP/CsA complex binds to and inhibits calcineurin (CN), a calcium-dependent serine/threonine phosphatase, also named protein phosphatase 2B (Friedman & Weissman, 1991; Liu et al., 1991). CN is a heterodimer composed of a 59 kDa A subunit (CNA) and of a 19 kDa B subunit (CNB). Four distinct functional domains have been identified in CNA: a catalytic domain, a CNB-binding domain, a calmodulin-binding domain, and an autoinhibitory domain (Klee et al., 1979; Hashimoto et al., 1990; Watanabe et al., 1995). The phosphatase activity of CNA is stimulated both by Ca2+ binding to CNB and by Ca2+-induced binding of calmodulin to CNA (Stemmer & Klee, 1994). The composite surface formed by the CyP/CsA complex contacts CN (Etzkorn et al., 1994) and even though CNA has been found to be the main partner, CNB appears also to be directly recognized. The ensuing noncompetitive inhibition of CNA phosphatase activity is instrumental in the immunosuppressive effects of CsA (Haddy et al., 1992; Husi et al., 1994).

Different members of the CyP family have been described. They all contain a conserved core domain carrying the CsA binding, CN interaction, and isomerase sites, flanked by distinct N- and C-termini accounting for their specificities (Gething & Sambrook, 1992; Galat, 1993). The prototype of this family is the abundant cytosolic 18 kDa form, now named CyPA (Handschumacher et al., 1984). Cyclophilin B (Spik et al., 1991; Arber et al., 1992) and CyPC (Schneider et al., 1994) are closely related, but their mRNA encodes a signal peptide that directs them to the secretory pathway. An amino acid sequence alignment reveals 65% identity between CyPA and CyPB (Spik et al., 1991; Gething & Sambrook, 1992) and more than 70% identity between CyPB and CyPC (Schneider et al., 1994). In the central core of the three forms, the amino acid conservation is over 80%, suggesting that the regions responsible for CsA binding, CN interaction, and isomerase activity are very similar. The three-
dimensional structure of human CyPA (Ke et al., 1991; Ottiger et al., 1997), human CyPB (Mikol et al., 1994), and murine CyPC (Ke et al., 1993) have been solved and, as expected, the central core is similarly shaped. The structure includes eight antiparallel \( \beta \)-strands forming a right-handed \( \beta \)-barrel overlayed by connecting loops and \( \alpha \)-helices (Ke et al., 1991).

Both the PPiase activity and the CsA-binding site of CyPA are closely localized in a large hydrophobic pocket formed by four \( \beta \)-strands and their connecting loops (Zydowsky et al., 1992), whereas the CN-binding region surrounds the CsA-binding pocket (Etzkorn et al., 1994). The corresponding regions of CyPB and CyPC have not yet been determined experimentally. In analogy to the enzymatic site and the CsA-binding pocket of CyPA where residues R55, F60, and W121 are involved (Zydowsky et al., 1992), and by using site-directed mutagenesis, we have previously demonstrated the enzymatic activity of CyPB to be dramatically reduced after substitution of the R62 and F67 residues, while the CsA-binding activity was lost by mutation of the W128 residue and strongly decreased after modification of the F67 residue (Carpentier et al., 1999).

Concerning the interaction between CyPA and CN, it has been found that the basic amino acids R69 and R148 located in the loops 69RHNG72 and 146GSRNG150, and K125 located in the \( \beta \)-helix 120EWLDGK125, which all reside on the protein surface around the CsA-binding site, are specifically implicated (Etzkorn et al., 1994). In vitro experiments have shown that CyPB and CyPC bound to CsA also form a complex with CN and block its phosphatase activity (Swanson et al., 1992; Bram et al., 1993; Ryffel et al., 1993). This inhibition is higher with CyPB/CsA (\( K_i = 20 \) nM) (Bram et al., 1993; Etzkorn et al., 1994) than with CyPC/CsA (\( K_i = 37 \) nM) (Bram et al., 1993) and with CyPA/CsA (\( K_i = 40 \) nM) (Bram et al., 1993) and 270 nM (Etzkorn et al., 1994), and might reflect varying binding affinities of the CyP/CsA complexes for CN, possibly due to fine structural differences between the CN-binding regions of the three isoforms.

In the present study, we aimed to delineate the CN-binding region of CyPB for a better understanding of the differences in CN inhibition displayed by the different CyP/CsA complexes. Based on the sequence homologies between CyP isoforms and on their three-dimensional configurations, we identified the potentially involved amino acids in CyPB. These residues were individually modified and the ability of the resulting mutant proteins complexed with CsA to bind to CN was assessed using an immunoblot assay developed for that purpose. This allowed us a precise comparison of the respective affinities of the complexes formed by CsA and wild-type or mutated CyPB.

Results

Comparative analysis of CyP sequence and conformation

A comparison of the three-dimensional conformations of human CyPA, human CyPB, and murine CyPC (Fig. 1) revealed that the eight antiparallel \( \beta \)-strands and the two \( \alpha \)-helices were all maintained. The amino acid sequence at the bottom of the CsA-binding pocket was highly conserved, while that of the surrounding loops showed more specificity between the three isoforms. In the case of CyPA, the two loops 69RHNG72 and 146GSRNG150, and the \( \beta \)-helix 120EWLDGK125 are involved in the ability to inhibit CN (Etzkorn et al., 1994). More precisely, the solvent-exposed basic residues R69, K125, and R148 were identified as interacting with

Fig. 1. Comparison of the three-dimensional structure of (A) CyPA, (B) CyPB, and (C) CyPC. The models were visualized with the WinMGM program using the coordinate files (A) ICWA, (B) ICYN, and (C) 2RMC from the Brookhaven National Laboratory Protein Data Bank. CsA is represented with yellow balls. A: The amino acid residues R69, K125, and R148 constitute the CN binding site of CyPA. The residue W121 is involved in the CsA binding site of CyPA. B: The corresponding residues of CyPB R76, K132, R157, and W128 are indicated. C: The corresponding residues of CyPC A103, K159, R186, and W155 are indicated.

CN (Fig. 1A). We have pinpointed the corresponding residues in CyPB, R76, K132, and R157 (Fig. 1B), and in CyPC, A103, K159, and R186 (Fig. 1C). The structures containing these particular
amino acids are the two loops 76RGDG79 and 155DSRDK159, and the 3_{10} helix 127AWLDGK132, and the two loops 103ARDG106 and 182DGHDR186, and the 3_{10} helix 154TWL

GK159 in CyPC (Fig. 2). The sequences 120EWLGDGK125, 127AWLDGK132, and 154TWLGDGK159 are almost identical in the three CyPs and form similar 3_{10} helices (Fig. 2B). More variation is found in the loop regions. Those with the sequences 76RGDG79 and 103ARDG106 in CyPB and CyPC, respectively, are slightly shifted compared to the corresponding loop 69RHNG72 in CyPA (Fig. 2A). More strikingly, the three-dimensional conformation of the β-turn–containing loops 155DSRDK159 and 182DGHDR186 of CyPB and CyPC, respectively, is quite different for the loop 146GSRNG150 of CyPA (Fig. 2C). Another important parameter for the ability of the CyP/CsA complex to interact with CN is the distance between the implicated loops and the α-helix. Here, also, CyPB and CyPC exhibit more resemblance to each other than to CyPA.

To identify the residues responsible for improved CN inhibition by the CyPB/CsA complex, we first compared the loops 69RHNG72, 76RGDG79, and 103ARDG106 of the three CyPs (Fig. 2A). This led us to replace R76 in CyPB with the neutral amino acid A, and G77 with H, the residue found in CyPA. The resulting mutants were termed CyPB_{R76A} and CyPB_{G77H}. The other amino acids of the loop seemed less likely candidates for the differential interactions with CN, as they are highly conserved.

Concerning the 127AWLDGK132 helix (Fig. 2B), we chose to analyze two different mutants. In CyPB_{W128A}, the residue W128, which is conserved in all eukaryotic CyPs and required for binding to CsA, was substituted by A. The second mutation concerned K132, a residue maintained in all three CyPs and potentially constituting an important component of the CN-binding region, as seen with the corresponding CyPA_{K125Q} mutant. Here, two amino acids with a comparable side-chain hindrance were exchanged, which diminished the inhibitory effects of the CyPA_{K125Q}/CsA complex on CN phosphatase activity compared to the wild-type form (Etzkorn et al., 1994). In analogy, we generated the mutant CyPB_{K132Q}, where K132 was replaced by Q.

Finally, we compared the loops 146GSRNG150, 155DSRDK159, and 182DGHDR186 (Fig. 2C). In the case of CyPA, the neutral substitution R148L has little effect on CN inhibitory activity, whereas the acidic substitution R148E leads to a marked increase of CN inhibition (Etzkorn et al., 1994). It has been postulated that CyPB/CsA is a better phosphatase inhibitor than CyPA/CsA due to the two negatively charged residues D155 and D158 flanking R157. Such residues are not found around the analogous residue R148 of CyPA. To check this hypothesis, we devised the mutants CyPB_{D155R} and CyPB_{D158R}, where D155 and D158 were replaced by the positively charged R residue.

**Enzymatic activity assay and CsA-binding analysis**

To ensure that the mutant proteins were all properly folded and functional, the PPIase activity of each of them was measured and compared to that of wild-type CyPB (Table 1). We found the enzymatic activity of the four mutants CyPB_{G77H}, CyPB_{K132Q}, CyPB_{D155R}, and CyPB_{D158R} to be in the range of 95–110% of that of wild-type CyPB. We also measured the interaction of each mutant with CsA (Table 1). CyPB_{G77H}, CyPB_{K132Q}, CyPB_{D155R}, and CyPB_{D158R} displayed 90 to 100% CsA-binding activity, respectively, compared to wild-type CyPB, indicating that the affinity for CsA had not been affected by the amino acid changes. Conversely, the CyPB_{R76A} mutant possessed only much diminished PPIase activity (15 ± 5%) and half-reduced CsA-binding efficiency (50 ± 7%). The CyPB_{W128A} mutant was used as a negative control in the assay (Carpentier et al., 1999). As expected, it exhibited only a reduced PPIase activity (60 ± 10% of wild-type

**Table 1.** PPIase activity and CsA binding efficiency of CyPB mutants compared with that of wild-type CyPB

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<tr>
<th></th>
<th>Relative PPIase activity</th>
<th>Relative CsA binding efficiency</th>
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<tr>
<td>CyPB</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>CyPB_{W128A}</td>
<td>60 ± 10</td>
<td>N.D.</td>
</tr>
<tr>
<td>CyPB_{R76A}</td>
<td>15 ± 5</td>
<td>50 ± 7</td>
</tr>
<tr>
<td>CyPB_{G77H}</td>
<td>100 ± 10</td>
<td>95 ± 8</td>
</tr>
<tr>
<td>CyPB_{K132Q}</td>
<td>95 ± 7</td>
<td>100 ± 9</td>
</tr>
<tr>
<td>CyPB_{D155R}</td>
<td>105 ± 15</td>
<td>90 ± 10</td>
</tr>
<tr>
<td>CyPB_{D158R}</td>
<td>110 ± 15</td>
<td>95 ± 10</td>
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*PPIase activity was measured with a UVP160 spectrophotometer as described (Fischer et al., 1984). CsA binding was determined using an automated LH-20 column binding assay (Handschochamer et al., 1984; Spik et al., 1991).  

*N.D., not detectable.*
CyPB levels), and no detectable interaction with CsA. Our results thus show that apart from CyPB<sub>R76A</sub> and the non-CsA binder, CyPB<sub>W128A</sub>, all CyPB mutants of the putative CN-binding region we generated were fully active PPIases with intact drug-binding properties.

Comparison of the affinities of CyPA/CsA, CyPB/CsA, and CyPB<sub>W128A</sub>/CsA for CN

To estimate the ability of each mutant CyPB/CsA complex to interact with bovine brain CN, we developed a specific immuno-assay for the detection of the formation of the CyP/CsA/CN/calmodulin complex in the presence of Ca<sup>2+</sup>, using an anti-CNA α-antibody directed against the C-terminal part of the protein. In the absence of protein/drug complex, the optical density (OD) at 490 nm was 0.2 ± 0.01, corresponding to nonspecific interactions (Fig. 3). A dose-dependent increase was noted up to 25 μg/mL of CyPB/CsA complex, above which a saturation effect gradually appeared. The maximal OD measured was 1 ± 0.1 for 100 μg/mL of CyPB/CsA. When a similar dose–response curve was generated for the CyPA/CsA complex, saturation of the signal was also attained at high concentrations but the maximal rise in OD only represented 31 ± 6% of that obtained with CyPB/CsA. This value directly reflects the difference of affinities between CN and the CyPB/CsA or CyPA/CsA complexes. In a control experiment, we have determined that in absence of CsA there is no significant interaction (2 ± 4%) between CyPB and CN (data not shown). We then looked at the mutant CyPB<sub>W128A</sub>, which is not bound by CsA. As shown in Figure 3, the affinity of the CyPB<sub>W128A</sub>/CsA complex for CN represented only 3 ± 4% of that of the CyPB/CsA complex, indicating that, as expected, this mutant did not significantly interact with CN.

Comparison of the affinities of CyPB mutants/CsA for CN

We next compared the binding to CN of the CyPB mutants of the putative CN interacting region (Fig. 4). The substitution of R76, G77, K132, D155, or D158 each led to a significant loss of binding to CN, with, however, some differences. The ability of CyPB<sub>G77H</sub>, CyPB<sub>K132Q</sub>, CyPB<sub>D155R</sub>, and CyPB<sub>D158R</sub> complexed to CsA to interact with CN represented 17 ± 3%, 28 ± 5%, 50 ± 10%, and 44 ± 9%, respectively, of that of CyPB/CsA, suggesting a direct implication of these four residues (Table 2). The mutation of G77 had a considerable impact, which underlines the importance of a neutral residue at this position for CN interaction. Mutation of the K132 residue also dramatically affected the affinity for CN, whereas less effects were seen following the substitutions of D155 or D158 by R. They, however, document the role of acidic residues in CN phosphatase activity was measured using the BIOMOL GREEN<sup>TM</sup> CN assay kit in the presence of a large excess of CsA and various concentrations of CyPA, CyPB, and mutated CyPB. The <i>K<sub>i</sub></i> was determined after linearization (1/V vs. [CyP/CsA]). The relative affinity of the CyP/CsA complex for CN was estimated using a detection method for pentamer formation.

![Fig. 3](image-url) Interaction of CyPA, CyPB, and CyPB<sub>W128A</sub> with CN. Recombinant human CyPA (○), CyPB (●), or CyPB<sub>W128A</sub> (△) were coated at the indicated concentrations before adding CsA, CN, and calmodulin. The amount of CN specifically bound was determined using an anti-CNA antibody and peroxidase-conjugated IgG as secondary antibody. Data are means ± SD (bars) of triplicate determination performed in four separate experiments.

![Fig. 4](image-url) Interaction of CyPB or its mutants of the putative CN-binding site with CN. Recombinant human CyPB (●), CyPB<sub>R76A</sub> (○), CyPB<sub>G77H</sub> (■), CyPB<sub>K132Q</sub> (▲), CyPB<sub>D155R</sub> (×), or CyPB<sub>D158R</sub> (○) were coated at the indicated concentrations before adding CsA, CN, and calmodulin. The amount of CN specifically bound was determined using an anti-CNA antibody and peroxidase-conjugated IgG as secondary antibody. Data are means ± SD (bars) of triplicate determination performed in four separate experiments.

<table>
<thead>
<tr>
<th>CyPB Mutant</th>
<th>&lt;i&gt;K&lt;sub&gt;i&lt;/sub&gt;&lt;/i&gt; (nM)</th>
<th>Relative affinity (%)</th>
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<tr>
<td>CyPB</td>
<td>19 ± 2</td>
<td>100 ± 12</td>
</tr>
<tr>
<td>CyPA</td>
<td>40 ± 5</td>
<td>31 ± 6</td>
</tr>
<tr>
<td>CyPB&lt;sub&gt;W128A&lt;/sub&gt;</td>
<td>N.C.</td>
<td>3 ± 4</td>
</tr>
<tr>
<td>CyPB&lt;sub&gt;R76A&lt;/sub&gt;</td>
<td>120 ± 20</td>
<td>14 ± 4</td>
</tr>
<tr>
<td>CyPB&lt;sub&gt;G77H&lt;/sub&gt;</td>
<td>130 ± 15</td>
<td>17 ± 3</td>
</tr>
<tr>
<td>CyPB&lt;sub&gt;K132Q&lt;/sub&gt;</td>
<td>70 ± 6</td>
<td>28 ± 5</td>
</tr>
<tr>
<td>CyPB&lt;sub&gt;D155R&lt;/sub&gt;</td>
<td>39 ± 4</td>
<td>50 ± 10</td>
</tr>
<tr>
<td>CyPB&lt;sub&gt;D158R&lt;/sub&gt;</td>
<td>36 ± 5</td>
<td>44 ± 9</td>
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</table>

*CN phosphatase activity was measured using the BIOMOL GREEN<sup>TM</sup> CN assay kit in the presence of a large excess of CsA and various concentrations of CyPA, CyPB, and mutated CyPB. The <i>K<sub>i</sub></i> was determined after linearization (1/V vs. [CyP/CsA]). The relative affinity of the CyP/CsA complex for CN was estimated using a detection method for pentamer formation.

N.C., no competition.
the loop 155–159 for CN interaction. Finally, the ability of the CyPB\textsubscript{R76A}/CsA complex to interact with CN represented only 14 ± 4% of that of CyPB/CsA, even though half-maximal binding to CsA was still observed.

**Inhibition of the phosphatase activity of CN**

The ability of CyPA, CyPB, and mutated CyPB complexed to CsA to inhibit the phosphatase activity of recombinant human CN was measured using the BIOMOL GREEN\textsuperscript{TM} calcineurin assay kit (Table 2). The $K_i$ obtained with the CyPA/CsA complex was 40 ± 5 nM, and with the CyPB/CsA complex 19 ± 2 nM, demonstrating that the CyPB/CsA complex was a twofold better inhibitor of CN phosphatase activity than the CyPA/CsA complex. The CyPB\textsubscript{G77H}, CyPB\textsubscript{K132Q}, CyPB\textsubscript{D155R}, and CyPB\textsubscript{D158R} were about seven-, 3.5-, two-, and twofold less effective ($K_i$ = 130 ± 15, 70 ± 6, 39 ± 4, and 36 ± 5 nM) than wild-type CyPB for phosphatase inhibition (Table 2). These differences in $K_i$ values correlate well with the respective affinities of the CyP/CsA complexes for CN that we measured (Figs. 3, 4) and confirm the implication of each of these residues in the interaction between CyPB/CsA and CN. The CyPB\textsubscript{R76A}/CsA complex had a $K_i$ similar to that of CyPB\textsubscript{G77H}/CsA, in line with its low affinity for CN but also with the impaired properties of the mutated protein (see Discussion below). As expected, the W128A mutation fully prevented the ability of CyPB to inhibit phosphatase activity, due to the inability to form a complex with CsA.

**Discussion**

Although CyPB is mainly localized in the endoplasmic reticulum and CN is essentially cytoplasmic, the two proteins display also a nuclear localization. Nuclear CyPB is able to capture CsA and to form the pentameric complex with CN (Le Hir et al., 1995). The immunosuppressive effect of CsA is most probably not only caused by the inhibition of cytoplasmic CN usually described, but also by the inhibition of nuclear CN. The inhibition of the phosphatase activity of nuclear CN may mediate the activity of phosphorylated regulatory proteins or transcriptional control elements involved in the activation of T-cells. The study of the better interactions between CN and the CyPB/CsA complex than with the CyPA/CsA complex may have an important physiological relevance to understand the effects of CsA.

We have adapted an immunooassay originally described for the measurement of the interaction of the FKBP12/FK506 complex with CN (Tamura et al., 1994) for a direct comparison of the affinities of CyP/CsA complexes with CN. This method determines the amount of pentameric complex formation between FKBP12, FK506, CN, calmodulin, and Ca\textsuperscript{2+}, using an anti-CNA \textalpha-antibody. We first verified that no significant pentamer complex was formed when using the mutant CyPB\textsubscript{W128A}, which is not bound by CsA and should therefore not interact with CN. We then compared the respective affinities of CyPA/CsA and CyPB/CsA for CN, and measured a threefold lower value for the former complex. We also found CyPA/CsA to be half as potent as CyPB/CsA for inhibition of CN. This is similar to previous results (Swanson et al., 1992; Bram et al., 1993) but different from those of (Etzkorn et al., 1994), who measured a lower, 13-fold reduced activity, altogether we may nonetheless postulate a direct correlation to exist between affinity of a CyP/CsA complex for CN and inhibition of CN phosphatase activity.

To delineate the CN interacting region of CyPB and try to explain the higher inhibitory effects on CN activity, we compared the sequence of the implicated regions from CyPA with the corresponding ones in CyPB and CyPC, and identified five amino acids likely to be involved. These residues were modified to generate the CyPB mutants R76A, G77H, K132Q, D155R, and D158R. The mutant proteins were overexpressed in Escherichia coli, purified, and tested. Each displayed impaired binding to CN.

The R76 residue of CyPB corresponds to R69 of CyPA, which is involved in CN interaction. Its mutation for an A residue in CyPB brought about a much reduced affinity for CN and a higher $K_i$ value for CN phosphatase activity. Though not directed against a residue of the central core, this mutation nonetheless led to impaired isomerase activity and diminished CsA binding. We have already reported that the substitution of the 76RGD78 tripeptide with AAA to similarly affect isomerase activity and completely suppress CsA binding (Carpentier et al., 1999), demonstrating the importance of this region in CyPB activity. The equivalent R69L mutation of CyPA also leads to reduced binding by CsA (Etzkorn et al., 1994). In CyPC an A residue is present at the equivalent position in mice and a T residue in humans (Friedman & Weissman, 1991; Schneider et al., 1994). A direct comparison between these two proteins has not been reported, but both display isomerase activity and CsA binding essentially comparable to CyPA (Bram et al., 1993; Ke et al., 1993; Schneider et al., 1994), suggesting that here the presence of either an A or a T residue is indifferent. Considering the interaction with CN, we cannot infer from our data whether or not R76 is directly implicated. It seems to be the case for the CyPA counterpart, R69, as the CyPAR69E mutant has intact CsA binding properties but a much higher $K_i$ for CN when complexed to CsA (Etzkorn et al., 1994). In FKBP12, however, substitution of the corresponding residue K44 by E has no effect on the inhibitory potential of the complex FKBP12 mutant/FK506 for CN (Aldape et al., 1992).

The presence of the neutral G77 residue in the 76–79 loop of CyPB seems crucial for CN interaction as its mutation had the most pronounced, specific effects. This residue is not found at the same location in murine CyPC or CyPA, both of which are weaker CN inhibitors than CyPB, in complex with CsA (Bram et al., 1993; Etzkorn et al., 1994). They are, however, still more effective than the CyPB\textsubscript{G77H}/CsA complex. Human CyPC has a G residue at the equivalent position but no comparable data about CN inhibition presently exist. The effects of the G77H mutation are therefore probably best explained by the sequence context of the 76–79 loop of CyPB and its three-dimensional structure, which constitute a unique feature essential for the interaction with CN. The presence of an RGD tripeptide in this region has previously been stated (Spik et al., 1991).

The residue K132 of CyPB corresponds to K125 of CyPA and K159 of CyPC. It belongs to a very conserved 3\textalpha helix of CsPs also harboring the residue W128, which is essential for CsA binding (Carpentier et al., 1999). Both the CyPB\textsubscript{K132Q} and CyPA\textsubscript{K125Q} mutants behaved similarly, as they still possessed intact CsA binding properties but a much reduced CN inhibitory activity.

The implication of the residues D155 and D158 of CyPB in the interaction with CN has been suggested by previous work on the CN interacting region of CyPA (Etzkorn et al., 1994). Here, the neutral substitution R148L brings little change in CN inhibition, whereas the acidic substitution R148E increases it. This was explained by the absence around R148 in CyPA of two negatively charged residues, as found flanking R157 in CyPB. The present
study confirmed the importance of the negatively charged residues D155 and D158 around R157 in CyPB, because their modification to R led to a 50% decrease in CN binding. In the corresponding loop of CyPC, there is no basic residue directly corresponding to R148 and R157, even though the three-dimensional model suggests that R186 is located at the equivalent position. The residues D182 and D185, which correspond to D155 and D158 of CyPB, are correctly exposed to permit an interaction with CN. Henceforth, the loops 155–159 of CyPB and 182–186 of CyPC most likely form an important interacting region for CN. Another relevant parameter is probably also the distance between the 3_10 helix and the loops. This distance is more similar between CyPB and CyPC than between CyPB and CyPA, and could be equally important to explain the differences in CN affinity.

Unpublished results from crystallography (generously communicated by Prof. H. Ke) confirm a molecular model of the interactions between CN and the CyPA/CsA complex (Ivery, 1999) based on the crystallographic data available about the interaction of CN with the FKBP12/FK506 complex (Griffith et al., 1995). Three-dimensional data largely corroborate earlier results (Eitzkorn et al., 1994), but are in disagreement with effects observed with some CyPA mutants and their ability to inhibit CN. In the three-dimensional structure, the residues R69 and R148 of CyPA are correctly located to allow a contact with CN, but the residue K125 is not. The structure also clarifies how an acidic residue substituting R148 in CyPA can increase the level of CN inhibition yielded by the corresponding complex with CsA, by analogy with the FKBP12/FK506 complex, as FKBP12 has an acidic E residue at the equivalent position. It does not, however, account for the effects of the K125Q mutation of CyPA, which result in a sixfold lower CN inhibition without affecting the CsA-binding ability, as K125 is not exposed to interact with CN. The author explains this by an indirect effect of the K125Q mutation on the conformation of bound CsA without alteration of the binding efficiency. Indeed, we have also observed a reduction of the affinity of the equivalent CyPB mutant for CN, probably due to an altered conformation of the complex drug. Such an effect was nonetheless not predictable. K125 and K132 are close to W121 and W128 in CyPA and CyPB, respectively, which are critical for CsA binding. In any case, we found that both the CsA binding efficiency and the PP2ase activity of the CyPB K125Q mutant were unchanged, suggesting that the observed effect on CN binding and inhibition was not due to an alteration of the protein configuration. CsA exists in diverse conformations in solution (Altschuh et al., 1994). Its conformation might, therefore, also differ, depending on its binding to a wild-type or mutant form of CyP, although this has not been reported until now.

In conclusion, we found the difference of inhibition of CN activity effected by CyPB/CsA and CyPA/CsA complexes to be directly related to their difference of affinities for CN. Our results with CyPB mutants are not in entire agreement with previous ones concerning CyPA (Eitzkorn et al., 1994) and underline the subtle differences existing between the CN-binding regions of CyPs. The loops 76RGDG79 and 155DSRDK159 of CyPB represent an important regions for CN binding that are more homologous to the loops 103ARDG106 and 182DGDHR186 of CyPC than to the loops 69RHNG72 and 146GSRNG150 of CyPA. The sequence of these regions and more specifically the residues G77, D155, and D158 might be mainly responsible for the better CN inhibitory activity exhibited by the CyPB/CsA complex. In addition, the local configuration and global charge of these regions are probably also of primary importance for the interaction with CN.

Materials and methods

Preparation of recombinant CyP

Recombinant human CyPA and CyPB were purified as previously described (Liu et al., 1990; Spik et al., 1991). For site-directed mutagenesis, a CyPB prokaryotic expression vector based on the pKK232-3 plasmid (Pharmacia, Uppsala, Sweden) was used as template (Mariller et al., 1996). The R76A, G77H, K132Q, D155R, and D158R mutants were generated with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, California) using the oligodeoxynucleotides 5’-gggccgagbdtactcaggagatgcca caggag-3’, 5’-gggccgagactcaccacagtaatgtgaggagaaag-3’, 5’-gcctgctagatgccagcatgtggtttgg-3’, and 5’-ggtggagagcaccaagagctcggcctgtt-3’, and their complementary regions. The DNA sequence of the inserts in the mutated plasmids was verified using the dyeoxy chain termination method (Sanger et al., 1977), and vector- and insert-specific primers. The generation of the CyPB W128A mutant has been described (Carpentier et al., 1999). Production and purification of recombinant wild-type and mutated CyPB were performed using the protocol described before (Spik et al., 1991). Protein separation was performed at pH 6.0 using a UnoS-12 cation exchange column (Bio-Rad, Richmond, California). As we have previously observed, a decrease in PP2ase activity of recombinant CyPB upon time (35–40% loss of enzymatic activity after a two-month storage), all experiments were performed quickly after purification.

Enzymatic activity assay and CsA binding analysis

PP2ase activity was assessed with a UVPC1605 spectrophotometer (Shimadzu, Japan) as described (Fischer et al., 1984), with the difference that the reaction was allowed to proceed for 90 s at 10 °C. For CsA binding determination, an automated LH-20 column (Pharmacia) binding assay was used as described (Handschumacher et al., 1984; Spik et al., 1991). The CsA binding capacity was calculated by comparing the initially applied amount of radioactive activity to that recovered in void volume fractions.

Interaction of CyP/CsA complexes with CN

The interaction between CyP/CsA and CN was determined by using a modification of a previously described method for the measurement of interactions between FKBP12/FK506 and CN (Asami et al., 1993; Tamura et al., 1994). Recombinant human wild-type or CyPB mutants were coated onto the solid surface of immuno-microtiter plates (Nunc Maxisorp, Polylabo, Strasbourg, France) by incubating the protein preparation at 30 °C for 2 h in phosphate buffer saline (PBS; 100 μL per well) at various concentrations. The residual binding sites were blocked by incubating the plate with 250 μL of 50 mM Tris/HCl (pH 7.5) buffer containing 0.5% bovine serum albumin (BSA), 0.1% Triton X-100, 0.5 mM dithiothreitol, 1 mM CaCl2, and 0.1 mM MgCl2 (CN assay buffer) for 30 min at room temperature. The solution was removed by aspiration. The plate was then incubated at 37 °C for 90 min with 100 μL of 5 μM CsA, 50 μL of 60 μg/mL bovine brain CN (Fluka, Buchs, Switzerland), and 50 μL of 10 μg/mL bovine testis calmodulin (Fluka). All preparations were made in the CN assay buffer. The plate was washed with the BSA-free CN assay buffer three times and then incubated with 100 μL of a goat polyclonal
anti-CNA α antisemur (SC#6123; Santa Cruz Biotechnology, Santa Cruz, California) diluted 1,000-fold in the CN assay buffer. Incubation was at 30°C for 90 min, after which the plate was washed three times with the BSA-free CN assay buffer. Following that, 100 μL of a polyclonal anti-goat IgG antibody conjugated to peroxidase (BioSys, Compiègne, France) diluted 2,000-fold in the CN assay buffer were added for a 1 h incubation at room temperature. After washing the plate three times with BSA-free CN assay buffer, 200 μL of 0.4 mg/mL ortho-phenylenediamine (Sigma, Saint-Louis, Missouri) in PBS/citrate pH 5 containing 4 μL/mL of 30% H₂O₂ were added. The plate was incubated at 37°C for 30 min before blocking the reaction with 50 μL of a 3 N H₂SO₄ solution. The coloration intensity was measured at 490 nm on a Bio-Rad microplate reader model 550.

CN inhibition assay
The phosphatase activity of CN was measured in the presence of calmodulin at 30°C, using the colorimetric BIOMOL GREEN™ CN assay kit (BIOMOL Research Laboratories, Plymouth, UK). Briefly, the amount of phosphate liberated from the RII phosphopeptide substrate by CN is determined in microtiter plates using a colorimetric method based on the Malachite green assay. Inhibition of the CN phosphatase activity was observed in the presence of a large excess of CsA (5 μM) and at various concentrations (up to 250 nM) of CyPA, CyPB, and mutated CyPB. The CyP solutions were dialyzed in Tris-buffered saline pH 7.4 beforehand to eliminate all traces of phosphate that could perturb the detection. The inhibition constant (Kᵢ) of the phosphatase activity was calculated after linearization (1/V vs. [CyP/CsA]).

Molecular modeling
Molecular modeling was carried out on a personal computer using the WinMGM software (Rahman & Brasseur, 1994). The structures for human CyPA, human CyPB, and murine CyPC were obtained from the Brookhaven National Laboratory Protein Data Bank as files 1CWA, 1CYN, and 2RMC, respectively.

Statistical analysis
Results are expressed as the mean values ± standard deviation from at least three separate experiments performed in triplicates. Statistical analysis was performed using a Student t-test, and a p-value less than 0.05 was considered as significant.

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References


