Structural Determinants of the Anti-HIV Activity of a CCR5 Antagonist Derived from *Toxoplasma gondii*†‡§

The protozoan parasite *Toxoplasma gondii* possesses a protein, cyclophilin-18 (C-18), which binds to the chemokine receptor CCR5, induces interleukin-12 production from murine dendritic cells, and inhibits fusion and infectivity of human immunodeficiency virus 1 (HIV-1) R5 viruses by co-receptor antagonism. Site-directed mutagenesis was employed to identify the domains in C-18 responsible for its CCR5 binding and antiviral functions. To do so we focused on amino acid differences with *Plasmodium falciparum* cyclophilin, which, although 53% identical with C-18, has minimal binding activity for CCR5, and we generated 22 mutants with substitutions in the regions of non-homology located on the putative surface of the molecule. Two mutations situated on the face of C-18, predicted to be involved in its interaction with the ligand cyclosporin A, were shown to be critical for CCR5-binding and the inhibition of HIV-1 fusion and infectivity. In contrast, four mutations in C-18 specifically designed to abolish the peptidyl-prolyl *cis*-trans-isomerase activity of the protein failed to inactivate its CCR5 binding and HIV inhibitory activities. Interleukin-12 induction by C-18, on the other hand, was abrogated by mutations effecting either the CCR5 binding or enzymatic function of the molecule. These findings shed light on the structural basis of the molecular mimicry of the chemokine function by a pathogen-derived protein and provide a basis for further modification of C-18 into an antiviral agent.

Blockade of viral infectivity is a major strategy for intervention in HIV infection (1). HIV entry into host T lymphocyte and monocyte/macrophages has been shown to be critically dependent on the interaction of the viral envelope with CD4 together with a chemokine co-receptor, CCR5 or CXCR4 (2–7). Although several other chemokine receptors (CCR2, 3, and 8, BOB, and others) can promote infection *in vitro* by specific HIV-1 variants (8), their role *in vivo* is limited (9). HIV-1 isolates that interact with CCR5 (R5 type) initiate most infections, and individuals with a genetic deletion (∆32) in the CCR5 open frame appear to be highly protected from HIV disease (10, 11). These observations suggest that agents that block R5 type HIV-CCR5 interaction may be particularly effective in preventing HIV-1 infection. Accordingly, a series of chemokine-based CCR5 antagonists have been developed and tested with partial success in clinical trials (12).

A number of pathogens have evolved molecules that can function as mimics of chemokines or chemokine receptors (13, 14). We have recently found that the protozoan parasite *Toxoplasma gondii* possesses a protein (C-18) that binds to the CC chemokine receptor CCR5, triggers CCR5-dependent chemotaxis, and induces the production of interleukin-12 (IL-12) by murine dendritic cells. In subsequent studies, C-18 was shown to inhibit both syncytia formation and infectivity of R5 but not X4 HIV viruses for human T cells (15, 16). Sequence analysis revealed C-18 to be an isoform of *T. gondii* cyclophilin (17). Because cyclophilins from mammalian species and the closely related apicomplexan protozoan parasite *Plasmodium falciparum* failed to bind significantly to CCR5, induce IL-12, or possess antiviral activity, the *T. gondii* cyclophilin protein appears to have acquired β-chemokine-like functions as a consequence of molecular mimicry (16). The structural basis of this mimicry is presently unclear, because C-18 has no sequence homology with the host CCR5 ligands MIP-1α/CCL3, MIP-1β/CCL4, RANTES (regulated on activation normal T cell expressed and secreted)/CCL5, or MCP-2/CCL8. Cyclophilins are peptidyl-prolyl isomerase, and their enzyme activity is inhibited by the drug cyclosporin A (CsA). Interestingly, CsA was found to block the antiviral and IL-12 inducing functions of C-18, suggesting that either the isomerase activity of the molecule or a structural motif conformationally altered by CsA ligation is required for C-18-chemokine receptor interaction.

C-18 has several properties that make it attractive as a candidate HIV co-receptor antagonist. Unlike other chemokine-based antagonists, C-18 blocks HIV interaction with T cells and macrophages with comparable efficiency (16). Moreover, the protein does not appear to induce significant CCR5 internalization, a potential disadvantage of a number of existing antagonists. Finally, recent experiments indicate that C-18 also binds to rhesus CCR5 and blocks fusion with the simian immunodeficiency viral envelope, thus allowing pre-clinical evaluation of its antiviral activity in an *in vivo* primate model. Nevertheless, because of its modest activity in both HIV fusion
and infectivity assays and probable immunogenicity, it is likely that the protein would need to be structured optimally before use as a clinical agent. To do so, an understanding of the requirements for the interaction of C-18 with CCR5 is necessary. We have approached this problem in the present study by site-directed mutagenesis of the C-18 protein. Our findings identify a region in the C-18 molecule involved in both CCR5 binding and viral inhibition and formally establish that its peptidyl-prolyl isomerase activity is not required for either biological function. At the same time, our data shed light on the molecular basis of host chemokine mimicry by this protozoan protein.

**EXPERIMENTAL PROCEDURES**

**Reagents and Experimental Animals—** Csa, a-chemotrypsin (type I-S from bovine pancreases), and N-succinyl-Ala-Ala-Pro-Phe-nitroani-

lilide were purchased from Sigma. Paired antibodies against IL-12 were obtained from BD Pharmingen for measurement of this cytokine by enzyme-linked immunosorbent assay. C57BL/6 (wild-type) and CCR5-deficient mice were obtained from the Jackson Laboratories (Bar Har-

bor, ME). TLR4-deficient mice were generously provided by Drs. S. Akira and T. Kaisho (Tokyo, Japan) and A. L. Beutler (Boston, MA, Massa-

chusetts, Worcester, MA). All animals were maintained at the American Laboratory Animal Care-accredited NIAID, National Institutes of Health animal facility, and 8–12-week-old male mice were employed in all experiments.

**Site-directed Mutagenesis of Candidate CCR5 Binding Determinants in C-18**—The structure of C-18 was modeled based on the crystal structure of *P. falciparum* cyclophilin with cyclosporin A bound (18) (PCyp19). Substitution of PCyp19 amino acid coordinates with a set representing the C-18 sequence were made using alignments generated with the automated Swiss-Model server. The initial model was energy-

minimized by 200 cycles of steepest descents followed by 200 cycles of conjugate gradient minimization using Discover. The quality of model geometry was checked using a Ramachandran plot. Coordinates for CsA were superimposed with PCyp19 and added to the C-18 model after superposition of the C-18 and PCyp19 polypeptide chains using Insight II. The goal of the modeling was not to predict specific binding interactions but rather to identify potential solvent-exposed amino acid side chains for site-directed mutagenesis experiments. Only putatively exposed amino acids that are not conserved between the *T. gondii* and *F. falciparum* cyclophilins C-18 and PCyp19, respectively, were selected for mutagenesis screening.

The generation of the C-18 expression construct in pCRT7/CT-TOPO vector (Invitrogen, NY) was performed using a QuikChange mutagenesis kit (Stratagene). The primers were used to generate the indicated C-18 mutants: Y10A, 5′-CAGAAAGC-

GCTATCGACGCACGCAG/CACG-3′; GEH11003-11013 (17, 18, 22), 5′-GCCATCGACGCACGCAGCTGCGCGGGC-3′; E29A, 5′-CGGCCGAAGATTTGCTGACGAAAA-

ATC-3′; D57A, 5′-CGAACGAAGATTTGCTGACGAAAAATC-3′. Substitution of PCyp19 amino acid coordinates with a set representing the C-18 sequence were made using alignments generated with the automated Swiss-Model server. The initial model was energy-

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ATC-3′; D57A, 5′-CGAACGAAGATTTGCTGACGAAAAATC-3′.
Measurement of IL-12 Inducing Activity of C-18 Proteins—Splenic dendritic cells (DCs) were partially purified from the spleens of C57BL/6 mice as described previously (15). For measurement of IL-12 production, DCs were resuspended in cell culture medium at 10^6 cells/ml and distributed in 96-well plates. Recombinant C-18 and mutant proteins were then added at 100 ng/ml, and the cultures were stimulated overnight. IL-12 p40 levels were measured by enzyme-linked immunosorbent assay (15). To rule out the effects of lipopolysaccharide contamination, splenic DCs from CCR5⁻/⁻/⁻ mice, which cannot be activated by C-18, and TLR4 knock-out mice, which are fully responsive to C-18 but non-responsive to lipopolysaccharide, were used as controls.

RESULTS

Selection of Sites for Mutagenesis Based on Structural Comparison of C-18 with P. falciparum Cyclophilin—We have shown previously that T. gondii but not P. falciparum or human cyclophilins display CCR5 binding and HIV-1 antiviral activity (16). This observation provided a strategy for the identification of the unique structural elements in C-18 that determine its chemokine-like activity. We first developed a model of the entire C-18 molecule based on homology with the known crystal structure PfCyp19 together with its CsA ligand (Fig. 1A) (18). We next identified those amino acids located on the putative molecular surface of C-18 that represent non-conservative substitutions of the corresponding amino acids in PfCyp19 (Fig. 1, A and B). Site-directed mutagenesis was then performed, substituting in each case an Ala for the C-18 residue or the consecutive residues targeted. An exception was mutant RP149-150 in which a double substitution was made to Tyr-Val, the corresponding amino acids present in PfCyp19. All of the mutant proteins were successfully expressed in E. coli, although mutant Arg^92 could not be refolded in soluble form (data not shown) and, thus, was eliminated from further analysis.

Specific Mutations in C-18 Reduce its CCR5 Binding Activity—Competition of I125-labeled C-18 interaction with CCR5-transfected cells was used to screen the mutants for altered chemokine receptor binding. This assay revealed significantly reduced competition by three of the 21 mutants tested, namely GEH17-19AAA (designated GEH17-19AAA throughout), D82A, and RP149-150YV (designated RP149-150YV throughout) (Fig. 2). To confirm the validity of the competition assay, direct binding assays were performed using representative mutants. As shown in Fig. 3, iodinated D82A and RP149-150YV each showed greatly reduced binding to CCR5-transfected cells in comparison to iodinated C-18 or K155A, a control mutant showing no loss in activity in the initial screen. Interestingly, two (GEH17-19AAA and RP149-150YV) of the three loss-in-function mutations identified in Fig. 2 are situated on adjacent loops in the C-18 model, with RP149-150YV bordering the CsA binding pocket (Fig. 1), implicating this region of the protein in CCR5 interaction. Indeed, as observed previously in other assays of C-18 function (15, 16), the addition of CsA dramatically inhibited the binding of labeled C-18 to the indicator cells (Fig. 3).

C-18 Mutants with Decreased CCR5 Binding Also Display Reduced Antiviral Activity—The same mutant proteins that were assessed for CCR5 interaction were also screened for their ability to inhibit HIV-1 envelope-dependent cell fusion in an assay employing CD4/CCR5—expressing PM1 target cells. Using 12E1 effector cells expressing the HIV-1 R5 envelope (JR-FL), wild-type C-18 blocked fusion with ID_{50} values ranging between 3 and 10 μg/ml in five experiments. The same three
mutants described above as showing impaired CCR5 binding showed decreased viral fusion inhibition with very significant increases in the ID₅₀ values (Fig. 4, A and B). The reduced antiviral activity of proteins GEH17-19AAA, D82A, and RP149-150YV was confirmed in a separate set of fusion assays employing a different R5 viral envelope (Ba-L). The findings in the fusion assays were reproduced in virus infectivity assays in which the ability of the proteins to block entry into human peripheral blood mononuclear cells was compared (Fig. 5).

Again, mutants GEH17-19AAA and RP149-150YV showed reduced activity. However, in this assay system we were unsuccessful in generating an ID₅₀ value for D82A even when using excessively high concentrations of the mutant protein.

The Peptidyl-prolyl Isomerase Enzymatic Activity of C-18 Is Not Required for CCR5 Binding or Antiviral Activity—As noted previously, the C-18 ligand CsA inhibits both the antiviral (16) and CCR5 binding (Fig. 3) activities of the protein. Because CsA is a potent antagonist of the peptidyl-prolyl isomerase
activity of cyclophilins, it was possible that this enzymatic function is required for the biological properties of *T. gondii* C-18. To test this hypothesis we generated four additional mutant proteins within the highly conserved substrate-binding pocket of C-18. These mutations had previously been shown to abrogate enzyme activity in human cyclophilin (23), and this loss in activity was confirmed in the C-18 mutants (Fig. 6A and supplemental Table I). Importantly, none of the four mutant proteins lost CCR5 binding (Fig. 6B) or antiviral activity as measured in the fusion and infectivity assays (Fig. 6, C and D). These data formally demonstrate that the CCR5-dependent biological functions of C-18 do not depend on its enzymatic activity.

In the same series of experiments we showed that the original series of C-18 mutant proteins studied above (Figs. 1–5) retain their enzymatic activity (Fig. 6A and supplemental Table I). The latter data argue that these recombinant molecules were properly folded following expression and purification. An exception however was mutant D82A, which, although displaying loss in fusion inhibition and CCR5 binding, also lacked enzymatic activity (data not shown) and, as described above, was unexpectedly totally lacking in activity in the inhibition of HIV infectivity assay. Based on this combined evidence we concluded that the loss in biological function observed with this mutant protein is likely to be the result of improper folding.

**IL-12 Induction by C-18 Requires Both the CCR5 Binding and Enzymatic Structural Domains of C-18**—C-18 was originally described as a parasite-derived inducer of IL-12 (15, 26), a cytokine involved in host resistance to *T. gondii* infection (27). To confirm the role of CCR5 binding in this activity, wild-type and mutant C-18 proteins were tested for their ability to induce IL-12 from cultures of murine splenic dendritic cells. As shown in Fig. 7 the same mutants (GEH17-19AAA and
RP149-150YV) that showed loss in CCR5 binding and antiviral function also showed impaired IL-12 inducing activity. Interestingly, however, the proteins with mutated peptidyl-prolyl isomerase enzymatic activity also showed decreased IL-12 induction despite their normal CCR5 binding.

We have demonstrated previously that T. gondii C-18 evolved the ability to bind both mouse and human CCR5 and to trigger chemokine-like functions in cells bearing this receptor (15, 16). The fact that the corresponding cyclophilin (PfCyp19) from P. falciparum, a closely related apicomplexan protozoan, lacks significant receptor binding activity suggested a molecular approach for understanding the basis of the structural changes leading to CCR5 targeting. Because C-18 and PfCyp19 share a 63% amino acid homology, we hypothesized that the critical sequence differences in C-18 responsible for its chemokine receptor interaction are located on the exposed surface of the molecule, and we identified a set of spatially distinct T. gondii specific amino acid residues as candidate determinants of CCR5 binding activity (Fig. 1). Amino acid differences in the internal region of the molecule were ignored in the present study but could conceivably contribute to its proper folding and receptor binding.

Site-directed mutagenesis revealed that two of the 22 non-homologous single amino acids or sequences chosen for analysis were critical for CCR5 binding as well as the inhibition of R5 viral fusion and infectivity. Interestingly, these two disabling mutations are located in the N and C termini of the protein. However, in the predicted three-dimensional structure of C-18, the amino acids in question lie in close proximity to each other (within 20 Å) on adjacent loops of a β-sheet structure (Fig. 1).

**DISCUSSION**

The peptidyl-prolyl isomerase activity of C-18 and representative mutants as expressed by the generation of p-nitroanilide measured at A390 from its substrate. The loss in enzyme activity of two mutants, R53A and W118A, is shown. An identical loss was observed with mutants F58A, H132Q, and D82A (not shown). The calculated K values for the remaining proteins are presented in supplemental Table I as evidence of proper refolding. B, the retention of CCR5 binding by the mutant proteins with inactivated enzymatic activity was confirmed based on their ability to compete the interaction of 125I-labeled C-18 with CCR5+ cells as described in the Fig. 2 legend. C and D, retention of the antiviral activity of C-18 proteins with mutated peptidyl-prolyl isomerase activity as evaluated by cell fusion (C) and infectivity (D) assays using similar protocols as described in the legends to Figs. 4 and 5.
The latter observation suggests that CCR5 binding may have evolved as a result of a limited number of mutations in regions that are separated in the primary structure of the molecule but adjacent in the tertiary structure. Given that CCR5 binding of conventional chemokine ligands involves multiple extracellular receptor loops, it is not surprising that spatially distinct structural elements on C-18 would be required for receptor interaction. Indeed, in previous studies multiple and spatially distinct amino acid substitutions were shown to abrogate the binding of MIP-1\(\beta\)/H9252 to human CCR5 (28, 29).

CsA is a known antagonist of the peptidyl-prolyl isomerase activity of cyclophilins, and we have demonstrated previously that CsA blocks the antiviral activity of C-18 and, as demonstrated here, inhibits its CCR5 binding. Nevertheless, the amino acid sequences that determine the substrate interaction are highly conserved between phylogenetically distinct cyclophilins, including those with no known CCR5 binding activity (22). This observation led us to hypothesize that the enzymatic activity of C-18 is not important for its mimicked chemokine functions (16). To directly test this hypothesis, we constructed four C-18 mutants with amino acid substitutions in the conserved substrate pocket that led to loss in enzymatic function. These mutations failed to diminish either the CCR5 binding or antiviral activities of C-18. Moreover, neither of the two mutations studied that did result in the loss in chemokine receptor binding altered the enzymatic activity of the protein. These findings formally establish that the peptidyl-prolyl isomerase activity of C-18 is not required for its antiviral activity.

Although in our experimental readout C-18 is the only cyclophilin tested with antiviral activity, other groups have described HIV inhibitory functions associated with mammalian cyclophilins. Host-derived cyclophilin A has been shown to be incorporated into HIV during virion assembly through interaction with a proline-rich domain in the capsid protein, and its presence is known to be essential for infectivity (30–32). Incubation with excess human cyclophilin has been shown in some (33, 34) but not all (35) reports to block HIV fusion and/or infectivity of both R5 and X4 viruses. This inhibition has been proposed to result from competition of the binding of cyclophilin on the viral particle with heparan and CD147 on the target cell membrane, leading to decreased virion attachment (34, 36). The antiviral activity of C-18 does not appear to involve the same mechanism, because its effects are restricted to R5 viruses and are due to specific interaction with CCR5, a property not shared by human and other cyclophilins. Moreover, the interaction of human cyclophilin with host cell CD147 has been
shown to require the peptidyl-prolyl isomerase activity of the protein can be deleted without any significant loss in antiviral activity. Because many of the potential unwanted side effects of cyclophilin administration are likely to result from the enzyme activity of the molecule, the latter observation is clearly of importance in reducing potential drug toxicity. In this context it should be pointed out that although C-18 is able to stimulate IL-12 production by murine CD8+ DCs, to date we have been unable to identify a responsive DC population in humans. Therefore, the IL-12 inducing activity of C-18 observed with murine DCs may be an irrelevant concern in designing a protein for human use. Moreover, as demonstrated here, mutation of the peptidyl-prolyl isomerase activity of C-18 was found to destroy its IL-12 inducing function without impairing its CCR5 binding and antiviral properties.

Having identified regions in C-18 that are critical for CCR5 binding, we can now examine sequence substitutions that might enhance this activity. In this regard, it was of interest that two of the mutations (E29A and K155A) resulted in small but reproducible increases in antiviral and CCR5 binding activity (Fig. 4). It is hoped that by continued mutational analysis, sequence alterations will be revealed that will further enhance the binding of C-18 to CCR5 and therefore lead to potentially increased efficacy of the molecule as an anti-retroviral agent.

Acknowledgments—We thank Edward Berger for helpful comments and Jose Ribeiro for his advice and encouragement of this project.

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