The chemotaxis and integrin-mediated adhesion of T lymphocytes triggered by secreted cyclophilin B (CypB) depend on interactions with both cell surface heparan sulfate proteoglycans (HSPG) and the extracellular domain of the CD147 membrane receptor. Here, we use NMR spectroscopy to characterize the interaction of CypB with heparin-derived oligosaccharides. Chemical shift perturbation experiments allowed the precise definition of the heparan sulfate (HS) binding site of CypB. The N-terminal extremity of CypB, which contains a consensus sequence for heparin-binding proteins, was modeled on the basis of the heparan sulfate proteoglycan core structure composed of eight antiparallel a-helices and a four-stranded a/3-barrel surrounded by a-helices and a/3-helices. The chemical shift perturbations and the derived structure show the same core structure composed of eight antiparallel a/3-strands forming a b-barrel surrounded by a-helices and loops. Whereas the nearly identical active site and CsA binding pocket further underscore their close relationship, both proteins do differ in their N and C termini. CypB containing two peptides of some 10 residues long that are lacking in CypA.

CypA and CypB act in the progression of inflammatory diseases such as rheumatoid arthritis and psoriasis, but are equally involved in the first steps of certain viral infections (8–10). Their inflammatory activity is conditioned by their interaction with heparan sulfate proteoglycans (HSPGs) and the membrane receptor CD147, two binding partners at the cell surface of T cell lymphocytes, granulocytes and macrophages (11–14). Significantly, both molecular partners have equally been described as co-receptors for the HIV-1 virus (10, 12, 15).

Both intact prolyl cis/trans activity of the cyclophilins and the presence of the Pro180 residue of CD147, located on one of the two extracellular immunoglobulin-like domains, are required for their chemotactic activity, raising the possibility that isomerization of the accessible Asp179-Pro180 peptide bond might be the molecular signal that translates ultimately in chemotactic activity (14, 16). Mutations in the catalytic site, with residues such as Trp129, Phe67, and Arg62 (17) negatively interfere with the signal transduction. Such a cyclophilin-dependent mechanism of regulation has already been demonstrated for the tyrosine kinase Itk (18), where CypA catalyzes the isomerization of the Asn286-Pro287 peptide bond. According to the isomerization state in trans or cis of this peptide bond, the Itk SH2 domain interacts with either its natural phosphotyrosine substrate or with its own SH3 domain (19).

Both CypA and CypB in vitro induce extracellular signal-regulated kinase (Erk) 1/2 phosphorylation, calcium flux generation and chemotaxis of responsive cells, although CypB is a more potent agonist and uniquely triggers integrin-mediated adhesion of T lymphocytes to fibronectin (11, 13, 14). Tight
binding of CypB to HS moieties of proteoglycans is one source for this increased potency, as mutations in the N-terminal 3KKK and 15YFD3 tripeptide motifs not only affect HS binding (16) but equally reduce CypB chemotaxis and abolish integrin-mediated adhesion (11, 17). Mutants deprived of enzymatic activity still bind to the cell surface of T lymphocytes, but are unable to induce biological responses, indicating that CypB has to interact simultaneously with both CD147 and HSPGs. Very recently, the interaction of CypB with the HS moieties of syndecan-1 was shown to promote and/or stabilize the complex between syndecan-1 and CD147, resulting in mitogen-activated protein kinase activation and subsequent pro-adhesive activity (20).

The minimal motif of HS interacting with CypB was mapped to an octasaccharide (21). However, the length is not the sole parameter defining the complexity of the sugar chains of HSPGs, as the exact sulfation pattern and the conformation of the glycanic moieties equally may contribute to the specificity of the interaction (22–26). Altogether, these data suggest that the high-affinity binding of CypB to specialized HS moieties stabilizes the interaction with its substrate or directly modulates its PPlase activity, resulting in an enhanced intracellular signaling via CD147.

We examine here by NMR spectroscopy the interaction between heparin-derived oligosaccharides and CypB. Whereas we confirm the direct implication of the N-terminal extension that distinguishes CypB from CypA in the HS binding, NMR chemical shift mapping and NOE data indicate a binding site of the glycanic moieties that distinguishes CypB from CypA in the HS binding, NMR structure due to proteolytic cleavages during the CypB purification procedure (7), we derive its structure based on NMR spectroscopy of soluble peptides allowed an estimation with NMR spectra of soluble peptides allowed an estimation with the SNARF program (van Hoesel FHJ, 2000 SNARF methodologies). The N-terminal α-strand containing the 15YFD3 motif. This novel identification of a HS binding patch close to the active site provides the possibility of a functional coupling between HS binding and prolyl cis/trans isomerase activity. We use EXSY spectroscopy in the absence or presence of an oligosaccharide to quantify the CypB isomerization efficiency toward the Asp179-Pro180 bond in a CD147-derived peptide. Finally, the N-terminal peptide responsible for the CypB-specific induction of T-lymphocyte adhesion to the extracellular matrix being absent from the x-ray structure due to proteolytic cleavages during the CypB purification procedure (7), we derive its structure based on NMR parameters, and investigate whether the heparin binding consensus sequence (3EKKGPKVK10 in CypB) adopts any regular heparin binding structure (23).

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Cyclophilin B**—A recombinant plasmid, pET15b-CypB, was constructed to increase the production of recombinant human CypB. The sequence coding CypB was amplified from the previously described plasmid pET15b (27), using the following forward primer 3′-acctctgccatgctgagaa-5′ and the reverse primer 5′-acaaggatcctactccttgctg-3′ and then inserted in a pET15b plasmid (Merck-Novagen, Darmstadt) between restriction enzyme sites Ncol and BamHI. The 24 first amino acids corresponding to the signal sequence were not included in recombinant CypB. Recombinant CypB starts with Ala1 and ends with Glu184. Our numbering is as in the x-ray Protein Data Bank file 1CYN (7). The pET15b-CypB plasmid was introduced in Escherichia coli BL21(DE3) pLysS cells (Novagen), and a 15N/13C-labeled sample was prepared by growing cells in M9 minimal medium with 15NH4Cl and 13C glucose as sole nitrogen and carbon sources, respectively. The 15N-2H-labeled sample was prepared by growing cells in a semi-rich deuterated medium (M9 medium in 99.5% D2O with 15NH4Cl, 2H7-glucose (2 g/liter) and 20% of deuterated 13N-rich medium (v/v) (Isogro, Cambridge Isotopes Laboratories). The cells were grown at 37 °C to reach an A600 nm = 0.8 and expression was induced at 20 °C with 0.4 mM isopropyl 1-thio-β-D-galactopyranoside. The cells were harvested after overnight induction and disrupted in lysis buffer (20 mM NaH2PO4/Na2HPO4, pH 6.8, 10 mM EDTA, Proteases inhibitor mixture (Roche), DNase I, RNase A) by sonication. Cell debris was removed by centrifugation at 20,000 × g for 30 min, then DNA was precipitated with streptomycin sulfate. After centrifugation at 15,000 × g for 30 min the supernatant was dialyzed (6–8 kDa cut-off) overnight against 20 mM NaH2PO4/Na2HPO4, pH 6.85. The recombinant CypB was sequentiaaullly purified by ion exchange (SP Sepharose Fast Flow) and gel filtration (Superose 12 PreP grade) chromatography. Finally the protein was dialyzed against 50 mM NaH2PO4/Na2HPO4, pH 6.3, 40 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol and concentrated by ultrafiltration (cut-off 10 kDa). Recombinant CypB was filtered (0.2 μ) and stored at −20 °C.

**Preparation of Heparin-derived Oligosaccharides**—The heparin-derived oligosaccharides were prepared as previously described (21). Briefly, heparin was enzymatically digested with heparinase 1 at 30 °C. The resulting digestion mixture was desalted on a Sephadex G-10 column (GE Healthcare), then fractionated by gel filtration (Superose 12 Prep grade) chromatography. Finally the protein was dialyzed against 50 mM NaH2PO4/Na2HPO4, pH 6.3, 40 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol and concentrated by ultrafiltration (cut-off 10 kDa). Recombinant CypB was filtered (0.2 μ) and stored at −20 °C.

**Peptide from CD147**—A 15-amino acid long peptide of CD147 centered around Pro180 (sequence 177NLMEADGQYRCNG187) was synthesized by classical solid phase chemistry (Neosystems, Strasbourg, France), and purified to homogeneity by high performance liquid chromatography. Upon dissolving this peptide in a phosphate buffer to a 1 mM concentration, some precipitate was observed. Comparison with NMR spectra of soluble peptides allowed an estimation of the concentration of the soluble fraction at 0.5 mM.

**NMR Spectroscopy**—All spectra were recorded on either a Bruker Avance 800 MHz spectrometer with standard triple resonance probe or a Bruker Avance 600 MHz equipped with a cryogenic triple resonance probe head, at 25 °C (Bruker, Karlsruhe, Germany). The proton chemical shifts were referenced using the methyl signal of TMS (sodium 3-trimethyl-silyl-[2,2,3,3-d4]propionate) at 0 ppm. The spectra were processed with the Bruker TOPSPIN software package and in-house routines with the SNARF program (van Hoesel FHJ, 2000 SNARF version 0.8.9, University of Groningen, The Netherlands). Resonance assignment of the CypB protein residues was performed by using the classical strategy of paired triple resonance experiments (28) on a 15N/13C CypB sample at 0.25 mM in a 50 mM NaH2PO4/Na2HPO4, pH 6.3, 40 mM NaCl, 1 mM EDTA, 1 mM
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The heteronuclear NOE effect was measured with standard refocused HSKC pulse sequence in the presence or absence of proton decoupling during the 5-s relaxation delay, on a 250 \( \mu \)M sample of \(^{15}\)N-CypB in the absence or presence of dp12. Hetero-NOE values were derived from the intensity ratios of the cross-peak with and without proton decoupling.

Residual dipolar couplings (RDCs) were collected on CypB and CypB-dp12 complex at 0.2 mM in 95% H\(_2\)O, 5% D\(_2\)O, 50 mM Na\(_2\)PO\(_4\)/Na\(_2\)HPO\(_4\), pH 6.3, 2.5 mM EDTA, 5 mM dithiothreitol, 85 \( \mu \)M TMSP-D4 (trimethyl-silyl propionate). RDCs were acquired on these uniformly \(^{15}\)N-labeled samples suspended in a liquid crystalline medium consisting of 5% (w/v) poloxymethylene 5-lauryl ether (C\(_{12}\)E\(_{5}\)) and 1-hexanol (Sigma) with a molar ratio of 0.85 (29). 1D\(_{\text{H}}\text{H}\) dipolar couplings were measured at 600 MHz and obtained using two-dimensional TROSY-type experiments (30, 31). Quadrature detection in the indirect dimensions of the multidimensional experiments was achieved by the echo/antiecho detection scheme for \(^{15}\)N, and by the TPPI States method for \(^{1}\)H. 64 scans were recorded per \((t_1, t_2)\) increment. Data processing and peak picking were performed using the software SNARF (van Hoesel FHJ, 2000 SNARF version 0.8.9, University of Groningen, The Netherlands). Because the complex was partially precipitated, RDC values on the isolated CypB were of better quality, and were used for the refinement of the core region (see below).

The PPlase activity of CypB on the CD147 peptide was assessed on a sample of 0.5 mM CD147 peptide and 25 \( \mu \)M CypB, in the absence or presence of dp14. EXSY spectra were acquired at 800 MHz with mixing times of 50, 100, 200, 300, and 400 ms, and 2k/256 complex points in the direct and indirect proton dimension, and Fourier transformed to 4k/1k complex frequency points after zero filling. Spectra at 100, 200, and 400 ms were repeated on an independent sample to evaluate the error margins. Because the exchange cross-peaks are close to the diagonal, the maximal peak intensity rather than the peak integral was measured for the Asp\(^{179}\) cis/trans cross-peaks, and normalized to the corresponding diagonal peak intensity. The exchange rate \(k_{\text{exch}}\) (s\(^{-1}\)) was calculated by fitting the theoretical curve given by Equation 1 (32) to the experimental data, where \(\%[\text{cis} \rightarrow \text{trans}]\) expressed as the intensity of the exchange cross-peak to the diagonal peak, corresponds to the fraction of molecules that undergoes a transition from cis to trans conformation during the mixing time, and 1/\(a\) is the excess of trans over cis forms, determined on the basis of the one-dimensional spectra of Fig. 1C.

\[
\%
[cis \rightarrow trans] = a \times \frac{1 - \exp(-(1 + 1/a)k_{\text{exch}} \times MT)}{1 + \exp(-(1 + 1/a)k_{\text{exch}} \times MT)}
\]

\[\text{Eq. 1}\]

**RESULTS**

**Molecular Characterization of the Partners**—Based on its high isoelectrolytical point, the recombinant human cyclophilin B, 184 amino acids residues, was purified in one step by ion exchange chromatography to above 95% based on SDS-PAGE. The protein eluted from gel filtration chromatography as a single peak with an elution volume corresponding to a monomer of 20 kDa, and the good dispersion of the methyl groups in the one-dimensional NMR spectrum indicated a globular tertiary folding (Fig. 1A). A doubly labeled \(^{15}\)N-\(^{13}\)C CypB was used for the NMR assignment strategy and all backbone resonances (except for Ala\(^{2}\) and Lys\(^{52}\)) and C\(_{\beta}\) carbons were fully assigned. To observe potential NOE contacts with heparin (see below), a deuterated \(^{2}\)H-\(^{15}\)N CypB was prepared. From the one-dimensional spectrum (Fig. 1A), the deuteration level was estimated to be around 95%. Even after 1 week in aqueous buffer, several amide functions from the core of the protein still did not exchange with protons from the solvent, thereby defining the rigid central core of the protein.

Previous gel mobility shift assays studies had determined an octasaccharide as the minimal length required for efficient binding of heparan sulfate to CypB (21). Therefore, we only considered oligosaccharides with a higher degree of polymerization in this work, and present the results with dp12 or dp14 oligosaccharides. As these molecules come from enzymatic digestion of heparin with heparinase I, there are several sources of heterogeneity, at the level of the sequence and the sulfation pattern. To minimize these heterogeneities for the NMR experiments, we selected for those dp12 oligosaccharides species that interact most tightly with CypB by mixing an excess of dp12 with CypB followed by purification of the complex by gel filtration chromatography. An even

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more stringent selection criterion was introduced by incubation of the oligosaccharide/protein mixture in 400 mM NaCl before and during the gel filtration (Fig. 1B). As the interaction between CypB and heparan sulfates is expected to be essentially driven by electrostatic forces, the high ionic strength should preclude binding of oligosaccharides species that weakly interact with CypB. Following the gel filtration, the buffer was exchanged to reduce the salt concentration to 40 mM. Despite this stringent procedure, we cannot exclude that our heparin oligosaccharides still contain some degree of structural heterogeneity.

To assess a potential enzymatic activity of CypB toward CD147 and quantify the modulation of this activity by the heparin oligosaccharides, we used a synthetic peptide of 15 amino acids, 173NLNMEADPGQYRCNG187, centered around the Pro180 CD147 residue. This CD147 peptide was characterized by homonuclear NMR spectroscopy, and both the absence of long range NOE contacts and JHN-HX constants around 6 Hz indicate the absence of stable secondary structure. Both the trans and cis forms of the central Pro180 could be identified, and from the intensity of the cis and trans forms of the Asp179 amide proton, we estimate a cis/trans ratio of 6% (Fig. 1C). The dual proline conformation shows up not only as distinct resonance frequencies of the flanking amide protons, but also as far as the Tyr184 amide group (supplemental Fig. S1).

Definition of the CypB Zone in Interaction with Oligosaccharide dp12—A first complex between 15N-labeled CypB and dp12 was obtained by mixing the two molecules in 400 mM NaCl to a molar ratio of 1:10, to ensure the ligand saturation of CypB (21). This complex was then purified by gel filtration chromatography using the same high ionic strength buffer (400 mM NaCl) to preferentially keep those dp12 species that strongly interact with CypB. To define the CypB residues involved in the interaction with heparin-derived dp12 oligosaccharides, we compared 1H-15N HSQC spectra of CypB alone and CypB in complex with dp12. Only a limited subset of CypB residues were affected by interaction with the dp12 oligosaccharide, excluding major conformational changes upon complex formation (Fig. 2). Previously, two CypB motifs, 4KKK6 and 15YFD17 (16), had been proposed to be directly involved in the interaction with heparan sulfates. Mapping the chemical shift changes along the sequence using our sequence-specific assignment confirmed the N-terminal 4KKK6 motif as an effective part of the heparin binding site. The Hn resonances of these three lysine residues undergo the most important shift upon heparin binding (Fig. 2A). However, the binding of dp12 to CypB had no influence on the NMR signals corresponding to the residues of the 15YFD motif (Fig. 2B), despite the fact that a 15YFD17 deletion mutant was previously found unable to bind efficiently heparan sulfates (16, 21). We did several additional chemical shift mapping experiments with CypB and different heparin-derived oligosaccharides (dp8, dp12, and dp14), but were unable to detect any perturbation of these YFD motif resonances. These data suggest an indirect participation of the YFD motif in the binding of the heparan sulfates, probably through destabilization of the N-terminal β sheet. Beyond the N-terminal KKK motif, three additional regions of the protein had their amide chemical shift affected upon binding of dp12. These regions correspond to the C-terminal strand, the 34–43 region, and the 95–102 region (Figs. 2E and 3D). The backbone amide proton from lysine 97, lysine 99, and furthermore, the He-1 from the side chain from tryptophan 129 shifted in the presence of dp12, extending the interaction zone toward the active site of CypB (Fig. 3D). The latter one is known to play a dual role in the binding of CypB to cyclosporin A and CD147. In conclusion, whereas the previous mutational analysis had positioned the heparan sulfate binding site and the substrate binding site of CypB at opposite sites of the protein, we show here that these two sites are contiguous. Our identification of 12 lysines of a total of 25 (but no arginine) in the full interaction zone confirms that the complex formation is mainly driven by ionic interactions between lysines side chains and sulfate groups of HS.

The gel filtration experiment should ideally yield a 1:1 complex, with selection for those oligosaccharides that contain an optimal binding pattern. However, going through this procedure precludes a simple titration experiment to derive an affinity constant. Therefore, to estimate the order of magnitude of the affinity in solution
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A

B

C

D

E

Combined Chemical Shift

Residues
between CypB and a dp12 heparin-derived oligosaccharide, we did a reverse titration experiment. On the sample with the complex CypB-dp12, purified by gel filtration chromatography, we removed half of the sample and replaced it by an equivalent volume of CypB alone at the same concentration, and then recorded a new $^1H$-$^15N$ HSQC spectrum. Resonances that were previously affected upon addition of dp12 shifted back to an intermediate position between free CypB and CypB bound to dp12 (Fig. 2D). This suggests that on the NMR time scale, the heparin fragment exchanges rapidly between bound and free states, corresponding to an interaction of CypB and dp12 with a dissociation constant in the micromolar range or even weaker.

NMR Characterization of the CypB/dp12 Complex—Our results, together with previous studies, point out an important role of the $^4$KKK motif from the CypB N terminus in the binding of heparan sulfates molecules. This N terminus is lacking in the x-ray structure of CypB (PDB code 1CYN) and structural data are only available starting from Gly$^7$, as the first 6 residues had undergone proteolytic cleavages during the purification process (7). In the absence of proteolysis of the N terminus during our purification, we recorded a three-dimensional NOESY-HSQC NMR experiment on the $^1H$-$^15N$ CypB alone or in complex with dp12 and compared the H$_{N}$-H$_{N}$ NOE patterns of both spectra. The near identity of the NOE patterns involving residues from the core region in both spectra confirms that dp12 binding does not induce major conformational changes in the CypB structure. Moreover, most of the NOEs observed could be predicted fromHN-HN distances derived from the x-ray structure, suggesting that this x-ray structure is a reasonably good starting point for the structure of the CypB bound to dp12. As for the C terminus, we detected NOEs between the Glu$^{184}$ and Tyr$^{101}$, Gly$^{102}$, Trp$^{105}$ side chain (He-1) and between Lys$^{183}$ and the Trp$^{105}$ side chain (Fig. 4A). The distance between the H$_{N}$ of Glu$^{184}$ and the He-1 proton of Trp$^{105}$ in the crystal structure being 9.2 Å, the observation of a clear NOE contact between both protons suggests that the C terminus of CypB in solution is closer to the core of the protein than in the x-ray structure. However, these structural differences for the C terminus are not induced by dp12 binding, as we did observe the same NOEs with comparable intensity in the NOESY-HSQC spectrum of the free protein. Finally, several NOEs were observed between residues in the 7–10 region and the 179–183 region. These observations correlate with the x-ray structure where these regions of CypB form a small $\beta$-sheet.

The absence of NOEs between the 3 lysines in the N terminus and the rest of the protein suggests that this motif is highly flexible, which might be a determining character for it being the initial and preferential binding site for HS. We measured this dynamical aspect of the CypB backbone by heteronuclear NOE experiments in the presence or absence of dp12 (Fig. 5). A significant increase of heteronuclear NOEs was observed for the

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**FIGURE 2. Chemical shift perturbations experiment.** A–D, superimposition of $^1H$-$^15N$ HSQC of free CypB (in blue) and CypB bound to dp12 heparin-derived oligosaccharides (in red). A, region of the spectra centered on the $^4$KKK CypB N-terminal motif. B, region of the spectra centered on the residue Tyr$^{15}$ from the $^15$YFD$^{17}$ CypB tripeptide. C, region of the spectra centered on residues Lys$^{96}$ and Tyr$^{161}$, which are close to the CypB active site. D, reverse titration experiment where half of the CypB-dp12 sample was removed and replaced by an equivalent volume of CypB alone at the same concentration. A new $^1H$-$^15N$ HSQC spectrum was recorded (in green) and compared with those of free CypB (in blue) and CypB-dp12 complex (in red). Resonances from the $^4$KKK motif that were previously affected upon addition of dp12 (A) shifted back to an intermediate position between CypB free and CypB bound to dp12. E, plot of the combined $^1H$ and $^15N$ chemical shift perturbations along the CypB sequence. The values were calculated with the following equation: Combined Chemical Shift ($\Delta\delta$) = ($\delta\Delta_{HN}$ + ($\Delta\delta_{SN}$/6.51)$^{1/2}$. The $\Delta\delta$ values in the interval 0.018–0.05 ppm are colored yellow, those with $\Delta\delta$ values in the interval 0.05–0.07 ppm colored orange, and those with $\Delta\delta$ values >0.07 colored red.
first 10 CypB residues upon binding of dp12, whereas a slight decrease of the heteronuclear NOE values was observed for residues 180–183. These observations suggest a direct interaction of the N-terminal lysine residues in the consensus sequence with the heparin-derived oligosaccharide, conferring a more rigid character upon binding. It further suggests that the chemical shift perturbations observed in the C terminus result from indirect effects rather than from a direct interaction with the oligosaccharide.

As the interaction between heparan sulfates and its binding partners involves negatively charged sulfate groups of HS and positively charged lysine side chains, backbone amide protons are seldom closer than 5 Å from the sugar protons, and 1H-1H intermolecular NOE correlations are not easily obtained (36–38). However, the use of a highly deuterated CypB limits spin diffusion (39), and moreover avoids confusion between heparin protons and aliphatic side chain protein resonances. We indeed detected some intermolecular NOEs between dp12 and Lys5/Lys6 (Fig. 4B). These NOEs, involving protons in the range of 3.8–4.3 ppm, are absent in the control experiment on the same protein preparation without dp12 (Fig. 4C), and probably correspond to protons from the carbohydrate rings of dp12. Due to the severe overlap of heparin protons and the additional molecular heterogeneity of heparin-derived oligosaccharide dp12, these signals could, however, not be assigned without ambiguity, but they do confirm the direct physical interaction between the 4KKK6 CypB motif and the dp12 molecule.

Because of the limited information that could be extracted from the NOEs involving residues in the N-terminal region, we assigned the 13C chemical shifts in the absence and presence of dp12, and obtained RDC values on a partially oriented sample of the isolated and dp12 complexed protein. These data yield constraints on the dihedral angles for the former, and long range orientational constraints for the latter. All experimental constraints were used in a refinement protocol aimed at completing the structure of CypB in its complex with dp12.

**Modeling of CypB in the CypB/dp12 Complex**—A multistep protocol starting from the x-ray structure completed with coordinates for the lacking N terminus (‘ADEKKK’6) was used to obtain a family of structures compatible with all experimental data. The 20 structures of lowest energy (Fig. 3A) well conserve the typical cyclophilin fold, and when superimposed on all Ca atoms, give an overall r.m.s. deviation of 0.87 ± 0.27 Å. When we superimpose the core regions of these structures, from residue 15 to 173, and calculate the r.m.s. deviation values for the isolated N- and C-terminal extensions, we find values of 2.39 ± 1.05 and 1.14 ± 0.48 Å, respectively, indicating still a reasonable definition of these fragments.

The different CypB regions involved in dp12 binding as defined by the chemical shift perturbation mapping are close in
space and form a well defined heparan sulfate binding site (Fig. 3D), which equally corresponds to the most electropositive area of CypB, containing 12 lysine residues (Fig. 3C). Closer examination of the N terminus in the CypB model showed that the structure of the consensus sequence $XBBBXXB^{'EKKGPK}X$ may lead to the suitable orientation of the lysine side chains for promoting interaction with HS, although experimental data to define these side chains lack. However, our resulting structures indicate the absence of the canonical $\alpha$-helix or $\beta$-strand structures that would project the basic side chains into the same direction (23). The strong $\text{NH}_{2}-\text{NH}_{3+1}$ contacts that would characterize such a helical conformation were indeed not observed in the three-dimensional spectrum of CypB/dp12. Moreover the absence of regular secondary structural elements in the N terminus was confirmed by the $^{13}$C chemical shift index method (40). Finally, our model shows that the N terminus is more surface accessible than the partially buried C terminus, in agreement with the experimental relaxation data.

**Enzymatic Activity of CypB on a CD147-derived Peptide—**

Chemical shift mapping suggested that the heparan sulfate binding site extends to the edge of the active site of CypB. Our NOE data further support this result, as dp12 binding on CypB affects the Trp$^{129}$ residue, which is part of the active site of CypB and plays a crucial role in the binding of cyclosporin A or the cell surface receptor CD147 (16). Indeed, the NOE patterns of the He-1 Trp$^{129}$ side chain in the presence or absence of dp12 are not identical. In the absence of dp12, no NOE correlations were detected, whereas in the presence of dp12 the He-1 of Trp$^{129}$ side chain correlates with the amide proton of the same residue and of two neighboring residues, Leu$^{130}$ and Asp$^{131}$.
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(supplemental Fig. S2). Upon binding of dp12, the Trp\textsuperscript{129} side chain could at least transiently be reoriented, opening up the possibility of a functional coupling between the binding of heparin-derived oligosaccharides and the enzymatic PPiase activity of CypB. To further assess this possibility, the enzymatic activity of CypB toward a CD147-derived peptide, centered on the CD147 Pro\textsuperscript{180} residue (in bold), \textsuperscript{173}NLNMEAD-PQQYRCNG\textsuperscript{187}, was characterized using EXSY NMR spectroscopy. The distinct trans and cis signals of the Asp\textsuperscript{179} amide protons were used to quantify the exchange process. Without CypB, both conformers are in very slow exchange, and no cross-peak between isoforms could be detected for mixing times up to 400 ms (Fig. 6A and supplemental Fig. S3A). However, when adding CypB in catalytic amounts to the same peptide sample, additional cross-peaks connecting the cis and trans amide protons of Asp\textsuperscript{179} are observed (Fig. 6B and supplemental Fig. S3B), confirming experimentally that CypB is able to catalyze the isomerization of Pro\textsuperscript{180} in CD147. Similar exchange peaks equally connected the cis and trans forms of Gly\textsuperscript{181} and Tyr\textsuperscript{184} by varying the mixing time of the EXSY spectra, an exchange rate \( k_{\text{ex}} = 51 \text{ s}^{-1} \) was found (Fig. 6D). Addition of CsA to the sample did abolish the exchange cross-peaks, confirming the overlap between the prolyl cis/trans isomerase active site and the CsA binding site (Fig. 6C and supplemental Fig. S3C). As the dp12 binding site extends to the active site of CypB, and as on the cell surface, the heparan sulfate chains of proteoglycans are longer than a dp12 oligosaccharide, we used the longest heparin oligosaccharide, a dp14 molecule, to investigate any functional implications. The same EXSY spectra were thus run with CypB in the presence of dp14. Prolyl cis/ trans isomerization was still present, and quantification of the rate led to a similar exchange rate of 48 s\(^{-1}\) (Fig. 6D). We therefore conclude that heparin binding extends up to the active site of CypB, but does not influence directly its enzymatic prolyl cis/trans isomerase activity.

**DISCUSSION**

Cyclophilins are proteins involved in several inflammatory diseases such as rheumatoid arthritis, and play a role in the HIV-1 viral infection process. It has been shown that both extracellular cyclophilin A and B are able to induce chemotaxis (13, 14, 41, 42) but that only CypB triggers T lymphocyte adhesion to fibronectin in the extracellular matrix (11). Although homologous, with more than 50% sequence identity for both full-length proteins, the main difference is in the N- and C-terminal extensions that characterize CypB but are lacking in CypA (43). The biological cyclophilin-mediated response requires both the cell surface HS of HSPG and the cell surface receptor CD147. CypB would bind to one or more cell surface HS moiety of syndecan I and subsequently promote the syndecan 1-CD147 association, resulting in an activation of p44/42 mitogen-activated kinases and a subsequent integrin-mediated induction of extracellular matrix adhesion (20). Precise molecular details of this activation mechanisms are as yet not available, be it for the early interaction steps or for the ensuing signal transduction.

Here, we use heparin-derived oligosaccharides to reproduce the physiological interaction between CypB and the glycan moiety of cell surface HSPG. The sulfated regions of heparin are similar to HS of HSPG. The use of oligosaccharides has already been validated as a good model to replace longer HS chains (44). Indeed, many structural and biological studies using oligosaccharides were consistent with the in vivo biological data (36, 45). The minimal binding unit for CypB has previously been shown to be an octasaccharide (dp8). Here, we used dp12 and dp14 oligosaccharides to study the interaction with CypB.

![FIGURE 6. EXSY data with the CD147 peptide. The H\textsubscript{N}-H\textsubscript{N} region of a 400-ms EXSY spectrum is shown for the isolated CD147 peptide (A), the peptide in presence of a catalytic amount of CypB (B), and the peptide with CypB/CsA (C). The exchange rate in the free peptide is too slow to give observable exchange cross-peaks. When catalyzed by CypB, we do observe such peaks, but these disappear upon addition of CsA. D, normalized intensity of the exchange cross-peaks as a function of mixing time, for CypB (triangles, solid line) or CypB/dp14 (squares, dotted line).](image)
Their enzymatic preparation from heparin followed by size exclusion chromatography leads to length-defined compounds with heterogeneous sulfation patterns. To minimize this heterogeneity, we purified the CypB-dp12 complex in high salt conditions, which should result in the selection for the stronger interacting species. The 0.4 M salt concentration used is lower than the 0.6 M concentration needed to elute CypB from a heparin-Sepharose column (46), and our gel filtration data show that we indeed form a complex (Fig. 1B).

The chemical shift perturbation strategy showed that only a defined subset of CypB residues are involved in the dp12 binding. Even though located in 4 different regions of the linear sequence of CypB, they are spatially close and form a well-defined HS binding site. NOE data confirm that at least the N-terminal 3KKK motif is involved in direct physical interaction with the sugar moiety, explaining why their triple mutation only the side chains of Tyr15 and Asp17 are solvent accessible, whereas the side chain of Phe16 is buried into the hydrophobic core of CypB. A plausible explanation for the fact that the CypB side chain of Trp129 not only undergoes chemical shift perturbations on each side. These lysine side chains constitute a positively charged patch on CypB that probably interacts with the bulky negatively charged sulfate groups of dp12 (22).

Chemical shifts in the 15YFD17 peptide, previously identified by site-directed mutagenesis as important for the interaction of CypB with HS, did not change upon interaction with dp12. However, only the side chains of Tyr15 and Asp17 are solvent accessible, whereas the side chain of Phe16 is buried into the hydrophobic core of CypB. A plausible explanation for the fact that the CypB mutant deleted of 15YFD17 does not directly bind to the heparan sulfate is that this deletion induces a destabilization of the first β-strand (Thr11–Arg19) and thus potentially disrupts the location of the N-terminal anchoring patch with respect to the rest of the binding site.

A final interaction zone is defined by the loop of residues 125–133 surrounding the active site. We show specifically that the side chain of Trp129 not only undergoes chemical shift perturbations through the addition of dp12, but that equally its orientation could be modified as witnessed by differential NOEs. This suggests that the HS chains of cell surface HSPG and is subsequently locally concentrated in the surrounding of the membrane receptor CD147 (20). Without a direct influence of the HS on its enzymatic activity, CypB can isomerize the Asp179–Pro180 peptidyl proline bond of the CD147 extracellular domain, which then triggers in an unknown fashion intracellular signaling events. Finally, we further validate the interaction of CypB with cell surface heparan sulfate as a potential therapeutic target to modulate the cyclophilin-mediated inflammation process.

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