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A Critical Site in the Core of the CCR5 Chemokine Receptor Required for Binding and Infectivity of Human Immunodeficiency Virus Type 1*

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Like the CCR5 chemokine receptors of humans and rhesus macaques, the very homologous (~98–99% identical) CCR5 of African green monkeys (AGMs) avidly binds β-chemokines and functions as a coreceptor for simian immunodeficiency viruses. However, AGM CCR5 is a weak coreceptor for tested macrophage-tropic (R5) isolates of human immunodeficiency virus type 1 (HIV-1). Correspondingly, gp120 envelope glycoproteins derived from R5 isolates of HIV-1 bind poorly to AGM CCR5. We focused on a unique extracellular amino acid substitution at the juncture of transmembrane helix 4 (TM4) and extracellular loop 2 (ECL2) (Arg for Gly at amino acid 163 (G163R)) as the likely source of the weak R5 gp120 binding and HIV-1 coreceptor properties of AGM CCR5. Accordingly, a G163R mutant of human CCR5 was severely attenuated in its ability to bind R5 gp120s and to mediate infection by R5 HIV-1 isolates. Conversely, the R163G mutant of AGM CCR5 was substantially strengthened as a coreceptor for HIV-1 and had improved R5 gp120 binding affinity relative to the wild-type AGM CCR5. These substitutions at amino acid position 163 had no effect on chemokine binding or signal transduction, suggesting the absence of structural alterations. The 2D7 monoclonal antibody has been reported to bind to ECL2 and to block HIV-1 binding and infection. Whereas 2D7 antibody binding to CCR5 was unaffected by the G163R mutation, it was prevented by a conservative ECL2 substitution (K171R), shared between rhesus and AGM CCR5s. Thus, it appears that the 2D7 antibody binds to an epitope that includes Lys-171 and may block HIV-1 infection mediated by CCR5 by occluding an HIV-1-binding site in the vicinity of Gly-163. In summary, our results identify a site for gp120 interaction that is critical for R5 isolates of HIV-1 in the central core of human CCR5, and we propose that this site collaborates with a previously identified region in the CCR5 amino terminus to enable gp120 binding and HIV-1 infections.

Infection by human immunodeficiency virus type 1 (HIV-1)[1] involves adsorption onto cell-surface CD4, followed by interaction of the viral gp120·gp41 envelope glycoprotein complexes with a coreceptor (1–6). Association of gp120 with CD4 induces a conformational change that exposes previously buried epitopes in gp120 and gp41 (7–9), including a site for gp120 interaction with the coreceptor (10–13). The latter interaction is thought to cause an additional conformational change that facilitates fusion of the viral and cellular membranes, resulting in transfer of the viral cores into the cytosol (14). The known coreceptors are all G protein-coupled receptors with seven transmembrane domains (TM) that normally signal in response to cognate chemokine ligands (15). The major coreceptor for macrophage-tropic (R5) isolates of HIV-1 is CCR5, a receptor for the β-chemokines MIP1α, MIP1β, and RANTES (1–5, 16). The coreceptor for T cell-tropic HIV-1 isolates is CXCR4, a receptor for the α-chemokine stromal cell-derived factor (6, 17, 18). R5 isolates are generally responsible for initial infection of individuals and predominate during the relatively early stages of disease, whereas T cell-tropic and dual-tropic viruses accumulate in the late stages of immune system demise (19–24).

Previous investigations have demonstrated that multiple extracellular regions of CCR5 may be important for infection by R5 isolates of HIV-1 (25–31). Studies using receptor chimeras (fusions between CCR5 and chemokine receptors unable to support HIV-1 infection) initially highlighted the contributions to infection of the amino-terminal region of CCR5 and extracellular loops (ECLs) 1 and 2 (25–30). Residues of the amino terminus of CCR5 critical to gp120 binding and infection (Y^{10}DINYY^{15}) have since been more precisely defined by other approaches including site-directed mutagenesis (25, 28, 32–36), whereas a potential role of ECL2 in mediating HIV-1 viral infectivity has been strengthened by a report by Wu and coworkers (31) that a monoclonal antibody whose epitope maps to a peptide derived from this domain (2D7) inhibits both infection and ^{125}I-gp120 binding. However, it is not known if 2D7 exerts its inhibitory effects by attaching to a site on CCR5 required for HIV-1 binding or if its binding globally alters CCR5 conformation or sterically interferes with gp120 interaction with another region of the receptor. Therefore, outside of an interaction with the amino terminus of the receptor, other

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[1] The abbreviations used are: HIV-1, human immunodeficiency virus type 1; AGM, African green monkey; ECL, extracellular loop; R5, macrophage-tropic; SIV, simian immunodeficiency virus; TM, transmembrane domain; gp, glycoprotein; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; MIP, macrophage inflammatory protein; RANTES, regulated on activation normal T cell expressed and secreted.

interactions between HIV-1 and CCR5 critical to viral infection are incompletely delineated.

Recently, we found a high frequency of heterozygosity for CCR5 substitution polymorphisms in African green monkeys (AGMs), a group of primate species believed to have been infected by immunodeficiency viruses since ancient times (28). These initially identified substitutions predominantly cluster in the amino terminus (D13N and Y14N) and in ECL1 (Q93R and Q93K), and they partially inhibit infections by multiple SIV_{agm} isolates.[2] Infectivities of R5 HIV-1 isolates were also inhibited by the Y14N and Q93R substitutions in the context of the wild-type AGM CCR5 and by Y14N in the context of human CCR5 (28). However, although wild-type AGM CCR5 is a strong coreceptor for SIV isolates, it is a relatively weak coreceptor for R5 HIV-1 isolates (28). This observation was surprising because rhesus macaque CCR5 is a strong coreceptor for R5 HIV-1 isolates (37) but differs from AGM CCR5 in only three amino acids. Indeed, the only extracellular amino acid substitution that is unique to AGM CCR5 and absent from rhesus and human CCR5s is G163R, which occurs at the juncture of TM4 and ECL2. We now describe evidence that this site is critical for gp120 binding and for infections by all tested R5 isolates of HIV-1.

EXPERIMENTAL PROCEDURES

Cells and Viruses—HeLa and HEK293T cells were from the American Type Culture Collection (ATCC, Rockville, MD). HeLa-CD4 (clone HI-J) and HeLa-CD4-CCR5 (clone JC.37) cells were described previously (38, 39). HeLa and HeLa-derived cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). HEK293T cells were maintained in the same medium supplemented with glucose (4.5 g/liter). The SF162, JRFL, ADA, and BaL R5 isolates of HIV-1 were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health. HIV-1 viruses were passaged in phytohemagglutinin-stimulated human peripheral blood mononuclear cells. Medium was harvested at times of peak reverse transcriptase release, passed through a 0.45-μm pore size filter, aliquoted, and stored at −80 °C. The JRCSF isolate was obtained as an infectious molecular clone, pYK-JRCSF, from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health, and transfected into HeLa cells. Culture medium was harvested after 72 h and used to infect HeLa-CD4-CCR5 cells (clone JC.37). Viral supernatants were harvested and filtered as above, and the supernatant from day 3 after infection was used in this study.

CCR5 Constructs and Mutagenesis—The rhesus macaque CCR5 expression plasmid was the generous gift of Zhiwei Chen and Preston Marx (Aaron Diamond AIDS Research Center) (37). Constructs containing human and AGM CCR5 were previously described (28). For some experiments, the human and AGM CCR5 plasmids were mutagenized to create the AGM (R163G) and human (G163R) CCR5s by the QuickChange mutagenesis kit (Stratagene, La Jolla, CA) as directed by the manufacturer; for the remaining experiments, mutants with identical coding sequences were created by swapping the *Bcl*I to *Bgl*II restriction fragment between AGM and human CCR5s. In either case, the entire coding sequence of CCR5 was sequenced to confirm that only the desired mutation was introduced. The chimeric CCR5s were created by splicing AGM and human CCR5 at either the *Bcl*I or *Bgl*II restriction site as described (28). The human (Y14N) site-directed mutant was described and characterized previously (28).

gp120 Expression and Purification—The molecular clone pYU2 was obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health, and the molecular clone SF162 was from Cecelia Chang-Meyer. The expression and purification of YU2 gp120 are described, with similar procedures used for expression and purification of SF162 gp120. Briefly, the YU2 envelope glycoprotein gp120 was polymerase chain reaction-amplified from proviral DNA using synthetic oligonucleotides designed according to the published sequence. The resulting polymerase chain reaction product was ligated into pSC11 (40), modified to contain a multilinker sequence, to generate pJL23, and was sequence verified. The 3′-antisense primer design appended a FLAG (Eastman Kodak Co.) epitope to the gp120 viral envelope protein following position Arg-498.

Plasmid pJL23 was used to generate recombinant vaccinia virus Venv-4 using standard techniques (41) for large scale expression of soluble gp120-FLAG. 16 T-225 flasks of CV-1 cells were seeded to contain approximately 1–2 × 10^7 cells/flask on the day of infection. Cells were infected with Venv-4 at a multiplicity of infection of 5 for 2 h in 0.1% bovine serum albumin in phosphate-buffered saline. After infection, cell monolayers were washed twice with phosphate-buffered saline and refed with 50 ml of Opti-MEM (Life Technologies, Inc.) per flask. After approximately 68 h, supernatants were harvested by centrifugation at 6000 rpm for 30 min at 4 °C. Clarified supernatants were supplemented with Triton X-100 (Boehringer Mannheim) to 0.5%, quick-frozen in liquid nitrogen, and stored at −70 °C.

Soluble gp120-FLAG proteins were purified by fast protein liquid affinity chromatography using M2-anti-FLAG affinity gel (Kodak) in an HR5/5 column (Amersham Pharmacia Biotech, bed volume ~1 ml) equilibrated in TBS (50 mM Tris-HCl, pH 8.0, 150 mM NaCl). Culture supernatants (500–1000 ml) were thawed at 37 °C, supplemented to 10 μg/ml each aprotinin and leupeptin (Boehringer Mannheim), filtered through a 0.22-μm filter, and then passed continuously over the M2 column at 1 ml/min at 4 °C for 24–28 h. The resin was washed extensively with TBS, and bound proteins were eluted with 100 μM synthetic FLAG peptide (Kodak) in TBS. Fractions (1 ml) containing gp120-FLAG were identified by Colloidal Blue staining of 10% SDS-polyacrylamide gel electrophoresis gels (Novex, San Diego, CA). Peak fractions were pooled, snap-frozen in liquid nitrogen, and stored at −70 °C.

Pooled fractions were separated from synthetic FLAG peptide and TBS by C4 reverse phase chromatography. Samples were loaded onto a 5-cm Vydac C4 analytical column at 1 ml/min in 10% acetonitrile, 0.1% trifluoroacetic acid. Using a 25–50% gradient, gp120 eluted at approximately 36–38% acetonitrile. Samples were collected on dry ice and were immediately lyophilized. Dried samples were resuspended in phosphate-buffered saline. Relative protein concentration determinations were made using a modified gp120 capture enzyme-linked immunosorbent assay (Intracel, Issaquah, WA) using anti-gp120 monoclonal antibody A32 from James Robinson at Tulane. Fractions with peak activity according to enzyme-linked immunosorbent assay were subjected to amino acid analysis for final concentration determinations.

BaL gp120 was purified as described previously from the culture medium of Schneider 2 *Drosophila* cells that were generously donated by Dr. Raymond Sweet (SmithKline Beecham) (42).

Binding Assays—^{125}I-MIP1α (2200 μCi/mmol) was purchased from NEN Life Science Products. Conditions for competition binding assays using chemokines are similar to those described previously (43).

YU2 gp120 was iodinated by the chloramine-T method according to the procedure of Rollins *et al.* (44). Assay conditions for measurement of direct binding of ^{125}I-YU2 gp120 to CCR5-expressing cells, in the presence of 10 nM soluble CD4, and inhibition of binding of ^{125}I-MIP1α to CCR5-expressing cells by YU2 gp120·sCD4 complexes have also been described previously (11).[3]

For gp120 binding assays done using cells expressing transmembrane-bound CD4, pcDNA3 expression vectors for CD4 and CCR5 were cotransfected into HEK293T cells by the standard DEAE-dextran/chloroquine method (45), except that the cells were plated in flasks that were treated with 0.1 mg/ml poly-L-lysine (Sigma) for 30 min, and no Me_2SO shock was used. Cells were seeded 48 h after transfection at 2 × 10^5 cells/well in poly-L-lysine-treated 24-well tissue culture cluster plates. 24 h later cells were incubated with the indicated concentration of BaL gp120 in DMEM, 10% FBS for 30 min at 37 °C. ^{125}I-MIP1β (2200 μCi/mmol, NEN Life Science Products) was added to a final concentration of 0.5 nM, and cells were incubated for an additional 30 min. The cells were washed, solubilized in 0.1 N NaOH, and counted in a gamma counter. Background counts were determined on vector-transfected cells and subtracted from the values obtained on CCR5-transfected cells. Counts were then expressed as percent binding by normalizing to values obtained with no added gp120.

Coreceptor Activity Assays—The assay to determine infectivities by R5 HIV-1 isolates was performed as described previously (28). Briefly, coreceptors were transiently expressed in HeLa-CD4 (clone HI-J) cells by the calcium phosphate transfection method (45). 48 h post-transfection the cultures were trypsinized and plated at 1.5 × 10^4 cells/well of a 24-well cluster plate for HIV-1 infection. 72 h post-transfection cells were pretreated with DEAE-dextran (8 μg/ml) at 37 °C for 20 min and

[2] S. E. Kuhmann and D. Kabat, manuscript in preparation.

[3] S. J. Siciliano, B. L. Daugherty, J. A. DeMartino, and M. S. Springer, manuscript in preparation.

TABLE I
Sequence differences between primate CCR5s

CCR5[a]	Amino acid and location[b]										
	9 NT	13 NT	49 TM1	52 TM1	78 TM2	123 ICL2	130 ICL2	163 ECL2	171 ECL2	198 ECL2	348 CT
Human	I	N	M	I	F	T	V	G	K	I	I
Chimp		D				S					
Rhesus	T	D	I	V	L		I		R	M	
AGM	T	D	I	V	L		I	R	R		T

[a] A blank space indicates that the sequence is identical to the reference human CCR5. The GenBank™ accession numbers for the CCR5 nucleotide sequences are as follows: human, U54994; chimpanzee, AF005663; rhesus macaque, U73739; and African green monkey, U83325.
[b] The abbreviations used are: NT, amino terminus; TM, transmembrane sequence; ICL, intracellular loop; ECL, extracellular loop; and CT, carboxyl terminus.

then incubated with 0.2 ml of virus diluted in DMEM, 0.1% FBS at 37 °C. After 2 h the cells were fed with 1 ml of DMEM, 10% FBS, and incubated at 37 °C for 3 days. The cells were then fixed in ethanol, and infected foci were visualized by an immunoperoxidase assay (46), using as primary antibody the 0.45-μm filtered supernatant from the anti-p24 hybridoma 183-H12–5C (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health). Stained foci were counted with a dissecting microscope under diffuse illumination, and values were normalized to those obtained using the same virus stock in the same experiment on cells transfected with wild-type human CCR5.

Signal Transduction—Xenopus laevis oocytes were collected and prepared as described previously (47). CCR5 cDNAs were subcloned into the oocyte expression vector pOG-1 at a site between 5′- and 3′-untranslated *Xenopus* β-globin sequences. Kir 3.1 and CCR5 cRNAs were prepared as described previously, and oocytes were microinjected with 5–50 ng of capped cRNA (47). Electrophysiological recording was done by two-electrode voltage clamp 2–5 days after cRNA injection as described (47). Briefly, the oocytes were clamped in a small chamber continually perfused with high K^+ Ringer's solution (100 mM KCl, 2 mM NaCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5), and recombinant human chemokines (Peprotech, Rocky Hill, NJ) were applied by bath perfusion. The holding potential was set at −30 mV, and current-voltage records were obtained during 250-ms voltage jumps to potentials between +40 and −100 mV. Desensitization kinetics were determined by least squares fit to single exponential functions.

Antibody Binding Assays—Binding of the mouse monoclonal antibody 2D7 was determined on HEK293T cells transfected as for gp120 binding (above). 72 h post-transfection cells were incubated with 2.5 μg/ml 2D7 (PharMingen, San Diego, CA) in DMEM, 10% FBS for 45 min at 37 °C, followed by goat anti-mouse IgG serum (Organon Teknika Corp., Durham, NC) at a 1:400 dilution for 45 min, followed by ^{125}I-protein A (0.4 μCi/ml, 2 to 10 μCi/μg; NEN Life Science Products) for 45 min. The cells were then washed, solubilized in 0.1 N NaOH, and counted in a gamma counter. Background counts were determined on vector-transfected cells and subtracted from the values obtained on CCR5-transfected cells. To correct for differences in CCR5 expression, values for 2D7 binding were divided by the counts bound to duplicate wells incubated for 1 h at 37 °C with 0.5 nM ^{125}I-MIP1β (2200 μCi/mmol, NEN Life Science Products).

RESULTS

Sequences of AGM, Human, and Rhesus Macaque CCR5s—Whereas human, rhesus macaque, and chimpanzee CCR5s are comparably able to support infection by R5 isolates of HIV-1 (28, 37, 48), AGM CCR5 is a weak HIV-1 coreceptor (28). Experiments analyzing AGM/human CCR5 chimeras have indicated that AGM residues responsible for poor coreceptor activity map within amino acids 1–168 (28). Table I shows a compilation of the amino acid substitutions that distinguish CCR5s from the aforementioned primates, and Fig. 1 shows a topological model of human CCR5 highlighting positions among the receptors that are divergent. Strikingly, the only amino acid substitutions unique to AGM CCR5 are G163R at the juncture of TM4 and ECL2 and I348T in the carboxyl-terminal cytosolic domain. Indeed, AGM and rhesus macaque CCR5s differ in only three positions, and G163R is the only difference in the critical region between amino acids 1 and 168.

gp120s from R5 Isolates of HIV-1 Compete Well for the Binding of MIP1α to Human and Rhesus CCR5s but Poorly for the Binding of MIP1α to AGM CCR5—As evidenced from an analysis of the binding of ^{125}I-MIP1α to HEK293T cells transfected with appropriate pcDNA3-CCR5 expression vectors, human, rhesus macaque, and AGM CCR5s were all highly expressed on the cell surface (Fig. 2). Moreover, these CCR5s all had strong binding affinities for human MIP1α (Fig. 2), with IC_{50} values, derived using unlabeled MIP1α to displace ^{125}I-MIP1α from CCR5-bearing cells, of 4.8, 0.7, and 0.3 nM for human, rhesus, and AGM, respectively.

Since we[3] and others (10, 11) have previously demonstrated that gp120 envelope glycoproteins from R5 isolates of HIV-1, when complexed to soluble CD4, compete for the binding of radiolabeled β-chemokines to human or rhesus CCR5, we asked if gp120·sCD4 complexes would also inhibit the binding of MIP1α to AGM CCR5. Surprisingly, neither complexes of YU2 gp120·sCD4 (Fig. 3*A*) nor SF162 gp120·sCD4 (data not shown) were able to displace the binding of ^{125}I-MIP1α to AGM CCR5-expressing cells even at concentrations of gp120 as high as 500 nM. In contrast, the IC_{50} values of sCD4-complexed gp120s in displacing MIP1α from human and rhesus receptors was between 5 and 10 nM (Fig. 3*A*). The failure of ^{125}I-MIP1α to be displaced from AGM CCR5 in these experiments could be explained if ^{125}I-MIP1α and R5 gp120·sCD4 complexes bind simultaneously but noncompetitively to AGM CCR5. Alternatively, it is possible that the affinity of gp120·sCD4 for AGM CCR5 is sufficiently low so as not to be able to displace the radiolabeled chemokine.

To address this issue directly, we analyzed the binding of ^{125}I-YU2 gp120·sCD4 complexes to the receptor-bearing cells. As shown in Fig. 3*B*, despite the fact that cells expressing AGM CCR5 were competent to bind ^{125}I-MIP1α (Fig. 3*A*), complexes of ^{125}I-YU2 gp120·sCD4 bound specifically only to cells expressing human or rhesus CCR5s but not to cells expressing AGM CCR5.

Role of Gly-163 in Binding and Infection of R5 Isolates of HIV-1—The above results suggested that AGM CCR5 functions as an attenuated coreceptor for R5 HIV-1 isolates and that it binds with relatively poor affinity to the viral gp120 envelope glycoproteins. To determine if the G163R substitution unique to the extracellular surface of AGM CCR5 was responsible for its deficits in HIV gp120 binding and coreceptor function, we constructed and analyzed the human (G163R) and AGM (R163G) CCR5 mutants.

Fig. 4 shows analyses of the coreceptor activities of the wild-type and mutant CCR5s in mediating infections by five different R5 isolates of HIV-1. Equivalent levels of surface expression of receptor on transfected HeLa-CD4 cells were indicated by binding of ^{125}I-MIP1β and/or antibodies directed against the human receptor (see Fig. 4 legend, and data not shown). Infections were quantitated by a focal assay on HeLa-CD4 cells transfected with the corresponding pcDNA3-CCR5 expression vectors (28) and data normalized relative to the activity of wild-type human CCR5 in each assay. From this figure it is clear that as compared with human CCR5, AGM

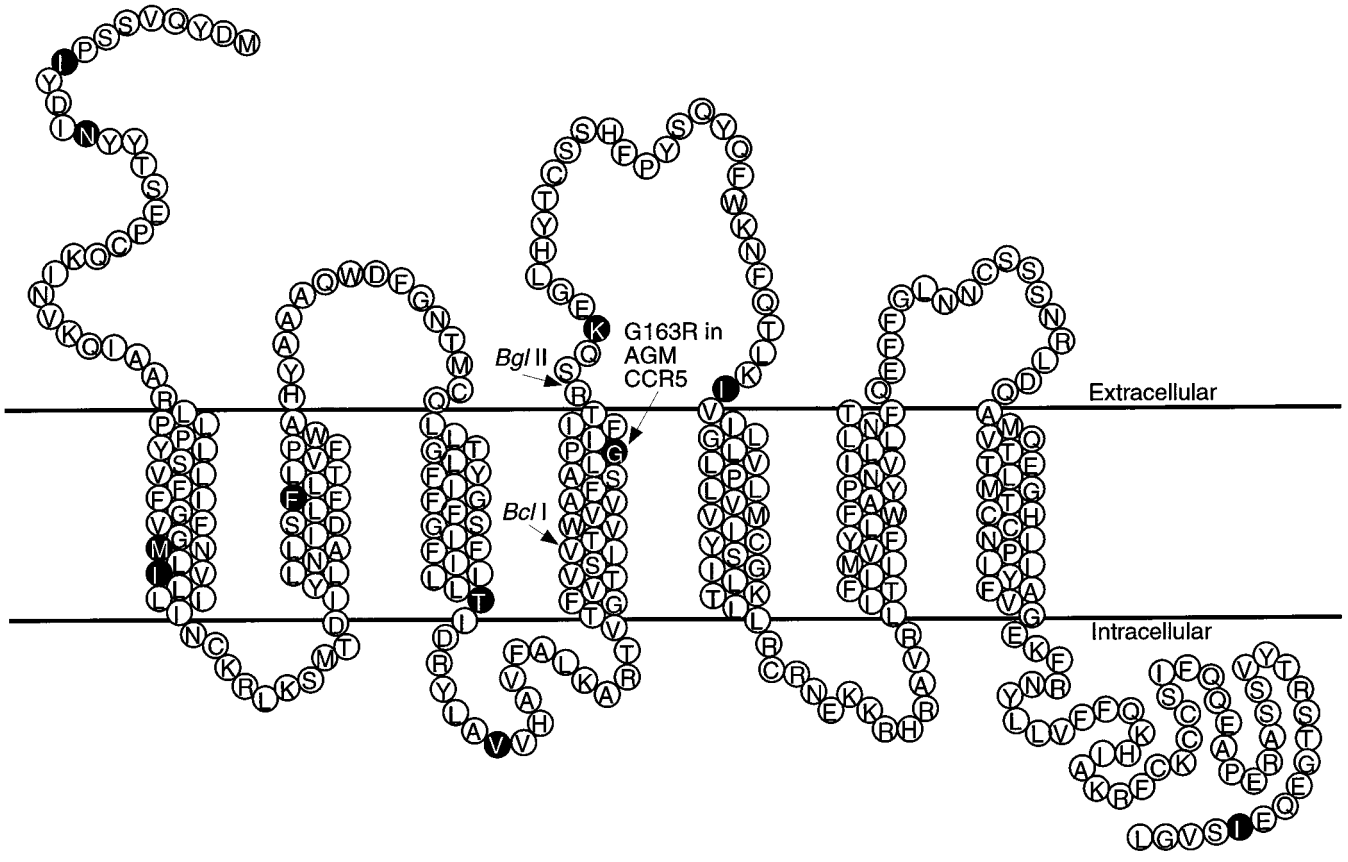

FIG. 1. **Topology of human CCR5 highlighting sites of primate sequence variations.** The extracellular membrane face is above the membrane which is indicated by *solid parallel lines*, whereas the intracellular face is *below*. The splice sites used to make CCR5 chimeras are indicated by *arrows* with the names of the restriction enzymes that were employed. The highlighted amino acids are positions that differ among the human, AGM, rhesus macaque, and chimpanzee CCR5 proteins (see Table I).

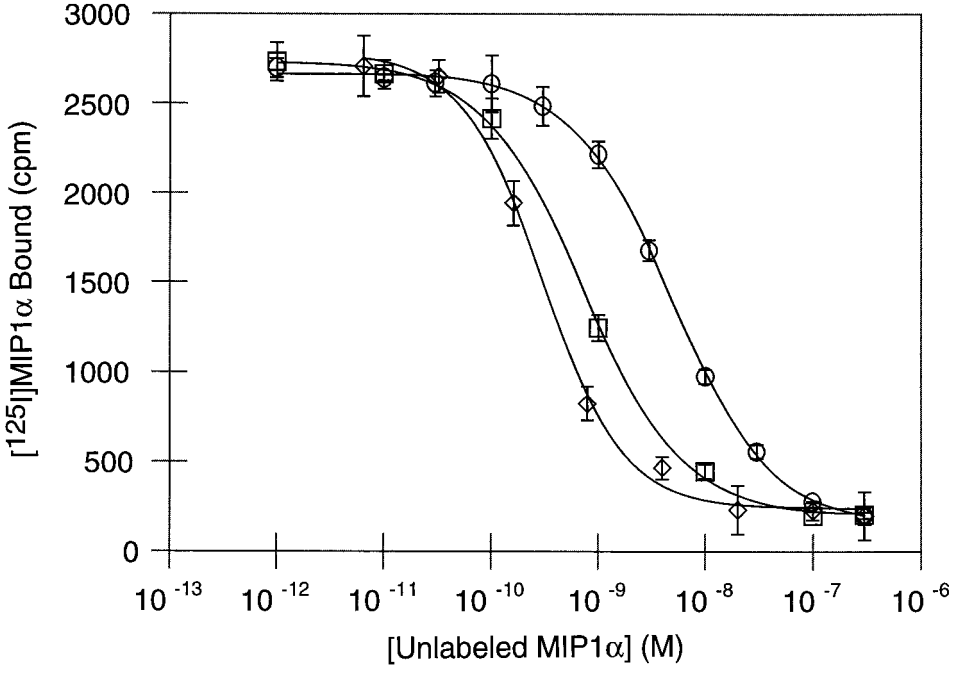

FIG. 2. **Human, AGM, and rhesus CCR5s bind MIP1α with high affinity.** Human (○), rhesus (□), and AGM (◇) CCR5s were transiently expressed in HEK293T cells. Affinities were determined by competition binding between a fixed concentration of ^{125}I-MIP1α and increasing concentrations of unlabeled MIP1α. Results are the average of triplicate determinations from a single representative experiment. 3×10^5 cells transfected with human or rhesus CCR5 or 7.5×10^4 cells transfected with AGM CCR5 were used per determination.

CCR5 is a much weaker coreceptor for all examined HIV-1 isolates. However, the AGM (R163G) substitution restores the efficiency with which the mutant AGM receptor is able to mediate viral infection almost to wild-type human CCR5 levels. Reciprocally, the human (G163R) substitution reduces the coreceptor activity of the mutant human receptor to the level of

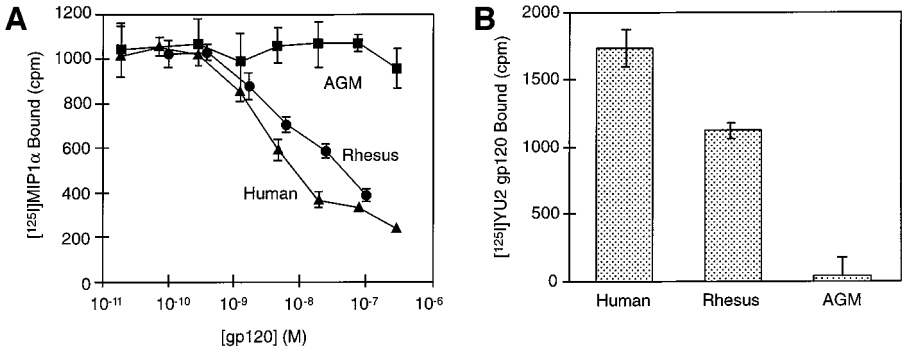

FIG. 3. **YU2 gp120·sCD4 complexes bind poorly to AGM CCR5.** *A,* competition binding between unlabeled YU2 gp120·sCD4 complexes and a fixed concentration of ^{125}I-MIP1α to HEK293T cells expressing human (▲), rhesus (●), or AGM (■) CCR5s. Results are the average of triplicate determinations from a single representative experiment. 3×10^5 cells transfected with human or rhesus CCR5 or 7.5×10^4 cells transfected with AGM CCR5 were used per determination. *B,* direct binding of ^{125}I-YU2 gp120·sCD4 complexes to transiently transfected HEK293T cells expressing human, rhesus, or AGM receptors as indicated (10^6 cells per determination). Results are the average of triplicate determinations from a single representative experiment.

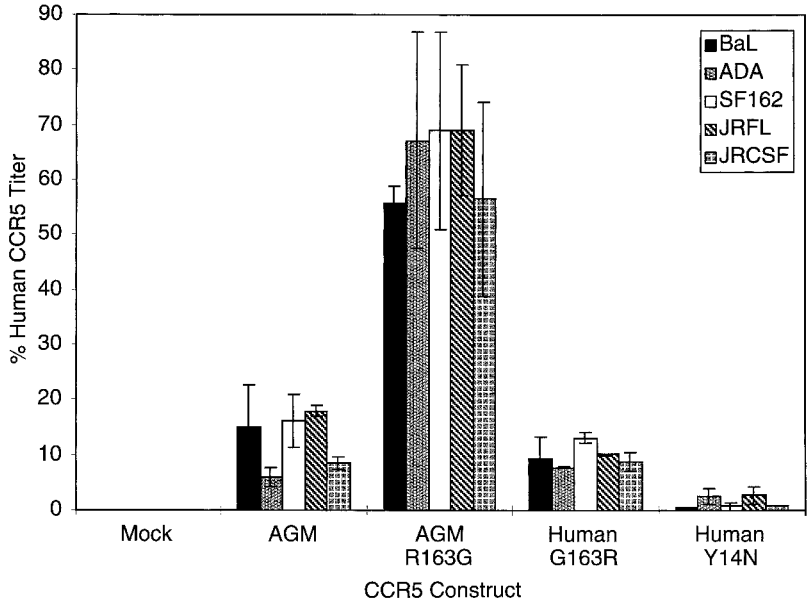

FIG. 4. **Coreceptor activities of human, AGM, and mutant CCR5s.** Relative coreceptor activities of the CCR5 constructs were determined in HeLa-CD4 cells for each of the R5 HIV-1 isolates indicated. The coreceptor activities were normalized to the activity of human CCR5 in the same experiment. The values are the averages of two experiments, and the *error bars* represent the range of values obtained. Mock-transfected cells were transfected with pcDNA3 with no insert. The human-derived coreceptors were expressed in the HeLa-CD4 cells as indicated by binding of rabbit anti-CCR5 serum as described previously (28). The values for binding from a representative experiment were as follows: human, 15.0 ± 0.9 cpm/μg of protein; human (G163R), 8.4 ± 2.3 cpm/μg of protein; and human (Y14N), 5.0 ± 1.6 cpm/μg of protein. This antiserum does not efficiently recognize the AGM-derived CCR5s, largely due to the N13D substitution in the amino terminus (S. E. Kuhmann and D. Kabat, unpublished observations). Likewise, these coreceptors also bound the 2D7 monoclonal antibody: human, 33.3 ± 1.0 cpm/μg of protein; human (G163R), 11.6 ± 0.7 cpm/μg of protein; and human (Y14N), 26.2 ± 0.7 cpm/μg of protein. This antibody also does not efficiently recognize the AGM-derived CCR5s, largely due to the K171R substitution in ECL2 (see Table III). Thus, expression of AGM derived CCR5s was inferred from binding of MIP1α and MIP1β (see Figs. 5 and 7, and data not shown).

wild-type AGM CCR5.

We also analyzed the binding of β-chemokines and of YU2 and SF162 gp120·sCD4 complexes to these wild-type and mutant CCR5s. As shown in Fig. 5 (*C* and *D*), the substitutions at amino acid 163 had no significant effect on the affinities of MIP1α for the different CCR5s. Likewise, these substitutions had no effect on the affinities of human or AGM CCR5s for MIP1β (see below). However, YU2 gp120·sCD4 complexes were able to displace ^{125}I-MIP1α much more readily from human CCR5 than from the human CCR5 (G163R) mutant (Fig. 5*A*). Similarly, YU2 gp120·sCD4 complexes displaced chemokine more efficiently from the AGM (R163G) mutant than from wild-type AGM CCR5 (Fig. 5*B*). These results suggest that the G163R amino acid substitution found in AGM CCR5 reduces its affinity for gp120s derived from R5 isolates of HIV-1.

We substantiated this conclusion by directly analyzing the binding of ^{125}I-YU2 gp120·sCD4 complexes to cell-surface CCR5. As shown in Fig. 6, the binding of ^{125}I-YU2 gp120·sCD4 complexes to cells transfected with human CCR5 was substantially attenuated by the G163R mutation, whereas the ability to bind to AGM CCR5 was restored by the reciprocal R163G mutation. Results similar to those in Figs. 4 and 5 were also obtained using gp120 derived from the R5 isolate SF162 (data not shown).

The previous results were derived by measuring the binding of cell-surface CCR5 to soluble complexes of monomeric gp120 and CD4. In contrast, HIV-1 infections involve cooperative viral attachment onto cell-surface CD4 followed by interactions with a coreceptor in the same membrane. Presumably, these alternative pathways for gp120 interaction with CCR5 would differ energetically, in part because the gp120 of virus adsorbed onto cell-surface CD4 would be confined in a small space at a

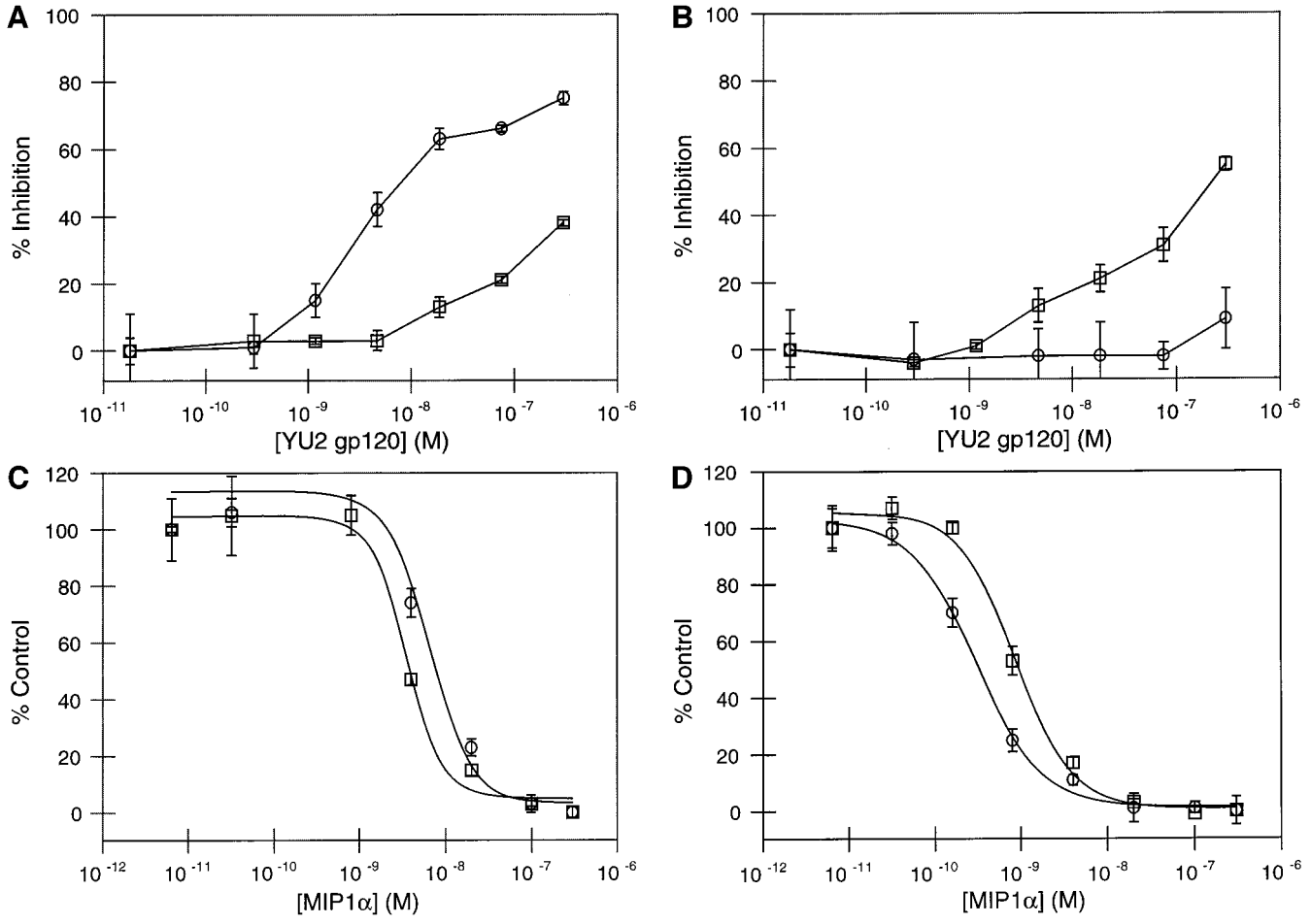

FIG. 5. **CCR5 substitutions at amino acid 163 alter the binding properties of YU2 gp120·sCD4 complexes but not MIP1α.** For *A* and *B*, YU2 gp120·sCD4 complexes were used to compete for binding of ^{125}I-MIP1α to HEK293T cells. Results are the average of triplicate determinations from a single representative experiment using cells transfected with human CCR5 (○) or the human (G163R) receptor mutant (□) in *A*, or cells transfected with AGM CCR5 (○) or the AGM (R163G) receptor mutant (□) in *B*. *C* and *D*, unlabeled MIP1α competes for the binding of ^{125}I-MIP1α to transiently transfected HEK293T cells expressing one of four CCR5 variants. *C*, data are from cells transfected with human CCR5 (○) or the G163R human receptor mutant (□). *D*, data are from cells transfected with AGM CCR5 (○) or the AGM (R163G) receptor mutant (□). In all cases, 10^5 cells were used per determination.

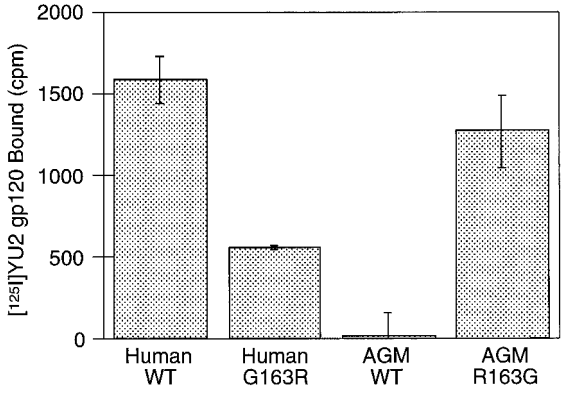

FIG. 6. **CCR5 substitutions at amino acid 163 alter the binding properties of ^{125}I-YU2 gp120·sCD4 complexes.** The direct binding of ^{125}I-gp120·sCD4 complexes was measured using transiently transfected HEK293T cells expressing human, human (G163R) mutant, AGM, or AGM (R163G) mutant receptors as indicated. Results are the average of triplicate determinations from a single representative experiment, using 10^6 cells per determination.

relatively high concentration. For these reasons, we coexpressed full-length human CD4 with the CCR5s in HEK293T cells, and we analyzed the displacement of ^{125}I-MIP1β by gp120 derived from an R5 HIV-1 isolate (BaL). ^{125}I-MIP1β was used for this analysis because it binds with equivalent affinity to all of the CCR5s being tested, with IC_{50} values, derived using unlabeled MIP1β to displace ^{125}I-MIP1β from CCR5-bearing cells, of 6.9 ± 1.3, 3.9 ± 0.8, 5.8 ± 1.5, and 4.4 ± 0.9 nM for human, AGM, human (G163R), and AGM (R163G) CCR5s, respectively (data not shown). As shown in Fig. 7, the resulting ^{125}I-MIP1β displacement data were qualitatively similar to the previous results obtained using YU2 or SF162 gp120·sCD4 complexes (*i.e.* Fig. 5, *A* and *B*). Importantly, these data corroborate our original findings by demonstrating that gp120·CD4 complexes on cell surfaces bind more avidly to human CCR5 than to the human (G163R) CCR5 mutant and more avidly to AGM (R163G) CCR5 than to wild-type AGM CCR5.

Signal Transduction by Wild-type and Mutant AGM and Human CCR5s—To learn further if mutations at position 163 cause major disruption of CCR5 structure or function, we quantitatively analyzed the signal-transducing properties of these receptors in response to MIP1α, MIP1β, and RANTES (see "Experimental Procedures"). As illustrated by the representative results in Fig. 8, human and AGM CCR5s were highly responsive to β-chemokines, and the signaling was not significantly affected by the substitutions at position 163. As shown previously, continued exposure to chemokines in this system is followed by down-modulation of signaling responses (47). The extents of down-modulation and the time constants for desensitization of the responses were also not significantly altered by

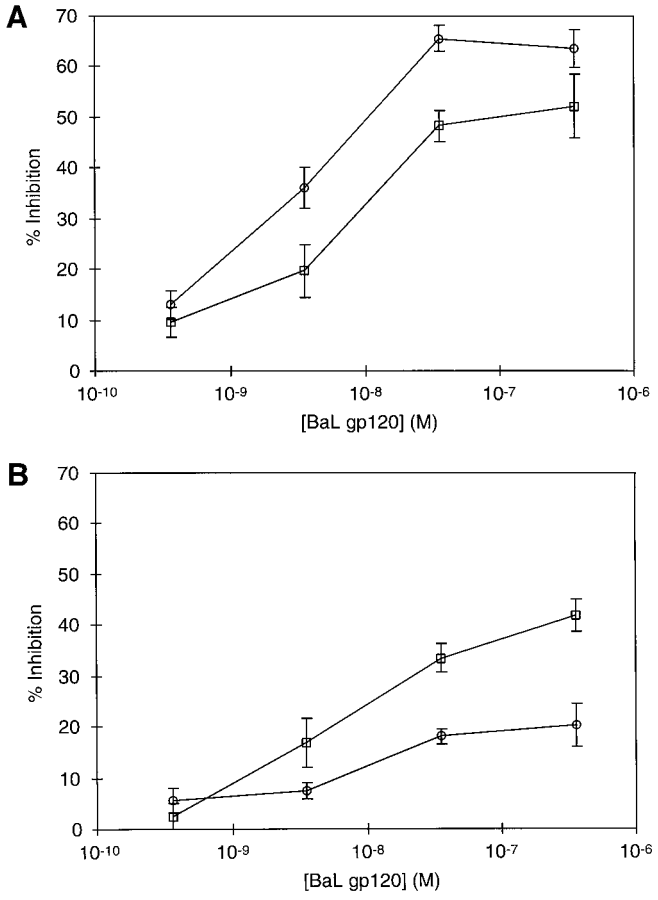

FIG. 7. **CCR5 substitutions at amino acid 163 alter the binding of BaL gp120 to cells coexpressing CD4 and CCR5.** BaL gp120 was used to compete for binding of ^{125}I-MIP1β to HEK293T cells cotransfected with CCR5 and CD4 expression plasmids. *A,* ^{125}I-MIP1β binding to wild-type human CCR5 (○) and human (G163R) mutant CCR5 (□) in the presence of the indicated concentrations of BaL gp120. Each point is the average from 9 experiments. *B,* ^{125}I-MIP1β binding to wild-type AGM CCR5 (○) and AGM (R163G) mutant CCR5 (□) in the presence of the indicated concentrations of BaL gp120. Each point is the average from 6 experiments. The *error bars* represent the S.E.

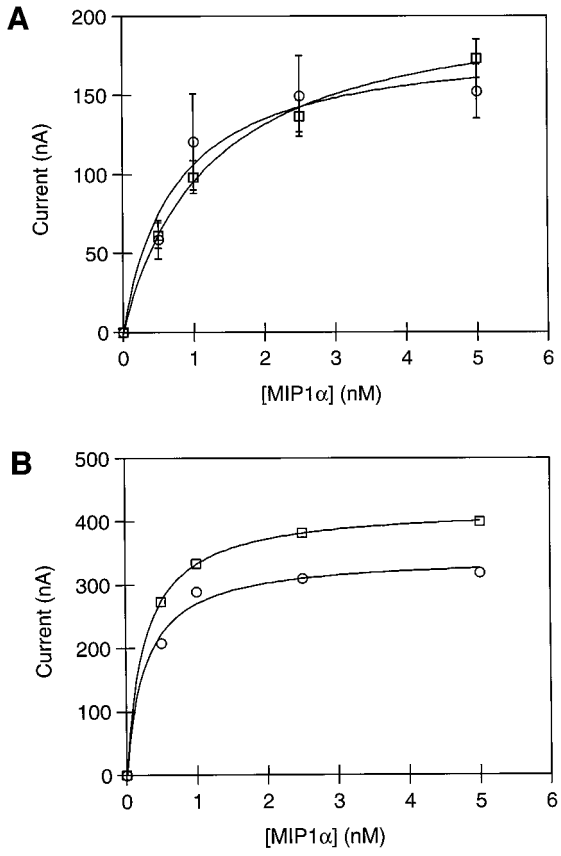

FIG. 8. **Effect of substitutions at amino acid 163 on activation of CCR5 by MIP1α.** Inward currents were measured by two-electrode voltage clamp in oocytes coexpressing CCR5 and Kir 3.1. *A*, activation of human CCR5 (○) by MIP1α was compared with the corresponding activation of human (G163R) mutant CCR5 (□). *B*, activation of AGM CCR5 (○) by MIP1α was compared with AGM (R163G) mutant CCR5 (□). Inward K^+ currents were measured at −80 mV during voltage pulses in two (*B*) or three (*A*) oocytes. *Error bars* in *A* represent the S.E. The EC_{50} values of activation were as follows: human, 0.74 ± 0.28 nM; human (G163R), 1.22 ± 0.13 nM; AGM, 0.30 ± 0.06 nM; and AGM (R163G), 0.30 ± 0.01 nM.

the G163R mutation in human CCR5 (results not shown).

Binding of the 2D7 Monoclonal Antibody to CCR5 Extracellular Loop 2—It has previously been reported that murine monoclonal antibody, 2D7, binds to a peptide corresponding to ECL2 of human CCR5 and neutralizes chemokine interaction with CCR5 as well as HIV-1 infections by R5 isolates (31). Indeed, we found that when HeLa-CD4 cells transfected with human CCR5 were pretreated for 30 min with 2.5, 5, 12.5, and 25 μg/ml of 2D7, infections by the R5 isolate JRCSF were inhibited by 24, 58, 77, and 93%, respectively (data not shown). These results suggested that R5 gp120s may interact with CCR5 ECL2. Since the G163R AGM substitution is at the juncture of TM4 and ECL2 in CCR5, we analyzed the binding of 2D7 antibody to wild-type and mutant human and AGM CCR5s. We found that whereas 2D7 bound to human CCR5 expressed in HEK293T cells (Table II), it did not bind to AGM CCR5 when expressed in these cells (Table II) or in HeLa/CD4 cells (data not shown). We also analyzed binding of 2D7 antibody to mutants and chimeras (see "Experimental Procedures") of human and AGM CCR5. The results shown in Table II suggest that both Gly and Arg at position 163 are compatible with binding of 2D7. For example, both human CCR5 and human (G163R) CCR5 bound 2D7 antibody. In addition, amino acids 1–168 of CCR5 site did not contribute to 2D7 binding as both human CCR5 and the AGM/human CCR5 chimera spliced at this site bound 2D7 antibody. However, a highly conservative K171R substitution present in ECL2 of both AGM and rhesus CCR5 receptors destroyed the 2D7 epitope, as indicated by the absence of 2D7 binding to a human/AGM chimera spliced at position 168. Interestingly the K171R mutation does not significantly interfere with HIV-1 infections, as this chimera is an active coreceptor (data not shown). Taken together, our results suggest that 2D7 binds to a region of ECL2 in human CCR5 that encompasses Lys-171 and imply that the interaction of 2D7 with this portion of ECL2 may inhibit HIV-1 interaction with a nearby site, which presumably includes or whose conformation is influenced by Gly-163.

DISCUSSION

Importance of the Gly-163 Region of Human CCR5 for Binding and Infectivity of R5 Strains of HIV-1—In this investigation, we analyzed differences in the R5 gp120 binding affinities and coreceptor activities of closely homologous human and non-human primate CCR5 proteins. In particular, we found that R5 gp120·sCD4 complexes bind well to human and rhesus macaque but not AGM CCR5. In addition, as compared with human CCR5, AGM CCR5 is a poor coreceptor for R5 isolates of HIV-1 (*e.g.* see Fig. 4), despite the fact that these CCR5s do not significantly differ in their coreceptor activities for SIV_{mac251} (28) and SIV_{agm} isolates.[2] These observations were initially surprising because AGM CCR5 contains only two unique amino

TABLE II
Binding of monoclonal antibody 2D7 to human, AGM, mutant, and chimeric CCR5s

CCR5 construct	Amino-terminal residues 1–162	Amino acid		Ratio of 2D7 binding (cpm) to MIP1β binding (cpm)
		163	171	
Human	Human	G	K	1.5 ± 0.2
AGM	AGM	R	R	0
Human (G163R)	Human	R	K	0.6 ± 0.1
AGM (R163G)	AGM	G	R	0
Human/AGM (*Bgl*II)	Human	G	R	0
AGM/human (*Bgl*II)	AGM	R	K	0.4 ± 0.1
AGM/human (*Bcl*I)	AGM	G	K	0.6 ± 0.1

acid substitutions that are absent from either of these other CCR5 homologues (see Table I and Fig. 1), and only one of these, G163R, is expected to occur in an extracellular domain of the receptor where it has the potential to interact with the viral glycoprotein. More specifically, the G163R substitution is predicted to lie at the juncture of TM4 and ECL2, a region that has not previously been unambiguously implicated in HIV-1 infections.

By using mutant human receptors, we demonstrated that the G163R substitution attenuates CCR5 binding to monomeric gp120s derived from R5 strains of HIV-1 (see Figs. 3 and 5–7) and, additionally, is primarily responsible for lessened HIV-1 coreceptor activity (Fig. 4). Thus, the human (G163R) CCR5 mutant binds gp120 less avidly than wild-type human CCR5 and is a poor coreceptor. Similarly, the reciprocal (R163G) CCR5 mutation of the AGM receptor enhances both gp120 binding and HIV-1 coreceptor activity. The effects of these substitutions were similar for five different R5 HIV-1 isolates and three different monomeric R5 gp120s examined, indicating that the virus interaction affected by this substitution may be universal to R5 HIV-1 strains. Furthermore, as measured by binding of ^{125}I-MIP1β, ^{125}I-MIP1α, or CCR5-specific antibodies (*e.g.* see Figs. 2, 3, 5, and 7), the effects of the G163R substitution on gp120 binding and HIV-1 infection are not the result of inhibition of surface expression of the receptor or of a global alteration in receptor structure. Moreover, the G163R substitution does not alter CCR5-mediated signal transduction responses to MIP1α, MIP1β, or RANTES or the kinetics or extents of CCR5 desensitization caused by prolonged exposures to MIP1α. Consequently, this substitution does not significantly perturb the binding of β-chemokines or the alternative CCR5 conformations involved in signal transduction or in receptor down-modulation.

We conclude therefore that the core region of CCR5 including Gly-163 is critically involved in adsorption and infection of R5 HIV-1 isolates. Although we have not yet thoroughly analyzed this region by mutagenesis, we believe that it may overlap with an epitope in ECL2 defined by the 2D7 mouse monoclonal antibody that also is known to block gp120 binding and infectivity of R5 HIV-1 isolates (31). Our results suggest that 2D7 antibody binding is sensitive to a K171R substitution (see Table II), a position that is close in linear sequence to Gly-163 (see Fig. 1).

The particular mechanistic role of Gly-163 in viral interaction with CCR5 and the dimensions of the region within the receptor that it helps define remain unknown. However, as we have preliminary evidence suggesting that G163E and G163A human CCR5 mutants both bind R5-derived gp120s with high affinity, it is unlikely that the small, flexible nature of glycine at position 163 is required for effective interaction with the HIV-1 envelope glycoprotein. Instead, glycine, alanine, or glutamic acid at position 163 may all be permissive amino acids in the context of CCR5, with each able to play an indirect role in gp120 binding. In contrast, our data indicate that arginine at position 163 clearly is non-permissive to gp120 binding and HIV-1 infection. This may perhaps be due to the potentially positively charged, electrostatic nature of this amino acid, or alternatively to its unique ability to influence the conformation of a critically important receptor site that may be nearby. Further studies will be required to evaluate these possibilities.

Other Regions of Human CCR5 Are Also Essential for gp120 Binding and HIV-1 Infectivity—Although our results demonstrate that the G163R AGM CCR5 amino acid substitution plays a major role in inhibiting the binding of R5 gp120 glycoproteins to CCR5 and in impairing the ability of AGM CCR5 to mediate infection by R5 HIV-1 isolates, our data also suggest that other amino acid differences between human and AGM CCR5s may play a supporting role. Thus, AGM (R163G) CCR5 is only approximately 60–80% as active as human CCR5 in mediating HIV-1 infections (Fig. 4) and binds more weakly to YU2 gp120·sCD4 complexes (Figs. 5 and 6). Similarly, human (G163R) CCR5 binds YU2 gp120·sCD4 complexes much more avidly than AGM CCR5 which also contains Arg at position 163 (Figs. 5 and 6). Residue substitutions outside of Gly-163 in AGM CCR5 known to be inhibitory to HIV-1 infection and gp120 binding include I9T and N13D (28). Likewise, amino acids in the amino terminus of human CCR5, including amino acids Y^{10}DINYY^{15}, have been shown to be critical for R5 gp120 binding and HIV-1 infectivity (25, 28, 32–35). Based on these considerations, it appears that infection by R5 strains of HIV-1 likely requires binding interactions with two distinct regions of CCR5 as follows: the amino terminus of the receptor and a region of the receptor outside of the amino terminus that encompasses the Gly-163 region. Although it may be that these two domains of CCR5 act independently in this regard, we cannot rule out the possibility that they may cooperate to form a single viral interaction site.

It is intriguing, however, that infections by R5 HIV-1 isolates require interactions with both the Gly-163 and amino-terminal regions of CCR5, because these regions have also been implicated in agonist binding to related receptors (49). Indeed, it has been proposed that agonists associate with chemokine receptors by a two-step mechanism involving an initial interaction with the amino terminus followed by a conformational change that facilitates association with ECL2 (50). Our results are compatible with a similar mechanism of HIV-1 binding to CCR5.

CCR5 Coreceptor Activities Do Not Strictly Correlate with Their Affinities for gp120s of R5 HIV-1 Isolates—Although it has been known that gp120 binding to CCR5 is essential for infections by R5 strains of HIV-1, it has been difficult to measure gp120 affinities for CCR5 in a physiologically relevant manner. In part, this difficulty stems from the fact that infection involves trimeric gp120·gp41 complexes embedded in the virion membrane that associate with CD4 and then diffuse on cell surfaces to interact with the coreceptor (14, 38). In contrast, our binding assays employ purified soluble monomeric gp120s (Figs. 3 and 5–7). While taking this caveat into consideration in the interpretation of our data, our results still strongly suggest that CCR5 coreceptor activities do not corre-

late precisely with their affinities for R5-derived monomeric gp120s. For example, gp120·sCD4 complexes bind with higher affinity to human (G163R) CCR5 than to AGM CCR5 (see Figs. 5*A* and 6), yet these CCR5s have similar coreceptor activities (Fig. 4). Furthermore, AGM CCR5 mediates infections by R5 HIV-1 isolates 10–20% as well as human CCR5 (see Fig. 4) despite its exceedingly poor apparent affinity for gp120·sCD4 complexes. Therefore, it appears that HIV-1 infections may be surprisingly insensitive to factors that substantially decrease virus affinities for CCR5. This conclusion may have profound implications for the prospects of identifying CCR5-directed antivirals, as small molecule inhibitors of the interaction between gp120 and CCR5 which merely lower the affinity of gp120 for CCR5 may not then be able to ultimately block viral entry.

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