Receptor Type I and Type II Binding Regions and the Peptidyl-Prolyl Isomerase Site of Cyclophilin B Are Required for Enhancement of T-Lymphocyte Adhesion to Fibronectin†

Mathieu Carpentier,‡ Fabrice Allain,‡ Marie-Christine Slomianny,‡ Sandrine Durieux,‡ Christophe Vanpouille,‡ Bernard Haendler,§ and Geneviève Spik*,‡

Laboratoire de Chimie Biologique, Unité Mixte de Recherche No. 8576 du Centre National de la Recherche Scientifique, IFR 118, Université des Sciences et Technologies de Lille, F-59655 Villeneuve d'Ascq Cedex, France, and Research Laboratories of Schering AG, D-13342 Berlin, Germany

Received November 15, 2001; Revised Manuscript Received February 20, 2002

ABSTRACT: Cyclophilin B (CyPB), a cyclosporin A (CsA) binding protein, interacts with two types of binding sites at the surface of T-lymphocytes. The type I sites correspond to functional receptors involved in endocytosis and the type II sites to sulfated glycosaminoglycans (GAGs). Mutational analysis of CyPB has revealed that W128, which is part of the CsA-binding pocket, is implicated in the binding to the functional type I receptors and that two amino acid clusters located in the N-terminus ensure the binding to GAGs. The peptidyl-prolyl isomerase activity of CyPB is not required for receptor binding. We have recently demonstrated that CyPB enhances adhesion of peripheral blood T-lymphocytes to fibronectin, a component of the extracellular matrix. We intended to identify additional amino acids involved in the binding of CyPB to its functional type I receptor and to determine regions responsible for the stimulation of peripheral blood T-lymphocyte adhesion. We determined that residues R76, G77, K132, D155, and D158 of the calcineurin (CN) interacting region were implicated in the recognition of type I receptor but not of GAGs. We also found that two different changes in the N-terminal extension that abated binding to GAGs prevented adhesion of peripheral blood T-lymphocytes to coated CyPB, whereas abbrogation of the PPIase activity had no effect. On the other hand, the adhesion of peripheral blood T-lymphocytes to coated fibronectin was not stimulated by CyPB mutants devoid of either type I receptor or GAGs binding activity or by mutants of the PPIase site. Altogether, the results demonstrate that different regions of CyPB are involved in peripheral blood T-lymphocyte activation and imply a novel important physiological function for peptidyl-prolyl isomerase activity.

Cyclophilins (CyPs)[1] are highly conserved and abundant proteins which are bound by cyclosporin A (CsA), an immunosuppressant drug of fungal origin widely used in the prevention of graft rejection (*1*, *2*). The prototype of this family is the cytosolic 18 kDa form, named cyclophilin A (CyPA) (*3*, *4*). Cyclophilin B (CyPB) (*5*) and cyclophilin C (CyPC) (*6*) are closely related but possess an N-terminal signal peptide which directs them to the secretory pathway. Alignment of amino acid sequences reveals 65% identity between human CyPA (hCyPA) and hCyPB (*5*, *7*) and more than 70% identity between hCyPB and human hCyPC (*6*). CyPs exhibit peptidyl-prolyl *cis/trans*-isomerase activity, a rate-limiting step in protein folding which is inhibited by CsA binding. The CyP/CsA complex blocks the phosphatase activity of calcineurin (CN), an event responsible for the immunosuppressive effects of CsA (*1*, *2*, *8*).

CyPs contain a conserved core domain carrying both the CsA-binding site and the isomerase site. This core is flanked by distinct N- and C-termini accounting for the specificities of CyPs (*7*). Determination of the three-dimensional structures of hCyPA (*9*), hCyPB (*10*), and murine CyPC (mCyPC) (*11*) showed the central core to be of comparable overall configuration. Eight antiparallel β-strands forming a right-handed β-barrel overlayed by connecting loops and α-helices have been evidenced. The N- and C-termini also form β-sheets and extend outside the barrel structure with specific differences between CypA and the two other forms. The sites responsible for peptidyl-prolyl isomerase activity and for CsA binding are located in close vicinity inside a large hydrophobic pocket at the surface of the central core (*12*). Concerning the interaction of CyP/CsA complexes with CN, it was found that different residues present in two loops and in the 3_{10} helix residing near the CsA-binding site are involved (*13*, *14*). We demonstrated that subtle differences exist between CyPA and CyPB, in line with the differential interaction with CN (*14*).

† Supported in part by the Centre National de la Recherche Scientifique (UMR No. 8576; Glycobiologie Structurale et Fonctionnelle; Director, Dr. J. C. Michalski, Université des Sciences et Technologies de Lille, France).

* To whom correspondence should be addressed. Tel: 33 3 20 43 41 55. Fax: 33 3 20 43 65 55. E-mail: genevieve.spik@univ-lille1.fr.

‡ Université des Sciences et Technologies de Lille.

§ Research Laboratories of Schering AG.

[1] Abbreviations: BSA, bovine serum albumin; CN, calcineurin; CsA, cyclosporin A; CyP, cyclophilin; CyCAP, CyPC-associated protein; hCyPA, human cyclophilin A; hCyPB, human cyclophilin B; hCyPC, human cyclophilin C; mCyPC, murine cyclophilin C; DPBS, Dulbecco's phosphate-buffered saline; GAG, glycosaminoglycan; K_d, dissociation constant; PPIase, peptidyl-prolyl *cis/trans*-isomerase.

10.1021/bi015951j CCC: $22.00
Published on Web 03/27/2002

CyPB is present in biological fluids such as human milk (*5*) and blood plasma (*15*, *16*). We have previously demonstrated that Jurkat T-cells and human peripheral blood T-lymphocytes exhibit specific surface binding sites for CyPB (*17*). A dissociation constant (K_d) of 10−20 nM and about 30000−120000 binding sites per cell were determined (*18*−*20*). Two different types of CyPB binding sites with similar affinities were found (*21*). The type II sites represent about 70% of the total; they are sensitive to 0.6 M NaCl treatment and were identified as glycosaminoglycans (GAGs) (*21*). Recognition of type II sites is found exclusively for CyPB, since neither hCyPA nor mCyPC displays competition in the binding assay (*21*). The type I sites are insensitive to ionic strength and correspond to a functional receptor, since CyPB binding is followed by endocytosis of the ligand/receptor complex (*21*) and by Ca^{2+} mobilization in the first minute following the addition of ligand (*22*). CsA reduces the interaction of CyPB with the type I receptor (*21*). An interaction between mCyPC and the type I lymphocyte receptor was also found, however, with a 2-fold lower affinity (*21*). In contrast, no significant displacement of bound hCyPB is observed in the presence of hCyPA (*21*). We, however, recently demonstrated that hCyPA generates also intracellular Ca^{2+} rise (220 ± 60 nM) even though hCyPA appeared 2.5-fold less efficient than hCyPB (550 ± 240 nM) (*22*). Both act via interaction with the same signaling receptor (*22*). Taken together, these results indicate that fine differences in the three-dimensional structure and/or a few specific amino acids in the central core of the CyPs probably account for variations in type I receptor-binding affinity (*21*).

We have previously described that the isomerase activity of CyPB is dramatically reduced after substitution of the R62 and F67 residues, while the CsA-binding activity is destroyed by mutation of the W128 residue and strongly decreased after modification of the F67 residue (*23*). Of these mutations, only that of W128 has been found to impair the interaction of CyPB with the type I receptor (*23*). The catalytic site of CyPB therefore does not appear essential, and the CsA-binding region seems only partially involved in type I receptor binding. Concerning the type II binding sites, we have previously shown that the N-terminal region of CyPB and more precisely the 3KKK5 and 14YFD16 clusters are implicated (*23*, *24*). The highly accessible basic 3KKK5 cluster of CyPB can establish ionic interactions with sulfated GAGs. CyPC, however, though possessing a basic RKR cluster at a similar position, does not interact with GAGs. It is the second 14YFD16 cluster which allows the specificity and tightness of interaction between CyPB and GAGs. Additional mutagenesis studies have shown that residues E22 and D23 which belong to the specific loop 19−24 of CyPB are not involved in the binding to GAGs (*23*).

In the present study we analyzed binding capacity of CyPB mutants of the CN-binding region and provide evidence that the region interacting with the type I binding sites overlaps the CN-binding region. We also demonstrate that not only the type I receptor-binding region but also the GAG-binding region and the peptidyl-prolyl isomerase site of CyPB are necessary for biological activities i.e., enhancement in adhesion of peripheral blood T-lymphocytes to the extracellular matrix component fibronectin.

EXPERIMENTAL PROCEDURES

Materials. Prokaryotic expression vectors for CyPB based on the pKK233-2 plasmid (Amersham Pharmacia Biotech, Piscataway, NJ) were used. The generation of the mutants used in this study has been described (*14*, *23*). The mutants analyzed were CyPB mutated in the N-terminal sequence ($CyPB_{K3A;K4A;K5A}$ named $CyPB_{KKK-}$, $CyPB\Delta_{14YFD16}$ named $CyPB\Delta_{YFD}$, $CyPB_{E22K}$, and $CyPB_{D23P}$), CyPB mutated in the CN-binding region ($CyPB_{R76A}$, $CyPB_{G77H}$, $CyPB_{K132Q}$, $CyPB_{D155R}$, and $CyPB_{D158R}$), and CyPB mutated in the PPIase and CsA-binding sites ($CyPB_{R62A}$, $CyPB_{F67A}$, and $CyPB_{W128A}$). Production and purification of recombinant wild-type and mutated CyPB from *Escherichia coli* extracts were performed using previously described procedures (*5*). Protein separation was performed at pH 6.0 using a UnoS-12 cation-exchange column (Bio-Rad, Richmond, CA).

Determination of T-Lymphocyte Adhesion to Immobilized CyPB. CyPB or its mutants were coated to microwell plates (Nunc-Polylabo, Strasbourg, France) by incubating various concentrations of the protein solution overnight in Dulbecco's phosphate-buffered saline (DPBS) containing 2 mM $CaCl_2$ and 1 mM $MgCl_2$ (100 μL; Sigma, St. Louis, MO) at pH 7.4 and 4 °C. The plates were washed twice with DPBS and incubated at 37 °C for 30 min with 200 μL of DPBS containing 0.5% BSA and 1×10^6 peripheral blood T-lymphocytes per well, prepared as described (*18*). The plate was washed twice with DPBS, and the remaining cells were fixed for 30 min with 200 μL of 3% formaldehyde, pH 7.8 at 4 °C. The plate was then washed twice with 250 μL of 100 mM borate (pH 8.2) at room temperature. The remaining fixed cells were colored for 10 min at room temperature with 100 μL of 1% filtered methylene blue. The plate was washed with 250 μL of 100 mM borate (pH 8.2) as long as the borate was blue tinted. Blue coloration contained in adhered cells was liberated through 30 min incubation with 100 μL of 100 mM HCl under agitation. The coloration, which is proportional to the number of adhered cells, was measured at 590 nm with a Bio-Rad microplate reader, model 550. The use of a calibration curve giving the optical density as a function of a known number of T-cells allowed to calculate the number of adhered T-cells per well for the various CyPB concentrations.

Determination of Peripheral Blood T-Lymphocyte Adhesion to Immobilized Fibronectin. Fibronectin purified from human plasma was coated to microwell plates by incubating overnight a 10 μg/mL fibronectin solution in DPBS at pH 7.4 and 4 °C. After two washes with DPBS, the plates were incubated at 37 °C for 20 min with DPBS containing 0.5% BSA, 1×10^6 T-lymphocytes per well, and various concentrations of CyPB or CyPB mutants. The plate was then treated following the procedure described above to determine the cellular adhesion to coated fibronectin.

Molecular Modeling. Molecular modeling was carried out on a personal computer using the WinMGM software (*25*). The crystallographic coordinates of the N-terminal 1DGKKK5 of CyPB (*10*) are not available. The modeling of complete CyPB was performed on the basis of the partially known coordinates of the human CyPB obtained from the Brookhaven National Laboratory Protein Data Bank as file 1CYN and completed with the missing N-terminal sequence. The known coordinates of 6GPK8 indicate a β-sheet conformation. The

Table 1: Competitive Binding Studies with CyPB Mutated in the N-Terminal Region[a]

ligands	K_d (nM) type I binding sites (functional receptor)	K_d (nM) type II binding sites (glycosaminoglycans)
CyPB	20 ± 10	10 ± 5
$CyPB_{KKK-}$	23 ± 10	NC
$CyPB\Delta_{YFD}$	33 ± 11	NC
$CyPB_{E22K}$	24 ± 10	11 ± 7
$CyPB_{D23P}$	23 ± 15	12 ± 8

[a] The indicated ligands were used to compete with wild-type [^{125}I]CyPB for binding to T-lymphocytes. For the discrimination between type I and type II binding sites, cells were washed with DPBS containing 0.6 M NaCl. Radioactivity was then measured in the supernatants (ligand released from the type II sites) and in the 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. NC, no competition.

determination of the putative three-dimensional structure of the N-terminal part was performed by Monte Carlo searching and energy minimization on a Silicon Graphics Optane Workstation using the SYBYL program with the TRIPOS force field (Tripos, St. Louis, MO).

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

RESULTS

Cellular Binding Properties of CyPB Mutated in the N-Terminal Region. We first examined whether the competitive binding profiles of CyPB mutants of the N-terminal extension were comparable when peripheral blood T-lymphocytes and Jurkat T-cells were used. Competitive binding experiments were performed using peripheral blood T-lymphocytes by incubating [^{125}I]CyPB together with increasing concentrations of unlabeled wild-type or CyPB mutated in the N-terminal region. After washing, the cells were treated with 0.6 M NaCl, and the distribution of the remaining surface-bound (type I sites, corresponding to functional receptors) and released (type II sites, corresponding to GAGs) ligand was analyzed.

We found that $CyPB_{KKK-}$, $CyPB\Delta_{YFD}$, $CyPB_{E22K}$, and $CyPB_{D23P}$ were as efficient as wild-type CyPB in inhibiting the binding of [^{125}I]CyPB to the type I binding sites (Table 1). In contrast, $CyPB_{KKK-}$ and $CyPB\Delta_{YFD}$ did not affect the binding of [^{125}I]CyPB to the type II sites whereas $CyPB_{E22K}$ and $CyPB_{D23P}$ reduced it to an extent similar to that measured with the wild-type form. These data indicate that, as previously described for the binding to Jurkat T-cells, the tripeptides 3KKK5 and 14YFD16 are involved in the binding to type II but not to type I sites present at the surface of peripheral blood T-lymphocytes. They also show that residues E22 and D23 are not required for binding to type II sites although they are specific of the CyPB isoform.

Cellular Binding Properties of CyPB Mutated in the CsA-Binding Pocket. Competitive binding experiments using peripheral blood T-lymphocytes indicated that a 7-fold higher concentration of $CyPB_{W128A}$ (K_d = 140 ± 90 nM) than of wild-type CyPB (K_d = 20 ± 10 nM) was necessary to displace 50% of the [^{125}I]CyPB bound to the type I sites. The residue W128 of the CsA-binding pocket of CyPB was therefore most probably involved in the binding to the functional receptor. Residue F67 which is also involved in the binding to CsA was, however, not required for the interaction with the type I receptor since the binding parameters of $CyPB_{F67A}$ (K_d = 32 ± 15 nM) were in the same range as those of wild-type CyPB (K_d = 20 ± 10 nM). We can therefore conclude that the CsA-binding pocket of CyPB is only partially involved in the binding to type I sites. In hCyPA and mCyPC, a W residue is also found at the position equivalent to W128 of CyPB. hCyPA is, however, not able to significantly compete with hCyPB for receptor binding whereas mCyPC has a 2-fold lower affinity (*21*). All of these data strongly suggest that additional residues located near the CsA-binding pocket are involved in the binding of CyPB to its functional receptor. Concerning the type II binding sites, no effect was observed for either mutation, as expected.

Rationale To Identify Other Amino Acids Involved in Receptor Binding. We focused our attention on the characteristic features of the secondary structure surrounding the CsA-binding pocket. The exposed loops 76−79, 155−159, and the 3_{10} helix 127−132 of CyPB which are involved in the interaction with CN (*14*) are candidate regions for establishment of specific interactions with the receptors present on T-cells. Sequence comparison of the equivalent loops 69RHNG72, 76RGDG79, and 103ARDG106 of hCyPA, hCyPB, and mCyPC, respectively, prompted us to generate CyPB mutants where R76 was exchanged with the neutral residue A and G77 with the basic residue H present in CyPA, to generate $CyPB_{R76A}$ and $CyPB_{G77H}$. Concerning the conserved 127AWLDGK132 3_{10} helix of CyPB, we modified residue K132 which is homologous to K125 of CyPA and close to W128 known to be involved in receptor binding. We devised the $CyPB_{K132Q}$ mutant where K132 of CyPB was substituted for a Q residue, an amino acid with a comparable side-chain hindrance. Finally, the three-dimensional conformations of the β-turn-containing loops 155DSRDK159 and 182DGHDR186 of hCyPB and mCyPC, respectively, are totally different from the loop 146GSRNG150 of hCyPA (*14*). Two negatively charged residues, D155 and D158, or D182 and D185, are responsible for the specific conformation of this loop in hCyPB and mCyPC, respectively. To assess the potential importance of these two residues in the interactions of hCyPB with its receptors, we engineered the mutants $CyPB_{D155R}$ and $CyPB_{D158R}$, where an amino acid of the opposite charge was introduced.

Cellular Binding Properties of CyPB Mutated in the CN-Binding Region. Competitive binding experiments conducted with $CyPB_{R76A}$, $CyPB_{G77H}$, $CyPB_{K132Q}$, $CyPB_{D155R}$, and $CyPB_{D158R}$ showed that these mutants were as efficient as wild-type CyPB in inhibiting the binding of [^{125}I]CyPB to the type II binding sites (Table 2), in line with our previous results and demonstrating that only the N-terminal end of CyPB is involved in these interactions (*23*, *24*). In contrast, a 5-fold higher concentration of $CyPB_{K132Q}$, a 9-fold higher concentration of $CyPB_{R76A}$, an 11-fold higher concentration of $CyPB_{G77H}$, and a 20-fold higher concentration of $CyPB_{D155R}$ or $CyPB_{D158R}$ were required to displace 50% of radiolabeled CyPB bound to type I binding sites. The data showed that the five mutated residues were all implicated in the interac-

Table 2: Competitive Binding Studies with CyPB Mutated in the Central Core[a]

ligands	K_d (nM) type I binding sites (functional receptor)	K_d (nM) type II binding sites (glycosaminoglycans)
CyPB	20 ± 10	10 ± 5
$CyPB_{R76A}$	180 ± 70	12 ± 5
$CyPB_{G77H}$	220 ± 100	10 ± 5
$CyPB_{W128A}$	140 ± 90	10 ± 4
$CyPB_{K132Q}$	105 ± 55	9 ± 5
$CyPB_{D155R}$	410 ± 190	12 ± 4
$CyPB_{D158R}$	400 ± 150	10 ± 7

[a] The indicated ligands were used to compete with wild-type [^{125}I]CyPB for binding to T-lymphocytes. The binding curves for each mutant were fitted using the wild-type dissociation constants (K_d) as fixed parameters.

Table 3: Competitive Binding Studies with CyPB Mutated in the PPIase Site[a]

ligands	K_d (nM) type I binding sites (functional receptor)	K_d (nM) type II binding sites (glycosaminoglycans)
CyPB	20 ± 10	10 ± 5
$CyPB_{R62A}$	21 ± 12	10 ± 5
$CyPB_{F67A}$	32 ± 15	12 ± 6

[a] The indicated ligands were used to compete with wild-type [^{125}I]CyPB for binding to T-lymphocytes. The binding curves for each mutant were fitted using the wild-type dissociation constants (K_d) as fixed parameters.

tion with type I receptors. An indirect effect of the changes on the global protein conformation can probably be ruled out as the PPIase activity, and CsA-binding efficiency of the mutated forms is comparable to that of wild-type CyPB, except for $CyPB_{R76A}$ (*14*). This mutant exhibits strongly impaired PPIase activity and half-reduced CsA-binding efficiency even though the R76 residue is not known to be directly required for these activities. It is possible that the R76 mutation leads to improper folding of the central core of the protein so that a direct involvement of this amino acid in receptor binding cannot be inferred. A role of the neighboring G77 residue seems, however, likely, indicating that the configuration of the loop 76−78 is nonetheless important for type I receptor interaction.

Cellular Binding Properties of CyPB Mutated in the PPIase Site. Competitive binding of CyPB mutated in the PPIase site ($CyPB_{R62A}$ and $CyPB_{F67A}$) for either type of receptor was comparable to that of wild-type CyPB, implying that R62 and F67 were not required for type I or type II binding site interaction (Table 3). The PPIase active site is therefore not directly involved in the binding of CyPB to T-lymphocytes.

Location of the Receptor-Binding Regions in the Three-Dimensional Model of CyPB. Analysis of the CyPB structure revealed that the GAG-interacting N-terminal subregions 3KKK5 and 14YFD16 and the type I receptor-binding residues G77, W128, K132, D155, D158, and possibly R76 were located on opposite sites (Figure 1). It also showed that the residues involved in type I receptor binding were on each side of the PPIase site, which is not itself involved in receptor binding. Residues D155 and D158 of the loop 155−159 and R76 and G77 of the loop 76−78 are characteristic of CyPB and spatially close in the three-dimensional

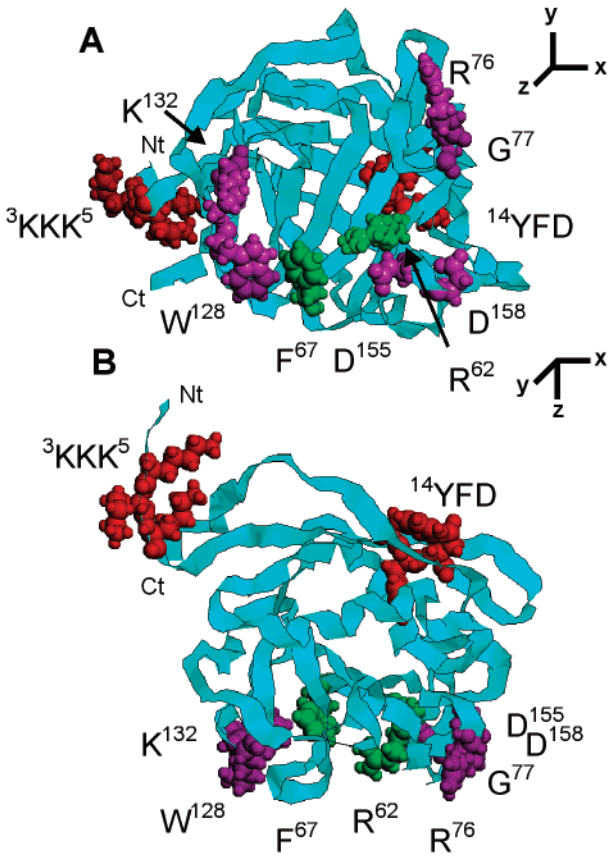

FIGURE 1: Localization of CyPB residues involved in T-lymphocyte receptor binding and in PPIase activity. The tripeptides 3KKK5 and 14YFD16 (red) interact with GAGs. Residue W128 (purple) of the CsA-binding pocket and residues R76, G77, K132, D155, and D158 (purple) of the CN-binding site are involved in type I receptor binding. Residues R62 and F67 (green) belong to the PPIase site. The three-dimensional modeling of CyPB was performed using the incomplete 1CYN PDB file. The missing coordinates of the N-terminal extremity 1DEKKK5 were calculated with the SYBYL software. CyPB is represented before (A) and after (B) a 90° rotation.

structure. The other amino acids, W128 and K132, are in the 3_{10} helix, which is almost entirely conserved among CyPs, and on the opposite side with respect to the PPIase site.

Identification of CyPB Regions Involved in Peripheral Blood T-Lymphocyte Adhesion. The adhesion of peripheral blood T-lymphocytes to immuno-microtiter plates coated with CyPB was analyzed at 37 °C (Figure 2). In the absence of coated CyPB, only about 20000 cells adhered per well. A steep dose-dependent increase of T-lymphocyte adhesion to coated CyPB was observed up to 10 μg/mL, after which the curve flattened to reach a maximum of 470000 cells/well at 30 μg/mL coated CyPB. To determine which CyPB regions were involved, we performed similar adhesion experiments with modified forms. The results obtained with mutants of the N-terminal GAG-interacting part (Figure 2A) of the type I receptor-binding site (Figure 2B) or of the PPIase site (Figure 2C) are shown. All mutants except $CyPB_{KKK-}$ and $CyPB\Delta_{YFD}$ allowed adhesion of T-lymphocytes in a manner similar to that of wild-type CyPB. For $CyPB_{KKK-}$ and $CyPB\Delta_{YFD}$ only a strongly reduced adhesion was observed in comparison to wild-type CyPB. The 3KKK5 and 14YFD16 clusters of the GAG-binding region were therefore important for T-lymphocyte adhesion to coated CyPB. Two other mutations in the N-terminal region, E22K

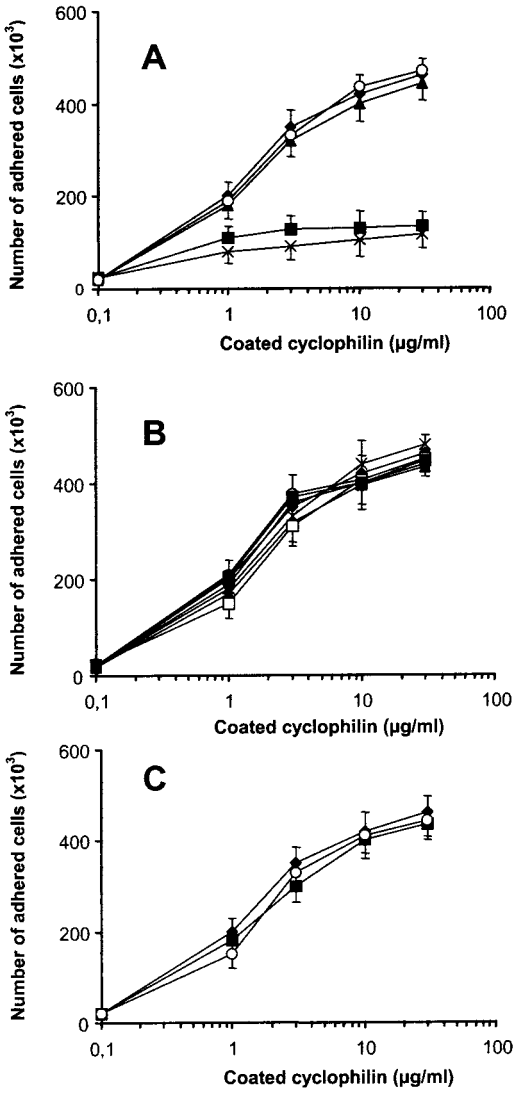

FIGURE 2: Adhesion of T-lymphocytes to coated wild-type or mutated CyPB. CyPB (◆) and (A) N-terminal mutant forms [$CyPB_{KKK-}$ (×), $CyPB\Delta_{YFD}$ (■), $CyPB_{E22K}$ (▲), and $CyPB_{D23P}$ (○)], (B) type I receptor-binding region mutant forms [$CyPB_{R76A}$ (●), $CyPB_{G77H}$ (○), $CyPB_{W128A}$ (□), $CyPB_{K132Q}$ (▲), $CyPB_{D155R}$ (×), and $CyPB_{D158R}$ (■)], and (C) PPIase site mutant forms [$CyPB_{R62A}$ (■) and $CyPB_{F67A}$ (○)] were coated to microwell plates at various concentrations. T-lymphocytes (1×10^6/well) were incubated at 37 °C for 30 min in DPBS/0.5% BSA, pH 7.4. The plate was washed with DPBS. Remaining cells were fixed at 4 °C with formaldehyde. The plate was washed with 100 mM borate, pH 8.2, at room temperature. Remaining fixed cells were colored with methylene blue at room temperature. The plate was washed with borate. Blue coloration was measured at 590 nm. A calibration curve expressing the optical density in function of known numbers of T-cells allowed the calculation of the number of adhered T-cells. Data are means ± SD (bars) of triplicate determinations performed in three separate experiments.

and D23P, which do not affect the binding to GAGs (*23*), had no effect on adherence of T-lymphocytes. CyPB mutated in the type I receptor-binding region ($CyPB_{R76A}$, $CyPB_{G77H}$, $CyPB_{K132Q}$, $CyPB_{D155R}$, and $CyPB_{D158R}$) stimulated T-lymphocyte adhesion as wild-type CyPB did, indicating that the mutated residues were not required for this function. Immobilized CyPB mutated at the PPIase site ($CyPB_{R62A}$ and $CyPB_{F67A}$) also allowed adhesion as well as wild-type CyPB, indicating that the R62 and F67 residues of the PPIase site were not involved either. Altogether, these data indicate that adhesion on coated CyPB was mediated by the type II binding sites of T-cells while the functional type I receptors were not implicated. Additional experiments performed at 4 °C indicated that adhesion was not inhibited at this temperature (data not shown). This strongly suggests that no active process but only ionic interactions between coated CyPB and the cell surface were essential for the adhesion of T-lymphocytes.

Identification of CyPB Regions Involved in the Enhancement of Cellular Adhesion to Coated Fibronectin. Fibronectin is a component of the extracellular matrix to which T-lymphocytes adhere, a phenomenon stimulated when CyPB is present in the medium (*22*). We found a dose-dependent stimulation of peripheral blood T-lymphocyte adhesion to fibronectin in the presence of soluble CyPB (Figure 3). The maximal increase occurred between 50 and 100 nM, above which a saturation was observed. To determine the CyPB regions required for these effects, we tested as above mutants of the N-terminal region (Figure 3A) of the type I receptor-binding sites (Figure 3B) or of the PPIase site (Figure 3C). As in Figure 1 and in our recent work (*22*), cellular adhesion induced by soluble $CyPB_{KKK-}$ or $CyPB\Delta_{YFD}$ was strongly reduced, demonstrating that interactions with GAGs are required for this biological activity of CyPB. $CyPB_{E22K}$ and $CyPB_{D23P}$ enhanced T-lymphocyte adhesion in a manner similar to that of CyPB, in accordance with their intact type II binding site properties (*23*). The $CyPB_{R76A}$, $CyPB_{G77H}$, $CyPB_{W128A}$, $CyPB_{K132Q}$, $CyPB_{D155R}$, and $CyPB_{D158R}$ mutants which possess only a low affinity for the type I receptor were unable to stimulate cell adhesion. These data demonstrate the involvement of the functional type I receptor in enhancing cellular adhesion by soluble CyPB and suggest an active process of downstream signal transduction. The existence of such an active process was further substantiated by the results observed with mutants of the PPIase site. The $CyPB_{R62A}$ and $CyPB_{F67A}$ mutants possess roughly 10% of the wild-type PPIase activity but display full binding to both binding sites types (*23*). Remarkably, they were both significantly impaired in their capability to enhance T-lymphocyte adhesion to fibronectin-coated plates. $CyPB_{R62A}$ had no stimulatory effect at all whereas $CyPB_{F67A}$ was 40-fold weaker at increasing adhesion than wild-type CyPB. The PPIase activity therefore appeared to be required but not sufficient to enhance cellular adhesion, suggesting that interaction of CyPB with its binding sites was a prerequisite for the stimulation of cell adhesion to fibronectin.

DISCUSSION

We have previously demonstrated that CyPB is a member of the heparin-binding protein family (*21*). The biological function of secreted CyPs are not yet defined. Increased CyPB levels have been measured in blood plasma originating from CsA-treated graft recipients (*20*) and from patients suffering from sepsis (*27*) or human immunodeficiency virus-1 infection (*28*), suggesting an involvement of this protein in inflammation. On the basis of our previous findings (*21*−*23*) we postulated that CyPB may act as a proinflammatory factor enhancing adhesion of cells exhibiting its receptors. Knowing the nature of the interactions of CyPB with its two types of cellular binding sites is a prerequisite for a better understanding of the function of this protein. In this study, we have delineated the type I receptor-binding

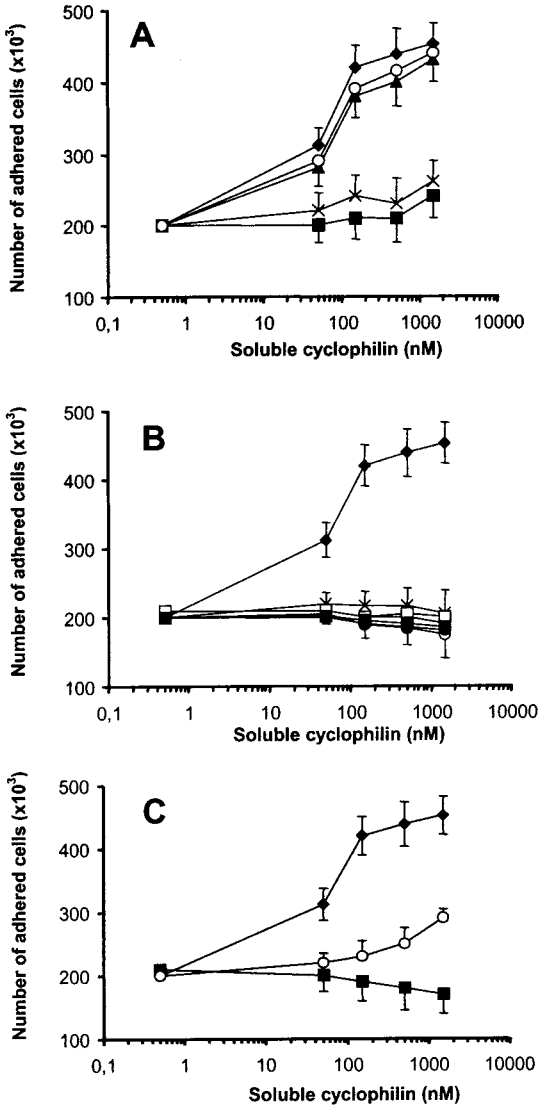

FIGURE 3: Enhancement of T-lymphocyte adhesion to coated fibronectin in the presence of wild-type or mutated CyPB. Fibronectin (10 μg/mL) was coated to microwell plates. T-lymphocytes (1×10^6/well) were incubated at 37 °C for 30 min in the presence of CyPB (◆) and (A) N-terminal mutant forms [$CyPB_{KKK-}$ (×), $CyPB\Delta_{YFD}$ (■), $CyPB_{E22K}$ (▲), and $CyPB_{D23P}$ (○)], (B) receptor-binding region mutant forms [$CyPB_{R76A}$ (●), $CyPB_{G77H}$ (○), $CyPB_{W128A}$ (□), $CyPB_{K132Q}$ (▲), $CyPB_{D155R}$ (×), and $CyPB_{D158R}$ (■)], or (C) PPIase site mutant forms [$CyPB_{R62A}$ (■) and $CyPB_{F67A}$ (○)] at various concentrations. The plate was washed with DPBS. Remaining cells were fixed with formaldehyde. The plate was washed with 100 mM borate, pH 8.2. Remaining fixed cells were colored with methylene blue. The plate was washed with borate. Blue coloration was measured at 590 nm. A calibration curve expressing the optical density in function of the known number of T-cells allowed the calculation of the number of adhered T-cells. Data are means ± SD (bars) of triplicate determinations performed in three separate experiments.

region of CyPB. Up to now only W128 was known to be involved (*23*). This residue is conserved in most eukaryotic CyPs and cannot account for the specificity of the CyPB isoform for the lymphocyte receptors. No affinity of hCyPA for the type I binding sites is determinable while the affinity of mCyPC for the functional receptor is 2-fold lower than that of CyPB (*21*). Additional CyPB residues must therefore be implicated. We surmised that candidate amino acids may be located near the W128 residue of the CsA-binding pocket, as CsA reduces the binding to T lymphocytes, probably due to steric hindrance (*21*). The sequence at the bottom of the CsA-binding pocket of CyPB is very conserved between CyPs while the flanking regions composed of two loops and a helix are more specific (*10*, *11*). The secondary structure around the CsA-binding pocket may explain that the affinity of hCyPB for CsA (K_d = 9.8 nM) is higher than that of hCyPA for CsA (K_d = 36.8 nM) and of mCyPC for CsA (K_d = 90.8 nM) (*29*). Similarly, these structural differences may explain that the affinity of CsA-complexed hCyPB for CN (K_i = 20 nM) is higher than that of complexed mCyPC (K_i = 37 nM) or of complexed hCyPA [K_i = 40 or 270 nM (*13*, *30*)]. We have previously demonstrated that mutation of residues R76, G77, K132, D155, and D158 of the loops 76−79, 155−159, and the 3_{10} helix 127−132 reduced the interaction of the resulting CyPB mutant/CsA complex with CN (*14*). Here we demonstrate that the same residues are also involved in the binding of CyPB to type I receptors at the T-cell surface. There are, however, notable differences in the interaction of CyPB with each partner. CyPB interacts with its type I receptor in absence of CsA, while formation of a complex with CsA is necessary for binding to CN. Moreover, the point mutations we introduced had different impacts on the interaction with the functional receptor or with CN. The D155R and D158R modifications led to a 2-fold lower affinity of mutant CyPB/CsA to CN while the binding affinity for type I receptor was 20-fold reduced. The affinity of $CyPB_{K132Q}$/CsA was only 3-fold reduced for CN while it was 5-fold lower in the case of binding to the functional receptor. Finally, the affinity of CsA-complexed $CyPB_{R76A}$ or $CyPB_{G77H}$ was only 5-fold lower for CN while that of mutated CyPB was 10-fold lower for the type I receptor. Altogether, the results showed a stronger impact of the mutations on receptor binding than on CN binding. Residues R76, G77, D155, and D158 may be instrumental in selective type I receptor recognition since they belong to specific regions of CyPB not maintained in other CyPs, while residues W128 and K132 are conserved among CyP isoforms.

A specific binding partner has been described for mCyPC. It is a 77 kDa membrane protein termed CyPC-associated protein (CyCAP) (*31*, *32*). CsA inhibits the interaction, and neither hCyPA nor hCyPB is able to bind to CyCAP. The regions of mCyPC that interact with CyCAP are thought to be localized in a loop close to the catalytic site and without sequence homology to the other CyPs, which explains the selective recognition (*31*, *32*). Such differences in the primary sequence and in the spatial conformation of the central core of the different CyPs might therefore explain the variations seen in the binding affinities of CyPB and CyPC for their respective interacting partners. The sequences of CyPB and CyPC are more similar compared to that of CyPB and CyPA, in line with a 2-fold lower affinity of mCyPC than of hCyPB for the type I receptor, whereas hCyPA exhibit no quantifiable affinity for the functional receptor (*21*).

A high molecular mass complex receptor for CyPA on T-lymphocytes has been described (*33*). It mediates intracellular Ca^{2+} flux induction, suggesting a transduction pathway to be activated upon CyPA binding (*33*). Most recently, we and others demonstrated that CyPB and CyPA are able to induce Ca^{2+} signaling and chemotaxis via the same functional receptor (*22*, *34*). By contrast, only CyPB is able to mediate integrin-mediated adhesion to the extra cellular

matrix. The unability for CyPA and $CyPB_{KKK-}$ to promote adhesion is relevant to failure in the high-affinity interaction with GAGs (*22*). Binding of CyPA to heparans via a C-terminal basic domain has been also reported (*35*), but we have demonstrated that the affinity of CyPA for heparin is very low compared to the affinity of CyPB (*23*). CD147 was described as a putative cell surface receptor for both CyPA and CyPB (*33*, *36*). Our recent data strongly suggest that CD147 is not directly involved in the binding of CyPB but presumably acts as a costimulatory molecule in cyclophilin-mediated signaling events (*22*). Whether the CyPB type I receptor is related to CyCAP, CD147, or other can only be clarified once it is purified and identified.

In the second part of this work, we focused on the biological activity of CyPB elicited upon lymphocyte receptor binding. We have previously shown that CyPB enhances adhesion of T-lymphocytes to fibronectin (*22*). Using various CyPB mutants, we found evidence for two different mechanisms of action. Concerning the adhesion of T-lymphocytes to coated CyPB, the two N-terminal GAG-binding clusters 3KKK5 and 14YFD16 of CyPB were the only areas required, suggesting a direct ionic interaction with type II binding sites. In the case of the enhancement of T-lymphocyte adhesion to fibronectin by soluble CyPB, we found that the type I receptor-binding residues (R76, G77, W128, K132, D155, and D158), the GAG-binding clusters (3KKK5 and 14YFD16) ,and the PPIase residues (R62 and F67) were required. These data suggest a complex mechanism for T-lymphocyte activation. The interaction of CyPB with GAGs does not only serve to increase the local concentration near the type I receptors but also affects the biological activity of the protein. Concerning other heparin-binding proteins, different situations have been described. The chemokines RANTES, MIP-1α, and MIP1-β elicit comparable effects on GAG-deficient and on wild-type Chinese hamster ovary cells, even though higher concentrations are needed (*37*). In the case of MIP-1α this can be explained by a greater affinity to the specific functional type I receptor expressed by wild -type as compared to GAG-deficient cells. This is, however, not the case for RANTES or MIP1-β, and it was suggested that the role of cell surface GAGs is mainly to present the chemokines to their functional receptors by increasing the local concentration of protein (*37*). Thus, they act as a selective antenna to recruit the chemokines close to their functional receptor (*38*). In the case of interleukin-8, however, the interaction with heparan sulfate plays a more fundamental part in the activity of the chemokine. Heparan sulfate allows the dimerization of interleukin-8, which modulates the binding to its receptor (*39*). Thus, the role of the interaction with GAGs may vary even between chemokines, either by modulating the binding to the functional type I receptor or by directly enhancing T-lymphocyte adhesion to fibronectin.

Amino acids of the PPIase enzymatic site of CyPB (R62 and F67) are not involved in the binding to receptors (*23*). They are, however, required for enhancement of adhesion to fibronectin. A possible scenario is that following binding of CyPB to the T-lymphocyte surface, isomerization of (an) exposed prolyl bond(s) present in the receptor takes place and induces the biological response. This would represent the first instance where the PPIase activity of a CyP is involved in a function other than chaperone or heat-shock protein.

In conclusion, our studies define the binding regions of CyPB to its T-cell receptors. The specific loops and the conserved 3_{10} helix bearing the CN-binding region as well as residue W128 in the CsA-binding region form the binding region of CyPB to type I receptors. We also demonstrate that binding to receptors is not sufficient for enhancement of T-cell adhesion. Although the interaction with GAGs does apparently not modulate the affinity of CyPB for its type I receptor, it is required for the biological activity. In addition, we found that residues in the PPIase site of CyPB are not involved in the binding but are nevertheless required for promoting cellular adhesion. The interactions between CyPB and its cellular binding sites are complex, and only identification of the functional type I receptor will allow to elaborate on the model proposed.

ACKNOWLEDGMENT

We are grateful to the Etablissement de Transfusion Sanguine de Lille (France) for the blood, to Prof. Ph. Delannoy from the UMR No. 8576 for human plasma fibronectin, and to Prof. G. Vergoten from the UMR No. 8576 for help in molecular modeling.

REFERENCES

1. Fruman, D. A., Burakoff, S. J., and Bierer, B. E. (1994) *FASEB J. 8*, 391−400.
2. Ho, S., Clipstone, N., Timmermann, L., Northrop, J., Graef, I., Fiorentino, D., Nourse, J., and Crabtree, G. R. (1996) *Clin. Immunol. Immunopathol. 80*, S40−S45.
3. Handschumacher, R. E., Harding, M. W., Rice, J., Drugge, R. J., and Speicher, D. W. (1984) *Science 226*, 544−547.
4. Haendler, B., Hofer-Warbinek, R., and Hofer, E. (1987) *EMBO J. 6*, 947−950.
5. Spik, G., Haendler, B., Delmas, O., Mariller, C., Chamoux, M., Maes, P., Tartar, A., Montreuil, J., Stedman, K., Kocher, H. P., Keller, R., Hiestand, P. C., and Movva, N. R. (1991) *J. Biol. Chem. 266*, 10735−10738.
6. Schneider, H., Charara, N., Schmitz, R., Wehrli, S., Mikol, V., Zurini, M. G., Quesniaux, V. F., and Movva, N. R. (1994) *Biochemistry 33*, 8218−8224.
7. Gething, M. J., and Sambrook, J. (1992) *Nature 355*, 33−45.
8. Liu, J., Farmer, J. D., Lane, W. S., Friedmann, J., Weissman, I., and Schreiber, S. L. (1991) *Cell 66*, 807−815.
9. Ke, H., Zydowsky, L. D., Liu, J., and Walsh, C. T. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 9483−9487.
10. Mikol, V., Kallen, J., and Walkinshaw, M. D. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 5183−5186.
11. Ke, H., Zhao, Y., Luo, F., Weissman, I., and Friedman, J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 11850−11854.
12. Zydowsky, L. D., Etzkorn, F. A., Chang, H. Y., Ferguson, S. B., Stolz, L. A., Ho, S. I., and Walsh, C. T. (1992) *Protein Sci. 1*, 1092−1099.
13. Etzkorn, F. A., Chang, Z. Y., Stolz, L. A., and Walsh, C. T. (1994) *Biochemistry 33*, 2380−2388.
14. Carpentier, M., Allain, F., Haendler, B., Slomianny, M. C., and Spik, G. (2000) *Protein Sci. 9*, 2386−2393.
15. Price, E. R., Jin, M., Lim, D., Pati, S., Walsh, C. T., and McKeon, F. D. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 3931−3935.
16. Allain, F., Boutillon, C., Mariller, C., and Spik, G. (1995) *J. Immunol. Methods 178*, 113−120.
17. Allain, F., Denys, A., and Spik, G. (1994) *J. Biol. Chem. 269*, 16537−16540.
18. Allain, F., Denys, A., and Spik, G. (1996) *Biochem. J. 317*, 565−570.
19. Denys, A., Allain, F., Foxwell, B., and Spik, G. (1997) *Immunology 91*, 609−617.
20. Denys, A., Allain, F., Masy, E., Dessaint, J. P., and Spik, G. (1998) *Transplantation 65*, 1076−1084.

21. Denys, A., Allain, F., Carpentier, M., and Spik, G. (1998) *Biochem. J. 336*, 689−697.
22. Allain, F., Vanpouille, C., Carpentier, M., Slomianny, M. C., Durieux, S., and Spik, G. (2002) *Proc. Natl. Acad. Sci. U.S.A.* (in press)..
23. Carpentier, M., Allain, F., Haendler, B., Denys, A., Mariller, C., Benaïssa, M., and Spik, G. (1999) *J. Biol. Chem. 274*, 10990−10998.
24. Mariller, C., Haendler, B., Allain, F., Denys, A., and Spik, G. (1996) *Biochem. J. 317*, 571−576.
25. Rahman, M., and Brasseur, R. (1994) *J. Mol. Graphics 12*, 212−218.
26. Wayner, E. A., Garcia-Pardo, A., Humphries, M. J., McDonald, J. A., and Carter, W. G. (1989) *J. Cell Biol. 109*, 1321−1330.
27. Tegeder, I., Schumacher, A., John, S., Geiger, H., Geisslinger, G., Bang, H., and Brune, K. (1997) *J. Clin. Immunol. 17*, 380−386.
28. Endrich, M. M., and Gehring, H. (1998) *Eur. J. Biochem. 252*, 441−446.
29. Husi, H., and Zurini, M. G. (1994) *Anal. Biochem. 222*, 251−255.
30. Bram, R. J., Hung, D. T., Martin, P. K., Schreiber, S. L., and Crabtree, G. R. (1993) *Mol. Cell. Biol. 13*, 4760−4769.
31. Friedman, J., and Weissman, I. (1991) *Cell 66*, 799−806.
32. Friedman, J., Trahey, M., and Weissman, I. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6815−6819.
33. Sherry, B., Zybarth, G., Alfano, M., Dubrovsky, L., Mitchell, R., Rich, D., Ulrich, P., Bucala, R., Cerami, A., and Bukrinsky, M. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 1758−1763.
34. Yurchenko, V., O'Connor, M., Dai, W. W., Guo, H., Toole, B., Sherry, B., and Bukrinsky, M. (2001) *Biochem. Biophys. Res. Commun. 288*, 786−788.
35. Saphire, A. C., Bobardt, M. D., and Gallay, P. A. (1999) *EMBO J. 18*, 6771−6785.
36. Pushkarsky, T., Zybarth, G., Dubrovsky, L., Yurchenko, V., Tang, H., Guo, H., Toole, B., Sherry, B., and Bukrinsky, M. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 6360−6365.
37. Ali, S., Palmer, A. C., Banerjee, B., Fritchley, S. J., and Kirby, J. A. (2000) *J. Biol. Chem. 275*, 11721−11727.
38. Kuschert, G. S., Coulin, F., Power, C. A., Proudfoot, A. E., Hubbard, R. E., Hoogewerf, A. J., and Wells, T. N. (1999) *Biochemistry 38*, 12959−12968.
39. Spillmann, D., Witt, D., and Lindhal, U. (1998) *J. Biol. Chem. 273*, 15487−15493.

BI015951J